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Determination of time dependent antibacterial activities of curcumin, carvacrol and styrax liquidus on *Salmonella* Enteritidis

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Abstract: *Salmonella* Enteritidis is amongst the most common causes of foodborne salmonellosis. Multi-drug resistant *Salmonella* strains has been associated with treatment failures. Plant-derived phytochemicals may be an alternative to antibiotics in combating these bacteria. The purpose of this study is to investigate the antibacterial activity of curcumin, carvacrol and styrax liquidus on *S.* Enteritidis and *S.* Enteritidis PT4. Minimum inhibitory concentration (MIC) values of these substances were detected at 1.5, 3, 7.5 and 24 h by broth microdilution method to evaluate their time-dependent antibacterial activities. The findings of the present study showed that MIC values of carvacrol, curcumin and styrax liquids for both *S.* Enteritidis and *S.* Enteritidis PT4 were 125.0 µg/mL, 132.5 µg/mL, 31.3 mg/mL for 24 h, respectively. Also, a time-dependent change was observed in the MIC values of curcumin. Carvacrol, curcumin and styrax liquidus can be used to provide antimicrobial effect on *S.* Enteritidis and *S.* Enteritidis PT4 in food applications, taking into account the MIC values and contact times.
Keywords: Carvacrol, curcumin, MIC, *Salmonella* Enteritidis, styrax liquidus.

Kurkumin, karvakrol ve sığla yağının *Salmonella* Enteritidis üzerine zamana bağlı antibakteriyel aktivitesinin belirlenmesi

Özet: *Salmonella* Enteritidis, gıda kaynaklı salmonellozisin en yaygın nedenleri arasındadır. Çoklu antibiyotiklere dirençli *Salmonella* suşları, tedavide başarısızlıklara neden olmaktadır. Bitki kaynaklı fitokimyasallar, bu bakterilerle mücadelede antibiyotiklere bir alternatif olabilir. Bu çalışmanın amacı, kurkumin, karvakrol ve sığla yağının *S.* Enteritidis ve *S.* Enteritidis PT4 üzerindeki antibakteriyel aktivitesini araştırmaktır. Bu maddelerin minimum inhibitör konsantrasyon (MİK) değerleri, zamana bağlı antibakteriyel aktivitelerini değerlendirmek için broth mikrodilüsyon yöntemi ile 1,5, 3, 7,5 ve 24. saatlerde belirlenmiştir. Çalışmanın bulgularında, hem *S.* Enteritidis hem de *S.* Enteritidis PT4 için karvakrol, kurkumin ve sığla yağının MİK değerlerinin 24 saat boyunca sırasıyla 125,0 µg/mL, 132,5 µg/mL, 31,3 mg/mL olarak tespit edilmiştir. Ayrıca, kurkuminin MİK değerlerinde zamana bağlı bir değişiklik de görülmüştür. MİK değerleri ve temas süreleri dikkate alındığında karvakrol, kurkumin ve sığla yağının, gıda uygulamalarında *S.* Enteritidis ve *S.* Enteritidis PT4 üzerinde antimikrobiyal etki sağlamak için kullanılabileceği sonucuna varılmıştır.
Anahtar sözcükler: Karvakrol, kurkumin, MİK, *Salmonella* Enteritidis, sığla yağı.

Introduction

Foodborne diseases resulting from ingestion of contaminated food by a defined list of microbes, parasites and chemicals are cause of morbidity and mortality worldwide (4). The World Health Organization (WHO) estimated that they affect 600 million people and 420,000 deaths occur annually in 2010, resulting in the loss of 33 million healthy life years. *Salmonella* species are responsible for a quarter of 550 million diarrheal illness worldwide each year (48). It is estimated that 155,000

deaths occur in the world each year due to bacteria *Salmonella* spp. (10). *Salmonella* spp. reside in the gastrointestinal tract of different domestic animals and are usually present in stool exerted by healthy animals and may contaminate fruits and vegetables and foods of animal origin (27, 46). Thus, *Salmonella* can be spread between human and animals, and cause disease. *Salmonella* Enteritidis and *S.* Enteritidis PT4 are most commonly isolated serotypes from foods of animal origin and are important causes of infections associated with these foods in humans (18, 47).

Antimicrobials play an important role in the control of bacterial foodborne infections. However, misuse and overuse of antibiotics in the management of human and animal diseases encourage the bacteria to develop resistance. The rapid emergence of antimicrobial resistance has been a global problem for managing the health care of people and animals (30). It has been reported that there is a link between the use of antimicrobials in livestock and the emergence of antimicrobial resistance in pathogenic bacteria (13, 27). Spread of resistant bacteria from animals to humans may occur through foods, environment, or direct interaction with animals and leads to great challenges in infection control (19). The emergence and spread of resistance to multiple antibiotics as well as a lack of new drug development by the pharmaceutical industry has led to an increase interest in medicinal plants. Various crude extracts or individual compounds and essential oils of the medicinal plants could serve as an alternative source of new antimicrobials due to a broad range of secondary metabolites (15, 23).

Several plant-derived compounds including carvacrol and curcumin have attracted the attention of the scientific community for their antimicrobial properties. Carvacrol, the main active ingredient of essential oils, are known by its broad-spectrum antimicrobial and antioxidant activities (6, 11, 33). Curcumin, a polyphenolic natural ingredient derived from *Curcuma longa* roots, is known to exert antimicrobial activity against a variety of bacterial species (2, 26). Styrax liquidus, locally named as “sığla yağı” is a resinous exudate (balsam) obtained from the incision trunk of *Liquidambar orientalis* Miller tree which is an endemic species in Türkiye. It has been used for the treatment of peptic ulcer in Turkish folk medicine (16). Styrax liquidus consists of resin, essential oils, styrene and cinnamic acid (16, 20). There are few studies investigated the antibacterial activity of styrax liquidus (35).

There is no research investigating antibacterial activity of these substances, becoming popular in the world and Türkiye, on *Salmonella* species. Hence, it was aimed to investigate of antibacterial activities of carvacrol, curcumin and styrax liquidus on *S*. Enteritidis and *S.* Enteritidis PT4 at different time parameters (1.5, 3, 7.5 and 24 h) in the present study.

Materials and Methods

Compounds and Materials: Carvacrol (Sigma, 282197) and curcumin (C1386, Sigma, ≥65%) were purchased from Sigma-Aldrich (Madrid, Spain), Styrax liquidus (Sweetgum) was purchased from local producers. Carvacrol and curcumin were dissolved in dimethyl sulfoxide (DMSO, Aldrich, 99.5%) to prepare stock solutions of 500 mg/mL for carvacrol (w/v, 1/1) and 3.6 mg/mL for curcumin (curcumin/DMSO, w/v, 8.33 mg/1.5 mL). Sweetgum was dissolved in absolute ethanol (Sigma, 1.02428) to prepare stock solution of 500 mg/mL (v/v, 1/1). Mueller Hinton broth (MHB, CM0405) and Mueller Hinton agar (MHA, CM0337) were purchased from Oxoid (Oxoid Ltd., Basingstoke, England).

Bacterial Strain: American Type Culture Collection (ATCC) standard *S.* Enteritidis (ATCC: 13076) and National Collection of Type Cultures standard *S.* Enteritidis PT4 (NCTC: 13349) were used for the screening antibacterial activities of sweetgum, carvacrol and curcumin. The strains were incubated at 37°C for 24 hours to evaluate the antibacterial activity of plant-derived compounds.

Evaluation of Antibacterial Activity: The MIC values of carvacrol, curcumin and styrax liquidus against *S.* Enteritidis and *S.* Enteritidis PT4 were determined according to the microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) (8). Briefly, the MICs of the carvacrol, curcumin and styrax liquidus were investigated at eight different concentrations ranged from 7.813 µg/mL to 1000 µg/mL, 5.07 µg/mL to 650 µg/mL and 1.95 mg/mL to 250 mg/mL, respectively. Serial 2-fold dilutions of carvacrol, curcumin and styrax liquidus were prepared in MHB. Two hundred microliters of each different concentrations of carvacrol, curcumin and styrax liquidus were added into the wells of a 96-well plate as separate study groups. Then, 20 µL containing 0.5 McFarland cell/mL bacteria was added each well (9). The well plates were incubated at 37°C under aerobic conditions for 24 h. The MIC was defined as the lowest concentration of the substance where bacterial growth was not detected. All trial groups were carried out in triplicate. Absorbance was measured at 600 nm (OD600) to monitor the microbial growth by using a microplate reader (Epoch, BioTek, USA) at 1.5, 3, 7.5 and 24 h of incubation. In the evaluation of optical density values, the average of the trials of each bacterium in triplicate were taken. It was found by taking the difference of the values given as "blank" from the reproductive optical density values of the relevant bacteria. The lowest substance concentration with no microbial growth and OD600 value of ≤0.1 was accepted as the MIC value (21). The effects of solvents used on bacterial growth was examined using the MIC method at the final concentration of solvents in each well. It was observed that the final concentration of the solvents in each well did not show any inhibitory effect against bacterial growth.

Results

The broth microdilution test was carried out to assess the antimicrobial activities of carvacrol, curcumin and styrax liquidus on *S.* Enteritidis and *S.* Enteritidis PT4 at 1.5, 3, 7.5 and 24 h. All MIC values are given in Table 1. Since bacterial growth could not be observed clearly, the MIC values of curcumin and styrax liquidus at 1.5 and 3 h could not be determined. Similarly, the MIC values of carvacrol could not be determined at 1.5 h. According to the results of the study, although there was no time-dependent change in the MIC values of carvacrol and styrax liquidus, a time-dependent change was observed in the MIC values of curcumin. Curcumin exhibited the excellent antimicrobial activity against these bacteria in 24 h of treatment. After 7.5 h of application, the MIC value of curcumin against *S.* Enteritidis was 650 µg/mL while it was 325 µg/mL against *S.* Enteritidis PT4. However, in 24-h application, curcumin presented similar MIC values against both bacteria (Figure 1).

According to the MIC values, Although *S.* Enteritidis and *S.* Enteritidis PT4 were found to be less susceptible to styrax liquidus (MIC value: 31.3 mg/mL), they were more susceptible to carvacrol (125 µg/mL). In the wells treated with curcumin, optical densities up to 0.25 (OD600 nm) were observed in the measurements made at 1.5 and 3 h. In this study, the solvents (Ethanol and DMSO) used to dissolve the substances did not show any antibacterial activity at the application doses.

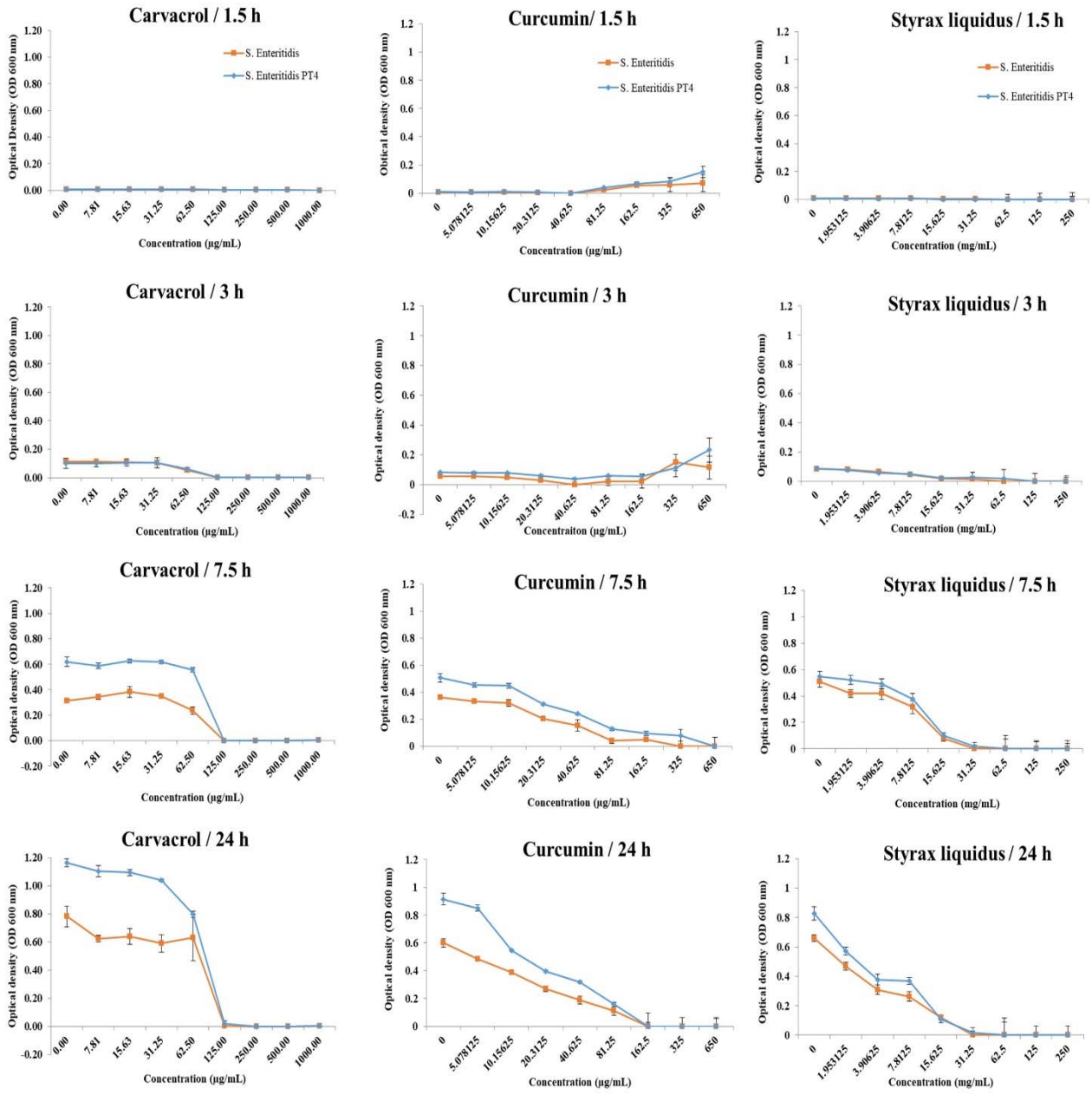

Figure 1. The growth rates of *S.* Enteritidis and *S.* Enteritidis PT4 according to exposure time and concentration of substances.

Table 1. MIC values of Carvacrol, Curcumin and Styrax Liquidus at 37ºC for 1.5, 3, 7.5 and 24 h.

Times	Trials	Carvacrol (µg/mL)		Curcumin (µg/mL)		Styrax Liquidus (mg/mL)	
		S. Enteritidis	*S.* Enteritidis PT4	*S.* Enteritidis	*S.* Enteritidis PT4	*S.* Enteritidis	*S.* Enteritidis PT4
1.5 h	1	*	*	*	*	*	*
	2	*	*	*	*	*	*
	3	*	*	*	*	*	*
3 h	1	125.0	125.0	*	*	*	*
	2	125.0	125.0	*	*	*	*
	3	125.0	125.0	*	*	*	*
7.5 h	1	125.0	125.0	650.0	325	31.3	31.3
	2	125.0	125.0	650.0	325	31.3	31.3
	3	125.0	125.0	650.0	325	31.3	31.3
24 h	1	125.0	125.0	162.5	162.5	31.3	31.3
	2	125.0	125.0	162.5	162.5	31.3	31.3
	3	125.0	125.0	162.5	162.5	31.3	31.3

Discussion and Conclusion

Antibacterial (antibiotic) drug resistance is a growing global problem and the number of new approved drugs is declining. Hence, the need for new antimicrobials becomes more pressing in bacterial infections (22, 31). Plant extracts and essential oils are known as a good source of antimicrobial substance effective on foodborne pathogens as they have antibacterial, antifungal, antiparasitic and antiviral properties (40, 41). There is a great number of natural compounds isolated from plants, a part of them has an important role in food, cosmetics, sanitary fields and oral-dental treatments (32, 37). Recently, there has been an increased interest in the assessment of the antimicrobial potential of natural plant compounds such as curcumin and carvacrol against pathogen bacteria (7, 23, 24).

Carvacrol has been reported as antibacterial agent. However, the reported values of MICs are widely divergent. Several studies have reported that carvacrol shows antibacterial activity against *S.* Enteritidis with MIC values ranging 156 to 331 µg/mL (5, 6, 11, 33). The result of this study showed a MIC value for carvacrol of 125 µg/mL for *S.* Enteritidis and *S.* Enteritidis PT4. The MIC and above concentrations of carvacrol inhibited the growth of *S.* Enteritidis and *S.* Enteritidis PT4 at 3, 7.5 and 24 h. The antibacterial activity of carvacrol has been attributed to its hydrophobic property that influences the fluidity and permeability of the bacterial cell membrane by changing the lipid fraction (34). Also, carvacrol leads to the leakage and loss of ATP from bacterial cells (44, 45). The membrane fluidity has been found to play an important role in the bactericidal activity of the carvacrol against *Bacillus cereus* (44). In this study, at concentration of 125 µg/mL and above, total inhibition of the growth was observed and carvacrol may be bactericidal towards *S.* Enteritidis and *S.* Enteritidis PT4. The bactericidal activity against these bacteria may be due to affecting the fluidity and permeability of bacterial cell membrane.

Curcumin, naturally found as a constituent of dietary species called turmeric (*Curcuma longa*), has been the subject of intensive investigation on its various activities including antiviral, antibacterial and anticancer (39). Contrary of these, there is an evidence that curcumin increases the resistance of *S.* Typhimurium resistance to antimicrobial agents such as antimicrobial peptides, reactive oxygen and nitrogen species. The tolerance developed may be attributed to the up-regulation of genes involved in resistance to some antimicrobial peptides and genes with antioxidant function (28). Adamczak et al. (1) reported that curcumin exhibit a significantly larger variation in the its antibacterial activity (MICs ranged from 31.25 to 5000 µg/mL against over 100 strains of pathogens belonging to 19 species) and suggested that curcumin can be considered as a promising antibacterial agent but, with a very selective activity. Several studies have reported that curcumin shows strong antimicrobial activity against Gram-positive than Gram-negative bacteria with MIC values ranged from 62.5 µg/mL to 5000 µg/mL (1, 14, 36, 38). Further detailed studies are needed to investigate its antibacterial activity. There are few studies investigating the antibacterial activity of carvacrol on *Salmonella* spp. A study reported that MIC value for curcumin were found to be 250 µg/mL for *S.* Typhimurium (36). In the current study, even though curcumin exhibited the excellent antimicrobial activity against both *S.* Enteritidis and *S.* Enteritidis PT4 (MIC value of 125 µg/mL) in 24 h exposure, the MIC values of curcumin against *S.* Enteritidis and *S.* Enteritidis PT4 were 650 µg/mL and 325 µg/mL, respectively, at 7.5 h of exposure. As the exposure time of curcumin to these

bacteria increased, its antibacterial activity strengthened. Curcumin shows it antibacterial activity through various mechanisms, including inhibiting bacterial DNA replication, altering gene expression and disrupting the bacterial cell membrane hence it can affect the cell division and proliferation of bacteria (1, 42). Increased antibacterial activity over time in this study might be attributed to its mechanism of action. Also, it was thought that the high OD observed at high concentration of curcumin (325 and 659 µg/mL) at 1.5 and 3 h measurements might be due to its coloring properties, low water solubility and poor chemical stability (25).

Styrax liquidus which is a resinous exudate obtained from the wounded barks of *Liquidambar orientalis* mainly consists of acid, ester, alcohol, phenolic and volatile compounds. Its main components are cinnamic acid, styracin, styrol, stoyrone, storesinol, storesin, cinnamyl cinnamate, 3-phenylpropyl cinnamate, benzyl cinnamate, styrene, trans cinnamyl alcohol, hydrocinnamyl and vanillin. Its composition may vary widely depending upon a number of factors such as collection site, processing and storage conditions (3, 16). It has been used against parasitic infections, for treatment of peptic ulcers and burns, and as antiseptic in Turkish traditional medicine (12, 17, 43). In an *in vitro* study investigating antibacterial activity against 20 different strains of bacteria using an agar diffusion method, the results showed that styrax liquidus inhibited completely the growth of 13 bacteria at a 10% concentration and did not inhibit the growth of any bacteria at a 0.1% concentration. All treatment concentration (10%-0.1%) were inactive against 7 bacteria (35). There is literature on the antimicrobial activity of Styrax liquidus on *Salmonella* spp. Our study showed that it has an inhibitory effect on *S.* Enteritidis and *S.* Enteritidis PT4 at high concentrations (MIC values of 31.2 mg/mL for both). Its antimicrobial activity may be attributed to the presence of substances with antimicrobial activity, such as cinnamic acid, in its composition.

In conclusion, carvacrol and curcumin have stronger antibacterial activities than styrax liquidus. While curcumin exhibit its strongest antibacterial effect at 24 h, carvacrol and styrax liquidus showed at 7.5 h. The antibacterial effect of carvacrol and styrax liquidus started at seven and a half hours of administration. Carvacrol and styrax liquidus can be used to provide antimicrobial effect on *S.* Enteritidis and *S.* Enteritidis PT4 in food applications at lower exposure times.

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Ethical Statement

This study does not present any ethical concerns.

Conflicts of interest

The authors declare no conflict of interest.

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Effects of dietary melatonin on broiler chicken exposed to continuous lighting during the first two weeks of life

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Abstract: Intensive broiler chicken production involves different lighting regimes, which affects a natural cycle of secretion of melatonin, a hormone included in multiple physiological processes in a bird's body. This research aimed to determine the effects of dietary melatonin supplementation during the first two weeks of broiler chickens' life, bred under constant 24 h lighting, on their health and some hematological, biochemical, and production parameters. The study lasted 6 weeks. Three hundred and twenty 1-day chickens were divided into two groups (control - C and experimental - M), with four replications and 40 chickens in each replica. In the first two weeks (the first phase of the study), a continuous lighting mode 24L: 0D was applied. Group M was receiving a diet supplemented with 30 mg/kg synthetic melatonin only during the first phase. Group C had a diet without melatonin supplementation throughout the whole study. Melatonin addition had a statistically significant effect ($P<0.01$) on the mean values of body weight and daily weight gain at the end of the 1st, 2nd, 3rd, 4th, 5th, and 6th week. The chickens of the experimental group have had a significantly higher value of the European Production Efficiency Factor (EPEF) ($P<0.05$). The results presented in this paper indicate a direct benefit in stress relief in broilers and a value of the organism's antioxidant system promotion, manifested by improved production performance and fattening efficiency.

Keywords: Broilers, light, melatonin, production, stress.

Introduction

Multiple physiological processes and forms of behavior of living beings show circadian rhythmicity (9). Synchronization of these processes in the organism is performed by biological pacemakers located in the retina, pineal gland, and hypothalamus of the birds (17). The pineal gland of birds comprises light receptors, pacemakers, and the outgoing signal – melatonin (18, 19). The circadian rhythm of melatonin is already established in the second trimester of chicken embryonic development, with the amplitude increasing until hatching (39). It is characterized by the increased synthesis during the night (darkness) and decreased production during the day (light).

Approximately 80% of melatonin in the bloodstream is produced by the pineal gland (14). Besides, melatonin is produced by cells of peripheral tissues and organs, such as enterochromaffin cells of small intestine mucosa. The production of melatonin in the gastrointestinal tract is associated with feed consumption, especially one rich in tryptophan (13) which is the precursor for melatonin synthesis (40).

Chickens take feed in the period of daylight. Therefore, there is the assumption that feed intake and growth are the highest in broilers reared in (nearly) continuous illumination (5). Broilers in intensive production are subjected to 23-24 h continuous artificial lighting in the first week of life. Continuous lighting at around 20 illuminance (lx) ensures that chickens acclimatize themselves properly to their environment, as indicated by optimal feed and water intakes (24). In the later stages of rearing, regimes with different photoperiods' duration are applied (6, 24, 32, 33).

Duration of lighting significantly affects melatonin synthesis, growth, and health status in chickens (41). It is expected that broilers exposed to continuous lighting have

a significant deficit of melatonin in serum, while performance and health are improved in broilers exposed to intermittent lighting (5). Sun et al. (32) have determined a significantly higher level ($P<0.05$) of melatonin in broilers exposed to continuous lighting in the 16L:8D regime, in contrast to chickens exposed to constant and intermittent lighting - with a shorter total period of darkness.

Melatonin has multiple roles in the organism, and the most important relates to the participation in the antioxidative system and protection of cells from the harmful effects of free radicals (40). Insufficient melatonin secretion in broilers is associated with metabolic and physiological disorders, leading to breeding diseases and deaths, thus with economic losses and several negative aspects of animal welfare (14), including fearful response in broiler chickens (19). The results of several studies have shown that in regime with controlled lighting, supplementation of melatonin in feed, drinking water or parenterally had positive effects on health and immune status of chicken, growth performance, feed intake and conversion, the morphology of intestine mucosa, and growth hormone levels along with others (1, 3, 12, 31, 38).

This research aimed to investigate the effects of melatonin food supplementation on broiler chicken bred under constant 24-hour lighting during the first two weeks of life on birds' health and some hematological, biochemical, and production parameters.

Materials and Methods

The trial was performed at the Experimental Broiler Farm of the Institute for Animal Husbandry, Belgrade, Serbia.

Housing conditions and nutrition of broilers: Three hundred and twenty (n = 320) 1-day-old Ross 308 broiler chickens of both sex and uniform body mass (41.1±3.1g) were used and kept in a floor system on a mat of chopped straw, in one room with group boxes for 40 broilers (10 heads/m^2). In the first two weeks, a continuous lighting (CL) mode 24L: 0D (light: darkness) was applied (a combination of the infrared bulb in the box and ordinary light bulbs in the facility, average light intensity 65 lx). From days 15 to 38 recommended (7) lighting regime with 16L: 4D: 2L: 2D was applied and from days 39 to 42, scheduled with 23L: 1D (light intensity 20 lx, ordinary light bulbs, measured by lux meter Testo 540, Testo, Germany). Feed and water were available ad libitum. For chickens' diets, three standard corn/soybean meal-based complete mash feeds were used (8): STARTER (from day 1 to 14) with 12.7 MJ/kg metabolic energy (ME) and 23.3% crude protein (CP); GROWER (from day 15 to 28) with 13 MJ/kg ME and 21% CP; and FINISHER (from day 29 to 42) with 13.4 MJ/kg ME and 18.7% CP. The health, behavior, and mortality of the chicken were monitored and recorded daily.

Experimental design: The experiment lasted 6 weeks and took place in two phases: from days 1 to 14 and from days 15 to 42. Chickens were divided into two groups (control - C and experimental - M), with four replications and 40 chickens in each replica. During the first phase, the experimental group (M) received a starter diet supplemented with 30 mg/kg synthetic melatonin, N-acetyl-5-methoxytryptamine (EINECS Number: 200-797-7; purity 99%; Elephant Co., Serbia), and during the second phase fed without melatonin. The control group (C) had a diet without melatonin supplementation throughout the whole period (6 weeks).

Blood parameters: At the end of the first phase**,** on day 14, blood samples, from five randomly chosen and stunned chickens from each group, were collected by heart puncture (25) and using heparinized vacutainers (Lithium heparin, Demophorius Ltd, Cyprus). Total erythrocyte count (RBC, x 10^{12}/L) was determined manually (28), using a hemocytometer (Neubauer-improved, Marienfeld, Germany) and with Hayem's solution addition (NRK Inženjering, Beograd). Hemoglobin concentration (Hb, g/L) on an ADVIA 120 Hematology System apparatus (Siemens Healthcare, Germany) on the principle of flow cytometry (36) was measured. Hematocrit (Hct, %) was determined by the microhematocrit method (28), by using Hawksley Micro Hematocrit Tube Reader (Hawksley, US). Blood samples previously were centrifuged at 13,000 rpm for 5 minutes (Becton Dickinson, USA). Hematological indices (MCV - Mean Corpuscular Volume; MCH - Mean Corpuscular Hemoglobin; MCHC - Mean Corpuscular Hemoglobin Concentration) were calculated by the following formulas (11):

MCV in fl = Hct (%) x 10/RBC (10^6/L)

MCH in pg = Hgb (g/L)/RBC (10^{12}/L)

MCHC in g/L= Hgb (g/L) x 100/Hct (L/L)

Total leukocyte number (WBC) was determined indirectly (28) from the relationship between the values obtained by counting the cells in the chamber and the stained blood smear (Wright solution, BioGnost, Croatia). Leukocyte formula (a percentage of heterophile, lymphocytes, monocytes, eosinophils, and basophils) was determined by the formula (28): Absolute number of leukocyte cell type = cell number in 1L x % leukocyte type cells/100.

The H/L ratio was calculated by dividing the number of heterophils by the number of lymphocytes (21). Catalase activity was measured by Perkin Elmer (USA) Lambda 35 spectrophotometer with a thermostated 1.00 cm quartz cell and using chemicals: EDTA (Sigma–Aldrich, Germany), hydrogen peroxide (33% H_2O_2), and potassium hydrogen phosphate (K_2HPO_4 x $3H_2O$), both from Merck (Germany). The samples were centrifuged at 2000 rpm for ten minutes. Plasma and buffy coat were removed by aspiration, and the erythrocytes were washed

three times with cold (4°C) saline and suspended in four volumes of deionized water. Erythrocyte debris was removed by centrifugation at 2000 rpm for 20 min. Erythrocyte lysates were diluted and stored at -70°C until assayed. Catalase activity was determined by measuring the decrease in absorbance (H_2O_2 degradation) at 240 nm for three minutes (10), and the values have been expressed as U/ml. One unit (U) of catalase activity is the amount of enzyme required to decompose one micromole of H_2O_2 per minute.

Production parameters: Individual body weight (BW, g) was measured when the chickens moved in and at the end of each week (7th, 14th, 21st, 28th, 35th, and 42nd day). By the values of individual body weights of birds and feed consumption per box between the two measurements, for different periods of fattening following parameters were calculated: daily gain (WG, g), daily feed intake (FI, g/day per chicken), and feed conversion ratio (FCR, kg of feed/kg of weight gain). For the entire duration of the experiment (42 days), the European Production Efficiency Factor (EPEF) was calculated, as a cumulative indicator of broiler fattening success based on average body weight, vitality, feed conversion, and fattening duration, according to the formula (7):

$$EPEF = \frac{\text{Live weight (kg) x Livability (\%) x 100}}{\text{Age in days x Feed Conversion Ratio (kg feed/kg weight gain)}}$$

Statistical analysis: For determination of the statistical significance of the results, appropriate parametric and nonparametric tests were used (Mann-Whitney test for blood and Tukey test for production parameters). The results were presented as text, graphs, and tables - as mean values and standard error (SE). Data processing was done using STATISTICA 8 (StatSoft, Inc., USA).

Results

Health status and mortality: Chickens of the control (C) and experimental group (M) have had a harmonious physique by the end of fattening, properly developed bone and muscle tissue, vivid temperament, and good condition. The feathers, skin, and visible mucous membranes were without peculiarities. Appetite was good, and the feces of the usual form. The ability to move actively and coordinate movements were harmonized, and muscle tone was expressed and preserved. Mortality was low and uniform between the groups (0.63% in both groups).

Blood parameters: The melatonin supplementation to chicken diet did not show a statistically significant effect on the difference in the average values of hematological parameters (P>0.05), although they were slightly higher in group M compared to C, as shown in the following order: RBC - 2.03: 1.94 x 10^{12}/L; Hb - 110.67: 103.5 g/L; Hct - 30.33: 28.75%; MCV - 150.67: 149.25 fL; MCH - 54.93: 53.75 pg; MCHC -364.97: 360.00 g/L; and WBC - 12.07: 10.25 x 10^9/L.

The difference between the groups in the leukocyte formula is more noticeable (Fig. 1) but also without statistical significance (P>0.05).

Values of the H/L ratio in groups M and C were 0.47 and 0.75, respectively (P>0.05).

Differences between mean values for catalase activity were not statistically significant (P>0.05). The more pronounced activity of catalase in the group (M) is noticeable (Fig. 2).

Production parameters: Melatonin addition had a statistically significant effect (P<0.01) on the mean values of BW and WG (tables 1 and 2) at the end of the 1st, 2nd, 3rd, 4th, 5th, and 6th week.

Regarding the mean values of FI and FCR, no significant differences were found (P>0.05), although birds of the M group had better feed conversion in all weekly measurement periods (Tab. 2).

EPEF values for the fattening period of 42 days were 287.98 ± 6.22 for group M and 259.58 ± 5.35 for group C, and these differences were statistically significant (P<0.05).

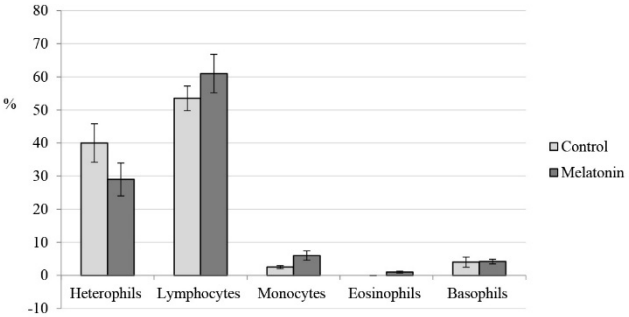

Fig. 1. Percentage distribution of individual leukocyte forms leukocyte formula (percentage±SE).

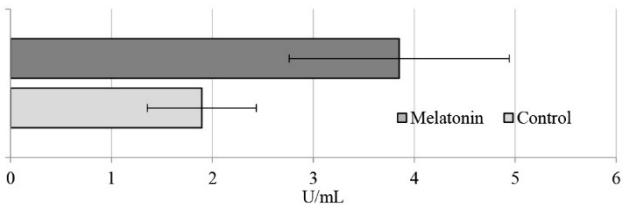

Fig. 2. Catalase activity (mean±SE).

Table 1. Mean body weight (BW) of chickens of different age.

Age of chicken (days)	BW (g)	
	Melatonin group	Control group
	Mean ± SE	Mean ± SE
1	$40.93^{a} \pm 0.27$	$41.33^{a} \pm 0.23$
7	$150.36^{a} \pm 1.30$	$140.70^{b} \pm 1.26$
14	$359.24^{a} \pm 4.48$	$336.75^{b} \pm 4.15$
21	$727.98^{a} \pm 11.09$	$649.68^{b} \pm 11.13$
28	$1128.68^{a} \pm 17.44$	$1032.32^{b} \pm 18.37$
35	$1671.09^{a} \pm 24.81$	$1537.92^{b} \pm 26.25$
42	$2278.67^{a} \pm 30.17$	$2114.05^{b} \pm 31.21$

a, b - Values within a row with different superscripts differ significantly at P<0.01.
SE – Standard Error.

Table 2. Mean values of daily weight gain (WG), feed intake (FI), and feed conversion ratio (FCR) by weeks.

Weeks	WG (g)		FI (g/day)		FCR (kg/kg)	
	Melatonin	Control	Melatonin	Control	Melatonin	Control
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
1	$15.64^{a} \pm 0.15$	$14.49^{b} \pm 0.15$	24.07 ± 0.64	22.65 ± 0.45	1.53 ± 0.05	1.56 ± 0.02
2	$29.84^{a} \pm 0.48$	$27.72^{b} \pm 0.42$	47.61 ± 0.71	45.47 ± 0.77	1.60 ± 0.04	1.64 ± 0.04
3	$52.98^{a} \pm 0.97$	$45.20^{b} \pm 1.01$	88.39 ± 0.70	80.72 ± 1.94	1.68 ± 0.03	1.81 ± 0.05
4	$57.24^{a} \pm 0.97$	$54.66^{b} \pm 1.07$	108.46 ± 2.25	106.80 ± 2.24	1.90 ± 0.02	1.95 ± 0.01
5	$77.49^{a} \pm 1.25$	$72.23^{b} \pm 1.33$	150.5 ± 5.71	147.16 ± 3.27	1.94 ± 0.01	2.04 ± 0.04
6	$86.80^{a} \pm 1.01$	$82.30^{b} \pm 1.00$	181.47 ± 2.57	168.74 ± 5.74	2.10 ± 0.08	2.06 ± 0.03

a, b - Values within a row with different superscripts differ significantly at P<0.01.
SE – Standard Error.

Discussion and Conclusion

Melatonin is a hormone of primary importance for the health and welfare of animals in all stages of life (2). Exposure to light is equally significant for young chicken organism's normal development and functioning as having a certain level of melatonin in the blood. According to welfare standards (27), broilers should not be exposed to light for a certain period during the day to ensure a diurnal melatonin cycle. In this experiment, after the broilers' prolonged exposure to the constant lighting, 14 days instead of the usual 7 (7), the adverse effects have not been recorded. Generally, the addition of melatonin has had a positive effect on blood parameters and production parameters. The absence of statistically significant differences in all blood parameters is probably a consequence of the small number of samples in this study.

The blood profile of poultry differs depending on age and many other factors (34, 35). Thus, the mean values of RBC (and other hematological parameters) in both groups can be considered as usual for young Ross hybrid chickens per Rusov (28) but are not consistent with data from other authors (4, 34, 35). Melatonin protects erythrocytes and the heme group from damage caused by oxidative stress and consequently from the shortening of life expectancy (20, 37), which might be the reason for slightly higher values of these parameters in the M group. An increase in the number of erythrocytes of the same volume (MCV) increases the hematocrit values. Higher hemoglobin values are associated with higher MCH and MCHC values, as seen from the calculation formulas.

A higher number of white blood cells in the M group may be related to the immunostimulatory effect of melatonin as in the experiment by Brennan et al. (12). Melatonin stimulates lymphocyte, monocyte, and eosinophil production (15, 16, 41). In an experiment on quails (22), similar results to ours have obtained: the addition of melatonin led to an increase in total leukocyte count (WBC), an increase in lymphocyte, and a decrease in heterophile percentages, and a decrease in heterophile/lymphocyte ratio; the shortening of the photoperiod had the same effect (which affected the physiological increase of melatonin in the organism). The ratio between heterophiles and lymphocytes (H/L ratio) indicates a stress response in poultry. In response to

stressors, the number of lymphocytes decreases, and the number of heterophiles increases (21, 30). Continuous lighting is stressful to broilers, but melatonin administration ameliorates the adverse effects of photo-stressful conditions in birds (19). A higher H/L value in the C group indicates a more pronounced stress response, so the values obtained in the M group show that birds who received melatonin tolerated the ambient conditions and the applied lighting regime better, compared to the chickens from the control group.

The fast growth rate in broilers is associated with rapid cell proliferation. Consequently, the higher level of reactive oxygen species (ROS) increasingly leads to oxidative stress, disturbing many metabolic and immunological pathways (23). One of the main antioxidative enzymes that are stimulated by melatonin under basal conditions is catalase (CAT). Melatonin supplement to the diet has had a stimulatory effect on CAT activity in group M, similar to that described by Reiter et al. (26).

The addition of melatonin to the diet of broiler chickens reared under 24L: 0D regime during the first 14 days of life showed a more pronounced positive effect on body weight and daily gain of broilers ($P<0.01$), which is consistent with the results of Akbarian et al. (3). Melatonin stimulates the synthesis of growth hormone (GH) in the pituitary gland and its secretion into the bloodstream by acting on the hypothalamus and thus has a positive effect on broiler growth performance (14) and energy metabolism (38). Considering the GH level in broiler plasma is the highest in the early phase of age (29) that probably manifested in our results. Decreased broiler activity also affects the gain of body weight (5), and it occurred from the third week of the experiment with a shortening of the photoperiod.

The positive effect of melatonin supplementation in the first two weeks on chicken growth continued throughout the end of the fattening period. Regarding feed consumption and conversion, there was no significant effect of melatonin supplementation. Still, it is evident that daily feed intake was higher, and the FCR was lower in the M group compared to the control group in all weeks of the experiment. FCR values are consistent with relevant data from other research (3). The treatment in group M led to more efficient fattening as compared to the control, which is confirmed by a significant difference in EPEF values ($P <0.05$).

Defining the optimal light and nutritional management is still a challenge in the poultry industry, where achieving high production goals is expected, along with preserving animal health and welfare. One way is the oral administration of melatonin and its precursor tryptophan, which has been recommended by many authors to alleviate the effects of stressors to which birds are exposed in conditions of intensive production (1, 3, 14, 23). Our results confirmed that melatonin feed supplementation to broilers enhanced their growth and productivity. Furthermore, melatonin administration alleviated the detrimental effects of continuous lighting on broilers. The presented results indicate a direct benefit of stress relief and a value of the organism's antioxidant system promotion, which was manifested by improved production performances and fattening efficiency in broilers.

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Ethical Statement

The trial was performed at the Institute for Animal Husbandry, Belgrade, Serbia, at the Experimental Broiler Farm. The experiment and experimental procedures were evaluated by the Ethics Committee of the Institute, and approved by the Veterinary Directorate in MAFW of the Republic of Serbia (Approval No: 323-07-00069/2017-05).

Conflict of Interest

The authors declare that there is no conflict of interest.

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Different coding systems for the modeling of lactation milk yields of Awassi sheep

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Abstract: This study evaluated the feasibility of using different coding systems for categorical variables when using continuous and categorical variables together for the modeling of the lactation milk yield of Awassi sheep. In the study, when all variables were included in the model, and Dummy Coding and Effect Coding methods were used for age, the effects of lactation duration, average daily milk yield, type of birth, and age 5 group were found to be statistically significant in addition to the constant term. When the Deviation Coding method was used for age, the effects of lactation duration and average daily milk yield were found to be statistically significant in addition to the constant term. On the other hand, when Forward and Backward Coding methods were used, the effect of the age 5 group was found to be statistically significant, along with the effects of lactation duration and average daily milk yield. The results of the study indicated that different results can be obtained depending on the various coding systems used. The results also indicated that the choice of coding system affected the interpretation of the obtained coefficients. Therefore, it can be stated that the aims of the researcher in the study should be defined clearly and the proper codding system should be selected according to the variables to be included in the model.
Keywords: Awassi sheep, milk yield, multiple regression, reference category, variable coding systems.

İvesi koyunlarında farklı kodlama sistemleri kullanılarak laktasyon süt veriminin modellemesi

Özet: Bu çalışmada, İvesi koyunlarda laktasyon süt verimi için sürekli ve kategorik değişkenlerin birlikte ele alınarak; kategorik değişkenler için farklı kodlama sistemlerinin uygulanabilirliği değerlendirilmiştir. Çalışmada ele alınan tüm değişkenlerin modele dâhil edilmesi durumunda, yaş için kukla ve etki kodlama yöntemleri kullanıldığında; sabit terim ile birlikte laktasyon süresi, günlük ortalama süt verimi, doğum tipi ve 5 yaş grubundan kaynaklanan fark istatistik olarak önemli bulunmuştur. Yaş için sapma kodlama yöntemi kullanıldığında; sabit terim ile birlikte, laktasyon süresi ve günlük ortalama süt verimine ait etkiler istatistik olarak önemli bulunmuştur. İleriye ve geriye dönük fark yöntemleri kullanıldığında ise laktasyon süresi ve günlük ortalama süt verimi ile birlikte 5 yaş grubunun negatif etkisi de istatistik olarak önemli bulunmuştur. Çalışmanın sonuçları, kullanılan kodlama sistemilerine göre farklı sonuçların elde edilebileceğini göstermiştir. Sonuçlar ayrıca, kodlama sistemi seçiminin, elde edilen katsayıların yorumlanmasını etkilediğini göstermiştir. Bu nedenle araştırmacının araştırmadaki amaçlarının açık bir şekilde belirlenmesi ve modele dahil edilecek değişkenlere göre uygun kodlama sisteminin seçilmesi gerektiği söylenebilir.
Anahtar sözcükler: Çoklu regresyon, değişken kodlama sistemleri, İvesi koyunu, referans kategori, süt verimi.

Introduction

The decision of which statistical methods are to be used for the analysis of data obtained from research in terms of variables or characteristics of interest is related directly to the variable type; in other words, the data structure and the means of acquisition. So, when deciding on the best statistical method for an analysis, a researcher should think about the types of variables and other environmental factors (11, 12, 13).

The yield and quality of economically important animal products such as meat, milk, eggs, fleece, and honey are affected by many factors, some of which are continuous, such as age and weight, while others are categorical, such as sex and birth type. When breeding to improve yield and quality, it is very important to use the right method of analysis for these economically important products (2, 9).

The direct inclusion of such categorical variables as gender, type of birth, and lactation order in standard multiple regression analysis models violates the assumptions of a regression analysis (14), and in these situations, different regression approaches may be used. The difficulties associated with the implementation of these approaches and the interpretation of their results lead few researchers to make use of them, and more often than not, these variables are included in the model after coding (3). When examining their relationship with the response variable, categorical variables can be included in the same model as continuous variables, and this makes it possible to identify the effects of the categorical and continuous variables included in the model on the response variable, as well as any potential interactions among the explanatory variables (3, 13, 14).

In the Dummy coding method, as one of the most frequently used coding approaches, the values of 1 and 0 are used to indicate whether individual observations belong to a particular group. The variables used in dummy coding are known as artificial variables, and do not exist in the original data, being created later for the transformation of categorical data into numerical data. Dummy coding is the preferred coding method when the goal is to compare multiple treatment groups with a single control group. In this case, the control group is called the reference group, and the differences between the regression coefficient of this group and those of other groups are examined. The statistical significance of the regression coefficients for these variables is tested using the t statistic (15, 18). In effect coding, dummy variables are assigned values of 1 or -1, which is a method that is similar to dummy coding, although there are differences in how the reference group is defined. The reference group is defined as "0" in dummy coding, and as "-1" in effect coding. The R^2 and F values in regression models are the same for the two coding methods, but the regression constant and regression coefficients are interpreted differently. In Alkharusi's (3) examination of the dummy coding and effect coding methods, it was reported that similar R^2 and F values were obtained through the two methods, although the interpretation was different, depending on the coding method used. When including categorical variables in a multiple regression model, the choice of the statistical software to be used is also important.

According to the literature review, it was observed that there were almost no studies about examining and interpreting different coding systems together in animal science. Therefore, in this study, various coding systems were examined together and their usability in modeling milk yield was evaluated, as well as the results obtained according to different coding systems were interpreted.

Materials and Methods

Material: Data on 287 Awassi sheep kept in the Şanlıurfa GAP Agricultural Research Institute (GAPTAEM) of the General Directorate of Agricultural Research and Policies (TAGEM) of the Ministry of Agriculture and Forestry were collected between 2013 and 2015.

Included in the study were ewes aged 3–5 years, whose lactation duration ranged from 30 days to 191 days, and lactation milk yield (LMY) varied between 27 and 248 kg. Ewe weight varied between 40 kilograms and 73 kilograms; 29% of the ewes gave birth to twins and 57% of the lambs were male. The lactation milk yields and lactation curves of ewes that lambed in different periods were examined. Lambing started in a different month in each year of the study, in November, December and January. The flock was cared for and feeding was performed with routine procedures in the Institute. Milk controls were performed on a 24-hour basis and repeated every 20 days, and the milk controls ended when two-thirds of the flock had finished lactating, upon which milking was ended for the entire flock.

Methods: The explanatory variables included in multiple regression models are usually continuous; although in many cases it is important to include also categorical variables in the model to improve its goodness of fit, to eliminate prediction errors, and to identify any potential interactions or joint effects (3, 10). Categorical variables are coded qualitatively, meaning that the assigned codes have no numerical values, and these variables can be included in standard regression analysis models as independent or explanatory variables. Regarding the coding system, if the categorical variable has "g" levels, it is possible to code "g-1" binary variables. For a gender variable with two categories (male, female), g = 2, and coding for either male or female would suffice (18, 19). The coding systems applied in the study are Dummy Coding, Effect Coding, Deviation Coding, Forward Difference Coding, Backward Difference Coding, Helmert Coding and Reverse Helmert Coding (1, 3, 6, 14). The coding schema for age categories was presented in Table 1.

The model used to examine the environmental factors that affect the observed lactation milk yield (OLMY), the estimated lactation milk yield (ELMY) and the model parameters was as follows: Y_{ijk}

$$Y_{ijk}= \mu+YA_i+ DT_j+b\ (X_{ijk})+ e_{ijk}$$

Yijk: ELMY, OLMY, model parameters, μ: Overall mean in terms of the analyzed trait, YA_i: i^{th} lambing year-Month, DT_j: J^{th} birth type, b: the partial regression coefficient of X_{ijk}, X_{ijk}: lactation length of the kth ewe, e_{ijk}: Residual associated with Y_{ijk}.

Table 1. Coding schema for age categories.

Age group	Dummy			Effect		Deviation			Forward		Backward		Helmert		R. Helmert	
	D1	D2	D3	E1	E2	S1	S2	S3	F1	F2	B1	B2	H1	H2	R1	R2
3	0	0	0	-1	-1	-1/3	-1/3	-1/3	1	0	-1	0	1	0	-1	-1/2
4	1	0	0	1	0	-1/3	2/3	-1/3	-1	1	1	-1	-1/2	1	1	-1/2
5	0	1	0	0	1	-1/3	-1/3	2/3	0	-1	0	1	-1/2	-1	0	1

R. Helmert: Reverse Helmert.

Table 2. Results of the regression model for lactation milk yield, including all variables, with Dummy coding and Effect coding for Age (kg).

Item	Dummy		Effect	
	b ± SE	t (P)	b ± SE	t (P)
Constant	-105.707 ± 5.969**	-17.708 (0.001)	-106.630 ± 6.062	-17.590 (0.001)
Birth Weight	-0.236 ± 0.751	-0.314 (0.754)	-0.204 ± 0.747	-0.273 (.785)
Ewe Weight	0.046 ± 0.081	0.571 (0.569)	0.036 ± 0.081	0.438 (.662)
Lac. Dur.	0.817 ± 0.019**	43.937 (0.001)	0.823 ± 0.019**	-43.897 (0.001)
ADMY	0.130 ± 0.002**	71.201 (0.001)	0.130 ± 0.002**	71.433 (0.001)
Sex	-0.622 ± 1.027	-0.606 (0.545)	-0.354 ± 0.511	-0.693 (0.489)
Type of Birth	-3.446 ± 1.310**	-2.630 (0.009)	-1.630 ± 0.653*	-2.494 (0.013)
Age 3	0.153 ± 1.370	0.112 (0.911)	-0.824 ± 0.903	-0.913 (0.362)
Age 4	0.763 ± 1.488	0.513 (0.608)	-0.526 ± 0.907	-0.580 (0.562)
Age 5	3.899 ± 1.795*	2.172 (0.031)	2.554 ± 1.156*	2.211 (0.028)
	R^2 = 0.977 F = 1186.665 P = 0.001		R^2 = 0.977 F = 1199.843 P = 0.001	

SE: Standard error, F: F statistic, t (P): t statistic (P value), Lac. Dur.: Lactation Duration, b: Coefficient, ADMY: Average Daily Milk Yield, R^2: Determination coefficient, *: P <0.05 , **: P<0.01.

The following model was used to analyze the factors (environmental and flock management) affecting lactation length (LD):

$$Y_{ijk} = \mu + YA_i + DT_j + e_{ijk}$$

Y_{ijkl}: ELMY, OLMY, model parameters, μ: Overall mean in terms of analyzed trait, YA_i: i^{th} lambing year-Month, DT_j: J^{th} birth type, e_{ijk}: Residual associated with Y_{ijk}.

Regression analyses were carried out for the study. The level of statistical significance was set as 5% and IBM SPSS Statistics software (Version 21.0. Armonk, NY: IBM Corp.) was used for all statistical computations.

Results

The results of the regression analysis are presented in Table 2, in which lactation duration, average daily milk yield, birth type, and age 5 can be seen to have had statistically significant effects (P<0.05), while the other variables did not. Of the variables with significant effects, all but birth type had positive coefficients. A one-day increase in lactation duration was thus predicted to increase the mean LMY by 0.817 kg, and a 1 kg increase in average daily milk yield was predicted to increase the mean LMY by 0.130 kg.

For the Dummy Coding of the birth type, the singleton category was used as the reference category. Thus, a value of 3.446 for type of birth denoted the difference between the groups of ewes that gave birth to single lambs and those that gave birth to twins. This coefficient had a negative sign, indicating that the ewes that gave birth to twins had a mean LMY of 3.446 kg lower than those that gave birth to single lambs. Similarly, for the variable of age, the "Age 3" group was used as the reference category. The regression coefficients for the differences between the mean LMY of the reference group and the mean LMY of the groups of ewes aged 3, 4, and 5 years were positive, indicating those with higher ages were associated with higher LMY. The differences between the mean LMY of the groups of ewes aged 3 and 4 and the reference category, that is to say, the group consisting of ewes aged 3, were not statistically significant (0.153 and 0.76 kg, respectively). In contrast, the difference between the mean LMY of the group Age 5 and that of the reference category (3.899 kg) was found to be statistically significant (P<0.05). Accordingly, the Age 5 group was predicted to have a mean LMY 3.899 kg higher than the mean LMY of the Age 2 group. When all variables were included in the model, the coefficient of determination (R2) was found to be 97.7%, which is higher. So, it was decided that the variables in the model could explain 97.7% of the change or variation in LMY. The other 2.3% could be explained by random environmental factors that were not part of the model.

To understand whether they affect LMY, the categorical variables were coded using the effect coding method and included in the model along with birth weight, lactation duration, and average daily milk yield. The results of the regression analysis for this model are presented in Table 2. As was the case with dummy coding, Table 2 shows that of the variables included in the model, only lactation duration, average daily milk yield, type of birth, and age 5 were found to have statistically significant differences (P<0.05). All of these variables had positive coefficients except birth type; thus, a one-day increase in lactation duration was predicted to increase the mean LMY by 0.823 kg, while a 1 kg increase in average daily milk yield was predicted to increase the mean LMY by 0.130 kg.For the effect coding of birth type, ewes that gave birth to single lambs were coded "-1" and those that gave birth to twins were coded "1". Thus, the mean LMY of ewes that gave birth to single lambs was predicted to be 106.630-1.630 = 105.00 kg, whereas the mean LMY of ewes that gave birth to twins was predicted to be 106.630 + 1.630 = 108.26 kg. For the effect coding of the variable age, the "Age 3" group was used as the reference category. Of the groups of ewes aged 3, 4, and 5, only the age 5 group had a positive regression coefficient, and this coefficient was statistically significant. This shows that the mean for the Age 5 group was higher than the overall mean in other words, the Age 5 group was predicted to have a mean LMY 2.554 kg higher than the overall mean. Similar to dummy coding, the coefficient of determination (R2) was found to be 97.7% when effect coding was used and all variables were included in the model.

Deviation Coding: In order to understand whether the age affects LMY or not, this variable was coded using the deviation coding method and included in the model together with birth weight, lactation duration and average daily milk yield. The results of the regression analysis are presented in Table 3. As Table 3 shows, only lactation duration and average daily milk yield had statistically significant effects (P<0.001), along with the constant term, whereas the effects of other variables were not (statistically) significant. All variables with significant effects had positive coefficients; thus, a one-day increase in lactation duration was predicted to increase the mean LMY by 0.816 kg, and a 1 kg increase in average daily milk yield was predicted to increase the mean LMY by 0.131 kg. Similar to the case in which all variables were included in the model together, R^2 was found to be 97.6% (P<0.001).

To understand whether it affects LMY, the age variable was coded using a forward (and backward) difference coding approach and included in the model along with birth weight, lactation duration, and average daily milk yield. The results of the regression analysis are presented in Table 3, in which it can be seen that lactation duration, average daily milk yield, and age 5 group had statistically significant effects (P<0.05), along with the constant term, as the effects of other variables were not significant. Of the variables with significant effects, all except Age 5 had positive coefficients. Thus, a one-day increase in lactation duration was predicted to increase the mean LMY by 0.809 kg, and a 1 kg increase in average daily milk yield was predicted to increase the mean LMY by 0.131 kg. Regarding the age variable, the coefficient for the category of age 3 was found to be significant, and this coefficient represented the difference between the means of the age 5 and age 4 groups. When the effects of other variables included in the model were taken into consideration, the difference between the mean LMY of the Age 5 and Age 4 groups was 2.537 kg, and this difference was statistically significant.

Backward difference coding: In backward difference coding for age, the same values obtained in forward difference coding were obtained but with opposite signs. Aside from that, the coefficients were identical.

Table 3. Results of the multiple regression model for lactation milk yield with Deviation and Forward (and backward) difference coding for Age (kg).

	Deviation		Forward (and backward) difference	
Traits	**b ± SE**	**t (P)**	**b ± SE**	**t (P)**
Constant	-109.003 ± 5.826**	-18.711 (0.001)	-108.505 ± 6.085**	-17.833 (0.001)
Birth Weight	0.542 ± 0.690	0.785 (0.433)	0.534 ± 0.695	0.788 (0.433)
Ewe Weight	0.030 ± 0.081	0.374 (0.709)	0.044 ± 0.081	0.541 (0.589)
Lac. Dur.	0.816 ± 0.019**	43.704 (0.001)	0.809 ± 0.002**	43.703 (0.001)
ADMY	0.131 ± 0.002**	72.161 (0.001)	0.131 ± 0.002**	71.867 (0.001)
Age 3	-0.689 ± 1.308	-0.527 (0.599)	-0.514 ± 0.896	-0.574 (0.567)
Age 4	-0.031 ± 1.461	-0.021 (0.983)	-2.031 ± 1.219	-1.666 (0.097)
Age 5	2.972 ± 1.760	1.689 (0.093)	-2.537 ± 1.165*	-2.178 (0.030)
	R^2 = 0.976 F = 1523.075 P = 0.001			

SE: Standard error, F: F statistic, t (P): t statistic (P value), Lac. Dur.: Lactation Duration, b: Coefficient, ADMY: Average Daily Milk Yield, R^2: Determination coefficient, *: P<0.05 , **: P<0.01.

Table 4. Results of the multiple regression model for lactation milk yield with Helmert and Reverse Helmert coding for Age (kg).

Item	Helmert		Reverse Helmert	
	b ± SE	t (P)	b ± SE	t (P)
Constant	-108.504 ± 6.085**	-17.830 (0.001)	-110.930 ± 5.862**	-18.925 (0.01)
Birth Weight	0.534 ± 0.695	0.768 (0.433)	0.610 ± 0.689	0.885 (0.377)
Ewe Weight	0.044 ± 0.081	0.541 (0.709)	0.054 ± 0.080	0.676 (0.500)
Lac. Dur.	0.809 ± 0.019**	43.743 (0.001)	0.817 ± 0.018**	45.317 (0.001)
ADMY	0.131 ± 0.002**	71.867 (0.001)	0.131 ± 0.002**	73.380 (0.001)
Age 3	-0.515 ± 0.898	-0.574 (0.567)	-0.113 ± 0.726	-0.156 (0.876)
Age 4	-1.688 ± 0.920	-1.834 (0.068)	0.238 ± 0.880	0.270 (0.787)
Age 5	-1.522 ± 0.853	-1.783 (0.076)	2.385 ± 0.870**	2.742 (0.007)
	R^2 = 0.976 F = 1517.303 P = 0.001		R^2 = 0.976 F = 1517.303 P = 0.001	

SE: Standard error, F: F statistic, t (P): t statistic (P value), Lac. Dur.: Lactation Duration, b: Coefficient, ADMY: Average Daily Milk Yield, R^2: Determination coefficient, *: P<0.05 , **: P<0.01.

To understand its effects on LMY, the age variable was coded using the Helmert coding method and included in the model along with birth weight, lactation duration, and average daily milk yield. The results of the regression analysis are presented in Table 4, in which it can be seen that of the variables included in the model, only lactation duration and average daily milk yield had statistically significant coefficients (P<0.001), along with the constant term. Thus, a one-day increase in lactation duration was predicted to increase the mean LMY by 0.809 kg, and a 1 kg increase in average daily milk yield was predicted to increase the mean LMY by 0.131 kg. The model's coefficient of determination (R^2) was found to be 97.6%.

To understand whether it affects LMY, the age variable was coded using the Reverse Helmert coding method and included in the model along with birth weight, lactation duration, and average daily milk yield. The results of the regression analysis are presented in Table 4, in which it can be seen that lactation duration, average daily milk yield, and age 5 had statistically significant effects (P<0.05), along with the constant term, while the effects of other variables were not (statistically) significant. Thus, a one-day increase in lactation duration was predicted to increase the mean LMY by 0.817 kg, and a 1 kg increase in average daily milk yield was predicted to increase the mean LMY by 0.131 kg. Regarding the age variable, the coefficient for the category of age 3 was found to be significant. In reverse Helmert coding, this figure represents the difference between the mean LMYs of the Age 5 group and the other three groups (ages 4, 3, and 2). When the effects of the other variables included in the model were taken into consideration, the mean LMYs of the Age 5 group and the other three groups was 2.385 kg. The model's coefficient of correlation (R^2) was found to be 97.6%.

Discussion and Conclusion

In the present study, different coding methods involving categorical variables that affect LMY were used. When the dummy coding and effect coding methods were used, the effects of lactation duration, average daily milk yield, type of birth and age 5 were found to be statistically significant, in addition to the constant term. After these variables were included in the model, a coefficient of determination (R^2) of 97.7% was obtained. The birth type coefficient was found to be negative in both models. When the deviation coding method was used for age, the effects of lactation duration and average daily milk yield were found to be statistically significant, in addition to the constant term. When forward and backward coding methods were used, on the other hand, the effect of age 5 was also found to be statistically significant, along with the effects of lactation duration and average daily milk yield. Both coding methods had R^2 values of 97.6%. When the Helmert and Reverse Helmert coding methods were used, the Helmert coding method was found to result in statistically significant coefficients for the variables of lactation duration and average daily milk yield, along with the constant term. Similar to the forward and backward difference coding methods, the Reverse Helmert coding method resulted in a statistically significant coefficient for the age 5 group, but with a positive sign. As directly related to our study, there are no studies in which different coding systems are used to predict lactation milk yield in sheep in the literature. However, although not directly related, in a study examining the effect of age on lactation the milk yields of Karakaş sheep kept by villagers, Gökdal et al. (8) reported 5-year old ewes to have a lactation milk yield around 20 kg higher than that of 2-year old ewes, and the difference was statistically significant. The differences between the other groups, however, were not statistically significant. Altın (5) examined the effects of sheep breeds,

type of lambing and age on the real lactation milk yields of Akkaraman sheep and Hamdani x Akkaraman hybrids (F1), and found that none of the three factors developed statistically significant differences. For the lactation milk yields, about 10-liter difference between the ewes that gave birth single (52 liters) and twins (62 liters) was not found to be statistically significant. The real lactation milk yields of the ewe groups aged 1, 2, 3, 4, and 5 (or more) years were, respectively, 58, 55, 56, 70, and 47 liters, on average, and the differences between these values were not significant. In Yılmaz et al.'s (19) examination of the effects of age, type of birth and weight on the lactation milk yields of Norduz sheep, the lactation milk yields of ewe groups aged 2, 3, 4, and 5 years were, respectively, 107, 122, 130, and 142 kg. The authors reported that the differences were statistically significant, and that each group was different from the others. However, the 8-kilogram difference between the average lactation milk yield (121 kg) of the ewes that had single births and the average lactation milk yield (129 kg) of the ewes with twins was not found to be statistically significant. In a study examining the effects of type of birth, lambing season and mother's age on lactation milk yields involving 77 ewes, Allah et al. (4) found the 5-year old ewes to have the highest mean yield at 73 kg, and those older than 5 years to have a mean yield of 68 kg, representing a statistically significant difference. The ewe group aged 2, 3 or 4 had a mean yield of are 70 kg, and the differences between this group (2, 3 or 4 years) and the other two groups (5 years or older) were not found to be significant. Allah et al. (4) also reported that ewes who gave birth to single lambs had a lactation milk yield that was some 12 kg higher than those who gave birth to twins, but this difference was not statistically significant. In Erol et al.'s (7) study of the effect of lactation order and year on lactation milk yield in Ankara goats, the reported difference of approximately 20 kilograms between the average lactation milk yield (approximately 73 kg) of the animals in the first lactation and the average lactation milk yield (approximately 92 kg) of the animals in the second lactation to be statistically significant. It was emphasized, however, that the difference of approximately 10 kg between the average lactation milk yield of the animals in the third lactation and the milk yield of the animals in the second lactation (mean lactation) was not statistically significant. The results from models that included categorical variables coded using different coding systems were similar to the findings reported previously in literature. Of the previous studies in literature that included the age variable in their models without coding, some found the effect of age on lactation milk yield to be significant (4, 7, 19), whereas others reported no significance (5, 8). This was the case with respect also to birth type.

Programs such as SAS, SPSS, and R use dummy coding, whereas JMP uses effect coding. The last category in alphabetical order is the reference category in SAS and SPSS, but the last category gets a value of "-1" in STATA and JMP (3).

The effect coding method is very similar to dummy coding, with the last group being coded as "-1". As is the case with dummy coding, this coding method is not appropriate when the goal is to make contrasts, but in such situations, effect coding is easier to understand and interpret than dummy coding, although dummy coding is the simplest of the coding systems. In dummy coding, the newly created binary variables take on values of 0 or 1, while in effect coding, different values may be assigned to categorical variables. Dummy coding only uses the numbers 1 and 0, but effect coding also uses the numbers 16 and 17.

In Helmert coding, the mean of a given category is compared with the overall mean of the following categories. As these codes are orthogonal, the regression coefficients represent the difference between the weighted means. If a matrix approach is to be used in Helmert coding, the Helmert contrasts are entered into the columns. For k common variables, a matrix of "k+1" columns and "n" rows is needed. The entry in the first row of the first column is k, and all other entries in this column are "-1". In the second column, the first entry is 0, the second entry is k+1, and all other entries are "-1". In the third column, the first two entries are 0, the third entry is-2, and all other entries are "-1". This operation continues until the kth column.

The Reverse Helmert Coding method is also known as the Difference Contrasts method, as the order of entries is the reverse of Helmert coding. In deviation coding, the mean of a given group is compared with the overall mean of the other groups. For example, when there are four groups, the mean of the first group is compared with the mean of the remaining three groups; the mean of the second group is compared with the mean of the remaining three groups; and the mean of the third group is compared with the mean of the remaining three groups.

The present study exhibited and explained various codding systems in regression models. In addition, the study also examined the usability of various codding systems for categorical variables with continuous variables in the modeling of the lactation milk yields of Awassi sheep. The results of the study indicated that different results can be obtained depending on the various coding systems used. The results also indicated that the choice of coding system affected the interpretation of the obtained coefficients. Therefore, it can be stated that the aims of the researcher in the study should be defined clearly and the proper codding system should be selected according to the variables to be included in the model.

This study is expected to make a significant contribution to the literature based on its detailed examination of the different coding systems used in regression models.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declare no conflict of interest.

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Intrahepatic branching of the portal vein in the Eurasian otter (*Lutra lutra*) and American mink (*Neovison vison*)

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Abstract: The study aimed to evaluate the comparative anatomy of the liver and intrahepatic branching of the portal vein of the Eurasian otter (*Lutra lutra*) and the American mink (*Neovison vison*). Due to their highly valuable fur, minks have expanded their range to many parts of Europe and become available for many biomedical studies. In this study, ten adult minks and five otters were used. The intrahepatic branching of the portal vein was studied by the combined injection and dissection technique. The macroscopic anatomy of the liver revealed that both species have six-lobed livers, although differences in shape, size and some additional fissures were documented. The portal vein, upon entering the liver, divides into the right and left branches. The branching pattern in otters had an additional branch at this level with a caudate process branch. The right branch of the portal vein ramified in the right lateral lobe and the caudate process in the mink livers, while the right branch in the otter livers only distributed functional blood to the right lateral lobe. The larger left portal branch, with its transverse and umbilical parts, ramified in the left liver portion, along with the quadrate, right medial lobe and papillary process.

Keywords: *Lutra lutra*, *Neovison vison*, portal vein, vascular casts.

Introduction

The Eurasian otter and American mink both belong to the large carnivore family of Mustelidae, which includes similar animals, such as the weasel, ferret, marten, badger and wolverine. They vary in size with similar long slender bodies, short legs and a long tail (13). The fully aquatic otters are mostly found in the wild and have developed adaptations to marine life, while the semi-aquatic mink has expanded its range due to the human interest in the fur industry, which has become very intense over the course of the last twenty years. Minks have become available for many studies as dissection models, in experimental surgery, as well as a potential biomedicine model (11). Some research has used mink as a model in toxicology (2), sinus inflammation (12), investigation of Aleutian disease (9), etc. The significance of the mink liver as a potential model for surgical resection, physiological, clinical and pathological investigation is great, due to its lobation similar to domestic canid species, and larger body in comparison with traditional laboratory animals. Smith and Schenk (21) gave a basic overview of the morphology, topography and lobation of the liver in the mink, while a detailed study of the intrahepatic ramification of hepatic veins and biliary ducts has been well documented (6). Basic insights into the otter liver revealed that the otter has six or seven hepatic lobes (1, 13). The intrahepatic branching of the portal vein was described in dogs (3, 10, 24) and cats (8), but also in other domestic animals such as sheep (7), rabbits (8), horse (23), buffalo (18), camels (22), as well as in humans (5). The aim of this study was to investigate the comparative macroscopic anatomy of mink and otter livers, and the intrahepatic branching of the portal vein. Potential use in the field of surgery requires a good understanding of the anatomy and vasculature of an organ. The available literature found about this specific field is quite unclear and poor.

Materials and Methods

Ten adult minks and five otters were studied. Minks of both sexes were obtained from a fur farm, where the animals were euthanized and the carcasses remained

undamaged. The animals were treated in accordance with the relevant legislation, Official Journal B&H No. 34/02. Of November 22 (16). Otters were obtained from the Department of Pathology, Veterinary Faculty, University of Sarajevo, where they were brought for the autopsy. The dissection was performed and connective tissue was removed for the macro morphological study of the livers. The blood vessels were identified on the visceral surface, and the portal vein was ligated and rinsed with 0.9% saline solution. The casts were obtained using a combined injection and dissection technique (17). The acrylic monomer (Interacryl cold - Interdent, Slovenia) powder and solvent forms were mixed in 1:2 proportions. The mass was colored with a polyurethane green pigment (Biodur, AC54) for the portal vein and injected manually. Upon injection, the livers were left at room temperature for 24 hours to complete the polymerization of the resin and then placed in a 5% formaldehyde solution for 10 days. The dissection was performed at the visceral surface of the liver until intrahepatic branches of the portal vein became visible. The terminology used in the study is in accordance with current anatomical terminology (14).

Results

The liver is a light brown gland with an average weight for minks of 51.75 ± 2.98 g and in otters of 151.6 ± 6.65 g. The diaphragmatic surfaces of both livers are smooth and convex, while the visceral surfaces are marked by the hepatic porta (porta hepatis) and the gall bladder (vesica fellea). The latter is located between the quadrate and right medial lobe of the liver, slightly dorsally in the otter. At the hepatic porta, the bile ducts leave and the portal vein and hepatic artery enter the liver. Several fissures extend from the ventral border and divide both livers into six lobes: left lateral lobe (LLL), left medial lobe (LML), quadrate lobe (QL), right medial lobe (RML), right lateral lobe (RLL) and caudate lobe (CL), subdivided into caudate (CP) and papillary process (PP) (Figure 1). Differences in the shape and size of the lobes were noticed. The right lateral lobe in the otter is subdivided by an additional fissure into two parts which resemble two separate lobes. Also, both processes of the caudate lobe are significantly larger than those in the mink liver. In most cases, the papillary process in the otter is twice as long and located over the hepatic porta. The caudate process forms a deep impression on the right kidney.

The portal vein enters the liver at the porta and divides into right (ramus dexter) and left (ramus sinister) branches in the liver of the mink (Figure 2). The branching pattern of the portal vein in otters is different because the caudate process branch ramifies from the portal vein at the same level as where the two major branches originate. The strong caudate process branch in otters, with 3-5 secondary branches, is directed dorsolaterally and distributes functional blood for that hepatic lobe. As for mink, the same branch originates from the right portal branch and has two smaller branches, due to the smaller size of the caudate process. Two or three branches from the ramus dexter are directed ventrolaterally toward the right lateral lobe - right lateral lobe branches (Figure 2). The vascular network of the right lateral lobe in otters is larger, because this lobe is separated into two parts by a deep fissure.

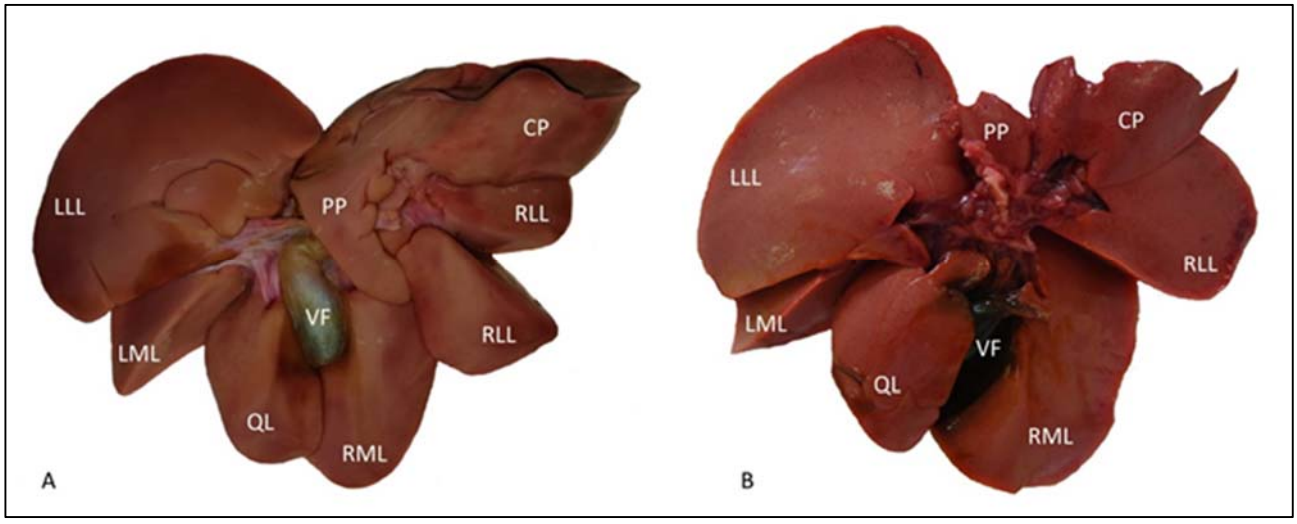

Figure 1. Visceral aspect of the liver. A - otter, B - mink.
LLL - left lateral lobe; LML - left medial lobe; QL - quadrate lobe; RML - right medial lobe; RLL - right lateral lobe; CP - caudate process; PP - papillary process; VF - gall bladder.

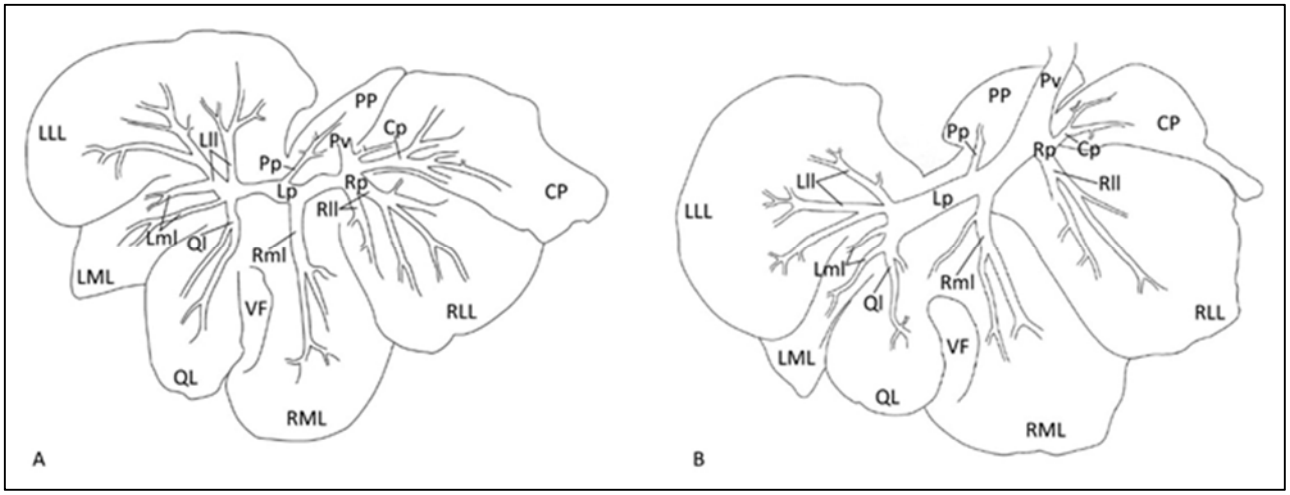

Figure 2. The portal vein ramification. A - otter, B - mink.

LLL - left lateral lobe; LML - left medial lobe; QL - quadrate lobe; RML - right medial lobe; RLL - right lateral lobe; CP - caudate process; PP - papillary process; VF - gall bladder; Pv - portal vein; Rp - right portal branch; Lp - left portal branch; Cp - caudate process branch; Pp - papillary process branch; Rll - right lateral lobe branches; Rml - right medial lobe branches; Ql - quadrate lobe branches; Lml - left medial lobe branches; Lll - left lateral lobe branches.

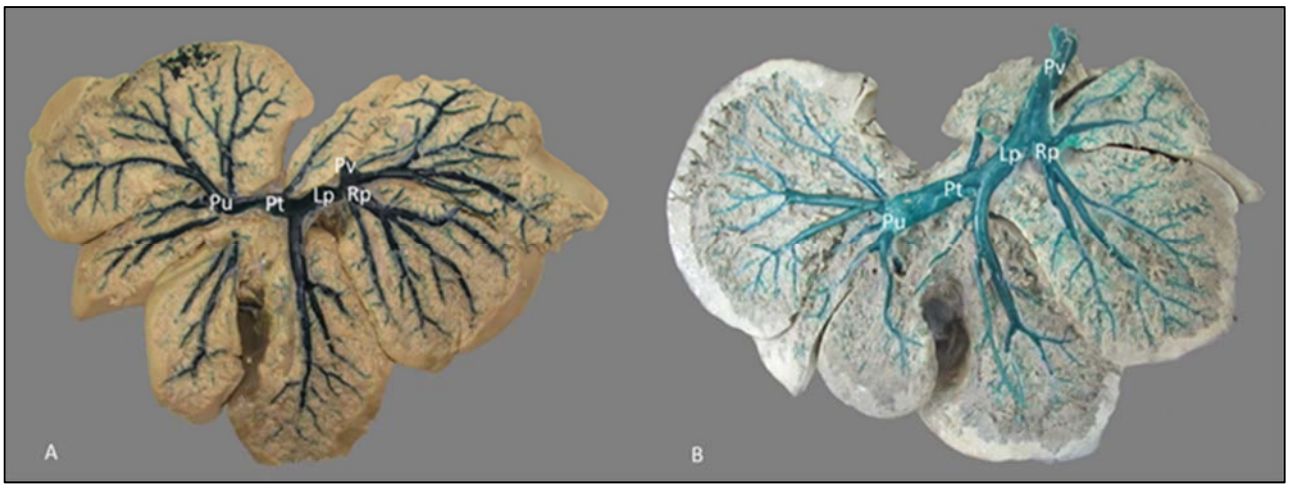

Figure 3. Corrosion cast of the portal vein. A - otter, B - mink.

Pv - portal vein; Rp - right portal branch; Lp - left portal branch; Pt - transverse part; Pu - umbilical part.

The left portal branch, in terms of size and direction, represents the continuation of the portal vein. The initial transverse part (pars transversa) is directed transversally and slightly ventrally, whilst the terminal umbilical part (pars umbilicalis) runs between the quadrate and left medial lobe in the form of an arc (Figure 3). This portal branch ramifies in the left liver portion, along with the quadrate, right medial lobe and papillary process. The first branch of the ramus sinister is directed ventrally and ramifies in the right medial lobe in both livers. The mink liver has an additional small branch directed towards the gall bladder from the right medial lobe branch. The single papillary process branch is directed dorsally from the transverse part. The final ramification of the left portal branch is at the level of the quadrate lobe, where the branches are directed towards the left lateral, left medial and quadrate lobes. The three branches are directed dorsolaterally and distributed in the left lateral lobe, two branches for the left medial lobe and two branches for the quadrate lobe. This branching pattern is similar in both the mink and the otter livers. The parietal surface of the liver exhibit terminal branches of the portal vessels. The position of the portal vein branches were visceral comparing to the branches of the hepatic veins which lies deeper and parietal.

Discussion and Conclusion

The liver, as the largest gland, has both exocrine and endocrine functions. The exocrine product, bile, is stored in the gall bladder, which is located between the quadrate

and right medial lobes of the liver. The six lobes of the carnivore liver are divided with several fissures from the ventral border (3, 4). Basic insights into mink livers reveal typical carnivore lobation (six lobes), shape and structure (21). The available literature on otter livers revealed a different lobation pattern. According to Baitchman and Kollias (1), the otter liver is grossly divided into six lobes, while a recent study (13) described otter liver as seven-lobed. We found that the otter liver has six lobes because the additional fissure of the right lateral lobe did not separate the lobe completely. Also, in terms of vasculature, the branching pattern showed two main branches that distributed portal blood to the complete right lateral lobe. The portal vein with its associated blood vessels forms a hepatic portal system. It collects blood from abdominal organs, except the terminal rectum, and terminates with a capillary bed in the liver (19). A detailed description of the portal vein distribution in dogs was given by Kalt and Stump (10). The portal vein divides at the porta into smaller right and larger left branches. The right branch ramifies in the caudate process of the caudate lobe and the right lateral lobe of the liver. The larger left branch gives off 5-7 branches for the right medial, quadrate, left medial, left lateral and papillary process of the caudate lobe. Similar ramifications in dog were described by Uršič et al. (24), and Sleight and Thomford (20). Our findings were in accordance with previous investigations in dogs regarding the individual branches entering the hepatic lobes. However, Uršič et al. (24) reported cases where the left portal branch supplied the medial part of the right lateral lobe of the liver, which was contrary to our findings and a previous study in dogs by Kalt and Stump (10). Also, the papillary process vein consists of 2-3 larger branches (24), while the present study describes a single papillary process branch, in both mink and otter. Heath and House (8) reported three main branches of the portal vein in cats. The caudate branch supplies the caudate lobe, the right branch supplies the right part of the liver from the gall bladder, and the left branch supplies the left lateral, left medial and quadrate lobes. In rabbits, the first branch of the portal vein emerges dorsally and supplies the caudate lobe. Then the portal vein continues in a cranioventral direction and divides into right and left branches (8). The portal vein ramification in the liver of horses is different from other mammals, as it divides into six branches: the caudate, dorsal diaphragmatic, right caudodorsal, right intermediate, right common and left branches (23). The portal vein of buffalo divides at the porta into short right and long left branches. The right branch gives off several interlobular branches that ramify in the right lobe, both processes of the caudate lobe and the quadrate lobe. The left branch consists of a transverse and an umbilical part. The transverse part has several branches for the papillary process and the quadrate lobe, while the umbilical part gives off three branches that ramify in the left and quadrate lobes (18). The portal vein is connected through the hepatic capillary system and the hepatic veins to the caudal vena cava (15) but no interconnecting branches was found between these vessels at our macroanatomic vascular study. The present study revealed detailed insights into the livers of two species belonging to the *Mustelidae* family. The results show that both livers consist of six lobes, although differences in the shape, size and additional interlobar fissures were documented. Both species exhibit similar intrahepatic distribution of the portal vein, which will be valuable for the further use in the field of surgery, pathology etc.

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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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Antimicrobial resistance of *Streptococcus* spp. and *Staphylococcus* spp. isolated from respiratory tract of race horses in Türkiye

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Abstract: As with other animal species, one of the most important challenges encountered in race horse breeding is performance and economic losses caused by infectious diseases. Antibiotics are the most important and common drugs used for protection against infectious diseases. Recently with the occurrence of antibiotic resistance, serious complications have emerged in terms of human and animal health. For this reason, to determine antibiotic resistance in bacteria, many research studies have been carried out in the world and Türkiye. However, in Türkiye no comprehensive study has yet been conducted on antibiotic resistance in race horses. Within the scope of this study, it was aimed to investigate antibacterial susceptibility and resistance in *Streptococcus* spp. and *Staphylococcus* spp. agents identified through isolation from tracheal aspiration fluid samples taken from thoroughbred horses brought to 75. Yıl Hippodrome Hospital. Disk Diffusion and Minimum Inhibitory Concentration (MIC) tests were used to determine antibacterial susceptibility. Susceptibility profiles of identified strains against some penicillin, cephalosporin, aminoglycoside, tetracycline, rifamycin and carbapenem group antibacterial drugs were determined. Also, it has been determined that 1 *Streptococcus* spp. and 10 *Staphylococcus* spp. agents are sensitive to amoxicillin and ampicillin-sulbactam; moderately sensitive to penicillin and amikacin and resistant to trimethoprim-sulfamethoxazole, kanamycin, gentamicin, enrofloxacin and rifampin.

Keywords: Antimicrobial susceptibility, disc diffusion, minimum inhibitory concentration (MIC), *Staphylococcus* spp. and *Streptococcus* spp., thoroughbreds.

Türkiye'deki yarış atlarının solunum yollarından izole edilen *Streptococcus* spp. ve *Staphylococcus* spp. etkenlerinin antibiyotik direnci

Özet: Diğer hayvan türlerinde olduğu gibi yarış atı yetiştiriciliğinde karşılaşılan en önemli zorluklardan birisi enfeksiyöz hastalıklardan kaynaklanan performans ve ekonomik kayıplardır. Antibiyotikler, enfeksiyöz hastalıklara karşı mücadelede kullanılan en önemli ve yaygın ilaçlardır. Son zamanlarda antibiyotik direncinin oluşması ile birlikte insan ve hayvan sağlığı açısından ciddi komplikasyonlar ortaya çıkarmaktadır. Bu nedenle, bakterilerde oluşan antibiyotik direncinin tespiti amacıyla dünyada ve Türkiye'de yapılmış bir çok araştırma bulunmaktadır. Ancak Türkiye'de, yarış atlarında antibiyotik direnci üzerine kapsamlı bir çalışmaya rastlanılmamıştır. Bu çalışma kapsamında 75. Yıl Hipodromu At Hastanesine getirilen safkan atlardan alınan trakeal aspirasyon sıvısı numunelerinden izole edilerek identifiye edilen *Streptococcus* spp. ve *Staphylococcus* spp. etkenlerinde antibakteriyel duyarlılık ve direncin araştırılması amaçlandı. Antibakteriyel duyarlılığın tespitinde Disk Difuzyon ve Minimum İnhibitör Konsantrasyon (MIC) testlerinden yararlanıldı. Tespit edilen suşların bazı penisilin, sefalosporin, aminoglikozid, tetrasiklin, rifamisin ve karbapenem grubu antibakteriyel ilaçlara karşı duyarlılık profilleri çıkarıldı. 1 *Streptococcus* spp. ve 10 *Staphylococcus* spp.'nin amoksisilin ve ampisilin-sulbaktam'a duyarlı; penisilin ve amikasine orta derecede duyarlı; trimetoprim-sülfametoksazol, kanamisin, gentamisin, enrofloksasin ve rifampine ise dirençli olduğu tespit edildi.

Anahtar sözcükler: Antimikrobiyel duyarlılık, disk difuzyon, minimum inhibitör konsantrasyon (MIC), *Staphylococcus* spp. ve *Streptococcus* spp., safkan at.

Introduction

Today, antibiotics have an important place in the prevention and treatment of diseases, and also in increasing the productivity power in animals. Especially after 1950, the widespread use of antibiotics as growth accelerators in food additives in animals promoted the development of resistance against antibiotics (1, 2, 14-16, 22).

According to the World Health Organization (WHO) Antimicrobial Resistance Monitoring Global Report, resistance against common bacteria has reached an alarming level in many parts of the world, and treatment options against these bacteria have gradually become ineffective. Furthermore, as a result of the systematic evaluation of scientific data, it has been concluded that antimicrobial resistance has negative effects on the outcome of patients and the costs of healthcare services (23). Average of 700,000 deaths seen every year in European countries, and 1.5 billion dollars spent on treatment are thought to be caused by resistance of bacteria to antibiotics over time (7).

In fact, it is known that the resistance against antibiotics started before the widespread use of the substances included in this group throughout the world. However, this "first resistance" form against these substances did not pose a threat to animal and human health. On the other hand, most of the resistant microorganisms that are mentioned to pose a threat today have formed due to genetic changes, mutation and selection (9). While one of the factors that may increase the resistance of bacteria is the direct use of antibacterial drugs, another reason is the indirect contact of the drugs with the bacteria. In studies, resistance genes have been isolated from human pathogens, animal-derived bacteria and even environmental bacteria (17, 19, 20).

Antibiotics are used for the treatment and control of various types of infections in many animal species as well as in humans. For this reason, resistance to antibiotics is a natural and inevitable situation. Multiple resistance cases occurring in factors that cause disease in humans have revealed the importance of the use of these substances both in the human and the veterinary medicine fields. Horses can be reservoir of antibiotic resistant organisms and genetic markers of resistance. This situation may affect animal welfare and the economy. This resistance may persist even in the absence of selection pressure (8).

Due to the close contact of humans and horses, the risks and consequences of human treatments and the application of common antimicrobial drugs used in horses is an issue that should be considered. It has been reported that common *E. coli* species in humans and animals show high resistance to common antimicrobial agents and there are antibiotic resistance genes such as dfrA17 and dfrA12 (12). These resistance genes are generally found on mobile genetic elements such as plasmids and integrons in Gram-negative microorganism isolates (3).

In this study, antibiotic susceptibility/resistance profile against the agents *Streptococcus* spp. and *Staphylococcus* spp. was determined in samples taken from thoroughbred British and Arabian horses brought to the 75. Yıl Hippodrome Hospital and due diligence has been made in this regard.

Materials and Methods

Tracheal aspiration fluid was collected by sampling method from a total of 133 thoroughbred horses, 93 thoroughbred British and 40 thoroughbred Arabian horses, aged 1 to 5 years old, registered in the studbook that are routinely brought to 75. Yıl Hippodrome Hospital between the dates of 24/04/2016 and 23/10/2017. Of these 93 British thoroughbred horses, 26 are female and 67 are male; out of 40 thoroughbred Arabian horses, 10 are female and 30 are male. The necessary Ethics Committee Approval was obtained from Ankara University Animal Experiments Local Ethics Committee for the study (Decision no: 2015-13-153).

The samples were brought to the Ankara University Department of Veterinary Medicine Pharmacology and Toxicology laboratory in the cold chain for bacterial isolation.

In the first isolation, 5% Sheep Blood Agar (KA) was used. After inoculation, the growth cultures were incubated at 37° C for 24-48 hours. After the incubation, colonies were purified in Tryptic Soy Agar (TSA) and stored at -20° C in TSA containing 15% glycerin to be used for identification studies, antibiogram and MIC (Minimum Inhibitory Concentration) determination (4). Strains isolated and stored at -20° C were reproduced for identification. Identification of bacteria was performed with Bruker Daltonik MALDI Biotyper (MALDI-TOF) (18).

Kirby-Bauer Disc Diffusion and Minimum Inhibitory Concentration (MIC) methods were used to determine the antibacterial resistance.

After incubation, inhibition zone diameters were measured in millimeters and standard zone diameters were compared with the standards specified by the "Clinical and Laboratory Standards Institute (CLSI)" and evaluated as “susceptible", “moderately susceptible" or "resistant" according to results. Each test was repeated three times to minimize the application errors that may occur at this stage, and the arithmetic mean of the obtained data was calculated.

The MIC value was determined by the microdilution method based on CLSI 2014. *S. aureus* ATCC 29213 standard strain was used as control strain (5).

Table 1. Comparison table for susceptibility of 11 identified agents to antibiotics.

Antibiotics	*Staphylococcus lentus*	*Staphylococcus sciuri*	*Staphylococcus sciuri*	*Staphylococcus lentus*	*Staphylococcus sciuri*	*Staphylococcus sciuri*	*Staphylococcus sciuri*	*Staphylococcus sciuri*	*Staphylococcus sciuri*	*Staphylococcus equorum*	*Streptococcus pneumonia*
Amoxicilline (25)	S	S	S	S	S	S	S	S	S	S	S
Penicillin G (10)	R	R	R							S	S
TMPS (30)			R	I	R	R	R	R	R	S	R
Canamycine (30)	R	R		R						I	
Gentamicin (10)	R	R		R						S	
Ampicillin-Sulbactam (20)	S	S	S	S	S	R	S	S	S	S	
Amikacin (30)				R						S	
Enrofloxacin (5)	R	R	R	R	R	R	R	R	R	S	
Rifampin (5)		R	I		R	R	R	R	R	S	

S: Susceptible; I: Intermediate Susceptible; R: Resistance.

Results

Bacterial growth did not occur in 3 out of 133 samples. More than one bacteria was isolated from some samples. The isolated bacteria were primarily stained by the Gram staining method. After staining, Gram positive cocci shaped bacteria were separated. Possible separation of *Streptococcus* and *Staphylococcus* was performed by catalase testing. Colonies were stored at -20° C for definitive identification, antibiogram, and MIC.

However; 4 Candida albicans, 155 Gram-negative bacteria, 2 *Streptococcus* spp. and 10 *Staphylococcus* spp. agents were determined. *Streptococcus* spp. and *Staphylococcus* spp. strains isolated from different horses were identified as following: 2 *Staphylococcus lentus*, 7 *Staphylococcus sciuri*, 1 *Staphylococcus equorum* and 1 *Streptococcus pneumonia*.

Susceptibility profiles of these strains against some penicillin, cephalosporin, aminoglycoside, tetracycline, rifamycin and carbapenem group antibacterial drugs [Amoxicillin, penicillin G, trimethoprim-sulfamethoxazole (TMPS), kanamycin, gentamicin, streptomycin, ampicillin-sulbactam, neomycin, amikacin, enrofloxacin, oxytetracycline, ceftiofur, ceftazidime, rifampin, imipenem and amoxicillin-clavulanic acid] were determined. Distribution of antimicrobial susceptibilities and antimicrobial resistance profiles of the strains were presented in Table 1.

It has been determined that agents are susceptible to amoxicillin and ampicillin-sulbactam; moderately susceptible to penicillin and amikacin; and resistant to trimethoprim-sulfamethoxazole, kanamycin, gentamicin, enrofloxacin and rifampin.

However, the susceptibility profile could not be revealed for streptomycin, neomycin, oxytetracycline, ceftiofur, ceftazidime, imipenem and amoxicillin-clavulanic acid.

Discussion and Conclusion

There are not many studies on horse diseases, rational drug use and especially antibiotic resistance regarding thoroughbred horses with high economic value. One of the basic principles of rational treatment is to ensure the correct and effective drug use for diseases.

Mir et al. (21) conducted a study on the isolation and identification of aerobic bacteria in the upper respiratory tract of horses with 88 healthy heads and 53 heads with respiratory diseases in India. As a result of this study, 84.11% of the total 321 isolates isolated from both groups were found to be Gram-positive where as 15.88% of them were determined as Gram-negative bacteria. Among them, 17.44% of them were identified as *Streptococcus equi subsp. Zooepidemicus and* 9.65% of them were Corynebacterium. 9.65% as *Staphylococcus intermedius*, 8.72% as *Staphylococcus aureus*, 7.16% as *Bacillus* spp., 5.60% as *Streptococcus pneumonia*, 5.60% as *Staphylococcus chromogens*, 5.29% as *Streptococcus equismilis*, 5.29% as *Pseudomonas aeruginosa*, 3.73% as *Rhodococcus equi*, 3.73% as *Escherichia coli*, 3.42% as *Klebsiella pneumoniae*, 3.42% as *Proteus vulgaris and* 1.24% as *Streptococcus equi subsp. equi* (21).

In a study conducted by Gutema et al. (10) in Ethiopia, isolation and identification were performed in an aerobic environment by taking the swabs from the nasopharynx region of 80 healthy donkeys and also of 20 donkeys with respiratory diseases. In this study, a total of 189 bacteria species were identified from both groups. Among them, 159 of them (84.1%) were gram positive and 30 of them are gram negative (15.9%). Bacteria were detected at a higher rate in animals with respiratory diseases than in healthy ones (10).

In a study conducted by Johns and Adams (11), susceptibility for *Escherichia coli* isolated from the respiratory tract of horses from 1999 to 2012 was inspected. This research was divided into two groups as 242 isolates detected between 1999-2004 (early period) and 222 isolates detected between 2007-2012 (late period). Periodic comparison of the rates of each antimicrobial-resistant and multi-drug resistant isolates was made accordingly. There was a significant increase in the rate of ceftiofur (%7.3-22.7; P=0.002), gentamicin (%28.5-53.9; P<0.001), tetracycline (%48.4–74.2, P=0.002) and MDR (%26.6–49.4, P=0.007) of *E. coli* isolates. In all *Streptococcus* spp. (*S. equi, S. zooepidemicus, S. equisimilis and unknown* β-hemolytic *Streptococcus* spp. (UBHS)) enrofloxacin resistance increased considerably from the early period (0%) to the late period (63%) depending on the species. It was understood that tetracycline and MDR resistance of S. zooepidemicus increased over time, trimethoprim-sulfamethoxazole resistance decreased over time, and resistance against commonly used antibiotics in pathogenic agents of horses increased. Susceptibility to antibiotics was evaluated by the Kirby-Bauer disk diffusion method and zones according to the contemporary Clinical and Laboratory Standards Institute (CLSI) (previously, NCCLS) (11).

Darien et. al. (6) conducted a study on false reporting of the increase in trimethoprim-sulfamethoxazole resistance of 50 *S. equi* isolates isolated from tracheal aspiration fluid taken from horses coming to Colorado State University Veterinary Training Hospital for both periods one of which is between the years 1987-1990 (1. Period) and the other is between the years 1997-2001 (2. Period). First, isolates tested in CSU-VDL (N 5 41) were analyzed by the Kirby-Bauer agar disc diffusion using Mueller-Hinton with 5% sheep red blood cells as culture medium. Originally resistant isolates (N 5 7) in CSU-VDL were retested using Mueller – Hinton agar without adding blood. Isolates tested in Purdue (N 5 41) were analyzed by the Sensititre / Trek Broth Dilution method (using hemolyzed horse blood in Mueller-Hinton broth medium for testing with SXT). Considering the results obtained in this study, resistance development between the two periods was compared and statistically analyzed. It was understood that there were statistically significant differences between the results obtained by using the Quantitative Micro broth Dilution method in the period 1987–1990 (8%) and the results obtained by using the Kirby-Bauer Agar Disc Diffusion method in the 1997-2001 period (42%). This study revealed that even small deviations in the established laboratory test practice guidelines may have a major impact on antimicrobial resistance test results. The study underlines the need for appropriate surveillance and monitoring methods of trends in antimicrobial resistance to detect and correct such problems (6).

In a study conducted by Karilyn (13), antibiotic resistance of isolated bacterial colonies from the samples collected from the nasopharyngeal cavity of 27 horses in various farms in England was tested with the BBL Sensi Disc™ antimicrobial resistance disc (Kirby Bauer antibiotic susceptibility test). Isolates obtained from 92.59% of the collected samples were Gram-positive coccus, 85.19% of them were Gram-negative coccus, 59.26% of them were Gram-negative bacilli and 25.93% of them were Gram-positive bacilli. *E. coli*, *Shigella* spp, *Enterobacter* spp and *S. aureus* which are found in the natural nasopharyngeal flora of horses were also isolated.

Karilyn (13), 13 different antibiotic discs were used. These antibiotics were 30 µg amikacin (AN), 10 µg gentamicin (GM), 5 µg ciprofloxacin (CIP), 30 µg ceftriaxone (CRO), 2 µg clindamycin (CC), 30 µg cefotaxime (CTX), 10 µg ampicillin (AM), 5 µg chloramphenicol (C), 75 µg ticarcillin (TIC), 25 µg trimethoprim-sulfamethoxazole (SXT), 30 µg tetracycline (Te), 10 µg bacitracin (B) and 30 µg vancomycin (Va).

According to the results obtained by measuring the zone diameters, 3.70% of isolates were susceptible to gentamicin, then 11.11% amikacin, 15.38% cefotaxime, 18.52% ciprofloxacin, 22.22% ceftriaxone, 28.00% clindamycin, 33.33% ticarcillin, 37.04% ampicillin, 44.44% tetracycline, 44.44% trimethoprim-sulfamethoxazole, 52.00% chloramphenicol, 62.96% vancomycin and 68.00% bacitracin respectively.

Amikacin, cefotaxime, ciprofloxacin and ceftriaxone were highly effective with values below 20% (Resistance values reflect the number of horses with antibiotic resistant bacteria compared to total sampled horses). Clindamycin, ampicillin, ticarcillin, trimethoprim-sulfamethoxazole and tetracycline had a resistance value between 20% and 40%. Chloramphenicol, vancomycin and bacitracin were relatively ineffective in killing normal horse flora at a rate of 50% to 70%, and these antibiotics are not used much in the veterinary field (13).

Within the scope of this study, important results have emerged on the both a scientific and economic scale. In bacteria with resistance to antibiotics, the effectiveness of the drug and the benefit of the treatment may decrease. Evaluation of resistance is extremely important, especially for animal species and disease diversity. For this reason,

countries have to do their own epidemiological studies. At this point, the results of the study reveal scientifically important data. The obtained data will also be a guide for veterinarians working in this field. It will be possible to choose antibiotics while creating treatment protocols in the field of veterinary medicine by considering these results.

In case of resistance, diseases that transmitted from animal to animal or from animal to human will become widespread. This situation is also important in terms of public health. Since the effectiveness of the drugs against resistant bacteria will decrease, they will be used with increasing doses.

Identifying the development of resistance to some commonly used drugs in veterinary medicine may help to find out the resistant strains in humans as well. Thus, this study may have a relevant importance in providing some data on the strains having a porentail to develop resistance against antibiotics in humans.

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Ethical Statement

Ethics committee approval of Ankara University Animal Experiments Local Committee was received for this research study (Approval No: 2015-13-153).

Conflict of Interest

The authors declare that they have no conflict of interest.

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Presence and virulence characterization of *Listeria monocytogenes* from fish samples in the Black Sea, Türkiye

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Abstract: *Listeria monocytogenes,* characterized by a high mortality rate in humans, is a bacterium that causes listeriosis and is found in various aquatic products. The aim of this research was to investigate the presence, serotype distribution, virulence factor genes, and antibiotic susceptibility of *L. monocytogenes* strains isolated from a total of 500 fish samples of whiting (*Merlangius merlangus euxinus*) (n:243) and striped red mullet (*Mullus surmuletus*) (n:257) caught in the Black Sea between the years 2013-2014. Only one (0.2%) *L. monocytogenes* strain (striped red mullet) was isolated according to the cultural method (EN ISO 11290-1) and confirmed by PCR analysis. The *L. monocytogenes* strain was identified as serogroup 4b-4d-4e. Furthermore, the strain harboured *hlyA*, *inlA*, *inlC*, *inlJ*, *plcA*, *plcB*, *prfA*, *mpl*, *actA*, *monoA-B*, *flaA*, *lip 1-2a*, *fri, iap, and gtcA* genes except the *dltA* gene. On the other hand, *L. monocytogenes* strain susceptibility to ampicillin, meropenem, erythromycin, trimetophrim/sulfamethoxazole, and penicillin G was evaluated with the disc-diffusion method. According to the results, serogroup 4b-4d-4e isolated from striped red mullet was found to be unique to raw fish and susceptible to all tested antibiotics. In addition, it is considered that carrying out this research in different seas and with different fish species would be appropriate for determining the prevalence and virulence characteristics of *L. monocytogenes*.
Keywords: Antibiotic susceptibility, fish, *Listeria monocytogenes,* virulence genes.

Karadeniz kaynaklı balıklardan izole edilen *Listeria monocytogenes* suşlarının varlığı ve virülens özelliklerinin incelenmesi

Özet: *Listeria monocytogenes*, insanlarda yüksek mortalite oranı ile karakterize listeriozise neden olan bir bakteri olup, su ürünlerinde çeşitli düzeylerde bulunabilmektedir. Bu çalışmanın amacı, Karadeniz'den 2013-2014 yılları arasında avlanan toplam 500 adet Tekir (*Mullus surmuletus*) (n: 257) ve Mezgit (*Merlangius merlangus euxinus*) (n: 243) balığından izole edilen *L. monocytogenes* varlığı, serogrup dağılımı, virülens faktör genler ve antibiyotik duyarlılığını araştırmaktır. Bir adet (%0,2) *L. monocytogenes* suşu (Tekir balığı) standart kültür yöntemi (EN ISO 11290-1) ile izole edilmiş ve PCR analizi ile doğrulanmıştır. Söz konusu, *L. monocytogenes* suşu 4b-4d-4e serogrubu olarak tespit edilmiştir. Ayrıca *L. monocytogenes* suşunun *hlyA*, *inlA*, *inlC*, *inlJ*, *plcA*, *plcB*, *prfA*, *mpl*, *actA*, *monoA-B*, *flaA*, *lip 1-2a*, *fri, iap, gtcA* genlerini içerdiği, fakat *dltA* genini içermediği belirlenmiştir. Diğer taraftan, *L. monocytogenes* suşunun ampicillin, meropenem, erythromycin, trimetophrim/sulfamethoxazole ve penicillin G antibiyotiklerine karşı duyarlılığı disk diffüzyon testi ile ölçülmüştür. Elde edilen sonuçlara göre, Tekir balığından izole edilen 4b-4d-4e serogrubunun çiğ balığa özgü olduğu ve test edilen antibiyotiklerin hepsine duyarlı olduğu bulunmuştur. Ayrıca, bu araştırmanın farklı denizlerde ve farklı balık türleri ile yapılmasının *L. monocytogenes*'in prevalans ve virülens özelliklerinin belirlenmesi için uygun olacağı düşünülmektedir.
Anahtar sözcükler: Antibiyotik duyarlılığı, balık, *Listeria monocytogenes,* virülens genleri.

Introduction

Considering that the majority of fish such as anchovy, bonito, horse mackerel, whiting, striped red mullet, etc., which are mostly hunted in Türkiye, are caught in the Black Sea. It is reported that the Black Sea has a very important place in fishing. Especially in the period after 2000 and still, 70-80% of the total seafood fishing is provided from the Black Sea (43). In addition, whiting fish first level with 8,941 tons and striped red mullet fish with 2,342 tons in the production of demersal fish, which are the most hunted fish as of 2019 in Türkiye, reveals that these fish are among the fish species that are popularly consumed in Türkiye (18).

Aquaculture is an increasingly important source of food suitable for human consumption. The common seafood bacterial pathogens are *Vibrio* spp., *L. monocytogenes*, *Aeromonas hydrophila* and *Salmonella* spp. (31). During 2010-2017 in EU/EAA, the ranking of the food vehicles implicated in strong evidence of Foodborne Outbreak was as follows: first "mixed food" followed by "fish and fish products" and then "vegetables and juices" and other products such as crustaceans, shellfish, and molluscs (10). Although the contamination level tends to be low in raw fish, this has been changing in previous years (44). However, The prevalence of *Listeria* spp. was observed 30% in freshwater fish samples and 10.4% in marine fish samples in Türkiye (50). On the other hand, in aquaculture, improper and mostly use of antibiotics can increase the prevalence of antibiotic resistance of zoonotic pathogens (4).

L. monocytogenes, which causes listeriosis, is common in the environment. It has been isolated from soil, water, wastewater, faeces, food, agricultural environment, food processing plants (42). The genus *Listeria* has been recognized as having 17 species. Only two of these species, *L. monocytogenes* and *L. ivanovii,* are considered human pathogens (34). *L. monocytogenes* strains are classified into 13 serotypes. At least 95% of the strains isolated from foods and humans are serotypes 1/2a, 1/2b, 1/2c, 4b. Although human listeriosis in sporadic cases has been mostly caused by serotypes 4b, 4e, 1/2a, 1/2c; serotypes 4b, 4e commonly have been detected, lesser 1/2b (37). Further typing and characterization steps are requirement to precisely determine the virulence factors caused by *L. monocytogenes* strains.

The pathogenicity of *L. monocytogenes* depends on various virulence factors. There are many virulence genes identified in *L. monocytogenes*. Among these genes, *hlyA* is one of the essential pathogenic factors and is responsible for the escape from the phagosomes and invasion of host cells (9). The *actA* gene encodes the surface protein ActA and is associated with cell-to-cell spread (8). The internalin genes are involved in the internalization and adhesion of *L. monocytogenes* (2). The *prfA* gene regulates and controls the expression of a number of virulence genes. The pathogenicity of *L. monocytogenes* depends mainly on the *prfA* virulence gene cluster (9).

The aim of the study was to determine the occurrence, virulence properties (*hlyA*, *inlA*, *inlC*, *inlJ*, *plcA*, *plcB*, *prfA*, *mpl*, *actA*, *monoA-B*, *flaA*, *lip 1-2a*, *fri, gtcA*, *dltA*, *iap*), serotyping (*Imo0737, Imo1118, ORF2819, ORF2110)* and antimicrobial susceptibility of *L. monocytogenes* isolated from fishes caught in the Black Sea in Türkiye.

Materials and Methods

Sampling: A total of 500 fish samples were collected from December 2013 to May 2014 from Kumkapı Commercial fish collection area, Istanbul. These fishes originated from the selected areas of the Black Sea in Türkiye (Figure 1). The fishes were brought to the commercial fish collection area after hunting from the Black Sea. Tested fresh fish samples (n=500) were striped red mullet (257 samples), whiting (243 samples) (Table 1). Each sample consisted of one fish. All samples were transported to the laboratory in sterile plastic bags (one sample per bag) in a thermos box at 4°C and analysed immediately.

Table 1. Distribution of fish caught in coded coordinate regions of the Black Sea in 2013-2014 fishing season by months**.**

Years	**2013**		**2014**								
Months	**December**		**January**		**February**		**March**		**April**		**May**
Type of Fishes	**[a]SRM**	**[b]Wh**	**SRM**	**Wh**	**SRM**	**Wh**	**SRM**	**Wh**	**SRM**	**Wh**	**SRM**
Coordinate areas and numbers of collected fish samples	[c]K-1 (10)	K-1 (10)	K-1 (20)	K-1 (24)	K-1 (18)	K-1 (22)	K-1 (65)	K-1 (80)	K-1 (27)	K-1 (24)	K-3 (9)
	K-3 (3)	K-3 (3)	K-3 (2)	K-3 (6)	K-3 (4)	K-3 (4)	K-3 (18)	K-3 (17)	K-3 (4)	K-3 (7)	K-11 (9)
	K-5 (2)	K-5 (2)	K-11 (6)	K-11 (10)	[d]K-9 (3)	K-9 (3)	K-9 (8)	K-7 (4)	K-9 (8)	K-9 (4)	K-15 (10)
	K-9 (4)	K-11 (1)	K-15 (2)		K-11 (10)	K-11 (14)	K-11 (4)		K-11 (4)	K-11 (8)	K-25 (2)
	K-11 (1)										
	K-19 (4)										

[a.]SRM, Striped Red Mullet; [b.]Wh, Whiting; [c.]K, Coordinate Area; [d.]K-9, The area where the fish isolated from *Listeria monocytogenes* was hunted

Isolation and identification of L. monocytogenes: The samples were analysed for detection of *L. monocytogenes* according to the EN ISO 11290-1 (19). Twenty-five grams of each fish samples (skin, gill and intestine) was transferred to 225 ml of Half Fraser Broth (Oxoid CM 895). And the suspension was incubated at 30°C for 24 h. Then 100 µl of the culture was transferred to 10 ml of Fraser Broth (Oxoid CM 895) and performed at 37°C for 48 h. An aliquot of 10 µl from the Fraser Broth was spread on the surface of an ALOA agar (Oxoid CM 1084) plate using a sterile loop. The ALOA plates were incubated at 37°C for 24 – 48 h. A sample was considered positive if there were one or more typical colonies (blue-green with an opaque halo) on ALOA agar plates.

The single colony isolate was purified by streaking onto ALOA agar plates and incubated at 37°C for 48 h. Following this, a single colony was subcultured on Tryptic Soy Agar (Oxoid CM 131) plate and incubated at 37°C for 24 h. The culture was transferred with a loop in Tryptic Soy Broth (Oxoid CM 129) and incubated at 37°C for 18 h. This culture was frozen in 20% glycerol containing 1 ml of Tryptic Soy Broth first at -20°C and then at -80°C (stepwise to prevent shock).

***Confirmation of isolates as L. monocytogenes and molecular serotyping with PCR*:** DNA was prepared using the Liu et al. (25) method. *monoA* and *monoB* primers were used for the specific identification by conventional PCR of all serotypes of *L. monocytogenes* and the PCR program was set as follows: initial denaturation 1 cycle of 94°C for 5 min; annealing 35 cycles of 95°C for 1 min, 53°C for 45 s, and 72°C for 1 min; extending 72°C for 7 min and 10°C limitless (3).

The conventional PCR technique was used to determine the main *L. monocytogenes* serogroups (1/2a-3a; 1/2b-3b; 1/2c-3c; 4b-4d-4e) (7). Additionally, PCR was designed to identify the *Imo0737*, *Imo1118*, *ORF2819*, and *ORF2110* genes. The procedure was performed according to following conditions: initial denaturation 1 cycle of 94°C for 3 min; annealing 35 cycles of 94°C for 0.40 min, 53°C for 75 s and 72°C for 75 s; extending 72°C for 7 min and 10°C limitless.

Imo0737 (691bp) 5'- AGGGCTTCAAGGACTTA CCC-3' ve 5'-ACGATTTCTGCTTGCCATTC-3',

Imo1118 (906bp) 5'- AGGGGTCTTAAATCCTG GAA-3' ve 5'- CGGCTTGTTCGGCATACTTA-3',

ORF2819 (471bp) 5'-, AGCAAAATGCCAAAACT CGT-3' ve 5'- CATCACTAAAGCCTCCCATTG-3',

ORF2110 (597bp) 5'- AGTGGACAATTGATTGG TGAA-3' ve 5'- CATCCATCCCTTACTTTGGAC-3'

***Identification of virulence genes*:** PCR technique was designed to identify the presence of virulence genes in *L. monocytogenes.* The mixture contained: 5 µl DNA, 3 µl $MgCl_2$ (25 mM) (Thermo), 1 µl reverse primer (10 µM), 5 µl dNTP (Thermo), 5 µl 10X buffer KCL (Thermo), 1 U (0.24 µl) Taq DNA polymerase (Thermo), 1 µl forward primer (10 µM) and dH_2O. The total volume was 50 µl. PCR reactions were performed using obtained genomic DNA. The primer sequences and amplification conditions were presented in Table 2. The electrophoretic separation of PCR products was carried out in 1-1.5% agarose gel.

Table 2. Virulence genes and primers sequences.

Virulence genes (bp)	Primer sequences (5'-3')	References
prfA (571)	GGTATCACAAAGCTCACGAG CCCAAGTAGCAGGACATGCTAA	(33)
mpl (1473)	GGCTCATTTCACTATGACGG GCTTCCCAAGCTTCAGCAACT	(33)
plcA (129)	CGAGCAAAACAGCAACGATA CCGCGGACATCTTTTAATGT	(24)
plcB (261)	GGGAAATTTGACACAGCGTT ATTTTCGGGTAGTCCGCTTT	(47)
hlyA (234)	CGGAGGTTCCGCAAAAGATG CCTCCAGAGTGATCGATGTT	(30)
actA (268 or 385)	GACGAAAATCCCGAAGTGAA CTAGCGAAGGTGCTGTTTCC	(21)
inlA (800)	ACGAGTAACGGGACAAATGC CCCGACAGTGGTGCTAGATT	(26)
inlC (517)	AATTCCCACAGGACACAACC CGGGAATGCAATTTTTCACTA	(26)
inlJ (238)	TGTAACCCCGCTTACACAGTT AGCGGCTTGGCAGTCTAATA	(26)
Lip1-2a (274)	GATACAGAAACATCGGTTGGC GTGTAACTTGATGCCATCAGG	(5)
iap (453)	GGGCTTTATCCATAAAATA TTGGAAGAACCTTGATTA	(40)
gtcA (251)	TGGGTTACTACAAGAAGAG AGTACTGATGCGATAAAAGCA	(35)
dltA (1000)	AAGTAGTGCAGTTTAGGAGAGGA AGATTGTACCACCGGATGTC	(23)
fri (471)	ATGAAAACAATCAACTCAGT CTACTCTAATGGAGCTTTT	(40)
flaA (864)	ATGAAAGTAAATACTAATATC TTAGCTGTTAATTAATTGAGT	(40)

***Antibiotic susceptibility testing*:** The antibiotic susceptibility of isolated *L. monocytogenes* strains was performed by the disc-diffusion method (13). Suspension of isolated *L. monocytogenes* strains was prepared according to the optical density of 0.5 in MacFarland standard in 0.9% saline solution. The suspension was performed on the Mueller Hinton Agar (Oxoid CM 337) plate with 5% defibrinated horse blood and 20 mg/L β-Nicotinamide Adenine Dinucleotide (Biolab Inc., NAD10025) (14) and then ampicillin (CT0002B, 2 µg), penicillin G (CT0152B, IU), erythromycin (CT0020B, 15 µg), meropenem (CT0774B, 10 µg), trimethoprim-

sulfamethoxazole (co-trimoxazole) (CT0052B, 25 µg) antibiotic discs (Oxoid) were included. The plate was incubated at 35±1°C in 5% CO_2 in air for 16-20 h. *Streptococcus pneumoniae* ATCC 46919 was used as quality control strains. Then inhibition zones around antibiotic discs were analyzed according to EUCAST (2018) Version 8.0 (15). Minimum inhibitory concentrations were determined using E-test (BioDisc) and MICE strips (Thermo) (Figure 8).

Results

***Isolation of L. monocytogenes strain*:** One *L. monocytogenes* strain was isolated from only one (1/500, 0.2%) fish sample. The fish sample belonged to the striped red mullet (1/257, 0.3%) originated from the K-9 area of The Black Sea (Figure 1).

***Confirmation and Serotyping*:** The identification of *L. monocytogenes* was performed by PCR amplification of the *monoA-B* gene (3). The strain possessed *monoA-B* gene (Figure 2). According to the results of PCR, the strain was identified as *L. monocytogenes*.

The strain harboured *Imo0737* (Figure 3) and *Imo1118* genes (Figure 4), except *ORF2110* (Figure 5) and *ORF2819* genes (Figure 6). Therefore, the strain was classified as serogroup 4b-4d-4e.

***Presence of selected virulence genes*:** The strain possessed the *hlyA*, *inlA*, *inlC*, *inlJ*, *plcA*, *plcB*, *prfA*, *mpl*, *actA*, *flaA*, *lip 1-2a*, *fri, gtcA*, and *iap* genes (Figure 7). But it did not have the *dltA* gene.

***Antibiotic susceptibility*:** The *L. monocytogenes* isolate was all susceptible to ampicillin, penicillin G, erythromycin, meropenem, and trimethoprim-sulfamethoxazole.

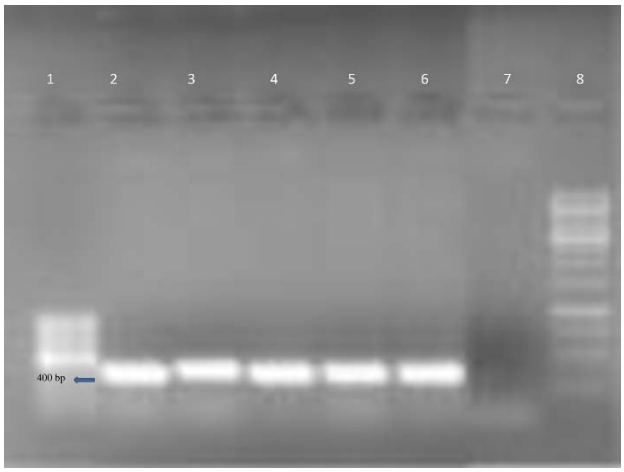

Figure 2. PCR gel vision of *monoA-B* (400 bp) gene.
From left to right: 1. 100 bp DNA Marker; 2. 186; 3. ATCC 7644; 4. ATCC 19111; 5. ATCC 19115; 6. ATCC 13932; 7. negative control (sterile ultra pure water); 8. 1 kb DNA Marker.

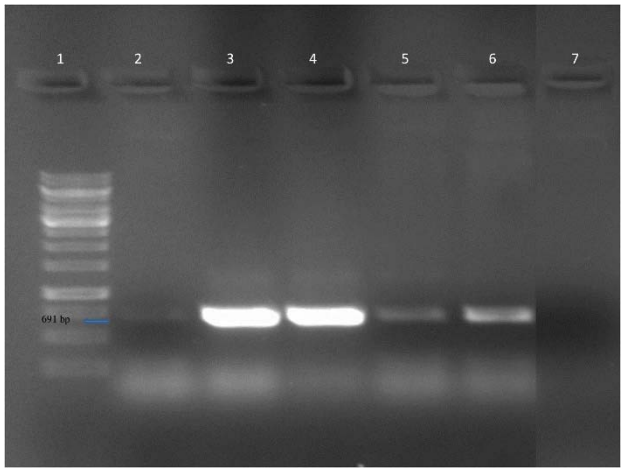

Figure 3. PCR gel vision of *Imo0737* (691 bp) gene.
From left to right: 1. 1 kb DNA Marker; 2. 186; 3. ATCC 7644; 4. ATCC 19111; 5. ATCC 19115; 6. ATCC 13932; 7. negative control (sterile ultra pure water).

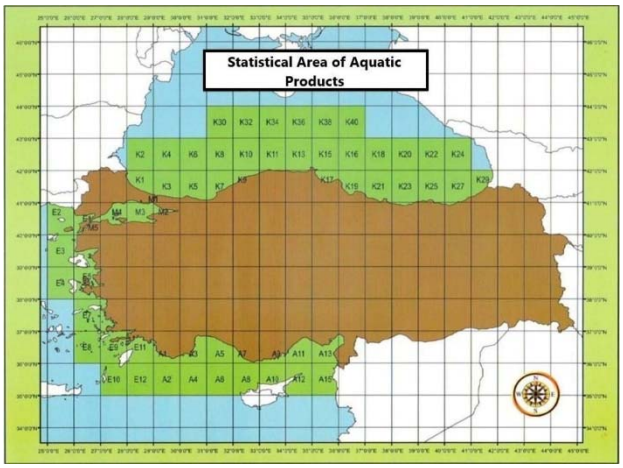

Figure 1. Statistical area of aquatic products (17).

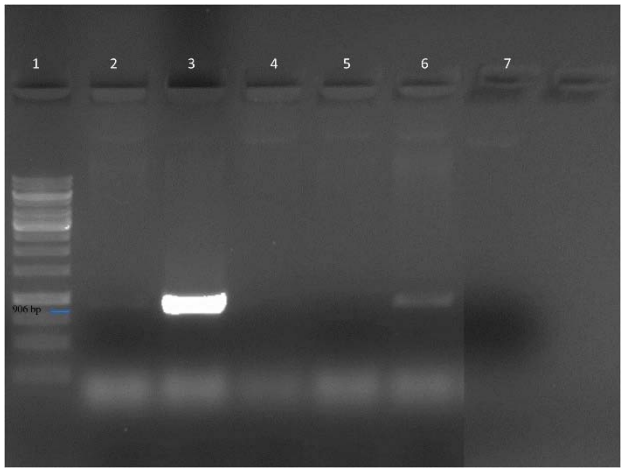

Figure 4. PCR gel vision of *Imo1118* (906 bp) gene.
From left to right: 1. 1 kb DNA Marker; 2. 186; 3. ATCC 7644; 4. ATCC 19111; 5. ATCC 19115; 6. ATCC 13932; 7. negative control (sterile ultra pure water).

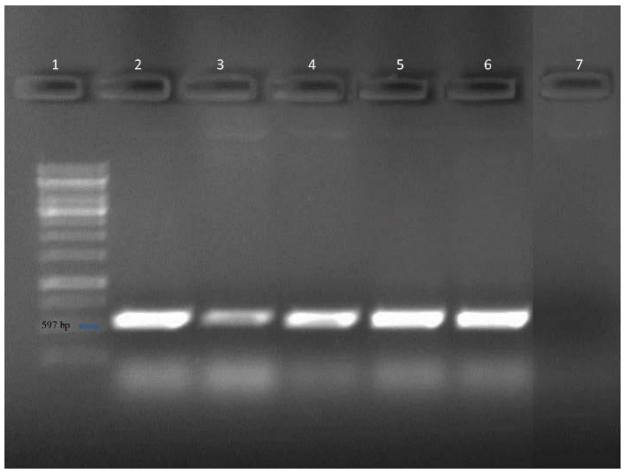

Figure 5. PCR gel vision of *ORF2110* (597 bp) gene.
From left to right: 1. 1 kb DNA Marker; 2. 186; 3. ATCC 7644; 4. ATCC 19111; 5. ATCC 19115; 6. ATCC 13932; 7. negative control (sterile ultra pure water).

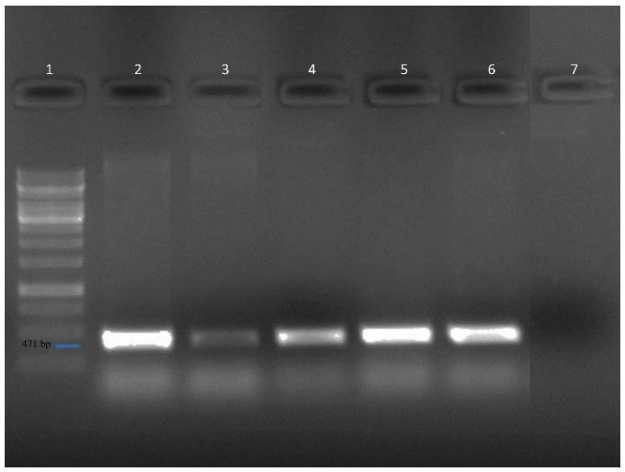

Figure 6. PCR gel vision of *ORF2819* (471 bp) gene.
From left to right: 1. 1 kb DNA Marker; 2. 186; 3. ATCC 7644; 4. ATCC 19111; 5. ATCC 19115; 6. ATCC 13932; 7. negative control (sterile ultra pure water).

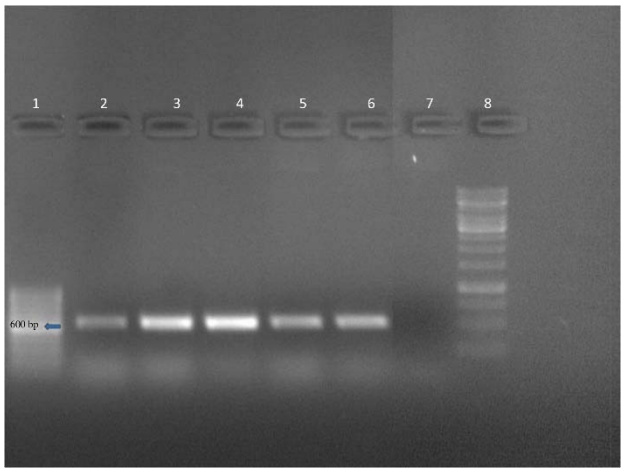

Figure 7. PCR gel vision of *prfA* (600 bp) gene.
From left to right: 1. 100 kb DNA Marker; 2. 186; 3. ATCC 7644; 4. ATCC 19111; 5. ATCC 19115; 6. ATCC 13932; 7. negative control (sterile ultra pure water); 8. 1 kb DNA Marker.

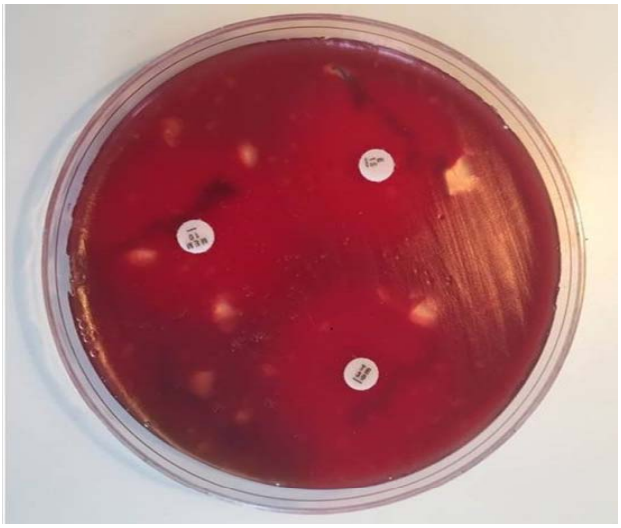

Figure 8. Antibiotic disc-diffusion test on Mueller-Hinton Agar with 5% defibrinated horse blood.

Discussion and Conclusion

L. monocytogenes is a ubiquitous bacterium isolated from freshwater and coastal water. In contrast, it is rarely isolated from deep seawater. Environmental conditions, such as waterfall, can affect the number of *Listeria* in the water, and as a result, *Listeria* may be present on the skin surface of fish and in aquaculture in the natural environment (44). In the study, *L. monocytogenes* was isolated from only one sample (0.2%) of the 500 fish samples. The strain belonged to the fish sample originated from the K-9 area in February 2014.

The quantities of *L. monocytogenes* in fish are highly determined by the amount of *L. monocytogenes* of water. Therefore, there are numerous studies conducted on seawater. In a study by El Marrakchi et al. (11) in seawater, it was shown that five out of 161 tested seawater samples were contaminated with *L. monocytogenes*. In Mexica, in an investigation of seawater 12 out of 144 samples were positive for these bacteria (36).

A high prevalence of *L. monocytogenes* in fresh fishes and sea fishes has been reported in previous studies. High percentage of contaminated fishes was detected, e.g. 8.8% (28 out of 317) in Estonia (22), 7.6% (37 out of 488) in Iran (20). In previous investigations in Türkiye, 6.6% of samples of freshwater fish (n=150) (12), 4% of samples of fresh fish (n=100) (1) were contaminated by these bacteria. Our results were consistent with other investigations (6, 32). Siriken et al. (38) isolated *L. monocytogenes* from one of 50 samples of raw anchovy (*Engraulis encrasicolus*) collected from the retailers and small-scale producers from the Black Sea region of Türkiye. In Greece, Soultos et al. (41) isolated *L. monocytogenes* from one of 120 marine fish samples. Thirty of these samples were whiting. Similarly, *L. monocytogenes* was not isolated from whiting samples examined in our study.

Marian et al (29) did not detect L. monocytogenes in fish samples. Similarly, Yucel and Balci (50) showed that none of the skin and gill samples of marine fish (n=48) was positive for *L. monocytogenes* in Türkiye.

In our study, the *L. monocytogenes* strain had *ORF1118* and *ORF2819* genes. The obtained results showed that the strain belonged to serogroup 4b-4d-4e. Serogroup 4b-4d-4e was isolated from a striped red mullet sample collected in February 2014. Striped red mullet is a fish that lives in demersal water in the Black Sea. *L. monocytogenes* determine lesser in fresh fishes that live in in-depth seas (44).

Ruminants faeces on surface waters flowing from agricultural and urban areas are identified as the potential source of *L. monocytogenes* (28). In our study, *L. monocytogenes* strain was isolated from the fish sample that originated from the K-9 area. This area contains Bartın Harbor and the place where the Bartın river flows into the Black Sea. The isolation of *L. monocytogenes* strain from a fish sample collected from this area can give information about the level of contamination of water in the area. In this context, there are publications about the pollution and microbial load of the Bartın River (45).

In the current study, the *L. monocytogenes* strain had *hlyA*, *inlA*, *inlC*, *inlJ*, *plcA*, *plcB*, *prfA*, *mpl*, *actA*, *monoA-B*, *flaA*, *Lip1-2a*, *fri, gtcA* and *iap* genes, except *dltA* gene. Wieczorek and Osek (49) and Skowron et al. (39) found virulence genes (*hlyA*, *plcA*, *plcB*, *iap*, *inlB*, *actA* and *prfA* genes) in *L. monocytogenes* strains obtained from the fish samples. These studies showed that *L. monocytogenes* strains obtained from fishes could be a threat to public health.

Most infections caused by *L. monocytogenes* are due to serotypes 1/2a, 4b, 1/2b. Whereas, serotype 1/2c is rarely found in clinical cases (27). On the other hand, in the present study, the serogroup of *L. monocytogenes* strain isolated from the fish sample was classified as 4b-4d-4e by PCR.

The serogroup 4b-4d-4e in different countries was observed in previous investigations of fish (46, 48). Whereas, serotype 1/2a was predominantly found in several countries (22, 39, 49). Fallah et al. (16) observed that serotype 4b was predominantly at cold conditions, although serotype 1/2a was predominantly at warm conditions. According to the reporting result, the growing ability of serotype 4b is better than 1/2a at cold conditions (16). Similarly, in our study, serogroup 4b-4d-4e was isolated from striped red mullet collected in winter (February 2014).

Jamali et al. (20) observed that 43 strains of *L. monocytogenes* were isolated from 488 raw fish samples and 374 swab samples. In this study, serotype 1/2a was predominant, but only serotype 4b was isolated from raw fish samples. Similarly, our study was carried out in raw fish samples and the strain was identified as serogroup 4b-4d-4e. In Türkiye, *L. monocytogenes* was isolated from eight out of 50 salted anchovies samples, 50 raw anchovies and 50 raw mussels collected from the retailers and small-scale producers from the Black Sea region. Serotype 1/2b (3b) was detected in six salted anchovies and a fish sample. On the other hand, Serotype 1/2b (3b) and 4b (4d or 4e) were obtained together from a raw mussel sample (38).

In most studies, isolated *L. monocytogenes* strains showed resistance to tested antibiotics at different rates (20, 49). In the Skowron et al. (39) study on fish and swab samples, serogroup 1/2a-3a among 70 *L. monocytogenes* strains was highly resistant to penicillin (44.4%). Furthermore, serogroup 1/2c-3c to ampicillin (30%), serogroup 1/2c-3c to erythromycin (60%), serogroup 1/2b-3b to trimethoprim/sulfamethoxazole (52.2%), serogroup 1/2c-3c to meropenem (40%) were highly resistant. Serogroup 4b-4d-4e was most susceptible among serogroups. Similarly, in our study serogroup 4b-4d-4e was susceptible to all tested (penicillin, ampicillin, meropenem, erythromycin, and trimethoprim-sulfamethoxazole) antibiotics.

In the present investigation, the low number of *L. monocytogenes* strains in tested fish samples is substantial for public health. The *L. monocytogenes* strain was isolated from a fish sample that originated from the K-9 area. This area contains Bartın Harbor and polluted Bartın river. In this context, the presence of *L. monocytogenes* in the area indicates that the microbiological quality of the water in the area should be low. On the other hand, it is consistent with our findings that *L. monocytogenes* serogroup 4b-4d-4e is mostly isolated from raw fish collected during the cold season. Furthermore, the present strain in our study was susceptible to all tested antibiotics, similar to other studies. This result obtained in terms of Public Health of Türkiye; showed that fish samples that were caught from the Black Sea and sampling directly from the boat were largely safe in terms of antibiotic resistant aquatic *L. monocytogenes* strains. However, to reliably determine antibiotic susceptibility and the presence of *L. monocytogenes* in fish, it is significant to carry out studies on different fish species in other seas in the future.

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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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Operative treatment of splint bone fractures in horses

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Abstract: In this study; it is aimed to present the operative treatment and results, which are indicated for the continue of their racing life in splint bone fractures which are frequently occurred in race horses. The 2nd and 4th metacarpal (Mc-II and Mc-IV) and metatarsal bones (Mt-II and Mt-IV) of the horse are accessory structures that support the carpus and tarsus. Fractures of these bones in horses are called "splint bone fractures". Splint bone fractures are relatively common in horses and usually seen in forelimbs. Fractures of the proximal part are rarer and represent 2.7-5% of all splint bone fractures, but challenging to manage. In the present study, 5 cases of splint bone fractures in different horses were evaluated. The distribution of the fractures were Mc-II in three, Mc-IV and Mt-IV in one each. Localization of the fractures were distal 1/3 in 4 and diaphyseal in 1 horse. For the management of these fractures, surgical removal of the fractured fragments were performed under general anaesthesia. Functional limb usage was achieved following the 30th day postoperatively in all cases.
Keywords: Fracture, horse, metacarpus, metatarsus, splint bone.

Atlarda splint kemik kırıklarının operatif sağaltımı

Özet: Bu çalışmada**; y**arış atlarında sıklıkla karşılaşılan splint kemik kırıklarında, yarış hayatlarını sürdürebilmeleri için endike olan operatif sağaltım ve sonuçlarının aktarılması amaçlanmıştır. İkinci ve dördüncü metakarpal (Mc-II ve Mc-IV) ve metatarsal kemikler (Mt-II ve Mt-IV) karpus ve tarsusu destekleyen aksesuar yapılardır. Atlarda bu kemiklerin kırıkları "splint kemik kırıkları" olarak adlandırılır. Splint kemik kırıkları atlarda sık ve özellikle de ön ekstremitede yerleşim gösterirler. Proksimal bölge kırıkları daha nadir olup, tüm splint kemik kırıkları içerisinde %2,7-5'lik bir orana sahiptir. Bu çalışmada farklı atlardaki 5 splint kemik kırığı değerlendirildi. Kırıkların dağılımı, üç olguda Mc-II, birer olgulda da Mc-IV ve Mt-IV olarak belirlenirken, lokalizasyonu ise dört olguda distal 1/3, bir olguda diafizer bölge olarak tespit edildi. Bu kırıkların sağaltımında, distal kırık fragmentleri genel anestezi altında operatif olarak uzaklaştırıldı. Olguların tümünde postoperatif 30. günde ilgili bacağın fonksiyonel olarak kullanıldığı gözlendi.
Anahtar sözcükler: At, kırık, metakarpus, metatarsus, splint kemik.

Introduction

In horses, metacarpus (Mc) / metatarsus (Mt) II-IV, also known as "splint bones" which anatomically localized caudo-lateral surface of the Mc-II both laterally and medially (1, 3). These bones take an important role in structures of the carpo-metacarpal and tarso-metatarsal joints (1, 3, 7, 18-21, 23, 24).

Splint bone fractures are mostly seen in forelimbs. Mc-II articulates with second carpal bones proximally and supports the load of this bone (1, 3, 5, 7). Mc-IV articulates with the fourth carpal bone proximally, but the load comes from the carpal bone both supported by Mc-III and Mc-IV (23, 24). An interosseous ligament spans the proximal two-thirds of the splint bone and maintains its attachment to Mc-III (21).

Small metacarpal bones fractures are relatively common in horses and may occur as a result external trauma, hyperextension of the metacarpophalangeal (MCP) joint, or as sequela to localized osteomyelitis caused by penetrating wounds (19, 20). In racehorses, these fractures may occur from acute overload (18-21).

Fracture of the small metacarpal bones can occur in proximal, middle, or distal third, and can be open or closed, and simple or comminuted. These fractures can be managed surgically or conservatively, depending on the location of the fracture along the bone, and whether it is open, closed, simple, or comminuted (2, 5, 16-18). Sequestration rarely occurs with closed fractures, even when multiple fragments are present. Osteomyelitis at the site and sequestration of the fracture fragments commonly

occur when fractures are open or caused by penetrating objects. Culture of the contaminated site often yields *Escherichia coli* or *Streptococus spp;* less frequently, *Staphylococci spp.* and *Pseudomonas spp.* are encountered (21, 23, 24).

Clinical signs of uncomplicated proximal and distal Mc-II or Mc-IV fractures include lameness, swelling at the fractures site, pain on digital palpation, and often, concurrent suspensory desmitis (9, 15). Chronic splint bone fractures can develop a subperiosteal exostosis as the fracture attempts to heal. Impingement by the exostosis on the suspensory ligament may limit the horse's return to athletic fitness and necessitate the removal of the exostosis to achieve soundness (5, 17).

The definitive diagnosis of a splint bone fracture is based on clinical signs and radiographic evidence of a fracture. MRI, which is one of the advanced diagnostic methods, is also effective in diagnosis (4). Diagnostic nerve blocks may be necessary to rule out other causes of lameness and to determine whether or not the fracture is responsible for the observed lameness. Associated suspensory desmitis is diagnosed by detecting enlargement and tenderness of the body and/or branches of the suspensory ligament (19, 21, 22).

Distal fractures: Fractures of the distal third of the splint bone is the most common type observed (1, 3, 7, 15). Thoroughbreds and forelimbs are more frequently affected than hind limbs (18-21). Horses 6 to 8 years old are more frequently affected with fractures of distal splint bone. In the previous studies distal splint bone fractures were concluded as fatigue fractures caused by the pulling of the interosseous ligament, which creates an increased force on the splint bone, especially during hyperextension of the MCP joint (23, 24).

Amputation of the fractured distal splint bone is the treatment of choice for both complicated and uncomplicated fractures (2, 5, 16). Although many splint bone fractures heal with rest and topical therapy alone, nonunion or formation of a large callus is common. A large callus may impinge on the suspensory ligament and cause lameness. Surgery hastens convalescence, prevents the formation of a large callus, and for open fractures, allows aggressive debridement of infected soft tissue. The horse should be confined to a stall for 30 days and hand walked 30 minutes twice daily. The limb should remain bandaged for 21 days. Following 30 days of confinement, the horse can be allowed to exercise in a small paddock or pasture for 3 to 6 months before training is resumed (1-3, 5, 7).

Proximal fractures: Proximal splint bone fractures can be closed or open and have been classified as comminuted, articular oblique, associated with osteomyelitis or chronic nonunion fractures (1-3, 5, 7). Subsequent exostosis and callus production following proximal splint bone fractures are most commonly associated with direct external trauma such as kicks from other horses, interference during work, or self-inflicted trauma. Amputation of more than two-thirds of the splint bone can result in excessive motion of the remaining segment during weight-bearing (5, 7, 9). This instability may lead to comminution or displacement of the remaining bone, periosteal reaction at the osteotomy site and chronic lameness. Chronic lameness can also result from interosseous desmitis and degenerative joint disease of the carpometacarpal joint due to articular instability.

The aim of this study is to share the clinical and surgical experience of five cases of splint bone fractures that been unreported before in our country.

Materials and Methods

Horses with complaints of lameness after trauma and diagnosed with splint bone fracture with clinical and radiographic examinations were included in the study. For each horse; age, sex, breed, localization and type of fractures were recorded. As a result of detailed clinical and radiographic examinations, it was determined whether the fracture was open, there was an open wound or there was a fracture complication. At least two-sided radiographs of all patients were obtained (Figure 1). A temporary elastic bandage was applied to the legs with splint bone fractures until the operation. For general anesthesia, following premedication with acepromazine (0.04 mg/kg, IV) injection, horses were sedated with xylazine %2 (0.6 mg/kg, IV) and inducted with ketamine %10 (2 mg/kg, IV). After lying down the ground, horses intubated and connected to anesthesia machine and anesthesia was continued with isoflurane. Caudo-medial approach for Mc-II and caoudo-lateral approach for Mc-IV was performed under general anesthesia on lateral recumbence. Flunixin meglumine (1 mg/kg, IV) injections were performed on all horses for 3 to 5 days following surgery. Conservative bandages were done to the horses for 7 to 14 days. The recovery period was determined as the period of time in which the horses could trot in a healthy way after they were put in stall rest following the operation.

Results

Study materials were composed of 4 Thoroughbreds and 1 Arabian horse of different ages and sexes. The mean age of the horses was 4.5 (ranged between 3-7 years). Four of the cases were male (80%) and the resting one was female (20%). Fractures were occurred related to kicks in 3 (case 2, 4, 5) cases and aetiology was unknown in 2 (case 1, 3). Fractures were localized to the distal part of the bone in 4 cases (case 1-4) and diaphysis in one case (case 5) (Figure 2). All of the distal fragments were surgically removed with the limited open approach.

Table 1. Clinical data of cases in this study.

Case no.	Breed	Age/Gender	Bone	Localization
1	Thoroughbred	3/male	Mc II	Distal 1/3
2	Thoroughbred	3.5/male	Mc IV	Distal 1/3
3	Thoroughbred	7/male (castrated)	Mt IV	Distal 1/3
4	Thoroughbred	5/female	Mc II	Distal 1/3
5	Arabian	4/male	MC II	Diaphyseal

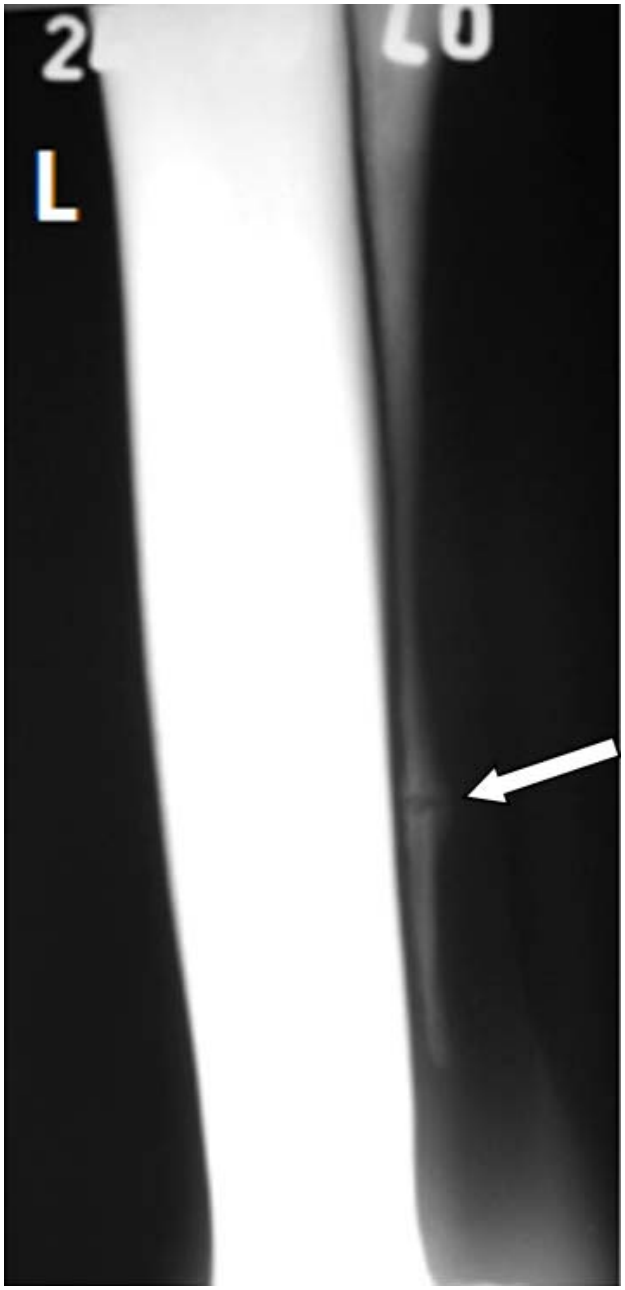

Figure 1. Preoperative oblique radiography of case 1. The white arrow: Fracture side of splint bone and a callus formation observed in chronic period.

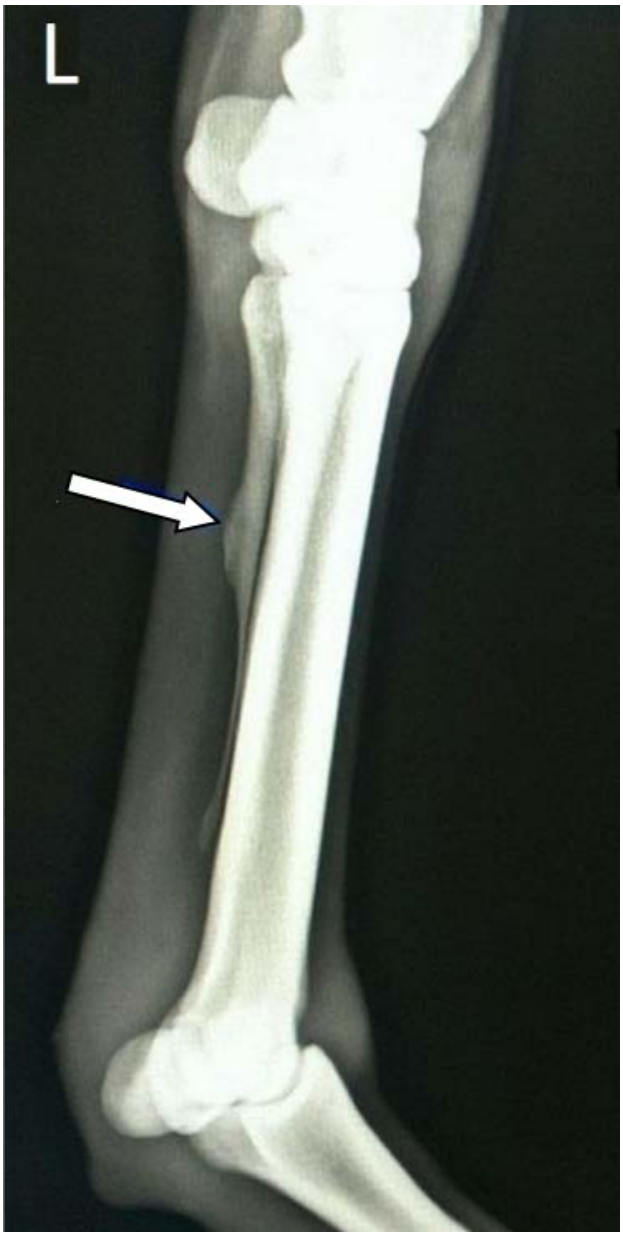

Figure 2. Preoperative lateral radiography of case 5. The white arrow: A callus formation observed in chronic period.

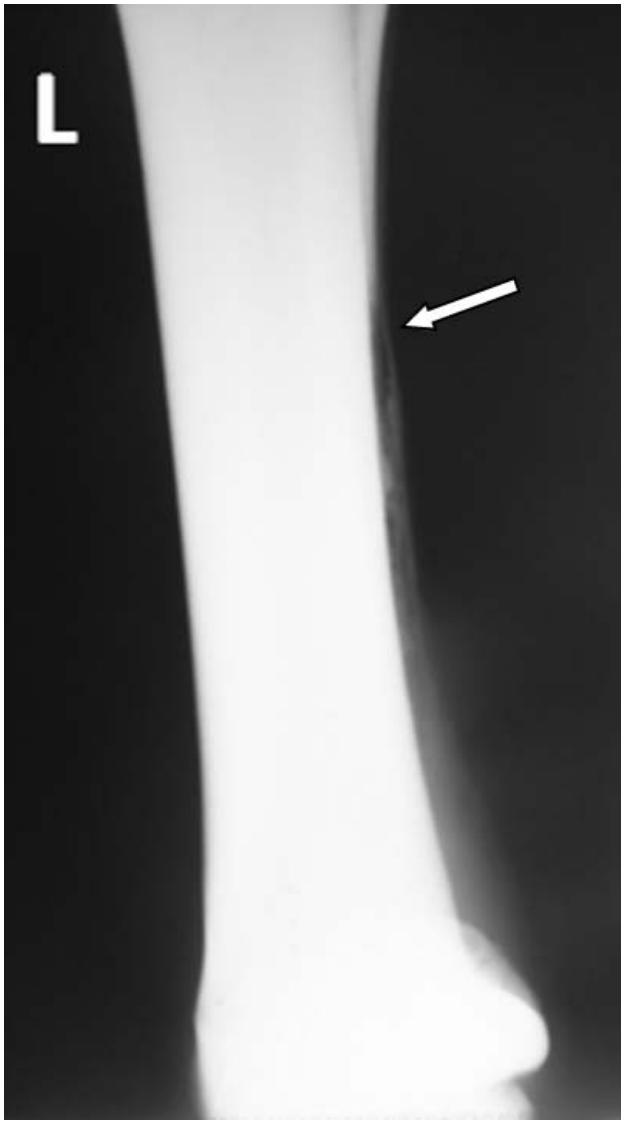

Figure 3. Postoperative oblique radiography of case 1.

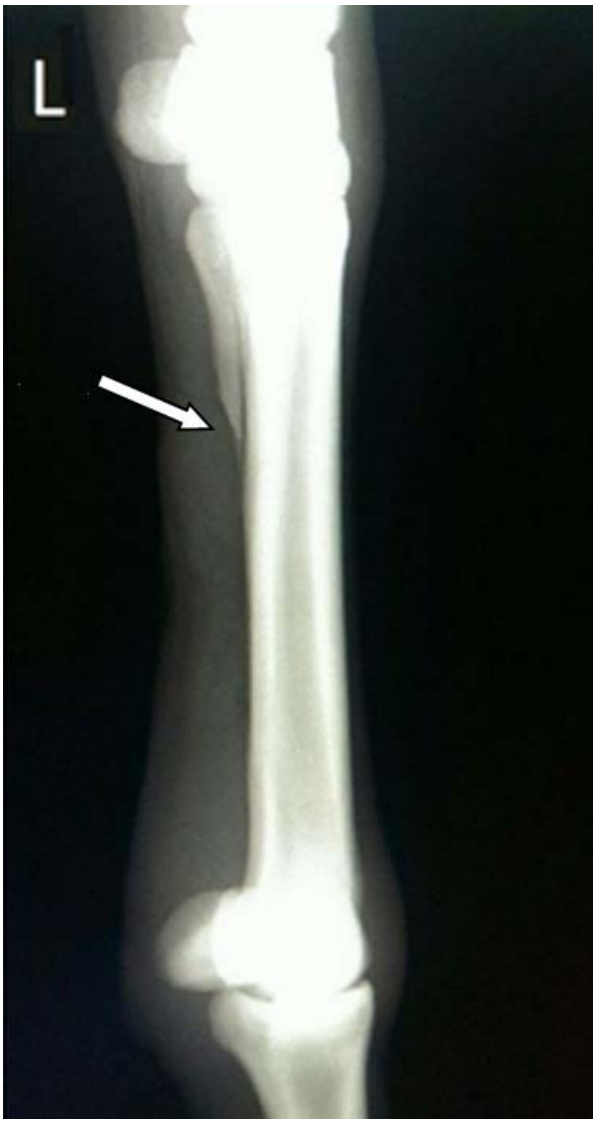

Figure 4. Postoperative lateral radiography of case 5. The white arrow: Blunted end of proximal fragment.

In clinical examination, all cases were lame when walking and reluctant to run. The pain was observed in palpation and nerve block was carried out for the differential diagnosis of distal region problems. The mean lameness score of the horses was 2 over 5 (1 to 3). Because all fractures were acute, there was no exuberant callus interfered with the suspensory ligament.

Because the surgical approach to the splint bones is safe and no obvious bleeding was observed during and after surgical removal of the fractured bones. Following removal of the fragments, sharp ends of the remaining bones were blunted with roungeurs in all cases. Closure of the skin and subcuticular tissues were done routinely. After the removal of the fragment (Figure 3, 4), exercise restriction and soft padded bandage applications were performed for two weeks postoperatively. After 20 days postoperatively, controlled walking exercises were performed twice daily and at the end of the first-month functional limb activities were observed in all of the horses. In the final clinical follow-ups no observable pain or lameness was achieved any of the cases. According to long-term telephone questionaries made 6 months after discharge, all clients were satisfied with the results.

Discussion and Conclusion

Splint bone fractures are usually seen in forelimbs (1-3, 5, 7, 9, 15-17) and Mc-II, because Mc-II directly supports the load of the second carpal bone. Eighty percent of the fracture cases in present study were seen in metacarpals which support the previous reports. However, there are studies reporting that metatarsal fractures, especially Mt-IV, are observed more frequently (6, 23). Therefore, it would not be right to talk about a special predisposition.

The main cause of the splint bones is direct trauma and hyperextension injuries but in racehorses, acute overload takes an important role in the etiology (2, 5, 18-21). The most common type of fracture is the fractures localized to the distal region. In this study, distal splint bone fractures constituted 80% of the cases. Although most reported etiology for these fractures is external trauma such as kicking, usually uncomplicated closed fractures are encountered.

Two types of treatment options have been reported for distal fractures which are conservative and surgical treatments, respectively (8, 10, 12-14). Different fractures of forearm bones in foals can be treated with modified Thomas splint applications (11). Suspensor desmitis risk is mentioned as a complication after both treatment options. In addition, in cases where a comminuted fracture is formed in the splint bone, exuberant callus formation caused by excessive movement in the fracture line are also mentioned (8, 10, 12-14). In the long-term, excessive exuberant callus compresses the surrounding soft tissues and results with local pain and lameness. In the distal region fractures, because the bone is not thick enough to allow internal fixation, bone plating is not possible as in proximal fractures. Therefore, the best option for distal fractures is to remove the free distal fragment with a minimal invasive limited open surgical approach (8, 13, 14). Considering the risks mentioned above, we performed the amputation of the free distal fragment in all cases and did not encounter complications such as suspensory desmitis or exuberant callus formation due to early admission to the clinic. In this study, complications such as osteomyelitis or sequesterization were not encountered since all fractures in this study were closed fractures and the patients were brought to the clinic in a short time following the formation of the fractures.

Open fractures are most frequently observed in the proximal region and osteomyelitis is inevitable in cases that early intervention is not performed. After the bone infection takes shape, both the treatment to be performed is more difficult and the possibility of the patient regaining his old athletic performance decreases (8, 9, 19, 23, 24). Long-term administration of antibiotics following sequesterectomy may not always result in a successful recovery. Therefore, open fracture cases should be intervened as soon as possible. Meticulous lavage is a must after shaving, asepsis and antisepsis of the area. After each application, the area should be taken into wet dressing in order to protect it from external factors, and the patient owners should be informed that the treatment period may be prolonged (6, 8, 13, 20).

In conlusion, splint bone fractures, especially in the distal region, can be successfully treated with early intervention and minimally invasive surgery. In cases where conservative treatment will be tried, possible suspensor ligament desmitis and the risk of exuberant sequestration should always be considered.

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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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Very cool white LED light improves reproductive performance and up-regulates expression of reproductive genes in layers

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Abstract: This study was carried out to evaluate the effect of light color temperature on reproductive performance and the expression pattern of GnRH-1 and FSH β genes in layers. At lay, 165 Fayoumi healthy pullets were separated into well ventilated, environmentally controlled rooms. Birds were allocated into three groups with 55 birds each (5 males and 50 females) till 3 months after laying, representing base generation (F0). Fertile eggs were collected and incubated, and the newly hatched chicks were also divided into three groups from the first day of life till 3 months after laying, representing the first generation (F1). In the two generations, the first group was exposed to cool white LED light (day light) (6500 kelvin); the second group was exposed to very cool white LED light (sky blue light) (10000 kelvin); and the third group was exposed to warm white LED light (yellow light) (2700 kelvin). The expression profiles of GnRH-1 and FSH β genes and the serum level of FSH were evaluated in birds of the two generations. Blue light-exposed groups exhibited better reproductive performance, higher up-regulation of GnRH-1 and FSH β genes and increased FSH levels than those exposed to either the white or yellow light colors. A comparison between F0 and F1 revealed an improved profile for F1 birds. It therefore appears that blue LED light could improve the reproductive status of laying chickens.

Keywords: Gene expression, layer, light, reproductive performance.

Introduction

Poultry production is an important component of agriculture all over the world. Recently, more attention has been given to poultry, mainly due to the quality of their meat and sustainable production (24). Chickens provide more than 67.6 percent of the daily recommended value of protein, and more than 72 percent of the daily requirement for Niacin in a 4-ounce portion for an adult human (13). Eggs are also high in nutrition and protein and are a convenient, low-cost food. Eggs contain B12, riboflavin, and choline, which have been positively linked to the development of memory functions in fetuses. Meat from native chickens is preferred by more consumers than meat from commercial broilers due to their superior taste, meat texture, low fat and cholesterol, and high protein content (36).

Light is an important environmental factor for manipulating the behavioural activity (20), reproductive characteristics (5), growth performance (40) and immunity of birds. Light-emitting diodes (LED) are very energy efficient and hence provide sufficient brightness (22). Thus, most poultry producers have replaced ICD (inductively coupled discharges) lamps with LEDs. Moreover, LEDs are potentially beneficial to the poultry industry due to their long life span, moisture resistance, and narrow spectrum (31). An important aspect of light characters that could have an impact on an animal's productivity and reproduction is light color. Light color is described by chromaticity. Chromaticity is the measure of a light source's warmth (warm light) or coolness (cool light) expressed in degrees Kelvin. The scale runs from 2000 to 7000K. Light with chromaticity values of 4000 K or higher is considered cool (as blue light), light with chromaticity values of around 3500 K or 3600 K is called neutral, and light of about 3000 K or less is considered warm (as red light) (25).

Photoreceptors of the deep brain of birds are stimulated into action only by very specific colors of light. Light is received through retinal and extra-retinal

photoreceptors. Photoreceptors are composed of large protein particles called opsins attached to an aldehyde of vitamin A, referred to as a chromophore (41). When a chromophore absorbs light, the photoreceptor goes through a conformational change, triggering a biochemical signal that is responsible for the release of neurotransmitters from the photoreceptor cell (16). Photoreceptors receive light signals and transmit them to oscillators, which control the bird's circadian rhythm via the synthesis and release of melatonin. The stimulation of photoreceptors is very important since it signals the production and release of certain hormones responsible for sexual development, aggressive behaviour and egg production (11).

GnRH1 is the gene that plays an important role in the generation of peptides responsible for the secretion of GnRH. GnRH1 is located on chromosome 8 (10). Gonadotropin-releasing hormone (GnRH) is a neurohormone produced in a specific neural cell and released at its neural terminal (35). A key area for the production of GnRH is the preoptic area of the hypothalamus. GnRH neurons originate in the nose and migrate into the brain, where they are scattered throughout the medial septum and hypothalamus and connected by very long dendrites (7). GnRH plays a fundamental role in the release of gonadotropins from the pituitary gland and is considered the primary hormone that regulates reproduction (46).

The follicle-stimulating hormone (FSH) is another fundamental hormone for reproduction. FSH is a member of the glycoprotein hormones secreted by gonadotrophs in the anterior pituitary gland. It is comprised of two dissimilar subunits, alpha and beta, encoded by separate genes (14). FSH can stimulate the proliferation of granulosa cells (9) and is required for the long-term culture of granulosa cells. Moreover, FSH plays an important role in stimulating the release of progesterone (21). In chicken, the FSH β precursor molecule consists of 131 amino acids with a signal peptide of 20 amino acids followed by a mature protein of 111 amino acids that is encoded by FSHβ cDNA, which is composed of 2,457 bp nucleotides, including 44-bp nucleotides of the 5`- untranslated region (UTR), 396 bp of the open reading frame, and a long 3`- UTR of 2,001 bp nucleotides followed by a poly (A) tail (42).

Very few studies have considered how light color temperature could affect reproductive performance and the expression pattern of reproductive genes, particularly in laying chickens (15). Moreover, there is much controversy between the findings of literature regarding this. Therefore, the objective of the present study was to quantify the effects of different light color temperatures on reproductive performance traits, and the expression pattern of GnRH and FSH genes in native Egyptian Fayoumi layers (Gallus gallus domesticus).

Materials and Methods

Experimental birds and design

Base generation (F_0): One hundred sixty-five native Egyptian Fayoumi healthy pullets at 17 weeks of age with similar body weight (900 ± 30 gram) were used in this experiment. Pullets were purchased from an experimental farm for poultry breeding in Fayoum Governorate, Egypt. All birds were housed under the same environment till the time of laying at a density of 8 birds/m^2, 12L: 12D light cycle, 67-77 % RH, and 28 °C. From the 19th week the lighting schedule was gradually increased half an hour every week till it reached 16L: 8D at laying time. At laying, 24th weeks, birds were randomly assigned into three groups of 55 birds each, in separate, well-ventilated, environmentally controlled rooms according to the light color temperature. Each room had a floor area of 9 m^2 and was used for housing 50 females and 5 males. The first group (control) was exposed to cool LED white light (daylight) (6500 kelvin). The second group (sky blue light) was exposed to very cool LED white light (10000 kelvin) and the third group (yellow light) was exposed to warm LED white light (2700 kelvin) till the end of the experiment. Light intensity was 25 lx (1.4-ft candle) during light phase and 0 lx during dark phase of the photoperiod and was recorded near the floor, approximately at the bird's height. Artificial light systems were placed 10 cm above the birds using plastic crosses attached to the ceilings of the rooms. Food and drinking water were allowed ad-libitum throughout the experimental period.

First generation (F_1): Eggs were collected daily, and egg numbers and egg weights were recorded daily for each group. Eggs were incubated at 37 °C and 70 % RH, and the newly hatched chicks (F_1) were marked using wing bands. Chicks were vaccinated using the program of vaccination of the Local Veterinary Organization. Equal number of chicks to that of F_0 was selected and used for the F_1 generation. Chicks were divided into three groups of 55 birds each (5 males and 50 females) from the first day of life similar to those of the base generation; control cool white, very cool sky-blue light and warm yellow light but were subjected to continuous artificial lighting during the first 8 weeks of age. This artificial light was decreased to 12 hours light and 12 hours dark in 17th week of age, then was gradually increased one hour/month till reached 16 hours light at the 21st week of age.

Reproductive performance traits: Fertility % and hatchability % were estimated according to the method described previously (32).

- Fertility percentage % (F %) = (No of fertile eggs/total No of eggs set) x 100.
- Hatchability % = (No of hatched chicks/total No of fertile eggs) x100.

Egg number and egg weight were estimated weekly for each group as per a previously described method (43). EW was recorded by weighing all eggs of each group using an electric digital balance with a range from 0.1 to 200 g. The mean egg number per hen was calculated by dividing total egg number of eggs per group by number of birds. Mean egg weight was calculated by dividing the total egg weight per hen per week by the number of eggs per hen per week (19). Eggs were weighed individually and recorded to the nearest 0.1 g using an electric digital balance with a range from 0.1 to 200 g. Egg length and egg width were measured using a Vernier caliper by carefully placing the internal content of an egg on a smooth glass plate to avoid rupture of the vitelline membrane. Egg shape index was calculated as egg width percent divided by length percent according to (2). Albumen weight was measured and recorded to the nearest 0.1 g using an electric digital balance. Albumen height was measured by placing a tripod at different places of thick albumen and an average of three readings was considered as albumen height. Haugh units % (HU %) were estimated according to a previous method (18).

HU = 100 log (H+7.57-1.7W $^{0.37}$) where; HU = Haugh units, H = albumen height (mm) and W = egg weight in gram.

Yolk weight was measured and recorded to the nearest 0.1 g using an electric digital balance. Yolk index % = yolk height /yolk diameter (12). Shell weight was measured and recorded to the nearest 0.1 g using an electric digital balance. Shell thickness (mm) was measured in micrometers as an average of 3 points (top, medial and base).

Sample collection: In both F_0 and F_1, tissue and blood samples were taken from all female and male birds. Tissue samples were taken from the hypothalamus and pituitary gland for RNA extraction. Samples were put in Eppendorf containing RNA later (Qiagen, Germany), to minimize the action of endogenous Rnase. Blood samples were collected without anticoagulant from wing veins into clean dry centrifuge tubes, were left to clot at room temperature and were then centrifuged at 3000 rpm for 15 min. Serum was stored at -20 ^{o}C until biochemical analysis.

RNA extraction and real-time PCR: RNA extraction was done using Rneasy Mini Kit (Qiagen, Germany), following the manufacturer protocol. RNA was treated with RNAse free-DNAse I (Qiagen, Germany), to remove any contaminating genomic DNA. The expression profile of GnRH *and* FSH genes was carried out in hypothalamus and pituitary gland respectively. The relative expression was quantified using SYBR Green PCR Master Mix (2xSensiFast™ SYBR, Bioline) and carried out according to primers described previously (49). Primer sequences and annealing temperatures are shown in Table 1. The housekeeping β-actin gene was used as an internal control. Reverse transcription of extracted mRNA and real-time PCR program schedule for each gene is illustrated in Table 2. CT values were determined by stratagene MX3005P software. To estimate the variation of gene expression in RNA of different samples, CT of each sample was compared with that of the control group according to the "ΔΔCt" method (49).

Table 1. Oligonucleotide primers sequence, accession number, annealing temperature and PCR product size of *GnRH-I, FSH-β and β-Actin* genes.

Gene	Primer (forward)	Product length (bp)	Accession number	Reference
GnRH-I	F:5-ATCTGCTTGGCTCAACACTG-3 R: 5-ATCAGGCTTGCCATGGTTTC-3	191	NM_001080877.1	(15)
FSH-β	F:5-GCCATCCTACTGCTCCTTCA-3 R:5-GCTTGGCAGTTTCTCGGTAC-3	154	NM_204257.1	(15)
β-Actin	F:5-GAGAAATTGTGCGTGACATCA-3 R:5-CCTGAACCTCTCATTGCCA-3	152	NM_205518.1	(49)

Table 2. Reverse transcription and real-time PCR program for *GnRH-I, FSH-β and β-Actin* genes.

Gene	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)		
			Secondary denaturation	Annealing	Extension	Secondary denaturation	Annealing	Final extension
GnRH-1 *FSH- β*	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	60˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	60˚C 1 min.	72˚C 1 min
β. actin	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	51˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	51˚C 1 min.	72˚C 1 min.

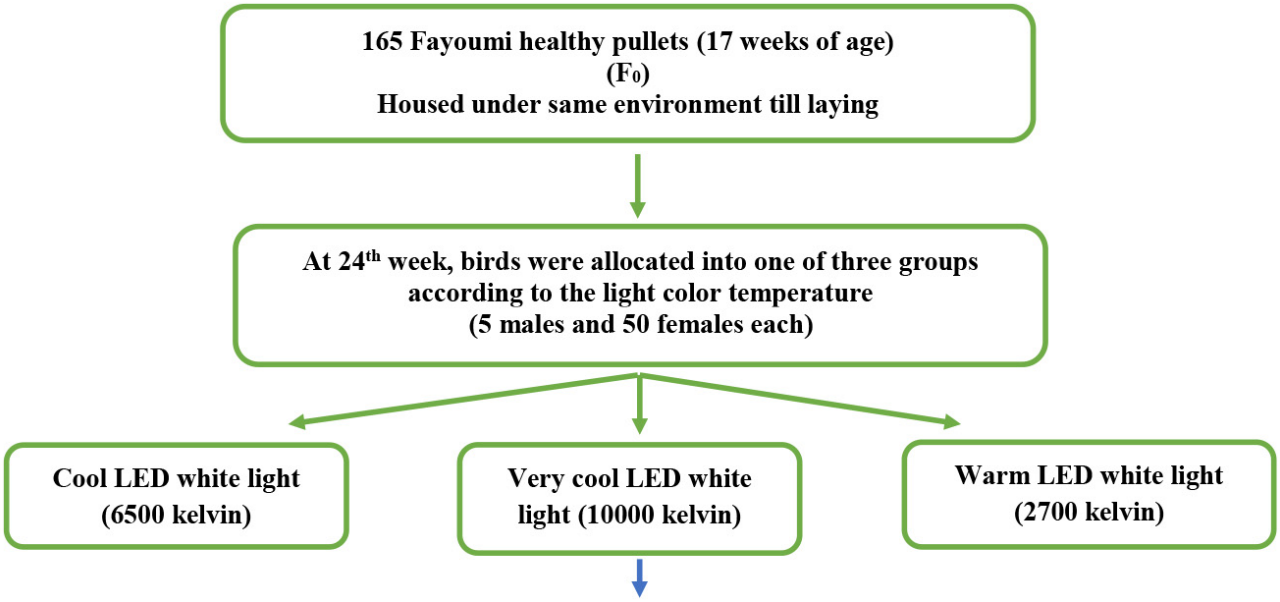

Figure 1. Experimental design, measures and samples collected from both male and female birds of base (F_0) and first (F_1) generation throughout the study.

Biochemical analysis: Serum values of FSH were determined according to the Siemens health diagnostics – USA kits protocol as previously described (3). The producer depends on using IMMULITE/IMMULITE 2000 rapid FSH, which is a solid-phase, enzyme labelled chemiluminescent immunometric assay. Experimental design, measures and samples collected from birds of both F_0 and F_1 throughout the study period are presented in Figure 1.

Data analysis: Data were tested for distribution normality, linearity and homogeneity of variance. Data were analysed using SPSS (Statistical Package for Social Science) version 12 and all results are reported as means ± SEM. Analysis of egg number, egg weight, egg mass, fertility %, hatchability % and egg quality traits were performed using General Linear Model (GLM) procedures-repeated measures. Remaining data were analysed using one-way analysis of variance (ANOVA) to test all groups unpaired values. Duncan Multiple Range Test was used to separate means among the treatment groups. Differences were considered to be significant at the level of ($P \leq 0.05$).

Results

Effect of light color temperature on reproductive traits: Females of base (F_0) and first (F_1) generations under different color light temperatures were evaluated for the following traits; egg number, egg weight, egg mass, fertility %, hatchability %, and egg quality traits including; egg shape index % (ESI), albumen weight, Haugh unit, yolk weight, yolk index %, shell weight and shell thickness (Table 3). Birds of blue light-exposed group exhibited a better performance for all reproductive traits compared to those of white light and yellow light-exposed groups. Comparison between F_0 and F_1 revealed that females of F_1 had a better reproductive performance than those of F_0.

Effect of light color temperature on expression pattern of reproductive genes*:* The effect of light color temperature on expression pattern of reproductive genes (*GnRH-1* and FSH β) was investigated in both males and females of F_0 and F_1 (Figure 2). Blue color light-exposed groups exhibited a significant up-regulation of FSH β and *GnRH-1* in both males and females compared to white (control) and yellow color light-exposed groups. Comparison of F_0 and F_1 generations demonstrated that F_1 had a higher up-regulation of FSH β and *GnRH-1* genes than F_0 in both males and females.

Effect of light color temperature on serum biochemical analysis: The effect of light color temperature on serum profile of FSH was explored in both males and females of F_0 and F_1 (Figure 3). Blue color light-exposed groups exhibited a significant increase in serum FSH values in both males and females compared to white (control) and yellow color light-exposed groups. Comparison of F_0 and F_1 generations elucidated that F_1 generation had higher values of FSH than F_0 in both males and females.

Table 3. Mean (± SE mean) reproductive performance parameters of female Fayoumi layers reared under monochromatic cool white (Control), very cool white (Blue sky), warm white light (Yellow) at base (F_0) and first (F_1) generation.

Group	Cool white (Control)		Very cool white (Blue sky)		Warm white (Yellow)	
Traits	F_0	F_1	F_0	F_1	F_0	F_1
	Means ± SE					
Egg number	$1.97^{bB} \pm 0.32$	$3.88^{bA} \pm 0.18$	$4.82^{aA} \pm 0.54$	$4.93^{aA} \pm 0.15$	$2.26^{bB} \pm 0.37$	$3.94^{bA} \pm 0.26$
Egg weight	$36.58^{aB} \pm 0.42$	$51.7^{aA} \pm 0.10$	$37.84^{aB} \pm 0.19$	$55.1^{aA} \pm 0.06$	$37.94^{aB} \pm 0.45$	$42.3^{bA} \pm 0.04$
Egg mass (g)	$72.06^{cB} \pm 0.40$	$200.59^{bA} \pm 0.09$	$182.33^{aB} \pm 0.23$	$271.60^{aA} \pm 0.32$	$85.74^{bB} \pm 0.25$	$166.66^{cA} \pm 0.13$
Fertility %	$87.00^{bA} \pm 0.57$	$89.9^{bA} \pm 0.66$	$98.87^{aA} \pm 0.53$	$100.00^{aA} \pm 0.66$	$90.06^{bA} \pm 0.50$	$93.3^{bA} \pm 0.60$
Hatchability %	$92.6^{bA} \pm 0.15$	$93.03^{bA} \pm 0.70$	$99.00^{aA} \pm 0.09$	$100.00^{aA} \pm 0.55$	$97.33^{aA} \pm 0.40$	$98.76^{aA} \pm 0.55$
Egg shape index % (ESI)	$76.85^{aA} \pm 0.82$	$76.65^{aA} \pm 0.37$	$74.14^{aB} \pm 0.36$	$75.00^{aA} \pm 0.24$	$75.14^{aA} \pm 0.55$	$76.40^{aA} \pm 0.86$
Albumen weight (g)	$21.00^{bA} \pm 0.87$	$22.00^{bA} \pm 0.82$	$21.14^{bA} \pm 0.40$	$22.13^{bA} \pm 0.49$	$25.56^{aA} \pm 0.56$	$26.56^{aA} \pm 0.68$
Haugh unite % (HU)	$81.91^{abA} \pm 0.22$	$82.91^{abA} \pm 0.22$	$84.75^{aA} \pm 0.23$	$85.75^{aA} \pm 0.23$	$81.14^{bA} \pm 0.60$	$81.64^{bA} \pm 0.39$
Yolk weight (g)	$15.57^{aA} \pm 0.47$	$16.57^{aA} \pm 0.34$	$15.42^{aA} \pm 0.75$	$15.80^{aA} \pm 0.75$	$14.42^{aA} \pm 0.49$	$15.42^{aA} \pm 0.42$
Yolk index %	$49.00^{aA} \pm 0.75$	$49.80^{aA} \pm 0.56$	$52.85^{aA} \pm 0.75$	$52.80^{aA} \pm .57$	$51.85^{aA} \pm 0.68$	$50.85^{aA} \pm 0.56$
Shell weight (g)	$6.14^{aA} \pm 0.34$	$6.05^{aA} \pm 0.27$	$6.57^{aA} \pm 0.20$	$6.90^{aA} \pm 0.30$	$5.00^{bA} \pm 0.20$	$5.00^{bA} \pm 0.30$
Shell thickness (mm)	$1.00^{bA} \pm 0.10$	$1.00^{bA} \pm 0.10$	$1.42^{aA} \pm 0.20$	$1.50^{aA} \pm 0.20$	$1.00^{bA} \pm 0.23$	$1.00^{bA} \pm 0.60$

Means of different levels within the same column having different superscripts are significantly different (P<0.05). Small letter indicates a significant difference between groups at the same generation. Capital letter indicates a significant difference between generations.

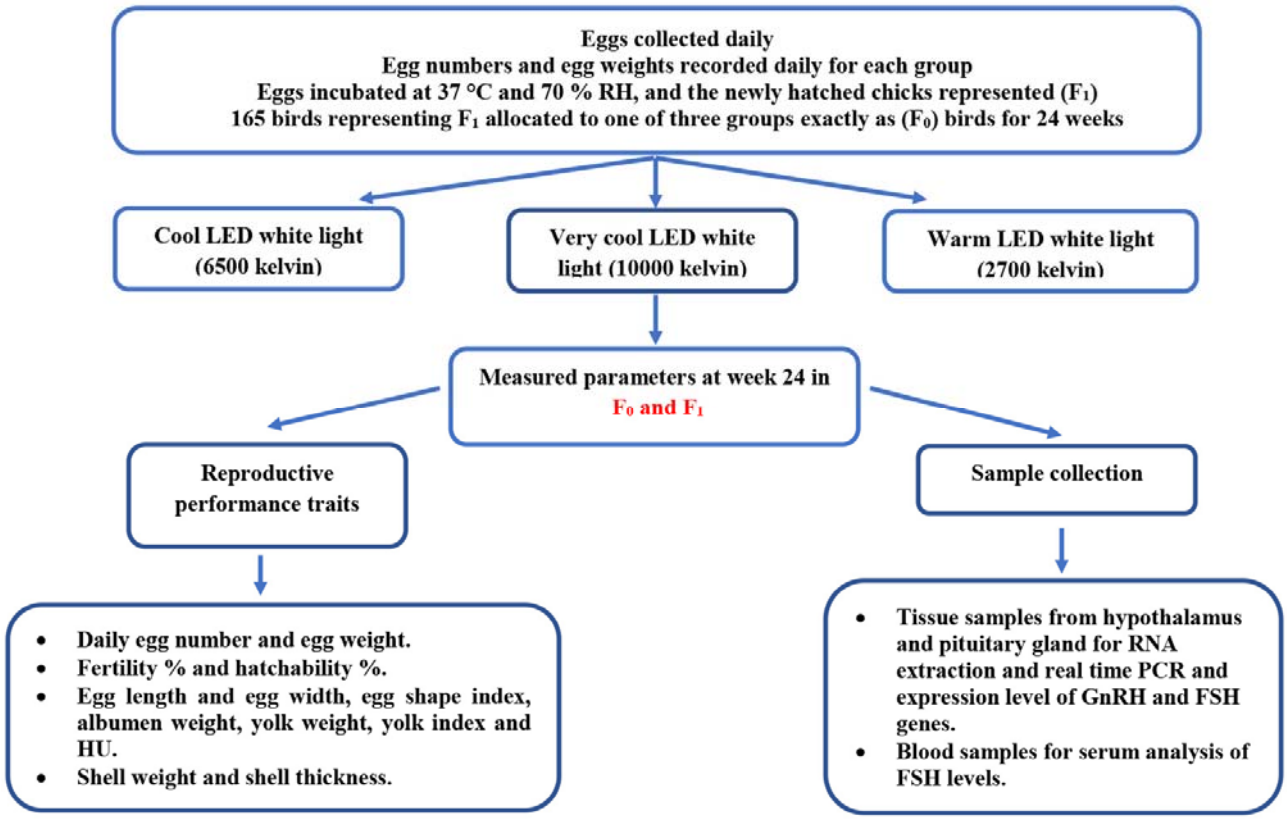

Figure 2. Relative expression of *FSH β* (a) and *GnRH1* (b) in both males and females of base (F_0) and first (F_1) generation. Small letter indicates a significant difference (P<0.05) between groups at the same generation. Capital letter indicates a significant difference (P<0.05) between generations.

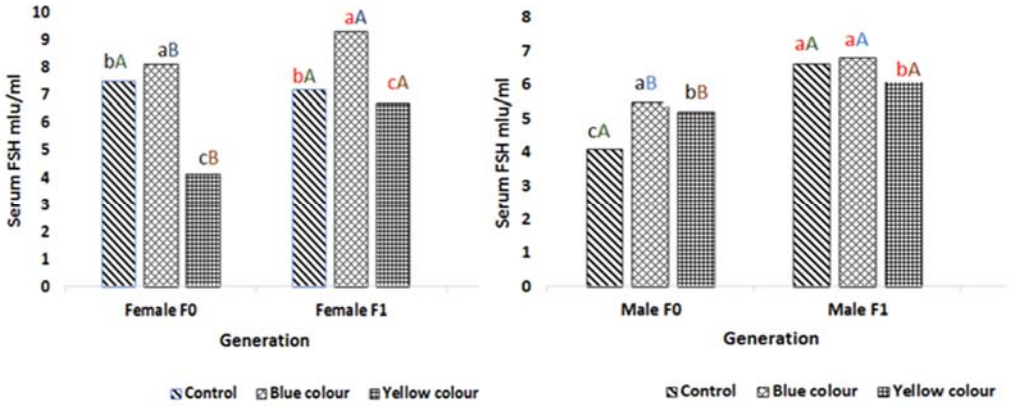

Figure 3. Serum levels of FSH in both males and females of base (F_0) and first (F_1) generation. Small letter indicates a significant difference ($P<0.05$) between groups at the same generation. Capital letter indicates a significant difference ($P<0.05$) between generations.

Discussion and Conclusion

Poultry has a major role to play in developing countries because it is relatively inexpensive and widely available. Chickens are the most popular poultry worldwide irrespective of culture and religion (1). This is because poultry products have a very high nutritive value and a low detrimental impact on the environment than other livestock and uses less water (44). Layers have been submitted to genetic improvement to produce more eggs at lighter body weight and with a lower feed intake. So, layer farms need to supply high nutritional requirements of birds and to apply management practices that can adapt to automated and environmentally controlled facilities, and high stocking densities (33). In the current study, the effects of light color temperature on the reproductive performance traits were investigated in F_0 and F_1 Fayoumi layers. The results of this study showed that very cool LED light improves reproductive performance traits of laying hens, serum FSH levels and up-regulation of mRNA level of *GnRH-1* and *FSH β* genes. The effects of light on reproductive performance may be related to the wavelength of light. One reason for the improved reproductive performance of birds experienced blue LED color could be their improved behaviour and temperament e.g., calming effect of the blue light *perse*. It has been demonstrated that birds reared under blue LED light were less active, less aggressive, less fearful and more docile compared to those reared under other light color environments (30). This reduced level of activity and fear might have redirected energy expenditure towards efficient reproductive traits. This could be supported by the fact that chickens demonstrated higher preferences for both blue and green light than for red and white light (34). However, the improved reproductive performance of laying chickens reared in monochromatic blue LED light could be also due to the increased estradiol and progesterone levels, and the decreased levels of melatonin hormone. It has been shown that blue light decreased plasma concentrations of melatonin, and increased plasma concentrations of estradiol and progesterone at 28 wk. of age in Erlang Mountainous Chinese laying hens (27). It could also be due to the up-regulated expression of reproductive genes and the increased serum levels of FSH as illustrated in our findings.

Findings of previous studies that investigated the effects of light color temperature on reproductive performance of chickens demonstrated controversial results. On one hand, some experiments concluded chicken who experienced blue color light demonstrated improved reproductive performance compared to those who received either white or yellow color light. Improved reproductive performance due to blue light included higher egg weights (17), higher levels of egg production (27, 28), and extended peak laying (27). On the other hand, other experiments showed that reproductive performance of birds represented by higher egg numbers was improved when birds were reared in red color light compared to other colors of lights (47). Other research has given the advantage of improving reproductive performance e.g., fertility and hatchability to white light rather than red, yellow or green (48). However, other studies observed no differences in productive performance of chicken e.g., egg weights and egg numbers (26), or fertility and hatchability (8) under different color lights. Others have reported that egg weight depended particularly on hen's age and nutritional factors rather than on light color (6). One reason for the controversy between results reported in the current experiment and those of previous work could be differences in light source, light color temperature, species/strain of birds, light intensity, and number of generations of birds investigated.

Results of the current experiment demonstrated also that very cool white LED (blue) light up-regulated *GnRH-1* and *FSH β* gene expression in both males and females of F_0 and F_1. To the best of our knowledge, the effect of light color on the expression pattern of reproductive genes in

layers has received little investigation. However, beneficial effects have been attached to the blue monochromatic LED light on reproductive traits and expression patterns of estradiol (ERα and ERβ) mRNA and progesterone mRNA in granulosa layers (27). Extra-retinal photo-stimulation combined with non-photo-stimulatory conditions to the retina caused a significant elevation in mRNA expression of hypothalamic *GnRH-I*, pituitary LH and FSH, plasma LH and gonadal steroids in broiler breeders (38). Similarly, *GnRH-I* mRNA expression in female turkey was sensitive to light stimulation during the photosensitive period (23) and was therefore used to characterize their different reproductive stages.

On the other hands, other experiments, despite lacking blue LED light in its lighting schedule, have reported a higher hypothalamic *GnRH-I* mRNA expression was observed in birds under red light had than those exposed to green light in white leghorn birds and broiler breeder hens (Cobb) respectively (29, 37). However, light source was not found to impact gene expression of *GnRH-I* and *FSH-β* in chickens (4).

Results of the current study also indicated that very cool white LED light (blue) elevated serum levels of FSH in both males and females of F_0 and F_1. This increase in FSH levels could be a reflection to the increased expression level of *GnRH-1* and *FSH β* gene. However, it could have also resulted as a reflection to the increased levels of estradiol and progesterone, and the upregulated expression patterns of estradiol and progesterone mRNA. Similar findings of increased serum FSH under monochromatic blue LED light have been previously demonstrated (26, 39, 45).

Light color temperature has a remarkable effect on reproductive performance of laying chickens particularly Egyptian Fayoumi layers. Blue LED light improved bird reproductive performances via increasing reproductive performance traits, serum FSH levels and up-regulation of mRNA level of reproduction-related genes. These findings recommend blue LED light in farms of layer chicken to improve their reproductive performance. Further studies should be carried out to investigate the effects of light color temperature on other chicken breeds. An expression profile of other reproductive genes is also needed to understand their regulation mechanisms on other productive and reproductive traits.

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Ethical Statement

The collection of samples and care of birds used in this study followed guidelines of Mansoura University and the protocol of the study and the protocol of the study was approved by the Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University.

Conflict of Interest

The authors declare no conflicts of interest.

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Molecular, biochemical, and histopathological effects of long-term low and high-percentage fructose consumption on the liver in rats

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Abstract: The aim of this study was to investigate the lipogenic and inflammatory effects of low and high percentage fructose solutions in rats. Wistar albino rats were fed with fructose solutions for 10 weeks. The groups were as follows: Cont (Control), F15 (Fructose 15%), F30 (Fructose 30%), and F60 (Fructose 60%). Rats' body weights were measured weekly. Also, lipogenic and inflammatory gene expression levels, biochemical parameters, and histopathological changes in the liver were investigated. After 10 weeks, it was observed that the animals in the F60 were the heaviest, while the animals in the F30 were the lightest. In all experimental groups, triglycerides were significantly higher than those of controls ($P<0.05$). In F30 and F60, *TNFα*, *IL-6,* and *IL-1β* were upregulated in the liver compared to control ($P<0.05$). In addition, *SREBP-1c, ChREBP, FAS, ACACA,* and *SCD-1* were upregulated in all fructose feeding groups compared to Cont ($P<0.05$). The livers of rats in the F30 and F60 groups had degenerative changes and steatosis. The most detrimental effects of fructose were observed in F60. The concentration of fructose was found to be a very important factor for maintaining normal liver physiology at the molecular level.
Keywords: Fatty liver, fructose, inflammation, lipogenesis, NAFLD.

Ratlarda uzun süreli düşük ve yüksek doz fruktoz tüketiminin karaciğerde moleküler, biyokimyasal ve histopatolojik etkileri

Özet: Bu çalışmanın amacı ratlarda düşük ve yüksek konsantrasyondaki fruktoz solüsyonlarının lipojenik ve inflamatuar etkilerini araştırmaktır. Çalışmada Wistar albino ratlar 10 hafta boyunca fruktoz solüsyonları ile beslenmiştir. Gruplar: Kont (Kontrol), F15 (Fruktoz %15), F30 (Fruktoz %30), F60 (Fruktoz %60) şeklinde olmuştur. Ratların vücut ağırlıkları haftalık olarak ölçülmüştür. Ayrıca lipojenik ve inflamatuvar genlerin ekspresyon seviyeleri, biyokimyasal parametreler ve karaciğerdeki histopatolojik değişiklikler araştırılmıştır. F60 en hafif grupken, F30 en ağır grup olarak belirlenmiştir. Trigliserit seviyeleri tüm deneme gruplarında Kont grubundan önemli ölçüde yüksek olmuştur ($P<0,05$). F30 ve F60'da, *TNFα*, *IL-6* ve *IL-1β* gen ekspresyon seviyelerinin Kont grubuna kıyasla arttığı belirlenmiştir ($P<0,05$). Bununla birlikte tüm fruktoz gruplarında *SREBP-1c*, *ChREBP*, *FAS*, *ACACA* ve *SCD-1* Kont grubuna kıyasla artmıştır ($P<0,05$). F30 ve F60 gruplarında karaciğerde dejeneratif değişiklikler ve steatoz belirlenmiştir. Fruktozun en zararlı etkileri F60 grubunda gözlenmiştir. Fruktoz konsantrasyonunun, normal karaciğer fizyolojisini moleküler seviyelerde sürdürmek için çok önemli bir faktör olduğu belirlenmiştir.
Anahtar sözcükler: Fruktoz, inflamasyon, lipogenez, NAFLD, yağlı karaciğer.

Introduction

Regulation of energy metabolism is controlled by numerous complex mechanisms. Obesity and diabetes have become global problems, causing high costs in healthcare today, as they also trigger cancer and cardiovascular diseases (29, 33). Studies on the metabolism have reported that simple sugars, particularly fructose, found in diets are major risk factors in developing metabolic diseases (10). The most important source of fructose is high fructose corn syrup (HFCS), which is used in soft drinks and many packaged foods with different percentages of fructose. (31). Fructose is metabolized independently of insulin and most of the fructose is transferred to the liver through the portal vein (35). The fructose-rich diet increases the levels of proteins involved in de novo lipogenesis formation in the liver

compared to oil-rich diets (31, 33). Non-alcoholic Fatty Liver Disease (NAFLD) may be caused by eating too much fructose, according to a report (22).

NAFLD is characterized by the storage of fat in the liver and is not dependent on intensive alcohol consumption. Excessive fructose consumption has been identified as the primary cause of this disorder (35).It has also been reported that chronic fructose consumption can trigger inflammation as well as fatty liver, leading to irreversible liver disorders (35). Chronic fructose consumption has been shown to induce lipid synthesis by triggering SREBP-1c (Sterol Regulatory Element Binding Transcription Factor 1c), ChREBP (Carbohydrate-Responsive Element-Binding Protein) transcription factors and thus target genes such as FAS (Fatty Acid Synthase), ACACA (Acetyl-CoA Carboxylase Alpha), and SCD-1 (Stearoyl-CoA Desaturase) (14).

Scientific debates on fructose consumption have been ongoing. However, molecular studies on the rate of fructose in diets are limited. In general, variable fructose solutions (mostly 10-30%) are used for the investigation of the metabolic effects of fructose on laboratory animals (2, 7). In this study, besides standard chow, consumption of 15%, 30%, and 60% concentration fructose solutions for 10 weeks was examined at the biochemical and histopathological levels. Expression levels of TNFα (Tumor Necrosis Factor Alpha), IL-1β (Interleukin 1 Beta) and IL-6 (Interleukin 6) genes in the inflammatory pathway and SREBP-1c, ChREBP, LXRα (Liver X Receptor alpha), FAS, ACACA, and SCD-1 genes in the lipogenesis pathway in the liver were also investigated.

Materials and Methods

Animals and experimental protocol: The study was ethically approved by the Animal Experiments Local Ethics Committee of Hatay Mustafa Kemal University (Decision number: 2018/2-8). Four groups were formed using 32 male Wistar albino rats. The rats were allocated in such a way that there was no statistical difference between the groups in terms of body weight averages (n = 8). The groups were as follows: Cont (Control group, standard chow), F15 (Fructose 15%, standard chow + 15% fructose solution), F30 (Fructose 30%, standard chow + 30% fructose solution), F60 (Fructose 60%, standard chow + 60% fructose solution). Rats in the Cont group consumed standard chow and water (31). On the other hand, standard chow and 15%, 30%, and 60% fructose solutions were presented to F15, F30, and F60 group rats, respectively. The feeding period was maintained for 10 weeks, and during the feeding period, the ambient light was set to be bright for 12 hours and dark for 12 hours (07:00–19:00 bright, 19:00–07:00 dark), and the humidity and ambient temperature were set at 55% and 21 ± 2 °C, respectively.

Euthanasia, blood, and tissue sample collection: At the end of the feeding period, the rats were fasted for 12 hours and euthanized by taking blood from their hearts under anesthesia (80 mg/kg Ketamine and 12 mg/kg Xylazine, IP). The liver tissues were divided into two pieces for molecular and histopathological analysis. Pieces taken for molecular analysis were frozen in liquid nitrogen and stored at - 86 °C. The other pieces were fixed with 10% formalin for histopathological analysis. Plasma analysis: Blood samples were centrifuged for 15 min at + 4 °C and plasma samples were stored at - 86 °C for analysis of glucose, insulin, HDL, LDL, total cholesterol, and triglycerides. While all biochemical parameters were detected with an auto-analyzer (Gesan Chem 200, Italy), insulin levels were determined with an ELISA reader (Multiskan GO Microplate Spectrophotometer, ThermoFisher Scientific, USA) using a rat-specific ELISA kit (SunRed Bio, China).

RNA isolation and cDNA synthesis: Total RNA isolation was performed according to the Trizol method (32). Following chloroform, isopropyl alcohol, and ethyl alcohol stages, RNA pellets were allowed to dry for about 10 min at room temperature as stated by the protocol of the TRI-Reagent kit (Sigma-Aldrich, USA, Cat. No: T9424). Pellets were diluted with 30-100 µL nuclease-free water (NFW). The purity and concentration of the samples were determined by the nucleic acid meter (Merinton, SMA-1000). Prior to cDNA conversion, samples were treated with DNase I (Thermo Fischer Scientific, USA) for possible genomic DNA contamination. For cDNA synthesis, the thermal cycler (Biorad T100, USA) protocol was 60 min at 42 °C, 5 min at 25 °C, and 5 min at 70 °C. The final volumes of cDNA samples were completed to a volume of 200 µL with NFW. RT-qPCR analysis: Expression levels of target genes were determined by qPCR (Rotorgene Qiagen, USA). The qPCR protocol was as follows: After 10 min for denaturation, 15 sec at 95 °C, 60 sec at 60 °C and 30 sec at 72 °C and 40 cycles. PPIA was used as an internal control, and all samples were studied in triplicate. A kit containing SYBR Green Dye was used for amplification of genes (Power SYBR Green PCR Master Mix, ThermoFisher Scientific, USA). The primers for target genes were checked at NCBI-Primer Blast and then used for amplification (Table 1).

Histopathological analysis: The fixed tissues were washed overnight to remove formalin under tap water. The routine tissue process was followed. Then, tissues were embedded in paraffin, sectioned at 5µm thickness from each block and deparaffinized in xylol, then passed through a series of 100%, 96%, 80%, and 70% alcohol, respectively. After the tissues were stained with Hematoxylin and Eosin (H&E), microphotographs (Olympus DP12) of the tissues were obtained under a light microscope (Olympus CX31) (23).

Table 1. Forward and reverse sequences of genes primers for qPCR.

Genes	Forward and Reverse Primer Sequences	Reference
PPIA	F: 5'-CAGACAAAGTTCCAAAGACAGCA-3' R: 5'-CACCCTGGCACATGAATCCT-3'	(8)
FAS	F: 5'-GCTGCTACAAACAGGACCATC-3' R: 5'-TCCACTGACTCTTCACAGACCA-3'	(26)
ACACA	F: 5'-CAATCCTCGGCACATGGAGA-3' R:5'-GCTCAGCCAAGCGGATGTAGA-3'	(11)
SCD-1	F: 5'-CCTTAACCCTGAGATCCCGTAGA-3' R: 5'-AGCCCATAAAAGATTTCTGCAAA-3'	(37)
ChREBP	F: 5'-CGGGACATGTTTGATGACTATGTC-3' R: 5'- AATAAAGGTCGGATGAGGATGC-3'	(13)
SREBP-1c	F: 5'-GCAACACTGGCAGAGATCTACGT-3' R: 5'-TGGCGGGCACTACTTAGGAA-3'	(13)
LXRα	F: 5'-CCTGATGTTTCTCCTGACTC-3' R: 5'-TGACTCCAACCCTATCCTTA-3'	(27)
TNFα	F: 5′-GGCATGGATCTCAAAGACAACC-3′ R: 5′-CAAATCGGCTGACGGTGTG-3′	(4)
IL-1β	F:5′-ACAAGGAGAGACAAGCAACGAC-3′ R: 5′-TCTTCTTTGGGTATTGTTTGGG-3′	(4)
IL-6	F: 5'-TGATGGATGCTTCCAAACTG-3' R: 5'-GAGCATTGGAAGTTGGGGTA-3'	(19)

Histopathological findings were evaluated according to the following criteria: Grade 0: Histopathological changes below 5%; Grade 1: Slight histopathological changes in between 5% and 33% of the total area; Grade 2: Moderate histopathological changes in 33% to 66% of the total area; Grade 3: Severe histopathological changes in more than 66% of the total area (12).

Statistical analysis: For statistical calculations, the SPSS package program (Version 22.0) was used. Before performing the statistical analysis, the variables were examined using the Shapiro-Wilk test for normality and the Levene test for homogeneity of variance as parametric test assumptions. Differences between groups were determined by One-Way ANOVA and the Tukey test. $P<0.05$ was used as the cutoff for significance. For gene expression calculations, the method of $2^{-\Delta\Delta Ct}$ was used and the results were given as fold change (21). Peak values in histopathological findings were taken as score averages in groups.

Results

The consumption of chow, water, and fructose solution and amounts of consumed energy were given in Table 2. Compared to F60, body weight averages (BW) in F15 and F30 increased at the end of the first week ($P<0.05$). The third week was the first week in which a significant difference occurred between F60 and the other groups in terms of BW. This significant difference continued until the 8th week. The BW in the F15 and F60 were similar from the 8th week to the end of the study. However, the F30 was found to have the heaviest animals, while the rats in the F60 were the lightest animals (Table 2, Figure 1a-d).

The highest liver weight average was found in F30 ($P<0.05$). LDL was highest with the approximately 20 mg/dL levels in F60 ($P<0.01$). While triglyceride was approximately 50 mg/dL in the Cont, it was two folds more in the fructose fed groups ($P<0.05$). Glucose, insulin, HDL and total cholesterol levels were similar in all groups.

Isolated total RNA purity (A260/A280: 1.94 ± 0.02) and concentration (322.57 ± 26.02 ng/µL) were appropriate quality. *TNFα*, *IL-6, and IL- 1β* genes expression levels were upregulated in F30 and F60 ($P<0.05$) (Figure 2a, Figure 2b, Figure 2c, respectively). While *LXRα* was similar (Figure 2f), *SREBP-1c* and *ChREBP* were upregulated all fructose feeding groups (Figure 2d, Figure 2e) ($P<0.05$). In addition, *FAS, ACACA* and *SCD-1* levels were found to increase at varying levels (Figure 2g, Figure 2h, Figure 2i, respectively).

Macroscopic examination showed that the livers of rats in the F30 and F60, which were more severe in the F60, were swollen, dark red in color and the edges were blunt. Moreover, there was blood on the cross section of the liver.

The histopathologic injury scores of the liver was given in Table 3. Hepatocytes, Remark cords, and hepatic central vein of Cont and F15 were in the normal histological structure (Figure 3a, 3b and 3c). In F30, Remark cords in some areas were detected to lose normal regularity. There were fatty changes which characterized by large empty vacuoles of sharp borders and degenerative

changes from parenchyma to hydropic in the cytoplasm of hepatocytes in focal areas (Figure 3d). In F30, portal veins and sinusoidal regions enlarged due to erythrocyte deposition (passive hyperemia). Moreover, mononuclear cell infiltrations were observed as small foci, especially in periportal areas (Figure 3e). In F60, dissociation in Remark cords was observed. Comparing to F30, larger fat vacuoles with diffuse distribution (steatosis) were detected (Figure 3f). Similarly, more severe passive hyperemia and mononuclear cell infiltration were observed especially in perivascular regions compared to F30 (Figure 3g). Hydropic degeneration in hepatocytes was present especially in periportal regions (Figure 3f, 3h). Focal necrosis of 2 rats was noted in F60, as well as necrotic changes in some hepatocytes (Figure 3i).

Table 2. Average of body weights (g), consumption of chow (g/rat) and water/solution (mL/rat) in groups.

Wks	Tra	Cont	F15	F30	F60	P
0	**BW**	209.38±7.50	208.50±7.89	206.88±9.82	206.75±9.75	-
	BW	254.04±11.16^{ab}	261.31±7.99^{a}	260.05±10.67^{a}	220.36±9.95^{b}	*
1	**Ch**	253.13	160.72	144.38	80.00	
	W/S	426.25	375.56	277.22	177.22	
	BW	296.12±14.22	297.16±8.52	297.78±14.14	255.55±13.88	-
2	**Ch**	248.50	156.78	143.22	86.67	
	W/S	471.25	405.56	298.33	202.78	
	BW	338.63±14.40^{a}	352.21±6.63^{a}	348.24±14.22^{a}	276.40±15.95^{b}	**
3	**Ch**	256.38	171.11	152.22	89.56	
	W/S	463.13	528.89	345.00	196.11	
	BW	369.15±16.00^{ab}	380.10±7.68^{a}	387.11±14.55^{a}	320.51±13.60^{b}	**
4	**Ch**	258.90	150.21	146.62	99.56	
	W/S	471.25	541.11	327.22	216.67	
	BW	394.19±18.57^{ab}	401.18±5.28^{a}	417.19±14.42^{a}	342.13±14.28^{b}	**
5	**Ch**	270.63	153.33	137.38	93.16	
	W/S	481.25	443.89	348.89	212.22	
	BW	418.61±19.94^{a}	425.58±6.35^{a}	436.85±15.74^{a}	359.89±15.38^{b}	**
6	**Ch**	260.29	138.72	134.67	87.56	
	W/S	438.13	685.00	335.00	221.67	
	BW	425.03±19.04^{ab}	444.93±5.42^{a}	457.00±16.14^{a}	376.30±15.89^{b}	**
7	**Ch**	265.06	152.80	128.44	90.71	
	W/S	482.50	598.33	290.56	211.11	
	BW	439.91±21.31^{ab}	454.98±5.80^{ab}	463.73±16.68^{a}	393.61±16.92^{b}	*
8	**Ch**	251.13	134.56	125.56	88.56	
	W/S	480.63	524.44	350.00	211.67	
	BW	458.15±23.41	468.79±6.65	485.15±17.33	423.44±15.43	-
9	**Ch**	247.71	139.10	115.91	78.30	
	W/S	465.63	638.33	327.78	215.00	
	BW	460.56±23.15^{ab}	475.93±4.98^{ab}	494.00±17.51^{a}	424.15±15.72^{b}	*
10	**Ch**	273.44	145.61	127.31	85.62	
	W/S	500.00	805.56	391.67	252.78	

$^{a, b}$: Means with different letters in rows differ significantly, **Wk**: Weeks, **Tra**: Traits, **BW**: Body Weights, **W/S**: Water/Solution, **Ch**: Consumption of chow, -: P>0.05, *: P<0.05, **: P<0.01.

Table 3. Histopathological findings in liver.

Histopathological Changes	Cont	F15	F30	F60
Inflammation	0	0	1	1
Passive hyperemia	0	0	1	2
Fatty degeneration	0	0	1	2
Hidropic degeneration	0	0	0	1
Necrose	0	0	0	1

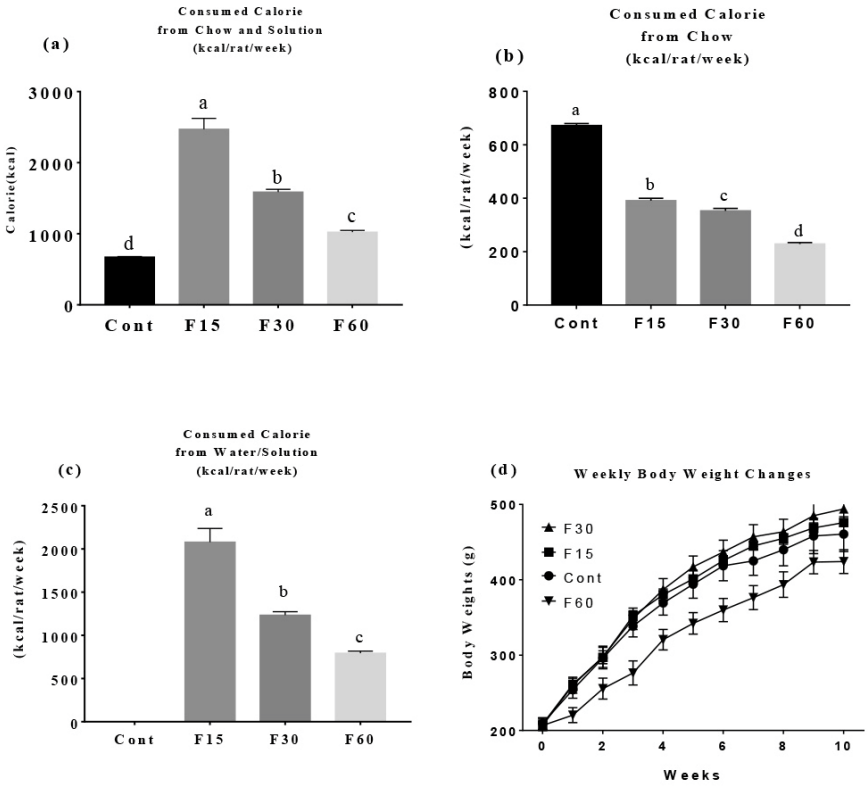

Figure 1. Average weekly consumed calorie in groups.
a) Weekly consumed total calorie (from chow + solution); **b)** Weekly consumed calorie from Chow; **c)** Weekly consumed calorie from Solution (In Cont group, there is no any fructose in water); **d)** Weekly body weight changes in groups.

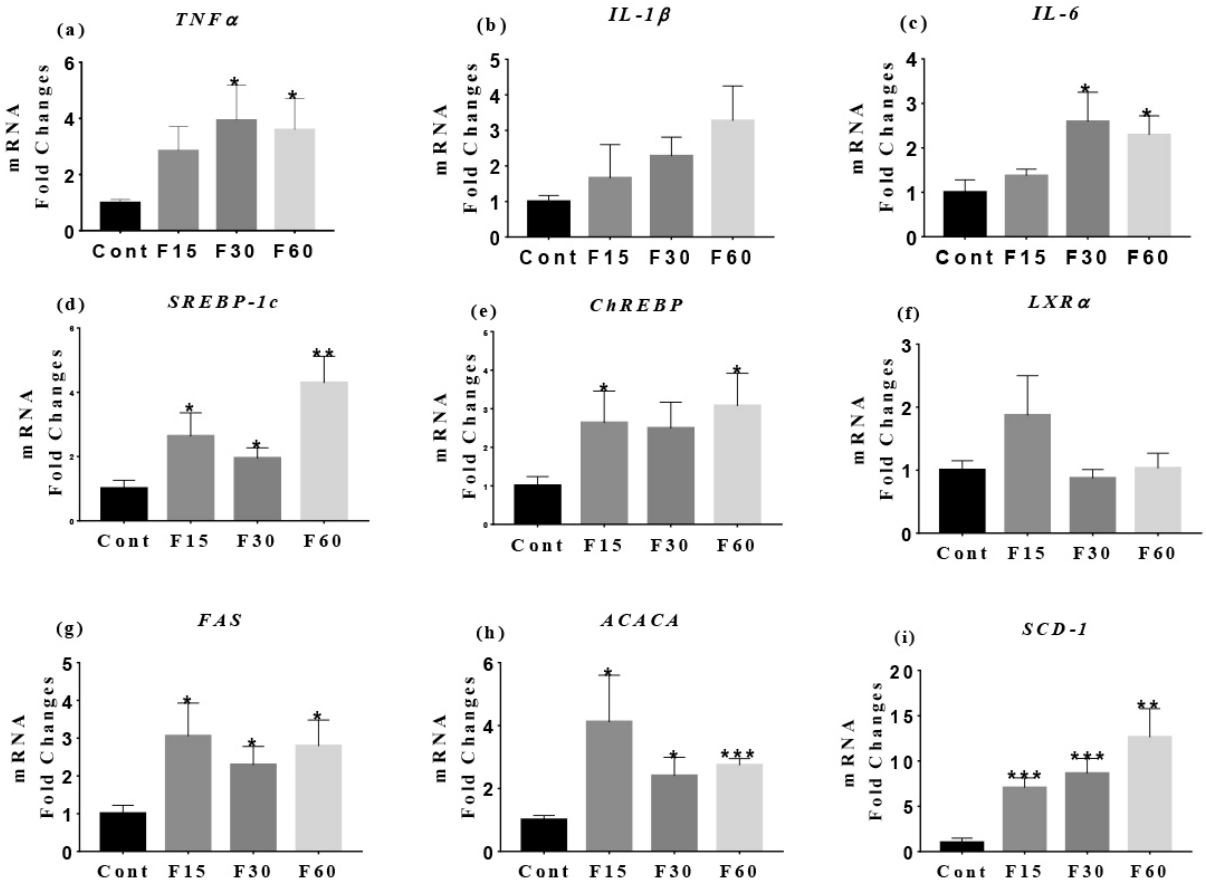

Figure 2. mRNA fold changes of genes in liver. *: P<0.05, **: P<0.01, ***: P<0.001.

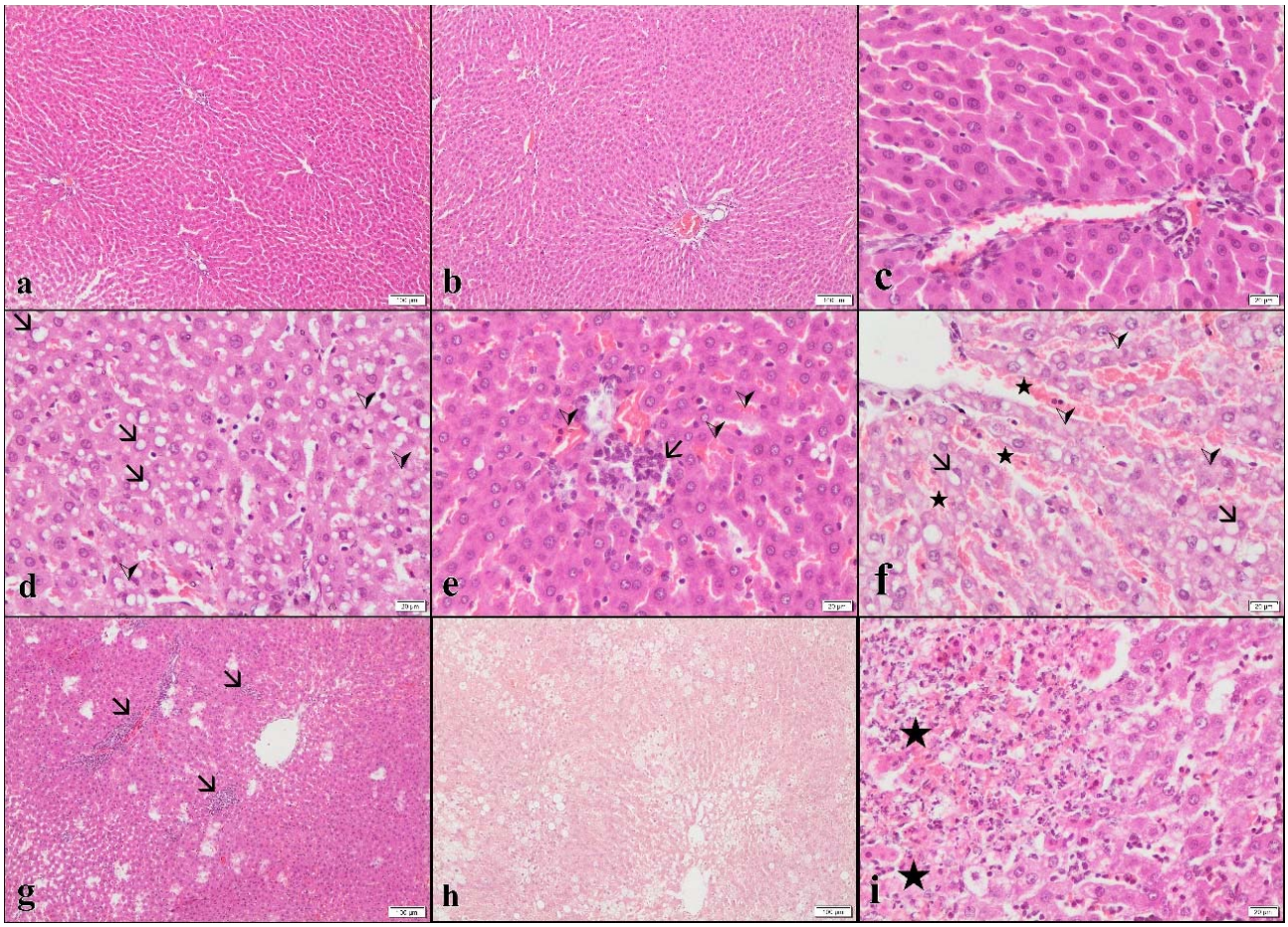

Figure 3. a: Normal histological structure of the liver in the Cont, H&E, ×100 µm. **b:** Normal histological structure of the liver in F15, H&E, ×100 µm. **c:** Normal histological structure of the liver in F15, H&E, ×20 µm. **d:** Fat vacuoles (arrows) and hydropic degeneration (arrowheads) in hepatocytes in F30, H&E, ×20 µm. **e:** Erythrocytes (arrowheads) and perivascular inflammatory cells (arrow) in sinusoids in F30, H&E, ×20 µm. **f:** Expansion with congestion in the sinusoidal spaces (stars), fat vacuoles (arrows) and hydropic degeneration (arrowheads) in F60, H&E, ×20 µm. **g:** Perivascular mononuclear cell infiltration in periportal areas (arrows) in F60, H&E, ×100 µm. **h:** Hydropic degeneration in hepatocytes in F60, H&E, ×100 µm. **i:** Focal necrosis in the liver (stars) in F60, H&E, ×20 µm.

Discussion and Conclusion

Studies for about 30 years have reported an increase in the incidence of obesity and complications in connection with the consumption of fructose (7). Although fructose is metabolized independently of insulin, as the concentration of fructose in solution increases, the amount of consumed chow decreases (35). In addition, the total amount of energy taken by diet varies as expected depending on fructose concentrations. The 60% fructose solution was consumed less by the F60 depending on the amount of energy in mL, and also chow consumption was suppressed in this group.

In a study of adult rats fed with 10% and 60% fructose solutions in addition to standard chow, it was reported that BW of both groups was the same as those of Control similar the results of our study (7). Although the F15 had the highest energy consumption, the BW were similar to those of the other groups. The BW of the F60 was lower than those of the F30, but were similar to those of the other groups. The results show that 30% fructose solution causes a significant increase in BW. It was reported that 20% fructose solution did not cause change in both BW and biochemical parameters (2). In this study, as well as BW, liver weights were similar in F15 and F60. However, the heaviest liver was in F30.

Glucose, insulin, HDL, and total cholesterol were found similar in all groups. These results were similar to those of some studies (17, 31). Although a high concentration of fructose solution does not cause a significant change in BW and other parameters, it does increase LDL and metabolic disorders are triggered (17, 31). Hypertriglyceridemia was observed in all fructose consuming groups. Fructose increased plasma triglyceride levels even though the consumption was low (7). The findings indicate that the percentage of fructose in the solution may be an important factor for hypertriglyceridemic effect.

Increased *TNFα* expression in liver triggers triglyceride production and leads to steatosis (9, 18). It has been shown that fructose at a concentration of 30% and above can upregulate *TNFα* expression (18). In a study, it has been found that 4-week consumption of 60% fructose solution increased *TNFα* expression in liver (36). We found that *TNFα* was upregulated in F30 and F60. The activity of this gene was found to be consistent with biochemical parameters as triglyceride and LDL and histopathological findings. *IL-1β* and *IL-6* genes also upregulated in F30 and F60. It has been reported that the expression level of *IL-1β* increased 3-fold in fructose fed rats (4). In our study, *IL-1β* was upregulated in F60 with the highest levels (3-fold). *IL-6*, usually co-regulated with *TNFα* and *IL-1β*, has also been reported to be involved in the regulation of triglyceride secretion (3). Plasma triglyceride levels and expression levels of this cytokine in fructose-fed groups have been confirmed this information. *TNFα, IL-1β* and *IL-6* expressions confirmed the histopathological findings of inflammation in the liver of F30 and F60.

SREBP-1c increased approximately 2-fold in F15 and F30 but the most upregulation was in F60. However, *ChREBP* upregulated in all experimental groups. Although SREBP-1c was reported to be affected by the activation of *ChREBP* and *LXRα*, it was thought that this increase might have been influenced by additional factors with fructose consumption (5). *LXRα* has been reported to be involved in lipid biosynthesis in the liver and regulate the amount of triglyceride (24). *LXRα* activity varies depending on the amount of insulin and increased approximately 2-fold in F15. However, expression levels of *LXRα* in other experimental groups were similar. It was thought that standard chow consumption and fructose concentrations might be caused these results.

Jegatheesan et al. (17) were reported that the body weight was lower in rats fed with 60% fructose for 8 weeks compared to the normally fed animals (17). However, triglyceride and LDL levels were significantly higher, similar to the F60 in this study. In the same study, it was reported that *SREBP-1c* and *ChREBP* expression levels increased, although BW was lower than those of the control (17).

FAS, ACACA and *SCD-1* were upregulated in all experimental groups. Similar to our study, it was reported that without changes in BW, triglyceride levels and lipogenic genes expression levels were increased in rats fed with 60% fructose for 4 weeks (16). The lipogenic effect of fructose is mostly seen by the effect of *SREBP-1c* and *ChREBP*. But also, *FAS, ACACA* and *SCD-1* activities are conspicuous. Fatty acid production during the lipogenesis process is caused by *ACACA*-mediated *FAS* catalysis (6, 30).

Studies conducted with animal models showed that high fructose consumption significantly affects *ACACA* in the liver and lipogenesis is significantly influenced by activity in this gene (15, 20, 34). Fructose induces more *ACACA* than other energy sources in the lipogenesis pathway (33). According to our study, although the low concentration of fructose solution affects the activity of this gene, increased lipogenic activity was found in F30 and F60.

SCD-1 has a central role in energy metabolism and is required for lipogenesis in combination with dietary fructose in SREBP-1c-dependent and independent pathways (25). *SCD-1* has been reported to increase energy consumption and insulin sensitivity, and the activity of this gene is closely associated with liver steatosis (25, 28). It has been understood from the results of this study that *SREBP-1c* has a close relationship with *SCD-1*. This gene upregulated more than 10-fold in F60. This result confirmed the histopathological findings of liver steatosis.

According to the histopathological examination, although the most severe changes were in F60, livers in F30 were also damaged. The storage of excessive fat increases liver sensitivity and makes it more susceptible to damaging agents and causes fibrosis and hepatosteatosis (1). In F30 and F60, lipogenic genes and histopathological results showed that high fructose caused liver damage due to fattening. Inflammatory genes results confirmed the damage in F30 and F60. However, it was found that animals fed with 15% fructose solutions were similar to the Cont in terms of liver histopathology. Although it was determined that feeding with 15% fructose solution for 8 weeks increased plasma triglyceride levels, it did not lead to significant pathological changes. Histopathological and biochemical results in F30 were similar to those of other studies (30, 31). Inflammation, fatty degeneration and passive hyperemia parameters were found to have the highest values in F60. Also, degenerative and necrotic changes were at the highest levels in F60.

Recently, with the consumption of fructose the activity of lipogenic genes in other tissues has been investigated as well. In a study, *SREBP-1c* and *ACACA* gene expression increased in the hypothalamus with 20% fructose consumption, while *FAS* was downregulated (2). In addition, it was reported that food intake was suppressed through *FAS* inhibitors in the hypothalamus, preventing body weight gain (2). With this study, lipogenic genes activity has been determined that strongly changed with the fructose consumption in liver. However, further study is needed on the activity of lipogenic genes in other tissues.

In conclusion, the results of this study show that NAFLD can occur without increasing overall body

weight. In addition, high fructose solution consumption causes significant histopathological changes with lipogenic and inflammatory effects at the molecular levels in the liver. The concentration of the fructose solution was found to be a very important factor. This study is thought to provide important information about production animals modeling metabolic syndrome with fructose.

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Ethical Statement

The study was ethically approved by the Animal Experiments Local Ethics Committee of Hatay Mustafa Kemal University (Decision number: 2018/2-8).

Conflict of Interest

The authors declared that there is no conflict of interest.

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Evaluation of the compatibility between corrosion casts and 3D reconstruction of pig head arterial system on cone beam computed tomography

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Abstract: This study aimed to compare the corrosion cast models of the porcine head arterial system with three-dimensional (3D) reconstructions using cone beam computed tomography (CBCT) of these cast models. Six heads from sows were simultaneously injected through both carotid arteries with Duracryl Plus for corrosion cast technique and an additional head, also from another one sow head, was filled with saturated lead tetroxide (Pb_3O_4) in a 10% hot water solution (40°C) of gelatin for CBCT study. Two-dimensional (2D) images were stored in Digital Imaging and Communications in Medicine (DICOM). Subsequently, segmentation and post-processing of these images were performed by using various software programs. The 3D models were found to be compatible with the corrosion cast models. It was observed that osseous structures and arteries were clearly identified on CBCT images. Specimen scan, segmentation, and post segmentation had a duration of 10-15 minutes, 4 hours, and 15 minutes, respectively. The internal carotid artery, external carotid artery, and its main branches were seen well on 3D models. In conclusion, it is considered that 3D models and images can be effectively used in anatomy education, radiological evaluations, pathological and variational investigations.
Keywords: Artery, cone beam computed tomography, corrosion cast, pig, three-dimensional reconstruction.

Konik ışınlı bilgisayarlı tomografide domuz kafa atardamar sisteminin üç boyutlu rekonstrüksiyon ile korozyon kast arasındaki uyumluluğun değerlendirilmesi

Özet: Bu çalışma, domuz kafa atardamar sisteminin korozyon kast modelleri ile bu modellerin konik ışınlı bilgisayarlı tomografisi (KIBT) kullanarak üç boyutlu (3B) rekonstrüksiyonlarının karşılaştırması amaçlanmıştır. Dişi domuzlara ait altı baş, aynı anda her iki şahdamarından, korozyon kast tekniği için Duracryl Plus enjekte edildi ve ayrıca bir başka dişi domuza ait bir baş, KIBT çalışması için %10 jelatinli sıcak su çözeltisi (40°C) içinde doymuş kurşun tetroksit (Pb_3O_4) ile dolduruldu. İki boyutlu (2B) görüntüler Tıpta Dijital Görüntüleme ve İletişim (DICOM) formatında saklandı. Daha sonra bu görüntülerin segmentasyonu ve son işlemleri çeşitli yazılım programları kullanılarak gerçekleştirildi. 3B modellerin, korozyon kast modelleriyle uyumlu olduğu görüldü. KIBT görüntülerinde kemik yapılar ve atardamarlar net olarak belirlendi. Örnek görüntüleme, segmentasyon ve segmentasyon sonrası işlemleri sırasıyla 10-15 dakika, 4 saat ve 15 dakika sürdü. İç karotis arter ile dış karotis arter ve ana dalları 3B modellerde iyi görüldü. Sonuç olarak anatomi eğitiminde, radyolojik değerlendirmelerde, patolojik ve çeşitli incelemelerde 3 boyutlu model ve görüntülerin etkin bir şekilde kullanılabileceği düşünülmektedir.
Anahtar sözcükler: Atardamar, domuz, konik ışınlı bilgisayarlı tomografi, korozyon kast, üç boyutlu rekonstrüksiyon.

Introduction

Recently studies have examining the vascular component of the head region of different animal species by using the corrosion cast technique (1, 19). This technique is used to visualize hollow or virtual anatomical spaces. The cavity is filled with a liquid or at least malleable substance, that is subsequently allowed to solidify, after which the surrounding tissues are removed by biological, enzymatic, or chemical maceration (4). When the morphology of the vascular system is examined with this technique, accurate diagnosis and follow-up of the disease are performed, as well as surgical planning and evaluation are possible. In addition, this technique is beneficial for both students and researchers in anatomy education, research, and clinical applications (5, 6, 18). However, its application is limited due to the application difficulties and repeatability problems. (9, 11, 12). For this reason, it is considered that the models obtained by the

corrosion casting technique can be used in anatomy education by using a different method.

The adversities encountered in the corrosion casting technique can be prevented by using three-dimensional (3D) reconstruction models of cross-sectional images obtained using imaging systems (6, 11). It is necessary to scan the whole structure by one of the imaging systems (Magnetic resonance imaging, computed tomography, or micro-computed tomography) to obtain two-dimensional (2D) cross-sectional images of the structure, and combine these images by using different programs to create 3D models (6, 14, 16). Although the general anatomy of the vessels in the porcine head is known, there are no studies in which the techniques of corrosion casting and modeling with 3D reconstruction are applied.

Therefore, it was aimed to investigate the head arteries of porcine (*Sus scrofa domestica*) with 3D reconstructions models from cone beam computed tomography (CBCT) images and evaluate the compatibility with corresponding corrosion casting specimens.

Materials and Methods

Seven mature sow heads were obtained immediately after the slaughtering of animals (Six months old, 95 – 105 kg body weight) for meat consumption in a licensed abattoir (Han Asparouhovo, Stara Zagora, Bulgaria) in accordance with the Bulgarian respective legislation. The head arterial system was washed with distilled water through the common carotid arteries approximately twenty hours after the death until leakage of the residual blood via jugular veins for avoiding obstruction in downstream channels by blood clots (4). Subsequently, two syringes were mounted on both carotid arteries of six heads for simultaneous introduction of 100 ml of Duracryl Plus (Spofa Dental, Czech Republic) solution (50 g cold polymerizing mixture + 150 ml methacrylate) using a special device constructed by us for such purposes (Figure 1). After that, the filled specimens were left at room temperature for 24 hours to complete the resin's polymerization. Then the heads were placed into 5% Potassium hydroxide for 10 days at 45^0C for removing the soft tissue. Finally, the corrosion specimens were washed with slow running water with some detergent to removing the remaining fat. One additional head was injected in the same way with a saturated solution of 120 g lead tetroxide (Pb_3O_4) in 100 ml 10% gelatin in hot water (40^0C).

Following injection, specimens were scanned with the CBCT at the Trakia University Faculty of Veterinary Medicine in Stara Zagora, on a Fidex I (Fidex, Animage LLC, California, USA) which is a unique three-modality diagnostic imaging system (Figure 2a, Figure 2b and Figure 2c). Scan parameters for all specimens were 0.46 mm slice thickness, 110 kV and 150 mA, field of view 23 cm diameter, and total scanning time approximately 15 minutes. After the scanning process was performed all images were taken and transferred to the high-quality computer allowing the segmentation process. 2D images were uploaded to the 3D Slicer software program (3D slicer, 4.11.0 version, GitHub, San Francisco, USA). The 3D reconstruction models were segmented and created through all levels of the coronal, transversal, and sagittal cross-sections. During the segmentation process, the head skeleton and arteries were rendered in every section based on distinctive grey-scale values to separate the different tissues (Figure 2d). After the head skeleton and arteries were manually segmented, the 3D models were created one by one (8). Subsequently, the post-segmentation process was performed on the 3D digital models with Meshmixer software (Autodesk Inc., San Francisco, version 3.5). The final 3D models of the head skeleton and the arteries were achieved after corrections were applied (Figure 2e). The terms used in the descriptions below are from the Nomina Anatomica Veterinaria (15).

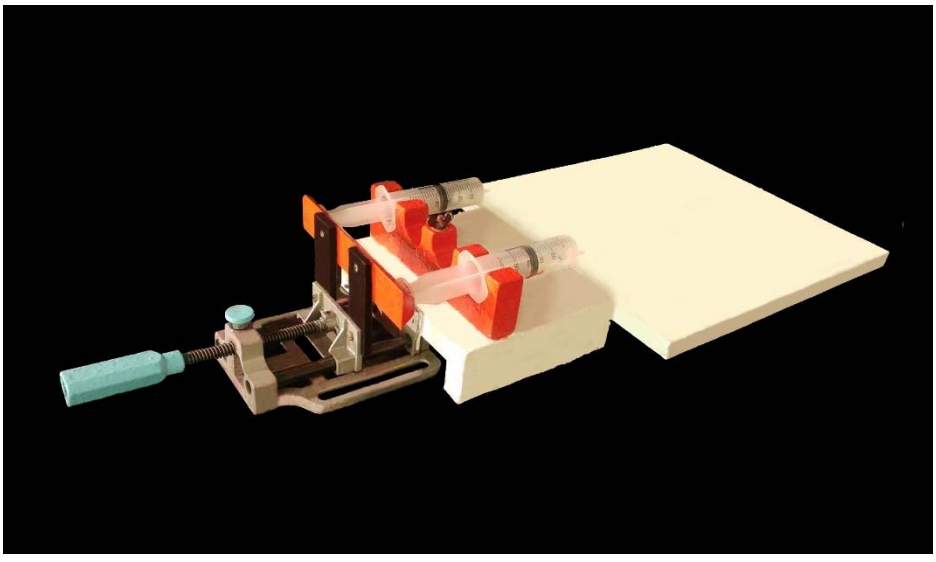

Figure 1. The device designed to fill the arteries of the samples simultaneously.

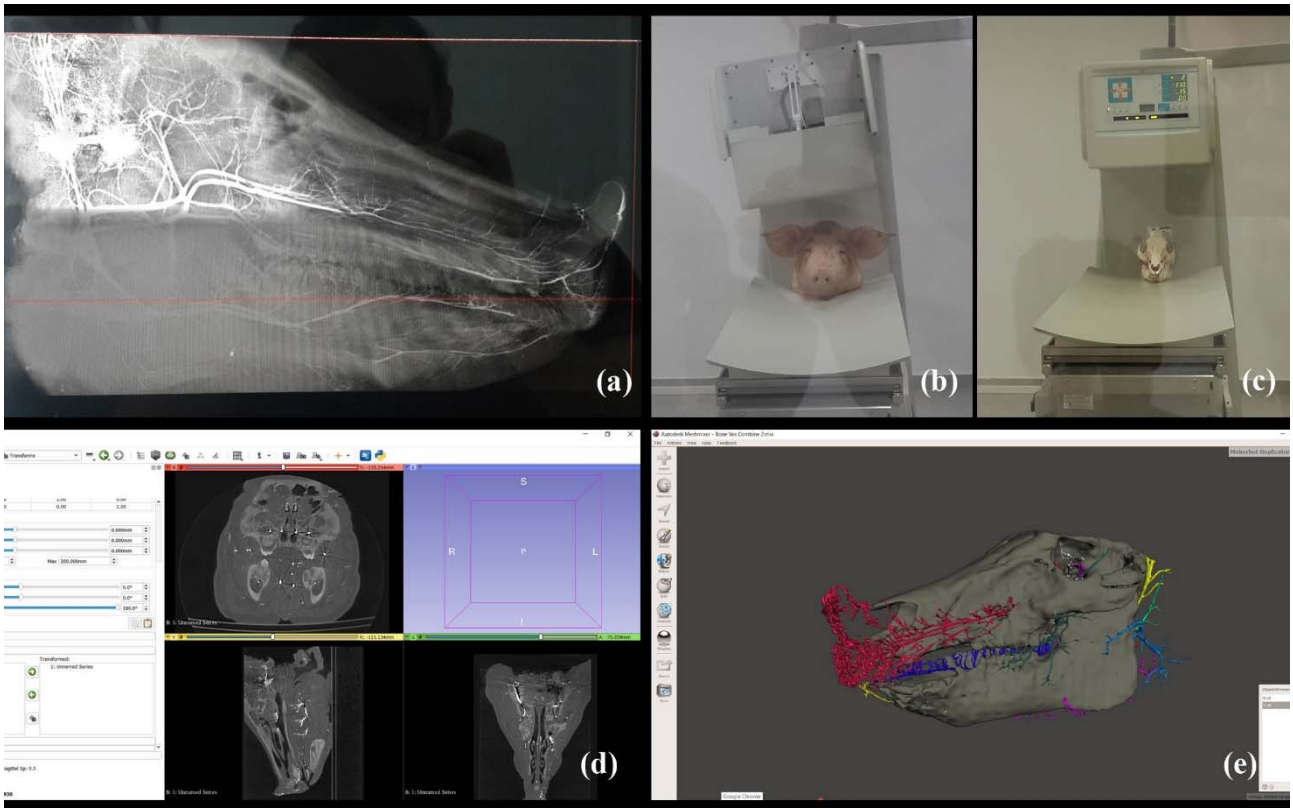

Figure 2. Stages of three-dimensional reconstruction of the specimens.
(a) Control for positioning of the corrosion cast specimens by radiography, (b) and (c) Scanning stage with cone-beam computed tomography of different corrosion casts, (d) Segmentation stage of 3D reconstruction models from different cross-sections, (e) Post-segmentation stage of the 3D models.

Results

It was seen that osseous structures and arteries could be well identified on CBCT images on both gelatin and the Duracryl corrosion casting specimens (Figure 2d, Figure 3b and Figure 3d). The combination of corrosion casting and CBCT scanning results in a visible arterial circulatory system that can be well separated from bone tissue (Figure 3). Specimen scanning, segmentation, and post segmentation had a duration of 10-15 minutes, 4 hours, and 15 minutes, respectively. Using these procedures, the 3D models of these anatomical casts of the vessels were displayed (Figure 3b and 3d) and it was seen that these models allow even better visualization of the morphology of the arterial circulatory system. The 3D model of the gelatin-filled cast (Figure 3d) and the Duracryl corrosion cast are shown in Figures 3d and 3b. It should be noted that gelatin gave better contrast on CBCT.

Almost all of the cranial head arteries, including branches of the internal carotid artery and external carotid artery were well visible on the 3D models of gelatin specimen. These arteries can be seen in Figure 4. It was also clearly seen that the right and left lingual arteries were anastomosed at the tip of the tongue (Figure 4, arrow). The 3D models were found to be compatible with the corrosion cast models. However, it was observed that the arteries were seen much better in gelatin specimens than the Duracryl corrosion cast specimens (Figure 3). Two different models of the cranial head arteries were given in Figures 3b and 3d.

Discussion and Conclusion

In literature, there are studies on the cranial head vessels of different animal species visualized by various chemicals and techniques that mostly concern the variety of brain vascularization (1, 2, 10, 16, 17, 19-21). However, there are no studies investigating the technique of corrosion casting combined with 3D modeling. In this study, for the first time, we rendered two different types of 3D reconstruction models of porcine cranial head arteries from two different corrosion casting types. Both techniques revealed clearly, instructive pictures of filled vessels, including also such delicate networks as these of rete mirabile. Also, anastomosing structures that are very difficult to detect or easily damages during dissection can be easily identified at the tip of the tongue. In our opinion, this is seriously advantage of that technique and highlights its suitability for education and other demonstration. Such 3D STL files can be used to produce 3D printed models, as well. In this way, it is thought the problem of replication in corrosion casting will be overcome.

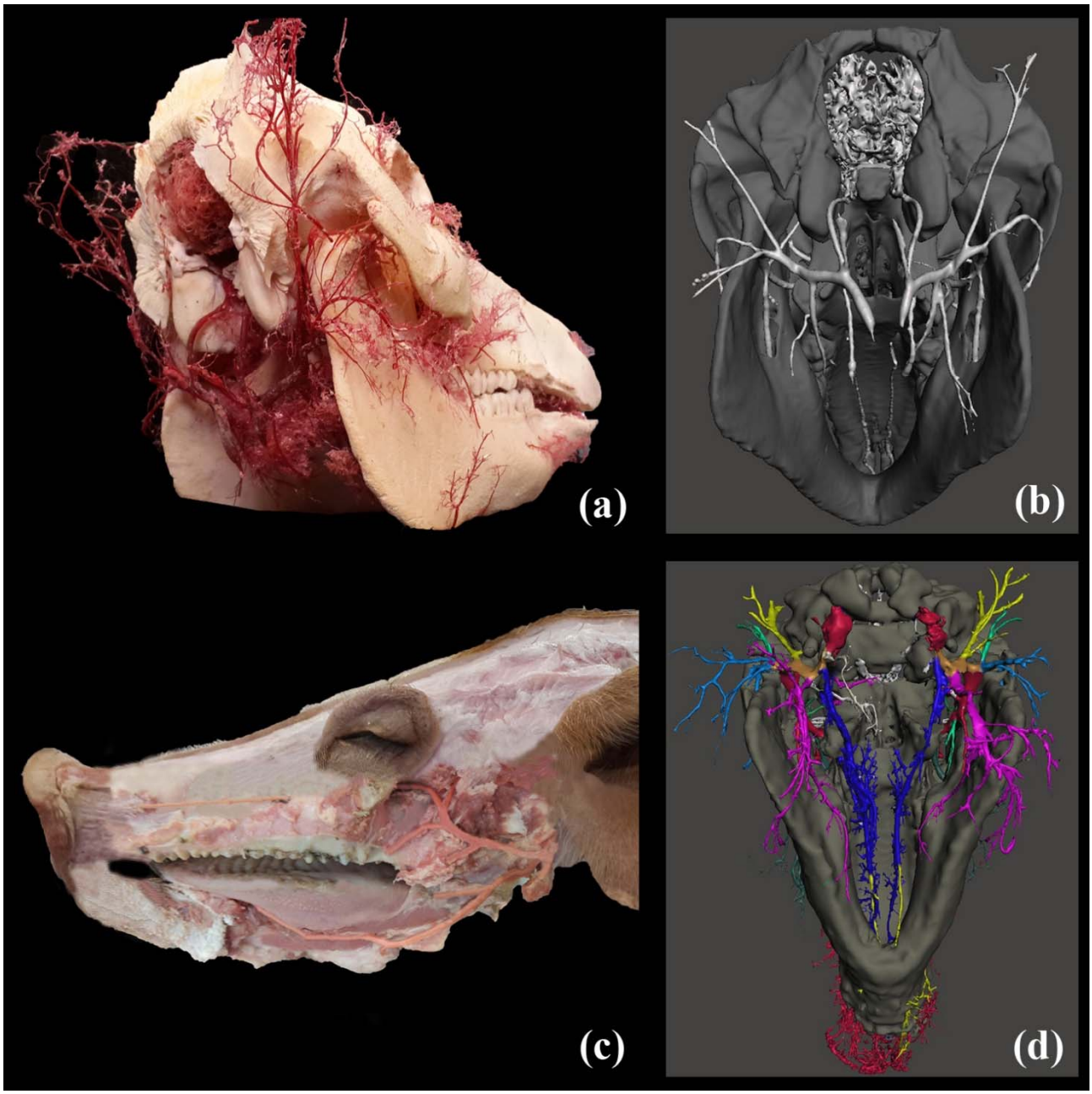

Figure 3. Comparison of the visualization of different corrosion cast and 3D models.
(a) Latero-caudal aspect of the Duracryl Plus corrosion cast specimen, (b) Caudo-ventral aspect of the 3D model of the Duracryl Plus corrosion cast specimen, (c) Lateral aspect of the gelatin corrosion cast specimen after some dissection, and (d) Ventral aspect of the 3D model of the gelatin corrosion cast specimen.

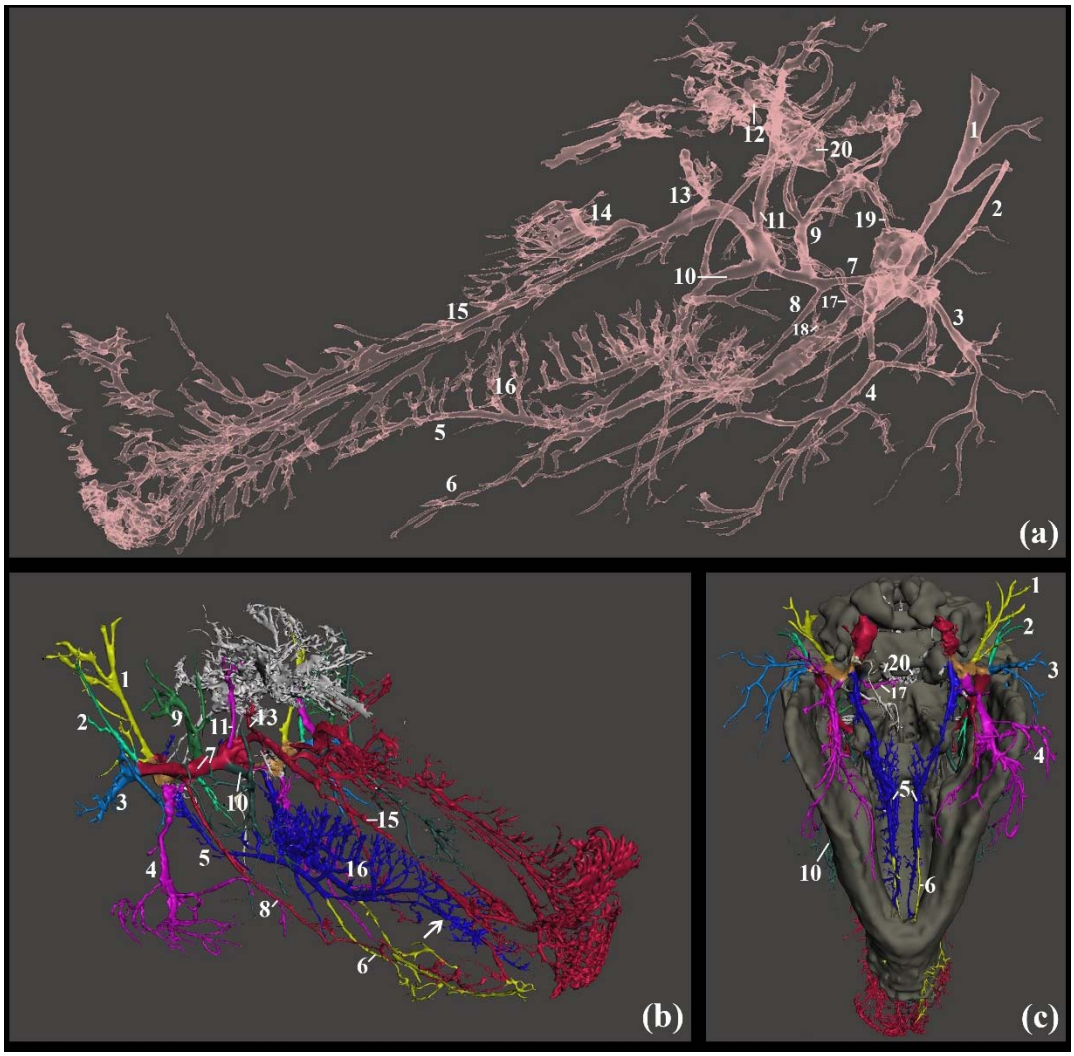

Figure 4. Three-dimensional reconstructed images of the arteries and bones of the pig head.
(a) Latero-cranial aspect of the branches of the left a. carotis communis, (b) Latero-cranial aspect of the colored branches of the arteries and (c) Ventral aspect of the arteries and bones. 1, a. auricularis caudalis; 2, a. auricularis rostralis; 3, a. transversa faciei; 4, a. facialis; 5, a. lingualis; 6, a. sublingualis; 7, a. maxillaris; 8, a. alveolaris mandibularis; 9, a. temporalis profunda caudalis; 10, a. buccalis; 11, a. ophtalmica; 12, a. supraorbitalis; 13, a. malaris; 14, branches of the a. palatina descendens; 15, a. infraorbitalis; 16, a. profunda linguae; 17, a. palatina ascendens; 18, a. pharyngea ascendens; 19, a. carotis interna; 20, rete mirabile epidurale rostrale; anastomose of left and right a. lingualis were indicated by white arrow.

One of the previous studies emphasized that computer-based learning was becoming popular because of decreasing education time, increasing student numbers, and education material costs (7). Parallel to these advantages, it was thought that 3D reconstruction models are useful education material for both theoretical and practical lessons for students in this study. These 3D models can be oriented in any desired position, useful cross-sections can be taken from the desired points and also length and volume measurements can be made. It can be summarized that these are the most important advantages compared to the original casts in this study.

It was also known that the cadaver dissection is the traditional learning material for anatomy and it has some limitations such as financial, ethical, and cultural problems (3, 7). As a matter of fact, the samples obtained by the Duracryl corrosion casting technique are quite fragile. Small vessel structures can be easily broken down when used in education halls. Therefore, these models are usually stored in areas such as museums and exhibition halls (13). On the other hand, the most prominent disadvantage of the gelatin corrosion casting model is that dissection should be performed to make the vessels visible.

As expressed before, this 3D reconstruction CBCT model may contribute to the corrosion cast methodologies and investigations in the pig's head. We concluded that the gelatin corrosion cast technique was much better detail quality on cone-beam computed tomography images than the Duracryl corrosion cast.

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Ethical Statement

Ethical approval is not applicable for this article.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Molecular diagnosis of neglected infectious agents of heep and attle abortions: the prevalences of *Coxiella burnetii, Francisella tularensis* and *Chlamydophila abortus* at a glance

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Abstract: Bacterial abortive agents such as *Brucella* spp., *Salmonella* spp., *Campylobacter* spp., *Listeria* spp. cause serious infections that lead to significant economic losses in cattle and sheep breeding. These bacteria can be easily cultured under *in vitro* laboratory conditions. Abortions caused by intracellular bacteria such as *Coxiella burnetii (C. burnetii), Chlamydophila abortus (C. abortus)* and *Francisella tularensis (F. tularensis)* are less prevalent in abortive cases and the diagnosis of these bacteria, many of which need to cell culture for cultivation and biosafety level-3 laboratory facilities for safe working, can be made by PCR. In this study, it was aimed to determine the molecular prevalence of *C. burnetii, C. abortus* and *F. tularensis* agents, which were neglected during the diagnosis of abortions, in cattle and sheep collected from different regions of Türkiye. A total of 395 clinical materials were analyzed via agent-specific commercial Real-Time PCR. As a result, the molecular prevalence of *F. tularensis, C. burnetii* and *C. abortus* was determined as 14%, 2.9% and 2.28%, respectively. *F. tularensis* in cattle clinical samples and *C. burnetii* in sheep clinical samples were not found. This is the first report that *F. tularensis* was found as an agent in an abortive material in Türkiye. Although it differs in terms of prevalence, it has been determined that these microorganisms, which are neglected in routine diagnosis, can be spread with aborted materials, especially vaginal discharge, which may pose a risk of transmission.
Keywords: Abortion, *Chlamydophila abortus*, *Coxiella burnetii*, *Francisella tularensis*, real-time PCR.

Koyun ve sığır abortlarında ihmal edilen enfeksiyöz ajanların moleküler tanısı: bir bakışta *Coxiella burnetii, Francisella tularensis* ve *Chlamydophila abortus* prevalansları

Özet: *Brucella* spp*., Salmonella* spp*., Campylobacter* spp*., Listeria* spp*.* gibi bakteriyel atık etkenleri sığır ve koyun yetiştiriciliğinde önemli ekonomik kayıplara yol açan infeksiyonlar oluşturmaktadır. Bu etkenler *in vitro* laboratuvar koşullarında kolaylıkla kültüre edilebilmektedirler. *Coxiella burnetii (C. burnetii), Chlamydophila abortus (C. abortus), Francisella tularensis* (*F. tularensis*) gibi intrasellüler etkenlerden kaynaklanan atık olguları ise daha az sıklıktadır. Kültürel analizleri için çoğunun hücre kültürüne ihtiyaç duymaları ve güvenli çalışma için biyogüvenlik seviyesi-3 laboratuar gereksinimleri nedeniyle bu etkenlerin teşhisi genellikle PCR ile yapılmaktadır. Bu çalışmada, Türkiye'nin farklı bölgelerinden toplanan, atık yapmış sığır ve koyun örneklerinden, teşhiste göz ardı edilen, *C. burnetii, C. abortus* ve *F. tularensis* etkenlerinin moleküler prevalanslarının belirlenmesi amaçlandı. Toplam 395 klinik materyal, etken spesifik ticari Real-Time PCR ile analiz edildi. Çalışma sonucunda, atık olgularında *F. tularensis, C. burnetii* ve *C. abortus* moleküler prevalansları sırasıyla %14, %2,9 ve %2,28 olarak belirlenirken, sığır klinik örneklerinden *F. tularensis* ve koyun klinik örneklerinden *C. burnetii* etken varlığına rastlanılmadı. Bu, *F. tularensis*'in Türkiye'de abort materyalinde etken olarak bulunduğuna dair ilk rapordur. Yaygınlık açısından farklılık gösterse de rutin teşhiste göz ardı edilen bu etkenlerin bulaş riski oluşturabilecek şekilde atık materyalleri ile özellikle de vajinal ekstretleri ile saçılabilecekleri belirlenmiştir.
Anahtar sözcükler: Abort*, Chlamydophila abortus, Coxiella burnetii, Francisella tularensis*, real-time PCR.

Introduction

Infectious abortions are the main problems that are encountered in cattle and sheep husbandry and cause significant economic losses. Infections caused by many bacteria, viruses and parasites are not only limited to abortion cases but also cause problems such as embryonic deaths, weak offspring or stillbirths and infertility in pregnant animals. Although their prevalence varies in cattle and sheep, abortion cases in the case of bacterial agents such as *Brucella* spp*., Salmonella* spp*.,*

Campylobacter spp., *Listeria monocytogenes* rank first in terms of prevalence (13, 28). The fact that these microorganisms can be easily cultured under *in vitro* laboratory conditions has contributed positively to the increasing reports on their prevalence. Cattle and sheep abortions caused by obligate intracellular bacteria such as *Coxiella burnetii (C. burnetii), Chlamydophila abortus (C. abortus)* and facultative intracellular pathogens such as *Francisella tularensis (F. tularensis)* are mostly ignored in diagnosis since they cannot be cultured in routine laboratories and therefore their reports are less prevalent (19, 22, 24).

The importance of these microorganisms, which lead to early embryonic deaths, stillbirths or abortions, is based not only on economic losses in animal production but also on their zoonotic potential (16).

Chlamydia are obligate intracellular bacteria and cause several clinically and economically important diseases such as abortion, urinary system infections, pneumonia, conjunctivitis and, enteritis in livestock (12). The species responsible for chlamydial infections are *C. abortus*, *C. pecorum* and, *C. psittaci. C. abortus* is commonly responsible for sheep and cattle abortions. Abortions generally coincide with the last few months of gestation and are formed as a result of diffuse placentitis. The agent, in the form of elementary bodies that are infectious but non-replicating, is scattered by abortive materials such as placenta, uterine and vaginal fluids and fetal materials. The scattered agent is usually taken through the digestive system and can cause new infections (25).

Q fever is a common zoonotic disease in humans and animals caused by *C. burnetii* which is an obligate intracellular bacterium (14). The agent can be scattered abundantly with the milk, vaginal secretions, feces and, placenta of the aborted animals (8, 15). The disease can be transmitted by direct contact with these aborted materials, especially in sheep, cattle and goats. Several arthropods, primarily ticks, also play a role in the epidemiology of *C. burnetii* (23). Although they are widely asymptomatic carriers, *C. burnetii* causes placentitis, abortion, stillbirth and infertility in cattle and sheep (15).

Tularemia is a zoonotic disease caused by *F. tularensis*, a facultative intracellular bacterium, that causes different clinical findings in many animal species such as rabbits, sheep, cats, dogs, horses and pigs, especially rodents, and in humans. The presence of the disease in sheep, which ranks first among farm animals in terms of susceptibility to *F. tularensis*, has mostly been reported by serologically (20).

Infection in humans has a widespread transmission characteristic through the handling of infected animals, contact with or consumption of contaminated food or water, aerosol pathway and vector (ticks, flies, and mosquitoes) bites (9, 18). *F. tularensis* infections in humans are mostly case reports involving clinical complaints and cultural, serological or moleculardiagnosis of risky contacts. There are scarcely any studies on the adverse effects of *F. tularensis* on fetal development during the pregnancy. Although there are few reports of abortion and premature birth in untreated cases of *F. tularensis* in humans (5, 10), most of the cases consist of concerns of the characterization of complications and limiting the use of antibiotics due to their teratogenic effects (35). There is a similar situation in sheep as well, and late term abortions or neonatal lamb deaths caused by *F. tularensis* have been reported (27). The abortion and lamb deaths caused by this infectioncan reach 50% if left untreated. Although seroprevalence studies in cattle, which are more resistant to the infection than sheep, suggest *F. tularensis* infection, common clinical findings were not encountered in these animals and most of the cases were asymptomatic (20, 32). Considering the difficulties encountered in isolation and the drawbacks such as the presence of cross- reactions in serological analysis, Real-Time PCR can be used as a reliable, highly sensitive and practical diagnostic method in diagnosis (2, 6, 11).

In this study, it was aimed to investigate the molecular prevalence of *Chlamydophila abortus*, *Coxiella burnetii*, *Francisella tularensis* by Real-Time PCR in clinical materials of aborted cattle and sheep collected from different regions of Türkiye.

Materials and Methods

Study material: Clinical samples of cattle and sheep with abortion encountering in the first three months of 2021 which were sent to the laboratories of the Veterinary Control Central Research Institute of the Ministry of Agriculture and Forestry (Türkiye) were examined. The samples represented the three geographic regions of Türkiye, Central Anatolia Region, Black Sea Regionand and Eastern Anatolia Region. In this context, 345 cattle samples including 92 blood sera, 99 vaginal swabs and 154 aborted fetus stomach contents and 50 sheep samples including 28 blood sera, 10 vaginal swabs and 12 aborted fetus stomach contents were analyzed (Table 1).

Cultural analysis: The abortive materials (vaginal swabs and fetal stomach contents) were examined for the presence of abortive bacterial agents such as *Brucella* spp., *Campylobacter* spp., *Salmonella* spp. and *Listeria* spp. that could be cultured *in vitro* on the agent specific media reported before (1, 7). The samples were cultured for *F. tularensis* on Francis media which was prepared with Brain Heart Infusion Agar (Oxoid, UK), 8-9% defibrinated sheep blood, 1% Dextrose (Difco, USA), 0.1% L-Cysteine (Sigma-Aldrich, USA), *Helicobacter pylori* Selective Supplement (Dent) (Oxoid, UK) and antibiotics (Penicillin G 1ml/100 IU, Cycloheximide

L/100mg, Polymixin B l/8×10[4]). *F. tularensis* subsp. *holarctica* (NCTC 10857) was used as positive control (19).

Real-Time PCR analysis: DNA extraction from clinical samples was carried out using the cador Pathogen 96 QIAcube HT Kit (QIAcube HT Plasticware, Qiagen) in accordance with the manufacturer's instructions. The Real-Time PCR process was performed with CFX96 Touch Real-Time PCR Detection System (Bio-Rad). In Real-Time PCR analyzes, Bio-Speedy® Tularemi Real-Time PCR detection kit (Bioeksen R&D Technologies Ltd.® Istanbul) for *F. tularensis* and Bio-Speedy® Chlamydia-Coxiella Real-Time PCR detection kit for *C. burnetii* and *C. abortus* (Bioeksen R&D Technologies Ltd.® Istanbul) were used. Real-Time PCR analyzes were performed in the presence of positive controls (*C. abortus* S26/3, *C. burnetii* NM/2017-11 P6, *F. tularensis* NCTC 10855) and negative control (nuclease-free water). Real-Time PCR reaction was created with 11 µL volume for each sample and consisted of 5 µL qPCR mix (2X), 3 µL agent specific oligo mix (*F. tularensis* or *Chlamydia-Coxiella* spp.), 1 µL internal control and 2 µL template DNA. Real-Time PCR was performed as pre-denaturation at 95 °C for 5 min, 45 cycles consisting of denaturation at 95 °C for 15 sec and binding and elongation at 60 °C for 40 sec. When reading the results, *F. tularensis* targeted reactions in the FAM channel of the Real-Time PCR instrument were evaluated for *F. tularensis*. For *Chlamydia-Coxiella* spp., FAM was evaluated for *C. abortus* and ROX for *C. burnetii*. All samples were evaluated for the internal control target in the HEX channel. At the end of the Real-Time PCR analysis, samples was evaluated according to the amplification curves and Ct (threshold value cycle) data. Amplification with a sigmoidal curve and a significant logarithmic phase at Ct ≤ 37 was directly evaluated as positive. The result was considered negative as indicated in the kit procedure when amplification did not occur or when the cycle threshold (Ct) value exceeded 37 cycles (Figure 1).

Statistical analysis: Data were analysed via IBM SPSS Statistics 20.0. program. Chi-square test was used and a P-value of <0.05 was considered statistically significant.

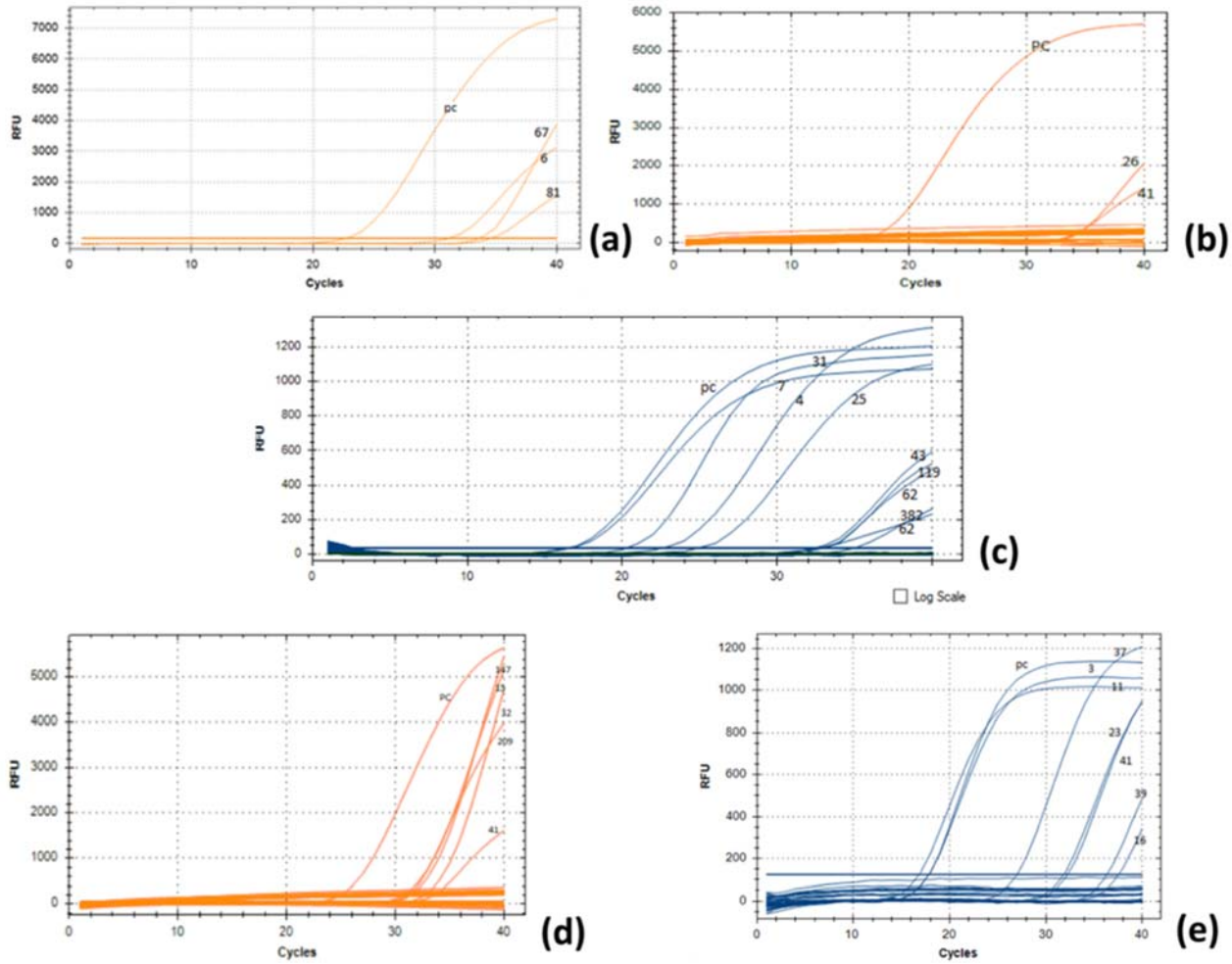

Figure 1. Amplification curves of Real-Time PCR reactions of cattle and sheep clinical materials.
(a): *C. burnetii* positivity in cattle vaginal swabs, (b): *C. burnetii* positivity in cattle blood serum, (c): *C. abortus* positivity in cattle and sheep vaginal swab and cattle fetus stomach content, (d): *C. burnetii* positivity in cattle fetus stomach content, (e): *F. tularensis* positivity in sheep vaginal swab and blood sera.

Table 1. Sample types and agent-specific RT-PCR positive results.

The microorganism	Cattle			Sheep			Total
	V. swab (n: 99)	Fetus (n: 154)	Blood sera (n: 92)	V. swab (n: 10)	Fetus (n: 12)	Blood sera (n: 28)	
F. tularensis	-	-	-	2 (20%)	-	5 (17.86%)	7 (14%) *
C. burnetii	3 (3.03%)	5 (3.25%)	2 (2.17%)	-	-	-	10 (2.9%) **
C. abortus	5 (5.05%)	2 (1.3%)	-	2 (20%)	-	-	7 (2.03%) ** 2 (4%) * 9 (2.28%) ***
Total	8 (8.08%)	7 (4.55%)	2 (2.17%)	4 (40%)	-	5 (17.86%)	26 (6.58%)

* Only for sheep samples, ** Only for cattle samples, *** Values determined for cattle and sheep samples.

Results

All aborted materials cultured *in vitro* on the relevant media in terms of specific bacteria were found to be culture negative. As a result of the Real-Time PCR analysis, the individual molecular prevalence of obligate or facultative intracellular agents in abortion was calculated as 4.93% in cattle and 14% in sheep, and it was determined as 6.58% in total. The findings in terms of the presence of all the neglected abortive agents were statistically significant based on the host preference ($P<0.05$). In total, the prevalence of *F. tularensis*, *C. burnetii* and *C. abortus* was 14%, 2.9% and 2.28%, respectively (Table 1). However, no significant relationship was found on the basis of a host in terms of the individually carrying these microorganisms ($P>0.05$).

C. burnetii was detected in 10 (2.9%) clinical samples of cattle, including 3 (3.03%) vaginal swab, 5 (3.25%) fetal stomach content and 2 (2.17%) blood serum (Table 1, Figure 1a,b,d). *C. abortus* was detected in 7 (2.03%) clinical samples of cattle, including 5 (5.05%) vaginal swab and 2 (1.3%) fetal stomach contents (Table 1, Figure 1c). *F. tularensis* DNA was not detected in any of the material of cattle (Table 1).

F. tularensis was detected in 7 (14%) of the clinical specimens belonging to sheep, including 2 (20%) vaginal swabs and 5 (17.86%) blood serum (Table 1, Figure 1e). *C. abortus* was detected in 2 (20%) vaginal swab samples of sheep (Table 1, Figure 1c). No *C. burnetii* DNA was detected in any of the abortive materials of sheep (Table 1).

Discussion and Conclusion

C. burnetii, F. tularensis and *C. abortus* infections are common worldwide and cause important zoonotic diseases. However, research of these microorganisms on the epidemiology of abortions in domestic animals is extremely limited. This situation is valid in both research laboratories and veterinary services of Türkiye and other countries. With obligate or facultative intracellular localization, these microorganisms can lead to infectious abortions, early embryonic deaths and infertility in cattle and sheep. Since most laboratories that make routine diagnosis do not have living environments such as cell or tissue culture and experimental animals, the diagnosis of these agents is inadequate or often overlooked. However, it is certain that agent isolation to be achieved after cultural analysis will be beneficial for further identification, characterization of molecular and antigenic, virulence trials and vaccine studies. PCR techniques based on enzymatic amplification and imaging of agent-specific protected gene regions provide more practical and reliable results in the diagnosis of obligate intracellular bacteria such as *F. tularensis* whose *in vitro* culture is quite difficult or requires high biosecurity measures. In this context, Real-Time PCR method is seen as an advantageous technique that provides results simultaneously with the amplification of gene regions (2, 6, 11, 33).

In a study in Switzerland, the analysis of *C. burnetii, C. abortus* and *Leptospira* spp., which were ignored in the diagnosis of cattle abortions, was performed by the serological, molecular, bacteriological, histopathological and immunohistochemical methods and the Real-Time PCR supported by the histopathological analysis was found the recommended method in diagnosis (33). As a result of this study, the molecular prevalence of obligate and facultative intracellular bacteria was found to be 6.58% from various clinical samples of aborted cattle and sheep. The presence of these neglected agents was significantly higher in cattle ($P<0.05$). Interpretations regarding the agent-specific prevalence and probabilities were discussed in the following sections.

Among the chlamydial agents, *C. abortus* is the species that it is widely responsible for sheep and cattle abortions. Abortions caused by *C. abortus* can sometimes occur as abort storms and the rate can reach up to 30% in naive herds. The advantages of PCR techniques have been reported both in terms of diagnosis and in the management of such outbreaks (7). In a study conducted by Kılıç et al. (21) on *C. abortus* in aborted cattle fetuses by PCR, a 6.3% positivity was obtained. Aras et al. (4) detected *C. abortus* in 2 (3%) of 65 stomach contents of aborted cattle fetuses by PCR in Konya region (Türkiye). Livingstone et al. (24) evaluated the epidemiology and potential effect of an enzootic abortion caused by *C. abortus* in sheep, and a small number of *C. abortus* specific genomes were detected by the Real-Time PCR in vaginal swabs of sheep

after abortion. In the studies, it has been emphasized that *C. abortus* should also be taken into account in combating infectious abortion in cattle and sheep. In this study, *C. abortus* DNA was detected in 9 (2.28%) of the samples belonging to cattle and sheep and these findings are relatively similar to other studies (4, 21, 24). The samples with *C. abortus* showed diversity known as important sources of infection in the transmission of the disease, including the vaginal swab of aborted animals and the stomach contents of the aborted fetus.

Q Fever cases caused by *C. burnetii* have been reported from all over the world except Antarctica and New Zealand (3, 26). An important part of these reports are studies in which the agent analysis is carried out by PCR from various aborted materials (tissue, blood, blood serum and milk samples) belonging to cattle and sheep. Erdenliğ et al. (11) obtained a 1.5% *C. burnetii* positivity in aborted cattle samples and 2.7% in aborted sheep samples by Real-Time PCR. In addition to the zoonotic importance of Q Fever, the researchers emphasized the diagnostic advantage of the Real-Time PCR in rapid and safe detection of this agent. In a study conducted by Selim et al. (30) in sheep, Q Fever was investigated by both ELISA and RT-PCR. Interestingly, 42 samples detected seronegative by ELISA were found to be positive by RT-PCR and thus, although ELISA is more advantageous as a screening test in the preliminary diagnosis of Q Fever, RT-PCR has been reported to give more reliable and accurate results. Although it is a generally low amount (4.3-6%), the molecular prevalence of Q Fever has been reported at different rates in abortion in ruminants (8, 22). In the detection of *C. burnetii*, which is an absolute intracellular agent, from abortions, blood serum samples have been reported to be indispensable clinical materials in addition to placenta, vaginal secrets and fetal tissue (29). Hence, in this study, *C. burnetii* positivity was obtained from 2 blood serum samples in cattle. Considering its general prevalence, the prevalence of *C. burnetii* in abortive cases was found to be low, similar to other studies (8, 22). The absence of sheep was interpreted as the abortion cases evaluated in this study may not be caused by *C. burnetii.*

Although *F. tularensis* is listed among the agents that can cause abortion and premature birth (35), it is not included in the diagnostic manual of many countries. Moreover, *F. tularensis* infections are contented with being defined as episodes that can occur during pregnancy in both humans and animals and have not been associated with the disease (27, 35). Nevertheless, it was emphasized that *F. tularensis* should be considered in the differential diagnosis in cases of abortion and neonatal death corresponding to the late period of pregnancy in places where the disease is endemic and tick activity is intense (27).

In the differential diagnosis, the histopathological changes observed in sheep abortions, alleged to be caused by *F. tularensis*, were described cursory. Macroscopic lesions consist of focal necrosis found in many organs such as the lung, liver, kidney, however, the bacteria could not be identified in smear prepared from these areas and stained with hematoxyline-eosin or Gram stain. Diagnosis can only be made on a limited number of samples by immunohistochemical (IHC), serological, PCR, and cultural methods (27). This confusion regarding gross macroscopy and other analysis methods could not be interpreted in this study because of the low culturability even if positive by PCR (17, 31). *F. tularensis* positivity has already been detected only by the Real-Time PCR.

Detecting of *F. tularensis* in intravascular monocytic cells with IHC in some abortive materials and the presence of its extracellular niches in the circulatory system indicates that the agent can be found in the circulatory system in abortion cases (27). Therefore, detection of *F. tularensis* from sheep blood serum not only serologically, but also by PCR in blood serum samples of 5 sheep, representing the circulatory system, showed the clinical importance of these samples in diagnosis. Although the histopathological analysis of aborted materials is not performed, the determination of the agent in blood will contribute to solving the mystery of the role of *F. tularensis* as a constructive agent in sheep abortions, which is theoretically known but scarcely encountered as clinical cases.

In the study, the agent was detected by the Real-Time PCR in the vaginal discharges of 2 aborted sheep. This is the first report that *F. tularensis* was found as an agent in an abortive material in Türkiye. In line with these findings, we think that *F. tularensis*, which is not a vaginal floral agent, may be scattered with vaginal secretions following the abortion, similar to that the probability of bacteria scattering with nasal or ocular discharges in the form of oculoglandular tularemia infection in human (34), and thus may be a possible abortion agent unless otherwise specified.

In conclusion, the molecular prevalence of *C. abortus, C. burnetti* and *F. tularensis* in cattle and sheep abortion was determined as 6.58%. Although their prevalences are similar to that obtained from many countries, it was determined that these agents, which are mostly ignored during the diagnosis, can be scattered with aborted materials especially vaginal discharges in a way that may pose a risk for animal and human. In areas where infections are endemic, these agents should be considered as a part of the diagnosis in cases with a history of disease and gross pathology. It can be also said that the Real-Time PCR is a usable method that provides fast, sensitive and reliable results in this direction.

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Ethical Statement

The ethical permission was ensured from The Animal Experiments Local Ethics Committee of Veterinary Control Central Research Institute (Türkiye) with a code of "2021-01". A publication permit was granted by the Ministry of Agriculture and Forestry (Türkiye) with the document dated 26.06.2020 and numbered 71037622-824.01.03-E.1750551.

Conflict of interest

The author(s) declare no potential conflicts of interest.

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Evaluation of HMF levels in unbranded flower honeys in terms of food safety

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Abstract: 5-hydroxymethylfurfural (HMF) is formed by reducing sugars in honey in acidic environments by the Maillard reaction and is known as a carcinogenic, mutagenic and genotoxic compound. The aim of this study is to investigate the toxic HMF content of unbranded flower honey samples sold under the sun under inappropriate conditions and unsupervised on the highway sides and to make an evaluation in terms of food safety. The quality of the analysis results was guaranteed by participating in an international proficiency test. Analysis was performed on a High-Performance Liquid Chromatography (HPLC) device with Diode Array Detector (DAD) and Refractive Index (RI) detectors- The HMF levels of 5 honey samples were determined to exceed the legal limit (40 mg kg^{-1}). It is revealed by the current study that the honey sold on the roadside is not safe in terms of toxic HMF. In addition, 5 samples were not found to comply with the regulation in terms of glucose + fructose content and 2 samples in terms of fructose/glucose ratio. Thus, it has also been determined that these honeys are not reliable in terms of purity.
Keywords: Bee product, food safety, HMF, honey, toxicity.

Markasız çiçek ballarındaki HMF düzeylerinin gıda güvenliği açısından değerlendirilmesi

Özet: 5-hydroxymethylfurfural (HMF), Maillard reaksiyonu ile baldaki şekerlerin asidik ortamlarda indirgenmesiyle oluşur ve karsinojenik, mutajenik ve genotoksik bir bileşik olarak bilinir. Bu çalışmanın amacı, karayolu kenarlarında güneşin altında uygunsuz koşullarda, denetimsiz satılan markasız çiçek balı örneklerinde toksik HMF içeriğini araştırmak ve gıda güvenliği açısından bir değerlendirme yapmaktır. Analiz sonuçlarının kalitesi, uluslararası bir yeterlilik testine katılarak garanti altına alınmıştır. HMF ve şeker seviyelerinin belirlenmesi için sırasıyla, Diyot Dizisi Dedektör (DAD) ve Refraktif İndeks (RI) dedektöre sahip bir HPLC cihazı kullanılmıştır. Beş bal numunesinin HMF seviyelerinin yasal sınırı (40 mg kg^{-1}) aştığı belirlenmiştir. Bu çalışma, yol kenarlarında satışa sunulan balların toksik HMF bakımından güvenli olmadığını ortaya koymaktadır. Ayrıca, 5 numune glukoz + fruktoz içeriği bakımından, 2 numune de fruktoz/glukoz oranı bakımından yönetmeliğe uygun bulunmamıştır. Böylece, bu balların saflık bakımından da güvenilir olmadığı belirlenmiştir.
Anahtar sözcükler: Arı ürünü, bal, gıda güvenliği, HMF, toksisite.

Introduction

Honey is a natural food containing around 80% carbohydrates (most importantly glucose, fructose, and sucrose) and 20% water. It also contains more than 180 bioactive components, including vitamins, minerals, amino acids, organic acids, enzymes, and phenolic compounds (8, 11, 14). Honey is classified as flower and secretion honey. Flower honey is obtained from plant nectars, and honeydew honey is obtained from the secretions of plant-sucking insects living on plants or from the secretions of living parts in plants according to Turkish Food Codex Honey Communique (21). Heat treatment can be applied to the honey in order to provide sufficient fluidity during filling, to delay or prevent crystallization and to stop microorganism development (9, 11). Acid catalyzed dehydration of hexoses (1, 3) and the Maillard reaction (1, 22) during heat treatment are the main routes for spontaneous formation of 5-hydroxymethylfurfural (HMF). Long-term storage is another factor that contributes to the increase of HMF amounts. Therefore, the determination of the amount of HMF is an indicator for excessive heat treatment or improper storage of honey (6, 11). HMF, which induces reactive oxygen species, has toxic effects (7). Current studies show that HMF can be considered as a potential carcinogen for humans or an agent that may be metabolized to carcinogenic compounds (1, 13). 5-sulfoxymethyfurfural, a conversion product of HMF, is a cytotoxic and mutagenic compound (5). If the literature is taken into account, HMF is a compound that should not only be considered as a quality criterion in

honey, but also be considered in terms of health risks as it is a compound with mutagenic and genotoxic metabolite. Commercial honeys are controlled for HMF levels, purity and other quality criteria by regular sampling of the authorities. However, the honey that is put up for sale on the side of the roads under the sun on makeshift benches by producers and/or people claiming to be producers without legal sales permission, is not followed by the state's control mechanisms. On the other hand, consumers passing through the national parks such as Ilgaz and Küre, buy these honeys considering that they are pure, natural, even organic and very healthy. Examining the HMF levels and compositions of these honeys - under the above-mentioned sales conditions is also extremely important in terms of consumer health and food safety. Therefore, the aim of this study was to examine toxic HMF content and composition of the unbranded flower honey samples sold in Kastamonu province in order to reveal whether consumers are under risk related to food safety and health.

Materials and Methods

***Collection of honey samples*:** A total of 22 unbranded flower honey samples were collected from Kastamonu province in the summer season. The samples (about 1 kilogram each) were obtained from small-scale traditional beekeepers selling on the sides of highways in the Ilgaz and Küre mountain regions. Flower honey samples were stored at room temperature (22 ± 2 °C) in a dark place until analysis.

Chemicals: HMF (≥ 99%), glucose, fructose, and sucrose were provided by Sigma Aldrich (Saint-Louis, MO, USA), AFG Bioscience LLC (Northbrook, USA) and Merck (Darmstadt, Germany). Methanol and acetonitrile (HPLC grade) were obtained from Sigma Aldrich. Ultrapure water was produced using Human Power 2 system (Seoul, Republic of Korea). All the other reagents used were of analytical purity and were obtained from Merck and Sigma Aldrich.

Methods: All analyses were carried out in Kastamonu University Central Research Laboratory Application and Research Center. The quantities of HMF and sugars were determined according to International Honey Commission (IHC) Methods (4) using a High-Performance Liquid Chromatography (HPLC) device (Shimadzu LC-20A Prominence series, Kyoto, Japan) with Diode Array Detector (DAD) and Refractive Index (RI) detector, respectively. Conditions for chromatographic analyses are given in Table 1. In addition, the free acidity and moisture analyses of the samples were completed according to IHC methods (2009).

Sample extraction for chromatographic analyses: A sample of 2.5 g of homogenized honey was accurately weighed into a 50 mL beaker. Then, the sample was dissolved in approx. 25 mL of water and transferred quantitatively to a 50 mL volumetric flask. The volumetric flask content was diluted to 50 mL with water and filtered through a 0.45 μm membrane filter to provide a sample solution ready for chromatography.

Method performance characteristics: Linearity was assessed with a calibration curve that was constructed using standard solutions of HMF and sugars in the ranges of 0.8, 4.0, 8.0, 12.0, 20.0, 40.0, 80.0 μg mL^{-1} and 15, 20, 40, 60, 80%, respectively. Ten replicates of HMF solution (in ultra-pure water spiked at the 0.8 μg mL^{-1} concentration) and sugar solution (glucose, fructose, and sucrose in ultra-pure water spiked at 0.5%; 1%, and 1% concentrations) analyses were performed. The limit of detection (LOD) and limit of quantification (LOQ) of the methods were expressed as 3 × standard deviation (SD) and 10 × SD, respectively, according to Eurachem Guide (2014) (17). To establish repeatability (intra-day precision) and reproducibility (inter-day precision), ten replicates of a homogenized flower honey sample analyses were performed on two different days, separately. Intra-day and inter-day precisions were expressed as the percentage relative standard deviation (RSD %). To establish the accuracy, recovery assays were carried out. For this purpose, homogeneous flower honey was diluted 4 times with ultrapure water. Standard HMF was spiked into this dilute solution with a concentration of 24 and 48 μg mL^{-1}, respectively. Likewise, standard sugars were added so that the in order to reach 5% and 30% dilutions, respectively. Six replicates of these samples were prepared and analyses were performed. Recovery is expressed in percent and calculated using the formula below.

Table 1. Conditions for chromatographic analyses.

Analysis Parameters	Column	Wavelength	Column and Detector Temperature	Mobile Phase	Flow Rate	Sample Volume
HMF	Inertsil C18; (5 μm, 4.6 × 150 mm)	285 nm	Room temperature	Water:Methanol (90:10, v/v)	1.0 (mL min^{-1})	20 μL
Sugars (Glucose, Fructose, Sucrose)	Inertsil NH2 ; (5 μm, 4.6 × 250 mm)	-	40 °C	Acetonitrile:water (80:20, v/v)	1.3 (mL min^{-1})	10 μL

Table 2. LOD, LOQ, intra-day, inter-day precision and recoveries for chromatographic analysis of flower honey.

Analyte	LOD/LOQ	Intra-day precision, %RSD (n=10)	Inter-day precision, %RSD (n=20)	Spiked Levels for recovery assays	Recovery of spiked samples, % ± SD (n=6)
HMF	0.036/0.121 (mg kg^{-1})	1.40	1.69	24 mg kg^{-1} 48 mg kg^{-1}	99.83 ± 1.65 100.65 ± 0.34
Fructose	0.278/0.927 (%)	1.54	0.94	5 % 30 %	91.47 ± 14.24 90.37 ± 1.26
Glucose	0.238/0.732 (%)	1.23	1.03	5 % 30 %	107.40 ± 11.52 99.99 ± 2.33
Sucrose	0.189/0.639 (%)	1.03	3.31	5 % 30 %	96.80 ± 2.56 99.40 ± 1.20

SD: Standard deviation
RSD: Relative standard deviation.

Table 3. Analysis results for flower honey samples.

No	HMF (mg kg^{-1} ± SD)	Free acidity (meq kg^{-1} ± SD)	Moisture% (w / w ± SD)	Fructose% (w / w ± SD)	Glucose% (w / w ± SD)	Sucrose% (w / w ± SD)	Total sugar% (w/w)	Fructose + glucose%	Fructose / glucose
1	**42 ± 1**	10.8 ± 0.1	9.8 ± 0.1	40.8 ± 0.2	26.4 ± 0.1	0.3 ± 0.0	67.5	67.2	**1.55**
2	10 ± 0.3	13.6 ± 0.1	13.9 ± 0.0	43.0 ± 0.3	36.0 ± 0.0	1.2 ± 0.1	80.2	79.0	1.19
3	2 ± 0.1	5.1 ± 0.0	15.4 ± 0.0	38.3 ± 0.15	27.3 ± 0.1	0.4 ± 0.05	66.0	65.6	1.40
4	<LOD	20.4 ± 0.15	15.8 ± 0.0	32.1 ± 0.2	25.4 ± 0.15	0.1 ± 0.0	57.6	**57.5**	1.26
5	7 ± 0.1	30.7 ± 0.1	15.8 ± 0.1	30.4 ± 0.3	22.3 ± 0.2	<LOD	52.7	**52.7**	1.37
6	2 ± 0.0	20.4 ± 0.2	14.4 ± 0.1	40.1 ± 0.1	32.3 ± 0.1	<LOD	72.4	72.3	1.24
7	26 ± 0.5	15.3 ± 0.1	16.6 ± 0.0	36.5 ± 0.1	34.6 ± 0.2	0.1 ± 0.0	71.2	71.1	1.05
8	8 ± 0.2	20.4 ± 0.1	15.8 ± 0.1	36.7 ± 0.2	32.0 ± 0.2	0.6 ± 0.0	69.3	68.7	1.15
9	**60 ± 1.3**	35.7 ± 0.05	17.8 ± 0.0	38.8 ± 0.4	32.6 ± 0.1	0.3 ± 0.0	71.7	71.3	1.19
10	10 ± 0.3	20.4 ± 0.1	15 ± 0.0	42.7 ± 0.2	38.9 ± 0.3	<LOD	81.6	81.6	1.10
11	11 ± 0.1	20.4 ± 0.0	13.1 ± 0.1	42.4 ± 0.2	34.7 ± 0.25	<LOD	77.2	77.2	1.22
12	**55 ± 3.2**	30.7 ± 0.2	14.0 ± 0.1	36.2 ± 0.3	38.3 ± 0.2	<LOD	74.5	74.5	0.95
13	1 ± 0.0	37.5 ± 0.1	16.0 ± 0.0	37.6 ± 0.25	33.4 ± 0.0	0.2 ± 0.0	71.1	71.0	1.13
14	7 ± 0.1	30.7 ± 0.3	15.0 ± 0.0	42.1 ± 0.3	32.7 ± 0.1	0.3 ± 0.05	75.1	74.8	1.29
15	**43 ± 2.4**	30.7 ± 0.1	15.8 ± 0.0	39.8 ± 0.1	32.1 ± 0.1	0.2 ± 0.0	72.1	71.9	1.24
16	**44 ± 1.9**	13.6 ± 0.0	17.0 ± 0.0	32.5 ± 0.1	25.4 ± 0.2	<LOD	57.9	**57.9**	1.28
17	7 ± 0.2	27.9 ± 0.1	15.8 ± 0.0	39.7 ± 0.35	34.0 ± 0.4	<LOD	73.7	73.7	1.17
18	10 ± 0.2	20.4 ± 0.1	13.3 ± 0.1	41.1 ± 0.15	29.8 ± 0.15	<LOD	70.9	70.9	1.38
19	<LOD	37.7 ± 0.05	10.8 ± 0.2	28.7 ± 0.1	17.5 ± 0.1	<LOD	46.2	**46.2**	**1.64**
20	37 ± 1.7	29.8 ± 0.1	13.7 ± 0.1	39.1 ± 0.25	32.1 ± 0.1	<LOD	71.2	71.2	1.22
21	16 ± 0.8	6.8 ± 0.1	13.7 ± 0.0	21.5 ± 0.15	24.6 ± 0.2	0.1 ± 0.0	46.2	**46.1**	0.90
22	5 ± 0.1	17.0 ± 0.2	13.4 ± 0.1	36.5 ± 0.3	30.3 ± 0.1	0.1 ± 0.0	66.8	66.8	1.20
Mean	18.32	22.55	14.63	37.12	30.58	0.18	67.87	67.69	1.23

SD: Standard deviation
Note: Results shown in bold are incompatible with the Turkish Food Codex Honey Communique.

$$\text{Recovery (\%)} = [(C1-C2) / C3] \times 100$$

C1 = Measured concentration of sample and added standard
C2 = Concentration of the sample
C3 = Concentration of the added standard

Analytical quality assurance: The laboratory where this study was carried out participated in "FAPAS® (accredited by UKAS as complying with the requirement of EN ISO/IEC 17043:2010) Proficiency Test No. 2839" for the evaluation of analytical quality assurance.

Statistical analysis: Statistical analysis was performed using IBM SPSS 22.0 software. The results of the analysis parameters were not normally distributed; the non-parametric Spearman correlation test was performed to evaluate the possible association between HMF levels and other analysis parameters.

Results

Method performance characteristics: The calibration curves were linear in the working range of the standards with coefficient of determination (R^2) values of 0.99997, 0.99995, 0.99999, and 0.99993 for HMF, glucose, fructose, and sucrose, respectively. The LOD and LOQ values, and the results of repeatability, reproducibility, and recovery, were shown in Table 2. The percentages of RSDs were < 20% in this study and were below the maximum acceptable RSD values calculated by Horwitz equation (12). The recoveries obtained for all concentration levels were acceptable.

Analytical quality assurance: Assigned values of the FAPAS test sample and z-scores for each analysis parameter of the laboratories participating in the international proficiency tests were determined by FAPAS. The z-score values of Kastamonu University Central Research Laboratory Application and Research Center ranged from 0.5 to 0.9 for all the parameters analysed in this study. Considering that the acceptable range of z-scores must be $-2 \leq z \leq 2$, the quality and reliability of our analytical method was proved.

Analysis Results for Flower Honey Samples: The analysis results of the flower honeys examined in our study and their comparison with the limits in the Turkish Food Codex, Honey Communique were given in Table 3.

Discussion and Conclusion

Turkish Food Codex Honey Communique (21) and European Union Directive (110/2001/ CE) (8) recommend a limit of 40 mg kg^{-1} for HMF in honey. The level of HMF was detected in the range from undetectable to 60.2 mg kg^{-1} (see Table 3) in this study and HMF levels of 5 honey samples were determined to exceed the legal limit given in Turkish Food Codex, Honey Communique. Similar to the results of our study, in a study examining 8 flower honey samples obtained from traditional producers in Bingöl province, HMF value (42.22 mg kg^{-1}) of a Honey sample - exceeded 40 mg kg^{-1} (24). In a study conducted with honey samples obtained from 46 members registered to the beekeepers' association in Gaziantep province, the average HMF value was determined as 27.690 mg kg^{-1}, but the HMF levels of three samples were out of the standard (16). If the measured value of HMF is above legal limit, it indicates that the honey has been stored in an unsuitable hot environment or may have been exposed to heat treatment (6, 9, 11). In contrast, in a study with honeys supplied directly from the producer in the eastern Black Sea Region, HMF was detected in the range from undetectable to 11 mg kg^{-1} (10) and the range of HMF values were 2.06-3.43 mg kg^{-1} for multifloral honeys from the Central Anatolia and 2.03-3.29 mg kg^{-1} for sunflower honeys from the Trakya Region (25). Unlike these studies, the reason for HMF values of some honey samples of this study being above the legal limits, may be exposure to the sun because they are sold on the sides of highways in the Ilgaz and Küre mountains.

In this study, some quality criteria and components of honey were also analyzed. In our study, mean fructose + glucose content was determined to be and 67.69% (see Table 3). But the fructose + glucose% (inverted sugar) content of 5 flower honeys was less than the minimum limit value (60%) determined by the relevant legal regulations. The reason for this situation may be that such honeys sold under the name of flower honey are actually mixed with honeydew honeys (the minimum limit value 45%) or produced with sugar syrups. In the literature, there are other studies in which some of the honeys purchased directly from the producers do not meet the requirements of the regulation in terms of glucose + fructose (10, 11). The mean fructose/glucose value was determined to be 1.23 (Table 3) in this study. While the fructose/glucose ratio should be 0.9-1.4 in flower honey according to Turkish Food Codex Honey Communique, this ratio was significantly higher than 1.4 in 2 samples in our study. This shows that the amount of fructose in the samples is higher than the amount required in these honeys. This suggests the possibility that fructose syrup was added to these honeys. The average sucrose amount was detected to be 0.18% in this study. The sucrose content did not exceed the limit value of 5% in any of the samples. Similarly, in the studies of Batu, Küçük and Çimen (2) and Güzel and Bahçeci (11), the results were found to be within the limits. In contrast, the content of sucrose in 14 of 20 flower honey samples from Erzurum Province exceeded the limit of Turkish Food Codex Honey Communique (20). The free acidity and moisture content of all flower honeys investigated in this study were below the maximum limit values given in the Turkish Food Codex, Honey Communique. The acidity and moisture content of flower honeys examined in other

studies (2, 11, 16, 20, 25) mostly abided by the limits of the relevant communique, in line with our study.

The samples examined in this study were obtained from traditional manufacturers that selling in makeshift huts without legal permission at different points on the highways in Ilgaz and Küre mountains. Thus, it is obvious that these samples were exposed to significant levels of daylight and summer heat. Some of these honeys may probably be produced in previous seasons. High temperatures and long-term storage are known to significantly increase the HMF levels (6, 9, 11). Korkmaz and Küplülü stored flower and honeydew honey samples produced by different companies at different temperatures and examined the effect of this situation on HMF levels (15). In their study, while the average HMF values of the samples kept at 10 ± 2 °C and 22 ± 2 °C for a year did not exceed 40 mg kg^{-1} that is the limit value in the Turkish Food Codex Honey Communique, the HMF level increased rapidly and was above 40 mg kg^{-1} (from 53.1 to 83.7 mg kg^{-1}) starting from the 6th month in samples stored at 35 ± 2 °C. These temperatures are common in the summer months in Kastamonu province, and it is possible that honeys sold on the sides of the highway might be left at this temperature and in the sun for weeks or even months. It is highly probable that the samples with high HMF levels in this study were stored for a long time or exposed to sunlight and/or heat treatment or produced in previous years. Although, moisture and free acidity contents which are important for the Maillard reaction are other parameters affecting HMF levels -(1, 18, 19), no significant relationship between these parameters and HMF levels was determined ($P > 0.05$) in the current study. The reasons for this may be that these parameters do not show much variation in honey samples, and most importantly, the heat and sunlight parameters are more effective on HMF levels under the conditions in which these samples are sold. Honey offered for sale by traditional beekeepers is not inspected and the conditions under which they are stored and sold are unknown, and HMF levels are questionable. The results of our study revealed that unbranded flower honeys sold by beekeepers on the roadside may be problematic in terms of HMF level and also revealed that their purity is questionable. Although the Ministry of Agriculture and Forestry of the Republic of Türkiye increases its legal inspections day by day, it is still not possible to examine all of the honey offered for sale in large number of commercial shops. For example, the average HMF value of honeys obtained from the markets was 56.70 ± 3.83 mg kg^{-1}, in the province of Aydın (23). Even the honeys sold in commercial shops cannot be fully inspected, and for this reason, the sale of honey that does not comply with the criteria in legal regulations cannot be prevented. Therefore, it does not seem possible for the authorities to inspect the honey produced by all traditional producers. That's why it is important that the authorities take the necessary precautions, increase the awareness of producers and consumers and ensure that they are informed about the health risks caused by consuming these products.

Consequently, it is thought that brand-free honeys sold on the sides of the highway by traditional beekeepers are not reliable in terms of HMF levels and purity. Unfortunately, these producers and their products are not inspected by the authorities responsible for monitoring compliance with the provisions of the Turkish Food Codex, Honey Communique. Authorities should make consumers aware of the safety and health risks of unbranded honey sold by the roadside, and producers should be trained on appropriate production, storage and sale conditions. Consumption of packaged and branded honeys is recommended, because these honeys are subject to inspections by authorities in terms of food safety and reliability.

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Ethical Statement

Since the study contains only food analyzes, ethics committee approval is not required.

Conflict of Interest

The author declared that there is no conflict of interest.

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Case Report / *Olgu Sunumu*

Exploratory laparotomic diagnosis of renal cystic echinococcosis in a domestic cat from Hatay province of Türkiye and its molecular confirmation

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Abstract: This case report was prepared to provide information about cystic echinococcosis detected in a twelve years old domestic cat during experimental laparotomy. In the anamnesis, there was a complaint of progressive abdominal swelling. As a result of clinical and radiological examinations, unknown intraabdominal formations were detected. At laparotomy, multiple cysts were detected on the right and left kidneys. Molecular analysis revealed that these cystic structures are larval forms of *Echinococcus granulosus*. The cysts are often found in the liver and lungs but they can arise less commonly in the brain, kidneys, muscle, bone and heart. Renal cystic echinococcosis is rare and this note describes it, confirmed by molecular analysis in a domestic cat. For this reason, it is thought that this note will contribute to the literature.
Keywords: Experimental laparotomy, kidney, Persian cat, renal cyst.

Türkiye'nin Hatay ilinden evcil bir kedide renal kistik ekinokokkozis'in eksploratif laparotomik tanısı ve moleküler doğrulaması

Özet: Bu olgu sunumu on iki yaşında evcil bir kedinin deneysel laparotomisi esnasında tanısı konan kistik ekinokokkozis hakkında bilgi vermek için hazırlandı. Anamnezde giderek büyüyen bir karın şişliği şikâyeti vardı. Klinik ve radyolojik incelemeler sonucunda karın içinde bilinmeyen oluşumlar tespit edildi. Laparotomide sağ ve sol böbrek üzerinde çok sayıda kistle karşılaşıldı. Yapılan moleküler analiz ise, kistik yapıların *Echinococcus granulosus*'un larvası olduğunu gösterdi. Kistler genellikle karaciğer ve akciğerlerde bulunurlar ancak daha az yaygın olarak beyin, böbrekler, kas, kemik ve kalpte ortaya çıkabilirler. Renal kistik ekinokokkozis nadirdir ve bu not evcil bir kedide moleküler analizle doğrulanmış olarak bunu açıklamaktadır. Bu nedenle bu notun literatüre katkı sağlayacağı düşünülmektedir.
Anahtar sözcükler: Böbrek, böbrek kisti, deneysel laparotomi, İran kedisi.

Cystic echinococcosis (CE) is an important parasitic disease caused by the dog tapeworm, *Echinococcus granulosus.* This parasite is widespread worldwide. The life cycle of *E. granulosus* occurs between two mammalian hosts. Definitive hosts are mainly dogs and the other canids. Intermediate hosts are sheep, goats, pigs, horses, cattle, and humans. Sometimes, dogs and cats act as intermediate hosts of *E. granulosus.* Thus, the disease can also occur in these animals. Intermediate hosts become infected by ingestion of parasite eggs, shed in the feces of the definitive host. Then oncosphere hatched from the eggs are passively transported via blood or lymph vessels to the liver, lungs or other organs and develop to hydatid cysts. Infection occurs in definitive hosts by ingestion of intermediate hosts' offals containing the larval stage of *E. granulosus* (8, 17).

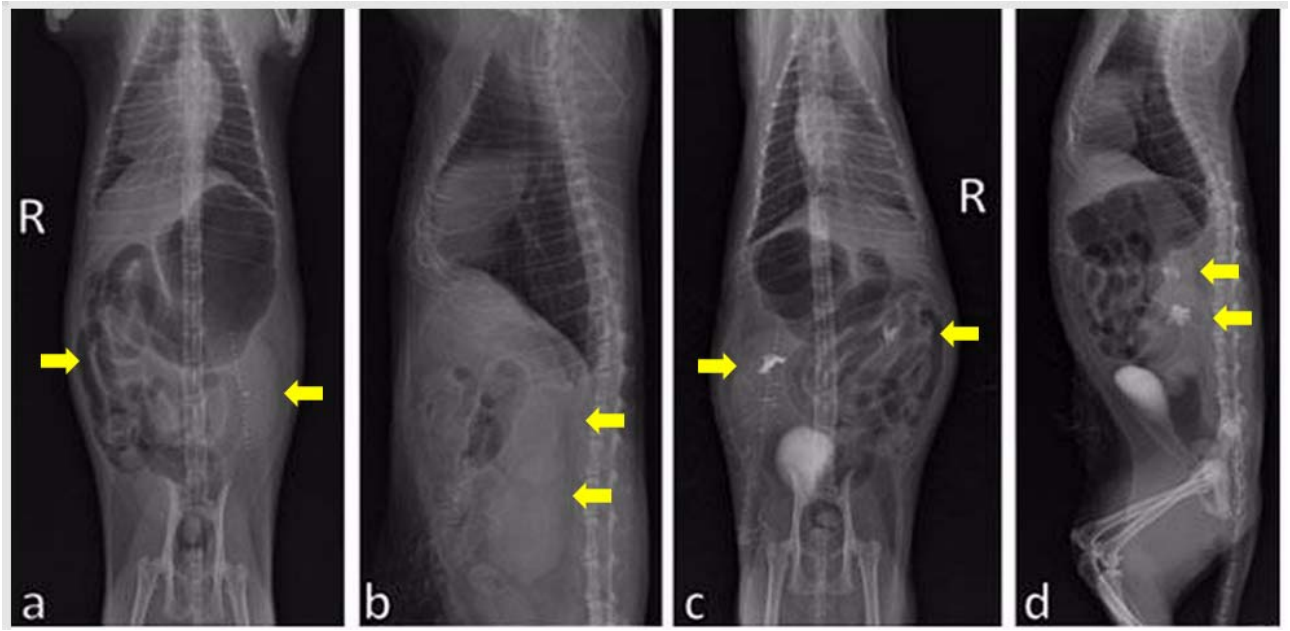

Figure 1. Preoperative direct abdominal VD (a) and LL (b) radiographs and appearance of the patient's enlarged and deformed kidneys on urinary system contrast (urography) abdominal VD (c) and LL (d) radiographs.

Echinococcus genus comprises different genotypes; *E. granulosus sensu stricto* (*s.s.*) (G1-G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6-G7, G8, G10), *E. felidis*, *E. oligarthrus*, *E. vogeli*, *E. multilocularis* ve *E. shiquicus* (3, 11, 14). In the world, *E. granulosus sensu stricto* G1 was detected in cats by molecular analysis (1-3, 8). Oguz et al. (12) also reported that this genotype in a 2-year-old stray cat, in the Van province of Türkiye for the first time.

A 12 years old, female Persian cat (neutered, 3.5 kg) comprised the material of this case study. The cat was brought to the Department of Surgery in Veterinary Health Practice and Research Hospital, Hatay Mustafa Kemal University. After the anamnesis of abdominal swelling, unknown intraabdominal formations were detected by clinical and radiological examinations (Figure 1). Iohexol (400 mg/kg IV, 350 mg / 100 ml, Opakim, Türkiye) was used in urography. After all diagnostic procedures, it was decided to perform an exploratory laparotomy. General anaesthesia was applied to the cat by using xylazine HCl 2 mg/kg IM and ketamine 10 mg/kg IM (after 5 minutes), and maintained with isoflurane inhalation anesthesia. The abdominal region of the patient was prepared for the surgery in the dorsal recumbency position. The abdominal cavity was accessed through incisions to skin, subcutaneous connective tissues, *linea alba*, and peritoneum with a ventral midline surgical approach. Intraabdominal organs and surrounding structures were examined. During exploration, multiple cysts were detected on the right and left kidneys. Significant enlargement of both kidneys and darkening in color were observed macroscopically. The cyst samples were taken by ligating with absorbable (4-0 no.) suture from the root so that the integrity of the cysts was not disrupted and the liquid content did not disperse. After the process, the peritoneum, abdominal muscles and subcutaneous connective tissues were closed with a simple continuous suture and the skin with simple interrupted sutures using absorbable (2-0 no.) suture. These cysts were sent to the parasitology laboratory. During the recovery, vital function monitorization revealed the patient died due to respiratory and cardiac arrest within 12 hours. The animal owner refused the offer to perform an autopsy.

The cysts were thoroughly smashed with a sterile scalpel and washed 5 times with phosphate buffered solution. DNA extraction was performed using the tissue kit and the extracts were stored at -20°C until used. Polymerase Chain Reaction (PCR) was applied as described by Eslami et al. (5). The nucleotide sequences of the primers targeting 882 bp region of the Cox 1 mitochondrial DNA gene of *E. granulosus* were F1 5'-GAATTTA CCGCGTTTGAA -3' and R 5'-CTTATATAAGAACCTAACGAC-3'. Primers were obtained from a commercial company in a lyophilized form. DNase-RNase-free water was added to the F1 primer 568 µl and to the R primer 517 µl and diluted to 100 pmol/1 µl. 2.5 µl 10X PCR buffer, 2.5 µl 25 mM $MgCl_2$, 2.5 µl 2.5 mM dNTP, 5U/µl Taq DNA Polymerase, 20 pmol from each of the primer pairs and 5 µl of template DNA were added to the PCR mix. The preliminary denaturation stage was 5 min at 95ºC in PCR amplification. It was carried out 35 PCR cycles as 45 sec

at 94ºC denaturation, 60ºC annealing and 72ºC elongation. The final elongation was done 10 min at 72ºC after the last cycle. PCR products were run on a 2% agarose gel, at room temperature, 100 V and 500 mA, for 45 minutes and the gels were evaluated under UV light and photographed (Figure 2).

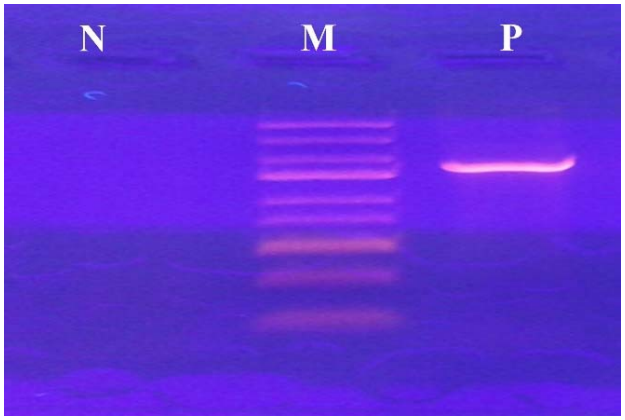

Figure 2. PCR product of the mitochondrial Cox1 gene, 882 bp, N: Negative control, M: Marker (100 bp), P: PCR product isolated from hydatic cysts.

There are very few studies on CE in cats in the world. CE was reported in a cat of unknown age in New Zealand, the cysts measuring 0.1-0.3 cm in diameter (10). Two dogs were tried to be infected with cysts from a cat with CE in Germany, one of which was infected. Only a few of these larval forms reached sexual maturity in the dog and were determined to be *E. granulosus* by measurement of adult parasites (16). In another case diagnosed morphologically in Ankara in 2002, free cystic structures ranging in size from 0.3-5.0 cm diameter were determined in the abdominal cavity of a two years old female stray cat with progressive abdominal swelling. However, a molecular diagnosis was not made in that case (4). *Echinococcus granulosus sensu stricto* G1, which is responsible for human CE cases worldwide (88.44%), was detected in cats by molecular analysis (1-3, 8, 13). Oguz et al. (12) reported that this genotype in a 2-year-old stray cat, in the Van province of Türkiye for the first time. Here, it was reported molecular detection of CE in a 12 years old, female domestic cat with abdominal swelling, in Hatay, Türkiye as a second case report.

In the case reports presented, cats generally domestic cat (1-3, 8) and keep indoors. Therefore, cats were infected maybe in these ways; contaminated food and water, consuming mechanical vectors such as houseflies crawling on dog feces, poorly washed raw vegetables, or unhygienic owner who has come into contact with an *E. granulosus*-infected dog.

Cats are not normally an intermediate host for *E. granulosus*. However, sometimes hydatid cysts can be coincidentally encountered in these animals. Although cats are in contact with the eggs of *E.granulosus*, which is thought to be commonly found in the external environment, the incidence of hydatid cyst in cats is low since cats are not the main hosts of the parasite. Indeed, Lizardo-Daudt et al. (9) tried to infect 11 domestic cats but was unsuccessful. In a case study reported by Armua-Fernandez et al. (1), an infected cat with CE was found to be highly positive for Feline Infection Virus, which is an immunosuppressive disease. Therefore, it can be suggested that CE may develop in immunocompromised cats. In many cases, the gender of the cat with hydatid cyst was determined as female (2, 3, 8, 12). It was also seen in a female cat in our case. Hormonal differences between male and female cats may trigger susceptibility to this disease. There is insufficient data on the epidemiology of the disease in stray and domestic cats. The prevalence of the disease is perhaps not as low as we think. Therefore, further studies are required to expand our knowledge regarding the epidemiology of feline cystic echinococcosis in Türkiye. In future studies, it will be useful to obtain information about the immune status of patients in terms of epidemiological perspective.

Cysts were found either free inside the abdominal cavity (1, 10) or attached to the liver (2, 8, 12) and spleen (3) in cats. In this case report localization of the cysts were kidneys. The cysts are often found in the liver and lungs but they can arise less commonly at the brain, kidneys, muscle, bone and heart (15). Kidneys are rare anatomic location for the cystic echinococcosis. In the world and Türkiye, renal cystic echinococcosis was reported in humans (6, 7, 15) but there is no record of renal echinococcosis in cats. This note describes the renal cystic echinococcosis, confirmed by molecular analysis in a domestic cat. For this reason, it is thought that this note will contribute to the literature.

As a result, CE should be taken into account in the differential diagnosis of abdominal distention cases in cats. Further studies concerning the prevalence of cystic echinococcosis in cats are required.

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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*

Efficiacy of ivermectin solution against Hirstiellosis in green iguanas-case report

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Abstract: Interest in green iguanas (*Iguana iguana*) as pet animals is increasing day by day. Compared to other pet animals, the diagnosis and treatment methods of reptilian diseases are limited. Considering that these diseases will also concern public health, new research is required. Hirstiellosis is a zoonotic ectoparasitic disease seen in green iguanas (*Iguana iguana*). Recommended therapies are mostly empirical, some of which can be low-impact or toxic. The objective of this study is to determine the effectiveness of ivermectin solution (5 mg/l) in the treatment of hirstiellosis and to provide preliminary data for further studies. Ivermectin solution was found to be effective in the treatment of three iguanas with the aforementioned disease, and no clinical side effects were found in the control examinations made at regular intervals.
Keywords: Hirstiellosis, iguana, ivermectin.

Yeşil iguanalarda Hirstiellosis'e karşı ivermektin solüsyonunun etkinliği-olgu sunumu

Özet: Pet hayvan olarak yeşil iguanalara (*Iguana iguana*) olan ilgi gün geçtikçe artmaktadır. Diğer pet hayvanlara kıyasla reptil hastalıklarının tanı ve tedavi yöntemleri sınırlıdır. Bu hastalıkların toplum sağlığını da ilgilendireceği göz önüne alındığında yeni araştırmalar yapılması gerekmektedir. Hirstiellosis yeşil iguanalarda (*Iguana iguana*) görülen zoonotik karakterde ektoparaziter bir hastalıktır. Önerilen tedaviler çoğunlukla ampiriktir ve bazıları düşük etkili veya toksik olabilmektedir. Bu çalışmanın amacı ivermektin solüsyonunun (5 mg/l) Hirstiellosis tedavisindeki etkinliğini belirlemek ve daha sonraki çalışmalar için ön veri sağlamaktır. Söz konusu hastalığa sahip üç iguanada yapılan tedavide ivermektin solüsyonu etkili bulunmuş, belirli aralıklarla yapılan kontrol muayenelerinde klinik yan etkiye rastlanmamıştır.
Anahtar sözcükler: Hirstiellosis, iguana, ivermektin.

In recent years, the growing interest in reptiles in our country has made it necessary to carry out more research into the diseases of these animals. Diagnosis and treatment methods in reptiles are quite limited compared to other spp. (11). Most of the reptiles that are brought uncontrolled from natural habitats can be infested by parasites and some of these parasites may be of zoonotic significance and closely related to public health (6). Parasites can transmit serious diseases, especially in reptiles living in poor sanitary and stress conditions (3).

Ectoparasites, especially mites, are important factors causing skin diseases in reptiles. The genus *Hirstiella* (*Acari, Prostigmata: Pterygosomatidae*) is common ectoparasites of green iguanas (*Iguana iguana*) . The mites are mostly localized around the eyes, neck, under the chin, axilla, inguinal area, tail and folded areas of the skin (7). These mites cause anemia, itching, crusting, dysecdysis and ulcerative skin lesions (3). Clinical symptoms such as anorexia, depression and lethargy are also found in the affected animals (2) . Some species act as a vector for protozoal infections and some species may also infect humans (3). These mites are known to cause the transmission of pathogens such as *Leishmania sp*. and *Haemogregarins sp*. (6) . In intensive infestation, mites are visible. Samples taken with adhesive tape in mild infestations can be examined under a light microscope (10). Repeated applications of fipronil and ivermectin are effective in treatment (8).

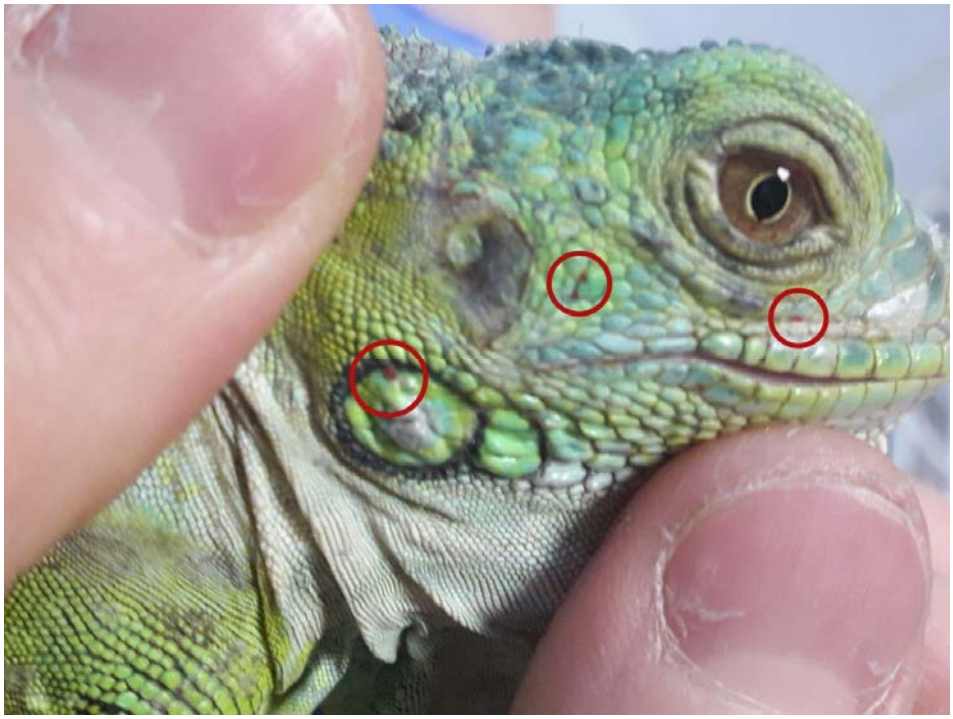

Figure 1. Red mites around eyes.

Figure 2. Dysecdysis in the dorsal area.

In our first case, a two years old green iguana was brought to Ankara University Animal Hospital with the complaint of itching, discoloration of the skin, loss of appetite and weakness. In the examination, it was observed that there were red mites around the eyes, under the neck and tail (Fig. 1). *Hirstiella sp*. was detected in the microscopic examination of the sample taken with adhesive tape. Topical ivermectin-water solution (5 mg/L, Vilmectin®) was applied every 4 days for 2 weeks for treatment (5). Ivermectin solution was also used for environmental disinfection. Two weeks later, it was found that the complaints were gone and recovery started. No clinical side effects such as anorexia and nervous system symptoms were found in the control examination performed after 1, 6 and 12 months.

In our second case, one year old green iguana was brought to hospital with the complaint of skin discoloration and dysecdysis (Fig. 2). It was observed that color changes intensified especially in the folded areas of the skin. Mites were detected in samples taken from suspicious areas. In microscopic examination, *Hirstiella sp*. was diagnosed (Fig. 4). Topical ivermectin-water solution (5 mg/L) was applied every 4 days for 2 weeks for treatment (5). Ivermectin solution was also used for environmental disinfection. It was observed at the end of the second week that the patient recovered. No clinical side effects such as anorexia and nervous system symptoms were found in the control examination performed after 1, 6 and 12 months.

Figure 3. Red mites around eyes and neck.

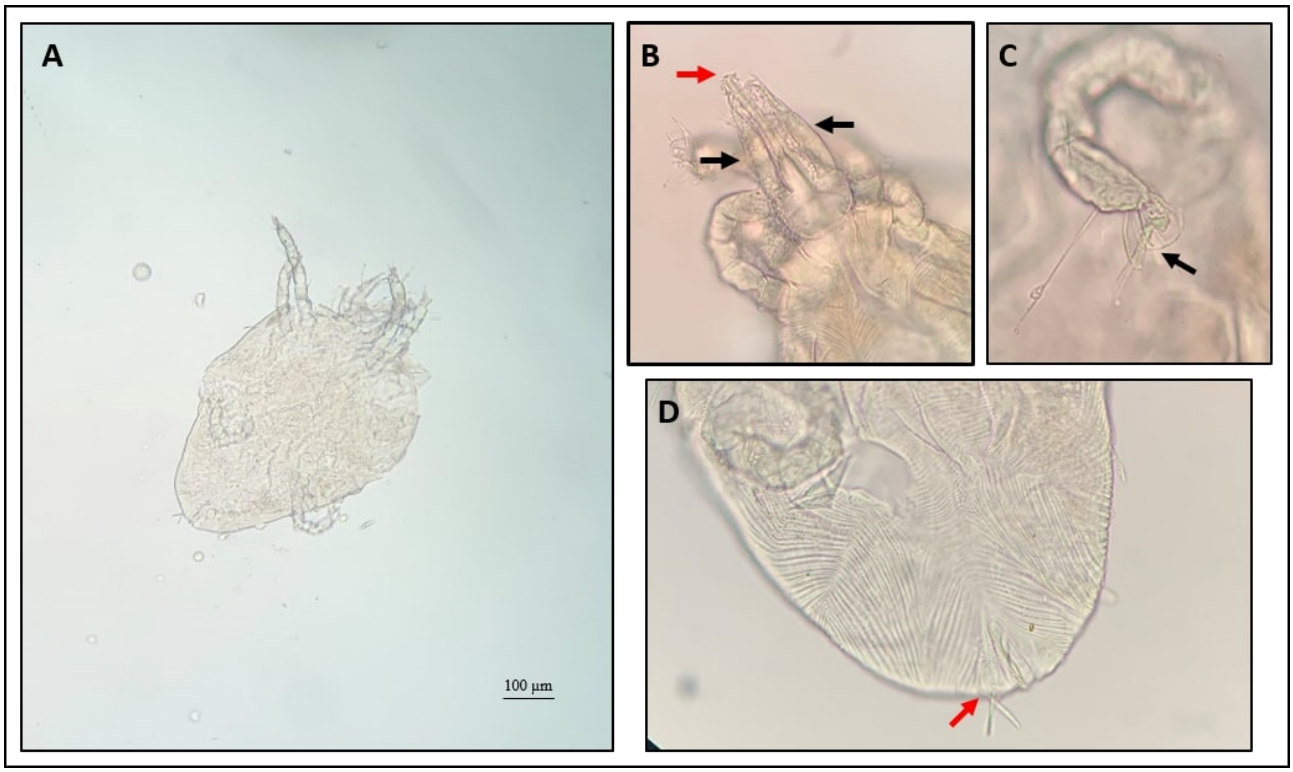

Figure 4. A, Hirstiella sp, ventral view; **B,** gnathosoma, black arrow: palps, red arrow: chelicerae; **C**, details of tarsus I, black arrow: claw; **D,** red arrow: details the anogenital area in ventral view.

Our third case, five years old green iguana was brought to hospital with complaint of itching (Fig. 3). The owner reported that the complaints started after putting the piece of wood they brought from outside into the terrarium. Upon skin examination, color changes, erythema, dysecdysis in the dorsal area and red mites were observed. In the microscopic examination, *Hirstiella sp*. was diagnosed. Topical ivermectin-water solution (5 mg/L) was applied every 4 days for 2 weeks for treatment. Ivermectin solution was also used for environmental

disinfection and a piece of wood was removed. It was observed that recovery started at the end of the second week. No clinical side effects such as anorexia and nervous system symptoms were found in the control examination performed after 1, 6 and 12 months.

The family of *Pterygosomatidae* parasites live on reptiles. The nine genera of mite include *Cyclurobia, Geckobia, Geckobiella, Hirstiella, Ixoderma, Pterygosoma, Scaphotrix, Equisistlana*, and *Zonurobia* known to infest reptiles (7). *Hirstiella sp*. is more common in reptiles (9). *Hirstiella sp*. has been reported from as first Türkiye, Iran, Florida and Italy (2, 4, 7, 12).

In studies of external parasite infections of reptiles, most of the time Hirstiella has been found in green iguanas. (3, 6, 9). The mites are mostly localized around the eyes, neck, under the chin, axilla, inguinal area, tail and folded areas of the skin (7). In all our cases, it was determined that mites were dense, especially in the folded areas of the skin. These mites cause anemia, itching, crusting, dysecdysis and ulcerative skin lesions (3). Itching was the first symptom to be noted by patient owners. In clinical examination, skin discoloration and dysecdysis were identified as common symptoms in all cases.

In intensive infestations, while mites can be detected visually, in cases where the number of parasites is low, examination of skin scraping under a light microscope is required (9). In two of our cases, mites were detected visually, while one was diagnosed with microscopic examinations.

Whole body application of olive oil, organophosphates, carbamates, pretrin or pretroid sprays/shampoos, ivermectin injection/spray and fipronil (%0.25) were used in the treatment (2, 6, 9). There are very few studies on the effects, safety and toxic effects of the drugs in the treatment protocol (5). Some drugs are even known to be toxic to lizards (1, 11). Farmaki et al. (6) used fipronil solution for the treatment of mite in green iguanas, found it effective and did not encounter any side effects. Gazyağcı et al. (7) have used fipronil solution in treatment and have been successful. Not much is known about the efficacy and reliability of the ivermectin. In a case report, it was reported to be effective in treatment (2). In our cases, ivermectin solution (5 mg/L) was used in treatment every 4 days for 2 weeks and provided clinical improvement (5). This solution has also been used in environmental disinfection. No side effects were encountered in follow up examinations.

It was concluded that the ivermectin solution (5 mg/L) can be used for the treatment of *Hirstiella sp*. for both green iguanas and their environments (5). However, we also believe that more comprehensive studies are needed to understand the therapeutic effect of ivermectin solution.

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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*

Monkeypox and other zoonotic poxviruses

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Abstract: Poxviruses have caused the most important diseases for humanity for a long time. An important triumph was achieved with the eradication of smallpox, defined by the World Health Organization in 1979. Poxviruses include significant agents that cause important animal diseases that are non-zoonotic and zoonotic. While humanity has been battling COVID-19, a new battle against monkeypox has recently emerged due to an increase in case numbers and the outbreak's global spread. The other points of the 2022 monkeypox outbreak that make it more serious than previous outbreaks are severe clinical outcomes such as encephalitis and death, and also the higher transmission rate, which occurs at approximately 99% in men, especially those who have sex with men. The 2022 monkeypox virus outbreak has focused public and scientific attention on poxviruses and potential bioterrorism risks posed by poxviruses. Therefore, it is aimed at writing a review that compiles information about monkeypox, cowpox, vaccinia, bovine papular stomatitis, orf, pseudocowpox, gray seal pox, and red deerpox viruses.
Keywords: Monkeypox, poxvirus, zoonotic infection.

Maymun çiçeği ve diğer zoonotik poksvirüsler

Özet: Poksvirüsler uzun zamanlardan beri insanlık açısından çok önemli hastalıklara yol açmıştır. Dünya Sağlık Örgütü'nün önderliğinde Poksvirüslerle özdeşleşen çiçek hastalığının (smallpox) 1979 yılında eradikasyonu ile önemli bir zafer kazanılmıştır. Poksvirüs ailesinde sadece hayvanlarda hastalık oluşturan zoonotik olmayan etkenler ile bazı zoonotik etkenler yer almaktadır. Günümüzde insanlık COVID-19 ile mücadele ederken, son zamanlarda artan vaka sayıları ve salgının dünya çapındaki dağılımı nedeniyle maymun çiçeği ile de savaşmak zorunda kalmaktadır. 2022 maymun çiçeği salgınını önceki salgınlardan daha ciddi hale getiren diğer noktalar, ensefalit ve ölüm gibi ciddi klinik sonuçlar ve erkeklerle seks yapan erkeklerde yaklaşık %99 oranında görülmesidir. 2022 yılı maymun çiçeği salgını hem halkın hem de bilim camiasının ilgisinin tekrar poksvirüslere ve bunlarla ilgili potansiyel biyoterörizm risklerine yönelmesine sebep olmuştur. Bu nedenle bu virüs ailesi içerisindeki zoonotik etkenler arasında yer alan maymun çiçeği, sığır çiçeği, vaccina, sığır papüler stomatit, orf, yalancı sığır çiçeği, gri fok çiçeği ve kızıl geyik çiçeği virüsleri hakkında bilgi veren bir derleme yazılması amaçlanmıştır.
Anahtar sözcükler: Maymun çiçeği, poksvirüsler, zoonotik infeksiyon.

Introduction

Poxviruses belong to the family *Poxviridae*, which is classified into the *Varidnaviria* realm, *Bamfordvirae* kingdom, *Nucleocytoviricota* phylum, *Pokkesviricetes* class, and *Chitovirales* order. The *Poxviridae* family consists of two subfamilies: *Chordopoxvirinae* (includes poxviruses of vertebrates) and *Entomopoxvirinae* (includes poxviruses of insects). There are 18 genera in the *Chordopoxvirinae* subfamily and they are summarized in Table 1. Virions of poxviruses are large (220-450 nm× 140-260 nm), pleomorphic, and brick-shaped with an irregular arrangement of surface tubules (most genera) or ovoid with a regular crisscross arrangement of surface tubules (*Parapoxvirus* genus). The genome of poxviruses consists of 130 to 360 kilobase pairs (kbp) linear double-stranded DNA and encodes 130 to 320 proteins. Unlike other DNA viruses, poxviruses replicate in the cytoplasm of the host cell due to encoding all the enzymes required for transcription and replication (83, 97).

Table 1. Classification of *Poxviridae* family.

Family: ***Poxviridae***	
Subfamily: ***Chordopoxvirinae***	
Genus: *Avipoxvirus*	Species: *Canarypox virus, Flamingopox virus, Fowlpox virus, Juncopox virus, Mynahpox virus, Penguinpox virus, Pigeonpox virus, Psittacinepox virus, Quailpox virus, Sparrowpox virus, Starlingpox virus, Turkeypox virus*
Genus: *Capripoxvirus*	Species: *Goatpox virus, Lumpy skin disease virus, Sheeppox virus*
Genus: *Centapoxvirus*	Species: *Murmansk microtuspox virus, Yokapox virus*
Genus: *Cervidpoxvirus*	Species: *Mule deerpox virus*
Genus: *Crocodylidpoxvirus*	Species: *Nile crocodilepox virus*
Genus: *Leporipoxvirus*	Species: *Hare fibroma virus, Myxoma virus, Rabbit fibroma virus, Squirrel fibroma virus*
Genus: *Macropopoxvirus*	Species: *Eastern kangaroopox virus, Western kangaroopox virus*
Genus: *Molluscipoxvirus*	Species: *Molluscum contagiosum virus*
Genus: *Mustelpoxvirus*	Species: *Sea otterpox virus*
Genus: *Orthopoxvirus*	Species: *Abatino macacapox virus, Akhmeta virus, Camelpox virus, Cowpox virus*, Ectromelia virus, Monkeypox virus*, Raccoonpox virus, Skunkpox virus, Taterapox virus, Vaccinia virus*, Variola virus, Volepox virus*
Genus: *Oryzopoxvirus*	Species: *Cotia virus*
Genus: *Parapoxvirus*	Species: *Bovine papular stomatitis virus*, Grey sealpox virus*, Orf virus*, Pseudocowpox virus*, Red deerpox virus**
Genus: *Pteropopoxvirus*	Species: *Pteropox virus*
Genus: *Salmonpoxvirus*	Species: *Salmon gillpox virus*
Genus: *Sciuripoxvirus*	Species: *Squirrelpox virus*
Genus: *Suipoxvirus*	Species: *Swinepox virus*
Genus: *Vespertilionpoxvirus*	Species: *Eptesipox virus*
Genus: *Yatapoxvirus*	Species: *Tanapox virus*, Yaba monkey tumor virus**

* Indicates zoonotic poxviruses.

Poxviruses are ancient viruses for humanity. Poxviral DNA is found in ancient human remains dated as far back as AD 600 (175). The method of cutaneous inoculation, called variolation, was practiced frequently in the Ottoman Empire. Mary Wortley Montague had written letters about variolation in Istanbul, the Ottoman Empire's capital city, which led to the introduction of variolation in England. The variolation had also introduced to Europe in the 18th century by the travellers returning from Istanbul (16, 159). Edward Jenner used the cowpox virus for vaccination against smallpox. Jenner termed this inoculation procedure "vaccination" due to the Latin word "vaccinia" that means cowpox (159).

Orthopoxvirus and *Parapoxivirus* genera contain significant zoonotic agents: monkeypox, cowpox, vaccinia, bovine papular stomatitis, orf, pseudocowpox, grey sealpox, and red deerpox viruses (Table 1). This review aims to substantially focus on monkeypox and the beforementioned zoonotic orthopoxviruses and parapoxviruses, considering the recent scientific data and global health emergency.

Monkeypox virus

Etiology: Monkeypox virus (MPXV), with another called the human monkeypox virus (hMPXV), has a 197 kb linear DNA genome and contains close to 190 non-overlapping ORFs, each longer than 60 amino acid residues (169). The virus is phylogenetically classified into 3 clades: Clade I (formerly 'Central African' or 'Congo Basin' clade), clade IIa ('West African' clade), and clade IIb ('West African' clade, and also including clade IIa). The clade IIb includes A.1, A.2, A.1.1, and B.1 lineages. Lineage A.1 consists of the MPXVs that are from 2018 and 2019 cases, mainly from the United Kingdom, Israel, and Singapore. The viruses of the 2022 monkeypox outbreak belong mainly to the lineage B.1, which is a descendant of lineage A.1 and some of the current isolates are determined to be classified in lineage A.2 (77, 85, 89).

The lineage B.1 viruses contain approximately 50 single-nucleotide polymorphisms (SNPs) in comparison to the MPXVs from the 2018–2019 outbreaks, indicating that lineage B1 has an increased mutation rate (85). A recent study reported that 9 MPXV isolates from the 2021-2022 cases in the USA, India, and Thailand are classified as lineage A.2 and have 16 distinct genetic variations when compared to other lineages, including 9 nonsynonymous, 3 synonymous, 1 stop gained variation, and 3 amino acid deletion in the OPG174 gene (89). The lineage A.2 has a lower nucleotide substitution rate than the B.1 lineage. The mean nucleotide substitution rates of

the A.2 and B.1 lineages are determined as 5.53×10^{-5} and 1.13×10^{-4} substitutions per base/year, respectively. The higher mutation rate of lineage B.1 viruses may be the reason for the increasing number of monkeypox cases and the accelerated transmission speed of the virus in the 2022 monkeypox outbreak (85, 89).

Recent studies reported that the majority of the current MPXV isolates have GA > AA and TC > TT nucleotide replacement motifs that are typical for a host enzyme called apolipoprotein B mRNA editing catalytic polypeptide-like3 (APOBEC3). The genome of MPXV isolates between 2017 and 2022 in the clade IIa has more GA > AA mutations in comparison to the clade I and the isolates prior to 2017 in the clade IIa. Most of the SNPs detected in the genomes of 2022 MPXV isolates are also GA > AA and TC > TT nucleotide replacements (69, 85). In another study, it was reported that a genomic comparison of viral isolates from 2015 to 2022 showed that 2022 monkeypox isolates have 30 T bases in length in the middle of the viral genome, which of the role is unknown (149). A study showed that D2L-like, OPG023, OPG047, OPG071, OPG105, OPG109, A27L-like, OPG153, OPG188, and OPG210 proteins of 2022 MPXV isolates have the highest number of mutations throughout the whole viral genome (197). Alignment against the first public sequence of the 2022 monkeypox outbreak (ON563414.3) indicated that novel mutations had occurred, but the viral genes involved in immune evasion, host range, cell proliferation such as A45L, C1L, D7L, D10L genes, drug resistance such as L3R, L6R, and A25R genes, and vaccine development such as the A25R gene have lower mutation rates (114). All these cumulative changes in the viral genome might be responsible for the recent rapid evolution of hMPXV1 and a possible explanation of the faster human-to-human transmission (11, 122). The virus is characterized as a Biosafety Level 3 (high threat) pathogen in the European Union and is on the list of selected agents in the United States following the recent situation (103).

Epidemiology: In 1958 in Denmark, "pox-like" non-fatal outbreaks occurred in cynomolgus monkeys, and the causative agent was named monkeypox virus (195). The first confirmed monkeypox virus human case was a 9-month-old indigenous boy hospitalized because of fever and rashes in the Democratic Republic of the Congo in 1970 (100). Monkeypox viruses were isolated from the kidneys of healthy chimpanzee and cynomolgus monkeys in the Democratic Republic of the Congo and the Netherlands, respectively, and from monkeys with clinical monkeypox disease in the USA (120). In 1970 and 1971, human cases of monkey pox were reported in Liberia, Nigeria, and Sierra Leone. When the humans (most of whom are aged 4–9 years) with monkeypox virus infection were epidemiologically investigated, most of them revealed that they occasionally consumed monkeys for food and played with the internal organs of the killed monkeys (62, 110). In total, 48 confirmed and suspected monkeypox virus cases were reported in the Democratic Republic of the Congo, Cameroon, Côte d'Ivoire, Liberia, Nigeria, and Sierra Leone between 1970 and 1979 (25) (Table 2).

In the 1980s and 1990s, monkeypox cases have only been reported from African countries, and human monkeypox virus infection has been accepted as endemic to some African countries since 2003 (Table 2). The Centers for Disease Control and Prevention (CDC) declared that some human cases with fever, papular rash, respiratory symptoms, lymphadenopathy, and sore throat were confirmed as monkeypox virus infection in 2003 in some states (Illinois, Indiana, Kansas, Missouri, Ohio, and Wisconsin) of the United States of America (USA). All of the patients have had contact with animals such as prairie dogs, Gambian giant rats, and rabbits (animal-to-human transmission), and some have been infected through direct contact with other infected humans (human-to-human transmission). Following traceback investigations, the CDC has reported that the source of the monkeypox virus that was introduced to the USA is Gambian giant rats that were exported from Ghana and co-housed with prairie dogs (28, 29).

In the 2010s, besides endemic countries, monkeypox cases were reported from non-endemic countries such as Israel and the United Kingdom (UK), which were related to the export of travellers (55, 191) (Table 2). On May 6, 2022, a patient with rashes who travelled from the UK to Nigeria and returned to the UK had been confirmed as monkeypox virus, and on May 7, 2022, the World Health Organization (WHO) was informed of this confirmed case of monkeypox in the UK (200). Despite the fact that the number of monkeypox cases has been increasing globally, WHO announced that monkeypox was not a global health treat on June 25, 2022, after more than 4100 cases were recorded in 46 countries (162). Alas, contrary to this announcement, on July 23, 2022, WHO declared monkeypox a public health emergency of international concern (116). On August 4, 2022, the USA declared the monkeypox outbreak a national public health emergency as the virus has infected more than 6,600 people and cases have been recorded in 48 states (95). According to the report of WHO on September 5, 2022, there have been a total of 52,015 laboratory confirmed cases and 395 probable cases (including 18 deaths) of monkeypox virus (204) (Figure 1, Table 2). Türkiye has reported the first case of human monkeypox virus infection on June 30, 2022, and five monkeypox cases in total since August 2, 2022 (157, 186). The USA, where the majority of global monkeypox cases have been reported, confirmed the first death of a monkeypox-infected patient recently (12).

Table 2. Human monkeypox virus infection cases in between 1970 and 2022 by year and country.

Years	Number of cases*	Countries reported cases	References
1970-79	48	Democratic Republic of the Congo, Cameroon, Côte d'Ivoire, Liberia, Nigeria, Sierra Leone	
1980-89	357	Democratic Republic of the Congo, Cameroon, Côte d'Ivoire, Gabon, Central African Republic	
1990-99	520	Democratic Republic of the Congo, Gabon	
2000-09	10,166	Democratic Republic of the Congo, Congo, South Sudan, United States of America	
2010-19	19,071	Democratic Republic of the Congo, Congo, Cameroon, Central African Republic, Nigeria, Liberia, Sierra Leone, Singapore, Israel, United Kingdom	
2020	6,257	Democratic Republic of the Congo	
2021	3,190	Democratic Republic of the Congo, Nigeria, The United Kingdom, United States of America	4, 25, 41, 156, 201, 203
2022**	52,015 laboratory confirmed cases and 395 probable cases	Andorra, Argentina, Aruba, Australia, Austria, Bahamas, Barbados, Belgium, Benin, Bermuda, Bolivia, Bosnia and Herzegovina, Brazil, Bulgaria, Cameroon, Canada, Central African Republic, Chile, China, Colombia, Congo, Costa Rica, Croatia, Cuba, Curaçao, Cyprus, Czechia, Democratic Republic of the Congo, Denmark, Dominican Republic, Ecuador, El Salvador, Estonia, Finland, France, Georgia, Germany, Ghana, Gibraltar, Greece, Greenland, Guadeloupe, Guatemala, Guyana, Honduras, Hungary, Iceland, India, Indonesia, Iran, Ireland, Israel, Italy, Jamaica, Japan, Latvia, Lebanon, Liberia, Lithuania, Luxembourg, Malta, Martinique, Mexico, Monaco, Montenegro, Morocco, Netherlands, New Caledonia, New Zealand, Nigeria, Norway, Panama, Paraguay, Peru, Philippines, Poland, Portugal, Puerto Rico, Qatar, Republic of Korea, Republic of Moldova, Romania, Russian Federation, Saint Martin, Saudi Arabia, Serbia, Singapore, Slovakia, Slovenia, South Africa, Spain, Sudan, Sweden, Switzerland, Thailand, The United Kingdom, Türkiye, United Arab Emirates, United States of America, Uruguay, Venezuela	

*Monkeypox virus cases which are laboratory confirmed or suspected are given in the numbers.
**Case numbers are given according to 5 September 2022 report of the WHO (200).

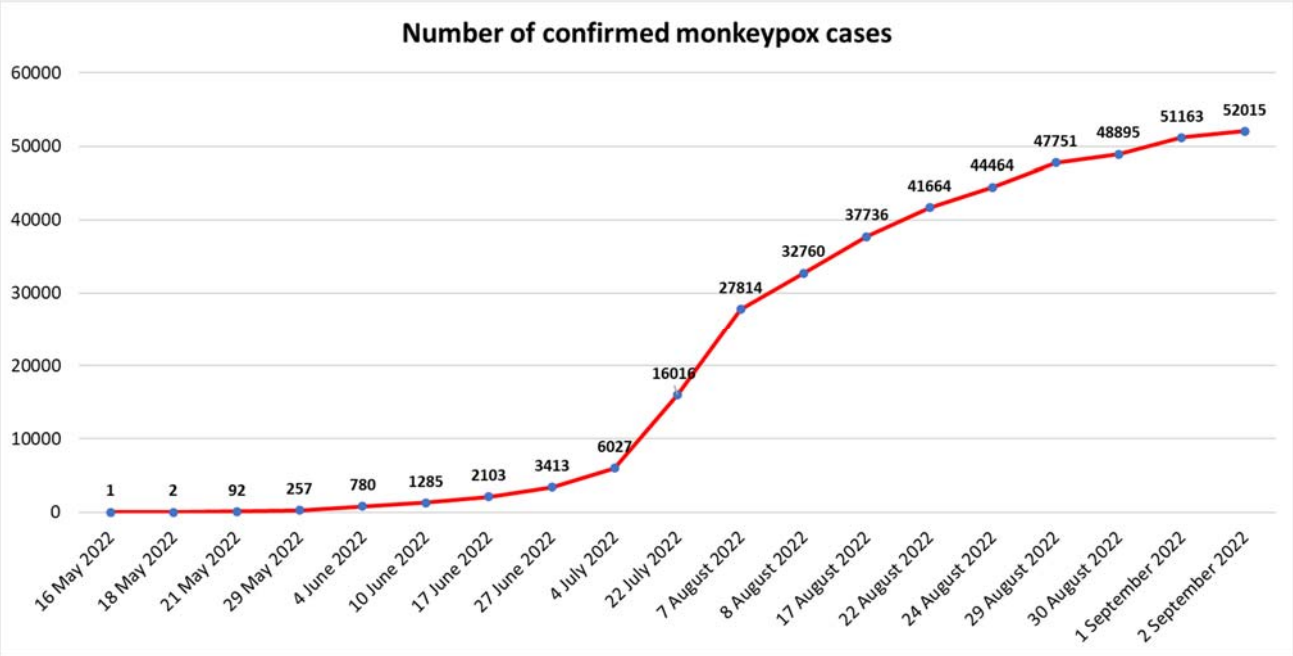

Figure 1. Number of confirmed monkeypox cases reported by WHO between 16 May 2022 and 2 September 2022.

Many mammals and non-human primates (i.e., sooty mangabey monkey, Gambian-pouched rat, rhesus macaques, cynomolgus macaques, Asian monkeys, Southern opossum, sun squirrel, African hedgehogs, jerboas, woodchucks, shot-tailed opossum, porcupines, giant anteaters, prairie dogs, elephant shrew, domestic pigs, rope squirrel, and African dormice) may have a role in transmission of monkeypox virus infection and can be a reservoir of the virus (8). Several outbreaks and cases were recorded in different monkey species both in laboratories and in the wild (132, 144, 147, 153).

The monkeypox virus can be transmitted by animal-to-human and human-to-human. Animal-to-human transmission can occur via direct contact with an infected animal's blood, body fluids, and pox-associated skin or mucosal lesions. Another possible route of transmission of monkeypox virus is through the bites of infected non-human primates or mammals (108, 131).

Human-to-human transmission of monkeypox virus can be achieved through close and direct contact with an infected person's respiratory secretions or droplets, skin or mucosal lesions, body fluids, or indirectly via virus-contaminated fomites (8, 192). Blood, skin and mucosal lesions, saliva, nasal/nasopharyngeal/throat swabs, rectal swabs, faeces, urine, and semen of the infected person are found to be positive for monkeypox and this may play a role in viral transmission (4, 134, 145, 184). Infectious virus is isolated from the semen of monkeypox-infected men, indicating the potential transmission via semen (104). The monkeypox virus is found in the household environment of an infected person and is infectious mainly on porous surfaces such as bedding, underwear, and towels. Although low viral titres were determined, which indicate a limited potential for indirect transmission, the household environment may play a role in transmission of the virus via contaminated fomites (129).

Recently, evidence for human-to-animal transmission of monkeypox has been reported in an Italian greyhound dog whose owner had monkeypox infection (165). The CDC is suggesting that monkeypox infected people should avoid contact with animals, including pets, domestic animals, and wildlife, in order to prevent the spread of the virus. If pets had close contact with monkeypox-infected people, they should be quarantined at home for 21 days, away from other animals and people (33).

Sexual transmission is reported as an important route for monkeypox infection, especially in people who have had close contact with an infected individual and in men who have sex with other men (75, 143). According to recent research, monkeypox is most common in men, particularly men who have sex with men, bisexual men, and men who have multiple sexual partners (107, 196). Interview-based epidemiological data from a recent study indicated that monkeypox transmission in the UK has been related to the sexual networks of gay and bisexual men who are all cisgender (196). An epidemiological study showed that 99% of the monkeypox cases were men in the USA and 94% of them had sexual or close intimate contact with men (152). The risk factors for monkeypox are determined as having sex with men, engaging in condomless sex, human immunodeficiency virus (HIV) positivity, and a story of previous sexually transmitted infections (23).

Clinical Signs: The incubation period of monkeypox is an average of 13 days but can be up to 34 days. The studies about the 2022 monkeypox outbreak reported that the incubation period is approximately 9–10 days and can be prolonged to 17–20 days. So, one can speculate that the lineage B.1 viruses have a shorter incubation period than other clades and lineages (128, 135, 184, 206). Even though 13% of the people who were sick were hospitalized, most of those who were admitted did not have serious problems (168).

Clinical manifestations of monkeypox virus infection vary, including gastrointestinal (diarrhoea, nausea, vomiting), upper and lower respiratory (runny nose, sore throat, wheeze, cough, respiratory distress), and systemic (lymphadenopathy, fever, sweats, chills, pruritus, myalgia, back pain, headache, asthenia, and abdominal pain) symptoms. However, the first clinical signs and common are usually fever, lymphadenopathy, exanthema, asthenia, fatigue, and headache. Skin and mucosal lesions can appear anywhere on the body, but they are most common on the face, limbs, palms of the hands, soles of the feet, oral and genital mucosa, and, in rare cases, conjunctivae and cornea. Lesions consist of rashes, macules, papules, vesicles, pustules, crusts, ulceration (23, 137, 158).

Interestingly, it is reported that monkeypox can be asymptomatic in some men who have sex with men, and these cases remain undiagnosed (45). Monkeypox lesions can also be without exanthem and a robust cellular immune response can be determinative for the occurrence of skin lesions (108). Following healing of skin lesions, sequelae can be observed, such as hyperpigmented or hypopigmented atrophic scars, patchy alopecia, hypertrophic skin scarring, and deformities of facial muscles following healing of ulceration (137). According to an observational study in the Democratic Republic of Congo, monkeypox virus infection of pregnant women can lead to miscarriage and foetal death with diffuse cutaneous maculopapillary skin lesions or healthy birth (123).

Monkeypox virus infection can cause genital lesions such as painless genital rash, macular rash, painful inguinal lymphadenopathy, ulcerated lesions, umbilicated pustules, painless white pustules on the penis, and pruritis

(75, 143). HIV positive men who have sex with men are reported to be at more risk for monkeypox virus infection and develop monkeypox-associated genital lesions (75, 127, 143).

Monkeypox lesions can be clinically misdiagnosed because of their resemblance to the lesions that are caused by other viral infections or may remain undiagnosed. Differential diagnosis for monkeypox and coxsackie virus infection should be performed for suspected lesions by virological diagnosis (107). Another recently published retrospective study from Belgium showed that men who had been misdiagnosed with gonorrhoea and/or chlamydia could be monkeypox positive with or without any lesions (45).

Some monkeypox cases can be fatal (86, 154). The mortality rate of monkeypox infection is in the range of 1-10%, but recently has been around 3-6% (21, 26, 202). The clade I viruses are associated with more severe disease and higher case fatality than the clade IIa viruses (177). The clade IIb viruses that drive the current monkeypox outbreak have case fatality ratios below 1% (85).

Control and Treatment: There is no specific therapy for monkeypox virus infection in humans. Some governments carry out 21 day mandatory quarantines for monkeypox infected people in order to control transmission of the disease. Several cases are recovered without any specific antiviral therapy (9, 23, 56). Non-specific therapies such as antibiotics and analgesics can be administered to monkeypox virus-infected patients. Antibiotics are frequently used for prevention or treatment of secondary bacterial infections (75, 143). Antiviral agents such as tecovirimat, a p37 protein inhibitor that is used for orthopoxvirus infections, and brincidofovir, a replication inhibitor of a variety of DNA viruses, can be used for monkeypox virus infection (4, 52, 156). Treatment of brincidofovir can lead to an increase in liver enzymes, whereas no adverse effects occurred with Tecovirimat treatment (4).

Due to cross-protection between orthopoxviruses, people vaccinated against smallpox are 85% protected against monkeypox virus infection. Vaccines containing live, replication-competent, different vaccinia virus strains, called first-generation smallpox vaccines, were used for smallpox eradication between 1967 and 1979, which was coordinated by the WHO. The U.S. Food and Drug Administration (FDA) has approved three smallpox vaccines: JYNNEOS (Imvamune or Imvanex/replication-deficient live virus vaccine), ACAM2000 (Live cowpox Vaccinia virus vaccine), and Aventis Pasteur Smallpox Vaccine (APSV/replication-competent vaccinia virus vaccine) (6, 60). Because of some concerns about first-generation vaccines, such as inadvertent transmission to other people, re-emergence of live viruses, and vaccine-associated adverse effects, third-generation smallpox vaccines that contain replication-deficient vaccinia viruses (e.g., modified vaccinia Ankara (MVA) and LC16m8) are preferred to prevent and control orthopoxvirus infections. In some countries, healthcare workers and laboratory personnel are vaccinated with third-generation smallpox vaccines (i.e., modified vaccinia Ankara (MVA) and LC16m8) for protection against orthopoxviruses including monkeypox virus (32, 150, 202). In a multi-center cohort enrolling in France, JYNNEOS (Imvanex or Imvamune) has begun to be used for vaccination for post-exposure prophylaxis to unvaccinated adults over 18 years who have been exposed to monkeypox less than 14 days ago or adults who have been vaccinated with a first dose less than 28 days earlier and have been exposed to monkeypox (112).

Other Zoonoses in *Orthopoxvirus* and *Parapoxvirus* genus

Cowpox virus

Epidemiology: Cowpox virus, an Orthopoxvirus, infection has been reported in many animal species including primates (e.g., Barbary macaque, marmoset, cotton-top tamarins), felids (e.g., cats, cheetah, lynx, lion, black panther, jaguar, puma), dog, fox, wild boar, cow, llama, horse, elephant, anteaters, beaver, bank voles, gray-sided vole, red-backed voles, field vole, root vole, wood mice, yellow-necked mice, house mice, common rat, giant gerbil, gerbil, ground squirrel, Patagonian cavy, common shrew, etc. (57, 92). Unlike many other animal species that can be infected by cowpox virus, direct transmission to humans has only been reported by infected cats, rats, cows, cheetahs, and Asian elephants (27, 74, 78, 133, 182). Infected rats can also infect other animal species (121). Possible human-to-human transmission was reported (183). However, this transmission route is not proven. Human cowpox virus infection is rare and there have been less than 150 reported cases.

Clinical Signs: Symptoms of cowpox virus infection in cats include oedema, hyperaemia, ulceration, exudation, and plaque-like alterations in the hindlimbs (91). Captive banded mongooses in a zoological park in Germany have exhibited papular, vesicular, or pustular skin lesions, crusts, anorexia, lethargy, imbalance, severe dyspnoea, wheezing, and death due to generalized cowpox virus infection (164). Virus infections in captive cheetahs in zoos and parks lead to skin (including ulcerations) and mucosal lesions, exudative pleuritis, and acute hemorrhagic pneumonia, with high morbidity and mortality (18, 119, 178). Cowpox virus infection of a pregnant mare can culminate in abortion of a foal with cutaneous papules throughout the entire skin and the oral mucosa (65).

Lesions in human cases of cowpox are macules, papules, vesicles, pustules, and black crusts and are usually restricted to the hands and face. Cowpox virus infection typically causes one lesion, but multiple inoculations, autoinoculation, and immunesupression can lead to multiple lesions and generalized infections (44). Fever, lymphadenitis (sometimes necrotizing), cellulitis, ulcerated and necrotizing skin lesions are reported in patients who have had direct contact with infected domestic cats (142, 207). Cowpox cases generally recover in 6–8 weeks, but it can take 12 weeks to heal in some cases (44). A generalized infection caused by cowpox virus was reported in a patient with haemorrhagic and ulcerated nodules, oedema, fever, and lymphadenopathy (73). Some human cases of generalized cowpox virus infection in kidney transplant patients who were scratched by their cats with ulcerating nodules culminated in death (68, 198). Ocular cowpox virus infection can lead to necrosis of the eyelid, necrotic granulomatous conjunctivitis, keratitis, leucomatous opacity, conjunctival oedema, necrotic eschar, and vision loss (51, 98). An atypical cowpox virus infection was recorded in a smallpox vaccinated patient with clinical manifestations such as painful cellulitis, multiple subcutaneous abscesses, and axillary adenopathy (10). Foetus can be infected by cowpox virus during pregnancy and can result in miscarriage (61).

Control and Treatment: Attenuated modified vaccinia virus Ankara (MVA) strains are used for vaccination against cowpox virus and are shown to be protective in elephants, rhinos, and captive cheetahs (53, 178). There is no specific vaccine against the cowpox virus for humans.

Vaccinia virus

Epidemiology: Vaccinia virus was used in the WHO eradication campaign for smallpox, which is an acute contagious disease caused by the variola virus of the orthopoxviruses. The Vaccinia virus strain Ankara, which was developed at the vaccine institute in Ankara, Turkey by propagating the virus on the skin of calves and donkeys (through donkey-calf-donkey inoculation), was passaged in chicken embryo fibroblast culture in Germany and, after the 516th passage, the virus was named Modified Vaccinia virus Ankara (MVA). Preferably safer smallpox vaccines were used during the last years of the smallpox eradication campaign (180, 194). Moreover, MVA serves as a safe and effective vector platform, such as in vaccines for rabies, Chikungunya, malaria, etc. (67, 115, 166).

In countries such as Uruguay, Brazil, and Colombia, reported cases of vaccinia virus infection (63, 190). Bovine vaccinia virus outbreaks that occurred in Brazil caused zoonotic infection of humans (13, 125, 173). Human-to-human transmission of vaccinia virus can be directly or indirectly (37, 146). Vaccinia virus can be isolated from the household environment of infected people, and this may be a route of human-to-human transmission (13). Another potential route of infection is consuming contaminated raw milk or raw milk products, and these can play a role in occupational infection of cheesemakers (37, 47).

Clinical Signs: According to the results of experimental infection of milking cows with vaccinia virus, lesions started at 2-4 days post-inoculation, healed averagely in 18 days, all infected cows exhibited DNAemia, some of them had viremia, and animals shed vaccinia virus in their faeces (160). Infectious vaccinia virus is detected in milk samples of naturally infected cows, and in milk samples, milk products, and even in pasteurized milk of experimentally infected cows (1, 47). Furthermore, vaccinia virus DNA was detected in dogs, cats, horses, wild coatis, and opossums, but transmission of the virus from these animals has not been reported yet (38, 39, 147, 148).

Vaccinia virus typically infects farmers, milkers, and their close contacts. Vaccinia virus-infected people showed skin lesions mainly on the hands, forearms, legs, and face, and generalized outcomes such as fever, headache, malaise, myalgia, and lymphadenopathy (173). Skin lesions usually start with itching, local oedema, pustules, vesicles, and ulcers (49, 173). Ocular lesions can occur (109). HIV positive patients who are vaccinia virus infected can develop progressive and more severe clinical outcomes (102). A patient who was in contact with cows that had lesions on their teats and udders, developed an ulcerated-pustule skin lesion, fever, headache, malaise, myalgia, and lymphadenopathy and was diagnosed as co-infected with vaccinia and pseudocowpox viruses (2). Occupational vaccinia virus infections can occur. Employees of a biopharmaceutical laboratory where vaccinia virus was inoculated to rabbits developed clinical infection in China (111).

Control and Treatment: Vaccinia virus has been used as a vaccine in both humans and animals against orthopoxviruses (32, 150, 178, 202). There is no specific treatment for vaccinia virus infection.

Bovine papular stomatitis virus

Epidemiology: The host spectrum of bovine papular stomatitis virus (BPSV), a member of the Parapoxvirus family, is restricted to cattle and humans. Humans can be infected through direct contact with infected animal lesions (22, 80). Occupational infections of BPSV were reported in milkers, veterinarians, and veterinary students. BPSV infection is frequently transmitted to milkers, especially if the teats of dairy cows are affected. (22, 48). Some tick species are reported as BPSV PCR positive and houseflies can be a mechanical vector for BPSV,

indicating that vectors may play a role in transmission and epidemiology of the disease (141, 171).

Clinical signs: BPSV infection in cattle leads to lesions such as papules, nodules, pustules, vesicles, ulcers, erosions, scabby proliferative lesions, crusts, and scabs that are usually on the muzzle, lips, gingiva, palate, tongue, and teats (88, 93, 126, 136, 170). Affected calves with oral lesions may refuse to be fed, and affected dairy cows with teat lesions may not allow milking because of local pain (48, 126). The erosive lesions on the oral mucosa can be observed as a ring or a horseshoe (43) In some cases, there could be papules and ulcers in the oesophagus, rumen, and omasum in cattle (88, 126, 170). Extensive proliferative scabby lesions and dermatitis of the teats were also reported in cows, sometimes leading to occlusion of the teat canal (84, 106). Infected cattle generally recover in 13–18 days with no treatment (136).

Symptoms of BPSV infection in humans include painful papules, nodules, pustules, vesicles, ulcers, and scars that are mainly restricted to the hands and rarely on the face and arms (48, 80, 124, 136).

Diagnosis and treatment: There is no specific treatment or vaccine for BPSV in animals or humans. Hygiene, disinfection, and isolation of infected animals can be the main procedures for preventing the spread of the virus to other animals and workers (93).

Orf virus

Epidemiology: Orf virus is a parapoxvirus and the causative agent of contagious pustular dermatitis (syn. contagious ecthyma, orf, sore mouth, scabby mouth) that results in infection of primarily sheep and goats, and also cattle, camels, other wild ruminants, and cervids (7, 167, 176, 187). Orf has a worldwide distribution with high morbidity and low mortality. Animals younger than 1 year of age are more susceptible to the disease and their inability to feed due to severe lesions leads to loss of weight gain and even death, and as a consequence, economic loss (15).

Between animals, orf is transmitted via direct contact and contaminated fomites. A recent report showed that the infectious orf virus is present in saliva and milk samples of goats without clinical symptoms, and the isolated virus is infectious to other orf-free goats under experimental conditions (113).

Sheep and goats, and rarely camels and cats can infect humans (7, 31, 64, 99). Orf virus is transmitted by direct contact with infected animals, contaminated fomites and, especially for children, infected animal bites can be the cause of infection (105, 185). Orf infection mostly occurred in farmers, animal workers, shepherds, wool shearers, veterinarians, butchers, and hunters, or was sometimes observed in people after Eid al-Adha (19, 82, 96, 193). Household injuries that happen during meat preparation and animal slaughter are reported to cause the development of orf infection in humans (31). Orf can be transmitted human-to-human via commonly used contaminated fomites (189). In a case of human orf, mother-to-child transmission has occurred and exophytic nodules and papules have developed on the scrotum and buttocks of the child (155). Autoinoculation is a possible way to spread the orf virus from lesions to other parts of the body (50, 179).

Clinical signs: Symptoms of orf in animals are characterized by erythematous macules, papules, vesicles, pustules, ulcers, scabbing and proliferative skin lesions which are mostly formed on the mouth, gums, lips, muzzle, nostrils, face, eyelids, ears, udder, and teats, and sometimes on the inner thigh, abdomen, axilla, tail, perineum, and extremities (46, 99, 174). In some cases, necrotizing cheilitis and dermatitis, crusting, hyperpigmentation, and oedema can develop and may result in partial obstruction of the nostrils (42). Wild ruminants can exhibit orf lesions on nostrils, lips, eyelids, face, chin, ear, nares, neck, leg, hooves/coronary band, interdigital space (187).

In humans, orf virus causes vesicles, pustules, painful erythematous, violaceous plaques, erythematous maculopapular lesions, targetoid bulla, weeping nodule, crusted papule, papilloma, ulceration, desquamation, or sometimes non-pruritic purulent yellow-whitish nodules, which usually occur on hands, fingers, and arms and can be single or multiple (70, 99, 117, 163, 185, 193). Orf may lead to the formation of hyperkeratotic nodules underneath fingernails, and erythematous, centrally ulcerated, hemorrhagic fragile nodules, and papillomatous projections on the face (71, 72). A nodular mass with erythema on the nose caused by orf virus infection is also reported (14). In a human case (140), Orf lesions that are formed as fungating and painful masses and cause obliteration of the toenail can be observed on the toe in a human case (140). Erythema multiforme development is reported in several human cases following orf infection (66, 90). Orf infection does not result in any pathology in the foetus during pregnancy and culminates with healthy labour and babies (24, 181).

Control and treatment: There are vaccines against orf in animals, but despite vaccination, there can be outbreaks of orf in small ruminant herds (42). Local antiseptics can be applied to the lesions of an affected animal, antibiotics can be used for secondary bacterial infection, and supportive treatment should be administered to young animals that cannot be fed due to oral lesions (176).

For humans, there is no specific orf vaccine. The orf lesions in humans generally spontaneously heal within 2-8 weeks without any specific treatment (30, 189) and local antiseptic therapy is usually sufficient in order to prevent

secondary infections (99). For exophytic lesions of the orf in humans, curettage and cautery can be an alternative treatment (94).

Pseudocowpox virus

Epidemiology: Pseudocowpox virus is a parapoxvirus and its infection, usually referred to as milker's nodule, affects cattle, other wild ruminants, and humans. Veterinarian, milkers, farmers, and animal workers are occupationally predisposed to pseudocowpox virus infection (5, 87, 199). Infection develops by direct contact with the lesions of an affected animal, usually during milking or following a bite of infected cattle (5, 34, 87, 118, 199).

Pseudocowpox virus is detected in cattle, American bison, water buffalo, camels, and cats (3, 58, 101, 172). Some tick species that are collected from cattle are found to be positive for pseudocowpox virus by PCR, indicating that ticks can play a role in pseudocowpox virus epidemiology (35, 141). Houseflies on barns can be a mechanical vector for the pseudocowpox virus (171).

Clinical signs: Pseudocowpox virus infection leads to lesions described as "ring" or "horseshoe", which are pathognomonic for the disease, and nodules, pustules, and ulceration, usually on the teats and udders (97, 138). Rare cases show vesicles, erosions, papules, and scabs on the vulva and vaginal mucosa, and also, development of hyperemia and white vesicles on the sublingual mucosa is reported in pseudocowpox virus infection in cattle (139). In experimental infection, hyperemic foci, ulceration, fibrinotic and scabby lesions were observed on the muzzle and lips of calves (54). Pseudocowpox virus infection is exhibited as multiple cutaneous nodules on the skin which are wart-like, proliferative, and keratinized in an American bison (172). In water buffalos, pseudocowpox symptoms are peeling of the tongue epithelium and oral ulcers (101). Pseudocowpox virus infection in camels causes papules, pustules, ulcers, and scabs on the lips, muzzles, and eyelids (3).

Milker's nodule has 5-15 days of incubation period, and lesions progress through six stages: maculopapular (erythematous nodule), targetoid (central papule with an erythematous outer ring and a pale inner ring), acute (ulceration and draining of nodule), regenerative (firm nodules, black papules, and crusts develop), papillomatous, and regression (76). Milker's nodule is clinically presented as erythema, small red macule, papule, nodule (can be with blue-violet bulla or crusted), blue-black nodulopustule, yellowish plaque, papulovesicule, erosion, crusting, and usually heals without eschar, but sometimes discoloration may develop (5, 34, 87, 118, 199). Complications such as lymphadenopathy, lymphadenomegaly, lymphangitis, erythematous papular rash, erythema multiforme, bacterial infection, etc. may occur (34, 151, 205).

Control and treatment: There is no specific vaccine for humans or animals against the pseudocowpox virus. Because most cases heal spontaneously, there is no need for treatment. However, if needed, infected humans and animals can be treated with antiseptics and antibiotics in order to prevent secondary bacterial infections (3, 76).

Grey sealpox virus

Sealpox virus is in the Parapoxvirus genus. Its infection is zoonotic and it primarily infects pinnipeds (seals and sea lions). The high-risk groups of humans are the marine mammal workers and handlers in rehabilitation facilities and parks, veterinary technicians, and veterinarians. The virus is transmitted from animal-to-human via direct contact or biting of an infected animal. The lesions of sealpox in pinnipeds are firm skin nodules that develop throughout the body, such as on the head, neck, thorax, abdomen, flippers, and white-gray verrucose nodules on the oral mucosa and tongue. The lesions usually heal spontaneously with a slightly raised, grey, furless scar formation. Infection of young animals that causes oral lesions may be fatal due to aversion to food intake. The sealpox virus causes lesions like orf and milker's nodule in humans. Tender nodule, grey bullous lesion, lesion with a red center and a pale margin, and scab are the most common lesions on the hands. There is no vaccine or specific treatment against sealpox infection for both humans and animals (20, 36, 40, 79, 130, 161, 188).

Red deerpox virus

Deerpox virus, which is a parapoxvirus, is a very rare zoonotic agent that causes lesions on the muzzle of deer. A possible transmission route is direct skin-to-skin contact with an affected animal. In deer, papillomatous lesions, alopecic, flat, proliferative dermal lesions, ulcers, and scabby lesions may develop in a variety of parts of the body, i.e., lips, oral cavity, tongue, muzzle, velvet, nose, ears, ventral thorax, limbs, abdomen. In human cases of deerpox virus infection, tumour-like painless greyish necrotic lesions, haemorrhagic crust, and granulomatous lesions with greyish spots are developed on the hands and face. Some human cases may have fever, lymphadenopathy, and nausea. There is no vaccine or specific treatment against deerpox virus infection (17, 59, 81).

Conclusion

The monkeypox outbreak in 2022 showed that challenges between humanity and viruses continue and will go on. The monkeypox outbreak that makes it more serious than previous outbreaks are severe clinical outcomes such as encephalitis and death, and also the higher transmission rate, which occurs at approximately 99% in men, especially men who have sex with men. The

presence of the aforementioned zoonotic infections in many different animal species is a problem and a big challenge to fighting the diseases. It is a matter of debate why the monkeypox virus 2022 outbreak spread so quickly but not in other poxviruses. Variables such as increased mutation rate due to natural evolution, suspicion of bioterrorism, overpopulation, the possibility and ease of travel between continents and countries, and changes in lifestyle may have influenced the epidemiology and treatment of the monkeypox virus 2022 outbreak. The availability of an effective vaccine against monkeypox virus provides a good protection and control strategy. However, mandatory or optional use of the vaccine or difficulties in accessing the vaccine in all countries should be a topic for discussion by health organizations, scientists, and policy makers.

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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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