ISSN 1300-0861
E-ISSN 1308-2817

Ankara Üniversitesi Veteriner Fakültesi Dergisi

Veterinary Journal of Ankara University

Cilt/Volume 66 • Sayı/Number 3 • 2019

Ankara Üniversitesi Veteriner Fakültesi Dergisi
Cilt / *Volume*: 66 • Sayı / *Number*: 3 • 2019
Veterinary Journal of Ankara University
Üç ayda bir yayımlanır / *Published three monthly*
E-ISSN 1308-2817

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İnternet Adreslerimiz / *Web Addresses*
http://vetfakderg.ankara.edu.tr
http://vetjournal.ankara.edu.tr
Yayım Tarihi: 14/06/2019

Ankara Üniversitesi Veteriner Fakültesi Dergisi
Cilt / *Volume*: 66 • Sayı / *Number*: 3 • 2019
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Ankara Üniv Vet Fak Derg, **66**, 221-229, 2019
DOI: 10.33988/auvfd.434359

Effects of dietary supplementation of betaine and sepiolite on performance and intestinal health in broilers

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Received date: 18.06.2018 - Accepted date: 02.05.2019

Abstract: The aim of the study was to explain the effects of betaine and sepiolite in diets on performance, carcass characteristics, some blood parameters and some intestinal health parameters in broilers. A total of 192 daily Ross 308 male broiler chicks were divided into one control group and three experiment groups each containing 48 chicks. Betaine and sepiolite was added as top dressed to the diets of experimental groups at the level of 0.15% betaine (1st group), 1.5% sepiolite (2nd group) and 0.15% betaine + 1.5% sepiolite (3rd group). Sepiolite and betaine were not added to the diet of control group. The experimental period was 6 weeks. No differences were observed in body weight, overall body weight gain, feed intake, feed conversion ratio, carcass yield and the relative weights of gizzard, heart, liver, spleen and bursa of Fabricius among groups. The relative weight of abdominal fat decreased with betaine usage. Supplemental betaine increased relative percentage of kidney weight. Dietary supplementation of betaine with sepiolite had some positive effects on intestinal histomorphology characteristics. The level of serum triglyceride was reduced with 0.15% betaine inclusion in the diet. Albumin, cholesterol, protein, uric acid, AST, ALP and ALT levels of blood serum were not affected by betaine with and without sepiolite supplement. Blood serum IgG level was increased by dietary supplementation of betaine with and without sepiolite. It is concluded that the dietary usage of betaine with sepiolite in broilers can be useful in the field due to having some improvements in intestinal histomorphology and immunity.

Keywords: Betaine, broiler, intestinal histomorphology, performance, sepiolite.

Broyler rasyonlarına betain ve sepiyolit ilavesinin performans ve bağırsak sağlığı üzerine etkileri

Özet: Bu araştırmanın amacı, broyler karma yemlerine betain ve sepiyolit ilavesinin performans, karkas özellikleri, bazı kan parametreleri ve bazı bağırsak sağlığı parametreleri üzerine etkilerini belirlemektir. Toplam 192 adet günlük Ross 308 erkek broyler civciv her biri 48 adet içeren bir kontrol ve üç deneme grubuna ayrılmıştır. Betain ve sepiyolit deneme grubu yemlerine dökme olarak %0.15 betain (1. grup), %1.5 sepiyolit (2. grup) ve %0.15 betain + %1.5 sepiyolit (3. grup) düzeylerinde ilave edilmiştir. Kontrol grubu konsantre yemine betain ve sepiyolit ilave edilmemiştir. Deneme süresi 6 haftadır. Betain ve sepiyolit ilavesi canlı ağırlık, canlı ağırlık kazancı, yem tüketimi, yemden yararlanma, karkas randımanı ile karaciğer, kalp, taşlık, dalak ve bursa Fabricus relatif ağırlıkları bakımından farklılık yaratmamıştır. Abdominal yağ relatif ağırlığı betain katkısı ile düşmüştür. Betain katkısı böbrek relatif ağırlığını artırmıştır. Betain ve sepiyolit katkısının bağırsak histomorflojisi özellikleri üzerine pozitif etkisi gözlenmiştir. Betainin %0.15 düzeyinde ilavesi kan serumu trigliserit değerini düşürmüştür. Kan serumunda protein, albumin, ürik asit, kolesterol, AST, ALT ve ALP düzeyleri betain ve/veya sepiyolit ilavesinden etkilenmemiştir. Kan serum IgG düzeyi betain ve/veya sepiyolit ilavesi ile artmıştır. Sonuç olarak bağırsak histomorfolojisi ve immunite üzerine olumlu etkilerinden dolayı betain ve sepiyolitin broyler rasyonlarında kullanımının sahada yararlı olabileceği kanısına varılmıştır.

Anahtar sözcükler: Bağırsak histomorfolojisi, betain, broyler, performans, sepiyolit.

Introduction

Betaine, a trimethylglycine, is created by choline in combination with the glycine. Betaine has been shown to have potential benefits for protecting intestinal cells and thus counteracting performance losses during heat stress and coccidiosis. Betaine is considered to be a methyl donor and osmoprotectant to improve, growth performance, nutrient digestibility and feed conversion ratio of poultry (29).

Betaine supplementation improved performance and carcass characteristics in broilers (29, 50). The increase in mineral absorption and retention affected water retention capacity of muscle tissue (13). High water retention capacity could be explained by higher meat/fat ratio in the body.

Sepiolite, one of the clay minerals is a magnesium silicate having fibre structure and internal crystal ducts ($Si_{12}Mg_8O_{30}(OH_2)_4(OH)_4.8H_2O$). Sepiolite has amorf, compact and lump types in the nature. Sepiolite has been approved as binding, anti- caking agent and coagulating agent for all animal types in European Union as E562 (10). Sepiolite is an ideal carrier for vitamins, minerals and other feed additives. Also it shows homogenizing characteristics by preventing seperation of feed components. Sepiolite has a low cation change capacity thus it is a good premix tranporter. Being an inert material it refers to have high chemical stability (16). It has a high water absorption capacity and prevents loss of dust. Avcılar et al. (5) reported that the addition of sepiolite at the levels of 25% and 50% in the broiler litter may use to improve the litter quality. Sepiolite increases growth performance due to increasing digestion and absorption of endogen enzymes, fats, carbonhydrate and proteins (33, 41).

Betaine supplementation to methyl group donor-adequate diets improved weight gain, feed conversion, carcass and breast muscle yield by 3-15% in poultry (31, 43). Ismail and Ahmad (20) concluded that the dietary betaine supplementation have beneficial effect on growth performance and some blood parameters without any negative effects on physiological responses in broilers. Some researchers (28, 46) have also reported that dietary betaine had positively affected the breast meat yield but reduced abdominal fat pad in broilers. Transitory inflammatory responses during the pre-starter and starter period was reduced by the presence of betaine in the diet of broilers (37).

Sepiolite can be used as therapeutic agents since it most likely replaces the growth factors in poultry diets (16, 41, 44). Sepiolite inclusion at 1% increased weight gain and reduced abdominal fat percentage and the levels of triglyceride and cholesterol in blood serum (12). Yalçın et al. (49) reported that 1% sepiolite supplementation was an effective additive in broilers due to its beneficial effects on performance, abdominal fat, viscosity of ileal digesta, ileal digestibility and duodenal villus height.

However, a published report regarding the interaction of sepiolite and betaine in poultry was not found. Furthermore, no studies about the effects of 1.5% sepiolite on broiler nutrition were seen. It was assumed that sepiolite and betaine which are given in combination might improve performance and carcass characteristics by influencing the nutrient metabolism and immune system. Therefore, this experiment was aimed to determine the effects of sepiolite and betaine supplementation on performance, carcass characteristics, some blood parameters and some intestinal health parameters in broilers.

Materials and Methods

All study were approved by the Animal Ethics Committee of the Ankara University (2012-16-100).

Animals and diets: A total of 192 daily Ross 308 male broiler chicks were divided to four groups and each group had 8 replicates of 6 chicks each. Each replicates were placed in separate floor pen having 80 cm width x 90 cm length x 80 cm height. As a litter, wood shavings was used. There were two nipples and one hanging suspended feeder in each pen. Water and mash feed were *ad libitum* during 6 weeks. Lighting was permanently applied. Temperature of room was 32±2°C on the first week and then gradually reduced to 24-26°C and this temperature was maintained upto slaughtering. The ingredients and chemical composition of the basal diets were given in Table 1. The diets were formulated to meet or exceed the nutrient requirements of broilers based on the management guide of Ross 308. Basal diets were supplemented with 0 (control), 0.15% commercial betaine (^{TNI}betain96 AN, Trouw Nutrition, Turkey), 1.5% sepiolite (Exal TH, Tolsa Turkey, Turkey) and 0.15% betaine + 1.5% sepiolite. Control group diet consisted of only basal diet. The composition of sepiolite used in this present study was shown in Table 2 (7).

Table 1. The ingredients and chemical composition of the basal diets (as-fed basis).
Tablo 1. Karma yemlerin bileşimi ve besin maddesi içerikleri.

Ingredients (g/kg)	Starter diet 0-21 days	Grower diet 22-42 days
Corn	474.0	493.0
Soybean meal	265.0	190.0
Full fat soya	210.0	237.0
Sunflower seed oil	10.0	40.0
Limestone	13.0	13.5
Dicalcium phosphate	19.0	18.0
Methionine	2.0	2.0
Lysine	1.0	1.0
Sodium bicarbonate	1.0	1.0
Salt	3.0	3.0
Vitamin mineral premix[1]	1.5	1.5
Salinomycine	0.5	-
Chemical composition (Analyzed)		
Metabolizable energy[2] (MJ/kg)	12.96	13.85
Crude protein (g/kg)	234.8	204.0
Calcium (g/kg)	11.3	10.4
Total phosphorus (g/kg)	7.1	6.4

[1]: Provides 1.5 kg of premix: 9000000 IU vitamin A, 4000000 vitamin D_3, 50000 mg vitamin E, 2000 mg vitamin K_3, 2000 mg vitamin B_1, 5000 mg vitamin B_2, 40000 mg niacin, 15000 mg calcium D pantothenate, 2000 mg vitamin B_6, 10 mg vitamin B_{12}, 1500 mg folic acid, 100 mg D-biotin, 120000 mg Mn, 40000 mg Fe, 100000 mg Zn, 16000 mg Cu, 1250 mg I, 200 mg Co, 300 mg Se, 125000 mg antioksidant (ethoxyquin, BHA).
[2]: Metabolizable energy content of diets was calculated (27).

Table 2. The composition of sepiolite[1].
Tablo 2. Sepiyolit bileşimi[1].

Sepiolite, %	65
Attapulgite, %	9
Dolomite, %	18
Calcit, %	8
Humidity, %	8.23
Heavy metals	
As, mg/kg	2.6
Cd, mg/kg	<1
Pb, mg/kg	1.16
Hg, mg/kg	0.02

[1]: Burçak and Yalçın (7).

Traits measured: Nutrient composition of basal diet was analyzed (3) for crude protein (CP, Method 968.06), ether extract (EE, Method 920.39), crude fiber (CF, Method 932.09) and ash (Method 967.05). Calcium (14) and total phosphorus (1) were analyzed. Metabolizable energy levels of diets were calculated (27).

Birds were individually weighed at the beginning and weekly to determine weight gain. Feed intake was determined weekly and feed conversion ratio was calculated as kg feed per kg weight gain. To determine mortality birds were followed up daily. The lesions of footpad (24) and breast (2) were evaluated and litter samples were taken to analyze for moisture content (3) on day 42nd of the experiment.

At the end all broilers were individually weighed and 8 broilers from each group were slaughtered by severing the jugular vein, internal organs, head and foot were removed manually after defeathering. Carcasses were weighed to calculate hot carcass yield. Abdominal fat, spleen, liver, heart, gizzard, kidney and bursa of Fabricius were weighed. Relative weights of them were calculated by dividing to slaughtering weight.

Histomorphological analysis were performed on day of 21 and 42 days of experiment, intestinal tissue samples were taken after slaughtering of 8 broilers (one from each replicate) from each group. About 2 cm segments were taken from the duodenum, jejunum and ileum and all divertikulum applications to intestinal segments were done as reported by Yalçın et al. (49). Intestinal sections were analyzed using Cellsens CS-ST-V1.8 software in digital microscope with camera control (Olympus BX51-DP71) (42). Ten measurements for each sample were made. Villus height and crypt depth were measured as mentioned by Yalçın et al. (49). The ratio of villus height to crypt depth was calculated. A geometric model $[2\pi \times villus\ height \times (villus\ width \div 2)]$ was used to calculate villus area as mm^2 (36).

Blood samples were taken from vena brachialis under the wing from eight fed broilers from each group on day 42 and centrifuged at 3.220 x g for 8 min to collect serum. The levels of total protein (ACN 678), uric acid (ACN 700), triglyceride (ACN 781), total cholesterol (ACN 433), alkaline phosphatase (ALP) (ACN 158), aspartate amino transferase (AST) (ACN 687) and alanine amino transferase (ALT) (ACN 685) by Abbott Aeroset autoanalyser with commercial Cobas kits (Roche Diagnostics). Serum IgG levels were determined with ELISA (17).

Statistical analyses: SPSS programme (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Kolmogorov-Smirnov test was applied for the normality of data distribution. One-way ANOVA was used to detect the differences among groups. The significance of mean differences between groups were analyzed using Duncan. Values were shown as mean±standard error in the tables. Statistical significance level was accepted as $P<0.05$ (9).

Results

Effects of dietary sepiolite and betaine supplementation on performance and litter moisture were given in Table 3. Final body weight, total weight gain, feed intake and feed conversion ratio were not affected by the inclusion of sepiolite and/or betaine. No significant interaction between sepiolite and betaine was found in these parameters. Carcass yield and the percentages of heart, spleen, liver, bursa of Fabricius, gizzard and proventriculus were not affected by the treatments (Table 4). No interaction effects were also seen in these parameters. Effects of sepiolite and betaine on duodenal, jejunal and ileal histomorphology on day 21 and 42 were shown in Table 5, Table 6 and Table 7, respectively.

Villus height, crypt depth and surface area were increased by dietary betaine supplementation ($P<0.05$) at day 21 and the ratio of villus height to crypt depth was increased by sepiolite supplementation at day 42 in duodenum ($P<0.05$). However no interaction was seen in these parameters. Jejunal villus height and vilus surface area were increased with only sepiolite or only betaine supplementation at day 21. Significant interactions in villus height ($P=0.035$) and the ratio of villus height to crypt depth ($P=0.021$) were also seen in villus height due to having increasing effect at day 21. There were significant interactions in villus width ($P=0.008$), crypth depth ($P<0.001$) and the ratio of villus height to crypt depth ($P=0.001$) in jejunum at day 42. İleal villus height ($P<0.001$) and villus surface area ($P=0.008$) was increased with only sepiolite supplementation at day 21. Significant interaction was observed in the ratio of villus height to crypt depth at day 21 ($P=0.020$) and at day 42 ($P<0.001$) in ileum and in the crypt depth at day 42 ($P<0.001$).

The levels of total protein, uric acid, albumin, total cholesterol, ALT, AST and ALP in blood serum were not

affected with sepiolite with/without betaine supplementation as given in Table 8. No interaction effects were also observed in these parameters. Serum IgG concentration was increased with sepiolite and betaine (P<0.001). There was a significant interaction of sepiolite and betaine (P=0.035).

Table 3. Effects of dietary sepiolite and betaine supplementation on performance and litter moisture.
Tablo 3. Karma yemlere betain ve sepiyolit ilavesinin performans ve altlık nemi üzerine etkileri.

Sepiolite %	Betaine %	BW day 0	BW day 42	BWG 0-21 day	BWG 22-42 day	BWG 0-42 day	FI 0-21 day	FI 22-42 day	FI 0-42 day	FCR 0-21 day	FCR 22-42 day	FCR 0-42 day	Litter moisture
		g	g	g	g	g	g	g	g	g/g	g/g	g/g	%
0		44.24	3024.61	839.42	2142.08	2981.50	1092.94	3476.64	4569.58	1.30	1.63	1.53	26.90
1.5		43.99	2970.50	819.66	2112.48	2932.13	1096.53	3476.87	4573.40	1.34	1.65	1.56	26.90
	0	44.19	3001.80	826.21	2134.77	2960.98	1100.34	3491.78	4592.12	1.33	1.64	1.55	26.86
	0.15	44.05	2993.32	832.87	2119.78	2952.65	1089.13	3461.73	4550.87	1.31	1.64	1.54	26.94
0	0	44.36	3016.96	830.68	2141.13	2971.81	1090.16	3478.00	4568.16	1.31	1.63	1.54	26.95
0	0.15	44.12	3032.27	848.16	2143.03	2991.19	1095.72	3475.29	4571.01	1.29	1.63	1.53	26.86
1.5	0	44.01	2986.64	821.74	2128.42	2950.16	1110.52	3505.55	4616.08	1.35	1.65	1.57	26.77
1.5	0.15	43.97	2954.36	817.57	2096.53	2914.11	1082.55	3448.18	4530.73	1.32	1.65	1.56	27.02
							P						
Sepiolite		0.403	0.272	0.035	0.485	0.298	0.819	0.996	0.932	0.084	0.416	0.214	0.989
Betaine		0.635	0.860	0.459	0.720	0.858	0.474	0.493	0.356	0.235	0.905	0.631	0.912
Sepiolite*Betaine		0.729	0.622	0.231	0.686	0.552	0.286	0.532	0.323	0.877	0.938	0.917	0.807

BW: body weight, BWG: body weight gain, FI: feed intake, FCR: feed conversion ratio

Table 4. Effects of dietary sepiolite and betaine supplementation on carcass yield and relative weight percentages of internal organs.
Tablo 4. Karma yemlere betain ve sepiyolit ilavesinin karkas randımanı ve relatif iç organ ağırlıkları üzerine etkileri.

Sepiolite %	Betaine %	Hot carcass yield	Heart	Liver	Spleen	Bursa fabricius	Gizzard	Kidney	Abdominal fat	Proventriculus
		%	%	%	%	%	%	%	%	%
0		73.43	0.524	1.972	0.113	0.22	1.245	0.52	1.195	0.337
1.5		73.00	0.505	1.955	0.105	0.21	1.247	0.51	1.192	0.341
	0	73.46	0.524	1.962	0.114	0.22	1.225	0.50	1.261	0.340
	0.15	72.98	0.504	1.964	0.104	0.21	1.267	0.53	1.127	0.338
0	0	73.45	0.536	1.963	0.116	0.23	1.242	0.51	1.390[a]	0.342
0	0.15	73.41	0.511	1.980	0.110	0.21	1.248	0.54	1.001[b]	0.332
1.5	0	73.47	0.513	1.961	0.112	0.22	1.208	0.49	1.131[ab]	0.337
1.5	0.15	72.54	0.497	1.948	0.098	0.20	1.285	0.53	1.253[ab]	0.344
							P			
Sepiolite		0.104	0.425	0.744	0.337	0.310	0.962	0.470	0.975	0.801
Betaine		0.067	0.379	0.972	0.232	0.060	0.294	0.030	0.220	0.898
Sepiolite*Betaine		0.088	0.865	0.771	0.556	0.830	0.369	0.680	0.023	0.532

[a,b]: Means with different letter in the same column are different at P<0.05 in instances with significant interaction.

Table 5. Effects of dietary sepiolite and betaine supplementation on duodenal histomorphology at day 21 and 42.
Tablo 5. Karma yemlere betain ve sepiyolit ilavesinin 21. ve 42. günlerde duodenum histomorfolojisi üzerine etkileri.

Sepiolite %	Betaine %	21 day					42 day				
		VH µm	VW µm	CD µm	VH/CD	VSA mm^2	VH µm	VW µm	CD µm	VH/CD	VSA mm^2
0		1074.51	158.32	109.85	10.15	0.54	1439.80	207.13	153.43	9.64	0.94
1.5		1090.07	159.46	118.29	9.63	0.54	1623.10	212.63	146.13	11.50	1.10
	0	1015.45	156.62	105.81	10.16	0.49	1479.91	208.68	150.56	10.06	0.98
	0.15	1149.13	161.16	122.32	9.63	0.59	1582.99	211.07	149.00	11.08	1.05
0	0	961.42	155.08	100.89	9.96	0.46	1395.12	208.86	149.50	9.55	0.91
0	0.15	1187.59	161.55	118.81	10.35	0.61	1484.49	205.40	157.37	9.73	0.96
1.5	0	1069.47	158.15	110.73	10.35	0.51	1564.70	208.50	151.63	10.58	1.05
1.5	0.15	1110.68	160.77	125.84	8.91	0.56	1681.49	216.75	140.63	12.43	1.14
						P					
Sepiolite		0.778	0.916	0.124	0.518	0.977	0.006	0.678	0.351	0.003	0.076
Betaine		0.020	0.675	0.004	0.513	0.022	0.109	0.856	0.840	0.093	0.444
Sepiolite*Betaine		0.101	0.859	0.794	0.260	0.228	0.828	0.658	0.230	0.163	0.772

VH: villus height, VW: villus width, CD: crypt depth, VSA: villus surface area

Table 6. Effects of dietary sepiolite and betaine supplementation on jejunal histomorphology at day 21 and 42.
Tablo 6. Karma yemlere betain ve sepiyolit ilavesinin 21. ve 42. günlerde jejunum histomorfolojisi üzerine etkileri.

Sepiolite %	Betaine %	21 day					42 day				
		VH µm	VW µm	CD µm	VH/CD	VSA mm^2	VH µm	VW µm	CD µm	VH/CD	VSA mm^2
0		773.71	167.86	132.52	5.98	0.41	1193.42	206.10	160.11	7.84	0.78
1.5		831.48	177.12	134.50	6.37	0.47	1156.30	188.78	153.83	8.19	0.68
	0	768.51	164.97	130.91	6.00	0.40	1175.70	202.38	153.72	8.17	0.75
	0.15	836.68	180.01	136.11	6.35	0.47	1174.02	192.50	160.22	7.86	0.71
0	0	763.28b	158.75	128.20	6.08^{b}	0.38	1176.38	225.96^{a}	180.85^{a}	6.74^{b}	0.85
0	0.15	784.14b	176.98	136.84	5.89^{b}	0.44	1210.46	186.24^{b}	139.38^{b}	8.93^{a}	0.72
1.5	0	773.75b	171.19	133.63	5.93^{b}	0.42	1175.03	178.79^{b}	126.60^{b}	9.59^{a}	0.66
1.5	0.15	889.21a	183.04	135.37	6.81^{a}	0.51	1137.58	198.76^{ab}	181.07^{a}	6.78^{b}	0.70
						P					
Sepiolite		0.011	0.262	0.586	0.091	0.032	0.603	0.109	0.421	0.617	0.143
Betaine		0.003	0.072	0.158	0.130	0.006	0.981	0.354	0.405	0.659	0.518
Sepiolite*Betaine		0.035	0.697	0.345	0.021	0.423	0.616	0.008	< 0.001	0.001	0.206

^{a,b}: Means with different letter in the same column are different at $P<0.05$ in instances with significant interaction.
VH: villus height, VW: villus width, CD: crypt depth, VSA: villus surface area

Table 7. Effects of dietary sepiolite and betaine supplementation on ileal histomorphology at day 21 and 42.
Tablo 7. Karma yemlere betain ve sepiyolit ilavesinin 21. ve 42. günlerde ileum histomorfoloji üzerine etkileri.

		21 day					42 day				
Sepiolite %	**Betaine %**	**VH µm**	**VW µm**	**CD µm**	**VH/CD**	**VSA mm²**	**VH µm**	**VW µm**	**CD µm**	**VH/CD**	**VSA mm²**
0		566.12	161.34	129.88	4.52	0.29	874.57	176.98	145.02	6.24	0,49
1.5		626.08	163.32	136.60	4.72	0.32	903.85	199.60	137.51	6.83	0,57
	0	587.50	160.37	134.04	4.51	0.30	917.80	190.05	142.09	6.7	0,56
	0.15	604.69	164.29	132.45	4.72	0.31	860.62	186.54	140.45	6.37	0,50
0	0	567.17	162.20	126.87	4.65^{ab}	0.29	870.32	174.33	158.09^{a}	5.59^{b}	0,48
0	0.15	565.06	160.48	132.89	4.39^{b}	0.28	878.81	179.63	131.96^{b}	6.89^{a}	0,49
1.5	0	607.83	158.54	141.20	4.38^{b}	0.30	965.27	205.77	126.08^{b}	7.81^{a}	0,64
1.5	0.15	644.32	168.11	132.00	5.05^{a}	0.34	842.43	193.44	148.94^{a}	5.85^{b}	0,51
							P				
Sepiolite		< 0.001	0.741	0.117	0.308	0.008	0.575	0.081	0.185	0.098	0.124
Betaine		0.253	0.515	0.707	0.277	0.227	0.277	0.781	0.770	0.347	0.292
Sepiolite*Betaine		0.201	0.350	0.078	0.020	0.083	0.214	0.487	< 0.001	< 0.001	0.246

^{a,b}: Means with different letter in the same column are different at P<0.05 in instances with significant interaction.
VH: villus height, VW: villus width, CD: crypt depth, VSA: villus surface area

Table 8. Effects of dietary sepiolite and betaine supplementation on blood serum parameters.
Tablo 8. Karma yemlere betain ve sepiyolit ilavesinin kan serum parametreleri üzerine etkileri.

Sepiolite %	**Betaine %**	**Protein g/l**	**Albumin g/l**	**Uric acid mg/dl**	**Cholesterol mg/dl**	**Triglyceride mg/dl**	**ALT IU/l**	**AST IU/l**	**ALP IU/l**	**IgG mg/dl**
0		26.53	10.86	3.86	62.84	61.27	40.71	130.56	794.50	5.14
1.5		27.87	10.66	4.16	58.36	57.39	38.78	131.06	830.94	6.36
	0	26.89	10.46	4.05	61.56	62.65	40.44	126.22	800.89	5.31
	0.15	27.50	11.05	3.97	59.64	56.02	39.05	135.39	824.56	6.19
0	0	26.51	10.26	3.81	65.66	65.93	41.92	123.44	790.44	4.89^{c}
0	0.15	26.54	11.45	3.90	60.02	56.61	39.50	137.67	798.56	5.38^{bc}
1.5	0	27.28	10.67	4.29	57.46	59.36	38.97	129.00	811.33	5.72^{b}
1.5	0.15	28.46	10.65	4.04	59.27	55.43	38.60	133.11	850.56	7.00^{a}
						P				
Sepiolite		0.115	0.783	0.076	0.138	0.173	0.363	0.941	0.268	<0.001
Betaine		0.469	0.417	0.615	0.521	0.023	0.510	0.184	0.470	<0.001
Sepiolite*Betaine		0.494	0.408	0.329	0.215	0.340	0.626	0.459	0.634	0.035

^{a,b,c}: Means with different letter in the same column are different at P<0.05 in instances with significant interaction

Discussion and Conclusion

During the first three weeks, body weight gain was decreased by the usage of sepiolite (P<0.05). However, supplementation of betaine or betaine with sepiolite did not affect weight gain during the first period. In contrast to the present results, some researchers (6, 12, 49) reported that body weight and weight gain were improved by dietary sepiolite. Endogenous digestive enzyme activity in the digestion of fats, proteins and carbohydrates were effective due to the sepiolite usage that increase digesta retention time (41). Body weight and weight gain in this experiment were not affected by 1.5% sepiolite. Some researchers (12, 33, 41) concluded that addition of sepiolite didn't affect feed intake and feed efficiency. Contrary to the present results, Yalçın et al. (49) indicated that 1% and 2% sepiolite supplementation decreased feed

intake by 4.7% and 1.8%, respectively compared to control group. Effects of sepiolite on broiler performance may be affected by many factors such as diet composition, sepiolite quality, water quality, weather and management conditions (49).

Similar to the present results, Schutte et al. (39) (level of 0.04%), Esteve-Garcia and Mack (13) (level of 0.5%) and Santos et al (37) (level of 0.1, 0.3, 0.5%) did not find any effects of betaine usage on body weight, weight gain, feed intake and feed efficiency. Ismail and Ahmad (20) reported that body weight gain and feed conversion ratio were not affected by dietary betaine supplementation during the 6 weeks experimental period. Some researchers (4, 43, 50) observed that daily weight gain and feed efficiency were improved by betaine supplementation. The improvement in performance due to betaine might be due to some factors such as donation of methyl groups (29), increasing growth hormone and insulin like growth factor I (19), enhancing intestinal immunity (25), increasing the nutrient digestibility and absorption ability of gut tract (18) and improving gut health and function (22, 29).

One chick in each group was dead during six weeks experiment. Similarly, some researchers observed that dietary supplementation of sepiolite (6, 12) and betaine (45, 51) didn't cause any differences in mortality in broilers. In the present study, sepiolite and/or betaine addition had no significant effect on litter moisture (Table 3), footpad lesions and breast burn of broilers. Footpad lesions and breast burn didn't seen due to the small number of birds in each pen and due to the optimal management and environmental conditions and proper litter moisture during the experiment. Similar results were observed by Yalçın et al. (49) in litter moisture. Nevertheless, addition of sepiolite may have beneficial effects against low digestible diets, litter quality, breast lesions and footpad dermatitis under conditions having large capacity and poor hygienic conditions (42, 49). Parizadian Kavan et al. (34) reported that the litter moisture in group fed the diet supplemented with 3% clinoptilolite, an aluminum silicate, was lower than that of control group. Olver (32) also showed that dietary zeolite supplementation leads lower litter moisture content. Mostashari-Mohases et al. (30) reported that excreta moisture content decreased with betaine supplementation.

The relative abdominal fat weight decreased with betaine ($P<0.05$) but didn't change with sepiolite or sepiolite with betaine supplementation. There is an interaction in relative abdominal fat weight between sepiolite usage and betaine usage ($P=0.023$). Betaine supplementation increased kidney weight percentages ($P<0.05$). Similar to the present results, some researchers reported that carcass yield (6, 12, 49) and the relative weights of liver (12, 41, 49), spleen (12, 41, 49), heart (49) and bursa of Fabricius (12, 49) were not affected by dietary sepiolite. In some studies (4, 8, 11, 13) carcass yield was increased by betaine supplementation. The improvement in carcass yield may be due to the osmotic effects of betaine that increases water retention (45). An interaction was observed in abdominal fat from betaine and sepiolite supplementation ($P=0.023$) in this present study. Some researchers reported that abdominal fat was decreased by sepiolite supplementation (12, 49) and betaine supplementation (21, 50). Betaine improves quality of lean meat by reducing amount of carcass fat. Zhan et al. (50) and Wang et al. (47) concluded that betaine addition decreased the abdominal fat due to the role in lipid metabolism in the body. However some researchers showed that betaine had no significant effect on the abdominal fat (8, 40, 51). Attia et al. (4) also indicated that there is an increase in abdominal fat by betaine supplementation. Santos et al (37) reported that betaine inclusion at the level of 0.1, 0.3 and 0.5% did not affect liver weight percentage as a proportion of body weight.

From all of the present results in intestinal histomorphology it can be concluded that dietary betaine with sepiolite may be positive effect on intestinal digestion. There was a reverse interaction ($P<0.05$) between sepiolite supplemented group and betaine supplemented group in crypt depth and the ratio of villus height/crypt depth in jejunum and ileum. Betaine affects intestinal development and intestinal functions. Betaine improves water holding capacity in intestinal cells (22) and affects intestinal epithelium. Klasing et al. (25) observed that dietary betaine supplementation could eliminate reduction of villus height due to coccidial infection in chicks. In another studies crpyt/villus ratio was decreased in both healthy and coccidial infected chicks (23) and lesion score was reduced (43) by dietary betaine supplementation. Dietary betaine supplementation with ionophore anticoccidials could help to protect intestinal health (23, 43) and reduce lesion score (39). Santos et al. (37) concluded that betaine supplementation reduced the severity of the apparent inflammatory response, decreased the width and increased the length of the villus and therefore improved the absorptive area of the duodenum. Yalçın et al. (49) stated that 1% and 2% sepiolite supplementation increased duodenal villus height in broilers.

In the present study blood serum triglyceride was decreased with betaine supplementation ($P=0.023$). Sepiolite with and without betaine significantly increased serum IgG concentration. In addition, additive effects were seen with the usage of sepiolite and betaine. In the study of Eser et al. (12), serum levels of cholesterol and triglyceride were decreased with 1% sepiolite supplement. Jahanian and Rahmani (21) reported that plasma

triglyceride was decreased with betaine supplementation but there was no effect on the level of plasma cholesterol. Reduction in triglyceride could be correlated with betaine usage in shynthesis of carnithine and lecithin for fat immobilization. El-Shinnawy (11) showed that betaine supplementation increased serum cholesterol, protein and albumin. Some researchers (8, 15) indicated that betaine supplementation improved the immunity of birds. Latshaw (26) also reported that betaine improves immune response due to increased digestibility and utilization of nutrients such as methionine, carotenoids, lysine, protein and fat (35).

In conclusion sepiolite and betaine supplementation didn't have any negative effects during 6 weeks experimental period. It is concluded that the improvement in intestinal health and immunity due to the dietary sepiolite with betaine supplementation can increase performance under industrial conditions especially under suboptimal conditions in broiler production.

Acknowledgement

This manuscript is inspired by PhD thesis of Kübra Uzunoğlu. This study was supported by Ankara University Research Fund (Project No: 13L3338004). The authors wish to acknowledge Beypi A.Ş. for chicks, Trouw Nutrition (Türkiye) for betaine and Tolsa-Turkey for sepiolite supply.

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Ankara Üniv Vet Fak Derg, **66**, 231-236, 2019
DOI: 10.33988/auvfd.436873

Determination of phthalates in some milk products by liquid chromatography/tandem mass spectrometry

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Received date: 25.06.2018- Accepted date: 01.05.2019

Abstract: This study was aimed to determine the presence and amount of the phthalates in yoghurt and ayran samples consumed in Turkey. For this purpose, 36 yoghurt and 24 ayran samples were used as materials in original packages collected from different markets in Turkey. The presence and the amounts of di-n-butyl phthalate (DBP), di (2-ethylhexyl) phthalate (DEHP), benzyl butyl phthalate (BBP), di-isononyl phthalate (DINP), di-n-octyl phthalate (DNOP) and diisodecyl phthalate (DIDP) were determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS). The results of the analysis howed that DBP, DEHP and BBP were present in the yoghurt samples whereas DINP, DIDP and DNOP were found to be lower than the detectable limit 20 µg/kg. The lowest and the highest mounts of DBP, DEHP and BBP in yoghurt samples are 6-229, 24-122, 22-63 µg/kg respectively. The lowest and highest phthalate amounts in ayran samples are determined to be 38-59 µg/kg for DBP and 26-81 µg/kg for DEHP. According to the results, although DEHP and DBP were the main phthalate esters in all milk products that were analyzed, the phthalate values of the samples were found to be less than the limit values ($P<0.001$).

Keywords: Dairy products, LC-MS/MS, phthalate, public health.

Bazı süt ürünlerinde likit kromatografi/tandem kütle spektrometresi ile fitalat tayini

Özet: Bu çalışma Türkiye'de üretim yapan farklı firmalara ait yoğurt ve ayranlardaki fitalat varlığı ve miktarının belirlenmesi amacıyla yapıldı. Bu çerçevede, farklı satış noktalarından orjinal plastik ambalajlarda temin edilen 36 yoğurt ve 24 ayran örneği materyal olarak kullanıldı. Örneklerden di-n-butilfitalat (DBP), di (2-etilhekzil) fitalat (DEHP), benzil butil fitalat (BBP), di-isononil fitalat (DINP), di-n-oktil fitalat (DNOP) ve diizodesil fitalat (DIDP) esterlerinin varlığı ve miktarı likit kromatografi/tandem kütle spektrometresi (LC- MS/MS) ile belirlendi. Analiz sonuçlarına göre, yoğurt ve ayran örneklerinde DBP, DEHP ve BBP esterleri saptanırken DINP, DIDP ve DNOP esterleri saptama sınırının (20 µg/kg) altında bulunmuştur. Yoğurt örneklerine ait en düşük ve en yüksek fitalat değerleri DBP, DEHP ve BBP için sırasıyla 46-229, 24-122, 22-63 µg/kg olarak saptanmıştır. Ayran örnekleri için en düşük ve en yüksek fitalat değerleri DBP için 38-59 µg/kg, DEHP için 26-81µg/kg olarak belirlenmiştir. Buna göre, analize alınan tüm süt ürünlerinde DEHP ve DBP en yüksek oranda saptanan temel fitalat esterleri olmasına ragmen, örneklere ait fitalat değerleri yasal değerlerin oldukça altında bulunmuştur ($P<0.001$).

Anahtar sözcükler: Fitalat, halk sağlığı, LC-MS/MS, süt ürünleri

Introduction

Phthalates are chemical compounds commonly used in the industry. Particularly it is often used as polymer additive in plastics because of providing the flexibility and softness to plastic material. For this reason, they are present in many products such as children toys, gloves, personal care products, printing inks, glues, solvents and including food packaging in particular (4,6). Today, phthalate acidic esters are mostly used as a plasticizer (softener). Di (2-ethylhexyl) Phthalate (DEHP) makes up 50% of the plasticizer used. DEHP is the most frequently used phthalate. It was started to be produced in United States of America (5). The main usage area is PVC production (95%). While it was being used in more than 50% of PVC products, this amount has decreased in recent years. The use of DEHP has been prohibited across Europe and America especially in food packaging containers, baby rattle and teether and baby toys upon determination of negative effects on human health at the beginning of 2000's (6, 11).

DEHP is mixed into the nature through all phases of production, use and disposal. Therefore a major part of the

substance is present in foods, interior room air and soil. It is biologically accumulated in invertebrate animals, fishes, and plants. However, biomagnifications are not seen in more sophisticated animals and humans due to being metabolized (14, 15).

All esters including DEHP in particular, which are added as softener to packaging material, creates an important danger in terms of food safety and public health. The effects of phthalates on human health were revealed with studies conducted. Phthalate esters are known to have endocrine disrupting effect by showing especially estrogenic characteristic. The effects on the organism may vary depending on the age, period, amount of being exposed to phthalates (14, 24). In this respect, the most sensitive periods are pregnancy, infancy and puberty periods (16). It is known that among phthalate esters demonstrate adverse effect as follows; DBH, DEHP on germ cell development, BBP on epididymal spermatozoa concentration, DIN and DIDP on liver cells (8). It is explained that the main intake way of phthalates entering into the organism through inhalation, parenteral attempts and skin contact with foods (7, 15). Foods are contaminated by plastic material thereby with DEHP for several times during production, packaging and preservation. It has been demonstrated with studies that foods are contaminated by migration of phthalates especially from packaging materials and printing inks. Phthalates may transfer to the food not only from packaging materials, but also from transport, production, storage as well as from soil, water, air and even during cooking in homes (7, 19). The phthalate amount in packaged food depends on phthalate concentration in the packaging materials, storage time and temperature, food's fat content and contact surface (3, 6, 13). The foods with high oil amounts pose an important risk due to phthalates show lipophilic characteristics. Transition occurs much more in oily foods such as milk and dairy products, olive, sunflower oil because of its obvious lipophilic characteristic, albeit the presence of DEHP has been found in many foods (10, 12, 20). Frequently, isolation in breast milk and from baby foods demonstrates that the interaction with this substance begins in the first years of the life. Determination of presence and amounts of phthalates in foods is essential with respect to public health. Studies conducted in various countries also show that milk and dairy products are major sources for phthalates (4, 15, 17, 24).

Legal regulations on the use of phthalates in many countries of the world have been introduced. The amount of daily intake to be tolerated by European Food Safety Authority (EFSA) was 0.01, 0.5, 0.05, 0.15 and 0.15 mg/kg/day for DBP, BBzP, DEHP, DiNP and DiDP, respectively (9). The legal limit values of BBP, DEHP, DnBP, DiNP and DiDP should be 30, 1.5, 0.3, 9, 9 mg/kg, respectively which can cause migration when contact with foodstuffs in EU directives; total phthalate levels that can pass from plastics to food are limited to 60 mg/kg (1). Similar practices and limits with the European Union have been adopted in Turkiye as well (2).

Yoghurt and ayran are sold in plastic packaging in Turkey are dairy products that are consumed commonly by every age segment. The purpose of this study was to determine whether there is a risk of public health or not in terms of phthalate contamination of food samples via analyzing yoghurt and ayran samples which were produced in Turkey.

Materials and Methods

36 full fat yoghurt and 24 ayran samples, which produced by manufacturing firms and were taken from different markets with original plastic packages used as materials. The samples with different brand and lot numbers were randomly taken from different markets, which were taken to the thermos (cold box) containers and brought to the laboratory immediately. It was kept in the refrigerator (+4°C) until analysis was made.

Among the samples, the presence and amounts of dibutyl phthalate (DBP), Di (2-ethylhexyl) phthalate (DEHP), benzyl butyl phthalate (BBP), di-'isononyl' phthalate (DINP), Di-n-oktil phthalate (DNOP) and di-'isodecyl' phthalate (DIDP) esters were determined by Liquid Chromatography/Tandem Mass spectrometry (LC-MS/MS)(Applied Biosystem-3200, USA) (22).

Standard solutions in 20-40-80-120-200 ve 400 µg/kg were extracted with the standard of DBP (cas no: 84-74-2), DEHP (cas no:117-81-7), BBP (cas no:85-68-7), DINP (cas no:28553-12-0), DNOP (cas no:117-84-0) and DIDP (cas no:26751-40-0) (Dr.Ehrenstorfer, Augsburg, Germany) esters having stock solutions prepared with hexane for calibration.

The lowest concentration standart was run 20 times to calculate standart deviation. LOD (Limit of detection) value was found as threefold of standart deviation of it. LOQ (Limit of Quantitation) value also came out as 3.3x LOD. For the % recovery value, mix standart was added to the blank sample to get the 50 µg/kg concentration. 6 separate spiked samples were prepared. According to these values % recovery and repeatability were calculated. LOQ, Recovery, Relative repeatability standard deviation (RSD), intra-laboratory reproducibility standard deviation (RSD, intra) and R2 values of the method were shown in Table 1.

Samples were homogenized by stirring with a glass stirring rod and weighted 2.00 g ±0.05 into a 10mL glass test tube. 2 mL methanol were added and the tube was vortexed 1 min. 2 mL hexane and 2 ml tert-butyl methyl ether were added to the solution and centrifuged for 2 minutes at 1500 rpm. After the centrifuging, hexane and

ether phase of the tube was transferred to another tube and hexane and tert-butyl methyl ether addition was repeated. Hexane-ether phase in the mixture was evaporated in a rotary evaporator (70°C). Residue was dissolved in 2 mL acetonitrile and was transferred to test tube. 3 mL hexane was added and mixture was shaken 2 min. Hexane phase in the tube was separated with aid of Pasteur pipette and acetonitrile phase was evaporated in rotary evaporator (70°C). Residue was dissolved in 0.5 mL acetonitrile and injected to LC-MS/MS. Analyses were carried out by using C 18-reverse phase (Zorbax Eclipse XDB 50x2,1 mm 1.8µ) column with an 80:20 water - methanol mobile phase as starting conditions and flow of 0.3 mL/min. Injection volume was 5 µl (22). MS parameters used for the analysis are given in Table 2.

Statistical analysis: One sample t-test was used to compare the DEHP and DBP values with the legal limit values.

Table 1. LOQ, Recovery %, Relative repeatability standard deviation (RSD), intra-laboratory reproducibility standard deviation (RSD, intraday) and R^2 values of the method.
Tablo 1. Metoda ait LOQ, % Geri Kazanım, Tekrarlanabilirlik (RSD, RSD, intra) ve R^2 değerleri.

Phthalate Esters	LOQ (µg/kg)	Recovery %	RSD (%)	RSD, intraday (%)	Calibration R^2 value
DBP	30	87.2	12	16	0.9961
DEHP	20	83.9	14	18	0.9907
BBP	20	90.9	12	18	0.9951
DINP	20	96.1	13	18	0.9895
DNOP	20	92.8	12	16	0.9918
DIDP	20	85.5	14	19	0.9972

Table 2. Instrument parameters for the analysis.
Tablo 2. Analiz için kullanılan sistem parametreleri.

Parameters	Description
Column	Agilent Zorbax SB-C18 column 2.1 mm x 50 mm x 1.8 µm
Column temp.	35 ◦C
Injection volume	5 µl
Mobile phase A	0.1 % Formic acid in water
Mobile phase B	Methanol
Flow rate	0.3 ml/min
Gradient	0 min- 20 % B
	0.5 min- 20 % B
	3 min- 95 % B
	6 min-95 % B
	6.1 min- 20 % B
	8.5 min- 20 % B
Run time	9 min
Ionisation mode	Positive electrospray ionisation mode with jet stream technology
Drying gas temp	325 ◦C
Drying gas flow	11 L/min
Nebuliser	35 psi

Results

Ionisation parameters for each compound were optimized to achieve better sensitivity and robustness. A total of 60 samples including 36 yoghurt, 24 ayran samples were analyzed in the study. Extracted Ion Chromatogram (EIC) of standard mixture of 6 phthalate compounds with a concentration of 40 µg/kg (Calibration Level 2) can be seen in Figure 1. Each compound has 2 MRM transitions. Transitions used for the analysis were given in Table 3.

According to results DBP, DEHP, and BBP were present in the samples whereas DINP, DIDP and DNOP esters were found to be lower than the detection limit 20 µg/kg. The highest values of DBP, DEHP, and BBP in yoghurt samples were found to be 229, 122 and 63 µg/kg respectively, the lowest values were 46, 24 ve 22 µg/kg (Table 4). The highest values of DBP and DEHP in ayran samples were found to be 59 and 81 µg/kg, the lowest values were 38 ve 26 µg/kg (Table 5). All the values found have been determined under the legal limit values ($P<0.001$).

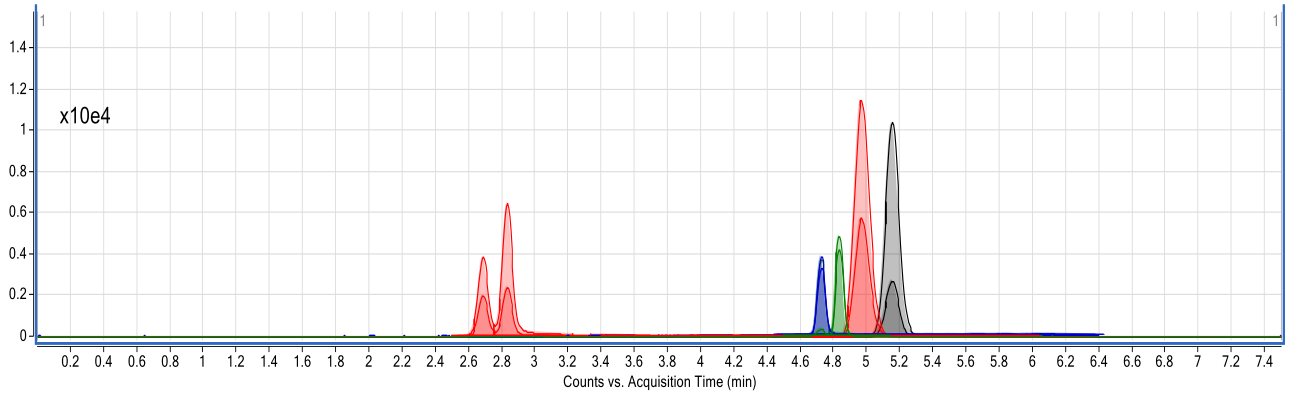

Figure 1. Extracted Ion Chromatogram, EIC, of standard mixture of 6 phthalate compounds with a concentration of 40 µg/kg (Calibration Level 2).
Şekil 1. Fitalat esterlerinin 40 µg/kg derişimde ekstrakte edilmiş iyon kromatogramları.

Table 3. MRM transitions and Retention Time values.
Tablo 3. MRM geçişleri ve alıkonma değerleri (µg/kg).

Phthalate esters	Precursor ion	Product ion1	Product ion2	Retention time (min.)
DBP	279	205	149	2.67
DEHP	391	167	149	4.72
BBP	313	205	149	2.85
DINP	419	275	149	4.96
DIDP	447	289	149	5.20
DNOP	391	121	261	4.83

Table 4. Phthalate values (µg/kg) of yoghurt samples.
Tablo 4. Yoğurt örneklerine ait fitalat değerleri (µg/kg).

No	DBP	DEHP	BBP	DIDP	DINP	DNOP	No	DBP	DEHP	BBP	DIDP	DINP	DNOP
1	89	35	ND	ND	ND	ND	**19**	113	76	ND	ND	ND	ND
2	136	53	ND	ND	ND	ND	**20**	166	92	ND	ND	ND	ND
3	77	ND	ND	ND	ND	ND	**21**	105	66	ND	ND	ND	ND
4	54	24**	ND	ND	ND	ND	**22**	213	122*	46	ND	ND	ND
5	172	90	33	ND	ND	ND	**23**	229*	109	33	ND	ND	ND
6	110	49	22**	ND	ND	ND	**24**	46**	ND	ND	ND	ND	ND
7	ND	ND	ND	ND	ND	ND	**25**	80	44	ND	ND	ND	ND
8	159	75	ND	ND	ND	ND	**26**	152	88	ND	ND	ND	ND
9	99	44	ND	ND	ND	ND	**27**	110	95	ND	ND	ND	ND
10	78	37	ND	ND	ND	ND	**28**	116	98	ND	ND	ND	ND
11	101	49	ND	ND	ND	ND	**29**	92	ND	ND	ND	ND	ND
12	188	96	40	ND	ND	ND	**30**	62	38	ND	ND	ND	ND
13	170	112	63*	ND	ND	ND	**31**	ND	ND	ND	ND	ND	ND
14	80	ND	ND	ND	ND	ND	**32**	105	52	ND	ND	ND	ND
15	181	86	24	ND	ND	ND	**33**	100	ND	ND	ND	ND	ND
16	91	46	ND	ND	ND	ND	**34**	85	35	ND	ND	ND	ND
17	81	40	ND	ND	ND	ND	**35**	142	68	ND	ND	ND	ND
18	196	101	ND	ND	ND	ND	**36**	102	60	ND	ND	ND	ND

*: The highest phthalate ester value, **: The lowest phthalate ester value. Detectable Limit: (20 µg/kg), ND: Not determined

Table 5. Phthalate values (µg/kg) of ayran samples.
Tablo 5. Ayran örneklerine ait fitalat değerleri (µg/kg).

No	DBP	DEHP	BBP	DIDP	DINP	DNOP	No	DBP	DEHP	BBP	DIDP	DINP	DNOP
1	ND	30	ND	ND	ND	ND	**13**	50	71	ND	ND	ND	ND
2	38**	51	ND	ND	ND	ND	**14**	ND	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND	ND	**15**	48	67	ND	ND	ND	ND
4	ND	ND	ND	ND	ND	ND	**16**	ND	45	ND	ND	ND	ND
5	45	60	ND	ND	ND	ND	**17**	ND	34	ND	ND	ND	ND
6	ND	26**	ND	ND	ND	ND	**18**	ND	54	ND	ND	ND	ND
7	59*	ND	ND	ND	ND	ND	**19**	46	63	ND	ND	ND	ND
8	46	67	ND	ND	ND	ND	**20**	55	70	ND	ND	ND	ND
9	57	81*	ND	ND	ND	ND	**21**	48	75	ND	ND	ND	ND
10	39	50	ND	ND	ND	ND	**22**	ND	ND	ND	ND	ND	ND
11	ND	ND	ND	ND	ND	ND	**23**	50	68	ND	ND	ND	ND
12	41	53	ND	ND	ND	ND	**24**	56	ND	ND	ND	ND	ND

* : The highest phthalate ester value, **: The lowest phthalate ester value. Detectable Limit: (20 µg/kg), ND: Not determined

Discussion and Conclusion

Tandem MS technique with being capable of more selective and more sensitive with respect to other single MS techniques let us analyse more complex matrices even without a significant sensitivity lost. The presence and quantity of 6 separate phthalate esters in yoghurt and ayran were researched in the study. According to analysis results DBP, DEHP, and BBP were present in the samples whereas DINP, DIDP and DNOP esters were found to be lower than the detection limit 20 µg/kg. While the highest values of DBP, DEHP, and BBP in yoghurt samples were found to be 229, 122 ve 63 µg/kg respectively, the lowest values were 46, 24, and 22 µg/kg. While the highest values of DBP and DEHP in ayran samples were found to be 59 and 81 µg/kg respectively, the lowest values were 38 and 26 µg/kg. Accordingly, DEHP and DBP were found to be main phthalate esters determined at the highest rate in all dairy products taken to the analysis.

Some studies were encountered in foods such as raw milk, pasteurized milk, butter, cheese, baby food related to searching phthalates in dairy products, but it was seen that no study was conducted related to ayran, and only one study was available related to yoghurt (10). It is stated that DEHP level in fruity yoghurt is 15-37 µg/kg, and DBP, BBP, DINP and DIDP are below the detectable limit in the research done by Sorensen on milk and dairy products. In this study, the following values were found in yoghurt samples as; DEHP 24-122 µg/kg, DBP 46-229 µg/kg, BBP 22-63 µg/kg, In this study, the results are higher than the values found by Sorensen, 2006 (22). Similarly to our study, Sireli et al. (2017) determined 6 phthalate esters (DEHP, BBP, DBP, DNOP, DEP and DMP) in yoghurt and fruits yoghurt samples by using GC-MS. According to this study, DEHP concentration values of the samples were similar to our results. However the concentration values of DBP and BBP were lower than our results (21). Zhoukun et al. (2011) in this study which they examined 9 types of phthalate esters in dairy products; in all the samples the main phthalate ester was DBP with a value of 100 ng / kg. The other results are similar to our study (24). It is thought that this difference might arise from air, water, storage conditions in addition to packaging material and printing ink used. Furthermore, fat content also affects the migrations of phthalates having lipophilic characteristic (10). The phthalate amounts in packaged foods depend on phthalate concentration in packaging material, storage time and temperature, fat content of the food and contact surface. The levels of phthalate esters which exist in ayran samples were found lower than yoghurt samples as expected because of water ratio of samples (20).

It was seen that phthalate esters were at different levels in other studies (13, 17) conducted on milk and dairy products. It is thought that this situation results from production, transport and storage conditions, including packaging material and food's fat amount in particular. In addition, the existence of different legal regulations concerning the packaging material in countries is among the causes of differences in studies.

In conclusion, considering the phthalate sources that milk products may be exposed to, it has been concluded that phthalate contamination that can reach on uncontrolled high level could pose a potential risk. DEHP, which is the most commonly used phthalate ester during production, processing and protection of foods, is considered among the group of "endocrine disrupting chemical substance" because of having estrogen agonist, testosterone antagonist. It is known that children

especially are more sensitive to this effect (8, 18). Additionally carcinogenic, teratogenic and mutagenic effect created by phthalates on animals is also a crucial threat for human health (16, 18, 23). Therefore, bringing the contamination by phthalates under control the contamination by from farm to table, and the use of alternative materials not containing phthalates in food's packaging are of great importance.

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Ankara Üniv Vet Fak Derg, **66**, 237-245, 2019
DOI: 10.33988/auvfd.441862

Effects of genotype and housing system on some bone biomechanical characteristics in broiler chickens

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Received date: 03.07.2018- Accepted date: 21.04.2019

Abstract: The aim of this study was to determine the effects of two genotypes (slow-growing and fast-growing) and three housing systems (deep litter, plastic slat and free-range) on some bone biomechanical properties of broiler chickens and to evaluate the interaction between genotype and housing systems. Broilers from two genotypes were reared at three different housing conditions. Fifteen bones were randomly selected from each housing system in both slow-growing and fast-growing groups, and the experiment was performed on 90 bones in total. To determine bone characteristic and biomechanical traits of tibiotarsi, bones collected from right leg and then weighed, cortical area measurements and three-point bending tests were applied. Both live body weight and carcass weight were significantly affected by genotype and housing systems. There was no interaction between genotype and housing system in terms of bone weight, cortical area, breaking strength, bending strength, and deflection. Also, housing systems had no statistical effect on these parameters. Fast-growing broilers were significantly had heavier tibiotarsi, larger cortical area and higher breaking strength than slow-growing broilers, while bending strength was significantly lower in fast-growing broilers. Deflection was not affected by genotype or by housing system. In conclusion, bone geometry and biomechanical properties were not affected by housing systems but by genotype. Fast-growing broilers had better bone morphology and stronger bones than slow-growing genotype. Therefore, fast-growing genotype can provide positive effects on bone growth and mechanical properties in broilers.
Keywords: Biomechanics, broiler, genotype, housing, tibiotarsus.

Etlik piliçlerde genotip ve barınma sisteminin bazı kemik biyomekanik özellikleri üzerine etkileri

Özet: Bu çalışmanın amacı, iki genotip (yavaş ve hızlı gelişen) ve üç barınak sisteminin (derin altlık, plastik ızgara ve serbest dolaşım) etlik piliçlerin bazı kemik biyomekanik özellikleri üzerindeki etkilerini belirlemek ve genotip ve barınak sistemleri arasında etkileşimi değerlendirmektir. İki farklı genotipe ait etlik piliçler üç farklı barınma sisteminde yetiştirildi. Hem yavaş gelişen hem de hızlı gelişen genotipe sahip etlik piliçlerde her bir konut sisteminden 15 kemik olacak şekilde rastgele seçildi ve deney toplam 90 kemik üzerinde gerçekleştirildi. Kemik özellikleri ve biyomekanik karakterlerinin belirlenmesi için sağ bacaklardan elde edilen tibiotarsuslar kullanıldı ve sonra bu kemikler tartılarak, kortikal alan ve üç nokta eğme testine tabi tutuldu. Hem canlı ağırlık hem de karkas ağırlığı, genotip ve konut sistemlerinden istatistiksel olarak etkilenmiştir. Kemik ağırlığı, kortikal alan, kırılma mukavemeti, eğilme gerilimi ve bükülme açısından genotip ve barınma sistemleri arasında herhangi bir etkileşim bulunamadı. Ayrıca, barınma sisteminin istatistiksel olarak bu parametreler üzerinde herhangi bir etkisinin olmadığı gözlendi. Yavaş gelişen etlik piliçlerin daha düşük eğilme gerilimi sahipken, hızlı gelişenlerin belirgin şekilde daha yüksek kemik ağırlığı, kemik kortikal alanı ve kırılma mukavemeti değerlerine sahip olduğu gözlendi. Ne genotipin ne de barınma sisteminin bükülme üzerine herhangi bir etkisi bulunamadı. Sonuç olarak, kemik geometrisi ve biyomekanik özellikler, barınma sistemlerinden değil, genotipten etkilenmiştir. Hızlı gelişen etlik piliçler, yavaş gelişenlerden daha iyi kemik morfolojisine ve daha güçlü kemiklere sahip olduğu gözlendi. Bu nedenle hızlı gelişen genotip, etlik piliçlerde kemik gelişimi ve mekanik özellikleri üzerinde olumlu etkiler sağlayabilir.
Anahtar sözcükler: Barınma, biyomekanik, etlik piliç, genotip, tibiotarsus.

Introduction

The demands of customers for different poultry products have been shaped by different commercial breeding systems (53). In recent years, organic and free-range (FR) products have become more available to customers (13, 18). However, environmental conditions,

human population density, and land availability limit the use of organic or FR farming systems (20). Confined systems such as caged rearing are still used in broiler production (9).

Although intensive genetic selection for fast-growing (FG) broilers has shortened the growing period considerably, some undesirable results, such as excessive feed intake and carcass fatness, have also occurred (41). Several factors for example nutrition, sex, strain, housing and stocking density affect growth performance and carcass characteristics of broiler chickens (3). Some studies have also reported that genotype affected body weight, body weight gain, feed intake and feed conversion ratio in broiler chickens (48, 52).

Most natural and organic poultry industries in the United States use an FG broiler genotype, which is the same as in classical production systems (20). Although growth performance of FG chickens is more efficient than slow-growing (SG) genotypes, some researchers suggested that SG chickens adapt to native systems more easily (6, 25). In general, broiler chickens have been bred for growing rapidly and they reach market weight by the age of 42 to 45 days. However, there is a rising trend to produce heavier chickens in the broiler industry (24, 60). Therefore, the poultry industry is based on intense production systems using FG genetic strains with high yield of breast muscle (34). For several decades, modern high-yield meat chicken has undergone very successful genetic selection to reduce the time and the feed required for reach the target body weight. However, it has been known for several years that rapid increase in body weight cause several undesirable effects on respiratory, reproductive and skeletal systems of broilers (12, 17, 24, 26, 31, 35). Such applications in the poultry industry pose a risk to animal welfare and increase the stress to the animals, potentially causing lameness, foot pad dermatitis and hock burn lesions, ascites and metabolic disorders (11).

Gait problems in broiler chickens has become a major problem, not only because they cause losses in the market and slaughterhouse, but also to affect the broilers' welfare negatively (1). However, for producing low cost meat, optimizing the production for high yield characteristics of those chickens has resulted with poor walking or locomotor ability, reduced viability and welfare (4). Obviously, skeletal leg health is important in terms of both economic considerations and the welfare of broiler chickens (31).

Some researchers reported that leg health and bone strength are poorer in modern wide-breast broilers than in unselected broilers (16, 22, 26, 35, 46). Kestin et al. (21) found significant differences in certain parameters of leg health in broiler chickens. In contrast, Yalcin et al. (57) did not observed any differences in bone breaking strength in the two different commercial broiler genotypes.

Housing systems also affect bone growth and breaking strength in poultry species. Conventional confined systems cause stress that may result in physiological and behavioral disorders (27, 38). FR systems may decrease stress and increase comfort and welfare (50). Tolon and Yalcin (49) reported that caged systems led to a decrease in humerus weight but did not affect tibia weight in broilers. Merkley and Wabeck (29) also observed that caged broilers had lower bone breaking strength than broilers grown in a conventional litter.

The objective of this study was to evaluate the effects of different housing systems on the biomechanical properties of tibiotarsus in broiler chickens with FG and SG genotypes.

Materials and Methods

This study was carried out at the Poultry Breeding Unit of Bursa Uludağ University Veterinary Faculty Animal Health and the Animal Production Research Center. The study was performed with the permission of the Bursa Uludağ University Animal Experimentation Local Ethics Committee (No: 01.09.2015–91).

Animals and groups: The study was designed to examine the effect of two broiler genotypes (SG and FG genotypes) with three housing systems [deep litter (DL), plastic slat (PS) and free-range (FR)] on the tibiotarsus of broiler chickens. One-day-old, Hubbard JA-57 SG and Ross 308 FG broiler chicks were purchased from a local commercial poultry farm. In the study, $2 \times 3 = 6$ main groups were formed, and each main group was composed of five replicates. Each replicated group had 10 male chicks. Therefore, each main group included 50 chickens. In total, 300 chickens (150 SG genotype and 150 FG genotype chickens) were used. Fifteen bones were randomly selected from each housing system in both SG and FG groups, and the experiment was performed on 90 bones in total. All broiler chickens used in the study were raised for 56 days and then they were slaughtered at 4403.30±77 g and 2385.66±77 g body weight for fast-growing and slow-growing broilers, respectively.

Housing systems and season: The study was carried out in May to June. Range area was covered by mesh canopy and no feed was provided in the range area. Deep litter was used as the confined system and shelter for the FR system. Plastic slat floor was used for the PS system. The DL system consisted of broilers reared indoors on rice husk litter, which was not changed throughout the rearing period, and the density of litter was 7 kg/m^2 and the depth of litter was 5 cm. Mean stocking density within the groups was 10 birds per 1 m^2 indoor and 5 m^2 outdoor for just the birds in free range group. The pasture consisted of alfalfa (*Medicago sativa*) 36%, pursley (*Portulaca*

oleracea) 4%, groundsel (*Senecio vulgaris*) 28%, cocklebur (*Xanthium Spinosum*) 32%. The PS system consisted of broilers reared indoors on plastic slat floors. The plastic slats measured 1.2 × 0.5 m (length × width) and were raised above the concrete floor by 0.5 meters to accumulate manure below.

Feed and lighting schedule: Hanging tube feeders (each 30 cm in diameter with 10–15 kg capacity) and bell drinkers were provided for the birds in all groups. All birds in all groups received a commercial multiphase diet (i.e., starter feed from days 0 to days 15, grower feed I from days 15 to days 30, grower feed II from days 30 to days 40, and finisher from days 40 to until the end of the experiment) (Table 1), which was produced and supplied by a commercial feed company in Turkey. Water and feed were provided ad libitum.

The birds were allowed access to range area at the beginning of 21 days of age in free range groups. During the experimental period, birds in free range groups had continuous access to outdoor range during daylight hours without any restrictions. The daily photoperiod consisted of 2 h of light and 2 h of darkness during the night and continuous day-light. During the day, daylight was used a light source and tungsten lights were used during the dark period. The lighting intensity was arranged as 5.0 lx m^2 for all groups.

Bone weight, cortical area and biomechanical tests: Right leg tibiotarsi were collected, dissected of surrounding soft tissues, weighed and wrapped with Phosphate-buffered Saline (PBS) soaked gauze then frozen at -20 ℃ until mechanical tests and cortical area analysis were conducted (47). The tibiotarsi were weighed with a Precisa XB4200C digital scale (Precisa Instruments Ltd., Switzerland). The bone cross section was considered an approximate hollow ellipsoid of varying thickness (Figure 1). To simplify the calculation of section properties as done in a previous study (32), the thickness value used in the following stress equation was calculated by taking the average of the measured thickness values of the four quadrants from lateral (t_1), anterior (t_2), medial (t_3), posterior (t_4) of the tibia.

Table 1. Composition and nutrient levels of the experimental diet.
Tablo 1. Yem kompozisyonu ve besin değerleri.

Ingredients	Unit	Starter (0-10 d)	Grower I (11-23 d)	Grower II (24-36 d)	Finisher (37-56 d)
Crude Protein	%	23.00	21.00	19.95	19.00
Crude Fiber	%	3.80	3.30	3.30	4.27
Crude Fat	%	5.46	6.40	6.50	8.01
Crude Ash	%	5.05	5.05	5.05	5.05
Calcium	%	1.05	0.90	0.85	0.85
Phosphorous	%	0.64	0.64	0.60	0.60
Sodium	%	0.16	0.17	0.16	0.16
Lysine	%	1.43	1.30	1.14	1.09
Methionine	%	0.51	0.54	0.48	0.41
E672 Vitamin A	IU	11000	10000	10000	10000
E671 Vitamin D3	IU	5000	4000	4000	4000
51774 - Nicarbazin	mg	125	125	-	-
E1 Iron (Ferrous sulphate 30%)	mg	50	50	50	50
E4 Copper (Cupric sulphate 25%)	mg	16	16	16	16
E6 Zinc (zinc oxide)	mg	100	100	100	100
Manganese (Manganous oxide 60%)	mg	100	100	100	100
E2 Iodine (Calcium iodate 6.2%)	mg	2	2	2	2
E8 Selenium (Sodium selenite 4.5%)	mg	0.20	0.20	0.20	0.20
EC 3.1.26 - 6 Phytase	FTU	1000	1000	1000	1000
EC 3.2.2.8 - Endo-1.4 beta xylanase	u/g	-	1500	1500	-
EC 3.2.1.1 - Alfa Amylase	u/g	-	2000	2000	-
EC 3.4.21.62 - Subtilisin (Protease)	u/g	-	20000	20000	-
E558 - Bentonite/Montmorillonite	mg	-	700	700	-
E776 - Salinomycin Sodium	mg	-	-	60	-

Diet and analysis nutrient composition were obtained from a commercial feed company.

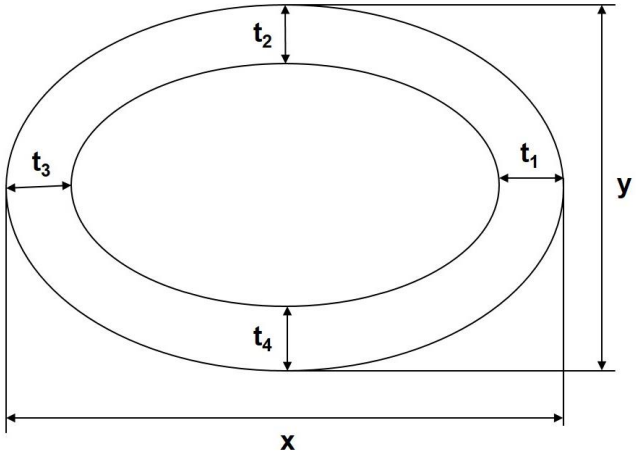

Figure 1. Geometry and dimensions of bone cross-section. t_1=lateral, t_2= anterior, t_3= medial, t_4= posterior.
Şekil 1. Kemik kesitinin geometrisi ve ölçümler. t_1=lateral, t_2= anterior, t_3= medial, t_4= posterior.

Figure 2. Three-point bending test apparatus.
Şekil 2. Üç nokta eğme testi aparatı.

Three-point bending tests were performed with a custom-made testing machine, which was designed for low strength materials (51). A load-cell (50 N, Tedea Huntleigh Malvern, USA) and a Linear Variable Differential Transformer (LVDT) (10-mm stroke, Novotechnik Tr10, Germany) were used to measure force and corresponding deflection during tests, respectively. Force and deflection measurements were recorded by using an oscilloscope (Nicholet-Oddysey XE, USA) at the rate of 100 data/sec. The experimental results can be affected by displacement rate; therefore, all tests were performed at a constant displacement rate of 10 mm/min, as suggested by a previous study (2). The span of the support roller was set to 70 mm, and the force was applied at the middle of the span (Figure 2).

Bone breaking strength was attained by reading the highest value of the load-deflection curve. By using recorded force data and geometrical properties of the damaged sample, bending strength (σ_{max}) was calculated as follows:

$$\sigma_{max} = \frac{8F_{max}Ly}{\pi[xy^3 - (x-2t)(y-2t)^3]}$$

Where F_{max} is the maximum load recorded from the load-deflection curve, L is span of the bending test fixture, t is average thickness value, and x and y are the outer diameters of the ellipse.

Bone breaking strength refers to the maximum load or force that a bone structure can withstand before fracture. In addition, maximum load is synonymous with whole-bone strength. Bending strength or ultimate stress is the highest load per unit area that bone-tissue can withstand before fracture. Deflection is the displacement of bone structure from the start of the bending process until fracture. Cortical area is important to define the total amount of bone at the diaphysis and to report. The engineering theory for compression or stress loading states that cortical area is the most appropriate morphological parameter (19).

Statistical analysis: Statistical analyses were performed using IBM® SPSS® (SPSS, Version 23.0; Chicago, IL, USA). To determine the main effects and interactions between groups, experimental groups considered as 2 × 3 factorial design and Two-way ANOVA was used for statistical evaluation. Linear

regression analysis was used to identify the regression equations between body weight and bone properties. Differences were considered statistically significant at the level of P = 0.05. Differences between groups were assessed by the Duncan multiple range test when statistically significant differences were found. Furtherly, One-way ANOVA was performed for the differences between interactive group data (45).

Results

The effects of genotype and housing systems on bone parameters (bone weight, cortical area, breaking strength, bending strength and deflection) are shown in Table 2.

The regression equations and coefficient of determination (R^2) between live body weight and bone properties are presented in the Table 3.

Table 2. Effects of genotype and housing system on live body weight, carcass weight, bone weight, cortical area, bone breaking strength, bending strength and deflection.
Tablo 2. Genotip ve barınma sisteminin canlı ağırlık, karkas ağırlığı, kemik ağırlığı, kortikal alan, kemik kırılma mukavemeti, eğilme gerilimi ve bükülme üzerine etkileri.

	Live body weight (g)	Carcass weight (g)	Bone weight (g)	Cortical area (mm^2)	Bone breaking strength (N)	Bending strength (N/mm^2)	Deflection (mm)
Genotype							
FG	4403.30±77	3588.815±62.688	31.169±0.683	68.820±2.103	438.009±19.576	104.253±6.961	2.877±0.090
SG	2385.66±77	1825.533±61.559	19.597±0.679	41.637±2.094	293.699±19.621	154.359±6.974	3.027±0.087
Housing system							
DL	3233.75±95^{b}	2572.122±77.459^{b}	24.707±0.836	51.700±2.604	346.203±24.037	132.357±8.532	2.770±0.112
PS	3306.50±93^{b}	2878.700±75.393^{a}	25.340±0.840	54.493±2.576	361.113±24.018	128.420±8.544	2.986±0.107
FR	3643.20±94^{a}	2670.700±75.393^{ab}	26.103±0.839	59.492±2.580	390.245±24.022	127.141±8.541	3.100±0.110
Genotype x Housing system							
FG × DL	4277.50±133^{b}	3534.000±106.62^{b}	30.032±1.183	63.614±3.643	421.504±33.923	108.890±12.079	2.736±0.148
FG × PS	4115.50±132^{b}	3324.444±112.39^{b}	31.194±1.170	66.984±3.621	434.910±33.897	111.184±12.089	2.902±0.153
FG × FR	4817.20±131^{a}	3908.000±106.62^{a}	32.282±1.186	75.862±3.656	457.612±33.946	92.686±12.083	2.994±0.149
SG × DL	2335.80±133^{c}	1807.400±106.62^{c}	19.382±1.184	39.786±3.648	270.902±33.984	155.824±12.072	2.804±0.159
SG × PS	2352.00±130^{c}	1819.800±106.62^{c}	19.486±1.177	42.002±3.635	287.316±33.967	145.656±12.076	3.070±0.150
SG × FR	2469.20±132^{c}	1849.400±106.62^{c}	19.924±1.172	43.122±3.620	322.878±33.960	161.596±12.084	3.206±0.147
P							
Genotype	0.001	0.000	0.000	0.000	0.000	0.000	0.250
Housing system	0.008	0.020	0.507	0.117	0.432	0.904	0.118
Genotype × Housing system	0.095	0.043	0.769	0.426	0.970	0.368	0.893

*a–c within rows; values with different superscript letters differ significantly (P < 0.001). FG: Fast-growing; SG: Slow-growing; DL: Deep Litter; PS: Plastic Slat; FR: Free-range.

Table 3. The regression equation and coefficient of determination between live body weight and bone properties in fast- and slow-growing broilers.
Tablo 3. Hızlı ve yavaş gelişen etlik piliçlerde canlı ağırlık ve kemik özellikleri arasındaki regresyon formülü ve determinasyon katsayısı.

Bone properties	Fast-growing genotype			Slow-growing genotype		
	R^2	Regression equation	P	R^2	Regression equation	P
Bone weight	0.282	21.906+0.002×BW	0.003	0.130	20.477+0.001×BW	0.548
Cortical area	0.168	115.626+0.005×BW	0.025	0.130	45.910+-0.002×BW	0.553
Bone breaking strength	0.142	265.121+0.040×BW	0.040	0.005	314.093+-0.008×BW	0.712
Bending strength	0.011	115.626+-0.003×BW	0.584	0.002	147.702+0.003×BW	0.828
Deflection	0.254	1.900+0.001×BW	0.005	0.001	3.059+-1.315×BW	0.924

R^2: Coefficient of determination; BW: Body weight.

Live body weight at slaughter (BW) age was significantly higher in FG broilers compare to SG broilers ($P < 0.05$). BW was also significantly different among housing systems and the FR group were heavier than the DL and PS groups. But the interaction (genotype×housing system) was not significant for body weight. In terms of carcass weight, significant differences were observed in both genotype and housing systems. Accordingly, the PS group were heavier than the DL group, but the difference was not significant for FR group. There was also significant interaction (genotype × housing system) for carcass weight. Housing systems had no statistical effect on bone weight, cortical area, breaking strength, bending strength, and deflection. Furthermore, there was no interaction between genotype and housing system in terms of these parameters. In contrast, bone weight, cortical area, breaking strength and bending strength were significantly affected by genotype ($P < 0.05$). According to this, FG broilers were significantly higher in bone weight, cortical area and breaking strength than SG genotypes, while bending strength was significantly lower in FG broilers ($P < 0.05$). But it was observed that deflection was not affected either by genotype or by housing system. In FG broilers, changes in body weight had significant positive effects on bone weight, cortical area, breaking strength and deflection ($P < 0.05$). But, bending strength was not affected from body weight changes. Changes in body weight did not affect the bone parameters in SG broilers.

Discussion and Conclusion

It is well known that fast-growing broilers have been selected and bred for rapid early growth and reach market weight in about 42 d. But slow-growing broilers reach market weight at 63 to 81 days of age (15). Because of their rapid development FG broilers obviously become heavier at slaughter age than slow-growing broilers. In the present study, FG broilers had higher live weight at slaughter age and carcass weight compare to SG broilers as expected.

Castellini et al. (7) suggested that organic free-range broilers had lower growth performance than broilers in conventional systems due to higher locomotor activity. In contrast, we observed FR broiler significantly higher body weight than those in other housing systems. Similar results were reported by Santos et al. (43) and Ponte et al. (36) showed significantly higher body weight in broiler chickens that had free access to pasture. Likewise, Bogosavljević-Bosković et al. (5) observed that broilers with free-range access showed better muscle gaining compared to those reared indoors only. In the present study, the increase in BW and carcass weight might be the result of several situations. First, while broiler chicks are generally reared 42 days, our broilers were raised 56 days. During this long time, FR broilers may have adapted to the environment and locomotor activity and they might have had better feed intake (50). Second, due to improving comfort and welfare of FR broilers compared to confined systems, FR broilers might increase their feed intake and ultimately body weight (36, 43). Third and last, FR broilers may have had access to the various forages, insects, and worms that may be available in the pasture (55).

In this study, the carcass weight of broilers in PS group was higher than in DL group. It is consistent with Enver et al. (8) who also reported that broilers reared on plastic slats has better carcass yield than the broilers raised on deep litter. PS group showed better performance as it had less contact with litter and more comfortable environment. Inconvenient and wet litter reduces overall health, welfare, performance and carcass yield in broilers reared on the deep litter (10, 33).

McDevitt et al. (28) reported that the FG broilers had significantly heavier tibiotarsi and more ash and organic matter per unit length of bone than SG broilers at the same age. Similarly, in the current study, FG broilers had significantly higher bone weight than SG broilers. Knowles and Broom (23) reported that the load on bone tissue stimulates bone growth and increases mineral density. It is thought that FG broilers had stronger, heavier tibiotarsi because of the higher mineral content of the bones and the heavier body mass being carried. Those findings were consistent with our study and explained the heavier tibiotarsi found in the FG broilers.

Bone growth and breaking strength may also be affected by housing systems. Tolon and Yalcin (49) reported that caging birds led to a decrease in the weight of the humerus, but caged systems did not affect the weight of the tibiotarsus. Merkley and Wabeck (29) reported lower bone breaking strengths in wings of broilers grown in cages than in broilers grown on deep litter. In addition, Vitorovic and Nikolic (54) reported that wing bones were more affected by the housing system than were leg bones. In the present study, tibiotarsus weight was not affected by the housing system. It is thought that bone development was not significantly affected by the housing system because the chickens were not kept in strictly caged so they had more free movement opportunity. These findings suggested that bone breaking strength and weight of the tibiotarsus were affected by the genotype but not by the housing system.

Yildiz et al. (58) suggested that significant differences were observed in cortical areas of humerus between caged, aviary, perchery and litter housing systems. That study stated that larger cortical area in aviary, perchery and litter was caused by greater activity of birds in these housing systems. Reiter and Bessei (40) also reported that active birds had thicker and denser cortical bone in the tibiotarsus than less active controls. As

explained by Wolff's Law (56), activity increases the thickness and density of cortical bone and increases the diameter of the diaphysis by deposition of bone in response to stress. In the present study, there was no significant difference in terms of cortical area among housing systems, whereas FG broilers had significantly larger cortical area than SG broilers. These results suggest that heavier bones in FG broilers have larger cortical area due to their faster growth. However, similar movement possibilities of the chickens in experimental housing systems did not significantly affect the bone cortical development.

According to bone breaking strength, bone weight and cortical area results, it was clear that the tibiotarsi of FG broilers were heavier, thicker and these broilers had a higher bone breaking strength than SG broilers. Similarly, McDevitt et al. (28) also reported that the mean breaking strength of the tibiotarsus of FG broilers at 42 days was greater than that of the SG broilers. These indicate that tibiotarsi of FG broilers were more grown and more durable. However, when bending strength values were examined, it was observed that FG genotype groups had lower bending strength values than the SG genotype groups. These results suggest that the bone composition of FG broilers was poorer than that of SG broilers, because the bending strength means the highest load per unit area where the bone tissue resists before breaking (19). In addition, the results of breaking strength and bending strength indicated that FG genotype showed the best bone geometry but not the best bone composition due to fast growth. It is well known that while breaking strength indicates that the strength of whole bone depends on geometrical and material properties, bending strength of bone depends only on material properties.

The deflection value was not affected by genotype or housing systems. But when this value is evaluated by force it is obvious that FG broilers had stiffer bones than SG broilers. This situation may be explained by the stiffer bones take more force to produce a given deflection (14).

The experimental results showed that the FG genotype causes an increase approximately 30% in bone geometric and material properties and a decrease approximately 10% in mechanical material properties. Thus, bone load carrying capacity have been increased approximately 20% in total.

Several housing or rearing system effects on bone strength have been studied on laying hens. According to these studies, hens in conventional cages have the highest fracture rates and the weakest bone resistance compared to all other housing systems. In contrast, hens in cage-free or free-range systems show the best musculoskeletal health (42, 44, 59). Although those researchers reported that cage-free or free-range hens had better bone morphological and biomechanical properties, we found that housing system did not affect bone morphology or biomechanical properties. Consistent with our study Moyle et al. (30) reported that access to pasture or to the outdoors had no effect on the tibial bone strength on fast growing broilers. It is thought that skeletal adaptation to housing systems will be shaped as the age progresses and thus the effects on the bone can be observed (37, 39).

In conclusion, genotype has influenced structural and biomechanical properties of the tibiotarsus in broilers. Broilers with fast-growing genotype had better bone geometrical properties and stronger bones compare to the broilers had slow-growing genotype. There was no significant effect of housing systems on bone morphology and biomechanical properties. It is thought that the impact of the housing systems on the skeletal system will occur as the age progresses like in laying hens. Therefore, further studies are needed to examine the effects of housing systems on the bone in broilers.

Acknowledgement

Materials used in this study were obtained from the animals used in the thesis titled "Effects of different housing systems and genotypes on slaughter properties, carcass characteristics and meat quality in broiler chickens."

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Ankara Üniv Vet Fak Derg, **66**, 247-254, 2019
DOI: 10.33988/auvfd.442735

Hayvan hastanelerinde acil, veteriner klinik hizmetleri ve hasta sahibi memnuniyet kriterleri ölçekleri: Geçerlik ve güvenirlik çalışması

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Geliş tarihi: 11.07.2018- Kabul tarihi: 20.04.2019

Özet: Veteriner klinikleri ve hayvan hastanelerinin hasta sahiplerine ait beklentileri en üst düzeyde karşılamaları ve memnuniyet düzeylerini artırmaları hayvan sağlığı hizmeti veren kurumların kârlılık, verimlilik ve sürdürülebilirliğinde önemli bir rol oynamaktadır. Bu kapsamda çalışmada amaç, hasta sahiplerinin memnuniyeti konusunda bir ölçme aracı geliştirmek ve bu ölçme aracının geçerlilik ve güvenirlik çalışmasının yapılmasıdır. Çalışmada Hasta Sahibi Memnuniyet Kriterleri Ölçeği (HSMK-Ö), Hasta Sahibi Memnuniyet Değerlendirme Ölçeği-Acil (HSMD-A), Veteriner Klinik Hizmet Memnuniyeti Ölçeği (VKHM-Ö) ölçme araçlarının yapı geçerliliği yapılmıştır. Çalışmaya başlamadan önce yapılan güç analizi (%80.121 güçle) sonucu belirlenen, 212 hasta sahibine uygulanmıştır. Yapı geçerliliği için; güvenirlik analizlerinde Cronbach Alfa (α) katsayısı kullanılmıştır. Geçerlik analizi için faktör analizi uygulanmıştır. HSMK-Ö; HSMD-A; VKHM-Ö maddeleri Varimax döndürme yöntemi sonucunda tek faktörlü bir yapı olarak belirlenmiştir. Geliştirilen ölçeklerde HSMK-Ö'de 14 madde, HSMD-A'da 8 madde, VKHM-Ö'de 18 madde için hesaplanan Cronbach Alfa (α) güvenilirliği katsayıları sırasıyla 0.759; 0.936; 0.778 çıkmıştır. Açıklanan toplam varyans incelendiğinde HSMK-Ö ve VKHM-Ö'de 4, HSMD-A'da tek faktörün bulunduğu ve bu ölçülen özelliğin her ölçek için sırasıyla %58.06; %61.375; %70.39' unun ölçüldüğü söylenebilir. HSMK-Ö, HSMD-A ve VKHM-Ö ölçme araçlarının geçerli ve güvenilir olduğu söylenebilir. Türkiye'de yapılacak farklı örneklem gruplarındaki hasta sahibi memnuniyetine ait çalışmalarda kullanımının yaygınlaştırılması önerilmektedir.

Anahtar sözcükler: Geçerlik, güvenilirlik, hayvan hastanesi, hasta sahibi, memnuniyet, ölçek geliştirme.

Emergency, veterinary clinical services and patient owners satisfaction criteria scales in animal hospitals: Validity and reliability study

Abstract: Veterinary clinics and animal hospitals need to be able to meet patient expectations at the highest level and increase their level of satisfaction. It plays an important role in the profitability, productivity and sustainability of animal health service providers. In this context, the aim of the study is to develop a measurement tool about the satisfaction of patient owners and to carry out the validity and reliability study of this measurement tool. The construct validity of the Patient Ownership Satisfaction Criteria Scale (HSMK-Ö), Patient Ownership Satisfaction Rating Scale (HSMD-A) and Veterinary Clinical Satisfaction Scale (VKHM-Ö). The power analysis (80.121% strength) performed prior to starting the study was applied to 212 patients whose outcome was determined. To ensure structural validity; Cronbach alpha (α) coefficient was used in reliability analyzes. Factor analysis was applied for validity analysis. HSMK-Ö; HSMD-A; VKHM-Ö items were identified as a one-factor structure as a result of the Varimax rotation method. In the developed scales, the cronbach alpha (α) reliability coefficients calculated for 14 items in HSMK-E, 8 items in HSMD-A and 18 items in VKHM-Ö were 0.759; 0.936; 0.778. When the total variance explained, there is only one factor in HSMK-Ö and VKHM-Ö in HSMD-A, and this measured value is 58.06% for each scale; 61.375%; 70.39% can be said to be measured. It can be said that HSMK-Ö, HSMD-A and VKHM-Ö measuring instruments are valid and reliable. Expanding the utilization of of satisfaction studies in patients with different sample groups is proposed to be held in Turkey.

Keywords: Animal hospital, patient ownership, reliability, satisfaction, scale development, validity.

Giriş

Veteriner hekimlik mesleği geçmişten günümüze hayvanların tedavi edilmesi ve iyileştirilmesinde, sürdürülebilir hayvansal üretim ile hayvansal gıda üretiminin güvence altına alınmasında, halk sağlığının korunmasında ve kırsal kalkınmanın dengeli bir şekilde gerçekleşmesinde önemli fonksiyonları üstlenmiş bir konumdadır (5). Veteriner hekimlik, istihdam alanının genişliği ve son yıllarda işsizlik oranının en düşük meslek grupları arasında olması gibi önemli avantajlara sahiptir (20). Veteriner hekimliğin istihdamında ilk sırada klinisyen veteriner hekimlik gelmektedir. Günümüz şehirleşme oranının artması ve yaşam tarzındaki değişiklere bağlı olarak pet hayvanları kentleşen toplumların bir parçası haline gelmektedir (2). Artan pet hayvanı sayısına bağlı olarak bu alt sektöre yönelik hizmet üreten ve hayvan sağlığını koruma hizmeti sunan veteriner klinik, poliklinik ile hayvan hastanelerinin sayısı son yıllarda hızla artmaktadır (1). Bünyesinde veteriner fakültesi bulunan üniversitelerin hayvan hastaneleri uzman personel ile nitelikli örnek bir veteriner hekimlik hizmeti sunulan, aynı zamanda uygulamalı eğitim veren kurumların başında gelmektedir. Bu birimler eğitim vermenin yanı sıra ilerde bu alanda çalışmayı düşünen veteriner hekim adaylarına örnek bir yapı oluşturmaları bakımından da önemlidir. Klinisyen veteriner hekimlerin mesleki bilgi yanında, müşteri ilişkileri, hizmet kalitesi yöntemleri, hasta kayıt sistemleri ve hasta takibi gibi konularda gerekli yeterliliklere sahip olmaları gerekmektedir. Ayrıca günümüzde hasta sahibi memnuniyeti konuları artan rekabet ortamında giderek önem kazanmaktadır. Veteriner klinikleri ve hayvan hastanelerinde kaliteli bir hizmetin önemli göstergelerinden birisi de hasta sahiplerinin memnuniyet düzeyidir. Memnuniyet düzeyinin bilinmesi ve periyodik olarak takip edilmesi, sunulmakta olan veteriner hekimlik hizmetlerinin iyileştirilmesine, hasta sahiplerinin beklentilerine yönelik niteliğin artırılması bakımından günümüzde önemli olup, öneminin yakın gelecekte de artacağı söylenebilir (9, 16).

Konuyla ilgili insan sağlığına yönelik verilen hastane hizmetleri ile ilgili ölçek çalışmaları sıklıkla karşımıza çıkmakta iken (9, 16, 22), veteriner klinikleri ve hayvan hastanelerinde hasta memnuniyeti ile Türkiye'de yapılmış sınırlı sayıda çalışma olup, ölçek geliştirilen bir çalışmaya rastlanmamıştır (3, 6).

Bu çalışma, Selçuk Üniversitesi Veteriner Fakültesi Hayvan Hastanesi'nden hayvan sağlığı hizmeti alan hasta sahiplerinin memnuniyetini ve beklentilerini ölçmek, bu konu ile ilgili bir ölçek geliştirmesine öncülük etmek ve geliştirilen ölçeğin ilerde bu alanda yapılacak çalışmalarda kullanılabilir olması amaçlarıyla yapılmıştır.

Materyal ve Metot

Bu çalışmada Selçuk Üniversitesi Veteriner Fakültesi Hayvan Hastanesi'nden hayvan sağlığı hizmeti alan hasta sahipleri ile yapılan yüz yüze görüşmede uygulanan formlardan elde edilen bilgiler araştırmanın materyalini oluşturmuştur.

Araştırmanın örneklem büyüklüğü, çalışmaya başlamadan önce yapılan güç analizi (priori power analysis) % 80.21 ile belirlenmiş olup, 2017 yılında Nisan-Aralık aylarında Selçuk Üniversitesi Veteriner Fakültesi Hayvan Hastanesi'ne gelen 212 adet hasta sahiplerine uygulanan formlardan oluşturulmuştur. Uygulanan formlarla hasta sahiplerinin memnuniyet düzeylerinin ölçülmesi için üç adet ölçek geliştirilmiştir. Bu ölçekler: 1. Hasta Sahibi Memnuniyet Kriterleri Ölçeği (HSMK-Ö) (14 Madde), bu ölçekle hasta sahibinin memnuniyet kriterlerinin neler olduğunun belirlenmesi, 2.Hasta Sahibi Memnuniyet Değerlendirme Ölçeği-Acil (HSMD-A) (8 Madde) olup, bu ölçekle hasta sahiplerinin veteriner acil klinikten aldıkları hizmetten memnuniyet düzeyinin tespit edilmesi, 3.Veteriner Klinik Hizmet Memnuniyeti Ölçeği (VKHM-Ö) (18 Madde) olup, hasta sahiplerinin hayvan hastanesi polikliniklerinden almış oldukları veteriner hizmetlerinden ne düzeyde memnun olduklarının belirlenmesi amaçlanmıştır. Ölçek maddeleri hazırlanırken DELPHI süreci ile yazarlar tarafından madde havuzu oluşturulmuştur. Sonraki aşamada hazırlanan form, 3 adet uzman tarafından değerlendirilmiş ve nihai form oluşturulmuştur. Hazırlanan soru formlarında negatif ifade bulunmamaktadır. Ölçek maddeleri 1 ile 5 arasında belirlenen Likert tipi ölçek skorlaması ile belirlenmiştir. Ölçeklerin geçerlik ve güvenilirlikleri test edilmiştir.

Ölçeklerin yapı geçerliliği için; güvenirlik analizlerinde Cronbach Alfa (α) katsayısı kullanılmıştır. Geçerlik analizi için her ölçeğe ayrı ayrı faktör analizi uygulanmıştır. Faktör analizine uygunluk Bartlett's küresellik testi, örneklem sayısının yeterliliğinde Kaiser-meyer-olkin örneklem yeterliliği istatistiği ile değerlendirilmiştir. Geliştirilen ölçeklerin maddeleri Varimax döndürme yöntemi sonucunda hasta sahibi memnuniyet kriterleri ve veteriner klinik hizmet memnuniyeti ölçeklerinde dört faktörlü, hasta sahibi memnuniyet değerlendirme ölçeği-acilde tek faktörlü bir yapı olarak belirlenmiştir. Çok faktörlü yapının belirlenmesi için Varimax döndürme yöntemi kullanılmaktadır. Varimax yönteminde basit yapıya ve anlamlı faktörlere ulaşmada faktör yükleri matrisinin sütunlarına öncelik verilir. Varimax yönteminde daha az değişkenle faktör varyanslarının maximum olması sağlanacak şekilde döndürme yapılır (13). Ölçeklerin toplanabilirliliği ise Tukey toplanabilirlik testi ile değerlendirilmiştir (7, 8, 15). Verilerin değerlendirilmesinde SPPS 22 istatistik paket programı kullanılmıştır (12).

Bulgular

Selçuk Üniversitesi Veteriner Fakültesi Hayvan Hastanesi'ne gelen hasta sahiplerine tek seferde 3 farklı form uygulanmıştır. Bunlardan ilki; Tablo 1'de hasta sahibinin alacağı veteriner hekimlik ve hastane hizmetlerinden memnun olabilmesi için hangi kriterlere dikkat ettiğinin belirlenmesine yönelik sorular sorulmuş ve geliştirilen Hasta Sahibi Memnuniyet Kriterleri Ölçeği'nin (HSMK-Ö) soru bazlı güvenilirlik katsayıları verilmiştir. İkinci hazırlanan form; hasta sahiplerinden hayvan hastanesi acil kliniğe gelenlere, acil klinikten almış oldukları hizmetin yeterlilik durumuna ilişkin formda acil klinik için hasta sahibi memnuniyet değerlendirme ölçeği (HSMD-A) geliştirilmiştir. Üçüncü formda yer alan maddeler; hasta sahiplerinden hayvan hastanesine gelenlere, klinikten almış oldukları veteriner hekimlik hizmetin yeterlilik durumunun tespiti amacıyla veteriner klinik hizmet memnuniyeti ölçeği (VKHM-Ö) geliştirilmiştir. Geliştirilen ölçeklerin soru bazlı güvenilirlik katsayıları Tablo 1'de sunulmuştur.

Tablo 1. Ölçeklerin soru bazlı güvenirlik katsayıları.
Table 1. Question-based reliability coefficients of scales.

	Ölçekten madde silinirse geçerli olacak ortalama	**Ölçekten madde silinirse geçerli olacak varyans**	**Madde toplam korelasyonları**	**Ölçekten madde silinirse geçerli olacak güvenirlik katsayısı (Cronbach α)**
Hasta sahibi memnuniyet kriterleri ölçeği (HSMK-Ö)				
Hastaneye ulaşım imkanları	54.27	31.54	0.25	0.76
Muayene öncesi ve sonrası bilgilendirme	54.07	31.84	0.36	0.75
Muayene için beklenen süre	54.30	31.47	0.34	0.75
Kayıt işlemlerinin kolay ve hızlı olması	54.36	30.67	0.40	0.74
Hastane içi temizlik	54.11	30.98	0.38	0.74
Hastane ortamının gürültüsüz olması	55.05	28.87	0.39	0.74
Yardımcı personelin davranışları	54.40	29.48	0.56	0.73
Tıbbi personelin davranışları	54.11	31.25	0.51	0.74
Tıbbi hizmetin kalitesi	54.05	31.52	0.36	0.75
Telefon vb iletişim araçları ve faydalanma	55.04	27.28	0.56	0.72
Kantin hizmetleri	55.66	28.83	0.35	0.75
Ambulans hizmetleri	54.88	28.64	0.38	0.75
Fiyatların bütçeye uygunluğu	54.28	30.89	0.36	0.75
Randevulu sisteme geçilmesi	54.88	30.86	0.23	0.76
Hasta sahibi memnuniyet değerlendirme ölçeği-Acil (HSMD-A)				
Acile ulaşım kolaydı	26.27	53.679	0.676	0.934
Acilde ilgili personelin karşılaması hızlıydı	26.39	49.748	0.886	0.920
İlk müdahale hızlıydı	26.34	49.542	0.878	0.921
Gerekli ilgi gösterildi	26.28	49.302	0.865	0.921
Acilde uzman personele kolay ulaşıldı	26.53	48.718	0.820	0.924
Acilde ilk aşamadaki teşhis ve tedavi hızlıydı	26.58	50.795	0.726	0.931
Acil fiyatı bütçeye uygundu	26.80	50.739	0.673	0.936
Ambulans hizmetleri yeterliydi	27.27	48.885	0.725	0.933
Veteriner klinik hizmet memnuniyeti ölçeği (VKHM-Ö)				
Hastaneye ulaşım kolaydı	59.25	93.27	0.28	0.77
Poliklinik kayıt işlemleri hızlı ve eksiksizdi	59.35	90.37	0.42	0.77
Bekleme salonu temiz ve oturmak için uygundu	60.27	82.73	0.55	0.75
Yardımcı personel saygılı ve ilgiliydi	59.36	88.00	0.50	0.76
Hastane içi aşırı gürültülüydü	60.24	96.60	0.03	0.79
Hastane iç düzeni yeterli	60.06	85.84	0.50	0.76
Muayene öncesi uzun süre bekletildim	60.25	99.15	-0.07	0.80
Muayene hastane dışı ortamda yapıldı	61.53	87.36	0.38	0.77
Muayene odası temiz ve düzenliydi	59.85	85.47	0.55	0.75
Muayene odasındaki görevlinin ilgisi yeterliydi	59.35	87.23	0.58	0.75
Muayene eden hekimin ilgisi yeterliydi	59.19	88.43	0.52	0.76
Hekim tarafından verilen tıbbi hizmet yeterliydi	59.27	88.40	0.53	0.76
Muayene süresi gereğinden fazla uzun ve sıkıcıydı	60.54	96.79	0.02	0.80
Gereğinden fazla ve detaylı muayene yapıldı	60.82	89.59	0.31	0.77
Muayene sonrası hekimin ilgisi ve verilen bilgiler yeterliydi	59.32	88.51	0.52	0.76
Operasyon öncesi ve sonrası verilen bilgiler yeterliydi	59.48	86.96	0.58	0.75
Masraflar hakkında önceden yeterli bilgi verildi	59.96	90.09	0.28	0.78
Tüm masraflar uygundu	60.06	88.50	0.35	0.77

HSMK-Ö'de Cronbach Alfa (α) değeri en düşük 0.72 bulunmuştur. Bu nedenle hiçbir soru formdan çıkarılmamıştır. Uygulamada kullanılan 14 soru için hesaplanan Cronbach Alfa (α) güvenilirliği katsayısı 0.759 hesaplanmıştır. HSMD-A ölçeğinde kullanılan 8 soru için hesaplanan Cronbach Alfa (α) güvenilirliği katsayısı 0.936 çıkmıştır. Bu sebeple hiç madde çıkarılmamıştır. VKHM-Ö'de uygulamada kullanılan 18 madde için hesaplanan Cronbach Alfa (α) güvenilirliği katsayısı 0.778 çıkmıştır bu sebeple hiçbir madde çıkarılmamıştır. Özetle geliştirilen her üç ölçekte Cronbach Alfa (α) güvenilirliği katsayının 0,70'in üstünde çıkması sebebiyle formların kullanımının oldukça uygun olduğu söylenebilir.

Ölçeklerin geçerliliğini belirlemek amacıyla sorular üzerinde Varimax yöntemiyle faktör analizi yapılmış ve bulguları Tablo 2'de gösterilmiştir. Açıklanan toplam varyans incelendiğinde HSMK-Ö'de soruların 4 faktör altında toplandığı ve bu 4 faktörlü ölçme aracı ile ölçülen özelliğin %58.06'sının açıklandığı belirlenmiştir. Kaiser-meyer-olkin örneklem yeterliliği istatistiği 0.77; Bartlett's küresellik testini ki kare değeri 650.224 hesaplanmıştır. HSMD-A'da açıklanan toplam varyans incelendiğinde 8 sorunun tek faktörde toplandığı ve tek faktörlü ölçme aracı ile ölçülen özelliğin %70.39'unun açıklandığı görülmüş olup; Kaiser-meyer-olkin örneklem yeterliliği istatistiği 0.912; Bartlett's küresellik testini ki kare değeri 504.377 hesaplanmıştır. VKHM-Ö'de toplam varyans incelendiğinde 18 sorunun 4 faktör altında toplandığı ve bu 4 faktörlü ölçme aracı ile ölçülen özelliğin %61.38'nin açıklandığı görülmüştür. VHKM-Ö için Kaiser-meyer-olkin örneklem yeterliliği istatistiği 0.854; Bartlett's küresellik testini ki kare değeri 1410.583 olarak hesaplanmıştır. Bu alandaki çalışmalarda toplam açıklanan varyansın en az % 55 olması yeterli olduğu bildirilmektedir (10,13). Üç ölçeğinde verilerinin faktör analizine uygun olduğu söylenebilir($P<0.05$). Genel olarak faktör analizi sonuçlarına göre ölçme aracının yapı geçerliliğinin sağlandığı söylenebilir.

HSMK-Ö'de faktörlere göre soruların dağılımı, faktör yükü tablosunda gösterilmiştir ve faktörler Tablo 3'teki gibi isimlendirilmiştir. Tablo 3 incelendiğinde hasta sahibi memnuniyet kriterleri ölçeği soruları 4 faktör altında toplanmıştır. Bu faktörler sırası ile Hizmet Kalitesi, Altyapı İmkânları, Harcanan Zaman ve Erişilebilirlik olarak adlandırılmıştır.

VKHM-Ö'de faktörlere göre soruların dağılımı aşağıdaki faktörü yükü tablosunda gösterilmiş ve faktörler Tablo 4'te adlandırılmıştır. Tukey toplanabilirlik testine göre HSMK-Ö, HSMD-A, VKHM-Ö ölçeklerin toplanarak bir ölçek toplam puanı elde edilmesi için Anova Tukey ölçeğin toplanabilirliği testi uygulanmıştır ve ölçeğin toplanarak bir ölçek toplam puanı elde edilmesi için uygun olduğu sonucuna varılmıştır ($P>0.001$).

Hasta Sahibi Memnuniyet Kriterleri, Hasta Sahibi Memnuniyet Değerlendirme-Acil, Veteriner Klinik Hizmet Memnuniyeti ölçeklerinde yer alan faktörlerin minimum-maksimum puanları, ortalama ve standart sapmaları Tablo 5'te verilmiştir. HSMK-Ö'den minimum 14 maksimum ise 70 puan alınmaktadır. HSMK-Ö'de Hizmet Kalitesi faktörü için minimum 6; maksimum 30 puan, Alt Yapı İmkanları faktörü için minimum 4; maksimum 20 puan, Harcanan Zaman faktörü ve Erişebilirlik için minimum 2; maksimum 10 puan alınabilmektedir. VKHM-Ö'den

Tablo 2. Faktör yüklerinin kareler toplamı HSMK-Ö, HSMD-A, VKHM-Ö.
Table 2. Sum of squares of total variance explained HSMK-Ö, HSMD-A, VKHM-Ö.

Faktör	Varimax döndürme sonucu faktör yüklerinin kareleri toplamı		
	Toplam	Açıklanan varyans %	Birkimli varyans %
Hasta sahibi memnuniyet kriterleri ölçeği (HSMK-Ö)#			
1	2.80	20.00	20.00
2	1.97	14.09	34.09
3	1.87	13.33	47.42
4	1.49	10.64	58.06
Hasta sahibi memnuniyet değerlendirme ölçeği-Acil (HSMD-A)##			
1	5.63	70.39	70.39
Veteriner klinik hizmet memnuniyeti ölçeği (VKHM-Ö)###			
1	3.789	21.052	21.052
2	3.207	17.818	38.870
3	2.447	13.595	52.464
4	1.604	8.911	61.375

#(Kaiser-meyer-olkin örneklem yeterliliği: 0.771; Bartlett's küresellik testini ki kare değeri 650.244; Serbestik derecesi 91 P=0.0001), ##(Kaiser-meyer-olkin örneklem yeterliliği: 0.912; Bartlett's küresellik testini ki kare değeri 504.377; Serbestik derecesi 28 P=0.0001) ### (Kaiser-meyer-olkin örneklem yeterliliği: 0.854; Bartlett's küresellik testini ki kare değeri 1410.583; Serbestik derecesi 153 P=0.0001)

Tablo 3. "Hasta sahibi memnuniyet kriterleri ölçeği" formu sorularının faktörlere dağılımı.
Table 3. Component of "Patient ownership satisfaction criteria scale" form questions to factors.

	Faktörler			
	1 (Hizmet kalitesi)	**2 (Altyapı/sosyal imkanlar)**	**3 (Harcanan zaman)**	**4 (Erişilebilirlik)**
Hastaneye ulaşım imkanları (4)				0.693
Muayene öncesi ve sonrası bilgilendirme (1)	0.503			
Muayane için beklenen süre (3)			0.779	
Kayıt işlemlerinin kolay ve hızlı olması (3)			0.823	
Hastane içi temizlik (1)	0.679			
Hastane ortamının gürültüsüz olması (1)	0.428			
Yardımcı personelin davranışları (1)	0.673			
Tıbbi personelin davranışları (1)	0.797			
Tıbbi hizmetin kalitesi (1)	0.712			
Telefon vb iletişim araçları ve faydalanma (2)		0.455		
Kantin hizmetleri (2)		0.744		
Ambulans hizmetleri (2)		0.556		
Fiyatların bütçeye uygunluğu (4)				0.636
Randevulu sisteme geçilmesi (2)		0.671		

Tablo 4. "Veteriner klinik hizmet memnuniyeti ölçeği (VKHM-Ö)" formu sorularının faktörlere dağılımı.
Table 4. Component of "Veterinary clinical satisfaction scale" form questions to factors.

	Faktörler			
	1 (Personel)	**2 (Beklenti)**	**3 (Gözetim)**	**4 (Ücretlendirme)**
Hastaneye ulaşım kolaydı		0.444		
Poliklinik kayıt işlemleri hızlı ve eksiksizdi		0.549		
Bekleme salonu temiz ve oturmak için uygundu		0.826		
Yardımcı personel saygılı ve ilgiliydi	0.503			
Hastane içi aşırı gürültülüydü			0.692	
Hastane iç düzeni yeterli		0.688		
Muayene öncesi uzun süre bekletildim			0.677	
Muayene hastane dışı ortamda yapıldı			0.653	
Muayene odası temiz ve düzenliydi		0.676		
Muayene odasındaki görevlinin ilgisi yeterliydi	0.709			
Muayene eden hekimin ilgisi yeterliydi	0.856			
Hekim tarafından verilen tıbbi hizmet yeterliydi	0.823			
Muayene süresi gereğinden fazla uzun ve sıkıcıydı			0.778	
Gereğinden fazla ve detaylı muayene yapıldı			0.625	
Muayene sonrası hekimin ilgisi ve verilen bilgiler yeterliydi	0.855			
Operasyon öncesi ve sonrası verilen bilgiler yeterliydi	0.610			
Masraflar hakkında önceden yeterli bilgi verildi				0.786
Tüm masraflar uygundu				0.748

minimum 18; maksimum 90 puan alınmaktadır. VKHM-Ö'de Personel faktörü için minimum 6, maksimum 30 puan, Beklenti ve Gözetim faktörleri için minimum 5, maksimum 25, Ücretlendirme faktörü için minimum 2, maksimum 10 puan alınabilmektedir.

Hasta Sahibi Memnuniyet Kriterleri-Ölçeği'ne bakıldığında Hizmet kalitesi faktöründen toplam 26.56±2.82; Altyapı imkanları faktöründen 14.43±2.98; Harcanan zaman faktöründen 8.73±1.25; Erişilebilirlik faktöründen 8.88±1.24 puan alınmıştır. Hasta sahiplerinin ortanın üzerinde bir memnuniyet kriterinin olduğu anlaşılmıştır. HSMD-A Ölçeğinden toplamda 31.03±6.72 puan alınmıştır. Veteriner Klinik Hizmet Memnuniyeti Ölçeği incelendiğinde ise Personel faktörü için 24.54±4.96, Beklenti faktörü için 11.45±2.53, Gözetim faktörü için 13.61±4.75, Ücretlendirme faktörü için 6.78±2.24, toplam ölçek puanı için minimum 18 maksimum 90 puan olarak belirlenmiştir. Toplamda ise 62.52±10.13 puan olarak belirlenmiştir.

Tablo 5. Faktörlere göre ölçeklerin tanıtıcı istatistikleri.
Table 5. Descriptives statistics of factor scores.

Faktör Adı	N	Minimum	Maksimum	Ortalama	Standart sapma
Hasta sahibi memnuniyet kriterleri ölçeği					
Hizmet kalitesi	207	15.00	30.00	26.56	2.82
Alt yapı imkanları	203	7.00	20.00	14.43	2.98
Harcanan zaman	210	4.00	10.00	8.73	1.25
Erişebilirlik	212	2.00	10.00	8.88	1.24
HSMK-Ö toplam	212	41	70	58.3	5.97
Hasta sahibi memnuniyet değerlendirme ölçeği-Acil					
Acil	72	12.00	40.00	31.03	6.72
Veteriner klinik hizmet memnuniyeti ölçeği					
Personel	193	9.00	30.00	24.54	4.96
Beklenti	206	4.00	15.00	11.45	2.53
Gözetim	195	5.00	25.00	13.61	4.75
Ücretlendirme	204	2.00	10.00	6.78	2.24
VKHM-Ö toplam	207	27.00	90.00	62.52	10.13

Tartışma ve Sonuç

Çalışmada hasta sahiplerine yöneltilen formlarda güvenilirlik ve geçerlilik analizleri yapılmış, veteriner kliniklerinde ve hayvan hastanelerinde hasta sahiplerinin memnuniyetlerinin ölçülmesine imkan veren üç ayrı ölçek geliştirilmiştir. Bu geliştirilen ölçekler KMO örneklem yeterliliği katsayısı, Cronbach Alfa (α) katsayısı ve faktörlerin birikimli varyansları bakımından ele alınarak yapılan araştırmalar ile değerlendirilmiştir.

Bir veri setine faktör analizi yapılıp yapılamayacağının önemli göstergelerinden birisi de değişkenler arası korelasyonun önemliliğinin yeteri sayıda olmasıdır. Bu yeterliliğin değerlendirilmesinde Kaiser Meyer Olkin (KMO) ölçüsü dikkate alınmaktadır (16). Faktör analizin uygun olması için KMO testinin 0.60'dan yüksek olması gereklidir (3, 19). Bu çalışmada veri setinin KMO istatistiği HSMK-Ö için 0.771, HSMS-A için 0.912, VKHM-Ö için 0.854 olarak bulunmuştur ve bu sonuçlar veri setinin faktör analizine uygun olduğunu göstermektedir. Hasta memnuniyetine etkili faktörlerin belirlenmesine yönelik bir çalışmada (16), KMO 0.936, sağlık alanında hizmet kalitesine yönelik bir çalışmada (22), 0.945, eğitim alanında yapılan bir çalışmada (18), 0.93, spor alanında ölçek uyarlaması yapılan bir araştırmada (17), 0.87, sosyal bilimlerde yapılan bir geçerlilik güvenilirlik araştırmasında (19), 0.80, başka bir geçerlik güvenilirlik çalışmasında (23), 0.942, kariyer alanında ölçek geliştirilen başka bir çalışmada (21), 0.87 olarak bulunmuştur.

Çalışmada geliştirilen birinci ölçek olan HSMK-Ö'ye ilişkin toplam güvenirlik katsayısı 0.759, ikinci ölçek olan HSMD-A için 0.936, üçüncü ölçek olan VKHM-Ö için 0.778 olarak bulunmuştur. Sağlık kurumlarında hizmet memnuniyeti ölçen bir çalışmada (9), güvenilirlik katsayısı 0.968, hasta memnuniyetini inceleyen Türkiye'de altı ilde gerçekleştirilen bir çalışmada (16), 0.945, hastanelerde hizmet kalitesinin ölçüne yönelik bir araştırmada (22), 0.960 olarak bulunmuştur. Sağlık alanında memnuniyet inceleyen çalışmaların araştırma kapsamında geliştirilen HSMD-A ölçeğinin güvenilirlik katsayıları benzer bulunmuştur. Müşteri memnuniyetine yönelik gerçekleştirilen bir çalışmada beş adet ölçek geliştirilmiş olup ölçeklerin güvenilirlik katsayıları 0.74-0.88 arasında bulunmuştur (10). Eğitim hizmetlerinde kalite ölçümünde 6 ölçekli bir çalışmada güvenilirlik katsayıları 0.68-0.91 arasında bulunmuştur (18). Sporda güdülenmeyi ölçeği ile ilgili bir çalışmada güvenilirlik katsayıları 0.72-0.76 arasında bulunmuştur (17). Sosyal bilimler alanında yapılan başka bir çalışmada ise 0.83 olarak bulunmuştur (19). Araştırma kapsamında elde edilen güvenilirlik katsayıları hasta memnuniyeti ölçen ve sağlık alanında ölçek geliştirilen çalışmalar ile HSMD-A ölçeği benzer bulunurken diğer HSMK-Ö ve VKHM-Ö ölçeklerinde biraz daha düşük olduğu belirlenmiş, bu iki ölçek bulgularının sosyal alanda gerçekleştirilen araştırmaların güvenilirlik katsayılarına benzer bulunmuştur. Bu durum veteriner kliniklerinde verilen hizmetin sosyal bir yönünün de olduğunu ortaya koymaktadır. Veteriner klinikleri ve hayvan hastanelerinin gerek planlama gerek uygulama aşamasında bunu göz ardı etmemelerini ortaya koymaktadır.

HSMK-Ö'de 14 madde 4 faktör altında toplanmış ve faktörlerin birikimli varyansı %58.06, HSMD-A 8 madde tek faktör altında toplanmış birikimli varyans %70.39, VKHM-Ö 18 madde 4 faktör altında toplanmış birikimli varyans %61.37 olarak hesaplanmıştır. Hasta memnuniyetini etkileyen faktörlerin belirlenmesine yönelik bir araştırmada 27 madde 5 faktör altında toplanmış,

açıklayıcılığı %57.64 bulunmuştur (16). Sağlıkta hizmet kalitesinin ölçümü ile ilgili bir çalışmada 33 madde 4 faktör altında toplanmış açıklayıcılığı her faktör için ayrı ayrı verilmiştir (22). Pazarlama alanında bir çalışmada 18 madde 6 faktör altında toplanmış birikimli varyans %74.55 bulunmuştur (10). Yapılan bir ölçek uyarlaması çalışmasında altı faktörlü ölçeğin açıklayıcı varyansının %66 olduğu bildirilmiştir (17). Sosyal bilimlerde yapılan geçerlilik güvenilirlik araştırmasında üç faktörlü ölçeğin açıklayıcı varyansının %58 olduğu belirlenmiştir (19). Başka bir geçerlilik güvenirlik çalışmasında 34 maddenin 3 faktör altında toplandığı açıklanan varyansın her faktör için sırasıyla %26.86, %14.12 ve %6.92 olduğu belirlenmiştir (23). Kariyer ile ilgili yapılan bir ölçek geliştirmesi çalışmasında 18 maddenin 4 faktör altında toplandığı ve açıklayıcı varyansın %47 olduğu belirlenmiştir (21).

Açıklanan varyansın çalışma alt alanları itibariyle farklı referans değerleri kabul ettiği bilinmektedir. Yapılan çalışmalarda davranış bilimleri açısından açıklanan varyans miktarının %40 olması yeterli iken, sosyal bilimlerde %55 ve üzeri değerler kabul görmektedir (11, 14). Çalışma kapsamında geliştirilen üç adet ölçeğin açıklayıcı varyanslarının kabul edilebilir seviyelerden yüksek olduğu ve diğer çalışmalarla da uyum gösterdiği söylenebilir.

Araştırma kapsamında faktör analizinin sonuçlarının genel değerlendirilmesinde, çalışma kapsamında geliştirilen bu ölçeklerin hasta sahiplerine uygulanması sonucu ölçeklerin her birinin toplam puanı hesaplanmıştır. Yapılan puanlamalar sonucunda hasta sahiplerinin fakülte hayvan hastanesinden almış oldukları hizmetten orta ve ortanın üstü düzeyde memnun oldukları belirlenmiştir. Hasta sahiplerinin memnuniyetini belirleyen kriterlerindeki puanlama değerlendirildiğinde, fakülte hayvan hastanesini tercih edenlerin öncelikli olarak alacağı tıbbi hizmetin kalitesini göz önünde bulundurdukları anlaşılmıştır. Bu durum polikliniklerden alınan hizmetlerin skorlamasına da yansımış, personel faktörü maksimum 30 puan üzerinden 24.54 gibi yüksek bir skor almıştır. Benzer şekilde beklenti faktörü de 15 üzerinde 11.45 puan almıştır. Yapılan faktör analizi ve skorlaması neticesinde, gözetim ve ücretlendirme faktörleri altındaki maddelerde sağlanacak iyileşmelerin hasta sahiplerinin toplam memnuniyet düzeylerinin artmasına imkân sağlayabileceği tespit edilmiştir. Ayrıca bu ölçekler serbest çalışan veteriner klinikleri ve hayvan hastaneleri tarafından da uygulanıp, kendi müşteri memnuniyetlerinin hangi düzeyde olduğunun tespitine de olanak sağlayacaktır. Böylelikle gerek kamuya ait gerekse girişimci veteriner hekimlere ait veteriner klinik ve hayvan hastaneleri müşteri memnuniyetinde hangi noktalarda düşük veya yüksek puan aldıklarını tespit edip ve sundukları hizmette ona göre bir iyileşme sağlayabileceklerdir. Gelecekte yapılması planlanan benzer ölçek geliştirme, geçerlilik güvenilirlik çalışmalarına bu araştırma referans bir çalışma olacaktır.

Bu çalışma ile hem araştırma kapsamında incelenen Selçuk Üniversitesi Veteriner Fakültesi Hayvan Hastanesi değerlendirilmiş hem de bu sektörde var olan benzer pet klinikleri ve hayvan hastanelerinde hasta memnuniyeti ve hizmet kalitesi açısından değerlendirme ve iyileştirmeler ön plana alınacak faktörler bilimsel olarak ortaya konulmuştur.

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Ankara Üniv Vet Fak Derg, **66**, 255-260, 2019
DOI: 10.33988/auvfd.456594

Molecular survey of *Anaplasma* and *Ehrlichia* species in cattle from Karaman of Turkey, including a novel tandem report of *Anaplasma marginale* msp1a gene

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Received date: 31.08.2018 - Accepted date: 06.05.2019

Abstract: Tick-borne pathogens cause serious health problems and loss of productivity in domesticated and wild animals. A molecular study was performed to detect the frequency of infection with *Anaplasma*/*Ehrlichia* (A/E) in cattle from Karaman province of Turkey. Venous blood samples were taken from 150 apparently healthy cattle in 2016. After amplification the hypervariable V1 region of the 16S rRNA gene of A/E species, a reverse line blot (RLB) assay was performed using species-specific probes. Since some samples gave signal only to A/E catch-all probe, the samples analyzed in terms of major surface proteins (MSPs) of *Anaplasma marginale*. Genetic diversity and tandem repeat analysis were made for msp1α gene sequences of *A. marginale*. *Anaplasma*-like bodies were detected in four (2.66%) animals via microscopic examination. *Anaplasma centrale* was detected in eight (5.33%) animals via RLB. When the samples were examined in terms of *A. marginale* msp1a gene with semi-nested PCR, a total of nine (6.00%) animals [six of them (4.00%) were positive for *A. centrale* with RLB] were found to be infected with *A. marginale*. In addition, the sequences of MSP1a amplicons revealed one new tandem repeat (Tr70). According to these results, it was determined that *A. marginale* and *A. centrale* were found in cattle in Karaman province and this study provided the first evidence of genetic diversity of *A. marginale* with one new tandem repeat in cattle in the region.
Keywords: *A. centrale, A. marginale,* cattle, Karaman, tandem repeat.

Anaplasma marginale msp1a geninin yeni bir tandem raporunu da içeren, Türkiye'nin Karaman yöresindeki sığırlarda *Anaplasma* ve *Ehrlichia* türlerinin moleküler araştırması

Özet: Kene kaynaklı patojenler evcil ve yabani hayvanlarda ciddi sağlık problemlerine ve verim kaybına neden olur. Karaman ilindeki sığırlarda *Anaplasma* / *Ehrlichia* (A/E) ile enfeksiyon sıklığını saptamak için moleküler bir çalışma yapıldı. Venöz kan numuneleri görünüşte sağlıklı olan 150 sığırdan 2016 yılında alınmıştır. A/E türlerinin 16S rRNA geninin değişken V1 bölgesi amplifiye edildikten sonra tür spesifik problar kullanılarak reverse line blot (RLB) deneyi gerçekleştirilmiştir. Bazı örnekler sadece A/E probuna sinyal verdiğinden, örnekler *Anaplasma marginale*'nin major surface proteinleri (MSPs) açısından analiz edilmiştir. *A. marginale*'nin msp1α gen dizileri için genetik çeşitlilik ve tandem tekrar analizi yapıldı. Mikroskobik inceleme ile dört (%2.66) hayvanda *Anaplasma* benzeri cisimler tespit edildi. *Anaplasma centrale* RLB ile sekiz (%5.33) hayvanda tespit edildi. Örnekler semi-nested PZR ile *A. marginale* msp1a geni açısından incelediğinde, toplam dokuz (%6.00) hayvanın [6'sı (%4.00) RLB ile *A. centrale* açısından pozitif olan] *A. marginale* ile enfekte olduğu bulunmuştur. Ek olarak, MSP1a amplikonlarının dizileri bir tane yeni tandem tekrarı (Tr70) ortaya çıkardı. Bu sonuçlara göre Karaman ilindeki sığırlarda *A. marginale* ve *A. centrale*'nin bulunduğu tespit edilmiş ve bu çalışma bölgedeki sığırlarda bir yeni tandem tekrarı ile *A. marginale*'nin genetik çeşitliliğinin ilk kanıtını sağlamıştır.
Anahtar sözcükler: *A. centrale, A. marginale,* Karaman, sığır, tandem tekrarı.

Introduction

Tick-borne diseases (TBDs) poses a great impact for animal and human health in tropical and subtropical climatic regions including Turkey. Turkey has a grand potential for animal breeding and livestock population comprises 14 million cattle, 29 million sheep and 9 million goats. Since TBDs (e.g. theileriosis, babesiosis and anaplasmosis) cause management problems due to significant economic losses, accepted as pre-eminent health trouble worldwide (21) and it is important that a real diagnosis and an effective treatment should be performed.

Anaplasma spp. are significant tick-borne bacteria because of medical and veterinary significance (16). Most common agent for cattle anaplasmosis is *Anaplasma marginale*. Biological transmission of *A. marginale* is associated with ticks mainly genera of *Rhipicephalus* and *Dermacentor* (22). *Anaplasma marginale* is highly pathogenic for cattle and the major signs are anemia, fever, icterus, weight loss and death (6). Until today, a large number of tandem repeats and genotypes have been identified based on the variability of tandem amino acid sequences in the msp1a gene region of *A. marginale* (13). More than two hundred and fifty tandem repeats have been reported in various parts of the world (11). It has been reported new tandem repeats in China, Turkey and Russia with recent studies (4, 18, 28).

Bovine anaplasmosis can be diagnosed on the basis of clinical symptoms and microscopic examination of Giemsa-stained blood smears (20, 29). It is adequate for the detection of acute infection, but not possible for detection of carrier animals. Serologic tests have been employed in diagnosing subclinical infections in epidemiological studies (9), but cross-reactions between species and false-negative results are potential restrictions. It is possible to eliminate these disadvantages with molecular techniques provide improved sensitivity and specificity than microscopy and serology. In addition to these, veterinary practitioners have a limited laboratory facility in field conditions, also early treatment is very important for TBDs.

Although clinical and subclinical infections have been reported data concerning genetic variants of these pathogens is scarce. This study provides information about the distribution and frequency of *Anaplasma*/*Ehrlichia* (A/E) species in cattle from Karaman province of Turkey with a novel genetic variant of *A. marginale*.

Materials and Methods

Study area and sample collection: This study was carried out in Karaman province (37º 11' N, 33º 15' E) located in the south of the Central Anatolia Region of Turkey (Figure 1) and it was conducted in compliance with the regulation issued by Karamanoğlu Mehmetbey University Animal Experiments Local Ethics Committee (2016/01). Sampling was performed in 2016. The Karaman province is 1033 meters above sea level and has an area of 8869 km^2. It has a continental climate with hot summers and cold winters. The mean annual rainfall and temperature are 336.3 kg/m^2 and 12 °C, respectively. Agriculture/animal husbandry and related industrial sector activities have an important place in the Karaman economy. One hundred fifty clinically healthy cattle from 21 different locations throughout Karaman were examined for clinical findings of anaplasmosis (body temperature, mucous membrane color and size of subcutaneous lymph nodes) between April and September 2016. Age, gender and breed of animals were saved. Five ml of blood sample were taken from the *vena jugularis* into tubes containing K_3EDTA-anticoagulant from each animal.

Preparation of blood smears and DNA isolation: Thin blood smears prepared from animals were fixed with absolute methanol for five minutes and stained with 5% Giemsa stain for 30 minutes. The slides were rinsed with water, and after drying in the air they were screened under oil immersion (×100 magnification) for the presence of *Anaplasma*-like bodies. At least 100 microscope fields have been examined and even if an agent has been found, the sample has been evaluated as positive.

Blood samples were defrosted at room temperature and vortexed for 15 seconds to homogenize. A commercial kit (QIAamp DNA Mini Kit, 51306) was used to isolate total genomic DNA. The DNA extraction was performed as described in the kit protocol using 200 µl blood sample. Genomic DNAs were stored at -20 °C until used as a template in the PCR.

Polymerase chain reaction and reverse line blot hybridization assay: Nested PCR was performed using two universal primers. EC9 (5′-TACCTTGTTACG ACTT-3′) and EC12A (5′-TGATCCTGGCTCAGAACG AACG-3′) which amplify 1450 bp fragment in the hypervariable V1 region of the 16S rRNA gene of A/E was used for the first amplification (12). For the second amplification, one µl of first round PCR products were used as a template DNA. To amplify 492-498 bp in the hypervariable V1 region in 16S rRNA gene of A/E, 16S8FE (5'-GGAATTCAGAGTTGGATCMTGGYT CAG-3') and BGA1B-new (Biotin-5'- CGGGATCCC GAGTTTGCCGGGACTTYTTCT-3') primers (8, 26) were used. To reduce non-specific amplification, a touchdown program was performed. DNA from positive control and distilled water were used. The PCR was performed in a final volume of 25 µl, containing PCR buffer [750 mM Tris–HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 5 mM $MgCl_2$, 125 µM deoxynucleotide triphosphates, 1.25 U Taq DNA polymerase, forward and reverse primers (10 pmol/µl), and template DNA. Five microliters of PCR product were visualized using UV transillumination in a 1.6% agarose gel stained with ethidium bromide and the remaining amplicons were stored for RLB until hybridization. Probes containing N-terminal N-(trifluoracetamidohexyl-cyanoethyl,N,N-diisopropylphosphoramidite [TFA])-C6 amino linker were synthesized by "Midland Certified Reagent Company, Inc." and used with a range of 200-900 pmol/150 µl concentration (Table 1). Preparation of biodyne C membrane, hybridization and rinsing were as previously described (7). Black spots in rows were evaluated by ChemiDoc™ MP System (Bio-Rad, UK) can make chemiluminescence detection.

Table 1. Sequences of oligonucleotides used in RLB.
Tablo 1. RLB'de kullanılan oligonükleotidlerin dizilimleri.

Oligonucleotide probe	Sequence (5'–′3)	Reference
Anaplasma/*Ehrlichia*	AmMC6-TTATCGCTATTAGATGAGCC	26
Ehrlichia/*Anaplasma* catch-all	AmMC6-GGGGGAAAGATTTATCGCTA	8
Anaplasma marginale	AmMC6-GACCGTATACGCAGCTTG	8
Anaplasma centrale	AmMC6-TCGAACGGACCATACGC	8
Anaplasma bovis	AmMC6-GTAGCTTGCTATGAGAACA	8
Ehtlichia sp. strain Omatjenne	AmMC6-CGGGTTTTTATCATAGCTTGC	8
Anaplasma phagocytophilum 1	AmMC6-TTGCTATAAAGAATAATTAGTGG	26
Anaplasma phagocytophilum 3	AmMC6-TTGCTATGAAGAATAATTAGTGG	26
Anaplasma phagocytophilum 5	AmMC6-TTGCTATAAAGAATAGTTAGTGG	26
Anaplasma phagocytophilum 7	AmMC6-TTGCTATAGAGAATAGTTAGTGG	26
Anaplasma phagocytophilum A-HGE	AmMC6-GCTATAAAGAATAGTTAGTGG	26
Anaplasma phagocytophilum A-D-HGE	AmMC6-GCTATGAAGAATAGTTAGTG	26

***Anaplasma marginale specific semi-nested PCR*:** A semi-nested PCR protocol for msp1α gene was conducted as described by Lew et al. (23). The primers 1733F (5'-TGTGCTTATGGCAGACATTTCC-3') and 3134R (5'-TCACGGTCAAAACCTTTGCTTACC-3') were used in the first PCR, and the primer pair 1733F and 2957R (5'-AAACCTTGTAGCCCCAACTTATCC-3') was used in the second reaction. For the second PCR amplification, one µl of the first product was used as a template. Amplification was carried out under conditions previously reported. PCR amplicons were separated by electrophoresis on 1.6% agarose gel (40 min, 100 V), stained with ethidium bromide, and visualized under ultraviolet light. *Anaplasma marginale* control DNA was previously isolated from a cow (GenBank accession no. GU201518).

DNA sequencing, MSP1a microsatellite and tandem repeat analysis: Five amplified fragments containing variable regions of *A. marginale* msp1α gene were purified from the agarose gel using a commercial PCR Clean up System (MinElute Gel Extraction Kit, 28604) and directly sequenced. The MAFFT (https://mafft.cbrc.jp/) and Emboss Transeq (https://www.ebi.ac.uk/Tools/st/emboss_transeq/) programs were used to conduct multiple alignments and translate nucleotide to an amino acid of MSP1a sequences respectively. Sequencing results submitted to GenBank after comparing with other sequences available in the NCB database (http://www.ncbi.nlm.nih.gov/nuccore). The isolates were identified according to the nomenclature as previously reported (10, 15). The nature of the microsatellite structure was GTAGG **(G/A TTT)** m **(GT)** n**T** ATG (17). Calculation of the SD-ATG distance was performed as (4 x m) + (2 x n) + 1. The microsatellite analysis was conducted using the RepeatAnalyzer (11), and the nature of tandem repeats was indicated as previously proposed (15).

Results

Microscopic examination of blood smears: *Anaplasma*-like bodies were detected in four (2.66%) Giemsa-stained blood smears. All of the animals evaluated as positive in the microscopic examination are from Karaman center.

Detection of Anaplasma spp. by RLB: One hundred and fifty blood samples were screened for the presence of bovine A/E species. According to RLB results, eight out of 150 cattle (5.33%) were found to be infected with *A. centrale*. Three samples gave positive signals to A/E catch-all probes and no species-specific probe signal received. No animals were infected with *Ehrlichia* spp. (Table 2).

Anaplasma marginale specific semi-nested PCR amplification: Nine samples (6.00%) [three of them gave positive signals to A/E catch-all probes and eight of them were found to be positive in terms of *A. centrale* via RLB] were positive in terms of *A. marginale* according to the semi-nested PCR with primers (1733F and 3134R - 1733F and 2957R) amplifying the msp1a gene.

DNA sequencing, MSP1a microsatellite and tandem repeat analysis: Five *A. marginale* positive samples were sent to sequence analysis and obtained sequences of msp1α gene were submitted to GenBank (accession numbers MG983513 to MG983517). Tandem repeat sequences and structure of the msp1α gene were analyzed to discover differences. Three different types of MSP1a tandem repeats with 23 to 29 amino acids for *A. marginale* strains were identified (Figure 2a). The MSP1a microsatellite analysis revealed that E genotype was detected in the analyzed sequences and microsatellite sequences produced SD-ATG distances 23 nucleotide. One new microsatellite structure designated as Tr70 [(m = 2, n = 7, SD-ATG distance = 23), (ADSSSAGGVLSQS GQASTSSQLG)] was described (Figure 2b). It was determined that *A. marginale* strains had 2 and 4 MSP1a repeat sequences in the studied area (Figure 2c).

Table 2. Distribution of *Anaplasma* species detected by microscopy, PCR and RLB.
Tablo 2. Mikroskop, PZR ve RLB ile tesbit edilen *Anaplasma* türlerinin dağılımı.

Province	n	Test			Overall results		
		Microscopy	RLB	*A. marginale* nPCR	*A. marginale*	*A. centrale*	*A. marginale* + *A. centrale*
		Anaplasma spp.	*A. centrale*	*A. marginale*			
Karaman center	42	4	2	3	2	1	1
Kazımkarabekir	16	-	3	3	-	-	3
Ayrancı	56	-	1	2	1	-	1
Ermenek	36	-	2	1	-	1	1
Total	150	4 (2.66%)	8 (5.33%)	9 (6.00%)	3 (2.00%)	2 (1.33%)	6 (4.00%)

Figure 1. Turkey map showing the study area.
Şekil 1. Çalışma alanını gösteren Türkiye haritası.

a)

Repeat form	Encoded sequence	Number of amino acid
Tr1	ADSSSAGDQQQESSVLSQSDQASTSSQLG	29
Tr70*	ADSSSAG------GVLSQSGQASTSSQLG	23
Is1, 73	TDSSSAGDQQQESGVSSQSGQASTSSQLG	29

b)

Isolates	Accesion number	Genotype	m	n	SD-ATG distance (nucleotide)
Kr3	MG983513	E	2	7	23
Kr7	MG983514	E	2	7	23
Kr37	MG983515	E	2	7	23
Kr58	MG983516	E	2	7	23
Kr78	MG983517	E	2	7	23

c)

Isolates	Structure of MSP1a tandem repeats			No. of repeats
Kr3	Tr1	Is1,73		2
Kr7	Tr8	Is1,73	Is1,73	4
Kr37	Tr1	Is1,73		2
Kr58	Tr8	Is1,73	Is1,73	4
Kr78	Tr8	Is1,73	Is1,73	4

Figure 2a. New repeat forms of *Anaplasma marginale* MSP1a (Tr70*) identified. The one letter amino acid code was used to depict the differences found in MSP1a repeats. 2b. The msp1α microsatellite and tandem repeat sequences in *Anaplasma marginale* isolates. The microsatellite (sequence in bold) was located between the Shine-Dalgarno and the translation initiation codon (ATG) with the structure: GTAGG (G/ATTT)m (GT)n T ATG. 2c. The structure of the MSP1a repeat regions, according to the nomenclature previously proposed (11, 15).
Şekil 2a. *Anaplasma marginale* MSP1a'nın (Tr70*) yeni tekrar formları belirlenmiştir. MSP1a tekrarlarında bulunan farklılıkları tasvir etmek için bir harfli amino asit kodu kullanılmıştır. 2b. *Anaplasma marginale* izolatlarında msp1α mikrosatellit ve tandem tekrar dizileri. Mikrosatellit (koyu sıralı) Shine-Dalgarno ile translasyon başlatma kodonu (ATG) arasında, yapı ile birlikte: GTAGG (G / ATTT)m (GT)n T ATG. 2c. MSP1a'nın tekrarlanan bölgelerinin yapısı, daha önce önerilen terminolojiye göre (11, 15).

Discussion and Conclusion

In recent years, studies related to ticks and TBDs has increased and TBDs cause a major health problem and loss of production in cattle in Turkey. *Anaplasma phagocytophilum*, *A. marginale*, *A. centrale*, *A. bovis* and *Ehrlichia* sp. strain Omatjenne have been reported in cattle from Turkey (1-3, 5, 19). Among *Anaplasma* species, *A. marginale* is the most pathogenic species causing infections in cattle and it is known to cause clinical infections resulting in death (22), while *A. centrale* causes milder infections (25).

Major surface protein 1a, an important protein to determine genetic diversity of *A. marginale* strains, interacts with the vertebrate and invertebrate host cells of the bacterium (14). Cattle movement is a prominent factor for the MSP1a genetic diversity in *A. marginale* worldwide (15, 27). Supported by the finding of one MSP1a genotype in Australia, where cattle entry is limited (23). To date, eleven different genotypes (A-K) of *A. marginale* msp1a gene in worldwide were described (10, 17). In this study, we present the genotypic variant E of *A. marginale* based on msp1a gene sequences. C, E, and G genotypes were previously reported in dairy cattle from Turkey (4). More than two hundred fifty *A. marginale* tandem repeats have been reported in various parts of the world (4, 13, 15, 18, 28). In studies on *A. marginale* msp1a gene conducted in China and Turkey, 21 and 3 new tandem repeats have been reported respectively. A computer program is prepared to prevent the confusion in tandem repeats (11). Tr1, Tr2, Tr3, 73, 74 and 76 tandem repeats have been reported in dairy cattle in Turkey (4). The one tandem repeat designated as ‘‘Tr70’’ in this study were not previously reported anywhere (11). Also, ‘‘Tr1’’ and ‘‘73’’ tandem repeats were reported with this present study.

The length of the microsatellite is related to the expression of the msp1a gene and affects the transmission of *A. marginale* and its infection (17). In this study, SD-ATG distances of 23 nucleotides is a higher expression level. This finding suggests a big capacity for infection and transmission of the *A. marginale* strains.

Studies conducted with RLB do not result in species level from time to time due to different genotypes among species. In a study conducted on *Theileria equi*, the sample was not signaled at the species level when signaling against catch-all probe due to different genotypes (24). Similarly, in this study, no signal was detected at the species level when three samples were signaled for A/E catch-all probe. As a result, msp1a gene of *A. marginale* was amplified and catch-all samples were confirmed as *A. marginale*.

In conclusion, the presence and distribution of A/E infections in cattle in Karaman province were investigated using microscopy, PZR and RLB methods in this study. *Anaplasma marginale* and *A. centrale* were detected in cattle. Three different types of MSP1a tandem repeats with one new microsatellite structures designated as Tr70 for *A. marginale* strains were identified. It is important to remember that there can be different genotypes and strains of *A. marginale* when A/E catch-all signal is received in similar studies. It is also expected that the diversity of msp1a genotypes can increase related to animal movements and animal imports from abroad.

Acknowledgement

This work was supported financially by a grant (41-M-16) from the Commission of Scientific Research Projects, Karamanoğlu Mehmetbey University.

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Ankara Üniv Vet Fak Derg, **66**, 261-265, 2019
DOI: 10.33988/auvfd.437314

Analysis of prion protein coding gene polymorphisms in Palestinian native sheep breeds

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Received date: 11.09.2018- Accepted date: 03.05.2019

Abstract: Prion protein coding gene (*PRNP)* is the genetic locus correlated with the greatest impact on classical scrapie susceptibility in sheep. At codons 136, 154, and 171 of *PRNP* alanine/arginine/glutamine (ARQ) and valine/arginine/glutamine (VRQ) haplotypes, in turn, are related to susceptibility to classical scrapie while alanine/arginine/arginine ARR haplotype is correlated with resistance. The aim of the present study was to genotype the Palestinian native sheep breeds for detection of genetic resistance. A total of 38 healthy sheep from Awassi and Assaf breeds were randomly sampled. Genomic DNA was isolated from blood samples. After PCR amplification and DNA sequencing, ARQ, ARR, ARH, AHQ, ARL and VRQ alleles and ARR/ARQ, ARQ/ARQ, ARQ/ARL, ARH/ARQ, ARH/ARL, AHQ/ARQ and ARQ/VRQ genotypes were detected in *PRNP* gene. ARQ allele was found as a predominant allele in this study with the frequency of 0.76 for Awassi and Assaf breeds while the uncommon allele ARL was identified at low frequencies in both breeds. In addition, two different polymorphisms were recognized (V12I and L23H) at different codons of *PRNP*. Results have indicated that most of the genotypes belong to risk group 3. The careful dissemination of ARR/ARR sheep is suggested to increase resistant allele frequencies in Assaf and Awassi breeds.
Keywords: Assaf, Awassi, Palestinian, *PRNP*, scrapie.

Filistin yerli koyun ırklarında prion protein kodlama geni polimorfizmlerinin analizi

Özet: Prion protein kodlama geni (PRNP) koyunlarda klasik scrapie duyarlılığıyla ilişkilendirilen genetik lokustur. Genin 136, 154 ve 171. kodonlarındaki ARQ ve VRQ allellerinin scrapie duyarlılığıyla, ARR allelinin ise scrapie dirençliliğiyle ilgili olduğu bilinmektedir. Bu çalışmanın amacı, scrapieye karşı genetik direncin belirlenmesi için Filistinli yerli koyun ırklarının genotiplendirilmesidir. Çalışmada, İvesi ve Assaf ırklarından, 38 sağlıklı ve rastgele seçilmiş koyun örnekleri kullanılmıştır. Koyun kan örneklerinden DNA izole edilmiş, PZR amplifikasyonu yapılmış ve sekans analizi gerçekleştirilmiştir. Prion protein kodlama geninde, ARQ, ARR, ARH, AHQ, ARL ve VRQ allelleri ve ARR/ARQ, ARQ/ARQ, ARQ/ARL, ARH/ARQ, ARH/ARL, AHQ/ARQ ve ARQ/VRQ genotipleri belirlenmiştir. Her iki ırkta da baskın olan ARQ alleli 0.76 ile en yüksek frekansta bulunurken, az görülen ARL alleli ise her iki ırkta da düşük frekanslarda belirlenmiştir. Ayrıca *PRNP* geninin farklı kodonlarında iki farklı polimorfizm (V12I ve L23H) tanımlanmıştır. Sonuçlar genotiplerin çoğunluğunun 3.risk grubuna dahil olduğunu göstermektedir. Assaf ve İvesi ırklarında dirençli allel sıklığını arttırmak için ARR/ARR koyunlarının dikkatli bir şekilde yaygınlaştırılması önerilmektedir.
Anahtar sözcükler: Assaf, Filistin, İvesi, *PRNP*, scrapie.

Introduction

Scrapie is a contagious prion disease in sheep which affects the central nervous system (24). The aggregation of the misfolded pathological form (PrP^{Sc}) of the normal cellular prion protein (PrP^{C}) causes the disease (15). It was the first diagnosed prion disease (17) to be regarded as a small ruminant disease in 1732 where the first examination carried out by veterinarians in Britain, Germany and France (27).

Prion protein coding gene (*PRNP)* is the genetic locus correlated with the greatest impact on classical scrapie susceptibility in sheep (6). The prion protein which is conserved in mammals is encoded by *PRNP* (31). While the normal prion protein is regarded as PrP^{C}, the misfolded pathological form is considered PrP^{Sc} (18, 30).

Even any genetic or posttranslational differences could not be found between PrP^{C} and PrP^{Sc}, the dissimilarities in C-terminal globular domain configuration and biochemical hallmarks such as protease resistance and solubility have been detected (4). The developmental preservation of the essential structure of prion protein (PrP) through mammals, additionally its

gene expression level in brain and localization in privatised membrane domains offer that the normal cellular protein (PrP^C) has a significant role in signaling, cell adhesion and differentiation (14,19).

In sheep, goats and cattle, the *PRNP* is found on chromosome 13 (3). The sheep *PRNP* has three exons (52, 98, and 4028 nucleotides in length) separated by two introns (2421 and 14,031 nucleotides in length) (20). In spite of the fact that scrapie is considering a contagious disease, the polymorphisms in *PRNP* strongly influence the susceptibility of scrapie in sheep. At codons 136, 154, and 171 of *PRNP* alanine/arginine/glutamine (ARQ) and valine/arginine/glutamine (VRQ) haplotypes in turn are related to susceptibility to classical scrapie while alanine/arginine/arginine *ARR* haplotype is correlated with resistance (16).

In Palestine, sheep were distributed across the breeds of Awassi (52.9%), Assaf (35.7%), crossbreed (11%) and other breeds (0.4%). The native Awassi sheep are used for meat, milk and wool production. The Assaf sheep which is a result of crossbreeding of local Awassi and Swiss East Friesian breeds in occupied Palestinian territories in 1955, produce high quality of milk and meat in comparison to Awassi (26). In 1993 the first diagnosis of scrapie in Assaf breed was detected in the north of Palestine and the whole flock was destroyed for precaution. After that in 1996, 2002 and 2005 scrapie cases were determined in different areas of Palestine and caused the cull of Assaf flocks.

According to our knowledge, there is only one research in the literature about ovine *PRNP* in Palestinian. Gootwine et al. (13) were genotyped only codons 136, 154 and 171 in Assaf sheep breed to find out the genetic resistance to scrapie. According to their results, ARR/ARR genotype could not be detected whereas ARR/ARQ, ARR/ARH, ARQ/ARQ were observed with frequencies of 0.177, 0.097, 0.613 respectively (13).

The aim of this study was to genotype the Palestinian native sheep breeds (Assaf and Awassi) by polymerase chain reaction (PCR) and DNA sequencing for detection of genetic resistance and possible additional polymorphisms and for comparing allele frequencies in addition to the previous study.

Materials and methods

Samples and DNA isolation: In this study, 38 clinically healthy sheep of native Palestinian sheep breeds from four towns were randomly sampled. 17 sheep of Awassi breed from four flocks and 21 sheep of Assaf breed from five flocks were randomly chosen. Blood samples were collected by Palestinian Veterinary Services (Gaza, Palestine) into tubes containing EDTA. Genomic DNAs were isolated from blood samples manually by using Qiagen DNeasy blood and tissue kit.

PCR amplification and sequencing: The Primer3web tool (http://bioinfo.ut.ee/primer3/) (25) has used for the design of primers for PCR amplification of prion protein coding gene (Gen-Bank accession number M31313 from nucleotide 24 – 912). The sequences 5'-CGTGGGCATTTGATGCTGACAC- 3' as a forward primer and 5'-GCTGCAGGTAGACACTCCCTC- 3' as a reverse primer were chosen. In PCR amplification, 30 µl final volume prepared which was including 6 µl 5X Master Mix, 5 µl (50-100 ng) genomic DNA and 0.3 µl (10 µM) of each primer. The PCR amplification was carried out in thermal cycler with a denaturation step 94°C for 3 min and 35 cycles of 94°C for 45 s, 57°C for 45 s, 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were conducted on a 1.5% agarose gel for electrophoresis and were visualized with a long wavelength UV transilluminator. The PCR products (889 bp) sequenced after purification and sequences were analysed by using MEGA 6 program (28) for detection of polymorphisms at codons 136, 154, 171.

Statistical analysis: According to results, the gene and genotype frequencies were figured out by using the formulas; (23)

$$Xij = nij / n \text{ and } x\hat{}i = 2nii + nij / 2n$$

where Xij indicates the genotypic frequency of AiAj; nij and nii show the number of individuals for homozygous (AiAi) and heterozygous (AiAj) genotypes, ; xˆi indicates the gene frequency of Ai and n shows the total number of individuals.

For assessing the potential deviations from Hardy – Weinberg equilibrium $\chi 2$ tests were conducted for each breed. The susceptibility to scrapie was evaluated by the grouping system which is proposed by Tongue et al. (29) according to each breed's genotypic distribution.

Results

The allele frequencies for 6 alleles (ARQ, ARR, ARH, AHQ, VRQ and ARL) in Awassi and Assaf breeds are shown in Table 1. ARQ allele which is associated with susceptibility to scrapie was found as predominant allele in two breeds, with the frequencies of 0.764 and 0.761 for Awassi and Assaf, respectively. The most resistant allele ARR was only detected in Awassi breed with the frequency of 0.205. AHQ allele which is also related to low resistance to scrapie was the lack in Awassi breed and observed at low frequency (0.071) in Assaf breed. Besides ARQ and AHQ alleles, ARH allele that is regarded as low resistance to scrapie as well as the lack in Awassi breed and detected at low frequency (0.095) in Assaf breed. The high susceptible allele VRQ was discovered with a frequency 0.023 in Assaf breed whereas was not detected in Awassi breed. In addition, ARL allele was determined at low frequencies of 0.031 and 0.050 in Awassi and Assaf respectively.

Table 1. Allele frequencies of *PRNP* in Palestinian sheep breeds.
Tablo 1. Filistin koyun ırklarında *PRNP* allel frekansı.

Allele	Awassi Breed	Assaf Breed
ARQ	0.764	0.761
ARR	0.205	0.000
ARH	0.000	0.095
AHQ	0.000	0.071
VRQ	0.000	0.023
ARL	0.031	0.050

Table 2. Genotype frequencies of *PRNP* in Palestinian sheep breeds.
Tablo 2. Filistin koyun ırklarında *PRNP* genotip frekansı.

Risk group	Genotype	Awassi	Assaf
2	ARR/ARQ	0.412	0.000
3	ARQ/ARQ	0.529	0.571
3	ARQ/ARL	0.059	0.048
3	ARH/ARQ	0.000	0.142
3	ARH/ARL	0.000	0.048
3	AHQ/ARQ	0.000	0.142
5	ARQ/VRQ	0.000	0.048

Table 3. Additional polymorphisms in *PRNP* of Palestinian sheep breeds.
Tablo 3. Filistin koyun ırklarında *PRNP* ek polimorfizmleri.

Breed	Sample	Haplotype	Polymorphism	Homozygote/ Heterozygote
Awassi	1	ARQ/ARQ	L23H	Homozygote
Awassi	4	ARQ/ARQ	L23H	Homozygote
Awassi	9	ARR/ARQ	L23H	Heterozygote
Awassi	10	ARR/ARQ	L23H	Heterozygote
Awassi	12	ARR/ARQ	L23H	Heterozygote
Awassi	15	ARR/ARQ	L23H	Heterozygote
Awassi	16	ARQ/ARQ	V12I	Homozygote
Awassi	16	ARQ/ARQ	L23H	Homozygote
Awassi	17	ARR/ARQ	L23H	Heterozygote
Assaf	18	ARQ/ARQ	L23H	Homozygote
Assaf	21	ARQ/ARQ	L23H	Homozygote
Assaf	24	ARQ/ARQ	L23H	Homozygote
Assaf	25	ARQ/ARQ	L23H	Homozygote
Assaf	26	ARH/ARL	L23H	Heterozygote
Assaf	28	AHQ/ARQ	L23H	Heterozygote
Assaf	29	ARQ/ARQ	L23H	Homozygote
Assaf	30	ARQ/ARQ	V12I	Homozygote
Assaf	30	ARQ/ARQ	L23H	Homozygote
Assaf	31	ARQ/ARQ	V12I	Homozygote
Assaf	31	ARQ/ARQ	L23H	Homozygote
Assaf	36	AHQ/ARQ	L23H	Heterozygote
Assaf	37	ARH/ARQ	L23H	Heterozygote

In *PRNP* gene the polymorphisms were detected which consist of seven genotypes (ARR/ARQ, ARQ/ARQ, ARQ/ARL, ARH/ARQ, ARH/ARL, AHQ/ARQ and ARQ/VRQ) (Table 2). The genotypes pertain to the second and fifth risk group. The ARQ/ARQ which is low resistance to scrapie was the dominant genotype in both Assaf and Awassi breeds and the high susceptible genotype ARQ/VRQ was observed only in one Assaf sheep.

Two additional polymorphisms were detected (V12I and L23H) moreover to determined polymorphisms at codons 136, 154 and 171 (Table 3). L23H polymorphism was detected in 8 and 11 sheep samples in Awassi and Assaf breeds, respectively, whereas V12I polymorphism detected in 1 and 2 sheep samples in Awassi and Assaf breeds, respectively. At codon 12, ATT instead of GTT caused V (Valine) to I (Isoleucine) alteration as well as at codon 23 CAC instead of CTC caused L (leucine) to H (Histidine) alteration.

According to the results of statistical analysis, P value was found greater than 0.05 for both breeds. Also $\chi 2$ was found 1.37 (degree of freedom 3) and 9.4 (degree of freedom 10) for Awassi and Assaf breeds respectively. These results implicate that the populations used in this study were in Hardy-Weinberg equilibrium.

Discussion and Conclusion

According to results in Awassi and Assaf breeds ARQ allele was discovered as a dominant allele in this study with a frequency of 0.76 which is compatible with the results of many previous studies performed for different sheep breeds in Turkey (21, 22), Iran (10), Greece (8), Italy (29) Portugal (11), Spain (1), Germany (7) and Britain (2) and also in previous study in Palestinian. In addition, ARQ is estimated to be wild-type allele of the *PRNP* and this study supports this prediction.

The association between Arginine amino acid at codon 136 and resistance to scrapie has shown before (9, 20). In spite of the fact that the significance of amino acid residue is not completely recognized for codon 154, H has a positive relation with resistance to scrapie at this position (9) while R amino acid was predominance at codon 154 in this study. R, Q, H and L were detected at codon 171 as different amino acid residues. The high frequency of Q and the low frequency of H amino acids are compatible with previously reported *PRNP* polymorphisms (5, 12).

In this study, ARR allele in Assaf breed was not detected. This is a major point of interest and may be a good explanation for some clinical scrapie cases that were detected in Assaf sheep previously (13). In comparison to the results of Gootwine et al. study in Awassi breed, ARR allele was detected with a higher frequency which is 0.205 and this can be the result of breeding programs. Also in

this study ARH and AHQ alleles could not be observed where in a previous study they were found with frequencies of 0.123 and 0.029 respectively in Awassi breed. However, ARL allele which is identified as uncommon in sheep populations and was observed in Iranian Zandi sheep previously (10) was also detected in this study in both breeds.

The highest resistance genotype ARR/ARR and the highest susceptibility genotype VRQ/VRQ were absent in Awassi and Assaf breeds matching with the results of the previous study in Awassi breed. The genotypes ARR/ARQ (risk group 2) and ARQ/ARQ (risk group 3) were detected with frequencies of 0.411 and 0,529, respectively. When these results were compared with the previous study, an increase in the frequency of the genotype in risk group 2 and a decrease in the frequency of the genotype in risk group 3 observed and this suggests the rising of resistant genotypes in Awassi breed (13). Also, the frequency differences shown by the results of the previous studies with the results obtained in this work reveal the importance of repeating these studies. The relation between additional polymorphisms (L23H,V12I) and the susceptibility to scrapie is yet to be investigated.

In conclusion, the results of this study demonstrate the significance of implementing appropriate breeding programs for increasing the genetic resistance against classical scrapie. According to low ARR allele frequency in Assaf breed, inbreeding has to be avoided in breeding programs. Also, insertion of sheep from different flocks with high ARR allele frequency for increasing resistance should be done carefully to avoid atypical scrapie and losses of genetic variations in breeds.

Acknowledgement

This study is the master thesis of the first author and was supported by Scientific Research Projects of Ege University with a project number 2016-FEN-013. The summary of this research was presented in 10th International Animal Science Conference, 26-28 October 2018, Antalya, Turkey.

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Ankara Üniv Vet Fak Derg, **66**, 267-272, 2019
DOI: 10.33988/auvfd.467062

The effect of melatonin in rats with uterine torsion on uterus contractions, and the levels of ADMA, SDMA, arginine, Hsp90, TLR4, and NF-κB

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Received date: 03.10.2018- Accepted date: 04.05.2019

Abstract: In this study was aimed at reducing uterine damage and increasing fertility after uterine torsion in pregnant animals. With this aim, uterine torsion was experimentally formed in 35 rats that were between 18-19 days pregnant. The animals were randomly divided into five groups, and melatonin was administered prior to torsion, at the time of torsion, and detorsion (10 mg/kg/gün IP). Ovario-hysterectomy operation was performed on all animals on the first day following parturition. Subsequently, from the obtained uterus samples, determination of the levels of asymmetrical dimethyl arginin (ADMA), symmetrical dimethyl arginin (SDMA), and arginine was made using the high-performance liquid chromatography (HPLC) and levels of Heat shock protein 90 (Hsp90), TLR4 (Toll Like Receptor 4) and NFκB (Nuclear factor kappa-light-chain-enhancer of activated B cells) were measured using the Western blot technique. The contraction-relaxation responses of the myometrium were also determined in the organ baths. According to the results of the western blot, higher protein expressions than those of the control group were determined in the second, third, fourth, and fifth groups in Hsp90, TLR4, NF-κB. The lowest values of arginine and ADMA were found in Group 3, whilst the lowest SDMA value was determined in Group 1. It was determined that melatonin reduces tissue damage secondary to torsio uteri and, furthermore, that administration of this hormone at the time of torsion formation was more effective than its administration at the time of detorsion.

Keywords: Melatonin, pregnancy, rats, torsion, uterine

Torsiyo uterili ratlarda melatoninin uterus kontraksiyonları, ADMA, SDMA, arjinin, Hsp90, TLR4 ve NFκB düzeylerine etkisi

Özet: Bu çalışmada gebe hayvanlarda torsiyo uteri sonrası şekillenen uterus hasarını azaltmaya ve fertiliteyi artırmaya yönelik bir çalışma planlandı. Bu amaçla 35 adet 18-19 günlük gebe ratda deneysel olarak torsiyo uteri oluşturuldu. Hayvanlar rastgele 5 gruba ayrılarak torsiyon öncesi, torsiyon anında ve detorsiyon sırasında melatonin (10 mg/kg/gün IP) uygulandı. Doğum sonrası ilk gün tüm hayvanlara ovariohisterektomi operasyonu yapılarak kornulardan numuneler alındı. Daha sonra elde edilen uterus dokularında asymmetrical dimethyl arginin (ADMA), symmetrical dimethyl arginin (SDMA) ve Arjinin miktar tayinleri yüksek performanslı sıvı kromatografisi (HPLC) ve Heat shock protein 90 (Hsp90), TLR4 (Toll Like Receptor 4) ve NFκB (Nuclear factor kappa-light-chain-enhancer of activated B cells) düzeyleri ise Western Blot ile belirlendi. Miyometriyumun in vitro kasılma gevşeme cevapları da organ banyosunda tespit edildi. Western blot sonuçlarına göre HSP90, TLR4, NFκB'da 2, 3, 4 ve 5. gruplarda kontrol grubuna göre daha fazla protein ekspresyonu olduğu belirlendi. Arjinin ve ADMA değerleri en düşük olarak 3. grupta bulundu. En düşük SDMA değeri ise 1. grupta tespit edildi. Sonuç olarak; elde edilen tüm bu veriler melatoninin torsiyo uteriye bağlı doku hasarını azalttığı ayrıca bu hormonun torsiyonun şekillendiği anda uygulanmasının detorsiyon anında uygulanmasına göre daha etkili olduğu kanaatine varıldı.

Anahtar sözcükler: Gebelik, melatonin, rat, uterus torsiyonu.

Introduction

Uterine torsion is one of the frequent causes of dystocia in all ruminants, although it is primarily found in cattle, and it constitutes 7% of all dystocias. Treatment applications are generally developed in order to correct the torsion and enable parturition (11), although no routine application exists for the maintenance of fertility in animals with uterine torsion. Nevertheless, more than half of the surviving animals with uterine torsion have been reported to become isolated from the herd in later stages due to infertility. The cause of infertility in these animals has been suggested to be the ischaemic/reperfusion damage as a result of torsion and detorsion (10).

It has been reported that the physiological and pharmacological concentrations of melatonin affect the free radicals that increase during ischaemic/reperfusion damage and reduce myocardial damage (mortality, arrhythmias, and infarction areas). This effect of melatonin may be related to its anti-adrenergic property, as well as its free-radical scavenging and anti-oxidant effects (7). The ischaemic/reperfusion preventive effect of melatonin is not limited to the heart, but has also been detected in hepatic (13) and cerebral (9) cells. This study set out to determine the effects of melatonin administrations on uterine tissue in pregnant rats in which uterine torsion was formed experimentally.

Materials and Methods

A total of 35 female Wistar rats aged 3-4 months, with a gestational age of 18-19 days and a weight of 200-250 g, were used. The animals were obtained from the Experimental Investigations Centre of Firat University, Turkey. They were kept in individual cages and were exposed to a rhythm of 12 hr dark and 12 hr light, and were fed *ad libitum*. Ethical approval was obtained from the Local Ethical Committee of Fırat University Laboratory Animals (17.12.2014 - 2014/128).

The animals were grouped as follows: Group 1: rats that were given only anaesthesia on their 18-19th gestational day (n=7). Group 2: rats with an experimentally formed uterine torsion of 360 degrees on their 18-19th gestational day, corrected six hr later (n=7). Group 3: rats with an experimentally formed uterine torsion of 360 degrees on their 18-19th gestational day, corrected six hr later, with melatonin (10 mg/kg IP) applied at the same time (n=7). Group 4: rats with an experimentally formed uterine torsion of 360 degrees on their 18-19th gestational day, with melatonin (10 mg/kg IP) administered six hr later along with torsion correction (n=7). Group 5: rats receiving melatonin on the 15-16th gestational day (10 mg/kg/day IP), and then uterine torsion of 360 degrees was formed experimentally in the uterus of these rats on their 18-19th gestational day, corrected six hr later (n=7).

Vaginal irrigations were performed as described by Risvanli et al., (12) and involved using elastic pipettes and tips with distilled water. Animals whose slides included spermatozoids were accepted as coitus positive. These dates were recorded as the zero day of pregnancy.

All operations were performed with the animals under ketamine/xylazine anesthesia, and animals with a gestational age of 18-19 days underwent laparotomy following routine procedures. The right cornu uteri of the animals were passed through the hole formed in the non-vascular region of *lig. lata uteri* of the left cornu uteri at the level of the urinary bladder. Then, the right cornu was passed through the same hole for the second time, and torsion was thereby formed. Following this procedure, the abdomens of the animals were closed with proper suturing material. Six hr after the operation, another laparotomy was performed on the same animals under anaesthesia and the torsion was corrected.

On the first day post-partum, all animals underwent ovariohysterectomy and the samples were obtained from uterine cornus and kept at -80°C. The contraction-relaxation responses of the myometrium were also determined in the organ baths. Within 24 hr after parturition/abortion a tissue section comprising all the layers of the uterus, with dimensions of 15x2 mm and of 23.4±6.67 mg weight, was obtained from the middle part of the uterine cornu parallel to the long axis (Figure 1).

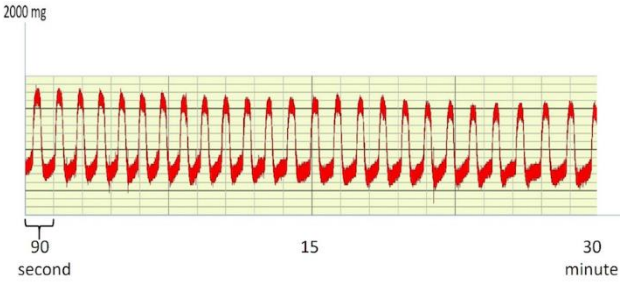

Figure 1. Spontaneous contraction within the first postpartum 24 hr.
Şekil 1. Postpartum ilk 24 saat içindeki kendiliğinden kasılma

The tissues that had been kept at -80°C in the deep freeze were removed and weighed on sensitive scales as pieces of 100 mg. Homogenization was performed by diluting 0.01 M phosphate buffer saline (pH 7.4) at a 1:10 ratio, and within ice blocks of 4°C. Following the homogenization procedure, centrifugation was carried out at 50.000 rpm for 10 min, and the supernatant parts were removed (5). The centrifugation procedure was repeated on the remaining pellet portions until these portions brightened up, and the obtained homogenates were apportioned. The amount of protein inside the homogenates was determined using the Lowry method

(µg/ml). A 20 µg total protein sample was loaded onto a 10% polyacrylamide gel and electrophoresed in SDS running solution (Running buffer; 2.4 mM Tris, 19.2 mM glisin, SDS of 0.01%) at 90 V for one hour. The separated proteins were transferred from the SDS-PAGE at 100 V in a period of one hour inside a transfer solution (25 mM Tris, 192 mM glisin, 20% methanol, pH 8.3) to the PVDF membrane. Following the transfer, the membrane was also blocked at 4°C inside the prepared milk powder of 5% inside PBS-T (PBS+ 1% Tween-20) solution. Following the blocking procedure, the membrane was treated with primary antibodies for two hours at room temperature. Subsequently, the membrane was washed four times with PBS-T for duration of 30 minutes. After the washing procedure, the membrane was treated with horseradish peroxidase conjugated secondary antibodies at a dilution of 1:10.000 for one hour at room temperature. The membrane was washed again four times with PBS-T for duration of 30 minutes. The protein bands were then visualized using the DAB chemical solution (5).

The analyses of ADMA, arginine, and symmetrical dimethyl arginine (SDMA) in the samples were carried out using the high performance liquid chromatography (HPLC) apparatus at the University of Firat's Department of Biochemistry (16).

In the organ bath experiments, the Kruskal-Wallis test, which is the equivalent of the one-way non-parametric analysis of variance, was performed in the statistical analyses of the data due to the presence of five groups, the use of different experimental subjects, and the lack of a normal distribution. The Kruskal-Wallis test was used in groups in which the medians were not equal, and the Bonferroni Corrected Mann-Whitney U test (P<0.01) was used as the post hoc multiple comparison method by lowering the level of significance in groups in which the significance was lower than 0.05. The Corrected Mann-Whitney U test was performed following the Kruskal-Wallis variance analysis in the evaluation of the distribution of arginine, SDMA, and ADMA values according to the groups. The SPSS for Windows version 22.0 (SPSS Inc. Chicago, Illinois, USA) program package was used for the statistical analyses.

Results

The parameters of the area under the contractile curve (AUC), amplitude, and frequency of spontaneously occurring contractions were determined in all five groups (Table 1). While the difference between the groups with regard to the parameters of amplitude and AUC was determined to be statistically significant (P<0.05), the difference regarding the frequency parameter was found to be insignificant (P>0.05).

When the difference between the groups with regard to AUC was addressed, the values belonging to Group 3 were determined to be higher than those of Groups 2, 4, and 5 (P<0.05). Still, the difference between Group 3 and Group 1 and 4 was found to be statistically insignificant (P>0.05) (Table 1).

When the difference between the groups with regard to amplitude was addressed, the values belonging to Group 3 were determined to be higher than those of Groups 2 and 5 (P<0.05). Still, the difference between Group 3 and Group 1 and 4 was found to be statistically insignificant (P>0.05) (Table 2).

Table 1. The 30-min analysis of spontaneous myometrial contractions.
Tablo 1. Spontan miyometriyal kasılmaların 30 dakikalık kasılma analizi

Groups	AUC (gXsec)	Amplitude (g)	Frequency
1	34.23±5.08^{a}	0.14±0.05^{a,b}	20.41±7.31
2	14.26±4.80^{b}	0.07±0.02^{c}	15.00±2.63
3	64.92±14.96^{a}	0.29±0.08^{a}	17.57±6.74
4	25.85±8.66^{c}	0.12±0.03^{a,b}	22.29±2.70
5	15.51±9.01^{b}	0.08±0.02^{c}	16.98±7.21
P	*	*	**

*P<0.05, **P>0.05, ^{a,b,c}: The difference between the frequencies demonstrated by different letters within the same column was significant (P<0.01).

Table 2. Distribution of the values of arginin, SDMA and ADMA according to the groups.
Tablo 2. Arjinin, SDMA, ADMA değerlerinin gruplara göre dağılımı

Groups	ARGININE µmol/L	SDMA µmol/L	ADMA µmol/L
1 (n=7)	8.74±1.13^{a}	0.32±0.07^{a}	1.38±0.34^{a}
2 (n=7)	20.50±3.66^{b}	1.59±0.32^{b}	2.27±0.53b^{d}
3 (n=7)	2.38±0.71^{c}	1.50±0.12^{b}	0.14±0.29^{bc}
4 (n=7)	8.05±2.30^{ad}	1.41±0.19^{b}	0.17±0.04^{c}
5 (n=7)	56.47±14.17^{e}	1.73±0.36^{b}	2.08±0.29^{d}
P	**	*	*

*P<0.01, **P<0.05 a, b, c, d, e: The difference between the frequencies demonstrated by different letters within the same column was significant (P<0.01).

The bands appearing as a result of the western blot procedure were visualized using the image-j program and the relative protein amounts were calculated. Accordingly, the protein expression values for Hsp90, TLR4, and NF-κB in Group 1, which was accepted as the control group, were observed to be 100%. For Hsp90, the protein expression values in the second, third, fourth, and fifth groups were determined to be higher than those of the

control group, in the order of 56.3%, 38.0%, 77.6%, and 70.1%, respectively. For TLR4, higher protein expression values than those of the control group were determined in the second, third, fourth, and fifth groups, in the order of 55.4%, 50.5%, 61.1%, and 47.9%, respectively. For NF-κB, higher protein expression values than those of the control group were determined in the second, third, fourth, and fifth groups, in the order of 65.5%, 54.5%, 62.7%, and 45.5%, respectively (Figure 2).

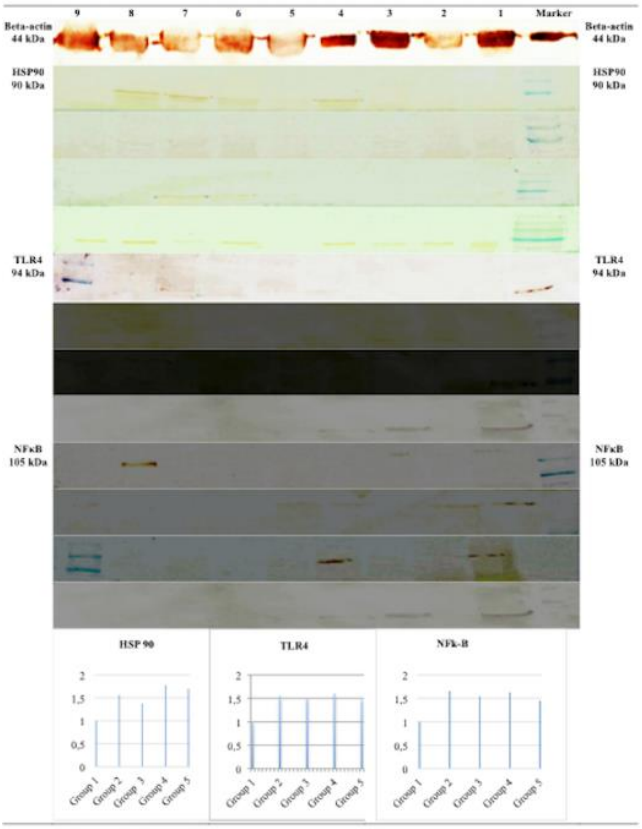

Figure 2. The expression values of HSP90, TLR4 and NFκB proteins between the groups.
Şekil 2. HSP90, TLR4 ve NFκB proteinlerinin gruplararası ekspresyon değerleri

The distribution of the arginine, SDMA, and ADMA values according to the groups has been summarised in Table 2. Accordingly, the lowest values of arginine (2.38±0.71 μmol/L; P<0.05) and ADMA (0.14±0.29 μmol/L; P<0.01) were determined in Group 3. The lowest SDMA value, however, was determined in Group 1 (0.32±0.07 μmol/L; P<0.01).

Discussion and Conclusion

Melatonin plays an important role in the TLR4/NF-κB pathway, especially in ischaemic/reperfusion damage. When the secretion of NF-κB and TLR4 are blocked, inflammation has been reported to be suppressed as well. NF-κB is activated in response to oxidative stress and is a redox sensible transcription factor, which is responsible for the production of inflammatory genes. In rats with TLR4 insufficiency, ischaemic/reperfusion damage-related inflammation has been suggested to decrease (6). The relationship between the Hsp90 and NF-κB molecules in various inflammatory disorders has been put forth in various studies. It has been reported that the transcription of many genes is up-regulated secondary to activation of NF-κB following cerebral ischaemia. Inhibition of the NF-κB pathway has been reported to be protective against cerebral ischaemic damage, both genetically and pharmacologically (8). The inhibitor is found through the formation of complexes with NF-κB kinase and Hsp90, and impairment of these complexes by Hsp90 inhibitors blocks the inhibitor NF-κB kinase function; subsequently, the NF-κB activation may be blocked. Secondary to this, an increase in ischaemic/reperfusion damage has been reported (14). In the present study, according to the results of the western blot, higher protein expressions than those of the control group for Hsp90 are found in the second, third, fourth, and fifth groups, in the order of 56.3%, 38.0%, 77.6%, and 70.1%, respectively. For TLR4, higher protein expression values than those of the control group were determined in the second, third, fourth, and fifth groups, in the order of 55.4%, 50.5%, 61.1%, and 47.9%, respectively. For NF-κB, higher protein expression values than those of the control group were determined in the second, third, fourth, and fifth groups, in the order of 65.5%, 54.5%, 62.7%, and 45.5%, respectively.

In ischaemic/reperfusion damage, ADMA reduces the activity of dimethylarginine dimethylaminohydrolase (DDAH), and increases its concentration. Furthermore, it also exerts an effect on the production of nitric oxide (NO) by competing with arginine for binding to the active point of the nitric oxide synthetase (NOS) center. However, the molecular mechanisms in the ischaemic/reperfusion damage have not been completely understood. Nonetheless, the ADMA/DDAH pathway has the potential ability to reduce the effects of ischaemic/reperfusion damages (4, 8, 18). In a study carried out by Ferrigno et al. (5) on male rats with regard to hepatic ischaemic/reperfusion damage, it was reported that the serum ADMA levels had increased and that the intracellular ADMA levels had decreased after a 60-min ischaemic attack. In the same study, following reperfusion, the DDAH activity and the mRNA and protein expression were reported to have decreased. In the present study also, the lowest values of arginine (2.38±0.71 μmol/L; P<0.05) and ADMA (0.14±0.29 μmol/L; P<0.01) were determined at the time of detorsion in Group 3, in which melatonin had been administered. The lowest SDMA value, however, was determined in Group 1, in which torsion had not been formed (0.32±0.07 μmol/L; P<0.01).

In spite of the fact that the mechanism of melatonin cannot be precisely explained, it has been reported to inhibit the spontaneous or oxytocin-stimulated myometrial contractions in pregnant and non-pregnant rats (2). However, it has also been reported that melatonin at micromolar doses does not inhibit the uterine smooth muscles stimulated with prostaglandin f_2-alpha in ovariectomized rats (1). Moreover, in a study performed on pregnant sheep, melatonin administration was reported to have no effect on the myometrial contractility. Still, the administration of melatonin has been reported to inhibit the oxytocin-stimulated myometrial activity in rats (15). Despite all the research carried out in the literature, no publication regarding the effects of melatonin on uterine contractility in produced ischaemic/reperfusion damage or torsion/detorsion was encountered. In the present study, however, the contractility in the uterus of rats, in which uterine torsion was produced experimentally in advanced pregnancy, was observed to be decreased compared with the control group. Of the AUC, amplitude, and frequency parameters evaluated in the organ bath experiments, the difference between the melatonin-administered groups with regard to amplitude and AUC was found to be statistically significant. However, the difference between the groups with regard to frequency was determined to be statistically insignificant. When the inter-group differences were addressed with regard to AUC, the values belonging to Group 3 were higher than those of Groups 2, 4, and 5. When the intergroup differences were addressed with regard to amplitude, the values in Group 3 were determined to be higher than those of Groups 2 and 5. Administration of melatonin at the time of detorsion positively affected the uterine contractions, and not at the time of torsion formation.

In previous studies on ovarian torsion (Adnexal torsion), various materials such as vitamins E and C, mannitol, melatonin, caffeic acid, and erythropoietin have been used to reduce the reperfusion damage, and as a result these chemicals have been reported to reduce the damage caused by ischaemia/reperfusion to the ovary (3), to a certain extent. In a study carried out by Turkoz et al., (17) in which they produced ovarian torsion in rats, melatonin was reported to protect the ovary against oxidative damage resulting from ischaemia/reperfusion. In the present study also, according to the results of the organ bath and western blot experiments, administration of melatonin was observed to reduce the damage resulting from ischaemia/reperfusion caused by torsion/detorsion in rats, in which torsio uteri was experimentally produced.

According to these experimentally obtained results, administration of melatonin at the time of torsion formation and not at the time of torsion reduces uterine damage caused by uterine torsion in advanced pregnancy. However, in animals such as sheep and cattle, generally due to the inability to determine the precise time of torsion, it was determined that administration of melatonin following the diagnosis of uterine torsion or just after correction of the torsion was beneficial.

Acknowledgement

This study was supported by the Scientific and Technological Research Council of Turkey (TUBITAK - 115O381).

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Ankara Üniv Vet Fak Derg, **66**, 273-280, 2019
DOI: 10.33988/auvfd.469526

Koyun orijinli *Toxoplasma gondii* izolatlarının multilokus PCR-RFLP yöntemi ile genotiplendirilmesi

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Geliş tarihi: 11.10.2018- Kabul tarihi: 04.05.2019

Özet: Bu çalışmada doğal enfekte koyunlardan elde edilen *Toxoplasma gondii* izolatlarının (n:10) genotiplendirilmesi amaçlanmıştır. Bu amaçla *T. gondii* izolatlarının dokuz lokusu (SAG1, SAG2, c22-8, L358, 5'-SAG2, 3'-SAG2, SAG3, BTUB and PK1) multipleks multilokus nested PCR-RFLP yöntemi ile amplifiye edilmiştir. Bunun sonucunda koyun orijinli *T. gondii* izolatlarının bir dizi lokusta birden çok allel barındırdığı tespit edilmiştir. Koyun izolatları (n: 8) iki lokusta (alt.SAG2 ve SAG3) tip II ve III, bir lokusta (c22-8) klonal tip II, bir lokusta (5'-SAG2) tip I ve III, bir lokusta (L358) I ve II ve bir lokusta (3'-SAG2) ise tip I/III alleli göstermiştir. Bir koyun izolatı; iki lokusta (alt.SAG2 ve L358) tip I, bir lokusta (c22-8) tip II, bir lokusta (BTUB) tip I ve II, bir lokusta (3'-SAG2) ise I/III alleli göstermiştir. Sonuncu koyun izolatı ise üç lokusta (SAG1, alt.SAG2 ve SAG3) tip III, iki lokusta (c22-8 ve L358) tip II, bir lokusta (3'-SAG2) I/III, bir lokusta (BTUB) ise tip I ve II allelleri göstermiştir. Bu çalışma Türkiye'de koyunlarda *T. gondii*'nin genotiplendirilmesine ilişkin ilk rapordur. Genotiplendirmeye yönelik olarak parazite arakonaklık yapan farklı hayvanlardan ve çeşitli bölgelerden elde edilen izolatlar üzerinde çalışılması Türkiye'de *T. gondii*'nin bulaşma yolları ve tipleri hakkında önemli bilgiler elde etmeye katkı sağlayacaktır.

Anahtar sözcükler: Genotiplendirme, koyun, multilokus nested PCR RFLP, *Toxoplasma gondii*, Türkiye

Genotyping of *Toxoplasma gondii* isolates originated from sheep by multilocus PCR-RFLP

Abstract: The aim of the study was to determine the genotype of *Toxoplasma gondii* isolates obtained from sheep naturally infected. Genotyping was performed on the sheep isolates (n: 10) using multiplex multilocus nested PCR-RFLP at nine loci (SAG1, SAG2, c22-8, L358, 5'-SAG2, 3'-SAG2, SAG3, BTUB and PK1)**.** *Toxoplasma gondii* isolates originated from sheep possessed multiple alleles at the same loci. The isolates (n:8) showed alleles of clonal type II and III at two loci (alt. SAG2 and SAG3), type II allele at the locus (c22-8), type I and III alleles at the locus 5'SAG2 and type I and II alleles at the locus L358, type I/III alleles at the locus 3'-SAG2. One isolate showed alleles of type I at four loci (alt. SAG2 and L358), type II allele at the locus c22-8 and type I and II alleles at the locus BTUB, type I/III alleles at the locus 3'-SAG2. One isolate showed alleles of type III at three loci (SAG1, alt. SAG2 and SAG3), type II allele at two loci (c22-8 and L358), type I/III alleles at the locus 3'-SAG2 and type I and II alleles at the locus BTUB. Genotyping may provide knowledge on infection route of *T. gondii* in people. To the author's knowledge, it is the first report on genotyping of *T. gondii* isolates originated from sheep in Turkey. Genotyping of isolates obtained from different hosts and from various regions will contribute to achieving the important knowledge about infection routes and clonal types of *T. gondii* in Turkey.

Keywords: Genotyping, multilocus nested PCR RFLP, sheep, *Toxoplasma gondii*, Turkey

Giriş

Dünya üzerinde en yaygın parazitik protozoonlardan biri olan *Toxoplasma gondii*'nin yaşam çemberinde kediler son konak, kedinin de dahil olduğu pek çok hayvan ve insan arakonaktır (8, 12). Toxoplasmatidae ailesi içinde yer alan tek tür olan *T. gondii*'nin dünya üzerinde farklı coğrafi bölgelerde subpopulasyonları vardır (30, 39). *Toxoplasma gondii* izolatlarının tiplendirilmesinde Polimeraz Zincir Reaksiyonu Restriksiyon Parça Uzunluk Polimorfizmi (PCR RFLP), multilokus enzim elektroforezisi veya satellit analizi gibi yöntemler kullanılarak çok sayıda belirteç kullanılarak parazitin üç temel suş grubuna sahip olduğu belirlenmiştir (tip I, 2 ve 3 suşları) (19, 39). Tip I suşları (RH ve GT-1) nadiren

izole edilmekte olup genelde insan orijinlidir (Avrupa ve ABD'den izole edilen *T. gondii*'ye ait suş koleksiyonunun %10'unu tip I suşları oluşturur). Bu tipte yer alan suşlar *in vitro* ortamda hızla çoğalır ve tachyzoit-bradyzoit dönüşümü nispeten daha azdır (24, 39). Tip II suşları (ME49, PDS, PLK, PTg) arakonaklardan (koyun, insan ve domuz) en yaygın izole edilen suşlardır. Bu suşlar *in vitro* ortamda yavaş çoğalır, tachyzoit-bradyzoit dönüşümü ve kist oluşumu kolaydır (20, 24, 39). Tip III suşları (CEP ve VEG) ise rekombinant genotipler ve atipik alleller barındıran yaygın olmayan bir genotiptir. Yaban hayvanlarından orijin alan izolatlarda daha sıktır (24, 39).

Konakta *T. gondii* ile enfeksiyonda gelişen tabloda parazit türü ve genotipi önemli role sahiptir. Farede, tip I suşları oldukça letal bir tablo şekillendirirken, aksine tip II ve III suşları nispeten daha az virulenttir (24, 33). İnsanda şekillenen toksoplasmosis esnasında asemptomatikten ciddi akut tabloya kadar değişen klinik görünüm izlenmektedir (39). İnsan toksoplasmosisinden genelde tip II suşlarının sorumlu olduğu belirtilmekle birlikte tip I veya tip I benzeri atipik *T. gondii* izolatları da insanda ciddi retinokoroiditis (16) ve akut toksoplasmosis sebebidir (7). Kuzey Amerika ve Avrupa'daki genotiplendirme çalışmalarında *T. gondii*'nin üç temel suş grubu (tip I, II ve III) tanımlanmıştır (19, 27). Benzer durum Afrika'dan da rapor edilmiştir (38). Ancak Güney Amerika'daki hayvan ve insan vakalarından elde edilen *T. gondii* izolatlarının Kuzey Amerika ve Avrupa'dakilerden oldukça farklı olduğu belirlenmiş ve bu farklı ve hiçbir tipe benzemeyen izolatlar "atipik ya da "ekzotik" izolatlar olarak tanımlanmıştır (14, 22, 23).

Türkiye'de koyunlarda *T. gondii* enfeksiyonlarının yaygınlığına ilişkin çeşitli seroprevalans çalışmaları bulunmaktadır (1, 3, 5, 31, 32, 40). Ülkemizde hem çiftlik hayvanlarında hem de insanlarda toksoplasmozis seropozitifliği giderek arttığı izlenmektedir (25, 41). Buna ilişkin olarak yakın gelecekte muhtemel klinik toksoplasmosis olgularının önlenmesine yönelik yeni stratejilerin belirlenmesi gerekliliğinin ortaya çıkacağı şüphesizdir. Dünyanın pek çok bölgesinde *T. gondii'*nin enfekte ettiği konaklarda şekillenen hastalığın birbirinden farklı seyrettiği dikkat çekmiştir. Bu durumun sebepleri arasında öncelikle parazit suşları arasındaki virulens farklılığının olduğu ileri sürülmüştür. Türkiye'de *T. gondii'*nin genotiplendirilmesine ilişkin insan, kedi ve yabani kuşlardan bildirimler (9, 11, 21) mevcut olmakla birlikte koyunlarda *T. gondii*'nin genotiplendirilmesine ilişkin henüz rapor bulunmamaktadır. Bu çalışmada doğal enfekte koyunlardan elde edilen *T. gondii* izolatlarının multipleks multilokus nested PCR RFLP yöntemi kullanılarak genotiplendirilmesi amaçlanmıştır.

Materyal ve Metot

Toxoplasma gondii izolatları: Bu çalışmada; Kırıkkale'de mezbahada rutin yapılan kesim sonrasında rastgele seçilen doğal enfekte koyunlara ait beyin dokularından elde edilen 10 *T. gondii* izolatına ait genomik DNA ile Türkiye Halk Sağlığı Kurumu'nda toksoplasmosis teşhisi amacıyla rutin uygulanan Sabin Feldman boya testi için fare peritonunda pasajlanan *T. gondii* RH suşuna ait tachyzoitlerden elde edilen genomik DNA kullanılmıştır.

DNA ekstraksiyonu ve nested PCR: Doğal enfekte koyunlara ait beyin dokularından DNA ekstraksiyon kiti (DNeasy Blood & Tissue Kits, Qiagen, Germany) firmanın belirtildiği biçimde uygulanarak genomik DNA'lar elde edilmiştir. *Toxoplasma gondii* B1 geninin varlığını doğrulamak için nested PCR yapılmıştır. İlk reaksiyonda B1 geninin 194 bp büyüklüğündeki kısmı Toxo 1 for: 5'-GGAACTGCATCCGTTCATGAG-3' ve Toxo 2 rev: 5'-TCTTTAAAGCGTTCGTGGTC-3' primerleri, ikinci reaksiyonda ilk reaksiyonda çoğaltılan DNA kısmı template olarak kullanılmış ve B1 geninin 97 bp'lik kısmı Toxo 3 for: 5'-TGCATAGGTTGCC AGTCACTG-3' ve Toxo 4 rev: 5'-GGCGACCAATCTG CGAATACACC-3' primerleri kullanılarak çoğaltılmıştır. Her iki PCR reaksiyonu 50 µl'lik hacimde olacak şekilde her bir primerden (30 pmol) 1.5 µl, 5 µl 10XPCR buffer, 5 µl 25mM MgCl2 , 4 µl 1mM dNTP mix, 0.25 µl of TaqDNA polymerase (1.25 IU; MBI Fermentas) ve template (ilk reaksiyonda 5 µl, ikinci reaksiyonda ise 2 µl) hazırlanmıştır. Reaksiyon karışımı 95°C'de 5 dk'yı takiben 35 siklus boyunca 95°C'de 1 dk, 55°C'de 1 dk ve 72°C'de 1 dk, takibinde 72°C'de 5 dk termal sıcaklık değişimine tabi tutulmuştur (Esco Swift minipro, Güney Afrika). *T. gondii* RH suşuna ait DNA ile ultrasaf su pozitif ve negative DNA olarak kullanılmıştır. PCR ürünleri ethidium bromid ile boyalı agaroz jelde (%1.5) yürütülmüş ve ultraviyole lamba ile izlenmiştir.

Genotiplendirme: *Toxoplasma gondii*'ye ait dokuz lokusun (SAG1, alt.SAG2, c22-8, L358, 5'-SAG2, 3'-SAG2, SAG3, BTUB ve PK1) multipleks multilokus nested (m-Mn) PCR RFLP yöntemi kullanılarak amplifikasyonu sonucunda genotiplendirme gerçekleştirilmiştir (35, 36). Her bir lokus için hedef DNA, Fast start DNA polimeraz kullanılarak multipleks PCR (m-PCR) ile amplifiye edilmiştir. Reaksiyon: 1xPCR buffer, 2 mM $MgCl_2$, 200 µM dNTP, 0,15 µM eksternal forward and reverse primerler, 1 U FastStart DNA polymerase and 1,5 µl DNA ekstraktı ile toplam 25 mikrolitre hacimde gerçekleştirilmiştir. Reaksiyon karışımı 95°C'de 4 dk'yı takiben 30 siklus 94°C'de 30 sn, 55°C'de 1 dk ve 72°C'de 2 dk termal sıcaklık değişimine tabi tutulmuştur.

Laboratuvar çalışmaları esnasında m-PCR ile çoğaltılan lokuslar 1:1 sulandırıldıktan sonra nested-PCR

aşaması için hedef DNA olarak kullanılmıştır. n-PCR 25 µl reaksiyon hacminde gerçekleştirilecek şekilde; 1xPCR buffer, 2 mM $MgCl_2$, 200 µM her bir dNTP'den, 0,30 µM her bir internal ileri (forward) ve geri (reverse) primerlerden, 1 U FastStart DNA polimeraz ve 1,5 µl sulandırılmış m-PCR ürünlerini içermiştir. Reaksiyon karışımına; 94°C 4 dk ve 35 siklus; 94°C 30 sn, 60°C 1 dk ve 72°C 1,5 dk sıcaklık uygulanmıştır.

Elde edilen n-PCR ürünlerinden alınan 5 µl'lik örnek, Tablo 1'de belirtildiği şekilde belirtilen restriksiyon enzimleriyle (NEB, ABD) kesilerek (Boeco Thermal Shaker, Germany) 0.3 mg/ml ethidium bromid içeren %2.5-3'lük agaroz jelde yürütülmüş ve oluşan bant desenleri ultraviyole lamba altında incelenmiştir. Elde edilen sonuçlar referans suşlara (GT1, PTG, CTG, TgCgCa1, Mas, TgCatBr5, TgCatBr64 ve TgRSCr1) ait DNA'larda görülen band desenleri ile kıyaslanmıştır.

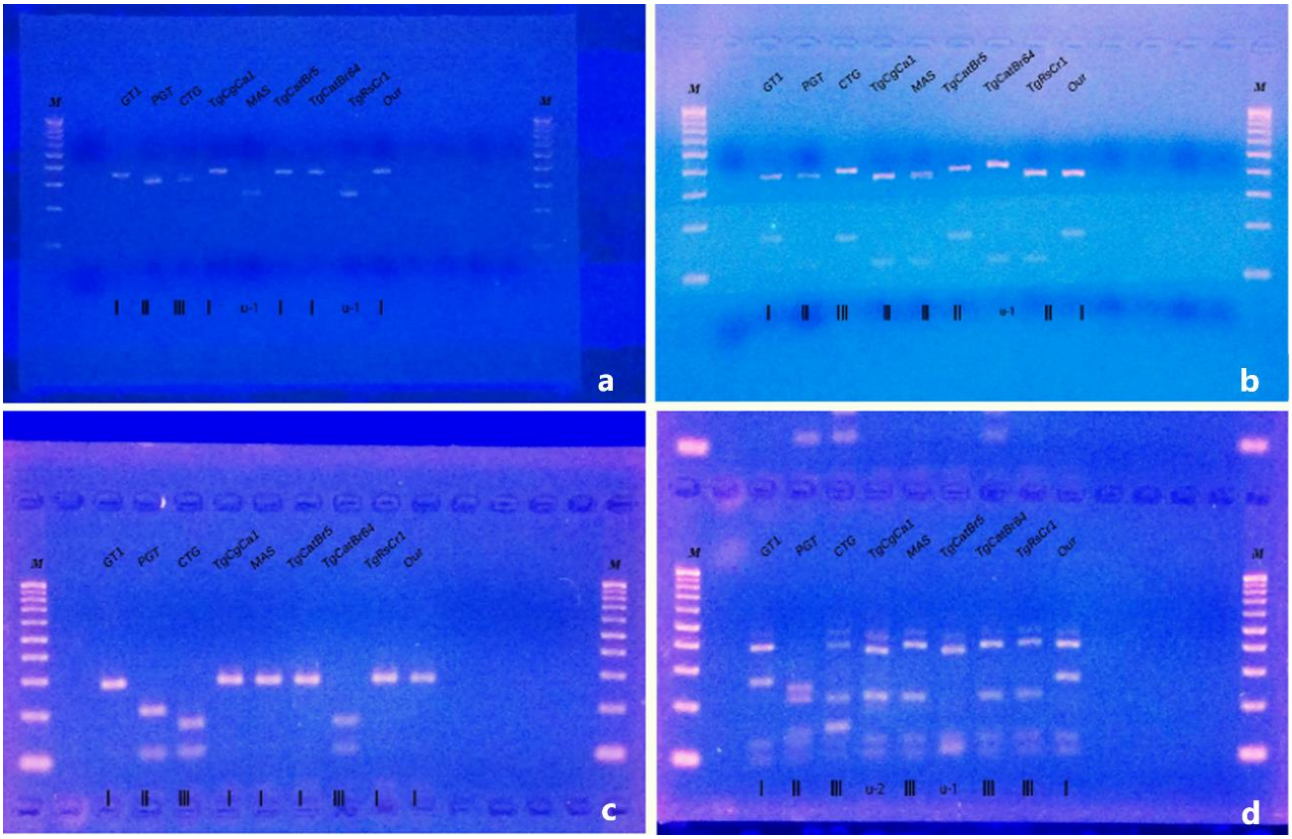
Bulgular

Çalışmada elde edilecek sonuçların güvenilirliği bakımından laboratuar çalışmaları Mn-PCR RFLP yönteminin standardize edilmesi ile başlamıştır. Bunun için öncelikle Tip I-III içinde yer alan referans suşlara (GT1, PTG, CTG, TgCgCa1, Mas, TgCatBr5, TgCatBr64 ve TgRSCr1) ait DNA'lar restriksiyon enzimleri ile kesilmek suretiyle deneyin standardizasyonu sağlanmış, reaksiyon sonucunda oluşan band desenleri ilgili literatür (35, 37) ile karşılaştırılarak doğrulanmıştır.

Türkiye Halk Sağlığı Kurumu'ndan temin edilen *T. gondii* RH suşuna ait tachyzoitlerden elde edilen genomic DNA, Mn-PCR RFLP kullanılarak genotiplendirilmiştir (Şekil 1). Mn-PCR RFLP metodunda restriksiyon enzimleri ile kesim sonrasında elde edilen ürünlerin agaroz jelde izlenen kesim profilinin, referans suşlar ile kıyaslanarak değerlendirilmesi sonucunda *T. gondii* RH suşunun 3'-SAG2 bölgesi dışında klonal Tip I alleli gösterdiği belirlenmiş, 3'-SAG2 bölgesinin ise tip I/III alleli gösterdiği izlenmiştir (Şekil 1).

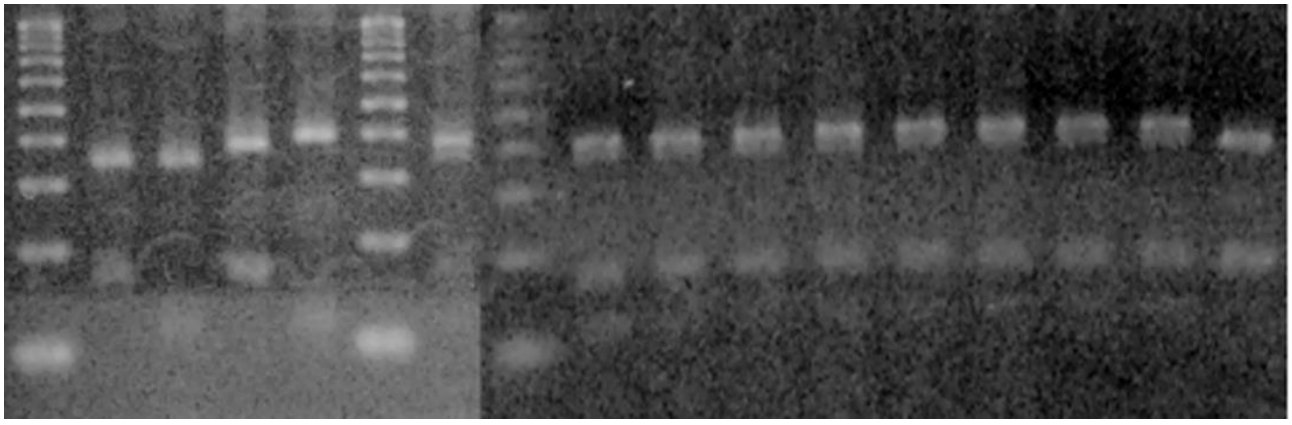
Tablo 1. Koyun orijinli *Toxoplasma gondii* izolatlarının multipleks multilokus nested PCR-RFLP yöntemi kullanılarak genotiplendirilmesinde kullanılan lokuslar, primerler ve restriksiyon enzimleri (37).
Table 1. Loci, primers and restruction enzymes used genotyping of *Toxoplasma gondii* isolates originated from sheep by multiplex multilocus nested PCR-RFLP (37).

Belirteç	Multipleks PCR primerleri (eksternal primerler)	Nested PCR primerleri (internal primerler)	Büyüklük (bp)	Restriksiyon enzimleri	Enzim sindirimi ve elektroforez
C22-8	F: TGATGCATCCATGCGTTTAT R: CCTCCACTTCTTCGGTCTCA	F:TCTCTCTACGTGGACGCC R:AGGTGCTTGGATATTCGC	521	BsmA I, Mbo II	NEB2, BSA, 37 C 30 dak 55 C 30 dak 2.5% jel
L358	F: TCTCTCGACTTCGCCTCTTC R: GCAATTTCCTCGAAGACAGG	F: AGGAGGCGTAGCGCAAGT R: CCCTCTGGCTGCAGTGCT	418	Hae III, Nla III	NEB4, BSA, 37 C 60 dak 2.5% jel
SAG1	F: GTTCTAACCACGCACCCTGAG R: AAGAGTGGGAGGCTCTGTGA	F: CAATGTGCACCTGTAGGAAGC R: GTGGTTCTCCGTCGGTGTGAG	390	Sau961+ HaeII (double digest)	NEB4, BSA, 37 C 60 dak 2.5% jel
alt.SAG2	F: GGAACGCGAACAATGAGTTT R: GCACTGTTGTCCAGGGTTTT	F: ACCCATCTGCGAAGAAAACG R: ATTTCGACCAGCGGGAGCAC	546	Hinfl+Taql, NEB3	BSA, 37 C 60 dak 2.5% jel
5'-SAG2	Gerekli değil. SAG2 external primerleri 5'-SAG2 için gerekli DNA fragmentini içerir.	F: GAAATGTTTCAGGTTGCTGC R: GCAAGAGCGAACTTGAACAC	242	Mbol, NEB4,	BSA, 37 C 1 saat, 2.5% jel
3'-SAG2	F: TCTGTTCTCCGAAGTGACTCC R: TCAAAGCGTGCATTATCGC	F: ATTCTCATGCCTCCGCTTC R: AACGTTTCACGAAGGCACAC	222	Hhal, NEB4	BSA, 37 C 1 saat, 2.5% jel
SAG3	F: CAACTCTCACCATTCCACCC R: GCGCGTTGTTAGACAAGACA	F: TCTTGTCGGGTGTTCACTCA R: CACAAGGAGACCGAGAAGGA	225	NciI, NEB4 NciI+Taq1 (double digest), NEB4	BSA, 37 C 60 dak 2.5% jel
BTUB	F: TCCAAAATGAGAGAAATCGT R: AAATTGAAATGACGGAAGAA	F: GAGGTCATCTCGGACGAACA R: TTGTAGGAACACCCGGACGC	411	BsiEI+TaqI (double digest), NEB4	BSA, 37 C 60 dak 2.5% jel
Apico	F:TGGTTTTAACCCTAGATTG TGG R: AAACGGAATTAATGAGATTT GAA	F: GCAAATTCTTGAATTCTCAGTT R: GGGATTCGAACCCTTGATA	640	Afl II+DdeI (double digest), NEB2	BSA, 37 C 1 saat, 2.5% jel



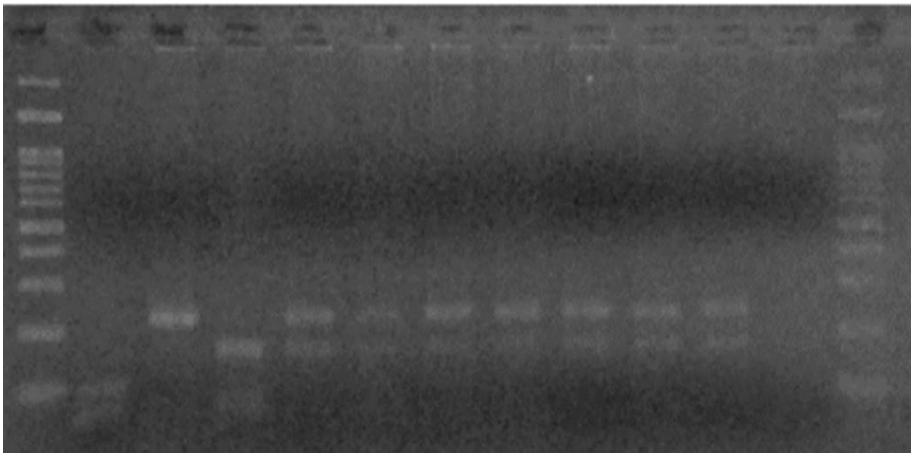
Şekil 1. *Toxoplasma gondii* RH suşuna ait dört lokusun (1.a: SAG1, 1.b:alt. SAG2, 1.c: L358, 1.d: PK1) kullanılarak yapılan multipleks multilokus nested PCR-RFLP sonuçları. GT1, PTG, CTG, TgCgCal, MAS, TgCatBr5, TgCatBr64 ve TGRsCr1, *T. gondii* tip I-III'e ait referans suşlardır. Our: RH suşu (Şekil 1.a, 1.b, 1.c ve 1.d'de en sağda yer almaktadır) tip I içinde yer alan suşlara ait band deseni göstermiştir. M: DNA ladder.

Figure 1. The multiplex multilocus nested PCR-RFLP results of four loci of *Toxoplasma gondii* RH isolate (1.a: SAG1, 1.b: alt.SAG2, 1.c: L358, 1.d: PK1). GT1, PTG, CTG, TgCgCal, MAS, TgCatBr5, TgCatBr64 and TGRsCr1 are reference strains of *T. gondii* clonal type I-III. Our: RH strain had uniform typing band patterns of type 1 strain (in the right column on Figure 1.a, 1.b, 1.c and 1.d). M: Low molecular weight DNA ladder.

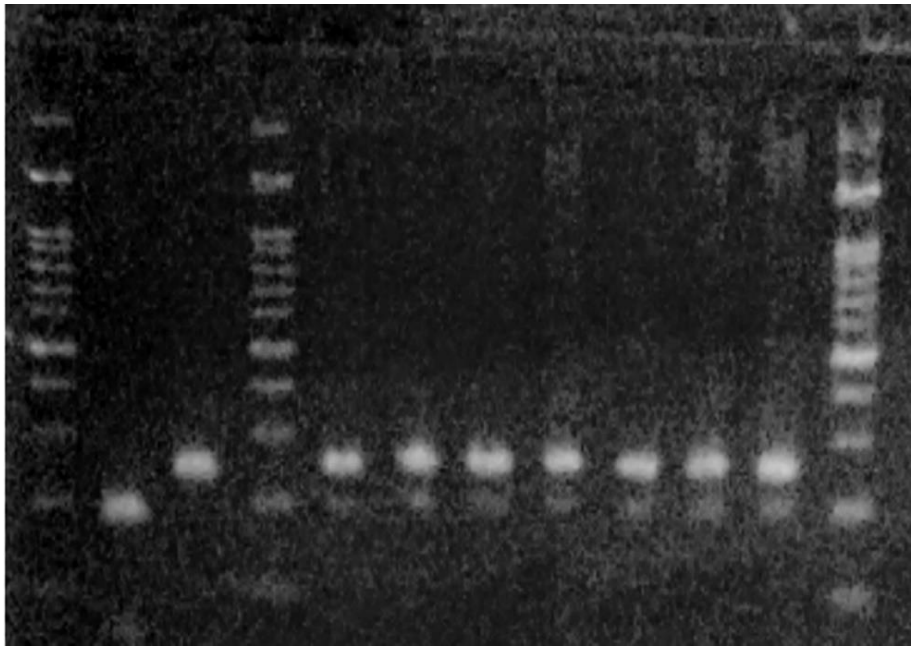


Şekil 2. *Toxoplasma gondii* koyun izolatlarının alt.SAG2 lokusunun multipleks multilokus nested PCR-RFLP sonucu. Soldan sağa sırayla: DNA ladder, CT1, PTG, CTG ve TgcatBr64 referans suşları, DNA ladder, *T. gondii* RH suşu, DNA ladder, 1-8 nolu koyun izolatları.

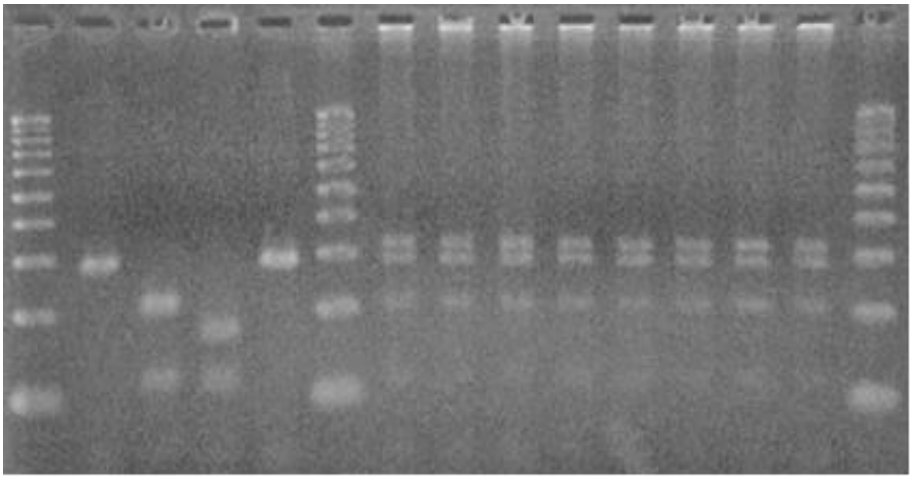
Figure 2. The multiplex multilocus nested PCR-RFLP results of alt.SAG2 locus of *Toxoplasma gondii* sheep isolates. From right to left, low molecular weight DNA ladder, CT1, PTG, CTG and TgcatBr64 reference strains, DNA ladder, *T. gondii* RH strain, DNA ladder, the sheep isolates (no: 1-8).



Şekil 3. *Toxoplasma gondii* koyun izolatlarının SAG3 lokusunun multipleks multilokus nested PCR-RFLP sonucu. Soldan sağa sırayla: DNA ladder, CT1, PTG ve CTG referans suşları, 1-8 nolu koyun izolatları ve DNA ladder.
Figure 3. The multiplex multilocus nested PCR-RFLP results of SAG3 locus of *Toxoplasma gondii* sheep isolates. From right to left, low molecular weight DNA ladder, CT1, PTG and CTG reference strains, the sheep isolates (no: 1-8) and DNA ladder.



Şekil 4. *Toxoplasma gondii* koyun izolatlarının 5'-SAG2 lokusunun multipleks multilokus nested PCR-RFLP sonucu. Soldan sağa sırayla, DNA ladder, CTG referans suşu, *T. gondii* RH suşu, DNA ladder, 1-7 nolu koyun izolatları ve DNA ladder.
Figure 4. The multiplex multilocus nested PCR-RFLP results of 5'-SAG2 locus of *Toxoplasma gondii* sheep isolates. From right to left, low molecular weight DNA ladder, CTG reference strain, *T. gondii* RH strain, DNA ladder, the sheep isolates (no: 1-7) and DNA ladder.



Şekil 5. *Toxoplasma gondii* koyun izolatlarının L358 lokusunun multipleks multilokus nested PCR-RFLP sonucu. Soldan sağa sırayla, DNA ladder, CT1, PTG ve CTG referans suşları, *T. gondii* RH suşu, DNA ladder, koyuna ait 1-8 nolu örnekler ve DNA ladder.
Figure 5. The multiplex multilocus nested PCR-RFLP results of L358 locus of *Toxoplasma gondii* sheep isolates. From right to left, low molecular weight DNA ladder, CT1, PTG and CTG reference strains, *T. gondii* RH strain, DNA ladder, sheep isolates (no: 1-8) and DNA ladder.

Tablo 2. Koyun dokularından elde edilen *Toxoplasma gondii* genomik DNA'ları (n: 10) ile *T. gondii* RH suşunun multipleks multilokus nested PCR-RFLP sonuçları.
Table 2. The multiplex multilocus nested PCR-RFLP results obtained from genomic DNA of *Toxoplasma gondii* originated from sheep (n:10) and *T. gondii* RH strain.

İzolat	Genotiplendirmede kullanılan lokuslar							
no.	SAG1	5'-SAG2	3'SAG2	alt.SAG2	SAG3	BTUB	c22-8	L358
RH suşu	I	I	I/III	I	I	I	I	I
1	PCR -	I+III	I/III	II+III	II+III	PCR -	II	I+II
2	PCR -	I+III	I/III	II+III	II+III	PCR -	II	I+II
3	PCR -	I+III	I/III	II+III	II+III	PCR -	II	I+II
4	PCR -	I+III	I/III	II+III	II+III	PCR -	II	I+II
5	PCR -	I+III	I/III	II+III	II+III	PCR -	II	I+II
6	PCR -	I+III	I/III	II+III	II+III	PCR -	II	I+II
7	PCR -	I+III	I/III	II+III	II+III	PCR -	II	I+II
8	PCR -	I+III	I/III	II+III	PCR -	PCR -	II	I+II
9	PCR -	III değil	I/III	I	PCR -	I+II	II	I
10	III	III değil	I/III	III	III	I+II	II	II

Koyundan elde edilen genomik DNA'ların (n:10) Mn-PCR RFLP sonuçları Tablo 2'de görülmektedir. Koyun orijinli *T. gondii* izolatlarının birden fazla klonal tip özelliği gösterdiği belirlenmiştir. *Toxoplasma gondii* izolatları (n:8) iki lokusta (alt.SAG2 ve SAG3) tip II ve III (Şekil 2 ve 3), bir lokusta (c22-8) klonal tip II, bir lokusta (5'-SAG2) tip I ve III (Şekil 4), bir lokusta (L358) tip I ve II, bir lokusta (3'-SAG2) ise tip I/III alleli göstermiştir (Şekil 5). Bu izolatlarda SAG1 ve BTUB lokusları PCR ile amplifiye edilememiştir (Tablo 2).

Bir *T. gondii* izolatı iki lokusta (alt.SAG2 ve L358) tip I, bir lokusta (c22-8) tip II, bir lokusta (BTUB) tip I ve II, bir lokusta (3'-SAG2) ise I/III alleli göstermiş, SAG1 ve SAG3 lokusları PCR ile amplifiye edilememiştir (Tablo 2).

Bir *T. gondii* izolatı ise üç lokusta (SAG1, alt.SAG2 ve SAG3) tip III, 2 lokusta (c22-8 ve L358) tip II, bir lokusta (3'-SAG2) tip I/III, bir lokusta (BTUB) ise tip I ve II allelleri göstermiş, 5'-SAG2 lokusu PCR ile amplifiye edilememiştir (Tablo 2). PK1 lokusu koyun orijinli genomik DNA örneklerinin hiçbirinden (n:10) amplifiye edilememiştir.

Tartışma ve Sonuç

Dünya üzerinde farklı ülkelerde *T. gondii* izolatları multilokus enzim elektroforezi, PCR RFLP veya satellit analizi gibi yöntemler kullanılarak genotiplendirilmektedir (2, 14, 22-24, 29, 38). PCR RFLP kullanılarak genotiplendirme yapılabilmesi için DNA ekstraksiyonu yapılacak örneğin yeterli miktarda parazit DNA'sını içermesi gerektiği ifade edilmiştir (6). *Toxoplasma gondii* ile ilgili olarak PCR RFLP yöntemi kullanılarak yapılan genotiplendirme çalışmalarında doğal enfekte konak dokularından elde edilen DNA'ların tümünün sonuç vermediği bildirilmiştir (6). PCR RFLP yöntemi ile sığır ve domuz orijinli genomik DNA'da *T. gondii* izolatlarının sadece bir ya da iki lokusu amplifiye edilebilmiş, aynı lokusta birden çok allel barındıran izolatların gözlenmesi ise araştırıcılar tarafından konağın farklı genotiplerdeki *T. gondii* ile enfekte olduğu şeklinde yorumlanmıştır (6). Ayrıca PCR RFLP sonucunda bir dizi lokusta farklı allellerin gözlendiği örnekler ise atipik *T. gondii* ile enfeksiyonun göstergesi olarak ileri sürülmüştür (6). Bu çalışmada, PCR RFLP yöntemi ile *T. gondii*'ye ait dokuz lokusun hepsi tüm koyun izolatlarında amplifiye edilememiştir. Bu durumun sebebinin doğal enfekte koyunlardan elde edilen genomik DNA içinde *T. gondii*'ye ait DNA miktarının az olması olabileceği düşünülmüştür. Ayrıca koyun kaynaklı *T. gondii* suşlarının bir dizi lokusta birden çok allel barındırdığı tespit edilmiştir. Bu koyunların genotipik olarak farklı *T. gondii* suşları ile enfekte olduğu düşünülmüştür.

İsviçre'de koyun diyafram örneklerinden elde edilen genomik DNA, PCR-RFLP ile genotiplendirildiğinde koyunlarda klonal tip II alellerinin baskın olduğu anlaşılmıştır, buna karşılık örneklenen koyunlarda miks ya da atipik alel kombinasyonları nadir görülmüştür (6). İsviçre'de sığırların tip I ya da II, atipik *T. gondii* ya da birden fazla tip ile enfekte olduğu bildirilmiştir (6). Benzer raporlar İngiltere (26), Fransa (15, 17) ve Danimarka (20) gibi diğer ülkelerden de kaydedilmiştir. İran'da koyun, sığır ve tavuk etlerinden elde edilen *T. gondii* izolatlarının SAG2 lokusu kullanılarak yapılan genotiplendirmesi sonucunda tip I özelliğinde olduğu bildirilmiştir (24). Hindistan'da SAG3 lokusu kullanılarak genotiplendirilen koyun ve keçi izolatlarının ise tip II ve tip III'e ait olduğu belirlenmiştir (24, 29). *T. gondii*'nin genotiplendirilmesinde sadece bir genetik lokus kullanılması (24, 29) elde edilen sonuçların güvenilirliği tartışmalıdır. Bu çalışmada ise koyun orijinli *T. gondii* izolatlarına ait dokuz lokus Mn-PCR RFLP ile amplifiye edilmiş ve koyunların birden fazla tip (I, II ve III) *T. gondii* ile enfekte olduğu tespit edilmiştir.

Türkiye'de *T. gondii'*nin genotiplendirilmesine ilişkin sınırlı sayıda bilgi görülmektedir (9, 11, 21). Yenidoğan kongenital toksoplasmosisli bebeklerden izole edilen Ankara ve Ege-1 olarak adlandırılan iki izolatının mikrosatellit analizi sonucunda Afrika 1 genotipinde yer aldığını belirlenmiştir (11). İzmir'de 22 sokak kedisi izolatının mikrosatellit analizi sonucunda 19'unun tip I, ikisinin tip II ve birinin ise Afrika 1 genotipinde olduğu bildirilmiştir (9). İzmir'de yabani kuşlardan elde edilen 14 izolat mikrosatellit markerı kullanılarak genotiplendirilmiş ve sekiz izolatın tip II, üçünün tip III, ikisinin tip II/II ve birinin ise tip II/III olduğu görülmüştür (21). Bu çalışmada ise koyunlardan elde edilen izolatların dokuz lokusunun Mn PCR RFLP yöntemi ile analizi sonrasında birden fazla tipte (I, II ve III) *T. gondii* genotipine sahip olduğu tespit edilmiştir.

Doğada *T. gondii*'nin farklı tiplerinde seksüel rekombinasyon nadiren görülmektedir. Yaşam çemberinde son konağın bağırsağında *T. gondii*'nin tek bir tipin kendi içinde eşeyli çoğalması sonucunda (self-mating) ürediği farz edilmektedir. Bu iddia hayvan ve insanlardan elde edilen *T. gondii* izolatlarının büyük oranda üç klonal yapıya ait olması ile açıklanmaktadır (4, 10). Ancak moleküler genotiplendirme yöntemleri kullanılarak yapılan çalışmalar arttıkça özellikle de Güney Amerika ve Asya'da bu klonal tiplerden farklı özellik gösteren, "atipik" ya da "ekzotik" olarak adlandırılan *T. gondii* izolatları rapor edilmektedir (13, 23, 30, 37). Atipik genotiplerin, sonkonak kedinin birden fazla tiple enfekte olmuş arakonağı yemesi sonucu ortaya çıktığı düşünülmektedir. Böyle bir durumda, farklı tipteki *T. gondii* kedi bağırsağında seksüel olarak rekombine olabilmekte ve sonucunda iki ebeveyn genotipinin karışımını gösterecek yeni bir nesil oluşmaktadır (23, 28, 30, 35). Bu durum hem birden fazla genotiple deneysel enfekte kedilerde (4, 10) hem de doğal enfekte kedilerde izlenmiştir (18). Doğal enfekte kedilerden elde edilen *T. gondii* oocystlerinin PCR RFLP ile genotiplendirilmesi sonucunda pek çok lokusta tip I ve tip II spesifik allellerin karışımını içeren band deseni gözlendiği bildirilmiştir (16). Doğada farklı parazit genotipleri arasında eşeyli çoğalma sonucunda daha fazla *T. gondii* suşu oluşma potansiyeli vardır ve bu durum gerek insan gerekse hayvanlar için virulensi artan, yeni biyolojik özelliklere sahip parazit suşlarını ortaya çıkarabilir (16). Ayrıca *T. gondii*'nin pek çok canlıyı arakonak olarak kullanabilmesi, ayrıca hem silvatik hem de evcil yaşam çemberine sahip olması nedeniyle farklı tipte yeni parazit suşları şekillenebilir (16). Bu çalışmada da koyundan izole edilen *T. gondii* izolatları pekçok lokusta tip I, II ve III spesifik alellerinin karışımını içeren band deseni göstermiştir. Çiftlik hayvanları ile iç içe yaşayan kedi hem arakonak canlılara paraziti bulaştırabilmekte hem de arakonak canlıların enfekte dokularına kolaylıkla ulaşabilmektedir. Dünyanın farklı coğrafi bölgelerinde yaşayan ve parazite arakonaklık yapan çiftlik hayvanları aracılığıyla farklı genotipteki *T. gondii'*nin Türkiye'ye girebilme ihtimali vardır. Arakonak canlıların dokularındaki farklı genotipteki parazitlerin yenilmesi sonucunda kedilerde yeni atipik suşların gelişmiş olmasından dolayı bu çalışmada koyun dokularından izole edilen *T. gondii* izolatlarının birden fazla tip özelliği gösterdiği düşünülmüştür.

Sonuç olarak, *T. gondii* önemli bir gıda kaynaklı parazittir (12). Türkiye'de insanlar için gıda kaynaklı toksoplasmosisin önemli kaynaklarından birisinin koyunlar olduğu düşünülmektedir. Genotiplendirme ile insanların toksoplasmosisle hangi yolla enfekte olabildiğine dair bilgi sağlanabilir. Buna ilişkin yapılan bir çalışmada aynı bölgede yaşayan insan ve hayvanlarda benzer *T. gondii* genotipleri bulunduğu bildirilmiştir (34). Bu çalışma Türkiye'de koyun orijinli *T. gondii* izolatlarında ilk genotiplendirme çalışmasıdır. Genotiplendirmeye yönelik olarak parazite arakonaklık yapan farklı hayvanlardan ve çeşitli bölgelerden elde edilen izolatlar üzerinde çalışılması Türkiye'de *T. gondii*'nin bulaşma yolları ve populasyon yapısı hakkında önemli bilgiler elde etmeye katkı sağlayacaktır.

Teşekkür

Genotiplendirmede kullanılan referans DNA'lar Tennessee Üniversitesi Mikrobiyoloji Departmanı'nda çalışan Dr. Chunlei SU'dan temin edilmiştir. Bu çalışma Kırıkkale Üniversitesi Bilimsel Araştırmalar Koordinasyon Birimi tarafından desteklenmiştir (Proje numarası: 2013-73).

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Ankara Üniv Vet Fak Derg, **66**, 281-288, 2019
DOI: 10.33988/auvfd.475104

Presence of *Listeria* species in ready-made meatballs offered by sale under freezing or cooling preservation

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Received date: 26.10.2018- Accepted date: 26.04.2019

Abstract: This study was conducted to detect contamination level of *Listeria* species in ready-made meatballs kinds that are stored under frozen or cooled conditions. In isolations and identifications of *Listeria* species from the samples, method approved and suggested by USDA/FSIS (United States Department of Agriculture/Food Safety and Inspection Service) was used. The strains that were identified to be *Listeria monocytogenes* with biochemical tests was verified as species through Real Time PCR method by using a primary pair specific to *hly A* gene location. In this study, a total number of 290 different type ready-made meatball samples were analysed. As a result of examining all samples was isolated *L. monocytogenes* in 32 (11.04%) samples, *L. ivanovii* in 9 (3.10%) samples, *L. innocua* in 22 (7.59%) samples, *L. welchimerii* in 8 (2.76%) samples and also *L. seeligeri* in 4 (1.38%) samples. In the serotyping of the 32 *L. monocytogenes* strains isolated from the samples; 15 isolated are found to be Type 1, where 3 strains are found to be Type 4, 11 strains to be type Poly and the rest 3 strains could not typified. The Mean pH and water activity values for the samples were found to be 6.62±0.56 and 0.985±0.007 respectively. In the result of the study, identifying *Listeria* species especially *L. monocytogenes* in cooled and frozen ready-made meatball samples studied suggest that such products whose consumption increased in the recent years pose important risk in terms of public health.

Keywords: *Listeria monocytogenes*, *Listeria* species, meatball, PCR.

Dondurma veya soğutma ile muhafaza edilerek satışa sunulan hazır köftelerde *Listeria* türlerinin varlığı

Özet: Bu çalışma, kasap, şarküteri ve süpermarketlerde dondurulmuş veya soğutulmuş olarak muhafaza edilen ve satışa sunulan hazır köfte çeşitlerinde *Listeria* türlerinin kontaminasyon düzeyini ortaya koymak amacıyla yapılmıştır. Örneklerden *Listeria* türlerinin izolasyon ve identifikasyonunda USDA/FSIS (United States Department of Agriculture/Food Safety and Inspection Service) tarafından önerilen yöntem kullanılmıştır. Biyokimyasal testlerle *L. monocytogenes* olarak identifiye edilen suşların *hly A* gen bölgesine spesifik bir primer çifti kullanılarak Real Time PCR yöntemiyle tür olarak doğrulaması yapılmıştır. Çalışmada toplam 290 adet hazır köfte örneği analize alınmıştır. Tüm örneklerin 32 (%11.04) tanesinden *L. monocytogenes*, 9 (%3.10) tanesinden *L. ivanovii*, 22 (%7.59) tanesinden *L. innocua*, 8 (%2.76) tanesinden *L. welchimeri* ve 4 (%1.38) tanesinden de *L. seeligeri* izole edilmiştir. Yapılan serotiplendirmede izole edilen 32 adet *L. monocytogenes* türünden 15 tanesi Tip 1, 3 tanesi Tip 4 ve 11 tanesi de Tip Poli olarak tanımlanmış, 3 izolat ise tiplendirilememiştir. Tüm örneklerde ortalama pH değeri 6.62±0.56 olarak bulunurken, su aktivitesi değeri ise ortalama 0.985±0.007 olarak saptanmıştır. Çalışma sonucunda incelenen örneklerde önemli oranlarda *Listeria* türlerinin, özellikle de *L. monocytogenes*'in tespit edilmesi, son yıllarda tüketimi gittikçe artan bu ürünlerin halk sağlığı açısından önemli bir risk oluşturabileceğini göstermektedir.

Anahtar sözcükler: Köfte, *Listeria monocytogenes*, *Listeria* türleri, PCR.

Introduction

Ready-made meatballs produced from red meat and poultry meat have become an ever increasing significant group of food in the recent years in parallel to the developments made in ready-made and semi-ready food industry. If these meatballs where various production techniques and different ingredients are used in production process are not going to be consumed immediately, they should be subjected to cold chain or commonly stored under frozen conditions (1, 19, 26).

Ready-made meatballs are often contaminated with microorganisms due to hygiene-related mistakes made during their production. Even if the meatballs are preserved by cooling or freezing once they are produced,

these pathogens, especially the ones with psychrophilic properties, are able to sustain their lives. If the cold chain is broken before the time the product is consumed, it may lead to a rapid multiplication of such pathogens, causing significant risks for the public health (8, 18, 19). Preservation by cooling and freezing are effective methods to limit the reproduction of pathogenic and non-pathogenic microorganisms in food. That being said, some psychrophilic microorganisms like *L. monocytogenes* are still able to multiply in cold-stored food and may cause infections (16, 19).

Various sources like additives, tools, equipment, personnel, water, environment, cross-contamination, and refrigerators may play a role in contamination of ready-made meatballs with *Listeria* species (11, 12, 18). Many studies in the past have revealed the fact that refrigerated or frozen ready-made meatballs offered to market are contaminated with substantial amounts of pathogenic and non-pathogenic microorganisms (8, 19, 24, 28, 29). This study aims to reveal the presence and prevalence of *Listeria* species especially that of *L. monocytogenes* in the refrigerated or frozen ready-made meatballs put up for sale in butchers, charcuteries and supermarkets in Van city (Turkey).

Materials and Methods

In this study, a total number of 290 ready-made meatball samples were analysed namely as follows; 50 pieces of Inegol kofte, 40 pieces of hamburger kofte; and 20 pieces each of; Tekirdag kofte, Akcaabat kofte, Adana kofte, Tire kofte, Izmir kofte, kasap kofte, cızbız kofte, odun kofte, satır kofte, and ızgara kofte (Kofte is a word in Turkish language and it is the counterpart of meatball in English language. Sample names were kept in their original marketed names). The samples were selected and collected by using simple randomly sampling method from the refrigerated or frozen ready-made meatballs offered by sale in butchers, charcuteries and supermarkets in Van city (Turkey) during January 2015-August 2015. All samples taken for analysis were consisted of the products of companies producing and selling throughout the country. The samples were collected 200 grams minimum in weight (about 10 samples each week), and they were transported to the laboratory within 2 hours max after collection in cold chain (+4 ºC), either in their original packaging or in aseptic sterile jars. Production dates, expiry dates and product batch numbers of samples of the same brand or same type were checked to ensure that they did not belong to the same production group.

Physicochemical analyses: The pH values of the samples were measured using calibrated pH-meters (Hanna® PH 221, Hanna Instruments, Italy), while their water activity was measured using a calibrated a_w device (Novasina® MS 1 Set, Switzerland).

Isolation and identification of Listeria species: For this purpose, pre-enrichment of the samples was conducted in *Listeria* Primary Selective Enrichment Broth (Oxoid CM863+SR142) where their selective enrichment was conducted in *Listeria* Secondary Selective Enrichment Broth (Oxoid CM863+SR143). From both of the enrichment broths, inoculation by a colony each is made by streaking method from *Listeria* Selective Agar (LSA) (Oxoid CM856+SR140). Typical colonies in 1-3 mm diameter with grey-brown colour surrounded by black colour are evaluated as *Listeria*-suspicious (9, 15).

The colonies growing in the LSA were purified on the Tryptone Soy Agar (containing 0.6% Yeast Extract) and identification of purified colonies were made by subjected to Henry's Oblique Illumination Test and other conventional tests of identification (Gram staining, catalase, oxidase, urea, motility, methyl red/Voges-Proskauer, β-hemolysis, CAMP, nitrate and glucose, sorbitol, D-mannitol, L-rhamnose, D-xylose fermentation) (14, 20, 22).

Confirmation of identified *L. monocytogenes* species with PCR: In order to confirm *L. monocytogenes* species with PCR, the modified method developed by Aznar and Alarcón's (6) from Border et al. (7) was used. In this purpose, in order to detect *hly A* gene in the samples, specific primary pair to this zone (LMF: CCTAAGACGCCAATCGAA; LMR: AAGCGCTTG CAACTGCTC) and instant commercial master mix (GeneAll®, Real. Amp™ SYBR qPCR Master Mix, Korea) was used. From the colonies identified as *L. monocytogenes*, DNA extraction was made by using commercial kits (GeneAll®, Exgene™ Cell SV). PCR tubes prepared separately for each *Listeria*-suspicious DNA extract are placed in real-time PCR device (Rotor GeneTM 6000 Corbett Research, Australia) and upon denaturation phase for 30 sec in 94 ºC, 45 sec of bonding in 55.5 ºC, 45 sec of extension in 72 ºC and 5 min of final extension in 72 ºC phases, totally 35 cycle of PCR amplification is applied. In the results of the application, locations of positive controls and negative control samples in graph were investigated.

Serological tests: In order to determine the serotypes of *L. monocytogenes* strains, was made aglutination test with commercial antiseras (BD Difco *Listeria* O Antisera Type 1, 4 and Poly) (4).

***Reference strains*:** The *L. monocytogenes*, *Staphylococcus aureus,* and *Rhodococcus equii* strains used in the research were obtained from Etlik Central Veterinary Control & Research Institute (Ankara, Turkey).

Statistical analysis: In this study, samples were selected and collected by using simple randomly sampling method. As the mean pH values determined in 12 different ready-made meatballs varieties showed normal

distribution in Kolmogorov-Smirnov test, one-way analysis of variance was applied for detection of statistically relation of between mean pH values of different kofte groups. The Duncan multiple comparison test was used to determine the significant differences as a result of the variance analysis. Since the a_w values determined in 12 different meatball groups did not show normal distribution as a result of Kolmogorov-Smirnov test, Kruskal-Wallis and Mann-Whitney tests were applied. Pearson correlation analysis was used to determine the relationship between pH and water activity (a_w) in each type of meatballs. The statistical analysis was performed using SPSS 23 statistical software program (2).

Results

Listeria species isolated from ready-made meatball samples and PCR verification with serotype distribution of *L. monocytogenes* isolated are given in Table 1 and Figure 1. Taking all the samples into consideration, *L. monocytogenes* is found to have the highest isolation rate (11.04%) and it is followed by *L. innocua* (7.59%), *L. ivanovii* (3.10%), *L. welchimeri* (2.76%) and *L. seeligeri* (1.38%) respectively. Table 2, Table 3 and Table 4 represents the levels of statistical significances of the ready-made meatball sample groups' and sub-groups' pH and water activity differences.

Table 1. The distribution of *Listeria* species in the samples and the serotyping of *L. monocytogenes* isolates.
Tablo 1. Örneklerde belirlenen *Listeria* türlerinin dağılımı ve *L. monocytogenes*'in serotiplendirilmesi.

Sample type	Meat species	n	Numbers of positive samples						Serotypes of *L. monocytogenes*			
			Listeria spp. positive samples	*L. monocytogenes*	*L. ivanovii*	*L. innocua*	*L. welchimeri*	*L. seeligeri*	Tip 1	Tip 4	Tip poli	Not typed
Inegol kofte	Chicken (F)	10	3 (30%)	2 (20%)	ND	**1** (10%)	**1** (10%)	ND	1 (10%)	-	1 (10%)	-
	Turkey (F)	10	3 (30%)	1 (10%)	1 (10%)	ND	1 (10%)	ND	1 (10%)	-	-	-
	Beef (C)	10	3 (30%)	ND	1 (10%)	2 (20%)	ND	ND	-	-	-	-
	Beef (F)	10	0 (0%)	ND	ND	ND	ND	ND	-	-	-	-
	Beef+Lamb (F)	10	2 (20%)	1 (10%)	ND	1 (10%)	ND	ND	-	-	1 (10%)	-
	Total samples	50	11 (22%)	4 (8%)	2 (4%)	4 (8%)	2 (4%)	0 (0%)	2 (4%)	0 (0%)	1 (2%)	0 (0%)
Tekirdag kofte	Beef (F)	20	5 (25%)	3 (15%)	**1** (5%)	**2** (10%)	ND	ND	1 (5%)	-	1 (5%)	1 (5%)
Akcaabat kofte	Beef (F)	20	4 (20%)	2 (10%)	1 (5%)	1 (5%)	ND	ND	2 (10%)	-	-	-
Adana kofte	Beef (F)	20	3(15%)	1 (5%)	ND	ND	2 (10%)	ND	-	-	1 (5%)	-
Tire kofte	Beef (F)	20	7 (35%)	3 (15%)	2 (10%)	**2** (10%)	**1** (5%)	ND	2 (10%)	1 (5%)	-	-
Izmir kofte	Beef (F)	20	6(30%)	3 (15%)	2 (10%)	ND	ND	1 (5%)	1 (5%)	-	2 (10%)	-
Hamburger kofte	Beef (F)	10	1 (10%)	1 (10%)	ND	ND	ND	ND	1 (10%)	-	-	-
	Beef (C)	10	2 (20%)	1 (10%)	ND	1 (10%)	ND	ND	1 (10%)	-	-	-
	Chicken (F)	10	1 (10%)	ND	ND	**1** (10%)	ND	**1** (10%)	-	-	-	-
	Chicken (C)	10	3 (30%)	2 (20%)	ND	1 (10%)	ND	ND	-	1 (10%)	-	1 (10%)
	Total samples	40	7 (17.5%)	4 (10%)	0 (0%)	3 (7.5%)	0 (0%)	1 (2.5%)	2 (5%)	1 (2.5%)	0 (0%)	1 (2.5%)
Butcher kofte	Beef (F)	10	2 (20%)	1 (10%)	1 (10%)	ND	ND	ND	1 (10%)	-	-	
	Beef (C)	10	3 (30%)	**1** (10%)	ND	**3** (30%)	ND	ND	-	-	1 (10%)	
	Total samples	20	5 (25%)	2 (10%)	1 (5%)	3 (15%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (5%)	0 (0%)
Cızbız kofte	Beef+Lamb (C)	10	3 (30%)	1 (10%)	ND	2 (20%)	ND	ND	-	-	1 (10%)	-
	Beef (F)	10	4 (40%)	1 (10%)	ND	2 (20%)	1 (10%)	ND	1 (10%)	-	-	-
	Total samples	20	7 (35%)	2 (10%)	0 (0%)	4 (20%)	1 (5%)	0 (0%)	1 (5%)	0 (0%)	1 (5%)	0 (0%)
Odun kofte	Beef (F)	20	5 (25%)	4 (20%)	ND	ND	ND	1 (5%)	1 (5%)	1 (5%)	1 (5%)	1 (5%)
Satır kofte	Beef (F)	20	2 (10%)	**2** (10%)	ND	ND	**2** (10%)	ND	-	-	2 (10%)	-
Izgara kofte	Beef (F)	20	5 (25%)	**2** (10%)	ND	3 (15%)	ND	**1** (5%)	2 (10%)	-	-	-
Total positive samples		**290**	**97 (33.45%)**	**32 (11.04%)**	**9 (3.10%)**	**22 (7.59%)**	**8 (2.76%)**	**4 (1.38%)**	**15 (5.17%)**	**3 (1.04%)**	**11 (3.79%)**	**3 (1.04%)**

F: Frozen, C: Cooled, ND: Not Detected, The bold numbers in the same column indicate that two different species were isolated from a single sample

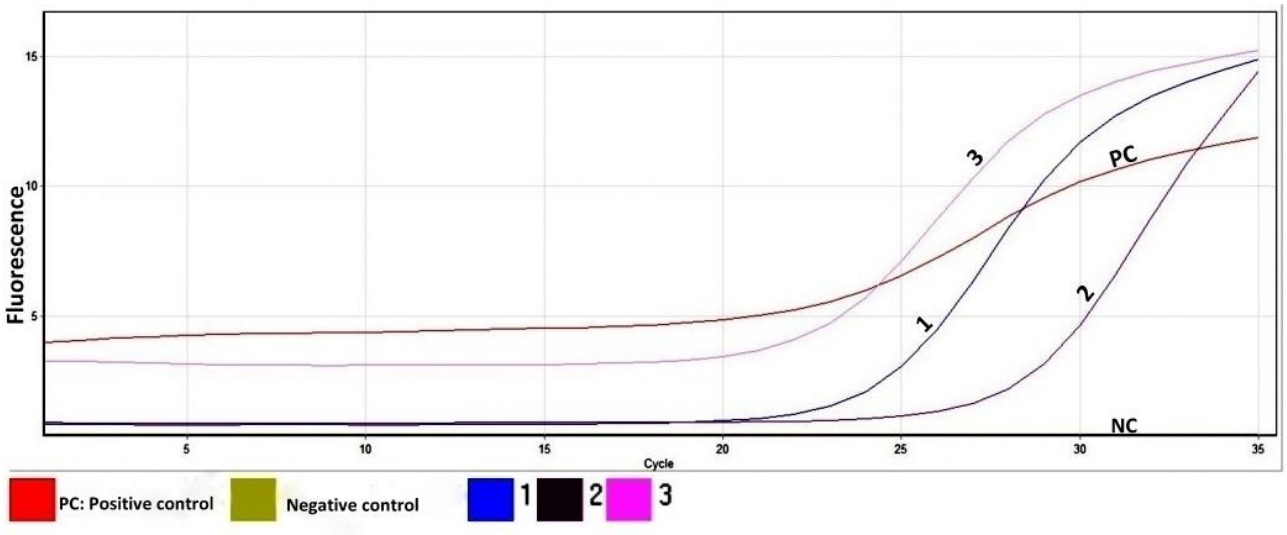

Figure 1. The graphics of PCR amplification of DNA extracts obtained from positive control, negative control, and some *L. monocytogenes* strains isolated from samples.
Şekil 1. Örneklerden izole edilen bazı *L. monocytogenes* suşları ile pozitif ve negative kontrolden elde edilen DNA ekstraktlarının PCR amplifikasyon grafikleri.

Table 2. The mean pH values of sample groups and the statistical significance of the differences in the between of mean pH value of the different types samples within the same sample groups and between the different sample groups.
Tablo 2. Örnek gruplarında belirlenen ortalama pH değerleri ve farklı örnek grupları arasındaki ve örnek grupları içindeki farklı tip hazır köfte örneklerinin ortalama pH değeri farklılıklarının istatistiksel önem dereceleri

Sample name	Meat and preservation type	n	pH
			Mean±SE
Inegol kofte	Chicken/Freezing	10	6.761 ± 0.047^{C}
	Turkey/Freezing	10	7.076 ± 0.093^{B}
	Beef/Cooling	10	6.564 ± 0.120^{xC}
	Beef/Freezing	10	7.426 ± 0.052^{aA}
	Beef +Lamb/Freezing	10	7.118 ± 0.089^{B}
Tekirdag kofte	Beef/Freezing	20	7.123 ± 0.079^{b}
Akcaabat kofte	Beef/Freezing	20	6.077 ± 0.046^{e}
Adana kofte	Beef/Freezing	20	6.471 ± 0.072^{d}
Tire kofte	Beef/Freezing	20	6.723 ± 0.061^{c}
Izmir kofte	Beef/Freezing	20	7.094 ± 0.052^{b}
Hamburger kofte	Beef/Freezing	10	6.013 ± 0.117^{efA}
	Beef/Cooling	10	6.206 ± 0.071^{yA}
	Chicken/Freezing	10	6.241 ± 0.087^{A}
	Chicken/Cooling	10	6.213 ± 0.099^{A}
Kasap kofte	Beef/Freezing	10	6.343 ± 0.128^{dA}
	Beef/Cooling	10	6.366 ± 0.099^{yA}
Cızbız kofte	Beef +Lamb/Cooling	10	6.149 ± 0.115^{A}
	Beef/Freezing	10	6.108 ± 0.126^{eA}
Odun kofte	Beef/Freezing	20	7.078 ± 0.052^{b}
Satır kofte	Beef/Freezing	20	5.840 ± 0.069^{f}
Izgara kofte	Beef/Freezing	20	7.262 ± 0.037^{ab}

The difference between the means marked with different small letters in the same column are statistically significant (P<0.05) (for the groups of frozen beef meatball). The difference between the means marked with different capital letters in the same column are statistically significant (P<0.05) (for different types of samples within Inegol kofte, hamburger kofte, kasap kofte and cızbız kofte groups).
The difference between the means marked with different x, y, z letters in the same column are statistically significant (P<0.05) (for the groups of cooled beef meatball).

Table 3. The statistical significance of the differences in the mean rank a_w values of ready-made beef meatballs groups offered by sale under cooling or freezing preservation.
Tablo 3. Soğukta veya dondurarak muhafaza altında satışa sunulan hazır sığır eti kofte örneği gruplarının ortalama rank a_w değerlerinin farklarının istatistiksel önem dereceleri.

Sample name	Meat and preservation type	n	a_w
			Mean rank
Inegol kofte	Beef/Cooling	10	16.450^{xy}
	Beef/Freezing	10	38.350^{e}
Tekirdag kofte	Beef/Freezing	20	63.300^{ed}
Akcaabat kofte	Beef/Freezing	20	46.800^{ed}
Adana kofte	Beef/Freezing	20	75.280^{cd}
Tire kofte	Beef/Freezing	20	119.850^{c}
Izmir kofte	Beef/Freezing	20	80.880^{bc}
Hamburger kofte	Beef/Freezing	10	153.550^{ab}
	Beef/Cooling	10	10.050^{y}
Kasap kofte	Beef/Freezing	10	145.550^{ab}
	Beef/Cooling	10	20.000^{x}
Cızbız kofte	Beef/Freezing	10	63.950^{ab}
Odun kofte	Beef/Freezing	20	110.130^{bc}
Satır kofte	Beef/Freezing	20	164.030^{a}
Izgara kofte	Beef/Freezing	20	144.050^{ab}

The difference between the mean ranks marked with different small letters in the same column are statistically significant (P<0.05) (for the groups of frozen beef meatball). The difference between the mean ranks marked with different x, y, z letters in the same column are statistically significant (P<0.05) (for the groups of cooled beef meatball).

Table 4. The statistical significance of the differences of mean rank a_w values within the group in the ready-made meatball sample groups offered for sale under cooling or freezing preservation.
Tablo 4. Soğukta veya dondurarak muhafaza altında satışa sunulan hazır kofte örneği gruplarında, grup içi ortalama rank a_w değerlerinin farklarının istatistiksel önem dereceleri

Sample name	Meat and preservation type	n	a_w
			Mean rank
Inegol kofte	Chicken/Freezing	10	20.450^{c}
	Turkey/Freezing	10	38.400^{a}
	Beef/Cooling	10	29.250^{b}
	Beef/Freezing	10	11.500^{d}
	Beef +Lamb/Freezing	10	27.900^{b}
Hamburger kofte	Beef/Freezing	10	32.400^{a}
	Beef/Cooling	10	21.000^{b}
	Chicken/Freezing	10	16.650^{bc}
	Chicken/Cooling	10	11.950^{c}
Kasap kofte	Beef/Freezing	10	12.220^{a}
	Beef/Cooling	10	8.800^{a}
Cızbız kofte	Beef +Lamb/Cooling	10	8.400^{a}
	Beef/Freezing	10	12.400^{a}

The difference between the mean ranks marked with different small letters in the same column are statistically significant (P<0.05).

Discussion and Conclusion

Meatballs represent an important group of ready-made foods. They are produced using minced meat, oil, various spices and filling materials, and are offered for sale either in raw form or after undergoing thermal semi-processing in oil. *Listeria* species, and especially the pathogenic species of *L. monocytogenes*, are readily encountered in meatballs, and cooling or freezing process doesn't affect their presence in foods drastically (16, 18, 19, 21, 27).

Many studies conducted in Turkey have displayed that hygienic quality in ready-made meatballs and other

similar products show variability and that these products can be contaminated with various pathogenic microorganisms (18, 24, 29).

Of the 290 meatball samples in this study, *Listeria* spp. was isolated from 97 (33.45%) of them, while *L. monocytogenes* was isolated from 32 (11.04%) of those (Table 1). These results are concordant to other studies in the literature which report very low hygienic quality for meatballs (8, 18, 24, 29).

When the results are inspected, it can be seen that *Listeria* spp. was isolated from all the samples, except for the Inegol kofte made from beef which were preserved by freezing. It can also be seen that *L. monocytogenes* was isolated from all the samples except for the Inegol kofte made out of beef, and hamburgers made out of chicken meat and preserved by freezing. The results indicate that there are no significant differences in terms of *L. monocytogenes* isolation based on the meat type and preservation method. This result implies that preservation by cooling or freezing the meatballs is not very effective. The factors effective in *L. monocytogenes* contaminations are mostly environmental contaminations and cross-contamination through the equipment used during the production stage (16, 17, 21, 28, 29). On the other hand, the fact that antibacterial properties of some spices like clove, thyme, onion, and garlic used in the production of meatballs having a negative effect on the *L. monocytogenes* count and vitality should also be kept in mind (5, 13).

The *L. monocytogenes* isolation prevalence of some samples was lower in our study compared to the literature (23, 28, 29) while others were higher (11). Such variations may be based on the differences between the production and preservation methods employed by the production companies, the inhibition effect of some of the additives and spices used during the production of the meatballs or the competing microflora, or due to the differences in analysis methods used (5, 13).

The equipment used, and the preferred hygiene protocols like HACCP and GM used by the production plants, also play an important part in the contamination of meat and meat products by *Listeria* spp. In the establishments that produce ready-made meatballs, the *Listeria* spp. that have spread to the equipment used can keep contaminating the products continuously, which may be a contributor to the high isolation rates revealed by the studies (12, 28, 29).

The species that was isolated the most in this study was *L. monoctogenes*, followed by *L. innocua*. Sharif and Tunail (23) isolated *L. monocytogenes* the most from more than 200 meat product samples they tested consisting of frozen cooked, half-cooked, thermally pre-processed, and raw products, and also found it was followed by *L. innocua*. Sireli et al. (28), on the other hand, found *L. innocua* was the predominant species for minced meat, meatball and burger samples, which was followed by *L. monocytogenes.*

In our study, the pH value for the meatball samples was found to vary between 5.16 and 7.75, with mean of 6.62±0.56. The pH range detected in our study is wider compared to the study of Soyutemiz (25) (which detected a pH range of 5.85-7.32 in ready-made meatballs), which may be due to the difference in regions from which the samples were collected from. Some researchers report that a high initial pH value in Inegol kofte's stored in +4 ºC is connected to the bicarbonate used in the production, and the drop at the 7th day is due to the acids produced by the micro-flora (27). The pH values in our study were found to be of alkali range for Tekirdag kofte, Izmir kofte, odun kofte, ızgara kofte, and some of the Inegol kofte samples, while it was found to be below pH 7.0 for the others (Table 2).

In the statistical analysis, some significant differences ($P<0.05$) were found in terms of pH value among the frozen ready-made beef meatball groups (Table 2). Among the groups of cooled ready-made beef meatballs a significant difference ($P<0.05$) was found only between Inegol kofte group and hamburger kofte/kasap kofte groups (Table 2). In groups contains more than one different types of meatballs, the differences between the pH values of some types of meatballs were found to be significant ($P<0.05$) only in Inegol kofte group (Table 2). No significant difference was found between meatball types in hamburger kofte, kasap kofte and cızbız kofte groups in terms of pH values (Table 2). These variations may be related to the difference of raw materials, additives, and micro-flora of the samples, to the preservation method employed, or to variances in the storage period length. Soyutemiz (25) has found that hamburgers had a lower pH value on average compared to other meatball types. While the pH values found in our study for the hamburgers were lower compared to the study of Soyutemiz (25) and similar to the study of Yörük (29), our study also has revealed that hamburger meatballs had a comparatively lower pH nonetheless.

The water activities of the samples were found to vary between 0.975±0.015 and 0.992±0.003, with mean of 0.985±0.007. In the statistical analysis, some significant differences ($P<0.05$) were found in terms of a_w value among the frozen ready-made beef meatball groups (Table 3). Also, among the groups of cooled ready-made beef meatballs a significant difference ($P<0.05$) was found only between a_w values of hamburger kofte group and kasap kofte group (Table 3). In addition, in the statistical analysis of the a_w values of the meatballs types in the groups, while there was no significant difference between the types of meatballs in the kasap kofte group and the cızbız kofte group, there were some significant differences

between the types of meatballs in the Inegol kofte group and hamburger kofte group (Table 4). When the water activity of the products are considered, some statistically significant differences ($P<0.05$) between both the groups and sub-groups can be observed (Table 3 and Table 4). It can be thought that this situation is due to the raw material, production and preservation differences for all product groups.

In the correlation analysis performed to determine whether there is a relationship between the pH values and a_w values of meatball groups, only in hamburger kofte samples were found to have a negative correlation ($P<0.05$) between pH and water activity values. This finding may be due to fact that half of the hamburger kofte samples were stored by cooling, where some psychrophilic microorganisms may have multiplied in the storage temperature, causing acidity (5, 26).

The Microbiological Criteria Code of the Turkish Food Codex mandates zero *L. monocytogenes* in 25 g of the sample for ready-made meatballs (3). Evaluated in this perspective, it is evident that 11.04% of the samples inspected in our study are in violation of the legal regulations. This situation suggests that these products may pose a serious public health hazard, in situation such as cooking with inadequate heat treatment and cross-contamination with uncooked foods.

As a result of this study, it has been revealed that meatballs preserved by cooling or freezing in the sale points are contaminated with *Listeria* species, especially with *L. monocytogenes*, and this contamination level is capable for forming a significant risk for the public health. This risk may further increase with inadequate cooking time or methods before consumption (10). The results clearly indicate that trusting the products preserved by cooling or freezing to be perfect in terms of food safety is a faulty perspective. To prevent these kinds of pathogenic risks, the ready-made meatballs have to be produced by paying the utmost attention to hygiene rules and must be delivered to the final consumer without breaking the cold chain.

Acknowledgement

This study was partially supported by the Van YYU Directorate of Scientific Research Projects under project number 2008-VF-B059.

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Ankara Üniv Vet Fak Derg, **66**, 289-296, 2019
DOI: 10.33988/auvfd.492584

Farklı sarımsak yağı dozlarının, korunga otunun *in vitro* gaz üretimi, rumen fermantasyonu ve metan üretimi üzerine etkisi

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Geliş tarihi: 05.12.2018- Kabul tarihi: 03.05.2019

Özet: Bu araştırma rumen sıvısına (RS) 0 (kontrol), 100, 200, 400, 800, 1200 ve 1600 mg/L sarımsak yağı (SY) ilavesinin korunga (*Onobrychis sativa* L.) otunun gerçek kuru madde sindirimi (GKMS), organik madde sindirimi (OMS), nötr deterjanda çözünmeyen lif sindirimi (NDFS), rumen sıvısı parametreleri ile karbondioksit (CO_2) ve metan (CH_4) gazı üretimi üzerine etkilerinin saptanması amacıyla düzenlenmiştir. Farklı SY dozlarının rumen fermantasyonu, OMS ve metabolik enerji (ME) düzeyinin saptanması için *in vitro* gaz üretim tekniği kullanılmıştır. Korunga otunun GKMS ve NDFS ise Daisy inkübatör tekniği ile saptanmıştır. Rumen sıvısına SY ilavesinin korunga otunun *in vitro* gaz üretimini, GKMS, OMS, NDFS ve metabolik enerji (ME) içerikleri ile rumen fermantasyonu sonucu oluşan toplam uçucu yağ asitleri (TUYA), asetik asit (AA), propiyonik asit (PA) ve butirik asit (BA) ve diğere uçucu yağ asidi düzeylerini azalttığı saptanmıştır ($P<0.01$). Ayrıca, rumen sıvısına farklı dozlarda SY ilavesi CH_4 ve CO_2 üretimini de düşürmüştür ($P<0.01$). Sonuç olarak, *in vitro* gaz üretimi, rumen fermantasyonu, besin maddeleri sindirimi, metan ve karbondioksit üretimi üzerinde, en fazla olumsuz etkili sarımsak yağı dozu 1600 mg/L RS olduğu saptanmıştır. Yüksek SY dozlarının rumen fermantasyonunu, yemlerin sindirimini olumsuz etkilemesi nedeniyle düşük dozlarda (400 mg/L RS) kullanılmasının uygun olacağı kanaatine varılmıştır.

Anahtar sözcükler: *İn vitro* gaz üretimi, korunga otu, metan, rumen fermantasyonu, sarımsak yağı

Effect of different garlic oil doses on *in vitro* gas production, rumen fermentation and methane production of sainfoin hay

Abstract: This study was conducted to determined, effects of addition of garlic oil (GO) 0 (control), 100, 200, 400, 800, 1200 and 1600 mg/L rumen fluid (RF) by *in vitro* gas production technique, on the true organic matter digestibility (TOMD), organic matter digestibility (OMD), neutral detergent fiber digestibility (NDFD), metabolizable energy (ME) compound and rumen fermentation parameters, carbon dioxide (CO_2) and methane (CH_4) gas production of the sainfoin (*Onobrychis sativa* L.) hay. *In vitro* gas production technique was used to determine the effects of different doses of GO on rumen fermentation, OMD and metabolic energy (ME) level. GKMS and NDFS contents of sainfoin hay were determined by Daisy incubator technique. The addition of GO significantly decreased the *in vitro* gas production, OMD, NDFD, ME, total volatile fatty acids content (VFA), acetic acid (AA), propionic acid (PA), butyric acid (BA) and the other volatile fatty acids levels of sainfoin hay ($P<0.01$). In addition, the addition of GO at different doses to the rumen fluid also reduced the production of CH_4 and CO_2 production ($P<0.01$). As a result, *in vitro* gas production, rumen fermentation, nutrient digestion, methane and carbon dioxide production on the most adverse effect garlic oil dose was found to be 1600 mg / L RF. It was concluded that it would be appropriate to use low doses (400 mg / L RF) because of the high GO doses that affect rumen fermentation and the digestion of feed.

Keywords: Garlic oil, *in vitro* gas production, methane, rumen fermentation, sainfoin hay

Giriş

Hayvancılık sektöründe hastalıkları, metabolik bozuklukları ve yemden yararlanmayı artırmak için antibiyotikler yaygın olarak kullanılmıştır (20, 23). Ancak son yıllarda hayvanlarda antibiyotik kullanımı, insan sağlığı için risk oluşturabilecek dirençli bakterilerin gelişmesine yol açması nedeniyle Avrupa Birliği 2003 yılında (EU regulation no.1831/2003 of the European Parliament and of the Council of 22 September 2003) aldığı karar gereği 2006 Ocak ayından sonra hayvan beslemede gelişmeyi teşvik edici antibiyotik kullanımını yasaklamıştır (15).

Bu sorunu çözmek için antibiyotiklere alternatif olabilecek yem katkı maddelerinin geliştirilmesine yönelik çalışmalar artmıştır. Bu çalışmalar sonucu aromatik bitki ve bu bitkilerden izole edilen esansiyel yağların antibiyotiklere alternatif olabilecekleri ortaya konulmuştur (11, 14, 23, 36). Esansiyel yağların çeşitli mikroorganizmalara karşı bakterisit, fungusit ve antiparazitik etkiye sahip oldukları bildirilmektedir (5, 10, 11, 12, 36). Bu yağların antimikrobiyal mekanizmaları henüz tam olarak aydınlatılmamasına rağmen, mikroorganizmaların hücre zarı lipid (lipofilik) tabakasının (3), kimyasal yapısı (5, 13) ile hücre duvarı enzimlerini olumsuz etkileyerek yapılarını bozdukları ve gelişmelerini sınırlamak suretiyle etki yaptıkları bildirilmektedir (4, 5). Esansiyel yağların antimikrobiyal, özellikleri dışında, antioksidan, antikanserojen ve immün sistemini geliştirici özellik göstermeleri, bu ürünlerin hayvan beslemede kullanımlarına imkan vermiştir. (5, 24). Ayrıca esansiyel yağların rumen fermantasyonunun kontrolünde kullanılması da gündeme gelmiştir (20, 36, 38). Bu esansiyel yağlardan birisini sarımsak yağı (SY) oluşturmaktadır. Sarımsak yağı ezilmiş sarımsağın (*Allium sativa*) 100^{o}C'de damıtılmasıyla elde edilmektedir. Sarımsak yağının gram-negatif ve pozitif bakterilere karşı antibakteriyel etki gösterdiği bildirilmektedir (10, 42). Sarımsak yağının ana bileşenini allisin ($C_6H_{10}S_2O$), dialil sülfür ($C_6H_{10}S$), dialil disülfür ($C_6H_{10}S_2$), allil merkaptan (C_3H_6S) ve tiyosülfinatlar gibi bileşikler oluşturmaktadır (10, 29).

Sarımsak yağı ile ilgili yapılan çalışmalarda SY'nın rumen mikrobiyal aktiviteyi uyardığı (31), bazı çalışmalarda ise rumen fermantasyonunu sınırlayarak olumsuz etki gösterdiği bildirilmektedir (10, 21, 24). Sarımsak yağının *in vitro* gaz üretimi (6, 26, 39), rumen sıvısı UYA (6, 10) ve metan (CH_4) üretimini azalttığını (6, 10, 28, 31, 39), rumen NH_3-N'nu ise etkilemediği bildirilmiştir (10, 20). Cardozo ve ark., (12) ile Blanch ve ark., (6)'ı ise SY'nın NH_3-N'nu düşürdüğünü bildirmişlerdir. Ayrıca SY'nın yemlerin sindirimini olumsuz etkilediği yapılan çalışmalarla ortaya konmuştur (6, 39).

Günümüze kadar yapılan çalışmalar SY'nın rumen ortamına etkisinin hem olumlu hem de olumsuz etki gösterdiği bildirilmektedir. Ayrıca SY'nın hangi dozlarda kullanılması gerektiğiyle ilgili sınırlı bilgi mevcuttur. Bu durumu ortaya koymak için SY ve farklı dozlarının (0, 100, 200, 400, 800, 1200 ve 1600 mg/L RS) *in vitro* gaz üretimi, besin maddeleri sindirimi, rumen fermantasyonu (pH, TUYA, NH_3-N) ile metan (CH_4) gazı üretimi üzerine olan etkilerini saptanmak ve ruminant beslemede önerilecek SY dozunu saptamak amacıyla düzenlenmiştir.

Materyal ve Metot

Yem ve hayvan materyali: Araştırmanın yem materyalini korunga kuru otu (KKO) oluşturmuştur. Korunga kuru otu 1 mm elek çapına sahip değirmende öğütülmüş ve analizlerde kullanılmıştır. Araştırmada kullanılan rumen sıvısı yaklaşık 1 yaşlarında kesimi yapılan 3 baş Kıvırcık ırkı koyundan Bursam Et Entegre San. Tic. Ltd Şti'nin 26.07.2018 yazılı kararı ile alınmıştır. Koyunlar kesim öncesi %70 kaba yem (çayır-mera otu) ve %30 yoğun yem temeline dayanan rasyonla yemlenmişlerdir.

İn vitro gaz üretim tekniğinin uygulanması: Korunga kuru otunun *in vitro* gaz üretimi, organik madde sindirimi (OMS) ve metabolik enerjilerinin (ME) saptanmasında Menke ve Steingass (33) tarafından geliştirilen "*in vitro* gaz üretim tekniği" kullanılmıştır. *İn vitro* gaz üretiminin saptanması için 100 mL'lik özel cam şırıngalar (Model Fortuna, Häberle Labortechnik, Lonsee-Ettlenschieβ, Germany) kullanılmıştır. Şırıngalara yaklaşık kuru madde üzerinden 200±10 mg yem örneği ile 0, 100, 200, 400, 800, 1200 ve 1600 mg/L RS düzeylerinde SY olacak şekilde hesaplanan miktarlarda 5 tekerrür olarak konmuştur. Cam şırıngaların üzerine Menke ve ark. (32)'nın bildirdikleri yönteme göre hazırlanmış RS/tampon çözeltisinden yaklaşık 30 mL ilave edilmiştir. Daha sonra cam şırıngalar 39°C'ye ayarlanmış su banyosunda inkübasyona alınmış ve sırasıyla; 3, 6, 12, 24, 48, 72 ve 96. saatlerdeki *in vitro* gaz üretim miktarları ölçülmüştür.

İnkübasyonun 96. saatinde şırıngalar içerisindeki rumen sıvısında pH, UYA ve NH_3-N ile karbondioksit (CO_2) ve metan (CH_4) gazları saptanmıştır. Karbondioksit ve CH_4 gazları rumen sıvısı bireysel UYA'leri kullanılarak aşağıdaki eşitliklerle hesaplanmıştır (7).

CO_2, mmol/L = Asetik asit/2 + Propiyonik asit/4 + 1.5 x Butirik asit

CH_4, mmol/L = (Asetik asit + 2 x Butirik asit) - CO_2

(UYA'nin konsantrasyonu mmol olarak alınmıştır).

Korunga otunun organik madde sindirimi (OMS) ve metabolik enerji (ME) içerikleri Menke ve Steingass (33)'ın bildirdikleri aşağıdaki eşitlikler ile hesaplanmıştır.

OMS, % = 15.38 + 0.8453 x GÜ + 0.0595 x HP + 0.0675 x HK

ME, MJ/kg KM = 2.20 + 0.1357 x GÜ + 0.0057 x HP + 0.0002859 x HY^2

(GÜ: inkübasyonun 24 saatindeki net gaz üretimi (mL/200 mg KM yem), HP: ham protein, HY: ham yağ ve HK: ham kül ise g/kg KM olarak alınmıştır).

Korunga kuru otunun gerçek kuru madde ve nötr deterjan lif sindirimi Ankom Daisy^{II} inkübatör cihazı kullanılarak saptanmıştır (ANKOM Technology Corp., Fairport, NY, USA, 2008).

Kimyasal analizler: Korunga kuru otunun kuru madde, ham protein, ham yağ ve ham kül analizi AOAC (2)'a göre, hücre duvarı bileşenlerinden nötr deterjan lif (NDF), asit deterjan lif (ADF) ve asit deterjan lignin (ADL) analizi Van Soest ve ark. (47) tarafından bildirilen

yöntemlere göre ANKOM 200 Fiber Analyzer cihazı ile saptanmıştır.

Rumen sıvısı pH'sı dijital pH metre cihazıyla (Sartorius PB-20), amonyak azotu (NH_3-N) kjeldahl metodu ile Blümmel ve ark. (7)'nın bildirdikleri yönteme göre, rumen UYA ise Wiedmeier ve ark. (48)'nın bildirmiş oldukları yöntem kullanılarak gaz kromotografi cihazıyla saptanmıştır.

İstatistik analizler: Araştırmadan verilerinin istatistiki olarak değerlendirmesinde varyans analizi (45), veriler arası görülen farklılıkların önem düzeylerinin saptanması Duncan Çoklu Karşılaştırma Testi ile belirlenmiştir (44).

Bulgular

Korunga kuru otunun kimyasal bileşimleri: Araştırmada kullanılan KKO'nun besin maddeleri bileşimi saptanmış ve Tablo 1'de verilmiştir. Korunga otu organik madde, ham kül, ham protein, ham yağ, NDF, ADF ve ADL içerikleri sırasıyla; %93.12, 6.88, 16.84, 3.50, 53.78, 37.97 ve 10.05 olarak saptanmıştır.

Tablo 1. Korunga otunun kimyasal bileşimi, %, (n=5).
Table 1. Chemical composition of sainfoin hay, %, (n=5).

Besin maddeleri	%
Organik maddeler	93.12
Ham kül	6.88
Ham protein	16.84
Ham yağ	3.50
Nötr deterjan lif, (NDF)	53.78
Asit deterjan lif, (ADF)	37.97
Asit deterjan lignin, (ADL)	10.05
Selüloz	15.81
Hemiselüloz	27.92

Sarımsak yağının in vitro gaz üretimi üzerine etkisi: Sarımsak yağı ve farklı dozlarının KKO'nun *in vitro* gaz üretimi üzerine etkisi Tablo 2'de verilmiştir. Rumen sıvısına farklı dozlarda SY ilavesi KKO'nun *in vitro* gaz üretimini önemli düzeyde düşürmüştür (P<0.01). Sarımsak yağı dozlarına bağlı olarak 96. saat *in vitro* gaz üretimi 76.65 ile 49.90 mL/200 mg KM arasında değişmiş ve en düşük 49.90 mL ile 1600 mg SY/L RS ilave edilen grupta saptanmıştır.

Sarımsak yağının besin maddeleri sindirimi ve ME üzerine etkisi: Sarımsak yağı ve farklı dozlarının KKO'nun *in vitro* koşullarda besin maddeleri sindirimi ve metabolik enerji (ME) üzerine etkisi saptanmış ve Tablo 3'te verilmiştir. *İn vitro* koşullarda rumen sıvısına SY ve farklı dozlarının ilavesi, KKO'nun GKMS, OMS ve NDFS içerikleri önemli düzeyde etkilemiştir (P<0.01). Rumen sıvısına artan dozlarda SY ilavesi yemlerin GKMS, OMS ve NDFS'in düşürmüştür (P<0.01).

Sarımsak yağının rumen fermantasyonu üzerine etkisi: Sarımsak yağı ve farklı dozlarının rumen sıvısına ilavesinin rumen fermantasyon özellikleri üzerine etkisi Tablo 4'te verilmiştir. Sarımsak yağı ve farklı dozlarının rumen sıvısına ilavesi TUYA, asetik, propiyonik ve butirik asitler ile NH_3N içeriklerini önemli düzeyde düşürmüştür (P<0.01). Rumen pH'sı ise artış göstermiş ve rumen fermantasyon parametreleri üzerine en etkili SY dozunun 1600 mg SY/L RS olduğu saptanmıştır (P<0.01).

Sarımsak yağının karbondioksit (CO_2) ve metan (CH_4) gazı üretimine etkisi: Rumen sıvısına SY ve farklı dozları ilavesinin CO_2 ve CH_4 gazı üretimi üzerine etkisi Tablo 5'te verilmiştir. Rumen sıvısına ilave edilen SY dozu artışına bağlı olarak *in vitro* CO_2 ve CH_4 gazı üretimi önemli düzeyde düşmüştür (P<0.01).

Tablo 2. Sarımsak yağı ve farklı dozlarının *in vitro* gaz üretimine etkisi, (mL).
Table 2. Effects of garlic oil and different doses on *in vitro* gas production, (mL).

İnkübasyon süresi, saat	Sarımsak yağı, mg/L RS							
	Kontrol (0)	100	200	400	800	1200	1600	SH
3	22.05^{a}	20.79^{a}	17.17^{b}	14.96^{c}	11.78^{d}	10.64^{de}	9.54^{e}	*0.678*
6	39.74^{a}	38.74^{a}	36.25^{b}	29.43^{c}	22.94^{d}	19.45^{e}	16.69^{f}	*0.963*
12	54.23^{a}	52.15^{a}	48.86^{b}	43.76^{c}	40.15^{d}	36.66^{e}	33.06^{f}	*1.029*
24	66.18^{a}	62.89^{b}	58.91^{c}	52.63^{d}	46.99^{e}	42.71^{f}	40.58^{f}	*0.945*
48	70.16^{a}	69.99^{a}	65.01^{b}	57.59^{c}	50.71^{d}	46.55^{e}	44.43^{e}	*0.899*
72	74.27^{a}	72.29^{a}	67.35^{b}	62.33^{c}	54.84^{d}	50.39^{e}	48.51^{e}	*0.828*
96	76.65^{a}	73.06^{b}	68.99^{c}	63.13^{d}	56.07^{e}	52.19^{f}	49.90^{f}	*0.996*

SH: Standart hata; Aynı satırda farklı harfler ile gösterilen ortalamalar arası farklılıklar önemlidir (P<0.01).

Tablo 3. Sarımsak yağı ve farklı dozlarının *in vitro* koşullarda besin maddeleri sindirimi ve ME üzerine etkisi.
Table 3. Effect of garlic oil and different doses on nutrient digestion and ME in *in vitro* conditions.

Parametreleri	Sarımsak yağı, mg/L RS							
	Kontrol (0)	100	200	400	800	1200	1600	SH
GKMS, %	73.56^{a}	71.89^{ab}	69.40^{b}	63.21^{c}	57.69^{d}	51.39^{e}	45.68^{f}	1.165
OMS, %	85.98^{a}	83.21^{b}	79.84^{c}	74,53^{d}	69.77^{e}	66.14^{f}	64.34^{f}	0.830
NDFS, %	66.59^{a}	66.95^{a}	60.07^{b}	53.06^{c}	48.91^{d}	43.83^{e}	42.27^{e}	1.272
ME, MJ/kg KM	12.49^{a}	12.04^{b}	11.50^{c}	10.65^{d}	9.89^{e}	9.30^{f}	9.01^{f}	0.133

GKMS: Gerçek kuru madde sindirimi; OMS: Organik madde sindirimi; NDFS: Nötr deterjan lif sindirimi; ME: Metabolik enerji. SH: Standart hata; Aynı satırda farklı harfler ile gösterilen ortalamalar arası farklılıklar önemlidir (P<0.01).

Tablo 4. Sarımsak yağı ve farklı dozlarının rumen fermantasyon özelliklerine etkisi.
Table 4. Effects of garlic oil and different doses on rumen fermentation properties.

Rumen sıvısı parametreleri	Sarımsak yağı, mg/L RS							
	0	100	200	400	800	1200	1600	*SH*
pH	6.05^{e}	6.14^{e}	6.16^{e}	6.34^{d}	6.43^{c}	6.52^{b}	6.64^{a}	*0.027*
NH_3-N, mg N/100 mL	38.05^{a}	36.43^{a}	31.49^{b}	27.50^{b}	24.95^{d}	21.47^{e}	18.43^{f}	*0.992*
TUYA, mmol/L	116.23^{a}	107.53^{b}	103.72^{b}	94.46^{c}	84.81^{d}	73.93^{e}	69.10^{e}	*2.496*
Asetik asit, mmol/L	62.20^{a}	57.64^{ab}	55.13^{b}	47.71^{c}	40.04^{d}	39.19^{d}	39.09^{e}	*2.279*
Propiyonik asit, mmol/L	26.06^{a}	23.72^{b}	22.86^{bc}	21.62^{cd}	20.81^{de}	20.22^{ef}	19.64^{f}	*0.679*
Butirik asit, mmol/L	17.58^{a}	16.55^{b}	16.43^{b}	17.10^{b}	16.31^{b}	10.99^{c}	7.55^{d}	*0.994*
DUYA, mmol/L	10.39^{a}	9.61^{ab}	9.30^{b}	8.03^{c}	7.64^{c}	3.54^{d}	2.86^{d}	*0.466*
Asetik asit/propiyonik asit	2.39^{ab}	2.43^{a}	2.41^{a}	2.20^{b}	1.92^{c}	1.94^{c}	1.99^{c}	*0.084*

NH_3-N: Amonyak azotu; TUYA: Toplan uçucu yağ asidi; DUYA: Diğer uçucu yağ asitleri; AA/PP: asetik asit/propiyonik asit. SH: Standart hata; Aynı satırda farklı harfler ile gösterilen ortalamalar arası farklılıklar önemlidir (P<0.01).

Tablo 5. Sarımsak yağı ve farklı dozlarının karbondioksit (CO_2) ve metan (CH_4) üretimi üzerine etkisi.
Table 5. Effects of garlic oil and different doses on carbon dioxide (CO_2) and methane (CH_4) production.

Parametreleri	Sarımsak yağı, mg/L RS							
	Kontrol (0)	100	200	400	800	1200	1600	SH
CO_2, mmol/L	63.99^{a}	59.58^{b}	57.93^{bc}	56.22^{cd}	55.64^{d}	41.13^{e}	35.76^{f}	0.878
CH_4, mmol/L	33.38^{a}	31.17^{b}	30.07^{b}	27.44^{c}	24.03^{d}	20.03^{e}	18.39^{e}	0.671

SH: Standart hata; Aynı satırda farklı harfler ile gösterilen ortalamalar arası farklılıklar önemlidir (P<0.01).

Tartışma ve Sonuç

Korunga otunun organik madde, ham kül, ham protein, ham yağ, NDF, ADF ve ADL içerikleri sırasıyla; %93.12, 6.88, 16.84, 3.50, 53.78, 37.97 ve 10.05 olarak saptanmış ve ham besin maddeleri bileşimi Deniz ve ark. (18), Karabulut ve ark. (25) ve NRC (35)'nin bildirdikleri besin maddeleri bileşimi ile benzer bulunmuştur.

Rumen sıvısına farklı dozlarda SY ilavesi KKO'nun *in vitro* gaz üretimini tüm inkübasyon saatlerinde düşürmüştür (P<0.01). Sarımsak yağı dozlarına bağlı olarak 96. saat *in vitro* gaz üretimi 76.65 ile 49.90 mL/200 mg KM arasında değişmiş ve en düşük 49.90 mL ile 1600 mg SY/L RS ilave edilen grupta, en yüksek ise SY ilave edilmeyen kontrol grubunda saptanmıştır. Tüm inkübasyon saatleri incelendiğinde 1200 ile 1600 mg SY/L RS dozlarının benzer etki gösterdiği saptanmıştır. Yapılan birçok çalışmada SY'nın *in vitro* gaz üretimini azalttığı bildirilmektedir (6, 26, 39, 43). Bu araştırmada saptanan bulgular Kılıç ve ark. (26) ve Blanch ve ark. (6)'nın bildirdikleri sonuçları desteklemektedir.

Rumen sıvısına SY ilavesinin *in vitro* gaz üretimini azaltması SY'da bulunan ve kükürt (S) içeren allisin ve dialil sülfitlerin antimikrobiyal aktivite göstermesi ile açıklanabilir (5, 19, 24). Sarımsak yağında bulunan organik sülfürlü (S) bileşiklerin (allisin, dialil sülfür, dialil disülfür ve dialil trisülfür) yapısında bulunan S atomlarının mikroorganizma hücre zarı ve içi protein ile amino asitlere bağlanarak fonksiyonlarını bozduğu, buna

bağlı olarak da gram pozitif ve negatif bakteriler ile bazı patojenlerin gelişmesini önlediği ortaya konmuştur (5, 10, 19, 21, 40, 43). Aynı mekanizma ile rumen mikroorganizmalarının gelişmesini de sınırladığı bildirilmektedir (5, 19, 31). Rumen mikroorganizmalarının sınırlanması *in vitro* gaz üretimini azaltmıştır.

İn vitro koşullarda rumen sıvısına SY ve farklı dozlarının ilavesi, KKO'nun GKMS, OMS ve NDFS içerikleri sırasıyla; %73.56-45.68, %85.98.79-64.34 ve %66.59-42.27 arasında değişmiş, SY ve dozları arasındaki farklılıklar önemli bulunmuştur (P<0.01). Rumen sıvısına artan dozlarda SY ilavesi yemlerin GKMS, OMS ve NDFS'ni düşürmüştür. Besin maddeleri sindirimi SY dozunun artışına bağlı olarak azalmış ve sindirimi 1200 ve 1600 mg SY/L RS dozları daha fazla olumsuz etkilemiştir. Sarımsak yağı dozunun artışına bağlı olarak GKMS, OMS ve NDFS'inde ki azalma sırasıyla; %37.90, %25.17 ve %36.52 olmuştur.

Korunga kuru otunun ME düzeyi de SY ve dozlarının artışına bağlı olarak 12.49 ile 9.01 MJ/kg KM arasında değişmiş ve SY dozu artışa bağlı olarak azalmıştır (P<0.01). En düşük ME düzeyi 1200 ve 1600 mg SY/L RS ilave edilen grupta saptanmış ve azalma oranı %27.86 olmuştur. Sarımsak yağı ve farklı dozlarının GKMS, OMS, NDFS ile ME içeriğindeki azalma SY'nın rumen fermantasyonunun sınırlanması ile açıklanabilir (5, 40, 43). Ayrıca OMS ve ME değerleri 24. saatte üretilen *in vitro* gaz hacmi (mL gaz/24 saat) temel alınarak hesaplanmıştır. Artan SY dozları rumen sıvısında antimikrobiyal etki göstererek (6, 26, 39, 43) daha az *in vitro* gaz oluşumuna yol açmış (*Tablo 2*) ve daha düşük OMS ve ME içeriğine neden olmuştur.

Sarımsak yağı dozu artışına bağlı olarak GKMS, OMS, NDFS ve ME içerikleri düşmüştür. Bu durumun sarımsak yağında bulunan kükürtlü bileşiklerin (allisin ve dialil sülfitlerin) antimikrobiyal etki göstererek rumen fermantasyonunu sınırlamasından ileri geldiği söylenebilir (5, 19, 24, 34).

Araştırmada saptanan GKMS'de ki düşüş farklı yem ve sarımsak yağı dozu ile çalışan Busquet ve ark. (10), Roy ve ark. (43) ve Patra ve Yu (40)'nun araştırmalarında da olmuştur. Aynı durum NDFS için incelendiğinde sarımsak yağı ile çalışan Busquet ve ark. (10) ile Patra ve Yu (40)'nun bulguları araştırma bulguları ile benzer saptanmıştır. Anassori ve ark. (1) ile Roy ve ark. (43)'nın sarımsak yağı ile yapmış oldukları çalışmalarda SY'nın OMS ve NDFS'ni düşürdüğünü bildirmişlerdir. Ayrıca KKO'nun kuru madde sindirimi, OMS, NDFS ile ME içeriği KKO ile çalışan Deniz ve ark. (18)'nın sonuçları ile benzer bulunmuştur.

Sarımsak yağı ve farklı dozlarının rumen sıvısına ilavesi TUYA ile asetik, propiyonik ve butirik asitler önemli düzeyde düşürmüş (P<0.01) ve SY dozu artışına bağlı olarak rumen sıvısı TUYA'leri 116.23 ile 69.10 mmol/L arasında saptanmıştır. Rumen sıvısı asetik, propiyonik ve butirik asit düzeyi ise sırasıyla; 62.20-39.09 mmol/L, 26.06-19.64 mmol/L ve 17.58-7.55 mmol/L arasında değişmiştir. Toplam UYA, asetik, propiyonik ve butirik asitler üzerinde en etkili olan SY dozunun 1600 mg SY/L RS olduğu saptanmıştır (P<0.01). Rumen sıvısına SY ilavesi rumen fermantasyonunu önemli düzeyde etkilemiştir. Bu durum SY'nın rumen mikroorganizmaları üzerine antibakteriyel (5, 19, 24) etki yapması ile açıklanabilir.

Roy ve ark. (43)'nın yapmış oldukları çalışmada rumen sıvısına 30, 300 ve 600 ppm SY ilave etmişler ve araştırma sonucunda SY dozları artışına bağlı olarak propiyonik asit artığı, TUYA'leri ile asetik asit oranının ise azaldığını bildirmişlerdir. Benzer bulgular Busquet ve ark. (10), Patra ve Yu, (39) ve Blanch ve ark. (6)'nın yapmış oldukları çalışmalarda da ortaya konmuştur. Araştırmada uçucu yağ asitlerine ait bulgular Busquet ve ark. (10), Patra ve Yu, (39) ve Blanch ve ark. (6)'nın bildirdikleri sonuçlarla uyumlu bulunmuştur. Araştırmada saptanan AA/PA oranı SY dozuna bağlı olarak 2.43 ile 1.92 arasında değişmiş ve SY dozları arası farklılıklar önemli bulunmuştur (P<0.01). Belirlenen AA/PA oranı SY ile çalışan Zhu ve ark. (49) (3.31-3.28) ile Mateos ve ark. (30)'nın saptadıkları (3.63-2.60) değerlerden düşük, Chaves ve ark. (14)'nın bildirdikleri değerlerden (1.8-1.7) daha yüksek bulunmuştur. Araştırıcıların AA/PA oranı ile ilgili bildirdikleri farklılıklar kullanılan rasyon ve yem farklılıklarından kaynaklandığı söylenebilir.

Sarımsak yağı dozu artışına bağlı olarak rumen sıvısı pH düzeyi 6.05-6.64 arasında değişmiş ve SY dozları arası farklılıklar önemli bulunmuştur (P<0.01). Sarımsak yağı rumen pH'sını artırmış ve en yüksek rumen pH'sı 1600 mg SY/L RS'da saptanmıştır. Sarımsak yağı dozu artışına bağlı olarak pH'nın artması, SY'nın rumen sıvısını asit karaktere dönüştüren uçucu yağ asitlerini azaltmasına bağlanabilir (*Tablo 4*). Araştırmada saptanan rumen pH değerleri SY ile çalışan Klevenhusen ve ark. (28), Anassori ve ark. (1), Patra ve Yu (40) ve Blanch ve ark. (6)'nın bulguları ile benzerlik göstermiştir.

Rumen sıvısı NH_3N düzeyi SY dozunun artmasına bağlı olarak düşmüştür (P<0.01). Amonyak azotu SY dozu artışına bağlı olarak 38.05 ile 18.43 mg N/100 mL arasında değişmiş ve en yüksek NH_3N 38.05 mg N/100 mL ile SY içermeyen kontrol grubunda, en düşük ise 1600 mg SY/L RS bulunan grupta (18.43 mg N/100 mL) saptanmıştır. Rumen sıvısı NH_3N düzeyindeki azalma başta rumen sıvısı mikroorganizmalarının etkinliğinin azalması yanında, esansiyel yağların amino asitlerin deaminasyonunu önlemesi ile açıklanabilmektedir (40, 43). Rumende amonyak (NH_3) şeklinde azot kaybının düşmesi yemin enerji ve azotundan yararlanmayı artıracağı ve bu yolla hayvan beslemeye yarar sağlayacağı bildirilmektedir (1, 6, 40). Araştırmada saptanan rumen

sıvısı NH_3N düzeyi SY ile çalışan Anassori ve ark. (1), Mateos ve ark. (30), Roy ve ark. (42), Patra ve Yu (36) ve Blanch ve ark. (6)'nın bildirdikleri sonuçlarda da olduğu gibi azalmış ve elde edilen bulgular araştırma sonuçları ile benzerlik göstermiştir.

Rumen sıvısına ilave edilen SY dozunun artması CO_2 gazı üretimini azaltmış ve SY dozları arasındaki farklılıklar önemli bulunmuştur ($P<0.01$). En yüksek 63.99 mmol/L ile kontrol grubunda, en düşük ise 35.76 mmol/L ile 1600 mg SY/L RS bulunan grupta saptanmıştır. *İn vitro* CH_4 gazı üretimi ise SY dozu artışına bağlı olarak azalmış ve CH_4 gazı üretimi dozlara bağlı bağlı olarak 33.38 ile 18.39 mmol/L arasında değişmiş ve SY dozları arası farklılıklar önemli bulunmuştur ($P<0.01$). Ruminantlarda CO_2 ve CH_4 üretimi rumende bulunan metanojenik bakteriler (arkealar) tarafından UYA ile hidrojen iyonlarını (H^+) kullanılarak üretilmektedir (6, 17). Sarımsak yağı diğer rumen bakterileri gibi metanojenik bakteriler üzerine antimikrobiyal etki göstererek CH_4 gazı oluşumu düşmektedir. Metan en önemli sera gazlarından birisi olup, sera etkisi CO_2'e göre 23 kat fazla olduğu bildirilmektedir (22, 27). Çiftlik hayvanlarının sera gazları emisyonuna katkılarının %18 olduğu bildirilmektedir. Bu kısmın yaklaşık %15'inin ruminant hayvanların rumen ve gübrelerinde gerçekleşen fermantasyon kaynaklandığı bildirilmiştir (46).

Rumende yemlerin fermantasyonu sonucu üretilen metan gazı ile yem enerjisinin %2-15'sinin kayba uğradığı bildirilmektedir (9, 16, 27). Metan gazı yoluyla enerji kaybını azaltmada ve sera gazı emisyonunu düşürmede esansiyel yağların kullanılabileceği bildirilmektedir (5, 41). Sarımsak yağı da rumende metanojenik bakterilerin sayısını sınırlayarak, metan üretiminin azalmasına yol açtığı birçok çalışma tarafından ortaya konmuştur (5, 40, 43). Özellikle dünya nüfusunun artmasına bağlı olarak ruminant hayvan sayısındaki artışta, ruminant kaynaklı sera gazlarının (CH_4, CO_2 ve azot-dioksit) artmasına yol açacağı düşünülmektedir. Sarımsak yağının metanojenik bakteriler başta olmak üzere, bazı rumen mikroorganizmalarına karşı antimikrobiyal aktiviteye sahip oldukları bildirilmektedir (6, 27, 30, 43). Bu antimikrobiyal etki nedeniyle rumende besin maddelerinin (selüloz, nişasta, protein vb.) parçalanması azalacağı ve buna bağlı olarak rumende metan üretimi sınırlayarak olumlu etkisi olacağı bildirilmektedir (30, 31, 40, 43). Bu açıdan ruminantlardan kaynaklı sera gazlarının azaltılmasında SY'nın önemli bir kaynak olacağı söylenebilir.

Araştırmada rumen sıvısına farklı dozlarda SY ilavesi *in vitro* metan gazı üretimini önemli düzeyde azaltmıştır. Araştırma bulguları SY'nın farklı dozları ile çalışan Patra ve Yu (39), Mateos ve ark. (30), Patra ve Yu (40) ve Blanch ve ark. (6)'nın bulguları ile paralellik göstermiştir.

Sonuç olarak, *in vitro* koşullarda rumen sıvısına farklı dozlarda SY ilavesi *in vitro* gaz üretimini, GKMS, OMS ve NDFS ile ME içeriklerini düşürmüştür ($P<0.01$). Aynı şekilde rumen sıvısına artan seviyede ilave edilen SY rumen metabilitlerinden TUYA ve bireysel uçucu yağ asitleri ile NH_3N, CO_2 ve CH_4 üretimini azaltmış, rumen pH'sını ise artırmıştır. Araştırmadan elde edilen sonuçlar değerlendirildiğinde, SY'nın NH_3-N düzeyini azaltarak azot kaybını önleyeceği ve metan şeklinde enerji kaybını azaltarak yem enerjisinden daha etkin yararlanılacağı söylenebilir. Ayrıca SY'nın ruminant beslemede kullanımı durumunda CO_2 ve CH_4 gibi sera gazları azalarak sera gazı emisyonuna katkı sağlayacağı düşünülmektedir. Araştırmadan elde edilen veriler ve bu alanda yapılan diğer araştırma verileri değerlendirildiğinde, ruminant beslemede verimi olumsuz etkilemeden kullanılacak SY dozunun ise düşük tutulması gerektiğini (100-400 mg SY/L RS) araştırma bulgularıyla ortaya konmuştur. Sarımsak yağının ruminant beslemede kullanımı ile ilgili çalışma sayısı son zamanlarda artmasına rağmen, bu konuda daha fazla sayıda *in vitro* ve *in vivo* çalışmaya gerek olduğu söylenebilir.

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Ankara Üniv Vet Fak Derg, **66**, 297-302, 2019
DOI: 10.33988/auvfd.488253

The effect of centrally and peripherally injected CDP-choline on plasma nesfatin-1 level in rats

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Received date: 27.12.2018- Accepted date: 03.05.2019

Abstract: Nesfatin-1 has a role in appetite control and energy balance. The activity of the cholinergic system also is able to affect feeding behavior. Moreover, the central cholinergic system interacts with central nesfatinergic systems. The main goal of the study was to determine the effect of intracerebroventricular (icv) and intravenous (iv) administrated CDP-choline (0.5 ve 1 µmol; icv ve 250 mg / kg; iv) on levels of plasma nesfatin-1 in the homogeneous number of male and female fasted and the satiated Wistar albino rats. The polyethylene cannula was inserted into the carotid artery and jugular vein of the rats anesthetized with sevoflurane (2–4%/100% O_2) to collect blood samples and to make iv injection, respectively. For icv treatment, the lateral ventricle of rats was cannulated with guide cannula. The basal levels of plasma nesfatin-1 in the satiated rats were higher than those observed in the fasted animals. While 0.5 and 1 µmol dose of icv and/or 250 mg/kg dose of iv injected CDP-choline increased the level of plasma nesfatin-1 in the satiated rats, plasma nesfatin-1 level of the fasted animals decreased after the same dose and route of CDP-choline injection. The current findings show that CDP-choline can influence the level of plasma nesfatin-1 in the rats. The effect of the drug was different according to the food intake of the rats. These data might suggest a potential role in CDP-choline on plasma nesfatin-1 concentration.
Keywords: CDP-choline, intracerebroventricular, intravenous, plasma nesfatin-1.

Merkezi ve periferik olarak enjekte edilen CDP-kolin'in sıçanlarda plazma nesfatin-1 seviyesi üzerine etkisi

Özet: Nesfatin-1, iştah regülasyonunda ve enerji homeostazında rol oynar. Kolinerjik sistemin aktivitesi de beslenme davranışını etkileyebilir. Ayrıca, merkezi kolinerjik ve nesfatinerjik sistemler arasında bir etkileşim vardır. Bu çalışmada homojen sayıda erkek ve dişi aç bırakılmış ve tok Wistar albino sıçanlara, serebral yan ventrikül (syv) ve intravenöz (iv) uygulanmış CDP-kolinin (0.5 ve 1 µmol; syv ve 250 mg / kg; iv) plazma nesfatin-1 düzeylerine etkisinin belirlenmesi amaçlanmıştır. Sevofluran (% 2-4 /% 100 O_2) anestezisi altında, sıçanların karotis arteri kan örnekleri toplamak için ve juguler veni ise iv enjeksiyon yapmak için kanüle edildi. Syv enjeksiyonlar için ise sıçanların lateral ventrikülüne kılavuz kanül yerleştirildi. Tok sıçanların bazal plazma nesfatin-1 seviyeleri, aç bırakılan hayvanlardakinden daha yüksek olarak gözlendi. 0.5 ve 1 µmol dozunda syv ve / veya 250 mg / kg dozunda iv enjekte edilen CDP-kolin, tok sıçanlarda plazma nesfatin-1 seviyelerini artırırken, aynı doz ve yolla CDP-kolin uygulaması, aç bırakılan hayvanların plazma nesfatin-1 düzeylerini azalttı. Sonuçlar, CDP-kolinin sıçanlarda plazma nesfatin-1 seviyelerini etkileyebileceğini göstermektedir. İlacın etkisi sıçanların besin alımına göre farklılık göstermektedir. Bu veriler, CDP-kolinin plazma nesfatin-1 konsantrasyonu üzerinde potansiyel etkisi olabileceğini göstermektedir.
Anahtar sözcükler: CDP-kolin, serebral yan ventrikül, intravenöz, plazma nesfatin-1.

Introduction

Nesfatin-1 is an anorectic neuropeptide with 82-amino acid and it is generated from a precursor protein, nucleobindin-2 (NUCB2) (23). Food intake affects nesfatin-1 expression (23). Significantly decrease in food intake following centrally nesfatin-1 injection in rodents was reported. Moreover, nesfatin-1 also decreased food intake when it was delivered peripherally (9, 23, 26).

CDP-choline is a nucleotide-synthesized endogenously. It has a number of cellular effects in various experimental conditions (34). When CDP-choline is administered, it is able to create cardiovascular, respiratory, neuroendocrine and neuroprotective responses. It also has beneficial effects in the treatment of some neurodegenerative and neurovascular diseases (6, 11, 30, 32, 34). CDP-choline in the body is rapidly metabolized to its metabolites such as choline (18, 35).

CDP-choline raised choline levels in the body and then enhanced cholinergic transmission by increasing the synthesis of acetylcholine (4, 33).

CDP-choline also is related to food intake behavior. In a previous study, CDP-choline-induced significantly declines in appetite ratings were shown (14). It is also known that centrally injected CDP-choline decreased levels of serum ghrelin but increased levels of serum leptin in rats (15). But there is no data on whether CDP-choline can affect plasma nesfatin-1 level or if there is any correlation between the effect of CDP-choline on plasma nesfatin-1 level and CDP-choline-evoked appetite. It is very well known that the hypothalamus has a dense cholinergic innervation as well as an intense nesfatinergic neuron. In consideration of the dense cholinergic innervation of hypothalamus (21, 22, 23), and the effect of CDP-choline on the vagal nerve, CDP-choline treatment could have a potential to affect on plasma nesfatin-1 levels in different food intake condition. Therefore, it was contemplated to indicate the effect of centrally or peripherally delivered CDP-choline on plasma nesfatin-1 levels in satiated and fasted rats in the present study.

Materials and Methods

Animals: A total of 63, 3 months old, female (n=30) and male (n=33) Wistar albino rats (250–300 g) were provided from Experimental Animals Breeding and Research Center, Uludağ University, Bursa, Turkey to use for the current study experiments. Animals housed in controlled conditions (20–22 ^{0}C room temperature, 60–70 % humidity and 12 h light/dark cycle), as four or five rats in per cage. Satiated rats were fed *ad libitum* with free access to food and water. Fasted rats were housed without food but free access water for 12 h overnight. The Animal Care and Use Committee of Uludag University, in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, approved all experimental procedures (2014-08/04). Each group has 7 animals, and each rat was used in a single experiment.

Surgical procedures: The animals anesthetized with sevoflurane (2–4% / 100% O_2). Under the anesthesia, to collect the blood sample and to make the intravenous injection, PE 50 tubing containing 100 U/ml heparin was used to cannulate the left carotid artery and jugular vein of rats, respectively. For central treatment, the guide cannula was inserted to the lateral cerebral ventricle. Briefly, a burr hole was drilled the skull according to 1.5 mm lateral to the midline and 1.0 mm posterior to bregma. The guide cannula was inserted into the hole and lowered 4.5 mm below the surface of the skull. And then the guide cannula was fixed to the skull with acrylic cement. Before the experiments, the rats were rested in separate cages for 4–5 h to be allowed to recover from anesthesia.

Drug and icv injection: CDP-choline was bought from Sigma-Aldrich Co. (Deisenhofen, Germany) the desired concentration of the CDP-choline dissolved in saline on the day of the experiment. Icv doses of CDP-choline were chosen from the study reporting the effect of central injected CDP-choline on serum ghrelin and leptin levels in rat (15) and iv dose of CDP-choline was chosen from the study showing the effect of CDP-choline on plasma vasopressin and catecholamines levels, and renin activity (28).

To perform icv delivery, the injection cannula was connected to a microsyringe with polyethylene tubing. The drug or its vehicle was delivered icv in a volume of 5 µl over a 60s time period. To ensure the drug was entirety delivered, the air bubble moving in the polyethylene tubing was tracked.

Experimental procedure: Firstly, to test how central injection of CDP-choline affected the plasma nesfatin-1 levels in both sexes of the fasted or the satiated rats, 0.5 (n=7 for fasted group; n=6 satiated group) and 1 µmol (n=7 for fasted group; n=6 satiated group) doses of CDP-choline or saline (5 µl; n=6 for fasted group; n=6 satiated group) were icv injected. 250 µl blood samples for plasma nesfatin-1 levels were collected via the arterial catheter before injection and at the 5, 10, 20, 40 and 60th min of injection.

Secondly, it was determined if peripherally injected CDP-choline had a role in the plasma nesfatin-1 levels in both sexes of the fasted or the satiated rats. For this reason, CDP-choline (250 mg/kg, iv; n=7 for fasted group; n=6 satiated group) or saline (1 ml/kg, iv; n=6 for fasted group; n=6 satiated group) was administered to the rats through venous catheter. Again 250 µl blood samples for levels of plasma nesfatin-1 were collected via the arterial catheter before injection and at the 5, 10, 20, 40 and 60th min of injection. Table 1 shows female and male animal number for each injection line.

Table 1. Female and male animal number for each injection line.
Tablo 1. Her enjeksiyon için dişi ve erkek hayvan sayısı

		Fasted rats number		Satiated rats number	
		Female	**Male**	**Female**	**Male**
Icv	Saline	3	3	3	3
	0.5 µmol	3	4	3	3
	1 µmol	3	4	3	3
Iv	Saline	3	3	3	3
	250 mg/kg	3	4	3	3

Determination of plasma nesfatin-1 level: To separate the plasmas, the blood samples in tubes containing EDTA were centrifuged at +4 ^{0}C, 1800 rpm for 20 min, and then plasma samples were kept at -80 ^{0}C up to plasma nesfatin-1 level measurements. Microplate enzyme immunoassay (ELISA), which was purchased from Phoenix Pharmaceuticals Inc. (CA, USA) was used to measure the plasma concentration of nesfatin-1. The measurements were performed according to the kit instruction. Level of plasma nesfatin-1 was expressed as ng/ml. Each sample was used for once to measure plasma nesfatin-1 level.

Statistical analysis: The results are given as mean ± standard error of the mean (S.E.M.). $P<0.05$ was considered as the level of significance. The repeated-measures two-way analysis of variance (RM-ANOVA; two-way) and the post-ANOVA test of Bonferroni were used for statistical evaluation.

Results

The fasted rats had 7.44 ± 0.82 ng/ml (7.38 ± 0.73 ng/ml for female rats and 7.50 ± 0.94 ng/ml for male rats) the basal levels of plasma nesfatin-1 (Fig 1). Levels of plasma nesfatin-1 were diminished significantly at 60 min following 0.5 and 1 µmol dose of icv (Fig 1A) or 250 mg/kg dose of iv (Fig 1B) CDP-choline injection in the fasted rats compared with saline-injected group. Both icv and iv injected CDP-choline-induced decreasing in plasma nesfatin-1 level started suddenly and lasted up to 60 min after the injections (Fig 1A, B).

The satiated rats had 13.37 ± 1.78 ng/ml (12.98 ± 1.24 ng/ml for female rats and 13.76 ± 2.32 ng/ml for male rats) the basal plasma nesfatin-1 levels (Fig 2). Administration of 0.5 and 1 µmol dose of icv (Fig 2A) or 250 mg/kg dose of iv (Fig 2B) CDP-choline significantly increased plasma nesfatin-1 levels of the satiated rats according to saline injected satiated rats' levels, respectively. While the increase in plasma nesfatin-1 level of the icv CDP-choline injected satiated rats continued up to 60 min after the injection (Fig 2A), the increase in plasma nesfatin-1 level of the iv CDP-choline injected satiated rats was observed only at 5th min of the injection (Fig 2B).

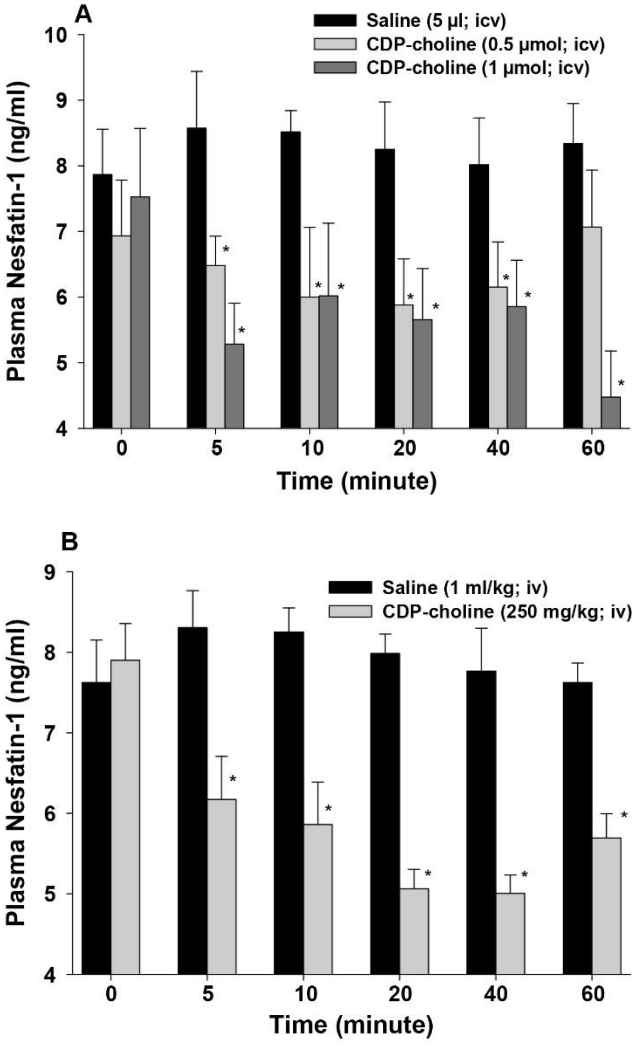

Figure 1. Effects of icv (A) and iv (B) administrated CDP-choline on plasma nesfatin-1 levels of the fasted rats. Data are given as means ± S.E.M. *P<0.05 significantly different from the saline group.

Şekil 1. Syv (A) ve iv (B) uygulanan CDP-kolinin aç bırakılmış sıçanlarda plazma nesfatin-1 seviyeleri üzerine etkileri. Veriler ortalama ± standart hata olarak verilmiştir. * P<0.05 tuzlu su grubuna göre anlamlı farkı göstermektedir.

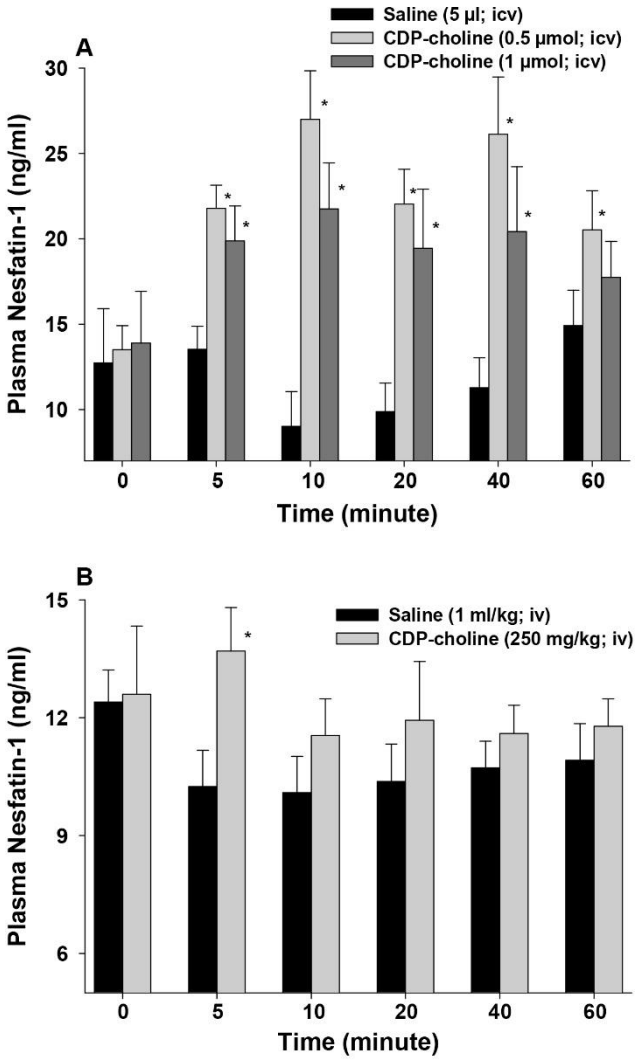

Figure 2. Effects of icv (A) and iv (B) injected CDP-choline on plasma nesfatin-1 levels of the satiated rats. Data are given as means ± S.E.M. * P<0.05 significantly different from the saline group.
Şekil 2. Syv (A) ve iv (B) uygulanan CDP-kolinin tok sıçanlarda plazma nesfatin-1 seviyeleri üzerine etkileri. Veriler ortalama ± standart hata olarak verilmiştir. * P<0.05 tuzlu su grubuna göre anlamlı farkı göstermektedir.

Discussion and Conclusion

The current findings report that CDP-choline differently affected plasma nesfatin-1 levels in fasted or satiated rats. The drug considerably enhanced levels of plasma nesfatin-1 in satiated conditions however it significantly suppressed the levels of plasma nesfatin-1 in fasted animals. Interestingly, basal levels of plasma nesfatin-1 were higher in satiated rats than those observed in fasted animals.

The data clearly show that 12 h fasting caused to decrease in levels of plasma nesfatin-1 in rats. The levels of plasma nesfatin-1 in the satiated rats were observed almost 2 times more than the fasted rats. It was explained that the hypothalamic NUCB2 mRNA expression was significantly decreased after the rats fasted for 24 h (23). On the other hand, refeeding after 48 h fasting period resulted in an increase in nesfatin-1 immunoreactivity in the hypothalamic area (16). It was also reported that gastric mucosa expressed NUCB2 mRNA almost 10 times more than the brain (31). Again 24 h fasting in rats caused decreasing in gastric mucosal NUCB2 mRNA expression. Those reports evidently indicate that the fasting led to suppress in the releasing of nesfatin-1 in central and peripheral and also those reports support our findings showing 12 h overnight fasting causing the decrease in plasma nesfatin-1 level. Moreover, in the current study, both sexes of rats were used in all groups and levels of plasma nesfatin-1 were quite similar in both sexes of rats. There is evidence supporting our current findings, which there are no differences between healthy male and female subjects in levels of plasma nesfatin-1 (17).

In the current study, the data show that the effect of CDP-choline on levels of plasma nesfatin-1 depends on satiety or fasting conditions. Although levels of plasma nesfatin-1 were suppressed by icv or iv CDP-choline injection in 12 h fasted rats, they were elevated by same routes and doses administered CDP-choline in satiated rats. CDP-choline is a highly bioavailable compound and it rapidly metabolized and increased acetylcholine concentration in the brain and, plasma and tissue, when it was injected (18, 25, 27, 35). Also, CDP-choline can cross the blood-brain barrier (29). Hence when it injected

peripherally, it could enhance cholinergic transmission in the brain and peripheral tissues. It was well known that activation of the hypothalamus or afferent/efferent vagal stimulus with cholinergic innervation has an important role in feeding control. Those hypothalamic or vagal innervations with icv or iv CDP-choline injection might cause the increase or decrease in plasma nesfatin-1 concentrations in the satiated or fasted rats, respectively. It has not been shown whether gastric mucosa is the source of the plasma nesfatin-1. However, there is a possibility that plasma nesfatin-1, at least in part, may be derived from gastric mucosa, because the abundance of the NUCB2 mRNAs (31) and the mRNAs for prohormone converting enzymes (20) in gastric mucosa have been described. Also, it was demonstrated that the nesfatin-1 concentrations in serum were raised after vagus nerve stimulation in high-fat-fed rats (8). This report is well compatible with the data showing an increase in levels of plasma nesfatin-1 after icv or iv injection of CDP-choline in satiated rats. Since icv or iv injection of CDP-choline causes to stimulate vagal nerve by activating cholinergic system it may increase in plasma nesfatin-1 levels, particularly in satiety condition.

Central and peripheral CDP-choline and choline treatments have some roles on glucose metabolism such as increase in plasma insulin and hyperglycemia depended on an increase in plasma levels of catecholamines (3, 10, 12, 13). Previous studies indicate that high levels of plasma glucose and insulin stimulated hypothalamic nesfatin-1 neurons so that meal ingestion was stimulated by nesfatin-1 neurons in the hypothalamus and thereby produces satiety (7). It was also reported that diabetic patients had lower plasma nesfatin-1 than healthy subjects (1). Considered the role of CDP-choline on glucose metabolism, the effect of CDP-choline on plasma nesfatin-1 concentrations might be secondarily effect depended on CDP-choline-induced glucose metabolism changes.

Hypothalamus plays vital roles in the control of appetite and energy metabolism. It is well known that hypothalamus has dense cholinergic innervations and is rich from nesfatinergic (23) and cholinergic neurons (21-23). It has been shown that both CDP-choline (14) and nesfatin-1 (23) have the anorexigenic effect. These results indicate that both cholinergic and nesfatinergic systems neuroanatomically originate from the same brain area and they have the same effect on food intake behavior. The central nesfatinergic and cholinergic systems interact with each other (2). Because centrally applied nesfatin-1 produced an increase in levels of the hypothalamic acetylcholine and choline and also central cholinergic receptors mediated the nesfatin-1-induced cardiovascular responses (2). Recently it has been reported that central injection of CDP-choline suppressed levels of serum ghrelin but increased levels of serum leptin in fed *ad libitum* rats (15). It was also demonstrated that leptin directly activated nesfatinergic neurons in the hypothalamus (5). It was known that obese subjects had high plasma nesfatin-1 concentration but low plasma ghrelin levels (24). When the role of CDP-choline on levels of plasma leptin and ghrelin, and its anorexigenic effect are taken consideration, CDP-choline might have a role on plasma nesfatin-1 level depends on satiety and fasting conditions. The effects of CDP-choline on plasma nesfatin-1 concentrations in the current study also support an earlier report showing that cholinergic mechanisms play a role in food intake behavior (14).

In conclusion, the current data demonstrate that the level of plasma nesfatin-1 was significantly lower in 12 h fasted rats than satiated rats. Furthermore, centrally and/or peripherally applied CDP-choline caused the additional decrease in plasma nesfatin-1 concentration of the rats fasted 12 h. In contrast, icv and/or iv CDP-choline injection increased extra the plasma nesfatin-1 levels in the satiety condition. Nevertheless, the current data did not explain which mechanism mediated CDP-choline related plasma nesfatin-1 levels change. However, the data might be conceded that the cholinergic system has a physiological role in nesfatin-1 release. The results may give an idea about CDP-choline usage for obesity treatment but future investigations are needed on the topic.

Acknowledgement

This study was supported by the Commission of Scientific Research Projects of Uludağ University, Project number HDP (T2012/10). This paper is the result of a Master thesis performed by Hikmet Aysin Usta at Bursa Uludağ University in Bursa.

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Ankara Üniv Vet Fak Derg, **66**, 303-309, 2019
DOI: 10.33988/auvfd.512959

A recursive path model for estimation of the live weight using some body measurements in Awassi sheep

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Received date: 15.01.2019- Accepted date: 30.04.2019

Abstract: Aim of this study was to investigate direct, indirect and total effects of body measurements on live weight in Awassi sheep and to derive prediction equations for estimation of the live weight. Live weight, head length, body length, withers height, chest depth, chest width and rump height measurements of 270 Awassi sheep at the age of 2-4 years raised at a private farm in Eskişehir Province, Turkey was used. A recursive path model was established to estimate the live weight of the Awassi sheep using these body measurements. Results showed that there was a significant indirect effect of withers height, rump height and body length and significant direct effect of chest depth, chest width and head length on live weight. The strongest predictor of live weight was chest depth, followed by chest width and head length. In conclusion, the equations given in this study could serve as a practical tool to predict the live weight of Awassi sheep.

Keywords: Awassi sheep, live weight, path analysis, structural equation modelling.

İvesi koyunlarda bazı vücut ölçüleri yardımıyla canlı ağırlığın tahmin edilmesinde kullanılabilecek rekursif bir path modeli

Özet: Bu çalışmanın amacı, İvesi ırkı koyunlarda çeşitli vücut ölçülerininin canlı ağırlık üzerine doğrudan, dolaylı ve toplam etkilerini incelemek ve bu ölçülerden yararlanarak canlı ağırlığı tahmin edecek bir model oluşturmaktır. Bu amaçla, Eskişehir ilinde özel bir çiftlikte yetiştirilen 2-4 yaşlarındaki toplam 270 İvesi ırkı koyunun canlı ağırlığı, baş uzunluğu, gövde uzunluğu, cidago yüksekliği, göğüs derinliği, göğüs genişliği ve sağrı yüksekliği ölçümleri elde edilmiştir. Bu ölçümler yardımıyla canlı ağırlığı tahmin etmek için rekursif bir path modeli oluşturulmuştur. Elde edilen sonuçlar, cidago yüksekliği, sağrı yüksekliği ve vücut uzunluğunun vücut ağırlığına dolaylı; göğüs genişliği, göğüs derinliği ve baş uzunluğunun ise doğrudan etkisinin olduğunu göstermiştir. Canlı ağırlığın en güçlü belirleyicisi göğüs derinliği, ardından göğüs genişliği ve baş uzunluğu olarak belirlenmiştir. Sonuç olarak, bu çalışmada verilen denklemlerin, İvesi koyunlarının canlı ağırlığını tahmin etmek için pratik bir araç olarak kullanılabileceği ortaya konmuştur.

Anahtar sözcükler: Canlı ağırlık, İvesi koyunu, path analizi, yapısal eşitlik modeli.

Introduction

Awassi is a multipurpose fat-tailed sheep breed with high adaptability and is one of the most widespread breeds in the Middle East and Southwest Asia (3, 25). The Awassi breed has been raised in South-eastern Anatolia, Syria, Iraq, Israel. It has also been introduced into many countries like Australia, India, Macedonia, Ethiopia, Spain, and New Zealand to study its performance and to be crossed with native breeds, showing its importance (34). Taking into consideration the place of lamb meat and milk in the food supply, the breed also has an essential role in Turkey, despite the declining numbers.

The body measurements of livestock give useful information about their morphological structure. Determination of live weight and body measurements in certain periods in sheep breeding are essential in terms of monitoring growth of the sheep so that it can be useful to make appropriate management decisions. Also, the estimation of live weight using various body measurements could be practical and relatively easy for breeders with insufficient resources (18).

Many studies showed the close relationship between live weight and various body measurements and also reported on the use of various body measurements of

animals to determine live body weight of cattle (1, 10, 21), sheep (2, 9, 14, 15, 24, 29) goat (16, 18) and pig (30). However, most of these authors deal with these linear measurements using correlation coefficients and multiple regression methods. On the other hand, examining only the direct effect of one variable on another may also not be optimal as in multiple regression model since some of the effects of an independent variable on a dependent variable may be transmitted through intervening variables. For that reason, a more suitable method to evaluate the relationship among these variables is needed.

Structural equation modeling (SEM) is a second-generation multivariate analysis technique that encompasses a broad array of statistical techniques such as path analysis, confirmatory factor analysis, latent growth models, item response theory and even including multiple linear regression (11). A Path model, which is the application of structural equation modeling without latent variables, could provide greater flexibility to test the structure coefficients on a theoretical basis. Although different models were used, the application of this technique is limited and reported in Karacabey Merino sheep (4), Yankasa sheep (33), Sahiwal cattle (17) and Landrace × Large White pigs (13).

The aim of this study was (1) to evaluate direct, indirect and total effects of various body measurements on live weight of Awassi sheep breed using structural equation modeling technique and (2) to show this technique's advantages over multiple regression analysis.

Material and Methods

In this study, a total of 270 Awassi ewes at the age of 2-4 years raised in a private enterprise at Eskişehir Province, Turkey was used (19). Eskişehir has a continental climate with very cold winters, hot summers and relatively sparse precipitations. The data collected after the shearing season and the temperatures range between 19.5^0C and 33^0C during the study period. The ewes were produced under semi-intensive management system and fed with the routine programme of the farm. Live weight was taken using a digital scale (kg) sensitive to 50g. The body measurements taken from animals were as follows:

Head Length: Frontal distance from mouth to poll,

Chest Depth: Vertical distance from the top of the withers to the xiphoid process,

Chest Width: Maximum intercostal diameter at the level of the 6^{th} rib, just behind the articulatio humeri,

Body Length: the distance from the tip of sternum to the base of the tail,

Withers Height: The distance from shoulder blades (the top of the withers) to the ground,

Rump Height: The distance from the top of the rump to the ground.

All body measurements were taken using tailor's tape measure and measuring stick while all animals were in standing position with head raised and weight on all four feet without body movement.

Statistical analysis: Descriptive statistics were calculated and presented as "Mean ± Standard error of the mean" for all variables. Pearson's correlation analysis was used to determine the strength and direction of the relationships among the variables. A preliminary assessment was performed using the multiple regression analysis with backward elimination method to identify the variables to be included in the path analysis. VIF and tolerance statistics were employed to analyse multicollinearity. In path analysis, the parameter estimates were obtained using the maximum likelihood method whereas observed information matrix (OIM) technique was used to obtain the variance-covariance matrix of the parameter estimates. Modification indices were used to give an empirical basis for making model modifications on theoretical sense to improve model fit. The goodness of fit testing of the model was evaluated by the following indexes: comparative fit index (CFI), Tucker-Lewis index (TLI), the root mean square error of approximation (RMSEA) and standardized root mean square residual (SRMR). STATA 12.1 MP4 statistical package was used for all statistical analysis. A p value less than 0.05 was considered statistically significant.

Results

Descriptive statistics for all variables in the model were calculated and presented in Table 1. The Pearson's correlation coefficients among live weight and the body measurements were given in detail in Table 2. The strongest correlation was between rump height and withers height ($r=0.764$, $P<0.001$).

Table 1. Descriptive statistics of the variables that are used in the model (n=270).
Tablo 1. Modelde kullanılan değişkenlere ilişkin tanımlayıcı istatistikler (n=270).

Variable	Mean	Standard deviation	Median	Minimum	Maximum
Live weight (kg)	47.2	4.8	47.4	37	64.1
Head length (cm)	19.4	2.02	19.5	15	24.5
Chest depth (cm)	30.2	1.91	30.5	24.5	35
Chest width (cm)	18.9	1.67	18.7	14.5	29.5
Body length (cm)	65.2	3.31	65.5	52.5	73
Withers height (cm)	61.4	2.46	61	51	67
Rump height (cm)	58.2	2.59	59	48	67

The preliminary multiple regression analysis completed after five steps. After the elimination of non-significant variables in each step, the final model included only head length, chest depth and chest width as the significant predictors of live weight. The R squared value of the final regression model was 0.270 (Table 3).

Using the information from the preliminary multiple regression, a structural equation model was established (Figure 1). The model included three exogenous variables: withers height, rump height, and body length. The only endogenous outcome variable in Figure 1 was live weight, the final outcome. The remaining variables, chest depth, chest width, and head length, were included as endogenous variables that mediate some part of the effect of antecedent variables on subsequent variables. Each of these endogenous variables was explained by other variables in the model (Table 4). R squared for equation level goodness of fit was 0.609. Relationship between exogenous variables was shown with a curved line. In concordance with the results of the modification indices, the model also considered any possible correlated residuals, assuming that some of the unexplained variances in chest width would be correlated with some of the unexplained variance in head length.

Table 4 shows the path coefficients of the four models for each endogenous variable. Considering the only direct relationship between endogenous mediator variables (chest depth, chest width, head length) and the endogenous outcome variable (live weight), the results were in agreement with preliminary regression model showing that the strongest predictor of live weight was chest depth, followed by chest width and head length (Table 4).

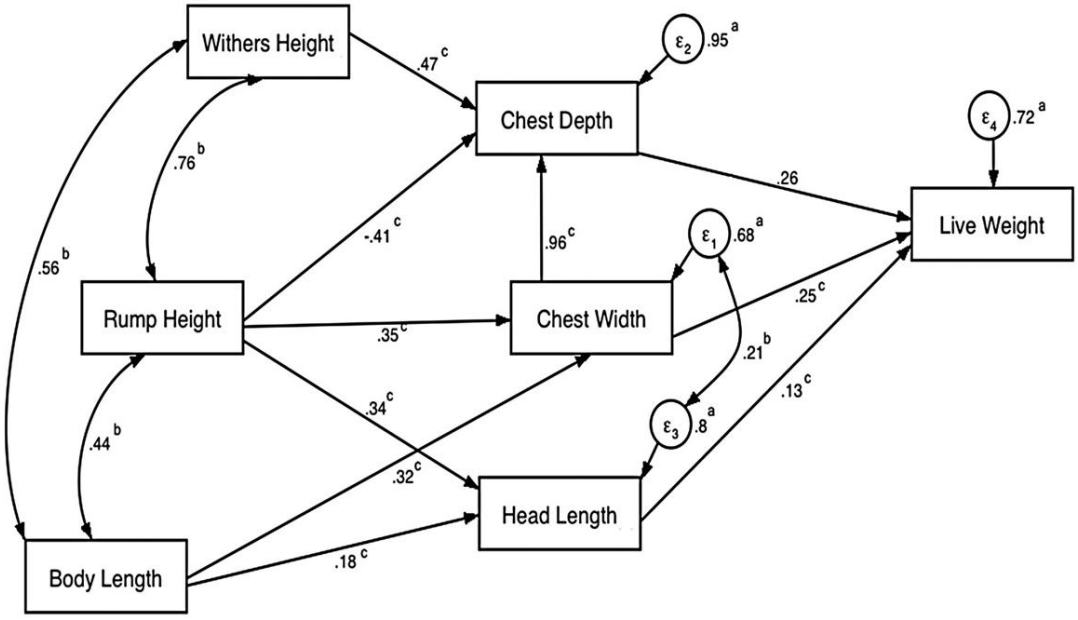

Figure 1. The recursive path model for estimating live weight of Awassi breed. In the figure, estimated variances were represented with the superscript letter a; standardized covariances with b and standardized path coefficients with c.
Şekil 1. İvesi koyunlarında canlı ağırlığın tahmini için oluşturulan rekursif path modeline ilişkin diyagram. Şekilde hesaplanan varyanslar a, standardize edilmiş kovaryanslar b, standardize path katsayıları ise c ile gösterilmiştir.

Table 2. Pearson's correlation coefficients among body measurements and live weight (n=270).
Tablo 2. Vücut ölçüleri ile vücut ağırlığı arasında hesaplanan Pearson korelasyon katsayıları (n=270).

	Live weight	Head length	Chest depth	Chest width	Body length	Withers height	Rump height
Live weight	1	0.347***	0.568***	0.542***	0.312***	0.390***	0.322***
Head length		1	0.503***	0.340***	0.327***	0.381***	0.417***
Chest depth			1	0.553***	0.603***	0.668***	0.504***
Chest width				1	0.518***	0.443***	0.486***
Body length					1	0.560***	0.437***
Withers height						1	0.764***
Rump height							1

*** P<0.001

Table 3. Results of the preliminary multiple regression analysis using live weight as dependent variable and the body measurements as independent variables. Analysis completed after five steps.
Tablo 3. Vücut ağırlığı bağımlı değişken, vücut ölçüleri bağımsız değişkenler olmak üzere, ön eleme çoklu regresyon modeli sonucu. Analiz toplam beş adımda gerçekleştirilmiştir.

Step no	Variable	B	SE	Std. B	t	P	95% CI		Collinearity statistics	
							LB	UB	Tolerance	VIF
Step 1	(Constant)	1.982	6.94		0.28	0.775	-11.68	15.65		
	Head length	0.343	0.15	0.14	2.29	0.023	0.048	0.64	0.69	1.43
	Chest depth	0.559	0.21	0.22	2.61	0.009	0.138	0.98	0.39	2.59
	Chest width	0.797	0.19	0.28	4.13	<0.001	0.417	1.18	0.58	1.70
	Body length	-0.056	0.11	-0.04	-0.52	0.603	-0.27	0.16	0.49	2.06
	Withers height	0.323	0.19	0.16	1.72	0.087	-0.05	0.69	0.28	3.47
	Rump height	-0.131	0.16	-0.07	-0.82	0.413	-0.44	0.18	0.36	2.75
Step 5 (Final Step)	(Constant)	7.194	4.12		1.75	0.082	-0.92	15.3		
	Head length	0.313	0.15	0.13	2.16	0.032	0.03	0.59	0.74	1.34
	Chest depth	0.682	0.17	0.26	3.92	<0.001	0.34	1.03	0.58	1.71
	Chest width	0.733	0.18	0.25	4.11	<0.001	0.38	1.08	0.69	1.45

B: Beta; SE: Standard error; Std. B: Standardized beta; LB: Lower bound; UB: Upper bound

Table 4. Unstandardized and standardized estimates for the recursive path model.
Tablo 4. Rekürsif path modeline ilişkin standardize edilmiş ve standardize edilmemiş tahminler.

Dependent (Endogenous) variables	Independent variables	Path coefficients			z	P
		Unstandardized		Standardized		
		Beta	SE	Beta		
Chest width	Rump height	0.224	0.035	0.346	6.29	<0.001
	Body length	0.163	0.027	0.321	5.85	<0.001
	Constant	-5.085	2.09		-2.43	0.015
Chest depth	Withers height	0.358	0.047	0.472	7.57	<0.001
	Rump height	-0.296	0.077	-0.411	-3.8	<0.001
	Chest width	1.271	0.197	0.961	6.29	<0.001
	Constant	2.05	2.61		0.79	0.432
Head length	Rump height	0.26	0.046	0.339	5.69	<0.001
	Body length	0.108	0.036	0.179	3.01	0.003
	Constant	-2.62	2.68		-1	0.318
Live weight	Chest depth	0.682	0.172	0.262	4.07	<0.001
	Chest width	0.733	0.177	0.253	4.056	<0.001
	Head length	0.313	0.144	0.128	2.183	0.029
	Constant	7.19	4.089		1.76	0.079

SE: Standard error

Although preliminary regression model showed no significant effect of withers height, rump height and body length on live weight, the path model revealed that there was a significant indirect effect of these three variables (withers height, rump height and body length) on live weight (Table 5). Considering both direct and indirect effects of the variables, the strongest total effect was from chest width followed by chest depth and head length.

The chi-square test of absolute model fit was reported as 12.83, along with 7 degrees of freedom returning with a probability value of P=0.076, which suggests that the model fits the data acceptable. Corroborating evidence is provided by other relative model fit statistics such as RMSEA, CFI, TLI and SRMR suggesting a close approximate model fit to data (Table 6).

Table 5. Direct, indirect and total effects of exogenous variables with their unstandardized beta coefficients.
Tablo 5. Ekzojen değişkenlere ilişkin standardize edilmemiş beta katsayıları ve doğrudan, dolaylı ve toplam etkiler.

Endogenous variables	Exogenous variables	Direct effect		Indirect effect		Total effect	
		Beta	SE	Beta	SE	Beta	SE
Chest depth	Chest width	1.27***	0.202	no path		1.27***	0.202
	Withers height	0.358***	0.047	no path		0.358***	0.047
	Rump height	-0.295***	0.078	0.284***	0.073	-0.011 NS	0.044
	Body length	no path		0.208***	0.026	0.207***	0.026
Chest width	Rump height	0.224***	0.036	no path		0.224***	0.036
	Body length	0.163***	0.028	no path		0.163***	0.028
Head length	Rump height	0.259***	0.046	no path		0.259***	0.047
	Body length	0.108**	0.036	no path		0.108**	0.035
Live weight	Chest depth	0.682***	0.173	no path		0.681***	0.172
	Chest width	0.733***	0.177	0.866***	0.138	1.599***	0.224
	Head length	0.313*	0.144	no path		0.313*	0.144
	Withers height	no path		0.244***	0.069	0.244***	0.069
	Rump height	no path		0.238**	0.073	0.238**	0.073
	Body length	no path		0.295***	0.048	0.295***	0.047

* P<0.05; ** P<0.01; *** P<0.001; NS: P>0.05; SE: Standard error

Table 6. The goodness of fit values of the specified model.
Tablo 6. Oluşturulan modele ilişkin uyum iyiliği kriterleri

Goodness of fit criteria	Close approximate fit	Reasonable approximate fit	Achieved Goodness of Fit Values for the Model
$\chi 2$	$0 \leq \chi 2 \leq 2df$	$2sd \leq \chi 2 \leq 3df$	11.591
P value	$0.05 < P \leq 1.00$	$0.01 \leq P \leq 0.05$	0.072
RMSEA	$0 \leq RMSEA \leq 0.05$	$0.05 \leq RMSEA \leq 0.08$	0.057 (PCLOSE=0.340)
CFI	$0.97 \leq CFI \leq 1.00$	$0.95 \leq CFI \leq 0.97$	0.994
TLI	$0.95 \leq TLI \leq 1.00$	$0.90 \leq TLI \leq 0.95$	0.978
SRMR	$0 \leq SRMR \leq 0.04$	$0.5 \leq SRMR \leq 0.10$	0.02

RMSEA: Root mean square error of approximation; TLI: Tucker-Lewis index; CFI: Comparative fit index; SRMR: Standardized root mean residual

Discussion and Conclusion

The relationship between live weight and various body measurements is well known in farm animals and it is one of the most important traits for the study of growth pattern and daily management. The accuracy of models used to predict the live weight of farm animals using various body measurements is of immense financial contribution to the enterprise (2).

The present study has shown a statistically significant correlation between live body weight and the body measurements supporting the hypothesis that the body weight could be predicted based on these measurements. The correlation of chest depth and chest width with live body weight were the strongest among other measurements. Previous studies also reported a high correlation between various body measurements and live weight in sheep and goats (4, 5, 12, 16, 20).

The most common predictive model used to interpret the relationship between live weight and body measurements are the multiple regression model (8, 23). A multiple regression represents a particular model of relationships in which all possible predictors are treated as co-equal and their interrelations are un-analyzed. So that the ability to obtain interpretable results from such models depends on a degree to which their structure matches the true relations among variables. Choosing a second-generation multivariate technique, however, could increase chances of discovering the relationship between dependent variables as well as between independent variables and dependent variables from one-time analysis simultaneously. Also, it is possible to show the direct effect, indirect effect and total effect via mediation. In this study, we have specified a mediational model where the effect of height at withers, rump, body length and head length on live weight flow through chest width and chest depth so chest width and chest depth are mediating variables between exogenous variables and the live weight. Although the provided model fits the data very well and ensures a theoretically consistent set of evidence, it is also possible that there may be other equivalent or non-equivalent models that fit the data well or even better.

One of the interesting and important findings of this study was a non-significant effect of withers height, rump height and body length in the preliminary multiple regression model. Topal et al. (28) also established a multiple regression model for Awasi breed in which there is no significant effect of these three variables in a similar manner. However, many previous studies with different breeds showed a significant direct effect of these three variables and these variables were included in their models (5, 15, 27). Using the advantage of structural equation modeling technique, it was revealed that these variables had no significant direct but indirect effects on live weight. This finding was also a good example to show the importance of the correct selection of techniques to reveal the relationship between variables. The final model revealed that the highest total effect to the live weight was chest width and chest depth which is inconsistent with the previous studies (4, 15, 28, 33).

It has been well known that there is a high phenotypic correlation between live weight and heart girth (2, 24). However, heart girth measurement was not included in the analysis, which could be considered as a limitation of the study. One of the issues with the current data was the high correlation of heart girth with live weight, chest depth and withers height measurements resulting in multicollinearity, which causes estimates of standardized partial regression coefficients and path coefficients to be less precise, less accurate and prone to rounding error (22). Collinearity implies that the effect of one predictor cannot be uniquely identified with the effect of another predictor. In such circumstances, the statistical model can include only one of the two predictors. Many alternative models such as PCA regression, PLS regression, Ridge regression, Lasso regression, etc have been proposed in the literature to address multicollinearity problem (7), however, this issue is out of the scope of this study. In this study, heart girth was excluded from the analysis among the collinear variables according to Weisberg's criteria (31). In this context, previous studies also reported collinearity between heart girth and withers height, despite the fact that different methodology was used to dealing with this problem (6, 26, 32).

According to the results of this study, it could be concluded that live weight can be predicted on the basis of linear body measurements in Awassi sheep breed. The established path model improved model fit compared to the multiple regression model and provided a great advantage by evaluating the indirect effects of some body measurements.

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Ankara Üniv Vet Fak Derg, **66**, 311-315, 2019
DOI: 10.33988/auvfd.436199

Short Communication / *Kısa Bilimsel Çalışma*

Detection of allele and genotype frequencies of bovine leukocyte adhesion deficiency, factor XI deficiency and complex vertebral malformation disease genes in Holstein cattle

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Received date: 25.06.2018- Accepted date: 01.05.2019

Abstract: Hereditary diseases cause yield and economic loses. It is important to examine hereditary diseases at the molecular level and to remove diseases from the herd. In our study, it was aimed to determine allele frequencies of genes that cause bovine leukocyte adhesion deficiency, factor XI deficiency and complex vertebral malformation diseases in Holstein cattle. Blood samples were randomly taken from 300 Holstein cattle in different dairy farms in Kocaeli, Sakarya and Balıkesir provinces. Deoxyribonucleic acid samples were isolated from blood samples by using the standard ammonium acetate salt-out method. The target regions were amplified by polymerase chain reaction to determine the mutant alleles causing bovine leukocyte adhesion deficiency, factor XI deficiency and complex vertebral malformation. According to the nucleotide chromotograms of the samples subjected to bovine leukocyte adhesion deficiency analysis, it was determined that 4 out of 300 cattle were heterozygous and 296 were homozygous. Polymerase chain reaction procedure for factor XI deficiency disease was sufficient, while samples amplified by polymerase chain reaction for complex vertebral malformation disease were subjected to restriction particle length polymorphism. Factor XI deficiency and complex vertebral malformation disease genes were all homozygous normal.

Keywords: Anaemia, β2 integrin glycoprotein, Holstein, point mutation, PCR-RFLP, SLC35A3.

Holştayn ırkı sığırlarda sığır lökosit bağlanma yetmezliği, faktör XI eksikliği ve kompleks vertebral malformasyon hastalıkları genlerinin allel ve genotip sıklıklarının tespit edilmesi

Özet: Kalıtsal hastalıklar verim ve ekonomik kayıplara neden olur. Kalıtsal hastalıkları moleküler düzeyde incelemek, hastalıkları sürüden uzaklaştırmak için önemlidir. Araştırmamızda, Holstayn ırkı sığırlarda sığır lökosit bağlanma yetmezliği, faktör XI eksikliği ve kompleks vertebral malformasyon hastalıklarına sebep olan genlerin allel sıklıklarının tespit edilmesi amaçlanmıştır. Kocaeli, Sakarya ve Balıkesir illerinde bulunan farklı çiftliklerdeki 300 adet Holştayn sığırdan kan örnekleri alınmıştır. Kan örneklerinden standart amonyum asetat çöktürme yöntemiyle deoksiribonükleik asit örnekleri izole edilmiştir. Sığır lökosit bağlanma yetmezliği, faktör XI eksikliği ve kompleks vertebral malformasyona sebep olan mutant allelleri belirlemek için hedef bölgeler polimeraz zincir reaksiyonu ile çoğaltılmıştır. Sığır lökosit bağlanma yetmezliği için dizin analizi işlemine tabi tutulan örneklerin nükleotid kromotogramlarına göre toplamda 300 adet sığırdan 4 tanesi heterozigot birey, 296 tanesinin homozigot birey olduğu tespit edilmiştir. Faktör XI eksikliği hastalığı için polimeraz zincir reaksiyonu işlemi yeterli olup, kompleks vertebral malformasyon hastalığı için ise polimeraz zincir reaksiyonu ile çoğaltılan örnekler restriksiyon parçacık uzunluğu polimorfizmi işlemine tabi tutulmuştur. Faktör XI eksikliği ve kompleks vertebral malformasyon hastalıkları genlerinin hepsi homozigot normal bulunmuştur.

Anahtar sözcükler: Anemi, β2 integrin glikoprotein, Holştayn, nokta mutasyonu, PCR-RFLP, SLC35A3.

About 70% of the cows utilized for the milk industry in the world are artificially inseminated (9). The artificial insemination method increases the yield per animal, while at the same time raising genetic similarity in breeds (28). Increasing genetic similarity within the breed has been shown to cause the spread of hereditary diseases, which rapidly reduces fertility in the population. It has been reported that these hereditary diseases tend to spread to the whole world in a very short time, even continents (23). For this reason, it is emphasized that the mutant alleles that

cause hereditary diseases can be cleared from the population only by determining the carrier individuals and removing them from the herd (1). It is stated that about 300 hereditary diseases have been detected in cattle breeds which have been extensively bred in the last fifty years (2).

Insufficiency of bovine leukocyte binding; was detected in the Holstein cattle. It can cause death due to the mucosal infections that appear at birth. Bovine leukocyte adhesion deficiency (BLAD) is a hereditary disease that is characterized by autosomal recessive inheritance (1,7). Bovine leukocyte binding deficiency disease is a point mutation that causes the substitution of the adenine nucleotide with the guanine nucleotide at position 383 of the gene that encodes the β2 integrin (CD18) glycoprotein, the subunit of the CD11/CD18 complex, which provides endothelial-leukocyte binding. The disease was reported in Turkey in 2004 (4). Factor XI, one of the proteins involved in blood coagulation, is also called serine protease (11). It has been determined that the disease results in a mutation that causes 76 bases to be added to exon 12 of factor XI gene (23). In cattle, this hereditary disease was first identified in US Holsteins in 1969. The mutant alleles causing factor XI deficiency (FXID) in Holsteins bred in Turkey were first reported in 2009 (19). There are several symptoms such as prolonged bleeding time after injection, bloody milk intake and anaemia (9, 18). In addition to these symptoms, calves show a lower birth weight and survival rate than the norm. It has been stated that the probability for affected animals to catch infectious diseases is higher than that of normal calves (18). FXID has reduced clotting activity in heterozygous individuals, causing it to completely disappear in homozygotes (19). Holstein cattle, a mutation is formed in the SLC35A3 gene that encodes the uridine diphosphate-N-acetylglucosamine transporter. Point mutation results the disease that causes the guanine nucleotide at position 559 of the SLC35A3 gene to change places with thymine (2). Complex vertebral malformation (CVM) is a lethal disease characterized by autosomal recessive inheritance in Holstein cattle. Approximately 80% of fetuses that are homozygous with the mutant alleles are discarded before the 260th day of gestation (3). The offspring that do complete the normal gestational period is mostly stillborn. Symptomatic joint curvatures, spinal cord curvature, malformations in the vertebral column, multiple spinal anatomical defects in the neck and back vertebrae of the spinal column extending from the skull to the tail spin, and adhesion in the vertebrae are characteristics of shortened neck and thoracic vertebrae in offspring that are not discarded (2, 3, 8). This is the most important of the clinical signs and the lethal shortness of the spine for the embryo (2). Holsteins are one of the most preferred cattle breeds around the world, also in Turkey. Autosomal genetic diseases are an important issue for dairy cattle owners. It is necessary to investigate the genetic structures of cattle in Turkey in terms of hereditary disorders. Due to the inadequacy of native gene resources, the genetic structures of many hereditary diseases thought to be unique to the race have not been fully brought to light. The main objective of this study was to identify hereditary disease carrier BLAD, FXID and CVM in Kocaeli, Sakarya and Balıkesir's dairy cattle population. The results of our study will be a resource for future studies.

This study was approved by the Local Ethics Committee on Animal Experiments of Istanbul University (Approval no: 09.09.2013/103). For the study, blood samples were collected randomly from 300 Holstein cattle in Kocaeli (Kandıra: Elite Agriculture and Livestock Milk Production Center (54 cattle), Aygaz (21 cattle), Asım Ağa Farm (25 cattle), Sakarya (Karapüçek town Ali Rıza Yıldız (20 cattle), İsmet Yıldız (19 cattle), İbrahim Kulaç (18 cattle), Yıldırım Özcan (17 cattle), Tacit Şahin (12 cattle), Hasan Burnaz (9 cattle), Aydın Özcan (5 cattle) and Balıkesir (Okyanus Farm, 100 cattle) provinces. Samples were taken from the tail vein, collected in vacuum tubes containing ethylene diamine tetra acetic acid (EDTA) and stored at -20ºC. Genomic deoxyribonucleic acid-(DNA) samples were isolated using the standard salt-out method (21). The Polymerase Chain Reaction (PCR) mixture volume of 25 µl contained the following: 1 U Taq deoxyribonucleic acid polymerase (Fermantas Life Sci., Canada), 2- 2.5 ml 10X PCR buffer, 1.5 mM $MgCl_2$, 50-100 ng DNA, 100 mM (dNTPs) (Fermantas Life Sci., Canada), and 10 pmol of each primer for all of the fragments. Designed primers (20), were used to reproduce the mutated region of BLAD; the order of primers was as follows: F: 5'GAATAGGCATCCT GCATCATATCCACCA3', R:5'CTTGGGGTTTCAGG GGAAGATGGAGTAG3'. Amplification consisted of, an initial denaturation 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, annealing at 61°C for 1 min and extension at 72°C for 1 min, with a final extension of 72°C for 10 minutes. Designed primers (31), were used to reproduce the mutated region of FXID; the order of primers was as follows: F: 5'CCCACTGGCTAGGA ATCGTT3', R: 5'CAAGGCAATGTCATATCCAC3'. Amplification consisted of an, initial denaturation 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min, with a final extension of 72°C for 10 minutes. Designed primers (17), were used to reproduce the mutated region of CVM: the order of primers was as follows: F: 5'CACA ATTTGTAGGTCTCACTGCA3', R: 5'CGATGAAAAA GGAACCAAAAGGG3'. Amplification consisted of an, initial denaturation 96°C for 5 min, followed by 30 cycles of 95°C for 30 sec, annealing at 56°C for 1 min and extension at 72°C for 30 sec, with a final extension of 72°C

for 10 minutes. PCR products were visualized after electrophoresis on a 2% agarose gel with a long-wavelength UV transilluminator (Thermo Fisher Scientific, Germany). Genotyping for CVM was done using PCR-RFLP methods. The PCR products were digested with the following fast digest restriction endonuclease: Eco T22 I (Ava III) for CVM disease. For RFLP analysis, 10 ml of the PCR products were digested for 60 minutes with 10 units of restriction enzyme at 37°C. The digested DNA fragments were separated by electrophoresis in 2% agarose gel. BLAD genotypes were identified by DNA sequencing. In our study, the sequence analysis process was carried out by Refgen-Gene Research and Biotechnology Ltd. Sti. (Gölbaşı-Ankara). Sequencing was performed by using an ABI-3100 sequencer (PE Biosystems) and the BiyDye Tm terminator cycle sequencing kit after the purification of the PCR products. The forward primer was used to sequence the PCR products of BLAD. The genotypes of FXID were detected by PCR methods. Genotype and allele frequencies of each genetic disease were calculated using the PopGene 32 software program and chi-square tests (χ^2) were also used to check whether the populations were in Hardy-Weinberg equilibrium using PopGene 32 software (32).

PCR amplification for BLAD, FXID and CVM was successful in all 300 samples that were analyzed. BLAD genotypes were identified by DNA sequencing. After sequencing, 4 BLAD carriers were found. The distributions of the CD18 protein allele and genotype frequency in Holstein cattle are shown in the Table 1. After the PCR, the normal FXID allele in unaffected animals produced a single 244 bp fragment. In homozygous affected animals, the fragment had a length of 320 bp and FXID carriers exhibited two fragments of 244 bp and 320 bp. The PCR products of CVM was digested with Eco T22 I (Ava III). After digestion, the normal CVM allele in unaffected cattle produced a single fragment of 233 bp. CVM carriers exhibit three fragments of 233 bp, 212 bp and 21 bp. Among the 300 Holstein cattle reared in Kocaeli, Sakarya and Balıkesir provinces 4 BLAD carriers found only, while carriers of FXID and CVM were not detected.

The incidence of BLAD carriers was found to be 1.37% in China (33), 6.6% in Iran (14), 5% (24) and 1.69% (25) in India. In Turkey, Şahin et al. (27) and Korkmaz Ağaoğlu et al. (16) reported the prevelance of BLAD carriers in Holsteins to be 2.18%, 2% respectively. In our study, the incidence of BLAD carriers among the Holstein cattle was estimated at 1.33%. According to the results we have obtained, our incidence is found to be lower than those reported in studies from China, India, Iran and Turkey. If we consider that there are more than 300 Holstein cattle in Kocaeli, Sakarya and Balıkesir provinces, the change of the incidence by increasing the number of animals in the study is possible.

According to the statistical result made, the number of individuals expected to be carriers in the future was calculated as 3.98. A change that would express a statistical significance of this ratio in the future is not expected and shows the distribution in accordance to the Hardy-Weinberg equilibrium and has not statistical significance. However, the removal of carrier individuals from the herd is of great importance in terms of preventing the emergence of sick individuals in the future. Carrier individuals must not be used in selection works. If the individuals are male, the sperm must not be used and if female, they must not be impregnated. Furthermore, these animals must be sent for slaughter in order to keep them away from the herd. There are some factors affecting diseases that occur in a region. Most significant are factors such as the effect of regional sperm export in the last 10 years, the frequency of use of this sperm in insemination, whether or not animals with genetic defects have been introduced, and whether or not artificial insemination or a stud bull was used in impregnation.

Previously, carriers of FXID have been identified in Turkish Holstein cattle (5, 15). The frequency of mutant FXID allele was estimated to be 1.69% (5) and 0.4% (15), similar to that the frequency reported by Mondal et al. 1.69% (22). Similarly, the percentage of carrier cattle was found to be 0.67% (31) and 1.8% (16). The prevalance of carriers found in this study was 0%, similar to previous studies (6, 10). The frequency in our study was found to be low compared to other studies. The low frequency is similar to results from studies in Iran, and this situation may be due to the fact that the embargo applied to Iran inhibited animal transportation.

Table 1 Detection of allele and genotype frequency of BLAD.
Tablo 1 BLAD'ın alel ve genotip sıklığının tespiti.

		Genotype				Allele frequency (%)		
		AA		AG		A	G	
Breed	**n[1]**	**Obs.[2]**	**Exp.[3]**	**Obs.[2]**	**Exp.[3]**			**(χ^2)[4]**
Holstein	300	296	296.01	4	3.980	0.9933	0.0067	0.0101 NS

[1]Number of the Holstein cattle, [2]Observed of values, [3]Expected of values, [4]Hardy-Weinberg equilibrium, NS: Not significant

The absence of animal transportation may prevent the same bulls from being used in artificial insemination, and the absence of the disease-causing genome in these bulls may prevent the disease from appearing in other animals.

Twenty-six and thirty-four CVM carriers were identified, corresponding to the heterozygote carrier frequency of 21.6% (26) and 34% (14) respectively in Iran, and no mutant homozygote was found. In China, it has been reported that the frequency of the carrier animals are 10.48% (33), 2.92 % (29) and 15.58% (30) in Holstein cattle. In our country, Kulaklı and Akyüz (17) did not find carriers of CVM. In our study, we did not find any carriers of CVM. Increasing the number of animals we use in our study may cause a change in the frequency of this disease. The prevention of genetic variations in species used for breeding farm animals has played a role in reducing economic losses. Studies carried out in the field of molecular genetics are useful in reducing anomalies that may occur at birth or in periods later in life (1). The importance of heterozygous animals can be easily understood considering the fact that many hereditary diseases follow recessive inheritance (12). Heterozygous animals can survive for many years without showing any symptoms. Therefore they need to be removed from the herd (13).

In our study, 300 Holstein cattle raised in the provinces of Kocaeli, Sakarya, and Balıkesir were examined and 4 BLAD disease carriers were found, while no carriers of the FXID or CVM diseases were found. In our study, the aim was to investigate mutant alleles causing BLAD, FXID and CVM diseases in Holstein cattle in Turkey, so as to screen candidate breeders for these diseases, and determine a feasible method to do so in the future. Given the size of the Holstein population in Turkey, the probability of having more individuals with BLAD, FXID, and CVM diseases arise. Therefore, the number of studies that screen candidate Holstein breeders in different regions of Turkey must be increased.

Acknowledgement

This study was supported by the Istanbul University BAP Project Application (Project number: 38954). This study was produced from a PhD thesis. We wish to thank the cattle Breeders of Kocaeli, Sakarya and Balıkesir.

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Ankara Üniv Vet Fak Derg, **66**, 317-320, 2019
DOI: 10.33988/auvfd.538077

Short Communication / *Kısa Bilimsel Çalışma*

Bir Avrasya porsuğunda (*Meles meles*) rastlanan parazitler

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Özet: Ondokuz Mayıs Üniversitesi Veteriner Fakültesi Parazitoloji Laboratuvarına getirilen, trafik kazasında ölmüş bir erkek porsuk parazitolojik açıdan sistematik olarak incelenmiştir. Parazitolojik inceleme sonucu porsuğun üç ektoparazit ve üç endoparazit türü ile enfekte olduğu saptandı. Ektoparazit olarak bir pire (*Paraceras melis*) ve iki kene türü (*Ixodes ricinus, Rhipicephalus sanguineus*), endoparazit olarak bir sestod (*Mesocestoides melesi*) ve iki nematod türü (*Aonchotheca putorii, Molineus patens*) belirlendi. Türkiye'de parazitlerin tümü için porsuk yeni konak, *M. melesi* ise Türkiye faunası için ilk bildirimdir.
Anahtar kelimeler: *Aonchotheca putorii, Mesocestoides melesi, Molineus patens, Paraceras melis,* porsuk.

Parasites found in a Eurasian badger (*Meles meles*)

Abstract: A male badger *(Meles meles)* died in a traffic accident admitted to the Parasitology Lab of the Faculty of Veterinary Medicine of Ondokuz Mayıs University was examined systemically. Parasitological examination revealed that the badger was infected with 3 ectoparasites and 3 endoparasites. Following ectoparasites were detected; one flea (*Paraceras melis*) and two ticks (*Ixodes ricinus, Rhipicephalus sanguineus*) and as endoparasites one cestode (*Mesocestoides melesi*) and two nematode species (*Aonchotheca putorii, Molineus patens).* Badger is a new host for all parasites in Turkey, while *M. melesi* is the first report for the helminth fauna of Turkey.
Keywords: *Aonchotheca putorii,* badger, *Mesocestoides melesi, Molineus patens, Paraceras melis.*

Avrasya porsuğu (*Meles meles*) sansargiller (Mustelidae) ailesinden, tombul gövdeli, kısa bacaklı, keskin dişli ve tırnaklı, memeli bir hayvan olup Asya ve Avrupa'nın belirli bölgelerinde yaşar. Türkiye'de özellikle ılıman iklime sahip kumlu bölgelerde, çalılık ve ormanlık alanlarda görülür. Boyu 60-70 cm, kuyruğu 15-20 cm, ağırlığı 15-20 kg dolayında, yeraltı tünellerinde yaşayan gececi bir hayvandır. Geceleri beslenmek amacıyla yuvasından çıkar ve böcek, kemirici, meyve, tahıl, mantar, bal gibi her türlü gıdayla beslenir. Bu nedenle birçok parazite ev sahipliği yapmaktadır (20).

Porsuk parazitleri hakkında Türkiye'de ilk bildirim, bu olguda bahsedilen parazitlerin 2015 yılında Erzurum'da 19. Ulusal Parazitoloji kongresinde sunulmasıyla yapılmıştır (4). Takiben Kars'ta dişi bir porsukta *Trichodectes melis* isimli bit türüne rastlanmıştır (18). Bununla birlikte bazı ülkelerde yapılan çalışmalarda porsuklarda onlarca parazite rastlanmış ve bazıları porsuklara özgü isimle adlandırılmıştır. Nitekim Avrasya porsuğunda ektoparazit olarak bit; *T. melis* (8, 18), pire; *Paraceras melis* (1) ve *Ixodes* türü kenelere (16) rastlanmaktadır. Helmint türleri daha çok görülmekte olup trematodlardan *Alaria alata, Echinostomum melis, Euryhelmis squamula, Isthmiophora melis* (20), sestodlardan *Atriotaenia incisa* (12, 14), *Mesocestoides* sp. ve *Taenia* sp. (12), nematodlardan *Angiostrongylus vasorum* (14, 20), *Aonchotheca putorii, Crenosoma melesi, Molineus patens, Pearsonema plica, Physaloptera sibirica, Spirocerca melesi* vb. (12, 14, 20) gibi onlarca türe rastlanmıştır. Türkiye'de bu parazitlerden bazılarına farklı hayvan türlerinde rastlanmış ve kızıl tilkilerde *P. melis* (2), evcil hayvanlar yanında domuz, keme, kirpi, sincap, tavşan ve tilkilerde *I. ricinus* (2, 5, 11), domuz, kirpi, tavşan ve tilkilerde *R. sanguineus* (11), sansarlarda *A. putorii* ve *M. patens* kaydedilmiştir (11).

Türkiye'de yabani hayvanların parazitleri yeterince bilinmediği için araştırma yapılan her hayvanda, Türkiye faunası için yeni parazit türlerine rastlanmaktadır. Bu

bildirimin amacı, ilk kez porsukta rastlanan ve Türkiye faunası için yeni kayıt olan türler hakkında bilgi vermektir.

Samsun, Ondokuz Mayıs ilçesinde bir köy yolunda trafik kazasında ölen bir porsuk, kaza yerinden geçen bir Veteriner hekim tarafından Anabilim Dalımıza getirilmiştir. Hayvana sistematik nekropsi yapılmış ve rastlanan parazitler ılık fizyolojik tuzlu su ile temizlendikten sonra, ektoparazit ve nematodlar kaynama sıcaklığındaki 70°C lik etil alkol ile sestodlar ise AFA ile tespit edilmiştir. Sonra keneler stereo mikroskopta direkt, pireler % 10 KOH ile saydamlaştırıldıktan sonra Hoyer mediumu ile monte edilerek, helmint türleri ise laktofenolde saydamlaştırılarak literatür (1, 6, 9, 14, 22) yardımıyla teşhis edilmiş, tanıda önemli bölgelerin ölçümü yapılmış, fotoğraflanmış ve kayıt numarası verilerek müzeye konulmuştur.

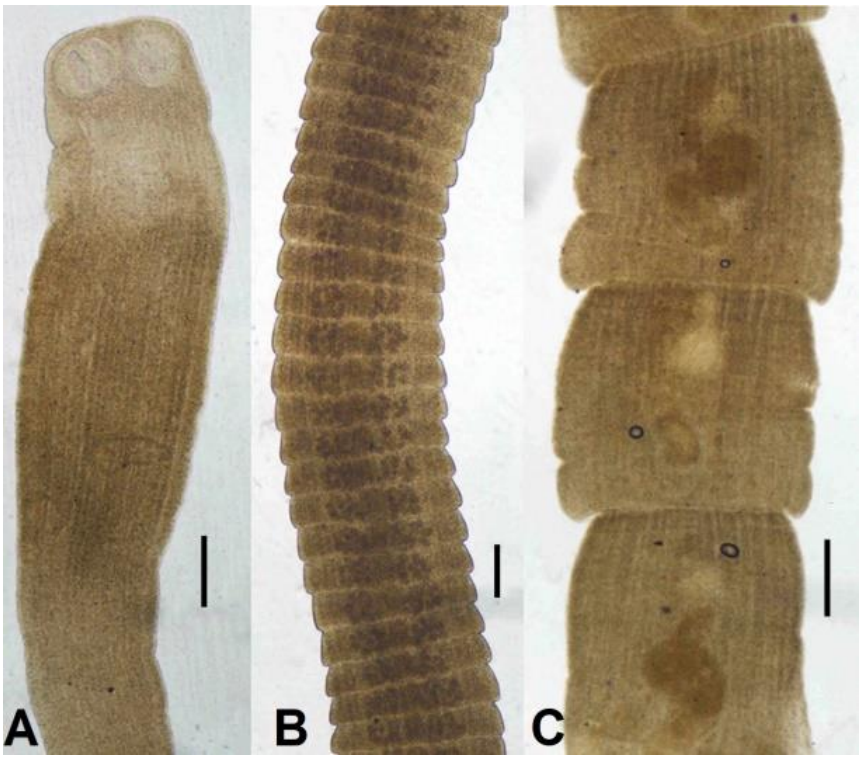
Ektoparazitlerden bir pire (bir dişi *P. melis*) ve iki kene türü (altı dişi, üç erkek *I. ricinus*; üç erkek, sekiz dişi *R. sanguineus*) saptanmıştır. Endoparazit helmintlerden bir sestod (dört adet *M. melesi*) ve iki nematod türü (dört erkek, dört dişi *A. putorii*; üç erkek, dört dişi *M. patens*) olmak üzere altı parazit türü saptanmıştır.

Porsuklara özgü bir pire olan *P. melis* kokarca, sansar, tilki, geyik gibi yabani memeliler ile bazen evcil kedi, köpek gibi hayvanlarda görülmekte ve birçok Avrupa ülkesinde rastlanmaktadır (1). Boyutları erkekte 2.5-3.5, dişide 3-4.5 mm kadar ve kırmızımtırak renkli olup parazitin morfolojisi ve ölçüm sonuçları literatüre uygun bulunmuştur (1).

Türkiye'de koyun, keçi, sığır gibi evcil hayvanlarda yaygın olan *I. ricinus* Karadeniz başta olmak üzere özellikle sahil bölgelerinde görülür. Bunun yanında tavşan, tilki, porsuk, kirpi gibi memeli hayvanlar ile fare, sıçan gibi kemiriciler ve hatta kertenkelelerde rastlanmaktadır (2, 5). Etken morfolojik olarak evcil hayvanlardaki örneklerle benzer özelliklere sahip olarak gözlenmiştir.

Köpek kenesi olarak bilinen *R. sanguineus* Türkiye'de tüm bölgelerde görülmekte, koyun, keçi, sığır, manda, at, eşek, kedi, köpek gibi evcil hayvanlar ile tilki, tavşan, kirpi gibi yabani hayvan ve insanlara tutunmakta, birçok riketsia ve protozoonun vektörlüğünü yapmaktadır (5). Morfolojik olarak evcil hayvanlardaki ile benzer özelliktedir.

Porsuklarda bulunan *Mesocestoides* türü *M. melesi* olarak kabul edilmekte, Avrupa'da porsuklarda sık görülmekte ve sansarlarda da rastlanmaktadır (10, 22). Parazit küçük yapılı olup, uzunluğu 5.6 (3.1-8.2) cm, skoleks çapı 0.45 (0.38-0.52) mm, çekmenler 0.16 (0.14-0.19) mm, boyundan sonraki genç halkalar 0.60 (0.55-0.63) mm genişlikte, ergin halkaların genişliği ise 0.61 (0.57-0.65) mm ölçülmüştür. Parazitler genç olduğu için son halkalarda paruterin organlar gelişmiş, ancak yumurtaların henüz tam gelişmediği görülmüştür. Bu nedenle genç ve küçük yapılı olan paruterin organlar genişliğine oranla daha uzun ve boyutları 0.23-0.25 x0.19-0.22 mm olarak saptanmıştır (Şekil 1).



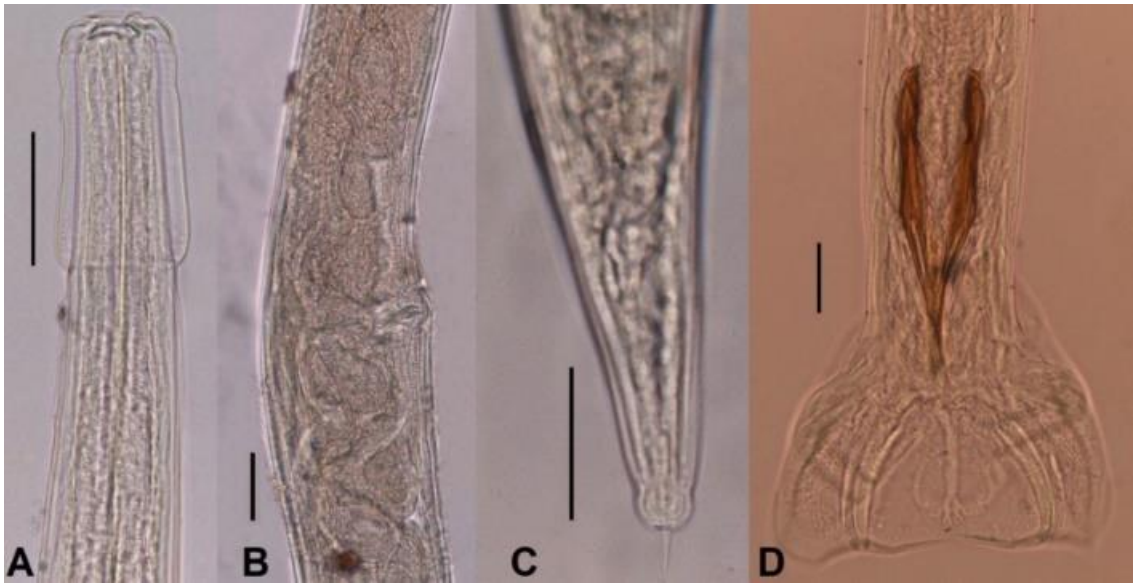
Şekil 1. *Mesocestoides melesi* (A: Skoleks ve boyun, B: Genç halkalar, C: Gebe halkalar, (Çubuk: 0 0.2 mm).
Figure 1. *Mesocestoides melesi* (A: Scolex and neck, B: Young proglottids, C: Gravid proglottids, (Bar: 0.2 mm).

Mesocestodies türlerinin erginleri evcil ve yabanıl karnivorlarda parazitlenir. Bu cinste geçerli 12 tür bulunmasına karşın, günümüzde morfolojik ve moleküler olarak ayrılabilen dört tür mevcut olup bunlar; *M. lineatus, M. litteratus, M. canislagopodis* ve *M. corti* (*M. vogae*)'dir (7, 17). Bununla birlikte Tenora (19), *M. melesi*'nin ilk tanımlanmasının yetersiz olduğu, bu nedenle *M. lineatus*'un sinonimi olabileceğini öne sürmüştür. Bu nedenle morfolojik olarak *M. melesi* olarak adlandırılan tür ve diğer türlerin moleküler olarak doğrulanmasında yarar bulunmaktadır. Yukarıdaki türlerden Türkiye karnivorlarında ergin *M. lineatus, M. corti* (*M. vogae*) ve vaşakta *Mesocestoides* sp.'ye rastlanmıştır (15). Bu nedenle *M. melesi* Türkiye parazitoloji faunası için ilk kayıttır. Erginden daha patojen olan larval tetrahyridosis, Türkiye'de son konak olan kedi, köpek gibi karnivorlar yanında kertenkele, tavuk, tavus kuşu gibi hayvanlardan da bildirilmiştir (3).

Aonchotheca (*Capillaria*) cinsi 30 dolayında tür içermekte olup bu cinsten Türkiye'de babunlarda *A. annulosa*, sığırlarda *A. bilobata*, sığır, koyun ve keçilerde ve *A. bovis* (*C. bovis*), kemiricilerde A. *putorii* ve *A. speciosa* saptanmıştır. Bunun yanında çeşitli memelilerde *Calodium hepaticum, C. papillosum, Capillaria* sp., *Eucoleus aerophilus, Pearsonema plica* gibi capillarid türler kaydedilmiştir (11, 21).

Capillarid bir parazit olan *A. putorii* kozmopolit yayılışa sahip olup Amerika ve birçok Avrupa ülkesinde kokarca, mink, porsuk, sansar gibi çeşitli yabani etçiller ve bazen evcil kedilerde görülür (21). Merdivenci (11), parazitin Türkiye'de sansarlarda görüldüğünü bildirmiş, ancak nerede, ne zaman ve ne kadar görüldüğü hakkında herhangi bir bilgi vermemiştir. Bu nedenle parazit Türkiye'de porsuk için ilk kayıt olmaktadır. Kıl şeklinde olan parazitin erkekleri 5-9, dişileri 9-15 mm olup kütikülası enine çizgili, iki lateral basiller bant mevcut ve anüs subterminaldir. Erkeğin arka ucunda geniş iki kanat mevcut olup, kaudal kanadın ucu parmağımsı iki çıkıntı taşır. Spikülüm kitinize yapılı ve distal ucu lanset şeklindedir. Dişide bazen vulva bölgesini kısmen kapatan bir kapak bulunabilir. Yumurtalar iki ucunda tıkaç olan limon şeklinde ve yüzeyi çizgilidir (Şekil 2).

Şekil 2. *Aonchotheca putorii* (A: Ön uç, B: Erkek, arka uç, C: Dişi, arka uç, D: Dişi, vulva ve uterus) (Çubuk: 0.05 mm).
Figure 2. *Aonchotheca putorii* (A: Anterior end, B: Male, posterior end, C: Female, posterior end, D: Female, vulva and uterus) (Bar: 0.05 mm).



Şekil 3. *Molineus patens* (A: Ön uç, B: Dişi, vulva, C: Dişi, arka uç, D: Erkek, bursa copulatrix, (Çubuk: 0.05 mm).
Figure 3. *Molineus patens* (A: Anterior end, B: Female, vulvar region, C: Female, posterior end, D: Male, bursa copulatrix, (Bar: 0.05 mm).

Porsuk, kokarca, sansar, tilki gibi hayvanlarda bulunan *M. patens* daha çok Kuzey Avrupa ülkeleri ve kutup bölgelerinde görülmektedir. Baş tarafında sefalik şişlik ve biraz geride kolayca görünen boşaltım deliği mevcuttur. Özofagus kassal ve bezsel olarak iki kısma ayrılır. Yapılan ölçümlerde ergin erkekler 7.38 (6.72-7.92) mm uzunlukta, bursa copulatrix başlangıcında genişliği 0.92 (0.89-0.96) mm saptanmıştır. Bursal kaburgalar 2–3 veya 2-4 şeklinde düzenlenmiş, dorsal kaburga ucu yanlara birer kısa dal verip ikiye ayrılır ve bunun uçları da iki parçalı olup 6 çatallı gibi görünür. Spikülümler 0.192 - 0.200 mm uzunlukta olup distal ucu üç dallı olup, bir kol diğer iki koldan uzundur. Gubernakulum dar ve uzun yapılı olup 0.1- 0.11 mm uzunluktadır.

Dişiler daha büyük olup 8.05 (7.24- 8.77) mm uzunlukta, vulva hizasında genişliği 0.14 (0.13-0.15) mm'dir. Vulva arka uca 1.8-2 mm mesafede olup üzerinde kapak yoktur. Leiper (9), vulvada kapak olduğunu öne sürmüş, ancak Popiołek ve ark. (13), benzer yapıdan söz etmemiştir. Anüs arka uca yakın olup kuyruk 0.13-0.14 mm uzunlukta ve ucunda 12.9 (11.6-14.7) µm uzunlukta dikenimsi yapı mevcuttur. Bulduğumuz tür literatürde (7) belirtilen özelliklere uygun olmakla birlikte dişide vulvada kapağa rastlanmamıştır (Şekil 3).

Sonuç olarak, Türkiye'de parazitlerin tümü için porsuk yeni konak, *M. melesi* ise Türkiye faunası için ilk bildirimdir.

Teşekkür

19. Ulusal Parazitoloji Kongresi'nde poster bildiri olarak sunulmuştur.

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Ankara Üniv Vet Fak Derg, **66**, 321-323, 2019
DOI: 10.33988/auvfd.455001

Case Report / *Olgu Sunumu*

The rarely seen congenital anomaly in a queen: Unilateral ovarian agenesis

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Received date: 11.09.2018- Accepted date: 03.05.2019

Abstract: Congenital malformations of the reproductive system are rarely encountered in queens and etiology is not known precisely. Agenesis of the ovary is one of the congenital anomalies and usually found incidentally during laparotomy operations. In this case, a 2-year-old female cat referred to the clinic for ovariohysterectomy is described. Before anesthesia and surgery, a routine examination was performed. In physical examinations and results of blood samples did not reveal any problem for the operation. After the pre-operative process, ovariohysterectomy was performed. Ovary and the uterine horn were identified on the right-side. However, only the uterine horn without any abnormality was detected on the left-side. The cat was diagnosed with unilateral ovarian agenesis. Then, a blood sample was taken to determine serum steroid hormone levels. Serum estradiol and progesterone levels were measured as 18 pg/ml and 1.4 ng/ml, respectively. As a conclusion, unilateral ovarian agenesis that is incidentally detected the laparotomy procedures were no adverse effect on sexual activity in queens.
Keywords: Cat, congenital anomaly, ovarian agenesis.

Bir kedide nadir görülen konjenital anomali: Tek taraflı ovaryum agenezi

Özet: Kedilerde üreme organlarının konjenital anomalisi nadiren görülür ve etiyolojisi tam olarak bilinmemektedir. Ovaryum agenezi konjenital anomalilerden biridir ve genellikle laparotomi ameliyatları sırasında rastlantısal olarak bulunur. Bu vakada ovariyohisterektomi için kliniğe getirilen 2 yaşlı dişi bir kedi sunuldu. Anestezi ve ameliyat öncesi rutin muayene yapıldı. Fiziki muayeneler ve kan örnekleri sonucunda ameliyat için herhangi bir sorun olmadığı belirlendi. Pre-operatif hazırlıklardan sonra operasyon gerçekleştirildi. Sağ tarafta kornu uteri ve ovaryum tespit edildi. Ancak sol tarafta sadece herhangi bir anormallik bulunmayan kornu uteri gözlendi. Olgu tek taraflı ovaryum agenezi olarak teşhis edildi. Daha sonra serum steroid hormon düzeylerini belirlemek için kan örneği alındı. Serum östradiol ve progesteron düzeyleri 18 pg / ml ve 1.4 ng / ml olarak ölçüldü. Sonuç olarak, laparatomi esnasında tesadüfen teşhis edilen tek taraflı ovaryum agenezinin kedilerde seksüel aktiviteye olumsuz etkisinin olmadığı belirlenmiştir.
Anahtar sözcükler: Kedi, konjenital anomali, ovaryum agenezi.

It is well known that congenital malformations of the reproductive system are rarely encountered in cats (3). And also, it is not adequately defined in the literature (6) and etiology is not known precisely. In addition, these anomalies are among the causes of reproductive disorders and cannot usually be detected before the puberty. Furthermore, the diagnosis of congenital abnormalities of reproductive organs such as the uterus and ovary relies on direct inspection through various techniques (1). Agenesis of the ovary is one of the congenital malformations and the diagnosis has usually been made incidentally during ovariohysterectomy/ovariectomy or during other laparotomy operations (9). Ovarian agenesis can affect one or both ovaries and accompany defects (absent or underdeveloped) of the tubular reproductive organs. In some cases, it can result in infertility and permanent anestrus which accompanied by an absence of cyclical behavior. And also, as it is known, treatment is not possible (1). In our knowledge, only one case of unilateral ovarian agenesis written by Pawar and Nadkarni (7) is reported so far.

The material of this case report was a female cat that 2-year- old, Mix Racial and weighs of 3 kg. The cat was fed with dry cat food. Due to prevention for estrus

behavior, the cat was presented for routine ovariohysterectomy to Clinic of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Ankara University, Turkey. As anamnesis, the cat owner reported that the cat was in heat period about 12 days ago, general health was good and has not ever been mated. On pre-operative physical examination, the pulse, rectal temperature, and respiratory rates of the cat were within normal ranges. A venous catheter (24-gauge, Mediflon) were placed in medial saphenous vein to permit postoperative blood sampling and drug infusion. Blood samples were taken for hematology tests. Results of complete blood count and serum biochemistry analysis were shown in Table 1 and Table 2, respectively. The cat was premedicated with atropine sulphate (Atropin 0.2%, Vetaş, Turkey) 0.045 mg per kg of BW subcutaneously followed by induction of anesthesia with Propofol (Fresenius, Fresenius Kabi, Australia GmbH) 6 mg per kg of BW intravenously. General anesthesia was maintained with isoflurane through inhalation. After preparation of the mid-ventral region for operation, a ventral midline celiotomy was carried out through about 2.5-cm incision to the skin at the umbilicus. After confirming, the uterine horns were raised to the abdominal wall. The ovary and uterine horn were identified on the right-side, however, only the uterine horn without abnormality was detected on the other side (Figure 1). The cat was diagnosed with unilateral ovarian agenesis. After confirming the unilateral ovarian agenesis, a blood sample was taken to evaluate hormonal status. Right ovarian pedicle was ligated and transected, right and left uterine horns were transected from slightly cranial to the cervix after application of essential ligations and clamping (Figure 1). The body wall was routinely closed in a simple suture pattern. For all surgery, 2/0 USP PGA suture material was used for ligations and closures. Serum estradiol and progesterone levels were measured as 18 pg/ml and 1.4 ng/ml, respectively. Post-operative treatment regimen included amoxicillin-clavulanic acid dosed at a per os dose of 25 mg per kg BW twice daily for 7 days. The wound was managed with povidone iodine and bandaged with daily changes. Skin sutures were removed 7^{th} days postoperatively. The cat recovered uneventfully.

Ovarian agenesis is extremely uncommon in cats (1) and it was reported that is more often seen in ruminants, pigs, and dogs (7). It can result in permanent anestrous and infertility (1,5) if both ovaries are affected. And also, it can accompany by an infantile genital tract beside absence of cyclical behavior (1). However, the findings of our case don't support this knowledge. Because of serum estrogen concentrations which above 15 pg/ml and serum progesterone concentrations which above 1 ng/ml are evidence of cyclic/sexual activity. Additionally, it is known that most of queens in inter-estrous period have serum estrogen concentrations below 12 to 15 pg/ml and in follicular phase have serum estrogen concentration above 20 pg/ml (2). In our case, it was thought that the patient in progression phase from inter-estrous period to follicular phase, because serum estradiol and progesterone levels were measured as 18 pg/ml and 1.4 ng/ml, respectively. This anomaly has only been reported in a paper (7), however, there was no evidence/information about the patient' sexual status or past reproductive events. This is the first reported case of ovarian agenesis including sexual activity and serum steroid hormone levels. The prevalence and genital/sexual activity are not reported because it is incidentally detected by laparotomy/ovariohysterectomy. It is too hard to diagnose in those patients suffered from ovarian agenesis had unaffected/normal cyclic activity.

Table 1. The results of complete blood count analysis.
Tablo 1. Tam kan sayımı analizinin sonuçları.

Parameter (Unit)	Results	Reference
WBC (10^9/l)	5.8	5.5- 19.5
LYM (10^9/l)	1.1	1.0- 7.0
MONO (10^9/l)	0.3	0.2- 1.0
NEUT (10^9/l)	3.9	2.8- 13.0
EOS (10^9/l)	0.5	0.1- 99.9
LYM (%)	19.1	15.0- 60.0
MON (%)	5.2	0.5- 11.0
NEU (%)	67.2	25.0- 85.0
EOS (%)	8.5	0.1- 12.5
RBC (10^2/l)	8.56	5.0- 11.0
HGB (g/dl)	14.8	8.0- 15.0
HCT (%)	33.2	25.0- 45.0
MCV (fl)	38.8	39.0- 50.0
MCH (pg)	17.3	12.5- 17.5
MCHC (g/dl)	44.6	31.0- 38.0
RDWa (fl)	19.7	20.0- 35.0
RDW (%)	15.9	14.0- 18.5
PLT (10^9/l)	182	200- 500
MPV (fl)	9.5	8.0- 12.0

Table 2. The results of serum biochemistry analysis.
Tablo 2. Serum biyokimya analizinin sonuçları.

Parameter (Unit)	Results	Reference
Glucose (mg/dl)	99.7	70.0- 110.0
Urea (mg/dl)	37.5	15- 64.2
Creatinine (mg/dl)	1.37	0.8- 1.8
Total protein (g/dl)	5.48	5.4- 7.8
Albumin (g/dl)	2.41	3.5- 4.5
Total bilirubin (mg/dl)	0.11	0.1- 0.2
D. bilirubin (mg/dl)	0.04	-
Cholesterol (mg/dl)	68.0	95.0- 130.0
Triglycerides (mg/dl)	36.2	50.0- 100.0
ALP (IU/L)	14.0	25.0- 93.0
ALT (IU/L)	31.3	6.0 - 83.0
AST (IU/L)	15.2	26.0- 43.0
CK (IU/L)	149.9	≤130
GGT (IU/L)	4.5	6.0- 28.0
LDH (IU/L)	125.0	63.0- 273.0
Total calcium (mg/dl)	7.5	20.0- 35.0
Phosphorus (mg/dl)	3.95	4.0- 7.3

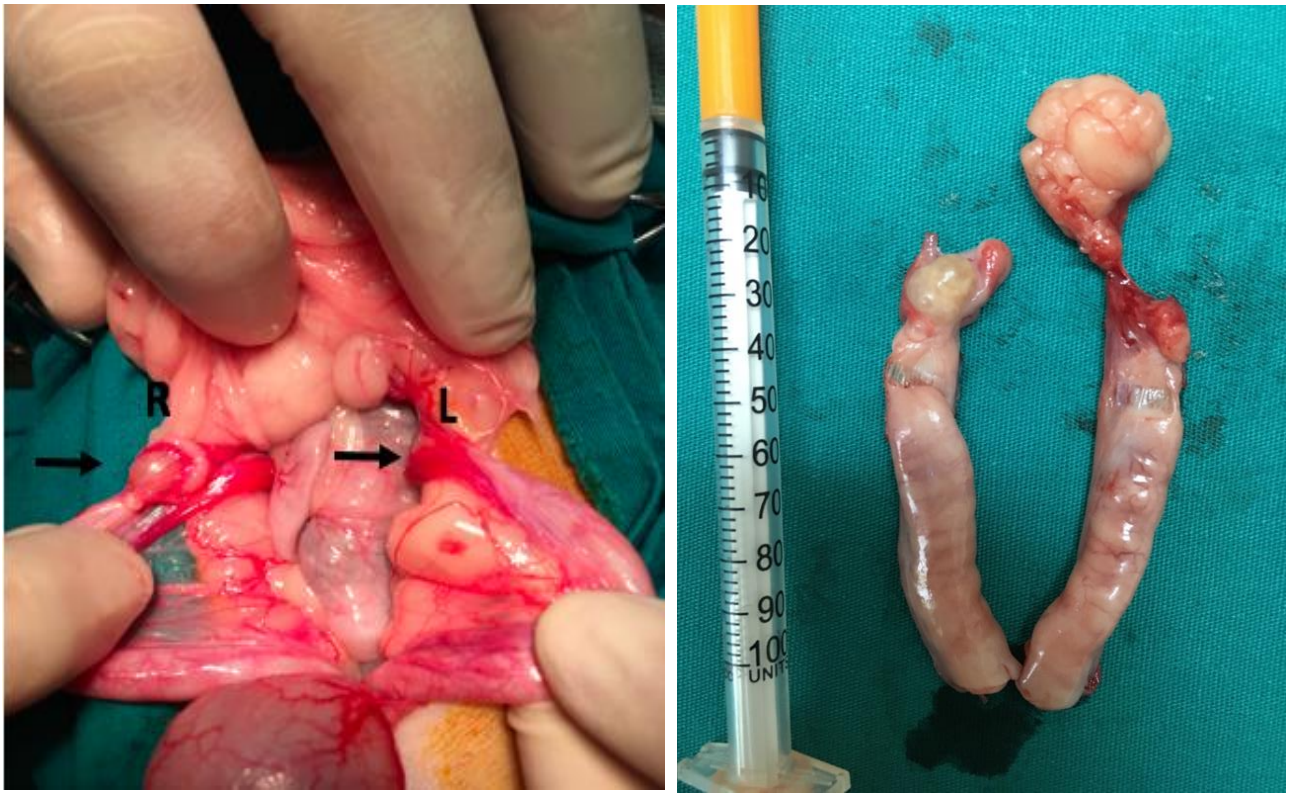

Figure 1. Left (L) unilateral ovarian agenesis in cat.
Şekil 1. Kedide sol (L)- tek taraflı ovaryum agenezi.

Gokulakrishnan and George (3) stated that ovarian agenesis could be directly related to genital malformations such as the absence of the ipsilateral uterine tube or uterine horn, however, no such abnormality was seen in the present the case. It was reported that ectopic ovary or a fibrotic part of ovarian remnants may be detected macroscopically at the site of ovary during surgery in some cases (1,5). It is necessary to perform a comprehensive histological investigation examination to reveal whether an ovary is completely absent or not, and presence of concomitant tract abnormalities.

It has been reported that chromosomal abnormalities and autosomal recessive/dominant genes may contribute to the etiology of ovarian agenesis in humans (8). Ovarian dysplasia with a chromosomal abnormality was also reported in a bitch (4). Therefore, karyotyping is generally recommended to obtain considerable information about exact mechanisms underlying this ovarian anomaly (1).

In summary, it was determined that unilateral ovarian agenesis events were no adverse effect on sexual activity in cats. And also, it was thought that it would be a normal pregnancy and parturition process in cats with unilateral ovarian agenesis. We think that this report could be useful for practitioners and our colleagues.

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Yayım Koşulları

1. Dergi, Ankara Üniversitesi Veteriner Fakültesi'nin hakemli bilimsel yayın organı olup, üç ayda bir yayımlanır. Derginin kısaltılmış adı "Ankara Üniv Vet Fak Derg" dir.
2. Derginin yayın dili Türkçe ve İngilizcedir.
3. Dergide, 250 kelimeyi geçmeyen özeti haricinde tamamı ya da bir kısmı daha önce başka bir yerde yayımlanmamış güncel, orijinal bilimsel araştırmalar, derlemeler, olgu sunumları, kısa bilimsel çalışmalar ve akademik haberler yayımlanır. Derleme niteliğindeki çalışmalar, ilgili bilim insanlarından davet usulü talep edilen yazılardan temin edilir.
4. Tüm yazılar, Microsoft Word yazılım programı ile Times New Roman 12 punto, çift aralıklı (5 mm) olarak, kenarlarda 30 mm boşluk bırakılarak ve A4 formunda (210 x 297 mm) tek sütun halinde ve iki yana yaslanmış olarak yazılmalıdır. Sayfa başlarına satır numarası eklenmelidir. Ayrıca ilk sayfa hariç her sayfa, sayfa numarası üst ortada olacak şekilde numaralanmalıdır. Yazılar, şekil, çizelge ve tablolar dâhil olmak üzere orijinal bilimsel araştırmalarda 15, derlemelerde 10, gözlemlerde ve kısa bilimsel çalışmalarda 7 sayfayı geçmemelidir.
5. Makaleler "vetjournal.ankara.edu.tr" dergi internet adresi üzerinden gönderilmelidir. Makale online olarak sisteme yüklendikten sonra yazar sırası (yazar ismi ekleme ve çıkarma dahil) değiştirilemez.
6. Orijinal çalışmalar konu başlığı, yazar/yazarların adları, adresleri, özet ve anahtar sözcükler, giriş, materyal ve metot, bulgular, tartışma ve sonuç, teşekkür ve kaynaklar sırası ile hazırlanmalıdır. Konu başlığı, özet, anahtar sözcükler ve tablo ve şekil başlıkları her iki dilde diğer kısımlar ise tercih edilen dilde yazılmalıdır. Ana dili Türkçe olmayan iletişim yazarının çalışmasında Türkçe özet şartı aranmaz. Kısa bilimsel çalışmalarda, giriş, materyal ve metot, bulgular, tartışma ve sonuç bölümlemesi yapılmaz. Teşekkür edilecek ise sadece teknik destek ile sınırlandırılmalıdır.
   Konu başlığı, kısa ve açık olmalı ve küçük harflerle koyu yazılmalıdır. Çalışmaya ilişkin açıklama dipnot işareti ile gösterilmelidir.
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   Özet, tek paragraf halinde en fazla 250 kelime olmalıdır.
   Anahtar sözcükler, alfabetik sıralanmış olarak yazılmalı ve 5 sözcükten fazla olmamalıdır.
   Giriş bölümünde, çalışma ile doğrudan ilgili kısa literatür bilgisi verildikten sonra, son paragrafta çalışmanın hipotezi ve amacı yazılmalıdır. Bu bölüm 2 sayfayı geçmemelidir.
   Materyal ve Metot, gereksiz ayrıntıya girilmeden, öz ve anlaşılır biçimde yazılmalıdır. Araştırmanın türü **(**Tanımlayıcı, Gözlem, Deneysel, Vaka-Kontrol, İzlem vb.), deneklerin karakteristikleri, deneklerin araştırmaya alınma ya da alınmama kriterleri ile birlikte veri toplama aşamasında, kullanılmışsa örnekleme yöntemi, örneklemin temsil yeteneğinin nasıl sağlandığı, olasılıksız örnekleme kullanılmışsa nedenleri yazılmalıdır. Örneklem büyüklüğü, hesaplama yöntemleri, kullanılmışsa güç değeri ve izlem çalışmalarında, sansürlü ve kayıp gözlem oranları ve nedenleri belirtilmelidir. İstatistiksel çözümlemelerde çözümleme yöntemleri ve kullanım nedenleri ile kullanılan istatistiksel işlemlere ilişkin kaynaklara yer verilmelidir.
   Bulgular bölümünde, veriler kısa bir şekilde açıklanmalıdır. Tablolarda verilen bulguların metinde tekrarlanmasından kaçınılmalıdır.
   Materyal ve Metot ile Bulgular bölümlerinde, alt başlıklar italik, ikinci alt başlıklar ise normal yazı tipiyle belirtilmelidir. İtalik alt başlık paragraf başında yer almalıdır. Resimler en az 1920 x 1280 dpi çözünürlükte olmalıdır. Tablo ve figürler yayın sonunda ayrı sayfalarda verilmelidir.
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   Aşağıda yaygın kullanılan referans tipleri verilmiştir;
   Kaynak bilimsel çalışma ise:
   Sandstedt K, Ursing J (1991): *Description of the Campylobacter upsaliensis previously known as CNW group*. Syst Appl Microbiol, **14**, 39-45.
   Sandstedt K, Ursing J, Walder M (1983): *Thermotolerant Campylobacter with no or weak catalase activity isolated from dogs*. Curr Microbiol, **8**, 209-213.
   Lamont LA, Bulmer BJ, Sisson DD, et al. (2002): *Doppler echocardiographic effects of medetomidine on dynamic left ventricular outflow tract obstruction in cats.* J Am Vet Med Assoc, **221**, 1276-1281.
   Kaynak kitap ise:
   Falconer DS (1960): *Introduction to Quantitative Genetics*. Oliver and Boyd Ltd, Edinburgh.
   Kaynak kitaptan bir bölüm ise:
   Bahk J, Marth EH (1990): *Listeriosis and Listeria monocytogenes.* 248-256. In: DO Cliver (Ed), Foodborne Diseases. Academic Press, San Diego.
   Kaynak internette yer alıyor ise erişim tarihi ile birlikte yazılmalıdır.
   Li G., Hart A, Gregory J (1998): Flokülasyona hız gradyanı etkisi. http://www.server.com/projects/paper2.html. (20 Mayıs 2004)
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7. Dergide yayımlanmak üzere Yayın Alt Komitesi'ne gönderilen makaleler tüm yazarlar tarafından imzalanan "Yayın Hakkı Devri Sözleşmesi" ile birlikte gönderilir. Çalışmada yazar olarak yer alabilmek için gereken şartlar "Yayın Hakkı Devri Sözleşmesi" formunda detaylı olarak açıklanmıştır. Gönderilen çalışmanın değerlendirilmeye alınıp alınmaması Editör'ün yetkisindedir. Yayıma ilişkin kabul veya ret kararı çalışmanın iletişim yazarına iletilir. Yayımlanması uygun görülmeyen çalışmalar iletişim yazarına iade edilmez.
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9. Ankara Üniversitesi Veteriner Fakültesi Dergisi'nde makale değerlendirmeleri kör hakemlik ve yazarlık esasına dayalı olarak yürütülmektedir. Bu sistemde hakem ve yazarların kimlikleri karşılıklı olarak gizli tutulur. Yazarlar makalelerini bu esasa göre hazırlayıp göndermeyi ve gizlilik kurallarına uymayı kabul ve taahhüt ederler.
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3. Original research articles, reviews, case reports and short communications on all aspects of veterinary science, which had not been previously published elsewhere in whole or in part except abstract not exceeding 250 words, are published in the journal. Review articles are only be submitted by invitation.
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   Lamont LA, Bulmer BJ, Sisson DD, et al. (2002): *Doppler echocardiographic effects of medetomidine on dynamic left ventricular outflow tract obstruction in cats.* J Am Vet Med Assoc, **221**, 1276-1281.
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Ankara Üniversitesi Veteriner Fakültesi Dergisi

Yayında yazar olarak yer alabilmek için aşağıdaki koşulların sağlanması gerekir.

1. Çalışmanın tasarımı, verilerin elde edilmesi veya bu verilerin analizi ve yorumlanması,
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Multidisipliner çalışmalarda ekibin başındaki kişi ile birlikte patolojik değerlendirmeleri ve istatiksel analizleri yapan kişilerle birlikte çalışma tasarımına veya çalışmaya önemli bir katkıda bulunan kişiler yazar olarak yer alır.

Aşağıda başlığı bulunan ve yazarları belirtilen makalenin tüm sorumluluğu Ankara Üniversitesi Veteriner Fakültesi Yayın Alt Komitesi Başkanlığı'na ulaşıncaya kadar yazar/larına aittir.

Yayının adı: .....

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Aşağıda isim ve imzaları bulunan yazarlar; yayınlamak üzere gönderdikleri makalenin orijinal olduğunu, daha önce başka bir dergiye yayınlanmak üzere gönderilmediğini ve 250 kelimeyi geçmeyen özeti haricinde kısmen ya da tamamen yayınlanmadığını, gerekli düzeltmelerle birlikte her türlü yayın hakkının, yazının yayımlanmasından sonra Ankara Üniversitesi Veteriner Fakültesi Dergisi'ne devrettiklerini kabul ederler. Yayımlanmak üzere gönderilen bu makalenin tüm sorumluluğunu da yazar/lar üstlenmektedir.

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