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ESER OFSET MATBAACILIK
BOSNAHERSEK CAD. ALTUNALEM YAPI KOOP. ZEMİN KAT - ERZURUM
Tel: +90 442 2334667 E-mail: eserofset25@hotmail.com

Address for Correspondence

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

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Research Article

The Effect of Mixture of Rapeseed Meal, White Lupin Seed, and Pea Seed in Rabbit Diets on Performance Indicators and Fatty Acid Profile of Meat and Fat

Dorota KOWALSKA [1,a] Janusz STRYCHALSKI [2,b] Cezary ZWOLIŃSKI [2,c]
Andrzej GUGOŁEK [2,d] Paulius MATUSEVICIUS [3,e]

[1] Department of Small Livestock Breeding, National Research Institute of Animal Production, Balice, 32-083 Kraków, POLAND
[2] Department of Fur-bearing Animal Breeding and Game Management, Faculty of Animal Bioengineering, University of Warmia and Mazury, 10-719 Olsztyn, POLAND
[3] Department of Animal Science, Veterinary Academy of Lithuanian University of Health Sciences, LT-47181, Kaunas, LITHUANIA
ORCIDS: [a] 0000-0002-4435-4244; [b] 0000-0003-0948-3072; [c] 0000-0002-7880-5867; [d] 0000-0002-5360-9755; [e] 0000-0002-6612-2479

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Abstract

Quality of rabbit's carcasses is largely determined by the composition of the lipid fraction. The objective of this study was to determine the effect of partial or complete substitution of soybean meal with a combination of rapeseed meal, white lupin seed and pea seed on the production results and fatty acid profile of meat and fat in rabbits. Ninety New Zealand White rabbits were divided into three feeding groups, 30 in each group: Control - C (mean protein source - 15% soybean meal in the diet), Experimental 1 - E1 (7.5% soybean meal, 5% rapeseed meal, 4% white lupin seed, 3% pea seed) and Experimental 2 - E2 (0% soybean meal, 10% rapeseed meal, 8% white lupin seed, 6% pea seed). No significant differences were determined in the final body weights of rabbits. Feed efficiency was better in both of the E groups than in group C. Dressing percentage was higher in group E2 than in group C. Also, protein content in thigh muscle was higher in groups E than in group C. As the dietary proportion of soybean decreased, the proportion of SFA in meat and in perirenal fat decreased, and that of PUFA increased. The obtained results indicate that soybean meal may be successfully replaced in rabbit diets by the combination of rapeseed meal, white lupin seed, and pea seed.

Keywords: *Brassicaceae, Fabaceae, Fatty acid profile, Growth performance, Meat composition, Rabbit feeding*

Tavşan Diyetlerinde Kolza Tohumu, Beyaz Acı Bakla Tohumu ve Bezelye Tohumu Karışımının Et ve Yağ Performans Göstergeleri ve Yağ Asidi Profili Üzerine Etkisi

Öz

Tavşan karkaslarının kalitesini büyük ölçüde lipit fraksiyonunun kompozisyonu belirler. Bu çalışmanın amacı, kolza tohumu küspesi, beyaz acı bakla tohumu ve bezelye tohumu kombinasyonu ile kısmen veya tam ikame edilmiş soya fasulyesi küspesinin tavşanlarda et ve yağın üretim sonuçları ve yağ asidi profili üzerindeki etkisini belirlemektir. Doksan Yeni Zelanda Beyaz tavşanı, her biri 30 tavşan içeren üç besleme grubuna ayrıldı: Kontrol - C (ortalama protein kaynağı - diyette %15 soya küspesi), Deneme 1 - E1 (%7.5 soya küspesi, %5 kolza tohumu küspesi, %4 beyaz acı bakla tohumu, %3 bezelye tohumu) ve Deneme 2 - E2 (%0 soya küspesi, %10 kolza tohumu küspesi, %8 beyaz acı bakla tohumu, %6 bezelye tohumu). Tavşanların final vücut ağırlıklarında anlamlı bir fark saptanmadı. Yemden yararlanma her iki E grubunda da C grubuna göre daha iyiydi. Tüylenme yüzdesi E2 grubunda C grubuna göre daha yüksekti. Ayrıca, but kasındaki protein içeriği E gruplarında C grubuna göre daha yüksekti. Soya fasulyesinin diyet oranı azaldıkça, et ve perirenal yağdaki SFA oranında azalmaa ve PUFA oranında artış görülmekteydi. Elde edilen sonuçlar, soya fasulyesi ununun kolza tohumu küspesi, beyaz acı bakla tohumu ve bezelye tohumu kombinasyonu ile tavşan diyetlerinde ikame olarak başarıyla değiştirilebileceğini göstermektedir.

Anahtar sözcükler: *Turpgiller, Baklagiller, Yağ asidi profili, Büyüme performansı, Et kompozisyonu, Tavşan besleme*

Correspondence
+48 89 5234442 Fax: +48 89 5234327
janusz.strychalski@uwm.edu.pl

INTRODUCTION

Complete pelleted mixtures are used in intensive rabbit production. As much as 19% of crude protein with an appropriate amino acid composition has to be supplied to the rabbits to ensure good fattening results. The most important limiting amino acids in rabbit nutrition are lysine, tryptophan, cystine, and methionine [1,2]. Soybean meal is currently the most common source of protein in the diets of rabbits from large-scale farms. However, soybean is also used in the countries where soybean is not grown and has to be imported. To become independent of soybean imports, research is being conducted to replace it with other components in rabbit diets. These include by-products of biofuel production, mainly rapeseed meal and rapeseed cake as well as dried distillers grains with solubles - DDGS [3-6]. Products such as sunflower cake and sunflower meal are also used for replacement [7-9]. Moreover, attempts to use the seed of white lupin [7,9-11], pea [12-14], and other plants of the *Fabaceae* family have been made [15,16]. All these products come from plants that have for years been grown in the transitional temperate climate zone. It was shown that even complete replacement of dietary soybean meal with a combination of rapeseed meal and the seeds of the pea and white lupin did not have a negative effect on production results, digestibility of nutrients nitrogen retention, and functioning of the gastrointestinal tract in rabbits [17,18].

However, regardless of the production parameters of rabbits, an important consideration is the quality of carcasses used for consumption, which is largely determined by the composition of the lipid fraction. Today, efforts are made to reduce the amount of saturated fatty acids and to increase the amount of unsaturated fatty acids in animal fat deposits [19]. There are plenty of studies indicating that rabbit nutrition has an effect on the fatty acid composition of the carcasses [5,20-22]. Therefore, when studying the usefulness of a component in rabbit nutrition, evaluation of production results should be followed by the investigation of its effect on fatty acid profile in muscle and adipose tissue.

It was hypothesized that replacement of soybean meal in the diets of meat rabbits with a mixture of *Brassicaceae* and *Fabaceae* plants seeds and by-products will have no adverse effect on the fatty acid profile of the carcasses. Thus, the objective of the study was to determine the effect of partial or complete substitution of soybean meal with a combination of rapeseed meal, white lupin seed and pea seed on the production results and fatty acid profile of meat and fat in New Zealand White rabbits.

MATERIAL and METHODS

Animal Care

The animal protocol used in this study was approved by the Local Institutional Animal Care and Use Committee in Olsztyn (Number: 24/2015), and the study was carried out in accordance with EU Directive 2010/63/EU for animal experiments.

Experimental Factor

The experimental factor was the contribution of rapeseed meal (RSM), white lupin seed (WLS) and pea seed (PS) in pelleted feed mixtures. The chemical composition and energy value of these components and of the soybean meal (SBM) are presented in *Table 1*. The control feed mixture (C group) contained 15% extracted soybean meal (SBM). In the first experimental group (E1), the diet contained 7.5% SBM, which was partially substituted with a mixture of RSM, WLS and PS. In the second group (E2), soybean meal was completely substituted with RSM, WLS and PS in pelleted feed mixtures. The formulation, chemical composition and energy value of feed mixtures are presented in *Table 2*, and the fatty acid content of these mixtures is given in *Table 3*. All rations met the nutritional requirements of growing rabbits [1].

Animals and Treatments

Ninety New Zealand White (NZW) rabbits were selected from 18 litters for the experiments. They were divided into 3 groups, 30 rabbits in each, being analogous in terms of origin, proportion of sexes, and body weight. The experiment was carried out from September to November and started when rabbits were weaned at 35 d of age (average body weight - 926.23±6.08) and terminated when they reached 90 d of age. Rabbits were kept in a closed experimental pavilion, in wire net flat deck cages (0.5´0.6´0.4 m; 2 animals each), and were fed pelleted diets *ad libitum*. They were kept under standard conditions: temperature of 18-20°C and relative air humidity of 60-75%, intensive ventilation of rooms, and regulated photoperiod (16-h lighting and 8-h darkness).

Table 1. *Chemical composition (%) and measured energy content of soybean meal, rapeseed meal, white lupine seed and pea seed*

Specification	SBM	RPM	WLS	PS
Dry matter	90.11	91.02	88.79	88.14
Crude ash	6.26	7.24	4.09	3.37
Crude protein	45.19	34.57	41.09	22.26
Ether extract	1.85	3.63	2.02	0.85
NDF	14.12	23.93	27.89	11.26
ADF	6.24	13.31	19.64	6.96
ADL	5.01	5.84	5.32	4.71
Lysine	2.38	1.67	1.37	1.51
Methionine + cystine	1.01	1.45	0.75	0.60
Threonine	1.37	1.64	1.12	0.84
Tryptophan	0.41	0.44	0.27	0.22
Gross energy (MJ/kg)	17.53	15.91	16.67	16.60

SBM: *soybean meal;* ***RPM:*** *rapeseed meal;* ***WLS:*** *white lupine seed;* ***PS:*** *pea seed;* ***NDF:*** *neutral detergent fibre;* ***ADF:*** *acid detergent fibre;* ***ADL:*** *acid detergent lignin*

Table 2. *Ingredients, chemical composition and measured energy content of feed mixtures*

Specification	Group		
	C	E1	E2
Ingredients (%)			
Soybean meal (48% CP)	15.0	7.5	-
Rapeseed meal	-	5.0	10.0
White lupine seed	-	4.0	8.0
Pea seed	-	3.0	6.0
Barley	14.5	12.5	10.5
Wheat	6.0	7.5	9.0
Corn	16.0	12.5	9.0
Dried alfalfa (18% CP)	23.0	23.0	23.0
Wheat bran	11.0	11.0	11.0
ARBOCEL*	6.0	5.5	5.0
Beet molasses	2.0	2.0	2.0
Skimmed milk powder	2.0	2.0	2.0
Dried brewer's yeast	1.0	1.0	1.0
Calcium carbonate	1.0	1.0	1.0
Dicalcium phosphate	1.0	1.0	1.0
Mineral-vitamin premix†	1.0	1.0	1.0
NaCl	0.5	0.5	0.5
Chemical composition (%)			
Dry matter	90.66	91.00	90.56
Crude ash	6.58	6.71	6.16
Crude protein	17.40	17.95	17.95
Ether extract	2.28	2.90	3.37
NDF	22.57	22.45	24.16
ADF	13.14	13.91	15.48
ADL	3.22	3.16	3.28
Lysine	0.78	0.85	0.79
Methionine + cystine	0.73	0.74	0.82
Threonine	0.70	0.79	0.77
Tryptophan	0.16	0.16	0.15
Gross energy (MJ/kg)	16.86	16.91	16.85

NDF: *neutral detergent fibre;* ***ADF:*** *acid detergent fibre;* ***ADL:*** *acid detergent lignin; * Crude fibre concentrate; † Composition mineral-vitamin premix 1 kg: Vit. A: 3.500.000 IU, Vit. D_3: 200.000 IU, Vit. E: 28.000 mg, Vit. K_3: 200 mg, Vit. B_1: 1.500 mg, Vit. B_2: 2.800 mg, Vit. B_6: 2.800 mg, Vit. B_{12}: 20.000 mcg, Folic acid: 200 mg, Niacin: 10.000 mg, Biotin: 200.000 mcg, Calcium pantothenate: 7.000 mg, Choline: 30.000 mg, Fe: 17.000 mg, Zn: 2.000 mg, Mn: 1.000 mg, Cu (copper sulfate x $5H_2O$, 24.5%): 800 mg, Co: 1.000 mg, I: 100 mg, Methionine: 150 g, Ca: 150 g, P: 100 g*

Table 3. *Fatty acid content in feed mixtures (% of total fatty acid pool)*

Fatty Acids	Group		
	C	E1	E2
SFA			
C12:0 (lauric)	0.43	0.35	0.35
C14:0 (myristic)	0.48	0.43	0.44
C16:0 (palmitic)	17.73	15.10	14.50
C18:0 (stearic)	2.99	3.00	2.94
C20:0 (arachidic)	0.51	0.97	1.23
Other SFA	0.32	0.34	0.33
Total SFA	22.46	20.19	19.79
MUFA			
C16:1 (palmitoleic)	0.22	0.27	0.34
C18:1 (oleic)	23.36	24.44	24.99
Other MUFA	0.71	0.94	1.17
Total MUFA	24.29	25.65	26.50
PUFA			
C18:2 (linoleic)	46.50	46.12	45.62
C18:3 (α-linolenic)	6.13	7.57	7.41
C20:4 (arachidonic)	0.40	0.27	0.48
C20:5 (EPA)	0.21	0.20	0.19
Other PUFA	0.00	0.00	0.01
Total PUFA	53.24	54.16	53.71

SFA: *saturated fatty acids;* ***MUFA:*** *monounsaturated fatty acids;* ***PUFA:*** *polyunsaturated fatty acids*

The rabbits were weighed individually on an electronic scale on days 35, 63 and 90. These data allowed calculating daily body weight gains (BWG) of the rabbits and the feed efficiency (FE) [BWG (g)/feed intake (g)]. At the end of the production trial, after 24-h fasting, the animals were weighed and killed according to the accepted recommendations for euthanasia of experimental animals (rabbits were stunned and bled, and the whole procedure took about 2 min). After the slaughter, the animals were skinned and eviscerated. After cooling the carcasses (for 24 h, at 4°C), tissue samples were taken for chemical analyses, and dressing percentage was calculated as follows:

DP (%) = Chilled carcass weight without head and giblets (kg)/Live weight (kg) x 100%

In addition, the percentage content of the primal cuts: forepart, loin and hind part, was calculated in the carcass. The carcasses were divided into the head (cut through the craniovertebral joint), the fore part (cut between the 7th and 8th thoracic vertebrae), the loin (cut between the 6th and 7th thoracic vertebrae) and the hind part (carcass section remaining after separation of the loin from the front, comprising the hindquarters and hind limbs). For fatty acids in the muscles and in adipose tissue, the saturation index (S/P) was calculated using equations presented by Peiretti and Meineri [21] and Volek and Marounek [9]:

$$S/P = (C14:0 + C16:0 + C18:0)/\sum MUFA + \sum PUFA$$

Chemical Analyses

The nutrient content of feed was determined by AOAC [23] standard methods in duplicate samples. Dry matter content (method 978.10) was determined in a laboratory drier, at

103°C. Crude ash content (method 942.05) was estimated by sample mineralization in a muffle furnace (Czylok, Poland) at 600°C. Total nitrogen content (method 984.13) was determined by the Kjeldahl method, in the FOSS TECATOR Kjeltec 2200 Auto Distillation Unit. Ether extract content (method 920.39) was estimated by the Soxhlet method, in the FOSS SOXTEC SYSTEM 2043. NDF (neutral detergent fiber), ADF (acid detergent fiber) and ADL (acid detergent lignin) were estimated in the FOSS TECATOR Fibertec 2010 System. NDF was determined according to the procedure proposed by Van Soest et al.[24]. ADF and ADL were determined according to procedures of AOAC [23] (methods 973.18 and 973.18D, respectively). The levels of amino acids in diets (method 982.30) were determined using the Biochrom 20 plus amino acid analyzer and Biochrom amino acid analysis reagents (Biochrom Ltd., Cambridge, England). Gross energy content was determined using a bomb calorimeter (IKA® C2000 basic, Germany).

Fat from ground samples of feed and animal tissues were extracted by the Soxhlet extraction procedure [23]. To determine fatty acid composition, all fat samples were methylated by the modified Peisker method [25] (1.5 cm^3 of a methanol:chloroform:concentrated sulfuric acid mixture, 100:100:1 v/v, was added to ca 150 mL fat, thermostat -80°C, 3 h), and fatty acid methyl esters were obtained. Fatty acids were separated and determined by gas chromatography: VARIAN CP-3800 gas chromatograph-Netherlands, flame-ionization detector (FID), capillary column (length - 50 m, f = 0.25 mm, film d = 0.25 µm), split injector, split ratio 50:1, 1 µL sample, detector temperature -250°C, injector temperature - 225°C, column temperature –200°C, carrier gas - helium, flow rate - 1.2 cm^3/min. Fatty acids were identified by comparing the retention times of individual fatty acid methyl ester standards (Sigma-Aldrich) and the retention times of peaks in the analyzed samples. The relative content of each fatty acid was expressed as percent of the total peak area of all fatty acids in the sample.

Statistical Analysis

Calculations were made with Statistica software [26]. Cage was considered the experimental unit. Data are expressed as means ± standard error of the mean (SEM). The results were processed statistically using least squares means in GLM procedures. For comparison of data, the $Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha_i\beta_j + \varepsilon_{ijk}$ model was used, where µ is the general mean, α_i is the effect of diet, β_j is the effect of sex, $\alpha_i\beta_j$ is the interaction effect between diet and sex, and ε_{ijk} is the random error. Analyses did not reveal significant effects of sex or significant interactions between fixed effects, therefore they are not reported in the tables.

RESULTS

Body weights and daily BWG of rabbits showed no significant differences among groups *(Table 4)*. Rabbits fed a control diet (C) consumed overall more feed (107.53 g/d) compared to rabbits fed E1 and E2 diets (99.91 g/d and 92.69 g/d, respectively), and the difference between groups C and E2 was statistically highly significant. Calculated FE parameter achieved more favorable values in E1 and E2 groups (3.38 g/g and 3.30 g/g, respectively) than in C group (3.78 g/g). DP was higher in group E2 compared with groups C and E1. The experimental factor appeared to have no impact on the content of the primal cuts (forepart, loin and hind part) in the carcass among rabbits' groups. Dry matter content (%) in thigh muscle did not differ between the groups, but crude protein content (%) was higher in both of the experimental groups than in the control group. A reverse trend was observed for ether extract, which was more abundant in the muscles of rabbits receiving 15% soybean meal in the diet compared to the experimental groups.

Table 5 shows the proportion of fatty acids in thigh muscle of the rabbits. Total SFA content was higher in the meat of rabbits from group C compared to those of groups E1 and E2. Among these fatty acids, the proportion of myristic

Table 4. *Productivity parameters of rabbits (mean±SEM)*

Specification	Group		
	C	E1	E2
BW at 35 d (g)	926.20±10.78	928.74±5.53	923.75±1.92
BW at 63 d (g)	1705.45±12.45	1699.92±16.82	1714.46±17.02
BW at 90 d (g)	2490.77±113.19	2554.42±134.36	2468.63±138.41
BWG 35-63 days od age (g/d)	27.83±3.86	27.54±3.75	28.24±4.14
BWG 63-90 days od age (g/d)	29.09±4.09	31.65±4.20	27.93±3.96
BWG 35-90 days od age (g/d)	28.45±3.92	29.56±4.46	28.09±4.41
FI 35-63 days od age (g/d)	96.05±11.53^{a}	89.59±10.87	83.71±10.34^{b}
FI 63-90 days od age (g/d)	119.43±13.26^{a}	110.61±12.09^{b}	101.99±11.70^{b}
FI 35-90 days od age (g/d)	107.53±12.83^{A}	99.91±11.52	92.69±11.18^{B}
FE 35-63 days od age (g/g)	3.45±0.04^{A}	3.25±0.03	2.97±0.03^{B}
FE 63-90 days od age (g/g)	4.10±0.05^{A}	3.50±0.04^{B}	3.65±0.05^{B}
FE 35-90 days od age (g/g)	3.78±0.05^{A}	3.38±0.03^{B}	3.30±0.04^{B}
DP (%)	45.33±0.28^{B}	46.39±0.47^{b}	48.46±0.41^{Aa}
Forepart (%)	36.65±0.18	35.52±0.16	35.37±0.18
Loin (%)	26.21±0.13	27.40±0.11	27.04±0.12
Hind part (%)	37.14±0.16	37.08±0.14	37.59±0.14
DM (%) in thigh muscle	28.68±0.37	28.02±0.21	28.43±0.13
CP (%) in thigh muscle	21.19±0.18^{B}	22.31±0.19^{A}	22.26±0.19^{A}
EE (%) in thigh muscle	4.96±0.54^{a}	3.58±0.46^{b}	3.78±0.32^{b}

SEM: *standard error of the mean;* ***BW:*** *body weight;* ***BWG:*** *body weight gains;* ***FI:*** *feed intake;* ***FE:*** *feed efficiency;* ***DP:*** *dressing percentage;* ***DM:*** *dry matter;* ***CP:*** *crude protein;* ***EE:*** *ether extract;* ^{a,b} *Values with different superscripts are significantly different at P<0.05;* ^{A,B} *Values with different superscripts are significantly different at P<0.01*

Table 5. *Fatty acid content in thigh muscle (% of total fatty acid pool; mean±SEM)*

Fatty Acids	Group		
	C	E1	E2
SFA			
C12:0 (lauric)	0.26±0.04	0.30±0.02	0.27±0.01
C14:0 (myristic)	$3.42±0.10^{Aa}$	$3.06±0.11^{b}$	$2.81±0.13^{B}$
C16:0 (palmitic)	$30.01±0.51^{Aa}$	$27.53±0.42^{Ab}$	$26.35±0.48^{B}$
C18:0 (stearic)	$5.94±0.17^{B}$	$6.63±0.31^{b}$	$7.01±0.15^{Aa}$
C20:0 (arachidic)	$0.10±0.00^{B}$	$0.11±0.00^{b}$	$0.13±0.00^{Aa}$
Other SFA	$0.19±0.03^{Bb}$	$0.32±0.04^{A}$	$0.39±0.03^{Aa}$
Total SFA	$39.92±0.53^{Aa}$	$37.95±0.40^{b}$	$36.95±0.53^{B}$
MUFA			
C16:1 (palmitoleic)	$4.57±0.28^{A}$	$4.45±0.24^{A}$	$3.14±0.22^{B}$
C18:1 (oleic)	25.85±0.49	25.64±0.45	24.97±0.38
Other MUFA	$0.02±0.00^{A}$	$0.01±0.00^{B}$	$0.01±0.00^{B}$
Total MUFA	$30.44±0.73^{a}$	$30.10±0.52^{a}$	$28.12±0.57^{b}$
PUFA			
C18:2 (linoleic)	$21.39±0.67^{B}$	$22.61±0.55^{b}$	$24.36±0.52^{Aa}$
C18:3 (α-linolenic)	$3.85±0.07^{Bb}$	$4.52±0.12^{a}$	$4.53±0.10^{Aa}$
C20:4 (arachidonic)	$3.49±0.34^{Bb}$	$3.80±0.23^{Ba}$	$4.81±0.35^{A}$
C20:5 (EPA)	0.21±0.01	0.24±0.01	0.23±0.01
Other PUFA	$0.70±0.04^{b}$	0.78±0.05	$1.00±0.07^{a}$
Total PUFA	$29.64±1.06^{B}$	$31.95±0.70^{b}$	$34.93±0.95^{Aa}$
PUFA 6/3	5.31±0.22	5.08±0.10	5.11±0.09
S/P	$0.66±0.06^{a}$	$0.60±0.05^{b}$	$0.57±0.05^{b}$

SEM: *standard error of the mean;* ***SFA:*** *saturated fatty acids;* ***MUFA:*** *monounsaturated fatty acids;* ***PUFA:*** *polyunsaturated fatty acids;* ***S/P:*** *saturation index;* ^{a,b} *Values with different superscripts are significantly different at P<0.05;* ^{A,B} *Values with different superscripts are significantly different at P<0.01*

Table 6. *Fatty acid content in loin (% of total fatty acid pool; mean±SEM)*

Fatty Acids	Group		
	C	E1	E2
SFA			
C12:0 (lauric)	0.22±0.03	0.29±0.03	0.22±0.02
C14:0 (myristic)	$3.53±0.07^{Aa}$	$3.20±0.13^{b}$	$2.92±0.10^{B}$
C16:0 (palmitic)	$30.15±0.55^{Aa}$	$28.55±0.49^{Ab}$	$26.36±0.42^{B}$
C18:0 (stearic)	5.64±0.13	5.98±0.26	6.38±0.22
C20:0 (arachidic)	$0.10±0.00^{Bb}$	$0.11±0.01^{Ba}$	$0.16±0.00^{A}$
Other SFA	$0.20±0.02^{B}$	$0.39±0.03^{Aa}$	$0.26±0.03^{b}$
Total SFA	$39.84±0.57^{A}$	$38.52±0.60^{a}$	$36.30±0.46^{Bb}$
MUFA			
C16:1 (palmitoleic)	$4.09±0.26^{A}$	$3.94±0.32^{A}$	$2.59±0.23^{B}$
C18:1 (oleic)	27.42±0.48	26.64±0.47	26.56±0.32
Other MUFA	$0.03±0.01^{A}$	$0.01±0.00^{B}$	$0.03±0.01^{A}$
Total MUFA	$31.54±0.58^{a}$	30.59±0.50	$29.18±0.41^{b}$
PUFA			
C18:2 (linoleic)	$21.77±0.77^{B}$	$23.20±0.87^{b}$	$25.79±0.79^{Aa}$
C18:3 (α-linolenic)	$4.23±0.12^{Bd}$	$4.82±0.22^{bc}$	$5.40±0.14^{Aa}$
C20:4 (arachidonic)	1.81±0.14	2.13±0.42	2.45±0.15
C20:5 (EPA)	$0.13±0.01^{B}$	$0.21±0.02^{A}$	0.16±0.02
C22:6 (DHA)	$0.03±0.01^{A}$	$0.03±0.01^{A}$	$0.00±0.00^{B}$
Other PUFA	0.66±0.07	0.50±0.06	0.73±0.07
Total PUFA	$28.63±0.84^{Bb}$	$30.89±0.68^{Ba}$	$34.53±0.76^{A}$
PUFA 6/3	5.40±0.23	5.04±0.12	5.09±0.09
S/P	$0.65±0.05^{a}$	0.61±0.05	$0.56±0.04^{b}$

SEM: *standard error of the mean;* ***SFA:*** *saturated fatty acids;* ***MUFA:*** *monounsaturated fatty acids;* ***PUFA:*** *polyunsaturated fatty acids;* ***S/P:*** *saturation index;* $^{a,b;c,d}$ *Values with different superscripts are significantly different at P<0.05;* ^{A,B} *Values with different superscripts are significantly different at P<0.01*

and palmitic acids was highest in group C, lower in E1, and lowest in E2. An opposite trend was found for stearic and arachidic acids, the proportions of which were highest in group E2 and lowest in group C. On the other hand, total MUFA proportion was higher in C and E1 than in E2 group. A similar relationship occurred for palmitoleic acid. The proportions of oleic acid did not differ significantly among the groups. Meanwhile, total PUFA showed high levels in group E2, insignificantly lower in E1, and significantly lowest in C group. The same relationship between the groups was observed for the two most important PUFA: linoleic and α-linolenic acids, in which highly significant differences between groups E2 and C were noted. The proportion of arachidonic acid was significantly higher in rabbit meat of group E2 compared to the other groups, and significantly higher in E1 than in C. There were no significant differences between the groups in the amount of EPA (eicosapentaenoic acid). Also, differences in PUFA 6/3 ratio were only numerical and statistically not significant. The S/P ratio was higher in group C than in both experimental groups.

Table 6 presents the proportions of fatty acids in the loin of the studied rabbits. Overall, the loin showed similar trends for fatty acids as did the thigh. The highest SFA content was noted in the loin of rabbits from group C, followed by groups E1 and E2, which was largely due to the differences in the amounts of palmitic acid and to a lesser extent in the amounts of myristic acid. However, the proportions of arachidic acid were lowest in group C and highest in group E2. Total MUFA was higher in group C than in group E2. In groups C and E1 there was more palmitoleic acid than in group E2. The proportions of PUFA were highest in the group of animals receiving no soybean, lower in the group fed 7.5% soybean, and lowest in the loin of animals from group C. This tendency was in relation to the proportions of linoleic and α-linolenic acids, for which the same intergroup relationships were observed. No significant differences between the groups were observed in the PUFA 6/3 ratio, just like in the thigh muscle. The S/P ratio was highest in group C and lowest in group E2, with a statistically significant difference between these groups.

Table 7. *Fatty acid content in perirenal fat (% of total fatty acid pool; mean±SEM)*

Fatty Acids	Group		
	C	E1	E2
SFA			
C12:0 (lauric)	0.36±0.07	0.52±0.06	0.35±0.03
C14:0 (myristic)	$4.96±0.14^{Aa}$	$4.38±0.18^{b}$	$4.04±0.12^{B}$
C16:0 (palmitic)	$32.68±0.66^{Aa}$	$30.62±0.44^{Ab}$	$28.32±0.35^{B}$
C18:0 (stearic)	5.25±0,10	5.61±0.29	5.83±0.26
C20:0 (arachidic)	0.03±0.00	0.04±0.01	0.07±0.01
Other SFA	$0.25±0.04^{Bb}$	$0.63±0.07^{A}$	$0.38±0.04^{Ba}$
Total SFA	$43.53±0.79^{A}$	$41.80±0.64^{A}$	$38.99±0.29^{B}$
MUFA			
C16:1 (palmitoleic)	$4.88±0.41^{A}$	$4.46±0.44^{a}$	$2.93±0.24^{Bb}$
C18:1 (oleic)	25.94±0.35	25.81±0.34	26.50±0.31
Other MUFA	0.02±0.01	0.01±0.00	0.01±0.00
Total MUFA	30.84±0.61	30.28±0.58	29.44±0.40
PUFA			
C18:2 (linoleic)	$20.82±0.76^{Bb}$	$22.49±0.70^{a}$	$25.22±0.60^{A}$
C18:3 (α-linolenic)	$3.93±0.10^{B}$	$4.43±0.25^{Ab}$	$5.10±0.08^{Aa}$
C20:4 (arachidonic)	0.33±0.02	0.40±0.05	0.45±0.02
C20:5 (EPA)	$0.03±0.00^{b}$	0.04±0.01	$0.05±0.01^{a}$
Other PUFA	$0.52±0.07^{b}$	0.56±0.08	$0.75±0.10^{a}$
Total PUFA	$25.63±0.84^{Bd}$	$27.92±0.97^{bc}$	$31.57±0.61^{Aa}$
PUFA 6/3	4.84±0.15	4.69±0.12	4.57±0.09
S/P	$0.76±0.07^{Aa}$	$0.70±0.06^{bc}$	$0.63±0.05^{Bd}$

SEM: *standard error of the mean;* ***SFA:*** *saturated fatty acids;* ***MUFA:*** *mono-unsaturated fatty acids;* ***PUFA:*** *polyunsaturated fatty acids;* ***S/P:*** *saturation index;* $^{a,b;\ c,d}$ *Values with different superscripts are significantly different at P<0.05;* ^{A,B} *Values with different superscripts are significantly different at P<0.01*

The proportion of fatty acids in perirenal fat of the studied rabbits is given in *Table 7*. Total SFA was higher in groups C and E1 than in group E2. Similar to the case of thigh muscle and loin, the proportion of myristic acid in perirenal fat was highest in the control group, lower in group E1, and lowest in group E2. Also, the proportions of palmitic acid in the studied rabbits reflected the situation observed in the muscles: they were highest in group C and lowest in group E2. No differences were found between the groups in total MUFA, although the proportion of palmitoleic acid was highest in the control group, lower in group E1, and lowest in group E2. In turn, total PUFA was highest in group E2 and lowest in the control group. Similar tendencies were observed for individual PUFA. Perirenal fat of the rabbits fed diets E1 and E2 contained more linoleic and α-linolenic acids compared to the rabbits fed the control diet. In addition, there was more EPA in group E2 than in group C. However, for the fatty acid profile, no differences between the groups were found for PUFA 6/3 ratio. The S/P ratio was highest in group C, lower in group E1, and lowest in group E2.

DISCUSSION

In our experiment we found that partial or complete replacement of soybean meal with the experimental components did not cause differences in body weight between the studied groups of rabbits, but it considerably improved feed efficiency. The experimental diets (E1 and E2) contained rapeseed meal, white lupin seed and pea seed. The proportions of barley, wheat and corn were manipulated to balance the diets. The content of protein, including lysine, methionine + cystine, and threonine in the experimental diets was higher than in the control diet and this probably caused differences in protein content in thigh muscle, the highest DP in group E2, and the lowest DP in the control group. The obtained content of dry matter, protein and ether extract in meat is characteristic of broiler rabbits. Chełmińska and Kowalska [27] observed a similar content of dry matter and protein to ours, and a slightly lower fat content in the meat of NZW rabbits. Also, the range of DP obtained in our study is consistent with the findings of Daszkiewicz et al.[28] and Chełmińska and Kowalska [27]. The BW of NZW rabbits in our experiment were slightly higher than those obtained by the previous studies. However, BWG of the rabbits investigated by Chełmińska and Kowalska [27] were similar to ours. Similar BWG in NZW rabbits, although calculated for the age range of 30-80 days, were reported by Cardinali et al.[29]. It is worth emphasizing that rabbits' body weights and BWG may differ according to the subject breed. In our previous experiment, Californian rabbits achieved body weights of 2291-2371 g at age 98 days, with BWG of 24.6-26.2 g [4]. Today, commercially bred hybrid rabbits may reach over 3000 g on day 84 of age [6,30].

Compared to the meat of other livestock species, rabbit meat is characterized by a low content of intramuscular fat, which has a beneficial composition of fatty acids for the consumer [19]. The diet of rabbits has a major effect on the subsequent fatty acid composition of the carcasses, because most fatty acids supplied to rabbits with the diet is not modified during the digestion and they quickly enter the fat depots with minor modifications [31,32]. Our results confirm this observation for total SFA and PUFA, although a different trend for total MUFA was observed. Nowadays, attempts are being made to reduce SFA levels and increase MUFA and PUFA levels in the human diet. In our study, relatively most PUFA in the thigh muscle of the rabbits was found in group E2, in which soybean meal was completely removed from the ration. Among PUFA, methylene-interrupted polyenes are considered particularly beneficial. Among these, special attention should be given to n-3 α-linolenic acid, which is usually consumed in inadequate amounts by humans. As reported by Dalle Zotte [19], its content in the loin of rabbits is 3.14% compared to 2.98% in the thigh muscle. The content of this acid in the thigh muscle of the rabbits studied by us ranged from 3.85 to 4.53% and it was much more favorable

in groups E1 and E2. A similar situation was found for the proportion of α-linolenic acid in perirenal fat, where the most favorable amount was noted in group E2, followed by E1, and the lowest amount in group C. This tendency is generally consistent with the proportions of this acid observed in the dietary mixtures, although in diet E1 it was slightly higher than in group E2. The dietary n-6/n-3 PUFA ratio is another important consideration. In most European countries, this ratio in the human diet is too high, at 10-20:1. The optimal n-6/n-3 PUFA ratio in the human diet should not be higher than 5-6:1. As stated by Newton [33], excessive consumption of n-6 fatty acids disrupts the metabolism of n-3 acids and the physiological balance of the compounds synthesized from these acids. In the present study, the n-6/n-3 PUFA ratio was relatively low in all carcasses, ranging from 5.08 to 5.31 and from 4.57 to 4.84 in thigh muscle and in perirenal fat, respectively; however, it did not differ statistically between groups. By way of comparison, in rabbits fed 10% of maize DDGS, the n-6/n-3 ratio was 6.51 [27]. We also calculated the simple saturation index (S/P) in the analyzed tissues *(Table 5, 6, 7)*. In general, both in muscles and in adipose tissue, this index was lower in groups E1 and E2 than in group C. Therefore, human consumers would benefit from eating rabbits in whose diets soybean meal was replaced with a combination of rapeseed meal, white lupin seed, and pea seed.

Based on the results obtained in our experiment, it may be concluded that substitution of soybean meal with white lupin and pea seeds improved the feed efficiency as well as dressing percentage and crude protein content of the carcasses. Importantly for human consumers, as the dietary proportion of soybean decreased, the proportion of SFA in meat and in perirenal fat decreased, and that of PUFA increased. As a result, the saturation index was more beneficial in the groups supplemented with rapeseed meal, white lupin seed and pea seed compared to group C. This indicates that soybean meal in rabbit diets may be replaced by the mixture of the above components.

Disclosure Statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

REFERENCES

1. Lebas F: Reflection on rabbit nutrition with a special emphasis on feed ingredients utilization. *Proceedings of the 8th World Rabbit Congress*, September 7-10, Puebla, Mexico, p. 686-736, 2004.

2. Carabaño R, Villamide MJ, García J, Nicodemus N, Llorente A, Chamorro S, Menoyo D, García-Rebollar P, García-Ruiz AI, De Blas JC: New concepts and objectives for protein-amino acid nutrition in rabbits: A review. *World Rabbit Sci*, 17, 1-14, 2009. DOI: 10.4995/wrs.2009.664

3. Gasmi-Boubaker A, Abdouli H, El Hichi M, Faiza K, Tayachi L: Feeding rapeseed meal to rabbits: Digestibility, performance and carcass characteristics. *Asian J Anim Vet Adv*, 2, 38-41, 2007. DOI: 10.3923/ajava.2007.38.41

4. Strychalski J, Juśkiewicz J, Gugołek A, Wyczling P, Daszkiewicz T, Zwoliński C: Usability of rapeseed cake and wheat-dried distillers' grains with solubles in the feeding of growing Californian rabbits. *Arch Anim Nutr*, 68, 227-244, 2014. DOI: 10.1080/1745039X.2014.921482

5. El-Medany A, El-Reffaei WHM: Evaluation canola meal on growing rabbits: Nutritionally and on their nutritional meat quality. *J Food Nutr Res*, 3, 220-234, 2015.

6. Gugołek A, Juśkiewicz J, Wyczling P, Kowalska D, Strychalski J, Konstantynowicz M, Zwoliński C: Productivity and gastrointestinal tract responses of rabbits fed diets containing rapeseed cake and wheat distillers dried grains with solubles. *Anim Prod Sci*, 55, 777-785, 2015. DOI: 10.1071/AN14206

7. Volek Z, Marounek M: Whole white lupin *(Lupinus albus* cv. Amiga) seeds as a source of protein for growing-fattening rabbits. *Anim Feed Sci Technol*, 152, 322-329, 2009. DOI: 10.1016/j.anifeedsci.2009.05.003

8. Matusevicius P, Zduńczyk Z, Juśkiewicz J, Jeroch H: Rapeseed meal as a partial replacement for sunflower meal in diets for growing rabbits – gastrointestinal tract response and growth performance. *Eur Poult Sci*, 78, 1-11, 2014. DOI: 10.1399/eps.2014.18

9. Volek Z, Marounek M: Effect of feeding growing-fattening rabbits a diet supplemented with whole white lupin *(Lupinus albus* cv. Amiga) seeds on fatty acid composition and indexes related to human health in hind leg meat and perirenal fat. *Meat Sci*, 87, 40-45, 2011. DOI: 10.1016/j.meatsci.2010.08.015

10. Al-harbi MS, Al-Bashan MM, Ali KH, Abu Amrah A: Impacts of feeding with *Lupinus albus* (white lupin) and *Lupinus termis* (Egyptian lupin) on physiological activities and histological structure of some rabbits organs, at Taif Governorate. *World J Zool*, 9, 166-177, 2014.

11. Volek Z, Bureš D, Uhlířová L: Effect of dietary dehulled white lupine seed supplementation on the growth, carcass traits and chemical, physical and sensory meat quality parameters of growing-fattening rabbits. *Meat Sci*, 141, 50-56, 2018. DOI: 10.1016/j.meatsci.2018.03.013

12. Seroux M: Spring peas as a source of protein for doe rabbits. *Proceedings of the 4th World Rabbit Congress*, Budapest, Hungary, pp.141-147, 1988.

13. Castellini C, Cavalletti C, Battaglini M: Pea protein in the feeding of rabbits during fattening. *Riv Coniglicoltura*, 28, 33-36, 1991.

14. Bonomi A, Bonomi BM, Quarantelli A: L'impiego della farina di semi di pisello *(Pisum Sativum L.)* Nell'alimentazione del coniglio da carne. *Ann Fac Medic Vet di Parma,* XXIII, 203-215, 2003.

15. Amaefule KU, Iheukwumere FC, Nwaokoro CC: A note on the growth performance and carcass characteristics of rabbits fed graded dietary levels of boiled pigeon pea seed *(Cajanus cajan)*. *Livest Res Rural Dev*, 17, 2005.

16. Lounaouci-Ouyed G, Berchiche M, Gidenne T: Effects of substitution of soybean meal-alfalfa-maize by a combination of field bean or pea with hard wheat bran on digestion and growth performance in rabbits in Algeria. *World Rabbit Sci*, 22, 137-146, 2014. DOI: 10.4995/wrs.2014.1487

17. Zwoliński C, Gugołek A, Strychalski J, Kowalska D, Chwastowska-Siwiecka I, Konstantynowicz M: The effect of substitution of soybean meal with a mixture of rapeseed meal, white lupin grain, and pea grain on performance indicators, nutrient digestibility, and nitrogen retention in Popielno White rabbits. *J Appl Anim Res*, 45, 570-576, 2017. DOI: 10.1080/09712119.2016.1233107

18. Gugołek A, Juśkiewicz J, Kowalska D, Zwoliński C, Sobiech P, Strychalski J: Physiological responses of rabbits fed with diets containing rapeseed meal, white lupine and pea seeds as soybean meal substitutes. *Cienc Agrotec*, 42, 297-306, 2018. DOI: 10.1590/1413-70542018423003318

19. Dalle Zotte A: Perception of rabbit meat quality and major factors influencing the rabbit carcass and meat quality. *Livest Prod Sci*, 75, 11-32, 2002. DOI: 10.1016/S0301-6226(01)00308-6

20. Szabó A, Romvári R, Fébel H, Nagy I, Szendrõ Z: Fatty acid composition of two different muscles in rabbits: Alterations in response to saturated or unsaturated dietary fatty acid complementation. *World Rabbit Sci*, 9, 155-158, 2001. DOI: 10.4995/wrs.2001.459

21. Peiretti PG, Meineri G: Effects on growth performance, carcass characteristics, and the fat and meat fatty acid profile of rabbits fed diets with chia *(Salvia hispanica* L.) seed supplements. *Meat Sci*, 80, 1116-1121, 2008. DOI: 10.1016/j.meatsci.2008.05.003

22. Alagón G, Arce O, Serrano P, Ródenas L, Martínez-Paredes E, Cervera C, Pascual JJ, Pascual M: Effect of feeding diets containing barley, wheat and corn distillers dried grains with solubles on carcass traits and meat quality in growing rabbits. *Meat Sci*, 101, 56-62, 2015. DOI: 10.1016/j.meatsci.2014.10.029

23. AOAC International. Official Methods of Analysis of AOAC International. 18th ed., Arlington, VA, USA, Association of Analytical Communities: Arlington, 2006.

24. Van Soest PJ, Robertson JB, Lewis BA: Methods for dietary fibre, neutral detergent fibre and non-starch polysaccharides in relation to animal nutrition. *J Dairy Sci*, 74, 3583-3597, 1991. DOI: 10.3168/jds.S0022-0302(91)78551-2

25. Peisker KV: A rapid semi-micro method for preparation of methyl esters from triglycerides using chloroform, methanol, sulphuric acid. *J Am Oil Chem Soc*, 41, 87-88, 1964. DOI: 10.1007/BF02661915

26. StatSoft Inc: STATISTICA (data analysis software system), Version 9.1., 2010. www.statsoft.com

27. Chełmińska A, Kowalska D: The effectiveness of maize DDGS in rabbit diets. *Ann Anim Sci*, 13, 571-586, 2013. DOI: 10.2478/aoas-2013-0032

28. Daszkiewicz T, Gugołek A, Janiszewski P, Kubiak D, Czoik M: The effect of intensive and extensive production systems on carcass quality in New Zealand White rabbits. *World Rabbit Sci*, 20, 25-32, 2012. DOI: 10.4995/wrs.2012.945

29. Cardinali R, Cullere M, Dal Bosco A, Mugnai C, Ruggeri S, Mattioli S, Castellini C, Trabalza Marinucci M, Dalle Zotte A: Oregano, rosemary and vitamin E dietary supplementation in growing rabbits: Effect on growth performance, carcass traits, bone development and meat chemical composition. *Livest Sci*, 175, 83-89, 2015. DOI: 10.1016/j.livsci.2015.02.010

30. Dänicke S, Ahrens P, Strobel E, Brettschneider J, Wicke M, von Lengerken G: Effects of feeding rapeseed to fattening rabbits on performance, thyroid hormone status, fatty acid composition of meat and other meat quality traits. *Arch Geflugelk*, 68, 15-24, 2004.

31. Lazzaroni C, Biagini D, Lussiana C: Fatty acid composition of meat and perirenal fat in rabbits from two different rearing systems. *Meat Sci*, 83, 135-139, 2009. DOI: 10.1016/j.meatsci.2009.04.011

32. Strychalski J, Gugołek A, Antoszkiewicz Z, Kowalska D, Konstantynowicz M: Biologically active compounds in selected tissues of white-fat and yellow-fat rabbits and their production performance parameters. *Livest Sci*, 183, 92-97, 2016. DOI: 10.1016/j.livsci.2015.11.024

33. Newton IS: Long chain fatty acids in health and nutrition. *J Food Lipids*, 3, 233-249, 1996. DOI: 10.1111/j.1745-4522.1996.tb00071.x

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Research Article

Occurrence and Molecular Characterization of Cephalosporin Resistant *Escherichia coli* Isolates from Chicken Meat

Nebahat BİLGE [1,a] Çiğdem SEZER [1,b] Leyla VATANSEVER [1,c] Sevda PEHLİVANLAR ÖNEN [2,d]

[1] University of Kafkas, Faculty of Veterinary Medicine, Department of Food Safety and Public Health, TR-36100 Kars - TURKEY
[2] Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, TR-31040 Antakya, Hatay - TURKEY

ORCIDS: [a] 0000-0002-5229-9847; [b] 0000-0002-9722-3280; [c] 0000-0002-5464-0834; [d] 0000-0002-8582-0598

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Abstract

Contamination of retail meat with extended spectrum beta-lactamase (ESBL) and/or AmpC type beta-lactamase (AmpC) producing *Escherichia coli* may contribute to increased incidences of infections in humans. Regular monitoring of these bacteria is required in the view of one health approach. In this study, 100 chicken meat samples obtained from Kars, Turkey were analysed and 214 isolates recovered from 72/100 samples were identified as *E. coli.* Antibiotic susceptibility tests showed that 15.42% of isolates (33/214) were resistant to at least one cephalosporin antibiotic, 20 (9.34%) were beta-lactamase producer. Among beta-lactamase producing *E. coli* isolates 8 had bla_{TEM}, 7 had bla_{CTX-M} and 3 had bla_{SHV} genes. Plasmid-mediated AmpC beta-lactamase (bla_{CMY}) gene was present in 13 isolates. Plasmid mediated quinolone resistance genes were also screened by polymerase chain reaction and identified by sequencing of the isolates. As a result, 3 isolates were found to be positive for *qnrB,* whereas the qnrS gene was detected in 4 isolates. Regarding the virulence genes 19 isolates were positive for *fimH* and 2 isolates were carrying *kpsMT* II. Phylo-group D2, A0, A1, B1 and D1 were detected in 13, 2, 2, 2 and 1 isolates, respectively. Our findings indicate that poultry meat could be an important carrier of ESBL positive *E. coli.*

Keywords: *Escherichia coli, Chicken, Cephalosporin, ESBL, AmpC*

Tavuk Etlerinde Sefalosporine Dirençli *Escherichia coli* Varlığı ve Moleküler Karakterizasyonu

Öz

Etlerin geniş spektrumlu beta-laktamaz (GSBL) ve/veya AmpC tipi beta-laktamaz üreten *Escherichia coli* ile kontaminasyonu, insanlarda meydana gelen enfeksiyonların insidensinde artışa neden olabilir. Bu nedenle bu bakterilerin düzenli olarak taranması, halk sağlığının korunması açısından önemlidir. Bu çalışmada Kars ilinden temin edilen100 adet tavuk eti incelenmiş ve bunlardan 72'sinden 214 adet *E. coli* izolatı elde edilmiştir. Yapılan antibiyotik duyarlılık testleri izolatlardan 33'ünün (%15.42) en az bir antibiyotiğe dirençli olduğunu, 20'sinin (%9.34) beta-laktamaz, 5'inin ise (%2.33) GSBL ürettiğini göstermiştir. Moleküler testler, beta-laktamaz üreten *E. coli* izolatlarından 8'inin bla_{TEM}, 3'ünün bla_{SHV}, 7'sinin bla_{CTX-M} ve 13'ünün plazmid aracılı AmpC beta-laktamaz (bla_{CMY}) genlerine sahip olduğuna işaret etmiştir. Plazmid aracılı kinolon direnç genleri de polimeraz zincir reaksiyonu ile görüntülenip sekanslanarak identifiye edilmiş; 3 izolatın *qnrB*, 4 izolatın da *qnrS* taşıdığı belirlenmiştir. Bunun yanında izolatlardan 19'unun *fimH* ve 2'sinin *kpsMT* II virulens genlerine sahip olduğu görülmüştür. Son olarak 13, 2, 2, 2 ve 1 izolatın sırasıyla filogrup D2, A0, A1, B1 ve D1'e dahil olduğu ortaya konmuştur. Bu çalışmadan elde edilen bulgular, kanatlı etinin GSBL pozitif *E. coli* açısından önemli bir taşıyıcı rolü üstlenebileceğini göstermiştir.

Anahtar sözcükler: *Escherichia coli, Tavuk, Sefalosporin, GSBL, AmpC*

INTRODUCTION

The *Enterobacteriaceae* are considered as a hygiene indicator in food industry [1]. This family possesses various bacterial species that can be pathogenic to humans and animals [2]. Among these species, *E. coli* strains in particular can cause postsurgical, urinary tract, blood-stream and central nervous system infections [3]. The successful treatment of bacterial infections commonly requires a careful usage of antimicrobials [4]. However, because of the several reasons including the use of antimicrobial agents in animal production, the resistance in this family has increased.

Correspondence
+90 5330301882 (GSM)
nebahatbilgeoral@hotmail.com

Addition of antimicrobial agents into animal feeds and veterinary use of cephalosporins and fluoroquinolones played a significant role in selecting resistant clones. The most important mechanism of that resistance is production of β-lactamases that hydrolyses the beta lactam ring and inactivates the β-lactam group antibiotics [1,5]. Among them, extended spectrum β-lactamases (ESBL) and AmpC are the most common enzymes around the world. ESBL producing microorganisms are resistant to 3rd and 4th generation cephalosporins but they are sensitive to carbapenems and cephamycins (cefoxitin), and are inactivated by clavulanic acid. On the other hand, the AmpC enzymes confer resistance to third generation cephalosporins and cephamycins but microorganisms having these enzymes are not inhibited by clavulanic acids and other β-lactamase inhibitors [6].

Extended spectrum β-lactamases genes are commonly located on plasmids that can be easily transferred between and within bacterial species. Some ESBL genes are mutant derivatives of established plasmid-mediated β-lactamases (e.g., $bla_{TEM/SHV}$), and others are mobilized from environmental bacteria (e.g., bla_{CTX-M}) [7].

Prevalence of ESBL producing *E. coli* infections are rapidly increasing in European countries causing longer hospital stays, increased costs and mortality [8]. They are considered as a serious threat causing therapy failure in human medicine [9].

Studies indicated that food producing animals carry increasing numbers of ESBL/AmpC producing *E. coli* isolates leading to the hypothesis that animals might become infection sources or even reservoirs contributing to the spread of these bacteria [10,11]. They also showed that ESBL producers could spread along the broiler production chain [12]. Other researchers reported that human and broiler ESBL *E. coli* were possibly associated and proposed broilers as a potential reservoir of foodborne resistant bacteria that infect human [13,14]. High levels poultry meat contamination with ESBL producers serving as a possible reservoir for human colonization has also been declared by other researchers [15]. According to The European Food Safety Authority (EFSA) the presence of ESBL producing *E. coli* in poultry meat is a significant threat to public health [16].

Regular monitoring of these bacteria is required in the view of one health approach. This study was conducted to understand the current situation of resistance to cephalosporins and its molecular mechanisms in *E. coli* isolated from raw chicken meat samples hoping to quantify the impact of the broiler meat production chain on consumer exposure.

MATERIAL and METHODS

Collection of Samples

One hundred retail packaged raw poultry meat samples were purchased from 47 markets in Kars Province, Turkey during the period from May to August 2017. The samples were transported to the laboratory on ice and processed within 24 h.

Bacterial Isolation

Two hundred and twenty-five millilitres of buffered peptone broth (Oxoid, UK) were added to 25 g of each sample and homogenized by stomacher blender. The homogenate was incubated at 37°C for 24 h. The next day 100 µL of enriched broth were inoculated on Violet Red Bile Lactose Agar (Oxoid, UK) and incubated at 37°C for 24 h [17].

Three colonies per plate exhibiting typical *E. coli* morphological appearance (pink to red, entire-edged colonies that are surrounded by a reddish zone of precipitated bile) were selected and phenotypically confirmed by evaluating catalase activity (positive), motility (positive), indole production from tryptophan amino acid (positive or negative), H_2S production (negative), urea hydrolyzation (negative) and utilization of citrate (negative). Voges-Proskauer (negative), triple sugar iron (glucose and lactose fermentation and CO_2 production positive) and methyl red (positive) tests were also applied to the isolates [18]. *E. coli* isolates were grouped phylogenetically by polymerase chain reaction (PCR) assay targeting the *chuA, yjaA* and *TESPE*4 genes [19,20] *(Table 1)*.

Resistance to Cephalosporin Antibiotics and Phenotypic ESBL Detection

Antimicrobial susceptibility testing for cephalosporins was performed using disc diffusion assay. Discs containing Cefpodoxime (10 µg), Ceftaximide (30 µg), Aztreonam (30 µg), Cefotaxime (30 µg) and Ceftriaxone (30 µg) were used alone and in combination with Clavulanate (10 µg). The phenotypic ESBL confirmation was carried out with a disc combination test, in which the isolates having ≥5 mm larger inhibition zone around the disc containing ceftaximide/clavulanate and cefotaxime/clavulanate than the one without clavulanate were considered ESBL producer. The results interpreted according to the Clinical Laboratory Standards Institute guidelines [21].

Molecular Detection of Antimicrobial Resistance Genes

PCR assay was conducted to determine whether the isolates harboured β-lactamase *(bla_{AmpC}, bla_{TEM}, bla_{SHV}* and *bla_{CTX-M})* [22-24] and plasmid-mediated quinolone resistance (PMQR) *(qnrA, qnrB, qnrC, qnrS* and *aac*[6′]-*Ib)* genes [25-29]. Specific ESBL and PMQR gene types were identified by amplicon sequencing, and then by comparing the obtained sequences with those submitted the online database *(http://blast.ncbi.nlm.nih.gov)*.

Determination of Virulence Genes

A total of 12 *E. coli* virulence genes including *papAH, papC, papEF, papG* allele II, *papG* allele III, *papG* allele II-III, *kpsMT*

Table 1. *PCR conditions and primers used in this study*

Target Gene	Primer Sequence	Amp. Size (bp)	PCR Conditions	Ref.
chuA	F: 5′-ATGGTACCGGACGAACCAAC-3′	288	Initial denaturation at 94°C for 4 min, followed by 30 cycles at 94°C for 5 s and at 59°C 20 s and final extension at 72°C for 5 min	[19,20]
	R: 5′-TGCCGCCAGTACCAAAGACA-3′			
yjaA	F: 5′-CAAACGTGAAGTGTCAGGAG-3′	211		
	R: 5′-AATGCGTTCCTCAACCTGTG-3′			
TESPE4	F: 5′-CACTATTCGTAAGGTCATCC-3′	152		
	R: 5′-AGTTTATCGCTGCGGGTCGC-3′			
bla_{AmpC}	F: 5′-GCACTTAGCCACCTATACGGCAG-3′	758	Initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, at 60°C for 30 s and at 72°C for 30 s and final extension at 72°C for 10 min	[22]
	R: 5′-GCTTTTCAAGAATGCGCCAGG-3′			
bla_{CTX-M}	F: 5′- ATGTGCAGYACCAGTAARGTKATGGC-3′	593	Initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, at 60°C for 30 s and at 72°C for 30 s and final extension at 72°C for 10 min	[24]
	R: 5′-TGGGTRAARTARGTSACCAGAAYCAGCGG-3′			
bla_{TEM}	F. 5′-TGAGTATTCAACATTTCCGTGT-3′	861	Initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, at 53°C for 30 s and at 72°C for 30 s and final extension at 72°C for 10 min	[23]
	R: 5′-TTACCAATGCTTAATCAGTGA-3′			
bla_{SHV}	F: 5′-CAAAACGCCGGGTTATTC-3′	937		
	R: 5′-TTAGCGTTGCCAGTGCT-3′			
qnrB	F: 5′-GG**M**AT**H**GAAATTCGCCACTG-3′	262	Initial denaturation at 95°C for 5 min, followed by 32 cycles at 94°C for 45 s, at 53°C for 45 s and at 72°C for 60 s and final extension at 72°C for 10 min	[25]
	R: 5′-TTTGC**Y**G**YY**CGCCAGTCGAA-3′			
qnrS	F: 5′-TCGACGTGCTAACTTGCG-3′	466	Initial denaturation at 95°C for 5 min, followed by 32 cycles at 94°C for 45 s, at 53°C for 45 s and at 72°C for 60 s and final extension at 72°C for 10 min	[26]
	R: 5′-GATCTAAACCGTCGAGTTCGG-3′			
aac[6′]-Ib	F: 5′-TTGCGATGCTCTATGAGTGGCTA-3′	482	Initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, at 56°C for 30 s and at 72°C for 30 s and final extension at 72°C for 10 min	[29]
	R: 5′-CTCGAATGCCTGGCGTGTTT-3′			
papAH	F: 5′-ATGGCAGTGGTGTCTTTTGGTG-3′	720	Initial denaturation at 95°C for 15 min, followed by 25 cycles at 94°C for 30 s, at 63°C for 30 s and at 68°C for 3 min and final extension at 72°C for 10 min	[30]
	R: 5′-CGTCCCACCATACGTGCTCTTC-3′			
fimH	F: 5′-TGCAGAACGGATAAGCCGTGG-3′	508		
	R: 5′-GCAGTCACCTGCCCTCCGGTA-3′			
papEF	F: 5′-GCGCATTTGCTGATACTGTTG -3′	336		
	R: 5′-CATCCAGACGATAAGCATGAGCA-3′			
iutA	F: 5′-GGCTGGACATCATGGGAACTGG-3′	300		
	R: 5′-CGTCGGGAACGGGTAGAATCG-3′			
papG allele III	F: 5′-GGCCTGCAATGGATTTACCTGG-3′	258		
	R: 5′-CCACCAAATGACCATGCCAGAC-3′			
kpsMT K1	F: 5′-TAGCAAACGTTCTATATTGGTGC-3′	153		
	R: 5′-CATCCAGACGATAAGCATGAGCA-3′			
hlyA	F: 5′-AACAAGGATAAGCACTGTTCTGGCT-3′	1.177		
	R: 5′-ACCATATAAGCGGTCATTCCCGTCA-3′			
papC	F: 5′-GTGGCAGTATGAGTAATGACCGTTA-3′	200		
	R: 5′-ATATCCTTTCTGCAGGGATGCAATA-3′			
kpsMT II	F: 5′-GCGCATTTGCTGATACTGTTG-3′	272		
	R: 5′-CATCCAGACGATAAGCATGAGC-3′			
papG allele II	F: 5′-GGGATGAGCGGGCCTTTGAT-3′	190		
	R: 5′-CGGGCCCCCAAGTAACTCG-3′			
papG allele II-III	F: 5′-CTGTAATTACGGAAGTGATTTCTG-3′	1070		
	R: 5′-ACTATCCGGCTCCGGATAAACCAT-3′			
univenf	F: 5′-ATCTTATACTGGATGGGATCATCTTGG-3′	1.105		
	R: 5′-GCAGAACGACGTTCTTCATAAGTATC-3′			
ireA	F: 5′-AAGTCAAAGCAGGGGTTGCCCG-3′	665		
	R: 5′-GACGCCGACATTAAGACGCAG-3′			

*K*1, *kpsMT* II, *hlyA, fimH*, *iutA, ireA* and *univenf* were detected with multiplex PCR [30].

RESULTS

In this study coliform bacteria were detected in 99 of 100 chicken samples and 214 isolates recovered from 72/100 samples were identified as *E. coli*. Antibiotic susceptibility tests showed that 33 isolates recovered from 20/100 samples (15.42%) were resistant to at least one cephalosporin antibiotic, 20 isolates recovered from 15/100 (9.34%) were β-lactamase producing and 5 isolates recovered from 4/100 (2.33%) were ESBL producing *E. coli* phenotypically *(Table 2)*. Based on the phylogenetic PCR analysis developed by Clermont et al.[20], phylo-group D_2, A0, A1, B1 and D1 were detected in 13, 2, 2, 2 and 1 isolates, respectively.

PCR was used to determine the ESBL and PMQR genes among the β-lactamase producing *E. coli* isolates (n=20); *(Table 2)*. The bla_{TEM} and plasmid-mediated bla_{CMY} genes were detected in eight (%40) and 13 (65%) of the isolates, respectively. Additionally, ten isolates (50%) harboured ESBLs encoding genes, of which the $bla_{CTX\text{-}M}$ genes were detected in seven isolates, whereas the $bla_{SHV\text{-}12}$ gene was present in three isolates. CTX-M type enzymes were further identified as CTX-M-1 (n=3), CTX-M-8 (n=2) and CTX-M-55 (n=2).

Among the PMQR genes tested, the *qnrS* and *qnrB19* genes were detected in four (20%) and three isolates (15%), respectively *(Table 2)*. However, none of the isolates were found to possess the other PMQR genes *(qnrA, qnrC,* and *aac*[6']*-Ib)*.

Of the virulence genes 19 isolates were positive for *fimH* and 2 isolates were carrying *kpsMT* II *(Table 2)*. Other virulence genes tested were not found among the *E. coli* isolates.

DISCUSSION

Because of plasmid-mediated acquisition of ESBL producing genes by *Enterobacteriaceae* family, especially *E. coli*, there has been increased resistance to beta-lactam antibiotics, lately. Although majority of the ESBL producers related studies are confined within the hospital premises, recent reports indicating ESBL positive microorganisms' presence in chicken meat and raw milk shows that focus should also be given on their occurrence and dissemination of in food producing animals [31].

Cephalosporin antibiotics are one of the most important class of antibiotics in human medicine and the development of resistance to this class antibiotics have been identified as the major concern during the last decade. In this study, 214 isolates recovered from 72/100 samples were identified as *E. coli*. Antibiotic susceptibility tests showed that 33 isolates recovered from 20/100 meat samples (15.42%) were resistant to at least one cephalosporin antibiotic. In fact, the findings of this study are not surprising in view of the high frequency of resistance to β-lactam antibiotics

Table 2. *Characteristics of β-lactamase producing E. coli isolates≠*

Isolate No	Phylogenetic Type	Antibiotic Resistance Genes	Virulence Genes
22a*	D2	$bla_{CTX\text{-}M\text{-}1}$ + $qnrB_{19}$	*fim*H
51a*	D2	$bla_{CTX\text{-}M\text{-}55}$	*fim*H
KH51b*	D2	$bla_{CTX\text{-}M\text{-}55}$	*fim*H
93a*	B1	bla_{TEM}	*fim*H
96a*	D2	$bla_{CTX\text{-}M\text{-}1}$ + $qnrB_{19}$	*fim*H
2b	D2	$bla_{CTX\text{-}M\text{-}1}$ + $qnrB_{19}$	*fim*H
6c	A1	bla_{TEM} + bla_{CMY}	*fim*H
8a	A0	bla_{CMY}	nd
11b	D2	bla_{CMY}	*fim*H + kpsMT II
13a	A1	bla_{TEM} + $bla_{SHV\text{-}12}$ + bla_{CMY} + *qnrS*	*fim*H
22b	D2	$bla_{SHV\text{-}12}$ + bla_{CMY} + *qnrS*	*fim*H
KH22c	D2	$bla_{SHV\text{-}12}$ + bla_{CMY} + *qnrS*	*fim*H
33a	A0	bla_{CMY}	*fim*H
36a	D2	bla_{TEM} + $bla_{CTX\text{-}M\text{-}8}$ + bla_{CMY}	*fim*H
K36b	D2	bla_{TEM} + $bla_{CTX\text{-}M\text{-}8}$ + bla_{CMY}	*fim*H
KH36c	D2	bla_{CMY}	*fim*H
39b	D2	bla_{CMY}	*fim*H + kpsMT II
40b	D2	bla_{TEM}	*fim*H
99a	D1	bla_{TEM} + bla_{CMY}	*fim*H
KH99c	B1	bla_{TEM} + bla_{CMY}	*fim*H

*≠ All isolates are phenotypically resistant to Cefpodoxime, Ceftriaxone, Cefotaxime, Ceftaximide and Aztreonam; * indicating the phenotypic ESBL positive isolates,* ***nd:*** *not detected*

as Kürekci et al.[32] already reported that the frequency of ESBL producing *E. coli* isolates in raw chicken meat samples reached 86.6% in Turkey. In addition, cephalosporin resistance has been frankly linked to the heavy field use of these antibiotics in animal husbandry for many years, and the occurrences of resistance frequency was markedly dropped when the usage of ceftiofur in animal production was discontinued in Japan [33]. Therefore, the perpetuation of cephalosporin resistance phenomenon would be expected, even though the use of antibiotics has not been allowed as a growth promoting agent in animal industry in Turkey since 2006.

In this study, based on the phylogenetic PCR analysis developed by Clermont et al.[19], phylo-group D_2, A0, A1, B1 and D1 were detected in 13, 2, 2, 2 and 1 isolates, respectively. Kürekci et al.[32] also indicated that phylo-group D was the most prevalent in isolates (82.7%) followed by A (15.3%), and B_2 (only 1 isolate). However, according to Tansawai et al.[34] phylogenetic grouping of the isolates showed that 51% belonged to group A, 27.5% to group B1 and 17.6% to group D. Only 4 isolates (3.9%) belonged to group B2. In addition, twenty isolates were further identified as β-lactamase producer and tested in details. Among these isolates only 5 isolates were found to be phenotypically ESBL positive, whereas ten isolates possessed ESBLs enzymes. This is not surprising, as the discrepancies between methods have already been reported for determination of ESBL producers [35]. In addition, it has been proven that combination of these methods might produce false negative results among the isolates having inducible AmpC enzyme [36]. The findings of this study in terms of ESBL production among *E. coli* isolates are in agreement with other published results from different countries including Nigeria (0.98%) [37], South Korea (2.43%) [38] Germany (5.4%) [39], and India (5.69%) [31].

Of the β-lactamase producing *E. coli* isolates, 50% had ESBLs enzymes with CTX-M type being the predominant, and followed by SHV-12. Overdevest et al.[15] also noted that the predominant ESBL genotype in chicken meat they analysed was $bla_{CTX\text{-}M\text{-}1}$ (58.1%) followed by $bla_{TEM\text{-}52}$ (14%) and $bla_{SHV\text{-}12}$ (14%). Similar results were also reported by Garcia-Graells et al.[6], who reported the frequency of CTX-M (48%), TEM (28%) and SHV (24%) in commensal *E. coli* with an ESBL phenotype. In the current study, the $bla_{CTX\text{-}M\text{-}1}$ gene was found to be predominant, followed by the $bla_{CTX\text{-}M\text{-}8}$ and $bla_{CTX\text{-}M\text{-}55}$ genes. Kürekci et al.[32] also indicated the occurrence of the $bla_{CTX\text{-}M\text{-}55}$ among ESBL producing *E. coli* isolates obtained from raw chicken meat samples. The $bla_{CTX\text{-}M\text{-}8}$ gene has also been identified among raw chicken meat products in Turkey, with low frequency rate (2.8%) [40]. It is a well-known fact that PMQR genes are commonly found on the same mobile elements carrying ESBL resistance genes, in particular $bla_{CTX\text{-}M}$ [32]. In the current study, we also demonstrated the presence of the *qnrB* and *qnrS* genes together with ESBLs enzymes. Other scientists also reported that they detected quinolone resistance gene *(qnrB)* [31], *qnrS* and *qnrB* [32].

In our study, 19 isolates were positive for the *fimH* gene with together the *kpsMT* II gene (n=2). Kürekci et al.[32] also indicated that the *fimH, iutA, iroN, kpsMT II, papC, papG allelle II-III* and *papEF* genes were present among the ESBL producer *E. coli* isolates obtained from the chicken samples. Similarly, Kim et al.[38] found that three isolates they obtained harboured, *fimH, fyuA, iutA, papC, pap EF, papG* allele II*, rfc* and *traT* virulence genes.

Taken together, our results showed poultry meat as an important carrier of ESBL positive *E. coli,* although more studies are needed to understand the dissemination of these clones among the poultry products.

Statement of Author Contributions

NB defined the research theme, designed the experiment, contributed to cultural analyses and wrote the manuscript. ÇS and LV conducted the cultural analyses and have made supervised the analysis of the results. SPÖ conducted the molecular analyses and has made a substantial contribution to interpretation of data. All authors discussed the results and contributed to the final manuscript.

REFERENCES

1. Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, Catry B, Herman L, Haesebrouck F, Butaye P: Diversity of extended-spectrum beta-lactamases and class C beta-lactamases among cloacal *Escherichia coli* isolates in Belgian broiler farms. *Antimicrob Agents Chemother*, 52 (4): 1238-1243, 2008. DOI: 10.1128/AAC.01285-07

2. Pitout JDD: Extraintestinal pathogenic *Escherichia coli*: A combination of virulence with antibiotic resistance. *Front Microbiol*, 3:9, 2012. DOI: 10.3389/fmicb.2012.00009

3. Clements A, Young JC, Constantinou N, Frankel G: Infection strategies of enteric pathogenic *Escherichia coli*. *Gut Microbes*, 3 (2): 71-87, 2012. DOI: 10.4161/gmic.19182

4. EFSA Panel on Biological Hazards (BIOHAZ): Scientific opinion on the public health risks of bacterial strains producing extended-spectrum beta-lactamases and/or AmpC β-lactamases in food and food producing animals. *EFSA J*, 9:2322, 2011. DOI: 10.2903/j.efsa.2011.2322

5. Hao H, Cheng G, Iqbal Z, Ai X, Hussain HI, Huang L, Dai M, Wang Y, Liu Z, Yuan Z: Benefits and risks of antimicrobial use in food-producing animals. *Front Microbiol*, 5:288, 2014. DOI: 10.3389/fmicb.2014.00288

6. Garcia-Graells C, Botteldoom N, Dierick K: Microbial surveillance of ESBL *E. coli* in poultry meat, a possible vehicle for transfer of antimicrobial resistance to humans. *WIV-ISP*, 13, 1-6, 2012.

7. Hawkey PM, Jones AM: The changing epidemiology of resistance. *J Antimicrob Chemother*, 64 (Suppl. 1): i3-i10, 2009. DOI: 10.1093/jac/dkp256

8. de Kraker MEA, Davey PG, Grundmann H: Mortality and hospital stay associated with resistant *Staphylococcus aureus* and *Escherichia coli* bacteraemia: Estimating the burden of antibiotic resistance in Europe. *PLoS Med,* 8 (10):e1001104, 2011. DOI: 10.1371/journal.pmed.1001104

9. Livermore DM: Has the era of untreatable infections arrived? *J Antimicrob Chemother,* 64 (Suppl. 1): i29-i36, 2009. DOI: 10.1093/jac/dkp255

10. Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, Herman L, Haesebrouck F, Butaye P: Broad-spectrum β-lactamases among *Enterobacteriaceae* of animal origin: Molecular aspects, motility and impact on public health. *FEMS Microbiol Rev*, 34 (3): 295-316, 2010. DOI: 10.1111/j.1574-6976.2009.00198.x

11. Aslantaş Ö, Elmacıoğlu S, Yılmaz EŞ: Prevalence and characterization of ESBL- and AmpC-producing *Escherichia coli* from cattle. *Kafkas Univ Vet Fak Derg,* 23, 63-67, 2017. DOI: 10.9775/kvfd.2016.15832

12. Projahn M, von Tippelskirch P, Semmler T, Guenther S, Alter T, Roesler U: Contamination of chicken meat with extended spectrum beta-lactamases producing- *Klebsiella pneumoniae* and *Escherichia coli* during scalding and defeathering of broiler carcasses. *Food Microbiol,* 77, 185-191, 2019. DOI: 10.1016/j.fm.2018.09.010

13. Ewers C, Bethe A, Semmler T, Guenther S, Wieler LH: Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: A global perspective. *Clin Microbiol Infect,* 18, 646-655, 2012. DOI: 10.1111/j.1469-0691.2012.03850.x

14. Kluytmans JAJW, Overdevest ITMA, Willemsen I, Kluytmans-van den Bergh MFQ, van der Zwaluw K, Heck M, Rijnsburger M, Vanderbroucke-Grauls CMJE, Savelkoul PHM, Johnston BD, Gordon D, Johnson JR: Extended-spectrum beta-lactamase producing *Escherichia coli* from retail chicken meat and humans: Comparison of strains, plasmids, resistance genes, and virulence factors. *Clin Infect Dis*, 56, 478-487, 2013. DOI: 10.1093/cid/cis929

15. Overdevest I, Willemsen I, Rijnsburger M, Eustace A, Xu L, Hawkey PM, Heck M, Savelkoul P, Vandenbroucke-Grauls C, van der Zwaluw K, Huijdens X, Kluytmans J: Extended-spectrum β-lactamase genes of *Escherichia coli* in chicken meat and humans, the Netherlands. *Emerg Infect Dis*, 17 (7): 1216-1222, 2011. DOI: 10.3201/eid1707.110209

16. EFSA: Scientific opinion on the public health hazards to be covered by inspection of meat (poultry). *EFSA J*, 10 (6): 2741, 2012. DOI: 10.2903/j.efsa.2012.2741

17. Ben Said L, Jouni A, Klibi N, Dziri R, Alonso CA, Boudabous A, Ben Slama K, Torres C: Detection of extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* in vegetables, soil and water of the farm environment in Tunisia. *Int J Food Microbiol*, 203, 86-92, 2015. DOI: 10.1016/j.ijfoodmicro.2015.02.023

18. Fattahi F, Mirvaghefi A, Farahmand H, Rafiee G, Abdollahi A: Development of 16S rRNA targeted PCR method for detection of *Escherichia coli* in Rainbow Trout *(Onchorynchus mykiss). Iranian J Pathol*, 8 (1): 36-44, 2013.

19. Clinical and Laboratory Standards Institute (CLSI): Performance standards for antimicrobial susceptibility testing. Twenty-fourth informational supplement. M100-S24, 2014.

20. Clermont O, Bonacorsi S, Bingen E: Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol*, 66, 4555-4558, 2000.

21. Escobar-Páramo P, Le Menac'h A, Le Gall T, Amorin C, Gouriou S, Picard B, Skurnik D, Denamur E: Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. *Environ Microbiol*, 8, 1975-1984, 2006. DOI: 10.1111/j.1462-2920.2006.01077.x

22. Hasman H, Mevius D, Veldman K, Olesen I, Aarestrup FM: β-Lactamases among extended-spectrum β-lactamase (ESBL)-resistant *Salmonella* from poultry, poultry products and human patients in the Netherlands. *J Antimicrob Chemother*, 56, 115-121, 2005. DOI: 10.1093/jac/dki190

23. Leinberger DM, Grimm V, Rubtsova M, Weile J, Schröppel K, Wichelhaus TA, Knabbe C, Schmid RD, Bachmann TT: Integrated detection of extended-spectrum-beta-lactam resistance by DNA microarray-based genotyping of TEM, SHV, and CTX-M genes. *J Clin Microbiol,* 48, 460-471, 2010. DOI: 10.1128/JCM.00765-09

24. Mulvey MR, Soule G, Boyd D, Demczuk W, Ahmed R, Multiprovincial *Salmonella* Typhimurium Case Control Study Group: Characterization of the first extended-spectrum beta-lactamase-producing *Salmonella* isolate identified in Canada. *J Clin Microbiol,* 41, 460-462, 2003. DOI: 10.1128/JCM.41.1.460-462.2003

25. Cattoir V, Poirel L, Rotimi V, Soussy CJ, Nordmann P: Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. *J Antimicrob Chemother*, 60, 394-397, 2007. DOI: 10.1093/jac/dkm204

26. Cavaco LM, Frimodt-Møller N, Hasman H, Guardabassi L, Nielsen L, Aarestrup FM: Prevalence of quinolone resistance mechanisms and associations to minimum inhibitory concentrations in quinolone-resistant *Escherichia coli* isolated from humans and swine in Denmark. *Microb Drug Resist*, 14, 163-169, 2008. DOI: 10.1089/mdr.2008.0821

27. Cavaco LM, Hasman H, Xia S, Aarestrup FM: *qnrD*, a novel gene conferring transferable quinolone resistance in *Salmonella enterica* Serovar Kentucky and Bovismorbificans strains of human origin. *Antimicrob Agents Chemother*, 53, 603-608, 2009. DOI: 10.1128/AAC.00997-08

28. Jacoby GA, Gacharna N, Black TA, Miller GH, Hooper DC: Temporal appearance of plasmid-mediated quinolone resistance genes. *Antimicrob Agents Chemother*, 53, 1665-1666, 2009. DOI: 10.1128/AAC.01447-08

29. Park CH, Robicsek A, Jacoby GA, Sahm D, Hooper DC: Prevalence in the United States of *aac (6')-Ib-cr* encoding a ciprofloxacin-modifying enzyme. *Antimicrob Agents Chemother*, 50, 3953-3955, 2006. DOI: 10.1128/AAC.00915-06

30. Chapman TA, Wu XY, Barchia I, Bettelheim KA, Driesen S, Trott D, Wilson M, Chin JJ: Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine. *Appl Environ Microbiol*, 72, 4782-4795, 2006. DOI: 10.1128/AEM.02885-05

31. Kar D, Bandyopadhyay S, Bhattcharyya D, Samanta I, Mahanti A, Nanda PK, Mondal B, Dandapat P, Das AK, Dutta TK, Bandyopadhyay S, Singh RK: Molecular and phylogenetic characterization of multidrug resistant extended spectrum beta-lactamase producing *Escherichia coli* isolated from poultry and cattle in Odisha, India. *Infect Genet Evol*, 29, 82-90, 2015. DOI: 10.1016/j.meegid.2014.11.003

32. Kürekci C, Osek J, Aydın M, Tekeli İO, Kurpas M, Wieczorek K, Sakin F: Evaluation of bulk tank raw milk and raw chicken meat samples as source of ESBL producing *Escherichia coli* in Turkey: Recent insights. *J Food Saf,* 39:e12605, 2019. DOI: 10.1111/jfs.12605

33. Shigemura H, Matsui M, Sekizuka T, Onozuka D, Noda T, Yamashita A, Kuroda M, Suzuki S, Kimura H, Fujimoto S, Oishi K, Sera N, Inoshima Y, Murakami K: Decrease in the prevalence of extended-spectrum cephalosporin-resistant *Salmonella* following cessation of ceftiofur use by the Japanese poultry industry. *Int J Food Microbiol*, 274, 45-51, 2018. DOI: 10.1016/j.ijfoodmicro.2018.03.011

34. Tansawai U, Sanguansermsri D, Na-udom A, Walsh TR, Niumsup PR: Occurrence of extended spectrum β-lactamase and AmpC genes among multidrug-resistant *Escherichia coli* and emergence of ST131 from poultry meat in Thailand. *Food Control,* 84, 159-164, 2018. DOI: 10.1016/j.foodcont.2017.07.028

35. Linscott AJ, Brown WJ: Evaluation of four commercially available extended-spectrum beta-lactamase phenotypic confirmation tests. *J Clin Microbiol*, 43 (3): 1081-1085, 2005. DOI: 10.1128/JCM.43.3.1081-1085.2005

36. Drieux L, Brossier F, Sougakoff W, Jarlier V: Phenotypic detection of extended-spectrum beta-lactamase production in *Enterobacteriaceae*: Review and bench guide. *Clin Microbiol Infect*, 14 (Suppl. 1): 90-103, 2008. DOI: 10.1111/j.1469-0691.2007.01846.x

37. Ojo OE, Schwarz S, Michael GB: Detection and characterization of extended-spectrum β-lactamase-producing *Escherichia coli* from chicken production chains in Nigeria. *Vet Microbiol,* 194, 62-68, 2016. DOI: 10.1016/j.vetmic.2016.04.022

38. Kim YJ, Moon JS, Oh DH, Chon JW, Song BR, Lim JS, Heo EJ, Park HJ, Wee SH, Sung K: Genotypic characterization of ESBL-producing *E. coli* from imported meat in South Korea. *Food Res Int,* 107, 158-164, 2018. DOI: 10.1016/j.foodres.2017.12.023

39. Müller A, Jansen W, Grabowski NT, Monecke S, Ehricht R, Kehrenberg C: ESBL- and AmpC-producing *Escherichia coli* from legally and illegally imported meat: Characterization of isolates brought into the EU from third countries. *Int J Food Microbiol*, 283, 52-58, 2018. DOI: 10.1016/j.ijfoodmicro.2018.06.009

40. Tekiner İH, Özpınar H: Occurence and characteristics of extended spectrum beta-lactamase-producing *Enterobacteriaceae* from foods of animal origin. *Braz J Microbiol*, 47, 444-451, 2016. DOI: 10.1016/j.bjm.2015.11.034

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Research Article

Carriage of Plasmidic AmpC Beta-Lactamase Producing *Escherichia coli* in Cattle and Sheep and Characterisation of the Isolates in Terms of Antibiogram Profiles, Phylogeny and Virulence [1]

Faruk PEHLIVANOGLU [1,a] Dilek OZTURK [1,b] Hulya TURUTOGLU [1,c]

[1] *The part of the data presented in this article was presented as oral presentation in Ecology and Safety 2019-28th International Conference (28 June-02 July 2019), Burgas, Bulgaria*

[1] Department of Microbiology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, TR-15030 Burdur - TURKEY
ORCIDS: [a] 0000-0001-9358-8007; [b] 0000-0002-9643-8570; [c] 0000-0003-0011-8597

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Abstract

AmpC type beta-lactamase enzyme production by *Escherichia coli* confers resistance to penicillin and cephalosporins including oxyimino-cephalosporin, cephamycin and aztreonam (variably). Screening of AmpC beta-lactamase determinants in both commensal and pathogenic *E. coli* isolates in livestock is important to reveal the resistance status of the bacteria. Therefore, we aimed to investigate the AmpC beta-lactamase producing *E. coli* isolates in cattle and sheep populations in Burdur, Turkey. The fecal samples were collected from 250 Holstein cows, older than 12 months of age and apparently healthy, and from 225 sheep from different breeds, older than 6 months of age and apparently healthy. After selective isolation and identification of the agent in coliform/*E. coli* selective medium supplemented with cefotaxime (2 µg/mL) or ceftazidim (2 µg/mL), the cefoxitin resistant *E. coli* isolates were determined by agar disc diffusion test (ADDT). Then, *pAmpC* beta-lactamase genes were determined by multiplex polimerase chain reaction (PCR) as gold standart test for *pAmpC* beta-lactamase producing *E. coli*. Finally, the isolates were characterized by PCR for phylogeny and Enterohemorrhagic/Shiga toxin producing *E. coli* (EHEC/STEC) related virulence genes. Totally 17 (6.8%) cattle fecal samples were found positive for *pAmpC* beta-lactamase producing *E. coli.* None of the sheep fecal samples yielded culture positive results for the bacteria of interest. Among the *E. coli* isolates, only *CIT* (origin, *Citrobacter freundii)* family *pAmpC* gene was found. The predominant phylogenetic group was found as group A and only *eae* gene was detected in only one *E. coli* isolate. Multidrug resistance (MDR) was observed in 7 (41.2%) isolates. Consequently, the present study revealed that *pAmpC* beta-lactamase producing *E. coli*, with MDR and low phylogenetic group diversity, exists in cattle population, but not in sheep population.

Keywords: *Beta-lactamase, Escherichia coli, intimin, pAmpC, Phylogeny, Ruminant*

Sığır ve Koyunlarda Plasmidik AmpC Beta Laktamaz Üreten *Escherichia coli* Taşıyıcılığı ve İzolatların Antibiyogram Profilleri, Filogenetik ve Virulans Yönünden Karakterizasyonu

Öz

Escherichia coli tarafından AmpC beta laktamaz enzim üretimi penisilinlere, oksiimino sefalosporinler dahil tüm sefalosporinlere, sefamisinlere ve değişken olmakla birlikte aztreonama direnç sağlar. Çiftlik hayvanlarında komensal ve patojenik *E. coli* izolatlarında AmpC beta laktamazların taranması bakterilerdeki antibiyotik dirençliliğinin durumunu göstermesi açısından önemlidir. Bu nedenle, Burdur ilindeki sığır ve koyun popülasyonunda AmpC beta laktamaz üreten *E. coli* yaygınlığını ortaya çıkarmayı amaçladık. Bu çalışmada, 12 aylık yaştan daha büyük ve sağlıklı görünümdeki 250 Holştayn ırkı sığır ve 6 aylıktan daha büyük ve sağlıklı görünümdeki 225 değişik ırktan koyundan dışkı örneği toplandı. Sefotaksim (2 µg/mL) veya seftazidim (2 µg/mL) ilave edilmiş Koliform/*E. coli* besi yerinde selektif izolasyon ve identifikasyon gerçekleştirildikten sonra, agar disk difüzyon testi (ADDT) ile sefoksitine dirençli *E. coli* izolatları belirlendi. Takiben *pAmpC* beta laktamaz genleri, *pAmpC* beta laktamaz üreten *E. coli* belirlenmesi için altın standart test olan multipleks polimeraz zincir reaksiyonu (PZR) ile belirlendi. Son olarak, izolatlar PZR ile filogenetik ve enterohemorajik/Siga toksin üreten *E. coli* (EHEC/STEC) virulans genleri açısından karakterize edildi. Toplam 17 (%6.8) sığır dışkı örneği *pAmpC* beta laktamaz üreten *E. coli* yönünden pozitif bulunurken koyun örneklerinin tümü negatif bulundu. Izolatlarda sadece *CIT* ailesi pAmpC geni tespit edildi. İzolatlarda en yaygın filogenetik grubun grup A olduğu ve sadece 1 izolatta eae virulans geninin olduğu tespit edildi. Çoklu antibiyotik dirençliliği 7 (%41.2) izolatta tespit edildi. Sonuç olarak, bu çalışma pAmpC beta laktamaz üreten *E. coli*'nin koyunlarda bulunmadığı ve sığırlarda çoklu antibiyotik dirençliliğine sahip ve az sayıda filogenetik çeşitlilikte var olduğu belirlendi.

Anahtar sözcükler: *Beta laktamaz, Escherichia coli, İntimin, pAmpC, Filogenetik, Ruminant*

Correspondence
+90 248 2132063
pehlivanoglu@mehmetakif.edu.tr

INTRODUCTION

Beta-lactamase enzymes that inactivate the beta-lactam antibiotics by hydrolysing the beta-lactam ring of the antibiotic [1,2] posses highly heterogenic character in nature. They were divided to several groups for classification that is updated regularly by the researchers [3,4]. Several classification schemes for bacterial beta-lactamases have been described. One of them was based on the activity of the beta-lactamases against different beta-lactam antimicrobials [5]. The other scheme developed by Ambler divides the beta-lactamase enzymes into four classes as A, B, C, and D, according to their amino acid sequence differences [6]. The group of beta-lactamases classified as Ambler Class C and named AmpC beta-lactamases can confer resistance to penicillins and cephalosporins including oxyimino-cephalosporins (e.g., cefotaxime, ceftazidime and ceftriaxone), cephamycins (e.g., cefoxitin and cefotetan), and aztreonam (variably) [5,7]. It has been reported that use of beta-lactams for treatment of several infections causes development of AmpC beta-lactamase producing *E. coli* isolates in animal and human intestinal microflora. This casual use also triggers an increase in AmpC beta-lactamase production in Gram-negative pathogens in humans and animals due to the horizontal transfer of resistance genes [8,9].

The genes encoding AmpC beta-lactamases can be located on a conjugative plasmid or chromosome of a Gram-negative bacterium [7]. Plasmid mediated AmpC beta-lactamases (pAmpC) are composed of 6 families which were formed based on amino acid sequences. These families are named as ACC (Ambler class C), CIT (origin, *Citrobacter freundii)*, DHA (site of discovery, Dhahran hospital in Saudi Arabia), EBC (origin, *Enterobacter claocae*), FOX (resistance to cefoxitin) and MOX (resistance to moxalactam). Unlike to extended-spectrum beta-lactamases (ESBL), AmpC beta-lactamases can not be inhibited by beta-lactamase inhibitors (clavulanic acid and tazobactam) [7,8].

Escherichia coli strains causing diarrhea in human have been classified into several pathotypes based on virulence characteristics and infection mechanisms. There have been described 5 main intestinal pathogenic *E. coli* strains named as enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC). EHEC strains are responsible from bloody diarrhoea, haemorrhagic colitis (HC) and the Haemolytic Uraemic Syndrome (HUS) in human and can be transmitted to human by consumed food [10]. All are life threating infections for human. On the other hands, animals especially cattle are known the reservoir of EHEC/Shiga toxin producing strains (STEC) and especially carriage status of O157:H7 strains have been revealed by several researchers in cattle [11]. Therefore, it is important to investigate the multidrug resistant *E. coli* isolates, such as ampC beta-lactamase producing *E. coli*, for EHEC/STEC virulence determinants to reduce the fecal shedding of such *E. coli* isolates in animals that prevents fecal contamination of food of animal origin.

The studies conducted in several countries in the world report the existence and prevalence of AmpC beta-lactamase producing Gram-negative bacteria isolated from livestock [2,12-15]. In Turkey, Pehlivanoglu [16] reported the presence of pAmpC producing *E. coli* in laying hens, Aslantaş et al.[17] reported in cattle, Gumus et al.[18] reported in dogs and cats and Aslantas and Yilmaz [19] reported in dogs, but more studies are needed to reveal the true prevelance of AmpC producing *E. coli* in Turkey. Hence, the present study was carried out to investigate the existence of pAmpC beta-lactamase producing *E. coli* isolates in healthy cattle and sheep in Burdur, Turkey and to characterize the isolates for antibiotic susceptibility pattern, phylogeny and virulence.

MATERIAL and METHODS

Sampling

Approximate sample size of the study was determined to be 138 using a 10% expected field prevalence [16,17] at the 95% confidence level and the desired absolute precision of 5% [20], but more animals were included to the present study for higher precise results. Twenty dairy cattle and 12 sheep farms with no close contact to cattle farms were selected randomly from different locations of Burdur. Approximately 50% of animals were selected randomly from each cattle and sheep farms. Total 250 Holstein cows, older than 12 months of age and apparently healthy, and 225 sheep from different breeds, older than 6 months of age and apparently healthy, were included in the study. Fecal samples (at least 5 grams) were collected from rectum of the animals by using separate disposable examination gloves for each animals. The fecal samples were put into sterile screw-top vials, transported to the laboratory on ice in a cooler within 2 h and kept at 4ºC until processing within 24 h. The protocol for fecal collection from animals in the present study was approved by Burdur Mehmet Akif Ersoy University (Turkey) Animal Care and Use Committee (approval number: 07.09.2012/05).

Selective Isolation

Isolation was initiated with preparation of a 10% suspension of each fecal sample in buffered peptone water (Lab M, UK) and incubation at 37ºC for 24 h under aerobic conditions. Fifty microliters from each suspension was plated onto Brilliance *E. coli*/coliform Selective Agar (Oxoid, UK) supplemented with cefotaxime (CTX, 2 µg/mL) (Sigma Aldrich, Germany) or ceftazidime (CAZ, 2 µg/mL) (Sigma Aldrich, Germany) and the plates were incubated at 37ºC for 24 h under aerobic conditions.

Presumptive *E. coli* colonies (purple or blue colour) from each plate (one colony from the selective agar with CTX and one colony from the selective agar with CAZ) per culture

positive fecal sample were selected randomly. Identification of the suspicious colonies were carried out by the biochemical tests [21]. Finally, molecular confirmation of the *E. coli* isolates were performed by PCR [22] after DNA extraction yielded by boiling method [23] *(Table 1)*.

Determination of the Presumptive AmpC Beta-lactamase Producing E. coli

Firstly, the *E. coli* isolates were tested in terms of ESBL production by agar disc diffusion test (ADDT) [24]. In this test, aztreonam (ATM, 30 µg), cefotaxime (CTX, 30 µg), cefpodoxime (CPD, 10 µg), ceftazidime (CAZ, 30 µg) and ceftriaxone (CRO, 30 µg) discs were used [24]. The isolate resistant to at least one of them were further tested with ESBL confirmatory test [24] and 34 isolates found positive for ESBL production were excluded from the study. Then, non-ESBL-producing isolates were tested for cefoxitin resistance by ADDT for phenotypic determination of AmpC beta-lactamase producers [24,25]. ADDT was performed by plating of each *E. coli* isolates with an inoculum (McFarland turbidity 0.5) on Mueller Hinton Agar (MHA) (Oxoid, UK) plates followed by the disc placement of cefoxitin (FOX, 30 µg) (Oxoid, UK). The plates were incubated at 37°C for 24 h. Inhibition zone diameter lower than 18 mm was accepted for the evidence of FOX resistance [24].

PCR Analysis of Plasmid Mediated AmpC Beta-lactamase Genes (pAmpC)

As the gold standard test for detemination of pAmpC producing *E. coli*, PCR was performed for *pAmpC* genes

Table 1. *Primers used in the present study*

Target Gene	Primer Sequence (5'----------3')	Amplicon (bp)	Reference
MOX	F-GCTGCTCAAGGAGCACAGGAT R-CACATTGACATAGGTGTGGTGC	520	Perez-Perez and Hanson [8]
CIT	F-TGGCCAGAACTGACAGGCAAA R-TTTCTCCTGAACGTGGCTGGC	462	Perez-Perez and Hanson [8]
DHA	F-AACTTTCACAGGTGTGCTGGGT R-CCGTACGCATACTGGCTTTGC	405	Perez-Perez and Hanson [8]
ACC	F-AACAGCCTCAGCAGCCGGTTA R-TTCGCCGCAATCATCCCTAGC	346	Perez-Perez and Hanson [8]
EBC	F-TCGGTAAAGCCGATGTTGCGG R-CTTCCACTGCGGCTGCCAGTT	302	Perez-Perez and Hanson [8]
FOX	F-AACATGGGGTATCAGGGAGATG R-CAAAGCGCGTAACCGGATTGG	190	Perez-Perez and Hanson [8]
chuA	F-GACGAACCAACGGTCAGGAT R-TGCCGCCAGTACCAAAGACA	279	Clermont et al.[26]
YjaA	F-TGAAGTGTCAGGAGACGCTG R-ATGGAGAATGCGTTCCTCAAC	211	Clermont et al.[26]
TspE4.C2	F-GAGTAATGTCGGGGCATTCA R-CGCGCCAACAAAGTATTACG	152	Clermont et al.[26]
16S rRNA	F-CCCCCTGGACGAAGACTGAC R-ACCGCTGGCAACAAAGGATA	401	Wang et al.[22]
rfbO157	F-CAGGTGAAGGTGGAATGGTTGTC R-TTAGAATTGAGACCATCCAATAAG	296	Bai et al.[27]
fliCH7	F-GCTGCAACGGTAAGTGAT R-GGCAGCAAGCGGGTTGGT	948	Wang et al.[22], Osek [28]
stx1	F-TGTCGCATAGTGGAACCTCA R-TGCGCACTGAGAAGAAGAGA	655	Bai et al.[27]
stx2	F-CCATGACAACGGACAGCAGTT R-TGTCGCCAGTTATCTGACATTC	477	Bai et al.[27]
eae	F-CATTATGGAACGGCAGAGGT R-ACGGATATCGAAGCCATTTG	375	Bai et al.[27]
ehxA	F-GCGAGCTAAGCAGCTTGAAT R-CTGGAGGCTGCACTAACTCC	199	Bai et al.[27]
espP	F-GATTACAGCACGCATTCATGGTAT R-TCCAGGCATCCTCAGTGACA	73	Posse et al.[29]
katP	F-GAAGTCATATATCGCCGGTTGAA R-GTCATTTCAGGAACGGTGAGATC	73	Posse et al.[29]
saa	F-CGTGATGAACAGGCTATTGC R-ATGGACATGCCTGTGGCAAC	119	Paton and Paton [30]

F: Forward, R: Reverse, bp: base pair

according to the method developed by Perez-Perez and Hanson [8]. The PCR protocol was modified slightly in our laboratory as follow: Two sets of triplex PCR (set 1: ACC, CIT, FOX and set 2: DHA, EBC, MOX) were established for detection of *pAmpC* genes. The first triplex PCR was adjusted as 25 µL consisted of; 50 mM KCl, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.4-0.6 µM primer sets (specific for FOX, ACC and CIT, respectively), 1.25 U of Taq DNA polymerase (Thermo Scientific) and 2 µL template DNA. The second triplex PCR was adjusted as 25 µL consisted of; 50 mM KCl, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.5-0.6 µM primer sets (specific for EBC, DHA and MOX, respectively), 1.25 U of Taq DNA polymerase (Thermo Scientific) and 2 µL template DNA. Thermal cycling conditions for both of triplex PCRs were 5 min at 94ºC for initial denaturation, followed by 35 cycles of 45 sec at 94ºC, 45 sec at 64ºC and 1 min at 72ºC, and a final elongation step of 7 min at 72ºC. The primer sequences used were presented in *Table 1*.

Antibiotic Susceptibility Profiles, Phylogroups and Virulence Genes of the Isolates

Susceptibility of pAmpC beta-lactamase producing *E. coli* isolates to beta-lactam antibiotics and to other classes of antibiotics were determined by ADDT [24,31,32]. The beta-lactams antibiotic discs (Oxoid, UK) tested were ampicillin (AMP, 10 µg), cefepime (FEP, 30 µg), cefuroxime sodium (CXM, 30 µg), cephalothin (CEF, 30 µg) and imipenem (IPM, 10 µg). The antibiotics (Oxoid, UK) from other classes tested were chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), enrofloxacin (ENR, 5 µg), florfenicol (FFC, 30 µg), gentamicin (CN, 10 µg), kanamycin (K, 30 µg), nalidixic acid (NA, 30 µg), streptomycin (S, 10 µg), sulfamethoxazole-trimethoprim (SXT, 25 µg) and tetracycline (TE, 30 µg). *E. coli* ATCC 25922 strain was used as the control strain in ADDT. The inhibition zone diameters were evaluated according to CLSI critical zone diameters in CLSI document M10 0-S26 [24], M31-A3 [31] and VET01-S2 [32]. Based on the results, the isolates were classified as resistant, intermediate or susceptible. In the present study, a pAmpC beta-lactamase producing *E. coli* isolate that was resistant to at least 3 different classes of antibiotics excluding beta-lactams was accepted as multidrug-resistant (MDR) strain.

The phylogroups (groups A, B1, B2 and D) of the *E. coli* isolates were determined according to a triplex PCR protocol as described elsewhere [26], with the modified PCR conditions by Higgins et al.[33]. The triplex PCR is based on the amplification of a 279 bp fragment of the *chuA* gene, 211 bp fragment of the *yjaA* gene and 152 bp fragment of TspE4.C2 (a noncoding DNA region of *E. coli* genome). The phylogenetic groups of the isolates were assigned according to following criteria: the phylogenetic group A (*chuA*-, TspE4.C2-), B1 (*chuA*-, TspE4.C2+), B2 (*chuA*+, *yjaA*+), or D (*chuA*+, *yjaA*-). Additionally, phylogenetic subgroups (A: A_0 and A_1; B2: $B2_2$ and $B2_3$; D: D_1 and D_2) were investigated as described by Escobar-Páramo et al.[34]. *E. coli* ATCC 25922 was used as positive control strain (*chuA*+, *yjaA*+ and TspE4.C2+) in the triplex PCR.

The pAmpC beta-lactamase producing *E. coli* isolates were screened by PCR for serotype O157:H7 (*rfbO157* and *fliCH7* genes) [27,28] and *eae* (intimin, attaching and effacing protein), *ehxA* (enterohemolysin), *espP* (extracellular serine protease), *katP* (catalase-peroxidase), *saa* (autoagglutinating adhesin), *stx1* (Shiga toxin 1) and *stx2* (Shiga toxin 2) to determine if the isolates were Enterohemorrhagic *E. coli* (EHEC) [27,29,30].

RESULTS

As the results of culture of cattle fecal samples, presumptive *E. coli* colonies were observed on at least one of both media (supplemented with CTX or CAZ) from 51 fecal samples. All colonies were identified as *E. coli* by phenotypic tests and PCR. After ESBL confirmatory test, *E. coli* isolates from 34 fecal samples were separated as ESBL-producing isolates. The remaining *E. coli* isolates (non-ESBL producers) were from 17 fecal samples and growth of *E. coli* colonies on these fecal samples (n=17) were observed on both medium (supplemented with CTX or CAZ) and therefore total 34 non-ESBL-producing *E. coli* were obtained. All of 34 non-ESBL- producing *E. coli* isolates were found to be resistant to cefoxitin by ADDT and therefore they were accepted as potential AmpC producers. In the present study, both potential AmpC beta-lactamse producing *E. coli* isolates (one from selective agar with CTX and the other one from the selective agar with CAZ) from a single fecal sample showed the same antibiotic susceptibility profile and the same phylogenetic group in all of the fecal samples. Therefore, the prevalence was estimated as 6.8% (17/250) for cattle in the study. *E. coli* was not isolated from sheep fecal samples.

All *E. coli* isolates from cattle harbored only *CIT* family *pAmpC* gene *(Fig. 1)*. In total, 5 (25%, 5/20) cattle herds were found positive in terms of carrying pAmpC beta-lactamase producing *E. coli* isolates *(Table 2)*.

According to phylogenetic analysis by PCR, 11 (11/17, 64.7%) *E. coli* isolates were found belong to group A (subgroup A_1), 3 (3/17, 17.6%) isolates to group D (subgroup D_1), 2 (2/17, 11.8%) isolates to group B2 (subgroup $B2_2$) and 1 (1/17, 5.9%) isolate to group B1 *(Table 2)*.

In total, 7 of 17 (41.2%) pAmpC beta-lactamase producing *E. coli* isolates were found MDR. Among the MDR isolates, 3 isolates belonged to phylogoup A (subgroup A_1), one isolate to phylogoup B1 and one isolate to D (subgroup D_1) *(Table 2)*. The highest resistance was found against TET (9/17, 52.9%) whereas the lowest resistance was against CIP, ENR and NA (1/17, 5.9%). All isolates were susceptible to FEP and IPM *(Table 3)*.

Among the virulence genes screened among the isolates, only *eae* (intimin) gene was detected in only one isolate

Table 2. *Distrubition of the pAmpC beta-lactamase producing E. coli isolates (cattle) according to herds, phylogenetic group and antibiogram profiles*

Farm No	*E. coli* Isolates (n)	*E. coli* Isolates (n): Phylogenetic Group	Resistance Profile	*blapAmpC* Gene Family
3	8	7:A (A_1)	-	CIT
		1:B2 ($B2_2$)	-	CIT
6	1	A (A_1)	CN, S, K, FFC, C, TE*	CIT
7	6	1:A (A_1)	TE	CIT
		1:B1	CN, S, K, SXT, NA, ENR, CIP, FFC, C, TE*	CIT
		1:B2 ($B2_2$)	S, K, TE	CIT
		3:D (D_1)	S, SXT, TE*	CIT
8	1	A (A_1)	CN, S, K, SXT, FFC C, TE*	CIT
9	1	A (A_1)	CN, S, K, FFC, C, TE*	CIT

** Multidrug resistant isolate (MDR, resistant to at least 3 classes of antibiotics except beta-lactam antibiotics). C: chloramphenicol, CIP: ciprofloxacin, ENR: enrofloxacin, FFC: florfenicol, CN: gentamicin, K: kanamycin, NA: nalidixic acid, S: streptomycin, SXT: sulfamethoxazole-trimethoprim, TE: tetracycline*

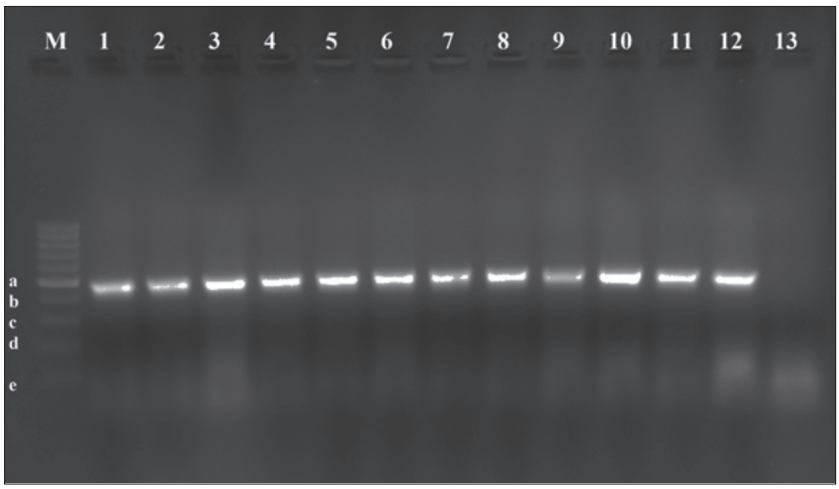

Fig 1. Results of PCR test performed for pAmpC beta-lactamase genes of *E. coli* isolates (cattle). M: Marker (a:500 bp, b:400 bp, c:300 bp, d:200 bp, e:100 bp), Lane 1-12: *E. coli* isolates positive for CIT type pAmpC beta-lactamase gene (462 bp), Lane 13: negative control

Table 3. *Antibiotic susceptibilities of pAmpC beta-lactamase producing E. coli isolates (cattle)*

Beta- Lactams	pAmpC Producing *E. coli* (n=17)		Other Antibiotics	pAmpC Producing *E. coli* (n=17)	
	R (n)	I (n)		R(n)	I (n)
AMP	17	0	CN	4	0
ATM	17	n/a	K	5	0
FEP	0	0	S	8	1
CTX	17	n/a	CIP	1	0
CPD	17	n / a	ENR	1	0
CAZ	17	n/a	NA	1	0
CRO	17	n /a	TE	9	0
CXM	13	0	SXT	5	0
CEF	17	0	FFC	4	0
IPM	0	0	C	4	0

n/a: not applicable, R: resistant, I: intermediate, AMP: ampicillin, ATM: aztreonam, CAZ: ceftazidime, FEP: cefepime, CPD: cefpodoxime, CRO: ceftriaxone, CXM: cefuroxime, CEF: cephalothin, CTX: cefotaxime, FOX: cefoxitin, IPM: imipenem, C: chloramphenicol, CIP: ciprofloxacin, ENR: enrofloxacin, FFC: florfenicol, CN: gentamicin, K: kanamycin, NA: nalidixic acid, S: streptomycin, SXT: sulfamethoxazole-trimethoprim, TE: tetracycline

from Farm 3. This *E. coli* isolate belonged to $B2_2$ phylogenetic group and was not resistant to any of the antibiotics (except beta lactams) tested. None of the pAmpC beta-lactamase producing *E. coli* isolates was O157:H7 serotype of *E. coli*.

DISCUSSION

The real prevalence of pAmpC beta-lactamase producing *E. coli* is still unknown in healthy cattle and sheep in Turkey. There is only one local study conducted on this topic in healthy cattle in Turkey and it was found only one *E. coli* isolate carrying *ampC (cmy)* gene out of 312 cattle rectal swab samples [17]. On the other hand, there is no report for pAmpC beta-lactamase producing *E. coli* in healthy sheep in Turkey. Even though some studies have shown the existence of AmpC beta-lactamase producing *E. coli* and *AmpC* genes on cattle origin food products (cheese, meat, and milk) in different parts of Turkey [35-37], these studies do not reflect exactly the extent of these isolates in live animals in Turkey due to possible contamination of the animal origin food during processing. Therefore, the present study conducted have given additional information about the presence of AmpC beta-lactamase producing *E. coli* in cattle and sheep in Turkey.

Although we detected AmpC beta-lactamase producing *E. coli* in 17 cattle, no sheep was detected positive for AmpC beta-lactamase producing *E. coli*. This can be attributed to use of the beta-lactams and other classes of antibiotics (aminoglycosides, beta-lactams, phenicols, quinolones, sulfamethoxazole-trimethoprim and tetracycline) more widely in the prevention and treatment of wide variety of infections (mastitis, lameness, calf diarrhea, metritis, arthritis, pneumoniae, salmonellosis, urinary tract infections, septisemia, etc.) in cattle population than sheep population in Turkey and some of them may cause co-selection of AmpC beta-lactamase producing *E. coli* isolates in gut microflora. It has been known that *ampC* genes are located on a large plasmid together with other antimicrobial resistance genes such as the genes responsible for aminoglycosides, phenicols, quinolones, sulfamethoxazole-trimethoprim and tetracycline resistances and frequent use of these antibiotics in livestock for several purposes leads the selection pressure for AmpC beta-lactamase producing Gram-negative bacteria in gut microflora [2]. In line with this it was detected co-resistance to at least one of these antibiotics in 9 (9/17, 52.9%) isolates and MDR in 7 (41.2%) isolates in this study.

Escherichia coli isolates obtained in this study were found fall into four main phylogenetic groups (A, B1, B2 and D). It is known that *E. coli* strains belonging to group A and B1 are primarily found in the commensal microflora [26,34]. The pathogen *E. coli* strains associated with extra-intestinal infections and diarrhea mainly belong to B2 and D groups [26,34]. Later, Escobar-Paramo et al.[34] stated phylogenetic subgroups (A: A_0 and A_1; B2: $B2_2$ and $B2_3$; D: D_1 and D_2). Likewise, in the present study, phylogentic analysis of *E. coli* isolates showed that group A (subgroup A_1) (n=11) is the predominant group, followed by group D (subgroup D_1) (n=3), group B2 (subgroup $B2_2$) (n=2) and group B1 (n=1). On the other hand, the reports indicate that *E. coli* strains from B2 and D phylogroups possess more virulence factors but less MDR pattern than A and B1 phylogroups [38,39]. Similarly, in the present study the *E. coli* isolates from A and B1 phylogroups showed resistance to more antibiotics than the B2 and D phylogroup isolates and EHEC/STEC related virulence gene (*eae*, intimin) detected was found in the *E. coli* isolate belonging to $B2_2$ phylogroup with no co-resistance to other classes of antibiotics. The *eae* gene encodes the intimin protein on the surface of EPEC/STEC/EHEC isolates and it is located on the locus of enterocyte effacement (LEE) pathogenicity island. The intimin protein is important for intimate attachment to the intestinal mucosa and the formation of the attaching and effacing lesions in EPEC and STEC/EHEC infections [40]. Therefore, it is possible to state that the *eae* gene positive and pAmpC beta-lactamase producing *E. coli* isolate from $B2_2$ phylogenetic group determined in cattle in this study can be pathogenic for both human and calf even thogh this isolate is not a STEC/EHEC isolate.

Overall, based on the similarity of phylogenetic analysis results and antibiogram profiles of the isolates, three farms (Farm 6, 8 and 9) had only one isolate, one farm (Farm 3) had 2 different isolates and one farm (Farm 7) had 4 different isolates. Additionally, all isolates had the same *blapAmpC* gene family (*CIT*). Hence, it can be stated that few parent *E. coli* strains with the same *pAmpC* gene (*CIT*) were circulating in the cattle farms in Burdur, Turkey.

It is generally accepted that food producing animals serve as reservoir for MDR *E. coli* strains and they can be transmitted to human by direct contact and/or via food chains. This issue has also been considered for AmpC beta-lactamase producing *E. coli* since there are many studies showing the similar *pAmpC* genes and plasmids in both animal and animal owners or farm workers [41-43]. Therefore, we can mention the possible health risk for people close contact to the cattle population in the present study.

In conclusion, the present study showed the absence of pAmpC beta-lactamase producing *E. coli* in sheep population and presence of few multi-drug resistant pAmpC beta-lactamase producing *E. coli* strains with only one type *pAmpC* gene family (*CIT*) in cattle population in Burdur, Turkey. However, more studies are needed to reveal and understand the course of prevalence of pAmpC beta-lactamase producing *E. coli* (pathogen and commensal) and diversity in *pAmpC* genes in livestock populations in Turkey. The other point that should be considered, as known, emergence of *E. coli* isolates possesing *pAmpC* genes may also bring the increase in production of the AmpC beta-lactamases in other Gram-negative bacteria due to horizontal

transfer of plasmids between Gram-negative bacteria species. Hence, the necessary preventive measurements should be taken, for examples, livestock sector workers and veterinarians should be informed regularly about increase in antimicrobial-resistant strains and proper selection of antimicrobials should be provided in the treatment of the infections in animals. Also, monitoring should be performed for antimicrobial resistance levels to limit the escalating trend in antimicrobial resistance and the emergence of resistance traits in genetic material of Gram-negatives.

Declaration of Conflicting Interests

The authors declare no conflict of interest.

REFERENCES

1. Frere JM: Beta-lactamases and bacterial resistance to antibiotics. *Mol Microbiol,* 16, 385-395, 1995. DOI: 10.1111/j.1365-2958.1995.tb02404.x

2. Seiffert SN, Hilty M, Perreten V, Endimiani A: Extended-spectrum cephalosporin-resistant gram-negative organisms in livestock: An emerging problem for human health? *Drug Resist Updat*, 16, 22-45, 2013. DOI: 10.1016/j.drup.2012.12.001

3. Bush K, Jacoby GA: Updated functional classification of beta-lactamases. *Antimicrob Agents Chemother* 54 (3): 969-976, 2010. DOI: 10.1128/AAC.01009-09

4. Vardanyan R, Hruby V: Antibiotics. **In,** Sysnthesis of Best Seller Drugs. 573-643, Elsevier B.V., 2016.

5. Bush K, Jacoby GA, Medeiros AA: Functional classification scheme for β-lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother*, 39 (6): 1211-1233, 1995. DOI: 10.1128/AAC.39.6.1211

6. Ambler RP: The structure of β-lactamases. *Philos Trans R Soc Lond B Biol Sci*, 289 (1036): 321-331, 1980. DOI: 10.1098/rstb.1980.0049

7. Jacoby GA: AmpC β-lactamases. *Clin Microbiol Rev*, 22, 161-182, 2009. DOI: 10.1128/CMR.00036-08

8. Pérez-Pérez FJ, Hanson ND: Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol,* 40, 2153-2162, 2002. DOI: 10.1128/JCM.40.6.2153-2162.2002

9. Lerner A**, Matthias** T**, Aminov** R**:** Potential effects of horizontal gene exchange in the human gut. *Front Immunol*, 8:1630, 2017. DOI: 10.3389/fimmu.2017.01630

10. Bugarel M, Martin A, Fach P, Beutin L: Virulence gene profiling of enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* strains: A basis for molecular risk assessment of typical and atypical EPEC strains. *BMC Microbiol,* 11:142, 2011. DOI: 10.1186/1471-2180-11-142

11. Venegas-Vargas C, Henderson S, Khare A, Mosci RE, Lehnert JD, Singh P, Ouellette LM, Norby B, Funk JA, Rust S, Bartlett PC, Grooms D, Manning SD: Factors associated with Shiga toxin-producing *Escherichia coli* shedding by dairy and beef cattle. *Appl Environ Microbiol*, 82, 5049-5056, 2016. DOI: 10.1128/AEM.00829-16

12. Asai T, Masani K, Sato C, Hiki M, Usui U, Baba K, Ozawa M, Harada K, Aoki H, Sawada T: Phylogenetic groups and cephalosporin resistance genes of *Escherichia coli* from diseased food-producing animals in Japan. *Acta Vet Scand,* 53:52, 2011. DOI: 10.1186/1751-0147-53-52

13. Hille K, Fischer J, Falgenhauer L, Sharp H, Brenner GM, Kadlec K, Friese A, Schwarz S, Imirzalioglu C, Kietzmann M, Von Münchhausen C, Kreienbrock L: On the occurence of extended-spectrum-and AmpC-beta-lactamase-producing *Escherichia coli* in livestock: Results of selected European studies. *Berl Munch Tierarztl Wochenschr,* 127 (9-10): 403-411, 2014.

14. EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control): The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017. *EFSA J,* 17 (2):e05598, 2019. DOI: 10.2903/j.efsa.2019.5598

15. Tewari R, Mitra S, Ganaie F, Das S, Chakraborty A, Venugopal N, Shome R, Rahman H, Shome BR: Dissemination and characterisation of *Escherichia coli* producing extended-spectrum β-lactamases, AmpC β-lactamases and metallo-β-lactamases from livestock and poultry in Northeastern India: A molecular surveillance approach. *J Glob Antimicrob Resist*, 17, 209-215, 2019. DOI: 10.1016/j.jgar.2018.12.025

16. Pehlivanoglu F: Existence of plasmid mediated AmpC beta-lactamase-producing *Escherichia coli* isolates in healthy laying hens. *Van Vet J*, 28 (2): 63-67, 2017.

17. Aslantaş Ö, Elmacıoğlu S, Yılmaz EŞ: Prevalence and characterization of ESBL-and AmpC-producing *Escherichia coli* from cattle. *Kafkas Univ Vet Fak Derg,* 23, 63-67, 2017. DOI: 10.9775/kvfd.2016.15832

18. Gumus B, Celik B, Kahraman BB, Siğirci BD, Ak S: Determination of extended spectrum beta-lactamase (ESBL) and AmpC beta-lactamase producing *Escherchia coli* prevalence in faecal samples of healthy dogs and cats. *Revue Med Vet*, 168, 46-52, 2017

19. Aslantas O, Yilmaz ES: Prevalence and molecular characterization of extended-spectrum beta-lactamase (ESBL) and plasmidic AmpC beta-lactamase (pAmpC) producing *Escherichia coli* in dogs. *J Vet Med Sci*, 79, 1024-1030, 2017. DOI: 10.1292/jvms.16-0432

20. Thrusfield M: Surveys. **In,** Veterinary Epidemiology. 3rd ed., 228-242, Blackwell Publishing, Ames, Iowa, 2007.

21. Winn W, Allen S, Janda W, Koneman E, Procop G, Schreckenberger P, Woods G: The Enterobactericeae. **In,** Koneman EW (Ed): Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed., 211-302, Lippincott Williams and Wilkins, Philadelphia, 2006.

22. Wang G, Clark CG, Rodgers FG: Detection in *Escherichia coli* of the genes encoding the major virulence factors, the genes defining the O157:H7 serotype, and components of the type 2 shiga toxin family by multiplex PCR. *J Clin Microbiol*, 40, 3613-3619, 2002. DOI: 10.1128/JCM.40.10.3613-3619.2002

23. Pehlivanoglu F, Turutoglu H, Ozturk D: CTX-M-15-type extended-spectrum beta-lactamase-producing Escherichia coli as causative agent of bovine mastitis. *Foodborne Pathog Dis*, 13 (9): 477-482, 2016. DOI: 10.1089/fpd.2015.2114

24. CLSI (Clinical and Laboratory Standards Institute): Performance standards for antimicrobial susceptibility testing. 26th Informational Supplement, CLSI Document M100-S26, Pennsylvania, USA, 2016.

25. Polsfuss S, Bloemberg GV, Giger J, Meyer V, Böttger EC, Hombach M: Practical approach for reliable detection of AmpC beta-lactamase producing *Enterobactericeae*. *J Clin Microbiol*, 49, 2798-2803, 2011. DOI: 10.1128/JCM.00404-11

26. **Clermont O, Bonacorsi S, Bingen E:** Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol*, 66, 4555-4558, 2000. DOI: 10.1128/aem.66.10.4555-4558.2000

27. Bai J, Paddock ZD, Shi X, Li S, An B, Nagaraja TG: Application of a multiplex PCR to detect the seven major shiga toxin-producing *Escherichia coli* based on genes that code for serogroup-specific O-antigens and major virulence factors in cattle feces. *Foodborne Pathog Dis*, 9, 541-548, 2012. DOI: 10.1089/fpd.2011.1082

28. Osek J: Development of a multiplex PCR approach for the identification of shiga toxin-producing *Escherichia coli* strains and their major virulence factor genes. *J Appl Microbiol*, 95, 1217-1225, 2003. DOI: 10.1046/j.1365-2672.2003.02091.x

29. Posse B, Zutter LD, Heyndrickx M, Herman L: Metabolic and genetic profiling of clinical O157 and non-O157 shiga-toxin-producing *Escherichia coli*. *Res Microbiol*, 158, 591-599, 2007. DOI: 10.1016/j.resmic.2007.06.001

30. Paton AW, Paton JC: Direct detection and characterization of Shiga toxigenic *Escherichia coli* by multiplex PCR for *stx*$_1$, *stx*$_2$, *eae, ehxA* and *saa*. *J Clin Microbiol*, 40, 271-274, 2002. DOI: 10.1128/JCM.40.1.271-274.2002

31. CLSI (Clinical and Laboratory Standards Institute): Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, Approved standard, 3rd Ed., CLSI document M31-A3, Pennsylvania, USA, 2010.

32. CLSI (Clinical and Laboratory Standards Institute): Performance

standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, 2nd informational supplement, CLSI document VET01-S2, Pennsylvania, USA, 2013.

33. Higgins J, Hohn C, Hornor S, Frana M, Denver M, Joerger R: Genotyping of *Escherichia coli* from environmental and animal samples. *J Microbiol Methods*, 70, 227-235, 2007. DOI: 10.1016/j.mimet. 2007.04.009

34. Escobar-Páramo P, Le Menac'h A, Le Gall T, Amorin C, Gouriou S, Picard B, Skurnik D, Denamur E: Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. *Environ Microbiol,* 8, 1975-1984, 2006. DOI: 10.1111/j.1462-2920.2006.01077.x

35. Pehlivanlar Onen S, Aslantas O, Yılmaz ES, Kurekci C: Prevalence of β-lactamase -producing *Escherichia coli* from retail meat in Turkey. *J Food Sci*, 80 (9): M2023-M2029, 2015. DOI: 10.1111/1750-3841.12984

36. Ozpinar H, Tekiner IH, Sarici B, Cakmak B, Gokalp F, Ozadam A: Phenotypic characterization of ESBL- and AmpC- type beta- lactamases in *Enterobacteriaceae* from chicken meat and dairy products. *Ankara Univ Vet Fak Derg*, 64 (4): 267-272, 2017.

37. Ozdikmenli Tepeli S, Demirel Zorba NN: Frequency of extended-spectrum β-lactamase (ESBL)- and AmpC β-lactamase-producing *Enterobacteriaceae* in a cheese production process. *J Dairy Sci*, 101, 2906-2914, 2018. DOI: 10.3168/jds.2017-13878

38. Smith JL, Fratamico PM, Gunther NW: Extraintestinal pathogenic *Escherichia coli. Foodborne Pathog Dis*, 4 (2): 134-163, 2007. DOI: 10.1089/fpd.2007.0087

39. Chakraborty A, Saralaya V, Adhikari P, Shenoy S, Baliga S, Hegde A: Characterization of *Escherichia coli* phylogenetic groups associated with extraintestinal infections in South Indian population. *Ann Med Health Sci Res*, 5 (4): 241-246, 2015.

40. McDaniel TK, Jarvis KG, Donnenberg MS, Kaper JB: A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc Natl Acad Sci USA,* 92 (5): 1664-1668, 1995. DOI: 10.1073/pnas.92.5.1664

41. Voets GM, Fluit AC, Scharringa J, Schapendonk C, Van Den Munckhof T, Leverstein-Van Hall MA, Stuart JC: Identical plasmid AmpC beta-lactamase genes and plasmid types in *E. coli* isolates from patients and poultry meat in the Netherlands. *Int J Food Microbiol*, 167, 359-362, 2013. DOI: 10.1016/j.ijfoodmicro.2013.10.001

42. Huijbers PMC, Graat EAM, Haenen APJ, Van Santen MG, Van Essen-Zandbergen A, Mevius DJ, Van Duijkeren E, Van Hoek AHAM: Extended-spectrum and AmpC β-lactamase-producing *Escherichia coli* in broilers and people living and/or working on broiler farms: Prevalence, risk factors and molecular characteristics. *J Antimicrob Chemother*, 69 (10): 2669-2675, 2014. DOI: 10.1093/jac/dku178

43. Ljungquist O, Ljungquist D, Myrenås M, Ryden C, Finn M, Bengtsson B: Evidence of household transfer of ESBL-/pAmpCproducing *Enterobacteriaceae* between humans and dogs - A pilot study. *Infect Ecol Epidemiol*, 6:31514, 2016. DOI: 10.3402/iee.v6.31514

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Research Article

The Effects of Propofol-Sevoflurane, Midazolam-Sevoflurane and Medetomidine-Ketamine-Sevoflurane Anesthetic Combinations on Intraocular Pressure in Rabbits [1]

Muharrem EROL [2,b] Hanifi EROL [1,a] Gültekin ATALAN [1,c] Cengiz CEYLAN [2,d] Muhammed Kaan YÖNEZ [1,e]

[1] *This study was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) (Project code: 215O249)*

[1] Department of Surgery, Erciyes University, Veterinary Faculty, TR-38250 Kayseri - TURKEY
[2] Department of Surgery, Balıkesir University, Veterinary Faculty, TR-10463 Balıkesir - TURKEY
ORCIDS: [a] 0000-0003-0780-9067; [b] 0000-0001-8140-3108; [c] 0000-0002-2613-5638; [d] 0000-0001-6509-4675; [e] 0000-0001-9160-6363

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Abstract

Intraocular pressure (IOP) is dependent on equilibrium between the rate of formation and outflow humor aqueous in the eye. Determination of IOP is one of the most important points and a very useful parameter in ophthalmic surgery. The aim of this study was to compare the effects of propofol + sevoflurane, midazolam + sevoflurane, medetomidine + ketamine + sevoflurane anesthetic combinations on IOP, and to find out suitable anesthesia protocol in rabbits for ophthalmic surgery. For this purpose, a total of 40 healthy four months old New Zealand female rabbits (Mean weight 2.34±0.67 kg) were used as subjects. The animals were divided into four groups; propofol + sevoflurane (PS), midazolam + sevoflurane (MS), medetomidine + ketamine + sevoflurane (MKS), and control (C), each having 10 rabbits. The IOP was measured from the right eyes before injection of anesthetics (0. min), during sevoflurane anesthesia at 5th, 10th, 15th, 20th, 25th and 30th min, and post anesthesia at 10th, 20th, 30th, 60th, 120th min and 24 h in the study groups. In PS group; IOP values decreased during anesthesia between 5th and 25th min. In general, the measured IOP values of MS group were higher than 0th min, and the increases continued at 10th min in post anesthesia. The measured IOP values of MKS group during anesthesia were higher than other time points. As a result of this study PS anesthetic combination is an ideal anesthesia for ocular surgery in rabbits. The increase in IOP is undesirable in ocular surgery due to operative and postoperative complications. It was also concluded that investigating the effects of anesthetic combination on IOP should continue in more detailed researches including in different species.

Keywords: *Propofol, Sevoflurane, Midazolam, Medetomidine, Ketamine, Anesthesia, Intraocular pressure, Rabbit*

Tavşanlarda Propofol-Sevofluran, Midazolam-Sevofluran ve Medetomidin-Ketamin-Sevofluran Anestezi Kombinasyonlarının Göz İçi Basıncına Etkileri

Öz

Göz içi basıncı (GİB), humor aközün dengeli bir şekilde üretimi ve drenajına bağlı olarak şekillenmektedir. GİB, oftalmik cerrahide önemli ve yararlı parametreler arasında yer almaktadır. Bu çalışmanın amacı tavşanlarda propofol + sevofluran, midazolam + sevofluran ve medetomidine + ketamin + sevofluran anestezi kombinasyonlarının GİB üzerine etkilerinin karşılaştırılması, oftalmik cerrahi için optimal anestezi protokolünün araştırılmasıdır. Çalışmada 40 adet 4 aylık sağlıklı dişi (ortalama ağırlık 2.34±0.67 kg) Yeni Zelanda tavşanı kullanıldı. Hayvanlar; propofol + sevofluran (PS), midazolam + sevofluran (MS), medetomidine + ketamin + sevofluran (MKS) ve kontrol (K) grubu şeklinde her bir grupta 10 adet tavşan olmak üzere toplam dört gruba ayrıldı. GİB ölçümleri çalışma gruplarında sağ gözden anestezi öncesi (0. dk), sevofluran anestezisi boyunca 5, 10, 15, 20, 25 ve 30. dakikalarda ve anestezi sonrası 10, 20, 30, 60, 120. dakikalarda ve 24 saat sonra yapıldı. PS grubunda anestezi süresi boyunca 5 ve 25. dk'lar arasında GİB değerlerinde azalma görüldü. Genel olarak MS grubunun ölçülen GİB değerlerinin 0. dk'dan yüksek seyrettiği ve anestezi sonrası 10. dk'ya kadar artışın devam ettiği görüldü. MKS grubunun anestezi sırasında ölçülen GİB değerinin anestezi öncesi ve sonrası değerlerden yüksek olduğu tespit edildi. Bu çalışma sonunda PS kombinasyonunun göz cerrahisi için daha uygun bir anestezi kombinasyonu olduğu görüldü. Oküler cerrahide GİB değerinin artışı operatif ve postoperatif olarak istenmeyen bir durumdur. Özellikle GİB üzerine anestezi kombinasyonlarının etkileri daha ayrıntılı ve türlere özel olarak araştırılması gerekmektedir.

Anahtar sözcükler: *Propofol, Sevoflurane, Midazolam, Medetomidine, Ketamine, Anestezi, Intraoküler basınç, Tavşan*

Correspondence
+90 352 3399484-29614
drhaneroll@yahoo.com

INTRODUCTION

The rabbit is widely used at researches on ophthalmic surgery due to the large size of its eyes, docile character, easiness at handle and economy. The use of general anesthesia is very important in ocular surgery for central positioning of the globe, relaxation of the extra ocular muscle and maintenance of intraocular pressure [1].

Intraocular pressure (IOP) is dependent on equilibrium between the rate of formation and outflow humor aqueous in the eye [2]. Determining IOP is one of the most important points and is a very useful parameter in ophthalmic surgery [3]. However, the use of certain drugs such as general anesthetics may cause an alteration in IOP. Alteration of IOP may cause complication after ophthalmic surgery. Because of this, investigations on an ideal anesthetic procedure for ophthalmic surgery were carried out by researchers [2,4,5].

Ketamine and propofol are widely used as anesthetic agents in rabbits. But their effects on IOP are different. For instance, propofol reduces IOP while ketamine increases IOP [6,7]. Midazolam causes decrease in IOP and has been used in general with ketamine in ocular surgery [1,8]. Medetomidine produces sedation and hypertension depending on the administration routes in animals. The prior studies have been demonstrated that medetomidine lowers the IOP after topical application in normotensive cats and rabbits [9]. Sevoflurane is an inhalation anesthetic and used widely for the maintenance of general anesthesia. Induction and recovery of sevoflurane anesthesia are rapid and safe in comparison to injectable anesthetics [10]. Furthermore, potent inhalation anesthetics decrease IOP by lowering humor aqueous rate formation and increase the trabecular outflow facility [2].

The aim of this study was to compare the effects of propofol + sevoflurane, midazolam + sevoflurane, medetomidine + ketamine + sevoflurane anesthetic combinations on IOP, and to find out suitable anesthesia protocol in rabbits for ophthalmic surgery.

MATERIAL and METHODS

Ethical Approval

Erciyes University Local Board of Ethics Committee for animal experiments has approved the study protocol of this research (EUHADYEK, decision no: 14/140).

Animals

Forty healthy four months old New Zealand female rabbits (Mean weight 2.34±0.67 kg) were used as the study materials. Each of rabbits was kept in a separate, maintained on a 12 h light/dark cage, cycle, 21±1°C temperature in the Balıkesir University, Veterinary Faculty, Surgery Department, before the study. The rabbits were fed with normal pellet diet and given water *ad libitum*. The animals were divided into four groups; as propofol + sevoflurane (PS), midazolam + sevoflurane (MS), medetomidine + ketamine + sevoflurane (MKS) and control (C) groups, each having 10 rabbits. Prior to anesthesia, the animals were transported to the examination table, and waited about 10 min to calm for accommodation to the environment conditions. Erol et al.[11] measured IOP with TonoVet from left and right eyes prior to anesthesia in healthy rabbits. They did not find significant differences between left and right eyes for IOP values. Therefore, we preferred the right eye for all the assessments. IOP was measured by TonoVet (RBT, I care Vet, Helsinki, Finland) with five repetitive measurements from right eyes (0. min) in all animals *(Fig. 1)*. Mean values of repetitive measurements were recorded.

Anesthesia Protocol

In PS group; 7 mg/kg Propofol (Propofol 10 mg/mL, PROPOFOL ABBOTT, Abbott Laboratories, USA) was applied intravenously (IV) from right *vena auricularis*. In MS group 0.3 mg/kg midazolam (Demizolam, DEM, Turkey) and in MKS group 0.3 mg/kg medetomidine (Domitor, Zoetis, Turkey) were applied intramuscularly (IM). After 5 min, 30 mg/kg ketamine (Ketasol 10%, Interhas, Turkey) was applied IM in MKS group. Group C received no anesthetic.

For endotracheal intubation of animals, the head and neck were held in atlantooccipital extension to displace the epiglottis to provide a straight passage for the endotracheal tube. The mouth was opened and local anesthetic, 2% lidocaine HCl, was sprayed into larynx. The neonatal intubation tube (2.5 mm diameter) was placed into trachea. After intubation the tube was connected to anesthetic machine (Non-breathing system, Magill circuit) and anesthesia was maintained 4% sevoflurane during 30 min in all study groups. At the end of anesthesia, the anesthetic machine was shut down and the system was washed with oxygen, then oxygen support was carried out for 2 min. The animals were followed until chewing reflex returned, then extubated.

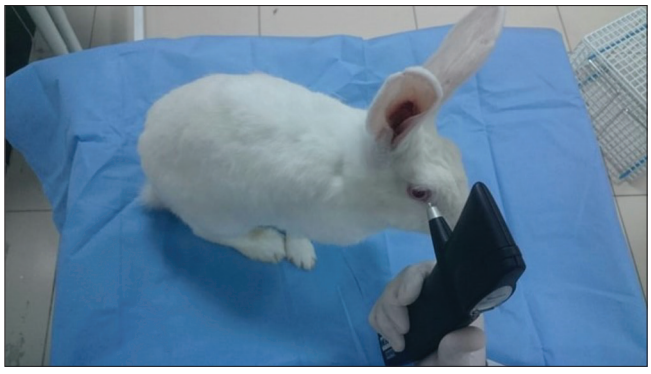

Fig 1. IOP measurement before anesthesia

IOP Measurement

All animals were placed on the operation table dorso-ventrally and fixed with hypoallergenic patch. The immobilization of animals was achieved by fixation, and the tightly fixing was avoided for the IOP measurement *(Fig. 2)*. The IOP was measured from right eyes before injectable anesthesia (0. min), during sevoflurane anesthesia at 5th, 10th, 15th, 20th, 25th and 30. min, and post anesthesia (after extubation) at 10th, 20th, 30th, 60th, 120. min and 24 h after in study groups. In group C, at the same time intervals IOP were also measured.

Statistical Analysis

The obtained data were statistically evaluated with IBM SPSS Statistics 21.0 (USA) program. Shapiro-Wilk test was used for normality. One-way ANOVA was used to compare values between groups', Student-Newman-Keuls test was used for multiple comparisons. The differences between repeated measurements were analyzed by repeated measure variance analysis and Bonferroni test. P<0.05 was accepted statistical significance and results are presented as Mean ± Standard error (SE).

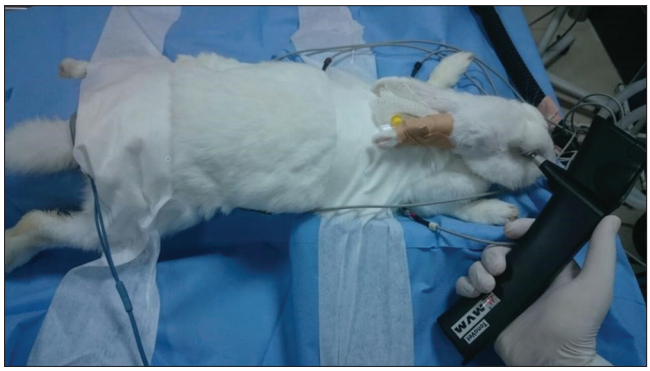

Fig 2. IOP measurement during anesthesia

RESULTS

In-group measurements and statistical evaluations of IOP are presented in *Table 1*. In PS group IOP values decreased during anesthesia between 5 and 25 min. The decreases between 0 and at 5th, 10th and 25th min during anesthesia were significant (P<0.05). In post anesthesia time intervals; significant alterations were recorded between 30 and 120th min (P<0.05). The measured value of IOP at 120th min was higher than the values measured at 30th min.

In general, the measured IOP values obtained from MS group were higher than the values obtained at 0 time, and the increase continued at 10th min during post anesthesia, which was statistically significant (P<0.05). At 30th min in post anesthesia the value of IOP approached to normal. Furthermore, the recorded values between 5th min during anesthesia and post anesthetic 30th min were higher than 0 time. The alteration in IOP were detected in MKS group. During anesthesia the measured values of IOP were higher than 0 time which were statistically different during anesthesia (between 5th, 10th and 15th, 20th, 30th) (P<0.05). In group C, there were some decreases and increases detected between measurements, but they were not significant.

When values obtain from different groups evaluated *(Table 2)*, MS and MKS values were higher and statistically different than PS group (P<0.05). Furthermore, increases in IOP were higher in MS than MKS group. In post anesthesia intervals the lowest values were recorded in PS group. Generally, IOP values approached to normal values at 60th min post anesthesia in MS and MKS groups. At the same time, it was also low in PS group.

Table 1. *Ingroup comparison of IOP measurements (Mean±SE)*

Groups	0th min	5th min	10th min	15th min	20th min	25th min	30th min	PA 10th min	PA 20th min	PA 30th min	PA 60th min	PA 120th min	PA 24th h
PS	13.1±2.18	10.6±0.96^{b}	10.4±0.84^{b}	9.7±1.41^{ab}	9.3±1.33^{ab}	8.6±1.34^{a}	9.2±2.2^{ab}	8.6±2.06^{a}	9.1±2.51^{ab}	8.7±1.25^{a}	8.8±2.78^{a}	10.4±2.06^{b}	14.1±1.91
MS	11.2±2.82	22.5±5.60^{a}	21.2±6.01^{a}	18.9±5.38^{a}	17.8±5.18^{a}	18.1±5.30^{a}	19.2±5.41^{a}	13.0±2.40^{b}	12.1±2.51	11.4±2.98	11.1±2.46	11.7±1.82	11.9±2.13
MKS	13.1±2.07	19.1±4.58^{a}	18.6±5.66^{a}	17.9±6.04^{b}	17.3±5.16^{b}	17.1±5.46^{b}	17.8±5.61^{b}	11.2±2.14	11.2±3.15	11.7±2.71	12.4±2.75	12.3±3.02	11.1±2.60
C	13.2±1.68	13.6±1.57	14.3±2.26	14.3±2.71	13.8±1.98	14.0±1.63	14.6±1.89	14.6±1.34	14.3±1.41	14.10±2.18	13.4±1.83	12.9±2.07	13.3±2.21

PS: *Propofol + Sevoflurane,* ***MS:*** *Midazolam + Sevoflurane,* ***MKS:*** *Medetomidine + Ketamine + Sevoflurane,* ***PA:*** *Post anesthesia, ^{a,b} in same line is statistically significant (P<0.05)*

Table 2. *Between groups comparison of IOP measurements (Mean±SE)*

Groups	0th min	5th min	10th min	15th min	20th min	25th min	30th min	PA 10th min	PA 20th min	PA 30th min	PA 60th min	PA 120th min	PA 24th h
PS	13.1±2.18	10.6±0.96	10.4±0.84	9.7±1.41	9.3±1.33	8.6±1.34	9.2±2.2	8.6±2.06	9.1±2.51	8.7±1.25	8.8±2.78	10.4±2.06	14.1±1.91
MS	11.2±2.82	22.5±5.60*	21.2±6.01*	18.9±5.38*	17.8±5.18*	18.1±5.30*	19.2±5.41*	13.0±2.40	12.1±2.51	11.4±2.98	11.1±2.46	11.7±1.82	11.9±2.13
MKS	13.1±2.07	19.1±4.58*	18.6±5.66$^{¥}$	17.9±6.04*	17.3±5.16*	17.1±5.46*	17.8±5.61$^{¥}$	11.2±2.14	11.2±3.15	11.7±2.71	12.4±2.75	12.3±3.02	11.1±2.60
C	13.2±1.68	13.6±1.57	14.3±2.26	14.3±2.71	13.8±1.98	14.0±1.63	14.6±1.89	14.6±1.34	14.3±1.41	14.10±2.18	13.4±1.83	12.9±2.07	13.3±2.21

PS: *Propofol + Sevoflurane,* ***MS:*** *Midazolam + Sevoflurane,* ***MKS:*** *Medetomidine + Ketamine + Sevoflurane,* ***PA:*** *Post anesthesia, $^{*,¥}$ in same column is statistically significant (P<0.05)*

DISCUSSION

Intraocular pressure is defined as the pressure of eye ball and its components, and the pressure against to the surrounding tunica fibrosa. It is affected by humor aqueous, choroid blood volume, volume of corpus vitreum, scleral flexibility, muscle tone surrounding the eye ball and external pressure [11]. At the same time, the laying position of the patient may cause the alteration in IOP. In the present study, the measurements of animals were done in sterno abdominal position by the same person (ME) to minimize the alterations in IOP. Blood pressure, diurnal cycle, posture and blood biochemistry may cause short time alteration in IOP. On the other hand, species, sex, age, season and weight may also affect the IOP in long time period [12-14]. In this study, same sex and species of animals were used, and the anesthesia was planned to coincide in the same hours during the day. In this way, the conditions that can cause intraocular pressure changes were minimized.

Usage of propofol for induction of anesthesia is known to reduce the intraocular pressure [6,15,16]. Sator-Katzenschlager et al.[15] emphasized that using sevoflurane and propofol anesthetic combination in humans decreases the IOP in non-ophthalmic surgery. They related this decrease to the potential effects of anesthetic agents on IOP.

Benzodiazepines such as midazolam are widely used with ketamine in laboratory animals' anesthesia. It decreases systemic arterial blood pressure, cerebral blood flow, cerebral pressure and IOP [2]. In general, benzodiazepines relax the extraocular muscles and increase outflow of humor aqueous. Thus, the relaxing and the other effects of benzodiazepines decrease the IOP. In ocular surgery, one of the essential aims of anesthetic management is to prevent the increase of IOP during anesthesia. The anesthesia related practices (Laryngoscopy, endotracheal intubation and extubation) cause increase in IOP through the stimulation of sympathetic nervous system. Additionally, hypoxia and hypercarbia also induce an increase in choroidal blood volume [17,18]. However, in the present study IOP values in MS group were higher than other groups during anesthesia. Statistically significant differences were detected among MS, PS and C groups. It has been thought that the rises in IOP at MS group might occur due to the tracheal intubation. Because benzodiazepines cause loss in airway muscle tone and results an increase in airway resistance [19]. In the present study, the intubation in MS group animals were more difficult than others. At the end of anesthesia, animals in all study groups were supported with oxygen to eliminate hypoxia and hypercarbia.

Alpha-2 adrenergic agonist such as medetomidine can affect IOP by several mechanisms. It decreases IOP by suppressing sympathetic neuronal function in rabbits [7]. Ketamine induces a dose dependent central nervous system depression that leads to a dissociative anesthesia. It increases cerebral blood volume, intracranial pressure and cerebrospinal fluid pressure. For all these reasons, the increase in IOP is a side effect of ketamine [8]. Hofmeister et al.[20] used ketamine alone in dogs for anesthesia, and investigated alteration in the IOP. They found that ketamine increases the IOP in dogs and suggested not to use alone in patients with glaucoma risk. Furthermore, Kılıç [21] investigated the clinical effects of medetomine + ketamine and xylazine + ketamine anesthetic combinations in rabbits. He found that the heart rate and venous partial oxygen pressure of medetomidine + ketamine groups were lower than that of xylazine + ketamine group during anesthesia. He explained this difference as the effects of medetomidine + ketamine on cardiovascular system in rabbits. The injection of ketamine (30-90 mg/kg, IM) increases 6 mmHg in IOP of rabbits that last for 2-4 h [4]. In the present study, 30 mg/kg ketamine injected IM to MKS group's animals, and the IOP increased about 6 mmHg during anesthesia. However, the increase did not continue for 2 h. In fact, the measured value at 10th min post anesthesia was lower than 0 time in MKS group. This situation showed that medetomidine causes suppression on sympathetic neuronal function in humor aqueous production and ciliary vasoconstriction in a short time period. Furthermore, reduction in IOP value at MS group at 10th min post anesthesia can be explained by the relaxing effect of midazolam on extraocular muscles and its increasing outflow effect of humor aqueous.

At the same time, sevoflurane causes dose dependent decrease in IOP [22]. In the present study, same dose sevoflurane was used in all study groups during 30 min anesthesia. The increase in IOP in MS and MKS groups supported above-mentioned literature [22]. There were no decreases recorded during sevoflurane anesthesia in MS and MKS groups. The decreases were detected only in PS group. These decreases can be explained by the effects of propofol on IOP.

As a result of this study, PS anesthetic combination is an ideal anesthesia for ocular surgery in rabbits. The increase in IOP is undesirable in ocular surgery due to operative and postoperative complications. It was also concluded that investigating the effects of anesthetic combination on IOP should continue in more detailed researches including in different species.

Conflict of Interest

None

REFERNCES

1. Hazra S, Palui H, Biswas B, Konar A: Anesthesia for intraocular surgery in rabbits. *Scand J Lab Anim Sci,* 38 (2): 81-87, 2011. DOI: 10.23675/sjlas.v38i2.230

2. Erol M, Erol H, Atalan G, Doğan Z, Yönez MK, Melek S: Effects of systemically used midazolam, ketamine and isoflurane anaesthetic agents on intraocular pressure and tear production in rabbits. *Harran Univ Vet Fak Derg*, 7 (1): 21-25, 2018. DOI: 10.31196/huvfd.458783

3. Qui Y, Yang H, Lei B: Effects of three commonly used anesthetics on intraocular pressure in mouse. *Curr Eye Res*, 39 (4): 365-369, 2014. DOI: 10.3109/02713683.2013.845224

4. Ghaffari MS, Rezaei MA, Mirani AH, Khorami N: The Effects of ketamine-midazolam anesthesia on intraocular pressure in clinically normal dogs. *Vet Ophthalmol*, 13 (2): 91-93, 2010. DOI: 10.1111/j.1463-5224.2010.00762.x

5. Dogan E, Yanmaz LE, Ersoz U, Senocak MG, Okumus Z: The effect of different doses of intramuscular xylazine HCl administration on intraocular pressure in rabbits. *Kafkas Univ Vet Fak Derg,* 22 (5): 801-803, 2016. DOI: 10.9775/kvfd.2016.15222

6. Dogan E, Yanmaz LE, Senocak MG, Okumus Z: Comparasion of propofol, ketamine and ketofol on intraocular pressure in New Zealand white rabbits. *Rev Med Vet*, 167 (1-2): 18-21, 2016.

7. Kovalcuka L, Ilgazs A, Bandere D, Williams DL: Changes in intraocular pressure and horizontal pupil diameter during use of topical mydriatics in the canine eye. *Open Vet J,* 7 (1): 16-22, 2017. DOI: 10.4314/ovj.v7i1.3

8. Verbruggen AMJ, Akkerdaas LC, Hellebrekers LJ, States FC: The effect of intravenous medetomidine on pupil size and intraocular pressure in normotensive dogs. *Vet Q,* 22, 179-180, 2000. DOI: 10.1080/01652176.2000.9695052

9. Allweiler S, Leach MC, Flecknell PA: The use of propofol and sevoflurane for surgical anaesthesia in New Zealand White rabbits. *Lab Anim*, 44, 113-117, 2010. DOI: 10.1258/la.2009.009036

10. Almeida DE, Nishimori CT, Oria AP, Paula DP, Nunes N, Laus JL: Effects of nitrous oxide on IOP and pupillary diameter in dogs anesthetized with varying concentrations of desflurane. *Vet Ophthalmol,* 11 (3): 170-176, 2008. DOI: 10.1111/j.1463-5224.2008.00616.x

11. Mori K, Ando F, Nomura H, Sato Y, Shimokata H: Relationship between intraocular pressure and obesity in Japan. *Int J Epidemiol*, 29, 661-666, 2000. DOI: 10.1093/ije/29.4.661

12. Komaromy AM, Garg CD, Ying GS, Liu C: Effect of head position on intraocular pressure in horses. *Am J Vet Res*, 67, 1232-1235, 2006. DOI: 10.2460/ajvr.67.7.1232

13. Wu SY, Nemesure B, Hennis A, Leske MC, Barbados Eye Studies Group: Nine-year changes in intraocular pressure: The Barbados eye studies. *Arch Ophthalmol*, 124, 1631-1636, 2006. DOI: 10.1001/archopht.124.11.1631

14. Güzel Ö, Olğun Erdikmen D, Yıldar E, Ekici A, Şaroğlu M, Ekiz B: The effects of propofol and a diazepam/alfentanil combination in dogs aged 10 years and above on heart rate, respiratory rate, pulse oximetry data, intraocular pressure, and body temperature. *Turk J Vet Anim Sci*, 37, 170-176, 2013. DOI: 10.3906/vet-1201-13

15. Sator-Katzenschlager S, Deusch E, Dolezal S, Michalek-Sauberer A, Grubmüller R, Heinze G, Wedrich A: Sevoflurane and propofol decrease intraocular pressure equally during non-ophthalmic surgery and recovery. *Br J Anaesth*, 89, 764-766, 2002. DOI: 10.1093/bja/aef249

16. Hofmeister EH, Williams CO, Braun C, Moore PA: Propofol versus thiopental: Effects on peri-induction intraocular pressure in normal dogs. *Vet Anaesth Analg,* 35, 275-281, 2008. DOI: 10.1111/j.1467-2995.2007.00385.x

17. Termühlen J, Gottschalk A, Eter N, Hoffmann EM, Aken HV, Grenzebach U, Prokosch V: Does general anesthesia have a clinical impact on intraocular pressure in children? *Pediatr Anesth*, 26, 936-941, 2016. DOI: 10.1111/pan.12955

18. Drummond GB: Comparison of sedation with midazolam and ketamine: Effects on airway muscle activity. *Br J Anaesth*, 76, 663-667, 1996. DOI: 10.1093/bja/76.5.663

19. Smith MD, Barletta M, Diehl KA, Hofmeister EH, Franklin SP: Effect of propofol and ketamine-diazepam on intraocular pressure in healty premedicated dogs. *Vet Anaesth Analg*, 46, 36-42, 2019. DOI: 10.1016/j.vaa.2018.09.043

20. Hofmeister EH, Mosunic CB, Torres BT, Ralph AG, Moore PA, Read MR: Effects of ketamine, diazepam, and their combination on intraocular pressures in clinically normal dogs. *Am J Vet Res*, 67 (7): 1136-1139, 2006. DOI: 10.2460/ajvr.67.7.1136

21. Kılıç N: A comparison between medetomidine-ketamine and xylazine-ketamine anaesthesia in rabbits. *Turk J Vet Anim Sci*, 28, 921-926 2004.

22. Artru AA, Momota Y: Trabecular outflow facility and formation rate of aqueous humor during anaesthesia with sevoflurane-nitrous oxide or sevoflurane-remifentanil in rabbits. *Neurosurg Anesth*, 88, 781-786, 1999. DOI: 10.1213/00000539-199904000-00019

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Research Article

Histologic and Electromyographic Evaluation of Neuroregenerative Effect of Stromal Vascular Fraction Following Neuroanastomosis [1]

Hilal ACAR [1,a] Ece ÇERÇİ [2,b] Marzieh Karimi KHEZRI [3,c] Melike ÇETİN [1,d] Uygur CANATAN [1,e] Mehmet Metin ŞEN [1,f] Vildan ASLAN [1,g] Canan ALTINCI SARIL [1,h] Elif MEKİK TEMİZ [1,i] Mevlüt Özgür TAŞKAPILIOĞLU [3,j] Hatice ERDOST [2,k] Hakan SALCI [1,l]

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[1] Department of Surgery, Faculty of Veterinary Medicine, Bursa Uludag University, TR-16059 Bursa - TURKEY
[2] Department of Histology and Embriology, Faculty of Veterinary Medicine, Bursa Uludag University, TR-16059 Bursa - TURKEY
[3] Department Neurosurgery, Faculty of Medicine, Bursa Uludag University, TR-16059 Bursa - TURKEY
ORCIDS: [a] 0000-0003-0154-9938; [b] 0000-0002-2740-4262; [c] 0000-0002-6397-0966; [d] 0000-0001-5424-3321; [e] 0000-0001-9650-0891: [f] 0000-0001-5962-0202 [g] 0000-0001-5323-6891; [h] 0000-0002-1418-3157; [i] 0000-0003-3407-4836; [j] 0000-0001-5472-9065; [k] 0000-0003-1547-7293: [l] 0000-0001-6548-8754

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Abstract

In this study, histologic and electromyographic (EMG) evaluation of neuroregenerative effect of stromal vascular fraction (SVF) following periferal nerve anastomosis was aimed. Totally, 31 Wistar Albino, male rats (weighing about 300 g) were studied, and these rats were grouped according to surgical techniques performed on the right sciatic nerve: group I (GRI) micro suture (n=7), group II (GRII) micro suture + SVF (n=7), group III (GRIII) fibrin glue (n=7) and group IV (GRIV) fibrin glue + SVF (n=7). Fat tissue was taken from 3 rats to prepare SVF, and SVF was produced by non-enzymatic method. The left sciatic nerve of all rats was evaluated for EMG as control. Under general anesthesia, after transversal incision of the sciatic nerve, microsurgical epineural repair technique was performed with 10/0 nonabsorbable suture. EMG examinations were performed in terms of conduction rate, amplitude, distal latency and spontaneous muscle activity at 0th day and postoperative (PO) 1st and 8th weeks. EMG results between and within the groups were statistically evaluated by one-way analysis of variance. Rats in all groups were sacrificed by decapitation at PO 8th week and histological examinations of the sciatic nerves were performed following preparation of the neural tissues. EMG examination results showed the highest nerve conduction in GRI, the highest amplitude in GRIII, normal latency in GRII and longer distal latency in GRIV at PO 8th week. Amplitude and conduction velocity increased gradually in all groups. In needle EMG, the best muscle membrane stabilization was achieved in GRII and GRIV at PO 8th week. Statistically, the values of amplitude, distal latency, conduction velocity, and spontaneous muscle activity were found to be at normal levels at PO 8th week in all groups (P>0.05). In the histological results, although fibroconnective tissue reactions in the anastomosis area had similar scores in GRII and GRIV, maximum fibroconnective tissue reaction and the best axonal regeneration was seen in GRI and GRIII, respectively. In addition, GRI and GRII had the most inflammatory cells accumulation in the suture region, and less inflammatory cells were seen in the anastomosis area of GRIII and GRIV. As a conclusion, fibrin glue presents good electrophysiological and histological results; however, it is clear that local SVF usage on the nerve anastomosis area can be a good choice to decrease fibroconnective tissue reaction and inflammation.

Keywords: *Electromyography, Histology, Neuroanastomosis, Rat, Stromal vascular fraction*

Nöroanastomoz Sonrası Stromal Vasküler Fraksiyonun Nörorejeneratif Etkisinin Histolojik ve Elektromiyografik Değerlendirilmesi

Öz

Bu çalışmada, periferal sinir anastomozunu takiben stromal vasküler fraksiyonun (SVF) nörorejeneratif etkisinin histolojik ve elektromiyografik (EMG) değerlendirilmesi amaçlandı. Toplamda, 31 Wistar Albino, erkek sıçanda (yaklaşık 300 gr ağırlığında) çalışıldı ve bu sıçanlar sağ siyatik sinirde uygulanan cerrahi tekniklere göre gruplandı: grup I (GRI) mikro dikiş (n=7), grup II (grup GRII) mikro dikiş + SVF (n=7), grup III (GRIII) fibrin yapıştırıcı (n=7) ve grup IV (GRIV) fibrin yapıştırıcı + SVF (n=7). SVF'yi hazırlamak için 3 sıçandan yağ dokusu alındı ve SVF, enzimatik olmayan bir yöntemle üretildi. Bütün sıçanların sol siyatik siniri EMG için kontrol olarak değerlendirildi. Genel anestezi altında, siyatik sinirin transversal ensizyonundan sonra 10/0 emilemeyen dikiş ile mikrocerrahi epinöral onarım tekniği uygulandı. EMG muayeneleri, 0. günde ve postoperatif (PO) 1. ve 8. haftalarda iletim hızı, amplitüt, distal latans ve spontan kas aktivitesi açısından yapıldı. EMG bulguları istatistiksel olarak gruplar arası ve grup içi tek yönlü varyans analizi ile değerlendirildi. Tüm gruplardaki sıçanlar PO 8. haftada dekapitasyon ile sakrifiye edildi ve sinir dokularının hazırlanmasından sonra siyatik sinirlerin histolojik incelemeleri yapıldı. EMG muayene sonuçları, GRI'de en yüksek sinir iletimini, GRIII'te en yüksek amplitütü, GRII'de normal latansı ve GRIV'te ameliyat sonrası 8. haftada daha uzun distal latansı gösterdi. Amplitüt ve iletim hızı, tüm gruplarda kademeli olarak arttı. İğne EMG'de, en iyi kas zarı stabilizasyonu PO 8. haftada GRII ve GRIV'te sağlandı. İstatistiksel olarak amplitüd, distal latans, ileti hızı ve spontan kas aktivitesi değerlerinin tüm gruplarda anlamlı olarak PO 8. haftada normal değerlerde olduğu görüldü (P>0.05). Histolojik sonuçlarda, anastomoz alanındaki fibrokonnektif doku reaksiyonları GRII ve GRIV'te benzer skorlara sahip olsa da, maksimum fibrokonnektif doku reaksiyonu ve en iyi aksonal rejenerasyon sırasıyla GRI ve GRIII'te görülmüştür. Ek olarak, GRI ve GRII dikiş bölgesinde en fazla enflamatuar hücre birikimine sahipti ve GRIII ve GRIV'ün anastomoz alanında daha az enflamatuar hücreler görüldü. Sonuç olarak, fibrin yapıştırıcısı iyi elektrofizyolojik ve histolojik sonuç sunar; ayrıca sinir anastomoz bölgesinde lokal SVF kullanımı fibrokonnektif doku reaksiyonu ve enflamasyonu azaltmak için iyi bir seçim olabileceği açıktır.

Anahtar sözcükler: *Elektromiyografi, Histoloji, Nöroanastomoz, Sıçan, Stromal vasküler fraksiyon*

Correspondence
+90 224 2940841
hsalci@uludag.edu.tr

INTRODUCTION

Peripheral nerve regeneration after surgical repair of nerve tissue is a complex process that continues as molecular healing. Neurorrhaphy, tissue adhesives, and grafting are the routine techniques of nerve anastomosis [1,2]. Neurorrhaphy is the most common choice, especially in situations where tension-free nerve endings are attached [1-7]. Fibrosis, adhesion neuroma and glioma formation after neurorrhaphy has a negative effect on nerve healing [1,4,5]. Because of the negative effects of neurorrhaphy on healing, tissue adhesives have been developed as an alternative [1,2,8] and fibrin glue, which is used for neural anastomosis, reduces local inflammation and fibrosis and provides neurotropic factors [2,6,9-11]. However, fibrin glue has been shown to inhibit local axonal regeneration by increasing local scar tissue formation and thus its usage has been limited [6,9].

In recent years, cell-based therapy techniques have been applied in the field of regenerative medicine. Stromal vascular fraction (SVF) obtained from adipose tissue is also among these treatment techniques [12-15]. SVF is a rich cellular source containing preadipocytes, mesenchymal stem cells, endothelial progenitor cells and adipose tissue-derived macropages [5,12,14,16-18]. SVF, composed of heterogeneous cell groups, contributes to nerve regeneration with its angiogenic and immunosuppressive effects [17,19]. Therefore, SVF has many indications such as lipotransfer, cardiac diseases, diabetes-related complications, nerve regeneration and burn wounds [12,14,16,17]. In addition, SVF is used as uncultured adipose-derived stromal cells and has advantages such as rapid derivation and reduction in transplantation, economize and reducing the risk of cell culture [5,13,20-23].

On the basis of the literature information given above, using histological and electromyographic (EMG) examinations, this presented study aimed to investigate the neuro-regenerative effect of locally applied SVF in neuro-anastomosis following suture and fibrin glue after sciatic nerve transection.

MATERIAL and METHODS

Animals and Grouping

This study was approved by Bursa Uludag University Animal Experiments Local Ethics Committee (Decision no: 2019-03/07).

The materials of the study consisted of 31 adult (n=31) Wistar Albino male rats weighing approximately 300 grams. All rats were allowed to illuminate for 12 h daytime and 12 h nighttime with an average temperature of 22°C, and the rats were fed *ad libitum*. Rats were randomly selected for the study groups. The study was performed on the right sciatic nerve: group I (GRI) micro suture (n=7), group II (GRII) micro suture + SVF (n=7), group III (GRIII) fibrin glue (n=7) and group IV (GRIV) fibrin glue + SVF (n=7). Fat tissue was taken from 3 rats to prepare SVF. The sciatic nerve of the left extremity of all rats was evaluated for EMG examination as control.

Experimental Procedure

The microsurgical procedure was performed under general anesthesia in accordance with the rules of asepsis and antisepsis. Xylazine HCl (9 mg/kg, IM) (Alfazine®, Egevet, Turkey) was used as preanesthetic and ketamine HCl (50 mg/kg, IM) (Alfamine®, Egevet, Turkey) was administered for induction. The sciatic nerve on the right extremity of the rats was reached by lateral approach. Transversal incision was made with scalpel one cm proximal to the bifurcation of the nerve. The surgical loop was used during the operation for magnification, so that the micro-imaging and nerve endings were confronted. In GRI and GRII, four micro sutures were applied by epineural repair technique using 10/0 non-absorbable suture material (Ethicon®, USA) *(Fig. 1-A)*. After the suturing procedure in GRII, the SVF prepared before the surgical procedure was absorbed into the surgicel (Ethicon®, USA) and delivered to anastomosis area locally *(Fig. 1-B)*. After transection in GRIII and GRIV, nerve endings were adhered with 4 mL fibrin glue (Tisseel®, Baxter, Austria) *(Fig. 1-C)*. After adhesion of nerve endings in GRIV, SVF impregnated with surgicel (Ethicon®, USA) was applied locally *(Fig. 1-D)*. After the procedures applied to the nerves in all groups, the surgical site was routinely closed.

In the postoperative (PO) period, meloxicam (1 mg/kg, IM) was used once daily for three days and enroflaxacin (10 mg/kg, IM, qd) was used for a week to all rats. All groups were sacrificed by decapitation at PO 8th weeks.

EMG Procedure

To assess functional nerve healing, EMG examination was performed at 0th day and PO 1st and 8th weeks using Medtronic Dantec Keypoint 4 EMG (Bourgogne, France). For the motor conduction velocity examination of the sciatic nerve, supra-maximal stimulation was given by monopolar needle electrodes from the back of genu and acetabulum. The ground electrode was placed between stimulator and recording electrode. The compound muscle action potential (CMAP) was recorded from gastrocnemius muscle. Distal latency, amplitude and nerve conduction velocity were evaluated. In needle EMG, tibialis cranialis muscle and gastrocnemius muscle, at 0.2 mV and 10 ms/D, spontaneous muscle activity was examined with concentric needle electrodes. Spontaneous activity of the muscles examined as reported previously [24]; +1: sparse fibrillation potentials or positive sharp waves, +2: fibrillation potentials or positive sharp waves in multiple sites in a muscle, +3: abundant spontaneous activity, +4: significant fibrillation potentials or positive sharp waves to fill the screen.

Collection of Adipose Tissue and SVF Preparation Protocol

For SVF preparation, the fat tissue was taken of the inguinal

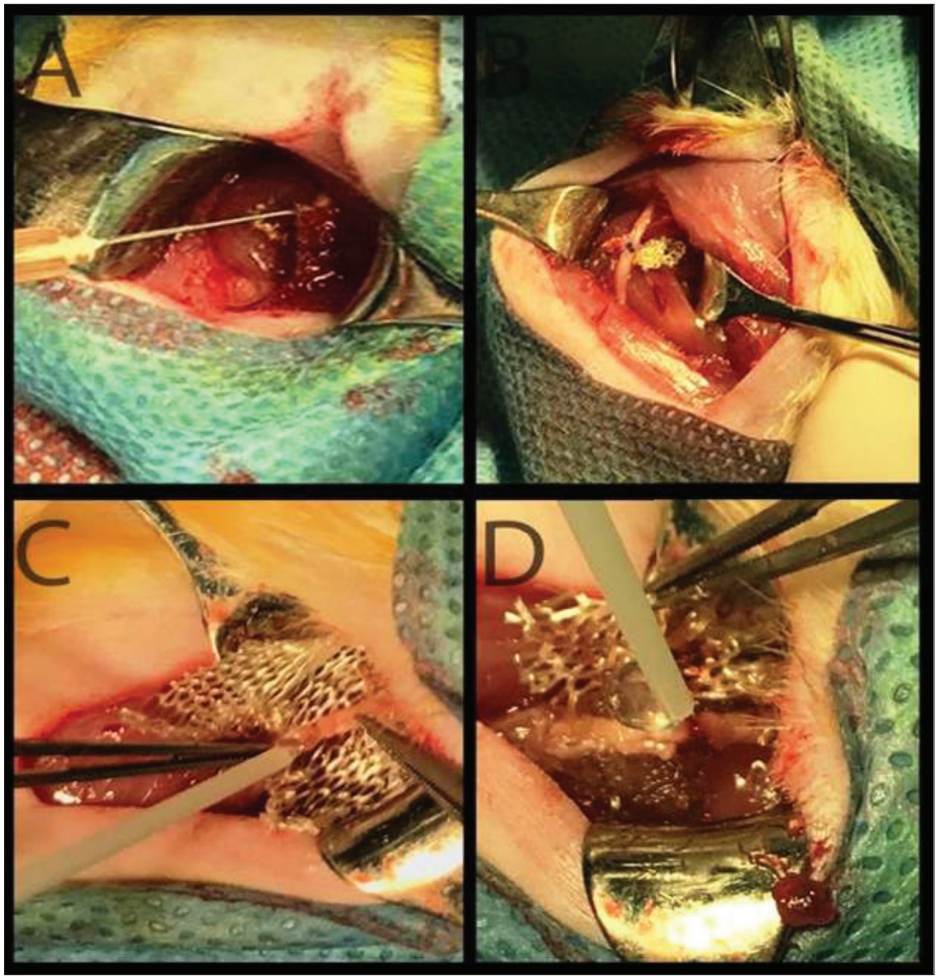


Fig 1. Intraoperative view of the procedures applied in GRI **(A)**, GRII **(B)**, GRIII **(C)** and GRIV **(D)**

region under general anesthesia from three rats. In total, 2.898 g of clean adipose tissue was harvested from the animals. Non-enzymatic SVF method (mechanic) was performed as described previously [25]. The adipose tissue was cut into small pieces then sterilized with alcohol for 2 sec in 50 mL falcon tube. Subsequent adipose tissues were washed with Dulbecco's phosphate buffered saline (D-PBS) separately performed in sterile petri dish. Adipose tissue samples was removed from D-PBS, placed into the 100 mm^2 petri dish and cut into the more small pieces (1-2 mm^2) for mincing process [26]. After this mincing process, 1.5 g of fat tissue + 1.5 mL of 0.08% isotonic content were added to 2 centrifuge tubes (15 mL volume). The centrifugation was carried out at 3500 rpm, 4°C for 4 min. After the supernatant was discarded, the pellet was filtered with 70-micron filter to use SVF. The 100 µL volume of fresh SVF was injected by insulin syringes into nerve anastomosis area of the rats.

Histological Procedure

After decapitation, nerve was quickly collected; soft tissues were removed from the nerve surfaces, and cut into small pieces. Specimens were fixed in 10% neutral buffered formalin for 3 days. When the fixation was completed, specimens were rinsed in water briefly. After dehydration in 70% ethanol, 80% ethanol, 96% ethanol, and absolute ethanol, specimens were embedded in paraffin wax and than 6-7 µm thickness tissue sections were cut and stained with Crossman's triple staining method [27]. Histology sections were examined microscopically (Nikon® Eclipse 80i Microscope, Netherlands). Photographs were taken with a camera (Nikon® Ds Camera Control Unit DS-L1, Japan).

The nerve regeneration was evaluated by nerve fiber alignment and fibrous tissue reaction in each section. To evaluate the axonal regeneration, fiber alignment of nerve was scored semiquantitatively: (+) weak, (++) moderate, (+++) strong, (++++) very strong. The fibrous tissue reaction in the lesion area, all slides were scored as no collagen and/or connective tissue (–), weak (+), moderate (++) and strong (+++) by two researchers in a blind manner. The analysis was performed as reported previously [28].

Statistical Evaluation

Statistical analyzes were done in SigmaStat verison 12.5 program (GmBH®, Germany). One-way analysis of variance (ANOVA) repeated measures test was used to compare electromyographic findings within and between groups. The results were evaluated at $P<0.05$ significance level.

RESULTS

The operation wound of all rats recovered without any complications and all rats survived until the end of the study. Nerve transection revealed right posterior limb paralysis in all rats which was evident in PO 1st week.

EMG Results

In GRI; conduction velocity was 31.6±1.6 m/s at PO 1st week and 71±5.8 m/s at PO 8th weeks. Amplitude was 0.6±0.5 mV at PO 1st week and 5.7±1.4 mV at PO 8th weeks; the distal latency was 4.95±0.05 ms at PO 1st week and 1.4±0.2 ms at PO 8th week. The amplitude, distal latency and conduction velocity were found to be increased when the values of PO 1st and 8th week were compared. Compared with the left extremity for control, conduction velocity was close to normal values at PO 8th weeks, but amplitude was lower than normal value and distal latency was longer. In needle EMG, severe fibrillation and positive sharp waves were observed in PO 1st week, denervation potentials became stable in PO 8th week.

In GRII, conduction velocity was 37.1±7.4 m/s in PO 1st week and 53±7.9 m/s in PO 8th week. The amplitude was 3±1.5 mV at PO 1st week and 4.1±0.7 mV at PO 8th week; distal latency was 1.7±0.6 ms at PO 1st week and 1.1±0.1 ms at PO 8th week. When compared with control, conduction rate and amplitude were significantly lower than normal value and distal latency was longer than normal. In needle EMG, severe fibrillation and positive sharp waves were observed in the PO 1st week, denervation potential was stable in many rats individually at PO 8th weeks.

In GRIII; conduction velocity was 59.2±7.5 m/s in PO 1st week and 68.3±3.1 m/s in PO 8th week. Amplitude was 1±0.5 mV at PO 1st week and 6.6±1.5 mV at PO 8th weeks; the distal latency was 1.3±0 ms at PO 1st week and 1.4±0.4 ms at PO 8th week. When compared to left extremity as control, conduction velocity was found to be close to normal value. Severe spontaneous activity was observed in needle EMG at PO 1st week. Fibrillation and positive sharp wave potentials decreased to the minimum and muscle membrane was stable at PO 8th weeks.

In GRIV; the rate of conduction was 39.1±8.2 m/s at PO 1st week and 52.7±9.3 m/s at PO 8th weeks. The amplitude was 1.4±0.3 mV at PO 1st week and 3.4±0.8 mV at PO 8th weeks; the distal latency was 1.6±0.4 ms in PO 1st week and 2.5±1.6 ms in PO 8th week. When compared to left extremity as control, conduction velocity and amplitude were lower than normal value and distal latency was longer than normal. Severe spontaneous activity was observed in needle EMG at PO 1st week. It was observed that fibrillation and positive sharp wave potentials minimized and membrane stability was not achieved in many rats at PO 8th week.

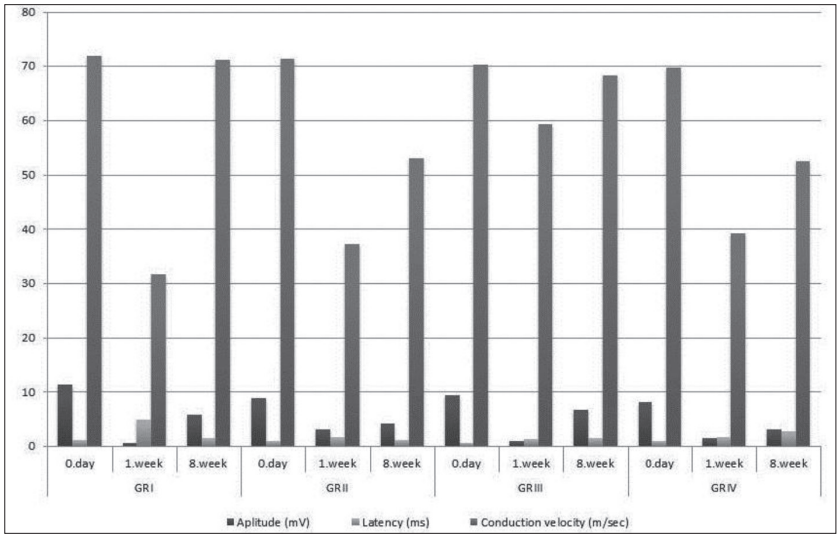


Fig 2. EMG examination results of all groups

Graphical changes in amplitude and conduction velocities of all groups at 0th day and PO 1st and 8th weeks were shown in *Fig. 2*.

Histological Results

Macroscopically, color change and thinning of the anastomosis area was observed in all groups. In GRI and GRII, suture materials were observed in the anastomosis area. Longitudinal continuity in the sciatic nerve was seen in all rats of all groups. Separation of the distal and proximal nerve segments and adherence of the segments to muscle tissues were not observed. During the study, ototomy was observed in one rat in GRII and four rats in GRIII.

Histological assessments are given in *Table 1*.

Table 1. *Score results of axonal regeneration of sciatic nerve*

Parameter of Histologic Evaluation	GRI	GRII	GRIII	GRIV
Fiber alignment	++	+	+++	++
Fibrous tissue reaction (anastomosis area)	+++	++	+	++

Fiber alignment: *(+) weak, (++) moderate, (+++) strong, (++++) very strong;* ***Fibrous connective tissue reaction:*** *(-) no collagen; (+) collagen and/or connective tissue between fibers; (++) connective tissue that partially interrupts fiber passage; (+++) connective tissue that totally interrupts fiber passage*

Regeneration of Ischiadic Nerve: Axonal regeneration and fiber alignment were found to be better in GRIII compared

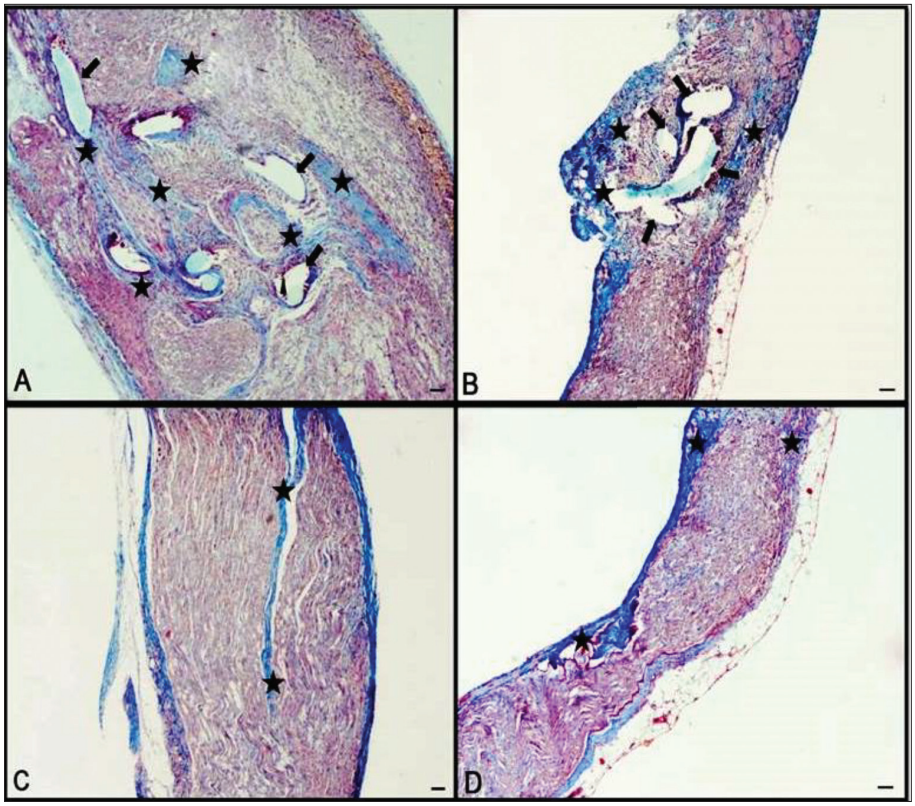
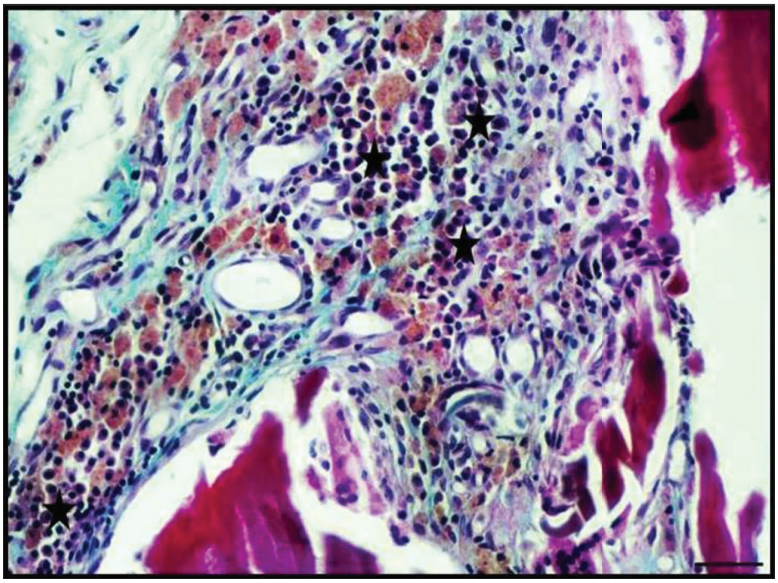


Fig 3. Longitudinal histologic section of GRI **(A)**, GRII **(B)**, GRIII **(C)** and GRVI **(D)**: experimental groups. Fibroconnective tissue reactions (*); decrease of fiber alignment and interruption-suture area *(arrows);* Triple stain, Bar 100 µm

Fig 4. Longitudinal histologic section. * High density of inflammatory cells observed in GRI; Triple stain, Bar 25 µm



to the other groups *(Table 1, Fig. 3-C)*. In GRIII, less fibroconnective tissue reactions were determined *(Fig. 3-C)*, and maximum fibroconnective tissue reactions were observed in GRI *(Fig. 3-A)*. Fibroconnective tissue reactions in the anastomosis area had similar scores (++) in GRII *(Table 1, Fig. 3-B)* and GRIV *(Table 1, Fig. 3-D)*. A significant decrease in fiber alignment was observed due to increased density of fibrous connective tissue reaction *(Fig. 3-A)*. Furthermore, it was found that the fiber alignment was partially and completely interrupted in GRI *(Fig. 3-A)*. There was a considerable connective tissue reaction in the perineural area of all groups compared with the controls. When the inflammation of the nerve fibers was examined, it was seen that GRI and GRII had the most inflammatory cells in the sutured area *(Fig. 4)*, and less inflammatory cells were observed in GRIII and GRIV *(Fig. 3-C,D)*.

Statistical Results

The statistical comparisons of amplitude, distal latency, conduction velocity, and needle EMG results are detailed in *Table 2* and *Table 3*.

Table 2. *This table points out the nerve conduction study values of the groups*

Group	Amplitude (mV) Mean±SE			Distal Latency (ms) Mean±SE			Conduction Velocity (m/s) Mean±SE		
	0. day	1. week	8. week	0. day	1. week	8. week	0. day	1. week	8. week
GRI	11.3 ± 1.6^{aA}	$0.6\pm0.5^{b**A}$	5.7 ± 1.4^{abA}	1.0 ± 0.3^{aA}	$4.9\pm0.0^{b**A}$	1.4 ± 0.2^{aA}	71.8 ± 5.1^{aA}	$31.6\pm1.6^{b**A}$	71 ± 5.8^{aA}
GRII	8.5 ± 2.1^{aA}	$3\pm1.5^{b*A}$	4.1 ± 0.7^{abA}	0.8 ± 0.1^{aA}	$1.7\pm0.6^{b**B**}$	$1.1\pm0.1^{b*A}$	70.5 ± 2.6^{aA}	$37.1\pm7.4^{b**A}$	53 ± 7.9^{abA}
GRIII	9.3 ± 2.6^{aA}	1 ± 0.5^{aA}	6.6 ± 1.5^{aA}	0.5 ± 0^{aA}	$1.3\pm0.0^{b*B**}$	1.4 ± 0.4^{abA}	70.3 ± 9.7^{aA}	59.2 ± 7.5^{aA}	68.3 ± 3.1^{aA}
GIV	7.7 ± 1.6^{aA}	$1.4\pm0.3^{b**A}$	$3.4\pm0.8^{b**A}$	0.8 ± 0.1^{aA}	$1.6\pm0.4^{B**}$	2.5 ± 1.6^{aA}	69 ± 3.6^{aA}	$39.1\pm8.2^{b**A}$	52.7 ± 9.3^{aA}

** P<0.05, ** P<0.01 and *** P<0.001;* ***Within groups:*** *Same letters (a, b and c) in the same line are not statistically significant (P>0.05);* ***Between groups:*** *Same letters (A and B) in the same column are not statistically significant (P>0.05)*

Table 3. *The mean and standart errors (SE) and also statistical analysis results of the needle EMG values in groups*

Group	Tibialis cranialis muscle Mean±SE			Gastrocnemius muscle Mean±SE		
	0. day	1. week	8. week	0. day	1. week	8. week
GRI	0 ± 0^{aA}	$4\pm0^{b***A}$	$2\pm0^{c***A}$	0 ± 0^{aA}	$4\pm0^{b***A}$	$1\pm0^{b***A}$
GRII	0 ± 0^{aA}	$3\pm0.3^{b***A}$	0.6 ± 0.3^{abA}	0 ± 0^{aA}	$3\pm0.2^{b***B*}$	$0.8\pm0.3^{c***A}$
GRIII	0 ± 0^{aA}	$3\pm1^{b*A}$	0.5 ± 0.5^{abA}	0 ± 0^{aA}	$3\pm0^{b**B*}$	0 ± 0^{aA}
GRIV	0 ± 0^{aA}	$2.4\pm0.3^{b***A}$	$1\pm0.2^{c***A}$	0 ± 0^{aA}	$3\pm0^{b***B*}$	$0.8\pm0.1^{c**A}$

** P<0.05, ** P<0.01 and *** P<0.001;* ***Within groups:*** *Same letters (a, b and c) in the same line are not statistically significant (P>0.05);* ***Between groups:*** *Same letters (A and B) in the same column are not statistically significant (P>0.05)*

In the Evaluation of Nerve Conduction Velocity

Amplitude Values: The decrease in the amplitude values of GRI and GRIV at PO 1st week was significant within the group comparison (P<0.05), it was close to normal values in GRI, GRII, GRIII at PO 8th week but there was no statistical significance (P>0.05). In the GRIV, the PO 8th week, amplitude value was not close to values of day 0 (P<0.05). There was no significant difference in the 8th week amplitude values between the groups (P>0.05).

Distal Latency: In the groups comparison, a significant difference was found between values of day 0 and PO 1st week of GRI, GRII and GRIII (P<0.01). In the PO week 1, GRI had a significant difference between the other groups (P<0.01), and there was no significant difference between the groups at PO week 8 (P>0.05).

Conduction Velocity: There was a significant difference in the comparison of GRI, GRII and GRIV within the group (P<0.01). In the comparison between the groups, no significant difference was determined between the PO 8th week values (P>0.05).

In the Evaluation of the Needle EMG

The increase in spontaneous activity of tibialis cranialis and gastrocinemius muscles at PO 1st week was found to be significant within grup evaluations of all groups (P<0.001). At PO 8th week, decrease in spontaneous activity of tibialis cranialis muscle was significant in GRI, GRII and GRV (P<0.001). While spontaneous muscle activity of gastrocnemius was significant only in the GRI at PO week 1 (P<0.001), it was statistically significant among all groups at PO 8 week (P>0.05).

DISCUSSION

In peripheral nerves, morphological and cellular changes occur in the nerve fragments as a result of partial or total damage [7,11]. The success of peripheral nerve repair depends on the density of regenerated axons and prominent regenerating myelin at the junction along with regenerating axons [11,29]. From past to present, many methods have been used to achieve optimal functional recovery in nerve regeneration [10,30]. Among these methods, micro suture technique is frequently preferred [3,5,8,31]. Epineural and perineural techniques provide better contact between nerve segments in nerve repair and cause nerve fibers to deteriorate and become more traumatized [8,9,11,31,32]. However, fibrin glue is an atraumatic technique that does not have foreign body effects on the nerve [31,32] and also reduces inflammation and fibrosis [8,10,32]. Fibers in fibrin glue-repaired nerves generally extend parallel to the axis, whereas suture-repaired nerves are known to interfere with axonal growth during regeneration [9,10,32]. In addition, suturing can prevent blood circulation by compressing the fascicles [11,32].

Thus, there is still a controversial statement, which surgical technique is ideal for peripheral nerve repair [10,31]. In this presented study, neuroanastomosis was performed in the

sciatic nerve using epineural micro suture and fibrin glue repair techniques. And the local effect of SVF on nerve regeneration, following to nerve repairement, was investigated by PO EMG examinations and nerve histology study, which was determined as 8 weeks based on axonal re-growth and functional regeneration time, as reported previously [33,34].

In comparative studies with fibrin glue and epineural repair, there is no detail in which the superiority was reported with histological findings [11,32]. It has been reported that fibrin glue may cause compression of the nerve by causing connective tissue reaction [11]. However, Breshah et al.[2] and Rafijah et al.[6], have been reported that fibrin glue has no negative effect on nerve regeneration and may even be functionally alternative to epineural suture repair. In addition, fibrin glue applied for neural anastomosis has better functional return and has superiority electrophysiologically [35,36]. In this study, there was a reduction in the continuity of the axons in group applied only micro suture. Moreover, comparing to the suturing groups (GRI and II), fibrin glue (GRIII) presented better electrophysiological results, and minimal inflammatory reaction and fibrosis on the anastomosis site of the nerve in histological evaluation.

The effect of SVF on nerve regeneration is not fully understood, but stromal, multipotent and hematopoietic cell populations are known to contribute to regeneration. It is also reported that regenerated axons heal faster and myelinated fibers have larger diameters [17]. Local neuroprotective effect of SVF was reported in diabetic rats [9]. In a nerve graft study, regeneration is better in SVF filled vein grafts and these grafts can be used in peripheral nerve regeneration instead of autologous nerve graft [15]. Mohammadi et al.[22] has been reported that SVF has favorable effects on nerve regeneration histologically. In SVF groups (GRII and GRIV) of this study, the inflammation was significantly minimal. Furthermore, SVF showed less fibroconnective tissue reaction compared to the group where only micro sutur was applied, and it was considered as an important result of reducing the fibrous tissue reaction of SVF.

Electromyografic examination provides important information for the determination of axonal regeneration and functional recovery after surgical repair of peripheral nerves [37]. In addition, Sta et al.[38], has been stated that the electrophysiologic evaluation is very sensitive in early stage nerve regeneration. Amplitudes, an indicator of regenerated axon count [7,9,19,32], increased gradually in all groups at PO 8th weeks. As informed by Martins et al.[39], the highest amplitude was determined in fibrin glue group (GRIII) at PO 8th week. The absence of statistical significance in amplitude values of GRIII during the study was interpreted that fibrin glue had a positive effect on nerve regeneration. CMAP latencies are also indicative of nerve conduction velocity and correlate with the level of axonal myelination and nerve regeneration. Low distal latency is revealing of higher conduction velocity and recovery [9]. Comparing to the and 8th week distal latency values of the groups, there was the longest distal latency in micro suture group at PO 1st week, which could be indicative for demyelinisation; however, the closest distal latency value was determined in micro suture plus SVF group (GRII) at PO 8th week. In the comparison between the groups, the message speed reached normal values in the 8th week EMG examination of all groups and there was no statistically significant difference between them (P>0.05).

Needle EMG is performed in the target organ muscles in order to evaluate reinnervation [7]. Electrophysiological methods have been reported to be the best indicator of nerve healing in the period from nerve repair to muscle re-innervation [28]. In addition, the fibrillation potentials used in clinical routine and the numerical evaluation scale of positive sharp waves ranging from +1 to +4 provide semi-measurable results about denervation in muscles [24]. In needle EMG, there was serious denervation potential in all groups at PO 1st week, but this activity decreased until the end of the study period, but the muscles were not completely stable. In the evaluation of spontaneous muscle activity, it was determined that gastrocnemius muscle activity was higher in the GRI at PO 1st week compared to the other groups (P<0.001), but there was no significant difference between the groups at the PO 8th week (P>0.001). The best recovery was the fibrin glue group as a result of semi-quantitative evaluation in parallel with histological findings. Muscle membrane stabilization was faster in SVF groups in the evaluation of re-innervation. Also, the fact that the potential for denervation in the groups using SVF started to decrease earlier may mean that SVF can contribute to renervation.

In conclusion, considering the presented study data, fibrin glue offers the best electrophysiological and histological results; SVF has been shown to reduce fibroconnective tissue reaction and inflammation, and may contribute to renervation by creating electrophysiological early denervation potential. Therefore, it is clear that SVF can be a potential alternative to the use of stem cells and growth factors.

REFERENCES

1. Barton MJ, Morley JW, Stoodley MA, Lauto A, Mahns DA: Nerve repair: Toward a sutureless approach. *Neurosurg Rev,* 37 (4): 585-595, 2014. DOI: 10.1007/s10143-014-0559-1

2. Breshah MN, Sadakah AA, Eldrieny EA, Saad KA: Functional and histological evaluation of rat sciatic nerve anastomosis using cyanoacrylate and fibrin glue. *Tanta Dent J,* 10 (2): 67-74, 2013. DOI: 10.1016/j.tdj.2013.08.005

3. Flores AJ, Lavernia CJ, Owens PW: Anatomy and physiology of peripheral nerve injury and repair. *Am J Orthop (Belle Mead NJ),* 29 (3): 167-173, 2000.

4. Özaydın İ, Ünsaldı E, Aksoy Ö, Yayla S, Kaya M, Ulkay Tunalı MB, Aktaş A, Taşdemiroğlu E, Cihan M, Kurt B, Yıldırım HC, Şengöz A, Erdoğan H: The effect of silicone tube and silicone tube + hyaluronic

acid application on adhesion formation in experimental peri- and epineurorrhaphy in a rat modal. *Kafkas Univ Vet Fak Derg,* 20 (4): 591-597, 2014. DOI: 10.9775/kvfd.2014.10583

5. Panagopoulos GN, Megaloikonomos PD, Mavrogenis AF: The present and future for peripheral nerve regeneration. *Orthopedics,* 40 (1): e141-e156, 2017. DOI: 10.3928/01477447-20161019-01

6. Rafijah G, Bowen AJ, Dolores C, Vitali R, Mozaffar T, Gupta R: The effect of adjuvant fibrin sealant on the surgical repair of segmental nerve defects in an animal model. *J Hand Surg,* 38 (5): 847-855, 2013. DOI: 10.1016/j.jhsa.2013.01.044

7. Wood MD, Kemp SWP, Weber C, Borschel GH, Gordon T: Outcome measures of peripheral nerve regeneration. *Ann Anat,* 193 (4): 321-333, 2011. DOI: 10.1016/j.aanat.2011.04.008

8. Bhandari PS: Use of fibrin glue in the repair of brachial plexus and peripheral nerve injuries. *Indian J Neurotrauma,* 10 (1): 30-32, 2013. DOI: 10.1016/j.ijnt.2013.05.008

9. Bhatnagar D, Bushman JS, Murthy NS, Merolli A, Kaplan HM, Kohn J: Fibrin glue as a stabilization strategy in peripheral nerve repair when using porpous nerve guidance conduits. *J Mater Sci Mater Med,* 28:79, 2017. DOI: 10.1007/s10856-017-5889-4

10. Childe JR, Regal S, Schimoler P, Kharlamov A, Miller MC, Tang P: Fibrin glue increases the tensile strength of conduit-assisted primary digital nerve repair. *Hand,* 13 (1): 45-49, 2018. DOI: 10.1177/1558944717691131

11. Suri A, Mehta VS, Sarkar C: Microneural anastomosis with fibrin glue: an experimental study. *Neurol India,* 50 (1): 23-26, 2002.

12. Aksu AE, Çalış M: Yağ dokusundan elde edilen mezenkimal kök hücreler ve stromal vasküler fraksiyon konsepti. *Türkiye Klinikleri J Plast Surg- Special Topics,* 4 (3): 19-26, 2015.

13. Matsumine H, Numakura K, Climov M, Watanabe Y, Giatsidis G, Orgill DP: Facial-nerve regeneration ability of a hybrid artificial nerve conduit containing uncultured adipose-derived stromal vascular fraction: An experimental study. *Microsurgery,* 37 (7): 808-818, 2017. DOI: 10.1002/micr.30060

14. Nguyen A, Guo J, Banyard DA, Fadavi D, Toranto JD, Wirth GA, Paydar KZ, Evans GRD, Widgerow AD: Stromal vascular fraction: A regenerative reality? Part 1: Current concepts and review of literature. *J Plast Reconstr Aesthet Surg,* 69 (2): 170-179, 2016. DOI: 10.1016/j.bjps.2015.10.015

15. Özkan HS, Silistreli ÖK, Ergür B, İrkören S: Repairing peripheral nerve defects by vein grafts filled with adipose tissue derived stromal vascular fraction: An experimental study in rats. *Ulus Travma Acil Cerrahi Derg,* 22 (1): 7-11, 2016. DOI: 10.5505/tjtes.2015.12612

16. Gentile P, Scioli MG, Bielli A, Orlandi A, Cervelli V: Concise review: The use of adipose-derived stromal vascular fraction cells and platelet rich plasma in regenerative plastic surgery. *Stem Cells,* 35 (1): 117-134, 2017. DOI: 10.1002/stem.2498

17. Guo J, Nguyen A, Banyard DA, Fadavi D, Toranto JD, Wirth GA, Paydar KZ, Evans GRD, Widgerow AD: Stromal vascular fraction: A regenerative reality? Part 2: Mechanisms of regenerative action. *J Plast Reconstr Aesthet Surg,* 69 (2): 180-188, 2016. DOI: 10.1016/j.bjps.2015.10.014

18. Mohammadi R, Sanaei N, Ahsan S, Rostami H, Abbasipour-Dalivand S, Amini K: Repair of nerve defect with chitosan graft supplemented by uncultured characterized stromal vascular fraction in streptozotocin induced diabetic rats. *Int J Surg,* 12 (1): 33-40, 2014. DOI: 10.1016/j.ijsu.2013.10.018

19. Shimizu M, Matsumine H, Osaki H, Ueta Y, Tsunoda S, Kamei W, Hashimoto K, Niimi Y, Watanabe Y, Miyata M, Sakurai H: Adipose-derived stem cells and the stromal vascular fraction in polyglycolic acid-collagen nerve conduits promote rat facial nerve regeneration. *Wound Repair Regen,* 26 (6): 446-455, 2018. DOI: 10.1111/wrr.12665

20. Bora P, Majumdar AS: Adipose tissue-derived stromal vascular fraction in regenerative medicine: A brief review on biology and translation. *Stem Cell Res Ther,* 8:145, 2017. DOI: 10.1186/s13287-017-0598-y

21. Harasymiak-Krzyzanowska I, Niedojadlo A, Karwat J, Kotuła L, Gil-Kulik P, Sawiuk M, Kocki J: Adipose tissue-derived stem cells show conciderable promise for regenerative medicine applications. *Cell Mol Biol Lett,* 18, 479-493, 2013. DOI: 10.2478/s11658-013-0101-4

22. Mohammadi R, Azizi S, Delirez N, Hobbenaghi R, Amini K: Transplantation of uncultured omental adipose-derived stromal vascular fraction improves sciatic nerve regeneration and functional recovery through inside-out vein graft in rats. *J Trauma Acute Care Surg,* 72 (2): 390-396, 2012. DOI: 10.1097/ta.0b013e31821181dd

23. Mohammadi R, Mehrtash M, Mehrtash M, Sajjadi SS: Nonexpanded adipose stromal vascular fraction local therapy on peripheral nerve regeneration using allografts. *J Invest Surg,* 29 (3): 149-156, 2016. DOI: 10.3109/08941939.2015.1093046

24. Öge AE, Matur Z: Sinir Iletim Çalışmaları ve Elektromiyografi Atlası. 20-25, Nobel Yayınevi, İstanbul, 2013.

25. Shah FS, Wu X, Dietrich M, Rood J, Gimble JM: A non-enzymatic method for isolating human adipose tissue-derived stromal stem cells. *Cytotherapy,* 15 (8): 979-985, 2013. DOI: 10.1016/j.jcyt.2013.04.001

26. Li YS, Chen PJ, Wu LW, Chou PW, Sun LY, Chiou TW: Investigating the mincing method for isolation of adipose-derived stem cells from pregnant women fat. *Cytotechnology*, 70 (1): 55-66, 2018. DOI: 10.1007/s10616-017-0162-8

27. Crossmann G: A modification of Mallory's connective tissue stain with a discussion of the principles involved. *Anat Rec,* 69, 33-38, 1937. DOI: 10.1002/ar.1090690105

28. Adams EJ, Gren JA, Clark AH, Youngson JH: Comparison of different scoring systems for immunohistochemical staining. *J Clin Pathol,* 52, 75-77, 1999. DOI: 10.1136/jcp.52.1.75

29. Wolthers M, Moldowan M, Binderup T, Schmalbruch H, Krarup C: Comparative electrophysiological, functional, and histological studies of nerve lesions in rats. *Microsurgery,* 25 (6): 508-519, 2005. DOI: 10.1002/micr.20156

30. Mohammadi R, Sanaei N, Ahsan S, Masoumi-Verki M, Khadir F, Mokarizadeh A: Stromal vascular fraction combined with silicone rubber chamber improves sciatic nerve regeneration in diabetes. *Chin J Traumatol,* 18 (4): 212-218, 2015. DOI: 10.1016/j.cjtee.2014.10.005

31. Menovsky T, Beek JF: Laser, fibrin glue, or suture repair of peripheral nerves: A comparative functional, histological and morohometric study in the rat sciatic nerve. *J Neurosurg,* 95 (4): 694-699, 2001. DOI: 10.3171/jns.2001.95.4.0694

32. Shamel J, Meyer VE, Bachem U: Glueing of peripheral nerves with fibrin: Experimental studies. *J Reconstr Microsurg,* 3 (3): 211-218, 1987. DOI: 10.1055/s-2007-1006987

33. Gramsbergen A, Ijkema-Paassen J, Meek MF: Sciatic nerve transection in the adult rat: Abnormal EMG patterns during locomotion by aberrant innervation of hindleg muscles. *Exp Neurol,* 161 (1): 183-193, 2000. DOI: 10.1006/exnr.1999.7233

34. Georgiou M, Golding JP, Loughlin AJ, Kingham PJ, Phillips JB: Engineered neural tissue with aligned, differentiated adipose-derived stem cells promotes peripheral nerve regeneration across a critical sized defect in rat sciatic nerve. *Biomaterials,* 37, 242-251, 2015. DOI: 10.1016/j.biomaterials.2014.10.009

35. Isaacs JE, McDaniel CO, Owen JR, Wayne JS: Comparative analysis of biomechanical performance of available "nerve glues". *J Hand Surg,* 33 (6): 893-899, 2008. DOI: 10.1016/j.jhsa.2008.02.009

36. Martins RS, Siqueira MG, Da Silva CF, Plese JP: Overall assessment of regeneration in peripheral nerve lesion repair using fibrin glue, suture, or a combination of the 2 techniques in a rat model. Which is the ideal choice? *Surg Neurol,* 64, S1:10-S1:16, 2005. DOI: 10.1016/j.surneu.2005.04.022

37. Hüseyinoğlu N, Özaydın İ, Yayla S, Yıldırım CH, Aksoy Ö, Kaya M, Şengöz A, Taşdemiroğlu E: Electrophysiological assessment of the effects of silicone tubes and hyaluronic acid on nerve regeneration in rats with sciatic neurorrhaphy. *Kafkas Univ Vet Fak Derg,* 18 (6): 917-922, 2012. DOI: 10.9775/kvfd.2012.6373

38. Sta M, Cappaert NLM, Ramekers D, Baas F, Wadman WJ: The functional and morphological characteristics of sciatic nerve degeneration and regeneration after crush injury in rats. *J Neurosci Methods,* 222, 189-198, 2014. DOI: 10.1016/j.jneumeth.2013.11.012

39. Martins RS, Siqueira MG, Da Silva CF, De Godoy BO, Plese JPP: Electrophysiologic assessment of regeneration in rat sciatic nerve repair using suture, fibrin glue or a combination of both techniques. *Arq Neuropsiquiatr,* 63 (3-A): 601-604, 2005. DOI: 10.1590/s0004-282x2005000400009

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Research Article

Serovars, Antimicrobial Susceptibility and Molecular Characteristics of *Haemophilus parasuis* Isolates in Southern China

Ling PENG [1,2,a] Xiaoqing YUAN [1] Ran FANG [1] Weizhen LIU-FU [1] Quan WEN [1] Xufu YANG [1,2]

[1] Yingdong College of biology and agriculture, Shaoguan University,Shaoguan 512005, CHINA
[2] Joint Laboratory of Animal Infectious Diseases Diagnostic Center-Harbin Veterinary research Institute of Chinese Academy of Agriculture Science, Shaoguan University, Shaoguan 512005, CHINA
ORCID: [a] 0000-0002-7798-703X

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Abstract

This study analyzed the characteristics of 133 *Haemophilus parasuis* isolates in southern China. These isolates belonged to eleven serovars (1, 2, 4-10, 13 and 15) with 12.0% of them being characterised as non-typable. A relatively high level in resistance was encountered for trimethoprim + sulfamethoxazole (89.9%), tetracycline (75.3%), amoxicillin (69.1%), streptomycin (63.6%), carbenicillin (60.2%), kanamycin (46.6%) and ampicillin (45.6%). A total of 60% of the isolates were negative for group 1 virulence-associated autotransporters *(vtaA)*. All group 1 *vtaA* negative isolates fell into polyacrylamide gel electrophoresis (PAGE) type I, while all group 1 *vtaA* positive isolates were classified as PAGE type II. The results of Multi-locus sequence typing (MLST) indicated a high degree of variation, 45 isolates in the study were assigned into 31 sequence types with 28 of these being new (not found in the MLST database). Antimicrobial resistance was observed in every serovar, there was no statistically significant correlation between the antimicrobial resistance and the serovars. The isolates allocated to clade 2 (based on MLST target sequences) showed the molecular characteristics of highly pathogenic strains in whole-cell protein profiling, *vtaA* groups 1, superoxide dismutase *(sodA)* sequence and MLST.

Keywords: *Haemophilus parasuis, Antimicrobial susceptibility, MLST, PAGE, vtaA, SodA*

Güney Çin'de Haemophilus parasuis İzolatlarının Serovarları, Antimikrobiyal Duyarlılıkları ve Moleküler Özellikleri

Öz

Bu çalışmada, Güney Çin'deki 133 H. parasuis izolatının özellikleri analiz edildi. Bu izolatlar, %12.0'ı tiplendirilemeyen olarak karakterize edilen on bir serovara (1, 2, 4-10, 13 ve 15) aitti. Trimetoprim + sülfametoksazol (%89.9), tetrasiklin (%75.3), amoksisilin (%69.1), streptomisin (%63.6), karbenisilin (%60.2), kanamisin (%46.6) ve ampisilin (%45.6) için nispeten yüksek bir direnç seviyesine rastlandı. İzolatların %60'ı, grup 1 virülansa bağlı ototransportörler (vtaA) yönünden negatifti. Tüm grup 1 vtaA negatif izolatları poliakrilamid jel elektroforezi (PAGE) tip I'e dahil edilirken, tüm grup 1 vtaA pozitif izolatları PAGE tip II olarak sınıflandırıldı. Multi-lokus sekans tiplendirmesinin (MLST) sonuçları yüksek derecede bir varyasyon gösterdi. Çalışmadaki 45 izolat 31 sekans tipine atanırken bunların 28'inin yeni olduğu belirlendi (MLST veritabanında bulunamadı). Her serovarda antimikrobiyal direnç gözlendi, antimikrobiyal direnç ile serovarlar arasında istatistiksel olarak anlamlı bir ilişki yoktu. Clade 2'ye ayrılan izolatlar (MLST hedef sekanslarına dayanarak) hücre proteini profillemesi, vtaA grupları 1, süperoksit dismutaz (sodA) dizilimi ve MLST sonuçları patojenitesi yüksek suşların moleküler özelliklerini gösterdi.

Anahtar sözcükler: *Haemophilus parasuis, Antimikrobiyal duyarlılık, MLST, PAGE, vtaA, SodA*

INTRODUCTION

Haemophilus parasuis (H. parasuis) is a Gram-negative bacterium that colonizes the upper respiratory tract of pigs. After invasion of the host, it causes Glasser's disease, which is associated with fibrinous polyserositis, meningitis, and arthritis. The infection of *H. parasuis* can be controlled by the use of serovar specific vaccines and antibiotics [1-3].

A total of 15 serovars and a large number of non-typable (NT) strains have been identified [3]. The high level of intrinsic diversity among *H. parasuis* populations has hindered

Correspondence
+86-1382-6319175
penglingfx@sgu.edu.cn

the development of effective cross-protective vaccines [3]. Although antibiotic treatment is the most common intervention in the control of Glasser's disease, the use of antibiotics may lead to increased antibiotic resistance [4], therefore, antimicrobial susceptibility testing is a crucial step prior to antibiotic prescription.

Multi-locus sequence typing (MLST) discriminates among isolates by comparing DNA sequences of six to ten house-keeping genes [5]. As an objective and highly standardized method, the MLST allows full characterization of all sampled isolates. In *H. parasuis* research, Olvera et al.[6] were the first to use the MLST method to genetically characterize *H. parasuis* isolates. Mullins et al.[7] then optimized the MLST-based analysis of population structure and genetic diversity among *H. parasuis* populations.

H. parasuis isolates range from highly virulent to non-pathogenic. Although the molecular basis underlying the virulence of many *H. parasuis* isolates has not been completely established, some virulence markers or factors have been proposed [7-11]. In this study, the antimicrobial susceptibility of 133 isolates of *H. parasuis* from southern China was tested against 26 antimicrobial agents. These isolates were characterized by serotyping, whole-cell protein profiling, identification of virulence-associated autotransporters *(vtaA),* and the sequencing of the superoxide dismutase *(sodA)* gene and by MLST. A phylogenetic analysis between the 45 isolates based on MLST target sequences was also conducted.

MATERIAL and METHODS

Serotyping of H. parasuis Isolates

A total of 133 *H. parasuis* isolates were collected either from the blood, joint fluid, lungs and nasal cavity of possibly diseased pigs accompanied by symptoms associated with Glässer's disease or from the nasal cavity of healthy pigs between 2007 and 2016 in southern China; A total of 88% of the 133 isolates were isolated from the nasal cavity of pigs, and 12% of the isolates were from blood, joint fluid, and lungs of pigs. Identification of the isolates was carried out by nicotinamide adenine dinucleotide (NAD) dependency, biochemical tests and using PCR [12]. The isolates were serotyped using a multiplex PCR method [13].

Antimicrobial Susceptibility

The antimicrobial susceptibility of the *H. parasuis* isolates was assessed by the disk diffusion method with the use of blood agar medium with 0.0025% of NAD [14]. Antimicrobial disks (Hangzhou Tianhe Microbiological Co., Hangzhou, China) and associated concentrations are described in *Table 1*. Growth inhibition was assessed against Clinical and Laboratory Standards Institute standards and the isolates were classified as susceptible, intermediate or resistant [15,16]. *Actinobacillus pleuropneumoniae* (ATCC 27090) and *Escherichia coli* (ATCC 25922) reference strains were used as experimental controls.

Whole-cell Protein Profiling and Identification of Virulence-associated Autotransporters

The whole-cell protein profiles of *H. parasuis* isolates were evaluated using the method described by Oliveira *and* Pijoan [9]. And the polyacrylamide gel electrophoresis (PAGE) types were identified. Isolates containing major proteins weighing between 36 and 38 kDa, were classified as PAGE type II and isolates lacking this group of proteins were classified as PAGE type I. All *H. parasuis* isolates were grown under the same culture conditions. The *vtaA* of the *H. parasuis* isolates were identified using the method described in Olvera et al.[10]. Accordingly, a multiplex PCR was used for the diagnosis of *H. parasuis* at the species level (group 3 *vtaA* positive) and to differentiate putative non-virulent isolates (group 1 *vtaA* negative).

Table 1. *Antimicrobial resistance profiles of H. parasuis isolates from southern China*

Antibiotic Tested (µg)	Level of Susceptibility		
	Sensitive (%)	Intermediate (%)	Resistant (%)
Erythromycin (15)	59.6	37.5	2.9
Tilmicosin (15)	100	0	0
Amoxicillin (10)	29.1	1.8	69.1
Ampicillin (10)	33.8	20.6	45.6
Carbenicillin (100)	30.5	9.3	60.2
Cefazolin (30)	91.3	2.5	6.3
Cefalexin (30)	80.4	8.4	11.2
Cefuroxime (30)	84.9	0.8	14.3
Ceftiofur (30)	100	0	0
Ceftriaxone (30)	100	0	0
Cefalotin (30)	100	0	0
Gentamicin (10)	79.6	4.9	15.4
Kanamycin (30)	41.0	12.4	46.6
Amikacin (30)	59.1	15.2	25.6
Spectinomycin (100)	95.1	0.0	4.9
Streptomycin (10)	22.7	13.6	63.6
Tetracycline (30)	17.8	6.8	75.3
Deoxytetracycline (30)	100	0	0
Enrofloxacin (5)	78.2	21.0	0.8
Ciprofloxacin (5)	66.5	26.1	7.5
Norfloxacin (10)	80.9	11.1	8.0
Ofloxacin (5)	100	0	0
Trimethoprim (1.25) + Sulfamethoxazole (23.75)	8.2	1.9	89.9
Lincomycin (2)	29.4	43.7	26.9
Rifampicin (5)	100	0	0
Albamycin (5)	100	0	0

MLST

A total of 45 isolates were selected for the MLST analysis and 7 housekeeping genes were used in the MLST analysis of the *H. parasuis* isolates: malate dehydrogenase gene *(mdh),* β chain of ATP synthase *(atpD),* translation initiation factor IF-2 *(infB),* ribosomal protein β subunit *(rpoB),* 6-phosphogluconate dehydrogenase(6pgd), glyceraldehyde -3-phosphate dehydrogenase *(g3pd)* and fumarate reductase B *(frdB)*. These genes were amplified with primers described in Mullins et al.[7]. The PCR reaction conditions were: 5 min at 95°C; 35 cycles of 95°C for 1 min, 48°C for 30 s and 72°C for 30 s, followed by a final elongation step of 72°C for 10 min. The PCR products were sequenced by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China), using the dideoxy chain-termination method. The housekeeping gene sequences, obtained for each isolate, were edited, assembled, and aligned by the software MEGA version 5.0 [17]. Then, the sequences were submitted to the MLST database *(http://pubmlst.org/hparasuis)* for the analysis of allele number and sequence type (ST) according to the methodology described by Jolley *and* Maiden [18]. A Neighbor-Joining tree, based on the Maximum Composite Likelihood distance estimation model with 1000 bootstrap replicates, was created from the concatenated sequences of the seven housekeeping genes [7].

Characterizing the Genetic Diversity of sodA

Le [19] and Chen et al.[20] reported there are some changs in *sodA* amino acid sites between virulent and avirulent strains of *H. parasuis*, focusing on changes in four *sodA* amino acid sites (at the 16th position: Asp-to-Glu, 49th position: Leu-to-Phe, 69th position: Arg-to-Gln, and 186th position Ile-to-Val). In the current study, the *sodA* gene of the 45 isolates were PCR amplified using the primers *sodA* F (5'ATG-GCATACACAT TACCTGAG T TAGA3') and *sodA* R (5'TTATG-CTTGGGAT TCAAAACGT3'). Subsequently, the PCR product obtained from each isolate was sub-cloned into the pUCm-T vector and sequenced using the dideoxy chain termination method (Sangon Biotech Ltd., Shanghai, China).

Statistical Analysis

A neighbour-joining tree derived from the MLST target sequences of 45 *H. parasuis* isolates was constructed by using MEGA version 5.0 software. An x^2-test was used to assess correlation between the antimicrobial resistance and the serovars, and a resulting P value lower than 0.05 was regarded as significant.

RESULTS

From the 133 serotyped *H. parasuis* isolates, eleven distinct serovars were identified. Serovar 10 (15.8%) was the most prevalent, followed by serovars 15 (12.8%), 6 (12.0%), 8 (9.8%), 5 (8.3%), 4 (7.6%), 9 (7.5%), 1 (6.8%), 7 (5.3%), 13 (1.5%) and 2 (0.8%). A total of 12.0% of the isolates tested were NT *(Table 2)*.

We tested the antibiotic resistance of the 133 isolates of *H. parasuis* against 26 different antibiotics in this study. The resistance rates to 7 antibiotics were over 40%, to 5 antibiotics were 10%-30% and to 6 antibiotics were below 10%, while, the resistance rates to 8 antibiotics were 0% *(Table 1)*. The resistance to 26 antimicrobial agents among all these isolates was counted and compared it with the distribution of serovars to the distribution of antimicrobial resistance. We found that the isolation frequency in serovar 5, 1, 7 and 8 was a little higher than the incidence of antimicrobial resistance in the corresponding serovar, while, the isolation frequency in serovar 6 and 9 was a little lower than the incidence of antimicrobial resistance in the corresponding serovar, but there was not significant difference (x^2-test, P>0.05). For other serovars, the isolation frequency of each serovar was very similar to the incidence of antimicrobial resistance in the corresponding serovar *(Fig. 1)*.

A total of 39.8% of the isolates were classified as PAGE type II, including some of those included in serovars 1, 2, 4, 5, 6, 7, 9, 13, 15 and some NT isolates *(Table 2)*. A total of 60.2% of the isolates were classified as PAGE type I, including some of those included in serovars 1, 4, 6, 8, 9, 10, 15, and some NT isolates *(Table 2)*.

In this study, all isolates were positive for group 3 *vtaA* and 60% of the isolates were negative for group 1 *vtaA*. All group 1 *vtaA* negative isolates were PAGE type I, while all group 1 *vtaA* positive isolates were classified as PAGE type II. All serovars 2, 5, 7, 13 isolates were group 1 *vtaA* positive and all serovars 8, 9, 10 isolates were group 1 *vtaA* negative *(Table 2)*.

Table 2. *Characteristics of the H. parasuis isolates in this study*

Characteristics	Serovars											
	1	2	4	5	6	7	8	9	10	13	15	NT
PAGE type I strains or Group 1 *vtaA* negative strains	1	0	1	0	15	0	13	8	21	0	12	9
PAGE type II strains or Group 1 *vtaA* positive strains	8	1	9	11	1	7	0	2	0	2	5	7
Serotypeable strains	9	1	10	11	16	7	13	10	21	2	17	16
Serotypeable strains frequency (%)	6.8	0.8	7.5	8.3	12.0	5.3	9.8	7.5	15.8	1.5	12.8	12.0

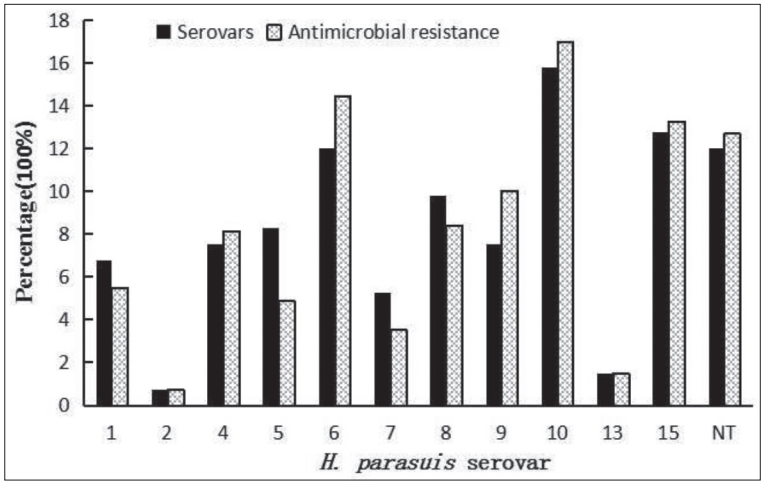
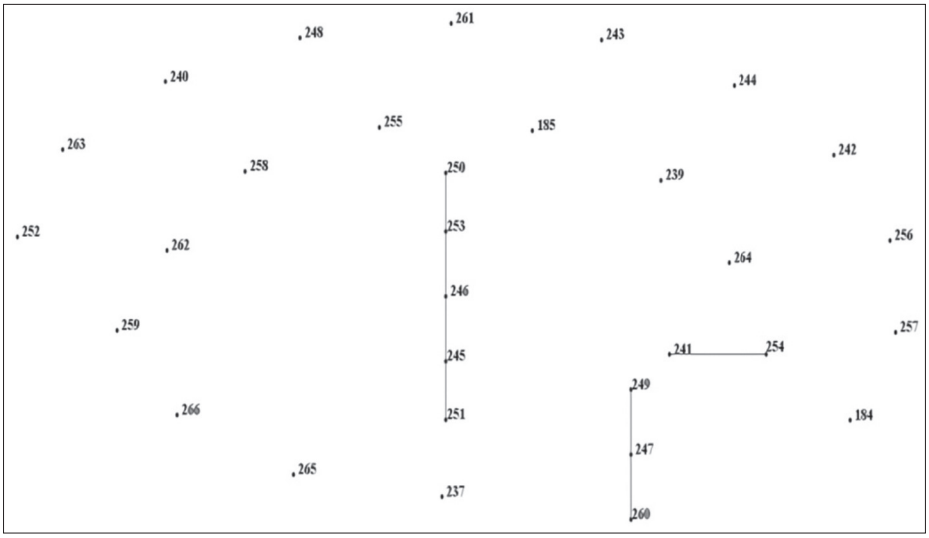


Fig 1. Distribution of *H. parasuis* serovars and antimicrobial resistance among each of these serovars.Serovars: frequency (%) = number of strains belonging to a certain serovar/total number of strains ×100%.Antimicrobial resistance: incidence (%) = number of resistances to all tested antimicrobial agents among strains of a certain serovars/total number of resistances to all tested antimicrobial agents across all strains×100%

Fig 2. Analysis of 31 *H. parasuis* STs in the study using eBURST



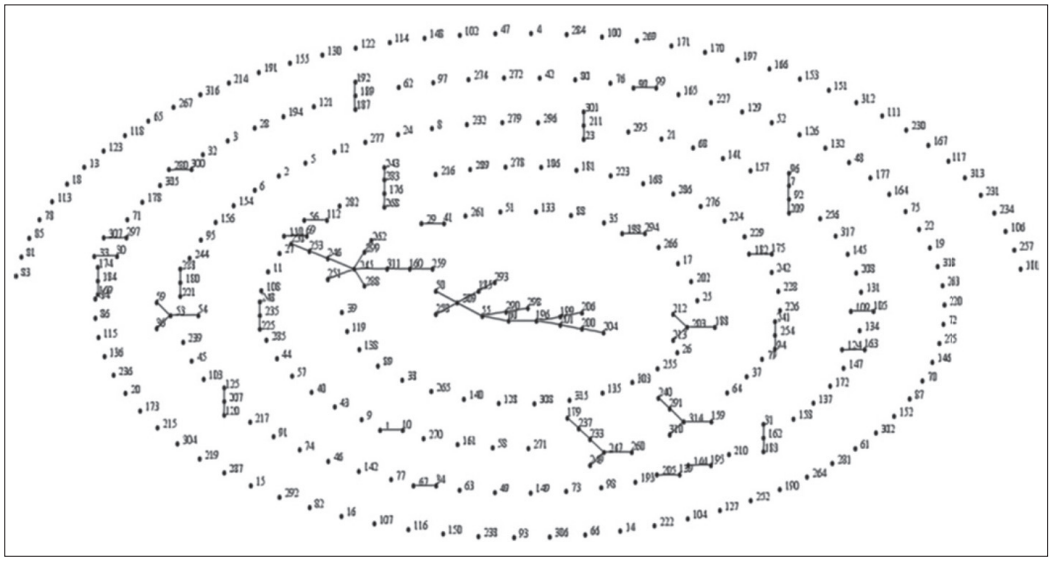


Fig 3. Analysis of all *H. parasuis* STs from the MLST database of *H. parasuis* using eBURST

In this study, we identified 13 new alleles across five of the seven housekeeping genes. Five new alleles were identified in 6 *pgd* (55-59), two in *atpD* (31-32), one in *frdB* (48), four in *infB* (51-54), and one in *mdh* (42). A total of 31 STs were found in this study, and 28 STs (ST239-ST266) were new MLST STs. clonal complex (CC)1 comprised ST241 and ST254; CC2 comprised ST245, ST246, ST250, ST251, and ST253; and CC3 comprised ST247, ST249

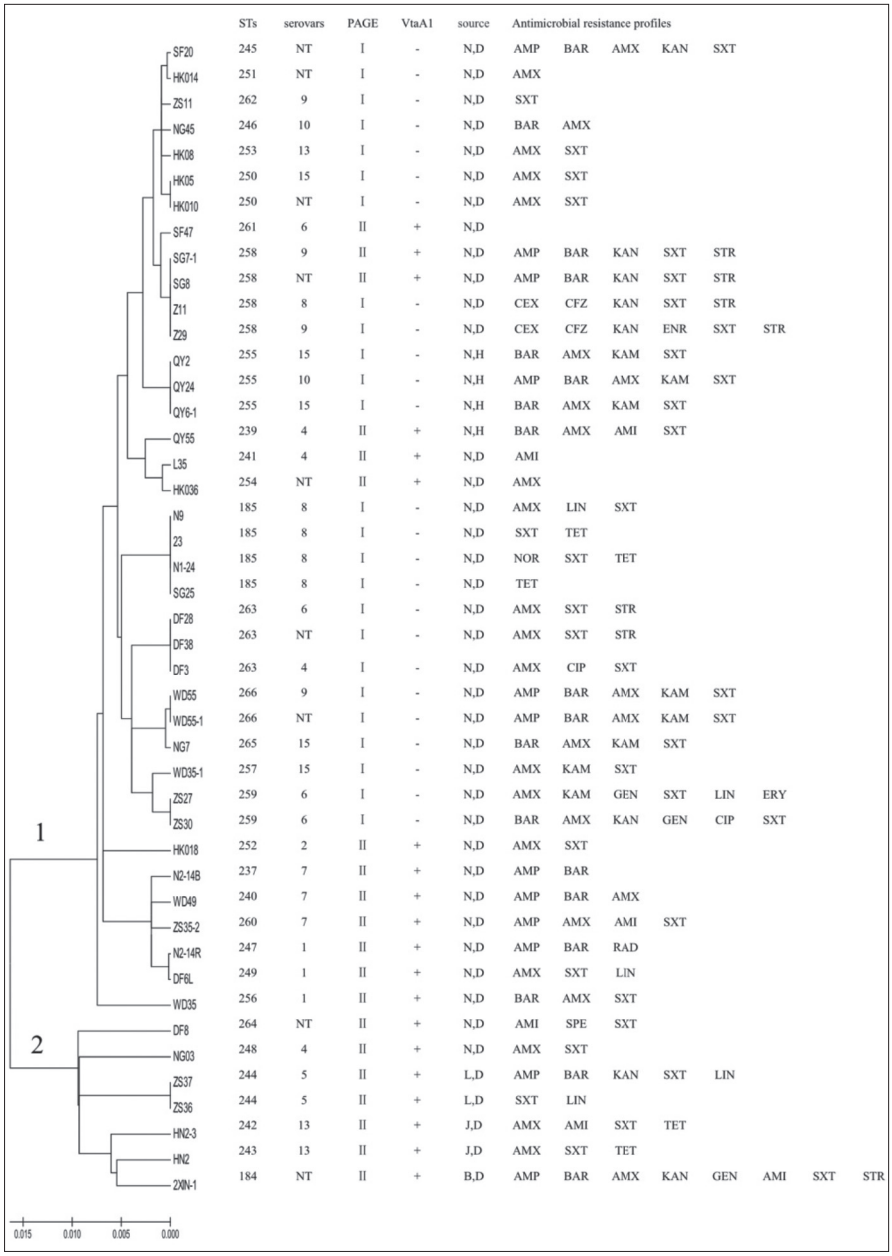


Fig 4. Neighbor-Joining tree derived from MLST target sequences of 45 *H. parasuis* strains. AMP: Ampicillin, BAR: Carbenicillin, AMX: Amoxicillin, CFZ: Cefazolin, CEX: Cefalexin, CXM: Cefuroxime, KAN: Kanamycin, GEN: Gentamicin, AMI: Amikacin, SPE: Spectinomycin, ENR: Enrofloxacin, NOR: Norfloxacin, CIP: Ciprofloxacin, SXT: Trimethoprim + Sulfamethoxazole, LIN: Lincomycin, ERY: Erythromycin, TET: Tetracycline, STR: Streptomycin; N: nasal, B: blood, J: joint, L: lung, H: healthy pig, D: diseased pig

and ST260 *(Fig. 2)*. ST245 was the predicted founder.

We also analyzed the population structure based on 318 STs from the MLST database of *H. parasuis*. The eBURST organized the 318 STs into 31CCs and 209 singletons. The predicted founders of CC1, CC2, CC4, CC6, CC9, CC13, CC14 and CC16 were ST309, ST245, ST314, ST203, ST211, ST180, ST184 and ST207, respectively. The predicted founders of CC3, CC5, CC7, CC10 and CC11 were multiple candidates. There were no predicted founders in other CCs *(Fig. 3)*.

The *sodA* gene of 45 *H. parasuis* isolates were sequenced. The results identified only 7 *H. parasuis* isolates (HN2-3, HN2, 2XIN-1, ZS36, ZS37, DF8 and NG03) with all four highly pathogenic sites of amino acids: aspartic acid at position 16 (Asp-16), leucine at position 49 (Leu-49), arginine at position 69 (Arg-69) and isoleucine at position 186 (Ile-186), other isolates with 1 to 2 changes at position 16, 49 and 69.

A neighbour-joining tree derived from the MLST target sequences of 45 *H. parasuis* isolates was constructed. Two major clades were obtained (clade 1and clade 2). Clade 1 includes the majority of isolates (84.4%) and STs (80.6%), including serovars 1, 2, 4, 6, 7, 8, 9, 10, 15, and some of the NT isolates. Clade 2 includes a minority of the isolates (15.6%) and STs (19.4%), including serovars 4, 5, 13 and some NT isolates *(Fig. 4)*. These isolates allocated to clade 2 all classified as PAGE type II (whole-cell protein profiling), contained all four highly pathogenic amino acids sites *(sodA* sequencing), and were positive for *vtaA* groups 1 *(vtaA* characterization). However, 65.8% of the 45 isolates allocated to clade 1 classified as PAGE type I and were negative for *vtaA* groups 1 *(Fig. 4)*.

DISCUSSION

This work was based on 133 *H. parasuis* isolates from southern China. These isolates belonged to eleven serovars (1, 2, 4-10, 13 and 15) with 13% of them being characterized as NT. Previous studies have reported that different *H. parasuis* isolates may belong to the same MLST ST, even if they consist of different serovars. Olvera et al.[21] and Olvera et al.[22] found that although *H. parasuis* isolates CD7-3 (serovar 14), CD9-1(serovar15), and CD10-4 (serovar 10) belonged to different serovars, they were all assigned to *H. parasuis* ST 46. Similarly, Wang et al.[11] found that although *H. parasuis* isolates H33 (NT), H35 (serovar 15), and H36 (serovar 14) belonged to different serovars, they were all assigned to *H. parasuis* ST 181. In this study, we found a similar result *(Fig. 4)*, which illustrates a lack of correlation between the *H. parasuis* STs and serovars.

In this study, 28 out of the 31 STs identified with the MLST method were novel. The eBURST organized the 31 STs into 3 CCs and 21 singletons, and organized all STs from the MLST database of *H. parasuis* into 31 CCs and 209 singletons, which illustrates the high heterogeneity of the population structure of *H. parasuis*.

In China, Zhou et al.[23] reported that 44.5% and 70.9% of 110 isolates were resistant to trimethoprim + sulfamethoxazole and enrofloxacin, respectively. Xu et al.[24] reported that most of the tested 112 isolates in theirs study were resistant to nalidixic acid (84.8%), TMP (67.9%), trimethoprim + sulfamethoxazole (58%), enrofloxacin (45.5%) and ciprofloxacin (41.1%). Zhao et al.[25] reported that 82.5% and 55.9% of 143 isolates were resistant to nalidixic acid and enrofloxacin. In our study, we observed a relatively high level of resistance to trimethoprim + sulfamethoxazole (89.9%), tetracycline (75.3%), amoxicillin (69.1%), streptomycin (63.6%), carbenicillin (60.2%), kanamycin (46.6%), and ampicillin (45.6%). This high frequency of resistance may be attributed to the intensive use of these antibiotics in the hog industry in China. We compared the distribution of serovars with the distribution of the incidence of antimicrobial resistance, and concluded that there was no statistically significant correlation between the antibiotic resistance and the serovars (x^2-test, $P>0.05$).

The association between drug resistance and the *H. parasuis* MLST STs was ambiguous. For example, although the isolates of N9, N1-24, 23 and SG25 were isolated from different farms and belonged to the same ST (ST185), they had different resistances *(Fig. 4)*. Similarly, the isolates ZS27 and ZS30, isolated from same farm and assigned to the same ST (ST259), also had different drug resistance patterns.

The isolates allocated to PAGE type II (whole-cell protein profiling), group 1 *vtaA* positive *(vtaA* characterization) and clade 2 (based on MLST target sequences) are potentially virulent strains of *H. parasuis*; whereas the isolates allocated to PAGE type I, group 1 *vtaA* negative and clade 1 are generally avirulent [7,9-11]. In the study, the majority of the isolates were classified as 'non-pathogenic, one explanation for this finding can be that the majority of the isolates (88%) were isolated from the nasal cavity. The isolates allocated to clade 2 all classified as PAGE type II and group 1 *vtaA* positive, these isolates showed the molecular characteristics of highly pathogenic strains in whole-cell protein profiling, *vtaA* groups 1 and MLST.

Previous research has suggested that the mutations of in *sodA* amino acid sites can influence the activity of *sodA* [26,27]. Le [19] further reported there are four changs in *sodA* amino acid sites between virulent and avirulent strains of *H. parasuis*. In the study, the isolates allocated to clade 2 (potentially virulent isolates) contained all four highly pathogenic amino acids sites in *sodA*, the isolates allocated to clade 1 (potentially avirulent isolates) were identified with 1 to 2 mutations in four highly pathogenic amino acids sites in *sodA,* which illustrates the *sodA* sequence may be uesd to predict the virulence of *H. parasuis* isolates, which requires further testing.

In summary, the current findings illustrates a high levels of drug resistance in *H. parasuis* isolates in Southern China, a high heterogeneity of the population structure of *H. parasuis*, a lack of correlation between the *H. parasuis* STs and serovars, there was no statistically significant correlations between antimicrobial resistance and the serovars and the *sodA* sequence may be uesd to predict the virulence of *H. parasuis* isolates, just as whole-cell protein profiling, *vtaA* groups 1 and MLST can be uesd to predict the virulence.

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REFERENCES

1. Miani M, Lorenson MS, Guizzo JA, Espíndola JP, Rodríguez-Ferri EF, Gutiérrez-Martín CB, Kreutz LC, Frondoloso R: Antimicrobial susceptibility patterns of brazilian *Haemophilus parasuis* field isolates. *Pesqui Vet Brasil,* 37 (11): 1187-1192, 2017. DOI: 10.1590/s0100-736x2017001100001

2. Liu H, Xue Q, Zeng Q, Zhao Z: *Haemophilus parasuis* vaccines. *Vet Immunol Immunopathol*, 180, 53-58, 2016. DOI: 10.1016/j.vetimm.2016.09.002

3. Oliveira S, Pijoan C: *Haemophilus parasuis*: New trends on diagnosis, epidemiology and control. *Vet Microbiol*, 99 (1): 1-12, 2004. DOI: 10.1016/j.vetmic.2003.12.001

4. Zhao Y, Guo L, Li J, Huang X, Fang B: Characterization of antimicrobial resistance genes in *Haemophilus parasuis* isolated from pigs in China. *PeerJ*, 6:e4613, 2018. DOI: 10.7717/peerj.4613

5. Maiden MCJ: Multilocus sequence typing of bacteria. *Annu Rev Microbiol,* 60, 561-588, 2006. DOI: 10.1146/annurev.micro.59.030804.121325

6. Olvera A, Cerda-Cuellar M, Aragon V: Study of the population structure of *Haemophilus parasuis* by multilocus sequence typing. *Microbiology*, 152 (12): 3683-3690, 2006. DOI: 10.1099/mic.0.29254-0

7. Mullins MA, Register KB, Brunelle BW, Aragon V, Galofré-Mila N, Bayles DO, Jolley KA: A curated public database for multilocus sequence typing (MLST) and analysis of *Haemophilus parasuis* based on an optimized typing scheme. *Vet Microbiol*, 162 (2-4): 899-906, 2013. DOI: 10.1016/j.vetmic.2012.11.019

8. Nicolet J, Paroz P, Krawinkler M: Polyacrylamide gel electrophoresis of whole-cell proteins of porcine strains of *Haemophilus*. *Int J Syst Bacteriol*, 30 (1): 69-76, 1980. DOI: 10.1099/00207713-30-1-69

9. Oliveira S, Pijoan C: Computer-based analysis of *Haemophilus parasuis* protein fingerprints. *Can J Vet Res*, 68 (1): 71-75, 2004.

10. Olvera A, Pina S, Macedo N, Oliveira S, Aragon V, Bensaid A: Identification of potentially virulent strains of *Haemophilus parasuis* using a multiplex PCR for virulence-associated autotransporters *(vtaA)*. *Vet J*, 191 (2): 213-218, 2012. DOI: 10.1016/j.tvjl.2010.12.014

11. Wang LY, Ma LN, Liu YG, Gao PC, Li YQ, Li XR, Liu YS: Multilocus sequence typing and virulence analysis of *Haemophilus parasuis* strains isolated in five provinces of China. *Infect Genet Evol*, 44, 228-233, 2016. DOI: 10.1016/j.meegid.2016.07.015

12. Angen O, Oliveira S, Ahrens P, Svensmark B, Leser TD: Development of an improved species specific PCR test for detection of *Haemophilus parasuis*. *Vet Microbiol,* 119 (2-4): 266-276, 2007. DOI: 10.1016/j.vetmic.2006.10.008

13. Howell KJ, Peters SE, Wang J, Hernandez-Garcia J, Weinert LA, Luan SL, Chaudhuri RR, Angen Ø, Aragon V, Parkhill J, Williamson SM, Langford PR, Rycroft AN, Wren BW, Maskell DJ, Tucker AW: Development of a multiplex PCR assay for rapid molecular serotyping of *Haemophilus parasuis*. *J Clin Microbiol*, 53, 3812-3821, 2015. DOI: 10.1128/JCM.01991-15

14. Dayao DAE, Kienzle M, Gibson JS, Blackall PJ, Turni C: Use of a propose antimicrobial susceptibility testing method for *Haemophilus parasuis*. *Vet Microbiol*, 172 (3-4): 586-589, 2014. DO I: 10.1016/j.vetmic.2014.06.010

15. Clinical and Laboratory Standards Institute: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals: Approved Standard. 4th ed., Wayne, PA, USA, 2013.

16. Clinical and Laboratory Standards Institute: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for *Haemophilus influenzae* and *Haemophilus parainfluenzae*. M02 and M07, Wayne, PA, USA, 2015.

17. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S: MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance,and maximum parsimony methods. *Mol Biol Evol*, 28 (10): 2731-2739, 2011. DOI: 10.1093/molbev/msr121

18. Jolley KA, Maiden MCJ: BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics*, 11:595, 2010. DOI: 10.1186/1471-2105-11-595

19. Le M: Genomic approach to study virulence associated factors of *Haemophilus parasuis*. DSc, Huazhong Agricultural University, Wuhan, China, 2010.

20. Chen D, Chi Y, Ai CH, Dong XP, Gao SY, Liu LY, Zhou TZ, Wang JL: Sod A gene sequence analysis of *Haemophilus parasuis* named LN/111013 and pathogenic preliminary judgment. *Chin J Vet Sci*, 35 (8): 1223-1227, 2015.

21. Olvera A, Cerda-Cuellar M, Nofrarias M, Revilla E, Segales J, Aragon V: Dynamics of *Haemophilus parasuis* genotypes in a farm recovered from an outbreak of Glässer's disease. *Vet Microbiol*, 123 (1-3): 230-237, 2007. DOI: 10.1016/j.vetmic.2007.03.004

22. Olvera A, Segales J, Aragon V: Update on the diagnosis of *Haemophilus parasuis* infection in pigs and novel genotyping methods. *Vet J*, 174 (3): 522-529, 2007. DOI: 10.1016/j.tvjl.2006.10.017

23. Zhou XL, Xu XJ, Zhao YX, Chen P, Zhang X, Chen H, Cai XW: Distribution of antimicrobial resistance among different serovars of *Haemophilus parasuis* isolates. *Vet Microbiol*, 141 (1-2): 168-173, 2010. DOI: 10.1016/j.vetmic.2009.05.012

24. Xu CG, Zhang JM, Zhao ZQ, Guo LL, Zhang B, Feng S, Zhang L, Liao M: Antimicrobial susceptibility and PFGE genotyping of *Haemophilus parasuis* isolates from pigs in South China (2008-2010). *J Vet Med Sci*, 73 (8): 1061-1065, 2011. DOI: 10.1292/jvms.10-0515

25. Zhao YD, Guo LL, Li J, Huang XH, Fang BH: Characterization of antimicrobial resistance genes in *Haemophilus parasuis* isolated from pigs in China. *Peer J*, 6:e4613, 2018. DOI: 10.7717/peerj.4613

26. Kwasigroch JM, Wintjens R, Gilis D, Rooman M: SODa: An Mn/Fe superoxide dismutase prediction and design server. *BMC Bioinformatics*, 9:257,2008. DOI: 10.1186/1471-2105-9-257

27. Yikilmaz E, Rodgers DW, Miller AF: The crucial importance of chemistry in the structure-function link: Manipulating hydrogen bonding in iron-containing superoxide dismutase. *Biochemistry,* 45 (4): 1151-1161, 2006. DOI: 10.1021/bi051495d

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Research Article

Serotyping and Antibiotic Resistance Profile of *Listeria monocytogenes* Isolated from Organic Chicken Meat [1]

Ali GÜCÜKOĞLU [1,a] Özgür ÇADIRCI [1,b] Göknur TERZİ GÜLEL [1,c] Tolga UYANIK [1,d] Sibel KANAT [1,e]

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[1] Ondokuz Mayıs University, Faculty of Veterinary Medicine Department of Food Hygiene and Technology, TR-55200 Kurupelit/Samsun - TURKEY
ORCIDS: [a] 0000-0002-8465-7768; [b] 0000-0003-2018-2545; [c] 0000-0002-0011-0440; [d] 0000-0002-3181-3878; [e] 0000-0002-6181-7239

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Abstract

In this study, 240 organic chicken pieces (80 thighs, 80 wings, 80 skinless-breast meat) were analyzed for the presence of *Listeria monocytogenes.* Within the framework of the analysis findings; *L. monocytogenes* was detected in 60 (25%) of all 240 collected samples. In particular, *L. monocytogenes* was detected in 24 (30%) of 80 thigh samples, 20 (25%) of 80 wing samples and 16 (20%) of 80 skinless-breast meat samples. Serotyping distribution of 96 *L. monocytogenes* isolates determined as 71.8% serotype 1/2a, 21.9% serotype 1/2b, 4.2% serotype 4b and 2.1% serotype 1/2c. According to antibiotic resistance profile, 26 isolates (27%) were found to be resistant to ampicillin. The other isolates were found to be resistant to meropenem, tetracycline, sulfamethoxazole/trimethoprim, penicillin G, amoxicillin/clavulanic acid, vancomycin, oxytetracycline, erythromycin and chloramphenicol as 23 (23.9%), 14 (14.5%), 13 (13.5%), 12 (12.5%), 9 (9.3%), 7 (7.2%), 5 (5.2%), 4 (4.1%) and 3 (3.1%) respectively. Multiple antibiotic resistance profiles were determined in 12 of *L. monocytogenes* isolates. The findings of this study are thought to be unique data for serotyping studies that will help in revealing the epidemiology of *L. monocytogenes* in organic poultry meat, enterprises operating in food sector and diagnosis and treatment of listeriosis.

Keywords: *Listeria monocytogenes, Organic chicken, Serotype, mPCR, Antibiotic resistance*

Organik Tavuk Etlerinden İzole Edilen *Listeria monocytogenes* İzolatlarının Serotip ve Antibiyotik Direnç Profilinin Belirlenmesi

Öz

Bu çalışmada, 240 organik tavuk parça eti (80 but, 80 kanat, 80 derisiz-göğüs eti) *Listeria monocytogenes* varlığı yönünden analiz edildi. Analiz bulguları çerçevesinde; toplam 240 örneğin 60'ında (%25) *L. monocytogenes* saptandı. Bulgularının örneklere göre dağılımı incelendiğinde; but örneklerinin 24'ünde (24/80-%30), kanat örneklerinin 20'sında (20/80-%25), derisiz göğüs eti örneklerinin ise 16'sında (16/80-%20) *L. monocytogenes* tespit edildi. Serotip dağılımında ise but örneklerinden elde edilen 40 *L. monocytogenes* izolatının 31'inin *L. monocytogenes* 1/2a, 6'sının *L. monocytogenes* 1/2b, 3'ünün *L. monocytogenes* 1/2c, 1'inin ise *L. monocytogenes* 4b serotipinde olduğu, kanat örneklerinden elde edilen 34 *L. monocytogenes* izolatının 24'ünün *L. monocytogenes* 1/2a, 9'unun *L. monocytogenes* 1/2b, 1'inin ise *L. monocytogenes* 4b serotipi olduğu, derisiz göğüs eti örneklerinden elde edilen 22 *L. monocytogenes* izolatının 14'ünün *L. monocytogenes* 1/2a, 6'sının *L. monocytogenes* 1/2b, 2'sinin ise *L. monocytogenes* 4b serotipi olduğu belirlendi. Antibiyotik direnç profiline bakıldığında; 26 izolat (%27) ampisiline dirençli bulunurken, meropenem, tetrasiklin, sülfametoksazol/trimetoprim, penisilin G, amoksisilin/klavulanik asit, vankomisin, oksitetrasiklin, eritromisin ve kloramfenikole karşı dirençli izolat sayısı sırası ile 23 (%23.9), 14 (%14.5), 13 (%13.5), 12 (%12.5), 9 (%9.3), 7 (%7.2), 5 (%5.2), 4 (%4.1) ve 3 (%3.1) olarak saptandı. *L. monocytogenes* izolatlarının 12'sinde ise çoklu antibiyotik direnç profili belirlendi. Sonuç olarak bu çalışmada tespit edilen bulguların *L. monocytogenes*'in organik kanatlı etlerinde epidemiyolojisini ortaya koyacak serotiplendirme çalışmalarına, gıda sektöründe faaliyet gösteren işletmelere ve listeriyosizin tanı ve tedavisinde özgün veri niteliğinde olacağı düşünülmektedir.

Anahtar sözcükler: *Listeria monocytogenes, Organik tavuk, Serotip, mPCR, Antibiyotik direnç*

INTRODUCTION

The awareness of balanced nutrition, which emerged after the second half of the twentieth century, has led to drastic changes in people's lifestyles and food consumption. However, the increase in chronic diseases in recent years drove people's desire towards eating more reliable and healthier food, and it is observed that people prefer organic

Correspondence
+90 362 3121919-3286. Fax: +90 362 4576622
aligucuk@omu.edu.tr

products in their nutrition [1,2]. "Organic", "biological", "biodynamic" and "agricultural ecological production" is a production system in which animal welfare comes to the fore in a controlled and certified manner with appropriate breeding techniques for the consumer mass demanding of high quality, healthy and risk-free products [3].

Besides the high nutritional value of poultry meat, it is an ideal environment for the development of saprophyte and pathogenic microorganisms as a result of the shredding and possible cross-contamination due to the technological processes that applied. Poultry meat is most commonly contaminated with pathogens such as *Salmonella* spp., *Campylobacte*r spp., *Staphyloccus aureus*, *Escherichia coli*, *Listeria* spp., *Yersinia enterocolitica*, *Aeromonas* spp. and *Clostridium perfringens* [4].

Several studies have focused on the comparison of conventional and organic production techniques in the presence of pathogens in poultry. Control of pathogens cannot be guaranteed due to the fact that poultry in organic breeding is more likely to be released in the open environment by free release, and there are restrictions on antimicrobial agents in feed and therapeutic use [5-7]. However, it is emphasized that contamination in poultry meat produced by both organic and conventional methods occurs through cross-contamination in the slaughter-house and during the processing [8]. The presence and serotype distribution of *L. monocytogenes*, which is the most common cause of foodborne infections among pathogenic microorganisms in poultry meat, is of great importance. *L. monocytogenes* is one of the most emphasized microorganisms due to the occurrence of the sporadic or epidemic character of listeriosis in humans, especially through food of animal origin, and its presence as a common flora in the food production [9]. In various studies, it was reported that contamination with *L. monocytogenes* was mostly observed in slaughter-houses and in the processing of foodstuffs, and that the prevalence of *L. monocytogenes* increased after cutting by 70-100% compared to pre-slaughter [10].

This study was aimed to i) determine the incidence of *L. monocytogenes* in organic chicken piece meats by using classical culture and IMS techniques, ii) confirm the isolates by PCR, iii) make a serotyping of isolates by mPCR and iv) detect the resistance status of the obtained isolates to selected antibiotics.

MATERIAL and METHODS

In the study, 240 pieces of organic chicken meat (thigh, skinless-breast meat, wing) which were sold in Samsun province in packed form were used as material. Each month 80 samples were obtained between October and December of 2018. Samples were purchased at least 500 g and were brought to the laboratory under the cold chain as soon as possible.

Isolation and Identification of Listeria monocytogenes

The IMS-based culture technique recommended by ISO 11290-1 [11] and Dynal [12] was used for the isolation. 25 g of the samples were weighed under aseptic conditions and diluted with 225 mL of Half Fraser Broth (Oxoid-CM0895) and homogenized in the stomacher for 90 sec at medium speed and then incubated at 30°C for 24 h. Following pre-enrichment, 20 µL of the immunomagnetic microparticle solution (Dynabeads anti-Listeria 710.06) homogenized with vortex was placed into the 1.5 mL microcentrifuge tubes according to the manufacturer's instructions and placed into the Dynal magnetic particle port with the magnetic stick removed. Subsequently, 1 mL of homogenized pre-enriched in Half Fraser Broth was added and the ongoing steps were completed in accordance with the recommendations of the manufacturer. From the obtained 100 µL Dynabeads Listeria complex, 50 µL was streaked on MOX (Modified Oxford Agar, Oxoid-CM0856 + Modified Listeria Selective Supplement, Oxoid-SR0206) agar and plates were incubated at 35°C for 24-48 h. After incubation, up to 5 suspected colonies were selected from the plaques and these colonies were cultured into TSA-YE (Tryptic Soy Agar-Yeast Extract, Oxoid-CM131) for biochemical tests and plates were incubated at 30°C for 24 h. The colonies that breed in TSA-YE (Tryptic Soy Agar-Yeast Extract, Oxoid, CM131) were tested by using, respectively; Gram staining, catalase, oxidase activity in SIM medium (Sulphate Indole Motility Medium; Merck 5470), ß-hemolysis and CAMP tests, sugar fermentation and nitrate reduction tests.

Verification of Listeria monocytogenes by PCR

DNA extraction of identified isolates was performed according to the boiling method. In addition, PCR protocol was designed using the primer sequences shown in *Table 1* designed by Bohnert et al.[13] and Doumith et al.[14] for PCR confirmation and serotyping. Electrophoresis of amplicons were performed in 2% agarose at 80 volts.

Antibiotic Resistance

Antibiotic resistance of the isolates were determined by the disc diffusion method on Mueller Hinton Agar (Oxoid, CM0337) based on the methods reported by CLSI [15] and EUCAST [16]. Besides, minimum inhibition concentration (MIC) of isolates resistant to various antibiotics were determined by Etest (Epsilometer test) method.

RESULTS

According to analysis, 60 (25%) of 240 samples were positive for *L. monocytogenes*. Distribution of isolates regarding to sample types was shown in *Table 2* and *Fig. 1*. Consisting of a large amount of portion (71.8%), 1/2a was found to be the dominant serotype. Distribution of all serotypes according to sample type was shown in

Table 1. *Primer sequences used in the study* [13,14]

Target Gene	Primer Sequence	PCR Product (bp)	Serotype
hlyA	F:GAATGTAAACTTCGGCGCAATCAG R:GCCGTCGATGATTTGAACTTCATC	388	*L. monocytogenes*
Imo0737	F: AGGGCTTCAAGGACTTACCC R: ACGATTTCTGCTTGCCATTC	691	1/2a, 1/2c, 3a, 3c
Imo1118	F: AGGGGTCTTAAATCCTGGAA R: CGGCTTGTTCGGCATACTTA	906	1/2c, 3c
ORF2819	F: AGCAAAATGCCAAAACTCGT R: CATCACTAAAGCCTCCCATTG	471	1/2b, 3b, 4b, 4e, 4d
ORF2110	F:AGTGGACAATTGATTGGTGAA R: CATCCATCCCTTACTTTGGAC	597	4b, 4e, 4d

Table 2. *Incidence of L. monocytogenes in organic poultry*

Sample	Number of Samples	Number of *L. monocytogenes* Positive Samples (%)	Number of *L. monocytogenes* Positive Isolates
Thigh	80	24 (30%)	40
Wing	80	20 (25%)	34
Breast	80	16 (20%)	22
Total	240	60 (25%)	96

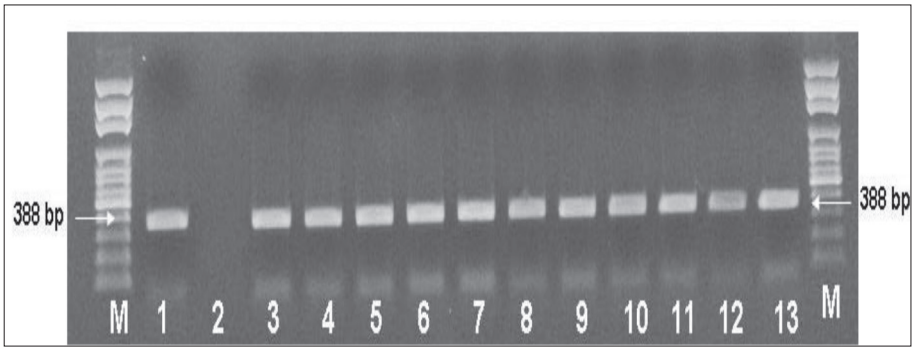

Fig 1. PCR electrophoresis image of *L. monocytogenes* isolates**:** [M: 100 bp DNA ladder, **lane 1:** *L. monocytogenes* positive control *(L. monocytogenes* RSKK 471), **lane 2:** negative control, **lane 3-13:** *L. monocytogenes* positive isolates]

Table 3. *Serotype distribution of L. monocytogenes isolates*

Number of Samples	Number of *L. monocytogenes* Positive İsolates Obtained by IMS-based Conventional Method	Number of *L. monocytogenes* Positive Isolates Verified by PCR (*hlyA* gene)	Distribution of *L. monocytogenes* Serotypes by PCR			
			1/2a (3a)	1/2b (3b)	1/2c (3c)	4b (4d,4e)
Thigh (n: 80)	40	40	31	6	2	1
Wing (n: 80)	34	34	24	9	-	1
Breast (n: 80)	22	22	14	6	-	2
Total (n: 240)	96	96	69	21	2	4

Table 3 and *Fig. 2*. The antibiotic resistance profile of our study revealed that 26 isolates (27%) were resistant to ampicillin. The other portion of the isolates were resistant to meropenem, tetracycline, sulfamethoxazole/trimethoprim, penicillin G, amoxicillin/clavulanic acid, vancomycin, oxytetracycline, erythromycin and chloramphenicol; as 23 (23.9%), 14 (14.5%), 13 (13.5%), 12 (12.5%), 9 (9.3%), 7 (7.2%), 5 (5.2%), 4 (4.1%) and 3 (3.1%) respectively. However, multiple antibiotic resistance profiles were determined in 12 of *L. monocytogenes* isolates *(Table 4, Table 5)*. Datas including the MIC levels were mentioned in *Table 6*.

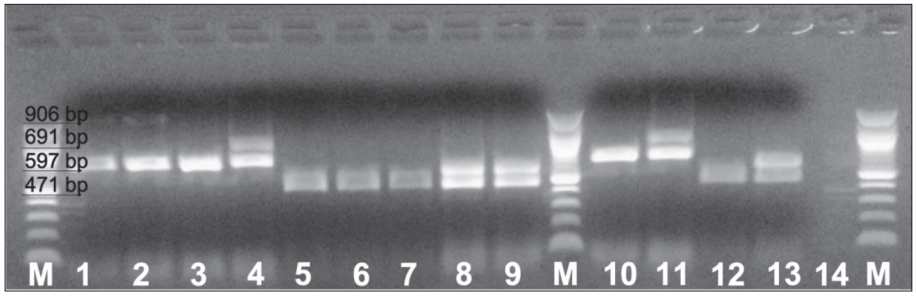

Fig 2. Multiplex PCR electrophoresis image of serotypes: [M: 100 bp DNA ladder, **lane 1-3:** *L. monocytogenes* serotype 1/2a, **lane 4:** *L. monocytogenes* serotype 1/2c, **lane 5-7:** *L. monocytogenes* serotype 1/2b, **lane 8-9:** *L. monocytogenes* serotype 4b, **lane 10:** *L. monocytogenes* serotype 1/2a positive control (*L. monocytogenes* RSKK 471), **lane 11:** *L. monocytogenes* 1/2c positive control (*L. monocytogenes* ATCC 7644), **lane 12:** *L. monocytogenes* serotype 1/2b positive control (*L. monocytogenes* RSKK 472), **lane 13:** *L. monocytogenes* serotype 4b positive control (*L. monocytogenes* RSKK 475), **lane 14:** negative control]

Table 4. *Phenotypic antibiotic resistance profiles of L. monocytogenes isolates*

Antibiotic	Number of Resistant Isolates (%)
Ampicillin (2 µg)	26 (27%)
Meropenem (10 µg)	23 (23.9%)
Tetracycline (30 µg)	14 (14.5%)
Sulfamethoxazol/Trimethoprim (1.25/23.75 µg)	13 (13.5%)
Penicillin G (1U)	12 (12.5%)
Amoxicillin/Clavulanic acid (30 µg)	9 (9.3%)
Vancomycin (30 µg)	7 (7.2%)
Oxytetracycline (30 µg)	5 (5.2%)
Erythromycin (15 µg)	4 (4.1%)
Chloramphenicol (30 µg)	3 (3.1%)

Table 5. *Phenotypic multiple antibiotic resistance profile of L. monocytogenes*

Number of Antibiotics	Antibiotic Profile*	Serotype	Sample Origin
4	AMP, TE, VA, SXT	1/2a	Thigh
4	E, PG, MEM, SXT	4b	Thigh
4	AMC, TE, VA, SXT	1/2a	Thigh
4	C, OT, VA, MEM	1/2a	Wing
3	AMP, OT, MEM	4b	Wing
3	PG, TE, SXT	1/2a	Breast
3	AMC, TE, VA	1/2a	Thigh
3	AMP, TE, MEM	1/2b	Thigh
3	AMC, E, MEM	1/2a	Wing
3	AMP, E, TE	1/2b	Breast
3	AMP, TE, SXT	1/2a	Wing
3	AMP, MEM, SXT	1/2a	Wing

AMC: *Amoxicillin/Clavulanic acid,* ***AMP:*** *Ampicillin,* ***C:*** *Chloramphenicol,* ***E:*** *Erythromycin,* ***OT:*** *Oxytetracycline,* ***PG:*** *Penicillin G,* ***TE:*** *Tetracycline,* ***VA:*** *Vancomycin,* ***MEM:*** *Meropenem,* ***SXT:*** *Sulfamethoxazole/Trimethoprim; * Only one of the same antibiotic group was evaluated*

DISCUSSION

From last few decades to present, studies on the presence of *L. monocytogenes* in poultry meat continue to be important in the worldwide. Unlikely to our findings, in many other studies a high-value prevalence was recorded, like Schafer et al.[17], who detected *L. monocytogenes* in 8.64-44.19% of chicken meat samples; Rahmat et al.[18], in

Table 6. *MIC values of L. monocytogenes serotypes with multiple antibiotic resistance profiles*

Serotype		1/2a	4b	1/2a	1/2a	4b	1/2a	1/2a	1/2b	1/2a	1/2b	1/2a	1/2a
Sample Origin		Thigh	Thigh	Thigh	Wing	Wing	Breast	Thigh	Thigh	Wing	Breast	Wing	Wing
Multiple Antibiotic Resistance Profile		AMP TE VA SXT	E PG MEMSXT	AMC TE VA SXT	C OT VA MEM	AMP OT MEM	PG TE SXT	AMC TE VA	AMP TE MEM	AMC E MEM	AMP E TE	AMP TE SXT	AMP MEM SXT
MIC (µg/mL)	AMC	-	-	16	-	-	-	16	-	16	-	-	-
	AMP	2	-	-	-	2	-	-	2	-	2	2	2
	C	-	-	-	32	-	-	-	-	-	-	-	-
	E	-	2	-	-	-	-	-	-	1.5	2	-	-
	OT	-	-	-	32	32	-	-	-	-	-	-	-
	PG	-	3	-	-	-	4	-	-	-	-	-	-
	TE	16	-	24	-	-	16	16	24	-	24	16	-
	VA	4	-	4	4	-	-	4	-	-	-	-	-
	MEM	-	0.5	-	0.5	0.5	-	-	0.75	0.75	-	-	0.5
	SXT	0.064	0.064	0.064	-	-	0.125	-	-	-	-	0.094	0.064

AMC: *Amoxicillin/Clavulanic acid,* **AMP:** *Ampicillin,* **C:** *Chloramphenicol,* **E:** *Erythromycin,* **OT:** *Oxytetracycline,* **PG:** *Penicillin G,* **TE:** *Tetracycline,* **VA:** *Vancomycin,* **MEM:** *Meropenem,* **SXT:** *Sulfamethoxazole /Trimethoprim*

62.5% of 24 carcass samples; Weis [19], in 62.5% of 8 chicken samples; Farber et al.[20], who reported the presence of 50% *L. monocytogenes* in 16 chicken meat; Elmalı et al.[21], who detected *L. monocytogenes* in 45% of chicken wing meat samples. On the other hand, many others reported the presence of *L. monocytogenes* in proportions similar to the findings of our study, like Bailey et al.[22] in 23% of 90 chicken carcasses; Rorvik et al.[23] in 20% to 100% of chicken carcasses from 5 slaughterhouses. In literature review, some of the studies that reported the presence of *L. monocytogenes* at lower values than the results of our study were as follows: Alsheikh et al.[24], 13.6% of the 250 ready-made chicken products; Alsheikh et al.[25], 12.8% of the 500 frozen chicken samples; Genigeorgis et al.[26] 12.5% of 160 chicken meats; Zeinali et al.[27] 18% of 200 fresh chicken carcasses; Basaran Kahraman et al.[28] 0% of 400 chicken carcasses. Although there were no studies on the presence of *L. monocytogenes* in organic poultry in Turkey, a limited number of studies are available in the literature. In a comparasive study of *L. monocytogenes* in organic and conventional poultry, the contaminations levels of products were reported 49.1% to 41% respectively [5].

In the present study, 71.8% of *L. monocytogenes* isolates were detected as serotype 1/2a. In other studies, Carvalho et al.[29] reported mostly 1/2a (94.6%) in chicken meat and chicken-meat processing environment, Oliveira et al.[30] identified 87% of the *L. monocytogenes* isolates as 1/2a in samples of chicken carcasses, and Zeinali et al.[31] determined that 52.77% of *L. monocytogenes* 1/2a serotype were predominant in chicken carcasses, followed by 4a and 4c serotypes (27.77%) in İran. Arslan and Baytur [32] revealed 57.6% of *L. monocytogenes* strains isolated from chicken meat were 1/2a. In contrast, Zeinali et al.[33] and Maung et al.[34] reported 1/2b dominance in samples of chicken meats. In addition, Ayaz and Erol [35] identified 4b as the dominant serotype (51.4%) in samples of turkey meats. Serotype dominance appears to be different due to changes in animal species, geography and seasonal parameters.

In terms of antibiotic resistance, in parallel to our study, in Ireland, Walsh et al.[36] reported that 351 *L. monocytogenes* isolates obtained from various foods were highly resistant to ampicillin, erythromycin, penicillin, and tetracycline. Davis and Jackson [37] investigated the antimicrobial resistance properties of *L. monocytogenes* isolates from human, environmental and food origin in the United States using Sensititre® method and similar to our study, isolates were found to be resistant against ampicillin, penicillin G, erythromycin and tetracycline. Harakeh et al.[38] revealed that 93.33% of *L. monocytogenes* isolates isolated from dairy products in Lebanon were resistant to oxacillin and 90% were resistant to penicillin. Similarly, Rahimi et al.[39] reported that *L. monocytogenes* isolates isolated from milk and dairy products in Iran were resistant to various antibiotics such as nalidixic acid, ciprofloxacin, erythromycin, tetracycline, gentamicin, ampicillin, penicillin, and chloramphenicol. Researchers have linked this high resistance to genetic material transfers that may occur between different species and unconscious drug use. Bilir Ormanci et al.[40], conducted the antibiotic resistance tests of *L. monocytogenes* isolates isolated from turkey meat by disk diffusion method and reported that the isolates they obtained were resistant to penicillin and ampicillin. Ayaz and Erol [35] reported that *L. monocytogenes* isolated from turkey meats were resistant to penicillin and ampicillin and were resistant to erythromycin but they couldn't

detect resistance to tetracycline, chloramphenicol and vancomycin. As an emerging problem of this century, increasing resistance to multiple antibiotics complicates the treatment of infections. In the present study, 12.5% of the isolates were found to be resistant to at least two different antibiotic type and MIC values were determined. In comparasion to other studies conducted, Lemes-Marques et al.[41] determined the MIC values of 13 *L. monocytogenes* isolates obtained from patients with listeriosis in Brazil against ampicillin and vancomycin by microdilution method. They identified that the isolates were not resistant to vancomycin and ampicillin. In our study, also resistance to vancomycin was not detected. Filiousis et al.[42] reported that one of the 30 *L. monocytogenes* isolates obtained from various foods in Greece was resistant to tetracycline and this MIC was determined to be 64 µg/mL. However, they reported that they could not detect any resistance to other antibiotics. Osaili et al.[43] reported that 11% of *L. monocytogenes* isolates obtained from ready-to-eat chicken products in Jordan were resistant to tetracycline and MIC values were determined as 16 µg/mL. Conter et al.[44] searched MIC values against 19 antibiotics including penicillin G, ampicillin, erythromycin, vancomycin and tetracycline by VITEK 2. The researchers reported that they could not detect any resistance to penicillin and erythromycin, but reported that 2% of their isolates were resistant to ampicillin and 0.8% to tetracycline and vancomycin. Yan et al.[45] investigated the antibiotic resistance profiles of 70 *L. monocytogenes* isolates obtained from various foods in China by microdilution method and according to their findings, 14 isolates were resistant to tetracycline, 2 isolates to ampicillin, erythromycin and chloramphenicol, and 1 isolate was resistant to penicillin and vancomycin. Okada et al.[46] mentioned in their study on 201 *L. monocytogenes* isolated from food, environment, animals, and humans in Japan, 31 of 32 isolates found to be resistant to chloramphenicol and had MIC values of 16 µg/mL and 1 isolate had MIC of 32 µg/mL. The researchers found that MIC of 1 isolate found to be resistant to oxytetracycline was 64 µg/mL. Despite the prohibition of antimicrobial use in organic poultry production, several studies have demonstrated that pathogenic and non-pathogenic bacteria have drug resistance properties. In the second half of the twentieth century, glycopeptide (vancomycin) resistance was not reported, but since the 1980s, staphylococci and enterococci suddenly developed resistance to vancomycin [47]. In studies, it was determined that enterococcal and streptococcal plasmids and transposons that carrying antibiotic resistance genes were transferred to *Listeria* species by conjugation. Charpentier and Courvalin [48] reported that plasmid pIP501, which is responsible for the resistance of chloramphenicol, macrolide, lincosamide and streptogramin was found in *Streptococcus agalactie*, and can be transferred to *L. monocytogenes* under *in-vitro* conditions. Similarly, Biavasco et al.[47] reported that the resistance gene from vancomycin-resistant Enterococci strains was transferred to *Listeria* species. In our study, the resistance that we detected for different antibiotics can be attributed to the occurring of mutations in bacteria and to genetic material transfers caused by the interaction between bacteria.

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REFERENCES

1. Fanatico AC, Pillai PB, Emmert JL, Owens CM: Meat quality of slow and fast growing chicken genotypes fed low nutrient or standard diets and raised indoors or with outdoor access. *Poult Sci*, 86, 2245-2255, 2007. DOI: 10.1093/ps/86.10.2245

2. Alali WQ, Thakur S, Berghaus RD, Martin MP, Gebreyes WA: Prevalence and distribution of salmonella in organic and conventional broiler poultry farms. *Foodborne Pathog Dis*, 7, 1363-1371, 2010. DOI: 10.1089/fpd.2010.0566

3. Food and Agriculture Organization of the United Nations (FAO): Organic agriculture and the law, 2012.

4. Rouger A, Tresse O, Zagorec M: Bacterial contaminants of poultry meat: Sources, species, and dynamics. *Microorganisms,* 5:50, 2017. DOI: 10.3390/microorganisms5030050

5. Miranda JM, Vazquez BI, Fente CA, Calo-Mata P, Cepeda A, Franco CM: Comparison of antimicrobial resistance in *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* strains isolated from organic and conventional poultry meat. *J Food Prot*, 71, 2537-2542, 2008. DOI: 10.4315/0362-028x-71.12.2537

6. Cui S, Ge B, Zheng J, Meng J: Prevalence and antimicrobial resistance of *Campylobacter* spp. and *Salmonella* serovars in organic chickens from Maryland retail stores. *Appl Environ Microbiol,* 71 (7): 4108-4111, 2005. DOI: 10.1128/AEM.71.7.4108-4111.2005

7. Rosenquist H, Boysen L, Krogh AL, Jensen AN, Nauta M: *Campylobacter* contamination and the relative risk of illness from organic broiler meat in comparison with conventional broiler meat. *Int J Food Microbiol*, 162 (3): 226-230, 2013. DOI: 10.1016/j.ijfoodmicro.2013.01.022

8. EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control): The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. *EFSA J*, 14:4634, 2016

9. Buchanan RL, Gorris LGM, Hayman MM, Jackson TC, Whiting RC: A review of *Listeria monocytogenes:* An update on outbreaks, virulence, dose-response, ecology, and risk assessments. *Food Control*, 75, 1-13, 2017. DOI: 10.1016/j.foodcont.2016.12.016

10. Jamshidi A, Zeinali T: Significance and characteristics of *Listeria monocytogenes* in poultry products. *Int J Food Sci*, 2019:7835253, 2019. DOI: 10.1155/2019/7835253

11. ISO 11290-1: Microbiology of the Food Chain -Horizontal Method for the Detection and Enumeration of *Listeria monocytogenes* and of *Listeria* spp. Part 1: Detection Method. 2017.

12. Dynal AS: Cell Separation and Protein Purification, Technical Handbook. 2nd ed., Dynal AS. Norway Printed. 02 96, 1996.

13. Bohnert M, Dilasser F, Dalet C, Mengaud J, Cossart P: Use of specific oligonucleotides for direct enumeration of *Listeria monocytogenes* in food samples by colony hybridization and rapid detection by PCR. *Res Microbiol*, 143, 271-280, 1992. DOI: 10.1016/0923-2508(92)90019-k

14. Doumith M, Buchrieser C, Glaser P, Jacquet C, Martin P: Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. *J Clin Microbiol*, 42 (8): 3819-3822, 2004. DOI: 10.1128/JCM.42.8.3819-3822.2004

15. Clinical and Laboratory Standards Institute (CLSI): Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. 3rd ed, CLSI Guideline M45. Wayne, PA; 2016.

16. The European Committee on Antimicrobial Susceptibility Testing (EUCAST): Breakpoint Tables for Interpretation of MICs and Zone Diameters. Version 7.1, 2017.

17. Schafer DF, Steffens J, Barbosa J, Zeni J, Paroul N, Valduga E, Junges A, Backes GT, Cansian RL: Monitoring of contamination sources of *Listeria monocytogenes* in a poultry slaughterhouse. *LWT- Food Sci Technol*, 86, 393-398, 2017. DOI: 10.1016/j.lwt.2017.08.024

18. Rahmat GR, Ibrahim A, Bakar FA: Prevalence of *Listeria monocytogenes* in retail beef and poultry. *Pertanika,* 14 (3): 249-255, 1991.

19. Weis J: Vorkommen von Listerien in Hackfleisch. *Tierarztl Umsch*, 52 (7): 456-458, 1989.

20. Farber JM, Sanders GW, Johnston MA: A survey of various foods for the presence of *Listeria* species. *J Food Prot*, 52 (7): 456-458, 1989. DOI: 10.4315/0362-028X-52.7.456

21. Elmalı M, Can HY, Yaman H: Prevalence of *Listeria monocytogenes* in poultry meat. *Food Sci Technol*, 35 (4): 672-675, 2015. DOI: 10.1590/1678-457X.6808

22. Bailey JS, Fletcher DL, Cox NA: Recovery and serotype distribution of *L. monocytogenes* from broiler chickens in southeastern United States. *J Food Prot,* 52 (3): 148-150, 1989. DOI: 10.4315/0362-028X-52.3.148

23. Rorvik LM, Aase B, Alvestad T, Caugant DA: Molecular epidemiological survey of *Listeria monocytogenes* in broilers and poultry products. *J Appl Microbiol,* 94, 633-640, 2003. DOI: 10.1046/j.1365-2672.2003.01895.x

24. Alsheikh ADI, Mohammed GE, Abdalla MA: Isolation and identification of *Listeria monocytogenes* from retail broiler chicken ready to eat meat products in Sudan. *Int J Anim Vet Adv*, 5 (1): 9-14, 2013. DOI: 10.19026/ijava.5.5570

25. Alsheikh ADI, Mohammed GE, Abdalla MA, Bakhiet AO: First isolation and identification of *Listeria monocytogenes* isolated from frozen and shock frozen dressed broiler chicken in Sudan. *Br Microbiol Res J*, 4 (1): 28-38, 2014. DOI: 10.9734/BMRJ/2014/2449

26. Genigeorgis CA, Dutulescu D, Garayzabal JF: Prevalence of *Listeria* spp. in poultry meat at supermarket and slaughterhouse level. *J Food Prot*, 52 (9): 618-624, 1989.

27. Zeinali T, Jamshidi A, Bassami M, Rad M: Isolation and identification of *Listeria* spp. in chicken carcasses marketed in northeast of Iran. *Int Food Res J*, 24 (2): 881-887, 2017.

28. Basaran Kahraman B, Issa G, Kahraman T: Prevalence, antimicrobial resistance and molecular characterization of *Salmonella* spp. and *Listeria monocytogenes* isolated from chicken carcass. *Kafkas Univ Vet Fak Derg*, 24 (5): 775-779, 2018. DOI: 10.9775/kvfd.2018.19754

29. Carvalho FT, Vieira BS, Vallim DC, Carvalho LA, Carvalho RCT, Pereira RCL, Figueiredo EES: Genetic similarity, antibiotic resistance and disinfectant susceptibility of *Listeria monocytogenes* isolated from chicken meat and chicken-meat processing environment in Mato Grosso, Brazil. *LWT- Food Sci Technol*, 109, 77-82. 2019. DOI: 10.1016/j.lwt.2019.03.099

30. Oliveira TS, Varjão LM, da Silva LNN, Pereira, RCL, Hofer E, Vallim DC, Almeida RCC: *Listeria monocytogenes* at chicken slaughterhouse: Occurrence, genetic relationship among isolates and evaluation of antimicrobial susceptibility. *Food Control*, 88, 131-138, 2018. DOI: 10.1016/j.foodcont.2018.01.015

31. Zeinali T, Jamshidi A, Bassami M, Rad M: Serogroup identification and virulence gene characterization of *Listeria monocytogenes* isolated from chicken carcasses. *Iranian J Vet Sci Technol*, 7 (2): 9-19, 2015. DOI: 10.22067/veterinary.v7i2.43658

32. Arslan S, Baytur S: Prevalence and antimicrobial resistance of *Listeria* species and subtyping and virulence factors of *Listeria monocytogenes* from retail meat. *J Food Saf*, 39:e12578, 2019. DOI: 10.1111/jfs.12578

33. Zeinali T, Jamshidi A, Rad M, Bassami M: A comparison analysis of *Listeria monocytogenes* isolates recovered from chicken carcasses and human by using RAPD PCR. *Int J Clin Exp Med*, 8 (6): 10152-10157, 2015.

34. Maung AT, Mohammadi TN, Nakashima S, Liu P, Masuda Y, Honjoh K, Miyamoto T: Antimicrobial resistance profiles of *Listeria monocytogenes* isolated from chicken meat in Fukuoka, Japan. *Int J Food Microbiol*, 304, 49-57, 2019. DOI: 10.1016/j.ijfoodmicro.2019.05.016

35. Erol I, Ayaz ND: Serotype distribution of *Listeria monocytogenes* isolated from turkey meat by multiplex PCR in Turkey. *J Food Saf*, 31, 149-153, 2011. DOI: 10.1111/j.1745-4565.2010.00278.x

36. Walsh D, Duffy G, Sheridan JJ, Blair IS, McDowell DA: Antibiotic resistance among *Listeria*, including *Listeria monocytogenes*, in retail foods. *J Appl Microbiol*, 90, 517-522, 2001. DOI: 10.1046/j.1365-2672.2001.01273.x

37. Davis JA, Jackson CR: Comparative antimicrobial susceptibility of *Listeria monocytogenes, L. innocua* and *L. welshimeri*. *Microb Drug Resist*, 15, 27-32, 2009. DOI: 10.1089/mdr.2009.0863

38. Harakeh S, Saleh I, Zouhairi O, Baydoun E, Barbour E, Alwan N: Antimicrobial resistance of *Listeria monocytogenes* isolated from dairy based products. *Sci Total Environ*, 407, 4022-4027, 2009. DOI: 10.1016/j.scitotenv.2009.04.010

39. Rahimi E, Ameri M, Momtaz H: Prevalence and antimicrobial resistance of *Listeria* species isolated from milk and dairy products in Iran. *Food Control*, 21, 1448-1452, 2010. DOI: 10.1016/j.foodcont.2010.03.014

40. Bilir Ormancı FS, Erol I, Ayaz ND, Iseri O, Sariguzel D: Immunomagnetic seperation and PCR detection of *Listeria monocytogenes* in turkey meat and antibiotic resistance of the isolates. *Br Poult Sci*, 49 (5): 560-565, 2008. DOI: 10.1080/00071660802298328

41. Lemes-Marques EG, Cruz CD, Destro MT: Pheno and ghenotypic characterization of *Listeria monocytogenes* clinical isolates from the south-western region of the state of Sao-Paolo, Brazil. *Braz J Microbiol*, 38, 287-292, 2007. DOI: 10.1590/s1517-83822007000200019

42. Filiousis G, Johansson A, Frey J, Perreten V: Prevalance, genetic diversity and antimicrobial susceptibility of *Listeria monocytogenes* isolated from open-air food markets in Greece. *Food Control*, 20, 314-317, 2009. DOI: 10.1016/j.foodcont.2008.05.018

43. Osaili TM, Alaboudi AR, Nesiar EA: Prevalence of *Listeria* spp. and antibiotic susceptibility of *Listeria monocytogenes* isolated from raw chicken and ready-to-eat chicken products in Jordan. *Food Control*, 22, 586-590, 2011. DOI: 10.1016/j.foodcont.2010.10.008

44. Conter M, Paludi D, Zanardi E, Ghidini S, Vergara A, Ianieri V: Characterization of antimicrobial resistance of foodborne *Listeria monocytogenes*. *Int J Food Microbiol*, 128, 497-500, 2009. DOI: 10.1016/j.ijfoodmicro.2008.10.018

45. Yan H, Neogi SB, Mo Z, Guan W, Shen Z, Zhang S, Li L, Yamasaki S, Shi L, Zhong N: Prevalence and characterization of antimicrobial resistance of foodborne *Listeria monocytogenes* isolates in Hebei province of Northern China, 2005-2007. *Int J Food Microbiol,* 144, 310-316, 2010. DOI: 10.1016/j.ijfoodmicro.2010.10.015

46. Okada Y, Okutani A, Suzuki H, Asakura H, Monden S, Nakama A, Maruyama T, Igimi S: Antimicrobial susceptibilities of *Listeria monocytogenes* isolated in Japan. *J Vet Med Sci*, 73 (12): 1681-1684, 2011. DOI: 10.1292/jvms.11-0051

47. Biavasco F, Giovanetti E, Miele A, Vignaroli C, Facinelli B, Varaldo PE: In vitro conjugative transfer of VanA vancomycin resistance between Enterococci and Listeria of different species. *Eur J Clin Microbiol Infect Dis*, 15, 50-59, 1996. DOI: 10.1007/bf01586185

48. Charpentier E, Courvalin P: Antibiotic resistance in *Listeria* spp. *Antimicrob Agents Chemother*, 43 (9): 2103-2108, 1999.

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Research Article

The Use of Alkyd Resin Method in Wistar Rats for the Preparation of Teaching Materials and Museum Exhibits [1]

Selim ÇINAROĞLU [1,a] Hacı KELEŞ [1,b]

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[1] Department of Anatomy, Faculty of Medicine, Niğde Ömer Halisdemir University, TR-51240 Niğde - TURKEY
ORCIDS: [a] 0000-0002-4495-6106; [b] 0000-0002-0770-8269

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Abstract

Alkyd Resin method, patented by the Turkish Patent Institute, is a cadaver preparation and preservation technique. This study examines whether exhibition-museum materials can be produced from rat cadavers processed with alkyd resin method in different postures and whether dissection and suturing can be performed on the samples. Besides, samples prepared with alkyd resin and samples prepared freshly were compared in terms of dissection and suturing. 14 Wistar Albino Rats were used in the study. Seven were prepared with alkyd resin method and the others were freshly prepared for comparison in terms of suturing and dissection. The alkyd resin method was applied to two cadavers as a pretrial and to five cadavers for them to be museum-exhibition and educational materials. Alopecia was detected in two cadavers. In some of the exhibition samples processed with the method mentioned, shrinkage and hardening of the skin was noticed. All the samples prepared with this method became products suitable for suturing and dissection. The exhibition-museum materials prepared with the alkyd resin method have maintained their first body postures. We believe that alkyd resin method can be applied to the entire body, and the products can be used for exhibition and educational purposes. Furthermore, the method should be further popularized, and it should be applied to cadavers of different species so that its applicability increases.

Keywords: *Alkyd Resin, Cadaver, Museum-exhibits, Rat*

Alkid Resin Metoduyla Hazırlanan Wistar Ratlarının Müze-Sergi ve Eğitim Materyali Olarak Kullanımı

Öz

Türk Patent Enstitüsü tarafından patentlendirilen Alkid Resin metodu, kadavra hazırlama ve muhafaza tekniğidir. Bu çalışmada Alkid resin yöntemi ile işlenen rat kadavralarından farklı postürlerde sergi-müze materyalleri üretilip üretilemeyeceği ve elde edilen örneklerin üzerinde diseksiyon ve dikiş uygulamalarının yapılıp yapılamayacağı araştırılmıştır. Buna ilaveten, alkid resinle hazırlanan örnekler ile taze olarak hazırlanan örnekler diseksiyon ve dikiş uygulamaları yönünden karşılaştırılmıştır. Çalışmada 14 adet Wistar Albino Rat kullanıldı. Bunlardan 7 tanesi alkid resin yöntemi ile geriye kalan 7 tanesi ise dikiş uygulaması ve diseksiyon açısından Alkid resinle hazırlanan örneklerle karşılaştırmak amacıyla taze olarak hazırlandı. Alkid resin yöntemi iki rat kadavrasına ön deneme amacıyla beş rata ise müze-sergi ve eğitim materyali olacak şekilde uygulandı. Deneme amacıyla işlenen 2 adet rat kadavrasında kıllarda dökülme belirlendi. Derisi üzerinde adı geçen yöntemle işlenen sergi örneklerinin bazılarında deride büzüşme ve sertleşme dikkati çekti. Bu yöntem ile hazırlanan tüm örnekler dikiş atmaya elverişli ve diseksiyona imkân tanıyan ürünlere dönüştü. Alkid resin metodu ile hazırlanan sergi-müze materyallerinin işlendiği günden beri verilen vücut pozisyonlarını koruduğu gözlendi. Alkid resin tekniğinin deri ve iç organlar dâhil tüm vücuda uygulanabildiği, elde edilen ürünlerin sergi ve eğitim amaçlı kullanılabileceği sonucuna varılmıştır. Ayrıca, adı geçen tekniğin yaygınlaştırılması ve uygulanabilirliğinin arttırılması için insan dâhil değişik canlı türlerinin kadavralarında çalışılması gerekmektedir.

Anahtar sözcükler: *Alkid resin, Kadavra, Müze-sergi, Rat*

INTRODUCTION

Anatomy is one of the oldest areas of medicine and one of the cornerstones of medical education. In addition to providing information to students and physicians, anatomy also designs emotions and thoughts regarding the dead body [1,2]. Cadavers have been used for anatomy education and training for centuries. Cadaveric dissection does not only

Correspondence
+90 388 2252587 Mobile: +90 533 1277799
selimcinaroglu@ohu.edu.tr

provide students with information about the shape and size of organs, but also gives an idea about how each organ is positioned relative to the rest of the body. Dissection is believed to support self-learning and teamwork [3]. However, in recent years, anatomy education has changed drastically due to financial and ethical concerns, developing technology and difficulties in procuring cadavers [4]. The importance of anatomy education in medical schools and concerns about teaching standards should be discussed persistently. After many years, Warner and Rizzolo [5] and Turney [6] brought forward how anatomy education has fallen below the sufficient level. The way to cope with modern practices is to strengthen the traditional dissection education by introducing innovations into it. Many institutions officially reported that traditional cadaver dissection, which replaced anatomical models and technology, is obsolete [3,7,8].

The contribution of different methods to education and training in medical schools has always been a research topic [9]. In schools, small animals such as rats and frogs were often used to teach simple aspects of anatomy. Due to the increasing recognition of animal rights laws, this practice has also decreased in recent years. This has led to the teaching of anatomy in a variety of ways, including Procedures, Problem Based Learning Scenarios (PBL) or more recently computer systems derived from imaging techniques [3]. In anatomy education, discussions arose about using models, videos, 3D reconstruction and technological devices instead of using cadavers [4]. However, the studies conducted increase the opinion that education with cadaver facilitates learning, increases respect for body, and positively affects sense of touch and feeling [10,11]. Animal models and human bodies are utilized in training with cadaver [12].

Today, studies for education, research and surgical experiences on laboratory animals have gained speed. The cadavers prepared for training are preserved with various solutions or alternative methods. Many of these methods offer excellent opportunities to learn tissues and organs [3,13].

The most up-to-date technique of preparing cadavers for education and exhibition is plastination developed by Gunther Von Hagens who preserved cadavers by keeping them in different positions [1]. Developed in the last 30 to 40 years, plastination is a method that allows cadavers to be used in education and training for a long time and that brings a new perspective to gross anatomy [14]. In particular, with the introduction of plastination technology, the limits of human anatomy samples available for teaching have begun to expand and the potential value of these samples in research has increased appreciably [15].

Plastinated body or organs do not emit fluids or odors and do not contain insects and bacteria. They do not need extra space for storage, do not require maintenance and are harmless [16].

As it was reported by the visitors, compared to the odor, wetness and disturbing appearance in human and animal cadavers prepared using traditional methods, there was no odor, wetness and disturbing appearances in bodies created using this method, which was displayed in museums with the name "Body Worlds" in many countries (Japan, Germany, Belgium, England and the USA) and attracted considerable attention of the participants [17].

Arı and Çınaroğlu [18] developed a cadaver preparation method where alkyd resin is used. This method includes various steps. Firstly, in the fixation step, muscles and tissues are embedded in formaldehyde solution. Secondly, in the dehydration step, tissues are dehydrated and de-fatted. The third step, which is the embedding step, reduces the volumetric loss and softens the cadaver so that surgical interventions on the cadaver can be performed. In the fourth step, the pre-drying step, the excess chemical is drained out. The fifth step, impregnation step, allows the tissues to retain their peculiar properties after the specimen is treated with a preservative solution containing Alkyd resin and a solution containing toluene and xylene. Finally, the post-drying step is the step when alkyd resin in the specimens are hardened after being dried. Since alkyd resin is a natural resin, cadavers prepared with this method provide a great advantage since they do not require any special storage-preservation conditions in addition to having normal color and consistency being elastic and odorless so that intervention can be performed on them [19-22].

Working with laboratory animals requires staff who is experienced, trained and knowledgeable in biosafety regulations. Well-trained and experienced staff is very important practically, theoretically and also in terms of implementing international ethical regulations (Three R's principle of Russell and Burch) [13,23,24].

In this study, we aimed to examine the applicability of the Alkyd resin method to the whole body, skinless or with skin, its usability in the preparation of educational and museum-exhibition materials, and whether it will allow suturing and dissection on the produced materials.

MATERIAL and METHODS

The study was approved by the Animal Experiments Local Ethics Committee of Van Yüzüncü Yıl University (Decision no: 275521-238 Date: 11.05.2015).

In the study, the use of living material and texture was planned in two different ways.

1. In the experimental animal unit, of the 14 adult *Wistar Albino Rats* (11 males, 3 females) weighing 250-350 g, which were excluded from production and intended for sacrifice at various times, seven of them were prepared with the alkyd resin method to be used as sample and seven of them (whole body cadaver, including internal organs) were freshly

prepared for comparison with those prepared with alkyd resin. The combination of 5-8 + 75-90 mg/kg IM Xylazine + Ketamine was used for euthanasia. The abdominal aorta of the animals deeply euthanatized were cut, and their blood was drained out.

2. In the study titled "Examination of the effects of selenium in protecting against the negative effects of amiodarone on the thyroid" conducted in the experimental animals unit, some of the body parts of the animals taken as control group were kept fresh for use in the panel, while others were prepared with alkyd resin in addition to the seven whole body cadavers again for comparison on the panel.

Comparison Panel

Seven whole body cadavers prepared with alkyd resin, seven fresh whole body cadavers taken out of cold storage and body parts were taken to the room where the panel would be held to perform the dissection and suture processes. The panel was held with a total 35 participants in total consisting of lecturers and instructors (PhD students) in the medical and veterinary faculties (eight surgeons, two of whom were general surgeons, seven anatomists, four pharmacologists, four zootechnicians, two histologists and three biochemists who did not know how to perform suture, three physiologists, two internal diseases specialists and two gynecologists). However, five people who did not know how to perform suture were removed from the evaluation. Suture was applied to cadavers' muscle tissue in the form of simple suture. The people participating in the panel were taken to the hall where the panel was held one by one and asked to answer the questions given in *Table 1*. The answers were analyzed with the Mann-Whitney U Test.

Preparation of Fresh Cadavers

In the aforementioned study, the sacrificed body parts of the rats used as the control group were kept in the cold storage at -18°C until the day that the panel was held.

Application of the Alkyd Resin Method

Two of the seven whole body cadavers were processed with the alkyd resin method for trial purposes (the application of the method by determining the waiting period in solutions). According to the trial results, five rats in different postures were prepared with the same method to be used as educational and exhibition-museum material. Fixation, washing, dehydration, embedding, pre-drying, impregnation and final post-drying procedures were applied to each sample prepared with the alkyd resin method.

Table 1. *Panel questions asked for materials prepared fresh and with alkyd resin*

Applicability characteristics: Dissection and incision
√ Dissection and incision are not applicable (0-2) √ Dissection and incision are very difficult (3-5) √ Dissection and incision provide limited opportunity (6-8) √ Dissection and incision are applicable (9-10)
Do you know how to perform suture?
Yes √ No √
If your answer is yes, please mark one of the following
√ Cannot be sutured (0-2) √ Very difficult to suture (3-5) √ Can be sutured (6-8) √ Can be sutured very easily (9-10)

Fixation

The first step of the alkyd resin method is fixation. A catheter was placed into the abdominal aorta of the materials to be prepared for demonstration. After the probing, the vessels were washed with the physiological saline solution administered. Then, the cadavers were brought to the desired position with the help of various ropes, fishing line, cloth and a small hanging apparatus *(Fig. 1)*. Following the position procedure, the animals were fixed by administering Spence's cadaver fixing fluid via the same vessel by means of the catheter. For a 64 kg living being, this fixing fluid is obtained by mixing 2 L formaldehyde, 4 L methyl alcohol, 600 mL glycerin, 800 g phenol and 3 L water [25]. The materials, which were applied the fixing fluid were placed in 10% formalin, and the fixing process continued for one month. Before proceeding from this step to the next, the cadavers were properly dissected.

Washing

Samples taken from the fixation were washed with tap water for 24 h, and the excess formaldehyde was rinsed out.

Dehydration

The washed samples were processed through 50%, 60%, 70%, 80%, 90% and 96% alcohol series. The samples were kept for approximately eight hours in each alcohol series. The samples taken out of the alcohol were then kept in alcohol-acetone (50% alcohol and 50% acetone) solution for 24 h. In the last part of the dehydration step, the materials were immersed in acetone at room temperature

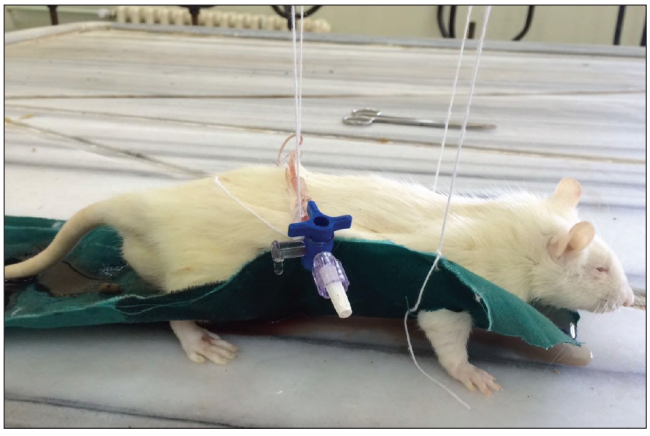

Fig 1. Rat cadaver, which was administered the fixation procedure

for 48 h. In this way, the fat and water levels in the samples were minimized.

Embedding

Dehydrated samples were placed in glycerol in vacuum desiccators and left under vacuum (220-380 mm Hg) for three days. Thus, the tissues hardened by acetone were partially softened.

Pre-drying

The samples taken out of glycerol were kept on a blotting paper for three days and cleared of excess glycerol.

Impregnation

Pre-dried materials were then immersed in alkyd resin solution in containers with a vacuum feature. They were kept in the vacuum (220-380 mm Hg) for 48 h. Thus, the alkyd resin was ensured to penetrated into the materials, and the impregnation phase ended.

Post-drying

The materials that thoroughly absorbed the alkyd resin solution were left to dry under normal room conditions for 15 days, and the alkyd resin method was finalized [18,19,21]. All the products were then taken onto the exhibition stand to protect them from damage after use.

RESULTS

The most challenging thing in the presented study was keeping the cadavers in the appropriate position while performing fixation. Alopecia, and shrinkage and hardening of the skin were detected in two rat cadavers used in the trial study *(Fig. 2)*. It is believed that the shrinkage and hardening of the skin, and alopecia are related to the waiting periods during the preparation of the materials. The preparation of the other five cadavers took 26 days, excluding the fixation period. The educational and exhibition-museum materials prepared with the alkyd resin method were found to have preserved their positions since the day they were processed. The original structure and size of the cadavers abdominal and chest organs were also observed to have been preserved *(Fig. 3, 4)*. The panel results for dissection and suturing applications are presented in *Table 2*. According to the results in *Table 2*, the difference between fresh and alkyd resin cadavers was revealed to be statistically insignificant in terms of dissection and suturing applications *(Fig. 5)*. The obtained samples have been preserved under room conditions for 46 months.

In terms of dissection characteristics, it was determined that the group with the alkyd resin behaved like fresh tissue and both groups were easily dissectable according to the participants' responses. Although there was no statistical difference between the responses given to the question regarding the applicability of dissection and incision for both groups, both groups were found to be 100% applicable regarding dissection and incision.

In the study, the participants of the panel stated that cadavers prepared with the alkyd resin behaved like fresh cadavers, and suturing was applicable for both groups.

DISCUSSION

Similar to other studies [18,19,21] conducted using the same alkyd resin method, in the present study, an apparent yellow color was not noticed among the observed colors of the samples obtained with the Alkyd resin method.

According to the anatomical study conducted by Thomas et al.[26], the presentation style of the anatomical study materials and the lack of the interrelationship between tissues and organs (the presentation of organs separately outside the cadaver) can be a disadvantage for training with cadaver. Whole body cadavers were prepared for the first time with this method, and it was concluded

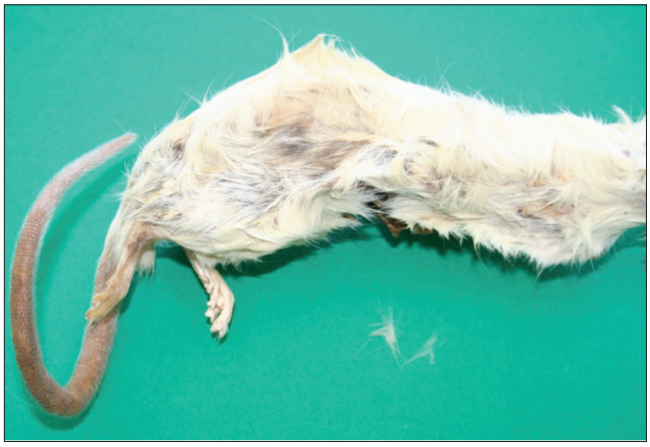

Fig 2. Alopecia and shrinkage of the skin

Table 2. *Panel comparison results of materials prepared fresh and with alkyd resin*

Processing Properties of Materials	N	Group	Median	*M*	*SD*	Min.	Max.	P
Dissection Characteristics	30	Fresh	10.00	9.30	1.70	4.00	10.00	.21
		A. Resin	10.00	9.80	0.76	7.00	10.00	
Can Be Sutured (Yes)	30	Fresh	7.00	7.73	2.34	2.00	10.00	.79
		A. Resin	7.00	8.11	1.49	7.00	10.00	

Fig 3. Products for educational and museum-exhibition purposes prepared with the alkyd resin method

Fig 4. General view of the internal organs in cadavers prepared with the alkyd resin method

that there was no change in the shape and size of the internal organs and the total structure of the cadaver. The cadavers prepared with alkyd resin method in this study, will eliminate the disadvantage of the lack of presentation stated in Thomas et al.[26] in terms of shape and image.

In their study, using an acetone meter, Ekim et al.[27] maintained the dehydration phase of the plastination method until the acetone concentration was balanced. In our study, it was revealed that there is more than one phase in the dehydration phase and an acetone meter cannot be used because of the alcohol use in these phases, and that keeping the samples in the dehydration phase longer causes shrinkage and hardening of the skin. However, it was also determined that establishing a standard in the application of this method for processing the skin of all other living groups was necessary.

Panel participants who knew how to perform sutures evaluated the samples prepared fresh and with the alkyd resin as "suturable". Only one panel participant (3.3%) emphasized that fresh tissues cannot be sutured. 100% of the participants stated that tissues with alkyd resin can be sutured. Panel participants expressed that dissection can be performed in both groups. This result coincides with the study results of Arı and Çınaroğlu [18], and Çınaroğlu and Arı [19] who stated that procedures such as dissection and suturing can be performed on tissues with alkyd resin.

Fig 5. Incision and suturing applied materials

Lewis et al.[28] argued that anatomical knowledge is required to learn surgery, that basic anatomical information and dissection times offered in the first year are decreasing, and that intern students do not remember this basic anatomical information. The authors state that detailed anatomical knowledge yields a high level of tissue and organ manipulation by improving the efficiency and safety of a surgeon in order to heal and save a patient, which is called anatomical engineering. They argue that while anatomy can also be learned from textbooks, atlases, computer models and projections, dissection is the most efficient method. In the same study, it was emphasized that the fact that endoscopic interventions, which largely replaced open surgeries, are performed on a two-dimensional screen limits the examination of the anatomical structures of the three-dimensional organs. Based on these arguments, they concluded that dissectible cadavers prepared fresh or prepared with alternative methods will increase knowledge and skill in surgical education and specific surgeries. There are also some other studies [29,30] where it is suggested that plastine rodents can be an excellent resource to learn their anatomy and to gain knowledge, and can be useful for innovative applications such as developing practice, comprehension, understanding complex anatomical relationships, surgical maneuvers, endoscopic trials, and developing autopsy protocols [13]. In addition to all these mentioned elements, it is reported that learning a new procedure with daily practices on living beings is quite dangerous for education and that practices on cadavers increase the education experience [31].

The present study proved that the alkyd resin method can be applied to the whole body and supported the arguments of Huri et al.[12], Lewis et al.[28] Stuart and Henry [29], Latorre et al.[30] and Tjalma et al.[31]. They argued that education with cadavers should not only be offered during schooling but should also be used in surgical training, the teaching of various surgical interventions, trial of new procedures and training of specialist physicians.

According to a study, it is estimated that 192.1 million experimental animals (including those that were produced for scientific studies but were not used or could not be used) were used worldwide in 2015 [32]. According to another study, it is stated that approximately one third of the experimental animals are lost as a result of education and wrong practices [33]. People working on laboratory animals should complete their education by avoiding toxic effects of the samples reflecting real tissue and by using the least number of animals [34]. Working with harmless materials reflecting the real tissue also reduces the anxiety that trainees and scientific researchers may have while handling live animals [34]. Within the framework of the 3R rule and due to ethical concerns, the alkyd resin method may be useful in order to prevent or reduce this number of animal losses in animal experiments. We produced rat cadavers that can be preserved in room conditions for 46 months. In studies conducted with lab animals, specifically with rats, these cadavers can be used for determining the places of organs and tissues, recognizing normal situses, marking the surgical intervention points, giving training before the study, teaching about anatomic structures, and preventing concerns that may occur with live animals.

With the innovations it will bring to anatomy education, the alkyd resin method may be an alternative to the old dissection methods mentioned by McLachlan et al.[3], Guttmann et al.[7] and McLachlan [8]. On this subject, the studies conducted by Arı and Çınaroğlu [18], Çınaroğlu and Arı [19], Çınaroğlu et al.[20], Keleş and Çınaroğlu [21], and Çınaroğlu and Keleş [22] contribute to gross anatomy. Similar to the abovementioned studies, this study also developed inexpensive anatomical models that can be preserved in room conditions for a long time. In addition to the mentioned studies, this study proved that these anatomical models can be dissected and incised and are suitable for suturing. Furthermore, with the six exhibitions opened in various regions in Turkey so far, this method is estimated to be a method that can be used together with the plastination method. These rat cadavers, which have been produced so far, have been presented to the scientific world for the purpose of exhibition and education, and academicians who want to conduct scientific studies and receive training visit the hall where cadavers are exhibited. In addition to the advantages brought by plastination of rodents stated in Ottone et al.[13], Stuart and Henry [29] and Latorre et al.[30], the alkyd resin method can offer similar advantages in anatomy classes and postgraduate studies.

In conclusion, given the purposes of the study, the alkyd resin method can be applied to the entire body, including skin and internal organs, and the products obtained can be used for exhibition and educational purposes. The technique in question should be applied to cadavers of different species to pursue its dissemination and to increase its applicability. Furthermore, it is believed that the examination of the alkyd resin method's dyeability characteristics like the ones in the plastination method, which we consider as a reference method in preparation of museum and exhibition materials is necessary.

REFERENCES

1. Al-Hossain MM: Museum and research center of anatomy. *PhD Thesis*, 28-31, BRAC University, Dhaka, Bangladesh 2015.

2. Çetkin M, Turhan B, Bahşi İ, Kervancıoğlu P: Tıp fakültesi öğrencilerinin anatomi eğitimi hakkındaki düşünceleri. *Gaziantep Med J*, 22 (2): 82-88, 2016.

3. McLachlan JC, Bligh J, Bradley P, Searle J: Teaching anatomy without cadavers. *Med Educ,* 38, 418-424, 2004. DOI: 10.1046/j.1365-2923.2004.01795.x

4. Estai M, Bunt S: Best teaching practices in anatomy education: A critical review. *Ann Anat*, 208, 151-157, 2016. DOI: 10.1016/j.aanat.2016.02.010

5. Warner JH, Rizzolo LJ: Anatomical instruction and training for professionalism from the 19th to the 21st centuries. *Clin Anat,* 19, 403-414, 2006. DOI: 10.1002/ca.20290

6. Turney BW: Anatomy in a modern medical curriculum. *Ann R Coll Surg Engl*, 89, 104-107, 2007. DOI: 10.1308/003588407X168244

7. Guttmann GD, Drake RL, Trelease RB: To what extent is cadaver dissection necessary to learn medical gross anatomy? A debate forum. *Anat Rec,* 281B, 2-3, 2004. DOI: 10.1002/ar.b.20042

8. McLachlan JC: New path for teaching anatomy: Living anatomy and medical imaging vs. dissection. *Anat Rec,* 281B, 4-5, 2004. DOI: 10.1002/ar.b.20040

9. Onuk B, Colak A, Arslan S, Sizer SS, Kabak M: The Effects of clay modeling and plastic model dressing techniques on veterinary anatomy training. *Kafkas Univ Vet Fak Derg,* 25 (4): 545-549, 2019. DOI: 10.9775/kvfd.2018.21304

10. Erbay H, Bilir A, Gönül Y, Turamanlar O, Songur A: Tıp fakültesi öğrencilerinin kadavra algısı ve eğitimde kadavra kullanımına yönelik yaklaşımları. *TJOB*, 2 (1): 63-72, 2015. DOI: 10.5505/tjob.2015.14633

11. Ögenler O, Kara A, Kadıoğlu S, Öztürk AH, Sungur MA: Bir grup anatomi öğretim elemanının kadavra ve eğitimde kadavra kullanma hakkındaki görüşleri. *TJOB*, 1 (1): 57-68, 2014. DOI: 10.5505/tjob.2014.29292

12. Huri E, Ezer M, Chan E: The novel laparoscopic training 3D model in urology with surgical anatomic remarks: Fresh-frozen cadaveric tissue. *Turk J Urol*, 42 (4): 224-229, 2016.

13. Ottone NE, Cirigliano V, Lewicki M, Bianchi HF, Aja-Guardiola S, Algieri RD, Cantin M, Fuentes R: Plastination technique in laboratory rats: an alternative resource for teaching, surgical training and research development. *Int J Morphol,* 32 (4): 1430-1435, 2014. DOI: 10.4067/S0717-95022014000400048

14. Bolintineanu SL, Pop E, Stancu G, Stancu G, Vaida MA, Sisu AM, Patrascu JM, Florescu S: Anatomical structures preservation using plastination techniques. *Mater Plast*, 54 (2): 221-224, 2017.

15. Priya K, Lama S, Magar A: Plastination--an unrevealed art in the medical science. *Kathmandu Univ Med J (KUMJ),* 5 (1): 139-141, 2007.

16. Ravikumar C: Plastination. *J Pharm Sci Res*, 6 (8): 271-273, 2014.

17. Moore CM, Brown CM: Experiencing body worlds: Voyeurism, education, or enlightenment? *J Med Humanit*, 28, 231-254, 2007. DOI: 10.1007/s10912-007-9042-0

18. Arı HH, Çınaroğlu S: A new approach to preservation of some organs using alkyd resin. *Res Vet Sci,* 1, 16-19, 2011. DOI: 10.1016/j.rvsc.2010.05.017

19. Çınaroğlu S, Arı HH: Investigation of macro anatomy of the urogenital system organs of norduz sheep by using the method of alkyd resin and preparation of their cadavers. *Van Vet J,* 26, 129-139, 2015.

20. Çınaroğlu S, Keleş H, Soygüder Z, Arı HH: Alkid resin yöntemi kullanılarak kadavra ruminant karaciğerinin hazırlanması. *9. Ulusal Veteriner Anatomi Kongresi,* Bildiri Kitabı, pp.49-50, 7-10 Eylül, Elazığ, 2015.

21. Keleş H, Çınaroğlu S: Taze ve alkid resinle hazırlanan rat sindirim sistemi organlarının tekstür ve renk değerlerinin karşılaştırılması. *Erciyes Üniv Vet Fak Derg,* 16 (3): 198-203, 2019.

22. Çınaroğlu S, Keleş H: Preparation of cadaver brain by using alkyd resin method. *20. Ulusal Anatomi Kongresi,* pp.146, 27-31 Ağustos, İstanbul, 2019.

23. Cornwall J, Hildebrandt S: Anatomy, education, and ethics in a changing world. *Anat Sci Educ*, 12, 329-331, 2019.DOI: 10.1002/ase.1898

24. Clark JM: The 3Rs in research: A contemporary approach to replacement, reduction and refinement. *Br J Nutr,* 120 (1): S1-S7, 2018. DOI: 10.1017/S0007114517002227

25. Yıldız B, İkiz İ: Kadavra yapımında ve korunmasında yaygın olarak kullanılan tespit sıvıları. *Uludağ Üniv Vet Fak Derg,* 12, 129-135, 1993.

26. Thomas RG, William John N, Delieu JM: Augmented reality for anatomical education. *J Vis Comm Med*, 33 (1): 6-15, 2010. DOI: 10.3109/17453050903557359

27. Ekim O, Tunalı S, Hazıroğlu RM, Ayvalı M: Evcil memeli hayvanlarda böbreklerin soğuk ortam tekniği ile silikon plastinasyonu. *Vet Hekim Der Derg*, 85 (2): 1-11, 2014.

28. Lewis CE, Peacock WJ, Tillou A, Hines OJ, Hiatt JR: A novel cadaver-based educational program in general surgery training. *J Surg Educ*, 69 (6): 693-698, 2012. DOI: 10.1016/j.jsurg.2012.06.013

29. Stuart MD, Henry RW: Plastinated specimens can improve the conceptual quality of biology labs. *Am Biol Teach,* 64 (2): 130-134, 2002. DOI: 10.1662/0002-7685(2002)064[0130:PSCITC]2.0.CO;2

30. Latorre RM, García-Sanz MP, Moreno M, Hernández F, Gil F, López O, Ayala MD, Ramírez G, Vázquez JM, Arencibia A, Henry RW: How useful is plastination in learning anatomy? *J Vet Med Educ,* 34 (2): 172-176, 2007. DOI: 10.3138/jvme.34.2.172

31. Tjalma WAA, Degueldre M, Van Herendael B, D'Herde K, Weyers S: Postgraduate cadaver surgery: An educational course which aims at improving surgical skills. *Facts Views Vis Obgyn*, 5 (1): 61-65, 2013.

32. Taylor K, Alvarez LR: An estimate of the number of animals used for scientific purposes worldwide in 2015. *Altern Lab Anim*, 47 (5-6): 196-213, 2019. DOI: 10.1177/0261192919899853

33. Taylor K, Gordon N, Langley G, Higgins W: Estimates for worldwide laboratory animal use in 2005. *Altern Lab Anim*, 36 (3): 327-342, 2008. DOI: 10.1177/026119290803600310

34. Streber ML, Davila T, Solano G, Esquivel L, Henry R: Production and use of plastinated rat models for teaching/Learning methods for bleeding. *ALTEX Proceedings 8th World Congress*, 21-25 Agust, Montreal, 2011.

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Research Article

Effects of Semen Extender Supplemented with Bovine Serum Albumin (BSA) on Spermatological Traits of Saanen Buck Semen Stored at +4°C

Asiye Izem SANDAL [1,a] Hatice SENLIKCI [1,b] Alper BARAN [1,c] Ozen Banu OZDAS [1,d]

[1] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Istanbul University - Cerrahpasa, TR-34320 Avcilar, Istanbul - TURKEY
ORCIDS: [a] 0000-0002-4952-7861; [b] 0000-0002-9568-2088; [c] 0000-0001-7053-3337; [d] 0000-0002-6867-5915

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Abstract

The aim of this study was to investigate the effects of two modified semen diluents on the spermatological parameters of Saanen goat sperm stored at 4°C. Ejaculates were obtained from five Saanen bucks by means of an electro-ejaculator and divided into five aliquots. Then, aliquots of ejaculates were used to create five experimental groups as follows: Group 1 (control), group 2A containing seminal plasma (SP) and bovine serum albumin (BSA; 10 mg/mL^{-1}), group 2B containing BSA without SP, group 3A containing egg yolk (EY; 17.00%) and SP and finally group 3B containing EY without SP. All aliquots of semen were extended with Tris-based extender. Spermatological parameters of experimental groups were observed at 0, 6, 12 and 24 h periods during storage at 4°C. Motility of semen in groups 2A and 2B was found to be best values compared to other groups. However, the motility of group 3A at 24 h was 0.00%. In conclusion, it was concluded that the addition of 10 mg/mL^{-1} of BSA to Tris-based extender could be useful in the storage of Saanen goat semen regardless of SP at 4°C.

Keywords: *Bovine serum albumin, BSA, Extender, Goat semen, Short term storage*

Sığır Serum Albumin (BSA) Katkılı Sulandırıcının +4°C'de Saklanan Saanen Teke Spermasının Spermatolojik Özellikleri Üzerine Etkileri

Öz

Bu çalışmanın amacı, modifiye edilmiş farklı iki sperma sulandırıcısının, 4°C'de saklanan Saanen ırkı teke spermalarının spermatolojik özellikleri üzerindeki etkilerini araştırmaktır. Ejakülatlar beş adet tekeden elektrojakülatör yardımıyla toplanmıştır ve beş eşit parçaya bölünmüştür. Daha sonra beş deney grubu oluşturmak üzere ejakülatlar şu şekilde porsiyonlanmıştır: grup 1 (kontrol), grup 2A seminal plazma (SP) ve bovine serum albumin içeren (BSA; 10 mg/mL^{-1}), grup 2B BSA içeren seminal plazma içermeyen, grup 3A yumurta sarısı (YS; %17.00) ve SP içeren ve son olarak grup 3B YS içeren SP içermeyen. Tüm semen porsiyonları Tris bazlı sulandırcılar ile sulandırılmışlardır. 4°C'ye soğutulma süresi boyunca deney gruplarının spermatolojik parametre gözlemleri 0., 6., 12. ve 24. saatlerde yapılmıştır. Grup 2A ve 2B'nin motilite değerlerinin diğer gruplarla karşılaştırıldığında en iyi sonuçları verdiği bulunmuştur. Fakat grup 3A'nın 24. saatteki motilitesi %0.00 olarak saptanmıştır. Sonuç olarak, Tris-bazlı sulandırıcıya ilave edilen 10 mg/mL^{-1} BSA'nın Saanen ırkı keçi spermasının soğutulmasında (4°C) seminal plazma farkı gözetilmeksizin kullanılabileceği sonucuna varılmıştır.

Anahtar sözcükler: Sığır serum albumini, *BSA, Sulandırıcı, Keçi semen, Kısa süreli saklama*

INTRODUCTION

The demand for goat milk has increased since processed goat milk form can be used as an alternative to cow's milk in terms of high levels of small fat spheres, calcium, iron, vitamin B12, vitamin C and vitamin D contents in the absence of breast milk [1]. Artificial insemination (AI) technique has an important place in goat breeding. It is one of the most frequently preferred biotechnological methods, especially in intensive cultivation production systems, to control reproduction and creation of high yield (meat, milk and wool) elite herds [2]. The AI has several advantages in terms of goat breeding. For example, transfer of the genes of an animal with high milk yield to another low-yielding animal with AI, transfer of genes between different farms and short-term storage options are a few

Correspondence
+90 212 473 7070/17263
izem@istanbul.edu.tr

of these advantages. By storing semen in the cold (+4°C) or transport of chilled semen from one place to another in suitable semen extenders requires less equipment and labor than cryopreservation and allows the transfer of good quality genes over long distances [2,3]. At the same time, since the metabolism of cooled spermatozoa is reduced to the basal level, sperms can maintain their viability for up to 24 h or more [4]. To support this, one of the studies reporting higher the pregnancy rate obtained using semen stored at room temperature or cooled to +4°C than that of frozen semen can be given as an example [4]. For example, Kulaksız et al.[5], have shown that domestic or exotic goat breeds' semen samples may have different sensitivities against cryopreservation. In addition, in a study conducted in Gurcu bucks, fresh and post-thawing spermatological parameters have been reported to vary depending on the season [6]. Of course, in chilled semen, problems like decreased motility, deterioration of structural integrity, decreased fertility and increased embryonic losses can occur [7]. However, all these complications due to spermatozoa damage are less than frozen semen. The most common method of protection against harmful effects that may occur during sperm chilling is to add egg yolk to the extenders [8]. However, egg yolk has a dis-advantage; each prepared extender with egg yolk may not have the same structure. That is, it is difficult to standardize it. Because eggs may contain various pathogens and also quality differences may occur depending on the time period after laying and storage conditions [9,10]. The presence of the egg yolk coagulation enzyme (EYCE), which is secreted from the bulbourethral gland and named as phospholipase A2, reduces the survival rate of goat spermatozoa [11]. The EYCE shows this effect by hydrolyzing egg yolk lecithin to fatty acids and lysolecithin [12]. Toxic substances released after hydrolysis cause damage to spermatozoa membrane and DNA by stimulating chromatin decondensation as a result of acrosome reaction. Considering all of these reasons, separation of seminal plasma may be a good choice with different solutions or it may be necessary to develop a different type of extender that does not contain egg yolk [13,14]. There are studies mentioning the use of bovine serum albumin (BSA) as an alternative protein source in goat semen extenders instead of egg yolk and it has been reported that it can be used not only for buck semen but also for ram semen extenders [10,15]. The BSA is a protein molecule with a large structure which is available in reproductive system fluids. Furthermore, in many research, it has been reported that BSA increases the viability and motility of spermatozoa after cryopreservation [16]. This study was conducted to investigate the usability of Tris-based semen extenders supported by BSA instead of egg yolk as a cryoprotectant in the cooling of buck semen.

MATERIAL and METHODS

Ethical Approval

All procedures on animals were carried out according to approval by the Local Ethics Committee for Animal Experiments of Istanbul University with the number of 2016/24 during this study.

Bucks and Semen Collection

In breeding season (September-December), semen samples were collected from five Saanen bucks (3-4 years of age) housed for this aim. The bucks belonged to the Reproduction and Artificial Insemination Department, Faculty of Veterinary Medicine, Istanbul University, Turkey and kept under regular nutrition and care conditions (roughage = 2.000 g, concentrated feed = 500 g; crude protein = 20.00%; crude cellulose = 6.80%; crude ash = 7.40%; crude fat = 3.20%; Calcium = 1.00%; Phosphorus = 0.60% and Sodium = 0.40%). Semen was collected from each buck by electro-ejaculation method and during this time, the bucks were sedated with 0.22 mg/kg^{-1} intramuscular injections of xylazine (Alfazyne, Ege Vet, Izmir, Turkey) and 1.10 mg/kg^{-1} flunixin meglumine (Finadyne, Intervet, Istanbul, Turkey). Semen was obtained with the aid of small electrical currents using an electro-ejaculator device (Ruakura, MK IV Ram Probe; Alfred Cox, Surrey, UK). This process was repeated 10 times, twice a week. During the semen collection procedure, the pre-lubricated rectal probe was placed in about 10 cm into the rectum of the sedated buck, laying on its side. Semen was collected into sterile tubes heated to 35°C at specific time intervals and in sets, as a result of a total of 10 electrical stimulations.

Evaluation of Semen

Immediately after ejaculates were obtained from each buck, they were stored in the water bath at 26°C and samples were taken for spermatological examinations from each ejaculate. Sperm volume, motility, and concentration were determined for ejaculates. Volume was measured with the help of an automatic pipette. Subjective motility was evaluated in a phase-contrast microscope and spermatozoa concentration was assessed with a Thoma cell counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) using Hancock solution [17]. As a result of all these examinations, only ejaculates with ≥80% motility rate and 2×10^9 per mL sperm concentration were used for dilution, cooling, and storage stages in the current study. All selected ejaculates were pooled to minimize individual differences among bucks before the study procedures.

Dilution and Cooling

Pooled ejaculates were divided into five aliquots and processed as follows: 1) Aliquot was diluted with Tris-based extender (TBE; Tris 273.70 mM, Fructose 55.50 mM, Citric acid 72.87 mM, Penicillin 1000 IU/mL^{-1} and Streptomycin 1 mg/mL^{-1}) with seminal plasma as control (group 1) [18]. All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, Missouri, USA); 2) An aliquot was diluted with TBE containing BSA (10 mg/mL^{-1}) with seminal plasma

in group 2A; 3) An aliquot was diluted with TBE containing BSA (10 mg/mL^{-1}) and without seminal plasma in group 2B; 4) An aliquot was diluted with TBE added with egg yolk (17.00%) with seminal plasma in group 3A; 5) An aliquot was diluted with TBE added with egg yolk (17.00%) and without seminal plasma in group 3B [17]. Aliquots from pooled ejaculate were centrifuged for 5 min at 1.500 g in group 2B and group 3B in order to separate seminal plasma. Instead of removed supernatants, extenders of their own group were added, as much as the volume they were removed. All semen samples were diluted, to reach final concentration of 800×10^6 per mL spermatozoa. Cooling was performed with Bio Cool® (BC-III-40; SP Industries, New York, USA) at a rate of 0.20-0.30°C per min. Motility, progressive motility, viability and morphology (percentage of abnormal spermatozoa) examinations were recorded at 0th, 6th, 12th and 24th h in semen cooled from 26°C to 4°C [2,19]. Post-cooling motility and progressive motility control were performed with a computer-assisted sperm analyzer (CASA, HTM-IVOS, version 12.3; Hamilton Thorne Biosciences, Beverly, USA). The ratio of live spermatozoa was determined using eosin-nigrosin staining method [19]. Dead/alive examination was evaluated under a light microscope at 400× [17,19]. Morphological examinations (acrosome, head, mid-piece and tail abnormalities) were performed on samples taken from the mixture prepared with Hancock solution with immersion oil technique under a phase-contrast microscope (1000×). At least 200 spermatozoa were evaluated for each sample in the calculation of morphological examination and viability percentage [17,19].

Statistical Analysis

All analyses were performed using IBM SPSS Statistics for Windows, Version 21.0. (Armonk, NY: IBM Corp.). All data were expressed as mean ± standard deviation. Two-way mixed ANOVA method was performed for motility, live spermatozoa and abnormal spermatozoa rates. Observations at 0th, 6th, 12th and 24th h were taken as within-subject variables, groups (1, 2A, 2B, 3A and 3B) were taken as between-subject factor and followed by Games-Howell post hoc tests. Shapiro-Wilk Normality Test was used to test the normality of distribution assumption, Levene's Test for homogeneity of variance assumption and Mauchly's Test of Sphericity for testing the sphericity assumption. All statistics were two-tailed and a P value of less than 0.05 was considered significant.

RESULTS

Motility Assessment

A two-way mixed ANOVA was conducted to examine the effect of experimental groups (1, 2A, 2B, 3A and 3B) and time (0th, 6th, 12th and 24th h) on motility rate (%). The analysis revealed a main effect of time (F (2.7, 121.56) = 479.72, P<0.001) and an interaction between time and groups (F (10, 8, 121.56) = 33.26, P<0.001).

Post hoc tests showed that motility rate was significantly higher in Group 1 compared to Group 3A (Mdiff = 21.4, P<0.01), in Group 2A compared to Group 3A (Mdiff = 28.4, P<0.001), in Group 2B compared to Group 3A (Mdiff = 25.5, P<0.001) and in Group 3B compared to Group 3A (Mdiff = 15.4, P<0.05).

Also, there was no statistical significance between Group 1-Group 2A, Group 1-Group 2B, Group 1-Group 3B, Group 2A-Group 2B, Group 2A-Group 3B, Group 2B-Group 3B (P>0.05). The worst results were found in Group 3A compared to all groups in terms of motility *(Table 1)*.

Viability Assessment

A two-way mixed ANOVA was conducted to examine the effect of experimental groups (1, 2A, 2B, 3A and 3B) and time (0th, 6th, 12th and 24th h) on live spermatozoa rates (%). The analysis revealed a main effect of time (F (3, 135) = 401.7, P<0.001) and an interaction between time and groups (F (12, 135) = 13.9, P<0.001).

Post hoc tests showed that live spermatozoa rates were significantly higher in Group 1 compared to Group 3A (Mdiff = 26.1, P<0.001), in Group 2A compared to Group 3A (Mdiff = 27.8, P<0.001) and in Group 2B compared to Group 3A (Mdiff = 32.7, P<0.001).

Also, there was no statistical significance between Group 1-Group 2A, Group 1-Group 2B, Group 1-Group 3B, Group 2A-Group 2B, Group 2A-Group 3B, Group 2B-Group 3B, Group 3A-Group 3B (P>0.05). The worst results were found in Group 3A compared to all groups in terms of live spermatozoa rates *(Table 2)*.

Morphological Assessment

A two-way mixed ANOVA was conducted to examine the

Table 1. *0, 6, 12 and 24 h motility results (%)*

Time	Group 1 Control	Group 2A BSA & SP+	Group 2B BSA & SP-	Group 3A EY & SP+	Group 3B EY & SP-
0 h	50.00±7.61	71.80±7.75	71.50±8.51	70.80±11.24	74.00±6.99
6 h	66.50±12.70	64.50±10.91	61.00±11.49	29.00±20.78	50.00±16.49
12 h	55.50±14.99	47.00±14.94	42.00±20.02	8.50±5.29	32.50±15.32
24 h	22.00±4.83	38.50±11.55	36.00±16.46	0.00±0.00	13.50±14.91

BSA: *Bovine Serum Albumin;* ***SP:*** *Seminal Plazma;* ***EY:*** *Egg Yolk; +/-: with/without*

Table 2. *0, 6, 12 and 24 h live spermatozoa rates (%)*

Time	Group 1 Control	Group 2A BSA & SP+	Group 2B BSA & SP-	Group 3A EY & SP+	Group 3B EY & SP-
0 h	73.60±10.47	68.00±11.82	76.60±10.69	67.60±14.51	71.20±9.97
6 h	56.90±14.11	58.10±12.87	68.80±15.61	24.80±20.16	50.60±22.18
12 h	54.50±16.16	53.30±15.83	49.50±10.12	12.50±3.27	41.10±21.58
24 h	24.60±8.69	36.90±7.27	40.90±18.42	0.00±0.00	13.80±15.31

***BSA:** Bovine Serum Albumin; **SP:** Seminal Plazma; **EY:** Egg Yolk; **+/-:** with/without*

Table 3. *0, 6, 12 and 24 h abnormal spermatozoa rates (%)*

Time	Group 1 Control	Group 2A BSA & SP+	Group 2B BSA & SP-	Group 3A EY & SP+	Group 3B EY & SP-
0 h	9.50±3.50	10.60±4.40	10.90±5.25	12.40±5.68	11.50±4.42
6 h	12.00±3.43	14.00±4.39	15.20±7.34	18.50±13.31	14.80±4.61
12 h	34.50±3.43	17.70±3.83	21.20±7.68	42.60±7.98	34.40±2.50
24 h	52.40±9.29	29.00±6.61	35.60±7.54	77.50±7.01	48.10±5.06

***BSA:** Bovine Serum Albumin; **SP:** Seminal Plazma; **EY:** Egg Yolk; **+/-:** with/without*

effect of experimental groups (1, 2A, 2B, 3A and 3B) and time (0th, 6th, 12th and 24th h) on abnormal spermatozoa rates (%). The analysis revealed a main effect of time ($F(2.5, 114.7) = 423.6$, $P<0.001$) and an interaction between time and groups ($F(10.1, 114.7) = 22.2$, $P<0.001$).

Post hoc tests showed that abnormal spermatozoa rates were significantly higher in Group 1 compared to Group 2A (Mdiff = 9.2, $P<0.001$), in Group 1 compared to Group 2B (Mdiff = 6.3, $P<0.05$), in Group 3A compared to Group 1 (Mdiff = 10.6, $P<0.001$), in Group 3A compared to Group 2A (Mdiff = 19.9, $P<0.05$), in Group 3A compared to Group 2B (Mdiff = 17, $P<0.001$), in Group 3A compared to Group 3B (Mdiff = 10.5, $P<0.001$), in Group 3B compared to Group 2A (Mdiff = 9.3, $P<0.001$) and in Group 3B compared to Group 2B (Mdiff = 6.4, $P<0.05$).

Also, there was no statistical significance between Group 1-Group 3B, Group 2A-Group 2B, Group 2A-Group 3B, Group 2B-Group 3B ($P>0.05$). The worst results were found in Group 3A compared to all groups in terms of abnormal spermatozoa rates *(Table 3)*.

DISCUSSION

In recent years, proteins that reduce cell damage and cell loss, such as BSA, have been added to freezing solutions as membrane stabilizers to optimize cryopreservation of cells. It has been reported that the use of 10% or 15% of BSA can replace egg yolk in ram semen diluents and can be used to increase sperm motility and viability after thawing [20]. Similar to this research, our study showed that the addition of BSA (10%) to two groups' extenders used in cooling activities of buck semen has a positive effect on motility, viability, and abnormal spermatozoa rates. It is thought that BSA can show this positive effect due to its protein structure, cryoprotective and cold protection properties. In other words, BSA, used instead of egg yolk, was found to be beneficial in terms of spermatological traits in the short-term storage of buck semen with or without seminal plasma. In the present study, motility and viability rates (%) were found to be 38.50±11.55 & 36.90±7.27 for group 2A and 0.00±0.00 & 0.00±0.00 for group 3A at 24th h, respectively. These results support the motility data of the study of Naijian et al.[15], in which they frozen and thawed buck semen using 10.00% BSA supplemented Tris-based extender. It is well known that egg yolk-based extenders are often used to protect spermatozoa against cold shock during short-term storage and it is recommended by many researchers that low-density lipoproteins (LDL) may be required for spermatozoa protection [21,22]. Some scientists also claim that LDL adheres to the cell membrane during cooling and freezing [23,24]. In this study, we aimed to investigate a substitute for egg yolk by considering different thoughts about egg yolk based extenders. Besides all these effects, there are a few important points to remember about egg yolk. That is since the egg yolk is a biological substance, it is unlikely that each egg will have the same characteristics. In such a case, the percentage of protein contained in each semen extender prepared with egg yolk will not be the same. In addition to this, it should be taken into account that individual egg yolk quality may vary depending on the number of days after laying and storage as well as the fact that egg yolk may be the carrier of various pathogens [9,10]. Moreover, the presence of EYCE, also known as phospholipase A, originating from the bulbourethral gland, is reported to reduce the survival rates of goat spermatozoa [11]. This function of EYCE is known due to the hydrolysis of egg yolk lecithins to fatty acids and lysolecithins [12]. This final component is harmful for the buck spermatozoa. For these reasons, it may be

necessary to develop a synthetic extender without egg yolk. The sudden drop in the motility rate (%) in group 3A (8.50±5.29) at the 12th h supports this idea. Additionally, the abnormal spermatozoa ratios given in *Table 3* were quite high in group 3A (77.50±7.01), although this ratio was lower in group 2A at 24th h (29.00±6.61) compared to group 1 (52.40±9.29). These results strongly support the harmful effect of EYCE and the positive effect of BSA on spermatological traits. At the 12th h, although there was no distinction in motility between the group 1 (55.50±14.99) and group 2A (47.00±14.94) or group 2B (42.00±20.02), a higher value was observed in the control group in terms of morphological disorder. This shows that the cryoprotective effect of BSA in seminal plasma or non-seminal plasma medium positively affects spermatozoon morphological integrity in cooling and storage of goat semen. BSA maintains membrane integrity by regulating the cholesterol flow from the plasma membrane of the mammalian spermatozoa to the outside of the cell to prevent fat accumulation on the cell membrane [25]. Cryoprotective effect of BSA is still continuing as the prolonged period of cold storage of sperm (by the 24th h) and synergistic effect especially in seminal plasma environment. In the group 3A there was a reduction more than 80% in motility within 12 h, whereas in group 2A there was no dramatic decrease like in group 3A. Similarly, when the motility ratio was evaluated in the seminal plasma groups [group 2A (47.00±14.94) and group 3A (8.50±5.29)] at the 12th h, there was a strong distinction between them in terms of motility. When the same groups were examined at 24th h, it was observed that the motility continued in group 2A but stopped completely in group 3A. As expected in live spermatozoa rates at 24th h, the best percentage values were found in groups 2A (36.90±7.27) and 2B (40.90±18.42) compared to group 3A (0.00±0.00). When the abnormal spermatozoa rates were examined at 24th h, the best value was found in group 2A (29.00±6.61) and the worst value was found in group 3A (77.50±7.01). Further, the BSA is a special and critical source of protein for the reproductive system. It has been preferred in many experiments related to *in vitro* embryo production of different species and successful results have been obtained [26-28]. Moreover, it has been reported by researchers that BSA has beneficial effects on sperm motility and viability in many experiments [10,15,16]. Many studies have supported that BSA-supplemented extenders reduce contact between buck seminal plasma proteins and seminal plasma components like spermatozoa membrane [28]. In a study by Beltran et al.[29], the use of 10 mg of BSA resulted in 32.91% motility after thawing. In the same study, they used different amounts of egg yolk to the diluent and found that the best motility result after thawing was found in the 5% egg yolk group. This result suggests that the low egg yolk ratio may cause less coagulation and may be higher for post-thaw motility. In our study, the best results in terms of motility, viability and abnormal spermatozoon after cooling in group 2A were similar to Beltran et al.[29]. As a result, the known disadvantages of EYCE on spermatozoa are tried to be overcomed with the use of BSA. Positive results such as enabling the transfer of Ca^{2+} ions to the plasma membrane, cholesterol and phospholipid ratio reduction in the external acrosomal membrane and sperm hyperactivity stimulation appear to be the evidence of the beneficial effect of using BSA as a supplement in semen extenders [30]. According to many works, it has been reported that BSA protects the integrity of the preserved membrane of sperm cells against unexpected temperature changes and oxidative stress [10,30]. For all these reasons, this study suggests the use of BSA instead of egg yolk in extenders used for cooling of buck semen. In conclusion, the best values found in the BSA-supplemented groups (groups 2A and 2B) in terms of motility, morphology, and viability. The harmful effects of egg yolk supplemented Tris-based extender on motility, morphology, and viability were observed intensely between 6 h and 24 h. In the cooling process up to the 6th h, when the groups 2A, 2B and 3B were examined, no sudden and rapid reduction was found in the motility rates, but it was observed that the motility decreased rapidly in group 3A. The reason for this is thought to be denaturation of proteins, agglutination and initiation of ROS activity as a result of the reaction of EYCE with egg yolk. If seminal plasma is removed by centrifugation, egg yolk-supplemented Tris-based extender can be used. However, since this method has a negative effect on spermatozoa viability and consequently causes time and material losses, it can be said that the use of BSA instead of egg yolk may be more beneficial in short-term storage of buck semen.

REFERENCES

1. Altun D, Sarıcı SU: Goat milk: Should it be the first choice for baby feeding? Çocuk *Sağ Hast Derg*, 60, 22-33, 2017.

2. Leboeuf B, Restall B, Salamon S: Production and storage of goat semen for artificial insemination. *Anim Reprod Sci*, 62, 113-141, 2000. DOI: 10.1016/S0378-4320(00)00156-1

3. Alamo D, Batista T, Gonzalez F, Rodriguez N, Cruz G, Cabrera F, Garcia A: Cryopreservation of semen in the dog: Use of ultra-freezers of -152°C as a viable alternative to liquid nitrogen. *Theriogenology*, 63, 72-82, 2005. DOI: 10.1016/j.theriogenology.2004.03.016

4. Salvador I, Yaniz J, Viudes-de-Castro MP, Gomez EA, Silvestre MA: Effect of solid storage on caprine semen conservation at 5°C. *Theriogenology*, 66, 974-981, 2006. DOI: 10.1016/j.theriogenology.2006.02.042

5. Kulaksız R, Ari UC, Daskin A, Güner AG: The effect of different glycerol concentrations on freezability of semen from Angora, Kilis and Saanen goats. *Slovak J Anim Sci*, 46, 39-44, 2013.

6. Kulaksız R, Ari UC, Kuru M, Yıldız S, Lehimcioğlu NC, Oztürkler Y, Atakişi E: Seasonal changes in testes size, fresh and post-thawing semen characteristics, serum testosterone level, and phospholipase A_2 activity in Gurcu male goats. *J Anim Plant Sci*, 29 (2): 353-358, 2019.

7. Yotov SA, Velislavova DV, Dimova LR: Pregnancy rate in Bulgarian white milk goats with natural and synchronized estrus after artificial insemination by frozen semen during breeding season. *Asian Pac J Reprod*, 5 (2): 144-147, 2016. DOI: 10.1016/j.apjr.2016.01.011

8. Valente SS, Pereira RM, Baptista MC, Marques CC, Vasques MI, Pereira MVCS, Horta AEM, Barbas JP: *In vitro* and *in vivo* fertility of ram semen cryopreserved in different extenders. *Anim Reprod Sci*, 117, 74-77, 2010. DOI: 10.1016/j.anireprosci.2009.04.007

9. Roy A: Egg-yolk coagulating enzyme in the semen and Cowper's gland

of the goat. *Nature,* 179, 318-319, 1957. DOI: 10.1038/179318b0

10. Matsuoka T, Imai H, Kohno H, Fukui Y: Effects of bovine serum albumin and trehalose in semen diluents for improvement of frozen-thawed ram spermatozoa. *J Reprod Dev,* 52, 675-683, 2006. DOI: 10.1262/jrd.18033

11. Bajuk BP, Pihlar T, Pogacnik N, Klinc P: Dialysis of the goat semen and its effect on the quality of frozen/thawed spermatozoa processed in the presence of egg yolk. *Anim Reprod Sci,* 198, 65-73, 2018. DOI: 10.1016/j.anireprosci.2018.09.001

12. Purdy PH: A review on goat sperm cryopreservation. *Small Ruminant Res,* 63, 215-225, 2006. DOI: 10.1016/j.smallrumres.2005.02.015

13. Ari UC, Daskin A: Influence of seminal plasma separation with different solutions on post-thaw sperm quality of Angora male goat semen. *Reprod Domest Anim,* 45 (3): 76, 2010.

14. Ari UC, Daskin A: Freezing of washed Angora goat semen with extenders added bull or ram seminal plasma. *Kafkas Univ Vet Fak Derg,* 16 (2): 233-237, 2010. DOI: 10.9775/kvfd.2009.591

15. Naijian HR, Kohram H, Zare Shahneh A, Sharafi M: Effects of various concentrations of BSA on microscopic and oxidative parameters of Mahabadi goat semen following the freeze-thaw process. *Small Ruminant Res,* 113, 371-375, 2013. DOI: 10.1016/j.smallrumres.2013.03.015

16. Alcay S, Toker MB, Gokce E, Onder NT, Ustuner B, Nur Z: Long term incubation resilience of post-thaw ram semen diluted with lecithin-based extender supplemented with bovine serum albumin. *Kafkas Univ Vet Fak Derg,* 25 (3): 291-297, 2019. DOI: 10.9775/kvfd.2018.20843

17. Salamon S, Ritar AJ: Deep freezing of Angora goat semen: Effects of diluent composition and method and rate of dilution on survival of spermatozoa. *Aust J Biol Sci,* 35, 295-304, 1982. DOI: 10.1071/BI9820295

18. Evans G, Maxwell WMC: Salamon's Artificial Insemination of Sheep and Goats. Butterworths, Wellington, Sydney, 1987.

19. Fukui Y, Kohno H, Togari T, Hiwasa M: Fertility of ewes inseminated intrauterinally with frozen semen using extender containing bovine serum albumin. *J Reprod Dev,* 53 (4): 959-962, 2007. DOI: 10.1262/jrd.19017

20. Alcay S, Toker MB, Gokce E, Ustuner B, Onder NT, Sagırkaya H, Nur Z, Soylu MK: Successful ram semen cryopreservation with lyophilized egg yolk-based extender. *Cryobiology*, 71, 329-333, 2015. DOI: 10.1016/j.cryobiol.2015.08.008

21. Allai L, Druart X, Louanjli N, Contell J, Nasser B, El Amiri B: Improvements of ram semen quality using cactus seed oil during liquid preservation in Tris egg yolk and skim milk based extenders. *Small Ruminant Res,* 151, 16-21, 2017. DOI: 10.1016/j.smallrumres.2017.02.001

22. Graham JK, Foote RH: Effect of several lipids, fatty acyl chain length, and degree of unsaturation on the motility of bull spermatozoa after cold shock and freezing. *Cryobiology,* 24, 42-52, 1987. DOI: 10.1016/0011-2240(87)90005-8

23. Liu CH, Dong HB, Ma DL, Li YW, Han D, Luo MJ, Chang ZL, Tan JH: Effects of pH during liquid storage of goat semen on sperm viability and fertilizing potential. *Anim Reprod Sci*, 164, 47-56, 2016. DOI: 10.1016/j.anireprosci.2015.11.011

24. Visconti PE, Kopf GS: Regulation of protein phosphorylation during sperm capacitation. *Biol Reprod*, 59, 1-6, 1998. DOI: 10.1095/biolreprod59.1.1

25. Pedersen ME, Ozdas OB, Farstad W, Tverdal A, Olsaker I: Effects of bovine oviduct epithelial cells, fetal calf serum and bovine serum albumin on gene expression in single bovine embryos produced in the synthetic oviduct fluid culture system. *Reprod Fertil Dev,* 17 (8): 751-757, 2005. DOI: 10.1071/rd05048

26. Enginler SO, Sandal AI, Ozdas OB, Arici R, Erturk E, Çınar EM, Mohammed IF, Baran A, Tek C, Gündüz MC: The effect of oviductal cells on *in vitro* maturation of canine oocytes in different culture media. *Turk J Vet Anim Sci*, 38, 14-19, 2014. DOI: 10.3906/vet-1306-57

27. Sandal AI, Ozdas OB: Vitrification of *in vitro*-produced bovine embryos matured in modified TCM-199 medium. *Turk J Vet Anim Sci,* 39, 688-692, 2015. DOI: 10.3906/vet-1507-105

28. Yamashiro H, Kumamoto K, Wang HF, Yamashita Y, Terada T: Effect of semen collection in extender solution on the characteristics of goat spermatozoa. *J Reprod Dev*, 52, 397-406, 2006. DOI: 10.1262/jrd.17104

29. Beltran BAG, Atabay EP, Atabay EC, Cruz EM, Aquino FP, Cruz LC: Optimized extenders for cryopreservation of buck semen for artificial insemination. *Philipp J Vet Anim Sci,* 39 (1): 1-10, 2013.

30. Uysal O, Bucak MN, Yavas I, Varisli O: Effect of various antioxidants on the quality of frozen-thawed bull semen. *J Anim Vet Adv*, 6, 1362-1366, 2007.

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Research Article

Ultrasonographic Examination of Sea Turtle Eyes *(Caretta caretta* and *Chelenoidas mydas)*

Cafer Tayer İŞLER [1,2,a]

[1] Department of Surgery, Faculty of Veterinary Medicine, University of Mustafa Kemal, TR-31040 Hatay - TURKIYE
[2] Department of Sea Turtles First Aid, Treatment and Resque Research and Aplication Center(DEKİYM), University of Mustafa Kemal, TR-31040 Hatay - TURKIYE
[a] ORCID: 0000-0002-1910-8316

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Abstract

Biometrical knowledge in both illness and health is important for correct diagnosis and treatment. There is little information on the biometric and disorders of sea turtles. In this study, we evaluated the use of orbital ultrasonographic examination in sea turtles *(Caretta caretta* and *Chelenoidas mydas)*. We used 10-12 MHz probes to examine clinically healthy eyes in the 10 *Caretta caretta* and 10 *Chelenoidas mydas* through ocular ultrasonography. Polar axis, equatorial axis, corneal thickness, anterior chamber, lens thickness and vitreous chamber were all determined. Statistical differences in the sea turtle were noted in the corneal thickness ($P<0.01$). Statistical difference was not found between *Caretta caretta, Chelenoidas mydas* and right or left eyes in the measurements of polar diameter, equatorial diameter, corneal thickness, anterior camera, lens thickness, vitreous space. In this study, ultrasonographic findings of the eye of sea turtles were evaluated. These data are the first in terms of literature and therefore, it is important.

Keywords: *Wild life, Sea turtle, Caretta caretta, Chelenoidas mydas, Eye, Ultrasonographic biometry*

Deniz Kaplumbağaları *(Caretta caretta* ve *Chelenoidas mydas)* Gözlerinin Ultrasonografik İncelenmesi

Öz

Hem hastalıklı hem de sağlıklı göze ait biometrik bilgi doğru tanı ve tedavi için önemlidir. Deniz kaplumbağalarının biometrisi ve hastalıkları hakkında çok az bilgi vardır. Bu çalışmada, *Caretta caretta* ve *Chelenoidas mydas* kaplumbağa gözlerinin ultrasonografik muayene bulguları değerlendirildi. On *Caretta caretta* ve 10 *Chelenoidas mydas* klinik açıdan sağlıklı gözlerinin ultrasonografik muayenesi için, 10-12 MHz'lik prob kullanıldı. Polar çap, ekvatoryal çap, korneal kalınlık, ön kamera, lens kalınlığı ve vitröz boşluk değerlendirildi. *Caretta caretta* ve *Chelenoidas mydas* deniz kaplumbağaları gözleri arasında, istatistiksel fark korneal kalınlıkta ($P<0.01$) belirlendi. Polar çap, ekvatoryal çap, korneal kalınlık, ön kamera, lens kalınlığı, vitröz boşluğun ölçümlerinde hem *Caretta caretta*, *Chelenoidas mydas* türleri arasında hem de sağ veya sol gözleri ölçümleri arasında istatistiksel fark bulunamadı. Bu çalışmada deniz kaplumbağalarına ait gözün ultrasonografik bulguları değerlendirilmiştir. Bu veriler literatür açısından ilk veridir ve bu nedenle önemlidir.

Anahtar sözcükler: *Yaban hayatı, Deniz kaplumbağaları, Caretta caretta, Chelenoidas mydas, Göz, Diagnostik ultrasonografi*

INTRODUCTION

The species of sea turtle examined lives in the Mediterranean Sea of Greece, Turkey, Italy and Africa. The main nesting sites are on the Sicilian and Calabrian coasts. Both juvenile and adult sea turtles forage in these rich shallow local habitats [1-3]. These animals are threatened and are in danger of extinction through the destruction of nesting sites, the pollution of the seas, from fish-hooks and other foreign bodies, including plastic bags, from propeller injuries and accidents with fishermen or boats [3-6]. Anatomical knowledge of the sea turtle using diagnostic imaging techniques can help to improve their preservation and their treatment following injury [7-9]. The turtle's sense organs function both in the water and out of it and include the orbit, ear and cloaca, but biologists and veterinarians,

Correspondence
+90 507 0200401 Fax: +90 326 2455704
cafer.isler@gmail.com, caferisler@mku.edu.tr

as yet, have little information about them. Turtles suffering from ocular illness and injuries are brought into the rescue centres; therefore, a knowledge of anatomical orbital features is of high importance. Ultrasonography has already been used to identify normal biometrics in many other species [10-12]. The aim of this study was to use ultrasonography (US) to describe the normal anatomical features of the eyes of the sea turtle.

MATERIAL and METHODS

Forty sea turtles' eyes were examined by US. The *Caretta caretta* and *Chelenoidas mydas* species of sea turtles living along the Samandağ coast were used in this study. The ultrasonographic examinations were applied by one researcher and the study was authorised by the Republic of Turkey Ministry of Agriculture and Forestry (11.10.2019 date and 3121635 protocol number). After completion of the ophthalmic examination such as rutin clinicial and ophthalmoscopic examination, measurement of intra-ocular pressure, schirmer tear test, all the turtles were found to have normal eyes. First, all the animals were gently restrained manually. A topical anaesthetic was then administered (intravenous, 1-2 mg/kg diazem and 4-5 mg/kg propofol), and the B-mode US process was then applied. The eye is closed during the ultrasound examination and useful a gel. All eye examinations were done using ultrasound devices, equipped with a 12-MHz linear probe (MyLab30©) (Esaote, Florence, Italy) at the hospital centre. The probe (Sterile Aquasonic 100; Parker La. Inc., Fairfield, NJ, USA) was used in a horizontal and vertical position until optimal images were obtained for the corresponding US scans. The ocular parameters were: polar axis (PA), equatorial axis (EA), corneal thickness (CT), anterior chamber (AC), lens thickness (LT), vitreous chamber, distance between the anterior and the posterior capsules, the distance from the posterior capsule of the lens to the retinal surface, the distance from the posterior line of the cornea to the anterior capsule of the lens, the distance between the two lines of the cornea, the maximal diameter perpendicular to the PA, and the distance from the corneal surface to the posterior wall of the retinal layer. These were all measured using the US device *(Fig. 1)*.

Data collected were analysed using the SPSS programme and were reported as Mean ± SD in the 10 CC and 10 CM [13]. Differences between the CC and CM eyes in PA, EA, CT, AC, LT, were analysed with the t-test or the Mann Whitney U test. The level of statistical significance used was $P<0.01$.

RESULTS

The turtles were all adults and females. There is a retraction reflex in this species, and for this reason some difficulties were encountered. However, the US exam of the eye was successfully performed within three minutes for each turtle. The anatomical structures of the eye were examined through the ultrasonographic examination. The optic disc was not visible. Regular retinal fundus was seen. The vitreous chamber (VC) was anechoic. The anterior chamber was somewhat thin and had an anechoic area. The lens contained two hyperechoic convex lines, presenting as the anterior and posterior capsules. The scleral appeared as a shadowing artefact as two hyperechoic lines at the level of the limbus. The cornea was seen as a slight anterior convexity with two hyperechoic parallel lines.

The ultrasonographic ophthalmic measurements for the sea turtle are summarized in *Table 1*.

In this species; statistical differences were not identified between PA, EA, AC, LT and right or left eyes. Statistical differences between the CC and CM eyes of the sea turtles were identified in CT ($P<0.01$).

DISCUSSION

In the sea turtle, the eye is placed dorsally and laterally in the skull [14], there are three keratinized eyelids, the dorsal

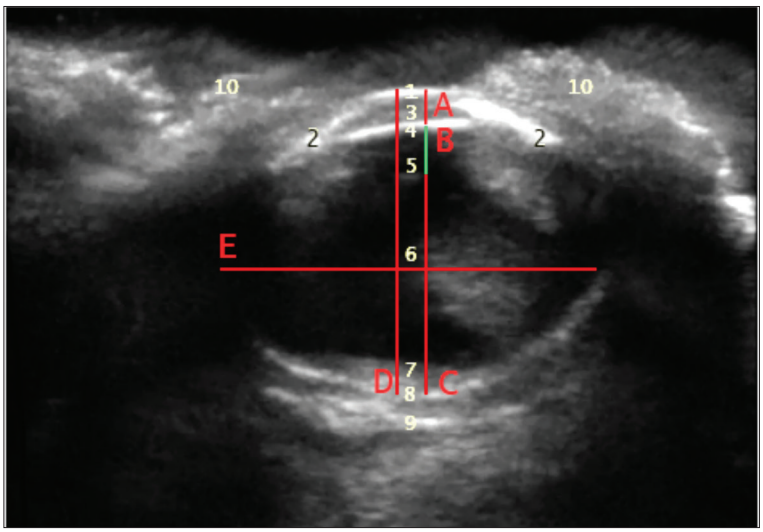

Fig 1. Loggerhead eye with the measurements performed and Ultrasonographic image of the Loggerhead eye; Legend 1= corneal thickness; Legend A = anterior chamber depth; B = lens thickness; C = vitreous chamber depth; D = polar axis; E = equatorial axis; 1 = cornea; 2 = scleral ossicle; 3 = anterior chamber; 4 = anterior capsule of the lens; 5 = posterior capsule of the lens; 6 = vitreous body; 7 = retinal layer; 8 = scleral cartilage; 9 = artifact shadowing from scleral ossicle; 10 = salt gland

Table 1. *Ultrasongraphic ophthalmic measurements in the sea turtle*

Measurement of the Eye	*Caretta caretta*	*Chelenoidas mydas*
Polar axis	17.61±2.71	17.30±2.33
Equatorial axis	20.81±3.90	21.13±3.01
Corneal thickness[a]	0.81±0.35[a]	0.63±0.11[a]
Anterior chamber	0.86±0.10	0.84±0.148
Lens thickness	4.60±0.50	4.50±0.50
Vitreous chamber	10.12±1.01	10.65±1.18

[a] *Statistical difference between the CC and CM eyes of the sea turtles (P<0.01)*

and ventral eyelids are mobile, the medial lid (secondary lid) is non-mobile [15,16]. The ocular globe is formed by three layers: 1) the sclera and the cornea, formed by connective tissue; 2) the intermediate and vascularized layer, the uvea; and 3) the inner sensory layer, the retina. Differing from other animals, in addition there are two supporting structures: posteriorly the scleral cartilage and anteriorly the scleral ossicle [16-18]. The study using US was very easy to perform but the scleral ossicle and the retraction reflex did give some difficulties. There are good correlations between the eye dimensions. This study determined the normal ocular ultrasonographic features of the sea turtle. We learned that the orbit has a polar axis that is shorter than the equatorial in addition to a slight corneal curvature, a very thin anterior chamber and a relatively small lens. The orbit and the equatorial border of the lens do not permit complete visualization due to the shadowing artefact of the scleral ossicle. This muscle determines the movement of the sclera towards the inside and the outside of the eye within its orbit.

Sea turtles can be affected by many eye diseases, which can make the anterior portions of the eye opaque, and these situations prevent normal ophthalmic examination [14,17-19]. Ultrasonography allows the evaluation of the direct visualization resulting from any disease that causes ocular opacity.

The results of Brudenall et al.[16] and Raposo et al.[10] are very close to each other and one of them reported in Leatherback sea turtle in post mortem examination. They reported significant differences among CT, anterior chamber depth, lens thickness, vitreal cavity depth (P<0.001). Our results may be slightly higher than those of the reported since our results were reported in healty sea turtles under anesthesia. We found a statistically significant difference between the CC and CM in the CT (P<0.001). This finding is first reported in the world.

We conclude that the technique of ultrasonographic evaluation is very useful for studying the eye in this species. The ultrasonographic features are useful for clinical evaluation and for presenting reliable information on the normal dimensions and intraocular anatomy of sea turtles' eyes. Use of the 10-12 MHz probes in US allows images to be obtained of all ocular structures. Ultrasonographical examination of the orbit should become routine in the technic of clinical examination. Diagnosis of diseases causing disruption of the normal biometry of the eye can be easily made using US.

REFERENCES

1. Mingozzi T, Masciari G, Paolillo G, Pisani B, Russo M, Massolo A: Discovery of a regular nesting area of loggerhead turtle *Caretta caretta* in southern Italy: A new perspective for national conservation. *Biodivers Conserv,* 16, 3519-3541, 2007. DOI 10.1007/s10531-006-9098-6

2. Hochscheid S, Travaglini A, Maffucci F, Hays GC, Bentivegna F: Since turtles cannot talk: What beak movement sensors can tell us about the feeding ecology of neritic loggerhead turtles, *Caretta caretta. Mar Ecol,* 34, 321-333, 2013. DOI: 10.1111/maec.12018

3. Maffucci F, D'Angelo I, Hochscheid S, Ciampa M, De Martino G, Travaglini A, Treglia G, Bentivegna F: Sex ratio of juvenile loggerhead turtles in the Mediterranean Sea: Is it really 1:1. *Mar Biol,* 160, 1097-1107, 2013. DOI: 10.1007/s00227-012-2160-x

4. Lazar B, Gracan R: Ingestion of marine debris by loggerhead sea turtles, *Caretta caretta*, in the Adriatic Sea. *Mar Pollut Bull,* 62, 43-47, 2011. DOI: 10.1016/j.marpolbul.2010.09.013

5. Santos RG, Andrades R, Boldrini MA, Martins AS: Debris ingestion by juvenile marine turtles: An underestimated problem, *Mar Pollut Bull,* 93, 37-43, 2015. DOI: 10.1016/j.marpolbul.2015.02.022

6. De Majo M, Macri F, Masucci M, Coci G, Pennisi MG: Clinical ultrasonography in loggerhead sea turtles *(Caretta caretta)*: Imaging of pathological features. *Vet Med-Czech*, 61, 155-161, 2019. DOI: 10.17221/8767-VETMED

7. Franchini D, Valastro C, Ciccarelli S, Caprio F, Lenoci D, Di Bello A: Ultrasonographic detection of ingested fishing lines in loggerheads *(Caretta caretta). J Wildl Dis*, 54 (4): 680-690, 2018. DOI: 10.7589/2017-12-302

8. Valente AL, Cuenca R, Parga ML, Lavín S, Franch J, Marco I: Cervical and coelomic radiologic features of the loggerhead sea turtle, *Caretta caretta. Can J Vet Res*, 70, 285-290, 2006.

9. Crognale MA, Eckert SA, Levenson DH, Harms CA: Leatherback sea turtle *Dermochelys coriacea* visual capacities and potential reduction of bycatch by pelagic longline fisheries. *Endang Species Res,* 5, 249-256, 2008. DOI: 10.3354/esr00112

10. Raposo ACS, Muramoto C, Pires TT, Dòrea Neto FA, Brito VJSC, Orià AP: Normal sonographic aspects of the eye in healthy sea turtles. *Vet Ophthalmol,* 20, E1-E14, 2017. DOI: 10.1111/vop.12498

11. Somma AT, Lima L, Lange RR, Turner-Giannico A, Montiani-Ferreira F: The eye of the red-eared slider turtle: Morphologic observations and reference values for selected ophthalmic diagnostic tests. *Vet Ophthalmol,* 18, 61-70, 2015. DOI: 10.1111/vop.12213

12. Williams SR, Dennison S, Dunnigan B, Moore B, Nicholson J, Zagzebski K, Ketten D, Cramer S, Arruda J: Diagnosis and management of intestinal partial obstruction in a loggerhead turtle *(Caretta caretta). J Zoo Wildl Med,* 44 (2): 457-461, 2013. DOI: 10.1638/2011-0179R1.1

13. Boyacıoğlu H, Güneri P: Sağlık araştırmalarında kullanılan temel istatistik yöntemler. *Hacettepe Dişhekimliği Fak Derg,* 30, 33-39, 2006.

14. Wyneken J: The Anatomy of Sea Turtles. 1-172,. U.S. Department of Commerce NOAA Technical, Memorandum NMFS-SEFSC-470, National Marine Fisheries Service, Southeast Fisheries Science Center, Miami, 2001.

15. İşler CT, Altuğ M, Cantekin Z, Özsoy ŞY, Yurtal Z, Deveci MZY: Evaluation of the eye diseases seen in loggerhead sea turtle *(Caretta caretta). Revue Méd Vét*, 165 (9-10): 258-262, 2014.

16. Brudenall DK, Schwab IR, Fritsches KA: Ocular morphology of the Leatherback sea turtle *(Dermochelys Coriacea). Vet Ophthalmol,* 11, 99-110, 2008. DOI: 10.1111/j.1463-5224.2008.00607.x

17. Altuğ ME, İşler CT, Yurtal Z, Deveci MZY, Kırgız Ö: Deniz kaplumbağalarında ilk yardım ve rehabilitasyon. *Türkiye Klinikleri J Vet Sci,* 8 (1-2): 42-50, 2017. DOI: 10.5336/vetsci.2017-59219

18. MacKay CS, Matoon JS: The eye. **In,** Matoon JS, Nyland TG (Eds): Small Animal Diagnostic Ultrasound. 3rd ed., 128-154, Saunders, Elsevier. St. Louis, Missouri, 2015.

19. İşler CT, Altuğ ME, Seçer FS, Cantekin Z: Treatment of bath with enrofloxacin in red-eared slider *(Trachemys scripta elegans)* suffer from conjunctivitis and its results. *Kafkas Univ Vet Fak Derg,* 21 (3): 429-431, 2015. DOI: 10.9775/kvfd.2014.12621

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Research Article

Geometric Morphometric Analysis of Cranium of Wolf *(Canis lupus)* and German Shepherd Dog *(Canis lupus familiaris)* [1]

İftar GÜRBÜZ [1,a] Ahmet İhsan AYTEK [2,b] Yasin DEMİRASLAN [1,c] Vedat ONAR [3,d] Özcan ÖZGEL [1,e]

[1] *This study was presented as a poster in 1st International Veterinary Anatomy Congress of Turkey (Xth National Anatomy Congress), 13-16 September 2017, Sandıklı/Afyonkarahisar, Turkey*

[1] Burdur Mehmet Akif Ersoy University, Department of Anatomy, Faculty of Veterinary Medicine, TR-15030 Burdur - TURKEY
[2] Burdur Mehmet Akif Ersoy University, Department of Antropology, Faculty of Arts and Science, TR-15030 Burdur - TURKEY
[3] İstanbul University-Cerrahpaşa, Department of Anatomy, Faculty of Veterinary Medicine, TR-34320 İstanbul - TURKEY
ORCIDS: [a] 0000-0001-9460-0645; [b] 0000-0002-7620-2333; [c] 0000-0003-3612-6142; [d] 000-0002-8359-243X, [e] 0000-0003-0394-5678

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Abstract

In the study, it was aimed to investigate by geometric morphometric analysis to shape of the skull and mandible in adult male wolf and German Shepherd Dog. The skulls and mandibles were photographed from left side. The shape of skulls and mandibles were optimized by using tpsDig programme with 20 landmarks (in skulls), and 13 landmarks (in mandibles) on 2-D images. The skulls and mandibles were superimposed via PAST software and the principale component analysis was conducted. How and where the shape changes occurs in the cranium was shown using MorphoJ software. Also, morphological differences of the cranium between two groups were determined. As a result, German Shepherd Dog's cranium were significantly seperated from the wolf's cranium in respect of cranium shapes. In skull and mandible, about 70% and 78% of the total shape variation, is reflected by the first three principal components, respectively. Shape differences were most distinct in parietal, occipital, zygomatic, temporal bone and posterior mandible between two groups. The findings of the study are important in terms of evaluating the materials to be extracted from the area of archeological excavation and creating a database that will provide to prominent information about the history of domestication.

Keywords: *Wolf, German Shepherd Dog, Mandible, Skull, Geometric morphometry*

Kurt ve Alman Çoban Köpeğinde Kafatasının Geometrik Morfometrik Analizi

Öz

Çalışmada Kurt ve Alman Çoban Köpeği'nin alt çene kemiği ve kafatası şeklinin geometrik morfometrik analiz yöntemi ile araştırılması amaçlandı. Bu amaçla kafatası ve mandibula sol taraftan fotoğraflandı. Kafatası ve alt çene kemiğinin şekli, 2D fotoğraf üzerinde 20 landmark (kafatasında) ve 13 landmark (alt çene kemiğinde) ile tpsDig programda optimize edildi. Kafatası ve mandibula'lar PAST yazılımı ile üst üste bindirildi ve temel bileşenler analizi yapıldı. Kafatası ve mandibula'daki şekil farklılıklarının nerede ve nasıl oluştuğu MorphoJ yazılımında görüntülendi. Ayrıca, iki grup arasında morfolojik şekil farklılıkları da belirlendi. Sonuç olarak, Alman çoban köpeği kafatasının şekilsel olarak kurt kafatasından önemli derecede ayrıldığı belirlendi. Kafatası ve alt çene kemiğinin ilk üç temel bileşeni toplam şekil varyasyonunun sırasıyla %70 ve %78'ini açıkladığı saptandı. İki grubun kafatasında şekil farklılıkları en fazla parietal, occipital, zygomatic, temporal kemiklerde ve mandibula'nın posterior'unda belirgindi. Çalışmanın bulguları arkeolojik kazı alanından çıkarılacak malzemelerin değerlendirilmesi ve evcilleştirme tarihi hakkında önemli bilgiler sağlayacak bir veri tabanı oluşturulması açısından önemlidir.

Anahtar sözcükler: *Kurt, Alman Çoban Köpeği, Mandibula, Kafatası, Geometrik Morfometri*

INTRODUCTION

Wolf *(Canis lupus* Linnaeus, 1758) is the first known animal to be domesticated as gray wolf or timber wolf [1]. Although archeological and genetic data inform us that domestication of wolves took place 16 thousand years ago, current studies also indicate that this period may date back to 36 thousand years ago [2]. The gray wolf *(Canis lupus)*

Correspondence
+90 248 2132166
iftargurbuz@mehmetakif.edu.tr

is a carnivore with a vast distribution range, occupying habitats in North America as well as in Europe and Asia [1]. Various subspecies of the gray wolf are identified due to morphological differences in color, size of body and bones, especially the skull, and behavior [3]. The German Shepherd dog is a breed of medium to large sized, slightly elongated, powerful and well-muscled with dry bone and firm overall structure and, originated from Germany [4].

The shape of the skull in dogs varies significantly among species and individuals in terms of its form and size [5]. The shape of the skull is the most important criteria in determining common dog breeds. Therefore, various researchers have examined the skulls of dog breeds anatomically [6]. Also, there are studies in which the skull and mandible of some dog breeds were examined using the classical morphometric method [7-10].

Geometric morphometric is a method of shape analysis defined as the analysis of all geometric information taken from Cartesian coordinates of anatomical points [11]. Geometric morphometry has also been proven to be a useful tool for the study of morphological evolution in mammals. This method is based on the analysis of data obtained from the anatomical points called landmark, which are identified in homologous regions which may reveal the shape and have reliability and reproducibility [11]. Many studies have been conducted on wolves and dogs using this method, which has been used extensively in scientific studies in recent years [12-16]. Also, there are geometric morphometric studies conducted on rodents [17], sheep [18], squirrels [19]. This study aimed to reveal similar or different aspects of wolf and German shepherd dog cranium, which are very similar in an external examination, with the geometric morphometric method.

MATERIAL and METHODS

Ethical Approval

The Local Ethics Committee for Animal Experiments of the Mehmet Akif Ersoy University has granted the required permission (Date: June 07, 2017; no: 299).

Animals

Cranium of the 5 adult male wolves and 5 adult male German shepherd dogs were used in the study. The study materials were obtained from the German shepherd dogs and the gray wolves collected from the Kars-Ardahan-Iğdır (in Turkey) region. The age and gender of the wolves and German Shepherd dogs were previously determined in the study. The animals used in the study were already death prior to the study and therefore no euthanize was needed to be performed.

Geometric Morphometric Analysis

In the study, 2-dimensional photographs of the cranium (18X205 Canon EOS 1000D with Sigma lens) were taken from the left side at a latero-lateral position for the geometric morphometric analysis.

The photographs were taken from a distance of 1 meter by a camera placed on a tripod with a water gauge. The landmarks were detected in two phases using Tps programs over 2-dimensional photographs. In the first phase, the photographs were introduced into the program named tpsUtil Version 1.60 [20] and were saved as tps files. In the second phase, the landmarks were marked on the photographs through tpsDig2 Version 2.18 program [21] and Cartesian coordinates were determined. Twenty landmarks were taken on the skull *(Fig. 1)* and 13 landmarks were taken on the mandible *(Fig. 2)*.

To remove the effect of factors such as direction, position,

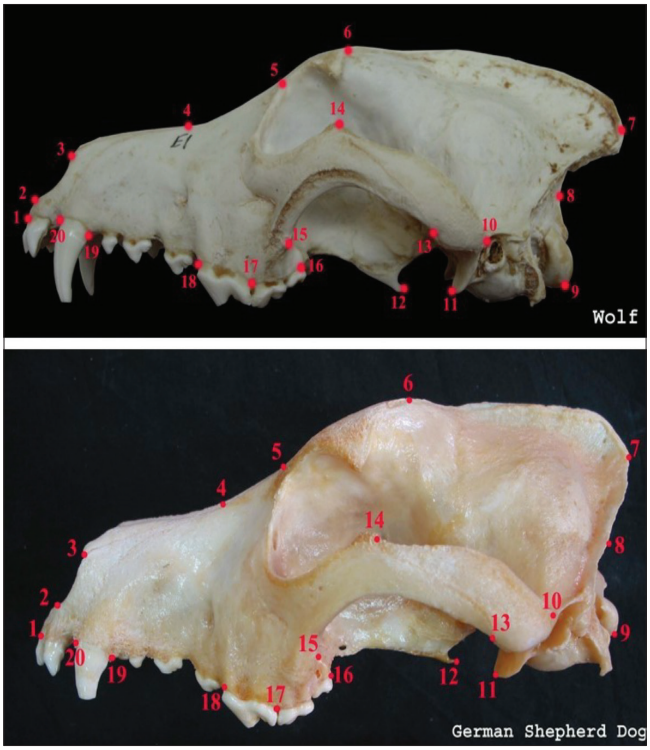


Fig 1. The points of landmarks on the cranium of the Wolf's and German Shepherd dogs

1. Anterior point of the incisive bone *(os incisivum)*, **2.** Anterior point of the nasal cavity *(cavitas nasalis)*, **3.** Anterior end of the suture between nasal bones *(os nasale)*, **4.** Suture between nasal bone and nasal branch of premaxilla, **5.** Level of angulus oculi medialis, **6.** Anterior point of external sagittal crest *(crista sagittalis externa)*, **7.** Posterior point of external sagittal crest, **8.** The midpoint of the posterior level of the external sagittal crest and the ventral level of occipital condyle *(condylus occipitalis)*, **9.** Most posterioventral point of the occipital condyle, **10.** Posterior point of the zygomatic process of temporal bone *(proc. zygomaticus ossis temporalis)*, **11.** Ventral point of retroarticular process *(proc. retroarticularis)*, **12.** Posterior end of the pterygoid process *(hamulus pterygoideus)*, **13.** The posterior point suture between the zygomatic process of temporal bone and temporal process of zygomatic bone *(proc. temporalis ossis zygomaticus)*, **14.** The superior point suture between the zygomatic process of temporal bone and temporal process of zygomatic bone, **15.** Anterioventral point of zygomatic arch *(arcus zygomaticus)*, **16.** Posterior edge of 3rd molar alveolus, **17.** Posterior edge of 1st molar alveolus, **18.** Anterior edge of 1st molar alveolus, **19.** Posterior edge of canine alveolus, **20.** Anterior edge of canine alveolus

and size on variation over the Cartesian coordinates that were obtained by marking of the landmarks, the data were overlapped by Generalized Procrustes Analysis and PAST 3.21 program [22]. Principal Component Analysis was performed over the new coordinates that were obtained by overlapping, and the shape variation was revealed in this way. Also, it was shown that the principal components caused what kind of shape changes on which landmarks using the MorphoJ 1.06 program [23]. The data on the landmarks were saved as a text file for statistical analyses.

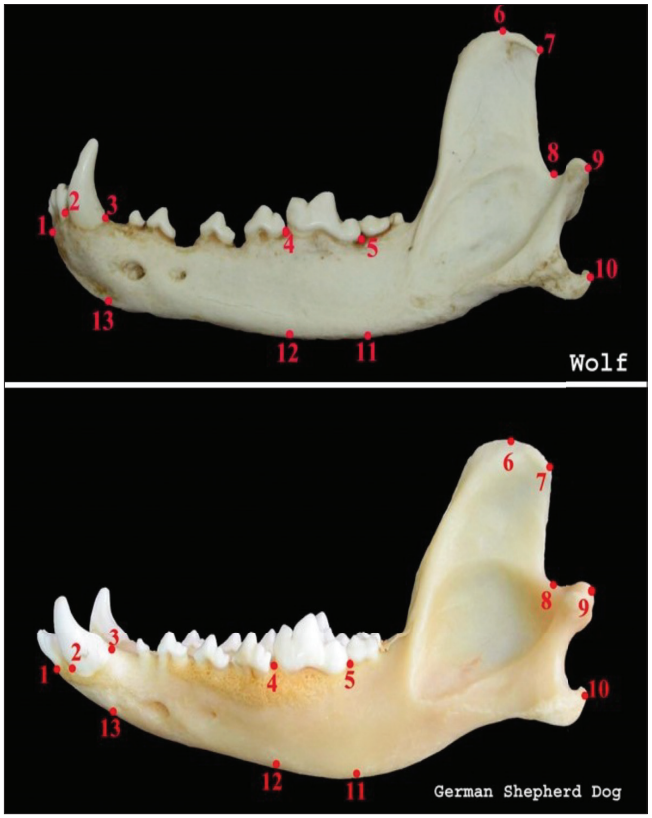


Fig 2. The points of landmarks on mandible of the Wolf's and German Shepherd dogs

1. Anterior end of the corpus mandibula, **2.** Anterior edge of the canine alveolus, **3.** Posterior edge of the canine alveolus, **4.** Anterior edge of the 1st molar alveolus, **5.** Posterior edge of the 1st molar alveolus, **6.** Top of the coronoid process *(proc. coronoideus)*, **7.** Dorsocaudal end of the coronoid process, **8.** Level of the mandibular incisura *(incisura mandibula)*, **9.** Caudal edge of the condylar process *(proc. condylaris)*, **10.** Caudal edge of the angular process *(proc. angularis)*, **11.** Level of ventral margin of the corpus mandibula at the level of posterior of the 1st molar alveolus, **12.** Level of ventral margin of the corpus mandible at the level of anterior of the 1st molar alveolus, **13.** Level of ventral margin of the corpus mandibula at the level of posterior of the canine alveolus

RESULTS

Geometric Morphometric Values of the Skull

The first principal component explained to 42% of the total shape variation in this geometric morphometric study conducted on 10 skulls with 20 landmarks as shown in *Table 1*.

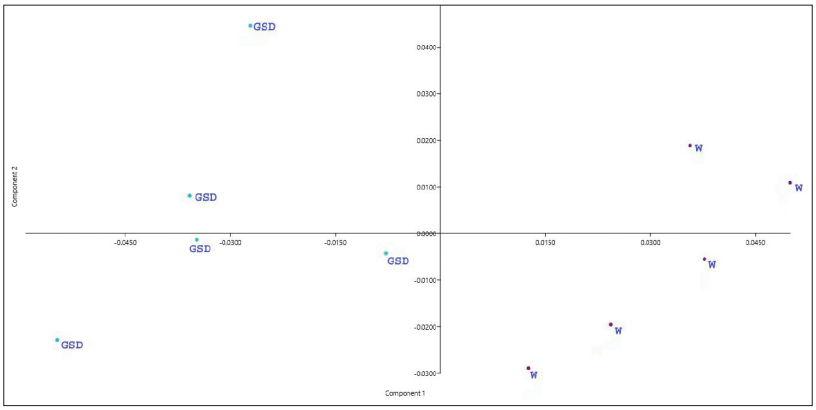
The skulls of wolves and German shepherd dogs were distinctly separated from each other for the first principal component according to the graph of principal component analysis in *Fig. 3*. When the groups were evaluated within themselves, German shepherd dogs showed a much greater variation than the wolves. Also, when all individuals were classified hierarchically, the individuals of both two groups were classified within their groups as indicated in *Fig. 4*.

Shape differences were the most distinct in os parietale, os zygomaticum, os temporale, and os occipitale in the analysis of the first principal component *(Fig. 5)*. A significant shape difference, which is less distinct when compared with the other bones, was in os maxilla between

Table 1. *The values of principal component analysis for the skull*

PC	Eigenvalue	% Variation
1	0.00135799	42.376
2	0.000481068	15.012
3	0.000434587	13.561
4	0.000319461	9.9687
5	0.000208181	6.4962
6	0.000158723	4.9529
7	0.000129919	4.0541
8	7.66E+00	2.3888
9	3.82E+00	1.1906

Fig 3. The graphic of principal component analysis for skull (GSD: German Shepherd dog, W: Wolf)



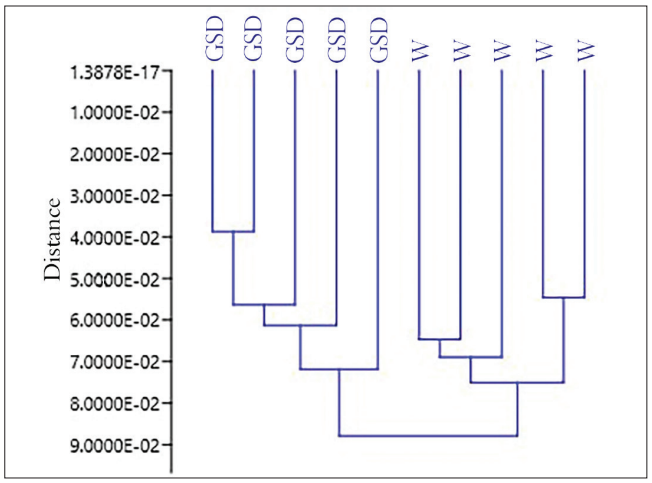


Fig 4. The graphic of hierarchical classification for skull (GSD: German Shepherd Dog, W: Wolf)

Table 2. *The values of principal component analysis for the mandible*

PC	Eigenvalue	% Variation
1	0.000973965	52.638
2	0.00029352	15.863
3	0.000228735	12.362
4	0.000172245	9.309
5	7.09E-02	3.8339
6	5.16E+00	2.7905
7	3.36E+00	1.8145
8	1.45E+00	0.7819
9	1.12E+00	0.60733

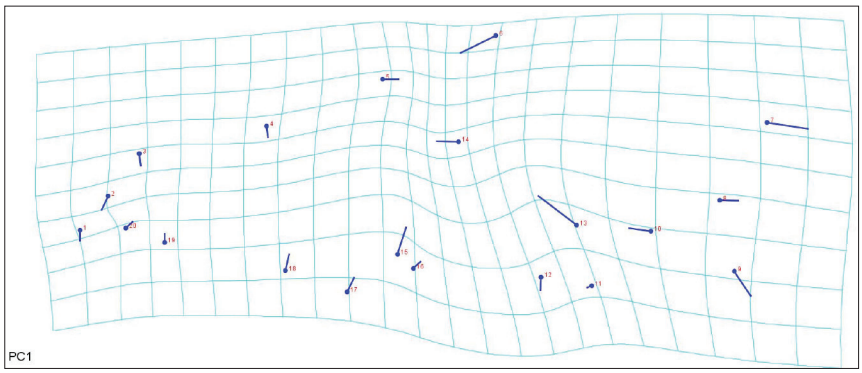
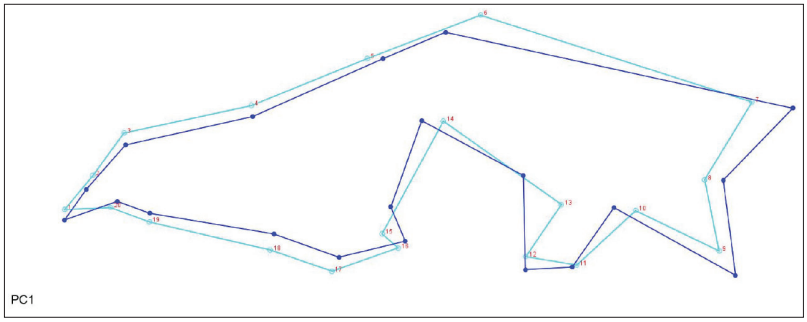


Fig 5. Landmark representation of shape differences of skull between wolf and German Shepherd Dog for the first principal component

Fig 6. Lineal representation of the shape differences of skull for the first principal component



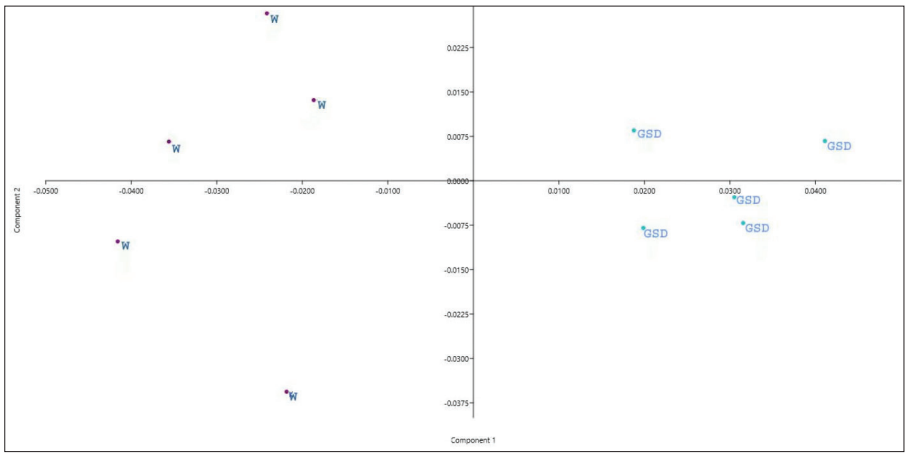


Fig 7. The graphic of principal component analysis for mandible (GSD: German Shepherd dog, W: Wolf)

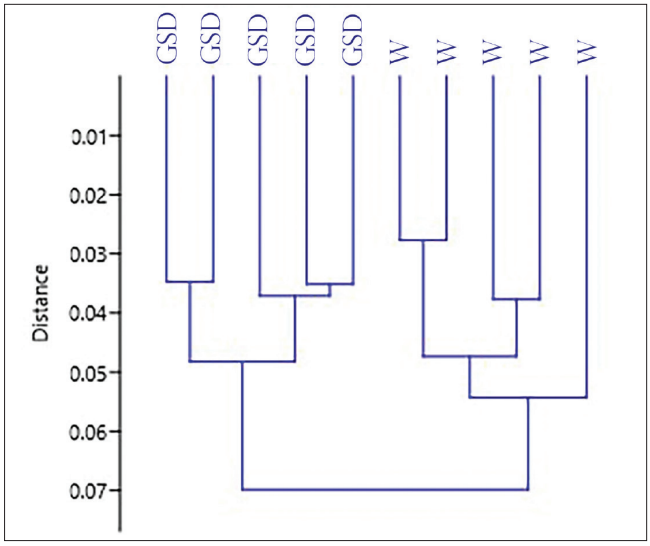


Fig 8. The graphic of hierarchical classification for mandibles (GSD: German Shepherd Dog, W: Wolf)

shape variation in this geometric morphometric study conducted with 13 landmarks as shown in *Table 2*.

In consequence of the analysis regarding the first principal component, the mandibles of the wolves and German shepherd dogs were distinguished from each other distinctly, according to the *Fig. 7*. When the groups were evaluated within themselves, in contrast to the skull, the mandible of wolves showed a much greater variation when compared with the German shepherd dogs. Also, when all individuals were classified hierarchically, the individuals of both two groups were classified within their groups *(Fig. 8)*.

There were significant shape differences in all of the regions of mandible, but they were more distinct in posterior mandible in the analysis of the first principal component *(Fig. 9)*.

Fig. 10 lineally showed how the overall skull shape varied between the groups.

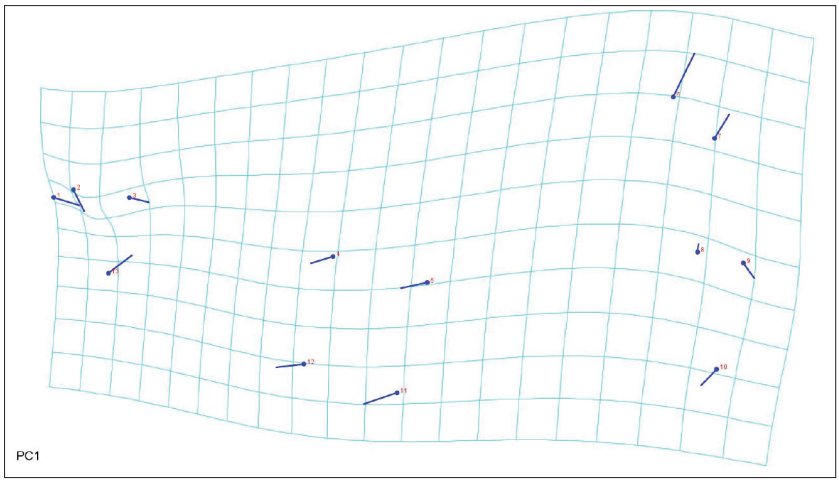
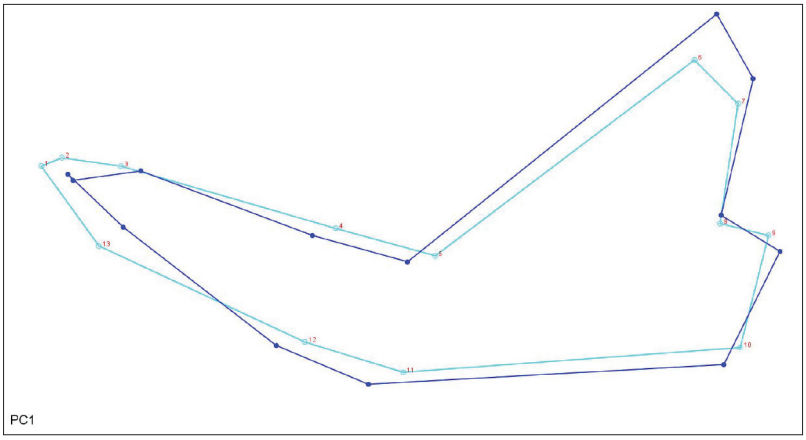


Fig 9. Landmark representation of shape differences of mandible between wolf and German Shepherd Dog for the first principal component

Fig 10. Lineal representation of the shape differences of mandible for the first principal component



the two groups. *Fig. 6* lineally showed how the overall skull shape varied between the groups.

The Geometric Morphometric Values of the Mandible

The first principal component explained to 53% of the total

Morphological Differences of the Skull

External sagittal crest *(crista sagittalis externa)* was observed to have a higher and caudoventral directional slope than os parietale in the wolves when compared with the German shepherd dogs at the level of landmark no. 6 and 7. The

caudal end of external sagittal crest reached occipital condyle *(condylus occipitalis)* with a concave curve in the wolves at the level of landmarks no.7 and 8. But the caudal end of external sagittal crest reached occipital condyle with a vertical slope in the German shepherd dogs *(Fig. 1, Fig. 5, Fig. 6)*.

When the skull was placed on a flat ground, the *hamulus pterygoideus* was inclined towards to caudoventral in the wolves, but it was relatively parallel to the ground in the German shepherd dogs at the level of landmark no.12 *(Fig. 1, Fig. 5, Fig. 6)*.

The concavity between the medial angle of the eye and frontal process of nasal congestion *(processus (proc.) frontalis ossis nasalis)* was relatively higher in the German shepherd dogs as shown in the landmark no.3, 4, 5 and 6 *(Fig. 1, Fig. 5, Fig. 6)*.

At the zygomatic arc, the upper edge of the zygomatic process of temporal bone *(proc. zygomaticus ossis temporalis)* was inclined towards to caudoventral in the wolves, but it was relatively parallel to the ground in the German shepherd dogs at the level of the landmarks no.13 and 14 *(Fig. 1, Fig. 5, Fig. 6)*.

Morphological Differences of the Mandible

The caudal end of the coronoid process was relatively inclined backwards in the wolf when compared with the German shepherd dog at the level of landmarks no. 7 and 8 *(Fig. 2, Fig. 9, Fig.10)*.

The ventral margin of mandible *(margo ventralis mandibula)* was more convex in the German shepherd dog when compared with the wolf at the level of landmarks no. 11, 12 and 13 *(Fig. 2)*.

The mental foramen *(foramen mentale)* in the caudal was placed at the ventral of 2nd and 3rd premolar tooth level in the wolves, but it was placed at the ventral of 3rd premolar tooth level in the German shepherd dogs *(Fig. 2)*.

The landmark no.8 accounted for the fact that the mandibular notch *(incisura mandibula)* was relatively deeper in the wolves when compared with the German Shepherd dogs *(Fig. 2, Fig. 9, Fig.10)*.

DISCUSSION

Wolves, which are used in hunting and for protection, have been transformed into dog breeds with different phenotypical characteristics in accordance with human needs with also the impact of artificial selection. Thanks to this transformation, it is known that 361 dog breeds, which have been defined by the World Canine Organization, exist today [4]. In the study, the cranium of the wolf and the German shepherd dog, a dolichocephalic breed, which has a similar skull image, have been analyzed in terms of their shapes. However, this study includes some limitations in terms of the number of wolf samples due to strict protection of this species by law, therefore the number of samples were kept minimum just to provide statistical meaning. However, in geometric morphometric studies, relatively small samples provide accurate results especially shape variation is in the case [24].

The shape of the cranium is associated with the differences in nutritional behavior along with the environmental and genetic factors [25]. The masticatory muscles, responsible for the feeding, form the main load of the skull and affect growth morphology [26]. The activation and coordination of the masticatory muscles determine the direction of the movement of the chin and control the masticatory force. At the same time, the difference in the skull and mandible in terms of shape affects the cross-sectional area of the masticatory muscles and therefore biting power [27].

External sagittal crest in carnivore is the anatomical structure which exists in the skull and constitutes a basis on the holding of especially masticatory muscles. Igado [28] reported that a distinct external sagittal crest existed in 57.14% of the males and 66.67% of the females in at least 2-years-old Nigerian Local dogs, but either it did not exist or was more flattened in the other skulls. Therefore, it was difficult to determine which gender had more powerful masticatory muscles [28]. However, the morphometric values of the skull increased with the age in the German shepherd dogs in the age group of 45-105 days was reported [7]. Based on this information, it was considered that external sagittal crest may become more distinct depending on the age [28]. Considering that the German shepherd dogs and the wolves that were employed in this study were adults, external sagittal crest was observed to have a higher than parietal bone and caudoventral directional slope in the wolves when compared with the German shepherd dogs at the level of the landmark no.6 and 7.

In a literature which examined the skulls and mandibles of Dinaric-Balkan and Carpathian grey wolves using the geometric morphometrics methods, it was reported that the difference in shape was statistically significant [29]. In the same literature, the most distinct difference of shape in the skull between the genders was an anterior-posterior shifting of zygomatic arches [29]. Milencovic et al.[29] also indicated that shift of zygomatic arches might have affected jaw movement. Similarly, in this study, the zygomatic arch was found to be significantly different in shape between the two groups, at the levels of landmarks no. 13 and 14. The top edge of zygomatic arc was inclined with caudoventral direction in wolves and relatively parallel to the ground in German Shepherd dogs.

In the morphological study on wolf and dog skulls, Janssens et al.[8] stated that canine tooth remained on dorsal position in wolves and ventral position in dogs when the skull was put on a flat ground in such a way that it contacted the

ground at the level of bulla tympanica and 1st premolar tooth. In this study, unlike the findings of the researchers [8], canine tooth remained in ventral position in the wolves and German Shepherd dogs. Also, Milencovic et al.[29] notified that the facial bone was in dorsoflexion position in Dinaric Balkan wolves, and in ventro-flexion position in Carpathian wolves. These changes in nonallometric cranial flexion are associated with the changes in the convexity or concavity of mandible [29].

Milencovic et al.[29] reported that combination of the 1st and the 2nd principal component of mandible explained to 64.7% of nonallometric shape variation in Dinaric Balkan wolves (female and male) and Carpathian wolves. In the literature, it was declared that mandible was more concave in Dinaric-Balkan wolves when compared with the Carpathian wolves [29]. In the present study, the first principal component in mandible explained to 53% of total shape variation and corpus mandible was determined to be more convex in German Shepherd dogs when compared with the wolves.

Olsen and Olsen [30] reported that the turned back morphological structure of dorsal part of ramus mandible was specific in domestic dogs and Chinese wolves *(Canis lupus chanco)* and did not exist in other dogs. On the other hand, Janssens et al.[31] stated that the coronoid process of mandibles of all dog species did not have a specific "turned back morphology" in consequence of their study on 384 dog skulls from 72 breeds and 60 wolf skulls from 4 wolf subspecies. Therefore, researchers [31] indicated that this feature could not be used in the distinction of dog or wolf species. In this study, at the levels of 7th and 8th landmarks, the caudal end of coronoid process was observed to be relatively "turn back morphology" in wolves when compared with German shepherd dogs.

In conclusion, it is a fact that the differences and data determined between the head bones of the wolf and the German shepherd dog will contribute to the interpretation of osteoarcheological remains excavated from archeological sites. Besides, it is considered that this study will support morphometric or geometric morphometric studies to be conducted on the other species of the family Canidae.

Author Contributions

Gürbüz İ, Aytek Aİ and Demiraslan Y designed and directed the study, Aytek Aİ conducted geometric morphometric application. Gürbüz İ and Demiraslan Y provided wolf skulls and Onar V provided German Shepherd dog skulls. Gürbüz İ, Aytek Aİ, Demiraslan Y, Onar V and Özgel Ö co-wrote the overall paper.

Conflicts of Interest

The authors declare no conflict of interest.

REFERENCES

1. Mech LD: The Wolf: The Ecology and Behavior of An Endangered Species. 3rd ed., 644-647, Natural History Press, Doubleday Publishing Company, New York, 1970.

2. Perri A: A wolf in dog's clothing: Initial dog domestication and Pleistocene wolf variation. *J Archeol Sci,* 68, 1-4, 2016. DOI: 10.1016/j.jas.2016.02.003

3. Wozencraft WC: Order carnivora. **In,** Wilson DE, Reeder DM (Eds): Mammal Species of the World: A Taxonomic and Geographic Reference. 3rd ed., 532-628, Johns Hopkins University Press, Baltimore, 2005.

4. St-Federation Cynologique Internationale: Federation Cynologique Internationale (AISBL), Secretariant General 13 Place Albert 1 B-6530 Thuin, Belgique, 2010.

5. Sisson S: Carnivore osteology. **In,** Sisson S, Grossman JD (Eds): The Anatomy of Domestic Animals. Vol. 2, 5th ed., 1474-1479, Saunders, 1975.

6. Brehm VH, Loeffler K, Komeyli H: Schädelformen beim Hund. *Anat Histol Embryol,* 14, 324-331, 1985. DOI: 10.1111/j.1439-0264.1985.tb00828.x

7. Onar V: A Morphometric study on the skull of the German Shepherd Dog (Alsatian). *Anat Histol Embryol*, 28, 253-256, 1999. DOI: 10.1046/j.1439-0264.1999.00202.x

8. Janssens L, Perri A, Crombe P, Dongen SV, Lawler D: An evaluation of classical morphologic and morphometric parameters reported to distinguish wolves and dogs. *J Archeol Sci Rep,* 23, 501-533, 2019. DOI: 10.1016/j.jasrep.2018.10.012

9. Onar V, Kahvecioğlu KO, Çebi V: Computed tomographic analysis of the cranial cavity and neurocranium in the German shepherd dog (Alsatian) puppies. *Vet Arhiv,* 72 (2): 57-66, 2002.

10. Onar V, Kahvecioğlu O, Mutuş R, Alpak H: Alman kurt köpeklerinde mandibula'nın morfometrik analizi. *Turk J Vet Anim Sci*, 23 (2): 329-334, 1999.

11. Slice DE: Geometric morphometrics. *Annu Rev Anthropol,* 36, 261-281, 2007.

12. Drake AG, Coquerelle M, Kosintsev PA, Bachura OP, Sablin M, Gusev AV, Fleming LS, Losey RJ: Three-dimensional geometric morphometric analysis of fossil canid mandibles and skulls. *Sci Rep*, 7, 9508, 2017. DOI: 10.1038/s41598-017-10232-1

13. Selba MC, Oechtering GU, Heng HG, Deleon VB: The impact of selection for facial reduction in dogs: Geometric morphometric analysis of canine cranial shape. *Anat Rec (Hoboken),* 303 (2): 330-346, 2020. DOI: 10.1002/ar.24184

14. Gieger M, Evin A, Sanchez-Villagra MR, Gascho D, Mainini C, Zollikofer CPE: Neomorphosis and heterochrony of skull shape in dog domestication. *Sci Rep,* 7, 13443, 2017. DOI: 10.1038/s41598-017-12582-2

15. Curth S, Fischer MS, Kupczik K: Can skull form predict the shape of the temporomandibular joint? A study using geometric morphometrics on the skulls of wolves and domestic dogs. *Ann Anat*, 214, 53-62, 2017. DOI: 10.1016/j.aanat.2017.08.003

16. Curth S, Fischer MS, Kupczik K: Patterns of integration in the canine skull: An inside view into the relationship of the skull modules of domestic dogs and wolves. *Zoology*, 125, 1-9, 2017. DOI: 10.1016/j.zool.2017.06.002

17. Boroni NL, Lobo LS, Romano PSR, Lessa G: Taxonoming identification using geometric morphometric approach and limited data: An example using the upper molars of two sympatric species of Calomys (Cricetidae: Rodentia). *Zoologia,* 34:e19864, 2017. DOI: 10.3897/zoologia.34.e19864

18. Yalçın H, Kaya MA, Arslan A: Comparative geometrical morphometries on the mandible of Anatolian wild sheep *(Ovis gmelini anatolica)* and Akkaraman sheep *(Ovis aries)*. *Kafkas Univ Vet Fak Derg*, 16 (1): 55-61, 2010. DOI: 10.9775/kvfd.2009.385

19. Lu X, Ge D, Xia L, Huang C, Yang Q: Geometric morphometric study of the skull shape diversification in Sciuridae (Mammalia, rodentia). *Integr Zool*, 9, 231-245, 2014. DOI: 10.1111/1749-4877.12035

20. Rohlf FJ: TpsUtil, Version 1.60. Department of Ecology and Evolution,

State University of New York, Stony Brook, NY, 2013.

21. Rohlf FJ: TpsDig2, Version 2.18. Department of Ecology and Evolution, State University of New York, at Stone Brook, USA, 2015.

22. Hammer Ø, Harper DAT, Ryan PD: PAST**:** Paleontological statistics software package for education and data analysis. *Palaeontol Electron*, 4 (1): 1-9, 2001.

23. Klingenberg CP: MorphoJ: An integrated software package for geometric morphometrics. *Mol Ecol Resour*, 11 (2): 353-357, 2011. DOI: 10.1111/j.1755-0998.2010.02924.x

24. Cardini A, Elton S: Sample size and sampling error in geometric morphometric studies of size and shape. *Zoomorphology*, 126, 121-134, 2007. DOI: 10.1007/s00435-007-0036-2

25. Figueirido B, Serrano-Alarcon FJ, Palmqvist P: Geometric morphometrics shows differences and similarities in skull shape between the red and giant pandas. *J Zool*, 286, 293-302, 2012. DOI: 10.1111/j.1469-7998.2011.00879.x

26. Russel AP, Thomason JJ: Mechanical analysis of the mammalian head skeleton. **In,** Hanken J, Hall BK (Eds): The Skull. Functional and Evolutionary Mechanisms. Vol 3, 345-383, The University of Chicago Press, Chicago, Illinois, 1993.

27. Herring SW: Masticatory muscles and the skull: A comparative perspective. *Arch Oral Biol,* 52, 296-299, 2007. DOI: 10.1016/j.archoralbio.2006.09.010

28. Igado OO: Skull typhology and morphometrics of the Nigerian Local Dog *(Canis lupus familiaris)*. *Niger J Physiol Sci,* 32, 153-158, 2017.

29. Milencovic M, Spedic VJ, Blagojevic J, Tatovic S, Vujosevic M: Skull variation in Dinaric-Balkan and Carpathian gray wolf populations revealed by geometric morphometric approaches. *J Mammal*, 91 (2): 376-386, 2010. DOI: 10.1644/09-MAMM-A-265.1

30. Olsen SJ, Olsen JW: The Chinese wolf, ancestor of new world dogs. *Science*, 197 (4303): 533-535, 1997. DOI: 10.1126/science.197.4303.533

31. Janssens L, Miller R, Dongen SV: The morphology of the mandibular coronoid process does not indicate that *Canis lupus chanco* is the progenitor to dogs. *Zoomorphology,* 135, 269-277, 2016.

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Research Article

Coherence of Clinical Symptoms at Antemortem Inspection and Pathological Lesions at Postmortem Inspection in Slaughter Pigs

Nikola ČOBANOVIĆ [1,a] Urška JAMNIKAR-CIGLENEČKI [2,b] Andrej KIRBIŠ [2,c] Manja KRIŽMAN [2,d]
Marina ŠTUKELJ [3,e] Ivan VIĆIĆ [1,f] Nedjeljko KARABASIL [1,g]

[1] University of Belgrade, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Bulevar Oslobodjenja 18, 11000 Belgrade, SERBIA
[2] University of Ljubljana, Veterinary Faculty, Institute of Food safety, Feed and Environment, Gerbičeva 60, 1000 Ljubljana, SLOVENIA
[3] University of Ljubljana, Veterinary Faculty, Clinic for Ruminants and Pigs, Clinic for Reproduction and Farm Animals, Cesta v Mestni log 47, 1000 Ljubljana, SLOVENIA

ORCIDS: [a] 0000-0003-2650-6272; [b] 0000-0002-3522-0551; [c] 0000-0003-2734-2222; [d] 0000-0002-5578-8486; [e] 0000-0003-2295-3788
[f] 0000-0001-8762-2811; [g] 0000-0001-6097-3216

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Abstract

The aim of this study was to examine the relationship between clinical symptoms recorded during the antemortem inspection in the lairage and pathological lesions at postmortem inspection of slaughter pigs. If clinical symptoms were an accurate indicator of pathological lesions at postmortem inspection it could be possible to incorporate only those parameters in the health and welfare monitoring system. The study was conducted on 1033 pigs originated from 39 small-scale farms. During the antemortem inspection, pigs were clinically inspected for the presence of coughing, sneezing and laboured breathing. The plucks of slaughtered pigs from each farm were examined for pneumonia, pleurisy and liver milk spots. No relationship was found between clinical symptoms and pathological lesions using Spearman correlation analysis. According to receiver operating characteristic curves and the area under the curves, 'positive farms' for pathological lesions at the postmortem inspection could not be accurately detected by the clinical symptoms recorded during antemortem inspection. These results suggest that the recording of pathological lesions at postmortem inspection is more reliable and feasible method for pig health and welfare monitoring than the recording of clinical symptoms during the antemortem inspection. Therefore, incorporating of pathological lesions scoring as part of the routine postmortem veterinary inspection process could function as iceberg indicators of underlying problems affecting pig health and welfare at farm level.

Keywords: *Coughing, Liver milk spots, Lung lesions, Receiver operating characteristic analysis, Sneezing*

Domuzlarda Antemortem Muayenedeki Klinik Bulgular İle Postmortem Muayenede Belirlenen Patolojik Lezyonların Tutarlılığı

Öz

Bu çalışmanın amacı, kesim domuzlarının barınakta antemortem muayenesi sırasında kaydedilen klinik semptomlar ile post-mortem muayenesinde belirlenen patolojik lezyonlar arasındaki ilişkiyi incelemektir. Böylece, klinik semptomlar postmortem muayenede belirlenen patolojik lezyonların doğru bir göstergesi ise, sadece bu parametreleri hayvan sağlığı ve refahı takip sistemine dahil etmek mümkün olabilir. Çalışma 39 küçük ölçekli çiftlikten temin edilen 1033 domuz üzerinde gerçekleştirildi. Antemortem muayene sırasında domuzlar öksürük, aksırma ve solunum güçlüğü yönünden klinik olarak incelendi. Kesilen domuzlar pnömoni, plörezi ve karaciğer süt lekeleri açısından incelendi. Spearman korelasyon analizi kullanılarak yapılan değerlendirmede klinik semptomlar ile patolojik lezyonlar arasında ilişki bulunmadı. Oluşturulan karakteristik eğrilere ve eğrilerin altında kalan alana göre, ölüm sonrası incelemede patolojik lezyonlar bakımından "pozitif çiftlikler", ön inceleme sırasında kaydedilen klinik semptomlarla doğru bir şekilde tespit edilemedi. Bu sonuçlar, domuz sağlığı ve refahının izlenmesinde postmortem muayenede patolojik lezyonların kaydedilmesinin, antemortem muayene sırasında klinik semptomların kaydedilmesinden daha güvenilir ve uygulanabilir bir yöntem olduğunu göstermektedir. Bu nedenle, rutin postmortem veteriner hekim muayene sürecinin bir parçası olarak patolojik lezyon skorlamasının kullanılması, çiftlik düzeyinde domuz sağlığını ve refahını etkileyen altta yatan sorunların göstergesi olarak işlev görebilir.

Anahtar sözcükler: *Öksürük, Karaciğer süt lekeleri, Akciğer lezyonları, Alıcı işletme karakteristik analizi, Aksırma*

Correspondence
+381 11 2685653
cobanovic.nikola@vet.bg.ac.rs

INTRODUCTION

Respiratory diseases and ascariasis are one of the major contributors to reduce health and welfare in pig production. Respiratory disorders in pigs may be accompanied by the clinical symptoms such as coughing, sneezing, laboured breathing, nasal discharge, lethargy, and fever [1,2], while *Ascaris suum*-induced respiratory distress in pigs can be followed by coughing and laboured breathing [3]. As a consequence of respiratory diseases and ascariasis in fattening pigs, pathological lesions at the postmortem inspection are frequently found, as an incidence between 12% and 45% for lung lesions and between 14.5% and 40% for liver milk spots [4-6]. These lesions are associated with significant economic losses for the pig producers, primarily due to a reduction in average daily weight gain, growth rate and feed conversion efficiency and increased morbidity, mortality, medication and veterinary expenses [7,8]. They also cause financial losses to the slaughterhouse as a consequence of reduced carcass and pork quality, increased carcass and viscera trimming procedures and disposal of organs unfit for human consumption [9-11].

A certain degree of contradiction exists between the studies whether the clinical examination is actually needed for a reliable health and welfare assessment at farm level or assessment of pathologic lesions at postmortem inspection would be a more sufficient way to assess pig health and welfare. Several studies [2,12-14] have reported that animal health and welfare at farm level can be estimated by calculating the frequency of clinical symptoms recorded during the antemortem inspection at the slaughterhouse and/or on the farm of origin. However, some authors [15-18] did not detect the relationship between clinical symptoms of respiratory diseases and *Ascaris suum* invasion and pathological lesions at postmortem inspection, indicating that the clinical observation during antemortem inspection was not a sensitive indicator of pig health and welfare. Therefore, the aim of this study was to examine the relationship between clinical symptoms (coughing, sneezing and laboured breathing) recorded during antemortem inspection in the lairage and pathological lesions obtained for the same batches of slaughter pigs during postmortem inspection. The hypothesis was that the level of clinical symptoms recorded in a given batch of pigs would be a good measure to detect 'positive farms' for pathological lesions at the postmortem inspection.

MATERIAL and METHODS

The study was conducted between 1 January 2016 and 1 January 2019 on 1033 slaughter pigs (539 barrows and 494 gilts), about six months old, with an average live weight of approximately 115 kg. All pigs were of the same genetics ([Yorkshire × Landrace] sows sired with Pietrain boars) and originated from 39 small-scale commercial farms. The study farms showed a large variability in housing conditions, microclimate control, feeding plan and management. On the day of slaughter, all pigs were subjected to similar pre-slaughter handling, transportation and lairaging in compliance with the standard marketing conditions for Southeastern Europe [19]. Slaughter procedure and carcass processing were identical for all pigs and were performed in accordance with the standard industry-accepted practices in the same low-input slaughter facility, with a weekly slaughter rate of 175 pigs.

Antemortem Inspection

One group of pigs for each of the 39 small-scale farms was selected during lairaging, whereby the animals were inspected for clinical symptoms by three trained assessors. At each sampling day, two pens which best represent the farm of origin, holding a minimum of 10 pigs were selected. Hospital pens were not included in the sampling plan. During clinical examination, assessors were stationed in the corridor with a clear view into all pigs in the selected pens. The pigs in the pens under surveillance are firstly roused and then have five minutes to calm down until their activity had gone back to normal.

Three symptoms were taken into account to evaluate the presence of clinical symptoms of respiratory diseases and *Ascaris suum* invasion: coughing, sneezing and laboured breathing. Coughing was recorded when pigs displayed an audible expulsion of air through the mouth. Sneezing was defined as a sudden involuntary expulsion of air from the nose and mouth due to irritation of one's nostrils. A pig exhibited laboured breathing when at least one of the following signs was observed: tachypnea (breathing frequency higher than 20 breaths/min), enforced abdominal breathing, breathing in a pumping way and excessive nostril movements. Coughing and sneezing were counted in each pen under surveillance for five minutes using the Welfare Quality® protocol [20]. In addition, the percentage of pigs showing coughing, sneezing and laboured breathing was also calculated. The farm level score was calculated based on the Welfare Quality® protocol [20].

Postmortem Inspection

The pluck from each slaughtered pig consisted of heart, lung and liver that are removed from the carcass by abattoir personnel and first visually and then by palpation assessed for macroscopically visible lesions of pneumonia, pleurisy and liver milk spots by the three trained investigators using the Welfare Quality® protocol [20]. Pigs that were inspected in the slaughterhouse were those which were clinically observed during an antemortem inspection in lairage. The assessment was performed directly at the slaughter line before the routine postmortem veterinary inspection, to include organs that would have been discarded during inspection. Pneumonia, pleurisy and liver milk spots were recorded as binary variables with the lesion being scored

as either present (score 2) or absent (score 0) in each organ. The farm level score was calculated based on the Welfare Quality® protocol [20].

Statistical Analysis

Statistical analysis of the results was conducted using SPSS software (Version 23.0, IBM Corporation, Armonk, NY, USA) [21]. The incidence of clinical symptoms and pathological lesions in slaughter pigs was calculated at the batch level. A batch was defined as a group of pigs belonging to the same farm that were killed on the same day at the same slaughterhouse. Batch size ranged from 20 to 35 pigs, with an average of 26.5 pigs per batch. The batch was used as an experimental unit for all statistical analyses. Data were described by descriptive statistical parameters as the mean value, standard deviation, standard error of means, and minimum and maximum range. A probability level of $P<0.05$ was chosen as the limit for statistical significance in all tests.

Spearman rank correlation analysis (r_{sp}) was run between the clinical symptoms and pathological lesions in slaughter pigs to numerically summarise the degree of association between any two variables. A further set of analysis compared the incidence of pathological lesions observed for the 39 batches (one per farm) with a benchmark value above which the health and welfare situation of the batch should be regarded as seriously compromised. Those farms with the incidence of pathological lesions that exceeded the alarm threshold set by the Welfare Quality® protocol [20] were considered as 'positive' farms (55% for lung lesions and 23% for liver milk spots). Threshold for lung lesions percentage was established based on alarm threshold for pleurisy [20], since it corresponded to the mean percentage of lung affected by lesions in the 39 screened farms. For each clinical symptom were created the receiver operating characteristic (ROC) curves, and the areas under the ROC curve (AUC) were calculated to acquire the accuracy of the prediction. The optimal cut-off points on the ROC curves (percentage of pigs expressing the clinical symptom) were determined by selecting the optimal sensitivity (SE) and specificity (SP) using the following formula: minimal value of $[(1 - SE)^2 + (1 - SP)^2]$ [22]. Sensitivity, specificity and positive predictive values were calculated for the selected cut-off point for each clinical symptom. Values of AUC were interpreted as follows: i) area greater than 0.9 indicates high accuracy; ii) area between 0.7 and 0.9 indicates moderate accuracy; iii) area between 0.5 and 0.7 indicates low accuracy; iv) area lower than 0.5 is interpreted as non-informative [22]. To determine if the detection of 'positive farms' for pathological lesions based on clinical examinations would improve when multiple clinical symptoms were considered, a logistical regression analysis was carried out with pathological lesion scores as a binary response variable (2 for an incidence above 55% for lung lesions; 23% for liver milk spots; and 0 for an incidence below aforementioned thresholds).

RESULTS

Incidence of Clinical Symptoms at Antemortem Inspection and Pathological Lesions at Postmortem Inspection

Incidence of clinical symptoms at antemortem inspection and pathological lesions at postmortem inspection calculated at farm level is shown in *Table 1*. Coughing was the most prevalent clinical symptom at antemortem inspection, with a mean value of 4.55% (0.06 coughs/pig) of the pigs affected, followed by sneezing (3.11%, 0.06 sneezes/pig) and laboured breathing (0.43%). The frequencies of coughing (ranged from 0.00 to 0.14 coughs/pig), sneezing (ranged from 0.00 to 0.24 sneezes/pig) and laboured breathing (ranged from 0.00% to 3.85%) at the farm level (n=39) were maintained under alarm threshold values set by the Welfare Quality® protocol [20].

The most prevalent pathological lesion at postmortem inspection was pneumonia (43.04%), followed by liver milk spots (31.83%) and pleurisy (21.68%) *(Table 1)*. According to Welfare Quality® protocol [20], each farm had the incidence of pneumonia above the alarm threshold set for this health criterion. In addition, of the 39 farms assessed, 30.77% exceeded the warning threshold and 5.13% exceeded the alarm threshold set for pleurisy. Of the 39 farms assessed, 33.33% exceeded the warning threshold and 48.72% exceeded the alarm threshold for liver milk spots.

Spearman Correlations Between Clinical Symptoms at Antemortem Inspection and Pathological Lesions at Postmortem Inspection

Spearman rank correlations between clinical symptoms at antemortem inspection and pathological lesions at the postmortem inspection are depicted in *Table 2*. No significant correlation was found between clinical symptoms during antemortem inspection and pathological lesions at the postmortem inspection ($P>0.05$).

ROC Curve Analysis for the Detection of 'Positive Farms' for Pathological Lesions at Postmortem Inspection based on Clinical Symptoms during Antemortem Inspection

When considering the alarm thresholds for pigs with pathological lesions at postmortem inspection set by the Welfare Quality® protocol [20], the incidence of 'positive farms' was 35.90% for lung lesions and 48.72% for liver milk spots.

The ROC curves for the detection of 'positive farms' for lung lesions using the clinical symptoms are shown in *Fig. 1*. The AUC were 0.63 for coughing (95% CI of 42.50-82.50), 0.66 for sneezing (95% CI of 47.60-84.20) and 0.67 for laboured breathing (95% CI of 50.00-84.70). For coughing the optimal cut-off value was 3.39% with a sensitivity of 68.20%, a specificity of 50.00%, and a positive predictive

Table 1. *Incidence of clinical symptoms at antemortem inspection and pathological lesions at postmortem inspection calculated at farm level (n = 39)*

Parameter		Mean	SD	SE	Minimum	Maximum	Warning Threshold [20]	Alarm Threshold [20]
Clinical symptoms	Coughing (%)	4.55	3.78	0.61	0.00	10.00	-	-
	Coughs/pig[1]	0.06	0.05	0.01	0.00	0.14	>0.15	>0.46
	Sneezing (%)	3.11	3.31	0.53	0.00	10.00	-	-
	Sneezes/pig[2]	0.06	0.07	0.01	0.00	0.24	>0.27	>0.55
	Laboured breathing (%)	0.43	1.15	0.18	0.00	3.85	>1.8	>5.0
Pathological lesions	Pneumonia %)	43.04	25.98	4.16	8.00	89.29	>2.7	>6.0
	Pleurisy (%)	21.68	18.99	3.04	0.00	71.43	>28.0	>55.0
	Liver milk spots %)	31.83	24.81	3.97	0.00	93.33	>10.0	>23.0

[1] number of coughs per pig during 5 min; [2] number of sneezes per pig during 5 min; ***SD:*** *standard deviation;* ***SE:*** *standard error of means*

Table 2. *Spearman rank correlations (r_{sp}) between clinical symptoms at antemortem inspection and pathological lesions at postmortem inspection*

Variables	Coughing	Sneezing	Laboured Breathing
Pneumonia	0.202	0.267	0.137
Pleurisy	0.034	0.248	0.120
Milk spots	0.017	-	0.108

** Statistical significance at (P<0.05)*

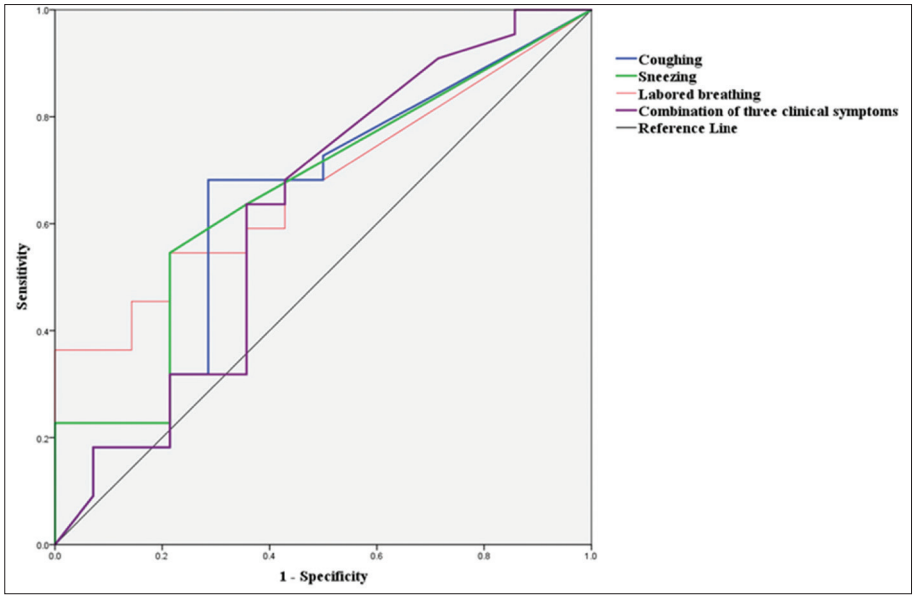

Fig 1. ROC curves for the detection of 'positive farms' for lung lesions using the clinical symptoms

value of 52.16%. For sneezing the optimal cut-off value was 1.67% with a sensitivity of 63.60%, a specificity of 35.70% and a positive predictive value of 44.00%. For laboured breathing, the optimal cut-off value was 1.47%, with a sensitivity of 68.20%, a specificity of 52.16% and a positive predictive value of 52.68%.

The logistic regression model that predicted high incidence of lung lesions best, comprised the following three clinical symptoms: coughing, sneezing and laboured breathing. As can be seen in *Fig. 1*, using this multivariable model for the detection of 'positive farms' for lung lesions did not significantly improve the quality of the ROC curve. The AUC using a multivariable model was slightly lower compared to the AUC for ROC curves obtained for single clinical symptoms: 0.62.

The ROC curves for the detection of 'positive farms' for liver milk spots using the clinical symptoms are shown in *Fig. 2*. The AUC were 0.63 for coughing (95% CI of 43.90-81.20) and 0.60 for laboured breathing (95% CI of 40.90-79.10). For coughing the optimal cut-off value was 1.67% with a sensitivity of 84.60%, a specificity of 52.20% and a positive predictive value of 47.71%. For laboured breathing the

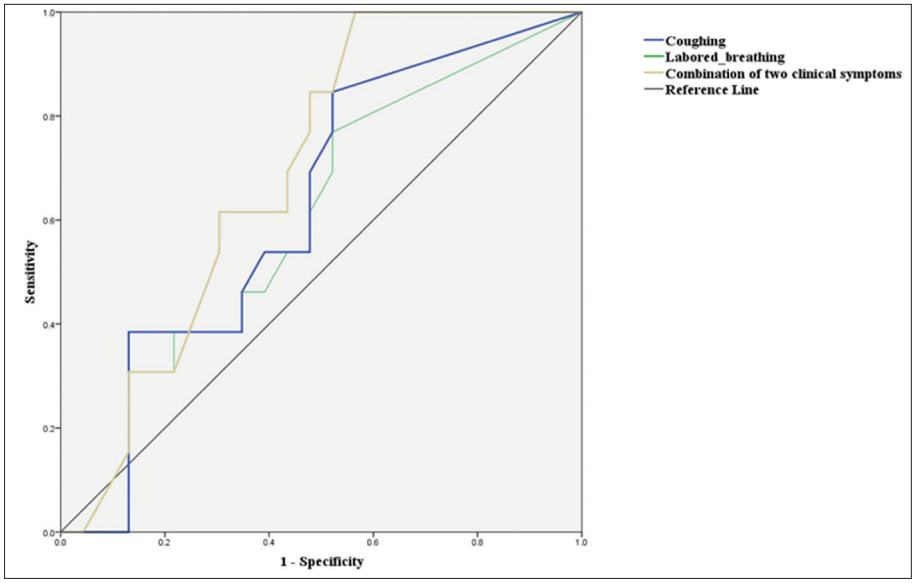

Fig 2. ROC curves for the detection of 'positive farms' for liver milk spots using the clinical symptoms

optimal cut-off value was 1.47% with a sensitivity of 76.90%, a specificity of 52.20%, and a positive predictive value of 45.25%.

The logistic regression model that predicted high incidence of liver milk spots best, comprised the following two clinical symptoms: coughing and laboured breathing. As depicted in *Fig. 2*, using this multivariable model for the detection of 'positive farms' for liver milk spots did not significantly improve the quality of the ROC curve. The AUC using a multivariable model was marginally improved compared to the AUC for ROC curves obtained for single clinical symptoms: 0.69.

DISCUSSION

The incidence of clinical symptoms recorded at antemortem inspection remained low throughout the entire study period. In addition, the frequencies of coughing, sneezing and laboured breathing were below both warning and alarm threshold values set by the Welfare Quality® protocol [20]. Therefore, according to the results of clinical examination, it can be argued that there was no indication of a health and welfare problem on the farm of origin. The incidence of pathological lesions detected at the postmortem inspection was much higher than the incidence of clinical symptoms recorded during the antemortem inspection. Furthermore, each pig farm had the incidence of at least one of the pathological lesions detected at the postmortem inspection above the alarm threshold set by the Welfare Quality® protocol [20]. Hence, according to the incidence of pathological conditions in slaughtered pigs, there was a strong indication of a serious health and welfare problem on the farm of origin.

The possibility to detect 'positive farms' for pathological lesions at postmortem inspection by recording the incidence of clinical symptoms during antemortem inspection was tested by ROC curve analyses. This statistical approach should not be interpreted as an assessment of the potential capability of clinical symptoms to be used in the diagnosis of pig diseases. ROC analysis was conducted to assess the potential use of clinical symptoms recorded at antemortem inspection as parameters able to discriminate between 'positive' and 'negative' farms for pathological lesions recorded at the postmortem inspection as indicators of health and welfare on the farm of origin. The positive predictive values found can be considered as low given the fact that only 44.00%-52.68% of the farms regarded as positive based on one of the clinical symptoms exceeded the positive threshold for pathological lesions at postmortem inspection. During this investigation, the incidence of 'positive farms' for lung lesions and liver milk spots was relatively high and, thus, it could be expected high positive predictive values [23]. Furthermore, AUC values between 0.63 and 0.67 for individual clinical symptoms and 0.62 for a multivariable model, indicating that 'positive farms' for lung lesions could not be accurately predicted by clinical observation. Likewise, AUC values between 0.60 and 0.69 for individual clinical symptoms and 0.62 for a multivariable model, indicating that the ability of the coughing and laboured breathing to detect 'positive farms' for liver milk spots was very low. Sensitivity between 63.60%-84.60% and specificity between 35.70%-52.20% were achieved for clinical symptoms in discriminating 'positive farms' for pathological lesions at the slaughter line. However, obtained specificity means that between two thirds and half of 'negative farms' would be incorrectly identified as 'positive farms' for pathological lesions at postmortem inspection. This indicates that this measure would not be of practical use in informing pig producers of potential health and welfare issues within the herd as feedback would be inaccurate in many cases. The results obtained by the ROC curve analyses were strengthened because in the present research, no relationship was found

between clinical symptoms and pathological lesions using Spearman correlations (r_{sp} ranged from 0.017 to 0.267; P>0.05) *(Table 2)*. Therefore, it can be considered that clinical symptoms recorded during antemortem inspection did not allow an accurate detection of 'positive farms' for pathological lesions at the postmortem inspection.

The results obtained in this study are comparable to several studies [15-18], who found a high prevalence of lung lesions in the herds without clinical symptoms. These results can be explained by the fact that respiratory diseases and ascariasis in fattening pigs are characterised by vague and nonspecific clinical symptoms [1,24]. These diseases occur in a subclinical form or as uncomplicated infections, and produce pathological lesions that can be only identified during postmortem inspection [1,15,17]. There is a possibility that sporadic clinical symptoms recorded in this study were not only provoked by lung inflammation, but also by inadequate environmental conditions in the lairage and/or on the farm of origin, such as high ambient temperature and relative humidity, presence of aerial dust and manure gases. In these situations, gross irritation of the nostrils and airways, together with suppression of the microscopic lung defense mechanisms resulted in sporadic coughing, sneezing and laboured breathing in finishing pigs. Thus, clinical examination cannot be regarded as an efficient and reliable method for the health and welfare assessment [16,25,26]. Accordingly, the evaluation of pathological lesions during postmortem inspection is of paramount importance to identify subclinical diseases, which are not possible to detect by clinical examination during an antemortem inspection at the slaughterhouse and/or on the farm of origin [26]. In addition, there are several advantages of pathological lesion assessment at the slaughter line compared with clinical examination at antemortem inspection at the slaughterhouse and/or on farm of origin. Although clinical examination is an inexpensive method for pig health and welfare assessment, it may be labour intensive, time-consuming and usually require pig handling or the pig needs to be forced to move [26]. The advantage of pathological lesion examination at the postmortem inspection is that the pigs from different farms can be examined on the same day, reducing travelling costs and minimising the risks of disease transmission within and between farms during assessments [26]. Also, postmortem health and welfare assessment avoid potential problems associated with having to assess pigs in crowded, dirty or poorly-lit conditions such as in the lairage pens and/or on the farm of origin [26].

Contrary to the findings of this study, some authors [2,12-14] found a positive association between the occurrence of pathological lesions at the postmortem inspection and clinical symptoms on farm during fattening. Several possible factors can be raised to explain this discrepancy. Data on clinical symptoms on farm during fattening and pathological lesions at the postmortem inspection are based on a slightly different sample of animals in the batches [13]. In addition, animals delivered to a slaughterhouse are clinically healthy, while those individuals who exhibit clinical symptoms during production cycle usually stay on farm and receive medical treatment until full recovery. Furthermore, some of the severely diseased pigs will not complete the production cycle because they will die or be culled during fattening prior to cohort slaughter. It is also possible that respiratory infections and ascariasis at the early stage of the fattening period will not necessarily result in pathological lesions as these might heal or become less evident at the postmortem inspection [13].

In conclusion, the results of this study showed that, in the context of pig health and welfare monitoring, the recording of pathological lesions at postmortem inspection is more reliable and feasible method than the recording of clinical symptoms during antemortem inspection. Therefore, incorporating of pathological lesions scoring as part of the routine postmortem veterinary inspection process could function as iceberg indicators of underlying problems affecting pig health and welfare on the farm of origin. However, before any firm conclusions can be drawn, further investigation is required to clarify the potential use of clinical symptoms in pigs during fattening on the farm of origin, concentration of acute phase proteins and serological testing for the most common respiratory pathogens and *Ascaris suum* to forecast the 'positive farms' for pathological lesions in slaughtered pigs.

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Conflicts of Interest

The author declares no conflict of interest.

Authors Contributions

NK and NČ defined the research theme, gave the conception of the research. NČ, IV and NK carried out experimental part of the study. UJC and MK have made supervised the analysis of the results, and contributed to the writing of the manuscript. AK and MŠ were involved in drafting the manuscript and revising it critically for important intellectual content and have made a substantial contribution to conception and design, analysis and interpretation of data. All authors discussed the results and contributed to the final manuscript.

REFERENCES

1. Jäger HC, McKinley TJ, Wood JLN, Pearce GP, Williamson S, Strugnell B, Done S, Habernoll H, Palzer A, Tucker AW: Factors associated with pleurisy in pigs: A case-control analysis of slaughter pig

data for England and Wales. *PloS One*, 7 (2): e29655, 2012. DOI: 10.1371/journal.pone.0029655

2. Nathues H, Spergser J, Rosengarten R, Kreienbrock L, grosse Beilage E: Value of the clinical examination in diagnosing enzootic pneumonia in fattening pigs. *Vet J*, 193 (2): 443-447, 2012. DOI: 10.1016/j.tvjl.2012.01.013

3. Dold C, Holland CV: Ascaris and ascariasis. *Microbes Infect*, 13 (7): 632-637, 2011. DOI: 10.1016/j.micinf.2010.09.012

4. Dalmau A, Temple D, Rodriguez P, Llonch P, Velarde A: Application of the Welfare Quality® protocol at pig slaughterhouses. *Anim Welfare*, 18 (4): 497-505, 2009.

5. Rocha LM, Velarde A, Dalmau A, Saucier L, Faucitano L: Can the monitoring of animal welfare parameters predict pork meat quality variation through the supply chain (from farm to slaughter)? *J Anim Sci*, 94 (1): 359-376, 2016. DOI: 10.2527/jas.2015-9176

6. Dalmau A, Nande A, Vieira-Pinto M, Zamprogna S, Di Martino G, Ribas JCR, da Costa MP, Halinen-Elemo K, Velarde A: Application of the Welfare Quality® protocol in pig slaughterhouses of five countries. *Livest Sci*, 193, 78-87, 2016. DOI: 10.1016/j.livsci.2016.10.001

7. Sanchez-Vazquez MJ, Smith RP, Kang S, Lewis F, Nielen M, Gunn GJ, Edwards SA: Identification of factors influencing the occurrence of milk spot livers in slaughtered pigs: A novel approach to understanding *Ascaris suum* epidemiology in British farmed pigs. *Vet Parasitol*, 173 (3-4): 271-279, 2010. DOI: 10.1016/j.vetpar.2010.06.029

8. Čobanović N, Jamnikar-Ciglenečki U, Kirbiš A, Križman M, Štukelj M, Karabasil N: Impact of various housing conditions on the occurrence of pathological lesions in slaughtered pigs. *Veterinarski Glasnik*, 73 (1): 17-29, 2019. DOI: 10.2298/VETGL190318010C

9. Karabasil N, Čobanović N, Vučićević I, Stajković S, Becskei Z, Forgách P, Aleksić-Kovačević S: Association of the severity of lung lesions with carcass and meat quality in slaughter pigs. *Acta Vet Hung*, 65 (3): 354-365, 2017. DOI: 10.1556/004.2017.034

10. Čobanović N, Vasilev D, Dimitrijević M, Teodorović V, Janković L, Karabasil N: Blood parameters, carcass and meat quality of slaughter pigs with and without liver milk spots. *Biotechnol Anim Husb*, 33 (4): 397-407, 2017. DOI: 10.2298/bah1704397c

11. Čobanović N, Janković Lj, Vasilev D, Dimitrijević M, Teodorović V, Kureljušić B, Karabasil N: Slaughterline records of various postmortem pathological lesions and their influence on carcass and meat quality in slaughtered pigs. *Anim Sci J*, 90 (11): 1475-1483, 2019. DOI: 10.1111/asj.13287

12. Jaeger HJ, Pearce GP, Tucker AW, Wood JLN, Done S, Strugnell B, Williamson S, Woodger N, Burling J, Habernoll H, Dewhirst J: Pleurisy in Pigs: Associated risk factors and impact on health, welfare and performance 2005-2008. Milton Keynes BPEX (British Pig Executive), 2009. [http://www.bpex.org.uk/KTRandD/ResearchAndDevelopment/Pleurisy.aspx]; *Accessed:* 15/11/2019.

13. Leruste H, Brscic M, Heutinck LFM, Visser EK, Wolthuis-Fillerup M, Bokkers EAM, Stockhofe-Zurwieden N, Cozzi G, Gottardo F, Lensink BJ, Van Reenen CG: The relationship between clinical signs of respiratory system disorders and lung lesions at slaughter in veal calves. *Prev Vet Med*, 105 (1-2): 93-100, 2012. DOI: 10.1016/j.prevetmed.2012.01.015

14. Becker PM, Van Wikselaar PG, Mul MF, Pol A, Engel B, Wijdenes JW, van der Peet-Schwering CMC, Wisselink HJ, Stockhofe-Zurwieden N: *Actinobacillus pleuropneumoniae* is impaired by the garlic volatile allyl methyl sulfide (AMS) *in vitro* and in-feed garlic alleviates pleuropneumonia in a pig model. *Vet Microbiol*, 154 (3-4): 316-324, 2012. DOI: 10.1016/j.vetmic.2011.07.011

15. Morris CR, Gardner IA., Hietala SK, Carpenter TE: Enzootic pneumonia: comparison of cough and lung lesions as predictors of weight gain in swine. *Can J Vet Res*, 59 (3): 197-204, 1995.

16. Ostanello F, Dottori M, Gusmara C, Leotti G, Sala V: Pneumonia disease assessment using a slaughterhouse lung-scoring method. *J Vet Med A*, 54 (2): 70-75, 2007. DOI: 10.1111/j.1439-0442.2007.00920.x

17. Baraldi TG, Neves Cruz NDR, Dalla Costa FA, Pereira DA, Storino GY, Da Silva AF, Panzardi A, De Oliveira LG: Association between coughing, pneumonia index and pleurisy. *In proceedings of the II Simpósio Internacional de Produção e Sanidade de Suínos*, Jaboticabal, Brazil, April 05-07, 2017.

18. Emikpe BO, Jarikre TA, Adediran OA, Olaniyi MO, Dikeogu TC: Haematology, bronchoalveolar cellular changes and pathology of swine pneumonia in Nigeria. *Sokoto J Vet Sci*, 16 (2): 1-9, 2018. DOI: 10.4314/sokjvs.v16i2.1

19. Čobanović N, Stanković, SD, Dimitrijević M, Suvajdžić B, Grković N, Vasilev D, Karabasil N: Identifying physiological stress biomarkers for prediction of pork quality variation. *Animals*, 10 (4):614, 2020. DOI: 10.3390/ani10040614

20. Welfare Quality®: Welfare Quality® assessment protocol for pigs (sow and piglets growing and finishing pigs). *Welfare Quality® Consortium*, L., The Netherlands, 2009.

21. SPSS: Statistical Package for Social Sciences for Windows (version S23.0). SPSS Inc., Armonk, NY: IBM Corp., USA, 2015.

22. Akobeng AK: Understanding diagnostic tests 3: Receiver operating characteristic curves. *Acta Paediatr*, 96 (5): 644-647, 2007. DOI: 10.1111/j.1651-2227.2006.00178.x

23. Akobeng AK: Understanding diagnostic tests 1: Sensitivity, specificity and predictive values. *Acta Paediatr*, 96 (3): 338-341, 2007. DOI: 10.1111/j.1651-2227.2006.00180.x

24. Kanora A: Effect on productivity of treating fattening pigs every 5 weeks with flubendazole in feed. *Vlaams Diergeneeskd Tijdschr*, 78, 170-175, 2009.

25. Scollo A, Gottardo F, Contiero B, Mazzoni C, Leneveu P, Edwards SA: Benchmarking of pluck lesions at slaughter as a health monitoring tool for pigs slaughtered at 170 kg (heavy pigs). *Prev Vet Med*, 144, 20-28, 2017. DOI: 10.1016/j.prevetmed.2017.05.007

26. Dalmau A, Fabrega E, Manteca X, Velarde A: Health and welfare management of pigs based on slaughter line records. *J Dairy Vet Anim Res*, 1 (3): 73-78, 2014. DOI: 10.15406/jdvar.2014.01.00016

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Research Article

Classification of Raw Milk Composition and Somatic Cell Count in Water Buffaloes with Support Vector Machines

Yalcin TAHTALI [1,a]

[1] Tokat Gaziosmanpasa University, Faculty of Agriculture, Department of Animal Science, TR-60250 Tokat - TURKEY
[a] ORCID: 0000-0003-0012-0611

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Abstract

The study investigates the classification of milk quality with support vector machines (SVM) using the raw milk composition and somatic cell count (SCC) data on buffalos. For this purpose, 11-variable (dry matter, fat-free dry matter, fat (%), protein, lactose, casein, urea, density, acidity, pH, freezing point) on milk composition and SCC of 288 buffalos were used. SVM is a classifier with a high generalization ability that is based on structural risk minimization with a statistical learning system and can be applied to both linear and non-linear data. The classification successes of some kernel functions used in the SVM (polynomial kernel, normalized polynomial kernel and radial basis kernel) were investigated and their classification performances were compared with a multilayer perceptron algorithm. The results showed that the classification successes of polynomial kernel, normalized polynomial kernel and radial basis kernel were 93.06%, 92.36% and 90.97%, respectively, while the classification success of the multilayer perceptron was 81.60%. The comparison of the results with respect to the root mean square error (RMSE) values revealed that the polynomial kernel had the lowest value (0.263), while the multilayer perceptron had the highest value (0.384). According to this criterion, the best classifier was the polynomial kernel function, while the weakest classifier was the multilayer perceptron (0.384). Considering the receiver operating characteristic (ROC) area values, with respect to the closeness to 1 criterion, normalized polynomial kernel was the best function, while the multilayer perceptron function was the weakest function. The separate evaluation of the precision, sensitivity and F-measure values showed that the polynomial kernel was the most successful function, while the multilayer perceptron was the weakest function.

Keywords: *Support vector machine, Somatic cell count, Kernel model optimization*

Mandalarda Çiğ Süt Bileşimi ve Somatic Hücre Sayısının Destek Vektör Makinaları İle Sınıflandırılması

Öz

Bu çalışmada amaç mandalarda çiğ süt bileşimi ve somatik hücre sayısı verilerini kullanarak süt kalitesinin destek vektör makineleri (DVM) ile sınıflandırılmasını araştırmaktır. Bu amaçla, 288 mandaya ait somatik hücre sayısı ve 11 değişkenli (kuru madde, yağsız kuru madde, yağ, protein, laktoz, kazein, üre, yoğunluk, asitlik, pH, donma noktası) süt bileşenleri kullanılmıştır. DVM, istatistiksel öğrenme sistemi ile yapısal risk minimizasyonuna dayanan, hem doğrusal hem de doğrusal olmayan verilere uygulanabilen yüksek genelleme kabiliyetine sahip bir sınıflandırıcıdır. DVM'de kullanılan bazı çekirdek fonksiyonlarının (polinom çekirdeği, normalleştirilmiş polinom çekirdeği ve radyal temel çekirdeği) sınıflandırma başarıları araştırılmış ve sınıflandırma performansları çok katmanlı bir algılayıcı algoritması ile karşılaştırılmıştır. Sonuçlar, polinom çekirdeğinin, normalize polinom çekirdeğinin ve radyal temel çekirdeğin sınıflandırma başarılarının sırasıyla %93.06, %92.36 ve %90.97 olduğunu, çok katmanlı algılayıcı algoritmanın sınıflandırma başarısının %81.60 olduğunu göstermiştir. Çekirdek fonksiyonlarının hata kareleri ortalamasının karekökü (RMSE) değerleri ile karşılaştırılması yapıldığında, polinom çekirdeğinin en düşük değere (0.263) sahip olduğunu, çok katmanlı algılayıcının en yüksek değere (0.384) sahip olduğu tespit edilmiştir. Bu kritere göre, en iyi sınıflandırıcının polinom çekirdek fonksiyonu, en zayıf sınıflandırıcının ise çok katmanlı algılayıcı (0.384) olduğu görülmüştür. ROC eğrisi altında kalan alan değerleri göz önüne alındığında, 1'e yakınlık kriteri açısından, normalleştirilmiş polinom çekirdeği en iyi fonksiyon, çok katmanlı algılayıcının en zayıf fonksiyon olduğu gözlenmiştir. Hassasiyet, duyarlılık ve F-ölçüm değerlerinin ayrı ayrı değerlendirilmesi sonucunda sınıflandırmada en başarılı fonksiyonun polinom çekirdeğini, en başarısız fonksiyonun ise çok katmanlı algılayıcı olduğu belirlenmiştir.

Anahtar sözcükler: *Destek vektör makinesi, Somatik hücre sayısı, Çekirdek model optimizasyonu*

INTRODUCTION

The studies regarding the solution of classification problems hold an important place in data mining. The generalization performance of an algorithm is an important criterion that should be considered in the selection of the machine

Correspondence
+90 356 2521616-2181
yalcin.tahtali@gop.edu.tr

learning algorithm that will be developed for the solution of classification problems. Generalization performance depends on factors such as training data, number/structure of independent qualities, model selection and parameter selection. Considering these factors, retrieving classified and meaningful information from the data and obtaining accurate information are directly proportionate to the generalization success of the algorithm. In other words, the better the generalization performance of the algorithm, the more realistic the retrieved information [1].

In recent years, Support Vector Machines (SVM) have become one of the most successful machine learning algorithms in the solution of the classification problems [1]. The method carries out classification either using a linear or non-linear function. The SVM method is based on estimating the more suitable function for separating the data.

The most prominent advantage of the SVM is they solve the classification problem by turning it into a quadratic optimization problem. Thus, they minimize the number of processes during the learning stage for the solution of a problem and reach solutions faster than other methods/algorithms [1]. They especially provide advantages in large scale datasets thanks to this feature. Moreover, as an optimization-based system, their classification performance, calculation complexity and practicality are better than other methods [2]. During the application of the SVM to the solution of the classification problems for various datasets, the selection of the kernel function and optimization of parameters play important roles. Based on data transformations, the solution of the kernel function finds the most suitable boundary among the possible outputs. This method is used in various fields including the classification of data in animal breeding.

Buffalo milk, the material of the study, contains relatively higher levels of protein, fat and mineral matters (especially calcium and phosphorus) and, thus, is more nutritious than cow milk [3]. Due to its high milk quality and availability for processing into other dairy products add to the demand for buffalo milk. Milk composition and somatic cell count (SCC) are important parameters in the determination of milk quality [4]. The number of somatic cells in normal milk is low and high number of somatic cells indicate that the milk is of low quality. In addition to its function as a quality measure, SCC in milk is an indicator of udder health in herd management and indicator in the diagnosis of mastitis [5,6]. The SCC limit for raw buffalo milk is 400.000 cells/ml according to the European Union (EU) directives (92/46 CEE and 94/71CEE) [7], while it is ≤500.000 cells/mL according to the Turkish Food Codex [8].

The study investigates the data mining applications for classification that is based on the critical SCC limit for animal breeding and especially for buffalo milk content using the SVM method.

MATERIAL and METHODS

The study material consisted of milk yield recordings on the 288 Anatolian buffalos that gave birth between 2011 and 2013 in Tokat and its counties, Turkey. The milk yields of the Anatolian buffalos were obtained with the help of the National Buffalo Improvement by the Public Project supported by the General Directorate of Agricultural Research and Policies. On the control days, the milk yields of the buffalos were recorded in kilograms in the morning and evening. The buffalo breeding in the research area is carried out under extensive conditions. The breeders usually do not use additional feeding especially during the foraging period, but additional feeding can be carried out during winter depending on available feed types (hay, dry clover, silage, etc.).

Support Vector Machines is controlled classification algorithm that is based on the statistical learning theory. The mathematical algorithms of SVM were first designed for the solution of the classification problem of two-class linear data and, then, generalized for the classification of multiclass and nonlinear data. The working principle of SVM is based on the estimation of the most suitable decision function for the distinction of two classes, in other words, it is based on the identification of the hyperplane in which the distance between two classes is the maximum and most appropriate distance [9,10]. There are two cases in the SVM: data are linearly separable or not linearly separable.

Linear Separability

Let's assume the data that will be used in training and contain N number of elements is $\theta = \{x_i, y_i\}, i = 1, 2\ldots, N$. Here, $y_i \in \{-1, 1\}$ is the label values and $x_i \in R^d$ is the feature vector. In the case of linear separation, these two-value data can directly be separated by a hyperplane. This hyperplane is called the separating hyperplane. The purpose of the SVM is to make sure the hyperplane is at the same distance to the sample groups in both classes.

If the training data comprising k number of samples in a two-class linearly separable classification problem is accepted as $\{x_i, y_i\}$, i = 1, .., k, the equations of the optimum hyperplane will be:

$$w.x_i+b \geq 1, \text{ for each } y= +1 \quad (1)$$

$$w.x_i+b \leq 1, \text{ for each } y= -1 \quad (2)$$

Here, $x \in R^n$ represents an N-dimension space, $y \in \{-1, +1\}$ represents class labels, w represents weight vectors (the normal of the hyperplane) and b represents the bias value [11]. To determine the optimum hyperplane, two hyperplanes that are parallel to this plane and form its boundaries should be created. The points forming these hyperplanes are referred to as support vectors and defined as $w.x_i+b<1$ or $w.xi+b>1$. *Fig. 1* shows the hyperplane for linearly separable datasets.

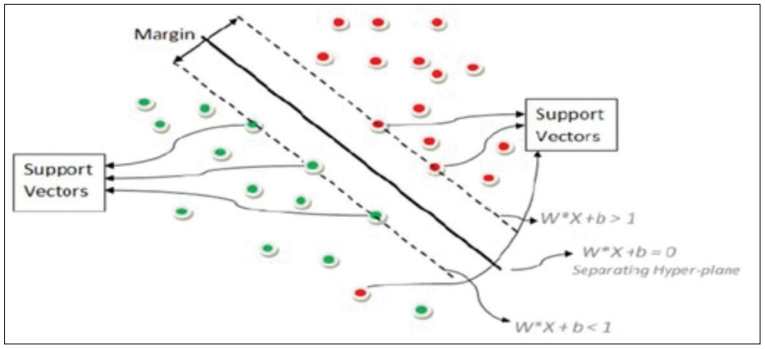
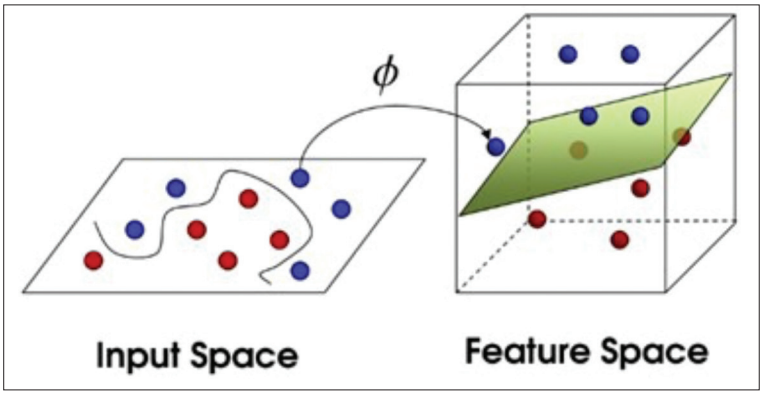


Fig 1. Hyperplane for linearly separable datasets

Fig 2. Transformation of the data into a higher dimension with the kernel function



To maximize the boundary of the optimal hyperplane, ||w|| should be minimized. In this case, determining the most suitable hyperplane requires solving the following limited optimization problem.

$$min\left[\frac{1}{2}\|w\|^2\right], \quad (3)$$

The corresponding boundaries are defined as [9].

$$y_i(w.x_i + b) - 1 \geq 0 \text{ and } y_i \in \{1, -1\} \quad (4)$$

The optimization problem can be solved using the Euler-Lagrange equations. After this process, equation 5 is obtained.

$$L(w, b, \alpha) = \frac{1}{2}\|w\|^2 - \sum_{i=1}^{k} \alpha_i y_i (w.x_i + b) + \sum_{i=1}^{k} \alpha_i \quad (5)$$

In conclusion, the decision function for a linearly separable two-class problem can be described as [11].

$$f(x) = sign\left[\sum_{i=1}^{k} \lambda_i y_i (x.x_i) + b\right] \quad (6)$$

Non-Linear Separability

As is the case in the classification of some data, the linear separation of many problems is not possible. In this case, the problem stemming from a portion of the training data that remains on the other side of the optimal hyperplane is solved by defining a positive dummy variable (ζ_i). The balance between maximizing the boundary and minimizing the inaccurate classification errors can be controlled by defining a regularization parameter that has positive values and is shown with C ($0 < C < \infty$) [12]. The optimization problem for the data that cannot be linearly separated using a regularization parameter or a dummy variable is:

$$min\left[\frac{\|w\|^2}{2} + C.\sum_{i=1}^{r} \zeta_i\right] \quad (7)$$

The corresponding boundaries are defined as:

$$y_i(w.\varphi(x_i) + b) - 1 \geq 1 - \zeta_i, \zeta_i \geq 0, \ i = 1, \ldots, N \quad (8)$$

As seen in *Fig. 2*, for the solution of the optimization problem given in Equation 7 and 8, the data that cannot be linearly separated in the input space is displayed in a high-dimension space defined as the feature space. Hence, the data can be separated linearly and the hyperplane between the classes are determined.

The SVM can make nonlinear transformations with the aid of a kernel function that is defined as fallow:

$$K(x_i, x_j) = \varphi(x_i).\varphi(x_j) \quad (9)$$

Thus, it allow the linear separation of the data in a higher dimension.

As a result, the decision rule for the solution of a two-class problem that cannot be linearly separated using the kernel function can be written as [11].

$$f(x) = sign\left[\sum_{i=1}^{k} \alpha_i y_i \varphi(x).\varphi(x_i) + b\right] \quad (10)$$

The most important point of a classification process that

Table 1. *Kernel functions and parameters used in support vector machines*

Kernel Function	Mathematical Statement
Polynomial Kernel (d)	$K(x,y) = ((x.y)+1)^d$
Normalized Polynomial Kernel (d)	$K(x,y) = \frac{((x.y)+1)^d}{\sqrt{((x.x)+1)^d((y.y)+1)^d}}$

will be carried out with SVM is the determination of the kernel function and optimum parameters of this function.

Table 1 shows the most commonly used kernel functions (polynomial, radial basis function and normalized polynomial kernels) in the literature.

A comparison of the kernel functions reveal that the polynomial and radial basis kernels are simpler and more understandable. Although it can appear mathematically simple, the increase in the degree of a polynomial function can complicate the algorithm, which both prolongs the process and reduces the accuracy of classification after a certain point. On the other hand, the effect of the changes in the kernel size parameter (γ) of the radial basis function on classification performance was determined to be relatively lower [13]. The normalized polynomial function was suggested by [14] to normalize the mathematical statement of the polynomial kernel instead of the normalization of the dataset. The normalized polynomial kernel can be viewed as a generalization of the polynomial kernel.

Multilayer Perceptron

Multilayer perceptron is a feedforward artificial neural network model in which the input data are adjusted on an appropriate output sequence. It is a non-parametric artificial neural network method that carries out various detection and estimation processes. In the multilayer perceptron, each j neuron in the hidden layer takes the sum of the multiplication of the input signals with the connection weight w_{ji} and calculates the y_j output as a function of this sum:

$$y_i = f(\sum w_{ji}x_i) \tag{11}$$

Here, *f* is an activation function that transforms the weighted sum of the signals affecting a neuron into the output value. The activation function can be a simple threshold function, sigmoidal or hyperbolic tangent function.

The sum of the quadratic differences between the calculated and desired values of the output neurons is defined as:

$$e = \frac{1}{2}\sum_j (y_j^* - y_j)^2 \tag{12}$$

Here, y_j* and y_j are the calculated and desired values of the j. output neuron, respectively. Each w_{ji} weight is adjusted to reduce the e value as fast as possible. How the w_{ji} value will be adjusted depends on the training algorithm.

Comparison of the Performances

Comparing the classifiers and determining the best classifier are of great importance in data mining [15]. The classification performances of algorithms are usually compared with respect to accurate classification percentage, accuracy rate, RMSE, ROC area, sensitivity, precision and F-measure.

Accuracy Rate: It gives the accurate classification percentages of observations.

$$Accuracy = \frac{TP+TN}{TP+FP+FN+TN} \tag{13}$$

Root Mean Square Error: Also known as the quadratic mean, it is a statistical method used in the measurement of the sizes of changing amounts.

$$\mathrm{RMSE} = \sqrt{\frac{1}{n}\sum_{i=1}^{n} e_i^2} \tag{14}$$

Here, *n*: number of samples, e_i: models error.

ROC Area: ROC area curve determines the estimation performances of different classification algorithms. The area below the ROC curve is one of the important measures in selecting the best classification algorithm. When the area below the curve approaches 1, it indicates that the classification is accurate.

Confusion Matrix: The confusion matrix shows the numbers of inaccurate or accurate classifications of data *(Table 2)*. The confusion matrix is used in the calculation of precision, sensitivity and F-measure, which measure the performances of classification algorithms.

The most popular and simple method in measuring the performance of a model is the accuracy and error rates of the model. Accuracy is the ratio of the accurately classified sample number (TP+TN) to the total sample number (TP+TN+FP+FN). This value is completed to 1 by the error rate. In other words, error rate is the ratio of the inaccurately classified sample number (FP+FN) to the total sample number (TP+TN+FP+FN) [16].

Table 2. *Classification according to the confusion matrix*

Accurate Class	Estimated Class	
	Class 1	Class 2
Class 1	a	b
Class 2	c	d

a: *TP (True-Positive),* ***b:*** *FN (False-Negative),* ***c:*** *FP (False-Positive),* ***d:*** *TN (True-Negative)*

$$Accuracy = \frac{TP + TN}{TP + FP + FN + TN}$$

$$Error\ rate = \frac{FP + FN}{TP + FP + FN + TN}$$

Sensitivity: Sensitivity is the ratio of the accurately classified positive sample number to the total positive sample number [16].

$$Sensitivity = \frac{TP}{TP + FN}$$

Precision: Precision is the ratio of the number of True Positive samples with a class estimated to be 1 to the total number of samples with a class estimated to be 1 [16].

$$Precision = \frac{TP}{TP + FP}$$

Precision and sensitivity are not separately sufficient to derive a meaningful comparison. Considering both measures together will yield more accurate results. The F-measure is defined for this purpose.

F-measure: F-measure is the harmonic mean of precision and sensitivity.

$$F - measure = \frac{2xSensitivityxPrecision}{Sensitivity + Precision}$$

In the SVM, the WEKA 3.8.3 (Waikato Environment for Knowledge Analysis) software and IBM SPSS 21.0 statistical package program were used for the analysis of the classification results. WEKA is a popular machine learning package written with Java and developed in Waikato University, New Zealand, and contains visualization tools and algorithms for data analysis and estimation modelling and graphical user interfaces for easy access to these functions.

RESULTS

Table 3 shows the descriptive statistics for the variables used in the study. Considering the SCC as a criterion of milk quality, the groups with high and low milk quality were formed with respect to these variables. As seen in *Table 3*, in terms of the -DM- and fat variables, there were statistically significant differences between low and high SCC according to the investigated criterion (P<0.05, P<0.01, respectively).

However, in terms of the fat-free dry matter (FDM), protein, lactose, casein, urea, density, acidity, pH and freezing point variables, there were no statistically significant differences between the low and high SCC (P>0.05). Moreover, the significant relationship between SCC and fat and dry matter was supported by the correlation matrix results in *Table 4*.

Table 4 shows the correlation matrix of the variables used in the study. As seen in *Table 4*, the highest correlation was between fat and -DM- (0.93, P<0.01), while the lowest correlation was between freezing point and pH (0.02, P>0.05). The relationship between the variables and SCC showed that it had the highest correlation with protein (0.69, P<0.01), while the lowest correlation was with urea (-0.02, P>0.05).

For the detailed analysis of the classification results, the performances of the algorithms were statistically compared. The WEKA 3.8.3 (Waikato Environment for Knowledge Analysis) software was used for classification with SVM. The SMO (Sequential Minimal Optimization) algorithm in the software and Multilayer perception algorithm were used for the multilayer perceptron. The 10-fold cross validation was selected and used for the data. *Table 5* shows the statistical results for the classification algorithms.

As seen in *Table 5*, the results for the classification algorithms revealed that, for the polynomial kernel, the accurate classification percentage was 93.06% and ROC area value was 88.7%, which agrees with the accurate classification percentage. Moreover, RMSE, precision, sensitivity and F-measure values were 0.263, 0.913, 0.995 and 0.931, respectively.

The accurate classification percentage of the normalized polynomial kernel was 92.36%. Furthermore, the ROC area value was 89.2%, which agrees with the accurate classification percentage. The RMSE, precision, sensitivity and F-measure values were 0.276, 0.924, 0.970 and 0.922, respectively.

As seen in *Table 5*, the accurate classification percentage of radial basis function kernel was 90.97%. In a similar fashion, the ROC area value was 86.6% and agreed with the accurate classification percentage. In addition, the RMSE, precision, sensitivity and F-measure values were 0.301, 0.903, 0.975 and 0.904, respectively.

The accurate classification percentage for the multilayer perceptron was 81.60%. The ROC area value was 81.2%, which is in keeping with the accurate classification percentage. Moreover, the RMSE, precision, sensitivity and F-measure values were 0.384, 0.843, 0.866 and 0.816, respectively.

Table 3. *Descriptive statistics for the milk characteristics*

Milk Characteristics	Low (n=76) Mean ± Std. Deviation	High (n=212) Mean ± Std. Deviation	P-value
DM	16.35±2.53	17.29±2.78	**0.011**
FDM	11.07±1.21	10.87±0.83	0.179
Fat	5.18±2.43	6.25±2.85	**0.002**
Protein	4.90±1.47	4.91±0.95	0.981
Lactose	5.23±0.61	5.16±0.53	0.366
Casein	3.54±1.12	3.63±0.87	0.542
Urea	0.04±0.02	0.04±0.02	0.833
Density	1029.45±8.21	1028.97±7.47	0.651
Acidity	7.92±5.40	8.21±3.37	0.658
pH	6.56±0.15	6.55±0.14	0.704
Freezing Point	0.57±0.16	0.59±0.16	0.573

***DM:** dry matter; **FDM:** fat-free dry matter*

Table 4. *Correlation coefficients between the variables and significance test results*

Milk Characteristics	SCC	DM	FDM	Fat	Protein	Lactose	Casein	Urea	Density	Acidity	pH
DM	0.68**	1									
FDM	0.28*	0.21*	1								
Fat	0.66**	0.93**	-0.16*	1							
Protein	0.69**	0.58**	0.83**	0.29*	1						
Lactose	-0.55**	0.58**	-0.19*	-0.54**	-0.67**	1					
Casein	0.38**	0.69**	0.43**	0.59**	0.76**	-0.80**	1				
Urea	-0.02	-0.31**	0.39**	-0.51**	0.15*	0.17*	-0.32**	1			
Density	-0.05	0.08	-0.08	0.20*	0.09	-0.35*	0.63**	-0.41**	1		
Acidity	0.34**	0.57**	0.34**	0.49**	0.68**	-0.76**	0.92**	-0.21*	0.63**	1	
pH	-0.56**	-0.23*	-0.16*	-0.19*	-0.37**	0.44**	-0.32**	0.08	-0.06	-0.34**	1
Freezing Point	-0.04	0.05	-0.04	0.07	0.03	0.13*	0.08	-0.04	0.10	0.10	0.02

** significant at the 0.05 level (2-tailed); ** significant at the 0.01 level (2-tailed); **DM:** dry matter; **FDM:** fat-free dry matter*

Table 5. *Comparison of the classification algorithms*

Algorithms	Features					
	Accuracy	RMSE	ROC Area	Precision	Sensitivity	F-Measure
Polynomial kernel	93.06	0.263	0.887	0.913	0.995	0.931
Normalized polynomial kernel	92.36	0.276	0.892	0.924	0.970	0.922
Radial basis function kernel	90.97	0.301	0.866	0.903	0.975	0.904
Multilayer perceptron	81.60	0.384	0.812	0.843	0.866	0.816

***RMSE:** Root Mean Square Error*

Table 6 shows the confusion matrix results. As revealed by the table, for the polynomial kernel, the number of accurately classified observations in the high class was 201 and the number of observations that were in the low class while they should have been in the high class was 1; the number of accurately classified observations in the low class was 67 and the number of observations that were in the high class while they should have been in the low class was 19. In a similar manner, for normalized polynomial kernel, the number of accurately classified observations in the high class was 196 and the number of observations that were in the low class while they should have been in the high class was 6; the number of accurately classified observations in the low class was 70 and the number of observations that were in the high class while they should have been in the low class was 16.

Table 6. *Confusion matrix of the classifications*

Accurate Class		Estimated Class		Error Rates
		High	Low	
Polynomial kernel	High	201	1	0.071
	Low	19	67	
Normalized polynomial kernel	High	196	6	0.081
	Low	16	70	
Radial basis function kernel	High	197	5	0.092
	Low	21	65	
Multilayer perceptron	High	175	27	0.181
	Low	26	60	

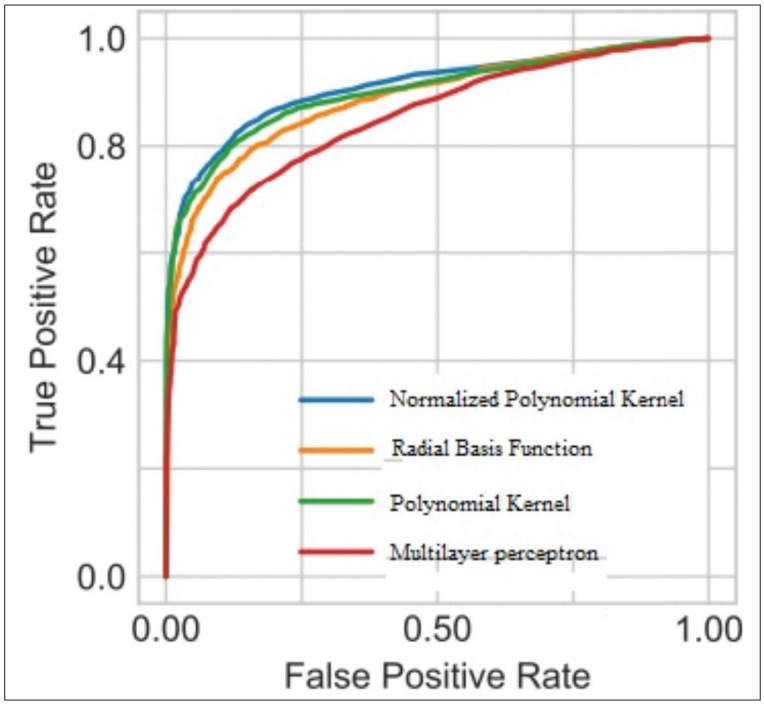


Fig 3. ROC curves of of different classification algorithms

For radial basis function kernel, the number of accurately classified observations in the high class was 197 and the number of observations that were in the low class while they should have been in the high class was 5; the number of accurately classified observations in the low class was 21 and the number of observations that were in the high class while they should have been in the low class was 65.

Furthermore, for the multilayer perceptron, the number of accurately classified observations in the high class was 175 and the number of observations that were in the low class while they should have been in the high class was 27; the number of accurately classified observations in the low class was 26 and the number of observations that were in the high class while they should have been in the low class was 60.

The error rates for the polynomial kernel, normalized polynomial kernel, radial basis function kernel and multilayer perceptron were calculated to be 0.071, 0.081, 0.092 and 0.181, respectively. The precision, sensitivity and F-measure values given in *Table 5* were calculated using the accuracy and error rates.

In the analysis using the WEKA data mining software, the classification algorithms were summarized in *Table 5* in light of various statistical criteria. According to the accuracy percentage in *Table 5*, the polynomial kernel function had the highest accuracy rate (93.06%), while the multilayer perceptron had the lowest accuracy rate (81.60%). When the functions were compared considering their RMSE values, the polynomial kernel had the lowest value (0.263), while the multilayer perceptron had the highest value (0.384). According to this criterion, polynomial kernel was the best classifier, while the weakest classifier function was the multilayer perceptron (0.384).

When the ROC area values are evaluated by their closeness to 1, normalized polynomial kernel was determined to be the best function, while the multilayer perceptron was

the weakest function. The separate examination of the precision, sensitivity and F-measure values revealed that the normalized polynomial kernel was the most successful function, while the weakest function was the multilayer perceptron. ROC curve are shown in *Fig. 3*.

DISCUSSION

Milk composition and the SCC in milk are important parameters in the determination of milk quality [4]. As SCC increases, the shelf-life and quality of milk decrease [17]. In addition to its role as a milk quality parameter, SCC is an indicator of udder health and used in the diagnosis of mastitis [5,6]. The SCC in milk is an important parameter in the determination of milk quality and early diagnosis of subclinical mastitis.

Although the number of studies on the issue is limited, SVM are used to the estimation of clinical and subclinical mastitis in dairy cows [18]. The researchers divided the animals into 2 groups as healthy and infected animals by determining the SCC in milks obtained monthly from Holstein cows for 12 months and reported that SVM achieved classification with an accuracy rate of 91% [18]. In the present study, accuracy rates were in the range of 81.59% and 93.05% for the kernel functions, which are close to the results found in the previous study.

Support vector machines are used to the diagnosis of clinical mastitis [19,20]. SVM are achieved classification with an 83.2% accuracy rate [19], while accuracy rate was 84.6% [20] These results are close to the results obtained with the multilayer perceptron in our study. SVM are used to develop a milk recognition system and classify milk with respect to its content after UHT [1]. In addition SVM are used to the estimation of rumen acidosis in dairy cattle and the classification of SCC [21,22]. Milk fatty acids and rumen pH value were taken as classification variables and linear kernel and radial basis kernel functions were used in classification with SVM [21].

Dry matter, fat, protein and lactose contents of milk obtained from 222 milch Murrah buffaloes were in the range of 16.94%-18.55%, 6.28%-8.38%, 4.05%-4.59% and 4.96%-5.34%, respectively, and the lactose content decreased as the SCC increased [23]. In this study, the dry matter, fat, protein and lactose contents of the milk obtained from 288 Anatolian buffaloes were close to those previously obtained by the researchers. As the SCC in milk increased, lactose content decreased (r=-0.55), while dry matter, fat and protein contents (r=0.68, r=0.66, 0.69) increased. Sekerden and Avsar [24] found that the ash, fat, dry matter, protein, acidity, density, pH and urea content of buffalo milk were 0.47%, 7.67, 17.55, 5.28, 0.17, 1.028, 6.61 and 3.78 mg/100 mL, respectively. Fernandes et al.[25] determined that the dry matter, fat, protein and lactose contents of milk were in the range of 14.5-17.1%, 6.1-6.9%, 3.9-4.2% and 4.5-5.2%, respectively, and stated that SCC did not affect the composition of buffalo milk. Ayasan et al.[26] investigated the effects of SCC on milk urea nitrogen and milk composition. 30 Holstein cows were divided into 2 groups based on their SCC. The researchers found that SCC (Group 1: x<268.000 cells/mL; Group 2: x>268.000 cells/mL) had a significant effect on the milk fat, milk lactose, fat-free dry matter and density (P<0.05) but did not have a significant effect on milk urea nitrogen, milk protein, milk casein, urea, dry matter, acidity, free fatty acid, citric acid and freezing point (P>0.05). In this study, SCC had a statistically significant effect on dry matter and fat (P<0.05), but did not affect other milk components (P>0.05). Furthermore, Ayasan et al.[26] found a significant relationship between SCC and milk fat (r=0.209; P=0.026), fat-free dry matter (r=-0.183; P=0.050), milk lactose (r=-0.196; P=0.037) and density (r=-0.281; P=0.002). In this study, there was a significant relationship between SCC and milk fat (r=0.68, P<0.01), fat-free dry matter (r=0.28, P<0.05), lactose (r=-0.55, P<0.01), but SCC did not have a statistically significant relationship with urea (r=-0.02, P>0.05), density (r=0.05, P>0.05) and freezing point (r= -0.04, P>0.05). Yesilova et al.[27] demonstrated that lactation milk yield in Anatolian buffaloes can be classified using mixture model. In our study, we investigates the classification of milk quality with SVM using the raw milk composition and SCC data on buffalos.

Somatic cell count is a good classifier in the determination of milk quality and mastitis. A review of the scientific literature revealed that although SVM was used in the determination of mastitis, the number of studies on its use in the determination of milk quality was limited. Within this context, in the study, SVM models were developed with polynomial, normalized polynomial and radial basis kernel functions. The classification performances of the kernel functions with SVM were compared with the multilayer perceptron method.

The study on SVMs analyzed the effects of critically important kernel functions on the classification results, i.e. performance, in detail and investigated the classification of milk quality with SVMs with three different kernel functions and multilayer perceptron using raw milk composition and SCC data. Compared with the results obtained with the multilayer perceptron method, the most commonly used kernel functions in SVM were more efficient and successful.

The dataset in the study comprised 12 variables including raw milk composition in buffaloes (DM, FDM, fat, protein, lactose, casein, urea, density, acidity, pH, freezing) and somatic SCC. The SCC in milk were divided into two classes as high and low in accordance with the Turkish Food Codex [28,29].

After the analysis of these variables, the performances of the algorithms were compared with respect to their accuracy, RMSE, ROC area, precision, sensitivity and F-measure and the results showed that the classification performances of SVMs were better than the multilayer perceptron algorithm.

Considering the kernel functions used in the study, the highest accuracy in SVM was obtained with the polynomial kernel function. The values obtained with the normalized polynomial kernel and radial basis function kernel were close to those obtained with the polynomial kernel. The algorithm of the multilayer perceptron had a lower accuracy value and thus, was not useful when compared with the kernel functions.

Considering the precision measure, the best result was obtained with the normalized polynomial kernel function. However, precision should not be interpreted separately; instead, it should be considered together with the sensitivity measure. As seen in *Table 3*, according to the sensitivity measure, the algorithms in descending order were polynomial kernel, radial basis function kernel, normalized polynomial kernel and multilayer perceptron. Evaluation with the F-measure in which the precision and sensitivity measures are considered together will yield better results.

In conclusion, using the performance measures for the models that were developed using different kernel functions for SCC revealed that higher or lower SCC than the specified criteria affected the quality of milk and other products obtained from milk. The systematic analysis and classification of the results are of great importance in the classification of milk quality. Future studies with larger datasets will add to the success of computer-based diagnosis systems.

REFERENCES

1. Abukhait J, Mansour AM, Obeidat M: Classification based on gaussian-kernel support vector machine with adaptive fuzzy inference system. *Prz Elektrotechnıczn*, 94 (5): 14-22, 2018. DOI: 10.15199/48.2018.05.03

2. Ghafouri-Kesbi F, Rahimi-Mianji G, Honarvar M, Nejati-Javaremi A: Predictive ability of random forests, boosting, support vector machines and genomic best linear unbiased prediction in different scenarios of genomic evaluation. *Anim Prod Sci,* 57 (2): 229-236, 2017. DOI: 10.1071/AN15538

3. Damé MCF, Lima CTS, Marcondes CR, Ribeiro MER, Garnero ADV: Preliminary study on buffalo *(Bubalus bubalis)* milk production in Southern Brazil. *Rev Vet*, 21 (1): 585-587, 2010.

4. Barth K: Evaluation of somatic cell count under automatic milking conditions. Physiological and technical aspects of machine milking. *Proceedings of an International Conference*, Nitra, Slovak Republic, 165-169, 26-27 June 2001.

5. Dhakal IP, Kapur MP, Anshu S: Significance of differential somatic cell counts in milk for the diagnosis of subclinical mastitis in buffaloes using foremilk and stripping milk. *Indian J Anim Health,* 31, 39-42, 1992.

6. Singh M, Ludri RS: Somatic cell count in Murrah buffaloes (Bubalus bubalis) during different stages of lactation, parity and season. *Asian Australas J Anim Sci,* 14, 189-192, 2001. DOI: 10.5713/ajas.2001.189

7. Sharma N, Singh NK, Bhadwal MS: Relationship of somatic cell count and mastitis: An overview. *Asian Australas J Anim Sci,* 24 (3): 429-438, 2011. DOI: 10.5713/ajas.2011.10233

8. Anonymous: Türkiye İstatistik Kurumu Hayvancılık İstatistikleri. http://www.tuik.gov.tr/hayvancilikapp/hayvancilik.zul"hayvancilikapp/hayvancilik.zul. *Accessed:* 12.05.2019.

9. Vapnik VN: The Nature of Statistical Learning Theory. 167-174, Springer-Verlag, New York, 1995. DOI: 10.1007/978-1-4757-2440-0

10. Vapnik VN: Statistics for Engineering and Information Science. The Nature of Statistical Learning Theory. 2th ed., 131-137, Springer, New York, 2000. DOI: 10.1007/978-1-4757-3264-1

11. Radhakrishnan S, Ramanathan R: A support vector machine with gabor features for animal ıntrusion detection in agriculture fields. *Procedia Comput Sci*, 143, 493-501, 2018. DOI: 10.1016/j.procs.2018.10.422

12. Udaya Shalika AWD, Seneviratne L: Animal classification system based on image processing & support vector machine. *J Comput Commun,* 4 (1): 12-21, 2016. DOI: 10.4236/jcc.2016.41002

13. Wang G: Machine learning for inferring animal behavior from location and movement data. *Ecol Inform*, 49, 69-76, 2019. DOI: 10.1016/j.ecoinf.2018.12.002

14. Zhao HT, Feng YZ, Chen W, Jia GF: Application of invasive weed optimization and least square support vector machine for prediction of beef adulteration with spoiled beef based on visible near-infrared (Vis-NIR) hyperspectral imaging. *Meat Sci*, 151, 75-81, 2019. DOI: 10.1016/j.meatsci.2019.01.010

15. Amraei S, Mehdizadeh SA, Sallary S: Application of computer vision and support vector regression for weight prediction of live broiler chicken. *Eng Agric Environ Food*, 10 (4): 266-271, 2017. DOI: 10.1016/j.eaef.2017.04.003

16. Ahmadi H, Rodehutscord M: Application of artificial neural network and support vector machines in predicting metabolizable energy in compound feeds for pigs. *Front Nutr*, 4:27, 2017. DOI: 10.3389/fnut.2017.00027

17. Harmon RJ: Physiology of mastitis and factors affecting somatic cell counts. *J Dairy Sci*, 77, 2103-2112, 1994. DOI: 10.3168/jds.S0022-0302(94)77153-8

18. Mammadova N, Keskin İ: Application of the support vector machine to predict subclinical mastitis in dairy cattle. *Sci World J,* 2013:603897, 2013. DOI: 10.1155/2013/603897

19. Cavero D, Tölle KH, Buxade C, Krieter J: Mastitis detection in dairy cows by application of fuzzy logic. *Livest Sci,* 105, 207-213, 2006. DOI: 10.1016/j.livsci.2006.06.006

20. De Mol RM, Ouweltjes W: Detection model for mastitis in cows milked in an automatic milking system, *Prev Vet Med,* 49, 71-82. 2001. DOI: 10.1016/s0167-5877(01)00176-3

21. Colman E, Waegeman W, De Baets B, Fievez V: Prediction of subacute ruminal acidosis based on milk fatty acids: A comparison of linear discriminant and support vector machine approaches for model development. *Comput Electron Agr*, 111, 179-185, 2015. DOI: 10.1016/j.compag.2015.01.002

22. Gao X, Xue H, Pan X, Jiang X, Zhou Y, Luo X: Somatic cells recognition by application of gabor feature-based (2D)^{2}PCA. *Int J Pattern Recogn,* 31 (12):1757009, 2017. DOI: 10.1142/S0218001417570099

23. Cerón-Muñoz M, Tonhati H, Duarte J, Oliveira J, Muñoz-Berrocal M, Jurado-Gámez H: Factors affecting somatic cell counts and their relations with milk and milk constituent yield in buffaloes. *J Dairy Sci*, 85 (11): 2885-2889, 2002. DOI: 10.3168/jds.S0022-0302(02)74376-2

24. Sekerden Ö, Avsar YK: Milk composition, rennet coagulation time, urea content and environmental factors affecting them in Anatolian Buffaloes. *J Anim Prod,* 49 (2): 7-14, 2008.

25. Fernandes SA, de Mattos WRS, Matarazzo SM, Gama MAS, Malhado CHM, Ferrão, SPB, Etchegaray MAL, Lima CGD: Effect of somatic cell count on Murrah buffaloes milk. *Prev Vet*, 21 (1): 552-553, 2010.

26. Ayasan T, Hızlı H, Yazgan E, Kara U, Gök K: The effect of somatic cell count on milk urea nitrogen and milk composition. *Kafkas Unıv Vet Fak Derg,* 17 (4): 659-662, 2011. DOI: 10.9775/kvfd.2011.4489

27. Yesilova A, Yilmaz A, Ser G, Kaki B: Modeling with Gaussian mixture regression for lactation milk yield in Anatolian buffaloes. *Indian J Anim Res*, 50 (6): 989-994, 2016. DOI: 10.18805/ijar.v0iOF.4545

28. Anonim: Türk Gıda Kodeksi Yönetmeliği. T.C. Resmi Gazete, 14 Şubat 2000, Sayı 23964, s. 35. Ankara, 2000

29. Beykaya M, Özbey A, Yıldırım Z: Determination of physical, chemical and microbiological properties of milk from some dairy plants in Sivas Province. *TURJAF*, 5 (4): 388-396, 2017. DOI: 10.24925/turjaf.v5i4.388-396.1172

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Short Communication

Novel Insights on the Pattern of Cough Associated with Tracheal Collapse in Griffon Dogs

Marwa HASSAN [1,a] Elham HASSAN [1,b] Faisal TORAD [1]

[1] Department of Surgery, Anesthesiology and Radiology, Faculty of Veterinary Medicine, Cairo University, Giza - EGYPT
ORDIDS: [a] 0000-0002-9260-4471; [b] 0000-0001-9873-144X

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Abstract

A prospective study was designed to test the hypothesis that the degree and location of tracheal collapse may influence its pattern of cough. Objective evaluation of cough was made in 45 dogs with tracheal collapse. The relationship between location and degree of collapse and cough was tested. Location of collapse did not have a significant effect on cough except for the moderate effect on the nature of cough (P=0.002; rs=0.45) while its degree had a significant effect (P<0.0001) on all cough parameters except for nature of cough (P=0.354). The pattern of cough associated with tracheal collapse is mainly influenced by the degree of collapse rather than its location.

Keywords: *Tracheal collapse, Cough, Dog*

Griffon Irkı Köpeklerde Trakeal Kollaps İle İlişkili Öksürük Modelinde Yeni Yaklaşımlar

Öz

Bu prospektif çalışma, trakeal kollaps derecesi ve lokalizasyonunun öksürük şeklini etkileyebileceği hipotezini araştırmak için tasarlandı. Trakeal kollaps şekillenmiş olan 45 köpekte öksürüğün objektif değerlendirmesi yapıldı. Lokasyon ve kollaps derecesi ile öksürük arasındaki ilişki incelendi. Kollaps lokasyonunun öksürük şekline orta düzeyde etkisi (P= 0.002; rs= 0.45) dışında öksürük üzerinde anlamlı bir etkisi olmamakla birlikte, derecesinin öksürük şekli (P=0.354) dışındaki tüm öksürük parametreleri üzerinde anlamlı etkisi (P<0.0001) olduğu belirlendi. Trakeal kollaps ile ilişkili öksürük modeli esas olarak kollapsın lokasyonu yerine derecesinden etkilenir.

Anahtar sözcükler: *Trakeal kollaps, Öksürük, Köpek*

INTRODUCTION

Tracheal collapse is a dynamic dorso-ventral reduction in the tracheal diameter which may involve isolated tracheal segment (cervical or thoracic) or the entire tracheal length [1,2]. The condition is commonly diagnosed in middle aged toy and miniature breed dogs with a prevalence of 0.5-2.9% [3-5]. The exact cause of tracheal collapse is unknown; the condition may arise due to flaccid dorsal tracheal membrane, weakened cartilaginous rings or both [6]. In lateral radiographs, tracheal collapse may be graded into mild, moderate and severe tracheal collapse based on the degree of reduction of the tracheal lumen diameter compared to the thoracic inlet width [7].

Tracheal collapse may remain asymptomatic till signs of respiratory distress appear including dyspnea, labored breathing, abnormal respiratory sounds and frequent cough. Cough is a protective air way function serving both corrective and preventive role [8]. It's a reflex triggered by compression or irritation of the airways and controlled by cough centers in the brainstem [9]. A characteristic paroxysmal goose-honking cough is the most common sign of tracheal collapse in order to overcome the resistance of air flow to maintain ventilation [2,10]. This pattern of cough may be a result of the action exerted by respiratory muscles to overcome the increased resistance of air flow at the area of the narrowed tracheal lumen. Based on *Poiseuille Law*, the resistance to air flow in straight circular tube is inversely proportional to the fourth power of its radius ($R=1/r^4$). This means that if the tracheal diameter is reduced by one half (50% reduction), the resistance of air within the tracheal lumen increases 16-fold [11].

Correspondence
+20 122 4068080
elhamhassan@cu.edu.eg

It is not clear whether the degree and possibly the location of tracheal collapse may have an influence on the pattern of cough associated with tracheal collapse. The aim of the present study was to test the hypothesis that the degree and location of tracheal collapse may have an influence on the pattern of cough in Griffon dogs with tracheal collapse.

MATERIAL and METHODS

Animals and Design

A prospective study was conducted on 45 Griffon dogs (32 males and 13 females), weighing 16.4±4.2 (mean±SD) kg and aging 7.5±3.2 (mean±SD) years. These dogs were admitted to clinic of the Department of Surgery, Anesthesiology and Radiology, Faculty of Veterinary Medicine, Cairo University. All study procedures were approved by Cairo University Institutional Animal Care and Use Committee (CU-IACUC) (approval No. CU/II/F/56/18). All dogs' owners were aware that their animals will be included in research purposes and signed a written consent indicating their approval.

Inclusion criteria of dogs included in the study are those dogs diagnosed with tracheal collapse based on clinical and radiographic examination and did not receive medicinal therapy during the last two weeks. The clinical manifestation of these dogs included signs of respiratory distress, periodical attacks of dyspnea and frequent coughing. Diagnosis of tracheal collapse was confirmed by right lateral inspiratory and expiratory radiographs.

Radiographic Evaluation

The location of tracheal collapse was categorized into cervical or thoracic tracheal collapse based on inspiratory and expiratory radiographs *(Fig. 1)*. The degree of tracheal collapse was graded based on the degree of reduction in tracheal lumen into mild, moderate and severe (25%, 50%, >75% respectively) based on Tanger and Hobson [7] grading system of tracheal collapse.

Objective Evaluation of Cough

Objective evaluation of cough was scored by the same examiner who was blind to the result of radiographic examination. Scoring was done based on clinical examination as well as owners' questionnaire designed to score cough evaluation parameters. Evaluation parameters included the onset of cough (acute: 0-3 weeks; subacute: 3-8 weeks; chronic: >8 weeks), frequency of cough (Grade I: 6 cough/h; Grade II: 6-12 cough/h; Grade III: >12 cough/h), nature of cough (Productive; Non productive), cough reflex (Present; Absent), control of cough (Voluntary; Involuntary), type of cough (Typical: initiated by inspiratory phase, pause then forced expiration; Atypical: missing inspiratory phase of cough) and wheezes (Present; Absent).

Statistical Analysis

Cough evaluation scores were tabulated for all dogs. A Spearman's correlation was run to determine the relationship between the degree and location of tracheal collapse with different cough evaluation parameters. Data were considered statistically significant when the P value <0.05. The strength of relationship was judged based on Spearman's correlation coefficient (r_s=0.00-0.19 very weak; 0.20-0.39 weak; 0.40-0.59 moderate; 0.60-0.79 strong; 0.80-1.00 very strong correlation). Data were analyzed using SPSS software 21 (IBM SPSS Inc., Chicago, IL).

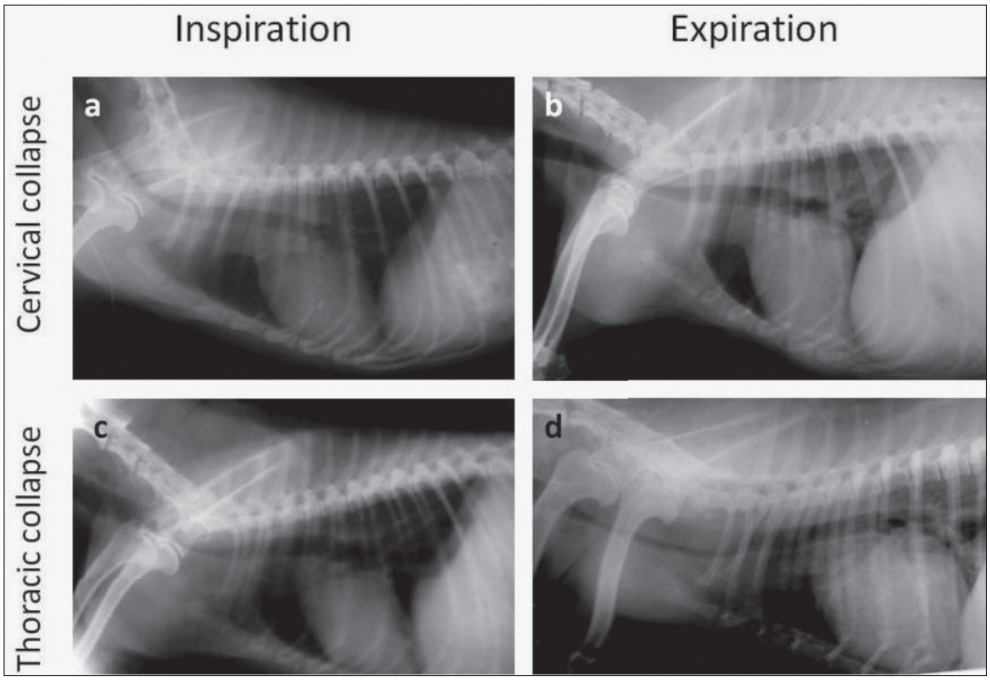

Fig 1. Right lateral inspiratory **(a,c)** and expiratory **(b,d)** thoracic radiographs demonstrating reduction in the tracheal diameter at the cervical **(a,b)** and thoracic **(c,d)** trachea

RESULTS

Radiographic Evaluation

Fortyfive Griffon dogs were diagnosed with tracheal collapse based on clinical and radiographic examination. The location of the collapsed trachea was in the cervical region in 33 (73.3%) dog and in the thoracic region in the remaining 12 (26.7%) dogs. Tracheal collapse was graded to be mild in 11 (24.4%), moderate in 22 (48.9%) and severe in 12 (26.7%) dogs. Distribution of the degree and location of tracheal collapse among presented dogs is demonstrated in *Table 1*.

Objective Evaluation of Cough

The characteristic cough associated with tracheal collapse had an acute onset in dogs with mild and moderate tracheal collapse, while dogs with severe tracheal collapse had a history of chronic cough lasting for more than 8 weeks *(Fig. 2)*. The frequency of cough increased with the more compromised tracheal lumen as manifested by increased frequency of cough in severe of degree of tracheal collapse *(Fig. 3)*. Productive cough was mostly prevalent in severe degree of tracheal collapse, while non-productive cough was predominant in mild and moderate degrees of tracheal collapse. In cervical tracheal collapse the nature of cough was mostly non-productive while productive cough was predominant in thoracic tracheal collapse *(Fig. 4)*. The presence of cough reflex and the voluntary control of cough were mostly associated with mild and moderate degrees of tracheal collapse. While dogs with severe tracheal collapse exhibited involuntary control of cough and absence of cough reflex *(Fig. 5, Fig. 6)*. Typical cough was only reported in mild tracheal collapse, while atypical form of cough was reported in moderate to severe tracheal collapse *(Fig. 7)*. Wheezes was absent in mild tracheal collapse, while it was recorded in moderate to severe degrees of tracheal collapse *(Fig. 8)*.

The location of tracheal collapse did not have a significant effect on all cough evaluation parameters except moderate effect on the nature of cough. A non significant correlation was reported between the location of tracheal collapse and

Table 1. *Degree and location of tracheal collapse among the presented 45 Griffon dogs*

Degree of Tracheal Collapse	Location of Tracheal Collapse		Total
	Cervical	Thoracic	
Mild (25% reduction)	8	3	11
Moderate (50% reduction)	17	5	22
Severe (75% reduction)	8	4	12
Total	33	12	45

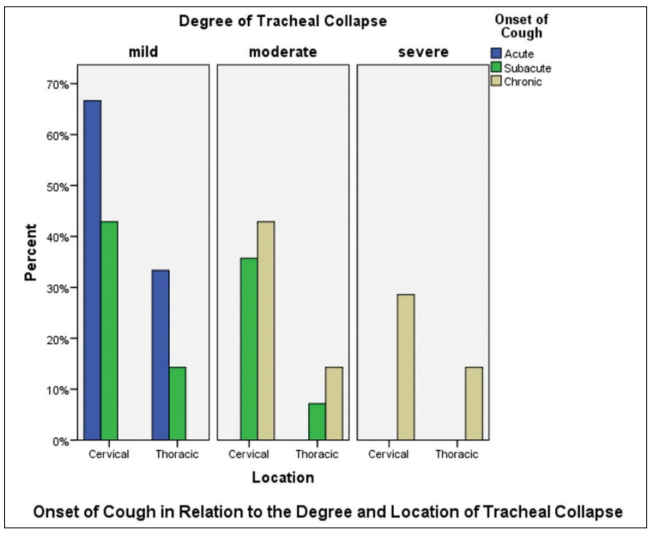

Fig 2. Evaluation of the onset of cough (acute-subacute-chronic cough) in relation to the location and degree of tracheal collapse

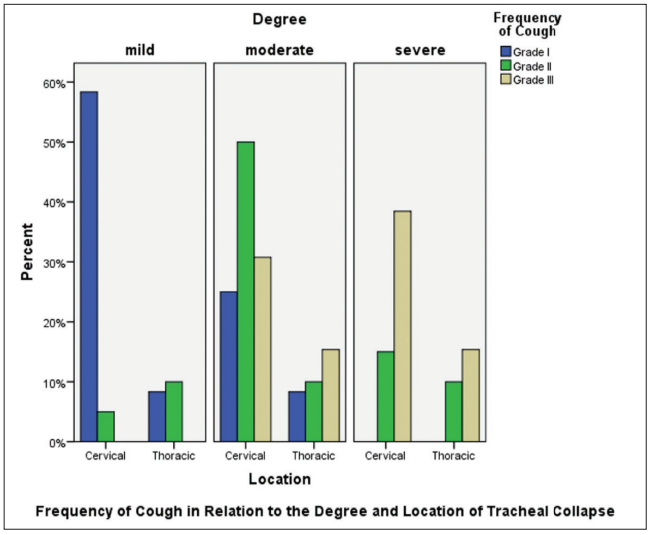

Fig 3. Evaluation of the frequency of cough (acute-subacute-chronic cough) in relation to the location and degree of tracheal collapse

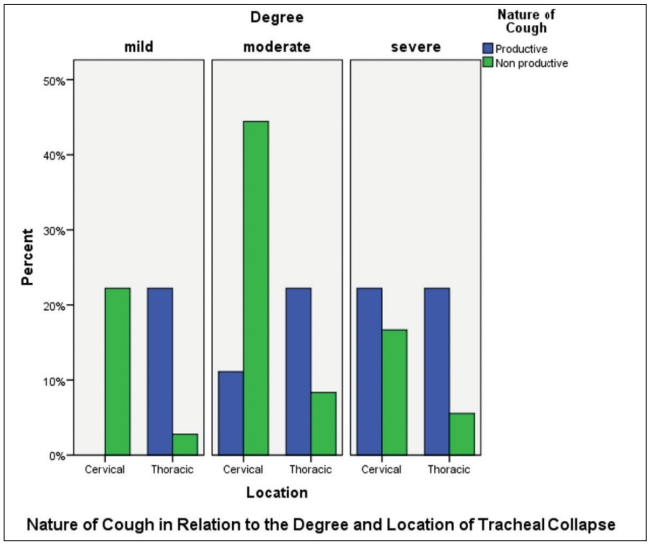

Fig 4. Evaluation of the nature of cough (productive-non productive cough) in relation to the location and degree of tracheal collapse

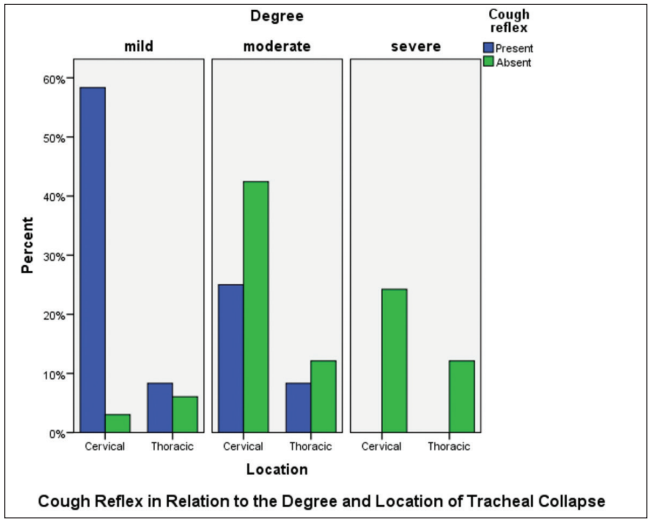

Fig 5. Evaluation of cough reflex (present-abscent) in relation to the location and degree of tracheal collapse

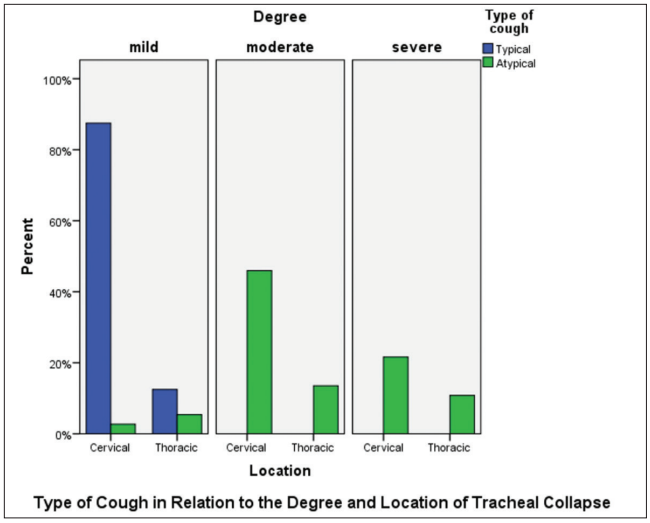

Fig 7. Evaluation of the type of cough (typical cough-atypical cough) in relation to the location and degree of tracheal collapse

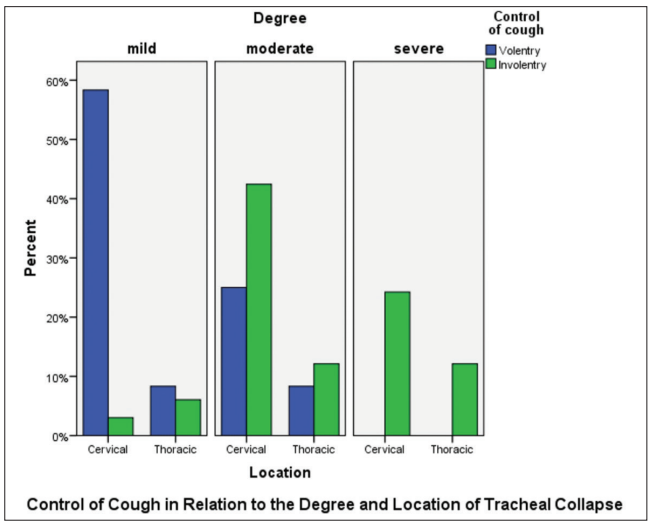

Fig 6. Evaluation of the control of cough (voluntary-invoulantry cough) in relation to the location and degree of tracheal collapse

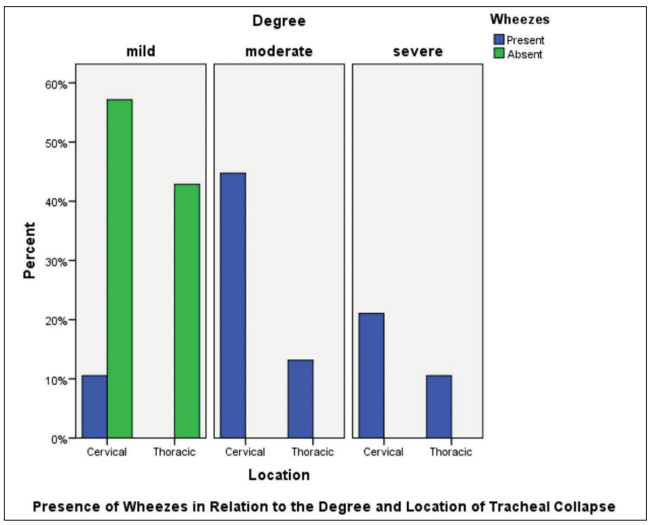

Fig 8. Evaluation of wheezes (presence-absence of wheezes) associated with cough in relation to the location and degree of tracheal collapse

duration of cough (P=0.78; r_s=0.04); frequency of cough (P=0.45*;* r_s=0.17); cough reflex (P=0.73*;* r_s=0.14); control of cough (P=0.37; r_s=0.14); type of cough (P=0.33*;* r_s=0.15) and wheezes (P=0.30*;* r_s=0.16). The only significant correlation between the location of tracheal collapse and the pattern of cough was on the nature of cough (P=0.002*;* r_s=0.45).

The degree of tracheal collapse had a significant effect (P<0.0001*)* on all cough evaluation parameters except for the nature of cough (P=0.354*)*. A strong positive correlation was reported between the degree of tracheal collapse and the duration of cough (r_s=0.74), frequency of cough (r_s=0.62), type of cough (r_s=0.66) and wheezes (r_s=0.61). Moderate correlation was reported between the degree of tracheal collapse and the presence of cough reflex and its voluntary control (r_s=0.58). No significant correlation between the degree of tracheal collapse and the nature (productive /non-productive) of cough (r_s=0.74).

DISCUSSION

The present study demonstrated that the pattern of cough associated with tracheal collapse is mainly influenced by the degree of the collapse rather than its location.

All presented dogs were of middle age (7.5±3.2 years) which is concurrent with the progressive nature of the disease. Advancement of age may results in lack of tracheal cartilage rigidity and laxity of the dorsal tracheal membrane resulting in dorso-ventral narrowing in the tracheal lumen. Ideally, normal trachea should be relatively firm to allow free movement of the air during respiration when negative pressure is induced by breathing in. Although numerous toy breeds may be affected with tracheal collapse [3-5], all presented cases were Griffon dogs which is mainly attributed to the over presentation of Griffon dogs among the admitted dog population.

In the present study, the diagnosis of tracheal collapse was made through radiographic examination. Inspiratory radiographs were more helpful in the diagnosis of cervical tracheal collapse while expiratory radiographs were recommended for diagnosing thoracic tracheal collapse. This supports the concept that tracheal collapse is a dynamic rather than static compression in the dorso-ventral diameter in tracheal lumen [12]. During inspiration, there is a negative pressure created within the pleural space, the wall of the cervical trachea tends to collapse as the surrounding pressure in the neck is relatively positive. In the mean time, the wall of the intrathoracic trachea tends to be held open. During expiration, the reverse process occurs, where the wall of the intrathoracic trachea tend to collapse and the cervical trachea are forced to be open by the change in the relative pressure [11].

The progressive nature of tracheal collapse was clearly manifested by the predominance of chronic cough in dogs with severe tracheal collapse while acute and subacute duration of cough were reported in dogs with mild and moderate degrees of tracheal collapse. Persistent chronic cough can seriously impair the quality of life and may result in vomiting and muscle pain [13]. The increased frequency of cough in dogs with severe tracheal collapse may be a result of the more forced action of respiratory muscles to maintain ventilation. The prevalence of productive cough in severe tracheal collapse may be a result of long standing inflammation of the tracheal mucosa resulting in loss of epithelium, fibrinous membrane formation, squamous metaplasia and increased subepithelial glands secreation [14]. Thoracic tracheal collapse was mostly productive which could be correlated to the predominance of subepithelial secretory glands and cilia in the thoracic part of the trachea [15].

The presence of cough reflex and its voluntary control in mild and moderate tracheal collapse is an attempt of the body to overcome the narrowed tracheal lumen. While severe tracheal collapse is a life threatening condition resulting in spontaneous involuntary cough to overcome the severe reduction in tracheal lumen. Typical cough initiated by inspiratory phase followed by pause and expiratory cough was only reported in mild tracheal collapse. Typically, cough is an aerodynamic sequence of inspiration, compression and forced exhalation against a closed glottis [8]. Atypical cough that was not preceded by inspiratory phase was reported in moderate and severe tracheal collapse to achieve faster maintenance of ventilation. The presence of wheezes in moderate and severe tracheal collapse could be attributed to turbulence of air within the reduced tracheal lumen.

The main limitations of the present study are the use of only one breed of dogs (Griffon dog) and the absence of follow up data of cough following medical or surgical treatment. Further studies should be directed to studying the pattern of cough following medical and surgical treatment in different breeds of dogs with tracheal collapse. In conclusion, the pattern of cough associated with tracheal collapse is mainly influenced by the degree of the collapse rather than its location.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Author Contributions

All authors contributed equally in conceiving and designing the study, analyzing the obtained data, critically revising the manuscript for important intellectual contents and approving the final version.

REFERENCES

1. Lindl Bylicki BJ, Johnson LR, Pollard RE: Comparison of the radiographic and tracheoscopic appearance of the dorsal tracheal membrane in large and small breed dogs. *Vet Radiol Ultrasound,* 56 (6): 602-608, 2015. DOI: 10.1111/vru.12276

2. Tappin SW: Canine tracheal collapse. *J Small Anim Pract,* 57 (1): 9-17, 2016. DOI: 10.1111/jsap.12436

3. Macready DM, Johnson LR, Pollard RE: Fluoroscopic and radiographic evaluation of tracheal collapse in 62 dogs. *J Am Vet Med Assoc,* 230 (12): 1870-1876, 2007. DOI: 10.2460/javma.230.12.1870

4. Marolf A, Blaik M, Specht A: A retrospective study of the relationship between tracheal collapse and bronchiectasis in dogs. *Vet Radiol Ultrasound,* 48 (3): 199-203, 2007. DOI: 10.1111/j.1740-8261.2007.00229.x

5. Heng HG, Lim CK, Gutierrez-Crespo B, Guptill LF: Radiographic and computed tomographic appearance of tracheal collapse with axial rotation in four dogs. *J Small Anim Pract,* 59 (1): 53-58, 2018. DOI: 10.1111/jsap.12679

6. Dallman MJ, McClure RC, Brown EM: Histochemical study of normal and collapsed trachea in dogs. *Am J Vet Res,* 49 (12): 2117-2125, 1988.

7. Tanger CH, Hobson HP: A retrospective study of 20 surgically managed cases of collapsed trachea. *Vet Surg,* 11 (4): 146-149, 1982. DOI: 10.1111/j.1532-950X.1982.tb00691.x

8. Hoffman Ruddy B, Nadun Kuruppumullage D, Carnaby G, Crary M, Lehman J, Ilegbusi OJ: Computational modeling of cough function and airway penetrant behavior in patients with disorders of laryngeal function. *Laryngoscope Investig Otolaryngol,* 2 (1): 23-29, 2017. DOI: 10.1002/lio2.44

9. Martin M, Pereira YM: Approach to the coughing dog. *In Practice,* 35, 503-517, 2013. DOI: 10.1136/inp.f5838

10. Maggiore AD: Tracheal and airway collapse in dogs. *Vet Clin North Am Small Anim Pract*, 44 (1): 117-127, 2014. DOI: 10.1016/j.cvsm.2013.09.004

11. Porth CM: Essentials of Pathophysiology. Concepts of Altered States. 6th ed., Wolters Kluwer Health, 2014.

12. Clarke DL: Interventional radiology management of tracheal and bronchial collapse. *Vet Clin North Am Small Anim Pract,* 48 (5): 765-779, 2018. DOI: 10.1016/j.cvsm.2018.05.010

13. Michaudet C, Malaty J: Chronic cough: Evaluation and management. *Am Fam Physician,* 96 (9): 575-580, 2017.

14. Suter PF, Lord PF: Diseases of the nasal cavity, larynx, and trachea. **In,** Suter PF, Lord PF (Eds): Thoracic Radiography: A Text Atlas of Thoracic Disease in the Dog and Cat. 237-240, Wettswil, Switzerland, 1984.

15. Breeze R, Turk M: Cellular structure, function and organization in the lower respiratory tract. *Environ Health Perspect,* 55, 3-24, 1984. DOI: 10.1289/ehp.84553

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Short Communication

Ovine Abortion Associated with *Campylobacter fetus* subsp. *fetus* ST2 in Turkey

Fuat AYDIN [1,a] Murat ABAY [2,b] Ayhan ATASEVER [3,c] Latife ÇAKIR BAYRAM [3,d] Emre KARAKAYA [1,e] Seçil ABAY [1,f] Görkem EKEBAŞ [3,g] Hamit Kaan MÜŞTAK [4,h] Kadir Semih GÜMÜŞSOY [1,i] Linda van der GRAAF-VAN BLOOIS [5,j] Kadir Serdar DİKER [6,k]

[1] Department of Microbiology, Faculty of Veterinary Medicine, Erciyes University, TR-38280 Kayseri - TURKEY
[2] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Erciyes University, TR-38280 Kayseri - TURKEY
[3] Department of Pathology, Faculty of Veterinary Medicine, Erciyes University, TR-38280 Kayseri - TURKEY
[4] Department of Microbiology, Faculty of Veterinary Medicine, Ankara University, TR-06110 Ankara - TURKEY
[5] Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, 3508 TC, Utrecht - THE NETHERLANDS
[6] Department of Microbiology, Faculty of Veterinary Medicine, Aydın Adnan Menderes University, TR-09010 Aydın-TURKEY

ORCIDS: [a] 0000-0002-5467-011X; [b] 0000-0003-2457-1919; [c] 0000-0002-6327-1604; [d] 0000-0001-9357-0755; [e] 0000-0003-2390-6190
[f] 0000-0001-5599-7539; [g] 0000-0001-9094-677X; [h] 0000-0002-3694-1959; [i] 0000-0001-6326-0377; [j] 0000-0001-8181-3393; [k] 0000-0003-2150-5553

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Abstract

In this study, we aimed to evaluate the microbiological, molecular and pathological findings of abortus cases detected in a sheep herd consist of 200 animals. Macroscopically, irregular necrotic foci were observed in the liver in aborted fetuses. Selective and non-selective media were used for the isolation of causative agent. Phenotypic and molecular tests were performed for identification. *Campylobacter fetus* subsp. *fetus* (Cff) was isolated from organs of four foetuses aborted. While the vaginal swap samples taken from sheep that did not abort, water, feed and litter samples produced negative results for Cff, 5 of the 20 faecal samples and 5 of the vaginal swaps taken from the sheep that had aborted, yielded Cff. Multilocus Sequence Typing (MLST) was used for genotyping and all isolates were detected as Sequence Type 2 (ST2). This is the first documented report of an ovine abortion caused by Cff ST2 in Turkey. It is considered that the development and use of vaccines, containing local *Campylobacter* species, would contribute to both prophylaxis and control of abortions caused by campylobacters including *Campylobacter fetus* subsp. *fetus*.

Keywords: *Campylobacter fetus subsp. fetus, MLST, Sheep abortion*

Türkiye'de *Campylobacter fetus* subsp. *fetus* ST2 İlişkili Koyun Abortusu

Öz

Bu çalışmada, 200 başlık bir koyun sürüsünde görülen abortus olgularının mikrobiyolojik, moleküler ve patolojik bulgularının değerlendirilmesi amaçlandı. Aborte fetuslarda makroskopik olarak karaciğerde düzensiz nekrotik odaklar gözlendi. Etken izolasyonu için seçici ve seçici olmayan besiyerleri kullanıldı. İdentifikasyonda fenotipik ve moleküler testlerden yararlanıldı. Atık dört fötüsun organlarından *Campylobacter fetus* subsp. *fetus* (Cff) izole edildi. Abort yapmayan koyunlardan alınan vajinal swap, su, yem ve altlık örnekleri Cff yönünden negatif bulunurken, abort yapan 20 koyunun dışkı örneklerinin 5'i ve vajinal swap örneklerinin 5'i Cff pozitif olarak saptandı. Genotiplendirmede Multilokus Sekans Tiplendirme (MLST)'den yararlanıldı ve tüm izolatların ST2 (Sekans Tip 2) olduğu belirlendi. Bu çalışma, Türkiye'de koyunlarda abort olgularından *Campylobacter fetus* subsp. *fetus* ST2'nin bildirildiği ilk rapordur. Yurdumuzda abort olgularından izole edilen *Campylobacter* türlerini içeren aşıların geliştirilmesi ve kullanımının, *Campylobacter fetus* subsp. *fetus*'un da neden olduğu abortusların hem profilaksisine hem de kontrolüne katkıda bulunacağı düşünülmektedir.

Anahtar sözcükler: *Campylobacter fetus subsp. fetus, MLST, koyun abortus*

Correspondence
+90 352 2076666/29912
sabay@erciyes.edu.tr

INTRODUCTION

The pregnancy and delivery rates of sheep and goats are quite high during the mating season (85-95% and 75-85%, respectively). Although factors that may show negative impact on fertility are rather few in sheep compared to cattle, still major problems are encountered in the maintenance of pregnancy in ewes and the delivery of healthy lambs [1,2]. The main problem in both sheep and cattle that is faced is abortion, which also causes grave economic loss. Infectious abortions are mostly observed at herd/flock level, rather than as sporadic cases [3,4]. *Campylobacter fetus* subsp. *fetus* (Cff) colonizes mainly in the intestinal tract of cattle and sheep and may cause sporadic abortion in both species [5]. Cff is recognised as a significant causative agent of ovine abortions.

Once a flock is exposed to the Cff, it rapidly spreads within the flock via the faecal-oral route, eventually causing a high abortion rate of 50-60% throughout the lambing season, and thus, heavy economic loss occurs [1,6].

It was aimed to determine the etiology of the abortion that was seen in a flock of 200 sheep and to report *Campylobacter fetus* subsp. *fetus* ST2 isolation.

MATERIAL and METHODS

Animals

In a flock of 200 Akkaraman sheep, 20 animals aborted in the 4th month of gestation, and four of the aborted foetuses were submitted to the Faculty of Veterinary Medicine of Erciyes University (ERU). The animals were vaccinated against enterotoxemia in the 2nd month of gestation, and it was informed that the flock was fed on beet pulp silage.

Necropsy

Necropsy was performed under aseptic conditions. The liver, spleen, stomach, and different parts of the small and large intestines, as well as the pancreas, kidneys, lungs, heart, and brain were dissected. Two specimens were taken from each tissue, one for histopathological analysis and the other for microbiological analysis.

Histopathological Analysis

For histopathological analysis, the tissue samples were fixed in 10% neutral buffered formalin solution, which was changed several times. The tissue samples were dehydrated through graded concentrations of ethanol prior to automated tissue processing, and then were embedded in paraffin wax. Deparaffinised sections were stained with haematoxylin and eosin stain and a modified Brown and Brenn method [7].

Bacteriological Analysis

The foetal organ specimens collected at necropsy and 40 faecal samples and 40 vaginal swap samples taken from 20 sheep that aborted and 20 sheep that did not abort, as well as 3 feed, 3 water and 3 litter samples underwent bacteriological analysis. Gram staining was used for slides prepared from the foetal tissue (liver, abomasum content, lung) samples. For the bacterial culture method, the foetal organ samples (liver, abomasum content, lung) were inoculated onto blood agar (containing 7% sheep blood), MacConkey Agar, and Eosin Methylene Blue Agar (EMB). Furthermore, in view of the hepatic lesions showing similarity to those observed in campylobacteriosis, the foetal liver samples were also inoculated onto blood agar base No: 2 (enriched with 7% sheep blood; Skirrow Selective Supplement, Oxoid, SR0069, UK). The inoculated plates were incubated under aerobic, microaerobic (Gas generating kits, Anaerocult C, Merck, Germany) and anaerobic (Gas generating kits, Anaerocult A, Merck, Germany) conditions for 48-72 h at 37°C. For the isolation of Cff from faecal samples, vaginal swap samples, feed, water and litter, a technique combining pre-enrichment and membrane filtration was used [8]. However, in the present study, we used Brucella broth supplemented with Skirrow's supplement (Skirrow Selective Supplement, Oxoid, SR0069, UK) and a 0.65 µm pore-size cellulose acetate membrane filter.

Phenotypic tests including Gram staining, motility test, oxidase and catalase activity, growth at 25°C and 42°C tests [8] and molecular analysis [9,10] were performed for identification of the isolates.

Molecular Analysis

DNA Extraction: Template DNA was prepared from pure cultures grown on blood agar by using boiling and centrifugation method.

Identification of the Isolates at Genus, Species and Subspecies Level: Phenotypic tests including Gram staining, motility test, oxidase and catalase activity, growth at 25°C and 42°C tests [8] and molecular analysis [9,10] were performed for identification of the isolates.

We performed three different PCR for the genus, species and subspecies identification of the isolates. Genus detection PCR was performed using C412F and C1288R primers described by Linton et al.[9], while species and subspecies detection PCR were carried out according to Schulze et al.[11] by using MG3F/MG4R and VenSF/VenSR primers. In addition, identification of other *Campylobacter* species were carried out according to method of Wang et al.[10].

16S Ribosomal RNA Gene Sequencing: In order to identify the species of the isolates, 16S rRNA gene sequencing was performed using the universal primers 27F and 1492R [12]. The amplified products were purified using the QIA-quick PCR Purification Kit (Qiagen, USA), and sequence analysis was performed using the Big Dye Direct Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions. After cycle sequencing, the amplicons were

purified with Sephadex G-50 (Sigma-Aldrich, USA) by using spin columns and sequenced on the Applied Biosystems 3500 Genetic Analyser (Applied Biosystems, USA).

All sequences were analysed with the CLC Main Workbench 6 and compared with reference sequences available on the website of the National Centre for Biotechnology Information using the Basic Local Alignment Search Tool for Nucleotides (BLASTn) programme *(https://blast.ncbi.nlm. nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome)*.

Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR): The ERIC primers 1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and 2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') were used for molecular typing of the isolates [8].

Multilocus Sequence Typing (MLST): The multilocus sequence type (ST) of the *C. fetus* subsp. *fetus* isolates was determined according to a previously described MLST protocol [13]. Briefly, seven housekeeping genes of the *C. fetus* subsp. *fetus* isolates (aspA: aspartase, glnA: glutamine synthetase, gltA: citrate synthase, glyA: serine hydroxy methyl transferase, pgm: phospho glucomutase, tkt: transketolase, and uncA: ATP synthase alpha subunit) were amplified.

The PCR products were sequenced in both forward and reverse directions. Alleles, STs, and clonal complex (CC) assignments were made using the PubMLST database *(https://pubmlst.org/bigsdb?db=pubmlst_campylobacter_nonjejuni_seqdef)*

RESULTS

Necropsy Findings

Macroscopic examination showed that irregular necrotic foci with a light brown dented centre and a pale periphery were scattered throughout the hepatic lobes *(Fig. 1-A)*. No characteristic lesions were observed in the other organs examined.

Histopathological Analysis

Histologically, the liver presented with multifocal areas of coagulative necrosis, mononuclear cell infiltration and numerous large bacterial colonies surrounding the necrotic areas, sinusoidal dilatation, and haemorrhage *(Fig. 1-B,C)*. *Fig. 1-C* shows many Gram negative curved-shaped bacteria within and around an area of necrosis. Different from the liver, the other organs that were examined did not present with any characteristic histopathological finding.

Bacteriological Analysis

Numerous Gram-negative spiral shaped bacteria were observed in the stained tissue preparations. At the end of the incubation period, no specific growth was observed on the aerobic and anaerobic incubated plates, while the blood agar plates incubated microaerobically showed the growth of smooth, translucent, non-haemolytic colonies, measuring 1-2 mm in diameter. These colonies were subcultured for pure culture of the isolates. Based on the results of the phenotypic tests, the isolates were identified as *Campylobacter* spp. Thus, organs belong to four foetuses analysed were found to be positive for *Campylobacter* spp.

Molecular Analysis

All isolates tested were identified as *Campylobacter* spp. according to Linton et al.[9]. 16S rRNA sequence analysis revealed that isolates were identified as *C. fetus*. However BLAST results showed that 16S rRNA sequencing could not differentiate between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. Next, the isolates were identified as *C. fetus* subsp. *fetus* according to the PCR method *(Fig. 2)* described by Schulze et al.[11]. In addition, the biochemical 1% glycine tolerance test was used for differentiation and isolates were found to be tolerant to 1% glycine.

While the vaginal swap samples taken from sheep that did not abort, water, feed and litter samples analysed with enrichment technique produced negative results for Cff, 5 (25%) of the 20 faecal samples taken from the sheep that had aborted, yielded Cff. Similarly 5 (25%) of the 20 vaginal swap samples taken from the sheep that had aborted, were positive for Cff. *Campylobacter* isolation results of the current study are presented in *Table 1*.

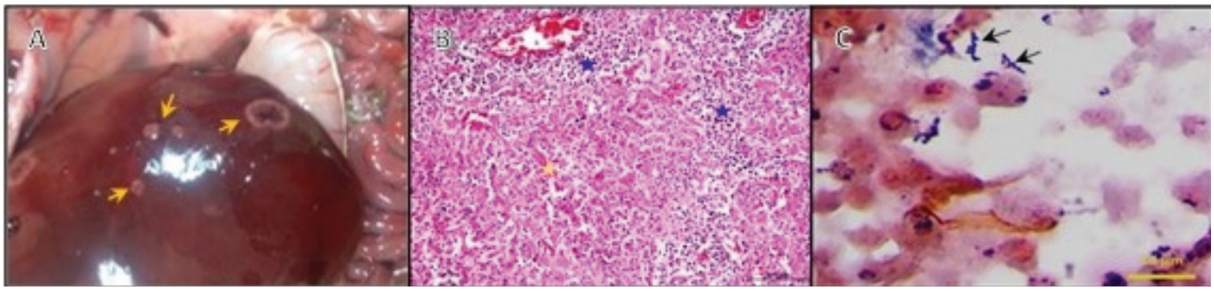

Fig 1. Pathology of the liver taken from the aborted foetus. **A)** Pale, necrotic areas scattered throughout the surface of the liver *(yellow arrows),* **B)** Multifocal moderate necrotizing hepatitis *(yellow star),* with neutrophil leukocyte and mononuclear cell infiltration *(blue stars);* Bar: 100 µm, Haematoxylin and eosin stain, **C)** Easily distinguishable clusters of spiral bacteria *(arrows)* in the hepatic lesions

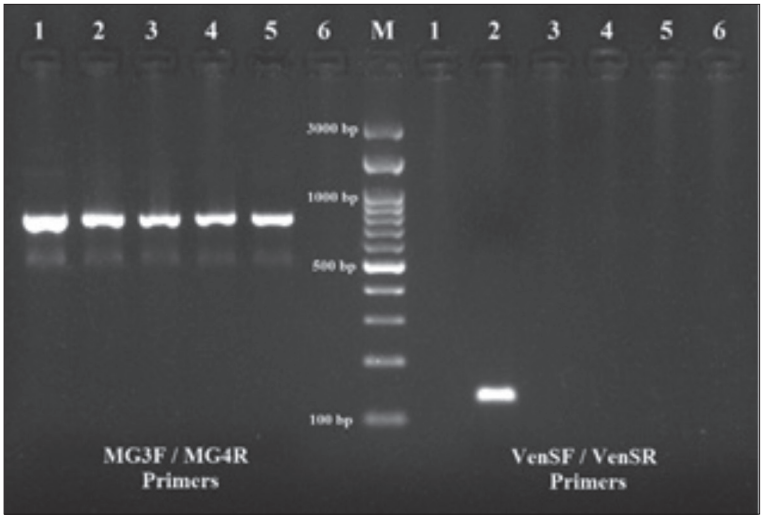

Fig 2. Agarose gel electrophoresis of PCR products obtained by using primer pairs MG3F/MG4R (750 bp) and VenSF/VenSR (142 bp); 1: *C. fetus* subsp. *fetus* control strain, 2: *C. fetus* subsp. *venerealis* control strain, 3: *C. fetus* subsp. *fetus* isolate recovered from aborted foetus, 4: *C. fetus* subsp. *fetus* isolate recovered from ovine feces, 5: *C. fetus* subsp. *fetus* isolate recovered from ovine vaginal swab, 6: Negative control, M: Marker, 100bp DNA Ladder H3 RTU, GeneDireX

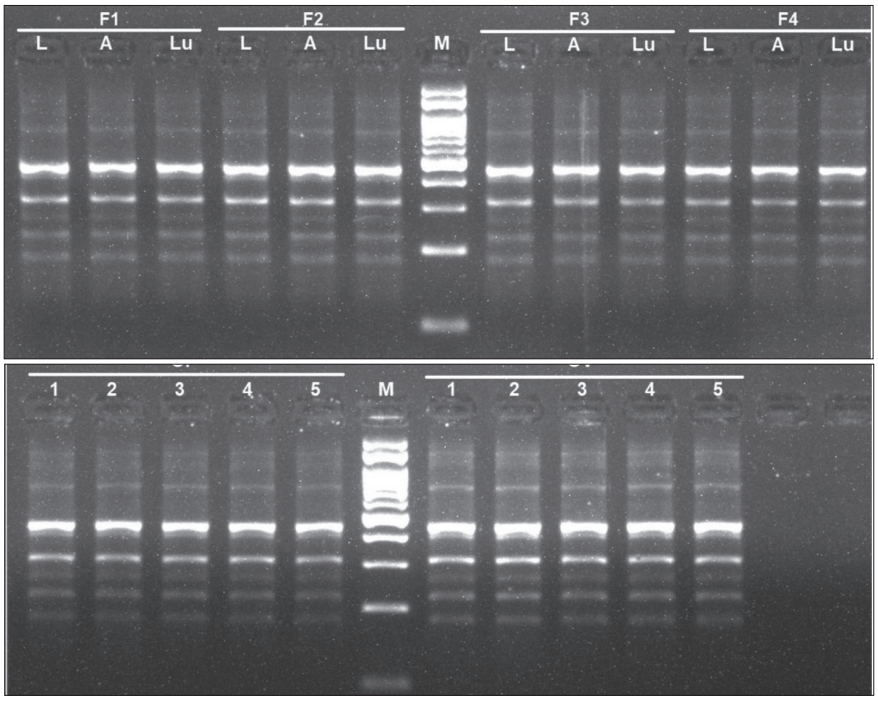

Fig 3. Agarose gel electrophoresis of ERIC-PCR products obtained by using 1R and 2 primer pairs. **F1:** Aborted foetus 1, **F2:** Aborted foetus 2, **F3:** Aborted foetus 3, **F4:** Aborted foetus 4, **L:** Liver isolate, **A:** Abomasum content isolate, **Lu:** Lung isolate, **OF:** Ovine faeces isolates (1-5), **OV:** Ovine vaginal swap isolates (1-5), **M:** Marker GeneDirex 100 bp DNA Ladder H3 RTU

In the genotyping performed by ERIC-PCR, it was determined that all of the 22 isolates *(Table 1)* obtained from aborted foetuses, sheep faecal samples, and sheep vaginal samples had the same band patterns *(Fig. 3)*; thus, a single representative Cff isolate belongs to each source was used in the MLST step of the study. The isolates which were deposited in the GenBank, were the representative Cff isolates.

16S rRNA Gene Sequencing

The 16S rRNA sequences of Cff isolates obtained from aborted foetus, ovine faeces and vaginal swaps were deposited in GenBank under accession numbers MK806573, MK818524 and MK818525 respectively.

Multilocus Sequence Typing (MLST)

In result, it was determined that the isolates obtained from the aborted foetuses, ovine faeces, and vaginal swaps were of the ST2 genotype *(Table 2)*.

DISCUSSION

Campylobacter coli [14], *C. jejuni* [15], and *C. fetus* subsp. *fetus* [16] are the main *Campylobacter* species isolated from ovine abortion cases, and several literature reports have been published on their isolation. In cases of ovine abortion associated with Cff, diagnosis is based on the results of phenotypic and molecular tests. In the ovine abortion case described in this report, the isolates obtained from the aborted foetuses and other material were identified as Cff on the basis of the results of phenotypic tests [8] and molecular analyses [9,17]. In the species identification of *C. fetus* isolates, different results have been reported for the

Table 1. *Number of samples analysed and distribution of Campylobacter species recovered*

Samples Analysed	n	*Campylobacter* Species Isolated		
		Cc	*Cff*	*Cj*
Aborted foetuses	4	-	4*	-
Faecal samples (from aborted sheep)	20	-	5	-
Faecal samples (from sheep that did not abort)	20	15	-	-
Feed	3	-	-	-
Litter	3	-	-	1
Vaginal swap samples (from aborted sheep)	20	-	5	-
Vaginal swap samples (from sheep that did not abort)	20	-	-	-
Water	3	-	-	-

Cc: *Campylobacter coli;* ***Cff:*** *Campylobacter fetus subsp. Fetus;* ***Cj:*** *Campylobacter jejuni;* ***-:*** *negative result; * number of positive samples*

Table 2. *Results from the sequence analysis of seven housekeeping genes and their allelic profiles based on MLST Database* regarding Campylobacter fetus*

Isolate Name	Housekeeping Genes Analysed and Allelic Profiles							ST
	ASP	GLN	GLT	GLY	PGM	TKT	UNC	
MK806573	1	2	2	2	1	1	2	2
MK818524	1	2	2	2	1	1	2	2
MK818525	1	2	2	2	1	1	2	2

** (https://pubmlst.org/bigsdb?db=pubmlst_campylobacter_nonjejuni_seqdef), MK806573, MK818524, and MK818525 are GenBank accesion number of the isolates whichwere recovered from aborted foetus, ovine faeces, and vaginal swaps respectively*

band size obtained with the use of MG3F/MG4R primers in previous studies. For example, while Hum et al.[17] reported to have obtained 960 bp bands, Schulz et al.[11] and Wagenaar et al.[18] reported to have obtained 750 bp bands with the use of these primers. Interestingly, the size of the bands obtained in the present case study was also 750 bp. The differences in the amplicon sizes in studies performed in different countries cannot be explained [11].

It is indicated that the reference test for the differentiation of *C. fetus* isolates at subspecies levels is the 1% glycine-tolerance test, and the results of this test are reported to be generally in agreement with PCR results [11]. Likewise, the results of these two methods were observed to be in agreement in the present study, both methods identified the isolates as Cff.

Only few literature reports are available on the MLST analysis of Cff isolates obtained from cases of ovine and bovine abortions [13]. There is no study performed on the typing of Cff with MLST in Turkey. However, in the study conducted by Van Bergen et al.[13], Cff isolates from several countries were genotyped by MLST, and of the 4 Cff isolates from Turkey that were tested, 3 were reported to have been identified as sequence type 2 (ST2), and 1 as sequence type 5 (ST5). In the current study, the isolates obtained from the aborted foetuses, vaginal swap samples and faecal samples were identified as ST2. However, there is no information on the isolation source (from abortion cases or intestinal carriage etc.) of the Turkish Cff isolates analysed in the study of Van Bergen et al.[13].

It is reported that, the macroscopic observation of multifocal necrotic areas, as if staple-punched, in the foetal liver, and the detection of coagulation necrosis at histopathological examination are specific to abortions associated with campylobacters. The macroscopic and histopathological findings detected in the foetal livers examined in the present study *(Fig. 1)* were in agreement with these specific findings reported in literature.

In conclusion, Campylobacters are frequently isolated from ovine abortions occurring in different locations in Turkey [14,15,19,20]. On the other hand, prophylactic vaccination is not periodically implemented against ovine abortions caused by campylobacters in Turkey. Therefore, it is considered that the development of inactive vaccines from local Campylobacter species isolated from the cases, and the immunisation of animals with these vaccines would contribute to both prophylaxis and disease control.

Conflict of Interest

The authors declared that there is no conflict of interest.

REFERENCES

1. Davies P: Infertility and abortion in sheep and goats. **In,** Noakes DE, Parkinson TJ, England GCW (Eds): Veterinary Reproduction and Obstetrics.

10th ed., 510-525, WB Saunders, Philadelphia, 2019.

2. Erdem H, Sarıbay MK: Gebelik ve tanı yöntemleri, **In,** Kaymaz M, Fındık M, Rişvanlı A, Köker A (Eds): Çiftlik Hayvanlarında Doğum ve Jinekoloji. 3. Baskı, 441-452, Medipress, Malatya, 2019.

3. Taşal I, Bozkurt G: Koyunlarda abort. *Türkiye Klinikleri J Vet Sci Intern Med-Special Topics*, 1, 11-17, 2015.

4. Rişvanlı A, Kalkan C, Doğan H, Öcal H: Koyun ve keçilere infertilite ve yavru atma. *Türkiye Klinikleri J Vet Sci Obstet Gynecol-Special Topics.* 2, 18-28, 2016.

5. Lastovica AJ, Allos BM: Clinical significance of *Campylobacter* and related species other than *Campylobacter jejuni* and *Campylobacter coli*. **In,** Nachamkin I, Szymanski CM, Blaser MJ (Eds): Campylobacter, 123-150, ASM Press, Washington DC, 2008.

6. Van Bergen MAP: Subspecies differentiation and typing of *Campylobacter fetus*. *Doctorate Thesis,* University of Utrecht, 2005.

7. Carson LF, Cappellano CH: Histotechnology: A Self-instructional Text. 4th ed., 98-241, ASCP Press, Chicago, 2015.

8. Aydin F, Gümüşsoy KS, Atabay HI, Içа T, Abay S: Prevalence and distribution of *Arcobacter* species in various sources in Turkey and molecular analysis of isolated strains by ERIC-PCR. *J Appl Microbiol*, 103, 27-35, 2007. DOI: 10.1111/j.1365-2672.2006.03240.x

9. Linton D, Owen RJ, Stanley J: Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. *Res Microbiol*, 147, 707-718, 1996. DOI: 10.1016/S0923-2508(97)85118-2

10. Wang G, Clark CG, Taylor TM, Pucknell C, Barton C, Price L, Woodward DL, Rodgers FG: Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni, C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. *J Clin Microbiol*, 40, 4744-4747, 2002. DOI: 10.1128/JCM.40.12.4744-4747.2002

11. Schulze F, Bagon A, Müller W, Hotzel H: Identification of *Campylobacter fetus* subspecies by phenotypic differentiation and PCR. *J Clin Microbiol*, 44, 2019-2024, 2006. DOI: 10.1128/JCM.02566-05

12. Lane DJ: 16S/23S rRNA sequencing. **In,** Stackebrandt E, Goodfellow M (Eds): Nucleic Acid Techniques in Bacterial Systematics. 115-175, John Wiley & Sons, New York, 1991.

13. van Bergen MAP, Dingle KE, Maiden MCJ, Newell DG, van der Graaf-Van Bloois L, van Putten JPM, Wagenaar JA: Clonal nature of *Campylobacter fetus* as defined by multilocus sequence typing. *J Clin Microbiol*, 43, 5888-5898, 2005. DOI: 10.1128/JCM.43.12.5888-5898.2005

14. Diker KS, Sahal M, Aydin N: Ovine abortion associated with *Campylobacter coli*. *Vet Rec*, 122:87, 1988. DOI: 10.1136/vr.122.4.87

15. Diker KS, Istanbulluoglu E: Ovine abortion associated with *Campylobacter jejuni*. *Vet Rec,* 118:307, 1986. DOI: 10.1136/vr.118.11.307

16. Gressler LT, Kirinus JK, Machado G, Libardoni F, de Vargas AC: *Campylobacter fetus* subspecies *fetus*: Abortion and stillbirths in sheep. *Cienc Rural,* 42, 697-700, 2012. DOI: 10.1590/S0103-84782012000400020

17. Hum S, Quinn K, Brunner J, On SL: Evaluation of a PCR assay for identification and differentiation of *Campylobacter fetus* subspecies. *Aust Vet J*, 75, 827-831, 1997. DOI: 10.1111/j.1751-0813.1997.tb15665.x

18. Wagenaar JA, van Bergen MAP, Newell DG, Grogono-Thomas R, Duim B: Comparative study using amplified fragment length polymorphism fingerprinting, PCR genotyping, and phenotyping to differentiate *Campylobacter fetus* strains isolated from animals. *J Clin Microbiol*, 39, 2283-2286, 2001. DOI: 10.1128/JCM.39.6.2283-2286.2001

19. Muz A, Ertaş HB, Öngör H, Gülcü HB, Özer H, Eröksüz H, Dabak M, Başbuğ O, Kalender H: Elazığ ve çevresinde koyun ve keçilerde abortus olgularının bakteriyolojik, serolojik ve patolojik olarak incelenmesi. *Turk J Vet Anim Sci,* 23, 177-188, 1999.

20. Gülmez Sağlam A, Akça D, Çelebi Ö, Büyük F, Çelik E, Coşkun MR, Şahin M, Otlu S: Isolation and molecular identification of *Campylobacter* spp. from vaginal swab sample obtained from sheep herds with abort history. *Kafkas Univ Vet Fak Derg*, 25 (5): 697-701, 2019. DOI: 10.9775/kvfd.2018.21654 *Ovine*

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Case Report

A Case of Polyostotic Fibrous Dysplasia in a Spider Monkey *(Ateles paniscus)* [1]

Gulbin SENNAZLI [1,a] Ozge ERDOGAN-BAMAC [1,b] Gulay YUZBASIOGLU-OZTURK [1,c] Lora KOENHEMSI [2,d]

[1] *Presented as an oral presentation at the 7th National Veterinary Pathology Congress, 08-10 September 2014, Kars, Turkey*
[1] Istanbul University-Cerrahpasa, Department of Pathology, Faculty of Veterinary Medicine, TR-34320 Istanbul - TURKEY
[2] Istanbul University-Cerrahpasa, Department of Internal Medicine, Faculty of Veterinary Medicine, TR-34320 Istanbul - TURKEY
ORCIDS: [a] 0000-0002-8419-2833; [b] 0000-0002-0352-4841; [c] 0000-0002-1761-0409; [d] 0000-0002-0352-4841

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Abstract

This paper presents the gross and histopathologic findings of polyostotic fibrous dysplasia which is rarely seen in non-human primates. A 3-year-old female spider monkey *(Ateles paniscus)* was referred to the clinics of our faculty with the complaints of severe dyspnea, lethargy and cough and was diagnosed with dilated cardiomyopathy following the radiographic examination. However, the monkey died due to depression of respiration before treatment was done. Necropsy was performed on the request of the owner. Dilated cardiomyopathy and irregularly shaped tumoral masses with diameters of 0.5-1.0 cm in the right and left ribs were detected. Following the histopathological examination, the lesions were diagnosed as fibrous dysplasia.

Keywords: *Fibrous dysplasia, Histopathologic findings, Spider monkey*

Bir Örümcek Maymununda *(Ateles paniscus)* Poliostotik Fibröz Displazi Olgusu

Öz

Bu bildiri, insan olmayan primatlarda nadir olarak görülen polyostotik fibröz displazinin makroskobik ve histopatolojik bulgularını sunmaktadır. Üç yaşındaki dişi örümcek maymunu *(Ateles paniscus)* şiddetli dispne, letarji ve öksürük şikayetleri ile fakültemiz kliniklerine getirilmiştir. Yapılan radyografik muayene neticesinde dilate kardiyomiyopati teşhisi konulmuştur. Ancak, herhangi bir tedavi uygulanmadan önce, solunum yetmezliği sonucunda ölüm gerçekleşmiştir. Hasta sahibinin isteği üzerine uygulanan nekropside, dilate kardiyomiyopatinin yanısıra, sağ ve sol kostalarda çapları 0.5-1.0 cm arasında değişen, düzensiz şekilli tümoral kitleler tespit edilmiştir. Histopatolojik inceleme sonucunda lezyonlar fibröz displazi olarak teşhis edilmiştir.

Anahtar sözcükler: *Fibröz displazi, Histopatolojik bulgular, Örümcek maymunu*

INTRODUCTION

Fibrous dysplasia (FD) is a tumor-like intramedullary fibro-osseous lesion of any bone in humans and animals [1-3]. FD is commonly described as the replacement of normal bone elements with benign cellular fibrous connective tissue within which irregular trabeculae of woven bone are haphazardly distributed [4]. The lesions of FD develop during skeletal formation and growth and have a variable natural evolution [2]. FD occurs throughout the skeleton with predilection for the long bones of extremities, ribs, pelvis and craniofacial bones [2,5], which leads to severe consequences including pathological fractures, impairment of limb function, facial and limb deformities, and compressive damage of sensory nerves resulting in blindness or deafness [4,6]. It has three clinical patterns namely; monostotic, polyostotic, and the McCune-Albright syndrome. In monostotic fibrous dysplasia, there is single bone involvement. Ribs and craniofacial bones are the most common sites. Polyostotic fibrous dysplasia involves two or more of the long bones, pelvis bones and ribs. In McCune- Albright syndrome polyostotic form is

Correspondence
+90 212 8663700/43660
ayyildiz@istanbul.edu.tr

accompanied by endocrine disturbances and cutaneous hyperpigmentation [3,7].

Although it has been documented in several animal species, including horse, pig, dog, and domestic cat, there are still few reports of FD in animals [1,8-10]. Only three cases have been reported in non-human primates up to date [11-13]. This paper presents a case of polyostotic fibrous dysplasia which was detected in the ribs of a spider monkey.

CASE HISTORY

A 3-year-old, 4.2-kg, female spider monkey which was housed in a private zoo was referred to the Clinics of Veterinary Faculty of Istanbul University- Cerrahpasa with the complaints of severe dyspnea, lethargy and cough. She was diagnosed with dilated cardiomyopathy following the radiographic examination. There were no distinct abnormalities that could be attributed to FD in the thoracic radiograph. The monkey died due to depression of respiration before treatment was done. Necropsy was performed at the Pathology Department of Veterinary Faculty of Istanbul University- Cerrahpasa on the request of the owner. Informed consent form was obtained from the owner for the procedures to be applied to this case.

On postmortem examination, severe edema, congestion and areas of consolidation in the lungs and dilated cardiomyopathy were observed. Irregularly shaped, nine tumoral masses with diameters of 0.5- 1 cm were detected in the left 3rd, 6th and 7th ribs and in the right 8th rib. They were moderately dense, gritty and whitish in colour. No gross lesions were noted in the skull or long bones.

Several sections of the tumoral masses and various organs were fixed in 10% neutral buffered formalin. Following decalcification in ethylenediamine tetraacetic acid (EDTA) solution the tissue samples were routinely processed, embedded in paraffin, cut at 5 µm, stained with hematoxylin and eosin (H&E), and evaluated under light microscopy.

Histopathological examination revealed severe areas of collateral hyperemia, edema and atelectasis in the lungs. The tumoral masses of the ribs consisted of thin, curved trabeculae of woven bone embedded in a loose fibrous connective tissue stroma. There was a variably cellular fibroblastic proliferation in storiform pattern with osteoid or bony trabeculae which was poorly formed *(Fig. 1-A)*. Collagen fibers in the stroma were generally delicate. Osteoblasts were not rimmed on trabecular surfaces *(Fig. 1-B)*. The hook-like, long crooked spicules of bone were typically seen in specimens *(Fig. 1-C)*. The degree of calcification of trabeculae was variable. Degeneration including hyalinization of stroma was seen in the primary lesion. There was no sign of atypia in the stroma. Clusters of giant cells resembling osteoclasts were often found in close proximity to the trabecule *(Fig. 1-D)*. Hemorrhagic

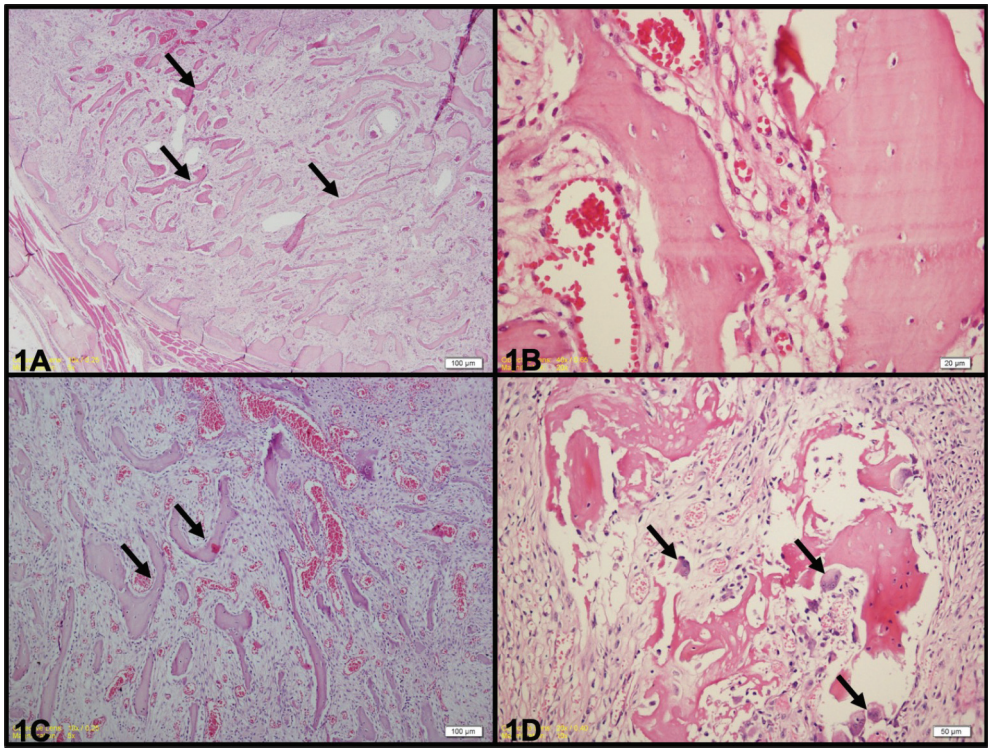

Fig 1. Osteodisplastic lesions, ribs; **A:** Cellular fibroblastic proliferation in storiform pattern with osteoid or bony trabecule which was poorly formed *(arrow),* **B:** Osteoblasts were not rimmed on trabecular surfaces, **C:** The hook-like, long crooked spicules of bone *(arrow),* **D:** Clusters of giant cells resembling osteoclasts *(arrow),* rib, (H&E)

scattered areas and cartilaginous foci were present, but cysts or necrotic foci were not seen in the lesions. The number of inflammatory cells and mitotic figures were rare. No sign of malignancy was observed in any of the specimens. The lesions were diagnosed as polyostotic fibrous dysplasia according to the histopathologic findings.

DISCUSSION

Tumor-like lesions of bone are uncommon in domestic animals and even less in non-human primates [11-14]. Bauer et al.[11] reported FD in the long bones of extremities (tibia, fibula and femur) in a cynomolgus macaque and Duncan et al.[12], reported FD in the maxilla and mandibula of a 4.5-year-old male spider monkey [11,12]. In veterinary literature, in comparison with the elder ones, young animals are reported more likely to be affected by FD [15]. The spider monkey of this report was a young individual like the ones described in previous reports [11-13]. Patients with FD are generally reported to have swellings, skeletal deformities and fracture symptoms in the localization sites. Generally, lesions are difficult to distinguish from malignancies on gross and radiologic examinations because of their infrequent clinical occurrence resulting in few specimens available for study by veterinary radiologists and pathologists [1]. In this case, those findings were not observed during physical examination. In routine radiographs, global cardiac enlargement was prominent, but no distinct abnormalities attributed to FD were noticed.

Common sites of skeletal involvement of FD in humans are long bones, ribs, craniofacial bones, vertebra and pelvis [2]. In domestic animals lesions have been found in sinuses, mandible and long bones [15]. In the case reports of non-human primates with FD, lesions were present in the maxilla and in the long bones [11-13]. In this case polyostotic lesions were observed only in the ribs. Although localization of FD lesions in the ribs have been reported in humans [16,17], it has not been reported in animals according to the authors knowledge.

The pathogenesis of FD suggests that it arises from a somatic mutation of the gene associated with cell differentiation from mesenchymal stem cells into mature osteocytes [18]. At necropsy, cytogenetic analysis on the monkey was not performed as the lesions were evaluated as neoplasia. As there were no endocrine disturbances involved such as cutaneous hyperpigmentation, hyperthyroidism, hyperparathyroidism, acromegaly and no pathologic lesions in thyroid and parathyroid gland, McCune-Albright syndrome was not considered as a potential diagnosis.

Fibrous dysplasia, fibro-osseous lesion of bone, is thought to be a developmental abnormality of bone-forming mesenchyme rather than a neoplasm. Benign fibro-osseous proliferations of bone in veterinary medicine include ossifying fibroma, osteoma, osteofibrous dysplasia [14]. Sometimes the tumor-like lesions can be confused with even malign neoplasia. Thus, FD can be distinguished histopathologically from ossifying fibroma, osteoma, osteosarcoma, and perhaps even from fibrous osteodystrophy [4,18]. FD consists of bone spicules that are usually more uniform and are not rimmed by osteoblasts, which is also a feature that helps to distinguish this lesion from other fibro-osseous lesions [18]. In the present case, collagen fibers in the stroma were generally delicate and the hook-like, long crooked spicules of bone were present in specimens and there were no osteoblasts on trabecular surfaces of immature woven bone. Thus, possibility of other fibro-osseous lesions for diagnosis were eliminated and the case was evaluated as polyostotic fibrous dysplasia. Since polyostotic fibrous dysplasia of ribs in a non-human primate is rarely seen, the authors believe that this case report is going to contribute valuable information to the veterinary literature.

REFERENCES

1. Fitzgerald W, Slocombe R, Caiafa A: Fibrous dysplasia of mandibular bone in a dog. *J Vet Dent*, 19 (2): 77-81, 2002. DOI: 10.1177/089875640201900203

2. DiCaprio MR, Enneking WF: Fibrous dysplasia. Patophysiology, evaluation, and treatment. *J Bone Joint Surg Am*, 87 (8): 1848-1864, 2005. DOI: 10.2106/JBJS.D.02942

3. Hartley I, Zhadina M, Collins MT, Boyce AM: Fibrous dysplasia of bone and McCune-Albright syndrome: A bench to bedside review. *Calcif Tissue Int*, 104, 517-529, 2019. DOI: 10.1007/s00223-019-00550-z

4. Riminucci M, Liu B, Corsi A, Shenker A, Spiegel AM, Robey PG, Bianco P: The histopathology of fibrous dysplasia of bone in patients with activating mutations of the Gsα gene: Site-specific patterns and recurrent histological hallmarks. *J Pathol*, 187, 249-258, 1999. DOI: 10.1002/(SICI)1096-9896(199901)187:2<249::AID-PATH222>3.0.CO;2-J

5. Dorfman HD: New knowledge of fibro-osseous lesions of bone. *Int J Surg Pathol,* 18 (Suppl. 3): 62S-65S, 2010. DOI: 10.1177/1066896910369924

6. Robinson C, Collins MT, Boyce AM: Fibrous dysplasia/McCune-Albright syndrome: Clinical and translational perspectives. *Curr Osteoporos Rep*, 14, 178-186, 2016. DOI: 10.1007/s11914-016-0317-0

7. Charpurlat RD, Orcel P: Fibrous dysplasia of bone and McCune-Albright syndrome. *Best Pract Res Clin Rheumatol,* 22 (1): 55-69, 2008. DOI: 10.1016/j.berh.2007.11.004

8. Wilson RB: Monostotic fibrous dysplasia in a dog. *Vet Pathol*, 26, 449-450, 1989. DOI: 10.1177/030098588902600513

9. Raval SH, Joshi DV, Patel BJ, Sutariya P, Patel JG, Parmar SN: Monostotic fibrous dysplasia in the nasal sinus of Marwari mare: A case report. *Indian J Vet Pathol*, 39 (4): 358-359, 2015. DOI: 10.5958/0973-970X.2015.00088.7

10. Soltero-Rivera M, Engiles JB, Reiter AM, Reetz J, Lewis JR, Sánchez MD: Benign and malignant proliferative fibro-osseous and osseous lesions of the oral cavity of dogs. *Vet Pathol*, 52 (5): 894-902, 2015. DOI: 10.1177/0300985815583096

11. Bauer C, Dunn BG, Brothman AR, Dick Jr EJ, Christensen C, Voges A, Moore CM: Polyostotic fibrous dysplasia in a cynomolgus macaque *(Macaca fascicularis)*. *Comp Med*, 62 (2):142-148, 2012.

12. Duncan JR, Lederer HA, Ramsey FK, Tyler DE: Fibrous dysplasia in a monkey. *Iowa State Univ Vet*, 25, 81-82, 1962.

13. Williams WM, Lombard LS, Firfer HS: Fibrous dysplasia in a monkey and a kudu. *J Am Vet Med Assoc*, 147, 1049-1052, 1965.

14. Slayter MV, Boosinger TR, Pool RR, Dammrich K, Misdorp W, Larsen S: Histological classification of bone and joint tumors of

domestic animals. **In,** World Health Organization. 2nd Series, Vol. 1, 9-11, Armed Forces Institute of Pathology, American Registry of Pathology, Washington DC, 1994.

15. Thompson KG, Dittmer KE: Tumors of bones. **In,** Meuten DJ (Ed): Tumors in Domestic Animals. 5th ed., 356-424, John Wiley&Sons, Inc., 2017.

16. Mahadevappa A, Patel S, Ravishankar S, Manjunath GV: Monostotic fibrous dysplasia of the rib: A case report. *Case Rep Orthop Res,* 1-5, 2012. DOI: 10.1155/2012/690914

17. Traibi A, El Oueriachi F, El Hammoumi M, Al Bouzidi A, Kabiri EH: Monostotic fibrous dysplasia of the ribs. *Interact Cardiovasc Thorac Surg,* 14, 41-43, 2012. DOI: 10.1093/icvts/ivr048

18. Maki M, Saitoh K, Horiuchi H, Morohoshi T, Fukayama M, Machinami R: Comparative study of fibrous dysplasia and osteofibrous dysplasia: Histopathological, immunohistochemical, argyrophilic nucleolar organizer region and DNA ploid analysis. *Pathol Int,* 51, 603-611, 2001. DOI: 10.1046/j.1440-1827.2001.01252.x

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Case Report

Ocular Transmissible Venereal Tumor in Two Dogs: Clinical and Cyto-histopathological Evaluation

F. Eser ÖZGENCİL [1,a] Fikret DİRİLENOĞLU [2,b] Deniz SEYREK İNTAŞ [1,c] A. Perran GÖKÇE [1,d]
Gül ÇIRAY AKBAŞ [1,e] Mehmet PİLLİ [1,f] Çağrı GÜLTEKİN [1,g] Mehmet Alper ÇETİNKAYA [3,h] Gamze MOCAN [2,i]

[1] Department of Surgery, Faculty of Veterinary Medicine, Near East University, 99138, Nicosia - CYPRUS
[2] Department of Pathology, Faculty of Medicine, Near East University, 99138, Nicosia - CYPRUS
[3] Hacettepe University, Laboratory Animals Application and Research Center, TR-06230 Sıhhiye/Ankara - TURKEY

ORCIDS: [a] 0000-0002-9893-0462; [b] 0000-0003-2021-6186; [c] 0000-0002-4872-6658; [d] 0000-0002-3033-7259; [e] 0000-0002-9893-0462
[f] 0000- 0003- 3036-4350; [g] 0000-0001-8586-1472; [h] 0000-0001-5097-6368; [i] 0000-0002-7625-4934

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Abstract

Transmissible venereal tumor (TVT) is a transmissible cancer that typically affects the external genital organs in canines. It can spread to other parts of the body via auto- and hetero-transplantation in a way that neoplastic cells are inoculated into the degraded mucosa or skin during the interaction of the animals, such as licking and sniffing, or less commonly via hematogenous or lymphatic routes. Here, we presented the clinical, cytological, and histopathological characteristics of two rare cases of TVT that developed ocular involvement and were associated with a poor prognosis. Fine needle aspiration biopsy was the initial method of choice in the diagnosis which is rapid, minimally invasive, and effective. It was diagnostic in one case and aided in the diagnosis in the other. The latter was considered a malignant round cell tumor in the cytological examination that needed further clinical and histopathological examination for definitive diagnosis. TVT is a round cell tumor that should be considered in the differential diagnosis of ocular lesions in canines.

Keywords: *Transmissible venereal tumor, TVT, Eye, Dog*

İki Köpekte Oküler Bulaşıcı Veneral Tümör: Klinik ve Sito-histopatolojik Değerlendirme

Öz

Bulaşıcı veneral tümör (BVT), köpeklerde tipik olarak dış genital organları etkileyen bulaşıcı bir kanser türüdür. Vücudun diğer kısımlarına oto- ve heterotransplantasyon ile yayılabilir. Bu yayılım hayvanların etkileşimi sırasında, yalama ve koklama gibi, hasarlı mukozaya veya deriye ekilen neoplastik hücreler ile ya da daha az rastlanan hematojen veya lenfatik yollarla olabilir. Bu yazımızda, oküler tutulum geliştiren ve kötü prognoz gösteren iki nadir BVT olgusunun klinik, sitolojik ve histopatolojik özelliklerini sunduk. İnce iğne aspirasyon biyopsisi hızlı, minimal invaziv ve efektif olmasıyla tanıda ilk tercih edilen yöntemdi. Bu yöntem, bir olgumuzda tanısal iken, diğer olguda tanıya yardımcı oldu. İkinci olgunun sitolojik incelemesinde malign yuvarlak hücreli tümör düşünüldü ve daha ileri klinik değerlendirme ve histopatolojik inceleme ile kesin tanıya gidildi. BVT, köpeklerde oküler lezyonların ayırıcı tanısında akılda tutulması gereken yuvarlak hücreli bir tümördür.

Anahtar sözcükler: *Bulaşıcı veneral tümör, TVT, Göz, Köpek*

INTRODUCTION

Transmissible venereal tumor (TVT) is a neoplasm that particularly involves the external genital organs in dogs. It is common among free-ranging dogs that suffer from malnutrition and live in groups in tropical and subtropical regions. The disease is transmitted to young and sexually mature dogs through coitus [1]. While spontaneous regression can be observed in some dogs infected with TVT, others may have a clinical picture that can lead to metastasis and even death [2]. When TVT cases are left untreated for a long time, metastases have been reported to occur usually to the lips and buccal and nasal mucosa; whereas metastases to the kidney, spleen, mesenteric lymph nodes, liver, pancreas, tonsils, lungs, brain, mediastinum, pituitary gland, skin, regional lymph nodes, and eyes were less

Correspondence
+90 392 6751000/3143
eserozgencil@yahoo.com

common [3-7]. We aimed to explain the clinical, cytological, and histopathological characteristics of TVT in two dogs located in the anogenital region, in addition to bilateral intraocular and palpebral conjunctiva involvement in one dog and multiple organ metastases including nictitating membrane and bilateral palpebral involvement in the other.

CASE HISTORY

Case 1: A 4-year-old non-castrated female pointer mix was admitted to the Near East University Animal Hospital with loss of vision and bilateral eye redness. Clinical examination showed bilateral chemosis, photophobia, epiphora, blepharospasm, and 180-degree corneal vascularization originating at the limbus. There were buphthalmos, corneal edema, and episcleral congestion due to bilaterally increased intraocular pressure. Physical examination revealed negative direct-indirect pupillary light reflexes and positive corneal reflex. In the direct ophthalmoscopic examination of the anterior chamber, neoplastic uveal masses were detected at 11-03 o'clock iridal quadrant in the right eye and at 08-12 o'clock iridal quadrant in the left eye in addition to a conjunctival mass in the left upper palpebra *(Fig. 1-a,b)*.

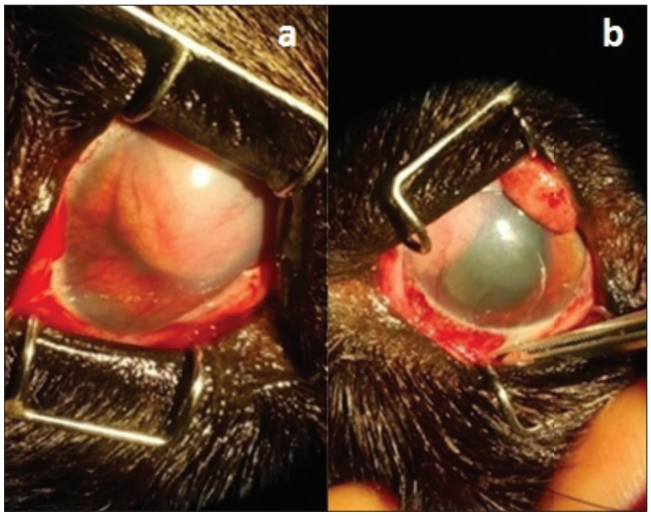

Fig 1. Appearance of uveal TVT in the right eye **(a)**; appearance of uveal, upper eyelid, and conjunctival TVT in the left eye of case 1 **(b)**

Abdominal and thoracic imaging results were normal; however, masses were detected in vaginal and rectal examinations. Ocular ultrasonography (USG) examination was performed with a transpalpebral approach using a 9 MHz microconvex probe under sedation, and sagittal, dorsal, and transversal sections were obtained. Ocular USG revealed a solid mass at the ciliary body level and retinal detachment in both eyes *(Fig. 2, Fig. 3)*. A complete blood count, biochemistry, and serology analyses were within the normal range.

Fine needle aspiration biopsy (FNAB) was performed in the anterior chamber and vitreous body by aspirating 0.1 mL material for cytological examination [8] *(Fig. 4)*. Touch and smear preparations were made from the aspirates, fixed in 96% ethanol and stained with Papanicolaou stain. Microscopically, the slides were sufficiently cellular and composed of monotonous neoplastic cells forming small, poorly cohesive groups or dispersed in an isolated fashion. The cells had round to oval nuclei and single prominent nucleoli. Although most of the tumor cells had large cytoplasm, occasional cells had an increased nuclear-cytoplasmic ratio. In some cells, intracytoplasmic brown pigment compatible with melanin was encountered. Several mitotic figures were identified. Cytomorphologic findings were consistent with a group of tumors known as "malignant round cell tumors," and melanoma was suspected primarily due to the tumor location and the presence of pigmented cells. At the time of cytological evaluation, a physical examination revealed two other masses in the anal and vaginal areas. With this clinical finding, TVT, another blue round cell tumor, was also considered at the top of the differential diagnosis list. The

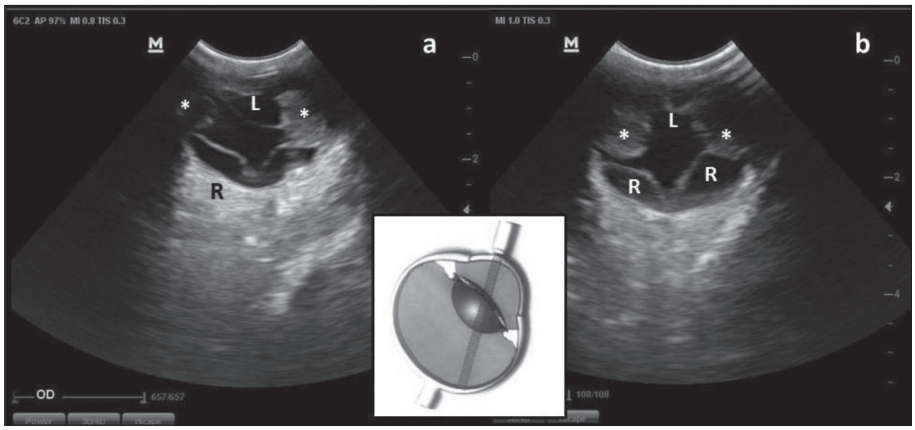

Fig 2. Homogenous and moderately echoic, solid masses (*) at the level of the ciliary body taken from right eye of case 1 and «flying seagull» appearance of the retinal (R) detachment **(a)**; Advanced retinal detachment (R) and echogenic masses (*) protruding from the caudal aspect of the ciliary body to the vitreous **(b)**; L: Lens

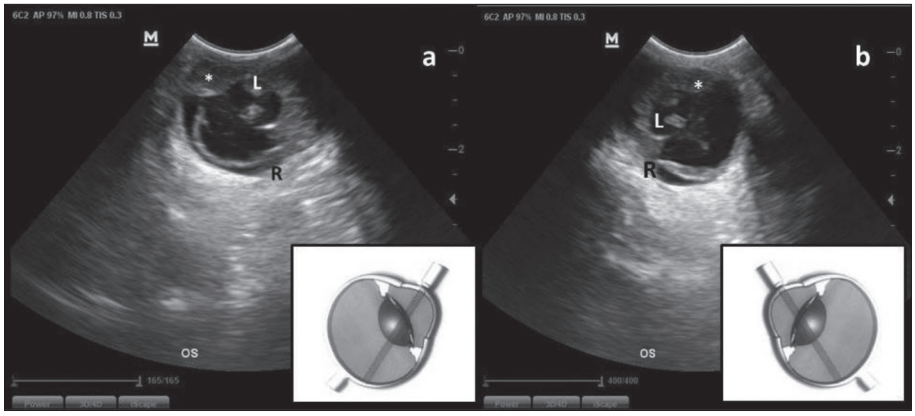

Fig 3. In the nearly sagittal sections of the left eye of case 1, a homogeneous, moderately echoic, solid structure (*) protruding towards the anterior chamber at the level of ciliary body was observed. The echogenic structures in the lens (L) were noted as two separate clusters. The retinal layer (R) appeared as a hyperechoic line that was thickened and separated from the ground **(a)**; The appearance of the left eye of case 1 from a different aspect, with an echogenic mass spreading into the anterior chamber (*). The mass may be originating from the iris or ciliary body. Hyperechoic structures within the lens (L) may indicate a cataract onset or swelling of the lens. Foci of irregular echogenic reflex and membranes are also seen in the corpus vitreum, which is expected to be anechoic. These are thought to be caused by degeneration or posterior separation of the vitreous. In addition, retinal detachment (R) and/or retinal retraction results in chorioretinal thickening **(b)**

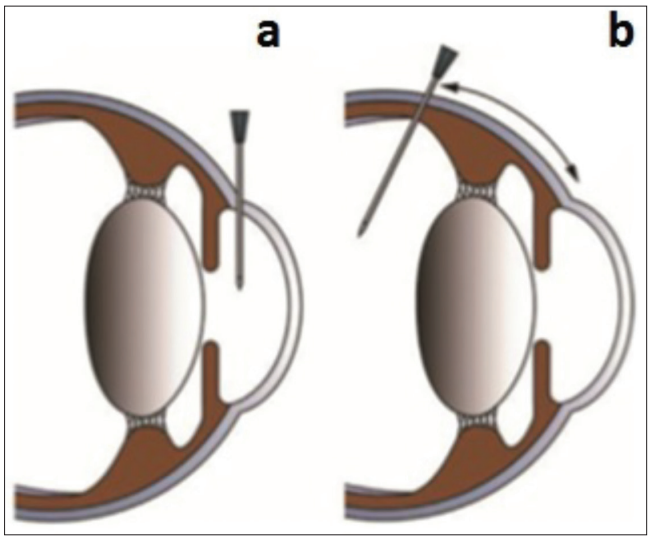

Fig 4. FNAB entrance points at the anterior chamber **(a)** and vitreus **(b)**

patient was considered to have malignant cytological findings consistent with a malignant round cell tumor *(Fig. 5-a)*.

Bilateral bulbus oculi extirpation was performed due to the rapid growth of intraocular masses and retinal detachment. Both eyes and two other anogenital masses were excised and immediately fixed in 10% neutral buffered formaldehyde solution. Grossly, the cut surface of the tumors was gray-white, solid, and nodular. In the left eye, the tumor was located predominantly in the anterior chamber, whereas the right eye was entirely involved and penetrated by the tumor *(Fig. 5-b)*. The sampled tissues were processed using a fully automated tissue processing machine, then embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin and eosin. Histological examination revealed solid or trabecular sheets of large and monotonous round cells with a single prominent nucleolus. The cells had a moderate amount of eosinophilic to clear cytoplasm. Some cells contained clear cytoplasmic vacuoles. There were an arborizing fibrovascular network and scant stroma among the tumor cells. Melanin pigment was found to be dispersed from the iris *(Fig. 5-c)*. The anal and vaginal tumors looked blander with very few mitoses and without necrosis, while the tumors located in the eyes showed more pronounced mitotic activity and foci of necrosis in the areas corresponding to fibrovascular septa *(Fig. 5-d)*. Clinical, cytological, and histopathological findings were consistent with a diagnosis of TVT. Vincristine was recommended; however, the patient died within 1 month after refusing treatment.

Case 2: A 5-year-old male dog was admitted to the Near East University Animal Hospital with a tumor located in bilateral palpebra and subcutaneous spread of the tumor in the whole body. Examination of the eyes revealed no intraocular problems; however, there was bilateral conjunctival hyperemia, chemosis, masses with varying sizes on the edges of lower and upper eyelids, hyperplasia and a subsequent prolapse in the left nictitating membrane *(Fig. 6-a, Fig. 7-a)*. As no intraocular lesion was detected in this case, FNAB was not performed. In the clinical examination, subcutaneous masses with varying sizes were detected in the head, neck, abdomen, extremities, and penis. Abdominal and thoracic radiographs and USG examination showed involvement of the liver and spleen as well as multilobular heterogenous masses in the vicinity of paraaortic, iliac, and inguinal lymph nodes. There was a prominent thickening of the submucosal layer

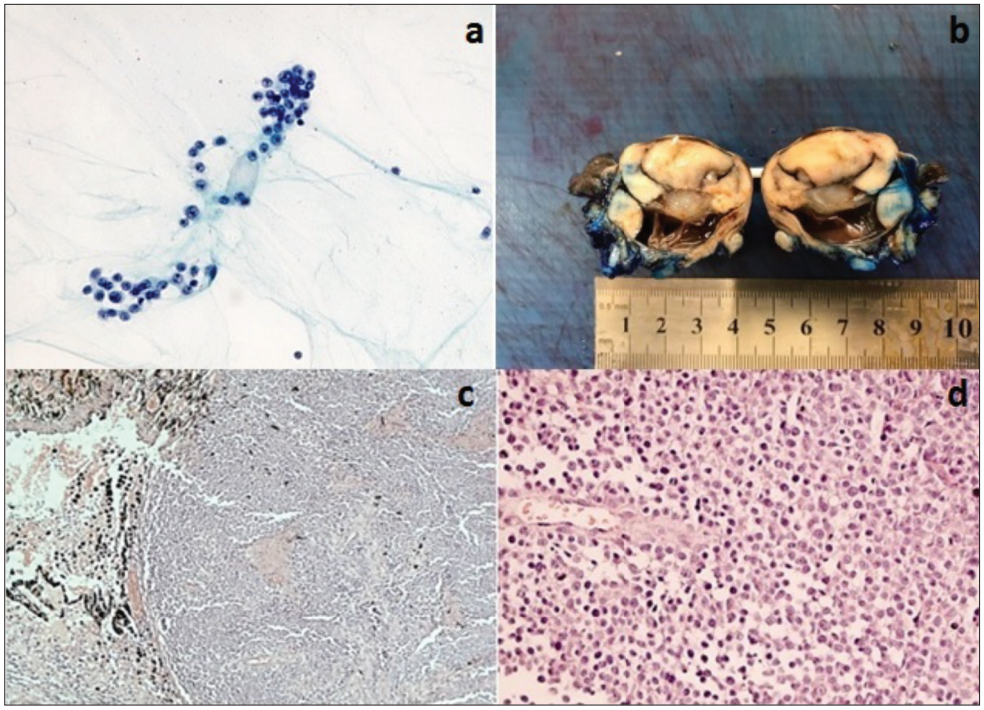

Fig 5. Cytologically, isolated or small groups of tumor cells with round nuclei, prominent nucleoli, and moderate cytoplasm are present **(a)**; Gray-white nodular tumor with a solid appearance. Extensive intraocular involvement is seen **(b)**; Histopathological examination of the excised eyes showed a round cell tumor in a sheet-like pattern. There are fibrovascular cores among tumor cells with foci of necrosis. Melanin pigment which was confusing in the cytologic examination found to be originating from the melanocytes in the iris **(c)**; Monotonous round tumor cells and rare mitoses in the histopathological examination of the masses excised from the anal and vaginal regions **(d)**

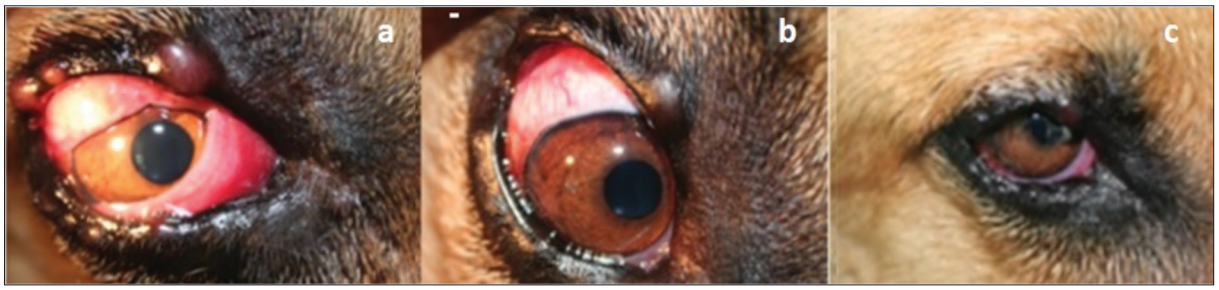

Fig 6. Significant tumor masses of TVT on the eyelid margin in the right eye of case 2 **(a)**; Shrinkage of the masses following the first **(b)** and second vincristin application **(c)**

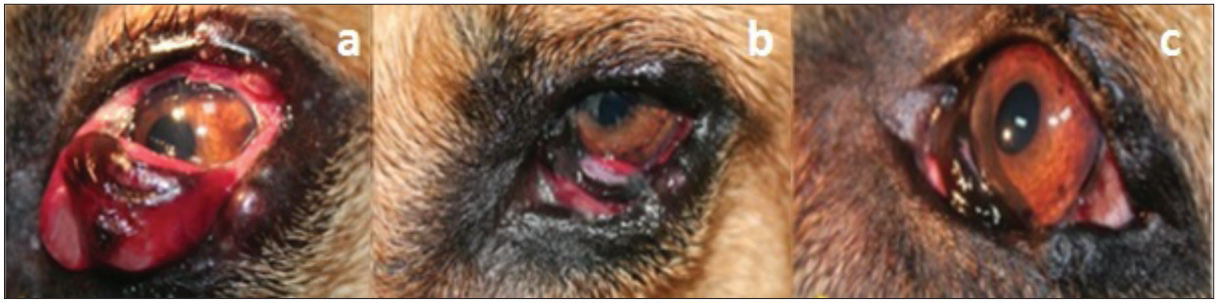

Fig 7. In the left eye of case 2, TVT-induced third eyelid hyperplasia and associated prolapse **(a)**; Shrinkage of the masses following the first **(b)** and second vincristin application **(c)**

of the stomach and small intestine. Ocular USG revealed irregularity and thickening in both bulbus oculi at the lateral ciliary body level. Fine needle aspiration biopsy was performed on one of the skin lesions, and slides fixed in 96% alcohol were stained with PAP, whereas air-dried slides were stained with Giemsa. Cytological evaluation revealed mostly isolated or sporadic groups of neoplastic cells with round nuclei and prominent nucleoli that exhibited slight pleomorphism. The cells had a moderate amount of eosinophilic cytoplasm and contained multiple small and clear vacuoles. There were occasional mitotic figures. Cytomorphologic findings were consistent with TVT. Vincristine (Vincristine 2 mg vial, Koçak Farma) 0.075 mg/m^2 was administered intravenously once a week for a total of 10 times and doxorubicin hydrochloride (Adriamycin 50 mg vial, Saba İlaç San.) 25 mg/m^2 iv was administered every

3 weeks for a total of three times. The masses were found to gradually shrink after the first and second administration *(Fig. 6-b,c, Fig. 7-b,c)*. Approximately three months after the start of treatment, the patient died following bulbus oculi extirpation due to intraocular metastasis of TVT, despite the treatment administered to treat severe uveitis that developed in both eyes and continued vincristine administration.

DISCUSSION

Although it has been reported that TVT primarily involves the genital region, it could also involve other parts of the body without genital findings when animals lick and sniff the genital areas of infected animals [5,7,9]. Metastases are caused by spread from a genital tumor primarily via autotransplantation or heterotransplantation due to neoplastic cells inoculated into the degraded mucosa or skin through licking, sniffing, itching, and biting, or less commonly via hematogenous or lymphatic routes [10,11]. Some researchers highlighted that TVT should also be included in the differential diagnosis if there are extragenital, especially ocular masses in dogs living in geographical regions with a high TVT prevalence to avoid such incidents [12,13]. Metastatic ocular TVTs involving the cornea and sclera and primarily ocular TVTs localized in the nictitating membrane and bulbar conjunctival tissue have been reported [8,10,14,15]. Although to a lesser extent, metastatic lesions resulting in widespread intraocular damage have also been reported [10,16,17]. TVT could be confused with various eye diseases such as severe uveitis and glaucoma when TVT is solely present in extragenital regions like the eyes in the absence of a simultaneous genital tumor [14,15,18]. In cases with intraocular neoplasms, USG examination is important for showing the involvement of the ciliary body and posterior structures by anterior uveal tumors. Bulbus oculi resection performed before the tumor spreads to the orbit in blind and painful eyes has been reported to be therapeutic [19,20]. In both cases, lesions were not noticed until palpebral and intraocular metastases occurred, which led to delayed admission and, in turn, delayed diagnosis and treatment. Intraocular USG; benefited in the decision of the extirpation of blind and painful eyes. It has been reported to cause tumor regression with TVT cell lysates by inducing canine dentritic cells [21,22]. In cases with conjunctival and palpebral TVT it has been stated that surgical excision and vincristine application may be curative [14]. Intratumoral therapy of vincristine and IL2 has also been reported to have impressive therapeutic effects on TVT [23,24]. Although chemotherapy is thought to be effective in preventing spread, it is believed that it does not benefit tumors with intraocular localization. In recent studies additionally to histological and cytological analyzes, LINE-c-myc PCR test is envisaged for the diagnosis of extragenital TVT [25]. The diagnosis of TVT relies on the clinical, cytological, and/or histopathological findings [1]. Grossly, TVTs in the genital area have a mean diameter of 4.0-7.5 cm, they bleed easily; have an irregular, cauliflower-like, pedunculated shape; and are friable. Extragenital lesions have an irregular nodular or multilobular appearance with a solid cut surface and whitish color [2]. Histopathologically, TVT is composed of monotonous tumor cells, which are poor in stroma and have sheet-like or trabecular alignment in the arborizing fibrovascular network. The tumor cells are sometimes accompanied by lymphocytes, plasma cells, and macrophages [1,6,14]. Cytomorphologically, TVT cells are typically uniform, large and round or slightly polyhedral. They have round to oval, centrally located nuclei, high nuclear-cytoplasmic ratio, and generally have one prominent basophilic nucleolus. The cytoplasm is generally large and typically has a varying number of clear vacuoles. Fine needle aspiration biopsy should be the primary choice in suspected TVT cases, as it is a minimally invasive, inexpensive, and fast technique that provides diagnostic information [11]. In cases where a definitive diagnosis cannot be made with cytology, diagnosis can be made through examining the tissue biopsy specimen with histopathologic and immunohistochemical stains. TVT must be distinguished from other round cell tumors, i.e., histiocytoma, lymphoma, mast cell tumors, poorly differentiated carcinoma, and melanoma [10,11,14]. In a study, an algorithm was used for the differential diagnosis of TVT. It was reported that the presence of cytoplasmic granules in the presence of discrete round cells indicated a mast cell tumor, whereas the absence of cytoplasmic granules along with the presence of vacuolization indicated TVT. In the absence of cytoplasmic granules in neoplastic cells, the presence of bean-shaped nuclei with indented appearance indicates histiocytoma, whereas the presence of giant cells indicates histiocytic sarcoma. Since there was no pronounced ocular mass in Case 2, FNAB samples were taken from one of the skin lesions and the typical cytomorphological findings were diagnostic. Whereas, in Case 1, a diagnosis of malignant round cell tumor could be made based on the cytological features of the tumor obtained via aqueocentesis and hyalocentesis. In this case, a straightforward diagnosis of TVT could not be made for three reasons: i) atypical location of the tumor, ii) lack of evident cytoplasmic vacuolization in tumor cells, which was previously reported as a diagnostic difficulty in cytology samples, and iii) the detection of pigments consistent with melanin in some cells led to a melanoma suspicion [10]. A subsequent physical examination and imaging studies revealed tumors in the anogenital region. In the histopathological examination of both intraocular and anogenital tumors, melanin pigment was found to be dispersed from the iris instead of being produced by tumor cells, and the tumor demonstrated typical features of TVT consisting of monotonous tumor cells with some of them having vacuolated large eosinophilic or clear cytoplasm, single prominent nucleoli in a background of thin fibrovascular network. The diagnosis of TVT was made based on the combination of clinical, cytological, and histopathological findings. In dogs, TVT can spread

to other parts of the body aside from the genital organs via auto- and hetero-transplantation. In patients with ocular involvement, the owners generally present to a clinic due to the presence of an externally visible tumor in the bulbus oculi and surrounding tissues or loss of vision. In the presence of intraocular masses larger than 2 mm, cytological examination of FNAB samples collected from the anterior and vitreous chambers has been reported to be practical and beneficial in the diagnosis. On the other hand, histopathological examination is recommended in cases when it is not possible to sample by FNAB or make a definitive diagnosis on cytological examination. In the presence of intraocular tumors, USG is useful in determining whether the eye is functional and deciding whether to perform a bulbus oculi extirpation. It was observed that chemotherapy provided a temporary improvement but failed to provide a cure in cases with metastases to the eye and the surrounding tissues.

REFERENCES

1. Mello MI, Gobello C, Ferreira F: The canine transmissible venereal tumor: Etiology, pathology, diagnosis, and treatment. **In,** Concannon P, England G, Verstegen I (Eds): Recent Advances in Small Animal Reproduction. International Veterinary Information Service, 2005.

2. Gülbahar M, Hazıroğlu R: Bir köpekte ekstragenital metastazlı transmissible venereal tümör olgusu. *Ankara Üniv Vet Fak Derg*, 42, 441–444, 1995.

3. Kabuusu RM, Stroup DF, Fernandez C: Risk factors and characteristics of canine transmissible venereal tumours in Grenada, West Indies. *Vet Comp Oncol*, 8 (1): 50-55, 2010. DOI: 10.1111/j.1476-5829.2009.00204.x

4. Park MS, Kim Y, Kang MS, Oh SY, Cho DY, Shin NS, Kim DY: Disseminated transmissible venereal tumor in a dog. *J Vet Diagn Invest,* 18 (1): 130-133, 2006. DOI: 10.1177/104063870601800123

5. Mozos E, Méndez A, Gómez-Villamandos JC, De Las Mulas JM, Pérez J: Immunohistochemical characterization of canine transmissible venereal tumor. *Vet Pathol,* 33 (3): 257-263, 1996.

6. Miller W, Albert R, Boosinger T: Ocular metastasis of a transmissible venereal tumour. *Canine Pract,* 15 (3): 19-21, 1990.

7. Featherstone H, Heinrich C, Donaldson D, Hartley C, Dietrich U, Ekesten B: Eye examination and diagnostics. **In,** Gelatt K (Ed): Essentials of Veterinary Ophthalmology. 103-145, Blackwell published, Ames, Iowa, USA, 2014.

8. Milo J, Snead E: A case of ocular canine transmissible venereal tumor. *Can Vet J*, 55 (1): 1245-1249, 2014.

9. Ferreira AJA, Jaggy A, Varejão AP, Ferreira MLP, Correia JMJ, Mullas JM, Almeida O, Oliveira P, Prada J: Brain and ocular metastases from a transmissible venereal tumour in a dog. *J Small Anim Pract*, 41 (4): 165-168, 2000. DOI: 10.1111/j.1748-5827.2000.tb03187.x

10. Das U, Das AK: Review of canine transmissible venereal sarcoma. *Vet Res Commun*, 24, 545-556, 2000. DOI: 10.1023/a:1006491918910

11. Amstutz H, Archibald J, Armour J, Blood D, Newberne, PM Snoeyenbos G: Diseases of the reproductive system transmissible canine venereal tumor. **In,** Fraser C, Mays A (Eds): The Merck Veterinary Manual. 662-663, Rahway, Merck & Co. Inc, New Jersey, USA, 1986.

12. Chikweto A, Kumthekar S, Larkin H, Deallie C, Tiwari KP, Sharma RN, Bhaiyat MI: Genital and extragenital canine transmissible venereal tumor in dogs in Grenada, West Indies. *Open J Vet Med,* 3 (2): 111-114, 2013. DOI: 10.4236/ojvm.2013.32018

13. Pereira JS, Silva ABF, Martins ALB, Ferreira AMR, Brooks DE: Immunohistochemical characterization of intraocular metastasis of a canine transmissible venereal tumor. *Vet Ophthalmol*, 3 (1): 43-47, 2000. DOI: 10.1046/j.1463-5224.2000.00097.x

14. Komnenou AT, Thomas ALN, Kyriazis AP, Poutahidis T, Papazoglou LG: Ocular manifestations of canine transmissible venereal tumour: A retrospective study of 25 cases in Greece. *Vet Rec,* 176 (20): 523, 2015. DOI: 10.1136/vr.102968

15. Rodrigues GN, Alessi AC, Laus JL: Intraocular transmissible venereal tumor in a dog. *Ciênc Rural,* 31 (1): 141-143, 2001. DOI**:** 10.1590/S0103-84782001000100023

16. Barron CN, Saunders LZ, Seibold HR, Heath MK: Intraocular tumors in animals. V. Transmissible venereal tumor of dogs. *Am J Vet Res*, 24, 1263-1270, 1963.

17. Pennick D, D'Anjou MA: Atlas of Small Animal Ultrasonography. Wiley and Blackwell, USA, 2008.

18. Veloso JF, de Andrade Oliveira TN, Andrade LP, Silva FL, Sampaio KMOR, Michel AFRM, de Lavor MSL, Carlos RSA: Three cases of exclusively extragenital canine transmissible venereal tumor (cTVT). *Acta Sci Vet*, 46 (Suppl. 1): 295, 2018. DOI: 10.22456/1679-9216.86846

19. Crossley R, Ramírez JA: Tumor venéreo transmisible canino de presentación atípica. *Rev Med Vet Zoot*, 64 (3): 78-90, 2017. DOI: 10.15446/rfmvz.v64n3.68695

20. Thangathurai R, Balasubramaniam GA, Dharmaceelan S, Balachandran P, Srinivasan P, Sivaseelan S, Manohar BM: Cytological diagnosis and its histological correlation in canine transmissible venereal tumour. *Vet Arhiv,* 78 (5): 369-376, 2008.

21. Hernández-Granados AJ, Franco-Molina MA, Coronado-Cerda EE, Zapata-Benavides P, Gamboa EM, Ramos-Zayas Y, Santana-Krímskaya SE, Rodríguez-Padilla C: Immunogenic potential of three transmissible venereal tumor cell lysates to prime canine-dendritic cells for cancer immunotherapy. *Res Vet Sci*, 121, 23-30, 2018. DOI: 10.1016/j.rvsc.2018.10.001

22. Vural SA, Haziroglu R, Vural MR, Polat IM, Tunc AS: Detection of progressive and regressive phase and LINE-1 retrotransposon in transfected dogs with transmissible venereal tumor during chemotherapy. *J Vet Sci*, 19 (5): 620-626, 2018.

23. Den Otter W, Hack M, Jacobs JJL, Tan JFV, Rozendaal L, Van Moorselaar RJA: Effective treatment of transmissible venereal tumors in dogs with Vincristine and IL2. *Anticancer Res,* 35, 3385-3392, 2015.

24. Den Otter W, Hack M, Jacobs JJL, Tan JFV, Rozendaal L, Van Moorselaar RJA: Treatment of transmissible venereal tumors in dogs with intratumoral interleukin-2 (IL-2). A pilot study. *Anticancer Res*, 35, 713-718, 2015.

25. Setthawongsin C, Techangamsuwan S, Tangkawattana S, Rungsipipat A: Cell-based polymerase chain reaction for canine transmissible venereal tumor (CTVT) diagnosis. *J Vet Med Sci*, 78 (7): 1167-1173, 2016. DOI: 10.1292/jvms.15-0710

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Case Report

Successful Treatment of Pyometra Concomitant with Diabetes Mellitus in a Bitch

Hande GÜRLER [1,a]

[1] Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, TR-55139 Samsun - TURKEY

[a] ORCID: 0000-0001-7239-5388

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Abstract

Pyometra, a diestrus period disorder with a long term of progesterone exposure to endometrium, is a common disease of nulliparous female dogs. Closed-cervix pyometra is an emergency medical condition that requires rapid intervention. The possibility of treatment is further limited when associated with a chronic disease such as diabetes. This case describes a closed cervix pyometra in a 4-year-old bitch with comorbid diabetes mellitus and long-term contraceptive therapy. Closed-cervix pyometra was diagnosed and confirmed by ultrasound examination typical of pyometra, vaginal smear indicating diestrus and serum progesterone level (52 ng/mL). Serum fructosamine level was 475 µmol/L, interpreting diabetes mellitus. Treatment included ovariohysterectomy and normoglycemia was achieved with glargine insulin. Although, reproductive hormones and diestrus are associated with Type II diabetes mellitus in bitches, there are no reports limited to our search, on pyometra concomitant with diabetes mellitus except Elkhounds. Since the situation has a vital clinical importance, this report aims to emphasize the insidious diabetes mellitus development potential and treatment in canine pyometra which is usually misdiagnosed or unconsidered in clinical practice.

Keywords: *Dog, Diabetes mellitus, Pyometra*

Dişi Bir Köpekte Piyometra ve Eşlik Eden Diyabetes Mellitus Olgusunun Başarılı Tedavisi

Öz

Piyometra hiç doğum yapmamış dişi köpeklerde sıklıkla görülen, endometriumun uzun süreli progesterona maruz kalması sonucunda şekillenen bir dioestrus dönemi hastalığıdır. Kapalı serviks piyometra ise prognozu zayıf, mortalite oranı yüksek hızlı müdahale gerektiren acil bir tıbbi durumdur. Diyabet gibi kronik bir hastalık ile ilişkili olduğunda tedavi olasılığı daha da sınırlıdır. Progesteron kaynaklı insülin direncinden dolayı köpeklerde diabetes mellitusun eşlik ettiği piyometra vakalarına rastlanması olası bir durumdur. Bu olgu, uzun süre kontraseptif uygulanmış 4 yaşlı bir köpekte diyabetes mellitus eşlik eden kapalı serviks piyometrayı tanımlamaktadır. Tanı, kapalı serviks piyometra için tipik ultrason görüntüsü, diöstrusu işaret eden vajinal smear bulgusu ve yüksek (52 ng/mL) serum progesteron seviyesi ile doğrulandı. Eşlik eden diabetes mellitus durumu, yüksek serum fruktozamin seviyesi (475 µmol/L) ile teşhis edildi. Tedavi amacıyla ovaryohisterektomi operasyonu ve normoglisemi sağlamak için glarjin insulin uygulaması gerçekleştirildi. Bu rapor, klinik pratikte köpeklerde piyometra olgularında genellikle eksik tanıya neden olan ve dikkate alınmayan, sinsi diyabetes mellitus gelişim potansiyelini vurgulamayı amaçlamaktadır.

Anahtar sözcükler: *Köpek, Diyabetes mellitus, Piyometra*

INTRODUCTION

Pyometra is the most common life-threatening uterine disorder of intact female dogs. Unlike most other female mammals, the female dog secretes ovarian progesterone for approximately 55-75 days after ovulation. It occurs due to prolonged exposure of the endometrium to progesterone during diestrus. Pyometra is classified as closed cervix pyometra in which purulent substance accumulates in the uterus and open cervix pyometra in with presence of vulvar discharge [1-5]. Vaginal discharge, inflammation, abdominal distension in the genital tract

Correspondence
+90 362 3121919/1234
handeulusoy@gmail.com

together with systemic disease are observed in dogs with pyometra [2]. Sepsis is a result of an exaggerated systemic inflammatory response (SIRS)and may commonly be a result of canine pyometra [3,4]. Endotoxemia and SIRS are life threatening probable results of pyometra [2]. The safest and most effective treatment of closed cervix pyometra is ovariohysterectomy (OHE) [5]. After surgical removal of the uterus, some complications may occur such as sepsis, septic shock, diffuse bacterial infection, peritonitis and bleeding. Diabetic patients who need any major surgical procedures require careful medical management [6,7].

In cases of closed cervix pyometra with serious metabolic load and risk of complications, treatment process is very difficult to manage. It is known that closed cervix pyometra more dangerous than open cervix pyometra [8]. One major complication of pyometra is development of canine diabetes mellitus (CDM), due to the insulin resistance creating effect of long-term progesterone exposure. In addition, the excessive uterine inflammatory actions resulting with inflammatory substances inducing the release of catecholamines and hormones, such as growth hormone, cortisol, glucagon causing hyperglycemia must be considered [9]. This report describes a closed cervix pyometra in a 4-year-old bitch with comorbid diabetes mellitus and long-term contraceptive therapy, and aims to emphasize the insidious diabetes mellitus development potential in canine pyometra that is usually misdiagnosed or unconsidered in clinical practice.

CASE HISTORY

A 4-year-old intact female Cocker Spaniel dog was admitted to the Animal Hospital of Ondokuz Mayıs University Faculty of Veterinary Medicine. The dog was kept indoor, with complete vaccination and deworming program and had no previous serious medical situation. On arrival at the hospital, the dog was clinically depressive. According to the history, the estrus cycle was postponed with synthetic progestogen medroxyprogesterone acetate (MPA) three times at an epicenter clinic.

Clinical presentation included polydipsia, polyurea, lethargy, abdominal distention and dehydration, sinus tachycardia, tachypnea and body temperature of 39.3°C. Whole blood count was performed with Mindray BC5000 Vet® (Mindray, China), indicating severe leukocytosis with WBC- 67×10^9 L^{-1} (reference interval 6-17 $\times$ 10^9 L^{-1}) and left shift. Serum biochemistry profile (total protein, albumin/globulin ratio, alanine aminotransferase, aspartate aminotransferase, gammaglutamyltransferase, urea, creatinine, glucose, lipase) was analyzed with Mindray BS 120® (Mindray, China) revealing severe hyperglycemia (520 mg/dL) and mild uremia (17 mmol L^{-1}). Pyometra was suspected despite the absence of vaginal discharge regarding the laboratory data. Closed cervix pyometra was diagnosed and confirmed by ultrasound (USG) examination (Esaote MyLab FiveVET® Esoate, Genoa). The vaginal smear and history indicated that the dog was in diestrus. Serum progesterone (P4) level was determined with ELISA, as 52 ng/mL. USG examinations of the abdominal viscera did not reveal any abnormal findings. Pancreas was also in normal clinical view, 6.5 mm thick with a pancreatic ductal diameter of 0.5 mm and normal echogenecity, as expected. Fructosamine level was analyzed by ELISA (Canine FTA ELISA Kit®, MyBiosource®, UK) in order to determine the etiology of hyperglycemia and demonstrated as 475 µmol/L (reference range: 200 to 375 µmol/L) indicating CDM and eliminating a pyometra-induced transient hyperglycemia.

The dog was hospitalized and treated presurgically with intravenous fluids and a subcutaneous injection of insulin glargine (Lantus Solostar®, Sanofi, China) 1 U/kg. Aggressive intravenous antibiotic therapy was started with metronidazole (Flagyl® i.v., Sanofi, Turkey) 10 mg/kg, BID and cefepime hydrochloride (Unisef® 1 mg IM/IV, Mustafa Nevzat, Turkey) 20 mg/kg BID, 14 days.

An OHE was performed the next day and the ovaries and uterus, which was severely distended with purulent material, was removed *(Fig. 1)*. Presence of corpus luteum on the right ovary was observed in post-operative ovary check. Normoglycemia was achieved during and after the operation, due to successful diabetes management. Regression of inflammatory process was achieved in 7 days with normal blood count, but normalizing blood glucose lasted longer with labile glucose curve and confusing

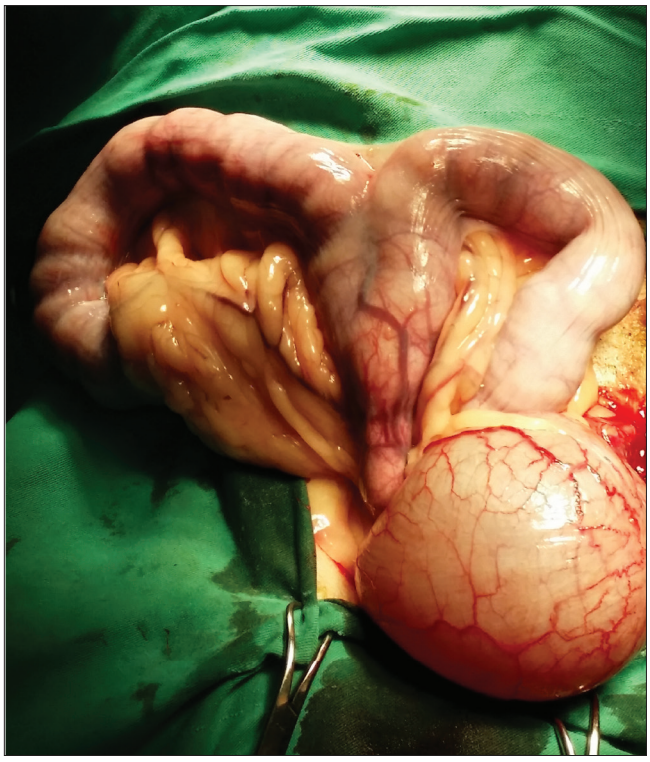

Fig 1. The image of uterus with closed cervix pyometra in OHE

resistance, but 16 U of glargine insulin (1 U/kg) at noon and total 10 U of glargine insulin at midnight, with the appropriate diet was determined as the ideal regimen for this individual patient. Blood urea level regressed following the operation. Follow up visits for 2 weeks demonstrated a fast-wound healing and recovery. Follow up for CDM after achieving the ideal regimen was carried out with monthly intervals and three months fructosamine measurements were back in reference levels within 6 months, indicating a successful CDM management.

DISCUSSION

It is reported that the incidence of pyometra is increased in nulliparous dogs older than 4 years [10]. The present bitch was a four years old nulliparous Cocker Spaniel.

Pyometra is defined as purulent fluid collection within the uterus with variable amounts of inflammatory cells in the uterine wall, that may or may not be preceded by cystic endometrial hyperplasia [11] associated with diestrus. Insulin resistance mediated by progesterone is well documented [1]. Fall et al.[12] described a diagnostic prevalence of 17% for pyometra with concomitant CDM during diestrus. The last report is concordant with our clinical observations and also the present case.

Arena et al.[9] reported 33% pyometra prevalence in dogs with CDM, although with limited subjects. Pöppl et al.[13] reported remission of CDM in 6 dogs, from 57 intact dogs with CDM after spaying. In the present case, CDM was not in a transient etiology, and insulin treatment continued in order to maintain normoglycemic status. As serum fructosamine concentration is not affected by acute increases in the blood glucose concentration, as occurs with stress- or excitement, measurement of a single serum fructosamine concentration demonstrates preceding 2-3 weeks status, high fructosamine concentration of the present dog shows a preceding CDM disorder [14], in which a regression would not be expected.

Moreover, increased risk of developing pyometra has been associated with hormone administration (estrogen and progestogen compounds) [2]. Progestagen contraceptives are predisposing for pyometra development which unfortunately is a frequent application in practice [6]. It is stated that dogs may have diabetes mellitus associated with progesterone intake [13,14]. It is thought that progesterone intake, as a contraceptive, may be effective in the formation of both pyometra and diabetes. In the present case, the etiology of CDM in a young dog is more likely to be due to this iatrogenic intervention of triple usage of progestogen to postpone of estrus.

Sepsis is a common condition in closed cervix pyometra presented with depression, leukocytosis, neutrophilia and monocytosis, with poor prognosis [8]. In the present case, a very fast wound healing process and clinical recovery, to a large extent due to an appropriate metabolic management, especially CDM, was achieved. Fructosamin level evaluation of the insulin-treated diabetic dog provides information on the status of glycemic control during the month prior to the evaluation and follow up checks in this case revealed the proper management of CDM [14,15]. Satisfactory metabolic equilibrium and tissue glucose feeding gives major contribution to healing processes.

Blood glucose levels under 300 mg/dL are generally neglected by the practitioner, but high fructosamine levels may be present in which urgent insulin therapy is indicated. Therefore, in similar cases of pyometra, fructosamin level analyze will be beneficial for diagnosing diabetes development due to high progesterone levels and must be included in conventional protocol in practice [16].

Although reproductive hormones and diestrus are associated with Type II diabetes mellitus in bitches, there are no reports limited to our search, on pyometra concomitant with diabetes mellitus except Elkhounds [12]. This case emphasizes the importance of blood glucose monitoring and if present, CDM management in the prognosis of canine pyometra, together with insidious diabetes mellitus development potential in canine pyometra, which is usually misdiagnosed or unconsidered in clinical practice.

REFERENCES

1. Batista PR, Blanco PG, Gobello C: Treatment of canine pyometra with the gonadotropin-releasing hormone antagonist acyline: A case series. *Top Companion Anim Med,* 30 (1): 25-27, 2015. DOI: 10.1053/j.tcam.2015.01.005

2. Hagman R: Clinical and molecular characteristics of pyometra in female dogs. *Reprod Domest Anim,* 47 (Suppl. 6): 323-325, 2012. DOI: 10.1111/rda.12031

3. Sant'Anna MC, Giordano LGP, Flaiban KKMC, Muller EE, Martins MIM: Prognostic markers of canine pyometra. *Arq Bras Med Vet Zootec,* 66 (6): 1711-1717, 2014. DOI: 10.1590/1678-6859

4. Rautela R, Katiyar R: Review on canine pyometra, oxidative stress and current trends in diagnostics. *Asian Pac J Reprod*, 8 (2): 45-55, 2019.

5. Jitpean S, Ström-Holst B, Emanuelson U, Höglund OV, Pettersson A, Alneryd-Bull C, Hagman R: Outcome of pyometra in female dogs and predictors of peritonitis and prolonged postoperative hospitalization in surgically treated cases. *BMC Vet Res,* 10:6, 2014. DOI: 10.1186/1746-6148-10-6

6. Wheaton LG, Johnson AL, Parker AJ, Kneller SK: Results and complications of surgical-treatment of pyometra- a review of 80 cases. *J Am Anim Hosp Assoc,* 25 (5): 563-568, 1989.

7. Schaer M: Surgery in the diabetic pet. *Vet Clin North Am Small Anim Pract,* 25 (3): 651-660, 1995. DOI: 10.1016/S0195-5616(95)50060-9

8. Jitpean S, Ambrosen A, Emanuelson U, Hagman R: Closed cervix is associated with more severe illness in dogs with pyometra. *BMC Vet Res,* 13:11, 2016. DOI: 10.1186/s12917-016-0924-0

9. Arena MN, Albino MVC, Botelho FA, Luchi RAS, Ponce FG, Severo JS: Evaluation of blood glucose in dogs with pyometra. **In,** *World Small Animal Association, World Congress*, Sao Paolo, Brazil, 21-24 July 2009.

10. Smith FO: Canine pyometra. *Theriogenology,* 66 (3): 610-612, 2006. DOI: 10.1016/j.theriogenology.2006.04.023

11. Feldman EC, Nelson RW: Canine and Feline Endocrinology and Reproduction. 3rd ed., 486-538, Saunders, Missouri, 2004.

12. Fall T, Hedhammar A, Wallberg A, Fall N, Ahlgren KM, Hamlin HH, Lindblad-Toh K, Andersson G, Kämpe O: Diabetes mellitus in elkhounds is associated with diestrus and pregnancy. *J Vet Intern Med,* 24 (6): 1322-1328, 2010. DOI: 10.1111/j.1939-1676.2010.0630.x

13. Pöppl AG, Mottin TS, González FHD: Diabetes mellitus remission after resolution of inflammatory and progesterone-related conditions in bitches. *Res Vet Sci,* 94, 471-473, 2013. DOI: 10.1016/j.rvsc.2012.10.008

14. Scott-Moncrieff JC: Feline hyperthyroidism. **In,** Canine and Feline Endocrinology. 4th ed., 136-196, Saunders Elsevier, St. Louis, Missouri, 2015.

15. Sloan JM, Path MRC, Oliver IM: Progestogen-induced diabetes in the dog. *Diabetes,* 24 (4): 337-344, 1975. DOI: 10.2337/diab.24.4.337

16. Fernoagă C, Codreanu MD, Cornilă M, Raluca Teodora NAE, Constantinescu R: Use of fructosamine in small animals with diabetes. *Scientific Works, Series C Vet Med*, 61 (2): 99-102, 2015.

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Letter to the Editor

A Case of Ectrodactyly and Micromelia with Flexural and Rotational Tarsal Deformity in a Simmental Calf

(Simental Bir Buzağıda Fleksural ve Rotasyonel Tarsal Deformite İle Birlikte Şekillenen Ektrodaktili ve Mikromeli Olgusu)

Uğur AYDIN [1,a] Uğur YILDIZ [1,b] Emin KARAKURT [2,c] Özgür AKSOY [1,d]

[1] Department of Surgery, Faculty of Veterinary Medicine, Kafkas University, TR-36100 Kars - TURKEY

[2] Department of Pathology, Faculty of Veterinary Medicine, Kafkas University, TR-36100 Kars - TURKEY

ORCIDS: [a] 0000-0001-5756-4841; [b] 0000-0002-4782-1012; [c] 0000-0003-2019-3690; [d] 0000-0002-4800-6079

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Dear Editor,

Congenital anomalies are common in cattle breeding and this problem causes great economic losses [1,2]. It has been determined that 26.6% of congenital anomalies occur in the musculoskeletal system in calves in Kars region, Turkey [2]. Micromelia is the small and imperfect development of one or more limbs [3,4]. Ectrodactyly is the congenital absence of all or part of one or more phalanges [1,3,4].

Many environmental factors and genetic disorders act alone or together in the formation of these malformations [2,3]. It is known that a dominant gene causes the formation of ectrodactyly in calves [3]. Also, nutritional disorders including some vitamin and mineral deficiencies, various teratogenic agents, stress factors, the use of bulls with genetic disorders, and not preferred artificial insemination in reproduction have important roles in etiology [2].

In congenital musculo-skelatal malformations, mostly dystocia occurs. Also, the calf has difficulty in moves and feeding. Even if existing disorders are treated and calves live, these animals have no reproductive value.

For the presented case, an informed consent form was taken from the owner.

A conformational disorder was observed in the back left leg of a 4-day, male, Simmental calf brought to the surgical outpatient clinic of Kafkas University Veterinary Faculty Animal Hospital. The tarsal joint was in the hyperflexion position in the cranial-dorsal direction, and it was found that the joint had no movement capacity during passive strains. It was determined that the entire part of the extremity at the distal of the tarsal joint was atrophic, and only the metatarsus-3 and its phalanges were shaped, and the metatarsus-4 and related fingers were absent, considering the rotational state of the limb. In addition, the limb section distal from the tarsal joint had a 180° rotational view. Due to this rotational structure, the solea part of the nail was located at the dorsal *(Fig. 1)*. Radiological examination revealed that metatarsus-3 did not have a proximal physical line and it was determined that tarsal bones showed an abnormal structure and localization. In addition, phalanx 1 and 2 were similarly fused to one bone appearance, fused together *(Fig. 2)*.

With the evaluations made, it was thought that keeping the relevant extremity under the abdomen in the flexion position without touching the ground would not cause much trouble in terms of sustaining the animal's life. The fact that the relevant limb was shaped in a rudimentary structure and rotationally created an unfavorable picture for operative intervention. In addition, operation was not required due to the known risks of amputation and not to cause extra economic losses.

Congenital anomalies are frequently encountered in calves, and many of them are considered unfavorable for operative treatment. Generally, it is known that the patient cannot stand and feed on its own in musculoskeletal anomalies. Although there are anatomical defects in the other extremity in our case, the fact that the patient can stand up and meet its needs until it can be cut is considered as an important criterion. The described case is considered as a congenital malformation whose multifactorial factors need to be determined, such as different, interesting and other patterns.

Correspondence
+90 474 2426807/5222
uguraydin076@hotmail.com

Fig 1. A, B: View of the patient from right and left side with the animal standing; The animal can easily stand and walk on three limbs; **C:** Excessive flexural deformity and appearance of torsioned joint *(arrow)*. The front face of the limb is at the back and the front of the back is at the distal part of the distal part of the tarsal joint (there is approximately 180° rotation); **D:** In the rotated limb, the solea part of the 3rd phalanx was located dorsally *(arrow)*

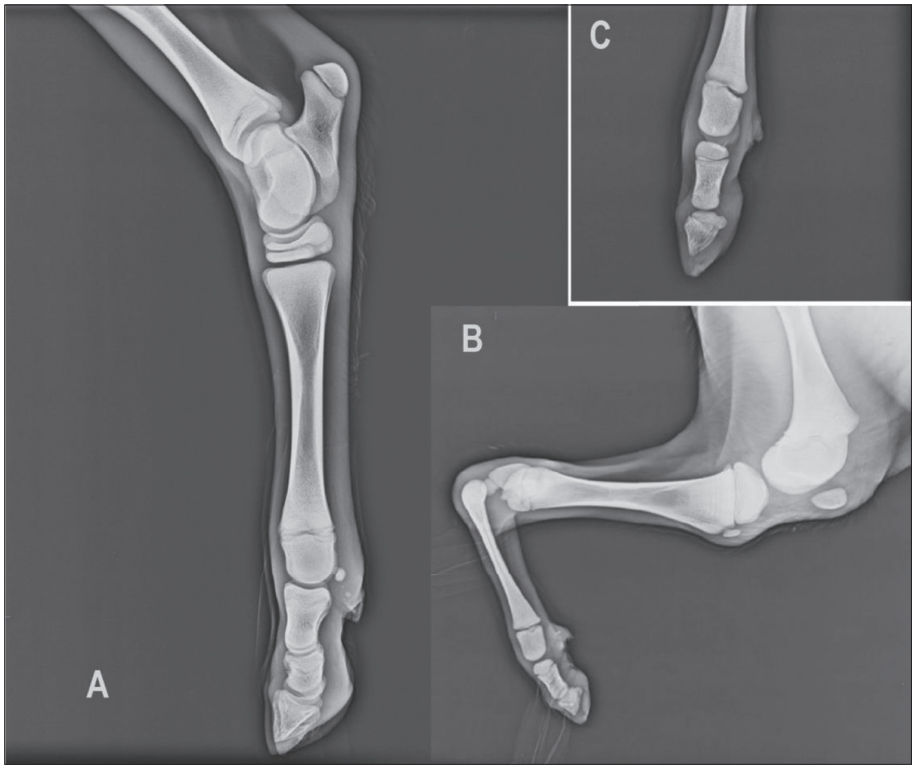

Fig 2. A: Normal appearance of tarsal joints, metatarsus and phalanges in intact limbs. It is noteworthy that the 2nd phalanx and proximal sesamoid bones are not normal in this extremity, even if the bone roof of the limb has a normal appearance; **B:** The appearance of the absence of proximal growth plate of metatarsus 3, with the abnormal localization of the tarsal bones in the limb with anomaly. Proximal sesamoid bones were not formed in the matatarsus 4 and its associated phalanges and anomaly leg; **C:** Phalanx 1 and 2 are formed together, but their borders are not clear. Phalanx 1 has a very small structure and seems to be fused with phalanx 2

REFERENCES

1. Leipold HW, Hiraga T, Dennis SM: Congenital defects of the bovine musculoskeletal system and joints. *Vet Clin North Am Food Anim Pract*, 9 (1): 93-104, 1993. DOI: 10.1016/S0749-0720(15)30674-5

2. Aksoy Ö, Kılıç E, Öztürk S, Özaydın İ, Kurt B, Baran V: Buzağı, kuzu ve oğlaklarda karşılaşılan doğmasal anomaliler: 1996-2005 (262 Olgu). *Kafkas Univ Vet Fak Derg*, 12 (2): 147-154, 2006.

3. Alkan F, Tuzcu M, Koç Y, Oğurtan Z: Bir buzağıda ectrodactyly ile birlikte şekillenmiş micromelia. *Vet Bil Derg*, 13 (2): 129-131, 1997.

4. Özenç E: Bir buzağının tüm ekstremitelerinde gözlemlenen peromelia olgusu. *Kocatepe Vet J*, 7 (2): 57-59, 2014.

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Letter to the Editor

A Rare Microfilaruria Case in a Dog Caused by *Dirofilaria immitis* [1]

(Bir Köpekte *Dirofilaria immitis*'in Neden Olduğu Nadir Bir Mikrofilarüri Olgusu)

Zeynep Nurselin COLAK [1,a] Emre KULLUK [1,b] Didem PEKMEZCI [1,c]

[1] *Presented in part in abstract form at the 2nd International Eurasian Conference on Biological and Chemical Sciences, Ankara, Turkey, 28-29 June 2019*

[1] Department of Internal Medicine, Faculty of Veterinary Medicine, University of Ondokuz Mayis, TR-55200 Samsun - TURKEY

ORCIDS: [a] 0000-0002-0631-5471; [b] 0000-0001-9128-355X; [c] 0000-0003-2072-8165

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Dear Editor,

Dirofilariasis is a vector borne disease "inexorable dreaded threadworm" accepted as the most important parasitic disease of canids caused by *Dirofilaria immitis* [1] with zoonotic potential and endemic in many parts of Europe, including Turkey [2-4]. The clinical symptoms and onset of the disease depend on of the adult worm burden, host-pathogen response, the duration of infection, and level of exercise [1]. Most of the cases have a history of weight loss, lethargy, diminished exercise tolerance, cough, dyspnea, syncope, poor condition, and abdominal distension [5]. Due to the right heart failure jugular venous distension and pulsation typically accompany hepato-splenomegaly, and ascites commonly seen in dogs with dirofilariasis [6]. However, to date presence of microfilariae in the urine has presented as a rare condition in two cases from dogs [7,8]. Thus, it is aimed to present this third case report of canine microfilaruria in the world within this lettering.

A male, 10-year-old, 20 kg, mix breed dog was brought to the Veterinary Teaching Hospital on November 2018 with the chief clinical signs of stranguria, hematuria, and constipation.

Besides the aforementioned symptoms the dog was in a good condition and any other abnormalities were seen in the physical examination. However, urinary bladder carcinoma was suspicioned because of the severe hematuria. Blood and urine samples were collected for routine analysis. The patient checked for hematological and biochemical analyzes. Same as thoracic and abdominal x-rays with echocardiography were performed. Laboratory findings both whole blood count and serum biochemical profile with revealed as normal. Only enlarged caudal lobar arteries with tortuous in appearance were seen in the lateral thoracic x-ray. The echocardiography of the dog assessed any right heart enlargement or abnormalities in the right ventricular end-diastolic dimension and septal or right ventricular free wall thickness.

On gross clinical examination, the urine was detected as brownish-red *(Fig. 1-a)*. During the microscopic examination live microfilariae were seen in the voided urine sediment *(Fig. 1-b)*. Later peripheral blood smears stained with Giemsa were examined for microfilaremia. Same as microfilariae were also seen in the stained blood smear *(Fig. 1-c)*. Finally, our diagnosis was serologically confirmed within the positive rapid heartworm antigen test *(Fig. 1-d)* and immediately a treatment protocol according to the "American Heartworm Society Guidelines for the Current Heartworm Infection Management in Dogs" was started to the patient [9]. The dog was being well after the second week of treatment and the urine sediment turned back to normal *(Fig. 1-e)*.

Contrary other cases of microfilaruria in dogs [7,8] our patient was not represented renal failure or lower urinary tract infection. Glomerulonephritis caused by antigen-antibody complex deposition in the kidneys is common in heartworm-infected dogs [1] and progression to renal failure, however, is uncommon [1,10]. This situation results in a measurable proteinuria (albuminuria), and heartworm antigen can be detected in the urine of infected dogs [1]. Therefore, microfilaruia could be expected as in the case herein due to this condition.

Correspondence
+90 362 3121919
dkazanci@omu.edu.tr

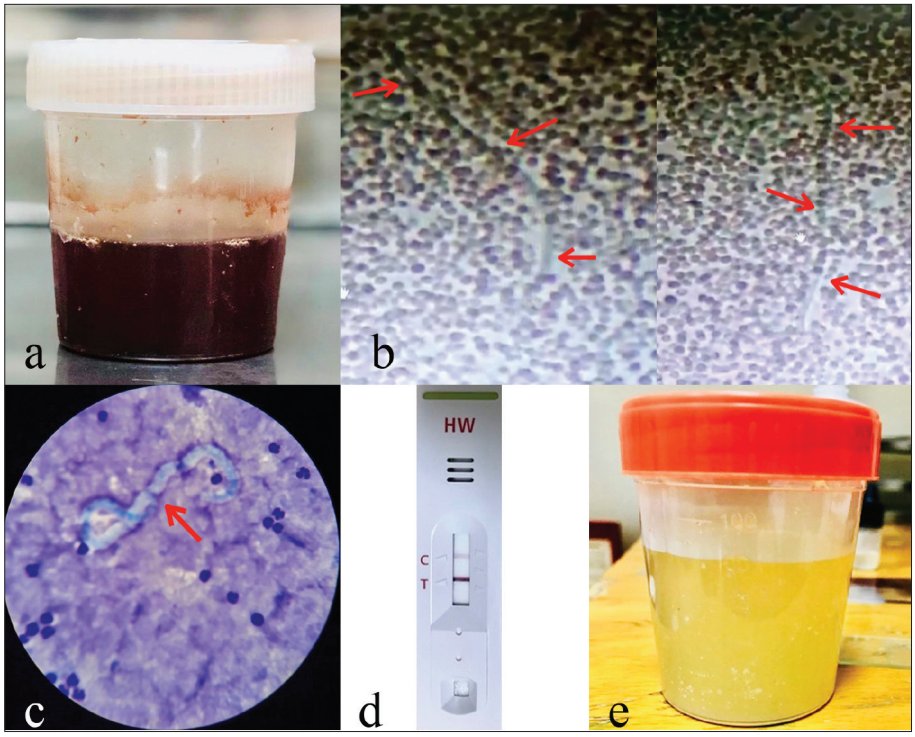

Fig 1. Severe hematuria of the patient (a), live microfilariae (red arrows) in the voided urine sediment (b), microfilariae in the stained blood smear (c), view of the result of rapid heartworm antigen test (d), post-treatment urine (e)

In conclusion, prevention with controlling strategies of dirofilariasis is quite important to reduce their diffusion in animals and humans. But at most, making the true diagnosis makes these controlling strategies vitally important. Therefore, we wanted to draw extra attention on urine analyzes in dogs suspected with dirofilariasis within this third canine microfilaruria case in the world.

REFERENCES

1. Bowman DD, Atkins CE: Heartworm biology, treatment, and control. *Vet Clin North Am Small Anim Pract*, 39, 1127-1158, 2009. DOI: 10.1016/j.cvsm.2009.06.003

2. Yildirim A, Ica A, Atalay O, Duzlu O, Inci A: Prevalence and epidemiological aspects of *Dirofilaria immitis* in dogs from Kayseri province, Turkey. *Res Vet Sci*, 82, 358-363, 2007. DOI: 10.1016/j.rvsc.2006.08.006

3. Sari B, Tasci GT, Kılıc Y: Seroprevalence of *Dirofilaria immitis*, *Ehrlichia canis* and *Borrelia burgdorferi* in dogs in Igdir province, Turkey. *Kafkas Univ Vet Fak Derg*, 19 (5): 735-739, 2013. DOI: 10.9775/kvfd.2012.8466

4. Simsek S, Ciftci AT: Serological and molecular detection of *Dirofilaria* species in stray dogs and investigation of *Wolbachia* DNA by PCR in Turkey. *J Arthropod Borne Dis*, 10, 445-453, 2016.

5. Nelson CT, McCall JW, Rubin SB, Buzhardt LF, Dorion DW, Graham W, Longhofer SL, Guerrero J, Robertson-Plouch C, Paul A: 2005 Guidelines for the diagnosis, prevention and management of heartworm *(Dirofilaria immitis)* infection in dogs. *Vet Parasitol*, 133 (2-3): 255-266, 2005. DOI: 10.1016/j.vetpar.2005.07.008

6. Traversa D, Di Cesare A, Conboy G: Canine and feline cardiopulmonary parasitic nematodes in Europe: Emerging and underestimated. *Parasit Vectors*, 3:62, 2010. DOI: 10.1186/1756-3305-3-62

7. Kaewthamasorn M, Assarasakorn S, Niwetpathomwat A: Microfilaruria caused by canine dirofilariasis *(Dirofilaria immitis)*: An unusual clinical presence. *Comp Clin Pathol*, 17, 61-65, 2008. DOI: 10.1007/s00580-007-0675-1

8. Monobe MM, da Silva RC, Araujo Junior JP, Takahira RK: Microfilaruria by *Dirofilaria immitis* in a dog: A rare clinical pathological finding. *J Parasit Dis*, 41, 805-808, 2017. DOI: 10.1007/s12639-017-0892-8

9. American Heartworm Society: Guidelines for the diagnosis, prevention, and management of heartworm *(Dirofilaria immitis)* infection in dogs and cats. 2018. https://www.heartwormsociety.org/veterinary-resources/american-heartworm-society-guidelines. *Accessed:* 20 November 2018.

10. Pekmezci D, Guzel M, Yildirim A, Ciftci G, Pekmezci GZ, Tutuncu M, Inci A: Evaluation of serum cystatin-C concentrations in dogs infected with *Dirofilaria immitis*. *Ankara Univ Vet Fak Derg*, 62, 303-306, 2015.

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