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Research Article

Effect of Thyme Species Extracts on Performance, Intestinal Morphometry, Nutrient Digestibility and Immune Response of Broilers

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Abstract

The study was performed to investigate the effect of hydroalcoholic extract of different thyme species on performance, intestinal morphometry, nutrient digestibility and immune response of male broiler chickens. A total of 160 day-old Ross 308 broiler chicks were randomly allotted to five treatments including a control basal diet or diets supplemented with 0.1% of four thyme extracts *(Thymus daenensis, T. kotschyanus, T. lancifolius,* and *T. transcaspicus)* with four replicates of eight birds each. The experiment was lasted for 28 days. There were no significant effects of treatments on broilers performance during the first three weeks of age. At fourth weeks of age and throughout the experimental period, the chickens on *T. daenensis* extract showed the best weight gain among the treatments ($P<0.05$), while feed intake and feed conversion ratio were not affected by the treatments throughout the experimental period. The birds receiving *T. daenensis* extract had higher jejunal villus height and villus height to crypt depth ($P<0.05$) than those on the control. All thyme extracts increased villus surface area ($P<0.05$). Supplementation of diet with *T. daenensis* extract improved digestibility of organic matter, dry matter, crude carbohydrate, crude ash and ether extract ($P<0.05$). The immune response to Newcastle disease (ND) vaccine and sheep red blood cell (SRBC) were not affected by the treatments. The results indicated that the response of broiler chickens to thyme extract depends on birds' age and genotype of the plant.

Keywords: *Chicken, Digestibility, Mucosal morphology, SRBC, Thyme spp.*

Etlik Piliçlerde Kekik Ekstraktının Performans, Barsak Morfometrisi, Besin Sindirilebilirliği ve Bağışıklık Yanıtı Üzerine Etkileri

Öz

Bu çalışma erkek broiler piliçlerde çeşitli kekik cinslerinin hidroalkolik ekstraktının performans, barsak morfometrisi, besin sindirilebilirliği ve bağışıklık yanıtı üzerine etkilerini araştırmak amacıyla yapılmıştır. Toplam 160 adet bir günlük Ross 308 broiler civciv her bir grupta 8 civciv ve dört tekrar olmak üzere rastgele olarak 5 gruba ayrılarak kontrol grubuna bazal diyet, diğerlerine ise %0.1 oranında farklı dört kekik *(Thymus daenensis, T. kotschyanus, T. lancifolius,*ve *T. transcaspicus)* ekstraktı verildi. Deney süresi 28 gün sürdü. İlk üç ay süresince broiler performansı üzerine denemelerin anlamlı bir etkisi olmadı. Dördüncü haftada ve deneysel süre boyunca *T. daenensis* verilen piliçler diğer denemeler arasında en iyi ağırlık kazanımını gösterirken ($P<0.05$) bu grupta yem tüketimi ve yem konversiyon oranında deneysel aşama süresince bir etki gözlemlenmedi. Kontrol grubu ile karşılaştırıldığında *T. daenensis* verilen piliçler daha yüksek jejunal villus yüksekliğine ve villus yüksekliği kript derinliği oranına sahiptiler ($P<0.05$). Tüm kekik ekstraktları villus yüzey alanında artmaya neden oldu ($P<0.05$). *T. daenensis* ekstraktı ilave edilen diyet organik materyalin sindirilebirliğini artıtdı ve kuru madde, ham karbohidrat, ham kül ve eter ekstraktı değerlerinde iyileşmeye neden oldu ($P<0.05$). Newcastle hastalığı aşısı ve koyun kırmızı kan hücrelerine bağışıklık cevabı deneysel uygulamalardan etkilenmedi. Elde edilen sonuçlar broiler piliçlerin kekik ekstraktından etkilenmesinin civcivin yaşına ve bitkinin genotipine bağlı olduğunu göstermiştir.

Anahtar sözcükler: *Tavuk, Mukozal morfoloji, Koyun kırmızı kan hücresi, Thyme spp.*

INTRODUCTION

Phytochemicals have been recently considered by researchers and producers as feed additives in poultry diets. It has been shown that adding medicinal plant extracts and essential oils into the diets can improve growth performance [1], gut function [2] and immune responses [3] of poultry.

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Thyme *(Thymus spp.,* L) is one of the aromatic plants that belong to the Lamiaceae family. It contains different essential oils such as thymol, carvacrol, linalool, α-terpineol, 1,8 cineole, α- pinen, p-simenn, camphene, limonene, and several other compounds [4]. Numerous beneficial properties such as antimicrobial [5-7], antifungal [8], anti-oxidant [4] and improved nutrients digestion [6] have been reported for thyme and its derivatives.

It is demonstrated that the quantity of bioactive components of medicinal plants and their activity varies by plant species [7,9], climatic and environmental conditions [10] harvesting stage, drying process and extracting methods [11].

The amount of carvacrol and thymol, as two major essential oils of thyme, is variable between thyme species (2 to 42 and 1 to 50 percent of total essential oils, respectively) [10] and even between populations of one species [7]. In one study, Amiri [9] has revealed the variation in terpene compounds by reporting the range of 16.4 to 42.6 percent for thymol, 7.6 to 52.3 percent for carvacrol and 3 to 11.4 percent for γ-terpinene of three wild-growing Thymus species in the west of Iran.

There are evidences that essential oils stimulate the secretion of digestive enzymes in broilers [1,5] and increase fat [12] and amino acid digestibilities [11]. The increase of *Lactobacillus* to *E. coli* ratio, bifidobacteria and propionibacteria populations have been also illustrated as the effect of essential oils in gastrointestinal tract of monogastric animals [13].

The modulatory effect of some medicinal plants [1], especially flavonoids rich plants such as thyme [14], has been shown on immune and defence system of poultry. The effect has been pronounced by increasing thymus, bursa of Fabricius and spleen weights [15], enhancing antibody titre against infectious bronchitis [16] and Newcastle disease (ND) vaccine [17] in broilers.

Although there are plenty of reports on thyme and its derivatives, to our knowledge, there is no study to compare the physiological effects of different species of thyme on broilers. Hence, the objective of this study was to investigate the influence of dietary supplementation with a hydroalcoholic extract of different thyme species on growth performance, gut morphometry, nutrient digestibility and immune response of broiler chickens.

MATERIAL and METHODS

Preparation of Plant Materials and Extraction

To minimize the environmental effects, the studied thyme species including *T. daenensis, T. kotschyanus, T. lancifolius* and *T. transcaspicus* were prepared from the same harvesting place, Agricultural and Natural Resources Research Centre of Ardabil, Iran. The plant materials were dried at room temperature in shade, and grounded using a laboratory mill. The hydroalcoholic extract of plants aerial parts was obtained by maceration technique using 50% ethanol as solvent at 10:1 solvent to plant ratio [18]. The extract was then concentrated in a rotary evaporator (Bibby RE 200 B, England) at 50°C and air-dried, then standardized based on 8% dry matter following the yield assessment [19]. The phenolic acid compounds of thyme extracts were evaluated by HPLC analysis [20,21] and expressed as µg/g dry weight. Total phenolic content of the extract was determined by folin-ciocalteau colorimetric method [22] and expressed as mg gallic acid equivalents (GAE)/L of extract *(Table 1)*.

Birds and Experimental Diets

A total of 160 one-day old Ross 308 male broiler chicks were used in an *in vivo* study. The birds were distributed into 20 wire floor cages with equal group weight, and were randomly assigned to one of five experimental diets. Therefore, a completely randomized design with five treatments including a control diet with no extract and four diets with 0.1% of four thyme species extract was employed with four replicates of 8 birds per each replicate. The diets were formulated according to the Ross 308 strain catalogue recommendations [23], and were fed to the chickens in three feeding phases of 1-10, 11-24 and 25-28 days of age *(Table 2)*.

The trial was lasted 28 days and conducted in an

Table 1. *Concentration of phenolic acid compounds (µg/g DW) and total phenolics (mg/L) of different thyme species extracts*

Phenlics	***T. daenensis***	***T. kotschyanus***	***T. lancifolius***	***T. transcaspicus***
Chlorogenic acid	187	129	175	110
Vanilic acid	175	48	107	66
Caffeic acid	190	162	242	117
Syringic acid	977	730	626	565
P_Cumaric acid	119	0	185	60
Rosmarinic acid	665	1047	870	1420
Sinapic acid	78	63	116	0
Total phenolics	707	615	570	666

Table 2. *Composition of experimental diets in different periods of the experiment*

Ingredient (%)	Starter (1-10 day)	Grower (11-24 day)	Finisher (25-28 day)
Maize	51.16	53.96	59.03
Soybean meal (44% CP)	40.00	36.17	31.40
Vegetable oil	4.07	5.76	5.65
Dicalcium phosphate	1.88	1.65	1.58
Oyester shell	1.34	1.11	1.09
Common salt	0.37	0.37	0.37
Vitamin premix[a]	0.25	0.25	0.25
Mineral permix[b]	0.25	0.25	0.25
DL-methionine	0.38	0.30	0.25
L-Lysine hydrochloride	0.30	0.17	0.13
Total	100	100	100
Calculated compositions			
ME, MJ/kg	12.66	13.18	13.39
Crude protein, %	22.41	21.00	19.28
Methionine, %	0.72	0.66	0.56
Methionine + cystine, %	1.07	1.00	0.87
Lysine, %	1.43	1.24	1.11
Arginine, %	1.45	1.33	1.21
Thereonine, %	0.83	0.83	0.75
Calcium, %	1.05	0.90	0.86
Available Phosphorus, %	0.50	0.43	0.43
Sodium, %	0.16	0.16	0.16

[a] Provided per kg of diet: vit. A, 9.000 IU; vit. D_3, 2.000 IU, vit. E, 18 mg; vit. K_3, 2 mg; vit B_1, 1.8 mg; vit B_2, 6.6 mg; Pantothenate calcium, 30 mg; Niacin, 10 mg; vit B_6, 3 mg; vit B_9, 1 mg; vit B_{12}, 0.015 mg; Biotin, 0.1 mg; Choline chloride, 500 mg
[b] Provided per kg of diet: Mn, 100 mg; Fe, 85 mg; Zn, 100 mg; Cu, 10 mg; I, 1 mg; Se, 0.2 mg

environmentally controlled room. Feed and water were provided *ad-libitum*, except at weighing times when feed was withdrawn for 4 h to ensure the emptiness of digestive system contents [24]. Feed intake and weight gain were measured weekly and feed conversion ratio was calculated. Light was provided for 23 h/day, while temperature was gradually reduced from the initial 31°C to approximately 21°C by day 21, and was kept constant until the end of the experimental period. All procedures used in the experiment approved by research council of the University of Mohaghegh Ardabili.

Nutrients Digestibility

To determine the total tract nutrient digestibility coefficients, 0.3 percent chromic oxide, as an inert marker, was added to the experimental diets, and fed to chickens from 25 to 28 days. The excreta samples of each replicate were collected twice daily and mixed together, then kept in the freezer (-20°C) for subsequent analyses. The samples were oven dried (60°C, 72 h) and ground prior to analysis. The chemical compositions of feed and excreta samples, including dry matter, organic matter, ether extract and crude protein were measured according to the AOAC [25] method. The crude carbohydrate content of samples was calculated by following formula [26]:

Crude Carbohydrate (%) = 100 - (% Nitrogen × 6.25) - % Crude Ash - % Ether Extract

The chromium oxide content of excreta and feed samples was measured according to the method described by Fenton and Fenton [27], then the apparent nutrients digestibility and nitrogen retention was determined using the following equation [28]:

$$\text{Digestibility (\%)} = [\,1 - (\frac{\%\ \text{diet}\ Cr_2O_3}{\%\ \text{excreta}\ Cr_2O_3} \times \frac{\%\ \text{excreta nutrient}}{\%\ \text{diet nutrient}})] \times 100$$

Morphometry

At the end of the experiment, two birds per cage (replicate) were randomly selected, weighed and euthanized by CO_2 asphyxiation. After eviscerating, a tissue sample (3 cm) from proximal jejunum was obtained and its digesta being washed with saline buffer was fixed in 10% buffered formalin. The fixed tissue samples in formalin solution were processed and embedded in paraffin. Sections were prepared at a thickness of 5 µm and stained with haematoxylin and eosin. The morphometric indices were determined using computer-aided light microscope image analyses (SPOT 3.1; Diagnostic Instruments, Sterling Heights, MI, USA). The measurements of villus height (from the tip of villus to the villus-crypt junction), villus width, crypt depth, villus height: crypt depth ratio and thickness of muscle layer were made. Apparent villus surface area was calculated from the villus height and width [29]. The values of means from 9 adjacent and vertically orientated villi and crypts from each bird were used for further analysis.

Immune Response to ND Vaccine and SRBC

All chicks were vaccinated against Newcastle disease (ND) at 7 days of age, by eye-drop and subcutaneous injection, simultaneously. Vaccination was carried out according to the regional vaccination program routine. At 14 days of age blood samples were collected from birds' wing in tubes to evaluate the immune response to ND virus by haemagglutination inhibition (HI) test according to Hossain et al.[30].

Sheep red blood cells (SRBC) were used to assay the antibody response. Two birds per cage were injected intramuscularly with SRBC (3% suspension in PBS, 1 mL/chick) at 17 days of age. Blood sampling of birds was done on the 7th days after injection. After inactivation of the serum at 56°C temperature for 30 min, 50 µL of serum was put in an equal amount of PBS in the first column of a 96-well V-shaped bottom plate, and the solution was incubated at 37°C for 30 min. Then a serial dilution was made (1:2), and 50 µL of

2% SRBC suspension was added to each well. After 30 min of incubation at 37°C, total antibody titre was read. The well immediately prior a well with a distinct SRBC button was considered as the endpoint titre for agglutination. To assess IgG titre, 50 µL of 0.01 M 2-mercaptoethanol in PBS was used, followed by the procedure mentioned above for total antibody titre. Antibody level of IgM was calculated from the difference between the total and the IgG [31].

Statistical Analysis

All collected data were subjected to statistical analysis using the General Linear Model procedure of SAS software 9.1 [32]. Duncan's Multiple Range test was used to detect the differences ($P< 0.05$) between different means.

RESULTS

Growth Performance

During the first 3 weeks of the experiment, none of the thyme extracts had a significant effect on chickens weight gain (WG), feed intake (FI) and feed conversion ratio (FCR) *(Table 3)*. In the fourth weeks of the experiment, supplementation of diet with *T. daenensis* extract increased WG of the chickens ($P<0.05$) and a significant decrease in FI and FCR of birds on *T. lancifolius* extract was also observed ($P<0.05$) although there was no significant difference between FCR of broilers on *T. daenensis* and *T. lancifolius* extracts. Throughout the entire period of the experiment (1 to 28 d), WG of chickens was only improved by *T. daenensis* extract ($P<0.05$). FI and FCR were not significantly affected by the treatments.

Morphometry

At 28 days of age, the birds on *T. daenensis* extract had significant high villus height and villus height to crypt depth ratio compared with control *(Table 4*; $P<0.05$). Jejunal crypt depth was increased by *T. kotschyanus* extract in comparison with control ($P<0.05$). Villus surface area

Table 3. *Effect of different thyme species extracts on growth performance of broilers during different weeks of age and throughout the experimental periods*

Period	Parameters	Treatments					Statistics	
		Control	*T. daenensis*	*T. kotschyanus*	*T. lancifolius*	*T. transcaspicus*	SEM	*P* Value
Day 1 to 7	WG (g)	104.70	104.40	108.12	110.83	106.57	3.013	0.559
	FI (g)	146.00	142.75	146.00	144.16	141.50	2.385	0.607
	FCR	1.40	1.37	1.35	1.30	1.33	0.033	0.339
Day 8 to 14	WG (g)	231.17	236.75	229.75	236.80	241.27	5.073	0.514
	FI (g)	346.50	353.25	340.00	345.50	355.50	5.243	0.275
	FCR	1.50	1.49	1.48	1.46	1.47	0.034	0.464
Day 15 to 21	WG (g)	367.30	386.85	372.73	363.33	371.70	7.566	0.285
	FI (g)	495.75	487.00	494.00	500.00	497.25	7.563	0.794
	FCR	1.35	1.27	1.33	1.38	1.34	0.036	0.284
Day 22 to 28	WG (g)	561.00 ^{b}	605.13 ^{a}	555.38 ^{b}	567.10 ^{b}	567.10 ^{b}	10.18	0.027
	FI (g)	826.50^{a}	849.50 ^{a}	825.50^{a}	779.25 ^{b}	830.75 ^{a}	12.37	0.015
	FCR	1.47 ^{a}	1.40 ^{ab}	1.49 ^{a}	1.37 ^{b}	1.47 ^{a}	0.028	0.043
Day 1 to 28	WG (g)	1263.43^{b}	1333.03^{a}	1265.93 ^{b}	1278.05^{b}	1284.13 ^{b}	15.37	0.037
	FI (g)	1815.00	1832.25	1805.25	1768.50	1825.25	16.89	0.120
	FCR	1.43	1.37	1.43	1.38	1.42	0.019	0.139

^{a,b} Means with different superscripts in the same row differ significantly (P≤.05); SEM: Standard error of means

Table 4. *Effects of different thyme species extract on broilers jejunal mucosal morphometry at 28 days of age*

Parameters	Treatments					Statistics	
	Control	*T. daenensis*	*T.kotschyanus*	*T. lancifolius*	*T. transcaspicus*	SEM	*P* Value
VH (µm)	865.75^{b}	1101.00^{a}	640.25^{b}	1036.75^{ab}	1098.00^{ab}	26.63	0.0001
CD (µm)	151.75 ^{b}	148.75 ^{b}	168.00 ^{a}	161.50 ^{ab}	159.75 ^{ab}	4.21	0.036
VS (mm^{2})	0.08^{c}	0.09^{b}	0.11 ^{a}	0.12 ^{a}	0.11 ^{a}	0.004	0.0001
VH to CD	5.72^{c}	7.43^{a}	5.61^{c}	6.43^{bc}	6.90^{ab}	0.286	0.0017
TML (µm)	152.75	161.250	175.75	165.50	171.25	5.91	0.109

^{a,b} Means with different superscripts in the same row differ significantly (P≤0.05); SEM: Standard error of means; VH: Villus height; CD: Crypt depth; VS: Villus surface area; VH to CD: Villus height to Crypt depth; TML: Thickness of muscle layer

Table 5. *The effect of different thyme species extract on apparent digestibility of nutrients (%) at 28 days of age*

Parameters*	Treatments					Statistics	
	Control	*T. daenensis*	*T. kotschyanus*	*T. lancifolius*	*T. transcaspicus*	SEM	*P* Value
DM	69.40 ^{bc}	73.57 ^{a}	68.39 ^{cd}	70.38 ^{b}	67.61 ^{d}	0.429	0.0001
OM	72.35 ^{bc}	76.09 ^{a}	71.51 ^{cd}	73.47 ^{b}	70.93 ^{d}	0.403	0.0001
EE	68.34 ^{c}	73.37 ^{a}	69.70 ^{bc}	71.87 ^{ab}	67.49 ^{c}	2.466	0.0002
NR	67.08 ^{abc}	70.10 ^{a}	63.53 ^{c}	68.19 ^{ab}	64.61 ^{bc}	1.431	0.026
CC	74.07 ^{bc}	78.91 ^{a}	73.69 ^{bc}	75.12 ^{b}	73.28 ^{c}	0.532	0.0001
ASh	32.39 ^{b}	39.86 ^{a}	27.04 ^{cd}	29.46 ^{bc}	23.23 ^{d}	1.295	0.0001

^{a,b} Means with different superscripts in the same row differ significantly (P≤.05); DM: Dry Matter; OM: Organic Matter; EE: Ether Extract; NR: Nitrogen Retention; CC: Crude Carbohydrate; SEM: Standard error of means

Table 6. *The effect of different thyme species extract on immune response of chickens to Newcastle disease (ND) vaccine and sheep red blood cell (SRBC) injection*

Parameters	Treatments					Statistics	
	Control	*T. daenensis*	*T. kotschyanus*	*T. lancifolius*	*T. transcaspicus*	SEM	*P* Value
ND titre (14 days)	2.25	2.00	3.00	2.00	2.00	0.270	0.319
Total Ig^{a} (28 days)	4.87	5.37	5.37	5.37	4.75	0.378	0.612
Ig G^{a} (28 days)	2.13	2.13	2.50	2.75	1.87	0.389	0.534
Ig M (28 days)	2.75	3.25	2.87	2.63	2.87	0.442	0.889

^{a} Inresponse to sheep red blood cells injection; SEM: Standard error of means

was increased by all thyme extracts (P<0.05). There were no significant differences between thyme extracts on the thickness of muscle layer.

Nutrients Digestibility

Interestingly, the highest digestibility of dry matter, organic matter, ether extract, crude carbohydrate and crude ash (P<0.05) was observed as the effect of *T. daenensis* extract *(Table 5)*.

Digestibility of ether extract was similar between *T. daenensis*and *T. lancifolius* extracts. Nitrogen retention was not changed significantly by different thyme extract compared with control.

Immune Response

Antibody titres against ND vaccine, total antibody titre, Ig G and Ig M were not significantly affected by the treatments *(Table 6)*.

DISCUSSION

Growth performance of broiler chickens was not influenced by thyme extract during the first three weeks of age. Such results have been also observed by other researchers who used thyme extract in drinking water [33] and diet [34] of chickens, and found no significant changes in performance parameters. However, at fourth weeks of age and within the experimental period, among four thyme extracts, *T. daenensis* extract improved birds growth response and *T. lancifolius* decreased FI and FCR compared with control. To compare the obtained results, there were no available reports on different thyme species supplementation in broilers diet. However, similar results have been reported by supplementation of a mixed essential oil, including thyme oil, to drinking water of quails [35]. There are contradictory effects of thyme on broiler performance in literatures. Beside the reports indicating affectless of thyme product, as mentioned above, in agreement with beneficial effect of one thyme extract in this study, there are some observations representing the positive effect of thyme on broilers growth response. Al-Kassie [1] used 100 and 200 ppm thyme essential oils and observed improved WG. Such finding was also reported by Hernandez et al.[36] using 5000 ppm *Labiatae* extract including thyme.

Most of herbal bioactive components leave their effects on broiler performance through their antimicrobial activity [5]. At an early stage of life, the population of microorganisms in digestive tract of broilers is limited and then develops with birds age [37,38]. For instance, the increases of *Streptococcus*, *Enterobacteriaceae* and *Clostridium perfringens* [37], Coli-forms, *Clostridium* species and total anaerobic bacteria numbers [38] have been reported. In digestive tract, the microorganisms compete with the host to get the nutrients such as carbohydrates and amino acids [39]. Thus, it seems that any factor could

eliminate the competition between the bacteria and the host, for example, by decreasing intestinal microbial population, can increase feed efficiency for the host. The antimicrobial property of thyme main active components including carvacrol and thymol has been illustrated [11,40]. Moreover, the differences in quantity of the active components between different thyme species have also been proven [7,9]. Therefore, the different growth response of broilers to the studied thyme species would be acceptable and a part of the beneficial effect of *T. daenensis* extract compared with other extracts on performance of aged birds might be explained due to relatively high total phelolics *(Table 1)*. The same explanation might be applied to different intestinal morphometry and nutrients digestibility as the effect of supplemental extracts because of their probable different phytochemicals *(Table 4* and *Table 5)*.

In contrast to some studies which used thyme phytochemicals [1,11] and observed their growth promoting effect, in this study, thyme extracts failed to show much improvement in growth response of broilers, except one case. The probable reason behind this observation might be related to housing system. Because in the mentioned studies, the birds were raised in deep litter pens, while in the present trial the wire floor cages were used for raising. It is well known that in deep litter systems, birds directly contacts with litter microorganisms which can affect their intestinal microbial population [41]. However, in cages the birds cannot access to the litter as an infection source.

Villus height to crypt depth ratio is considered as a health indicator for the gut. Increased crypt depth and decreased the ratio means high nutrients demand for intestine maintenance [42]. Therefore, the higher ratio indicates that more nutrients have been directed to body growth. Moreover, increased digestion and absorption of nutrients can also be expected by higher villus height and villus surface area. The higher digestibility of nutrients by *T. daenensis* extract compared with the control *(Table 5)* confirms this concept. Hence, the improved weight gain of birds fed with this extract might be described by this explanation too. The increased nutrients digestibility as the effect of thyme extracts have also been reported by other researchers [37,43].

High villus height induced by thyme extract, specifically *T. daenensis*, might be related to the presence of high antioxidant agents in this extract *(Table 1)*. Furthermore, thyme extract contains essential oils such as thymol and carvacrol which their antioxidant activity, similar to alpha tocopherol and butylated hydroxytoluene, has been elicited [4]. It is likely that the compounds with antioxidant property can trigger villus development by preventing the damage of free radicals produced from digestion processes. Moreover, it could be mediated by anti microbial activity of thyme phytochemicals [5-7]. Because shorter intestinal villi height and lower cell mitosis have been observed due to bacterial toxins such as ammonia [44].

In keeping with our results, application of thyme extract [16], thyme powder [45] and thyme essential oil [46] in broiler diet did not revealed any significant effects on antibody titre against ND vaccine. The finding on SRBC is similar to that obtained by Toghyani et al.[45], who also did not observe any improvement in broiler humoral immune response using thyme powder in the diet. It is noted that the plants rich in flavonoids such as thyme can stimulate immune system through increasing vitamin C activity and antioxidant properties [14]. The lack of positive result of using thyme extract in this experiment on immune response is probably related to the dose of supplemental extract [3,45] and absence of secondary challenge with SRBC [47]. Furthermore, preparing relatively high hygiene condition in wired cages for broilers might be another possible reason for our findings on bird's immunity. It is shown that the influence of essential oils is more pronounced in microbial challenged broiler chickens [48].

In summary, it can be concluded that the response of broilers to dietary inclusion of thyme extracts depends on the plant genotype. The role of bird age was also illustrated in this regard, as the response was more pronounced after 3 weeks of age. The improvement in broiler performance as the effect of thyme extract was well related to improved intestinal mucosal architecture and increased nutrients digestibility.

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Research Article

Ultrastructural and Immunohistochemical Investigations in Calves with Coronavirus Pneumoenteritis Syndrome

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Abstract

The aim of present studies was the structural and morphogenetic investigation of spontaneous pneumoenteritis syndrome in newborn and growing calves with regard to confirmation of some structural features of disease morphogenesis. The research was done with 370 calves from 6 cattle farms in 4 regions of the country, at the age of 24 h - 25 days. For rapid antigenic and viral detection of pathogens, Multiscreen Ag ELISA, Bovine respiratory, Pulmotest respiratory tetra ELISA kit for antigenic diagnosis of *BoHV-1, BVDV, BRSV,* and *BPI-3* sandwich test for tissue lysates (BIOX Diagnostics, Belgium) and Rainbow calf scour 5 BIO K 306 Detection of Bovine Rotavirus, Coronavirus, *Escherichia coli* F5, *Cryptosporidium parvum* and *Clostridium perfringens* in bovine stool (BIOX Diagnostics, Belgium) were used. In 5% of cases, laboratory antigenic tests of lung tissue lysates from pneumonic calves detected co-infections with *BoHV-1, BVDV, BRSV* and *BPI-3*. The utilised antigenic, ultrastructural and virological diagnostic methods allowed concluding that they could be successfully used in the diagnostics of pulmonary and gastrointestinal viral infections in juvenile calves. Electron microscopy and immunohistochemical methods of lung and intestinal tissue are also important and applicable for diagnostics and in differential diagnostic recognition of the condition from other common diseases as IBR, BVD, BRSV, *Mannheimia haemolytica, Cryptosporidium parvum*, BRV and *E. coli* K99 (F5).

Keywords: *Calves, Pathology, IHC, Ultrastructure, BCoV*

Coronavirus Pnömoenteritis Sendromlu Buzağılarda Ultrastrüktürel ve İmmunohistokimyasal İncelemeler

Öz

Bu çalışmanın amacı spontan pnömoenteritis sendromlu yeni doğan ve büyüme dönemindeki buzağılarda hastalığın morfogenezine yönelik bazı yapısal özellikleri incelemektir. Çalışma ülkenin 4 bölgesinden 6 çiftlikte toplam 370 adet 24 saatlik ile 25 günlük arasındaki buzağı üzerinde gerçekleştirildi. *BoHV-1, BVDV, BRSV* ve *BPI-3*'ün hızlı tespiti amacıyla Multiscreen Ag ELISA Bovine respiratory, Pulmotest respiratory tetra ELISA kit kullanıldı (BIOX Diagnostics, Belçika). Sığır dışkısında Rotavirus, Coronavirus, *Escherichia coli* F5, *Cryptosporidium parvum* ve *Clostridium perfringens* tespiti amacıyla Rainbow calf scour 5 BIO K 306 kullanıldı (BIOX Diagnostics, Belçika). Vakaların %5'inde *BoHV-1, BVDV, BRSV* ve *BPI-3* pnömonili buzağıların akciğer doku lizatlarında birlikte gözlemlendi. Çalışmada kullanılan antijenik, ultrastrüktürel ve virolojik tanı metotlarının başarılı bir şekilde pulmoner ve gastrointestinal viral enfeksiyonların tespitinde kullanılabileceği belirlendi. Akciğer ve barsak dokularında elektron mikroskopi ve immunohistokimyasal metotlar tanıda uygulanabilir olup IBR, BVD, BRSV, *Mannheimia haemolytica, C. parvum,* BRV ve *E. coli* K99 (F5) gibi diğer yaygın hastalıklardan ayırıcı tanıda önemlidirler.

Anahtar sözcükler: *Buzağı, Patoloji, İmmunohistokimya, Ultrastrüktür, BCoV*

INTRODUCTION

Bovine Corona Virus - BoCV is outlined as one of primary etiological agents causing gastrointestinal diseases in newborn calves (calf diarrhoea - CD), winter dysentery (WD) in adult cattle and respiratory infections in calves and

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cattle in fattening farms - pneumoenteritis syndrome [1]. As early as in 1972, Stair et al.[2] reported pneumoenteritis in calves induced by coronaviruses. The first isolation of BoCV from bronchial secretion and nasopharyngeal washings of calves with signs of pneumonia occurred in 1982 and later, it was determined as bovine respiratory coronavirus (BRCoV) [1,2]. Strains isolated from diarrhoeic newborn calves and cattle have been classified as enteric or enteropathogenic coronaviruses (BECoV) [3]. Thomas et al.[4] affirmed that enteric and respiratory BCoV were the same, although detected in different life cycle stages. They have been isolated from the intestines and lung of calves affected with pneumoenteritis syndrome, and were antigenically and genomically similar [3,4].

In the study of Zhang et al.[5] BCoV was the second most prevalent viral agent isolated from lungs of calves with respiratory infections after bovine herpesviruses BoHV-1.

Bovine coronavirus causes enterocolitis and pneumonia in calves from the 24th h to the 5th month of age. The disease is characterized by a high morbidity rate ranging from 50% to 100%. Mortality rate is usually low, typically less than 2%, with only a few reports describing case fatality associated with this virus [6-10]. The incubation period for BCoV-WD ranges from 2 to 8 days. In small housed herds, the incidence of diarrhea during an outbreak begins with the explosive appearance of signs in 10% to 15% of animals on the first day. The diarrhea may contain a slight to copious amount of mucus. The amount of blood varies from case to case and ranges from just visible flecks or streaks to large clots, or it may be uniformly mixed into the feces. Pyrexia is usually not present during the diarrheal phase of the disease, although it has been reported to precede it by 24 to 48 h or have no consistent relationship. Mild to moderate signs of respiratory disease (eg, cough, nasal discharge) have been inconsistently observed preceding or concurrent with the diarrhea [6,11].

The clinical manifestation of the syndrome depends not only on the BCoV itself, but also on the host, environmental factors, immunological status of the herd, ambient temperature and the occurrence of secondary co-infections with other pathogens [4-7].

According to Craig et al.[7] the most accurate methods of laboratory diagnostics of coronaviruses are immuno-electron, electron microscopy (EM) and enzyme-linked immunosorbent assay (ELISA) as histological changes in the lung and intestines are not always specific for a definite histopathological diagnosis of coronaviral infection. In formalin-fixed specimens from intestinal segment and lungs, an immunohistochemical method has been used [7-9], requiring a specific monoclonal antibody (MAb), bound to the nucleocapsid protein of BCoV. The latter is the most prevalent protein of the virus for its detection in formalin-fixed tissues [8,9]. In non-fixed intestinal and lung specimens, the direct immunofluorescence assay (IFA) was also often used [8]. Daginakatte et al.[9] reported haemagglutination test with mouse red blood cells and polymerase chain reaction as other important techniques for detection of BCoV in tissue samples.

On the basis of literature overview and the relevance of the pathology, we aimed to perform immunohistochemical and ultrastructural investigation of spontaneous cases of pneumoenteritis syndrome in newborn and growing calves with regard to confirmation of some structural features of disease morphogenesis.

MATERIAL and METHODS

Animals and Samplings

The study comprised 370 calves from six dairy cattle farms. The age of animals was from 24 h to 25 days. Samples of 18 dead calves were obtained for antigenic, histopathological, immunohistochemical, lectron microscopy studies. Data presented in *Table 1*.

Antigenic Studies

a) Multiscreen Ag ELISA: For detection of respiratory pathogens were used, Multiscreen Ag ELISA, Bovine respiratory, Pulmotest respiratory tetra ELISA kit for antigenic diagnosis of BoHV-1, BVDV, BRSV, and BPI 3 sandwich test for tissue lysates. (BIOX Diagnostics, Belgium). From all calves, lung samples measuring 0.5 x 0.5 cm were obtained.

b) Rapid Pentavalent Antigenic Strips Test: Rainbow calf scour 5 BIO K 306 Detection of Rotavirus, Coronavirus, *E. coli* F5, Cryptosporidium and *C. perfringes* in bovine stool (BIOX Diagnostics, Belgium) were used. From all calves, faecal samples about 3 g.

Gross Pathology Examination

Eighteen carcasses of calves dead after manifestation of

Table 1. *Samples from 18 calves for antigenic, histopathological, immunohistochemical, lectron microscopy studies*

Number of Calves	Samples for Multiscreen Ag ELISA	Samples for Antigenic Strips Test	Samples for Histopathological Examination	Samples for Immunohistochemical Studies	Samples for Electron Microscopy Examination
18	from all calves, lung samples measuring 0.5 x 0.5 sm were obtained.	from all calves, faecal samples about 3 g	from all calves, tissue samples (1 cm x 1 cm) from lungs and bronchial lymph nodes, abomasum, duodenum, jejunum with mesenteric lymph nodes, ileum, caecum, colon and rectum	from all calves, tissue samples (1 cm x 1 cm) were obtained from lungs and the ileum	from all calves, tissue samples (1 mm x 1 mm) were obtained from lungs and the ileum

enteritis and respiratory signs were submitted to gross anatomy study.

Histopathological and Immunohistochemical Examination

Tissue samples (1 cm x 1 cm) were collected for histopathological examination from lungs and bronchial lymph nodes as well as from affected proximal and distal gastrointestinal compartments: abomasum, duodenum, jejunum with mesenteric lymph nodes, ileum, caecum, colon and rectum - all intestinal parts with 2.5 cm length. Specimens were fixed in 10% neutral buffered formalin for 48-72 h and embedded in paraffin. Cross sections, 4 µm thick were cut from paraffin blocks on a Leica RM 2235 microtome and stained with haematoxylin-eosin (H/E). Furthermore, tissue samples were obtained from lungs and the ileum for immunohistochemical (IHC) studies. To this end, a monoclonal non-conjugated antibody Monoclonal Antibody anti-mouse unconjugated, Coronavirus pan Monoclonal Antibody (FIPV3-70): sc-65653, 1 mg/mL, (Santa Cruz Biotechnology, Germany) was used.

Protocol for ICH: De-paraffinize sections thoroughly in xylene/synthetic solvent, and hydrate through a graded series of alcohols. Wash twice in water. Outline section with PAP pen. If required, treat with 0.3% (w/v) H_2O_2 in methanol for 15 min to block endogenous peroxidase activity (2% (w/v) H_2O_2/methanol can be used for a shorter time if preferred; Bio-Rad offers peroxide blocking reagent (BUF017B). Wash 3 times in TBS.

If required, include an appropriate antigen retrieval step to enhance the immunostaining (see protocol: Antigen Retrieval Techniques for use with Formalin-Fixed Paraffin-Embedded Sections). Bio-Rad offers a variety of retrieval/antigen unmasking fluids. Wash once in water. Incubate sections for 10 min in 10% normal serum from the species in which the secondary antibody was raised. Tap excess serum off the slides before staining.

Incubate sections with primary antibody for at least 30 min at room temperature in a humid chamber, or overnight at 4°C. Wash 3 times in TBS. Add enzyme-conjugated secondary antibody at the recommended dilution (see specific datasheet for details). Incubate for at least 30 min at room temperature. Wash 3 times in TBS. Incubate with the appropriate substrate solution for the recommended period of time (Bio-Rad recommends the use of DAB substrate with HRP-conjugated antibodies, and Fast Red/Napthol AS-MX for Alkaline Phosphatase-conjugated antibodies). Wash once in water. Counterstain with hematoxylin for 1 min. "Blue" with running water for 5 min. Then wash. Mount in aqueous mounting medium, or alternatively dehydrate through a graded series of alcohols and xylene/solvent. Mount in synthetic mountant.

Electron Microscopy Examination

For detection of ultrastructural lesions, tissue samples were collected from the same sites. They were processed according to the routine electron microscopy technique - fixation for 24 h in 4% glutaraldehyde in 0.1 M phosphate buffer pH 7.3, post fixation for 24 h in 1% OsO_4, dehydration and embedding in Durcupan ACM Fluka. Ultrathin sections were cut on a Reichert ultratome and stained with uranyl acetate and lead citrate. For the TEM study, JEOL 1200 EX transmission electron microscope with accelerating voltage 80 kV was used. All reagents were purchased from Sigma Aldrich - Merck.

RESULTS

The epidemiological survey at the six farms identified that intestinal and respiratory infections as the major health problem in newborn and juvenile calves. In 75% of calves aged between 1 and 15 days, clinical signs comprised digestive disturbances

In the other 25% (mainly between 4 and 10 days of age), respiratory signs were also present along with gastrointestinal ones.

Antigenic Findings

The results from laboratory antigenic analyses of lung tissue lysates from pneumonic calves confirmed co infections involving Bo*HV*-1, BVDV, BRSV and BPI-3 in 5% of cases *(Table 2)*.

In 75% of gastroenteritis cases, bovine rotaviruses and cryptosporidiae were identified as etiological agents. In 20%

Table 2. *Optical density values higher than 6% were positive for BoHV-1, BVDV, BRSV and BPI-3*

Disease	Positive Control Values	Optical Density Values										
Bo*HV*-1	0.086	0.255	**0.954**	0.051	0.057	0.056	0.097	**0.657**	0.056	0.053	0.053	0.052
BVDV	2.391	0.059	0.056	**0.857**	0.057	0.058	0.061	0.074	0.460	0.060	0.054	0.052
BRSV	0.094	0.067	0.068	0.064	0.066	0.065	0.063	0.066	0.070	0.088	0.071	0.058
BPI-3	2.090	0.055	**0.864**	**0.761**	0.061	0.055	0.056	0.066	0.057	**0.669**	0.073	0.084
Bo*HV*-1	0.062	0.065	0.071	0.070	0.068	0.068	0.073	**0.980**	0.073	0.069	0.077	0.066
BVDV	2.443	0.080	0.070	**0.663**	0.080	0.072	0.071	0.088	0.068	0.107	0.061	0.065
BRSV	0.071	0.090	**0.775**	0.074	0.279	0.070	0.071	**0.666**	0.066	0.073	0.093	0.062
BPI-3	2.354	0.057	0.137	0.090	0.080	0.064	0.069	0.088	0.076	0.072	0.062	0.058

of complicated states with simultaneous occurrence of gastrointestinal and respiratory troubles (pneumoenteritis), bovine coronaviruses were the only pathogens implicated.

Clinical and Macroscopic Findings

In calves with pneumoenteritis, strong dehydration and emaciation were observed. The inspection of the head revealed sunken eyes with bilateral corneal opacity. Visible conjunctival and buccal cavity mucosae were pale and anaemic. The perianal region and tail base were extensively stained with orange-yellow to greenish diarrhoeic faeces with mucous consistency.

Gross changes were identified along the entire length of small and large intestines. The intestinal wall was flaccid and transparent. Intestinal serosa exhibited diffuse hyperaemia with local haemorrhages. The intestinal content was yellow and slimy. Caecal content was pale yellow mixed with blood and a large amount of gas bubbles. Small and large intestinal mucosae were strongly hyperaemic and oedematous, spattered with striated haemorrhages in some areas. The mesenteric lymph nodes of all calves were enlarged and juicy.

The palpation of lungs revealed that they were thickened, with marbled appearance, filled with large amount of cloudy foamy fluid reaching the trachea and primary bronchi. Regional lymph nodes were enlarged and juicy. Areas with interstitial emphysema were observed on the periphery of lung lobes.

The epicardial surface exhibited multiple haemorrhages, most extensive in the coronary margin area. The mucous coats of the trachea, larynx and epiglottis were hyperemic and spattered with single petechiae.

Histopathological Findings

Microscopic lesions were most prominent in the lung and the distal small intestinal compartment. Pulmonary lesions were concentrated in the interalveolar and peribronchial connective tissue. As a result of degeneration, epithelial cells were shed in the alveolar lumen among the serous exudate collection. Interstitial connective tissue was strongly oedematous due both to its impregnation with exudate and infiltration with lymphocytes and macrophages. Part of the small intestine (ileum) villi exhibited strong atrophy and their middle part was infiltrated with numerous lymphocytes. Degenerative and necrobiotic processes predominated in epithelial cells of villi and crypts. The submucosa was oedematous, diffusely hyperaemic and spattered with large haemorrhages, while glands were enlarged secondary to the profuse hypersecretion *(Fig. 1)*. Haemorrhages and hyperaemia were visible in the medullary part of mesenteric lymph nodes.

Immunohistochemical Findings

The used immunohistochemical (IHC) technique confirmed the presence of bovine coronavirus antigen in intestinal and pulmonary tissue cross sections *(Fig. 2, Fig. 3)*. This confirmed the field and laboratory antigenic results in calves with pneumoenteritis.

Electron Microscopy Findings

Ultrastructural changes in tissue morphology were detected both in specimens collected from lungs and distal small intestine.

The lung exhibited destruction and desquamation of the respiratory epithelium surrounding the alveoli, type I pneumocytes with enhanced cytoplasmic vacuolation among which a large amount of activated macrophages, lymphocytes and granulocytes had migrated *(Fig. 4-a,b,c)*. Coronavirus-like nucleocapsids in double-membrane vesicles were present in the respiratory epithelium of some of tissue samples *(Fig. 4-a)*. Apart the enhanced

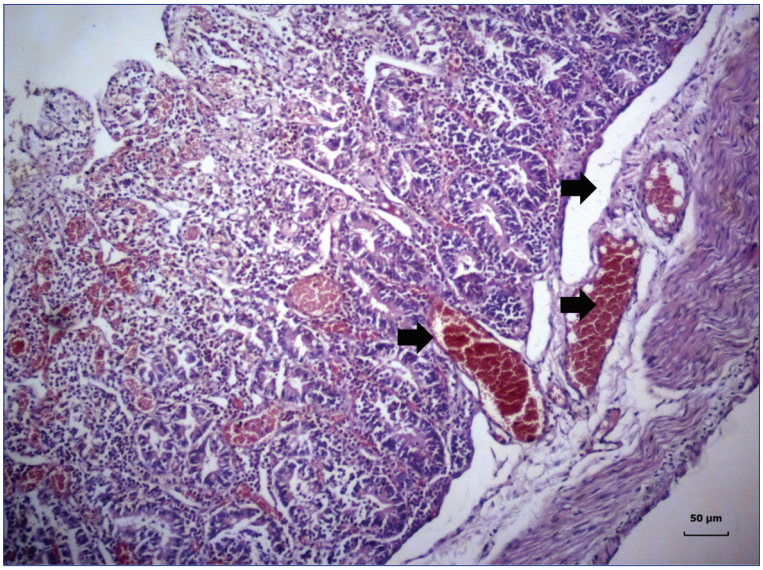


Fig 1. Catarrhal haemorrhagic enteritis, submucosal oedema *(arrow)* and vascular hyperaemia in the submucosa *(arrows)* of the ileum of a calf with pneumoenteritis syndrome, H/E, Bar=10 µm

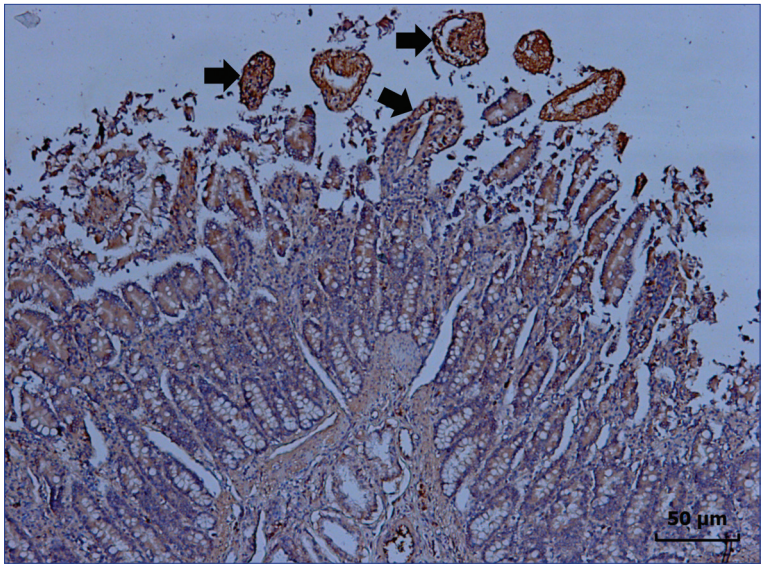
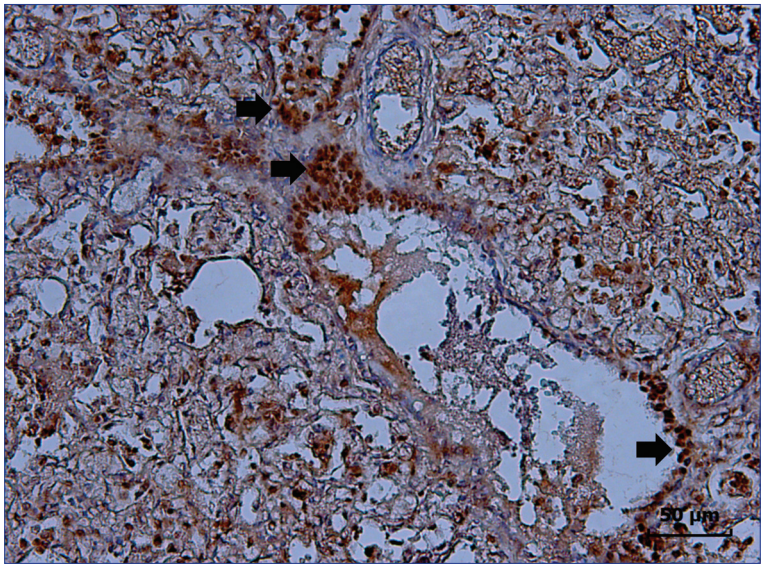


Fig 2. Immunohistochemical detection of coronavirus antigen with *MAb - FIPV3* in ileal enterocytes *(arrows)* of a calf with pneumoenteritis syndrome, ICH, Bar=10 µm

Fig 3. Immunohistochemical detection of coronavirus antigen with *MAb - FIPV3* in bronchial epithelium - lung *(arrows)* of a calf with pneumoenteritis syndrome, ICH, Bar=10 µm



destruction of cytoplasmic structures, many pneumocytes had altered nuclei - chromatin margination or chromatin condensation.

Presence of virus-like particles with morphological features and size 80-100 nm similar to that of coronaviruses was detected within the strongly destructed enterocytes and in the interface of adjacent intestinal cells *(Fig. 5-a,b)*. Affected cells had microvilli shed from the apical end, strong vacuolation and lack of clearly defined cell organelles, but with distinct electron-dense intracytoplasmic inclusion bodies.

DISCUSSION

The results of the present study confirmed that bovine coronaviruses (BCoV) were the primary viral agents causing pneumoenteritis syndrome in calves.

Furthermore, we suggest that the occurrence of respiratory and intestinal diseases in calves is associated with the lack of immunoprophylaxis of pregnant cows as well as to faulty elements of the rearing technology of newborn calves [10].

We share the opinion of researchers [5-10] affirming that the clinical manifestation of BCoV-associated pneumoenteritis syndrome is due to the tropism of the virus to the gastrointestinal tract, nasal cavity, proximal trachea and lungs. We agree with the belief [10,11] for a mutual relationship between BCoV and BRDC (bovine respiratory disease complex), causing severe respiratory infections with stunted growth and development of dairy and feedlot calves. Expectedly, this pathology incurs considerable economic losses to farmers [11].

The established macro- and microscopic lesions, as well as ICH findings in the respiratory and digestive tract samples of studied calves due to BCoV replication are large comparable to those reported by others [12]. In this

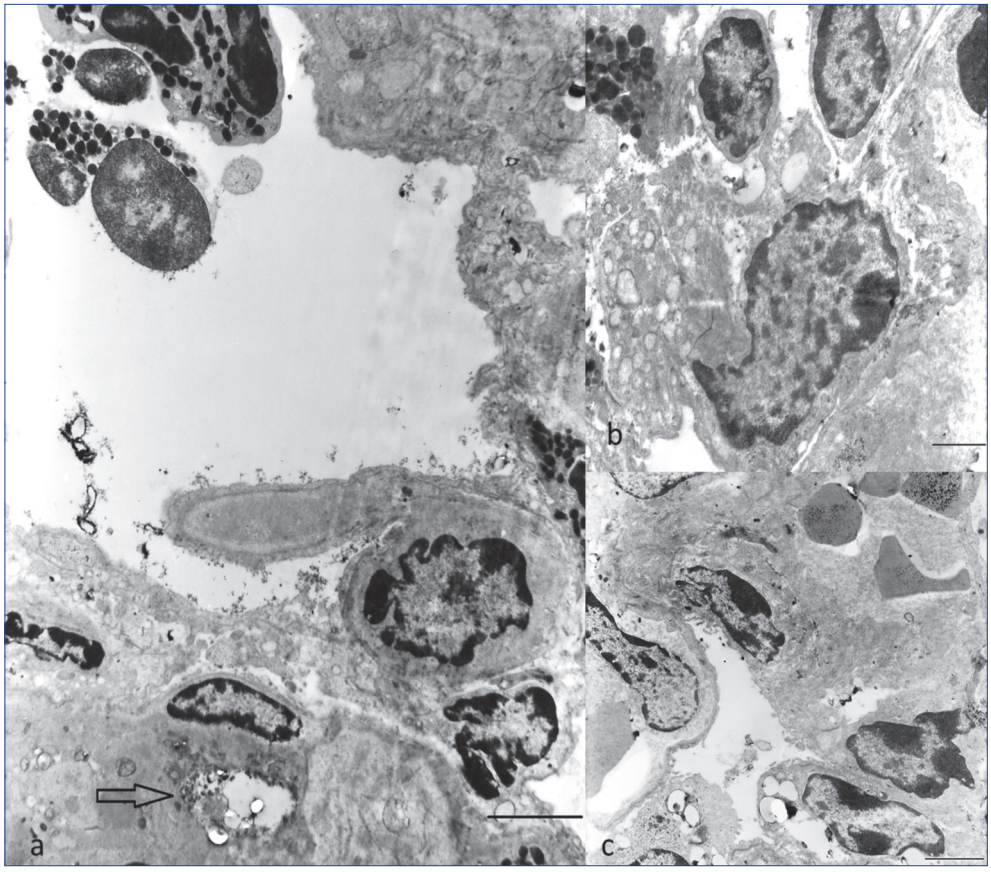


Fig 4. Alveoli with respiratory epithelium and presence of agranulocytes and granulocytes (a, b, c). Pneumocytes with enhanced cytoplasmic vacuolation, nuclear chromatin condensation and margination, cellular detritus to the lumen of the alveolus (a, b, c), activated macrophages (with pseudopods) and granulocytes (a, b); coronavirus-like particles (nucleocapsids) in membrane-confined vesicles *(arrow)* (a). Durcupan, Bar=3 µm (a), Bar=1 µm (b), Bar=2 µm (c)

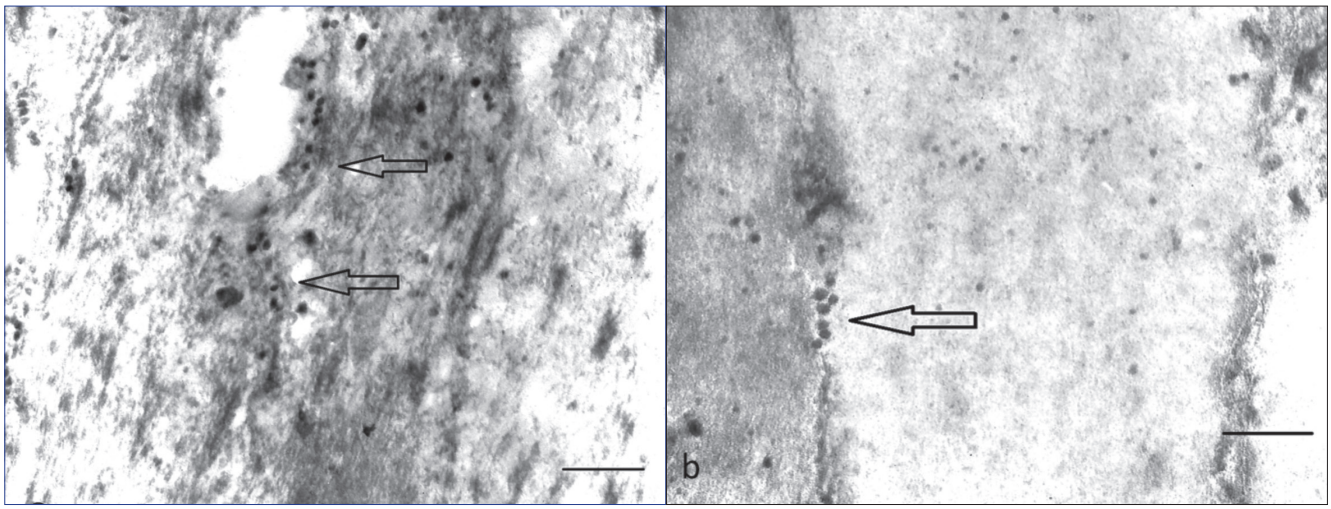


Fig 5. Enterocytes from *lamina epithelialis mucosae*, part of ileal *villi intestinales*. Presence of virus-like particles *(arrow)* in strongly destructed enterocytes (a). No distinct cell organelles are visible (a, b). Presence of virus-like particles at the interface of two adjacent enterocytes (b). Durcupan, Bar=800 nm(a), Bar=600 nm (b)

and other studies [7-12] co-infections with BCoV and other bacterial or viral agents result in higher morbidity and mortality rates in calves up to 14 days of age. BCoV (calf diarrhea, pneumonia in calves and adult cattle, winter dysentery, and combined pneumonia and diarrhea in young and adult cattle) are due to the virus tropism for the intestinal tract, nasal passages, proximal trachea, and lungs [12].

Lung microscopic lesions were indicative for interstitial pneumonia specific for viral respiratory infections [4-12]. In studied samples, there were no intracytoplasmic inclusion bodies in bronchial epithelial cells as reported by others [9-13] in infections with bovine respiratory syncytial virus (BRSV) and bovine parainfluenza 3 virus (BPI3V). We also confirm that interstitial pneumonia is of marked viral etiology unlike bronchopneumonia whose etiology is mainly bacterial *(E. coli, Mannheimia haemolytica, Pasteurella multocida, Mycoplasma bovis, Histophilus somni)* [13].

Microscopic lesions in intestines (atrophy and superficial catarrhal desquamative inflammation) can be associated with viral replication. Unlike these findings, such changes in rotaviral enteritis are observed in the middle part of villi of both small and large intestines [13,14]. The macro- and micro lesions in the lung and the gastrointestinal tract of calves affected by pneumoenteritis are relevant with regard to the differential diagnosis of the syndrome and its differentiation from respiratory (IBR, BVD, BRSV, *M. haemolytica* etc.) and intestinal *(Cryptosporidium parvum*, bovine rotaviruses, bovine coronaviruses and *E. coli* K99 [F5]) diseases in this category of animals.

The present study confirmed that the used antigenic and ICH laboratory tests are of good diagnostic value with respect to viral infections in ruminants [11-14]. IHC can be a very useful test to confirm the etiology. IHC can also be useful in cases with histologic lesions of viral enteritis but for which other microbiologic tests have not confirmed viral infection. Use of IHC for confirming the presence of these enteric pathogens is most successful when tissues are collected and placed in formalin. This sampling method ensures preservation of potentially virus-infected epithelium covering intestinal villi [14,15].

The electron microscopy findings for polymorphonuclear cells and agranulocytes in alveolar spaces and among the respiratory epithelium support the viral etiology of the infection [11-13]. Viral particles whose morphology corresponded to that of the coronaviral agent have been found out both in pneumocytes and enterocytes in some of studied animals. These data evidence that pneumocytes and enterocytes are the primary target cells for coronavirus infection [7-14]. We also affirm that predominating pathological findings were diffuse lesions in alveolar spaces, obliteration and interstitial changes and damage of apical surfaces of intestinal villi resulting from lining epithelium destruction [14,16]. The targets are the conserved nucleocapsid gene for detection of the virus and spike gene for epidemiologic investigation and strain differentiation. At present, there is no commercial test available for BCoV antigen detection in the United States. However, lateral flow immunoassays (LFT) are useful cow and calf-side tests, and are available in European Union for BCoV antigen detection in the feces [11].

The utilised antigenic, ultrastructural and virological diagnostic methods allowed concluding that they could be successfully used in the diagnostics of pulmonary and gastrointestinal viral infections in juvenile calves. Electron microscopy and IHC methods of lung and intestinal tissues are also important and applicable for diagnostics and in differential diagnostic recognition of the condition from other common diseases as IBR, BVD, BRSV, *M. haemolytica, C. parvum*, BRV and *E. coli K99 (F5*).

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Research Article

Evaluation of Genotoxic Effects of C_{60} Fullerene-γ-Fe_2O_3 and Multi-Wall Carbon Nanotubes-γ-Fe_2O_3 Nanoparticles

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Abstract

The use of magnetic nanoparticles in nanomedicine applications has increased significantly in recent years. Genotoxic evaluation of the nanomaterials used for this purpose is therefore very important. In our study, the genotoxic effect of C_{60} fullerene-γ-Fe_2O_3 and multi-wall carbon nanotubes-γ-Fe_2O_3 magnetic nanoparticles over a wide concentration range (0.1, 1.0, 5.0, 10.0, 25.0, 50.0, and 100.0 µg/plate) was investigated using the Bacterial Reverse Mutation Test. These magnetic nanoparticles did not cause genetic damage to *Salmonella typhimurium* TA100 and TA98 in the presence and absence of metabolic activation. Due to the rapid increase in the presence of nanoparticles in our daily lives, mutagenicity and toxicity data related to nanoparticles are quite valuable. For this reason, *in vivo* and *in vitro* studies that allow for effective evaluation of these compounds is of the utmost importance.

Keywords: *Genotoxicity, Nanotoxicity, Multi-wall carbon nanotubes-γ-Fe_2O_3, C_{60} Fullerene-γ-Fe_2O_3, Salmonella/Microsome mutagenicity assay*

C_{60} Fullerene-γ-Fe_2O_3 ve Çok Duvarlı Karbon Nanotüpler-γ-Fe_2O_3 Nanopartiküllerinin Genotoksik Etkilerinin Değerlendirilmesi

Öz

Nanotıp uygulamalarında manyetik nano parçacıkların kullanılması son yıllarda önemli ölçüde artmıştır. Bu amaçla kullanılan nano malzemelerin genotoksik değerlendirilmesi bu nedenle çok önemlidir. Çalışmamızda, C_{60} fullerene-γ-Fe_2O_3 ve çok duvarlı karbon nanotüpler γ-Fe_2O_3 manyetik nanopartiküllerin geniş bir konsantrasyon aralığında (0.1, 1.0, 5.0, 10.0, 25.0, 50.0 ve 100.0 µg/plaka) genotoksik etkisi Bakteriyel Geri Mutasyon Testi kullanılarak araştırıldı. Bu manyetik nanopartiküller, metabolik aktivasyonun varlığında ve yokluğunda *Salmonella typhimurium* TA100 ve TA98'de genetik hasara neden olmamıştır. Günlük yaşamlarımızda nanopartiküllerin varlığındaki hızlı artış nedeniyle, nanopartiküller ile ilgili mutajenite ve toksisite verileri oldukça değerlidir. Bu nedenle, bu bileşiklerin etkili bir şekilde değerlendirilmesine olanak sağlayan *in vivo* ve *in vitro* çalışmalar oldukça önemlidir.

Anahtar sözcükler: *Genotoksisite, Nanotoksisite, Çok duvarlı karbon nanotüpler-γ-Fe_2O_3, C_{60} fullerene-γ-Fe_2O_3, Salmonella/Mikrozom mutajenite testi*

INTRODUCTION

Nanotechnology deals with the production of nanometer-scale (1-100nm) materials with the appropriate size and features to suit the potential area of use [1,2]. Nanotechnology products such as polymeric nanomaterials, fullerenes, single-walled carbon nanotubes, multi-walled carbon nanotubes, magnetic nanoparticles, and quantum dots have a wide range of applications [3]. As the particle size of a material decreases to the nanometer range, its physical and chemical properties vary considerably from those of its larger counterparts [4]. With these developments, it

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is important to examine the effects of nanomaterials on living organisms, such as their toxicity and mutagenicity.

It is difficult to design multifunctional nanoparticles with the necessary properties to be effective for both the diagnosis and treatment of diseases. One of the important precursors in this area is magnetic nanoparticles. We have studied the mutagenesis of γ-Fe_2O_3 magnetic nanoparticles containing multi-wall carbon nanotubes and C_{60} fullerene, which are potentially useful in nanomedicine. In the recent past, nanomedical applications of magnetic nanoparticles have been investigated due to their superparamagnetic moments with high magnetic saturation [5].

Superparamagnetic iron oxide nanoparticles (SPION) with different surface chemistries are used in many *in vivo* nanomedical applications such as magnetic resonance imaging contrast enhancement, tissue repair, immunoassay, detoxification of biological fluids, hyperthermia, drug delivery, and in-cell separation [6].

Carbon nanotubes (CNTs) are nanomaterials with very specific physicochemical properties that can be used as biosensors in nanotechnology and medical applications, as new vehicles for diagnosis and treatment of diseases, and as molecular carriers for drug delivery [7]. However, research on CNTs is steadily on living organisms as new applications for these materials as medical devices for treatment and diagnosis are discovered [8]. However, little work has been done on the effects of CNTs on human and other living things, and this effect varies depending on the nature of the CNTs, the duration of exposure, and the dose [9].

Fullerenes, especially C_{60}, have very good physical and electrochemical properties for use in medical fields. Fullerenes may have different activity areas, including antiviral activity as an inhibitor of HIV protease and antioxidant activity as a radical scavenger. However, when fullerenes are exposed to light, singlet oxygen is produced, which can lead to DNA damage by direct electron transfer. At the same time, fullerenes can be used as a tool in gene and drug delivery. Although the nanoparticle properties such as the suitability for injection and high accumulation potential for target organs are important for medical applications, the most important property is nontoxicity [10]. Genotoxicity and cytotoxicity studies are common due to increased concern about the toxic potential of nanoparticles [11].

In our study, the genotoxic effect of C_{60}-γ-Fe_2O_3 and MWCNT-γ-Fe_2O_3 nanoparticles at a wide range of concentrations was tested using the OECD test guidelines 471 (Bacterial Reverse Mutation Test). The principle of this test is to detect mutations that inhibit the synthesis of certain essential amino acids and cause recurrence of mutations previously formed in test strains [12]. In the Bacterial Reverse Mutation Test (Salmonella/microsome mutagenicity assay), the S9 mix is used for metabolic activation. The S9 mixture acts as an imitator of the oxidative system responsible for the oxidative, peroxidative, and reductive metabolisms of various biological or chemical substances containing cytochrome P-450. Metabolic activation (S9) is therefore a valuable method for detecting changes in mutagenicity as a result of biotransformation of a compound during *in vitro* studies [13-15].

The main objective of this work is to determine the genotoxicity of C_{60} fullerene-γ-Fe_2O_3 (C_{60}-γ-Fe_2O_3) and multi-wall carbon nanotubes-γ-Fe_2O_3 (MWCNT-γ-Fe_2O_3) nanoparticles, which will be used in biomedical applications such as drug transport.

MATERIAL and METHODS

Nanoparticles

The C_{60}-γ-Fe_2O_3 and MWCNT-γ-Fe_2O_3 nanoparticles used in this work were synthesized by Dr. Kilinc [3,16]. In this study, Sodium azide was used as a positive control and DMSO/water was used as a negative control to determine the mutagenicity of the nanoparticles. The positive controls used in this study were 1 µg/plate Sodium azide (NaN_3) for TA100, 1.5 µg/plate 2-amino-fluorene (2-AF) for TA98.

Instrumentation

Concentration of Fe was measured by ICP-OES (Perkin Elmer, Optima 2100 DV) at a wavelength of 238.204 nm. Infrared spectra of cMWCNT and MNP at 4000-400 cm intervals were recorded by FT-IR (Mattson Model 1000). Model P525 Vibrating Sample Magnetometer (VSM) was used as a physical property measurement system (PPMS). To obtain SEM and HR-TEM images of MNPs, a LEO-Evo 40XVP scanning electron microscope and a Jeol JEM 2100 FHR TEM were used at 200 kV with a probe size below 0.5nm [3,16].

Synthesis of cMWCNT-γ-Fe_2O_3 Magnetic Nanoparticle

1.25g MWCNT was refluxed in 50 mL 1.0 mol/L HNO_3 for 24 h in order to expose the raw MWCNT to strong acidic conditions for carboxylation. Then, the dispersion was exposed to a mixture of H_2SO_4 and HNO_3 (3:1, v/v) at 30°C for 5.0 h. The cMWCNT obtained by filtration was then dried at 85°C for 24 h.

1.25g of MWCNT was refluxed in 50 mL of 1.0 mol/L HNO_3 for a day. Then it was dispersed in a mixture of H_2SO_4 and HNO_3 (3:1, v/v) and sonicated at 30°C for 5.0 h. Then, cMWCNT (oxidized and shortened) was filtered and dried in oven at 85°C for a day. $FeCl_3 \cdot 6H_2O$ and $FeCl_2$ (mole ratio 2:1) were dissolved in 40 mL of distilled water and 0.1g of cMWCNT was added to it by vigorously stirring. 30 mL of NH_3 was added dropwise for a time of 60 min at 80°C. Resulting black solution was filtered and cMWCNT-γ-Fe_2O_3 as black precipitate was washed with distilled water until it was neutral. Then, it was dried in oven at 90°C for a day [16].

Synthesis of C60-γ-Fe_2O_3 Magnetic Nanoparticle

γ-Fe_2O_3 (0.36g) was added to the solution of 0.17g of C60 fullerene dissolved in 100.0 mL of toluene. The mixture was sonicated for 5.0 min at 30°C and vigorously stirred for 3 days at room temperature. $FeCl_3.6H_2O$ and $FeCl_2$, at the molar ratio of 2:1, were dissolved in distilled water and stirred in a 100.0 mL three necked flask. 30.0 mL of 5% NH_4OH solution was added dropwise at 75°C with vigorous stirring for about 2.0 h under nitrogen purge. It was subsequently washed with distilled water, toluene, and absolute ethanol until the C60 absorption peak disappeared by monitoring UV-VIS spectra at 554nm [3].

Characterization of cMWCNT-γ-Fe_2O_3 and C60-γ-Fe_2O_3 Magnetic Nanoparticle

Approximately 0.01g of cMWCNT and cMWCNT-γ-Fe_2O_3 were weighed. First 3.0 mL concentrated HCl and then 0.5 mL H_2O_2 and 1.0 mL HNO_3 added into the beaker and heated to dryness. The resulting cMWCNT-γ-Fe_2O_3 MNPs and cMWCNT were dissolved in 50 and 5.0 mL of 1.0 mol/L HNO_3, respectively. The Fe concentrations in the cMWCNT-γ-Fe_2O_3 magnetic nanoparticle and raw cMWCNT from were measured by ICP-OES. Surface functionality were investigated by FT-IR spectra. The chemical structure of γ-Fe_2O_3 nanoparticles functionalized with cMWCNT was investigated by XRD. The magnetic saturation value of MNPs were determined using VSM. Macro-structures of nanomaterials were determined by SEM while morphology and microstructures were determined using HR-TEM [3,16].

Bacteria Strains

The strains TA98 and TA100 of *Salmonella typhimurium* were purchased from the Salmonella genetic stock center at University of Calgary, Canada and genetically controlled [17]. Salmonella strains were used with or without the S9 mix [18].

S9-based Metabolic Activation System

S9 was prepared using a mixture of metabolic activator, phosphate buffer (0.2 M), 130 µL deionized water, S9 fraction 100 µL, KCl (0.33 M), $MgCl_2$ (0.1 M), NADP (0.1M), and glucose-6-phosphate (0.1M). The supernatant from liver homogenate of rats exposed to phenobarbital was used for elution of S9 fractions [18,19].

Bacterial Reverse Mutation Assay

Bacterial reverse mutation assays were performed according to Maron and Ames [18]. Basically, histidine-independent and histidine-dependent mutations were detected using *S. typhimurium* TA98 and TA100 test strains in the presence or absence of S9 mix.

S. typhimurium TA98 and TA100 test strains supplied as lyophilized strains were diluted by adding 1 mL of nutrient broth under aseptic conditions. Then, the diluted culture was transferred into 4 mL of nutrient broth. Single colony planting on nutrient-scored plaques by taking a drop of diluted cultures. Plates were maintained at +4°C for genotyping strains. Control tests performed for mutant strains before mutation tests were performed include control of histidine requirement, control of R factor, control of Rfa mutation, control of *uvr*B mutation and control of the number of spontaneous return colonies. Master plates, which can be stored at +4°C, were also prepared simultaneously with the control of genetic properties. Plates containing selective nutrient media prepared for the control of each property were plated on the same column with sterile toothpicks from the test strips, which were reduced by one colony, to the plate of MGA, MGA + histidine/biotin, ampicillinous nutrient agar and two nutrient agar (for UV and master plaque) respectively. The plates were incubated at 37°C. After incubation, the colonies bearing the full genetic traits were labeled on the master plate and stored at +4°C for use in culture preparation. Positive mutagen control used to detect bacterial responses to known standard mutagenes was made in parallel with the essential experiment. *S. typhimurium* TA98 was used in the presence of the 2-Nitrofluorene S9 mixture, an indirect mutant for the strain, and in the absence of the direct mutant 2-Nitrofluorene S9 mixture. Sodium azide was used as a direct mutagen for *S. typhimurium* TA100 in the presence and absence of S9 mixture. The 'standard plate incorporation' method was used when running the Ames *Salmonella*/microsomal test system. In this technique; Two different experiments were carried out in the presence and absence of S9. In the mutagenicity/antimutagenicity studies performed with *S. typhimurium* standard test strains, it is predicted that 1-2x10^9 Colony forming unit (CFU) will be one mL of bacterial cultures. For the experiment, first night culture was prepared. The master platelet colony was inoculated into 40 mL of liquid medium and left in the shaker (1-2x10^9 cfu/mL, OD540=0.1-0.2) at 37°C at 120 rpm for 11-13 h. In the experiments in the absence of S9, 100 mL of soft agar, previously liquidified in a hot water bath and adjusted to a temperature of 45-50°C., were distributed in 2 mL of sterile glass tubes of 2 mL each with the addition of 10 mL of 0.5mM histidine/biotin solution. Then 0.1 mL of bacterial culture and 0.1 mL test compound (different concentrations of nanoparticules) were added to the tubes and vortexed for 3 seconds at low speed before spreading to plates containing MGA at room temperature. The mixing-pouring-spreading process is carried out so that the soft agar does not freeze and spread over the entire surface of the plaque, not exceeding 20 sec. The same procedure was applied to positive and negative controls. In experiments in the presence of S9, 100 mL of a soft agar, previously liquidified in a hot water bath and adjusted to a temperature of 45-50°C, was added to 10 mL of a 0.5mM histidine/biotin solution and 2 mL of sterile 13×100mm glass tubes It was distributed.

Antimutagenicity experiments were performed in the absence of S9 with *S. typhimurium* TA98 and TA100 strains.

In the antimutagenicity experiments, the overnight culture was firstly prepared as described in the mutagenicity test. 100 mL of a soft agar, which was liquidated in a hot water bath and adjusted to a temperature of 45-50°C, was distributed in sterile glass tubes of 2×3 mL of sterile glass. Then 0.1 mL of bacterial culture, 0.1 mL of mutagen (dounomycine for TA98, sodium azide for TA100), 0.1mL of test compounds at different concentrations and 500 µL of phosphate buffer were added to 2 mL of the top agar containing 0.5 mM histidine/biotin were added to the tubes and vortexed for 3 seconds at low speed before spreading to plates containing MGA (minimal glucose plates). Plates were allowed to incubate for 48-72 h at 37°C. The number of colonies formed after the incubation was recorded. Each dose was tested in parallel on three plates and three independent experiments were performed at different times. The decrease in the number of colonies was calculated according to the following formula as percent inhibition.

For the antimutagenicity assays, the inhibition % was calculated according the formula given below.

(Inhibition % = [1–T/M] ×100)

Where T is the number of revertants per plate in the presence of mutagen, and the test sample, and M is the number of revertants per plate in the positive control. The antimutagenic effect (% inhibition) between 25-40% defined as moderate antimutagenicity, 40% or more as strong antimutagenicity, and 25% or less inhibition as no antimutagenicity [20].

Statistical Analysis

All experiments were carried out in triplicate and the results are expressed as means ± standard deviation (SD). Statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's multiple comparison post-test to verify the significance of a positive response. SPSS software version 14.0 (Illinois, USA) was used for statistical tests, and a p value of $P<0.05$ was considered statistically significant.

RESULTS

Results of the Salmonella microsome test are given in *Table 1* and *Table 2*. Mutagenic assays of both MWCNT-γ-Fe_2O_3 and C_{60}-γ-Fe_2O_3 using a wide range of concentrations (100, 50, 25, 10, 5, 1, and 0.1 µg/plate). MWCNT-γ-Fe_2O_3 did not increase the number of revertant colonies in S. *typhimurium* TA98 and TA100 with and without the metabolic activation mix (S9) *(Table 1)*. Similarly, there was no significant change in the number of revertant colonies in *S. typhimurium* TA98 and TA100 used to investigate the mutagenic potential of C_{60}-γ-Fe_2O_3, both in absence and in presence of the metabolic activation system (S9) *(Table 2)*. However, Sodium azide (NaN_3; for TA100) and 2-amino-fluorene (2-AF; for TA98) used as positive controls significantly increased the number of revertant colonies statistically. Genotoxicity testing is considered a valuable tool for evaluating the carcinogenic risk of nanoparticles [12]. However, a single test cannot identify all relevant genotoxic substances. Therefore, several *in vitro* and *in vivo* tests are typically used to assess genotoxicity. Spontaneous mutational DNA damage in TA98 strain is reverted to wild-type by specific mechanisms of frameshift (templated mutations at CG sequence), which do not occur in TA100 (base substitution at CG sequence).

C_{60} fullerene-γ-Fe_2O_3 [3] and MWCNT-γ-Fe_2O_3 [16] were successfully used adsorptions of flurbiprofen, a non-steroidal anti-inflammatory drug and harmane, one of the most potent tremor producing β-carboline alkaloids. By considering, the results it could be concluded that both of nanomaterials could be used in biomedical application include adsorption and controlled release of drug as nanocarriers.

Table 1. *Spontaneous revertant colonies induced by MWCNT-γ-Fe_2O_3 to S. typhimurium (TA 98, TA 100) with and without metabolic activation (S9)*

Dose (µg/plate)	TA100		TA98	
	-S9	+S9	-S9	+S9
0	89.6±3.4	91.0±12.0	35.3±4.5	33.0±2.0
0.1	100.6±13.0	98.0±12.3	27.3±1.5	36.6±5.5
1	81.3±7.5	98.6±5.1	32.3±5.7	38.3±8.7
5	88.0±0	96.3±10.4	30.3±2.5	40.0±11.2
10	87.3±9.5	102.6±7.5	33.6±9.7	31.3±9.0
25	72.3±5.5	93.6±5.6	38.0±8.0	37.3±4.6
50	87.0±6.0	86.0±13.0	34.0±2.0	30.3±4.1
100	85.0±2.0	88.0±3.4	38.3±2.0	36.0±4.6
DMSO/water	89.0±6.8	89.6±8.0	35.3±4.2	44.3±4.8
Positive Control	960.6±30.4[b]	1615±45.9[b]	368.6±37.8[c]	704.3±24.5[c]

All values are expressed as mean±standard deviation (SD). *[a] DMSO/water, [b] Sodium azide (1 g/plate) (Positive control), [c] 2-Nitrofluorene (2 g/plate) (Positive control), * P<0.05 (ANOVA+Dunnett's multiple comparison post test)*

Table 2. *Spontaneous revertant colonies induced by C60-γ-Fe_2O_3 to S. typhimurium (TA 98, TA 100) with and without metabolic activation (S9)*

Dose (µg/plate)	TA100		TA98	
	-S9	+S9	-S9	+S9
0	89.6±6.0	91.6±8.3	30.3±4.5	44.6±2.0
0.1	67.0±5.5	92.3±11.2	38.6±11.7	39.3±3.7
1	81.3±8.5	70.3±6.8	37.6±10.6	35.0±5.5
5	85.0±9.8	89.3±8.6	34.6±8.5	33.6±6.1
10	117.3±29.8	80.0±11.0	36.3±2.0	32.0±2.0
25	119.0±13.4	92.3±10.6	31.6±3.7	47.0±11.5
50	87.6±4.9	88.0±5.2	34.6±4.6	35.6±5.5
100	88.6±16.0	91.0±4.0	38.3±3.2	34.3±3.5
DMSO/water	89.0±6.8	89.6±8.0	35.3±4.2	44.3±4.8
Positive control	960.6±30.4[b]	1615±45.9[b]	368.6±37.8[c]	704.3±24.5[c]

*All values are expressed as mean±standard deviation (SD). [a] DMSO/water, [b] Sodium azide (1 g/plate)(Positive control), [c] 2-Nitrofluorene (2 g/plate) (Positive control), * P<0.05 (ANOVA + Dunnett's multiple comparison post test)*

DISCUSSION

Recent biomedical studies have shown that magnetic nanoparticles may be an important tool for *in vivo* and *in vitro* applications. Most magnetic nanoparticles contain superparamagnetic iron oxides such as magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3). For medical use, it is very important that these particles do not show any toxic effects [21]. In this study, C_{60}-γ-Fe_2O_3 and MWCNT-γ-Fe_2O_3 nanoparticles were investigated for their genotoxic potential. The nanoparticles of superparamagnetic iron oxide nanoparticles used in nanomedicine should have diameters of 5-100 nm to ensure similarity to biological macromolecules and for cell compatibility.

In our study, we used nanoparticles appropriate for medical applications, although the two have different chemical structures. Therefore, this study on genotoxic potential of test samples is very important. Carbon nanotubes can exhibit toxic effects at different levels due to their different sizes and properties, such as different surfaces [22]. However, it may be suggested that similarities between the high aspect ratio nanoparticles (HARNs) and asbestos result in similar toxic potentials, as suggested by the HARNs theory [23]. According to the previous studies as the size of the nanoparticle decreases, the penetration and thus possibility of the toxicity increases [24,25]. Nanoparticle penetration mechanisms are thought to occur by means of adhesive interaction or passive diffusion without a specific receptor on their surface. Electrostatic charges, steric interactions, Van der Waals interactions or interfacial tension effects; can provide penetration of the nanoparticles without vesicles [26,27]. Penetration of the nanoparticles into the cell can occur without the utilization of phagosomes [28]. Previous studies have indicated that C_{60} molecules penetrate through the nuclear membrane after penetration into the cell. However, it has been demonstrated that fullerenes inhibit the activity of HIV protease by binding to the active site, which is a basic enzyme for HIV virus [29]. Nanoparticles used in our study were observed by TEM to have spherical sizes less than 10 nm and 5 nm for C_{60}-γ-Fe_2O_3 and MWCNT-γ-Fe_2O_3, respectively [3,16]. Although the nanoparticle sizes in our study were quite small, their physical and chemical should be evaluated in terms of toxicity.

The detailed mechanisms of nanoparticle-induced genotoxicity are not completely understood and it is furthermore not clear if there are any nano-specific effects on DNA [30]. The "nano-specific effect" means a mechanism of toxic action especially in particles whose initial sizes change 1-100 nm while not associated with those of different sizes and similar chemical compound. Genotoxicity mediated by particle can be categorized in either "primary genotoxicity" or "secondary genotoxicity". The former intends to self-genotoxicity from the nanoparticles while the latter intends to increase in genotoxicity induced by reactive oxygen species (ROS) accumulated in course of particle-revealed inflammation [31]. Not only *in vivo* but also *in vitro* studies have indicated that ROS are produced by nanoparticles of diverse compounds (fullerenes, carbon nanotubes, quantum dots, and automobile exhaust). They cause oxidative damage in cellular components such as lipid peroxidation, protein carbonylations, DNA oxidation, interference in signaling functions, and modulations in gene transcription [32,33].

Previous studies of genotoxicity using the bacterial reverse mutation assay have shown that the increase in the number of returning colonies in bacteria exposed to multi-wall carbon nanotubes is not significant and does not cause mutagenic effects [1,19,22]. Similarly, in this study, the MWCNT-γ-Fe_2O_3 magnetic nanoparticles did not cause mutation under any conditions tested genotoxicity of *(Table 1)*. Jia et al.[34] compared the genotoxic potential of single-wall nanotubes (SWNTs), multi-wall nanotubes

(with diameters ranging from 10 to 20 nm), and C_{60} fullerenes, and determined that C_{60} fullerene and multi-wall nanotubes showed lower cytotoxicity than single-wall nanotubes based on MTT assays. Based on these results, it can be concluded that the cobalt and iron traces of the MWCNT-γ-Fe_2O_3 magnetic nanoparticles we use do not generate genotoxic responses in this test system.

Fullerenes, especially C_{60} attractants, have widespread use in nanomedicine due to their physical and chemical properties [35-37]. Studies investigating whether cytotoxic C_{60} fullerene (C_{60}) is useful for various mammalian cells are available in the literature [38-40]. Studies on the genotoxic effect of C_{60} with the bacterial reverse mutation test (AMES) are also available in the literature [1,37,41]. Shinohara et al.[41] investigated the genotoxic effect of C_{60} nanoparticles by the Bacterial Reverse Mutation Test and found that the number of revertant colonies in the group exposed to C_{60} was less than two times that of the negative control, even with metabolic activation. The negative control of the number of return colonies caused by mutagenic acceptance of a test sample should be more than two-fold [37].

However, studies on the evaluation of the mutagenesis of magnetic γ-Fe_2O_3 (metal oxide) nanoparticles using AMES bioassay are limited [4,42]. Whereas the magnetic forms of nanomaterials are used for many nanomedical applications, Pan et al.[4] evaluated the mutagenesis of different metal oxide nanoparticles (Al_2O_3, Co_3O_4, CuO, TiO_2, and ZnO) by the Bacterial Reverse Mutation Assay. Their results showed that these metal oxide nanoparticles do not exhibit mutagenicity in the absence of S9 metabolic activation. However, in the presence of S9 activation, CuO, TiO_2, and ZnO showed mutagenic potential at different levels for some bacterial strains. In our study, it was observed that there was no significant change in the number of colonies returning by metabolic activation (S9 mix) of C_{60}-γ-Fe_2O_3 nanoparticles *(Table 2)*. This suggests that the nanoparticles used in the study do not cause oxidative or peroxidative effects. These results are reliable, given the authors' experience in the field [3,16]. C_{60} and MWCNT will continue to be valuable additions in the field of medicine, especially since Fe_2O_3-containing nanoparticles are magnetic and do not cause mutagenicity.

As a results of this study, the C_{60}-γ-Fe_2O_3 and MWCNT-γ-Fe_2O_3 magnetic nanoparticles tested in *Salmonella* microsome mutagenicity test have not been shown any muatagenic effect. Although these negative results observed in *Salmonella* microsome mutagenicity test, the further investigation needs to be done. The genotoxicity of C_{60}-γ-Fe_2O_3 and MWCNT-γ-Fe_2O_3 magnetic nanoparticles obtained both provide an overview of the current study and provide useful information for future investigations.

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Research Article

Mülki Tatbikat-ı Baytariyye Mektebi (Muavin Baytar Mektebi): Kuruluşu ve İşleyiş Esasları

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Öz

Mülki Tatbikat-ı Baytariyye Mektebi, Osmanlı Devleti'nin sivil veteriner hekimliği teşkilatını güçlendirecek uzman personel yetiştirmek amacıyla 1910 yılı Ekim'inde açılmış bir meslek yüksekokuludur. Kuruluşunda, salgın hayvan hastalıklarına karşı etkin bir mücadele sağlama hedefi temel etkendir. İlkin Muavin Baytar Mektebi adıyla kurulan okul vasıtasıyla kısa sürede veteriner hekim maiyetinde olmak üzere mesleki hizmet verebilecek uzman memurlar yetiştirilmesi, böylece salgın hastalıklarla daha aktif bir mücadele yapılması planlanmıştır. Ele alınan çalışma, söz konusu okulun iç hizmet yönetmeliği olarak günümüz Türkçesine aktarabileceğimiz "Dâhili Talimatnamesi"ni konu edinmektedir. Söz konusu talimatnamede okulun açılış amacı, akademik ve idari kadrosu, buraya ayrılan bütçe, öğrenci kabulündeki temel şartlar, derslerin idaresi ve içerikleri vb. muhtelif konu hakkında ayrıntılı bilgi mevcuttur. Buradan ve konuyla ilgili birincil kaynak niteliğindeki arşiv kayıtlarından hareketle okulun Türk veteriner hekimliği tarihine sunduğu katkıların değerlendirilmesi ve konu hakkındaki çalışmaların güncellenmesi amaçlanmıştır.

Anahtar sözcükler: *Mülki Tatbikat-ı Baytariyye Mektebi, Veteriner hekimliği tarihi*

An Evaluation of the Internal Service Regulation of the Mülki Tatbikat-ı Baytariyye Mektebi (Muavin Baytar Mektebi)

Abstract

Mülki Tatbikat-ı Baytariyye Mektebi is a vocational college opened in October 1910 with the aim of educating specialist staff to strengthen the organization of the Ottoman State's civilian veterinary medicine. In the establishment of the school, the main objective was to provide an effective fight against epidemic animal diseases. It was planned to train specialist officers who would be able to provide vocational services in the veterinary profession in a short time through this school which was established firstly as Muavin Baytar Mektebi. Thus, a more effective struggle against epidemic diseases would be achieved. The main subject of this study is the School's Internal Service Regulation (Partial Instruction). In the work supported by the archival records of the primary source, the contributions of Mülki Tatbikat-ı Baytariyye Mektebi to the history of Turkish veterinary medicine are tried to be evaluated.

Keywords: *Mülki Tatbikat-ı Baytariyye Mektebi (Civil Veterinary Health Technician School), History of veterinary medicine*

GİRİŞ

Türk sivil veteriner hekimliği tarihinde bir milat olarak kabul edilebilecek olan sivil veteriner hekimlik eğitiminin başlangıcından II. Meşrutiyet'in ilanına kadar geçen sürede veteriner hekimliği hizmetlerinin organizasyonuna yönelik çok sayıda yasal düzenleme yapılmıştır. Askeri öğrenciler ile birlikte sivil öğrencilerin de veteriner sınıfına kabul şartlarını belirleyen 1873 tarihli nizamname [1], sivil veteriner hekimlerin istihdam şartlarını belirleyen 1876 tarihli Mülkiye Baytarları Nizamnamesi [2] ve 1893'te veteriner merkezi teşkilatına şekil veren nizamnamenin [3] bunların en önemlileri olduğu söylenebilir. Yine 1888'de Nafia Nezareti'ne bağlı Umur-ı Baytariyye Müfettiş-i Umumiliği'nin kurulması ve 1889'da Sivil Veteriner Hekimliği Okulu'nun açılışı bu alandaki gelişimi hızlandıran diğer önemli adımlar olmuştur [4]. Ne var ki tüm bu girişimlere rağmen salgın hayvan hastalıklarının, özellikle de sığır

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vebasının, İttihat ve Terakki hükümetinin iktidara geldiği 1908 tarihinde henüz önü alınamamıştır [5].

Sığır vebası başta olmak üzere çeşitli hayvan hastalıklarının Basra'dan Rumeli'ye uzanan geniş bir coğrafyada çoğu kez salgın halinde hüküm sürmesi, Osmanlı merkezi otoritesi için yıkılışa kadar uzanan uzun zaman zarfında etkin bir sonuç alamadığı ve hazineye büyük yük getiren temel mücadele alanlarından biri olmuştur [6,7]. Taşrada görevli memurların 1900'lerin başından itibaren merkeze gönderdikleri resmi yazılarda salgınların şiddet ve nüfuzuna odaklanılmasının [8-12] bunun açık bir kanıtı olduğu söylenebilir.

Tecrübe edilen örnekler[1], günümüz meslek yüksekokullarına denk gelecek bir okul kurarak devletin sivil sahadaki veteriner hekimliği teşkilatlanmasını güçlendirme düşüncesini doğurmuştur. Bu çalışmada, söz konusu okulun iç hizmet yönetmeliği temel alınarak Türk veteriner hekimliği tarihine sunduğu katkıların değerlendirilmesi amaçlanmıştır.

MATERYAL ve METOT

Çalışmanın ana kaynağı, Tatbikat-ı Baytariyye Mektebi'nin *(Şekil 1)* iç hizmet yönetmeliği olarak günümüz Türkçesine aktarabileceğimiz "*Dâhili Talimatnamesi*"dir [13]. *Çalışma ayrıca i*lk el kaynak niteliğindeki döneme ait arşiv kayıtları ile desteklenmiştir. Cumhurbaşkanlığı Devlet Arşivleri Başkanlığı'nın Osmanlı Arşivi kısmına ait muhtelif fonda yer alan söz konusu arşiv belgesi aracılığıyla Okulun kuruluş ve gelişimi ile ilgili verilerin tespitine çalışılmıştır. Arşiv belgelerinin metin içerisindeki kullanımında günümüz Türkçesindeki karşılıkları tercih edilmiştir. Araştırma, tarih araştırmalarında uygulanan temel yöntemler olan analiz ve sentez yöntemleri ile değerlendirilmiş, konu kronolojik olarak ele alınmıştır.

BULGULAR

Bir Meslek Yüksekokulu Kurma Düşüncesinin Doğuşu

Salgın hayvan hastalıklarının neden olduğu sorunlardan dolayı, II. Meşrutiyet idaresinin hemen başlarında günümüz meslek yüksekokullarına denk gelecek bir âli mektep (yüksekokul) kurarak devletin sivil sahadaki veteriner teşkilatlanmasını güçlendirme düşüncesi doğmuştur [14]. Gerekli müzakereleri 1909 yılı sonbaharında yürüterek bu düşünceyi somutlaştıracak kararları almak üzere Şura-yı Devlet bünyesinde özel bir komisyon oluşturulur. Komisyon, Mülkiye Baytar Mektebi Müdürü ile Orman Maadin ve Ziraat Nezareti Baytar Umum Müdürü'nün görüşleri de alınarak ilk yıl 50 öğrenci kabul edecek ve takip eden yıllarda ihtiyaca göre öğrenci sayısını arttırabilecek iki yıllık bir meslek yüksekokulunun açılmasına karar verir [15]. Dâhiliye Nezareti'ne sunulan karar tezkeresine bakıldığında, kuruluş düşüncesinin ortaya çıkışındaki temel dinamiği bulaşıcı hayvan hastalıklarının oluşturduğu açıkça görülmektedir [15].[2] Devletin bu mücadelede temel dayanak olarak gördüğü sivil personelin arttırılması amacıyla, kurulacak olan okula kadro dışı kalan memurlar kayıt olacaklardır. Böylece belli bir eğitim seviyesine ulaşmış olduğu halde memuriyet elde edememiş olan kesimin de istihdamı ve dolayısıyla girişimden çok yönlü fayda sağlanması hedeflenmiştir [15,16].

Komisyon kararları Dâhiliye Nezareti kanalıyla Sadaret Makamına sunularak gerekli izin talep edilir. Ancak Sadaret, kuruluş onayından önce okulun nerede açılacağı, hangi birime bağlı olacağı, idaresinin nasıl olacağı gibi başlıkların açıklığa kavuşturulmasını ister [15,16]. Dolayısıyla konu devletin bununla doğrudan ilgili birimi olan Orman Maadin ve Ziraat Nezareti'ne[3] havale edilir. Nezaret bünyesinde Umur-ı Baytariyye Müdüriyeti tarafından gerekli talimat ve nizamname tasarıları hazırlanarak okulun kuruluş ve idaresi ile ilgili temel unsurlar netleştirilir. Söz konusu tasarılardan ilki, çalışmanın ana konusunu oluşturan *Tatbikat-ı Baytariyye Mektebi Dâhili Talimatnamesi* (Okulun İç Hizmet Yönetmeliği) [13], ikincisi ise okul mezunlarının istihdam şartlarını düzenleyen *Tatbikat-ı Baytariyye Memurları Nizamnamesi*'dir [17]. Hazırlanan metinler Şura-yı Devlet'te[4] konuyla ilgili oluşturulan özel komisyona sunulur [16].

Ziraat Nezareti, okulun Mülkiye Baytar Mektebi yakınında kiralanacak bir konakta açılabileceğini, böylece öğrencilerin ameliyatlar ile uygulamalı dersleri burada görebileceklerini öngörmektedir. Okulun idaresi ise Mülkiye Baytar Mektebi'nde olduğu gibi Ziraat Nezareti'ne bağlı olacaktır. Kuruluşu için 2500 liralık bir masraf belirlenmiştir. Nezaret bunun 1910-1911 (Rumi 1326) mali yılı bütçesinde *Ziraat ve Umur-ı Baytariyye Kalemi* başlığı altında kendisine tahsis edilen ödeneğe ilavesini ister [18].

[1] Örneğin, 1901 yılında Basra Vilayetine bağlı Müntefik Sancağında görülen hayvan hastalığı nedeniyle vilayetten gelen talebe bağlı olarak buraya bir askeri baytar gönderilmiştir [8]. Yozgat Sancağına 1903 yılında hayvan hastalığı ihbarı üzerine gönderilen baytar müfettişi, burada şiddetli bir sığır vebası salgınının yaşanmakta olduğunu merkeze bildirmektedir [9]. Mamuretülaziz (Elazığ) Vilayetinden 1905 yılında merkeze gönderilen bir telgrafta ise hastalığın büyük yayılım gösterdiği dile getirilmekte ve tek bir veteriner hekimin buna karşı mücadelede yeterli olamayacağı beyan edilerek iki hekim talep edilmektedir [10]. Yanya Sancağından Haziran 1906'da gönderilen başka bir yazıda salgının Berat'ta hızla yayılmakta olduğu bildirilmektedir. Etkin bir mücadele yapılabilmesi buraya ivedi bir baytar müfettişinin gönderilmesine bağlanmıştır [11]. Aynı yılın kış aylarında ise Başkentte bilad-ı selase (Eyüp, Galata, Üsküdar) ile Beykoz, Kartal, Makriköy (Bakırköy) taraflarında ortaya çıkan bir salgın için tedbir alınmasına çalışıldığı görülmektedir [12].

[2] Okulun açılış gerekçesi, karar tezkeresinde şu ifadelerle dile getirilmektedir; *"Baytar memurlarının adem-i kifayetinden naşi taşrada zuhur eden hayvan hastalıkları tevsi-i daire-i sirayet etmekde ve birçok telefatı mucib olmakda olmasına nazaran baytar memurlarının tezyid-i mikdarıyla taşrada icab eden mahallere izam ve istihdamları elzem görünmektedir".*

[3] Osmanlı Devleti'nin tarım ve hayvancılık politikalarını yürütmekle sorumlu olan ve Tanzimat Dönemi bürokratik reformu sonucu kurulan temel kurumu Ziraat Nezareti'dir. Muhtelif defalar isim değişikliğine konu olan Nezaretin ele alınan tarihlerdeki adı Orman Maadin ve Ziraat Nezareti'dir.

[4] Şura-yı Devlet, Osmanlı Devleti'nin Tanzimat Döneminde yürütülen bürokratik reform sonucu oluşturulan ve fonksiyonları bakımından günümüzdeki Danıştay'la benzerlik gösteren devlet kurumudur.

Şekil 1. Tatbikat-ı Baytariyye Mektebi öğrencileri öğretmenleri ile [14]

Umur-ı Baytariyye Müdüriyeti tarafından hazırlanan ödenek talebi onaylanarak 1910-1911 mali yılı genel bütçesine dâhil edilir. Okul, Ziraat Nezareti'nin bütçe taslağına kıyasla birkaç küçük değişiklik yapılarak 1470 liralık (147 bin kuruş) bir masrafla 14 Ekim 1910'da İstanbul'da Ahırkapı mevkiinde açılmıştır. Bu tarihteki resmi adı "*Mülkiye-i Baytariyye Muavinlerine Mahsus Mekteb*"; yani Muavin Baytar Mektebi'dir [12,14].[5] Kaynaklar, bir sonraki mali yıl için okul ödeneğinin 2700 liraya yükseltildiğini (270 bin kuruş) göstermektedir [13,18].

Muavin Baytar Mektebi, kuruluşundan yaklaşık bir yıl sonra *Tatbikat-ı Baytariyye Mektebi* adını alır. Bu değişikliğin tarihi tam olarak tespit edilemese de 1911 yılına ait bazı arşiv belgeleri böyle bir isim değişikliğine gidildiğini açıkça göstermektedir [19,20]. Ancak bu, uzun soluklu bir düzenleme olmamış, yaklaşık iki yıllık kısa aradan sonra eski ismin kullanılmasına devam kararı alınmıştır [21].[6] Yine 1911'de yapılan bir düzenleme ile Muavin Baytar Mektebi (diğer adıyla Tatbikat-ı Baytariyye Mektebi) öğrencileri, öğrenimleri devam eden idadi ve âli mektepler (lise ve yüksekokullar) öğrencileri gibi askerlikten muafiyet hakkı kazanmışlardır [19,20,22-24]. Tanzimat'ın eğitim alanındaki reformlarının bir ürünü olarak 1912 yılında Okulun ilk mezunları Mülkiye Baytar Mektebi mezunlarıyla birlikte diğer yükseköğretim kurumlarındaki benzerleri gibi yurtdışına uzmanlık eğitimi almak üzere gönderilmişlerdir. Bunların kimler oldukları saptanamamakla birlikte, 1912 yılı ortalarına doğru yurda döndükleri bilgisi mevcuttur [25,26].[7]

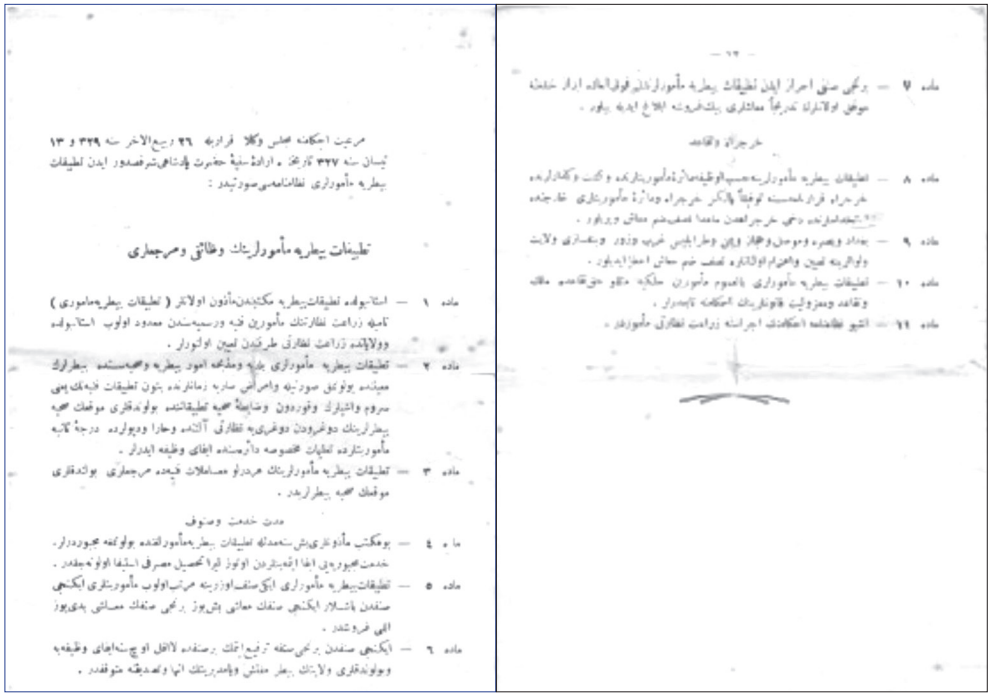
Okul mezunlarının istihdam şartları ile görev ve sorumlulukları ise 26 Nisan 1911 tarihli *Tatbikat-ı Baytariyye Memurları Nizamnamesi* ile belirlenmiştir [13,17]. Nizamnamede istihdam edilecek tatbikat memurlarının görevleri günümüz ifadesiyle; *"veteriner hekim maiyetinde olarak belediyelerin veya mezbahaların veteriner hekimliği hizmetlerini görmek, salgın vakalarında serum ve aşıların hazırlanıp uygulanmasında, kordonlarda ve zabıta-i sıhhiye uygulamalarında doğrudan bulundukları bölgenin sıhhiye veteriner hekimi denetim ve emrinde; haralarda ve depolarda ise ikinci derece memuriyetlerde ilgili yönetmeliklerle çerçevelenmiş görevleri yerine getirmek"* şeklinde gösterilmektedir [madde. 2]. Bağlı bulundukları idari bölgenin sıhhiye baytarları, uyguladıkları her türlü fenni işlem için tatbikat memurlarının yetkili mercileri tayin edilmişlerdir [madde. 3] [13,17].

Bu suretle görev ve hizmet tanımı yapılan baytar tatbikat memurları (diğer söylemiyle muavin baytarları) ilgili yönetmelik gereği iki sınıf olarak şekillendirilmiş ve beş yıl zorunlu hizmete tabi tutulmuşlardır. Bağdat, Basra, Musul, Hicaz, Yemen, Trablusgarp, Zor ve Bingazi gibi Osmanlı

[5] Ziraat Nezareti'nin öngördüğü bütçeye kıyasla elde edilen ödenekteki en büyük fark, muallim maaşlarının oranıdır. Nezaret, muallim maaşlarını iki sınıf üzere değerlendirerek birinci sınıf muallimlere 1500, ikinci sınıf muallimlere 1000 kuruş maaş öngörürken, bu rakamlar onaylanan ödenekte birinci sınıflar için 1350 ve ikinci sınıf muallimler için ise 1200 kuruş olarak yer almıştır.

[6] Okulun bağlı olduğu birim olan Ticaret ve Ziraat Nezareti'nin resmi yayın organı olan Ticaret ve Ziraat Nezareti Mecmuası'nın 6 Aralık 1913 tarihli bir yazısında Nezaret tarafından alınmış olan söz konusu karar okuyucuya duyurulmuştur.

[7] Biri Meclis-i Vükelâ tutanağı olmak üzere 1912 yılına ait iki arşiv kaydı, Tatbikat-ı Baytariyye Mektebi'nin aynı yıl mezun olup yurtdışına eğitimlerini tamamlamak amacıyla gönderilmiş olan talebelerinin yurda dönmek üzere olduklarını göstermektedir.



تعليمات بيطريه مأمورلرينك وظائف ومرجعلري

مرتبت احكامه عطس وكلا قراريله ٢٦ ربيع الاخر سنه ٣٢٩ و ١٣ ثيان سنه ٣٢٧ تاريخلي ارادهٔ سنيهٔ حضرت پادشاهي شرفصدور ايدن تعليمات بيطريه مأمورلري نظامنامه‌سي صورتيدر :

ماده ١ — استانبولده تعليمات بيطريه مكتبندن مأذون اولانلر (تعليمات بيطريه مأموري) نامله زراعت نظارتنك مأمورين فنيه ورسيه‌سندن معدود اولوب استانبولده وولايتلرده زراعت نظارتي طرفندن تعيين اولنورلر .

ماده ٢ — تعليمات بيطريه مأمورلري بيطريه وظيفه‌سنه دائر بيطرلرك مبينه‌سنه يوكلنمش صورتيله واجرآسي سايه زمانده بنون تعليمات فنيه‌يه تمامي سروم واشيانك وفوردون وضايعهٔ صحيه تعليماتنده يولندقلري موقعلرده صحيه بيطرلرينك دوغرودن دوغريه نظارتي آلتنده وحارا ويولارده درجهٔ كافيه مأمورلرندن تعليمات مخصوصه دائرسنده ايفاي وظيفه ايدرلر .

ماده ٣ — تعليمات بيطريه مأمورلرينك هردرلو معاملات فنيه‌ده مرجعلري بولندقلري موقعلك صحيه بيطرلريدر .

مدت خدمت وصنوف

ما ده ٤ — يوكسكتب مأذونلري بش سنه‌دنبري تعليمات بيطريه مأمورلقنده بولونمغه مجبوردرلر . خدمت مجبوريه‌لري ايفا ايتمدكجه اونلره تحصيل مصرف استيفا اولونه‌مجدر .

ماده ٥ — تعليمات بيطريه مأمورلري ايكي صنف اوزرينه مرتب اولوب مأمورلقلري ايكنجي صنفدن باشلار ايكنجي صنفك معاشي بش يوز رقمي صنفك معاشي بدي يوز الي فرو شدر .

ماده ٦ — ايكنجي صنفدن برنجي صنفه ترفيع ايتمك ايچون صنفده لااقل اوچ سنه ايفاي وظيفه ويولندقلري ولايتلرك بيطر معلمي ويامديريتلرك انها وتصديقه متوقفدر .

ماده ٧ — برنجي صنفي احراز ايدن تعليمات بيطريه مأمورلرندن فوق العاده ارار خدمتلرنه موافق اولانلرك تدريجاً معاشلري بش شر ليره قدر ترفيع ايدر .

خروج وتقاعد

ماده ٨ — تعليمات بيطريه مأمورلريه حسب الوظيفه مأموريتلرنده وكلت وكفالتلرنده خروج قرار لحسبته كوچك بالكز خروج ويا مأموريتلري خارجنده استعفالرنده دخي خروجلرنده نصف معاش ويريلور .

ماده ٩ — بغداد وبصره ومومي وعفار وزين وطرابلس غرب ووزون وسفاقس ولايات ولوازمه تعيين واعزام اولنانلره نصف يوم معاش اعطا ايدلور .

ماده ١٠ — تعليمات بيطريه مأمورلري نامنه مأمورين حكميه مكلو حقوقلرنه مالك وقائد ومسئوليت قانونيه احكامه تابعدرلر .

ماده ١١ — اشبو نظامنامه احكامنك اجراسنه زراعت نظارتي مأمورلر .

Şekil 2. Tatbikat-ı Baytariyye Memurları Nizamnamesi [13]

Devleti'nin uzak coğrafyalarında görevlendirilenlerine yarım maaşlık teşvik primi öngörülerek mesleğe ilginin artması hedeflenmiştir [madde. 4,5,9] [13,17].

Balkan Savaşları ile başlayıp I. Dünya Savaşı'na dek uzanan, neredeyse kesintisiz savaş sürecinde, Askeri Veteriner Hekimliği Teşkilatının yetersiz kalması üzerine Tatbikat-ı Baytariyye Mektebi mezunları da orduda hizmet vermişlerdir. Zira ihtiyacı karşılamak amacıyla emekli veteriner subayların göreve çağrılması ve sivil veteriner hekimlerin askeri veteriner hekimler yanında istihdamı yönündeki acil durum tedbirleri yetersiz kalmıştır [27,28].[8] Bunun üzerine Harbiye Nezareti'nin talebiyle Okul mezunlarının seferberlik süresiyle sınırlı olmak koşuluyla istihdamları yoluna gidilmiştir [28]. İlgili Kanun hükümlerinden anlaşıldığı kadarıyla Tatbikat-ı Baytariyye Mektebi mezunları mülazım-ı sânî (teğmen) rütbesiyle orduya alınmışlar ve bu rütbenin her türlü hak ve hukukuna sahip olmuşlardır [28,29].[9]

Tatbikat-ı Baytariyye Mektebi, eğitim-öğretim hizmetlerini I. Dünya Savaşı yıllarında da sürdürmüş, daha sonra kapanmıştır [30]. Hizmet verdiği yıllar içinde 160'ı aşkın muavin baytar yetiştirmiştir. Muzaffer Bekman, *Veteriner Tarihi* başlıklı eserinde [31] bunların bir listesini sunmaktadır. Listede verilen tarihler incelendiğinde, Okulun son mezunlarını 1924'te verdiği görülür. Kapanışı da bu tarihte olmuştur. Bekman, söz konusu tarihe dek özellikle sığır vebası ile mücadelede veteriner hekimler yanında verdikleri hizmetlerde büyük yararlıkları görülmüş olan veteriner tatbikat memurlarına Cumhuriyet Döneminde Müşavere Heyeti kararıyla veteriner hekim olma şansı verildiğini kaydetmektedir. Böylece Okul öğrencilerinden gönüllü olanlar Yüksek Baytar Mektebi (Baytar Mekteb-i Âlîsi)'ne kayıt yaptırmışlar ve eğitimlerine burada devam ederek veteriner hekim olmuşlardır [14,31].

Tatbikat-ı Baytariyye Mektebi İç Hizmet Yönetmeliği

Söz konusu çalışmaya kaynak teşkil eden Yönetmelik, *Tatbikat-ı Baytariyye Mektebi* başlığıyla 1911 (R.1327) yılında Ahmed İhsan ve Şürekâsı Matbaacılık Osmanlı Şirketi tarafından İstanbul'da yayımlanmış 16 sayfalık Eski Harfli Türkçe kitapçık içinde yer alan nüshadır. Kitapçıkta ayrıca bu Okuldan mezun olanların istihdam şartları ile görev ve sorumluklarını düzenleyen (yukarıda bahsedilmiş olan) 11 maddelik Tatbikat-ı Baytariyye Memurları Nizamnamesi *(Şekil 2)* de yer almaktadır. Kitapçığın son iki sayfasında ise Okulun ilk iki yıla ait idari ve akademik kadrosu, istihdam edilen diğer personel ve bunlara ait maaşlar, Okula ayrılan iki yıllık ödenekle birlikte tablolar halinde verilmiştir [13].

Çalışmanın odak noktasını oluşturan İç Hizmet Yönetmeliği, konularına göre ana başlıklar halinde toplanmış 29 maddeden oluşmaktadır. Hükümler söz konusu başlıklar altında madde madde sıralanmıştır. Bu çerçevede sekiz

[8] 1914 yılı Ağustos ayına gelindiğinde Mülkiye Baytar Mektebi'nin 270 dolayındaki toplam mezunundan 200'ü aşkını bu doğrultuda istihdam olunmuştur.

[9] Söz konusu Kanun, "Seferberlikte Orduya Alınan Mükellef Sivil Me'murin-i Baytariyye ile Muavin Baytar Mektebi Me'zunu Hakkında Kanun-ı Muvakkat"tir. Geçici nitelikte (süresi seferberlikle sınırlı) olan bu Kanunun ilk maddesinde mükellef sivil baytar ve muavin baytar tabirinden, 20-45 yaş aralığında bulunan ve askerlik hizmeti ile mükellef olan Mülkiye Baytar Mektebi mezunu baytar tabipler ile Tatbikat-ı Baytariyye mezunu muavin baytarlar olduğu ifade edilmektedir. İkinci madde hükümlerine göre, bunların seferberlik süresince ihtiyaç oranında ve en gençlerinden başlanarak istihdam olunacakları anlaşılmaktadır.

ana başlık görülmektedir. Günümüz Türkçesiyle bunlar; Okulun Kuruluş Amacı ve Eğitim Süresi [madde. 1-2], Öğrencinin Kabul Şartları [madde. 3-8], Okul Müdürünün Görevleri [madde. 9-11], Müdür Yardımcısı [madde. 12-13], Öğretmenlerin Tayin Usulleri ve Görevleri [madde.14-20], Sınavlar [madde. 21-25], Ders Programları [madde. 26-28] ve İdari Kadro [madde. 29]'dur [13].

Yönetmelikle, belirtilen başlıklar altında Okulun işleyişiyle ilgili olarak genel hatlarıyla şu esaslar karara bağlanmıştır: Okul, 'Devletin veteriner hekimlere olan şiddetli ihtiyacını karşılamak' ve özellikle 'ülkenin ekonomik refahı önündeki en büyük engellerden biri' olarak gösterilen salgın hayvan hastalıkları ile mücadeledeki etkinliğini arttıracak tatbikat memurları yetiştirmek amacıyla Orman Maadin ve Ziraat Nezareti tarafından kurulmuştur. Nezarete bağlı olan ve gündüz eğitimi veren iki yıllık bu okula 18-25 yaş arası Osmanlı tebaası gençlerin, İstanbul ve taşra idadilerinden mezun olmaları halinde sınavsız, rüşdiye çıkışlı olmaları halinde ise sınavla kabul edilmeleri esas alınmıştır. Giriş sınavları okul müdürü başkanlığında ve okul muallimlerinden oluşan bir heyet tarafından yapılır. Yabancı dil bilmek tercih sebebi sayılmıştır. Mezunlar, Ziraat Nezareti'nin resmi ve fenni memurları olarak *tatbikat-ı baytariyye memuru* unvanı ile İstanbul veya taşrada beş yıl süre ile hizmet vermekle yükümlü tutulmuşlardır [13].

Okul kadrosu, akademik ve idari olmak üzere iki kısım olarak belirlenmiştir. İdari kadro, *heyet-i idare* adıyla aylık 2500 kuruş maaşla bir müdür, aylık 1500 kuruş maaşla bir müdür yardımcısı ve birinci kademeden muallim maaşı olan 1350 kuruş maaşla bir muallimden ibarettir. Bunlar, okulun yönetim ve idaresi yanında yapılacak satın alımlardan ve hazırlanacak istatistiklerden sorumlu tutulmuşlardır. Yönetmelik metninin yer aldığı Tatbikat-ı Baytariyye Mektebi başlıklı kitapçık sonundaki tablolardan, ilk heyetin kimlerden oluştuğu saptanabilmektedir. Buna göre okulun ilk idari kadrosunu müdür sıfatıyla Ali Rıza (Erem) Efendi, müdür muavini sıfatıyla Bohor Efendi ve muallim Seyfeddin Efendi meydana getirmektedir [13].

Akademik kadroya gelince, bunu da Umur-ı Baytariyye Müdüriyeti'nin talebi üzerine Ziraat Nazırı tarafından ataması yapılan ders muallimleri oluşturmaktadır. Bunlar, sorumlu oldukları derslere ait müfredatta açıkça gösterilmiş olan konuları eğitim dönemi sonuna kadar tamamlamakla yükümlü tutulmuşlardır. Derse ait her türlü eğitim uygulamasını bizzat yapmak ve yazılı yazısız her türlü ders materyali hazırlamak muallimlerin görevi olarak belirlenmiştir. Tatbikat-ı Baytariyye Mektebi Yönetmeliği, müfredatta adı geçen derslerden birinci sınıfta okutulanlar için muallimlere 1350 kuruş, ikinci sınıfta okutulanlar için 1200 kuruş aylık bağlamıştır. Serîriyyât (klinik) uygulamaları muallimi için öngörülen işaret edilen aylık ise 500 kuruştur. Yukarıda sözü geçen tablolardan bu ödeneklerle birlikte çalışmaya başlayan ilk yılın akademik kadrosunu da belirlemek mümkündür. Bunlar; *Hıfzü's-sıhha ve İdâre-i Hayvânât* muallimi, aynı zamanda okul müdürü olan Ali Rıza (Erem) Efendi; *Tedâvî ve İspençiyârî* muallimi, aynı zamanda müdür muavini olan Bohor Efendi; *Mebâdî-i Hikmet ve Kimyâ-yı Tıbbî* muallimi, aynı zamanda idare heyeti üyesi olan Seyfeddin Efendi; *Ma'lûmât-ı Teşrîhiyye ve Fisyolojiyye* muallimleri ise Neşet ve Ali Rıza Beylerdir. İkinci yıl kadroları münhal, yani boş görünmektedir. Bunların hangi isimlerce doldurulduğu belirlenememiştir [13].

Akademik ve idari kadronun haricinde Tatbikat-ı Baytariyye Mektebi'nde ayrıca aylık 500 kuruş maaşla bir eczacının, 800 kuruş maaşla bir muhasebe kâtibinin, yine 500 kuruş maaşla bir dâhiliye zabitinin ve 250'şer kuruş maaşla beş hademenin hizmet vermesi planlanmıştır. İlk yıl için bu kadroları dolduran isimler de bellidir [13].

Yönetmeliğin 26 ve 27. maddelerinde iki senelik eğitim için belli bir müfredat ve bunun dâhilinde okutulacak ders içerikleri ayrıntılı olarak tayin edilmiştir *(Tablo 1)*. Bu çerçevede ilk yıl *Ma'lûmât-ı Teşrîhiyye ve Fisyolojiyye, Mebâdî-i Hikmet ve Kimyâ-yı Tıbbî, Tedâvî ve İspençiyârî, Hıfzü's-sıhha ve İdâre-i Hayvânât* derslerinin, ikinci yıl ise *Emrâz-ı Hayvânât* ve *Emrâz-ı Sâriye* derslerinin uygulamalı olarak okutulmasının planlandığı görülmektedir. Klinik uygulamaları şeklinde günümüz Türkçesine çevrilebilecek ve hayvanlar üzerinde tecrübeye dayalı olarak yürütülen amelî serîriyyât (klinik uygulama) dersi de yine ikinci sınıf dersi olarak planlanmıştır [13].

Teorik ve uygulamalı olmak üzere iki kısımda yürütülmesi tasarlanan müfredat derslerinin yıllık ders saati planlaması ise *Tablo 1*'deki gibidir.

Yönetmeliğin 28. maddesinde, yukarıda adı geçen derslerin her yılın sonunda akademik kadro tarafından gözden geçirilerek bilimsel gelişmeleri takiben güncellenmesinin uygun olacağı ibaresi yer almaktadır. *'İşbu programın terakkiyât ve keşfiyyât-ı cedîde-i fenniye üzerine ta'dîli câiz olub'* ifadesi ile dile getirilen bu esas, dinamik ve modern bir eğitim çizgisinin takibinin hedeflendiğini göstermesi bakımından dikkat çekicidir [13].

Tatbikat-ı Baytariyye Mektebi Yönetmeliği hükümleri çerçevesinde karara bağlanan bir diğer başlık da sınavlardır. Yönetmelik bunları özel-yazılı ve genel-sözlü olmak üzere ikiye ayırmaktadır. Özel-yazılı olan sınav, bir dersin hem birinci ders dönemi sonuna işaret eden Şubat ayı sonunda hem de ikinci ders dönemi sonuna işaret eden eğitim yılı sonunda yazılı olarak yapılan ve not hesaplaması 23. madde kapsamında ayrıntılı olarak gösterilmiş, o derse özel sınavıdır. Genel-sözlü sınav ise her eğitim yılı sonunda Ziraat Nezareti tarafından tayin olunan bir kurul tarafından yapılan ve o yıla ait tüm dersleri kapsayan uygulamalı-sözlü sınavıdır. Bu sınavdan geçiş koşulları da 24. madde kapsamında netleştirilmiştir. Her iki sınavı da başarıyla veren öğrencilere *Tatbikat-ı Baytariyye Memuru* unvanı alma hakkı tanınmıştır. Bunlar, taşıyacakları unvana ait her türlü hak ve imtiyaza sahip olacaklardır [13].

Tablo 1. *Tatbikat-ı Baytariyye Mektebi'nin iki yıllık müfredat programı* [13]

Dersin Adı		Günümüz Türkçesindeki Karşılığı	Ders Saati (teorik + uygulamalı)
Birinci Sene Dersleri	Emrâz-ı Hayvânât	Hayvan Hastalıkları	96
	Emrâz-ı Sâriyye (zâbıta-i sıhhiyye, 'amelî mikrobî ve muâyene-i lühûm ile birlikte)	Bulaşıcı Hastalıklar (Hayvan sağlık zabıtası, uygulamalı mikrobiyoloji ve et muayenesi ile birlikte)	112
	Serîriyyât ('amelî ve tecrübî olarak hayvânât üzerinde tatbîk olunur)	Klinik Uygulamaları	138
	Toplam (saat)		**346**
İkinci Sene Dersleri	Ma'lûmât-ı Teşrîhiyye ve Fisyolojiyye	Anatomi ve Fizyoloji Bilgisi	96
	Mebâdî-i Hikmet ve Kimyâ-yı Tıbbî	Bilime Giriş ve Tıbbi Kimya	96
	Tedâvî ve İspençiyârî	Tedavi ve (Veteriner) Eczacılık	84
	Hıfzu's-Sıhha ve İdâre-i Hayvânât	Hayvan Sağlığının Korunması ve Hayvan İdaresi (Yetiştiriciliği)	64
	Toplam (saat)		**320**

TARTIŞMA ve SONUÇ

Tatbikat-ı Baytariyye Mektebi, Osmanlı dönemine mahsus meslek yüksekokullarından biri olarak değerlendirilebilir. Ortaya çıkışında, Sivil Veteriner Okulunun açılışına da temel oluşturan 'salgın hayvan hastalıklarıyla mücadele etme düşüncesi' başlıca etken olmuştur [7,14]. Zira, her ne kadar 1893-1908 arası süreçte Osmanlı topraklarında başta sığır vebası olmak üzere salgın hayvan hastalıklarıyla mücadele konusunda önemli adımlar atılmış ve bu çerçevede bir yandan hayvan sağlık zabıtası teşkilatlandırılmaya çalışılıp bir yandan da ülke sınırları dahilinde sığır vebası serumu imal edilmesine başlanmışsa da[10], II. Meşrutiyet'in ilanını takip eden yıllarda salgınlar tekrar nüfuz kazanmıştır [7]. Muhtelif kaynak [5-7], bu süreçte Osmanlı coğrafyasının farklı noktalarında büyük zarar veren epidemiler yaşandığını kaydetmektedir. Çalışmaya konu olan Okul da 1908 sonrasında yeniden şiddetlenen söz konusu salgınlara karşı etkin ve hızlı bir müdahale sağlayacak mesleki bilgi sahibi yardımcı personel yetiştirilmesini hedeflemiştir. Okul, veteriner hekimliği alanına yardımcı personel yetiştirme hedefindeki ilk yapılanma [5,14] olması bakımından da Türk veteriner hekimliği tarihinde ayrı bir yere ve öneme sahip görünmektedir.

Tatbikat-ı Baytariyye Mektebi, bir yüksek öğretim kurumu olması ve en kısa sürede bir yardımcı meslek grubunu yetiştirmeyi hedeflemiş olması [5] dolayısıyla, buraya öğrenci seçiminde bazı kriterler aranmış olmalıdır. Zira okula alımda belli bir eğitim seviyesindeki gençler hedef kitle olarak belirlenmişlerdir. İki yıllık bir mesleki eğitimin ardından -Okulun kuruluş amacı gözetilerek- bunların seri biçimde yurt çapındaki muhtelif hizmet alanlarında görevlendirilmeleri öngörülmüştür.

Okul öğrencilerine askerlik muafiyeti getirilmesi de yine ülkenin baytar muavinlerine olan şiddetli ihtiyacını ve bu sorunu çözmek için Hükümet tarafından ortaya konulan hızlı refleksi göstermektedir. Ne var ki I. Dünya Savaşı'nın neden olduğu olağanüstü koşullar, orduda veteriner hekimlik hizmetlerine olan ihtiyacı önemli ölçüde artırdığından, mülki ve askeri veteriner okulları yanında diğer yüksek okullardaki akademik kadro ile birlikte Mülki Tatbikat Mektebi öğrencileri de savaşa katılmışlardır [32].[11]

Okul mezunları, tatbikat-ı baytariyye memuru unvanını almışlar ve ilgili yönetmelik hükümlerince atanmışlardır. Bunların beş yıl kadar zorunlu hizmete tabi tutulmaları, uygulamanın acil ihtiyaçtan kaynaklandığını teyit eden diğer bir unsur olarak görülebilir. Uzak coğrafyalarda görevlendirilen tatbikat-ı baytariyye memurlarına yarım maaşlık teşvik primi verilmiş, böylece mesleğe rağbetin arttırılmasına çalışılmıştır. Bu, hizmetin taşraya taşınması açısından da olumlu bir adım olarak değerlendirilebilir. Mezunların yönetmelik metninde açık biçimde saptanan görev ve sorumluluklarının günümüz veteriner sağlık teknisyenleri ve teknikerlerinin yetki ve sorumlulukları ile paralellik göstermesi ise ayrıca dikkat çekicidir [33]. Nitekim günümüzde artık sağlık teknikeri, veteriner sağlık teknisyeni veteriner sağlık teknikeri gibi muhtelif unvan almış olan ve hayvan sağlık hizmetleri alanında görev yapmakta olan yardımcı hizmet grubu, veteriner hekimlerin belirlemiş olduğu enjeksiyon tatbiki, dezenfeksiyon işlemleri vb. hayvan sağlık hizmetlerini yürütmektedirler. Dene-

[10] Bekman, Cumhuriyet dönemine uzanan süreçte veteriner hekimliğin en büyük sıkıntılarından birini yasal düzenlemelerin eksikliği olarak göstermektedir [14]. Bu nedenle, özellikle salgınlarla mücadelede bir yaptırım gücü oluşturabilmek adına 1873 yılında hayvan sağlık zabıtası teşkilatlanmasının çekirdeğini oluşturan ilk talimatname hazırlanmış ve Hüdavendigar vilayetinde uygulamaya konmuştur [7]. Erk, ilgili çalışmasında konuyla ilgili ayrıntılı bilgi vermektedir. Yine buradan öğrendiğimiz kadarıyla Osmanlı topraklarında sığır vebası için serum imaline 1897 yılında başlanmıştır [7]. Ancak Bekman'ın işaret ettiği üzere sınırlı miktarda hazırlanabilen serumla Erk'in işaret ettiği büyük epidemilerin önüne geçilememiş olmalıdır. Zira bunları uygulayabilecek sayıda veteriner hekim de yoktur [14].

[11] Muavin Baytar Mektebi öğrencilerinden kaçının askere alınmış olduğu net olarak tespit edilememektedir. Konuyla ilgili bilgimiz Dinçer'in ilgili çalışmasında vermiş olduğu rakamlarla sınırlıdır. Dinçer, 1914 yılında Mülki ve Askeri Veteriner Okulları öğrencileri ile birlikte Mülki Tatbikat Mektebi öğrencilerinden toplam 192 öğrencinin, bir aylık eğitimin ardından orduya yollandıklarını kaydetmektedir.

timleri de veteriner hekimlerce yapılmaktadır [33]. Bu da ele alınan yasal düzenlemenin modern meslek etiği ve deontoloji algısıyla uyumlu bir içeriğe sahip olduğunu göstermektedir.

Okulda takibine çalışılan müfredat, günümüz veteriner sağlık teknikerlerinin ders programıyla[12] büyük ölçüde benzerlik gösteren ve ayrıca aynı dönemde Askeri ve Sivil Baytar Mekteplerinde olduğu üzere uygulama ve teorik eğitimin bir arada yürütüldüğü [34], dönemi itibariyle bilimsel bir program olarak değerlendirilebilir. Ders içeriklerinin de, muhtelif kaynakta ifade edildiği üzere geniş ve zengin olduğu görülmektedir [14].[13] Müfredatın her yılın sonunda akademik kadro tarafından gözden geçirilerek bilimsel gelişmeleri takiben güncellenmesi zorunluluğunun da bilimsel bir eğitim açısından olumlu bir karar olduğunu söylemek mümkündür.

Sonuç itibariyle denebilir ki; veteriner hekimlik hizmetlerine duyulan ihtiyaca bağlı olarak kurulan bu okulla birlikte, dönemin hükümeti oldukça geniş bir coğrafyada veteriner sağlık hizmeti verecek yardımcı personelin en kısa zaman zarfında yetiştirilmesini sağlayarak özellikle salgın hayvan hastalıklarıyla olan mücadelesinden etkin bir sonuç alabilmeyi hedeflemiştir.

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Research Article

Effect of Cholesterol and 7-Dehydrocholesterol on Bull Semen Freezing with Different Rates of Glycerol

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Abstract

The aim of this study was to evaluate the effect of 7-dehydrocholesterol loaded cyclodextrin (7-DHCLC) and cholesterol loaded cyclodextrin (CLC) with the addition of different glycerol (G) rates on bull semen cryopreservation. Semen samples of three bulls were pooled and divided into seven groups; control group (C) and the other six were extended with standard Tris extender including different G rates (3, 6 and 9%) with CLC or 7-DHCLC to reach 1.5 mg/120x10^6. Extended samples were frozen in 0.25 mL straws. After thawing, motion characteristics and motility, viability, acrosome integrity, mitochondrial activation, functional membrane integrity and abnormal spermatozoa rates were assessed. The highest membrane integrity, viability and total motility were detected in 7-DHCLC with 9% of G group (P<0.001). The lowest progressive motility was detected in 3% G groups (P<0.001). 7-DHCLC with 9% G had higher mitochondrial activity compared with 3% G groups. In addition, no statistical difference was observed between groups in terms of acrosome integrity and abnormal spermatozoa rates (P>0.05). According to these results, 7-DHCLC with 9% G addition maintained some spermatological parameters better than other groups after thawing in bull semen thus, it was concluded that 7-DHCLC could be used instead of CLC in bull semen cryopreservation.

Keywords: *Cholesterol 7-dehydrocholesterol, Cyclodextrin, Glycerol, Bull semen, Freeze-thawed*

Kolestrol ve 7-Dehidrokolestrolün Farklı Gliserol Oranları İle Boğa Sperması Dondurulmasına Etkisi

Öz

Bu çalışmanın amacı, 7-dehidrokolesterol yüklü siklodekstrin (7-DHCLC) ve kolesterol yüklü siklodekstrin (CLC) 'in farklı gliserol (G) oranları ilavesi ile boğa sperması dondurulması üzerindeki etkilerini değerlendirmektir. Üç adet boğaya ait sperma örnekleri toplandı, birleştirildi ve yedi gruba ayrıldı; biri kontrol (C) ile, diğer altı grup ise CLC veya 7-DHCLC (1.5 mg/120x10^6) ve farklı G oranlarını (%3, 6 ve 9) içeren standart Tris sulandırıcısı ile sulandırıldı. Sulandırılan spermalar 0.25 mL'lik payetlerde dondurulduu. Çözdürme sonrası gruplar hareket karakteristikleri ve motilite, viabilite, akrozom bütünlüğü, mitokondriyal aktivasyon, fonksiyonel membran bütünlüğü ve anormal spermatozoa oranları yönünden değerlendirildi. En yüksek membran bütünlüğü, viabilite ve motilite %9 G ilavesi ile 7-DHCLC grubunda tespit edildi (P<0.001). En düşük progresif motilite %3 G gruplarında saptandı (P<0.001). %9 G içeren 7-DHCLC grubu, %3 G gruplarına kıyasla daha yüksek mitokondriyal aktivite belirlendi. Ayrıca, gruplar arasında akrozom bütünlüğü ve anormal spermatozoa oranları açısından istatistiksel bir fark gözlenmedi (P>0.05). Bu sonuçlara göre, %9 G ilave edilen 7-DHCLC grubunun, diğer gruplara kıyasla eritme sonrası bazı spermatolojik parametreleri daha iyi koruduğu ve bu nedenle boğa sperması dondurulmasında CLC yerine 7-DHCLC kullanılabileceği sonucuna varıldı.

Anahtar sözcükler: *Kolesterol, 7-dehidrokolesterol, Siklodekstrin, Gliserol, Boğa sperması, Dondurma-çözdürme*

INTRODUCTION

Detrimental effects of cryopreservation on functional characteristics of spermatozoa have been well documented. During the process of semen freezing, spermatozoa have to endure a series of harmful events, including the osmotic

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pressure stress that occurs due to addition and removal of cryoprotectants [1,2], extracellular and intracellular ice formation [3], and variations in the plasma membrane lipids stimulated by shift of liquid crystalline phase to gel phase [4,5]. The most common membrane permeable cryoprotectant is glycerol (G), which restricts the ice formation within the cell [6,7]. Removal and addition of cryoprotectants, for example G, was stated to induce the osmotic damage [1,2,4]. Swelling and shrinking are responses of sperm to osmotic change that can lead to cell death and/or significant loss of membrane integrity [2].

In the female reproductive tract, the primary step of sperm capacitation is the cholesterol efflux from the plasma membrane [8]. When adequate cholesterol is removed, the membrane becomes stabilized and the acrosome membrane gains the ability to bind thus resulting in acrosome reaction [9]. Cholesterol addition to semen extender or incubation medium restricts the acrosome reaction [10,11]. On the other hand, the lipid components of spermatozoa membranes affect not only the way that sperm replies to freezing, but also the ability of sperm to capacitate and undergo the acrosome reaction [12-15]. In addition, cholesterol has a complex effect on plasma membrane property. For instance, it decreases membrane permeability and phase changes, provides a proper physical and/or chemical microenvironment for membrane proteins, regulates morphological characteristics and acts as a membrane antioxidant [16,17].

Cyclodextrins are cyclic oligosaccharides, which are the main degradation products of starch. One of the frequently used cyclodextrins, methyl-beta-cyclodextrin can solubilize hydrophobic molecules such as cholesterol. Some studies have reported that adding cholesterol loaded cyclodextrin (CLC) into bull, ram, and stallion semen extenders doubles, even triple the sperm cholesterol content [14] as well as the cholesterol-phospholipid ratio. As a result of this increase, the semen gets the least damage due to the temperature changes at the freezing stage. It is reported that addition of CLC into semen extender generally increases total motility, mitochondrial activity, membrane integrity and viability. These improvements generally observed ranging from 1-2% to 24%, mostly between 10-20% [14,18]. In addition, there are some reports indicating that treatment of sperm with CLC decreases the damage in acrosome, abnormal spermatozoa rate [19], and DNA fragmentation [20]. Additionally, Lopez-Revuelta et al.[21] stated that cholesterol modifications in cell membrane prevent the oxidation and the spreading of Reactive Oxygen Species (ROS).

In some studies demonstrated that CLC treatment with semen affect sperm membrane potential and permeability [22]. Post-thaw sperm quality positively correlated with G level and CLC. Because, CLC could be due to the changes in osmotic tolerance and permeability of the membrane to G [23]. But there have not any study about 7-DHCLC with G levels. At this points different rate of G levels were evaluated with the different cyclodextrin compounds.

7-dehydrocholesterol consists of a cholesterol upper stage (intermediate product) and cholesterol conjugates in the biochemical diagram. Therefore, it is a cholesterol conjugate, which is formed before the production of cholesterol. Amorim et al.[24] cryopreserved bull semen with the addition of cholesterol conjugates (palmitate, pelorganate, stearate and heptanoate) and obtained improved sperm quality after thawing. Likewise, Moraes et al.[25] cryopreserved bull semen with cholestenol and desmosterol, which are intermediate products of cholesterol and after thawing, the viability and motility results were increased, compared to the control group. Even though there has been only one study reported for the short-term preservation of bull semen with 7-DHCLC [26], there have not been any studies regarding bull semen cryopreservation with 7-DHCLC addition yet.

With this information taken into consideration, the aim of this study was to investigate the effect of CLC and 7-DHCLC in Tris-egg yolk extender with different G levels on bull semen cryopreservation. In addition, this study investigates the suitability of 7-DHCLC addition as a replacement for CLC since it is the first study conducted with the addition of 7-DHCLC for cryopreservation purpose of bull semen.

MATERIAL and METHODS

Cyclodextrin Preparation

Methyl-beta-cyclodextrin was loaded with cholesterol as described by Purdy and Graham [14]. A working solution of the cholesterol-loaded cyclodextrin was prepared by adding 50 mg of CLC and 7-DHCLC to 1 mL stock Tris at 37°C and mixing the solution briefly using a vortex mixer.

Animals and Semen Collection

Semen samples were collected from three Simmental bulls (2-3 years old), regularly used for breeding purpose in International Center for Livestock Research and Training (Ankara, Turkey) under uniform feeding and housing conditions. Animals were being fed with 43% barley, 10% wheat bran, 35% corn, 5% soy pulp, ad libitum salt, minerals, vitamins, dry yeast and water. A total of 30 ejaculates were collected via an artificial vagina twice a week. Following the collection, ejaculates were evaluated and only the ones with $1.0x10^9$ spermatozoa/mL sperm concentration and >80% motility were pooled and used. Pooled semen was divided into seven groups and extended in Tris egg-yolk extender (Tris 30.7 g, citric acid 16.4 g, fructose 12.6 g, 1000 mL distilled water, 20% egg yolk); one as control (C, 6% G), and the other six including different rates of G (3, 6 and 9%) with either CLC or 7-DHCLC, reaching the dose

of 1.5 mg/120x10^6. After this step, standart breeding protocol were used in this study at labarotory. Briefly, the diluted sperm samples were cooled slowly in a water bath (22°C) inside the cold cabinet and equilibrated for 4 h at 4°C [27,28]. Following the equilibration, diluted semen samples were loaded into 0.25 mL French straws and frozen in a programmable digital freezing machine (Digitcool 5300 ZB 250, IMV, France) at 3 programmed rates: -3°C/min from +4°C to -10°C, -40°C/min from -10°C to -100°C, and -20°C/min from -100°C to -140°C. Then, the straws were plunged into liquid nitrogen at -196°C.

Evaluation of Microscopic Sperm Parameters

Motility and Motion Characteristics: Computer-assisted sperm analysis (SCA, Microptics) was used to examine motion characteristics and motility. 6 µL of frozen-thawed sample was put onto a slide, mounted with a cover slide and analyzed with a 10×objective at 37°C. The total sperm motility (%), progressive motility (%), VCL (curvilinear velocity, µm s^{-1}), VSL (straight linear velocity, µm s^{-1}), VAP (average path velocity, µm s^{-1}), LIN (linearity index (LIN = (VSL/VCL) x 100), WOB (Wobble, µm), and STR (Straightness, VSL/VAP x 100) were recorded. For each evaluation, at least 250 cells in six different microscopic fields were analyzed.

Functional Membrane Integrity: The hypo-osmotic swelling test (Hos Test) was used to evaluate the integrity of the sperm membrane, based on swollen and curled tails with 100 mOsm hypoosmotic solution at 37°C for 60 min. After this period, 10 µL of the sample was put onto a slide, mounted with a cover slide and analyzed on a warm stage. Three hundred spermatozoa were evaluated under bright-field microscopy. Spermatozoa with coiled or swollen tails were recorded [29].

Abnormal Sperm Assessment: Abnormal sperm assessment was performed in accordance with sperm blue staining procedure in CASA system. Abnormal spermatozoa rate was examined by using "Sperm Blue®, Microptics®, Spain" kit [30].

Semen Viability: The kit for viable spermatozoa rate, SYBR-14/PI (Invitrogen, L-7011), was used to perform the assessment. The method previously described by Garner and Johnson [31] was used. After staining, at least two hundred sperm cells were examined under a fluorescence microscope (Leica DM 2500).

Acrosome Status: Spermatozoa acrosomal integrity was assessed using FITC-PNA (Invitrogen, L7381) and PI staining methods previously explained by Garner and Johnson [31]. After the staining, at least two hundred spermatozoa per sample were evaluated under a fluorescence microscope (Leica DM 2500).

Mitochondrial Activity: Spermatozoa mitochondrial activity was assessed using JC-1/PI (Invitrogen, T3168) staining method previously described by Garner et al.[32]. After the staining, at least two hundred spermatozoa were evaluated under a fluorescence microscope (Leica DM 2500). In the present study, the mitochondrial potential was evaluated based on total mitochondrial activity in accordance with JC-1/PI staining method. This signified that if sperm mid-piece displayed dark green to yellow/orange fluorescence, it had a mitochondrial activity, on the contrary, if it displayed pale green or there was no fluorescence in its mid-piece, the sperm had no mitochondrial activity.

All the experimental work on animals were conducted according to the regularly used for breeding purpose application by the same expert and certified veterinarians/researchers in line with the laws and regulations of the local ethical committee.

Statistical Analysis

Before performing the statistical analysis, data was examined with Shapiro-Wilk test for normality and Levene test for homogeneity of variances as parametric test assumptions. Data was subjected to one way analysis of variance (ANOVA) when the parametric test assumptions are met. Tukey test was used as post hoc procedure for significant differences. Kruskal Wallis test was used to test the difference between groups when the parametric test assumptions are violated. Dunn's multiple comparison test was used as post hoc procedure for the significant differences. A probability value of less than 0.05 was considered significant, unless otherwise noted. SPSS 14.01 was used for statistical analysis.

RESULTS

The increasing percentage of different G rates resulted in enhanced motility (56.80%) and membrane integrity (58.57%) particularly in the group 9% G with 7-DHCLC (P<0.001) *(Table 1)*. The lowest G concentration (3% G) caused a significant decrease in progressive motility (P<0.001). However, in terms of abnormal spermatozoa rate and acrosome integrity, there was no statistical significance among the groups with three different G rates (P>0.05). These results may refer to the membrane protective properties of CLC and 7-DHCLC. With increased G concentrations, the velocity parameters were affected positively as well, in particular, the VCL value, although interestingly, the highest STR values were obtained from the 6% G groups of CLC and 7-DHCLC (P<0.001) *(Table 2)*. These correlations were also confirmed by viability and mitochondrial activation values. While the viability percentage was higher in the 7-DHCLC 9% G group than control, groups with 3% G rates have shown the lowest viability (P<0.001) and mitochondrial activation results (P<0.05), indicating that a certain G threshold rate was necessary to obtain a proper amount of viable sperm after thawing *(Table 3)*.

Table 1. *Mean (±SEM) CASA motility, abnormal spermatozoa rate and membrane integrity after thawed the bull semen*

Group	Total Motility (%)	Progressive Motility (%)	Total Abnormality (%)	Hos Test (%)
CLC 3G	9.07±5.56^{c}	2.74±2.40^{c}	26.43±5.38	43.43±8.56^{bc}
CLC 6G	36.31±7.35^{b}	14.64±3.02^{ab}	25.71±3.40	47.14±7.08^{bc}
CLC 9G	36.17±9.80^{b}	11.63±4.93^{b}	28.29±9.03	53.00±4.58^{b}
7-DHCLC 3G	13.13±8.38^{c}	3.23±2.95^{c}	31.29±6.90	36.14±3.44^{c}
7-DHCLC 6G	35.93±9.23^{b}	14.99±5.08^{ab}	25.71±5.02	41.86±4.67^{c}
7-DHCLC 9G	56.80±4.34^{a}	18.31±8.78^{ab}	22.29±7.39	58.57±4.65^{a}
Control	45.33±12.33^{b}	19.84±3.39^{a}	29.71±8.62	44.29±10.48^{bc}
P	*	*	-	*

* *P<0.001, Different superscripts within the same column demonstrate significant differences*

Table 2. *Mean (±SEM) CASA kinetic parameters after thawed the bull semen*

Group	VCL (μm s^{-1})	VSL (μm s^{-1})	VAP (μm s^{-1})	LIN (μm s^{-1})	STR (μm)	WOB (μm s^{-1})
CLC 3G	77.87±3.96^{c}	36.61±9.06	49.47±6.82	47.03±11.79	73.03±10.19^{ab}	63.56±8.62
CLC 6G	84.99±2.95^{b}	41.79±8.31	54.10±6.11	49.34±10.65	76.70±6.29^{a}	63.80±8.09
CLC 9G	95.11±5.67^{a}	34.11±6.13	54.09±7.38	35.76±5.28	62.87±5.11^{b}	56.70±5.12
7-DHCLC 3G	76.81±4.64^{c}	33.20±7.76	48.60±5.58	43.20±9.85	68.59±9.34^{ab}	62.34±5.81
7-DHCLC 6G	83.26±3.32^{bc}	41.20±7.52	52.47±4.96	49.80±10.36	75.70±8.08^{a}	65.09±7.49
7-DHCLC 9G	93.69±5.42^{a}	41.61±9.38	58.80±8.50	44.20±8.25	71.70±5.58^{ab}	61.23±7.09
Control	92.47±2.92^{a}	39.71±4.64	57.11±4.21	43.03±5.38	69.43±4.45^{ab}	61.81±4.67
P	*	-	-	-	*	-

* *P<0.001, Different superscripts within the same column demonstrate a significant difference*

Table 3. *Mean (±SEM) viability, acrosome integrity and mitochondrial activity after thawed the bull semen*

Groups	Viability (%)	Acrosome Integrity (%)	Mitochondrial Activation (%)
CLC 3G	23.92±8.31^{d}	53.55±5.67	36.80±11.66^{b}
CLC 6G	41.02±5.67^{b}	52.27±10.02	41.38±9.80^{ab}
CLC 9G	40.52±5.19^{b}	51.25±7.33	41.59±9.43^{ab}
7-DHCLC 3G	28.52±2.87^{cd}	53.74±9.27	34.23±9.89^{b}
7-DHCLC 6G	37.05±8.60^{bc}	46.12±1.63	42.40±6.75^{ab}
7-DHCLC 9G	58.72±8.16^{a}	49.66±6.22	53.78±4.73^{a}
Control	46.83±2.87^{b}	48.32±7.87	44.94±3.62^{ab}
P	*	-	**

* *P<0.001,* ** *P<0.05 Different superscripts within the same column demonstrate a significant difference*

DISCUSSION

In this study, cryopreservation of bull semen with the supplementation of 7-DHCLC and CLC with different rates of G were evaluated. After thawing, semen was assessed using an objective and highly technological approach in terms of sperm quality (CASA motility and motion characteristics, acrosome integrity, mitochondrial activation, viability, functional membrane integrity and abnormal spermatozoa rate).

Motamedi-Mojdehi et al.[33] observed that viability and motility were higher in all groups with different rates of CLC (1, 1.5, 3, 4.5 mg/120x10^{6}) than untreated (0 mg) groups in post-thaw ram semen. They also reported that there was no significant difference between the CLC groups (0 and 4.5 mg) in functional membrane integrity between the treated groups at different G levels (3, 5, 7%). In the present study, 1.5mg/120x10^{6} of 7-DHCLC and CLC were used with different ratios of G (3, 6, 9%) prior to freezing and after thawed HOS test was highest in 9%

G concentration. Anel et al.[34] have stated that the low level of CLC gives the possibility to decrease the required level of G for freezing. Although generally low level of G was found to preserve the volume of sperm cells and protect the cells from osmotic stress, in the current study, the highest total motility, viability and sperm membrane integrity values were observed in 9% G (high level G) group treated with 7-DHCLC when compared to other groups (P<0.001). The lowest progressive motility values were observed in 3% G (low-level G) groups both with CLC and 7-DHCLC addition (P<0.001). In addition, these results have shown that the 7-DHCLC addition has been more effective than CLC considering the cryotolerance of sperm cells, protection of their morphologic and metabolic properties from toxicity and high osmotic pressure that have been caused by G. Similar to our study, Thomas et al.[22] have demonstrated that CLC treatment affects the sperm membrane permeability. In the current study, 7-DHCLC 9% G had higher mitochondrial activation compared to both 3G groups. In accordance with our study, Blanch et al.[23] gradually increased the G concentration in CLC-treated semen, and they concluded that the post-thaw quality of sperm positively correlated with the G level. In most studies [35-37], when cryoprotectant rate is increased in the freezing media, detrimental effects induced by osmotic stress become more visible. However, the higher survival rate of sperm treated with CLC could be due to the changes in osmotic tolerance and permeability of the membrane to G [34]. In the present study, besides the cholesterol, we loaded 7-dehydrocholesterol with cyclodextrin. 7-dehydrocholesterol is a cholesterol conjugate that is formed before cholesterol is produced. Though no published data is available regarding bull semen cryopreservation with 7-DHCLC; according to our motility, membrane integrity and viability results 7-DHCLC can be used with 9% G in bull semen cryopreservation. In addition, other than VCL and STR, there was no significant difference in terms of post-thaw sperm kinetic parameters (VSL, VAP, WOB, LIN, WOB) (P>0.05). The lowest VCL values were detected in CLC and 7-DHCLC with 3% G. In bulls, the importance of VCL in fertilization capacity of spermatozoa has been well documented [31]. Therefore, these values indicate that sperm classified as highly mobile swims faster than those classified to have lower mobility [38]. Thus, the role of VCL in sperm transportation may be during the passage through the female reproductive tract and penetration to the oocyte vestments.

In several types of research, it has been demonstrated that treating spermatozoa with CLC prior to freezing increases the motility and viability post-thawed when compared to the untreated groups. Moreover, it has been stated that the addition of ≤2 mg CLC/120x10^6 has given the best results [14,18,39,40]. Although, in one study, treatment of sperm with 6 mg/mL (1.8 mg/120x10^6) CLC did not show a positive effect on sex-sorted semen after thawing, this result may be due to the harmful effect of the process on high membrane fluidity before flow cytometry sorting [39]. In a research on bull sperm with induced capacitation and acrosome reaction after thawing, *in vivo* fertilization rates were found similar in CLC-treated spermatozoa (1.5 mg CLC/120x10^6) and control group [42]. Additionally, there was no significant difference between the acrosomal integrity and abnormal spermatozoa rates of the groups. Thus, it may be asserted that cholesterol and 7-dehydrocholesterol could not decrease the percentage of morphological defects. Purdy and Graham [14] detected that when cyclic oligosaccharides of glucose with a hydrophobic center capable of incorporating lipids or cyclodextrins [43] are loaded with cholesterol and incubated with bull sperm prior to semen freezing; higher viability and motility results are observed than control groups after thawing. This cholesterol incubation may have a protective effect at low temperatures as the cells are freezing, at which the plasma membranes of spermatozoa undergo the lipid-phase transition from fluid to gel state [42]. In addition, CLC treatment could also modify the membrane permeability to penetrating cryoprotectants, since cholesterol is one of the most important regulators of membrane fluidity and permeability [44,45].

In conclusion, 7-DHCLC that has been used for the first time in bull semen cryopreservation, and better results have been obtained with the high level of G (9% G). It can be noticed that bull semen frozen with 7-DHCLC and 9% G had higher kinetic traits such as motility, progressive motility and various kinetic parameters. In addition, viability, membrane integrity and mitochondrial activity values of this group were the highest as well. According to the present study, it may be thought that membrane stabilizers such as cholesterol or its subunits could have the ability to reduce the toxic effect of G at high levels in bull semen. On the other hand, this kind of stabilization agents could allow bull spermatozoa to tolerate high osmotic pressure caused by G as well. Thus, it can be concluded that the 7-DHCLC provides protection on cellular energy metabolism, as well as structural and motion mechanism; 7-DHCLC can be used instead of CLC for bull semen cryopreservation.

Conflict to Interest

We have no *conflict of* interest to *declare.*

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Research Article

Y-Chromosome Polymorphisms in 12 Native, Karagül, Karacabey Merino Breeds from Turkey and Anatolian Mouflon *(Ovis gmelinii anatolica)* [1]

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Abstract

In this study, 182 male animals from 12 native sheep breeds, as well as Karacabey Merino and Karagül breeds of Anatolia, wild sheep Anatolian Mouflon *(Ovis gmelinii anatolica)* were used as the study material. Based on SRY and SRYM18 regions on the Y-chromosome, haplotypes of the populations were analyzed using DNA sequence analyses. The SRY region, A-oY1 allele was observed in all of the individuals studied. On the other hand, four different alleles corresponding to four Y-chromosome haplotypes were detected at the SRYM18 microsatellite region. Among native Anatolian breeds (n=143), H6 haplotype (80.41%), H4 haplotype (9.09%), H8 haplotype (8.40%) and H12 haplotype (2.1%) were identified. H6 haplotype was observed in all 16 individuals of Ovis gmelinii anatolica. Pairwise F_{ST} values based on haplotype frequencies were calculated for domestic sheep, and the highest F_{ST} value was observed between Karagül and Kıvırcık along with Karagül and *Ovis gmelinii anatolica* with pairwise F_{ST} value of 0.43202 ($P<0.01$). Y chromosome polymorphism of sheep from Turkey were examined comparatively with the accumulated data in the literature. Out of seven haplotypes (H4, H5, H6, H7, H8, H12, H19) observed in Europe and Asia, 4 haplotypes (H4, H6, H8 and H12) were observed in Anatolia. H12 was a private haplotype of Sakız, H6 seems to be the predominant haplotype of domestic sheep (79.51%) as well as being the only haplotype observed in *Ovis gmelini anatolica*. H4 haplotype seemed to be associated with fat tailed sheep migrating to Turkey, entering from south east of Turkey, which may be related with the arrival of nomadic Turks.

Keywords: *Y-Chromosome haplotypes, SRY, SRYM18, Ovis gmelinii anatolica, Native sheep breeds of Turkey*

Türkiye'den 12 Yerli, Karagül, Karacabey Merinosu ve Anadolu Yaban Koyununda *(Ovis gmelinii anatolica)* Y-Kromozom Polimorfizmleri

Öz

Bu çalışmada, Anadolu'dan yerli evcil koyun *(Ovis aries)* ırklarından 13 ırk ile kökeni Avrupa'dan olan Bandırma Merinosu koyun ırkı ve Anadolu Yaban Koyunu *(Ovis gmelinii anatolica)*'na ait olmak üzere toplam 182 erkek birey materyal olarak kullanılmıştır. Y kromozomunda bulunan SRY 5'promotor bölgesi ve Y kromozomuna özel bir mikrosatellit olan SRYM18 bölgeleri DNA dizi analizi yöntemleriyle incelenmiştir. SRY bölgesine ait analizlerde tüm bireylerde SNP A-oY1 alleline rastlanmıştır. SRYM18 mikrosatellit bölgesinde ise 4 farklı allel görülmüş ve toplamda 4 farklı babasal soy, haplotip, tespit edilmiştir. Yerli Anadolu ırklarında (n=143), H6 haplotipi (%80.41), H4 haplotipi (%9.09), H8 haplotipi (%8.40) ve H12 haplotipi (%2.1) belirlenmiştir. *Ovis gmelinii anatolica*'nın 16 bireyinde de sadece H6 haplotipi gözlenmiştir. Evcil koyunlar için haplotip frekanslarına dayalı F_{ST} değerleri hesaplanmış ve en yüksek F_{ST} değeri Karagül-Kıvırcık ve Karagül-*Ovis gmelinii anatolica* arasında F_{ST} değeri 0.43202 ($P<0.01$) gözlenmiştir. Türkiye koyunlarının Y kromozomu polimorfizmi literatürdeki birikmiş verilerle karşılaştırmalı olarak incelenmiştir. Avrupa ve Asya'da gözlenen yedi haplotipten (H4, H5, H6, H7, H8, H12, H19) dördü Anadolu'da (H4, H6, H8 ve H12) gözlenmiştir. H12, Sakız'ın özel bir haplotipi, H6 ise evcil koyunların baskın haplotipi (%79.51) ve *Ovis gmelini* anatolica' nın tek haplotipi olarak görülmektedir. H4 haplotipinin Türkiye'ye güneydoğudan girmiş olabileceği düşünülen yağlı kuyruklu koyunla ilişkili olduğu görülmüştür ve bu koyunların göçebe Türklerin gelişi ile ilişkili olabileceği düşünülmektedir.

Anahtar sözcükler: *Y-Kromozom haplotipleri, SRY, SRYM18, Ovis gmelinii anatolica, Türkiye'nin yerli koyun ırkları*

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INTRODUCTION

Sheep, goat, cattle and pigs, which are farm animals of great importance for human beings, were domesticated in the area spanning between Central Anatolia and North of Zagros Mountains [1] and domesticated sheep spread to Asia, Africa and Europe in the following few millennia. During the spread, in accordance with the multiple founder effects, only subset of the variation that was present in the center of domestication must have reached to the peripheries of the domestic sheep distribution. Thus, at least part of the native breeds and wild sheep of Turkey distributed in or close to the center of domestication may have unique genetic variations of special value [2].

While husbandry of farm animals can improve the desired characteristics, these attempts can also cause deterioration in some other characteristics, such as adaptation to environmental conditions and resistance against diseases. Native breeds from Anatolia deserve special conservation efforts due to being free from systematic selection and isolation and their endurance against challenging environmental conditions (e.g. surviving under extreme temperatures with low-quality feed, covering long distances for grazing, resisting parasites) [3]. However, since there are many candidates of native breeds and wild stocks to be conserved, for a sensible and sustainable conservation plan there must be a prioritization scheme [4]. Genetics-based information will reveal the evolutionary history of breeds and will also provide information on their genetic composition, thus will contribute for the prioritization in their conservation.

Sheep is one of the first domesticated farm animals [5]. Various genetic markers of Y chromosome [6-9], mitochondrial DNA (mtDNA) [10-17] and autosomal markers [5,15,18,19] of domestic sheep are used to understand the evolutionary history of the domestic sheep genome.

Previous studies examined domestic sheep and wild sheep individuals from *Ovis canadensis, Ovis dalli spp., Ovis vignei, Ovis ammon, Ovis musimon, Ammantragus lervia* [6-8]. These studies revealed that sex-determining gene (SRY) from the male specific region of the sheep Y chromosome had 8 polymorphic sites (oY1-oY8). It was observed that SNP site oY1 is polymorphic within the domestic sheep as A/G with A being the predominant one [7]. Other 7 SNP sites were polymorphic between the wild sheep species but not within the species [8]. When another region, SRYM18 microsatellite region, was sequenced from the male specific region of the sheep Y chromosome three polymorphic sites were detected: a pentanucleotide (TTTTG), an indel and a dinucleotide (TG) sites (8). Based on two regions 18 Y chromosome haplotypes (H1-H18) were defined in sheep [8].

Regarding the wild sheep, mtDNA based evidence indicated that *Ovis orientalis (Ovis gmelini* by the new nomenclature) [20] was suggested as the ancestral source of domestic sheep. While *Ovis gmelini musimon* was accepted as the early feral forms of domesticated sheep, wild *Ovis gmelini* populations were found in Anatolia, Iran, Azerbaijan and Armenia [21].

Ovis gmelini musimon samples: 10 individuals from Spain [7] and 19 individuals from Ukraine [8] were studied previously based on the Y chromosome markers. However, none of the wild *Ovis gmelini anatolica* samples were examined based on these markers.

An Anatolian wild sheep, *Ovis gmelinii anatolica*, populations is located in the 42.000 km^2 Bozdağ protection area in Konya province of Central Anatolia. *Ovis gmelinii anatolica* went through a recent bottleneck with only 15-20 individuals left at 1970s [22]. Currently, their population size is approximately 500 individuals [16].

The present research aims to determine the genetic diversity of native sheep breeds and the wild sheep *(Ovis gmelinii anatolica)* of Turkey in terms of Y chromosome based genetic markers. It is believed that results of the present study will contribute to the understanding of evolutionary history of domestic sheep and sheep domestication in Turkey.

MATERIAL and METHODS

Ethics Statement

Blood samples from modern sheep were collected with the approval of Istanbul University Veterinary Faculty Ethics Committee (permit number: 2006/172). *Ovis gmelinii anatolica* blood samples were collected with the approval of both Selçuk University Veterinary Faculty Ethics Committee (permit number: 2009/041) and the General Directorate of Nature Conservation and National Parks, Turkish Republic Ministry of Forestry and Hydraulic Works. The samples were studied with the approval of the Middle East Technical University Ethics Committee (permit number: 2011/02). Thus, all necessary permits were obtained for the described study, which fulfilled all of the regulations.

Samples and Sampling

In our study, selection of native sheep breeds (for each breed more than 3 flocks were sampled), their blood sampling and DNA isolation were carried out within the scope of the national project TÜRKHAYGEN-I (http://www.turkhaygen.gov.tr). Karacabey Merino, samples were collected by Sheep Breeding Research Institute of Bandırma district. Blood samples of Anatolian Mouflon *(Ovis gmelinii anatolica)* individuals were collected by Ministry of Forestry and Water Affairs of the Republic of Turkey, Directorate General for Nature Protection and National Parks.

The numbers of individuals examined in each breed and their tail types were as follows: Thin-tailed sheep breeds; Karayaka (n=15), Gökçeada (n=16), Kıvırcık (n=16), Karacabey

Merino (n=12); Semi-fat-tailed sheep breeds; Sakız (n=15) which is similar to Chios breed of Greece, Hemşin (n=14), Herik (n=11); Fat-tailed sheep breeds; Akkaraman (n=11), Dağlıç (n=10), Çineçaparı (n=12), İvesi (n=7) which is similar to Awasi breed of Middle East , Morkaraman (n=9), Norduz (n=7), Karagül (n=11). Herik and Hemşin were hybrids of the breeds from Turkey. Karagül was brought from Russia in 1926, Karacabey Merino was a hybrid between German meat Merino (rams) and Kıvırcık (ewes) and it has been in Anatolia since 1935. Although, all of the breeds were in Turkey for many generations the last two breeds were not considered as native breeds of Turkey. Additionally, 16 individuals of Anatolian Mouflon *(Ovis gmelinii anatolica)* were examined. Sites of the breeds and *Ovis gmelini anatolica* were indicated on the map presented in *Fig. 1*. Blood samples (10 cc) were drawn by specialized veterinarians from the jugular vein into K3 EDTA tubes. DNA isolations were performed by using phenol-chloroform isoamyl alcohol (25:24:1) extraction method [23].

Y-Chromosome Markers

In the present study, 5′ promoter region of SRY (Gene Bank No: AY604734) and SRYM18 (EU980105.1) loci of Y chromosome were amplified by PCR. Primer sequences for amplification of the SRY region were as follows: Forward; 5′-TCA GTA GCT TAG GTA CAT TCA-3′ and Reverse: 5′-GTG CTA CAT AAA TAT GAT CTG C-3′ [6]. Primer sequences for the amplification of SRYM18 microsatellite locus were; Forward; 5′-GGC ATC ACA AAC AGG ATC AGC AAT-3′ and Reverse: 5′-GTG ATG GCA GTT CTC ACA ATC TCC T-3′[7].

All PCR amplifications were performed in a volume of 25 µL containing 10 pmol/µL of each primer, 0.2 mM of each dNTP, 1X Taq buffer, 2.5 mM $MgCl_2$, 1 unit Taq polymerase and approximately 90 ng of template DNA. The PCR thermocycling conditions were as follow: initial denaturation at 94°C for 3 min, and 35 cycles of denaturation at 95°C for 25 s, annealing at 56°C for 35 s, extension at 72°C for 50 s, and a final extension at 72°C for 10 min. PCR products were purified using FavorPrep Gel/PCR purification kit (Favorgen) prior to Sanger sequencing, which was performed by REFGEN Gene Research and Biotechnology Limited Inc. (Ankara, Turkey).

Raw sequences were assembled by using Chromas Pro version 1.5 (http://www.technelysium.com.au/ChromasPro.html) and consensus sequences were aligned by ClustalW algorithm [24], implemented in Bioedit version 7.1.3 [25].

Haplotypes were determined in accordance with the *Table 1* of Meadows and Kijas's [8] study.

Statistical Data Analyses

In order to display relationships between haplotypes based on mutations, a median joining network (MJ) [26] was constructed with NETWORK 4.6.1.0 (http://www.fluxus-engineering.com), by using the default settings. Genetic distances between sub-populations were obtained by calculating pairwise F_{ST} values based on haplotype frequencies using Arlequin 3.11 [27]. Significance of the results was tested with 1000 permutations.

RESULTS

In the present study, SRY and SRYM18 sequences were analyses for a total of 182 sheep including 166 individuals from native sheep breeds (including a breed imported from Russia and a hybrid of a merino breed) and 16individuals from *Ovis gmelinii anatolica*.The analysis of SRY showed that all individuals had allele A in terms of SNP oY1 (A/G). The whole SRY region was reanalyzed in all of the individuals but no polymorphism was found. In SRYM18 microsatellite locus no polymorphism was observed between individuals in terms of pentanucleotide (TTTTG) repeat number. Furthermore, the presence of G nucleotide between TTTTG and dinucleotide (TG) repeats was the same in all individuals. Considering TG repeat numbers, 13 TG, 14 TG, 15 TG and 16 TG repeats were found. The classification of haplotypes was performed in accordance with Meadows et al.[7] study using the oY1 SNP in the SRY gene and the SRYM18 microsatellite locus.

In this study, H4, H6, H8 and H12 haplotypes were found as shown in *Table 1*. All individuals of *Ovis gmelinii anatolica* and Karacabey Merino had H6 haplotype.

Among the 14 domestic breeds (n=166), H6 was the most common haplotype observed in 132 individuals (79.51%) from Turkey. H12 haplotype was found in only 3 individuals (1.81%) of Sakız breed. H4 and H8 haplotypes were found in 17 (10.24%) and 14 individuals (8.44%), respectively. Haplotypes H4 and H8were observed in various breeds with different tail types. Similarly, among 12 Anatolian native domestic sheep breeds (n=143), the most frequent haplotype was H6 haplotype (80.41%) followed by H4 haplotype (9.09%), H8 haplotype (8.40%) and H12 haplotype (2.1%).

Y-chromosome haplotypes found in native breeds, Karacabey Merino, Karagül breeds and *Ovis gemelinii anatolica* from Turkey are shown in *Fig. 1*. It is observed that embedded in H6 haplotype distribution the frequency of H4 haplotype seemed to decrease from east to west, whereas, H8 haplotype is mainly in the central parts of Anatolia.

Statistical Analyses

Median-joining network analysis based on Y chromosome polymorphisms: The molecular proximity (mutational differences between haplotypes) of the haplotypes we observed was visualized in *Fig. 2*. In this figure, the relationship between haplotypes as well as tail types (fat-tailed, semi fat-tailed and thin-tailed) of the sheep breeds are displayed.

Table 1. *Y-chromosome haplotypes observed in this study in each of the sheep breeds*

Sheep Breeds		Chromosome				
		H4	H6	H8	H12	Total
Thin-tailed sheep breeds	Karayaka	1	10	4	-	15
	Gökçeada	1	14	1	-	16
	Kıvırcık	-	16	-	-	16
	Karacabey Merino	-	12	-	-	12
Semi-fat-tailed sheep breeds	Sakız	-	12	-	3	15
	Hemşin	6	8	-	-	14
	Herik	1	9	1	-	11
Fat-tailed sheep breeds	Akkaraman	1	10	-	-	11
	Dağlıç	-	8	2	-	10
	Çineçaparı	-	9	3	-	12
	İvesi	-	6	1	-	7
	Morkaraman	1	8	-	-	9
	Norduz	2	5	-	-	7
	Karagül	4	5	2	-	11
Total		17	132	14	3	166

Sheep breeds were classified based on their tail types

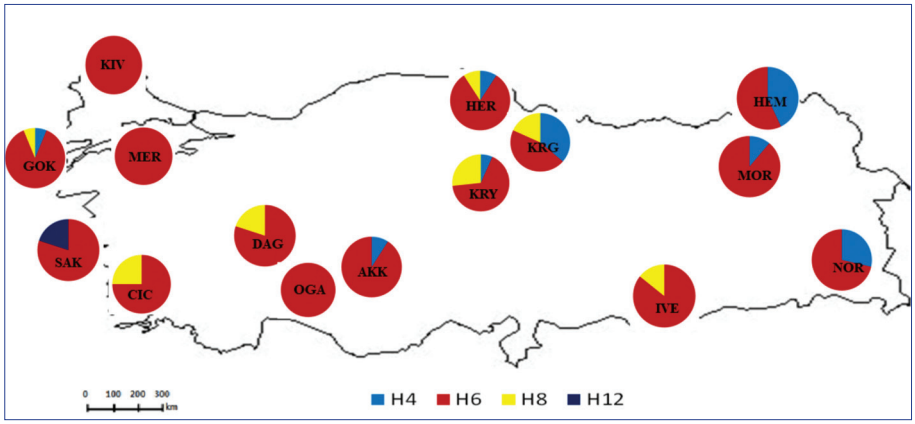

Fig 1. Distribution of Y chromosome haplotypes of domestic sheep breeds and Anatolian Mouflon in Turkey. Abbreviations: Karayaka (KRY), Akkaraman (AKK), Gökçeada (GOK), Dağlıç (DAG), Morkaraman (MOR), Kıvırcık (KIV), Ivesi (IVE), Herik (HER), Karagül (KRG), Hemşin (HEM), Çineçaparı (CIC), Sakız (SAK), Norduz (NOR), Karacabey Merino (MER), *Ovis gmelinii anatolica* (OGA)

In *Fig. 2*, it can be seen that H4, H6 and H8 haplotypes were found in sheep breeds with all types of tails, while H12 was found only in a semi fat-tailed sheep breed (Sakız). Thin tail breeds seemed to display H4 relatively less than breeds with other tail types. Furthermore, based on *Fig. 2* it is possible to suggest that H4 and H8 haplotypes were genetically related to H6 haplotype and evolved from H6 independent of each other. Similarly, the H12 haplotype could have been evolved from H8.

Pairwise F_{ST} values between the breeds: Pairwise F_{ST} values between the breeds based on haplotype frequencies are presented in *Table 2*. According to the results, Hemşin is significantly different from Karayaka, Gökçeada, Kıvırcık, Sakız, Çineçaparı, OGA and Merinos. Karagül is also significantly different from Gökçeada, Kıvırcık, Sakız, OGA and Merinos. Most distant breeds on the basis of their Y chromosome haplotype frequencies are Karagül and Kıvırcık as well as Karagül and OGA with pairwise F_{ST}=0.43202 (P<0.01). The most similar breeds, however, seemed to be the Akkaraman and Morkaraman with pairwise F_{ST} value of - 0.10984 (non-significant).

Turkey, Asia and Europe in the range of the Y chromosome haplotype distribution: Y chromosome haplo-type distributions of domestic sheep from different countries in Europe and Asia along with different breeds within Turkey (the present study) are shown in *Fig. 3*.

DISCUSSION

In this study, 182 male individuals from 12 native sheep breeds as well as Karacabey Merino, and Karagül breeds from Anatolia and male sheep from Anatolian Mouflon *(Ovis gmelinii anatolica)* were subjected to sequence analysis of SRY and SRYM18 loci of Y chromosome. Previously in Öner

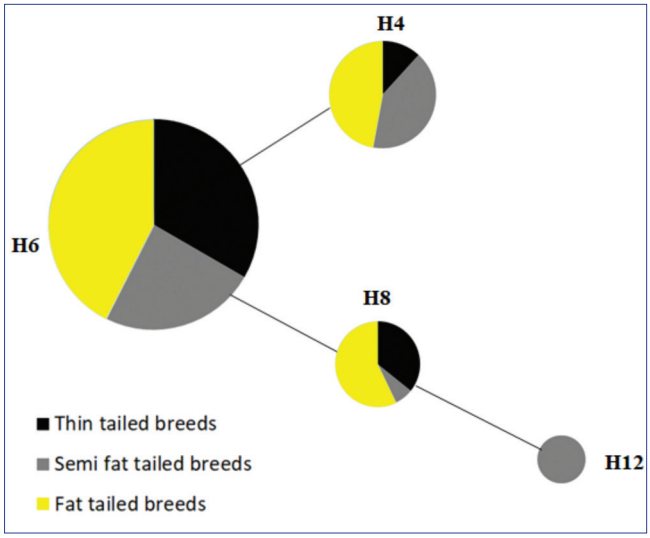

Fig 2. Relationship between haplotypes based on median joining network, distribution of haplotypes and tail types of native sheep breeds of Turkey by using pie charts

gmelinii anatolica individuals examined in this study. Early feral forms of domestic sheep from Ukraine (n=19) [8] and Spain (n=10) [7] also displayed only A-oY1 allele. In fact, all other wild sheep samples exhibited A-oY1 allele [8]. Thus, the results proposes that A-oY1 allele is the ancestral form of whereas G-oY1 allele that is observed in domestic sheep with H5 and H7 haplotypes may be, the derived form. However, this hypothesis must also be confirmed by examining wild *Ovis gmelinii* populations from Armenia, Iran and Cyprus [21] which have not been studied yet.

SRYM18 microsatellite region sequences revealed no polymorphism among *Ovis gmelinii anatolica* individuals When analyzed along with the SRY locus, they displayed H6 haplotype which was also observed uniformly in *Ovis musimon* [7,8]. Absence of polymorphism in *Ovis gmelinii* might be an ancestral state. If this is the case, then haplotypes observed in domestic sheep breeds emerged after the sheep domestication. Alternatively, wild populations in the Middle East [21] might be exhibiting polymorphism(s)

Table 2. *Pairwise F_{ST} values and their significances between the breeds on the basis of their Y-chromosome haplotype frequencies*

	KRY	GOK	KIV	SAK	HEM	HER	AKK	DAG	CIC	IVE	MOR	NOR	KRG	OGA	ME
KRY	0.00000														
GOK	0.04709	0.00000													
KIV	0.23742*	0.03333	0.00000												
SAK	0.08163	0.02666	0.15044	0.00000											
HEM	0.11796*	0.19376*	0.40584**	0.20031*	0.00000										
HER	-0.01779	-0.07314	0.09315	0.00386	0.10961	0.00000									
AKK	0.08661	-0.06748	0.03562	0.02941	0.18107	-0.06333	0.00000								
DAG	-0.05691	-0.03226	0.17949	0.03069	0.17768	-0.07089	0.01811	0.00000							
CIC	-0.06671	0.01437	0.22372	0.06203	0.17792*	-0.03806	0.06764	-0.09310	0.00000						
IVE	-0.04371	-0.08893	0.12840	-0.00707	0.17323	-0.10811	-0.04804	-0.12544	-0.08896	0.00000					
MOR	0.05764	-0.07881	0.06796	0.01172	0.13332	-0.08400	-0.10984	-0.00269	0.04327	-0.06305	0.00000				
NOR	0.01566	0.02553	0.33214	0.05987	-0.07207	-0.04671	0.01282	0.03686	0.05204	0.01754	-0.03616	0.00000			
KRG	0.03903	0.20482*	0.43202**	0.20249*	-0.04017	0.10429	0.21194	0.12378	0.10650	0.13612	0.16301	-0.03047	0.00000		
OGA	0.23742*	0.03333	0.00000	0.15044	0.40584**	0.09315	0.03562	0.17949	0.22372	0.12840	0.06796	0.33214	0.43202**	0.00000	
MER	0.19941	0.01101	0.00000	0.11765	0.36122*	0.05979	0.00826	0.13669	0.18182	0.08197	0.03356	0.27147	0.38128**	0.00000	0.0000

** P<0.05 ** P<0.01 *** P<0.001*
Karayaka (KRY), Akkaraman (AKK), Gökçeada (GOK), Dağlıç (DAG), Morkaraman (MOR), Kıvırcık (KIV), Ivesi (IVE), Herik (HER), Karagül (KRG), Hemşin (HEM), Çineçaparı (CIC), Sakız (SAK), Norduz (NOR), Karacabey Merino (MER), Ovis gmelinii anatolica (OGA)

et al.'s [9] study 10 native breeds from Turkey were examined and only A-oY1 allele in the SRY gene region was observed. In conformity to the Öner at al.'s [9] result, only A-oY1 allele was observed among the domestic breeds we studied. However, previously, in a single breed (Karakaş) from Turkey, G-oY1 allele was observed in two individuals [8]. Karakaş was not examined in the present study and in Öner et al.'s [9] study and it was not reported as one of the native sheep breeds of Turkey [28].

Ovis gmelinii anatolica, was not previously studied in terms of SRY locus. A-oY1 allele was found in all (n=16) *Ovis* while, *Ovis gmelinii anatolica* might have lost its variability in the previous century due to a severe bottleneck [22]. However, the frequency of most common Y chromosome haplotype(s) found in different countries were as follows: 72.8% H6 [29] in African breeds; 60.7% H6 [7,8] in sheep breeds from all over the world; 65.0% [30] in Northern Eurasian breeds; 55.5% H8 and 34.5% H6 [31] in Tunisian breeds; 43.4% H6 [32] in Chinese breeds. Thus, it seems that H6 haplotype is the most frequent haplotype (except in Tunisia) in most of the places of the old continents. These results also support the proposal that H6 might be the

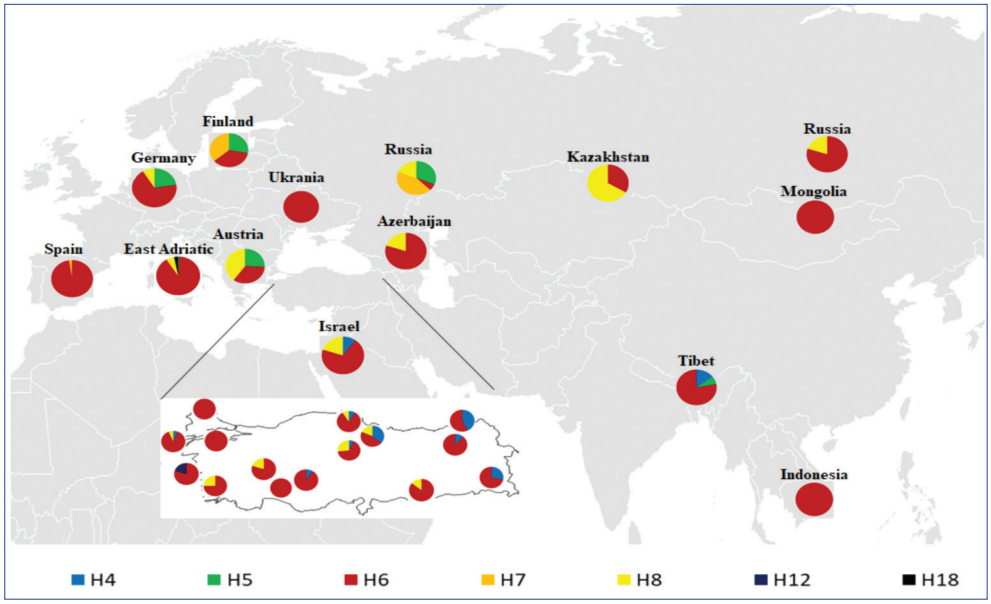

Fig 3. Y-chromosome haplotype distributions in domestic sheep breeds and wild sheep from Turkey (the present study) shown together with domestic sheep populations from Asia and Europe

ancestral haplotype. Similarly, among the haplotypes found in Anatolian native domestic sheep breeds, the most frequent was H6 haplotype (80.41%).Then followed by haplotypes H4 (9.09%), H8 (8.40%) and H12 (2.1%) among 143 individuals. All individuals of *Ovis gmelinii anatolica* had H6 haplotype. Central location of H6 in the present study and in the previous study [8] also supports the proposition.

Haplotypes H6 and H5 were observed in German Merino but only H6 in Merino Long wool from Germany [7]. H6 was the only haplotype observed in merino breeds from Australia and Mexico [8]. Despite the fact that rams of German meat merino were used for the Karacabey merino, only H6 haplotype was found in our study in 12 individuals of Karacabey Merino breed in Turkey.

To our knowledge, Herik and Karagül breeds were analyzed for the first time in our study and H4, H6 and H8 haplotypes were found in both breeds. In our study, only 1 individual fromeach of thin-tailed Karayaka and Gökçeada breeds exhibited H4 haplotype However, this haplotype was not observed in other studies that focused on thin-tailed sheep breeds in Turkey [7,9]. Presence of H4 in thin tailed breeds can be taken as the evidence of the week isolation between the breeds. Differences between the haplotype frequencies of the same breeds in different studies for instance presence of private haplotype H12 in Sakız only in the present study can be attributed to the sampling effect. Since more than 3 flocks were visited during the sampling of native breeds in the present study, results could be used in further comparisons.

Studies on Y chromosome haplotype distributions in domestic sheep breeds and wild sheep from Turkey (the present study) in Europe and Asia show in the *Fig. 3* [7,8,33]. According to *Fig. 3*, it can be seen that sheep samples examined were scattered and scarce over the vast geography. Therefore, conclusions based on the haplotype distributions should be interpreted cautiously. Nevertheless, it is seen again that H6 haplotype is the most prominent haplotype, in every breed or country except in the western Russia, Finland, Kazakhstan and Austria. However, H5 and H7 (both having G-oY1 allele) seemed to be common in Northern Europe (Russia and Finland) and in Austria. H7 seemed to be associated with H5 in Russia and Finland. Just 2 samples out of 19 sheep from Tibet also displayed H5 haplotype (H5 may be reached to Tibet by sheep trading). Therefore, as a first approximation it can be suggested that G-oY1 allele and thus H5 and H7 emerged later in the spread of domestic sheep and may be confined in the northern and central Europe. H8 existing from east to west in the northern Asia and Europe is also seen in Azerbaijan, Austria, East Adriatic and in Israel as well as in the breeds of Anatolia. However, H8 is neither present in the most eastern and most western breeds (except in GOK with a low frequency, 6.25%) of Turkey. The emergence and direction of spread of H8 cannot be determined by the distribution of the haplotype. Haplotype H4 is seen in Tibet and Israel and in Eastern breeds of Turkey as well as in Akkaraman, a central Anatolian breed. Furthermore, these breeds do not have haplotypeH8 *(Fig. 3)*. This observation may suggest that, there was a massive wave of male sheep migration partly harboring H4 arrived to Turkey from the south of Caspian Sea (since sheep of Azerbaijan is not exhibiting H4). This migration might be relatively recent because although they were surrounded by breeds having H8 haplotype they did not exhibit H8. These sheep might be represented by Morkaraman, Hemsin, Norduz and Akkaraman breeds of Turkey. The Norduz is believed to be a variety of Akkaraman breed [34]. Indeed, these two breeds have non-significant pairwise F_{ST} value (F_{ST}=0.01282). Also Morkaraman and Akkaraman have non-significant pairwise F_{ST} value (F_{ST}=-0.10984).These observations support

the implication that Akkaraman, Morkaraman, Norduz and Hemşin might be sharing a common evolutionary history. These 4 breeds (fat tailed or semi fat tailed) seemed to have a different evolutionary history than those of İvesi, Dağlıç, Çineçaparı and Karagül (fat tailed or semi fat tailed), Gökçeada (thin tailed) breeds all harboring H6 and H8 but rarely H4 (1 individuals from each of Karagül and Gökçeada). It is well known that nomadic Turks arrived to Anatolia in the 11th century, originally spreading from East of Aral Sea [35] travelling through Iran. Together with their sheep they settled mostly in Eastern and Central Anatolia [36] and migration of nomadic Turks continued for two hundred centuries [36,37]. Perhaps their sheep were the ones with H4 and without Y Chromosome H8 haplotype. In this case, for conservation studies of native breeds of Turkey, 'sheep of nomadic Turks' must also be considered as a group from which breed(s) must be chosen for conservation.

In the present study, we focused on domestic breeds and wild sheep of Turkey and studied their Y chromosome haplotypes in order to contribute to the paternal evolutionary history of sheep by combining the available data from the literature. Further genetic studies utilizing higher resolution genetic markers on ancient as well as modern samples of *Ovis gmelini* and domestic sheep from different regions and time periods of Anatolia, the Middle East and other regions of the old continents will expand the understanding of both the early stages of the domestication process and the evolutionary history of domestic sheep.

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Research Article

Babesia spp. in Dogs from Córdoba, Colombia

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Abstract

Canine babesiosis is a tick-borne disease with worldwide distribution, caused by intra-erythrocytic protozoa of the genus *Babesia*. The present study describes the clinical features and molecular detection of *B. canis vogeli* in dogs in Córdoba, Colombia. Blood samples were taken from dogs with clinical signs compatible with tick-borne diseases. Peripheral blood samples were examined microscopically for the presence of *Babesia* spp. merozoites and trophozoites. Blood sample obtained by jugular or cephalic venipuncture were used for DNA extraction. PCR assay was carried out using primers for the 18S rRNA gene of *Babesia* spp. of the 42 dogs suspected of babesiosis, 23 were females and 19 males. Peripheral blood examination demonstrated intraerythrocytic piroplasms compatible with *Babesia* spp. in 23 of 42 (54.7%) dogs. The morphometric study showed that 73% (17/23) were large babesias (2.4x5 µm) and 26% (6/23) were small *Babesia* (1x3.2 µm). Eleven of 42 (26%) blood samples were positive for *B. canis vogeli*. Clinical signs found in positive dogs were: anorexia 63% (7/11), lethargy and apathy 63% (7/11), fever 54% (6/11), pale mucous membranes 54% (6/11), lymphadenomegaly 18% (2/11), vomiting 9% (1/11) and diarrhea 9% (1/11). Blood counts showed that 70% (8/11) dogs had anemia. Leukocyte disorders were variable; 27% (3/11) had leukocytosis and 46% (5/11) had leukopenia. Sequenced samples of the 18S rRNA gene showed 99% identity with *B. canis vogeli* sequences from several countries.

Keywords: *Animal diseases, B. canis vogeli, Colombia, 18S rRNA gene*

Kolombiya, Cordoba'daki Köpeklerde *Babesia* spp.

Öz

Köpek babesiosisi (Canine babesiosis), *Babesia* cinsinin intraeritrositik protozoası tarafından oluşturulan ve dünya çapında yaygınlık gösteren kene kaynaklı bir hastalıktır. Bu çalışmada, Córdoba, Kolombiya'daki köpeklerde *B. canis vogeli*'nin moleküler tespiti ve hastalığın klinik özellikleri tanımlanmıştır. Kene kaynaklı hastalıklarla uyumlu klinik bulguları olan köpeklerden kan örnekleri alındı. Periferal kan örnekleri *Babesia* spp. merozoit ve trofozoitlerinin mevcudiyetini belirlemek amacıyla mikroskopik olarak incelendi. DNA ekstraksiyonu için juguler veya sefalik venipunktür ile elde edilen kan örnekleri kullanıldı. PCR analizi, *Babesia* spp. 18S rRNA genine yönelik primerler kullanılarak gerçekleştirildi. Babesiosis şüphesi olan 42 köpeğin 23'ü dişi ve 19'u erkek idi. Köpeklerin 42'sinin 23'ünde (%54.7), periferik kan muayenesinde *Babesia* spp. ile uyumlu intraeritrositik piroplazmalar saptandı. Morfometrik çalışma, %73'ünün (17/23) büyük *Babesia* (2.4x5 µm) ve %26'sının (6/23) küçük *Babesia* (1x3.2 µm) olduğunu göstermiştir. Kan örneklerinin 42'sinden 11'i *B. canis vogeli* için pozitif idi. Pozitif köpeklerde gözlenen klinik bulgular: anoreksiya %63 (7/11), uyuşukluk ve apati %63 (7/11), ateş %54 (6/11), soluk mukozal zarlar %54 (6/11), lenfadenomegali %18 (2/11), kusma %9 (1/11) ve ishal %9 (1/11). Kan sayımı, köpeklerin %70 (8/11)'inde anemi olduğunu gösterdi. Lökosit bozuklukları değişkendi; %27'sinde (3/11) lökositoz ve %46'sında (5/11) lökopeni vardı. 18S rRNA geninin sekanslanmış örnekleri çeşitli ülkelerden elde edilen *B. canis vogeli* sekansları ile %99 benzerlik göstermiştir.

Anahtar sözcükler: *Hayvan hastalıkları, B. canis vogeli, Kolombiya, 18S rRNA geni*

INTRODUCTION

Canine babesiosis is a tick-borne protozoa disease caused by different *Babesia* species. The disease produces hemolytic alterations of variable intensity, which include fever, lethargy, anorexia, anemia, hemoglobinuria, and may even compromise other organ systems, thereby causing a wide variety of clinical signs [1]. The pathogenicity of the agent involved, the immune competence of the infected dog and the possibility of co-infections with other agents are the main factors that cause this variability in clinical presentation [2].

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At a global level, 12 piroplasm species capable of infecting dogs have been identified [2]; the identification is based on host specificity and the morphology of the intraerythrocytic forms [1]. However, this method fails to identify individually and accurately the species or subspecies that causes the infection, whereas the use of molecular techniques has allowed an accurate identification of the species and subspecies of *Babesia* and an understanding of their dynamics and distribution [3].

The species *Babesia canis* and *Babesia gibsoni* traditionally have been considered the only piroplasms that parasitize dogs. *Babesia canis* is classified as a large piroplasm and *B. gibsoni* as a small piroplasm, both have being reported in all five continents [2].

Currently, three subspecies of *B. canis* are recognized. *Babesia canis canis* is found in Europe and transmitted by *Dermacentor reticulatus*, *Babesia canis vogeli* is found in Europe, North Africa, America, Asia and Australia and is transmitted by *Rhipicephalus sanguineus*. *Babesia canis rossi* found in South Africa and North America is transmitted by *Haemaphysalis leachi* [4]. A new large *Babesia* sp. has also been reported in United States [5]. Within the small piroplasms, there are further reports of *B. gibsoni* on five continents. Moreover, recent molecular characterization has identified other small piroplasms, called *Theileria annae* endemic to Spain [6] and *Babesia conradae* reported in Southern California [7]. These species are morphologically identical, but have different vectors and variations in pathogenicity and clinical manifestations [8].

The aim of this study was to characterize the clinical presentation and molecular species of *Babesia* spp. collected from infected dogs in Cordoba, Colombia.

MATERIAL and METHODS

Dog and Blood Examination

Between November 2013 and December 2014, 42 dog blood samples were collected at the veterinary clinic of University of Cordoba and three private clinics. The dogs had clinical signs of ticks-borne disease, including fever, pale mucous membranes, lethargy, anemia, apathy, muscle tremors and hematuria and reported a diagnosis positive to *Babesia* spp. by microscopic examination with Wright-stained blood smears.

Anticoagulated blood samples were collected from all dogs for complete blood counts. The blood was centrifuged, separating plasma from erythrocytes; the white cell layer was removed by pipetting, obtaining a concentrate of erythrocytes which was divided into two aliquots and stored at -90°C for further processing for nucleic acid extraction.

Samples of peripheral blood from the ear tip were taken from each animal to prepare thin smears. The slide smears were Wright-stained and examined under light microscopy (magnification 1000X) for detection of intraerythrocytic piroplasms. A scale to establish the degree of parasitemia was used: + (1-5 erythrocytes parasitized per blood smear), ++ (6-20 erythrocytes parasitized per blood smear), +++ (21-50 erythrocytes parasitized per blood smear) and ++++ (>50 erythrocytes parasitized per blood smear) [9]. The parasitic morphometry was determined using the Leica Application Suite version 3.1.1 software (LAS EZ, Leica Microsystems, Switzerland).

DNA Extraction

DNA from 200 µL of concentrated erythrocytes was extracted using the QIAmp blood kit (Qiagen, Chatsworth, CA) kit, according to the manufacturer's instructions. The extraction product was stored at -20°C.

Amplification and Sequencing

Amplification of the 18S ribosomal RNA (rRNA) gene of *Babesia* spp. was performed using primers as shown in *Table 1*. The PCR assay for each subspecies of *Babesia canis* PCR assay was evaluated using the following combinations of primer pairs: BAB1/BAB3 *(B. canis canis),* BAB1/BAB4 *(B. canis vogeli)* and BAB1/BAB5 *(B. canis rossi)* [10]. Amplification was carried out under the following conditions: an initial denaturation step at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 20 sec; a final extension step at 72°C for 5 min.

PCR for detection of *B. gibsoni* was performed using primers GIB599/GIB1270 [11] *(Table 1)* with the following conditions: initial denaturation step at 95°C for 2 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C

Table 1. *Primers used for amplification for PCR Babesia spp.*

Name	Description	Sequence (5'-3')	Product (pb)	Ref.
BAB 1	All *Babesia canis* spp.	GTGAACCTTATCACTTAAAGG (forward)		[10]
BAB 3	*B. canis canis*	CTACACAGAGCACACAGCC (reverse)	746	
BAB 4	*B. canis vogeli*	CAACTCCTCCACGCAATCG (reverse)	590	
BAB 5	*B. canis rossi*	AGGAGTTGCTTACGCACTCA (reverse)	342	
GIB599	*B. gibsoni*	CTCGGCTACTTGCCTTGTC (forward)	665	[11]
GIB1270		GAAGCCGAAATAACGGC (reverse)		

for 30 sec and extension at 72°C for 90 sec; a final extension step at 72°C for 5 min.

Amplifications were performed using a programmable thermocycler. The PCR mixture contained 0,4Nm of each dNTP (Invitrogen, California), 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris - HCl pH 9.0 (GenTaq®, Colombia), 0.4 mm each primer (Oligo Macrogen, Korea), 1.5 U polymerase GenTaq (GenTaq®, Colombia) and 5 µL of DNA previously extracted, to a final volume of 25 µL.

The PCR products were visualized following electrophoresis in 1.5% agarose gel stained with ethidium bromide (0.4 mg/mL) under UV transillumination. Results were captured and recorded using a digital imaging system. For sequencing, amplicons with high DNA concentration were selected. Both strands of amplified fragments were directly sequenced using ABI3730XL (Life Technologies, Applied Biosystems). The sequences were compared for similarity to sequences in GenBank, using the BLAST program hosted by NCBI, National Institutes of Health, USA http://www.ncbi.nlm.nih.gov. Alignments and phylogenetic analyses using the MEGA 6 software package were conducted [12]. A phylogenetic tree was constructed using the Neighbor-Joining model.

Statistical Analysis

The Chi-squared or Fisher's exact tests were used to compare proportions. Differences between independent groups were analyzed with the Mantel-Haenszel test [13]. Analyses were performed with SAS 9.1 software for Windows, with a probability (P) value <0.05 as statistically significant.

RESULTS

Epidemiological and Blood Smear Examination

Forty-two dogs suspected of having babesiosis were included; 23 were females and 19 males, with an age range of 12-84 months. There were 23 dogs from 11 defined breeds and 18 mixed. The most represented breed was Labrador (8/23).

Peripheral blood smear evaluation showed intraerythrocytic piroplasms morphologically consistent with *Babesia* spp. in 54.7% (23/42) of the clinically suspected dogs *(Fig. 1)*. In the morphometric study, 73% (17/23) had dimensions corresponding to large babesias (2.4x5 µm) and 26% (6/23) were classified as small babesias (1x3.2 µm).

Babesia spp. *PCR Analysis*

For differentiation of the subspecies of *B. canis*, PCR was performed separately with each pair of primers. No amplification with primers for *B. canis canis* or *B. canis rossi* was detected. However, PCR with primer of *B. canis vogeli* yielded an amplicon for 26% (11/42) of the samples. No amplification with primers for the species *B. gibsoni* was detected.

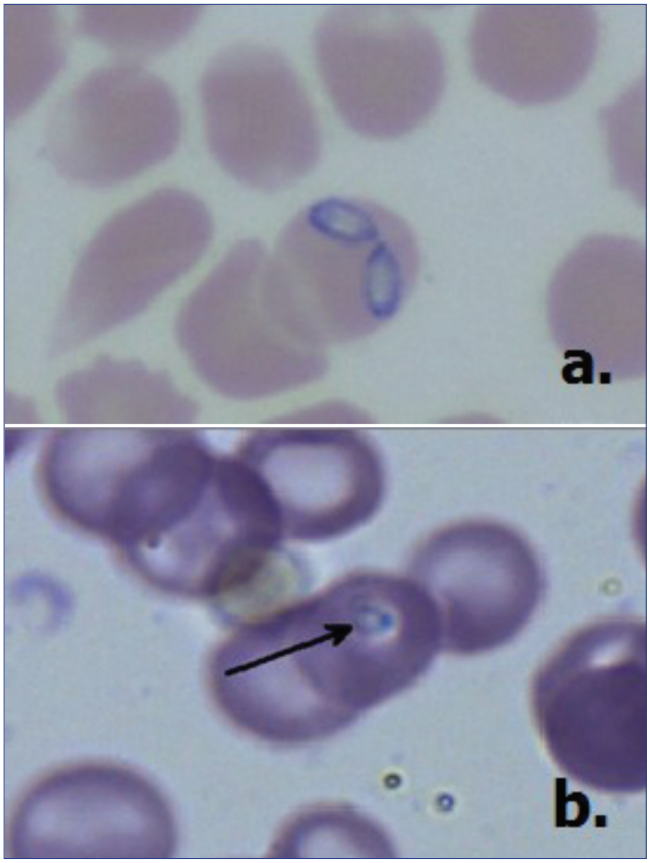

Fig 1. Intraerythrocytic forms of *Babesia* spp. observed in the extended blood of patients (1000x). a. Large *Babesia* sp. (4.4 µm x 2.2 µm); b. Small *Babesia* sp. (1.6 µm x 1 µm)

Clinical and Pathological Findings in Dogs Infected with B. Canis Vogeli

Seven dogs were female and four were male. Ages ranged from 3 to 84 months, with three puppies and eight adults. The patients belonged to various breeds: mixed (4/11), Labrador (1/11), Schnauzer (1/11), Pitbull (1/11), Bullterier (1/11), Beagle (1/11), Pug (1/11), Fila Brasileiro (1/11) *(Table 2)*. Statistically significant differences for these variables (Fisher's exact test; P=0.72; P=0.45; P=0.08 respectively) were not found.

The clinical signs on physical examination of 11 dogs with *B. canis vogeli* were: anorexia 63% (7/11), lethargy and apathy 63% (7/11), fever 54% (6/11), pale mucous membranes 54% (6/11), lymphadenomegaly 18% (2/11), vomiting 9% (1/11) and diarrhea 9% (1/11). Statistically significant differences for the clinic signs were not found (P=0.02).

Complete blood counts showed that 70% (8/11) of the dogs had anemia, with a hematocrit value below the reference interval (37-45%). The leukocyte disorders were variable; 27% (3/11) of the patients had leukocytosis and 46% (5/11) had leukopenia *(Table 2)*.

Table 2. *Clinical and pathological findings in 11 dogs infected with B. canis vogeli*

Dog ID/sex/age (m)	Breed	Clinical Signs	Hematological Abnormalities	Parasitemia Degree
25/F/36	Mixed	Anorexia, lethargy and apathy, pale mucous membranes, vomiting	Anemia	+
26/M/24	Mixed	Fever, anorexia, lethargy and apathy, pale mucous membranes	Anemia, thrombocytopenia	++
27/F/4	Fila Brasileiro	Anorexia, lethargy and apathy, pale mucous membranes, demodicosis	Anemia, leukocytosis	+++
30/F/3,5	Bulterrier	Lymphadenomegaly and demodicosis	Thrombocytopenia	++
31/F/24	Mixed	Fever, anorexia, lethargy and apathy	Leukopenia, thrombocytopenia	++
32/F/3	Schnauzer	Fever, anorexia, lethargy and apathy, pale mucous membranes,	Anemia, leukopenia	++++
34/F/48	Mixed	Pale mucous membranes	Anemia, leukocytosis	+
37/M/30	Pug	Fever, lethargy and apathy, pale mucous membranes	Anemia, leukopenia	++
38/F/48	Beagle	Anorexia, lethargy and apathy, diarrhea	Leukopenia, thrombocytopenia	+++
41/M/84	Piltbull	Health check for infertility	Anemia, leukopenia, thrombocytopenia	+
43/M/48	Labrador	Fever, anorexia, lymphadenomegaly	Anemia, leukocytosis, thrombocytopenia	+

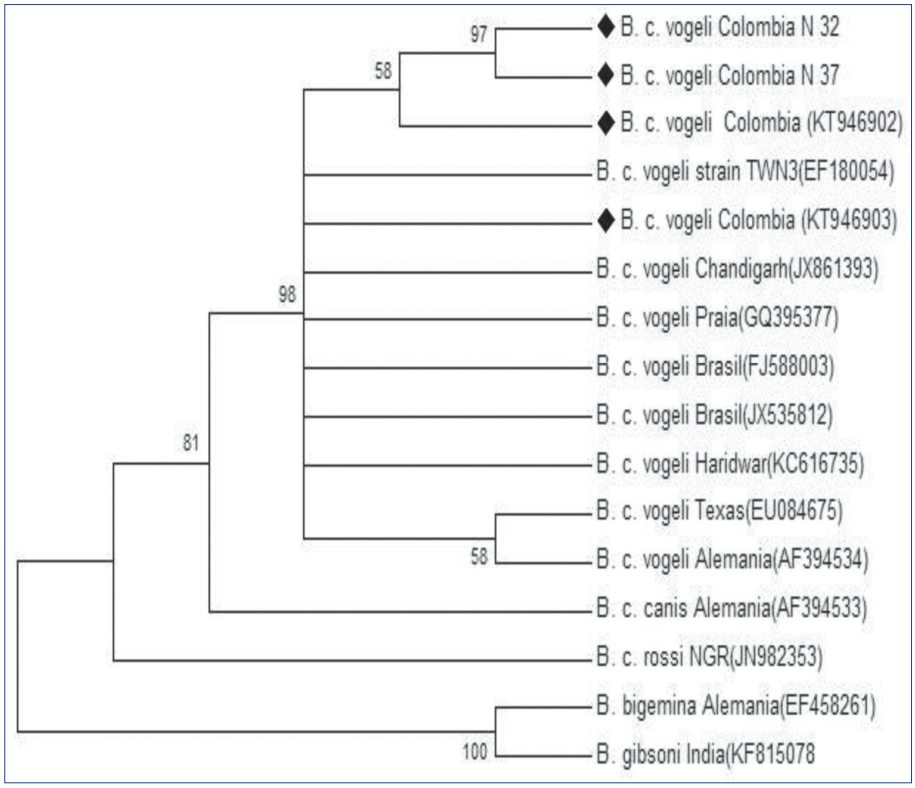

Fig 2. Phylogenetic analysis showing the position of the group of *B. canis vogeli* found in this study, obtained by comparision of partial sequence of the 18S rRNA gene with the sequences of *Babesia* spp. available in GenBank (accession numbers in parentheses). The neighbor-joining method was used, based in the kimura 2-parameter model. The numbers on the tree indicate bootstrap values for branch points (500 replications). The evolutionary analysis was made with the MEGA 6 program [12]

Phylogenetic Analysis

Five of the 11 *B. canis vogeli* samples were sequenced and compared with the sequences available in the GenBank database. The samples showed 99% identity with the sequences of *B. canis vogeli* Chandigarh (India, GenBank JX861393), Praia (Cape Verde, GenBank GQ395377), Recife (Brazil, GenBank FJ588003), Taiwan (China, GenBank EF180054), Brazil (GenBank JX535812), Haridwar (India, GenBank KC616735) and Texas (GenBank EU084675). Sequence analysis of *B. canis vogeli* found in this study grouped with *B. canis vogeli* sequences available in GenBank with high bootstrap levels of 98% *(Fig. 2)*. Two *canis vogeli* strain Colombia 18S rRNA partial sequence were deposited in GenBank database under access numbers KT946902 and KT946903.

DISCUSSION

This study demonstrated for the first time in Córdoba, that

canine babesiosis is mostly caused by large piroplasms (73%). Molecular characterization confirmed that *B. canis vogeli* is the most common cause of babesiosis, and also showed high genetic similarity with *B. canis vogeli* in other countries. However, in 6 of 42 patients the morphological study showed characteristics of small *Babesia* sp. *B. gibsoni* is a small *Babesia* transmitted by *R. sanguineus* and reported to affect dogs in South America; it has been reported in countries like Brazil [14] and recently in Nicaragua [15]. In this study, there was no evidence of its presence and there are no reports in Colombia. However, its existence cannot be excluded due to the presence of the vector.

Clinical manifestations of *B. canis* in patients vary depending on the subspecies involved [1]. In the present study, only *B. canis vogeli* was found and patients presented with variable clinical signs; fever (T>39.5°C) (6/11), anorexia (7/11), lethargy and apathy (7/11), pale mucous membranes (6/11). The clinical variability presentation of the diseases in the present study is consistent with other studies, in which the same subspecies of *Babesia* was detected [8,16,17].

In the present study *B. canis vogeli* was frequently found in adults; however, higher levels of parasitemia were observed in the three puppy patients (≤1 year), confirming the increased susceptibility of these to the *Babesia* spp. [1,18]. This result is similar to reported by Solano Gallego in Italian puppies (1-2 months); in four of 11 dogs with *B. canis vogeli,* the puppies developed severe hemolytic anemia with fatal outcome for two puppies [8].

The clinicopathological data of dogs infected with *B. canis vogeli* of this study were similar to those reported by other authors, without observing a homogenous clinico-pathological pattern as reported by Solano et al.[8] and Cardoso et al.[4] in their studies in dogs in Italy and Portugal, respectively. In the present study, most patients had alterations erytrhocyte (73%: 8/11), similar results were found by Carli et al.[19] (67%). However, our results were lower than those reported by Solano Gallego et al.[8] (93%) in dogs of Italy.

Alterations in the white cell profiles, as leukocytosis and leukopenia, have been described irregularly in babesiosis, with leukopenia being more common. In this study, 46% of patients had leukopenia, similar to that reported by [1,8,9,19]. Leukocytosis in babesiosis is less frequent [1]; in our study it was observed in 27% (3/11) of patients. However, it has also been reported in other studies worldwide [8,9,20]. Leukopenia is a component of the systemic inflammatory response syndrome (SIRS) described in babesiosis and it is included in the criteria for the diagnosis of sepsis in dogs [9].

Thrombocytopenia is a typical finding in canine babesiosis; immune-mediated platelet destruction, co-infections and sequestration of platelets in the spleen are possible mechanisms [1,2]. In this study, 46% of the positive patients showed thrombocytopenia; similar findings have been reported in other studies worldwide, such as Ruiz de Gopegui et al.[20] in Italy and Inokuma et al.[11] in Japan, with 100% and 60% thrombocytopenia respectively. The results reported for these authors are higher than those in this study. However, Solano Gallego et al.[2] reported a 36% thrombocytopenia in dogs in Italy, lower that the value found in our study.

B. canis vogeli is the most globally distributed subspecies of *Babesia*; it is found in Africa [21,22], Europe [4,16], Asia [11,23] and Australia [24,25]. In South America, *B. canis vogeli* has been reported in Colombia [26], Venezuela [17], Brazil [27,28] and Argentina [29]. This discovery is supported by the geographical distribution of tick *R. sanguineus* as vector, being the Ixodide species most widely distributed in domestic dogs in different regions of the country [30].

The presence of *B. canis vogeli* and absence of *B. canis rossi* and *B. canis canis* in naturally infected dogs in Colombia contrasts with the results reported by Düzlü et al.[31], where *B. canis canis* (12.0%) and *B. gibsoni* (9.0%) were the most prevalent Babesia species in blood samples of dogs in Turkey, *B. canis vogeli* (2.3%) was found in lower proportion.

B. canis vogeli sequences found in this study, are closely related to isolates from Asia (India), Africa (Praia), North America (United States) and South America (Brazil). Despite the proximity with Venezuela, *B. canis vogeli* sequences found in the present study could not be compared with isolates reported in Venezuela [17]. The same is true for isolates reported by Vargas et al.[26] in the central area of Colombia, because the amplified gene fragment of 18S ribosomal does not correspond to the amplified fragment of this study.

In conclusions, the situation of the absence of others species or subspecies of *Babesia* may change in the future due to different factors. These include climate change, especially global warming and anthropogenic factors such as permanent travel companion animals, the resistance of vectors and pathogens to products and medications for control as well as failures in treatment and prevention. These factors are responsible for the spread of tick borne diseases to non-endemic areas.

This work demonstrated for the first time in the Caribbean the presence of *B. canis vogeli* in dogs and the clinical and epidemiological characteristics associated with infection by this hemoparasite. The molecular identification was extremely valuable as a tool for epidemiological analysis, allowing the inference that there are other species in the Caribbean that require more extensive studies in the region both in dogs and other domestic species.

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Statement of Animal Rights

This article does not contain any studies with animals performed by any of the authors.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Research Article

Whole Genome Sequencing of the Dzo: Genetic Implications for High Altitude Adaptation, Sterility, and Milk and Meat Production

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Abstract

The Dzo, a hybrid between the domestic Yak and cattle found on the Qinghai-Tibetan plateau are commercially important owing to increased meat and milk production, as well as their adaptation to extremely high-altitude environments. To better understand the genomic architecture and adaptive capabilities of this unique domesticated hybrid, we performed whole genome resequencing and compared the genomic architecture and variation of the Dzo to its progenitors: the cattle and Yak. In total, 33.17 M single nucleotide variations (SNVs) were detected between the Dzo and cattle reference genomes. Even though the Dzo is known to be sterile, no genetic signatures associated with sterility were found on the Dzo Y chromosome. On the contrary, our results suggest that the autosomal *DMC1* locus (Chromosome 5. 110729098. C>CT) plays a role in the sterility of Dzo, which warrants further exploration of its functions. We integrated the whole genome resequencing data of cattle and Yak to obtain candidate genes with a high degree of variation that might be associated with altitudinal adaptation. We found that the *EPAS1* gene, which encodes hypoxia-inducible factor 2α, exhibited significant variation (Chromosome 11.28664187 C>T) between Yak and cattle, and may play a key role in the genetic basis of altitudinal adaptation. In addition, in analyzing differences between the Dzo, Yak, and cattle genomes, we uncovered several additional genomic signatures relevant to high altitude adaptation and meat and milk production. These findings underscore the need for further studies to improve ruminant stock for sustainable agriculture on the Qinghai-Tibet plateau..

Keywords: *Dzo: Genome sequencing, Dzo, Altitude adaptation, Sterility, Milk and meat production*

Dzo Sığırlarında Tüm Genom Sekanslaması: Yüksek Rakıma Adaptasyon, Sterilite İle Süt ve Et Verimine Etkisi

Öz

Evcil Yak ile Qinghai-Tibet platosunda bulunan sığırın bir hibriti olan Dzo artmış et ve süt üretimi ile birlikte aynı zamanda oldukça yüksek rakımlı bölgelere adaptasyonu nedeni ile ticari olarak önem arz etmektedir. Bu özgün evcil hibritin genomik yapısını ve uyum kapasitelerini daha iyi anlamak amacıyla tüm genom sekanslaması yapılarak genomik yapısı ve varyasyonlar Dzonun progenitörleri olan sığır ve Yak ile karşılaştırıldı. Dzo ile sığır referans genomları arasında toplam 33.17 M tek nükleotid varyasyonu belirlendi. Dzo steril olarak bilinmesine rağmen Dzo Y kromozomunda sterilite ile ilgili hiç bir genetik işarete rastlanmadı. Aksine, elde edilen bulgular otozomal *DMC1* lokusunun (Kromozom 5. 110729098. C>CT) Dzonun sterilitesinde rol oynadığına işaret etmekte ve bu nedenle de daha ileri araştırılmaların yapılması gerekmektedir. Yüksek rakıma adaptasyon ile ilişkili yüksek oranda varyasyona sahip genlerin araştırılması amacıyla sığır ve Yakın tüm genom sekanslama verilerinin entegrasyonu gerçekleştirildi. Hipoksi ile indüklenebilir faktör 2α'yı kodlayan *EPAS1* geni Yak ve sığır arasında yüksek varyasyon (Kromozom 11.28664187 C>T) göstermekteydi ve bu nedenle yüksek rakıma adaptasyonun genetik temelinde rol oynayabileceği düşünüldü. Ayrıca, Dzo, Yak ve sığır genomları arasındaki farklılıklar analiz edildiğinde, yüksek rakıma adaptasyon ile birlikte et ve süt üretimine etki eden bazı ilave genomik işaretlere rastlandı. Bu bulgular Qinghai-Tibet platosunda daha ileri ve devam ettirilebilir ruminant yetiştiriciliği için daha fazla çalışmalara ihtiyaç olduğunu göstermektedir.

Anahtar sözcükler: *Dzo: Genom sekanslama, Dzo, Yüksek rakım adaptasyonu, Sterilite, Süt ve et üretimi*

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INTRODUCTION

The Yak *(Bos grunniens)* inhabits the Qinghai-Tibetan plateau of China and the adjacent highland regions of Central and East Asia, at altitudes of 2000-5500 m. These animals possess numerous anatomical features and physiological traits that facilitate life at high altitudes, including enlarged lungs and hearts, and the lack of hypoxic pulmonary vasoconstriction. In addition, these animals are efficient foragers in the extreme environment that characterizes livestock production in this challenging landscape. Currently, more than 14 million domestic Yak provide meat, milk, transportation, dung for fuel, and hides for Tibetans and other nomadic pastoralists living in Central Asia, while approximately 15.000 wild Yak also inhabit this region. In addition, the Yak of the Qinghai-Tibetan plateau exhibits high degree of genetic and phenotypic diversity, where this diversification has driven their development as a genetic resource, including the formation of various locally-adapted phenotypes and hybrid forms. In regions of mixed pastoralism and agriculture at lower altitudes in Central Asia, the deliberate hybridization of Yak and cattle is now widespread [1], where the Dzo *(Bos taurus x Bos grunniens)*-the first-generation male hybrid of male cattle and female Yak-has become an economically important resource. The Dzo adapts well to local environmental conditions and retains important production characteristics from both parental species: the ability to adapt to a harsh environment from the Yak and increased productivity from cattle. As a result, the Dzo exhibits higher productivity than the Yak in terms of both milk and meat yields [2,3], yet possesses better physiological adaptation to high altitude environments than cattle.

However, although these F_1 interspecies hybrids exhibit clear production advantages, male hybrids are sterile, and thus phenotypic quality and genetic variation in the Dzo is currently a result of parental genotypic combinations and their segregation patterns alone [4]. In recent years, although a number of studies have been carried out to investigate the sterility of the Dzo-such as studies involving breeding, cytogenetics, tissue morphology, endocrinology, biochemistry, and molecular biology-the underlying mechanisms of male sterility remain unclear, despite the potential importance of solving this problem for future agricultural development. Furthermore, understanding the genetic architecture of cattle, Yak, and Dzo from a genomic standpoint could provide important insights into understanding the quantitative trait loci involved in the survival and performance of hybrids, as well as the interactions between genes and pathways that allow hybrids to maintain high levels of productivity in the face of multiple environmental challenges [5,6].

In the present study, we sequenced the genomes of both the Dzo and wild Yak and compared these sequences to previously published domestic Yak genome data and bovine single nucleotide polymorphism (SNP) data from the 1.000 bull genomes project [7,8]. Thus, the objectives of this investigation were to 1) improve our understanding of the mechanisms of adaptation to the plateau environment in the Yak, and 2) to analyze how loci associated with adaptation to this extreme environment are configured in the Dzo, with the goal of elucidating both the basis of trait differences (milk and meat production) in cattle, Yak, and Dzo, and mechanisms of male sterility in the Dzo. As a result, by characterizing the genetic architecture of the Dzo, we have highlighted genetic differences between these regionally important livestock and have begun to explore roles of these differences in local adaptation, and their importance for genetic resource management.

MATERIAL and METHODS

Ethics Statement: Methods of animal care and use were approved by the Institution of Animal Care and Use Committee of Southwest Minzu University and Qinghai University.

Samples, Sequencing, and Library Construction: All Dzo and Yak used in the present study were selected at the age of three from a slaughterhouse in Tongde County (100°63'E; 35°24'N) and the Datong Yak breeding farm (101°67'E; 36°92'N), both in Qinghai province, China. After injecting Dzo with 2×10^{-3} mL xylazine hydrochloride per kg body weight, Dzo were slaughtered and pooled Dzo (PI) samples of testicular tissue from six F1 individuals were obtained. Wild Yak (YA) blood was taken from the jugular veins of six males, and DNA subsequently isolated using the Aidlab Genomic DNA Extraction Kit (Aidlab Biotechnologies Co., Ltd, Beijing, China). For the purposes of the present study, Yak was considered to be wild based on the following taxonomic characters: long hair and large skeleton. Genomic DNA was extracted (TaKaRa, Dalian, China) for library construction, and Illumina paired-end sequencing libraries (insert sizes of 500 bp) were constructed according to the manufacturer's instructions (Illumina, San Diego, California, USA). Sequencing was performed on an Illumina HiSeq 2000 (carried out at BGI, Shenzhen, China). In addition, sequence data from three wild Yak (W1, W2, and W3 from Hoh Xil National Nature Reserve) and three domestic Yaks (D1, D2, and D3, collected from Gansu, Sichuan, and Tibet, respectively) were downloaded from the *Bos grunniens* Genome sequencing BioProject (NCBI Accession No.: PRJNA217895) for comparative analyses.

Read Filtering and Alignment: Raw sequencing reads from a total of eight samples (six Yaks from NCBI and two pooled samples from the present study) were filtered on the basis of chastity score, and trimmed on the basis of quality score, using Fast QC (v0.10.1) and Trimmomatic (v0.32) [9,10] in four steps: 1) removal of adapters; 2) removal of bases from the start or end of a read, if the quality threshold fell below 3; 3) scanning the read with a 4-base pair sliding

window, and removal when the average Phred quality per base fell below 15; and 4) removal of reads shorter than 50 bases or average Phred quality below 20 for the whole read. Clean reads were mapped to the *B. taurus* reference genome (assembly UMD3.1) [11] using BWA (0.7.10-r789) [12] using the BWA-MEM algorithm. Detailed parameters were as follows: java-jar trimmomatic-0.32.jar PE ILLUMINACLIP: TruSeq3-PE. fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW: 4:15 MINLEN:50./bwa mem -t 4 -k 32 -M.

Variant Identification and Annotation: After sequences were aligned, files were converted to BAM format using SAMtools (v1.0) [13]. BAM files were sorted, and duplicate reads were filtered using Picard. Single nucleotide variations (SNVs) and the detection of insertions or deletions (INDELs) were performed using the Genome Analysis Toolkit (GATK, version 2.4-9) [14-16]. SNVs data were downloaded from the 1000 bull genomes project [7] (http://www.1000bullgenomes.com/doco/all_snps_annotated_2013_04_25.tab.gz). Structural variants (SVs) with a confidence score ≥ 40 (Phred quality score) in the YA and PI genomes were detected using Breakdancer (v1.1) [17]. The annotation of SNVs, INDELs, and SVs were conducted using an in-house perl script. Sequence alignments were constructed between proteins that were altered by missense SNVs and proteins encoded by orthologous genes and were scored using the SIFT algorithm to predict the functional impact of protein substitutions [18]. A Y chromosome from cattle *(B. taurus)* was downloaded from NCBI (GenBank No.: CM001061) as a reference sequence. Clean reads were mapped to the Y chromosome reference sequence using BWA (0.7.10-r789) with a BWA-MEM algorithm. The identification of variation and steps for annotation were performed as previously mentioned.

Population Structure: We constructed a phenetic tree using all SNVs with genetic distance matrices between individuals. A neighbor-joining algorithm was used in TreeBeST (https://github.com/Ensembl/treebest) with uncorrected p-distances and bootstrapping (1.000). MEGA5 [19] was used to visualize the tree, and FRAPPE (v1.1) [20] was utilized to infer population structure and ancestry. This analysis was based on 33 M SNVs, and no prior information regarding ancestry was assumed. We ran 10.000 iterations on a pre-defined number of clusters (K; 2-7).

RESULTS

We obtained a total of 230.43 Gb of data from the pooled genome sequences of eight samples, including the Dzo (PI) and wild Yak (YA) collected for this study. The PI and YA samples were sequenced to a depth of approximately 24X and 26X respectively, while the other six Yaks were sequenced to a depth of 6X. In total, 33.17 M single nucleotide variations (SNVs) were identified between the genome sequences of the eight samples and the cattle reference genome (UMD3.1), while 238,188 SNVs were found in coding sequence (CDS) regions, where 99.409 non-synonymous SNVs were annotated. In addition, 13.04 M SNVs were observed in the seven Yak genome sequences, and 30.95 M SNVs were found when comparing the seven Yak genomes to the bovine reference genome. When comparing the Yak data to the 1.000 bull SNP data (26.72 M), we detected 7.58 M SNV sites, where 221.42 K sites exhibited different allelic types (2.92%). As expected, a very high percentage (91.23%) of the 27.50 M SNVs were heterozygous in the Dzo when compared to the bovine reference genome, while 27.63% of the 27.61M SNVs were heterozygous between the YA and bovine reference genome. Furthermore, 3.57 M insertions and deletions (INDELs) were detected between the bovine reference genome and the eight samples (PI, YA, and the six Yak sequences from NCBI [W1, W2, and W3; and D1, D2, and D3]), 7.323 of which were in CDS regions. In total, 58.514 structural variants (SV) were detected in comparison PI and the reference genome, while 73.221 SVs were identified between the YA and the reference genome.

As shown in *Fig. 1A*, the neighbor-joining tree demonstrated that the Dzo (PI) is positioned between the cattle and the Yak. For Bayesian clustering, when K = 2, the cattle and Yak were identified as separate clusters *(Fig 1B);* however, when K = 3, the domestic and wild Yak cluster was divided into two groups. Interestingly, the wild Yak (YA) sample in this study featured approximately 24% of the domestic Yak genomic proportions, although the observed phenotypes of domestic Yak could not be distinguished from those of typical wild Yak. For the three wild Yak sequenced from the Hoh Xil National Nature Reserve, no genomic signals of the domestic Yak could be detected. The finding that there

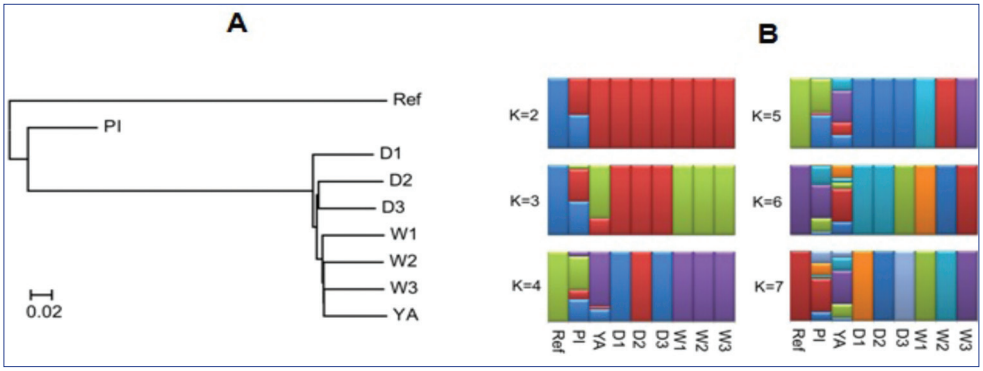

Fig 1. Neighbor-joining tree and population structure for Yak and Dzo. (A) The neighbor-joining tree for Yak and Dzo; (B)The population structures for Yak and Dzo. Ref: Cattle; PI: Dzo; YA: wild yak; W1 to W3 and D1 to D3: The three wild Yaks and domestic Yaks which sequence data were downloaded from *Bos grunniens* Genome Sequencing Bio-project, respectively

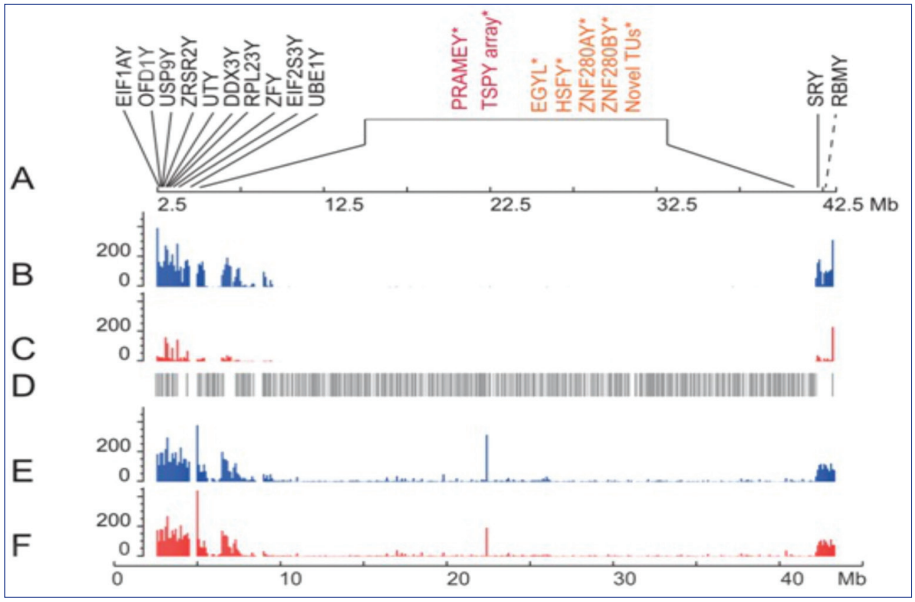

Fig 2. Genome landscape of the bovine male-specific region of the Y chromosome (bMSY). (A) Gene map of bMSY. X-degenerate single-copy genes or transcripts (black) are clustered at either end of the bMSY, whereas multicopy genes/transcripts (red and orange) are present in the majority of the bMSYs. RBMY was missing from the draft assembly. The relative position of RBMY (dashed line) was determined based on a RH-mapping analysis. (B) The SNV distribution in YA chromosome. (C) The SNV distribution in the PI chromosome. (D) A gene map of bMSY. Gray lines indicate the gene region distributed on bMSY. (E) The read depth of YA. (F) The read depth of PI. Window size was set to 100 Kb. The scale on the bottom of the ideogram is based on the bovine Y chromosome assembly

Table 1. *Alignment results of nine X-degradation genes of Y chromosomes between PI and cattle*

Gene ID	Gene Start	Gene End	Gene Covered Base	Gene Depth	Exon Depth
EIF1AY	2678121	2664668	13450	46.56	11.35
OFD1Y	2760114	2824003	63890	313.92	N/A
USP9Y	2907549	3033182	125615	209.99	15.38
ZRSR2Y	3112384	3172379	59968	543.71	18.91
UTY	3224590	3409324	184678	233.05	11.86
DDX3Y	3458510	3468728	10219	33.19	17.31
ZFY	3727723	3748502	20780	125.14	23.55
EIF2S3Y	3815112	3862568	47444	235.17	32.32
SRY	42225210	42225899	690	11.04	11.04

N/A represents no exon information for OFD1Y in NCBI

was a substantial degree of overlap between the genomic sequences of the supposedly wild Yak (YA) sequenced in the present study and the accessed domestic Yak sequences indicates that the wild Yak used in the present study were likely wild/domestic Yak hybrids.

Although the bovine genome project included the Y chromosome, resequencing data for the Y chromosome has largely been ignored owing to its abundance of repetitive and palindromic sequences. In this study, we mapped the sequence data from PI and YA to the cattle Y chromosome (GenBank No.: CM001061). Based on this alignment, we inferred that domestic Yak D1 and D2 are females, which was not elucidated in the original study [8] owing to the lack of alignments involving the key sex determination (*SRY*) gene used in determining the sex of most mammals. The alignment performed in the present study also highlighted the abundance of repetitive sequences on the Y chromosomes, especially at the telomeres *(Fig. 2)*, where at the ends of the Y chromosome, genes mainly originate via X chromosomal degradation [20]. The *SPY* gene is important in sex determination, while the highly repetitive sequences around these single copy genes may provide protection against degradation [20,21]. The average sequencing depth of the nine single copy genes analyzed in PI and YA in the present study (Eukaryotic translation initiation factor 1A Y-linked *(EIF1AY)* gene, the Y-linked oral-facial-digital, type 1 *(OFD1Y)* gene, the ubiquitin-specific protease 9, Y chromosome *(USP9Y)* gene, the RNA-binding motif and serine/arginine rich 2 *(ZRSR2Y)* gene, the ubiquitously transcribed tetratricopeptide repeat gene on Y chromosome *(UTY) gene*, the DEAD box helicase 3 Y-linked *(DDX3Y)* gene, zinc-finger protein gene on Y-chromosome *(ZFY)* gene, the eukaryotic translation initiation factor 2, submit 3 and structural gene Y-linked *(EIF2S3Y)* gene and *SRY)*, including introns and intergenic regions, were 180X and 197X, respectively *(Table 1* and *Table 2)*. However, it is important to note that except for STY, some sequences are likely to have been erroneously attributed to these genes owing to the abundant repetitive elements in the introns, which could not be filtered. In contrast, the sequencing depths for the exonic regions in PI and YA were 17X and 20X, respectively. Here, we detected 3.219 SNVs between the Dzo (PI) and cattle Y chromosome,

Table 2. *Alignment results of nine X-degradation genes of Y chromosomes between YA and cattle*

Gene ID	Gene Start	Gene End	Gene Covered Base	Gene Depth	Exon Depth
EIF1AY	2678121	2664668	13358	51.26	15.69
OFD1Y	2760114	2824003	63650	340.97	N/A
USP9Y	2907549	3033182	125601	225.72	19.74
ZRSR2Y	3112384	3172379	59995	608.52	19.41
UTY	3224590	3409324	184735	251.36	16.19
DDX3Y	3458510	3468728	10219	36.78	17.09
ZFY	3727723	3748502	20780	134.22	22.56
EIF2S3Y	3815112	3862568	47438	262.80	43.73
SRY	42225210	42225899	690	8.36	8.36

N/A represents no exon information for OFD1Y in NCBI

Table 3. *SNV annotation results of Chromosome Y of PI*

Chr ID	Loci	SNV	Amino Acids	Syn/Non	Gene_ID	Protein_Id
ChrY	3171104	G<->A	R<->H	1	100306950	XP_003584415.1
ChrY	3171086	G<->C	S<->T	1	100306950	XP_003584415.1
ChrY	4394284	G<->A	A<->T	1	100849661	XP_003584416.1
ChrY	4394211	A<->T	E<->V	1	100849661	XP_003584416.1
ChrY	4394169	A<->G	Y<->C	1	100849661	XP_003584416.1
ChrY	3728235	G<->T	V<->L	1	280962	NP_803457.1
ChrY	3506348	A<->G	M<->V	1	790278	XP_001256796.3
ChrY	2544329	G<->A	A<->A	0	100849399	XP_003584412.1
ChrY	5114940	A<->G	T<->T	0	100849792	XP_003584418.1
ChrY	3641828	A<->G	P<->P	0	100849362	XP_003584411.1
ChrY	3641999	C<->T	Y<->Y	0	100849362	XP_003584411.1
ChrY	3642115	C<->T	T<->I	1	100849362	XP_003584411.1
ChrY	3860224	C<->T	Y<->Y	0	100271755	XP_003584417.1
ChrY	3860206	T<->C	S<->S	0	100271755	XP_003584417.1

where only eight SNVs were non-synonymous *(Table 3)*. For wild Yak (YA), 14.630 SNVs were detected, 58 of which were non-synonymous.

We analyzed 52 autosomal genes known to be related to meiotic processes in order to explore male sterility in the Dzo (PI). For example, the positive regulatory domain zinc finger protein 9, encoded by the *PRDM9* gene, is a major determinant of meiotic recombination in humans and mice [22], where variation within this gene strongly influences recombination hotspot activity and meiotic instability in humans [23]. Previous studies have shown that mRNA levels of *PRDM9* are much lower in the testes of sexually immature Yak calves and sterile male cattle-Yaks in comparison to those of normal adult Yaks, suggesting that *PRDM9* might be associated with male fertility in the Dzo. In total, 513 SNVs were detected in these 52 genes, 251 of which were non-synonymous, and 44 of which were found in the SNV set from the 1.000 bull genome project. Of the non-synonymous SNVs, 97 SNVs were fixed, were observed to be different between the Yak and cattle sequences and were found to be heterozygous in the Dzo genome. For the *PRDM9* gene, we found 38 SNVs, where 26 of these were non-synonymous. Interestingly, we also found an INDEL (Chromosome 5.110729098. C>CT) located in the CDS region of the meiotic recombinase *(DMC1)* gene, and seven non-synonymous SNVs in the replication protein A *(RPA)* gene family *(Table 4*, we only present the non-synonymous also in the 1.000 bull project SNV set). The annotated SNVs for these genes may be a starting point for further detailed research into male sterility in the Dzo.

Four genes related to altitudinal adaptation *(EPAS1*, *EGLN1*, *HYOU1*, and *HMBS)* were examined to explore adaptive differences between Yak, Dzo, and cattle. These genes have previously been identified in Tibetan people, and have been shown to help humans adapt to high altitude conditions [24-27], hypoxia, and myocardial infarction [28]. Previous studies of these genes in local domestic livestock were performed either solely within cattle [29] or Yak. In total, 44 SNVs were found in the CDS region of these four genes, including 16 non-synonymous SNVs. Interestingly, only two of these non-synonymous SNVs were in the *EPAS1* gene from the 1.000 bull project SNV set. Moreover, while 14 of the non-synonymous SNVs in the aforementioned genes could be distinguished between cattle (reference

Table 4. *SNP annotation of male-sterile*

ChrID	Location	Pop_SNP	Gene Name	Codon	CDS_ID	Protein_ID	1000BullDB
Chr1	45021322	A G G G R G G G G	PRDM9	AGA<->GGA;	cds276	XP_002683648.2	A/G
Chr1	45028357	T Y Y Y Y Y Y Y Y	PRDM9	TTT<->TCT;	cds276	XP_002683648.2	T/C
Chr1	45028408	C S S S S S S S S	PRDM9	TCC<->TGC;	cds276	XP_002683648.2	C/G
Chr1	45028440	G R R R R R R R R	PRDM9	GGA<->AGA;	cds276	XP_002683648.2	G/A
Chr1	45028459	G R R R R R R R R	PRDM9	GGA<->GAA;	cds276	XP_002683648.2	G/A
Chr1	45029328	T K K K K K K K K	PRDM9	CTC<->CGC;	cds276	XP_002683648.2	T/G
Chr1	45029346	A G G G G G G G G	PRDM9	GAA<->GGA;	cds276	XP_002683648.2	A/G
Chr1	45029661	G A A A R A A R A	PRDM9	AGC<->AAC;	cds276	XP_002683648.2	G/A
Chr1	45033708	G G S G S G S S S	PRDM9	GCA<->CCA;	cds276	XP_002683648.2	G/C
Chr1	45033759	C Y Y Y Y Y Y Y Y	PRDM9	CGA<->TGA;	cds276	XP_002683648.2	C/T
Chr19	23508655	A G G G R G G G G	RPA1	ACC<->GCC;	cds33180	NP_001068644.1	A/G

Table 5. *SNP annotation of EPAS1, EGLN1, HYOU1 and HMBS*

ChrID	Location	Ref D1 D3 D2 PI W1 W2 W3 YA	Gene Name	Codon	Non/Syn	CDS_ID	Protein_ID	1000BullDB
Chr11	28641695	A G G G G G G G G	EPAS1	ACA<->ACG	0	cds20127	NP_777150.1	A/G
Chr11	28650973	C G G G G G G G G	EPAS1	CAA<->GAA	1	cds20127	NP_777150.1	C/G
Chr11	28659125	T C C C Y C C C C	EPAS1	TTT<->TTC	0	cds20127	NP_777150.1	T/C
Chr11	28659176	G G G G R G G G G	EPAS1	GCG<->GCA	0	cds20127	NP_777150.1	/
Chr11	28660443	A G G G R G G G G	EPAS1	ACA<->ACG	0	cds20127	NP_777150.1	A/G
Chr11	28662841	T C C C Y - - C C	EPAS1	CTC<->CCC	1	cds20127	NP_777150.1	T/C
Chr15	30201004	C T T T Y T T T T	HMBS	CCC<->CCT	0	cds25736	NP_001039672.1	C/T
Chr15	30201004	C T T T Y T T T T	HMBS	CCC<->CCT	0	cds25737	XP_005216001.1	C/T

sequence) and Yak, there were no polymorphisms detected within the Yak population. It is notable that the only variant (Chromosome11.28664187C>T) between Yak and cattle is predicted to be deleterious using SIFT and was found to be located in the *EPAS1* gene *(Table 5,* we only present the SNP annotation also in the 1.000 bull project SNV set).

We examined 52 genes related to production traits, including growth, milk, and meat characteristics. Of these genes, 537 SNVs were found in CDS regions, 189 of which were non-synonymous, and 57 of which were found in the 1.000 bull data set. Hormone-sensitive lipase *(LIPE)*, which is considered to be an important candidate gene associated with meat traits, is also involved in free fatty acid mobilization [30]. Here, we found six non-synonymous SNVs in the *LIPE* gene that exhibited fixed differences between Yak and the cattle reference sequence. Of these, three were found in the 1.000 bull project data set (Chromosome18. 51226081G>A; Chromosome18. 51226221A>G; Chromosome18. 51226425A>G), and three were not found in the 1.000 bull SNV set (Chromosome18. 51221047C>T; Chromosome18. 51222967G>A; Chromosome18. 51226270C>A). For fatty acid binding protein *(FABP)* genes (a family of transport proteins for fatty acids and other lipophilic substances, such as eicosanoids and retinoid [31,32], 24 non-synonymous SNVs were found in the CDS region, 11 of which were fixed between Yak and the bovine reference sequence *(Table 6,* we only present the non-synonymous also in the 1.000 bull project SNV set). These fixed SNV differences between Yak and cattle populations could be associated with observed meat quality differences, and thus warrant further investigation. Interestingly, in four genes related to growth and milk production *(GH1*, *GHR*, *PRL*, and *PRLR),* we did not detect any non-synonymous SNVs in *GH1* and *PRL* but did find eight non-synonymous SNVs in *GHR* and *PRL* between cattle and Yak *(Table 6)*. These results indicate that the receptor genes for growth hormone and prolactin secretion may play an important role in differences in growth and milk production between the congeners.

DISCUSSION

Although the draft genome sequence of the Yak has been available for four years, and resequencing studies have since been undertaken [6,33,34], genetic variation in the Yak-most notably structural variation and genetic comparisons with other bovid species-Remains poorly understood. Moreover, the genome sequence of the Dzo has not been reported until now. Although the Dzo is the F1 hybrid between cattle and Yak, and the SNPs found in the Dzo indeed derive from that simple cross, it unclear which parental gene combinations produce viable Dzo, or how this variation segregates across individuals. Thus, not only did our study focus on the Dzo, but on the relationships

Table 6. *SNP annotation of milk-meat-growth*

ChrID	Location	Pop_SNP	Gene Name	Codon	CDS_ID	Protein_ID	1000BullDB
Chr6	7105326	G C C C S C C C C	FABP2	CAA<->GAA	cds11066	XP_005207643.1	G/C
Chr6	7105350	T Y Y Y Y C Y Y Y	FABP2	AAA<->GAA	cds11066	XP_005207643.1	T/C
Chr6	7105359	A C C C C C C C C	FABP2	TAA<->GAA	cds11066	XP_005207643.1	A/C
Chr6	7105759	A R A A A A A A A	FABP2	ATA<->ACA	cds11066	XP_005207643.1	A/G
Chr6	7107541	T Y C S S G S S S	FABP2	ATC<->CTC;ATC<->GTC	cds11066	XP_005207643.1	T/G
Chr6	7108785	A W W W W A A W W	FABP2	ATG<->AAG	cds11066	XP_005207643.1	A/T
Chr6	7108842	A R A R R A R R R	FABP2	TTT<->TCT	cds11066	XP_005207643.1	A/G
Chr14	46817895	G C C C C C C C C	FABP9	CTG<->GTG	cds25027	NP_001179339.1	G/C
Chr14	46817903	C T T T Y T T T T	FABP9	TGT<->TAT	cds25027	NP_001179339.1	C/T
Chr14	46819115	G G G G S G G G G	FABP9	GAC<->GAG	cds25027	NP_001179339.1	G/C
Chr14	46835065	T C C C C C C C C	FABP4	ATC<->GTC	cds25028	NP_776739.1	T/C
Chr18	51226081	G A A A R A A A A	LIPE	CGG<->CAG	cds31387	NP_001073689.1	G/A
Chr18	51226081	G A A A R A A A A	LIPE	CGG<->CAG	cds31386	XP_005219231.1	G/A
Chr18	51226221	A G G G R G G G G	LIPE	ATC<->GTC	cds31387	NP_001073689.1	A/G
Chr18	51226221	A G G G R G G G G	LIPE	ATC<->GTC	cds31386	XP_005219231.1	A/G
Chr18	51226425	A G G G R G G G G	LIPE	AAC<->GAC	cds31387	NP_001073689.1	A/G
Chr18	51226425	A G G G R G G G G	LIPE	AAC<->GAC	cds31386	XP_005219231.1	A/G
Chr20	31891025	G A A A A A A A A	GHR	ACC<->ATC	cds35638	NP_788781.1	G/A
Chr20	31891050	T Y T T Y T T T T	GHR	AGC<->GGC	cds35638	NP_788781.1	T/C
Chr20	31891130	T G T T K T T T T	GHR	AAC<->ACC	cds35638	NP_788781.1	T/G
Chr20	39115345	T C C C Y C C C C	PRLR	AGT<->AAC	cds35722	NP_001034815.1	T/C
Chr20	39115345	T C C C Y C C C C	PRLR	AGT<->AAC	cds35723	XP_005221632.1	T/C
Chr20	39115345	T C C C Y C C C C	PRLR	AGT<->AAC	cds35724	XP_005221633.1	T/C
Chr20	39115345	T C C C Y C C C C	PRLR	AGT<->AAC	cds35725	XP_005221634.1	T/C
Chr20	39115345	T C C C Y C C C C	PRLR	AGT<->AAC	cds35726	XP_005221635.1	T/C
Chr20	39115345	T C C C Y C C C C	PRLR	AGT<->AAC	cds35727	NP_776580.1	T/C

between the three species, by comparing SNPs in cattle, Yak, and Dzo genomic sequences. In detecting genome-wide variation in this study, very large numbers of SNVs (>30 M) were uncovered between the Yak genomes and the *Bos taurus* reference, as well as between the genomes of eight individuals (Yak and Dzo) and the *Bos taurus* reference, implying that a very large number of variants are segregated within the Yak and between Yak, cattle, and Dzo. When comparing the Dzo to the cattle reference genome, >90% of SNVs were found to be heterozygous in the genome of the F1 Dzo samples. Homo- or heterozygosity in the Dzo genome likely exerts strong effects on gene expression and functionality in this hybrid, which could play an important role in the maintenance of traits of physiological and economic relevance, including adaptations to high altitude. However, this variation may also come at a cost via the incompatibility between alleles [35], a phenomenon that can only be further investigated using single individual sequences that can be phased and subjected to linkage and haplotype analyses.

Previously, Qi et al.[36] assessed the impact of cattle admixture on domestic Yak and concluded that admixture between Yak and cattle has impacted the contemporary genetic makeup of the domestic Yak. Recently, Medugorac et al.[1] inferred bovine haplotypes in the genomes of Mongolian Yaks using whole-genome sequencing and showed that these introgressed regions are enriched for genes involved in nervous system development and function (gene ontogenies often associated with domestication), which supports the idea that introgressive hybridization could have facilitated Yak management and breeding. However, until now, no studies have assessed the genetic implications of domestication on wild Yak. In our study, we detected the introgression of domestic Yak in the wild Yak (YA) genome for the first time. Although the phenotypes of the sampled individuals were superficially indistinguishable from wild Yaks, approximately 24% of their genome was inferred as having a domestic Yak origin, leading us to infer second-generation introgression. This may be related to the unique method of grazing in pastoral areas of the Qinghai-Tibetan plateau, where herders often manage domestic Yak adjacent to wild Yak populations. We were able to detect this introgression by showing that population differentiation can be easily and precisely identified between wild and domestic Yak at the genome level, potentially shedding light on the mechanism and spread of Yak domestication and its contemporary impact

on wild Yak populations-a process that clearly needs to be monitored as it potentially threatens their genetic integrity. Based on our results, it is clear that further range-wide studies of domestic/wild Yak introgression are necessary, and that hybridization between wild Yak and domestic Yak should be tightly controlled to maintain the genetic integrity of wild and domestic Yak populations, in addition, the mechanism of male sterility in the Dzo is one of the most pressing management questions in Yak science, and yet it remains unresolved. In combining genomic information from Y chromosomes and autosomal sequence from cattle, Dzo, and Yak, we were able to explore this problem by examining variations across the Y chromosome and in 52 autosomal genes known to be related to meiotic processes. Intriguingly, however, we only detected eight non-synonymous SNVs in coding regions of the Y chromosome between the Dzo and cattle, while 58 were found to distinguish Yak and cattle. As previously described in species (including cattle) [37,38], we found that both ends of the Y chromosomes in Yak and Dzo comprise a large number of repeat sequences, mainly distributed in intergenic regions and introns. These repetitive sequences have played a key role in the formation of the Y chromosome [20,21,37,38] by preventing recombination, thereby protecting important single copy genes at both ends [20,21,39]. However, we could not find strong evidence for the determination of Dzo sterility in genes along the Y chromosome based on our SNV results, suggesting that autosomal genes maybe hold the key to Dzo male sterility. Thus, discrepancies in the Y chromosome between cattle, Yak, and Dzo need to be further explored to elucidate the substantial difference between the expected (approximately 30 non-synonymous substitutions between Dzo and cattle) and observed (eight) non-synonymous SNVs. While this observation suggests that reproductively viable Dzo may require the presence of a strongly cattle-like Y chromosome segregating in the parental population to survive, variations in the Y-chromosomes of domestic Yak populations need to be further characterized.

The meiotic recombination protein DMC1 plays a central role in homologous recombination in meiosis by assembling the sites of programmed DNA double strand breaks and localizing allelic DNA sequences located on homologous chromatids. In the present study, the INDEL (Chromosome 5.110729098. C>CT) discovered in the CDS region of the *DMC1* gene in the Dzo is predicted to have a major impact on gene function. Replication protein A (RPA) not only binds to single-stranded DNA during replication and keeps DNA unwound during replication, it also binds to ssDNA during the initial phase of homologous recombination, including prophase I of meiosis [40]. variation of *PRDM9* also influence male recombination in cattle with variation in *REC8* and *RNF212*, which correlate with genome-wide recombination rates, while variation in *PRDM9* also influence genome-wide hotspot frequency [41]. Here, we detected seven non-synonymous SNVs in the *RPA* gene family, and 26 non-synonymous SNVs in the *PRDM9* gene *(Table 4)*, potentially leading to changes in gene function.

Previous research has indicated that first and second backcross Dzo generations are also sterile, where no spermatogonia were detected in the seminiferous tubules in first generation hybrids and other early backcross generations. By the third generation of backcrossing (with either Yak or cattle), some spermatocytes can be present and the occasional male is found to be fertile, although fertility is not assured until the fourth or fifth generation of backcrossing [4]. Possible causes for this phenomenon have been attributed to X- and Y-chromosome incompatibility. Although we only focused on the Y chromosome here, our results provide informative data for the further study of Dzo male sterility, which should focus on both sex-linked genes and functional studies of the autosomal genes highlighted here.

Linking genomic correlations to environmental parameters is of special importance in Yak science, given the unusual habitat that this species occupies. In humans, a SNV in *EPAS1* has been detected between Tibetan (high-altitude) and Han (low-altitude) populations, and is associated with erythrocyte abundance, and thus supports the role of *EPAS1* in the adaptation to hypoxia necessary for adaptation to high altitudes [24]. Newman et al.[29] found a high degree of association in the oxygen degradation domain of EPAS1 between an *EPAS1 (HIF-2α)* double variant in Angus cattle with high-altitude pulmonary hypertension (HAPH) and demonstrated that the variant appears to be prevalent only in lowland cattle. In Yak, three novel SNPs have also been identified in *EPAS1*, and have been genotyped in three breeds. In the present study, we predicted that one variant (Chromosome11.28664187C>T) in *EPAS1* between Yak and cattle is deleterious. However, we did not identify the same SNPs that have been previously identified in the *EPAS1* gene. This is likely a result of the fact that previous studies have been examining intraspecific variation (within cattle and Yak populations) but have not compared across species and did not specifically investigate interspecific differences in altitudinal adaptation. Overall, in the four genes that are known to be related to altitudinal adaptation and that have been closely examined here (*EPAS1*, *EGLN1*, *HYOU1* and *HMBS*), we found 16 non-synonymous SNVs in CDS regions, and 14 non-synonymous SNVs that distinguished cattle and Yak, suggesting that differences in high plateau adaptation between cattle and Yak could be linked to some of the substitutions detected in these genes. The further investigation of the potential roles of these substitutions will require functional analyses.

For genes related to growth, milk, and meat traits, six non-synonymous SNVs in the *LIPE* gene and 11 non-synonymous SNVs in *FABP* genes possessed fixed differences between Yak and the cattle reference genome. Some of these genes are known to be linked to leanness in

cattle, and therefore phenotypic differences between Yak and cattle may be important in this context. Interestingly, eight non-synonymous SNVs were found in genes for the growth hormone and prolactin receptors *GHR* and *PRLR*, respectively. Previous studies have shown that some homozygous, or compound heterozygous, mutations in *GHR* may induce a partial insensitivity to GH in humans [42]. Prolactin (PRL) is secreted from the anterior pituitary, and plays an extensive role in corpus luteum formation, mammogenesis, and lactogenesis; where the functional activity of PRL is mediated by its receptor (PRLR) in the PRL signal transduction cascade [43]. Moreover, SNVs in PRLR have also been detected in goat, and have been shown to influence milk yield in different goat breeds [44]. Our study indicates that GHR and PRLR may play an important role in differences in growth and milk traits between Yak, Dzo, and cattle.

To better understand genomic differences between cattle (assembly UMD3.1), Yak (GenBank No.: GCA_000298355.1), and Zebu *(B. indicus*; GenBank No.: GCA_000247795.1), we aligned the Zebu and Yak genomes to the cattle reference sequence. The results of this alignment showed that the Yak and Zebu genomic sequences covered 89.99% and 88.76% of the cattle genome, respectively (not present). Thus, a subspecies relationship between cattle and Zebu ancestors that is inferred and likely reflects the quality of the reference sequences [45]. The results of the genome coverage analysis showed large differences in the genomic sequences for Yak, Zebu, and cattle. One potential but important reason for this finding may be the integrity of the genome assembly. Resequencing-based assemblies are limited in establishing differences between bovine genomes alone. In the future, it will be important to complete the genomes for Yak and Zebu. Moreover, it should be noted that our results were based on a pooled sequencing strategy for six wild Yaks and six Dzo individuals. Although the pooling of samples has been successfully used in studies on dog and chicken domestication, the strategy of pooling samples has been shown to affect some statistical analyses, most notably haplotype and linkage analyses [46,47]. Therefore, the sequencing of multiple genomes separately for Yak and Dzo is desirable in the future.

In brief, our results provide basic high-value data for researchers studying Yak, cattle, and Dzo breeding. Comparative analyses of the genomic sequences of Dzo, Yak, and cattle using whole genome sequencing strategies, provide a more in-depth understanding of the genetic variations between Yak, cattle, and Dzo. Here, we have provided evidence of variation in genes related to meiosis and gametogenesis, substantial variation in genes related to high-altitude adaptation, as well as genes associated with meat and milk production between the Yak and cattle genomes. In summary, these genetic variations may be helpful in genotype-phenotype association analyses of Qinghai-Tibet plateau ruminants, most notably for Yak and Dzo, and could also help to shape the management practices of wild and domesticated ruminant populations to preserve the genetic integrity of both populations.

Availability of Supporting Data

All raw reads generated in this work have been deposited in the NCBI database under BioProject accessionPRJNA359997 (alias: SRP095965). The whole genome SNP and INDEL data set has been deposited in the following link: https://pan.baidu.com/s/1bzB1t8.

Competing Financial Interests

The authors have declared that no competing interests exist.

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Research Article

Brain Cells Death on Infant Mice *(Mus musculus)* Caused by Carbofuran Exposure During the Lactation Period

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Abstract

An exposure to insecticide carbofuran has been reported able to generate a reactive oxygen species (ROS) in mice brains. This study was undertaken to evaluate the oxidative damage, biochemical and histopathological alterations by respectively examining malondialdehyde (MDA), cholinesterase (ChE) levels, necrosis and apoptosis in the suckling mice whose mothers were exposed to the carbofuran. The carbofuran was exposed via an oral route at the doses of 0.0208 mg/kg and 0.0417 mg/kg BW from the first until the fourth postnatal day after the delivery (n=27). The six-day-old pups were examined for its brain's MDA and ChE levels as well as the necrotic and apoptotic Purkinje cell were counted using the Tunel assay and hematoxylin-eosin (HE) staining. The mothers' exposure to carbofuran caused an increase in MDA levels, necrosis of Purkinje cells and a decrease in ChE, but there was no significant apoptosis in lactating pups. Carbofuran altered the level of the marker parameters related to the MDA, ChE and necrosis of Purkinje cells. Consistent changes were found in MDA, ChE and necrosis of Purkinje cells of the subjected pups, especially between the control and treatment groups, and there was no change between treatments. In conclusion, the transfer of carbofuran intoxication through the mother's milk resulted in the oxidative stress, biochemical and histopathological alterations in the suckling pups.

Keywords: *Carbofuran, Purkinje cells, Infant mice brain, Lactation*

Laktasyon Döneminde Karbofurana Maruz Kalan Yavru Farelerde *(Mus musculus)* Beyin Hücreleri Ölümü

Öz

İnsektisit karbofurana maruz kalmanın farelerin beyinlerinde reaktif oksijen türleri (ROS) ürettiği bildirilmiştir. Bu çalışma, anneleri karbofurana maruz bırakılan ve anne sütü emen farelerde malondialdehid (MDA), kolinesteraz (ChE), nekroz ve apoptozis düzeyleri incelenerek oluşan oksidatif hasar ile biyokimyasal ve histopatolojik değişiklikleri değerlendirmek amacıyla yapılmıştır. Fareler (n=27) karbofurana, oral yoldan, doğumdan sonraki 1. günden 4. güne kadar 0.0208 mg/kg ve 0.0417 mg/kg BW dozlarında maruz bırakıldı. Altı günlük yavruların beyin MDA ve ChE düzeyleri yanı sıra, Tunel testi ve hematoksilen-eozin (HE) boyaması kullanılarak nekrotik ve apoptotik Purkinje hücre sayısı incelendi. Annelerin karbofurana maruz kalması, MDA düzeylerinde artışa, Purkinje hücrelerinin nekrozuna ve ChE'de azalmaya neden oldu, ancak emen yavrularda belirgin bir apoptoz gözlenmedi. Özellikle kontrol ve tedaviye tabi tutulan yavruların MDA, ChE ve Purkinje hücrelerinin nekrozunda tutarlı değişiklikler bulunmuş, tedavi grupları arasında arasında ise herhangi bir değişiklik olmadığı tespit edilmiştir. Sonuç olarak, anne sütü yoluyla karbofuran zehirlenmesi, emen yavrularda oksidatif stres, biyokimyasal ve histopatolojik değişiklikler ile sonuçlanmıştır.

Anahtar sözcükler: *Karbofuran, Purkinje hücreleri, Yavru fare beyni, Laktasyon*

INTRODUCTION

Insecticide carbofuran residues in food may be harmful to organisms which are actually not the target of insecticide itself [1]. In a flower plantation which was contaminated by carbofuran in Ecuador in 2001, there were several cases of babies born with abnormalities, such as declining reflexes and motoric skill. At the child stage, there were some brain function developmental abnormalities, such as the degeneration of memorizing and concentrating abilities [2]. In tested animals, carbofuran contamination causes an oxidative stress and weakens the motoric, memory, and cognitive functions [3]. Like organophosphate, carbofuran inductions result in a significant oxidative damage in the cerebral cortex, cerebellum, and brain-stem [4]. Carbofuran inductions in the cerebral cortex

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strongly correlate to the decline of cognitive and motoric functions [3].

An oral administration of carbofuran has been proven to strongly stimulate the reactive oxygen species (ROS) in mice brains and increase the levels of malondialdehyde (MDA) [3]. Intraperitoneal sub-acute administrations of carbofuran increase the brain's oxidative stress as the dose increases; and thus, improve MDA levels significantly. The increase of oxidative stress induces the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase in the brain [5]. The presence of ROS could trigger the formation of hydroxyl radicals (OH*) which breaks the DNA chains or changes the composition of nucleotides in DNA creating mutations and apoptosis [6]. Hydroxyl radicals (OH*) as a result of an oxidative stress could also damage all membrane systems in the cell, such as creating leaks in the lysozyme membrane which may cause the cell death (necrosis).

Uncontrolled increase in ROS causes the injury and death of neuron cells [7]. Cerebrum consists of 80% neuron cells and of 20% glial cells which are responsible for transmitting information to the spinal cord to control motoric functions [8]. During the embryonal stage of brain development, neuron cells develop earlier and reach their developmental peak at mid-pregnancy. Glial cells develop at mid-pregnancy until a few days before the fetus is born and reach the peak of development at the end of pregnancy [9]. The development of the cerebellum starts in the late period of pregnancy and develops at maximum (peak) at the beginning of birth (the beginning of lactation). The death of neuron cells, cerebral glial cells and Purkinje cells due to carbofuran exposure potentially degenerates reflexes and motoric functions.

A deeper understanding of the brain's cell death mechanism in mice pups due to carbofuran exposure during the lactation period is necessary to acquire the basic treatment and prevention during the lactation period. Besides, it is important to understand the brain's cell death mechanism in order to identify the most sensitive period as well as the type of cells affected by carbofuran exposure during the lactation period. If the mechanism and the type of dead cells are recognized, actions can be taken to prevent the degeneration of reflexes and motoric skills in infant mice.

The aim of this research was to determine the brain's cell death mechanism in mice pups whose mother were exposed to carbofuran during the lactation period by measuring the MDA levels as an indicator of ROS production, ChE levels as the indicator of a neural function response to carbofuran exposure, apoptotic and necrotic cerebral Purkinje cells. This study contributed in disclosing the prevention mechanism of brain's cell death in mice pups whose mother were exposed to carbofuran during the lactation period. In addition, this study also provided a scientific information of insecticide carbofuran exposure, especially during the lactation period related to the attempts of inhibiting brain's development disorders.

MATERIAL and METHODS

Ethics Approval

The study was approved by the Faculty of Veterinary Medicine's Animal Ethics Committee. All variables had been considered in accordance with the Ethics Committee related to the animal handling to ensure no discomfort or pain caused to the animals during sampling (2011/111-KE).

Laboratory Animals

The animals used in this study were 27 female mice *(Mus musculus),* 10 weeks old, with the weight range of 25-30 grams, and 12-weeks-old male mice. Environmental adaptation was done to female mice *(Mus musculus)* for 7 days. On the 8th day, pregnant mare serum gonadotropin (PMSG) with a dosage of 5 IU/mouse was injected into the female mice and followed by Human Chorionic Gonadotrophin (HCG) injections with a dosage of 5 IU/mouse which was performed on the 10th day. Afterwards, the female mice were mated with the 12-week-old male mice. On the 11th day, a gestation examination was carried out. The gestation of female mice was indicated by the visible mating plug covering the female mice vulva; and then the day was considered as the first day of gestation [10].

Carbofuran Exposure

Carbofuran exposure was targeted at the suckling mice's brain and this study examined the Purkinje cells in the cerebellum. The female mice were exposed to carbofuran with a dosage of 00.0208 mg/kg (1/24 LD_{50}) and 0.0417 mg/kg (1/12 LD_{50}) [10] on the 1st to 4th day of the lactation period given orally using a sterile disposable syringe. The six-day-old mice pups were then tested and measured for MDA and Cholinesterase (ChE) levels and histopathologic preparations were made. A microscopic examination was conducted to estimate the number of cells experiencing necrosis and apoptosis by using HE staining and an Apoptag Apoptosis Detection Kit.

Measuring Cholinesterase (ChE) Levels

Cholinesterase (ChE) was determined according to the manufacturer's instructions of Cholinesterase FS (DiaSys Diagnostic Systems, 11401) [11]. To measure the ChE levels on the six-day-old mice's cerebrospinal fluid, the following materials were used to create a substrate: S-Butyryl-thiocholine iodide Phosphate buffer pH 7.7, 5.5 dithiobis-2-nitrobenzoate. The principle of ChE level measurement was that the process of S-Butyrylthiocholine iodide + H_2O hydrolysis and with the help of ChE sample was converted to Thiocholine iodide + butyrate. Thiocholine iodide + 5,5-dithiobis-2 - nitro benzoate would transform into 5 - Mercapto - 2 - nitro benzoate - 5 Mercaptothiocholine. The

reaction solution is from Diluent 100 mL, 3 mL reagent mix (for 30 samples) with concentrations of Phosphate buffer pH 7.7 50 mmol/L, S-Butyrylthiocholine iodide 6 mmol/L, 5,5-dithiobis-2-nitrobenzoic 0.25 mmol/L. The standard wavelength of reaction solution was 405 nm and the length of translucent light was 1 cm measured at 25, 35 and 37°C. The 10 mL of sampled fluid was collected and mixed with 100 mL of reaction solution. The mixture was examined every minute for three minutes. To determine the sampled concentration, the enzyme activity of sample (U/L) = ΔA/min x 68500 was measured.

Measuring Malondialdehyde (MDA) Level

Malondialdehyde (MDA) was determined by the method of Conti et al.[12]. The measurement of malondialdehyde level on the six-day-old mice pups' brains was performed using the MDA/Thiobarbituric Acid Reactive Substance (TBARS). This method was performed by weighing 1 gram of the infant mice brain sample then putting it into a reaction tube, mixing it with 9 mL cold PBS and then crushing it with a spatula. The liquid was then centrifuged at 3000 rpm for 15 min. 4 mL of supernatant was collected and added to 1 mL of *trichloroacetic acid* (TCA) 15% solution. Then, 1 mL of 0.37% Thiobarbituric acid (TBA) solution was added into HCl 0.25 N and heated in a water bowl at 80°C for 15 min. After cooling the solution at room temperature for 60 min, the solution was centrifuged at 3000 rpm for 15 min. Finally, the value of the absorbance was read against the red lines which were formed using a spectrophotometer at λ = 532 nm.

Necrosis Examination Using HE Staining

Necrosis examination was performed using HE staining. The six-day-old mice's brains were fixated with 10% formalin buffer and brain tissues were processed in routine processing until they formed paraffin blocks. The paraffin blocks were cut using a microtome with a thickness of 5 µm in a series and then glued to the object glass. The observed area was the cerebellum which was obtained through the coronal section at the position 11/1.56 mm from the edge of the posterior lobe [13]. After 24 h' fixation, the mice brain was washed in a 70% alcohol solution three times and stained using Haematoxylin Eosin (HE). Purkinje cells which experienced necrosis were characterized by cells undergoing pyknosis and karyorrhexis.

Apoptosis Examination Using Tunel Assay

The examination of apoptotic cells was performed by Tunel assay. The paraffin blocks were cut using a microtome with a thickness of 5 µm in a series and then glued to the object glass using polylysine. For counting the apoptotic cells, pieces of the tissue were processed with S7101 Apoptag Plus Peroxidase. Apoptotic cells were identified by the color absorbent (dark brown).

Data Analysis

Data analysis and evaluation of statistical significance among different determined values was performed using one-way Analysis of variance (ANOVA) with post hoc analysis (Duncan test) [14]. The values were expressed as mean ± SD and considered significant at P 0.05. The statistical analysis was performed using Statistical Product and Service Solutions (SPSS) version 17.0.

RESULTS

Malondialdehyde (MDA) Level

This study showed that there was an increase in MDA levels, as the results of the ANOVA test indicated a sign value of 0.000 between the control and treatment group, which was less than the significance value α=0.05. It can be interpreted that there was a difference between the control and treatment groups. Thus, carbofuran administration in doses of either 0.0208 mg/kg or 0.0417 mg/kg might potentially produce the free radicals. The Duncan test indicated a sign value of 0.115 between 0.0208 mg/kg and 0.0417 mg/kg, which was more than the significance value α=0.05. There was no significant difference in pups whose mothers were exposed to carbofuran of 0.0208 mg/kg and those with 0.0417 mg/kg. Although there was no significant difference, the increase in MDA levels was very high at 58.95% and 247.47% compared to that the control group, and the increase in MDA levels reached 118% between 0.0208 mg/kg and 0.0417 mg/kg *(Table 1)*.

Cholinesterase (ChE) Level

This study presented the decreasing ChE levels and the ANOVA test result indicated a sign value of 0.001 between

Table 1. *Effect of carbofuran exposure in subacute doses for 4 days on the MDA, ChE levels, Purkinje necrotic cell and Purkinje apoptotic cell in mice pups' brains (n=27)*

Parameters	Control Group (mean±SD)	0.0208 mg/kg BW Carbofuran (mean±SD)	0.0417 mg/kg BW Carbofuran (mean±SD)
Malondialdehyde (nmol/mg)	25.53±3.02	40.58±5.77[a]	88.7±3.02[a]
ChE levels (U/L)	801.75±129.73	671.50±50.53[q]	606.75±28.45[q]
Purkinje necrotic cell	1.98±1.92	6.24±0.73[y]	7.68±1.01[y]
Purkinje apoptotic cell	11.00±1.92	13.00±0.73	14.13±1.01

Statistical difference from the control: [a,q,y] Significant at P≤0.05

the control and treatment groups, which was less than the significance value α=0.05. It can be concluded that there was a difference between control and treatment groups. Carbofuran exposure in the doses of 0.0208 mg/kg and 0.0417 mg/kg potentially lowered the ChE levels. The Duncan test analysis revealed a sign value of 0.707 between 0.0208 mg/kg and 0.0417 mg/kg, which was more than the significance value α=0.05. There was no significant difference in pups whose mothers were exposed to carbofuran of 0.0208 mg/kg and 0.0417 mg/kg. Although there was no significant difference, the decrease in ChE levels reached 16.25% and 24.32% compared to the control group, and the decrease in ChE levels reached 9.64% between 0.0208 mg/kg and 0.0417 mg/kg *(Table 1)*.

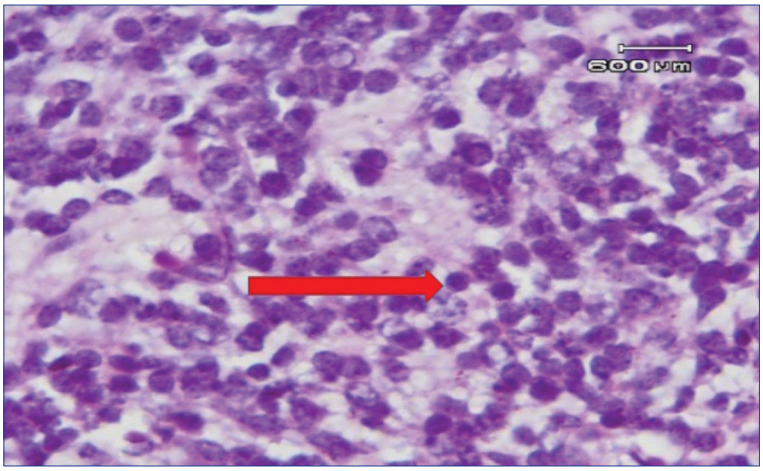
Necrotic Cell

In light microscopic examinations, histopathological changes were observed in mice pups' brains of all exposed groups compared to the control groups. This study found an increasing number of Purkinje necrotic cell in the brains of six-day-old mice pups whose mothers were exposed to carbofuran on the first and the fourth day of the lactation period. Carbofuran exposure in doses of 0.0208 mg/kg and 0.0417 mg/kg had enhanced Purkinje necrotic cell. There was no significant difference in pups whose mothers were exposed to 0.0208 mg/kg and 0.0417 mg/kg of carbofuran. Nevertheless, the increase in Purkinje necrotic cell was very high at 215.15% for a dosage of 0.0208 mg/kg and 287.87% for a dosage of 0.0417 mg/kg compared to the control group. Furthermore, the increase in Purkinje necrotic cell reached 23.07% between the dose administrations of 0.0208 mg/kg and 0.0417 mg/kg. An overview of staining results using HE showed necrosis of the Purkinje cells between the control and treatment groups *(Fig. 1,2,3; Table 1)*.

Apoptotic Cell

This study expressed that there was an increasing number of Purkinje apoptotic cell in the brains of six-day-old mice pups from mother mice which were exposed to the insecticide carbofuran from the 1st and 4th day of the lactation period. The results of the Kruskal-Wallis test indicated a significance level of 0.549>0.05. There was no significant difference in Purkinje apoptotic cell in lactating

Fig 1. Histopathology of the cerebellum of mice pups *(Mus musculus)* from the control group. H.E Staining. 1000x



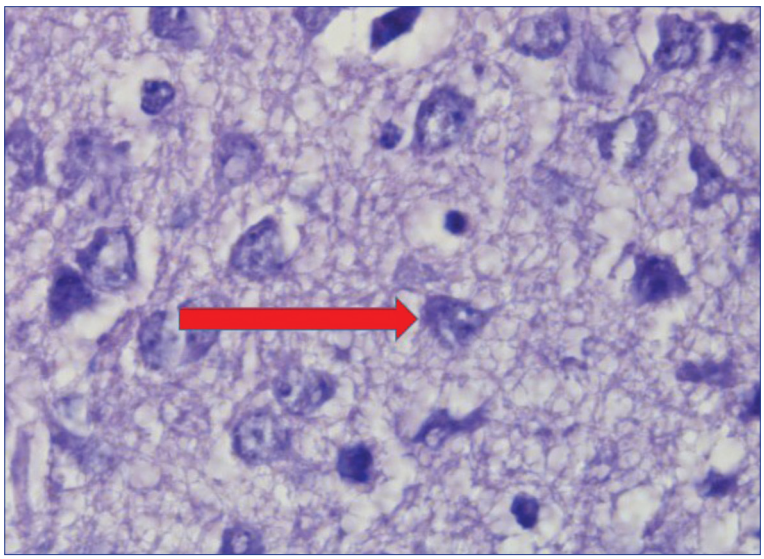


Fig 2. Histopathology of the cerebellum of mice pups *(Mus musculus)*, red arrow indicates the Purkinje necrotic cells. H.E Staining. 1000x

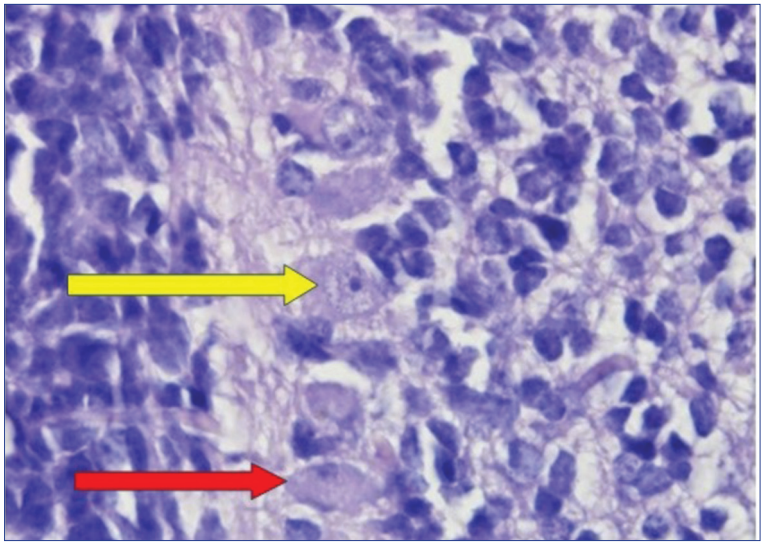


Fig 3. Histopathology of the cerebellum of mice pups *(Mus musculus)*, yellow arrow indicates the normal Purkinje cells, red arrow indicates the Purkinje necrotic cells. H.E Staining. 1000x

pups whose mothers received 0.0208 mg/kg and 0.0417 of carbofuran compared to the control groups *(Table 1)*. The estimated increase of Purkinje apoptotic cell in the carbofuran groups was 18.18% and 28.45%, while the increase in Purkinje apoptotic cell reached 7.99% between the dose administrations of 0.0208 mg/kg and 0.0417 mg/kg *(Table 1)*.

DISCUSSION

The metabolism of carbofuran has been well studied in rats, mice, and lactating cows. Carbofuran is rapidly absorbed, metabolized, and eliminated, primarily via urine, in the species investigated [15]. Carbofuran was altered by oxidation of the number 3 carbon and of the N-methyl group, hydrolysis of the ester linkage, and conjugation of metabolites containing a hydroxyl group. Carbofuran metabolites in the milk were the 3-hydroxycarbofuran, 3-keto-carbofuran, and 3-hydroxy-N-hydroxy methyl derivatives of carbofuran, which were found both in free and conjugated forms. Conjugated 2,3-dihydro-2,2-dimethyl-3-keto-7-hydroxybenzofuran was the major hydrolytic product of carbofuran in the milk. The same metabolites were also found in the urine and feces. 3-Hydroxycarbofuran was one of the most rapidly formed metabolites and 3-ketocarbofuran phenol was one of the end products [16].

3-hydrocarbofuran and nitrosocarbofuran could induce the micronucleus formation, while 3-ketocarbofuran could not but caused a significant DNA migration in SCGE test. Moreover, 3-ketocarbofuran caused an obvious increase in damaged cells accompanied with a great decrease in undamaged cells which displayed a higher degree of cell damage than other three compounds, especially seriously damaged cells increased in number and suggested a more serious DNA damaging effects. There has been no report on the mechanisms of DNA damaging effects induced by carbofuran and its metabolites. But in the oxidation and the hydrolyze of its transformation, free radicals are a potential outcome, which is widely known to reduce DNA damage. The presence of metabolic active system in organism could be degraded to less toxic phenols directly, while 3-hydrocarbofuran was metabolized to 3-ketocarbofuran with a high direct toxicity to cells, thus, 3-hydrocarbofuran revealed a stronger *in vivo* toxicity [17].

Purkinje cell is a cell-specific marker of the cerebellar Purkinje cell and a suitable indicator for observing the postnatal development of the cerebellum after birth. The Purkinje cell levels in the mice's cerebellum during the critical postnatal (4 days after birth) is to determine the effect of external exposure on cerebellar growth in the offspring during lactation [18].

Many pathophysiological conditions may have oxidative stress. In normal conditions, it is balanced by the antioxidant system and this balance is disturbed due to the increased oxidative stress. MDA is one of the fairly reactive metabolic products created from the effect of free oxygen radicals on tissues and from a series of reactions during lipid peroxidation. Malondialdehyde (MDA) is the best and a sensitive indicator of lipid peroxidation and so of oxidative stress [19,20].

The final result of lipid peroxidation is MDA and a high MDA level indicates the damaged oxidation processes or cell membrane due to free radicals [21,22]. The results of this study showed that carbofuran exposure at subacute doses (0.0208 mg/kg and 0.0407 mg/kg) during a lactation period caused a significant enhancement in MDA levels of mice pups' brains. The data suggested that the significantly elevated MDA levels in the brain in turn produced a reactive oxygen species (ROS) which caused an oxidative stress in this organ. The increase of MDA levels results in cell death which is triggered by toxicants, depending on the dose. In this study, carbofuran dose increased MDA levels but there was no significant difference in pups whose mothers were exposed to carbofuran of 0.0208 mg/kg and 0.0417 mg/kg. Although there was no significant difference, the increase

in MDA levels reached 118% between 0.0208 mg/kg and 0.0417 mg/kg *(Table 1)*. The increased doses could increase the significant MDA levels if exposed postnatally on the 1st until 20th day [23]. In another study, the increase in MDA levels due to an acute exposure of carbofuran may reach up to 175.04% for the dose administrations of 0.2 mg/kg which are compared to 0.4 mg/kg in mice [5]. Furthermore, oral sub-acute exposure of carbofuran for 28 days in male mice increases the MDA levels by 65% in other study [24].

This indicates that the increasing MDA levels due to carbofuran exposure depends highly on the dose, duration of exposure, and the type of affected organ. Vulnerability of brain development depends on the agent or the active metabolite which can be achieved during the development of nervous system and is associated with the exposure period. Exposure before or after the organ is fully developed makes the organ less vulnerable to inhibitions than if the exposure occurs during the organ development [25]. An adult mouse brain is protected by the blood-brain barrier (BBB) to chemicals, while such protection does not exist in mice fetus and 6 months old mice [26].

Neuronal membranes which are rich in polyunsaturated fatty acids are the source of lipid peroxidation reaction [27,28]. Lipid peroxidation causes destruction and damage to cell membranes and also changes the fluidity/membrane permeability [28,29]. Thus, the increase in MDA is caused by pesticides induction by forming ROS. Acute intraperitoneal carbofuran exposure may cause a significant increase in MDA levels of the brains and livers of adult mice. The increase in MDA levels is in line with the doses of carbofuran exposed. Carbofuran is proven to be efficiently absorbed and rapidly distributed to various organs of an organism. Lipophilic nature of carbamate also causes it to be able to interact with lipid serums and tissues [30]. The forming process of lipid peroxidation starts from hydrogen ions on the side chain of polyunsaturated fatty acids (PUFA), which construct the cell membranes by free radicals, form carbon radicals. Carbon radicals are oxidized to form peroxyl radicals. Furthermore, peroxyl radicals draw H^+ ion into the side chain of adjacent PUFA and form lipid peroxidation. This process is a chain reaction because the lipid peroxidation attracts more H^+ ions into the side chain of adjacent PUFA until the PUFA chain is finally split into other compounds, such as MDA, 9-hydroxy-nonenal, ethane and pentane [21,22].

Cholinesterase level measurements are often conducted to determine the exposure effect to insecticides. ChE used in this measurement was collected from tissues, plasma and red blood cells [31] and ChE collected from the brain was the best sample to be used as an indicator of the exposure to insecticides [32]. In this study, the insecticide carbofuran decreased the ChE levels between the control and the treatment groups. However, there was no significant difference in the ChE levels among lactating pups whose mothers received carbofuran of 0.0208 mg/kg and the ones with 0.0417 mg/kg *(Table 1)*. The increased doses could decrease the significant ChE levels if exposed postnatally on the 1st until the 20th day [23]. Almost all insecticide exposure resulted in the decrease of ChE levels, either during embryonic period, growth period, or adulthood. Generally, the response of the decreased ChE level is in accordance with the exposed dose. However, the decreased ChE levels due to the insecticide exposure in several phases of individual growth may induce varied responses.

There were correlations between the accumulation of acetylcholine and the extent of MDA. An increased oxidative stress by carbofuran might be a result of cholinergic hyperactivity or might be due to its direct effect on the production of reactive oxygen [33]. The peroxidation does not only alter lipid milieu, the structural and functional integrity of the cell membrane, but also affects the activities of various membrane-bound enzymes, including acetylcholinesterase (AChE) and different ATPases. The inhibition of ATPase activities may be a causative factor of neuronal/cellular dysfunction, due to an alteration in cationic transport across the membrane and a disturbance in uptake as well as a release of certain neurotransmitters [34].

The role of ChE is activated before the synaptogenesis during the formation of neural tube. The formation of ChE is in line with the axon growth [35]. The cholinergic system in early development acts as a regulatory growth and has morphogenetic functions [36] by controlling cell proliferation, motility, cell differentiation and genetic expression [37]. Thus, the cholinergic system has a very important role in the cell development and brain formation [38]. Although the brains of infant mice are extremely sensitive to carbofuran exposure during the lactation period and both treatments showed a decrease in ChE levels, all infant mice were still alive with symptoms of mild poisoning. The decrease in ChE levels indicates the response of brain or adult nervous system [35].

In this study, the insecticide carbofuran increased the necrotic death of Purkinje cells both in control and treatment group. However, there was no significant difference in necrotic cells among lactating pups whose mothers received carbofuran of 0.0208 mg/kg and the ones with 0.0417 mg/kg. In this study, we found an association between the increased MDA levels and the necrotic cells. The increased lipid peroxidation and lipid peroxidation products, such as MDA levels, contribute to neuronal loss in conditions associated with oxidative stress [39]. Increased MDA levels indicates a membrane damage and leads to a cellular necrosis. The attack of free radicals on a cell membrane makes it devoid of integrity and viability causing the cells to undergo necrosis [40]. Cell death caused by the swelling of cytoplasm, nucleus karyolysis and lysis are classified as necrosis [41,42].

The number of Purkinje necrotic cells was not as many as the number of cells which experienced apoptosis due to the carbofuran exposure. This was because during the neurogenesis period, the Purkinje cells had experienced more apoptosis physiologically through homeostasis efforts. However, when the number of Purkinje necrotic cells was compared to the control group, there was an increase in the number of Purkinje necrotic cells in the treatment group which was significantly higher than the number of Purkinje apoptotic cells (apoptosis increases 28.45% and necrosis increases up to 287.87%) *(Table 1)*.

In this study, the insecticide carbofuran could increase the apoptosis of Purkinje cells between control and treatment groups. However, there was no significant difference in the number of Purkinje apoptotic cells among lactating pups whose mothers received carbofuran of 0.0208 mg/kg and the ones with 0.0417 mg/kg. Such different results from those of a study by Luqman [10] could be because the brain development phases during the embryonal period and lactation period have different critical time. In addition, the duration of carbofuran exposure during embryonal period was longer (10 days), while during the lactation period the exposure lasted only for 4 days. Although cerebellum is the most sensitive organ to oxidative-stress causing neurotoxins, longer exposure time is needed for the agent to reach the target of nuclear DNA and mitochondria to induce apoptosis [43]. The exposure of carbofuran insecticide can increase the activity on cerebral ROS during embryonal period and the expression of p53, caspase 3 and apoptosis. The increasing expression of p53, caspase 3 and apoptosis indicated that the insecticide carbofuran caused an apotosis through an intrinsic pathway [10].

In conclusion, this study revealed that the carbofuran had been distributed in pups' tissues through the milk of lactating mothers and had caused an oxidative damage of pups' brains. Carbofuran exposure indicated that mice pups' brains were particularly more vulnerable to oxidative stress, which may eventually lead to neurobehavioral disorders. In this study, we also found that the insecticide carbofuran dose in lactating mice of 0.0208 mg/kg BW had been able to increase ROS activity and Purkinje cell death. with the same dose, if converted to humans, according to dose conversion by Laurence and Bacharach (1964), it will be equal to 0.115 mg/kg BW. This result of dose conversion can be applied as a carbofuran potential standard in increasing the ROS activity and Purkinje cell death since the residual level found in cows' meat and milk is around 0.17 mg/kg BW and 0.349 mg/kg BW [44].

The insecticide carbofuran exposure to the lactating mice made the mean of Purkinje apoptotic cell higher than the necrotic ones in all treatment doses. A high increase of apoptosis in Purkinje cells allowed an opportunity to prevent and manage the strategy to overcome Purkinje cell death due to carbofuran insecticide exposure during the lactation period, such as by providing antioxidant variations. Some efforts to prevent the formation of ROS can be done to inhibit and countermeasure the neuronal development cell death due to the exposure to carbofuran. The administration of antioxidants, such as vitamin C, curcumin, and allopurinol during lactation is possible to reduce the oxidative stress through the inhibitor xanthine oxidase and scavenger effects of free radicals. Therefore, there are still opportunities to improve the environment of neonate's nerve by increasing the growth of axons, dendrites and synaptogenesis and myelination of axons [45,46].

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Research Article

Preliminary Study on Association of *EDNRB* Gene with Heterochromia Iridis in Cats *(Felis catus)* [1]

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Abstract

This study conducted an investigation on three exons of the endothelin receptor type B *(EDNRB)* gene of Thai odd-eyed cats to find out the association between the variations in the gene and heterochromia iridis. DNA sequencing analysis was performed on 11 odd-eyed cats compared to 11 normal-eyed cats. Seven variations were found across the studied region (XM_003980457.2: c.610A>G, c.820+40C>T, c.821-14C>T, c.916A>G, c.1025+36G>T, c.1025+69A>T, and c.1025+138C>T) with two of them (c.610A>G and c.916A>G) causing amino acid changes (P.Asn128Ser and P.Val230Ala). There was no statistical association between the variations near the three exons of *EDNRB* and feline heterochromia iridis (chi-square test, P-value >0.05).

Keywords: *EDNRB, Heterochromia iridis, Odd-eyed cat, SNPs, Sequencing*

Kedilerde *(Felis catus)* EDNRB Geni ile Heterokromia İridis Arasında İlişkiye Dair Ön Çalışma

Öz

Bu çalışmada Tayland tek-göz kedilerinde endotelin reseptör tip B *(EDNRB)* geninin üç ekzonunda gen varyasyonu ile heterokromia iridis arasında bir ilişki olup olmadığı araştırıldı. On bir tek-göz kedide DNA sekans analizi yapıldı ve 11 normal gözlü kedi ile karşılaştırıldı. Çalışılan bölge itibarıyla yedi varyasyon (XM_003980457.2: c.610A>G, c.820+40C>T, c.821-14C>T, c.916A>G, c.1025+36G>T, c.1025+69A>T ve c.1025+138C>T) tespit edildi ve bunların ikisi (c.610A>G ve c.916A>G) amino asit değişimine (P.Asn128Ser ve P.Val230Ala) neden olmaktaydı. Çalışmada, üç *EDNRB* ekzonu yakınındaki varyasyonlar ile kedi heterokromia iridis arasında istatistiksel bir ilişki tespit edilmedi (ki kare testi, P-değeri >0.05).

Anahtar sözcükler: *EDNRB, Heterokromia iridis, Tek-göz kedi, SNP, Sekanslama*

INTRODUCTION

Heterochromia iridis is a condition of difference in iris coloration, which occurs in animals. There are three types of heterochromia: complete heterochromia (completely different iris colors of the two eyes), sectoral heterochromia (difference in color in some parts of the iris), and central heterochromia (rings of spikes which have lighter or different color from the rest of the iris, radiating from the pupil). This difference in coloration is either acquired

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(by accident, disease, or some drugs) or congenital [1]. Congenital heterochromia iridis found in mammals is usually associated with mosaicism and chimerism as well as an involvement with white skin/fur and complete heterochromia in which the individual has one iris blue in color and the other iris having some other color. Moreover, there are several reports that abnormality or dysfunction of some genes can cause heterochromia iridis through Waardenburg syndrome (WS) and skin/deafness disease. Examples of such genes include endothelin receptor type B (*EDNRB*), microphthalmia-associated transcription factor (*MITF*), pair-box 3 (*PAX 3*), and sex determining region Y-box 10 (*SOX10*) [2-5].

EDNRB is a gene that synthesizes the receptor protein located on the cell surface called endothelin receptor type B (OMIM: 131244) which interacts with the endothelin protein. The mechanism regulates some critical biological processes involving the stimulation of cell growth and division of some structure or molecules such as blood vessels and hormones [6]. This receptor has an important role during fetal development, which involves determining cell differentiation for melanocytes and regulating melanin which is also involved in the normal function of inner ear [7]. Some studies have reported that defects in this gene can cause heterochromia iridis in humans. There was a case in which mutation of *EDNRB* had caused Waardenburg's syndrome type IV in Chinese population and caused blue/brown complete heterochromia iridis as well as deafness and white patches of hair (NM_000115.3: c.-121G>T; c.-26G>A; c.552T>C; c.831A>G) [3]. There also had a report of heterochromia iridis that have been observing in a Brazilian family that had a defect in the *EDNRB* gene [8]. Moreover, homozygous mutation in the same region of *EDNRB* has been reported that can cause heterochromia and white hair , which are symptoms of the ABCD syndrome (WS type IV), without hearing impairment in a family's children[9]. These suggested that the dysfunction of *EDNRB* may relate to heterochromia iridis or odd-eye trait in other animals as it does in human.

Heterochromia iridis can be found in cats as well, usually as blue-yellow or blue-green irises with mostly with white coat. These cats are called odd-eyed cats, of which Turkish Van, Turkish Angora, Sphynx, Persian, Oriental Shorthair, Japanese Bobtail, and Khao Manee are the examples [10]. These cats have several conditions similar to those in other animals affected by dysfunction of genes that mentioned above, particularly the Waardenburg syndrome in humans and cattle [2,3,5,11]. Thus, the study of these genes should facilitate cat breeding for improving chances of the heterochromia trait together with decreasing or preventing chances of hearing impairment that could come with the trait. The objective was to investigate the association between feline *EDNRB* gene and heterochromia iridis in Thai domestic cats.

MATERIAL and METHODS

Collection of Samples

Phenotypic data and blood samples were obtained from 11 cats having normal eye color (male=8, female=3) and 11 white, heterochromia cats (male=5, female=6) *(Fig. 1)*. Blood samples from cats were collected in blood collecting tubes coated with EDTA as anticoagulant. Among the cats used in this study, there were three kittens born in the same liter in which two of them had heterochromia iridis while the other did not *(Fig. 2)*. The Ethics Committee of the Faculty of Veterinary Medicine, Chiang Mai University, Thailand, approved this study in 2016 (S35/2559).

DNA Extraction, Polymerase Chain Reaction (PCR), and Sequencing

Each blood sample (200 µL) was used for obtaining the genomic DNA. DNA extraction method was performed by phenol-chloroform extraction, which adapted from Taş (1990) [12]. The quality and quantity of the DNA were verified by 1% agarose gel electrophoresis and UV absorbance at 260 and 280 nm.

Three exons of feline *EDNRB* (exon 1, 2, and 3) were amplified by polymerase chain reaction (PCR) using two pairs of primers designed from feline genome (Abyssinian

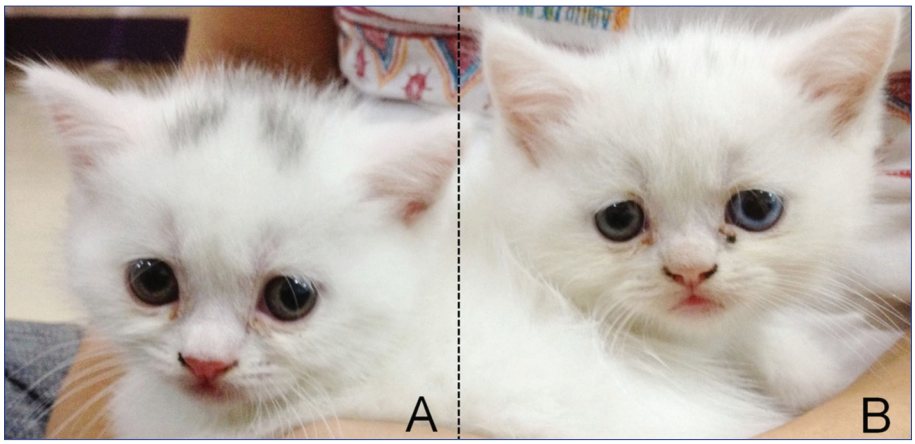

Fig 1. Representative of cats with normal eye color (A) and cats with heterochromia eyes (B)

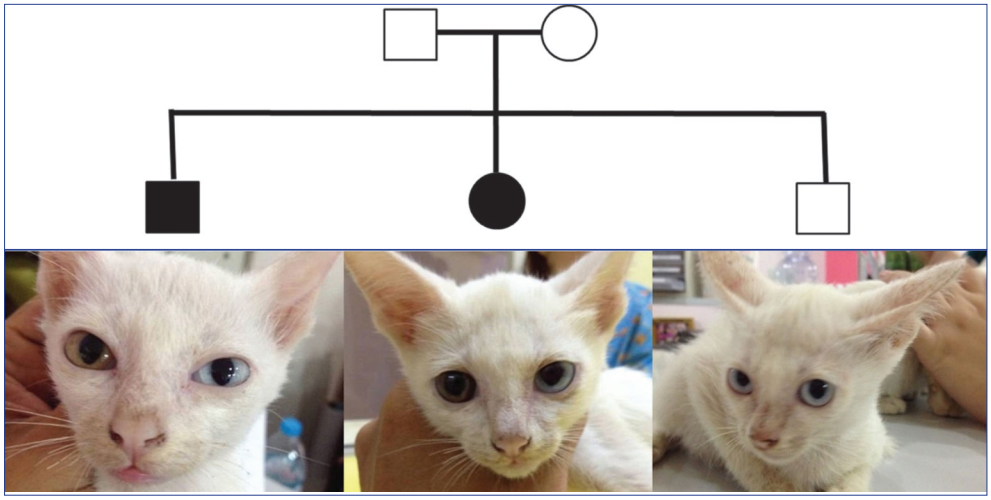

Fig 2. Three kittens born in the same litter: two of them had heterochromia iridis, while the other did not

Table 1. *Primers used in this study*

Region	Primer Sequence (5′→3′)	Product Size (bp)
EDNRB exon 1	Fw: TCCTAACTAGGCACCCTCCC	595
	Rw: CAGTCTTTCTTCCCTGCGGT	
EDNRB exon 2 and 3	Fw: TGGCAGTCCTTATGGAGGAGA	801
	Rw: AGGGCACCGTGTGAAAATCT	

cat, GenBank: GCF_000181335.2) by using BLAST PRIMER (http://blast.ncbi.nlm.nih.gov) *(Table 1)*. The location of studied region was chosen based on the orthologous loci of human *EDNRB* gene (GenBank: NM_000115.3), reported by Jiang et al.[3]. The PCR reaction contained 20 ng genomic DNA; 1X reaction buffer (RBC Bioscience, Taiwan); 0.25 mM dNTP (Vivantis Technologies, Malaysia); 0.25 µM primers (BioDesign, Thailand); 1 U *Taq* DNA polymerase (RBC Bioscience, Taiwan); and deionized distilled water with a total volume of 25 µL. The PCR was performed in Major Cycler, CYCLER-25 thermal cycler (Major Science, USA), with the following cycling profile: 95°C for 5 min; 45 cycles of 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec; and a final extension at 72°C for 10 min. The PCR products were detected by gel electrophoresis using 2% agarose gel as medium and GelRed (Biotium, USA) as visualizer. The amplified products were then scanned for variations by Sanger sequencing (1st Base, Malaysia). The secondary structures of the EDNRB polypeptide with/without mutations were predicted by using the SPIDER3 program (http://sparks-lab.org/server/SPIDER3).

Analysis of Association

The sequencing data were compared between the normal eye group and the heterochromia iridis group using *Felis catus* genome database version 8.0 on GenBank as the reference (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/181/335/GCA_000181335.3_Felis_catus_8.0). The range of locus that was searched for variation/mutation included 200 bp of intron regions adjacent to exon. The collected data were analyzed for correlation by using chi-square test.

RESULTS

Compared to reference data from brown coat Abyssinian cat genome (GenBank: GCF_000181335.2), analysis of the DNA sequences on exon 1, exon 3, and adjacent introns of *EDNRB* showed that there were two variations with amino acid changes found on odd-eyed cats and five variations found on both normal-eyed and odd-eyed cats *(Fig. 3)*. They were c.610A>G, c.916A>G, c.820+40C>T, c.821-14C>T, c.1025+36G>T, c.1025+69A>T, and c.1025+138C>T, respectively (GenBank: XM_003980457.2). Various allele frequencies were calculated, and they are shown in *Table 2*. On c.610, a heterozygous mutation (A/G) was found on one of the odd-eyed cats. The substituted base G changed amino acid from asparagine to serine (P.Asn128Ser). On c.916, one of the odd-eyed cats was found to have base transition from A to G, which caused amino acid to change from valine to alanine (P.Val230Ala), while there was no variation in the normal-eyed cats, which made allele frequency to be 1.0A (and 0.91A/0.9G for heterochromia cats). On c.820+40, the base substitution between C and T (reference allele=T) was found to be sporadic among normal-eyed cats and odd-eyed cats, and heterozygous genotype (C/T) was found in three heterochromia cats. On c.821-14, heterozygous genotype C/T was found in one normal cat and homozygous genotype T/T was found in one heterochromia cat, while the majority of genotype was C/C. On c.1025+36, however, the majority

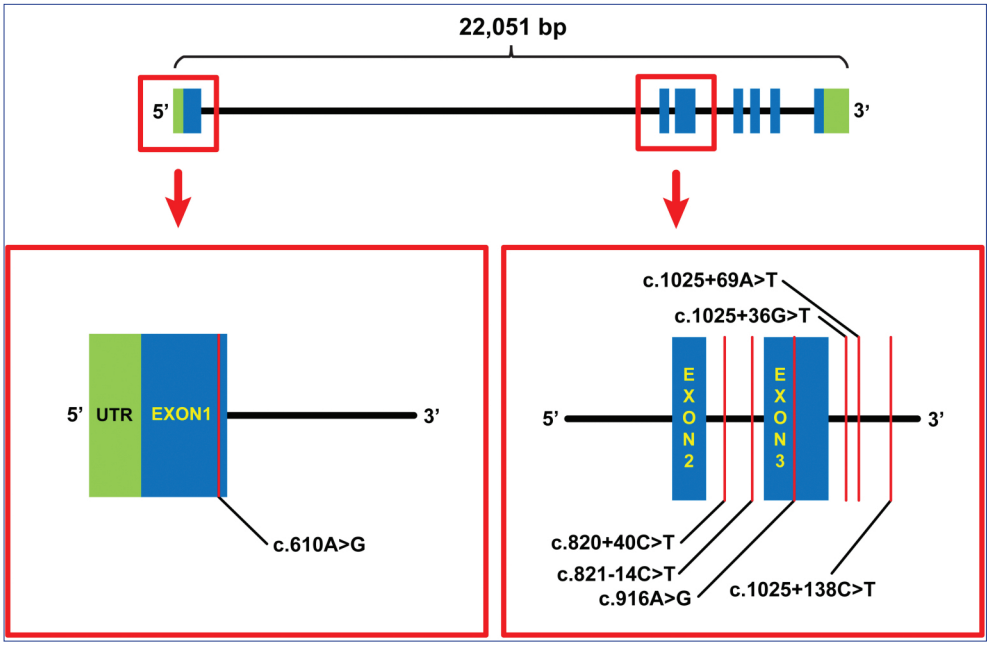

Fig 3. The seven variations found on exon 1, 3, and adjacent intron of the *EDNRB* gene (Feline *EDNRB*, GenBank: XM_003980457.2)

Table 2. *Allele frequencies and Chi-square results*

Locus	Allele Frequency				Chi-square* (*P*-value)	Association (*P*≤0.05)
	Normal-eyed Cats		Odd-eyed Cats			
	Allele 1	Allele 2	Allele 3	Allele 4		
c.610	1.00A	0.00G	0.95A	0.05G	0.311748	no
c.820+40	0.55C	0.45T	0.59C	0.41T	0.760858	no
c.821-14	0.95C	0.05T	0.91C	0.09T	0.549773	no
c.916	1.00A	0.00G	0.91A	0.09G	0.147759	no
c.1025+36	0.73G	0.27T	0.82G	0.18T	0.471847	no
c.1025+69	0.91T	0.09A	0.91T	0.09A	1.00	no
c.1025+138	0.91C	0.91T	0.91C	0.91T	1.00	no

**df*=1

of allele was G instead of T compared to reference data. Moreover, it was found that two odd-eyed cats had heterozygous G/T genotype on this locus, while there was no heterozygous genotype in normal-eyed cats. The variations found on c.1025+69 and c.1025+138 were same, with allele frequencies of 0.09A/0.91T for both normal-eyed cats and heterochromia cats, and 0.91C/0.09T for both normal-eyed cats and heterochromia cats, respectively. In addition, there were two samples of odd-eyed cats, which had heterozygous genotypes on both loci (A/T on c.1025+69 and C/T on c.1025+138, respectively). When analyzed for association using chi-square test, it was found that there was no association between those seven variant loci and heterochromia iridis in cats *(Table 2)*. Furthermore, the predicted secondary structures of three possible mutated amino acid sequences (EDNRB polypeptides with Asn128Ser: NS, Val230Ala: VA, and Asn128Ser-Val230Ala: MT) were slightly different from those in wild type: WT *(Fig. 4)*.

DISCUSSION

Since the heterochromia iridis involves with the embryonic development of neural crest, a transient, multipotent, migratory cell population unique to vertebrates that gives rise to a diverse cell lineage such as craniofacial cartilage and bone, smooth muscle cells, peripheral and enteric neurons, glial cells and melanocytes [13], a crucial factor in the development of skin/fur and eye color. The cascade of gene expressions in neural crest cells depends on the stage of neurulation as well. Several genes that link to heterochromia iridis were previously reported such as *EDNRB*, *PAX3*, *SOX10*, and *MITF* [2,3]. Paired box gene 3 (*PAX3*) is a gene that encoding one of the transcription factors called neural plate border specifier, which mediates influence of induction signals (Wnts, bone morphogenic proteins: BMPs, and fibroblast growth factors: FGFs expressions) and regulates a group of genes called neural crest specifiers that turns embryo cells into neural crest

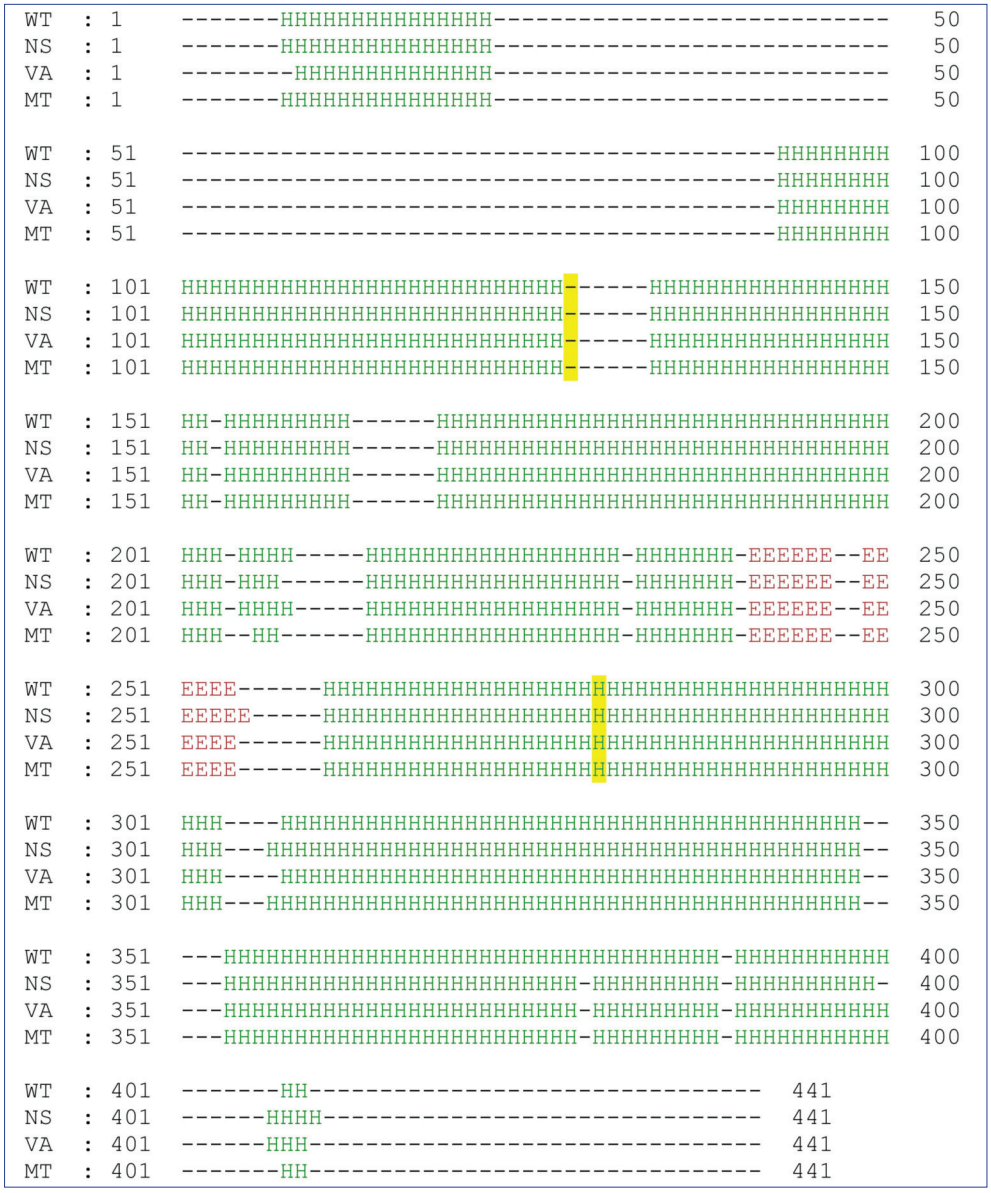

Fig 4. Comparison of the secondary structures of three types of possible mutated EDNRB proteins (NS, VA, and MT) with that of the wild type (WT); H, helix motif; E, strand motif. The yellow highlight indicates the location of the amino acid change (mutation)

cells. SOX10, one of neural crest specifiers, then may turn on most of neural crest effector genes including *MITF*, which is involved in the differentiation of melanocytes, by signaling to *EDNRB*, the receptor protein located on the cell membrane [14]. Hence, as one from many genes involving neural crest development of *EDNRB* gene, two variations (c.610A>G and c.916A>G) found only in heterochromia cats were not statistically related with heterochromia iridis. Moreover, for familial DNA sequence comparison, there was no difference between a white blue-eyed cat and its odd-eyed siblings, suggesting that *EDNRB* may not be the cause of their special eye colors, which is in contrast with other studies [3,8,9,15] that found the mutation at the same location (exon 1 and 3).

Interestingly, the amino acid changes in two exons may be associated with the change in protein properties and conformation due to changes inthe secondary structure of *EDNRB* protein *(Fig. 4)*. Although asparagine and serine are amino acids with uncharged polar side chains, the difference between asparagine and serine is that asparagine has one additional carbon chain with amine group at the end. Valine and alanine are also in the same circumstance: they are amino acids with hydrophobic side chains. Valine is larger than alanine by two methyl groups [16]. With regard to sizes of amino acids, the difference in sizes of the changed amino acids may result in change in protein structure and conformation, which would lead to loss of function of the endothelin receptor protein [17].

In conclusion, the following can be stated: exon 1, 2, and 3 of *EDNRB* were scanned in domestic cats and seven variations were found; two of them were found only in odd-eyed cats and had amino acid changes, but they had no significant relation to heterochromia iridis. Comparison of the DNA sequences between the two odd-eyed cats and their normal-eyed siblings showed no differences in nucleotide sequences as well. The findings of this study

was the first investigation of relationship between three exons of *EDNRB* and heterochromia iridis in odd-eyed cats. To find out genes or DNA regions which involving heterochromia iridis in cats, more samples and candidate genes such *PAX3*, *SOX10*, and *MITF* should be done including with using other novel approaches.

Conflict of Interest

The authors declare that there have no conflict of interests.

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Research Article

Determination of the Stages of Deep Pectoral Myopathy Induced in Broilers Fed with Supplemental Coenzyme Q_{10} [1]

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Abstract

The aim of this study was to examine the structural characteristics and incidence of different stages of deep pectoral myopathy (DPM) that was induced in broilers fed a coenzyme Q_{10} (CoQ_{10}) supplemented diet. A total of 288 1-day-old chicks (Cobb 500) were equally divided among 8 pens (pens 1 to 8). The diet was the same for all chicks until day 35 post-hatching. Subsequently, broilers in pens 5 to 8 were fed the 20 mg of CoQ_{10}/kg finisher diet until the end of the experiment (day 42 post-hatching). To induce DPM, 5 male birds from each of the pens 1 to 8 were subjected to encouraged wing flapping (EWF) at the end of their 37th day. At the end of the trial, the incidence of DPM stages in broilers was determined and an analysis of the histological parameters of deep pectoral muscles was performed. Results showed that, in the groups subjected to EWF, broilers with the CoQ_{10} supplement had a lower average DPM stage and volume density of necrotic muscle cells, as well as a higher volume density of non-necrotic muscle cells. These results can be related to the antioxidant properties of CoQ_{10}, which, in chickens subjected to EWF, reduced the effects of DPM on cell necrosis and muscle tissue damage.

Keywords: *Deep pectoral myopathy, Coenzyme Q_{10}, Broiler*

İlave Koenzim Q_{10} İle Beslenerek Derin Pektoral Myopati Oluşturulan Broiler Piliçlerde Miyopatinin Devrelerinin Belirlenmesi

Öz

Bu çalışmanın amacı; koenzim Q_{10} (CoQ_{10}) ilaveli diyet ile beslenerek derin pektoral myopati (DPM) oluşturulan broiler piliçte meydana gelen myopatinin farklı devrelerini ve yapısal özelliklerini araştırmaktır. Toplam 288 adet 1 günlük civciv (Cobb 500) 8 kafese eşit olarak dağıtıldı. Tüm civcivler için yumurtadan çıkmayı takiben 35. güne kadar diyet aynı tutuldu. Takibinde, 5'den 8'e kadar olan kafeslerdeki civcivler çalışmanın son gününe kadar (yumurtadan çıkmayı takiben 42. gün) 20 mg CoQ_{10}/kg bitirme diyeti ile beslendi. DPM oluşturmak amacıyla, 1'den 8'e kadar olan kafeslerin her birinde 5 erkek civciv 37. günlerinde kanat çırpmaya zorlandı. Deneme sonunda, broiler piliçlerdeki DPM devreleri belirlendi ve derin pektoral kaslarda histolojik parametreler analiz edildi. Elde edilen sonuçlar kanat çırpmaya zorlanan gruplarda CoQ_{10} ilavesi ile beslenenlerde daha az DPM devresi ve daha az nekrotik kas hücresi hacim yoğunluğu ile daha fazla nekrotik olmayan kas hücresi yoğunluğunun oluştuğunu gösterdi. Bu sonuçlar CoQ_{10}'nın antioksidan özellikleri ile ilişkili olup, kanat çırpmaya zorlanan piliçlerde DPM'nin hücre nekrozu ve kas dokusu hasarı etkilerini azaltabilir.

Anahtar sözcükler: *Derin pektoral myopati, Koenzim Q_{10}, Broiler*

INTRODUCTION

Several myopathies which occur in broilers, such as deep pectoral myopathy (DPM), white striping (WS), and wooden breast (WB), are thought to be related to modern trends in poultry production that target increased growth rate,

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body weight and muscle mass of chickens.While WS is characterized by the occurrence of white striations [1], and WB by hardening of the breast muscle [2], DPM is associated with ischemic necrosis ofthe deep pectoral muscles [3].

The function of the deep pectoral muscle is related to wing activity [4]; its location near the sternum, the non-elastic fascia which surround the muscle, and the great size of the breasts in broiler chickens, leave insufficient space for the muscle to expand in response to wing movements. This leads to increased pressure within the muscle, blood flow obstruction and inadequate oxygen supply [5]. Tissue necrosis occurs as a result of ischemia. Described processes represent main characteristics of DPM.

Among several types of stresses (technological, environmental, nutritional, and internal) that can occur in poultry production [6], some of them caused by, for example, increased stocking density, cathcing or carrying the chickens by their wings, inappropriate weighing methods, etc., could result in unnecessary wing movements. This contributes to the development of more or less pronounced degrees of DPM [7]. In recent studies, methods defined as forced wing flapping [8] and encouraged wing flapping [9] have been used for the induction and examination of DPM in broilers.

Oxidative stress is related to elevated production of reactive oxygen species [10,11] that are associated with tissue necrosis [12]. Oxidative stress can be induced during hypoxia [13,14]. Considering that hypoxia is one of the characteristics of DPM (hypoxia can be induced through the occlusion of blood vessels and ischemia), it is therefore important to examine the influence of antioxidants on DPM development.

Beside the antioxidant properties of coenzyme Q_{10} (CoQ_{10}) [15], it has an important role in oxidative phosphorylation and ATP synthesis [16]. CoQ_{10} is already used as a supplement in broiler diets for the treatment of certain diseases and to improve immune functions. Its supplementation has been found to decrease mortality due to pulmonary hypertension syndrome in broilers exposed to low ambient temperature [17]. Furthermore, in research on Newcastle disease, chicks antibody titer was higher on day 21 post-hatching in broilers supplemented with 20 mg of CoQ_{10}/kg of diet [18].

The purpose of this study was to identify and examine the stages of DPM induced in broilers fed with supplemental CoQ_{10}.

MATERIAL and METHODS

Animals and Experimental Design

For the purpose of this study, 288 1-day-old chicks (Cobb 500) were randomly distributed between 8 floor pens (pens 1 to 8), 36 chicks in each pen. Pens 1 to 4 represented 4 replicates, where chickens were fed with starter (days 1-14), grower (days 15-35) and finisher (days 36-42) diets *(Table 1)*. Meanwhile pens 5 to 8 represented 4 replicates where feeding programs were the same as in pens 1 to 4, but with the additional supplementation of 20 mg of CoQ_{10}/kg finisher diet (days 36-42). To induce DPM, 5 male birds from each of the pens 1 to 4, as well as from each of the pens 5 to 8, with a body weight nearest to the mean body weight of the male birds, were subjected to encouraged wing flapping (EWF) at the end of day 37 post-hatching.

The chickens of the control group were fed a basal diet, while the 3 treatments included: 1) chickens fed the basal diet and subjected to EWF; 2) chickens fed a basal diet with CoQ_{10} supplementation; and 3) chickens fed a basal diet supplemented with CoQ_{10} that were subjected to EWF. Food and water were available *ad libitum*. The use of animals in this experiment was approved by the decision of the Ministry of Education, Science and Technological Development of Serbia (Decision No. 401-00-9/2011 of 25 January 2011).

For DPM induction in broiler chickens, the EWF method as described by Lien et al.[9] was applied. This method was basically performed in several steps. First, the chicken was held in the palms of the hands at a height of 1 m, after

Table 1. *Composition of experimental diets*

Ingredients	Starter (0-14 d)	Grower (15-35 d)	Finisher (36-42 d)
Diet composition (g/kg)			
Corn	488.0	528.0	553.0
Full fat soybean	170.0	180.0	165.0
Soybean meal (44% CP)	270.0	220.0	200.0
Soy oil	20.0	20.0	30.0
Monocalcium phosphate	15.0	15.0	15.0
Limestone	18.0	18.0	18.0
Salt	2.5	2.5	2.5
Sodium bicarbonate	3.0	3.0	3.0
L-Lysine HCl	1.0	1.0	1.0
DL-methionine	2.5	2.5	2.5
Vitamin and mineral premix	10	10	10
Calculated nutrient composition			
AME (MJ/kg)	12.87	13.11	13.43
Crude protein (g/kg)	225.5	210.6	198.4
Lysine (g/kg)	13.4	12.4	11.5
Methionine (g/kg)	5.9	5.7	5.6
Ca (g/kg)	11.5	11.4	11.3
P total (g/kg)	7.5	7.3	7.2
P available (g/kg)	4.4	4.3	4.2

CP: crude protein, AME: apparent metabolizable energy

which it was raised to a height of 2 m. From that height, each chicken was allowed to fall freely back to a height of 1 m. Flapping of the chicken's wings occurred during the fall. One cycle of EWF consisted of the previously described procedure. In our study, broilers were treated with EWF at the end of day 37 post-hatching, with a total treatment duration of 45 seconds, which included about 20 cycles of EWF.

During the rearing period, which lasted 42 days, body weight of broilers was in accordance with performance objectives for Cobb 500, and there were no significant differences between treatments. Subsequently, the 5 male birds from each of the pens 1 to 8 that were subjected to EWF were sacrificed. From each of the pens 1 to 4, as well as from each of the pens 5 to 8, 5 male birds that were not subjected to EWF, with body weights nearest to the mean body weight of the male birds, were also sacrificed.

Detection of DPM Stages

In order to reach the deep pectoral muscle, a ventral cut was made through the breast tissue. A score of DPM 0 was used to indicate no signs of myopathy in deep pectoral muscles. Categorization of DPM into a further 4 stages was made according to Kijowski and Kupinska [8]. Hemorrhages and clotted blood in the vessels characterized stage 1 (DPM 1). In stage 2 (DPM 2), necrosis and fibrosis of muscle tissue were present, while the color of the deep pectoral muscle was pale pink. Stage 3 (DPM 3) was related to a change in the color of muscle tissue to green, particulary in the central muscle area. A high degree of muscle tissue necrosis characterized stage 4 (DPM 4), which was manifested by an intensive green color of muscle, which in some parts turned to white and gray.

Morphometric and Stereological Examinations

For the purpose of histological analysis, samples of deep pectoral muscle *(M. supracoracoideus)* were taken after determination of macroscopic DPM stage. Muscle tissue samples were fixed in a 10% buffered formalin solution, followed by several stages of tissue processing: dehydration, clearing, and embedding in paraffin [19,20]. Then, the samples were cut into serial 5µm thick sections using a microtome. Hematoxylin-eosin (H&E) and the Mallory trichrome staining method were performed [21,22] to observe the following parameters: diameter and volume density of non-necrotic and necrotic muscle cells, diameter and volume density of adipose cells, and volume density of connective tissue proper cells.

Samples were analyzed by light microscopy using a Leica DMLS microscope with a Leica DC 300 digital camera and Leica IM 1000 software (Leica Imaging Systems Ltd, Cambridge, UK). The diameter of muscle as well as of adipose cells was measured as the average of the longest lines drawn across the length and width of their cross-sections. The M42 multipurpose testing system was used to measure the volume density of certain cells (muscle cells, adipose cells, and connective tissue proper cells). This testing system represents a grid that consists of 21 straight-line segments, while the test points are located at both ends of each line, making 42 test points in total [23,24]. To calculate the volume density of certain cells, the following formula was used:

$$Vv(c) = \frac{P(c)}{P(m)} \cdot 100\ (\%)$$

where Vv(c) is the volume density of certain cells, P(c) is the number of test points lying over the certain cells, and P(m) is the number of test points lying over the muscle.

Statistical Analysis

Analysis of variance (ANOVA) and *post hoc* Tukey's test were used to determinate the influence of different treatments on the value of the observed histological parameters. To study the influence of different treatments on the incidence of DPM stages, chi-square test followed by the Bonferroni correction was used; a significance level of 0.05 was applied. Statistical tests were carried out using the software package Statistica 13.0 (Dell Software, Round Rock, Texas, USA).

RESULTS

Measurements showed that in those groups where chickens were fed a basal diet and treated with EWF, the incidence of DPM stage 0 was lower ($P<0.01$), while the incidence of DPM stage 3 as well as average DPM stage was higher ($P<0.01$) compared to groups without EWF treatment *(Table 2)*. Between groups with EWF treatment, average DPM stage was lower ($P<0.05$) in groupswhere chickens were fed a basal diet with CoQ_{10} supplementation.

All stages of DPM were detected during the examination of deep pectoral muscles in sacrificed birds *(Fig. 1)*. Deep pectoral muscles with no signs of DPM were labeled as DPM 0. Microscopically these muscles showed standard characteristics of skeletal muscles. DPM stage 1, characterized by red colored deep pectoral muscle with excessive fluid in the damaged area, histologically showed hyperemia, edema, and numerous leucocytes. DPM stage 2 was defined macroscopically by a pale plum color of the deep pectoral muscle with an accompanying fibrotic texture, and microscopically by structural alterations such as necrosis of muscle cells with pale cytoplasm and nuclei. Certain muscle cells were swollen during the early stages of necrosis. In DPM stage 3, muscle samples taken from the central, green parts of the deep pectoral muscle, histologically demonstrated an increased quantity of fibrous tissue with the occasional presence of adipose cells. Muscle tissue classed as DPM stage 4 macroscopically

Table 2. *Effects of treatments on the incidence of deep pectoral myopathy (DPM) stages in broilers at 42 days post-hatching*

Item	Groups			
	Control	Basal Diet with EWF Treatment	CoQ_{10} Supplementation	CoQ_{10} Supplementation with EWF Treatment
DPM 0 (%)	87.50^{A}	37.50^{B}	87.50^{A}	62.50^{AB}
DPM 1 (%)	6.25	6.25	12.50	18.75
DPM 2 (%)	6.25	12.50	0	6.25
DPM 3 (%)	0^{B}	37.50^{A}	0^{B}	12.50^{AB}
DPM 4 (%)	0	6.25	0	0
Average DPM stage	0.19^{Bab}	1.69^{Aa}	0.12^{Bab}	0.69^{ABb}

EWF: encouraged wing flapping. CoQ_{10}: Coenzyme Q_{10}
^{A,B} Values in the same row without a common superscript capital letter differ significantly (P<0.01)
^{a,b} Values in the same row without a common superscript lowercase letter differ significantly (P<0.05)

Table 3. *Effects of treatments on histological parameters of muscle, adipose, and proper connective tissue of M. supracoracoideus of broilers at 42 days post-hatching*

Parameter	Groups			
	Control	Basal Diet with EWF Treatment	CoQ_{10} Supplementation	CoQ_{10} Supplementation with EWF Treatment
Muscle tissue				
Diameter of non-necrotic muscle cell (µm)	62.06±1.86	56.40±1.66	62.17±1.47	60.10±1.90
Diameter of necrotic muscle cell (µm)	54.23±3.27	50.99±2.78	55.24±3.86	52.05±2.99
Volume density of non-necrotic muscle cells (%)	82.05 ± 0.70^{A}	47.61 ± 1.00^{C}	83.64 ± 0.77^{A}	64.16 ± 1.28^{B}
Volume density of necrotic muscle cells (%)	0.68 ± 0.15^{C}	21.55 ± 0.98^{A}	0.62 ± 0.18^{C}	8.79 ± 0.84^{B}
Adipose tissue				
Diameter of adipose cell (µm)	24.74±1.75	19.07±1.68	22.61±0.56	20.45 ±1.73
Volume density of adipose cells (%)	0.36 ± 0.1^{B}	3.05 ± 0.44^{A}	0.42 ± 0.1^{B}	2.54 ± 0.33^{A}
Proper connective tissue				
Volume density of proper connective tissuecells (%)	12.30 ± 0.45^{B}	22.36 ± 0.57^{A}	11.36 ± 0.41^{B}	20.97 ± 0.67^{A}

Values are presented as mean ± standard error of the mean
EWF: encouraged wing flapping. CoQ_{10}: Coenzyme Q_{10}
^{A,B} Values in the same row without a common superscript capital letter differ significantly (P<0.01)

contained whitish regions within areas of green necrotic tissue; in addition, partial replacement of damaged muscle tissue with fibrous tissue and in places with adipose tissue, were detected at the microscopic level.

Histological examination of muscle tissue showed that, in groups with EWF treatment, volume density of necrotic muscle cells was higher (P<0.01), while the volume density of non-necrotic muscle cells was lower (P<0.01) compared to other groups *(Table 3)*. Between groups with EWF treatment, volume density of necrotic muscle cells was higher (P<0.01), while volume density of non-necrotic muscle cells was lower (P<0.01) in groups fed the basal diet. Microscopic observations of adipose and connective tissue showed that the volume density of adipose cells as well as connective tissue proper cells were lower (P<0.01) in groups without EWF treatment compared to other groups.

DISCUSSION

Between the groups subjected to EWF treatment, the groups fed the CoQ_{10} supplemented diet had a lower volume density of necrotic muscle cells, as well as a higher volume density of non-necrotic muscle cells. The cause of this could be related to the antioxidant properties of CoQ_{10}. Excessive wing flapping is known to lead to ischemia and hypoxia [25].

Under hypoxic conditions, oxidative stress may be induced [14], which results in cell necrosis, tissue damage, and structural modifications within skeletal muscles [12,26]. These effects of oxidative stress can be reduced by anti-oxidants; so CoQ_{10} could decrease changes in tissue structure and cell necrosis and be responsible for the lower volume density of necrotic muscle cells in broilers fed the CoQ_{10} supplemented diet.

Fig 1. Macroscopic (left column) and microscopic (right column) characteristics of deep pectoral myopathy (DPM) stages in broiler chickens. A: No signs of DPM (DPM 0); B: no changes in the structure of deep pectoral muscle (DPM 0), H&E, Bar = 50 µm; C: red-colored areas in deep pectoral muscle (DPM 1); D: numerous leucocytes in damaged parts of muscle (DPM 1), H&E, Bar = 50 µm; E: pale plum colored muscle with a fibrotic structure (DPM 2); F: necrotic muscle cells *(arrows)* in deep pectoral muscle (DPM 2), H&E, Bar = 50 µm; G: greening in central parts of muscle (DMP 3); H: fibrous tissue surrounds necrotic muscle cells (DPM 3), Mallory trichrome, Bar = 50 µm; I: final stage of myopathy (DPM 4); J: replacement of damaged muscle cells *(arrows)* with fibrous tissue (DPM 4), Mallory trichrome, Bar = 50 µm. DP: deep pectoral muscle, SP: superficial pectoral muscle

The use of different classifications used by authors in determining the stage of DPM could lead to misunderstandings. In papers so far, 3-stage [3] and 4-stage [8] classifications can be found. In order to classify DPM stages more precisely, both macroscopically and microscopically, we applied the 4-stage classification as used by Kijowski and Kupińska [8].

In previous studies in which broilers were subjected to forced wing flapping [8] or EWF [9] at 5 days before slaughter (similar to the current experiment) results obtained were in agreement with ours. Similary, Kijowski and Kupińska [8] also observed all 4 stages of DPM in Ross 308 and Flex line broilers that were subjected to forced wing flapping. In male broilers (Ross x Cobb 500) subjected to EWF, a 71% prevalence of DPM lesions was detected by Lien et al.[9], which supports the current findings in the group fed a basal diet and subjected to EWF.

If we surmise that the antioxidant CoQ_{10} can reduce muscle tissue damage and cell necrosis caused by oxidative stress [10,15], it can be assumed that a consequence of that reduction could be a lower average DPM stage in groups of broilers fed a CoQ_{10} supplemented diet and treated with EWF, as compared to groups of broilers fed a basal diet and treated with EWF. This points out the direct relationship between DPM stage and the degree of tissue damage and cell necrosis. Our results were in agreement with the above mentioned theories; between the groups that were subjected to EWF, the volume density of necrotic muscle cells was lower in groups fed the CoQ_{10} supplemented diet.

Examination of DPM in house reared broilers [27] report similar observations as were noticed in our study, such as greenish, pale, and swollen muscles with the presence of necrotic and hemorrhagic tissue that were visible during dissection of the pectoral muscle. In addition, leucocyte infiltration, necrotic muscle fibers, large necrotic areas, and fibro-adipose tissue were detected during microscopic examinations.

The question arises, if the EWF treatment, which in our study lasted 45 seconds, is prolonged, how would this be reflected in the signs of DPM. Kijowski and Kupińska [8] show that increased duration of wing activity leads to greater fatigue of the pectoral muscle, which results in a reduced number of wing flaps in additional time. Therefore, it can be surmised that prolonged duration of EWF treatment will lead to enhanced signs of DPM, but not in proportion to the extended time.

Determination of the most suitable day of age of broilers for the application of EWF treatment is very important. In our study, broilers were subjected to EWF treatment at the end of their 37th day, based on previous findings [8,9] where treating broilers too early in their life resulted in almost complete absence of DPM; this was because of the low weight of the pectoral muscle in which symptoms of myopathy were unable to develop. Meanwhile the application of treatment a short time before slaughter, did not allow time for the later stages of DPM to develop.

Fathi [17] showed that differences in total mortality percentage of broilers due to ascites existed between 2 groups fed diets supplemented with 20 mg and 40 mg of CoQ_{10} per kg of feed. In previous studies, the age at which broilers were offered CoQ_{10} supplements differed. Huang et al.[28] offered broilers a CoQ_{10} supplement diet from 1 day post-hatching, while Fathi [17] offered CoQ_{10} supplement at day 15. As in our experiment, broilers were treated with 20 mg of CoQ_{10}/kg of finisher diet from days 36 to 42; in further research it would be useful to apply different levels of CoQ_{10} in treatments and/or to apply the supplemented diets to broilers of different ages.

We were mindful of animal welfare when selecting EWF in this study as the means for inducing DPM. In previous studies [29-31], the applied methods, such as surgical occlusion of the vascular supply, electrically-induced contractions of the muscle, and forced wing exercise, were more stressful and/or painful to broilers than EWF.

Intensive wing flapping is the main reason for the occurrence of DPM in broilers, and by reducing it the incidence of DPM could be decreased. In order to successfully reduce signs of DPM in broilers, several measures must be applied such as reduced human activity in broiler houses as well as animal activity around the house, refraining from catching birds by their wings, providing sufficient space for every bird during resting, moving, eating, or drinking, etc. These measures are in line with the flock management guidelines presented by Bilgili and Hess [3] in order to reduce the incidence of green muscle disease in broiler flocks.

In conclusion, results indicate that, in broilers fed supplemental CoQ_{10}, the average DPM stage as well as effects of DPM on histological parameters of deep pectoral muscle were reduced at 42 days post-hatching. These findings suggest that the antioxidant properties of CoQ_{10} could reduce the effects of DPM on cell necrosis and change in tissue structure, considering that between the groups subjected to EWF treatment, a lower average DPM stage and volume density of necrotic muscle cells, as well as higher volume density of non-necrotic muscle cells were detected in broilers fed supplemental CoQ_{10}.

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Research Article

The First Detection of anti-*Anaplasma phagocytophilum* Antibodies in Horses in Turkey

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Abstract

Anaplasma phagocytophilum, the causative agent of equine granulocytic anaplasmosis, affects several species of wild and domesticated mammals, including horse, besides human. In Turkey, there were many reports on *A. phagocytophilum* circulation among cattles, sheep, dogs, mice, humans, except horses. In this study, we aimed to inquiry whether *A. phagocytophilum* were circulating among the horse population or not. For this purpose, 105 mare horse blood sera were examined for the presence of Anti-*Anaplasma phagocytophilum* IgG antibodies by IFAT. The seroprevalance rate of 8.57% horse sera were found to be positive. This was the first report about the presence of anti-*A. phagocytophilum* antibodies in horses in Turkey..

Keywords: *Anaplasma phagocytophilum, Horse, IFAT*

Türkiye'de Atlarda anti-*Anaplasma phagocytophilum* Antikorlarının İlk Tespiti

Öz

Equine granulocytic anaplasmosis'in etkeni olan *Anaplasma phagocytophilum*, atların dahil olduğu pek çok vahşi ve evcil memeliyi, yanı sıra insanları etkilemektedir. Türkiye'de *A. phagocytophilum*'un atlar hariç, sığırlar, koyunlar, köpekler, fareler, insanlar arasında sirküle olduğuna dair birçok çalışma mevcuttur. Bu çalışmada, *A. phagocytophilum*'un at populasyonu arasında mevcut olup olmadığını araştırmayı amaçladık. Bu amaçla, 105 dişi at kan seumu Anti- *A. phagocytophilum* IgG antikorlarının varlığını tespit etmek için IFAT ile incelendi. At kan serumlarının %8.57'si pozitif bulundu. Bu çalışma, atlarda anti-*A. phagocytophilum* antikorlarının varlığı ile ilgili ilk rapordur..

Anahtar sözcükler: *Anaplasma phagocytophilum, At, IFAT*

INTRODUCTION

Anaplasma phagocytophilum (A. phagocytophilum), a member of Anaplasmataceae in the order of Rickettsiales is a causative agent of Equine Granulocytic Anaplasmosis (EGA) was defined in 2001 [1]. The agent is an obligate, intracellular, gram negative tick-borne, zoonotic rickettsiale bacterium of human and animals [2,3].

The family Anaplasmataceae contains arthropod-borne α-proteobacteria which causes important economic and health losts both in veterinary and human medicine relevant to endemic and emerging infectious diseases. Particularly, *Anaplasma* genera infects peripheral blood cells, *A. phagocytophilum* infects myeloid cells of bone marrow, especially neutrophills and sometimes eosinophils [3,4]. *A. phagocytophilum* is transmitted by ticks of Ixodes genera

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during seasons of tick activity [5]. Agent replication takes place within the vacuoles of phagocytes [3,6].

The first reported case of Equine Granulocytic Anaplasmosis (EGA) was in California, USA [7,8] in 1969, *A. phagocytophilum* infection in domesticated animals, ticks and people has a worldwide geographic distribution such as Europea [9-11], Great Britain [12], Asia [13,14] and Africa [15,16]. Also, infections of *A. phagocytophilum* have been reported in neighbouring countries of Turkey such as Greece [17], Bulgaria [18] and Iran [19]. Many available studies conducted on *A. phagocytophilum* infection on horses were present [20-25]. In Turkey, although studies conducted on *A. phagocytophilum* including a wide variety of animal species such as cattle, sheep, dog, mice, besides human [26-32] were exist, but there is no report about equine anaplasmosis. The aim of this study was to detect anti-*A. phagocytophilum* antibodies in horses in Nevşehir province of Turkey.

MATERIAL and METHODS

Ethical Approval

The ethics committee of Veterinary Control Central Research Institute (Date 27.11.2015, Report no: 2015/07) approved the protocol used in this study.

Samples

The material of this study was consist of 105 mares from different races in Nevsehir province of Turkey. Ages of animals were between 3 and 24. Horses were rising for touristic purposes. All animals were clinically healthy. Blood samples were collected between February-April in 2016. Blood samples were collected by jugular vein puncture into vacutainer tubes without anticoagulant and store at 4ºC until arrival at the laboratory. After arriving to the laboratory, blood samples were centrifuged at 5.000 rpm 10 min, subsequently sera were seperated. Serum samples were stored at -20ºC until analysis performed.

Serologic Analysis

Samples were screened for IgG against *A. phagocytophilum* according to the instructions of commercially available IFAT Kit *(Anaplasma phagocytophilum* IFA Equine Antibody kit; Fullerton, California, USA; Cat no: EEE-120) based on *A. phagocytophilum* HGE-1 isolate antigens derived from HL-60 cells. Slide examination was performed using fluorescence microscope at 400-fold magnification.

Interpretation of the Results

All samples tested at a 1:80 as starting dilution in phosphate buffer saline solution (PBS) pH 7.2 according to the manufacturer's protocol. IgG titers 1:80 and greater were considered as positive. Samples were considered positive when bright green flurescence of *A. phagocytophilum* morulae observed at 1:80 and greater IgG titers. Samples were considered negative if no flurescence was seen at 1:80 titer.

Statistical Analysis

The animals were divided into two age groups: animals aged equal and up to 7 years, and more than 7 years. Association between the presence of *A. phagocytophilum* and age of animals is evaluated by 2x2 contingency table and analyzed by using Fisher's Exact test. For statistical analysis, Statistical package IBM SPSS is used.

RESULTS

Anti- *A. phagocytophilum* IgG antibodies were detected in nine out of 105 (8.57%) horse sera.

The statistical analysis was shown in *Table 1*. N represented the number of animals from two age categories. Presence of *A. phagocytophilum* (in terms of proportion) was given in parenthesis. The results were showed that approximately 9% of all the blood samples of all animals aged equal and up to 7 years were positive for *A. phagocytophilum*. Additionally, this rate seemed similar for the animals aged more than 7 years, i.e., approximately 8%. For α=0.05, the result of the Fisher's Exact test *P*-value=0.545 indicated that the difference between the presence of *A. phagocytophilum* infection in respect to horse ages could not reach a statistical significance. In statistical sense, analysis of data revealed that there was not a significant relationship between the age of the horses and the infection of *A. phagocytophilum*.

DISCUSSION

As previously emphasized before, many studies published for the presence of *A. phagocytophilum* in various animals in Turkey [26,27,29,30]. Best of our knowledge, there is no study about the presence of *A. phagocytophilum* in horses and this will be the first study about this subject in Turkey.

In this study we determined the seroprevalence rate as

Table 1. *Results of the statistical analysis*

Age	N	Positive n (%)	Negative n (%)	Fisher's Exact Test *P-value*
Equal and up to 7 years	43	4 (9.30%)	39 (90.70%)	0.545*
More than 7 years	62	5 (8.06%)	57 (91.94%)	

** P>0.05 = Not significant*

8.57% which is lower than France (11.3%) [33], Guatemela (13%) [34], Sweden (16.6%) [35], Italy (17.03%) [36], Denmark (22.3%) [37], Tunus (16.3-67%) [15,38], USA (17-29%) [39], and Czech Republic (73%) [40], and higher than Korea (2.9%) [41], Sub-Saharan Africa (0%) [42], Taiwan (2.5%) [43], and Japan (3.4%) [44]. Our result was found to be approximately equal with the studies conducted in Italy (8.9-9%) [45,46].

Equine Granulositic Anaplasmosis is usually diagnosed by interpreting the combination clinical signs, results of laboratory tests and epizootic history. Various diagnostic methods can be used to determine the disease according to the course of infection [47]. Since having no argument about the existence of the disease in horses in Turkey, we first had to decide which diagnostic tests provided usefullness by revealing the advantages and disadvantages of them. In solely acute stage of the infection, appearing morula in granulocytes usually 2-4 days after infection can be seen from blood smears taken from the infected animals stained using Wright, Giemsa methods which is highly specific, but have a limited sensitivity was not preffered to inquire *A. phagocytophilum* due to the sampling group of healthy horses in the study [48,49]. In addition to this, lack of sensitivity observed in healthy carrier hosts which have low parasitaemia was also reported [50]. Another diagnostic tool, PCR is an excellent diagnostic tool for the detection of early stage of the infection fastly. PCR becomes positive between 1-21 days post infection, sporadic PCR positivenes can be observed after 21 days, but not for long time [50]. Due to short course of bacteriemia, while IgG antibodies were detectable, *A. phagocytophilum* DNA cannot. This situation correspond to past infection. Both serology and PCR positive results correspond to early infection [50]. Due to having no information about EGA in horses in Turkey, purposeful diagnostic tool for our study in our sampling group consisting of randomly selected healthy horses with no apparent clinical signs was seeking IgG against *A. phagocytophilum* by IFAT. Since it provides an excellent screening method to explore whether the circulation of bacteria exist or not among the horses.

Early production of specific IgG titers occurred during cell mediaed and humoral immun-response can be observed 19 days after the infection date with a peak being reached approximately 8 weeks after the infection [51]. In naturally infected horses, immunity persists for at least 2 years and does not appear to depend on latent infection and carrier status [52]. Nine out of 105 seropositivness with no clinical manifestation determined in the study showed that horses were somehow exposed to tick infestation and *A. phagocytophilum* circulated among horse population. Anaplosmosis is usually seen in Aegean, Black-Sea, Marmara region of Turkey, where humidity and dense vegetation provide good habitats for *Ixodes ricinus (I. ricinus)* [53,54]. On the other hand, the presence of *I. ricinus* was reported on sheep in all regions of Turkey [55,56]. There was also a report from the neighbouring province, Kayseri whom had the similiar geographic conditions to Nevşehir [57]. The authors reported 8% *A. phagocytophilum* rate in a study conducted on dogs, in Kayseri [57]. The Anatolian Plateau (Central Anatolia) is much more subject to extremes than are the coastal areas. Winters on the plateau are especially severe. Because of central Anatolia's geographical conditions, one cannot speak about a general overall climate. Hence, it was thougt that presence of *I. ricinus* was also exist in the Central Anatolia [53].

In this study seropositivness was attributed to sub-clinical EGA where the clinical signs of the disease mild and absent [58]. Persistent subclinical EGA was also hypothesized by Chang et al.[59] in experimental infections.

The severity of the disease varies according to the age of the horse. Horses less than 1 year old exhibit limited clinical signs, those younger than 4 years old and 4 years show mild clinical signs, horses older than 4 years develop characteristic symptoms of disease [60]. However, according to the statistical analysis, no significant relationship between the age of the horses and the infection of *A. phagocytophilum* in the study.

The outcome of the present study provided to obtain a knowledge about the presence of *A. phagocytophilum* circulating on horses, in Nevşehir province of Turkey. Asymptomatic animals may be reservoirs for humans and other animals. From this point of view, we think that; further studies in domestic and wild animals will help us for a better understanding the epidemiology and effective control strategies of the disease.

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Research Article

Effects of *GH-Alu*I and *MYF5-Taq*I Polymorphisms on Weaning Weight and Body Measurements in Holstein Young Bulls [1]

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Abstract

Live weight and body measurements are economically important quantitative traits that affect carcass yield and calf survival in cattle. Four genes, growth hormone gene *(GH),* myogenic factor 5 *(MYF5),* fatty acid binding protein 4 *(FABP4)* and signal transducers and activators of transcription 5A *(STAT5A)* were chosen as candidate genes for live weight and body measurements due to their important role in growth. The aims of this study were to genotype *GH-Alu*I, *MYF5-Taq*I, *FABP4-Hin*II and *STAT5A-Ava*I polymorphisms and to investigate their associations with live weights and body measurements in Holstein young bulls. Genotyping of the single nucleotide polymorphism (SNP) markers in these candidate genes was carried out using the restriction fragment length polymorphism (RFLP) analysis. Frequencies of L allele for *GH,* A allele for *MYF5*, G allele for *FABP4* and C allele for *STAT5A* were, 0.96, 0.61, 0.79 and 0.74, respectively in the examined animals. The regression analysis indicated that the *GH-Alu*I polymorphism showed an association with weaning weight (WW), 180th day weight and hearth girth from birth to 180 days of age. The *MYF5-Taq*I polymorphism was found to influence body length at birth and birth weight (BW). However, no significant association was detected between the *FABP4-Hin*II genotypes and measured traits. The *GH-Alu*I and *MYF5-Taq*I polymorphisms may be useful for selection on live weight and body measurement traits in Holstein young bulls.

Keywords: *GH, Live weight, MYF5, RFLP, Weaning weight*

*GH-Alu*I ve *MYF5-Taq*I Polimorfizmlerinin Erkek Holstein Buzağılarında Sütten Kesim Ağırlığı ve Vücut Ölçüleri Üzerine Etkileri

Öz

Canlı ağırlık ve vücut ölçüleri gibi önemli kantitatif özellikler, karkas verimi ve buzağı yaşama gücünü etkilemektedirler. Büyümedeki rolleri nedeniyle büyüme hormonu geni *(GH)*, miyojenik faktör 5 *(MYF5)*, yağ asidi bağlayıcı protein 4 *(FABP4)* ve sinyal dönüştürücü ve transkripsiyonu aktive edici faktör 5A *(STAT5A)* canlı ağırlık ve vücut ölçüleri için aday gen olarak önerilmiştir. Bu çalışmada erkek Holstein buzağılarda *GH-Alu*I, *MYF5-Taq*I, *FABP4-Hin*II ve *STAT5A-Ava*I polimorfizmleri belirlenerek, elde edilen genotip verileriyle canlı ağırlık artışı ve vücut ölçüleri arasındaki ilişkilerin araştırılması amaçlanmıştır. İncelenen örneklerin bu aday genlerdeki SNP markırları yönünden genotipleri restriksiyon parçacık uzunluk polimorfizmi (RFLP) analiziyle belirlenmiştir. İncelenen örneklerde *GH* için L allel, *MYF5* için A allel, *FABP4* için G allel ve *STAT5A* için C allel frekansı diğer allelden yüksek bulunmuştur (sırasıyla 0.96, 0.61, 0.79 ve 0.74). Elde edilen genotip verileri kullanılarak yapılan regresyon analizi sonunda *GH-Alu*I polimorfizmi ile sütten kesimdeki (WW) ve 6. ay canlı ağırlıkları ile tüm ölçüm dönemlerindeki gögüs çevresi uzunluğu ile ilişkili olduğu görülmüştür. *MYF5-Taq*I polimorfizmi ise doğumdaki vücut uzunluğu ve doğum ağırlığı (BW) ile ilişkili bulunmuştur. İncelenen örneklerde *FABP4-Hin*II polimorfizmi ölçülen özelliklerin hiç biri ile ilişkili bulunmamıştır. Çalışma sonunda *GH-Alu*I ve *MYF5-Taq*I polimorfizmlerinin canlı ağırlık ve vücut ölçüleri yönünden yapılacak seleksiyon çalışmalarında kullanılabileceği düşünülmüştür.

Anahtar sözcükler: *GH, Canlı ağırlık, MYF5, RFLP, Sütten kesim ağırlığı*

INTRODUCTION

Beef is an excellent source of protein for human nutrition but, the production costs is higher compared to poultry and pork [1]. Therefore, increasing the meat yield is one of the most important issue in cattle breeding specially in countries in which their consumer preferences depending on mainly ruminant meat. Genomic selection

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is an encouraging development in livestock, proposing improved production by deciphering molecular genetic markers to design novel breeding programs and to develop new markers-based models for selecting favorable genotypes [2].

One of the best known genetic marker for beef yield is the growth hormone gene *(GH)* [3,4] which is encoding for growth hormone. *GH* is located on cattle chromosome 19 (BTA19) [5], and consists of five exons and four introns [6]. It is playing role in numerous physiological processes such as growth traits, mammary gland development and lactation [7]. Although, studies have mostly focused on association of *GH* genotype with milk yield traits, association of *GH* genotypes with live weight and body measurement traits have been less investigated [8]. Several polymorphisms were detected in the bovine *GH* gene, however, the best known of these polymorphisms is the leucine (L) to valine (V) substitution at position 127 in exon 5 of *GH* gene which creates a cut point for *Alu*I restriction enzyme [4].

Skeletal muscles occur result of a series of physiological processes which is named as myogenesis, and it includes cell specification, proliferation and differentiation, in which multi-potential mesodermal cells are differentiated into myoblasts [9]. Myogenesis begins at embryonic stage and continues to postnatal maturation [10] and controlled mainly by myogenic determination *(MyoD)* gene family [11]. Myogenic factor 5 *(MYF5)* gene is one of the four members of *MyoD* family [12] and located on BTA5 in bovine [13]. It is evaluated as a candidate gene for growth traits in beef cattle breeding because of its tasks in muscle development and growth traits [13,14].

The fatty acid binding protein 4 *(FABP4)* has got important tasks in lipid hydrolysis and intracellular fatty acid uptake in different tissues [15,16]. This protein is encoded by *FABP4* gene and its mRNA expresses firstly in adipocytes [17] from a locus located on BTA14 in cattle [18]. In literature, few reports were shown the relationship between *FABP4* and carcass weight in native Korean cattle breed [19,20]. However, no study was devoted to association *FABP4* variants with live weight and body measurements in Holstein cattle.

Signal transducers and activators of transcription protein (STAT) is a family of transcription factors which is mediated the effects of some peptide hormones and cytokines. STAT family has seven members [21], of them *STAT5A* is an important mediator of growth hormone which is located on BTA19 in bovine [22]. Therefore, it was thought that *STAT5A* gene may be used as a marker for important yield traits such as growth and live weight gain in livestock [23].

Although functional relation of these genes in growth metabolism has been reviewed in literature, association of polymorphisms in these genes with live weight traits and body measurements have not been strongly taken into consideration in Holstein cattle breed. Therefore, aims of this study were to investigate SNPs in *GH*, *MYF5*, *FABP4* and *STAT5A* genes, and investigate their association with live weight traits and some body measurements in Holstein young bulls reared in Turkey.

MATERIAL and METHODS

The project was approved by the relevant Animal Ethics committee of Erciyes University (#13/72 10.04.2013). A total of 59 male Holstein calves, born between March 2013 and November 2014 were used in this study. Animals were not applied to a special feeding program. Calves were stay with their mother after birth and consume *ad-libitum* colostrum for three days. At the end of three days, calves were fed with milk 10% of their birth weight until weaning. At seven days old, calf starter feed which contains calve growing feed and forages were provided ad-libitum until weaning. After weaning, calves were fed with milk replacer for 40 days until milk replacer weaning day. Young bulls were fed with forage and beef cattle feed (concentrated protein) mixture until 180th day. As live weight traits; birth weight (BW), weaning weight (30. day) (WW), milk replacer weaning weight (70. day) (RW) and weight at 180th day after birth (SW) were measured. The animals were weighed to the nearest kilogram using an electronic weighing scale (EziWeigh 5i, Tru-Test, New Zealand) mounted on a concrete platform. For body measurements; body length, wither height and hearth girth were measured. Body measurements were taken by two observers using an ordinary measuring tape and recorded in centimeters. Body length was measured as the distance from *Atriculus huneri* to *Tuber ichii*; wither height was measured as the distance from the ground to the highest point of wither. Hearth girth was measured as behind the front shoulder at the fourth ribs, posterior to the front leg [24].

All blood samples were collected post-natal period, and genomic DNA was extracted by the phenol:chloroform:isoamlyalcohol method. The *GH*, *MYF5*, *FABP4* and *STAT5A* gene polymorphisms were genotyped by using PCR-RFLP. The PCR reactions mixtures of all genes were prepared as total volume of 25 µL, including 1.5 mM $MgCl_2$, 200 µM dNTP, 5 pmol of forward and reverse primer of each gene, 1×PCR buffer, 1U Taq polymerase and approximately 100 ng DNA. The PCR protocols for investigated genes were as follow: for *GH* gene, 4 min at 94°C for, then 40 cycles of 94°C for 40 s, 60°C for 40 s, 72°C for 40 s and the final extension at 72°C 10 min; for *MYF5* gene, initial denaturation at 94°C for 4 min, then 38 cycles of 94°C for 1 min, 64°C for 30 s, 72°C for 1 min and final extension at 72°C for 4 min; for *FABP4* gene, initial denaturation at 95°C for 4 min, then 32 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min; for *STAT5A* gene, pre-denaturation at 95°C for 4 min, then 34 cycles of 94°C for 1 min, 64°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. The obtained PCR products were digested by 5 U of restriction endonuclease enzymes according to

their own protocols (Fermentas, Thermo Fisher Scientific Inc., Waltham, MA, USA). After digestion procedures, the genotypes were detected on 3% (for *GH* and *STAT5A* genes) and 2% (for *FABP4* and *MYF5* genes) agarose (Prona Agarose; Basica Le, Burgos, Spain) gel electrophoresis. The nucleotide sequences of PCR primers and restriction enzymes used for genotyped amplification and RFLP process are presented in *Table 1*.

Allele and genotype frequencies of genes, investigated in the present study were calculated by using OEGE - Online Encyclopedia for Genetic Epidemiology studies online tool [29]. Statistical analyses were performed by using IBM SPSS Statistics 22.0 software. For *GH*, *MYF5*, *FABP4* and *STAT5A* genes genotype and phenotype associations were investigated by using independent general linear model (GLM) procedure and Duncan test for significance levels; $P<0.1$ and $P<0.05$. Sire (n=11) were assumed random factor in the GLM model. The statistical model used as follows:

$$Y_{ij} = \mu + S_j + G_i + e_{ij}$$

Where Y_{ij} is the observation of the weaning weight and body measurements traits; μ is the overall mean for each trait, S_j is the random effect of j^{th} sire, G_i is the fixed effect of i^{th} genotype for the relevant polymorphism and e_{ij} is the random residual error.

RESULTS

The 223 bp products were obtained after PCR process and PCR products were digested by *Alu*I restriction enzyme for *GH* gene. At the end of digestion, it was expected one band (223 bp) for VV genotype, three bands (223, 171 and 52 bp) for LV genotype, two bands (171 and 52 bp) for LL genotype. The band of 52 bp could not be observed on agarose gel electrophoresis. However, two bands (223 and 171 bp) were enough for genotyping *(Fig. 1a)*. The LL genotype was found to be the highest frequency, and the VV genotype was found to be the lowest frequency in our investigated Holstein population. The L allele frequency was higher than V allele *(Table 2)*.

The 490 bp products were obtained after PCR process and they were digested by *Taq*I restriction enzyme for *MYF5* gene. At the end of digestion, it was observed one band (490 bp) for AA genotype, three bands (490, 367 and 123

Table 1. *Primer sequence, accession number, PCR product size*

Gene	Accession Number	Sequence	Product Size	Restriction Enzyme	Reference
GH	EF592534.1	GCTGCTCCTGAGGGCCCTTCG GCGGCGGCACTTCATGACCCT	223 bp	*Alu*I	[25]
MYF5	M95684.1	AGAGCAGCAGTTTTGACAGC GCAATCCAAGCTGGATAAGG	512 bp	*Taq*I	[26]
FABP4	NC007312	ATTATCCCCACAGAGCATCG ACAAGACTTGGCCTCAAGGA	399 bp	*Hin*II	[27]
STAT5A	AJ237937	CTGCAGGGCTGTTCTGAGAG TGGTACCAGGACTGTAGCACAT	215 bp	*Ava*I	[28]

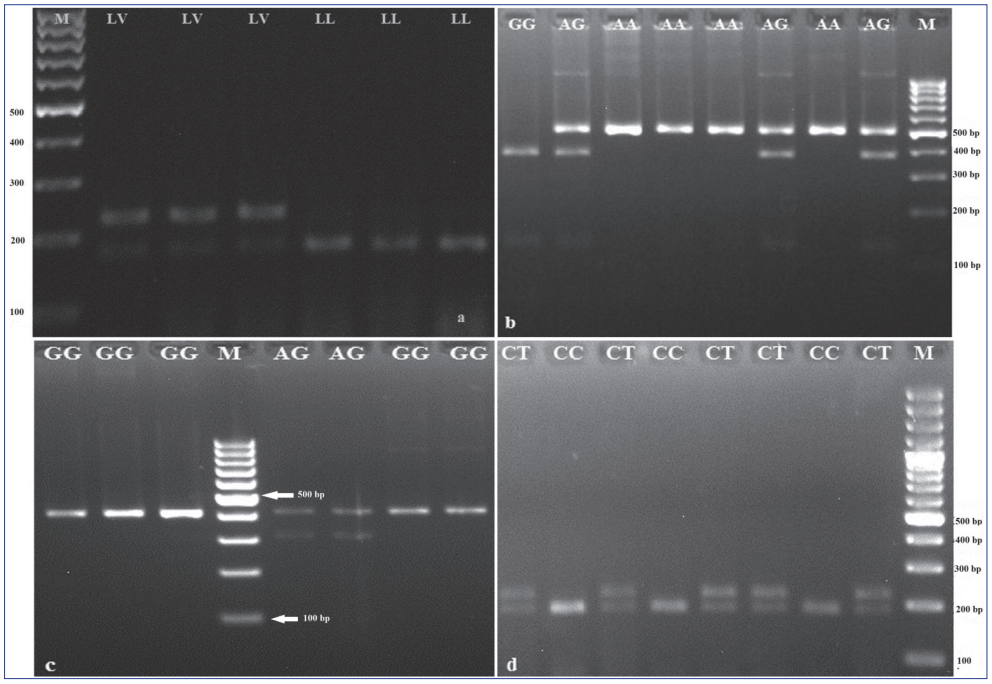

Fig 1. Agarose electrophoresis pattern of GH (a), MYF5 (b), FABP4 (c) and STAT5A (d). M: molecular marker (100 bp)

Table 2. *Genotype and allele frequencies of the GH, MYF5, FABP4 and STAT5A genes in beef Holstein cattle*

Gene	Genotype						Allele Frequency		Chi-squared
GH	LL		LV		VV		L	V	χ^2=0.12 P=0.734 (df=1)
	Obs (Exp)	F	Obs (Exp)	F	Obs (Exp)	F	0.96	0.04	
	54 (54.11)	0.915	5 (4.79)	0.085	0 (0.11)	-			
MYF5	AA		AG		GG		A	G	χ^2=1.16 P=0.2819 (df=1)
	Obs (Exp)	F	Obs (Exp)	F	Obs (Exp)	F	0.61	0.39	
	20 (21.97)	0.339	32 (28.07)	0.542	7 (8.97)	0.119			
FABP4	AA		AG		GG		A	G	χ^2=4.26 P=0.0389 (df=1)
	Obs (Exp)	F	Obs (Exp)	F	Obs (Exp)	F	0.21	0.79	
	0 (2.65)	-	25 (19.7)	0.424	34 (36.25)	0.576			
STAT5A	CC		CT		TT		C	T	χ^2=7.49** P=0.0062 (df=1)
	Obs (Exp)	F	Obs (Exp)	F	Obs (Exp)	F	0.74	0.26	
	28 (32.07)	0.475	31 (22.86)	0.525	0 (4.07)	-			

Obs: Observed genotype; Exp: Expected genotype; F: Frequency; df: degree of freedom

Table 3. *Least squares means and standard errors for BW, WW, RW and SW in male calves according to GH, MYF5, FABP4 and STAT5A genotypes*

Gene	Genotype	N	Traits			
			BW (kg) Mean (±SE)	WW (kg) Mean (±SE)	RW (kg) Mean (±SE)	SW (kg) Mean (±SE)
GH	LL	54	42.38±1.13	53.37±1.25[a]	73.21±1.71	188.57±4.78
	LV	5	39.80±2.70	46.89±2.98[b]	72.43±4.09	166.30±11.41
P			0.360	0.041	0.856	0.065
MYF5	AA	20	45.13±6.57	55.61±6.47	72.88±8.21	185.05±24.63
	AG	32	41.88±4.17	53.55±6.13	76.71±8.87	180.22±19.07
	GG	7	40.71±2.30	54.20±4.54	76.34±5.72	185.14±28.44
P			0.078	0.719	0.303	0.930
FABP4	AG	25	42.47±1.31	52.72±1.51	72.87±1.98	185.25±5.71
	GG	34	41.69±1.33	52.54±1.53	73.38±2.00	186.83±5.78
P			0.603	0.918	0.819	0.807
STAT5A	CC	28	42.23±1.33	52.68±1.53	75.59±1.90[a]	184.38±5.78
	CT	31	41.95±1.34	52.59±1.54	70.61±1.91[b]	187.69±5.81
P			0.858	0.959	0.028	0.622

Statistical differences among genotypes were shown as: [a] and [b] P<0.05

bp) for AG genotype, two bands (367 and 123 bp) for GG genotype *(Fig. 1b)*. The AG genotype was found to be the highest frequency whereas GG genotype was found to be the lowest frequency in our animals. The A allele frequency was found higher than G allele *(Table 2)*.

The 399 bp products were obtained after PCR process and they were digested by *Hin*II restriction enzyme for *FABP4* gene. At the end of digestion, it was observed one band (399 bp) for GG genotype, three bands (399, 302 and 97 bp) for AG genotype, two bands (302 and 97 bp) for AA genotype *(Fig. 1c)*. The GG genotype was found to be the highest frequency; however, the AA genotype was not found. The G allele frequency was higher than A allele *(Table 2)*.

The 215 bp products were obtained after PCR process and PCR products were digested by *Ava*I restriction enzyme for *STAT5A* gene. At the end of digestion, it was observed one band (215 bp) for TT genotype, three bands (215, 181 and 34 bp) for CT genotype, two bands (181 and 34 bp) for CC genotype *(Fig. 1d)*. The CT genotype was found to be the highest frequency, and the TT genotype was not detected. The C allele frequency was higher than T allele *(Table 2)*.

Association was found between *GH* and WW. The calf with *GH*-LL genotype had higher WW than other genotype (P<0.05) *(Table 3)*. *STAT5A* was found associated with RW. For *STAT5A* genotype CC had highest RW compared to other genotype CT (P<0.05) *(Table 3)*. Additionally, prospective associations were identified between *GH*-LL and higher SW

Table 4. *Least squares means and standard errors for body lengths in calves according to GH, MYF5, FABP4 and STAT5A genotypes*

Gene	Genotype	N	Traits			
			Body Length at Birth (cm) Mean (±SE)	Body Length at Weaning Old (cm) Mean (±SE)	Body Length at Milk Replacer Feed Old (cm) Mean (±SE)	Body Length at 180th Day (cm) Mean (±SE)
GH	LL	54	67.39±0.797	74.35±0.68	78.91±0.67	106.74±1.46
	LV	5	69.43±1.903	71.76±1.62	76.56±1.60	99.90±3.48
P			0.306	0.128	0.161	0.063
MYF5	AA	20	68.70±4.13[a]	74.15±4.11	78.35±3.36	106.65±6.72
	AG	32	67.03±2.82[ab]	74.31±2.40	78.59±3.36	106.34±6.37
	GG	7	65.29±3.45[b]	74.86±3.08	79.57±3.36	108.86±6.41
P			0.035	0.744	0.623	0.531
FABP4	AG	25	67.37±0.93	74.11±0.80	78.44±0.79	106.14±1.74
	GG	34	67.89±0.94	74.01±0.81	78.86±0.80	105.77±1.77
P			0.620	0.914	0.641	0.850
STAT5A	CC	28	67.98±0.94	73.97±0.81	78.62±0.80	104.28±1.72
	CT	31	67.25±0.94	74.15±0.82	78.66±0.80	107.66±1.73
P			0.505	0.850	0.966	0.095

Statistical differences among genotypes were shown as: [a] and [b] P<0.05

Table 5. *Least squares means and standard errors for HG in calves according to GH, MYF5, FABP4 and STAT5A genotypes*

Gene	Genotype	N	Traits			
			HG at Birth (cm) Mean (±SE)	HG at Weaning Old (cm) Mean (±SE)	HG at Milk Replacer Feed Old (cm) Mean (±SE)	HG at 180th Day (cm) Mean (±SE)
GH	LL	54	80.56±0.71	87.22±1.04	92.01±1.11	131.81±1.29
	LV	5	77.43±1.70	82.38±2.48	86.53±2.64	125.98±3.07
P			0.081	0.065	0.050	0.072
MYF5	AA	20	81.70±4.40	88.60±4.74	92.30±4.54	131.65±8.280
	AG	32	80.63±2.85	87.00±4.72	90.78±5.01	130.84±5.036
	GG	7	80.14±2.73	88.00±5.57	93.43±6.99	131.86±7.105
P			0.541	0.613	0.483	0.935
FABP4	AG	25	80.74±0.84	86.82±1.24	91.53±1.33	131.16±1.54
	GG	34	79.65±0.85	86.51±1.26	91.24±1.35	131.13±1.55
P			0.253	0.826	0.847	0.988
STAT5A	CC	28	80.12±0.86	85.77±1.24	90.32±1.32	130.39±1.55
	CT	31	80.29±0.86	87.57±1.24	92.47±1.33	131.91±1.55
P			0.863	0.213	0.165	0.396

(P=0.065) and *MYF5*-AA and higher BW (P=0.078) *(Table 3)*. Calves with *MYF5*-AA genotype had highest body length at birth than other genotypes (P<0.05) *(Table 4)*. Additionally, prospective association was identified between *GH*-LL (P=0.063) and *STAT5A*-CT (P=0.095) and longer body length at 180th day *(Table 4)*. Prospective association was identified between *GH* genotypes and hearth girth (HG) at different ages *(Table 5)*. *GH*-LL genotype was found associated with longer HG in all measured ages *(Table 5)*. No association was found between *FABP4* genotypes and investigated traits in this study.

DISCUSSION

To the best of our knowledge there is only few data about association between *GH*, *MYF5*, *FABP4*, and *STAT5A* genes polymorphisms with live weight and morphological measurements in Holstein cattle breed. In the present study genotype frequency of *GH*-LL was found most abundant compared to other genotypes *(Table 2)*. Similar results were observed in other Holstein populations from different countries. Frequency of genotype VV was found low or none in different Holstein populations [30-33].

These findings are consistent with our results, obtained in this study. Among investigated genes in our study, most promising association results were obtained from *GH* gene regarding live weight and body measurement traits in Holstein young bulls *(Table 3, 4* and *5)*. *GH*-LL genotype was associated with higher weaning weight compared to *GH*-LV *(Table 3)*. To the best of our knowledge, no association study was found regarding *GH-Alu*I polymorphism and WW in Holstein cattle breed. However, association between *GH-Alu*I polymorphism and weaning weight investigated in Canchim cattle [34] and Charolais and Zebu hybrid together with Charolais, Nelore and Canchim hybrid [35] and in all investigated genotypes no association was observed between *GH-Alu*I polymorphism and WW. Additionally, *GH-Alu*I polymorphism was found prospectively associated with SW *(Table 3)* and 180th day body length *(Table 4)*. In literature it was stated that *GH-Alu*I genotypes effects the growth and feed intake levels in cattle [36,37]. Additionally, it was shown that GH concentration is affected from *GH-Alu*I genotypes and age in cattle [36]. In the present study *GH-Alu*I genotypes found only associated with WW, SW and body length at 180th day but not with the other traits *(Table 3* and *4)*. We thought that this may be due to effects of environmental factors such as feed intake on *GH* genotypes. According to our literature search no study was found investigating effects of *GH-Alu*I genotypes with heart girth measurement. Heinrichs et al.[38] reported estimation of dairy heifer body weight from heart-girth measurements by using equations or tables. In the present study, heart girth was found prospectively associated with *GH-Alu*I genotypes in all periods *(Table 5)*, therefore it is thought that *GH-Alu*I polymorphisms might be used in selecting cattle with longer heart girth which may cause higher body weights.

In the current study all three genotypes for *MYF5-Taq*I polymorphism were observed in the investigated Holstein population *(Table 2)*. *MYF5-Taq*I AG genotype frequency was found most abundant compared to other two genotypes *(Table 2)*. Similar results were observed in different cattle breeds in Korean [39] and Turkish native cattle breeds [26] that genotype AG was found more abundant than two other genotype. There are not so many researches about association of *MYF5* genotypes with body weight in Holstein cattle. Nasr et al.[40] found an association between body weight and another *MYF5-Taq*I site. However, in the current study we only found association with birth weight in young Holstein bulls. As a member of myogenic regulatory factors, *MYF5* is an important transcription factor for skeletal myogenesis in mammalian embryos [41]. Because of its crucial role in embryonic growth, we thought that *MYF5* variants may affect birth weight and body measurements at birth. Supporting this idea above, in the present study, *MYF5-Taq*I polymorphism was found associated with body length at birth *(Table 4)* and prospectively associated with BW *(Table 3)*. Similarly, an association between *MYF5-Taq*I polymorphism and birth weight was reported in a Canadian commercial beef cattle population developed from various cattle breeds [13]. However, no association found in terms of same polymorphism in Korean and Chinese native cattle populations [39,42]. Our anticipation on inconsistent association results may resulted from breed differences. Because distinct genetic difference between European and Asian cattle breeds has been shown in literature [43].

In our study *MYF5-Taq*I GG genotype calves had lower birth weight and body length compared to other genotypes, however, animals those have low birth weight and body length are compensating these traits and reaching to similar body weight and length of animals with AA and AG genotypes. Probability of perinatal mortality was found higher in calves with heavier birth weight and birth weights above 42 kg shown as at high risk of perinatal mortality [44]. Therefore, selecting animals with GG genotype may lead us to select calves with lower risk of perinatal mortality without any economic loss at the slaughter age.

For *FABP4-Hin*II polymorphism genotype GG was the most abundant and genotype AA was none in the Holstein population *(Table 2)*. Similarly, in the Korean Hanwoo cattle genotype GG was also found as common genotype and frequency of AA was found as lowest (0.07) [18]. And no association was observed between *FABP4-Hin*II polymorphism and investigated phenotypes in Holstein young bulls.

In the present study, no *STAT5A-Ava*I TT genotype was observed in Holstein male calves. Similarly, this genotype was not found also in Polish Black-and-White [23] and Holstein cattle breeds [37]. However, TT genotype was observed in Podolica bulls [28] and Polish native cattle [23]. *STAT5A-Ava*I polymorphism was found associated with RW in our investigated animals *(Table 3)*, CC genotype had shown higher RW compared to CT genotype. In consistent with our results, *STAT5A-Ava*I CC genotype was found favorable for body weight in different ages from different cattle breeds [23,28,37].

Taken together, results in our study provide evidence that interaction between *GH-Alu*I and *MYF5-Taq*I polymorphisms have potential effects for growth and morphological traits which are correlated with economical traits in Holstein cattle. Further studies are ultimately needed to use the SNPs of these two candidate genes in larger populations for genomic selection and investigate other polymorphisms those are linked with *GH-Alu*I and *MYF5-Taq*I SNPs for growth related traits in cattle.

Conflicts of Interest

The authors declare no conflict of interest

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Authors' Contributions

MUC: Made experimental design and wrote the manuscript. JMK: Collected phenotype and samples and performed statistical analysis. KA: Performed laboratory experiments, responsible for chemical and reagents. EGA: Performed laboratory experiments. MK: Collected phenotype and samples. BA: Made experimental design and wrote the manuscript

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Research Article

Molecular Characterization of Infectious Bronchitis Virus Strains Isolated from Vaccinated Flocks in Serbia and Their Comparison with the Isolated Strains from Neighboring Countries

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Abstract

The aim of this study was to isolate, genetically characterize and determine phylogenetic relationship of strains detected in Serbia in comparison to other strains. A total of 480 samples collected from 13 different commercial layer flocks, obtained from tracheal swabs were included. Samples taken from 2016 to 2017 were molecularly analyzed by real-time RT-PCR, multiplex nested RT-PCR, and by sequencing of the S1 gene. Phylogenetic analyses based on partial S1 sequences revealed that six strains were classified as the D274 genotype, two strains as the QX genotype and two strains as the 4/91 genotype. The difference in nucleotide similarity between the Serbian isolates belonging to the D274 group ranges from 0 to 1.2%. Comparison of the obtained strains and D274 (X15832) showed differences from 0 to 0.9%. The greatest nucleotide similarity of detected QX strains was with Chinese QXIBV (KC795604), ranging from 98.8% to 99.1%. Two Serbian strains belonging to the 4/91 genotype had 99.7% and 98.8% nucleotide similarities with vaccine strain 4/91 (KF377577). This study has shown that viruses belonging to D274, QX, and 4/91 genotypes were circulating in poultry flocks in Serbia during 2016 and 2017.

Keywords: *IBV, Genotyping, QX, D274, 4/91*

Sırbistan'da Aşılı Sürülerden İzole Edilen Enfeksiyöz Bronşitis Virus Suşlarının Moleküler Karakterizasyonu ve Komşu Ülkelerden İzole Edilen Suşlar İle Karşılaştırılması

Öz

Bu çalışmanın amacı, Sırbistan'da Enfeksiyöz bronşitis virus suşlarını izole etmek, genetik karakterizasyonlarını yapmak ve diğer suşlar ile olan filogenetik alakasını belirlemektir. Çalışmada, 13 farklı ticari yumurtacı tavuk sürülerinden trakeal swab yoluyla toplanan toplam 480 örnek kullanıldı. 2016 ile 2017 arasında alınan örneklerin gerçek-zamanlı RT-PCR, multiple nested RT-PCR ve S1 gen sekanslaması ile moleküler analizi gerçekleştirildi. Kısmi S1 sekanslama temelli filogenetik analiz altı suşun D274 genotipi, iki suşun QX genotipi ve iki suşun 4/91 genotipi olduğunu gösterdi. D274 grubuna ait Sırp izolatları arasında nükleotid benzerliğindeki farklılık 0 ile %1.2 arasında değişti. Elde edilen suşlar ile D274 (X15832) karşılaştırıldığında 0 ile %0.9 arasında farklılık tespit edildi. Belirlenen QX suşlarının en yüksek nükleotid benzerliği %98.8 ile %99.1 arasında olmak üzere Çin QXIBV (KC795604) ileydi. 4/91 gentopine ait iki Sırp suşu ile aşı suşu olan 4/91 (KF377577) arasında %99.7 ile %98.8 nükleotid benzerliği gözlemlendi. Bu çalışma D274, QX ve 4/91 genotiplerine ait virusların 2016 ile 2017 yılları arasında Sırbistan'daki kanatlı kümeslerinde bulunduğunu göstermiştir.

Anahtar sözcükler: *IBV, Genotiplendirme, QX, D274, 4/91*

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INTRODUCTION

Infectious bronchitis (IB) is highly contagious viral disease of poultry affecting respiratory tracts, but the disease is also associated with the nephritis, poor weight gain, and reproductive signs as a decline in egg production and quality. The disease is caused by infectious bronchitis virus (IBV), of the family *Coronaviridae*. Its genome consists of positive sense single-stranded RNA, containing genes coding for four structural proteins: spike (S), membrane (M), nucleocapsid (N), and small envelope (E) proteins [1]. S protein is responsible for virus attachment and fusion of the virus with the host cell and it is cleaved into S1 and S2 subunits. S1 is highly variable, including three hypervariable regions (HVRs) [2] and induces neutralizing antibody production in the host [3]. The molecular identification of IBV is based mainly on the analysis of the S1 protein gene [4].

The disease can be managed through an adequate implementation of biosecurity measures and vaccination. Control of vaccination is complicated due to small changes in the amino acid sequences of the S protein that can result in the generation of new antigenic types [5]. For that reason and despite the use of live and inactivated vaccines, there is a continuous emergence of variants responsible for worldwide outbreaks of IB and economic losses of poultry production. It is, therefore, necessary to constantly monitor the field situation and identify circulating IBV genotypes to adequately adjust vaccination program, which will protect poultry flocks. Many different genotypes of IBV have been identified in the world. In Europe, the predominant ones are 793B (4/91), Massachusetts, Italy02, and QX [6].

In Serbia, IB is endemic and is controlled by the use of mainly Massachusetts strains, 4/91 and D274 vaccines. To date, there is no information available on the circulation of variant IBVs in Serbia, which makes this investigation especially important. Therefore, the objective of the present study was to isolate, genetically characterize and determine phylogenetic relationship of strains detected in Serbia in comparison to other strains reported in Europe and around the world.

MATERIAL and METHODS

Sampling

A total of 480 samples were included in this study. Samples were collected from 13 different commercial layer flocks in Central Serbia (Zlatibor, Morava, Raška and Rasina regions) from June 2016 to February 2017. All samples were obtained from tracheal swabs of layer flocks without clinical signs of IB, with the history of sporadic outbreaks previously. The age of the flocks and the vaccinating programs are shown in *Table 1*. Testing was carried out at the Veterinary Specialist Institute, Department for Laboratory Diagnostic in Kraljevo, Serbia. Samples were placed in sterile phosphate-buffered saline (Sigma-Aldrich, Schnelldorf, Germany).

RNA Extraction and Real-time RT-PCR

RNA was extracted directly from tracheal swabs by a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracted RNA was stored at -80°C until the use in the real-time RT-PCR reaction.

For the detection of IBV-specific nucleic acid, a TaqMan-probe based group-specific real-time PCR assay was used as previously described [7]. Forward primer IBV5_GU391 (5'-GCT TTT GAG CCT AGC GTT-3') located at nucleotide positions 391 to 408 of the IBV M41 strain genome, reverse primer IBV5_GL533 (5'-GCC ATG TTG TCA CTG TCT ATT G-3') located at nucleotide positions 533 to 512 of the IBV M41 strain genome, and TaqMan dual-labelled probe IBV5_G (5'-CAC CAG AAC CTG TCA CCT C - 3') located at nucleotide positions 494 to 473 of the IBV M41 strain genome were used to amplify and detect a 143-base-pair fragment of the 5'-untranslated region (UTR). PCR amplification was performed on Stratagene Mx3000P (Stratagene, USA), using SuperScript III Platinum Quantitative One-Step RT-PCR kit (Thermo Fisher Scientific, USA) under following conditions: 50°C for 30 min; 95°C for 2 min; 45 cycles of 95°C for 15 s followed by 60°C for 60 s. A total volume of 25 µL reaction mixture containing 9 µL nuclease-free water, 12.5 µL reaction Mix, 0.2 µL of each primer, 0.5 µL of Taq Mix enzymes and 0.125 µL of TaqMan probe for each tube. The limit of detection for this assay was 100 genome copies per reaction.

Multiplex Nested RT-PCR

Nested PCR were performed according to Worthington et al.[6]. The initial PCR used primers SX1+ (5'-CACCTAG AGGTTTG T/C T A/T GCAT-3') and SX2- (5'-TCCACCT CTATAAACACC C/T TT-3'). The amplicon was further amplified in a second internal PCR that used primers SX3+ (5'-TAATACTGG C/T AATTTTTCAGA-3'), SX4- (5'-AATAC AGATTGCTTACAACCACC-3'). In first round SuperScript III Platinum Quantitative One-Step RT-PCR kit (Thermo Fisher Scientific, USA) was used under following conditions: 50°C for 30 min; 95°C for 2 min; 30 cycles of 95°C for 15 s followed by 48°C for 1,5 min and 72°C for 2 min. Second round was performed using DreamTaq Hot Start Green PCR Master Mix (Thermo Fisher Scientific, USA): 95°C for 10 min; 30 cycles of 95°C for 15 s followed by 48°C for 1,5 min and 72°C for 2 min.

The amplified products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide. The gel purification process was carried out using the GeneJet Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The purified DNA was stored at -20° C until sequencing.

Sequencing

The sequencing of the obtained purified PCR product was carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Woolston, UK), according to the manufacturer's instructions. Sequences were analyzed with 3130 Genetic Analyzer (Applied Biosystems, Woolston, UK). The obtained results were processed using the SeqScape program (Applied Biosystems, Woolston, UK) and corrected in Chromas Lite program (Technelysium Pty, Ltd, Brisbane, Australia).

Phylogenetic Analysis

MEGA 7.0 software [8] was used for phylogenetic analysis and genotyping. Pairwise and multiple sequence alignment was done by Clustal O, a part of Unipro UGENE - a unified bioinformatics toolkit [9]. The evolutionary history was inferred by using the maximum likelihood method based on the general time reversible model [10].

Isolated IBV sequences were pairwise compared with the S1 gene as follows: Genogroup GI-1- GI-11 (Beaudette (M95169), Holte (GU393336), Gray (L14069), Holte (L18988), N1/62 (U29522), VicS (U29519), TP/64 (AY606320), L165 (JQ964061), ARK99 (M99482), B (AF151954), UFMG/G (JX182775)); GI-12 (D3896 (X52084), D274 (X15832); GI-13 - GI-27 (Moroccan-G/83, (EU914938), B1648 (X87238), B4 (FJ807932), IZO 28/86 (KJ941019), CA/Machado/88 (AF419315), JP8127 (AY296744), 58HeN-93II (KC577395), Qu_mv (AF349621), Spain/97/314 (DQ064806), 40GDGZ-97I (KC577382), Variant 2 (AF093796), V13 (KF757447), CA/1737/04 (EU925393), NGA/B401/2006 (FN182243), GA08 (GU301925)); Genogroup GII-1 (D1466 (M21971)); GIII-1 (N1/88 (U29450)); GIV-1 (DE/072/92 (U77298)); GV-1 (N4/02 (DQ059618)) and GVI-1 (TC07-2 (GQ26594)) [11], QXIBV (KC795604), Ark52930 (FJ899688), H120 (M21970), It/497/02 (DQ901377), L-1148 (DQ431199), D207 (M21969 J04329), and vaccine strains (H120 (FJ888351), M41 (DQ834384), MA5 (KU736747), and 4/91 (KF377355)).

GenBank Accession Number

Gene bank accession numbers of the Serbian S1 sequences used in this investigation are presented in *Table 1*.

RESULTS

The detection and quantifications limits were determined using cycle threshold (CT) values obtained for each reaction containing from 10^7 to 10^2 copies of the standard RNA. The assay was negative below 100 template copies. Therefore, the limit of detection and quantification were both determined to be 100 template copies. Results were analysed in terms of CT values. A CT value below 38 cycles was regarded as a positive result, and a negative result was represented by a CT value ≥38.

Among 480 examined samples from tracheal swabs of layer flocks, IBV was detected in 10 cases. Based on their partial S1 gene sequences, IBVs identified in Serbia during this study can be divided into three groups. The first group represents D274 genotype within isolates MH010330, MH010331, MH010332, MH010334, MH010335 and MH010336. The second genotype is 4/91 where isolates MH010333 and MH010339 are positioned. The third genotype belongs to QX with isolates MH010337 and MH010338 *(Fig. 1)*. Isolates MH010330, MH010331 showed 100% mutual nucleotide similarity, the same as isolates MH010332 and MH010336. Isolate MH010335 showed 99.7% similarity to MH010330, MH010331, and MH010334, and 98.8% to MH010332 and MH010336, respectively. Strain MH010334 showed 99.1% nucleotide similarity to MH010332 and MH010336. Strains MH010330 and MH010331 were also closely related (99.4% similarity) to the MH010332 and MH010336 isolates. The differences in nucleotide similarities between the Serbian D274 strains in this study varied from 0 to 0.9% comparing with D274 (X15832) and 2.4 to 2.7% comparing with D3896 (X52084) isolated in Netherland in 1978 *(Fig. 1)*. Our isolates, belonging to the QX genotype, showed 99.7%

Table 1. *Serbian IBV strains used in the study with the epidemiologic data, vaccination program and accession numbers*

Strain	Type of Chicken	Age (weeks)	Vaccination Program	Accession No.
1	Layer	25	1 d/MA5, 35d/4/91	MH010330
2	Layer	25	1 d/MA5, 35d/4/91	MH010331
3	Layer	26	1 d/MA5, 35d/4/91	MH010332
4	Layer	26	1 d/MA5, 35d/4/91	MH010333
5	Layer	26	1 d/MA5, 35d/4/91	MH010334
6	Layer	26	1 d/MA5, 35d/4/91	MH010335
7	Layer	26	1 d/MA5, 35d/4/91	MH010336
8	Layer	29	1 d/MA5, 10 d 4/91, 31d/MA5	MH010337
9	Layer	29	1 d/MA5, 10 d 4/91, 31d/MA5	MH010338
10	Layer	21	1 d/MA5, 10 d MA5, 56d/4/91	MH010339

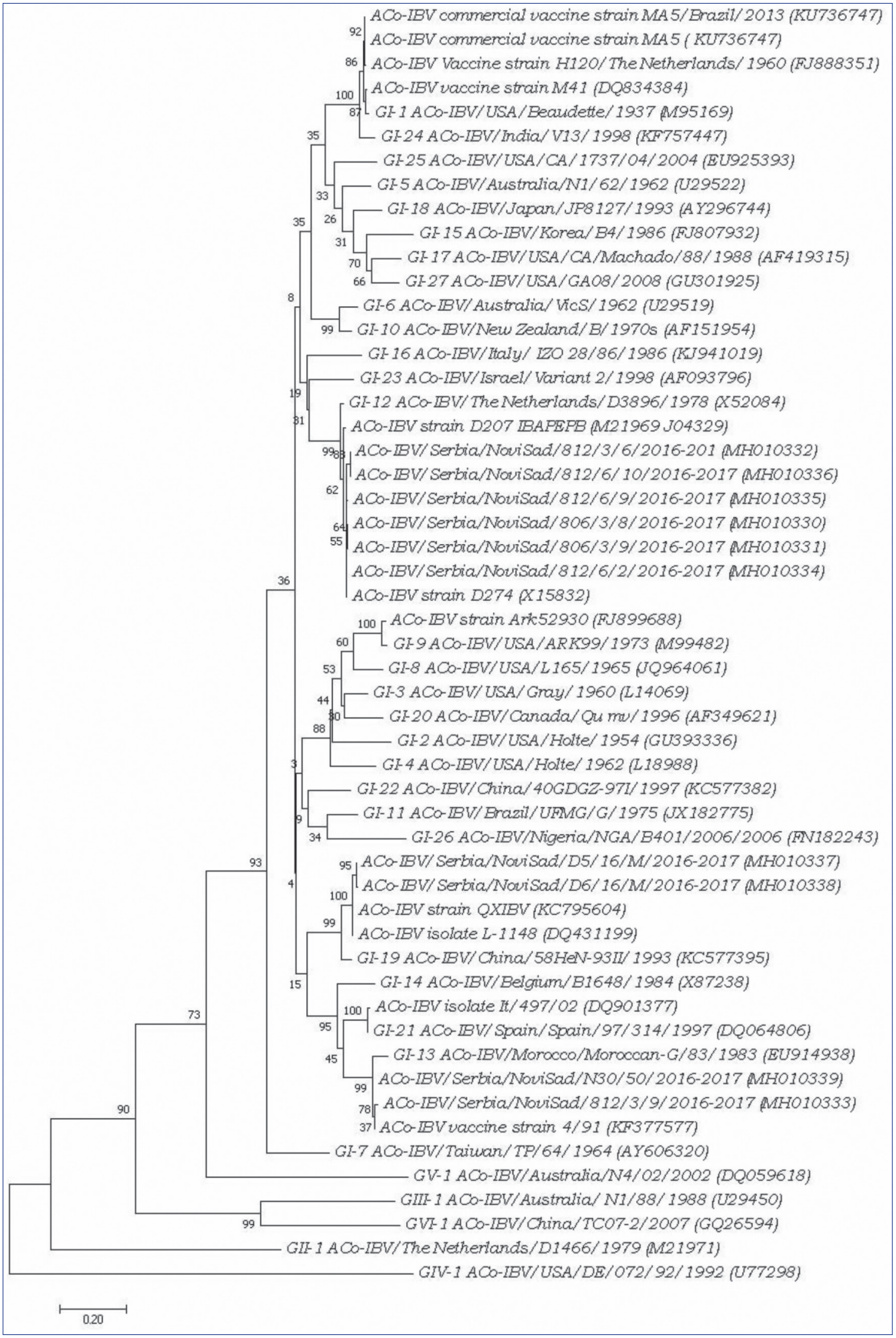

Fig 1. Maximum likelihood tree (1000 bootstop replicates) generated by neighbor-join method, representing the IBV isolates detected in Serbia and selected IBV reference strains

mutual nucleotide similarity. The difference in nucleotide similarity between the isolates belonging to the D274 group ranged from 0 to 1.2%. Serbian strains MH010337 and MH010338, closely related to QXIBV (KC795604) and L-1148 (DQ431199), varied from 0.9 to 1.2%, and QX (KC577395) varied from 5.8 to 6.1% *(Fig. 1)*. Serbian strains MH010333 and MH010339, belonging to 4/91 genotype, showed 98.5% mutual nucleotide similarity, and isolate MH010339 was 99.7% similar to 4/91 vaccine strain (KF377577), while isolate MH010333 was 98.8% similar to the same 4/91 strain *(Fig. 1)*.

DISCUSSION

The dominant genotype detected within this study was D274, where it was detected six out of 10 strains. The difference in nucleotide similarity between the isolates belonging to the D274 group ranged from 0 to 1.2%. Comparing the obtained Serbian strains and D274 (X15832) it can be seen that strain MH010334 showed 100% similarity with D274 (X15832); strains MH010330, MH010331, and MH010335 had 99.7% similar nucleotides and strains MH010332 and MH010336 showed 99.1% nucleotide similarity. This is probably not surprising given that D274 strains are widely used for vaccination. The disadvantage of live vaccines is that they spread easily in the field [12,13], which enables the survival and circulation of the vaccine virus in flocks. This is also supported by the fact that there were no clinical symptoms of the disease in the flocks from which the virus was isolated, but the virus circulated. Based on the data presented in *Table 1*, it can be seen that the flocks from which the virus was isolated were not vaccinated with the D274 vaccine. This vaccine is largely used in Serbia and, together with MA5 and 4/91, recommended for controlling IB in laying flocks. In the past, the D274 vaccine was used on the farms where Serbian strains originated from, and also on farms close to the farms from which Serbian strains belonging to D274 genotype were isolated. Also, strain D3896 (X52084) isolated in the Netherlands in 1978 had a high similarity with our strains ranging from 97.3-97.6% and strain D207 (M211969 J04329) ranging from 97.6% to 98.5%. The most common IBV genotype in some Western European countries in the early and middle 1980s was D274-like, and that was the reason for initiating vaccination [14,15].

Two QX strains (MH010337 and MH010338) were detected in this study. The greatest nucleotide similarity of detected strains was with QXIBV (KC795604) and L-1148(DQ431199), and there was 99.1% similarity for isolate MH010337 and 98.8% for isolate MH010338. Comparing our strains with QX (KC577395) strain MH010337 showed 94.2% similarity and strain MH010338 93.9%, respectively. The QX strain was initially isolated in China [16] during 1990s. This strain at first did not circulate outside Asia, but later was detected in broilers in Italy in 2011 [17]. Also, Chinese QX type was detected in Poland [18] and Hungary [19]. In Europe, beside Chinese strains, QX-like strains characterized as European QX was detected in the United Kingdom [20-22], Finland [23], Russia [24], Slovenia [25], Spain [26], and Sweden [27]. This is the first confirmation of QX in Serbia, so it is hard to say where it comes from, but most likely from neighboring countries. QX viruses have been isolated from a flock where vaccination with MA5 and 4/91 was performed. Since the virus has been isolated from a flock without clinical signs, this indicates that although the virus was present, due to adequate vaccination there were no clinical manifestations. Terregino et al.[28] showed that MA5 and 4/91 vaccines administered at 1 day and 14 day protect chickens from infections and QX-like IB disease, which this vaccinating protocol makes useful in reducing economic losses caused by QX strains.

Since it was first described in the early nineties in the UK [29], 4/91 IBV genotype spread over many other countries and became one of the most predominant in Europe [12,29-32]. The present study showed that 4/91 is also present in Serbia and demonstrated its circulation in our country. It is hard to say how long it has been present since this is the first study in Serbia dealing with the IBV genotyping. Vaccine 4/91 was used in both flocks from which the virus was isolated *(Table 1)*. Serbian strain MH010339 showed 99.7% and strain MH010333 98.8% similarity to vaccine strain 4/91 (KF377577). The differentiation of the vaccine strain from a wild strain is difficult and possible by nucleotide sequencing of the SI gene, where the percentage of nucleotide similarity is important. Worthington et al.[6] have categorized vaccine-related IBVs that have less than 99% part-S1 similarity as field IBV viruses. In our study, where isolate MH010339 showed above 99% similarity to vaccine strain, it was a vaccine virus. Strain MH010333 differed by 1.2% indicating that it can also be a field strain, especially considering the fact that such viruses may have emerged through antigenic drift over time, potentially encouraged by continuous use of homologous vaccine strains, resulting in vaccine pressure [33]. There was also a high similarity of our isolates to Moroccan-G (EU914938), which is 95.9% for isolate MH010333 and 96.2% for isolate MH010339.

In conclusion, this study has shown that viruses from D274, QX, and 4/91 genotypes were circulating in poultry flocks in Serbia during 2016 and 2017, and that this is the first genotyping of IBV in Serbia. Considering the fact that isolated strains originated from a flock without clinical symptoms, it can be said that the vaccination applied in Serbia, using MA5, 4/91, and D274 vaccines, represents a good protection for the present, because it protects flocks from IB and therefore prevents economic losses. Certainly, in the future, the situation with new genotypes in Serbia should be monitored and studies should be carried out involving a large number of farms and a wider area.

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Research Article

Comparison of Oxidative/Nitrosative Stress, Leptin and Progesterone Concentrations in Pregnant and Non-pregnant Abaza Goats Synchronized with Controlled Internal Drug Release Application

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Abstract

The aim of this study was to determine the oxidative/nitrosative stress, leptin and progesterone concentrations in pregnant and non-pregnant Abaza goats after synchronization with controlled internal drug release (CIDR) during the breeding season. For this purpose, 40 clinically healthy Abaza goats, aged 2-4 years, were intravaginally exposed to CIDR on day 0, and injected with equine chorionic gonadotropin and prostaglandin $F_{2\alpha}$ on day 9 of the experiment. CIDR was removed on day 11. At the end of the experiment, they were monitored for estrus and exposed to fertile males for mating. Blood samples were collected 8 days before synchronization, then on days 0, 11 of CIDR insertion and on mating day. A pregnancy diagnosis was conducted using transrectal ultrasonography 30 day after mating. The blood serum from 40 goats (30 pregnant + 10 non-pregnant) was used for biochemical analyses. Malondialdehyde (MDA), nitric oxide (NO), total oxidant capacity (TOC), total antioxidant capacity (TAC) and endothelial NO synthase (eNOS) activities were significantly higher on mating day in pregnant goats compared to non-pregnant goats ($P<0.05$). The eNOS activity and TOC concentrations were significantly higher on day 11 in pregnant goats compared to non-pregnant goats ($P<0.001$). Serum P4 concentration increased in pregnant group on day 11 and decreased all groups on mating day than day -8, day 0 and day 11 ($P<0.001$). In conclusion, the administration of CIDR to Abaza goats exacerbated oxidative and nitrosative stress.

Keywords: *Abaza goat, Synchronization, eNOS activities, Leptin, Nitric oxide, Progesterone*

Controlled Internal Drug Release Uygulaması İle Senkronizasyon Yapılan Gebe ve Gebe Kalmayan Abaza Keçilerinde Oksidatif/Nitrozatif Stres, Leptin ve Progesteron Konsantrasyonlarının Karşılaştırılması

Öz

Bu çalışmanın amacı üreme sezonunda controlled internal drug release (CIDR) ile senkronizyon sonrasında gebe ve gebe olmayan Abaza keçilerinde oksidatif/nitrosatif stres, leptin ve progesteron konsantrasyonlarının belirlenmesidir. Bu amaçla, klinik olarak sağlıklı 2-4 yaşlı 40 Abaza keçisine 0. gün CIDR intravaginal olarak yerleştirildi ve 9. gün equine chorionic gonadotropin ile prostaglandin $F_{2\alpha}$ enjekte edildi. CIDR 11. gün çıkarıldı. Uygulama sonrasında östrus takibi yapılarak keçiler fertil tekeler ile çiftleştirildi. Senkronizasyona başlamadan 8 gün önce, 0. gün, 11. gün ve çiftleşme günü kan alındı. Çiftleşmeden sonraki 30. gün transrektal ultrasonografi ile gebelik muayenesi yapıldı. Biyokimyasal ölçümler için 40 keçiden (30 gebe + 10 gebe olmayan) elde edilen kan serumu kullanıldı. Gebe olan keçilerle gebe olmayan keçiler karşılaştırıldığında malondialdehid (MDA), nitrik oksit (NO), total oksidan kapasite (TOC), total antioksidan kapasite (TAC) ve endotelyal NO sentaz (eNOS) aktiviteleri anlamlı olarak yüksek bulundu ($P<0.05$). eNOS aktivitesi ve TOC konsantrasyonları gebe olan keçilerde gebe olmayan keçilere göre 11. günde anlamlı olarak daha yüksekti ($P<0.001$). Serum P4 konsantrasyonu gebe olan grupta 11. günde arttı ve tüm gruplarda çiftleşme günü -8, 0. ve 11. günlere göre azaldı ($P<0.001$). Sonuç olarak, CIDR uygulaması Abaza keçilerinde oksidatif ve nitrozatif stresi arttırdığı tespit edildi.

Anahtar sözcükler: *Abaza keçisi, Senkronizasyon, eNOS aktiviteleri, Leptin, Nitrik oksit, Progesteron*

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INTRODUCTION

Vaginal inserts that contain progesterone (P4) [sponge or controlled internal drug release (CIDR)] can be used for estrus synchronization in ruminants [1-4]. These inserts are left in the vagina and can cause tissue damage and inflammation [5]. As a result, such applications can create stress for the animal [6]. A complex relationship has been reported between inflammation and reactive oxygen species (ROS) [7]. Intravaginal inserts in particular are reported to cause oxidative stress in goats [8]. However, there are few studies about the relationship between oxidative stress and CIDR applications [4,8].

Reactive oxygen species (ROS) are eliminated by mechanisms that are known as antioxidants in the organism. Malondialdehyde (MDA), the final product of lipid peroxidation and the most important indicator, is the most important molecule effective in cellular degeneration caused by free radicals [7]. Nitric oxide (NO) plays a role in several physiological events in the body and is produced by nitric oxide synthetase [9]. Endothelium-derived NO is synthesized by eNOS and is an important indicator of basal vascular tonus. Besides protecting vascular integrity and preventing leukocytes from attaching to endothelial cells and the proliferation of smooth muscle cells, endothelium-derived NO also acts to inhibit thrombocyte adhesion and aggregation [10]. A study conducted on dairy heifers reported that NO and MDA levels increased after application of intravaginal inserts and total antioxidant capacity (TAC) was decreased [6].

Leptin plays an important role in reproductive functions and nutritional condition [11,12]. According to Sarraf et al.[13], leptin concentrations and proinflammatory cytokines, such as acute tumor necrosis factor-α and interleukin-1 increased when inflammation is caused by administering substances such as endotoxin or turpentine. However, there is no information about serum leptin concentrations during estrus synchronization using intravaginal devices, especially in goats.

This study aims to determine oxidative and nitrosative stress in pregnant and non-pregnant Abaza goats following estrus synchronization using CIDR during the breeding season and the relationship between oxidative status, progesterone and leptin concentration that may occur after CIDR application.

MATERIAL and METHODS

This study was conducted with the approval of the Ethics Committee of Animal Experiments of Kafkas University, Kars, Turkey (KAÜ-HADYEK - 2016/020).

Location

This study was conducted in Kars province, Turkey. The research unit is located at 1751 m altitude and 40°34'23"N and 43°02'27"E latitude and longitude, respectively.

Animals and Ration (Diet)

Forty non-lactating Abaza goats aged 2-4 years and weighing 50-60 kg were selected. The animals were fed twice a day with dry clover, dry hay and concentrated feed (12% crude protein, 2600 kcal/kg). Goats were given *ad libitum* access to water.

Estrus Synchronization Protocol

This study began in September (breeding season). The progesterone-releasing device (CIDR, Eazi-Breed CIDR®, Zoetis, Turkey) was inserted into the vagina (day 0) and left there for 11 days. On day 9, all of the goats were injected with 400 IU equine chorionic gonadotropin (i.m., eCG, Chronogest®, MSD-Intervet, Turkey) and prostaglandin $F_{2\alpha}$ (i.m., 5 mg, dinoprost tromethamine, Dinolytic®, Zoetis, Turkey). The CIDR was removed on day 11, and estrus detection began 12 h later. A buck joined to goat herd every 6 h to test for estrus, and those in estrus were exposed to fertile Abaza bucks for mating. Transrectal ultrasonography with 5-7.5 MHz linear transducer (SonoSite Titan®, SonoSite, USA) was used for pregnancy diagnosis 30 days after mating [14].

Blood Sampling

Blood samples were collected 8 days before the beginning of estrus synchronization protocol (day -8), day 0, day 11 and mating day. The blood was collected from the *vena jugularis* and centrifuged for 15 min (3000 rpm). Serum samples were stored at -20°C until assays were performed.

Biochemical Analysis

Serum MDA concentration was determined using the method described by Yoshioka et al.[15] based on the reaction between MDA and thiobarbituric acid. The optical density was read at 535 nm (Epoch®, Biotek, USA). MDA concentration (µmol/L) was calculated from the standard curve obtained using 1,1,3,3- tetraethoxypropane (Sigma).

Nitric oxide measurement was performed according to the method described by Miranda et al.[16], where nitrate is reduced to nitrite by vanadium chloride ($VaCl_3$), and then in an acidic environment nitrite exposed to sulphanilamide to produce colored diazonium compound, which was read at 540 nm. Nitrite and nitrate concentrations calculated from the standard curve obtained using sodium nitrite ($NaNO_2$, Sigma) and sodium nitrate ($NaNO_3$, Sigma), respectively. After nitrate and nitrite concentrations were determined separately, the sum of nitrate and nitrite concentrations shows the amount of NO (µmol/L).

Total antioxidant capacity (TAC) was measured by commercial kits (TAC Assay Kit®, Rel Assay Diagnostic, Turkey). Antioxidants in the sample reduce dark blue-green colored

ABTS [2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] radicals to the colorless reduced ABTS form. The difference in absorbance is related to the total antioxidant concentration in the sample at 660 nm [17]. The reaction rate is calibrated with Trolox, which is widely used as a traditional standard for TAC measurement assays. The results were given in liters per millimolar equivalent of Trolox (mmol Trolox Eq/L).

Total oxidant capacity (TOC) was measured by commercial kits (TOC Assay Kit®, Rel Assay Diagnostic, Turkey). The ferrous ion-o-dianisidine complex is oxidized to the ferric ion by the oxidants present in the sample. It forms a colored complex with xylenol orange. The optical density of the color is related to the total oxidant molecules in the sample at 530 nm [18]. The measurement was calibrated with hydrogen peroxide (H_2O_2), and results were given in liters per micromolar equivalent of H_2O_2 (μmol H_2O_2 Eq/L).

Endothelial NO synthase (eNOS) activity was measured by a commercial goat ELISA kit (Goat eNOS ELISA Kit®, MyBioSource, USA). The assay sensitivity was 1.0 pg/mL. The intensity of color was measured in a microplate reader at 450 nm. A standard curve is plotted relating the optical density of the color to the concentration of standards. The eNOS activity was calculated from this standard curve in each sample.

Leptin assay was made by an ELISA kit purchased from Cusabio (Goat Leptin, LEP ELISA Kit®, Cusabio Biotech, China). The assay procedure was performed as described in the kit instruction manual. The detect range and min detection limit (sensitivity) of the kit were 0.625-40 ng/mL and 0.156 ng/mL, respectively. Determine the optical density of each well within 5 min, using a microplate reader set to 450 nm. The amount of leptin detected in each sample was compared to a leptin standard curve.

A commercial ELISA kit was used (DRG Progesterone ELISA Kit®, DRG Instruments GmbH, Germany) for the quantitative determination of P4. The range of the assay and sensitivity were 0-40 ng/mL and 0.045 ng/mL, respectively. Serum progesterone concentrations were determined according to the manufacturer's instructions. The optical density was determined within 10 min with a spectrophotometer at 450 nm. A standard curve was computed to determine the quantity of progesterone in each sample.

Statistical Analysis

Statistical analysis of the data was performed using the SPSS® 18.0 software (Chicago, IL, USA) program. Groups were compared with nonparametric tests because of the abnormal distribution (Shapiro-Wilk test) of the data. Statistical differences between pregnant and non-pregnant goats were evaluated using the Mann Whitney-U test. The non-parametric Friedman test and Wilcoxon test were also used for time periods (between days). Correlations between variables were identified with the Spearman correlation test.

RESULTS

When the CIDR was removed vaginitis was detected with speculum examination. Mucopurulent-purulent discharge was also seen on the CIDR when it was removed from the vagina. A pregnancy rate of 75% (30/40) was recorded through transrectal ultrasonography 30 days after mating.

Serum MDA, NO, TOC concentrations and eNOS activity were higher ($P<0.001$) in pregnant and non-pregnant goats on day 11 and mating day than day -8 and day 0. Leptin concentrations were higher ($P<0.01$) in the pregnant group on day 11 and mating day than day -8 and day 0. TAC concentrations were lower on day 11 when compare to -8, 0 and mating day in the pregnant group ($P<0.001$). Serum P4 concentration increased with the insertion of the CIDR in pregnant group on day 11 and decreased all groups on mating day than day -8, day 0 and day 11 ($P<0.001$, *Table 1*).

Statistically significantly higher in eNOS activity and TOC concentrations on day 11 in pregnant goats compared to non-pregnant goats ($P<0.001$, *Table 1*). MDA, NO, TOC, TAC and eNOS activity were statistically higher on mating day in pregnant goats compared to non-pregnant goats ($P<0.05$, *Table 1*).

A positive correlation was found between MDA and TOC ($r = 0.378$, $P<0.05$), MDA and leptin ($r = 0.384$, $P<0.05$) on day 11. There was a negative correlation between TAC and TOC ($r = -0.347$, $P<0.05$) on day 11. There was a strong positive correlation between eNOS activity and TOC ($r = 0.714$, $P<0.01$) and a strong negative correlation between leptin and TAC ($r = -0.554$, $P<0.01$).

DISCUSSION

Oxidative stress is a serious problem which is being studied extensively in human and animals, especially in cattle, sheep and goats in conditions of sepsis, mastitis, metritis, retentio secundinarum and genital tract inflammation [6,19]. There are few studies about fertility and the oxidative stress created by P4 sources used intravaginally for estrus synchronization in small ruminants [4,8]. Intravaginal sponge applications are known to be a common cause infection [20]. Results of the present study showed that the use of CIDR caused significantly increases in MDA, NO, TOC concentrations and eNOS activity on day 11. After the CIDR was removed, these concentrations declined but did not reach pre-treatment values. These increases may be the result of stress due to local irritation and inflammation caused by the CIDR in the vagina. Some studies have reported that administering P4 or estrogen can cause an increase in eNOS activity [21]. The present study also showed that eNOS activity increased with CIDR application on day

Table 1. *Changes in MDA, NO, TOC, TAC, P4 and leptin concentrations and eNOS activity on days -8, 0, 11 and mating day in pregnant and non-pregnant goats synchronized with CIDR (Mean ± SE)*

Parameters	N	Day -8	Day 0	Day 11	Mating Day	P Value	Pregnancy Status
MDA µmol/L	10	13.93±1.22^{a}	13.50±1.25^{a}	24.41±1.15^{b}	21.98±0.92^{c}	<0.001	Not pregnant
	30	14.21±1.79^{a}	14.14±2.59^{a}	22.79±4.87^{b}	22.31±4.19^{b}	<0.001	Pregnant
	P value	0.659	0.614	0.344	0.013	-	
NO µmol/L	10	12.32±2.47^{a}	11.61±1.37^{a}	22.40±1.24^{b}	18.99±0.45^{c}	<0.001	Not pregnant
	30	12.24±2.11^{a}	12.73±2.34^{a}	22.29±3.64^{b}	20.95±3.89^{b}	<0.001	Pregnant
	P value	0.975	0.28	0.571	0.005		
eNOS U/L	10	5.04±0.49^{a}	5.08±0.23^{a}	6.58±0.38^{b}	6.53±0.30^{b}	<0.001	Not pregnant
	30	5.13±0.33^{a}	5.39±1.48^{a}	8.46±0.71^{b}	7.58±0.48^{c}	<0.001	Pregnant
	P value	0.85	0.181	<0.001	<0.001	-	
TOC µmol H_2O_2 Eq/L	10	13.51±3.09^{a}	13.08±1.79^{a}	17.55±1.21^{b}	17.20±0.24^{b}	<0.001	Not pregnant
	30	13.53±1.58^{a}	13.68±2.59^{a}	26.69±0.48^{b}	23.54±0.78^{c}	<0.001	Pregnant
	P value	0.91	0.166	<0.001	<0.001	-	
TAC mmol Trolox Eq/L	10	1.67±0.13	1.57±0.10	1.63±0.10	1.62±0.08	0.429	Not pregnant
	30	1.64±0.12^{a}	1.61±0.08^{a}	1.58±0.10^{b}	1.73±0.11^{c}	<0.001	Pregnant
	P value	0.571	0.18	0.176	0.01	-	
P4 ng/mL	10	2.16±1.20^{a}	3.65±1.62^{b}	4.73±1.25^{b}	0.15±0.07^{c}	<0.001	Not pregnant
	30	2.49±1.26^{a}	3.10±0.58^{a}	5.35±1.27^{b}	0.13±0.06^{c}	<0.001	Pregnant
	P value	0.571	0.567	0.181	0.850		
Leptin ng/mL	10	0.71±0.24	0.75±0.11	0.84±0.28	1.09±0.38	0.237	Not pregnant
	30	0.82±0.19^{a}	0.73±0.11^{b}	1.00±0.24^{c}	1.02±0.95^{c}	<0.001	Pregnant
	P value	0.101	0.339	0.08	0.570	-	

^{a,b,c} The difference between values with different letters in the same row is significant (P<0.05). MDA: Malondialdehyde, NO: Nitric oxide, eNOS: Endothelial NO synthase activities, TAC: Total antioxidant capacity, TOC: Total oxidant capacity, P4: Progesterone, SE: Standard error

11 and mating day than day -8 and day 0 in pregnant goats *(Table 1)*. It is also possible that the physiological increased in the serum estrogen level on estrus day (mating day) affected the increase in eNOS activity.

Oxidative stress can be assessed in terms of certain biological markers. Of the antioxidant parameters, TAC measurement alone can be used to determine the dynamic balance between plasma oxidants and antioxidants [22]. Oral et al.[6] found that TAC declined after the use of an intravaginal progesterone device in heifers, while TOC values remained unchanged. In the present study, however, a decline was assessed in TAC in pregnant goats on day 11, but TOC increased in parallel with MDA, NO and eNOS activity on day 11 and mating day in all groups. It is thought that the rise in TOC occurred in response to the inflammation and stress caused by the application of CIDR.

Hormonal applications are frequently used in sheep and goats to induce and/or synchronize estrus in order to perform artificial insemination easily and to mitigate seasonal effects [1,4]. Plasma P4 concentrations are reported to be above 1 ng/mL 2 h, 4 h, 4 days and 13 days after using CIDR or P4 sponges in sheep and goats [23]. P4 concentrations decreased below 1 ng/mL after CIDR was removed and $PGF_{2\alpha}$ injection was given [24]. In the present study, P4 concentrations were increased when the CIDR was inserted in pregnant group on day 11 compared to day -8 and day 0. Serum P4 concentration decreased all groups on mating day than day -8, day 0 and day 11. On estrus day, however, it was found to be 0.13±0.06 ng/mL in pregnant group *(Table 1)*. P4 concentrations were above 1 ng/mL on application days (except for estrus day) because the study was conducted during breeding season.

Leptin concentrations are elevated in cases of inflammation/immune pathology and leptin can play a role, especially in intestinal inflammation. However, the exact nature of its role is not understood in such cases [25]. The innate immune system plays an important role in regulating leptin production. Leptin concentrations increased sharply when proinflammatories such as tumor necrosis factor-α and interleukin-1 are administered or with inflammation stimulants such as lipopolysaccharides and turpentine [26]. Rises in plasma estrogen concentrations in heifers at puberty also increase leptin concentrations [27]. Estrogen is thought to modulate the expression of leptin and its receptor in rodents as well [28]. Leptin has also been reported to play a role in gonadotropin-releasing hormone (GnRH) synthesis and therefore to raise estrogen

concentrations [29,30]. However, our study showed that leptin concentrations increased with the removal of CIDR (day 11) and mating day in pregnant goats, which was when oxidative stress indicators higher. This could be correlated with the oxidative status markers of leptin, which is associated with several inflammation markers. The correlations between MDA and leptin or TAC and leptin support this theory. Still, leptin concentrations tended to rise on day 11 and mating day when the estrogen concentrations increased. This may be because leptin stimulates GnRH synthesis and therefore increases estrogen synthesis.

It is reported that oxidative stress markers like NO and eNOS may play a role in luteinizing hormone peak or human chorionic gonadotropin synthesis. These mediators may also play a role in the process of oocyte maturation and ovulation [9,19]. Antioxidant system is active against oxidants during this process. In fact, it protects the oocyte from oxidative damage in follicular fluid [31]. Our study, however, showed a statisticaly significant increase in some oxidative status markers (eNOS activity and TOC) on day 11 and MDA, NO, TAC, TOC, leptin and eNOS activity statistically significantly higher on mating day in pregnant goats compared to non-pregnant goats *(Table 1)*. The exact reason for this difference is not known. But pregnancy status might be different because there was better oocyte maturation and a LH surge in the goats that got pregnant. Mating day oxidative status may have been better compensated for with antioxidant systems in the animals that would be pregnant. This may have affected fertilization.

In conclusion, the administration of CIDR to Abaza goats exacerbated oxidative and nitrosative stress and increased P4 concentrations (pregnant goats). However, serum leptin concentrations were increased on CIDR removal day and mating day in pregnant group. Measuring serum oxidant and antioxidant status markers on mating day may also provide information about the goats' ability to conceive and make fertility projections. However, more comprehensive studies are needed.

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Research Article

Immunological and anti-*Eimeria* Effects of Hot Water and Methanolic Extracts of *Pleurotus sajor-caju* in Broiler

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Abstract

Present study reports the immunomodulatory and anti-*Eimeria* effects of hot water and methanolic extracts of *Pleurotus (P.) sajor-caju* (a locally grown oyster mushroom). Mushrooms were processed to obtain hot water and methanolic extracts followed by lyophilization. The lyophilized extracts were subjected to proximate analysis followed by their evaluation for immunomodulatory and anti-Eimeria activities. Immunomodulatory evaluation of these extracts in industrial broilers revealed significantly higher ($P<0.05$) cell mediated immunity through lymphoproliferative response to phytohemagglutinin-P as compared to control. These extracts also showed higher humoral immune response through elicited total Ig, IgG, and IgM titers at day 7th and 14th after primary and secondary intramuscular injections of sheep red blood cells. Further, all the groups were orally inoculated with infective dose of sporulated oocysts of *Eimeria (E.)* species (including *E. tenella, E. maxima, E. acervulina* and *E. necatrix)* followed by monitoring of per cent mortality, oocysts per gram of feces (OPG) and intestinal lesion scoring. OPG values in birds of experimental groups administered with mushroom extracts were significantly lower ($P<0.05$) as compared to those of control group. Control group showed higher mortality ratio and lesions scores as compared to groups administered with extracts. In conclusion, hot water and methanolic extracts of *P. sajor-caju* showed significant immune boosting activity in broilers and subsequent protective efficacy against Eimeria infection.

Keywords: *Mushroom extracts, Pleurotus sajor-caju, Eimeria, Immunological, Broiler*

Etlik Piliçlerde *Pleurotus sajor-caju* Sıcak Su ve Metanolik Ekstraktının İmmunolojik ve Anti-Eimeria Etkileri

Öz

Bu çalışmada *Pleurotus (P.) sajor-caju* (yerel yetişen bir istiridye mantarı) sıcak su ve metanolik ekstraktının bağışıklık düzenleyici ve anti-*Eimeria* etkileri araştırılmıştır. Mantarlardan liyofilizasyonla sıcak su ve metanolik ekstraktı elde edildi. Liyofilize edilen ekstraktlar bağışıklık düzenleyici ve anti-*Eimeria* aktivitelerini test etmek amacıyla analiz edildi. Ticari broiler tavuklarda bu ekstraktların bağışıklık düzenleyici etkisinin değerlendirilmesi sonucunda kontrol ile karşılaştırıldığında fitohemaglutinine karşı lenfoproliferatif cevap ile karakterize hücre aracılı bağışıklığın anlamlı derecede yüksek olduğu belirlendi ($P<0.05$). Bu ekstraktlar, koyun kırmızı kan hücrelerinin primer ve sekonder kas içi enjeksiyonu sonrası 7. ve 14. günlerde total Ig, IgG ve IgM titreleri ile karakterize humoral bağışıklık cevabın da yüksek olmasına neden oldu. Tüm gruplara *Eimeria (E.)* türleri *(E. tenella, E. maxima, E. acervulina* ve *E. necatrix)* enfektif dozda oral yolla inokule edildi ve sonrasında mortalite yüzdesi, her bir gram dışkıda oosit miktarı ve barsak lezyonları incelendi. Kontrol ile karşılaştırıldığında mantar ekstraktı verilen hayvanların yer aldığı deney gruplarında her bir gram dışkıda oosit miktarı anlamlı derecede düşüktü ($P<0.05$). Kontrol grubunda mortalite oranı ve lezyon skorları ekstrakt verilen gruplarla karşılaştırıldığında daha yüksekti. Sonuç olarak, *P. sajor-caju* sıcak su ve metanolik ekstraktı etlik piliçlerde bağışıklığı artıcı ve *Eimeria* enfeksiyonuna karşı koruyucu etki gösterdi.

Anahtar sözcükler: *Mantar ekstraktı, Pleurotus sajor-caju, Eimeria, Bağışıklık, Broiler*

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INTRODUCTION

Pleurotus (P.) species are one of the 25 commonly consumed species of mushrooms worldwide and are becoming popular now a day because of excellent nutritional and medicinal properties [1]. In the world, button mushroom i.e. *Agaricus bisporous* is among the leading specie as far as cultivation is concerned, followed by shiitake *(Lentinus edodes)* and oyster *(P. ostreatus)* [2]. Commercially, mushroom production is dramatically increased in the world with mainly China and Europe contributing in its production to be increased up to almost 8 million tonnes, annually [3].

Among the oyster species, *P. sajor-caju* is gaining its due share because of its reported immunomodulatory, anti-inflammatory and anti-tumor activities [4,5]. There are several important nutrients including carbohydrates, proteins, dietary fibers, minerals and vitamins present in the mushrooms due to which they are considered as essential in the dietary composition of human beings [6,7]. For the last many years, research has been focused to explore the utility of mushroom compounds as immunomodulators in different animal models [8,9]. In this regard, several types of extracts from various mushroom species were evaluated for immune modulation in different animal models [10,11]. Moreover, various biologically active metabolites including glycoproteins, hydrolytic and oxidative compounds, phenolics and lipids had also been isolated from crude extracts of *Pleurotus* spp. to determine their biological activities [12]. These studies revealed immunomodulatory effects of these active substances through enhancing mitogenicity and activation of immune effector cells including macrophages, natural killer cells and lymphocytes. These immune cells further increase the production of cytokines including tumor necrosis factor (TNF-α), interleukins (ILs) and interferons (IFNs) [13].

In this regard, water soluble proteoglycans fractions from *P. ostreatus* had been reported for enhanced cytotoxicity of NK cells and stimulation of macrophages for increased production of nitric oxide with marked decrease in the quantity of sarcoma cells in sarcoma 180 bearing mice [14]. Similarly, methanol, dichloromethane and n-hexane extracts of *Agaricus blazei* were investigated against Ehrlich tumor bearing mice and revealed stimulated lympho-proliferative activity of splenocytes and enhanced antibody production [15]. Keeping in view the well documented pharmacological activities of *P. sajor-caju* in different disease models, this study was conducted to evaluate the immunotherapeutic activity of hot water and methanolic extracts of *P. sajor-caju* against *Eimeria* infection in chickens.

MATERIAL and METHODS

Procurement and Processing of Mushrooms

Fresh mushrooms *(P. sajor-caju)* were procured from the local grower at Millet Town, Faisalabad, Pakistan and its authenticity was confirmed by the concerned Botanist from University of Agriculture, Faisalabad, Pakistan (UAF), and a specimen (Voucher No. 175) was kept in Ethnoveterinary Research Center, Department of Parasitology, UAF.

Mushroom were dried at 50°C followed by grinding and passed through sieve (2 mm) to maintain the uniformity of the dried powder. The hot water extract was prepared by the methodology described previously [16]. In brief, the dried powder (500 g) was added in water (1500 mL) at temperature of 100°C in a water bath for 2 h. More water (500 mL) was added after each hour. The mixture obtained was centrifuged at 3000 rpm for 30 min and supernatant was collected. Supernatant thus obtained was subjected to lyophilization which yielded 85 g dried extract per 500 g of dried powder. The dried extract was stored at 4°C till further use in the experiment. The methanolic extract was prepared by the methodology described by Yang and his co-workers [17]. Briefly, dried mushroom powder (500 g) was vortexed thrice with 2000 mL of 80% methanol at room temperature for 48 h. Pooled extract obtained was subjected to rotary evaporator. Concentrated extract was lyophilized which yielded 25 gm of methanolic extract. These extracts (hot water and methanolic) were subjected to proximate analysis to determine the concentrations of crude protein, fiber, ash, ether and nitrogen free extracts [18].

Experimental Design

The experimental study was conducted at Experimental Station, Faculty of Veterinary Sciences, UAF. For the purpose, day old birds (n=150; Hubbard) purchased from local hatchery were reared under standard management conditions. Birds were given free access to fresh water and withdrawal feed. The study was approved for ethical consideration and humane handling of animals by Institutional Animal Care & Use Committee and Advance Studies & Research Board of UAF. After acclimatization for 5 days, the birds were divided into 3 groups i.e. 1, 2 and 3; each containing 50 randomly selected birds. On day 7th, 8th and 9th of the experiment, the groups 1 and 2 were administered with mushroom extracts as follows:

Group 1- Hot water extract of *P. sajor-caju* administered orally at the dose rate of 200 mg/kg body weight (BW)/day using phosphate buffered saline (PBS; 2 mL) as a solvent

Group 2- Methanolic extract of *P. sajor-caju* administered orally at the dose rate of 200 mg/kg BW/day using PBS (2 mL) as a solvent

Group 3- Control administered orally 2 mL of PBS.

On day 15th of the experiment, all three groups were subdivided into two equal groups (n=25 each) i.e. 1a, 1b; 2a, 2b and 3a, 3b, respectively. The subgroups 1a, 2a and 3a were designated to evaluate immunomodulatory effects through lymphoproliferative response to phyto-haemagglutinin-P (PHA-P), antibody response to sheep

red blood cells (RBCs) and organ (spleen, bursa of Fabricius and cecal tonsils) to body weight ratios. The subgroups including 1b, 2b and 3b were shifted to another shed and challenged with *Eimeria* infection to assess the anti-*Eimeria* efficacy of the extracts.

Immunological Evaluation

Classical toe web assay was performed to quantify cell mediated immunity. In this essay, PHA-P was injected intra-dermally in the right toe web of experimental birds. Meanwhile, PBS was also injected in the left toe web of same birds. Lymphoproliferative response was observed in terms of increase in the thickness of toe web after 24, 48 and 72 h by using the following formula:

Lymphoproliferative response = (Thickness of PHA-P injected toe web - Thickness of PBS injected toe web).

Microplate haemagglutination test was used to demonstrate humoral immune response. Sheep RBCs (5%) were injected twice as T-cell mitogen in the breast muscles of experimental birds at an interval of 14 days. Immunoglobulin (total Ig, IgG and IgM) titers were determined at day 7th and 14th after primary and secondary injections of sheep RBCs [19].

Lymphoid organs including spleen, thymus, bursa of Fabricius and cecal tonsils were collected from chickens of the experimental groups after humane slaughtering on day 42 of the experiment. All the organs were weighed to determine the per cent organ to live body weight ratios [20].

Evaluation of Anti-Eimeria Effects of Mushroom Extracts

To assess the anti-*Eimeria* effect, subgroups (1b, 2b and 3b) were challenged with infective dose (6.5×10^4-7.0×10^4) of sporulated oocysts of mixed *Eimeria (E.)* species viz. *E. tenella*, *E. maxima*, *E. acervulina* and *E. necatrix* (local isolates; maintained at Immunoparasitology Laboratory, UAF) on day 15th of experiment. These groups were monitored for oocyst per gram of droppings, lesion scoring and per cent protection from day 4th to 12th post challenge with *Eimeria* species [20-22].

Statistical Analysis

Data obtained was analyzed using completely randomized design and one-way analysis of variance (ANOVA) using SPSS ver. 16. The differences among mean values were determined using Tuckey's range test and differences were considered significant at P<0.05. For immunoglobulin titers, geometric mean titers (GMT) were calculated [23].

RESULTS

Proximate Analysis

Proximate analysis revealed 29.22% and 17.59% crude protein, 11.93% and 6.50% ash contents, 1.52% and 2.39% ether extract, 57.33% and 73.52% nitrogen free extract from hot water and methanol extracts of *P. sajor-caju*, respectively. However, no fibers were found in both extracts *(Table 1)*.

Immunological Evaluation

Lymphoproliferative Response to PHA-P: Cell mediated immune response was evaluated through Toe web essay. Thickness observed in the toe web of birds belonging to experimental groups treated with hot water and methanol extracts showed significantly higher values (P<0.05) at 24, 48 and 72 h post PHA-P injection as compared to control. The results revealed that both extracts showed excellent T-cell mitogen activity *(Fig. 1)*.

Humoral Immune Response Against Sheep RBCs: Total immunoglobulins, IgG and IgM titers observed in the hot water and methanol extracts at days 7th and 14th post-primary and post-secondary injections of sheep RBCs were significantly higher as compared to control. However, the titers (total Ig, IgG and IgM) observed in the methanolic extract treated groups were highest followed by those of hot water extract *(Table 2)*.

Lymphoid Organs to Body Weight Ratios: Birds belonging to experimental and control groups were weighed and

Table 1. *Results of proximate analysis of hot water and methanolic extract*

Extract Type	Hot Water Extract (%)	Methanol Extract (%)
Crude protein	29.22	17.59
Crude fiber	-	-
Ash	11.93	6.50
Ether extract	1.52	2.39
Nitrogen free extract	57.33	73.52

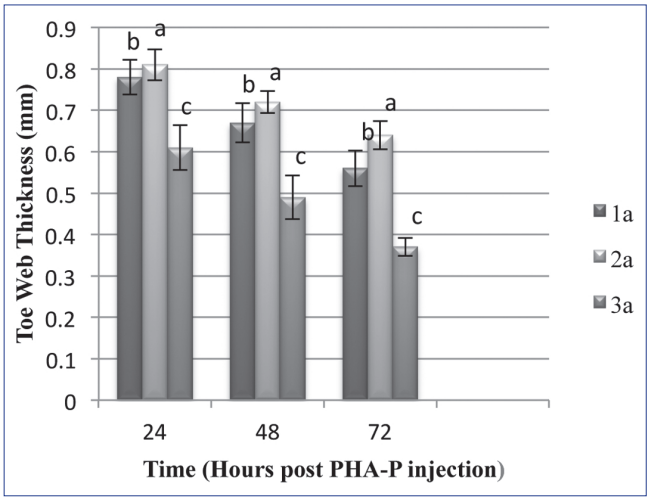

Fig 1. Lymphoproliferative response to Phytohaemagglutinin-P in mushroom extracts administered and control groups. Bars sharing different letters present a significant difference (P<0.05). Group 1a: Hot water extract of *Pleurotus sajor-caju,* Group 2a: Methanol extract of *Pleurotus sajor-caju,* Group 3a: Control

Table 2. *Anti sheep-RBCs antibody titers (Total Ig, IgG, IgM) after day 7 and day 14 post-primary and post-secondary injection of Sheep RBCs*

Total Immunoglobuln	7ppi	14ppi	7psi	14psi
Group 1a PSC/HWE	48.53	42.27	55.72	36.73
Group 2a PSC/ME	63.97	48.53	73.45	55.72
Group 3a Control	31.99	27.86	36.73	27.86
IgM				
Group 1a PSC/HWE	32.53	18.00	34.58	8.87
Group 2a PSC/ME	42.84	27.39	45.59	13.45
Group 3a Control	22.18	11.16	20.73	9.49
IgG				
Group 1a PSC/HWE	16.00	24.27	21.13	27.86
Group 2a PSC/ME	21.13	21.13	27.86	42.27
Group 3a Control	9.18	16.00	16.00	18.37

PSC/HWE - Pleurotus sajor-caju/Hot Water extract, PSC/ME = Pleurotus sajor-caju/Methanolic extract, 7ppi = day 7 post-primary injection of sheep RBCs, 14ppi = day 14 post-primary injection of sheep RBCs, 7psi = day 7 post-secondary injection of sheep RBCs, 14psi = day 14 post-secondary injection of sheep RBCs

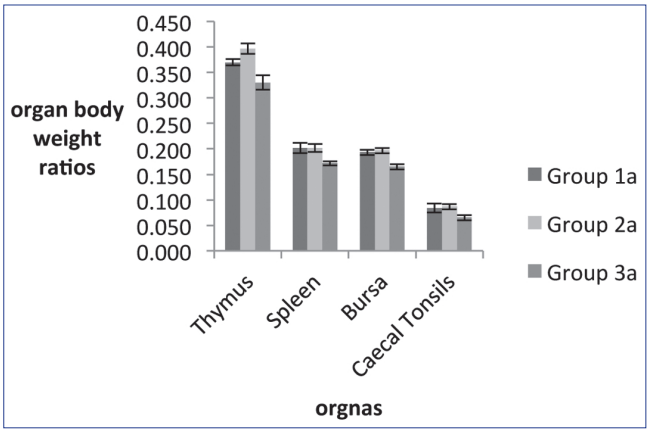

Fig 2. Organ to body weight ratios of experimental and control groups. Group 1a: Hot water extract of *Pleurotus sajor-caju*, Group 2a: Methanol extract of *Pleurotus sajor-caju*, Group 3a: Control

sacrificed to obtain lymphoid organs including thymus, spleen, bursa of Fabricius and cecal tonsils. Organ to body weight ratios of these organs were statistically non-significant (P>0.05) *(Fig. 2)*.

Evaluation of Anti-Eimeria Effects of P. sajor-caju Extracts

Post-challenge with *Eimeria* species, the birds in experimental groups administered with mushroom extracts and control group were monitored for OPG, percent protection and lesion scoring. In control group, significantly higher (P<0.05) OPG of droppings were observed as compared to *P. sajor-caju* extracts treated experimental groups. Among the mushroom administered groups, minimum OPG was observed in the methanolic extract followed by hot water extract *(Fig. 3)*.

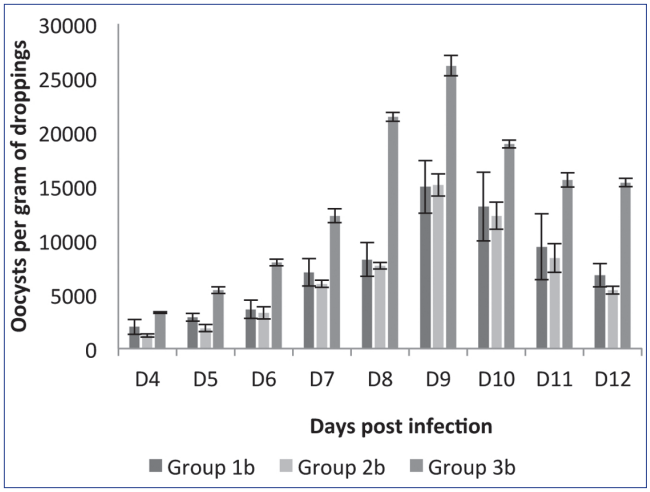

Fig 3. Oocysts per gram of droppings from day 4 to day 12 post challenge with *Eimeria* species. Group 1b: Hot water extract of *Pleurotus sajor-caju*, Group 2b: Methanolic extract of *Pleurotus sajor-caju*, Group 3b: Control

Table 3. *Lesion Scoring observed in intestines of experimental and control birds*

Groups	Lesion Scoring of the Birds				
	0	1	2	3	4
Group 1b	4	7	5	4	5
Group 2b	3	6	5	4	7
Group 3b	1	2	4	6	12

Group 1b: Birds given Hot water extract of Pleurotus sajor-caju, Group 2b: Birds given Methanol extract of Pleurotus sajor-caju, Group 3b: Control

Highest protection (45%) was observed in group treated with methanolic extract followed by hot water extract treated group (31%) and control group (24%). Birds of all the experimental groups designated for anti-*Eimeria* activities were evaluated for intestinal lesion scorings on a scale of 0 to 4. Results showed that severe lesion scores (3-4) were lesser in birds of groups administered with hot water (09) and methanolic extracts (11) as compared to those of control group (18) *(Table 3)*. It indicated the protective efficacy of *Pleurotus* extracts against adverse effects of *Eimeria* infection on the intestine of the affected birds.

DISCUSSION

Oysters *(Pleurotus)* are among the popular edible mushrooms consumed by human being worldwide. This group of mushroom is cosmopolitan for their nutritional and therapeutic properties. Several studies in the past revealed medicinal potentialities of different species of oyster mushrooms and classified them as "mushroom nutraceuticals" [14,24]. Keeping in view the medicinal properties of *Pleurotus* species, current study was designed to evaluate immuno-therapeutic activities of hot water and methanolic extracts of *P. sajor-caju* against coccidiosis in broiler.

In a recent study, proximate analysis of 10 mushrooms (*Cantharellus cibarius*, *Rusula delica var chloroides*, *Ramaria largentii*, *Hygrophorus russula*, *Amanita caesarea*, *Fistulina hepatica*, *Boletus aureus*, *Armillaria tabesceus*, *Armillaria mellea* and *Lepista nuda*) were evaluated to determine the concentration of carbohydrates (55.33-66.87), fats (2.10-6.00), proteins (21.57-34.77), and ash (5.61-9.44) [25]. In another study, nutritional composition of different *Pleurotus* species including *P. pulmonaris*, *P. floridanus*, *P. cystidiosus* and *P. sajor-caju* was demonstrated on dry weight basis. Carbohydrates, proteins, crude fat, crude fibers and ash were found in the range of 85.86-88.38%, 0.98-2.17%, 0.62-0.84%, 2.76-3.12% and 1.03-2.20%, respectively [12]. Results found in the current study have slight variations with the above-mentioned studies. It may be due to different ways and times of harvesting, different storage conditions, species differences, difference of compost used during mushroom growth, different conditions of management and other parameters including temperature, relative humidity, water availability and geographical distribution [26-28].

Mushroom extracts in the current study revealed remarkable cellular immune response in terms of increased thickness of toe web in birds treated with hot water and methanolic extracts of *P. sajor-caju*. These immunomodulatory activities may be due to activation of NK cells, higher production of interferon, enhanced complement activation, potentiation of phagocytic activities and prevention of leukocyte reduction [29,30]. In another study, after administration of *Lentinus edodes'* polysaccharides, chickens challenged and infected with Marek's disease showed significant increase in T-cell proliferation and interleukin production [31,32]. Studies in other animals including rats showed almost similar results as after administration of different mushrooms resulted in the enhancement of T-cell mediated immune responses including lymphocyte proliferation, increasing spleen thymus indexes and corrections of immunosuppression after administration of immune inhibitors such as cyclophosphamide and deltacortisone [33,34]. Moreover, mushroom extracts obtained from different mushrooms also enhanced the production of interleukin 1 and 8 [35]. Likewise, polysaccharide isolated from *Lentinus edodes* enhanced cell-mediated immune response through delayed type of hypersensitivity reaction shown through enhancement in proliferation of splenocytes and increased production of TNF-α and IFN-γ [36]. However, the actual mechanism involved in the enhancement of cellular immune response is still obscure.

Enhanced immunoglobulin production in chicken mucosa had been reported in the past after administration of different mushrooms including *Lentinus edodes*, *Tremella fuciformis* and *Fomitella fuciformis* [33,37]. This enhanced production may be due to proliferation and activation of immune effector cells including macrophages, T-helper, natural killer cells and dendritic cells. Enhanced activation and proliferation of these cells increase the production of certain cytokines such as IL-12 and IFN-γ after administration of different extracts of *Agaricus brazelliance* and *Antrodia camphorate*. These cytokines may be responsible for activation of effector phase of innate and adaptive immunity [38,39].

After challenge infection of sporulated oocysts of *Eimeria*, oocysts per gram of droppings, mortality percentage and lesion scores in experimental groups were observed. In this regard, limited research has been conducted on the effects of mushrooms particularly against *Eimeria* infection in chicken [40-42]. Birds administered with hot water and methanolic extracts of *P. sajor-caju*, showed excellent protection against coccidiosis in chicken. This might be due to polysaccharides present in the crude extracts [33-35]. Similar findings had also been reported in some previous studies [43,44]. In conclusion, results of this study revealed the immunopotentiating and anti-*Eimeria* efficacy of hot water and methanolic extracts of *Pleurotus sajor-caju*. Further studies to rule out the specific mechanism of action of these extracts for such activities and their commercial feasibility are underway in our lab.

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Short Communication

Identification and Genetic Characterization of Astrovirus in Wild Boar *(Sus scrofa)* in China

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Abstract

Porcine astrovirus (PAstV) is a frequently detected virus in pigs suffering from diarrhea worldwide. Here, we report the first identification and complete genome sequence of astrovirus in wild boar (Sus scrofa) in Jiangxi, China. The complete genome sequence of a representative astrovirus, WBAstV/CH/2015, was amplified and determined. Sequence homology analysis showed that WBAstV/CH/2015 had 40.8% to 79.7% homology with PAstVs worlwide, and shared the highest homology (79.7%) with another wild boar astrovirus (WBAstV) strain WBAstV-1/2011/HUN from Hungary. Phylogenetic analysis showed that WBAstV/CH/2015 was closely related to WBAstV-1/2011/HUN and located in the cluster of PAstV 4.

Keywords: *Porcine astrovirus (PAstV), Diarrhea, Wild boar (Sus scrofa), Genome, Phylogenetic analysis*

Çin'de Yaban Domuzunda *(Sus scrofa)* Astrovirusların Tanımlanması ve Genetik Karakterizasyonu

Öz

Domuz astrovirusu (PAstV) dünya çapında ishalli domuzlarda en sıklıkla belirlenen bir virustur. Bu çalışmada, Çin'in Jiangxi eyaletinde yaban domuzunda *(Sus scrofa)* astrovirusun ilk tespiti ve tüm genom sekans analizi gerçekleştirilmiştir. Astrovirus WBAstV/CH/2015'un tüm genom sekansı amplifiye edildi ve belirlendi. Sekans homoloji analizi, WBAstV/CH/2015'in dünye çapında PAstVları ile %40.8 ile %79.7 arasında benzerliğe sahip olduğunu ve Macaristan'da tespit edilen diğer bir yaban domuzu astrovirus (WBAstV) suşu olan WBAstV-1/2011/HUN ile en yüksek benzerliğe (%79.7) sahip olduğunu gösterdi. Filogenetik analiz WBAstV/CH/2015'in WBAstV-1/2011/HUN ile yakın akraba olduğunu ve PAstV 4 topluluğuna yakın olarak yer aldığını göstermiştir..

Anahtar sözcükler: *Domuz astrovirusu (PAstV), İshal, Yaban domuzu (Sus scrofa), Genom, Filogenetik analiz*

INTRODUCTION

Astrovirus is a non-enveloped, single-stranded, positive-sense RNA virus that belongs to the family *Astroviridae*. Members of *Astroviridae* are associated with gastroenteritis, diarrhea, encephalitis and respiratory symptoms as those viruses could infect series of mammalian species (*e.g.* humans, bats, cattle, dolphins, deer, mice, mink, sheep, cats, pigs, and marine animals) and birds (*e.g.* duck, turkeys, and chickens) [1-4]. The entire genome of astrovirus is about 7 kb in length that includes 3 open reading frames (ORFs), ORF1a, ORF1b and ORF2. The ORF1a encodes the non-structural polyprotein 1a, while the longer ORF1b encodes polyprotein 1b, including the RNA-dependent RNA polymerase (RdRp), and ORF2 encodes the viral capsid structural polyprotein [5]. Porcine astrovirus (PAstV), a member of

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Astroviridae, was first observed in diarrheal feces of weaning piglets in 1980 by electron microscopy, and then was identified in 1990 [6,7]. To date, PAstV has been detected in diarrheal and/or healthy pigs in several countries, including the United States, Canada, China, South Korea, and many other countries. PAstV is a widely distributed virus that causes diarrhea, dehydration, and congenital tremor in pigs. The infection rates of PAstV are 17.5% to 89% in domestic pigs, including pigs with diarrhea and healthy pigs [2,8-10]. In China, there have been studies concerning PAstV, but these were limited only to molecular epidemiology and domestic pigs [10,11]. In this study, we investigated the infection rate of PAstV in diarrheal wild boars. To elucidate the genetic characterization and evolutionary relationships with PAstVs from other countries/areas, we identified and analyzed the full-length genome sequence of a representative astrovirus in wild boar (WBAstV).

MATERIAL and METHODS

Diarrhea Outbreak Information

In June, 2015, a sudden outbreak of diarrhea occurred at a wild boar farm (about 100 sows) in Jiangxi, China. The wild boars had no contact with domestic pigs and other animals, and were breed and raised in a closed community. Pigs in different ages showed watery diarrhea, including breeding sows, suckling piglets, and weaned pigs, with a morbidity of 40% in all of the boars and a mortality of 20% in suckling piglets. A total of 20 diarrheal feces were collected and submitted to Animal Disease Diagnostic Center, Key Laboratory for Animal Health of Jiangxi Province, China.

Pathogen Detection

To confirm the cause of the diarrhea, common diarrhea-associated pathogens, including porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine rotavirus (PoRV), porcine deltacoronavirus (PDCoV), porcine circovirus type 2 (PCV-2), classical swine fever virus (CSFV), porcine kobuvirus (PKV), porcine bocavirus (PBoV) and pathogenic *Escherichia coli* and *Salmonella* were investigated based on the previous methods [12,13]. Porcine astrovirus was further tested as previous studies unveiled its association with diarrhea in pigs [14].

Complete Genome Sequencing of A Representative WBAstV

To understand the genetic information of those astroviruses in diarrhea wild boars, five couples of overlapping primers targeting the complete genome of WBAstV were designed based on the conserved regions determined by a multiple alignment analysis of the reference PAstVs retrieved from GenBank *(Table 1)*. Total RNAs were extracted from the feces by RNAplus Reagent (TaKaRa, Japan) according to the manufacturer's instructions. The first-strand cDNA synthesis was performed at 42°C for 50 min and then 95°C for 5 min to inactivate the M-MLV reverse transcriptase (TaKaRa, Japan) and followed by 4°C for 5 min. The entire genome was amplified by five pairs of primer. Fragments were amplified using rTaq DNA polymerase (TaKaRa, Japan) on the conditions of a denaturation at 94°C for 4 min, 35 cycles (94°C x 45 sec, 53°C x 45 sec, 72°C x 1.5 min), and then with a final extension at 72°C for 10 min. The 5'- and 3'- rapid-amplification of cDNA ends (RACE) for the determination of the terminal sequences of WBAstV were performed by using 5'/3' SMARTer RACE kit (Clontech, Beijing, China) following the manufacturer's instructions. Positive PCR products were subjected to gel purification, and afterwards cloned into pMD 18-T vectors (TaKaRa, Japan). Three to five positive clones of each amplicon were submitted to a commercial sequencing company (Sangon Biotech, Shanghai, China) for sequencing at both directions by Sanger sequencing methodology.

Sequence Analysis

The raw sequence fragments of the representative WBstV,

Table 1. *Primer information of complete genome of PAstV amplification*

Name	Sequence(5'-3')	Position	Product Size (bp)	Overlap (bp)
PAstv 1-F	CCAAGGTTGATTTAGCTGTC	44-63	1195	
PAstv 1-R	CCAGTTGGATCCCTTATCTC	1219-1238		
PAstV 2-F	CAGCAGCACTCATTTCCCTG	965-984	1282	273
PAstV 2-R	ATCGTCTTCAGGGTCACTCC	2228-2247		
PAstv 3-F	GAAGGGTAAGACCAAGCATGGC	2045-2066	1034	130
PAstv 3-R	ATTGCTGAAAAGGCAGACACATAGG	3055-3079		
PAstv 4-F	GTCATCCATATCTCAGCCACAGAG	2924-2947	1025	155
PAstv 4-R	ATCAGGAGTTGTAGGCTCAGGAG	3927-3949		
PAstv 5-F	AGGGATTATGACACCATCGTC	3657-3677	1755	292
PAstv 5-R	TGGCCAAGAGTATGGATTTCA	5349-5369		
5'RACE	GGTAACGTAGGGTCTCCTCAG	873-898	898	
3'RACE	GTGTGTGGGGTTTGGATTGGGGCCA	5300-5320	1345	

named as WBAstV/CH/2015, were assembled by SeqMan in DNAStar *Lasergene* V 7.10 (DNAStar, Inc., Madison, WI). Homology of nucleotide (nt) and deduced amino acid (aa) sequences of WBAstV/CH/2015 and reference PAstVs were comparatively analyzed. Phylogenetic trees based on the entire genomes, ORF1b and ORF2 of WBAstV/CH/2015 and reference astroviruses were constructed using the neighbor-joining method by the software of molecular evolutionary genetics analysis 6.0 (MEGA v. 6.0) (http://www.megasoftware.net/) with a bootstrap of 1.000 replicate datasets [5].

RESULTS

The diarrheal associated pathogens, PEDV, PDCoV, TGEV, PDCoV, PRoV, PCV-2, CSFV, PKV, PBoV, pathogenic *E. coli* and *Salmonella* were tested upon the 20 fecal samples from diarrheal wild boar, but all showed negative results. PAstV, a suspected diarrhea virus, was further tested. Unexpectedly, 60% (12/20) of these samples were found to be positive for PAstV. The weaning piglets showed the highest detection rate, 7 out of the 9 samples were positive; samples from the suckling piglets (2/5) and sows (3/6) were also found to be positive for PAstV, but with lower infection rates.

To investigate the genetic characterize and relationship of WBAstV with astroviruses from other countries/areas, a representative WBAstV, designated as WBAstV/CH/2015, was amplified and sequenced. Multiple sequences of WBAstV/CH/2015 were assembled and annotated using DNAStar *Lasergene* software. The entire genomic sequence of WBAstV/CH/2015 was 6, 644 nt in length, excluding the 3′ poly(A) tail, and the sequence was deposited in GenBank under the accession number KX033447. The genome structure of WBAstV/CH/2015 was typical of astrovirus, and was arranged in the order of the 5′ untranslated region (UTR) (nt 1 to 103), ORF1a (nt 104 to 2, 653), ORF1ab (nt 104 to 4, 099), and ORF2 (nt 4, 091 to 6, 572), and the 3′UTR (nt 6, 573 to 6, 644). The 5′UTR of WBAstV/CH/2015 was 103 nt; the replicase gene containing ORF1a and ORF1b was 3, 996 nt in length; ORF2, coding the capsid protein, was 2, 481 nt, and the 3′ UTR was 107 nt. Similar to the astroviruses from domestic pigs and wild boars, WBAstV/CH/2015 contained a conserved start pentamer (CCAAA) at the beginning of the 5′ terminus. We also found that the frameshift heptamer (AAAAAAC) followed by a stem-loop structure, present near the 3′ end of ORF1a in the PAstV-4 genome, which is a potential signal for a ribosomal frameshift during translation to generate the replicase polyprotein ORF1ab *(Fig. 1)*.

Sequence homology analysis showed that WBAstV/CH/2015 had 40.8 to 79.7 % homology to PAstVs, and shared the highest homology (79.7%) with a wild boar astrovirus, WBAstV-1/2011/HUN *(Table 2)*. The ORF1ab gene of WBAstV/CH/2015 was 3, 395 nt long, encoding a protein of 1, 132-aa, and with a 31.9% to 92.4% homology with the reference strains, shared the highest homology with PAstV4-CH-2014, a PAstV determined in domestic pigs in Jiangxi province in 2016.

Phylogenetic analyses of WBAstV/CH/2015 and astroviruses from domestic pigs, wild boars and other host species were conducted based on the sequences of complete genome, ORF1b and ORF2. The phylogenetic trees showed that WBAstV/CH/2015 was located in the cluster of type 4 astroviruses with 3 other porcine astroviruses and evolutionarily closed to WBAstV-1/2011/HUN, a astrovirus from wild boar in Hungary *(Fig. 2A)*. The phylogenetic results indicated that WBAstV/CH/2015 belongs to the lineage PAstV-4. Phylogenetic trees based on the sequences of ORF1ab and ORF2 also revealed the close relationship between strain WBAstV/CH/2015 and strain WBAstV-1/2011/HUN *(Fig. 2B,C)*.

DISCUSSION

Astroviruses have a wide range of host species. As emerging infectious diseases pose a continuous health

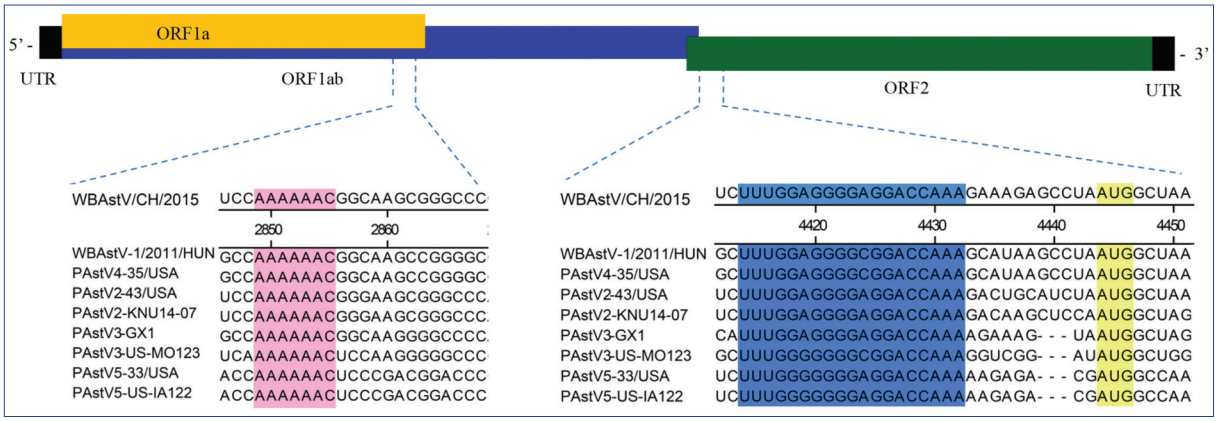

Fig 1. The schematic diagram of genomic structure, frameshift heptamer site and transcription initiation site of the subgenomic RNA of WBAstV/CH/2015. A frameshift heptamer AAAAAAC *(shaded in red)* and genotype conserved sequences *(shaded in green)* are present just upstream of the transcription initiation site *(shaded in yellow)* of the subgenomic RNA at the ORF1b and ORF2 junction

Table 2. *Nucleotide (nt) and amino acid (aa) sequence identities in percentage based on a comparison of the whole genome sequence*

Astrovirus Reference Strain	% Identity to WBAstV/CH/2015						
	Genome (nt)	ORF1a (nt)	ORF1a (aa)	ORF1ab (nt)	ORF1ab (aa)	ORF2 (nt)	ORF2 (aa)
PAstV2-43/USA	44.8	47	40.2	53.6	49.2	41.4	27.7
PAstV2-51/USA	44.5	47.1	40.2	53.6	49.1	40.1	25.9
PAstV2-US-IA122	44.2	46.8	39.8	53.4	49	39.6	26.8
PAstV2-KNU14-07	43.6	46.9	39.1	53.3	48.5	41.1	27.8
PAstV2-Bel-12R021	43.7	47.1	39.3	53.6	48.2	39.3	25.3
PAstV3-GX1	42.8	39.5	24.1	46	38.8	33.6	23.3
PAstV3-US-MO123	41.3	43.3	27.8	47.5	37.6	28	20
PAstV4-35/USA	77	81.6	84.8	85.9	90.4	65.3	58.4
PAstV4-US-IL135	76.7	80.6	81	84.9	87.7	66.2	58.5
PAstV4-CH-2014	79.7	85.4	88	88.4	92.4	67.9	62.4
WBAstV-1/2011/HUN	77.5	79.2	78.3	83.7	56.1	67.5	64.3
PAstV5-33/USA	40.8	41.4	21.5	45.2	32	37.1	22.4
PAstV5-US-IA122	40.9	41.8	21.5	45	31.9	31.8	20.9

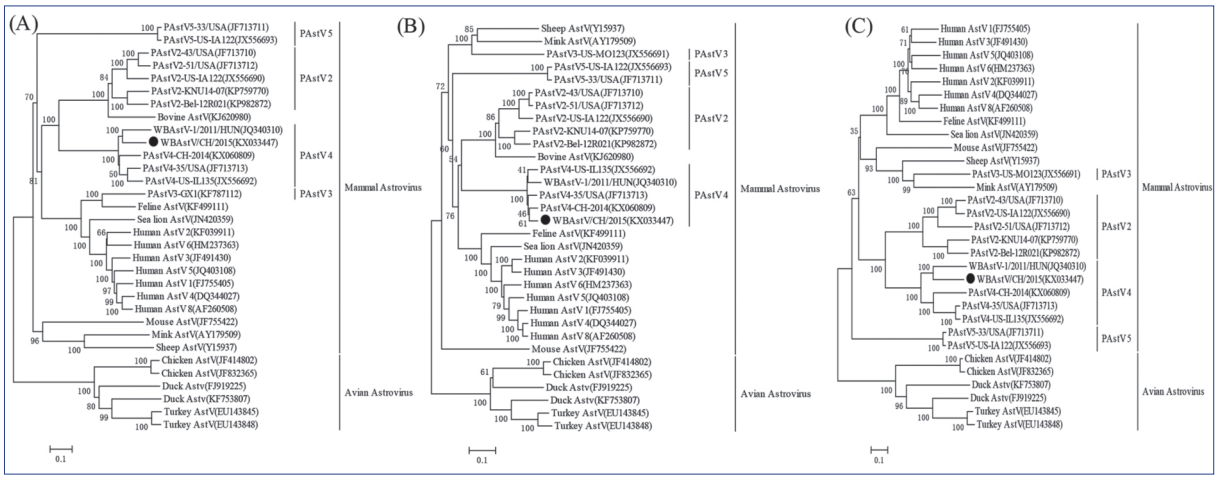

Fig 2. Phylogenetic trees of the entire genome (A), ORF1b (B) and ORF2 (C) sequence of Porcine Astroviruses. A bar of 0.1 indicates nucleotide or amino acid substitutions per site. "●" indicates the strain identified in this study

threat to wild and domestic animals as well as to humans, continued characterization of astrovirus in different host species and areas will help our understanding of their origin and the possible mechanism of cross-species transmission. The study presented here is based on a field outbreak of diarrhea in wild boar herd. Our investigation showed the presence of astrovirus in wild boar in Jiangxi, one of the main pork producing province in China. Pathogen detection showed negative of eight porcine diarrheal-associated viruses (PEDV, PDCoV, TGEV, PDCoV, PRoV, PCV-2, CSFV, PKV, and PBoV), pathogenic *E. coli* and *Salmonella* in these diarrheal samples, and only WBAstV was found in 60% of these samples. The result was unlike the previous report in diarrheal domestic pigs and wild boars in another main pork produce province, Sichuan, in China. They found frequent coinfections of PEDV and PAstV in diarrheal pigs [15-17]. Shan et al.[18] reported the high presence of astrovirus in healthy and diarrheal piglets in high density premises. Studies revealed astrovirus was associated with gastroenteritis and diarrhea in humans and animal species [19,20]. Our results revealed the astrovirus might be associated with diarrhea in wild boar. Further studies on virus isolation and pathogenesis are needed.

The entire genomic sequence of the representative astrovirus, WBAstV/CH/2015, was determined in this study. Sequence homology analysis showed WBAstV/CH/2015 was highly conserved with WBAstV-1/2011/HUN strain from wild boar in Hungry. The ORF2 showed high similarity to astroviruses in domestic pigs, but 20-aa shorter than the wild boar astrovirus from Hungry, WBAstV-1/2011/HUN [8]. The frameshift heptamer (AAAAAAC) at the ORF1a/1b junction was also found in WBAstV/CH/2015, and was conserved in PAstVs. The phylogenetic analysis

showed that WBAstV/CH/2015 was closely related to the wild boar astrovirus, WBAstV-1/2011/HUN from Hungary [8], and located in the cluster of type 4 astroviruses with 3 other porcine astroviruses. Recently, investigations have displayed up to five genotypes of PAstVs (PAstV-1~ PAstV-5) in domestic and wild pigs [17]. To our knowledge, this is the first report of type 4 astrovirus in wild boar in China. WBAstV/CH/2015 strain showed a 76.7%~79.7% nucleotide sequence identity to the recently discovered PAstV-4. Interestingly, we found the homology of ORF1ab of WBAstV/CH/2015 was highest with PAstV4-CH-2014, a astrovirus determined in domestic pigs in the same provine. Although the wild boars we investigated had not contacted with domestic pigs and other animals, we supposed humans, feeds or vechicles might carried this virus and transmitted to the wild boars. While further studies on the cross-species transmission of astrovirus are needed.

In conclusion, we firstly identified the presence of astrovirus in wild boars in Jiangxi, China. Then we determined and analyzed the genetic characterizations of the full-length genome sequence of a representative astrovirus in wild boar. Our results give further insight of into the presence of astroviruses in wild animals and provide information of the epidemiology and evolution of PAstV in China and other countries.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Case Report

Enterotoxemia Caused by *Clostridium perfringens* Type E in a Calf

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Abstract

Clostridial enterotoxemia caused by *Clostridium perfringens* type E was diagnosed postmortem in a 2-months-old calf in a family farm containing 20 cattle at different ages. Varying degrees of severity of segmental fibrino-hemorrhagic and necrotic enteritis was present at the necropsy. *Clostridium perfringens* was isolated from the intestinal tissue and intestinal content and the genes encoding alpha and iota toxins were detected by polymerase chain reaction (PCR). Histopathological examination showed the presence of adherent Gram-positive rods on the surface of villi and in poly morphonuclear leucocytes in the lamina propria of the intestinal mucosa.Overall, the results of the present study suggest that *C. perfringens* type E should be considered at differential diagnosis in fibrino-hemorrhagic enteritis and sudden deaths in post weaned calves.

Keywords: *Bovine, Clostridium perfringens type E, Enterotoxemia*

Bir Buzağıda *Clostridium perfringens* Tip E Nedenli Enterotoksemi Olgusu

Öz

Farklı yaş gruplarındaki 20 sığırdan oluşan bir çiftliğe ait 2 aylık buzağıda, postmortem olarak *Clostridium perfringens* tip E ye bağlı enterotoksemi tespit edildi. Nekropside, değişen derecelerde fibrino-hemorajik ve nekrotik enteritis tespit edildi. *Clostridium perfringens,* bağırsak dokusundan ve içeriğinden izole edildi, alfa ve iota toksinlerini kodlayan genler polimeraz zincir reaksiyonu (PZR) ile tespit edildi. Histopatolojik incelemede, bağırsak villuslarında, adherent Gram pozitif rodlar ve polimorfnükleer lökositler görüldü. Sonuç olarak, *C. perfringens* tip E'ye bağlı tokseminin, sütten kesilmiş buzağılardaki ani ölümlerin ve fibrino-hemorajik enteritislerin ayırıcı tanısında düşünülmesi gereken bir hastalık olduğunu söylemek mümkündür.

Anahtar sözcükler: *Sığır, Clostridium perfringens tip E, Enterotoksemi*

INTRODUCTION

Clostridium perfringens (CP) is a non-motile and ubiquitously distributed Gram-positive microorganism [1]. It is normally present in animals and humans intestinal contents but sometimes causes infection and proves highyl pathogenic regarding intestinal diseases [2]. Most of the infections caused by CP biotypes are mostly encountered in herbivores and human. Clostridial diseases of the intestinal tract are often evaluated under an umbrella term so called enterotoxemia, characterized by the intestinal and histotoxic tissue injury arising from 4 major exotoxins including alpha, beta, epsilon and iota together with 13 minor toxins. Each of the major toxin types encoded by different genes causes specific disease syndromes. There are 5 strain types of CP as classified by letters A, B, C, D, and E. These strain types are differentiated based on the 4 major antigenic lethal exotoxins that they produce. Alpha toxin is a lecithinase that affects cell membranes, causes hemolysis or cell necrosis and is produced by all 5 strain types [1]. The structure of the beta toxin is unknown. This toxin is produced by B and C strains, and causes enteritis. It has necrotic effect, leading to paralysis in the intestine. Epsilon toxin is activated by enzymatic digestion, and

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exerts its effects especially in the brain and kidneys. The iota toxin which increases the permeability of the cell membrane is released as a prototoxin and activated by proteolytic enzymes. CP type E causes intestinal disease in calves, lambs and rabbits. Illnesses with type A and E develop in the same way, but types B and C mostly affect newborns and have a much simpler pathogenesis [1,3]. In all of the enterotoxemia cases, the bacteria rapidly multiply in the intestine and the disease develop depending on the bacterial exotoxin. CP Type E isolates secrete two of these toxins, alpha toxin, single polypeptide with phospholipase C, sphingomyelinase, hemolytic, and lethal properties and iota toxin, a binary toxin consisting of two non-covalently associated components. The latter toxin is an uncommon and unique toxin that produced by type E *C. perfringens* isolates [1,4]. The disease caused by type E isolates of *C. perfringens* are the most poorly understood of all *C. perfringens* enteritis. The role of the iota toxin in pathogenesis is unknown [4] and it has rarely been reported in calves. The aim of this report is to diagnostic workup confirming the case of CP type E infection in a 2 months old calf.

CASE HISTORY

Two months old, male, Black Swiss calf was referred to Veterinary Teaching Hospital with the clinical signs of depression, diarrhea, anorexia and high body temperature (42°C) for treatment. The calf died before the clinical examination and the necropsy was carried out within 1 h of death. The calf was from a family farm containing 20 cattle at different ages. There was no any clinical finding of illness for the rest of animals.

Gross Findings

The gross examination revealed varying degrees of severity of segmental fibrino-hemorrhagic and necrotic enteritis *(Fig. 1A)*. The most severely affected segment was the ileum and the jejunum in which there were intraluminal fibrin tangles, hemorrhagic exudate, and necrosis *(Fig. 1B)*. Mezenterial lymph nodes were swollen approximately 2 times of normal size and were congested in cut surfaces *(Fig. 1C)*. The visceral organs including liver and lungs were pale in color, whereas kidneys showed diffusely cortical and medullar congestion.

Histopathological Findings

Tissue samples of small and large intestines, liver, lungs, spleen, pancreas, brain, heart, pericardium and kidneys were collected and fixed by immersion in 10% neutral formalin. All tissues were processed routinely by histological techniques for the production of 4-5 µm sections and stained with hematoxylin-eosine (H&E). Selected tissue sections were stained with Brown-Brenn's method. Histopathologically, there was necrotizing hemorrhagic enteritis at various severity *(Fig. 1D)*. Gram-positive rods were attached on the surface of villi and in polymorphonuclear leucocytes in lamina propria of muscosa were also observed. Follicular lymphoid hyperplasia, histiocytic hyperplasia and interstitial hemorrhage were detected in mesenteric lymph nodes. In liver, there was multifocal, moderately dense periportal lymphohistiocytic infiltrations.

Bacterial Isolation, Identification and Determining Antimicrobial Susceptibility

A 25 g of the affected intestinal sample was pummeled

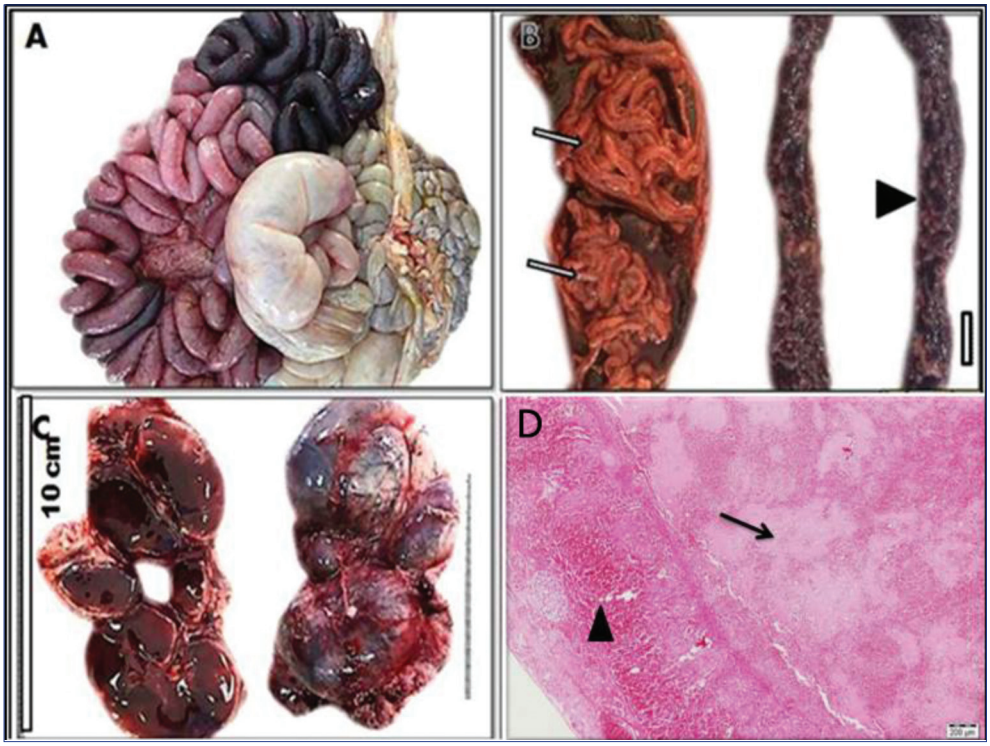

Fig 1. Gross and histopathological findings. A: Gross appearance of intestinal tract showing segmental, moderate to severe enteritis. B: Intraluminal fibrinous *(arrow)* and hemorrhagic exudation in cecum and necrotic changes *(arrow head)* in ileum. C: Cut and external surface of a cranial mesenteric lymph node having hyperemic and edematous appearance. D: Severe necrotic *(arrow)* and hemorrhagic enteritis *(arrow head)* with focal neutrophilic and few histiocytic infiltrates

within 225 mL of sterile peptone water for 2 min in a stomacher. Decimal dilutions were made from the resulting homogenate and pour-plated to Tryptose Sulphite Cyloserine (TSC) agar. The petri plates were incubated at 46±1°C for 20-24 h under anaerobic conditions (anaerobic jar with Anaerocult). Five colonies were selected at random among the typical black colored colonies and purified for identification [5].

The suspected isolates were in grown in Brucella blood agar and the Thioglycolate broth (enriched with 5% sheep blood, hemin (5 mg/L) and vit K1 (1 mg/L) (Becton Dickinson, Heidelberg, Germany) at 37°C for 72 h in a jar with an anaerobic gas-pack (Thermo Fisher Scientific, USA). The colonies were subjected to Matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF MS) based VITEK MS (database v2.0) (Bio-Mérieux, France) system identification. Results showed that the isolates were identified as *C. perfringens* with a high score value (99.90%).

Antimicrobial susceptibility test was performed on Brucella agar by modified Kirby-Bauer disk diffusion method [5,6]. The isolate was susceptible to imipenem, vancomycin, tetracycline, florfenicol, teicoplanin, linezolid, chloramphenicol, cefepime, but resistant to penicillin, ampicillin, trimethoprim-sulfamethoxazole, streptomycin, levofloxacin, erythromycin and clindamycin.

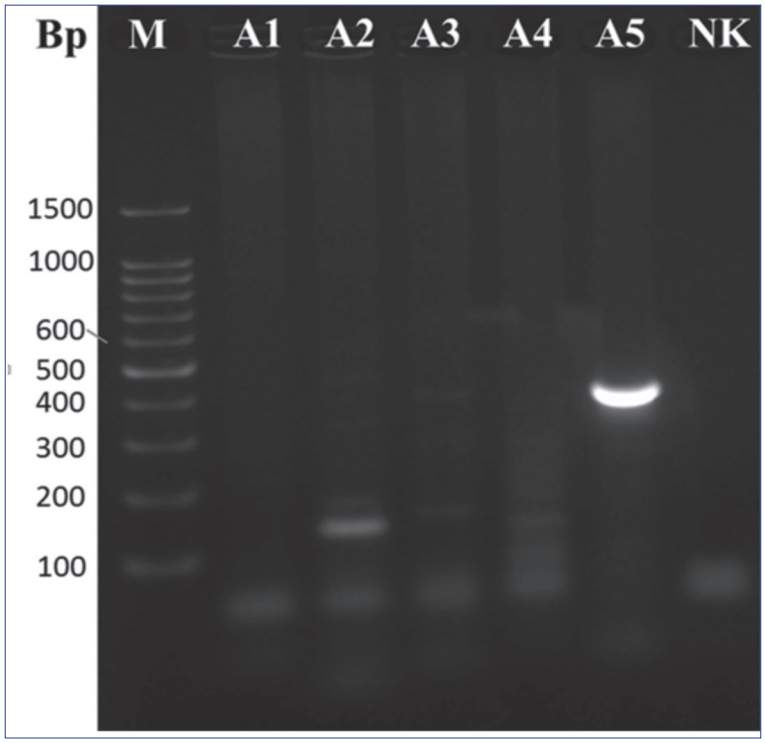

Fig 2. PCR result of C. perfringens' toxin genes. M: 100 bp DNA Ladder (NEB, USA), NK: Negative Control, A1: Enterotoxin; Negative, A2: cpi - iota toxin; Positive, A3: cpb-beta toxin; Negative, A4: cpe - epsilon toxin; Negative A5: cpa - alpha toxin; Positive

PCR for the Detection of Toxin Genes

The *C. perfringens* isolate was analyzed for the presence of alpha, beta, epsilon and iota toxin genes by PCR using the primers and protocol as described previously [7]. The primers are shown in *Table 1*. Briefly, DNA was extracted from the isolate using a column-based DNA isolation kit (DNA mini kit, Qiagen, Germany). The PCR run was performed using the GeneAmp PCR System 9700 device (Applied Biosystems) and amplicons were photographed with UV illumination after electrophoresis.

The identities of the amplicons were confirmed by comparison of the sequence with previous reports obtained from Gen-Bank. The isolate was found positive alpha and iota toxins by PCR *(Fig. 2)*.

DNA and RNA Isolation for Bovine Herpesvirus and Bovine Virus Diarrhea Virus

Amplification conditions: Following initial denaturation step for 3 minutes at 94°C, 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, and a final elongation step at 72°C for 10 min.

Viral Genome Amplification: Tissue samples from mesenterial lymph nodes and liver were homogenized in which of 50 mg were taken

Table 1. *Sequences of the PCR primers*

Primer	Sequence 5'-3'	Product Size (bp)	Gene	Reference
cpb F	GCGAATATGCTGAATCATCTA	196	*cpb*	[7]
cpb R	GCAGGAACATTAGTATATCTTC			
cpe F	GGGGAACCCTCAGTAGTTTCA	506	*cpe*	[8]
cpe R	ACCAGCTGGATTTGAGTTTAATG			
cpi F	AAACGCATTAAAGCTCACACC	293	*iap*	[8]
cpi R	CTGCATAACCTGGAATGGCT			
cpa F	GTTGATAGCGCAGGACATGTTAAG	402	*cpa*	[9]
cpa R	CATGTAGTCATCTGTTCCAGCATC			

for RNA and DNA isolation. DNA isolation was performed using DNAzol (ThermoFisher, USA), and RNA isolation using EZ-RNA Total RNA Isolation Kit (BI Biological Industries, Israel). Positive control bovine herpesvirus type 1 (BHV-1), bovine virus diarrhea virus, and Colorado strain (BVDV), strain NADL with DNA and RNA isolation was performed.

In order to determine the presence pestivirus from the suspected tissue, the isolated RNAs and their panpestivirus gene-specific primers of RT-PDiscCR was performed according to a previously defined procedure [10] to determine the presence of BHV-1 from the suspicious tissue using BHV-1 specific primers. The PCR analysis was carried out as described earlier [11].

No evidence for the presence of pestivirus or BHV-1were found in RT-PCR and PCR analysis (data not shown).

DISCUSSION

Infection caused by *C. perfringens* type E has not been reported in calves in Turkey. However, a case of enterotoxemia due to *C. perfringens* type A in a cow was reported earlier with only microbiological findings [12]. *C. perfringens type E* was found to be responsible for 4.0% (45 out of 1113 strains) of all clostridial enterotoxemia cases in calves in the USA [3]. Absence of complete immunoprophylaxis and lack of drug treatment are the issues that make the type E enterotoxemia important in animals. The important factor in the pathogenesis of clostridial enterotoxemia is the presence of starch forming a suitable environment for the propagation and proliferation of such saccharolytic bacteria in the small intestine [1,4]. When the calves were weaned, enough intestinal nutrients might pass through the intestines as it takes time for the rumen flora adaptation. This assumption might explain the higher prevalence of toxin type E in weaning calves as in the presented case. For differential diagnosis and/or concurrent occurrence of BVD and herpesvirus were excluded by PCR examination as these viruses have immunosuppressive feature.

Overall, toxinotype E could be considered in differential diagnosis in fibrino-hemorrhagic enteritis and sudden deaths in calves. Pathological examination would be highly important to reveal the importance of type E toxinotype in cattle industry. Further studies are necessary to clarify the mechanisms involved in the pathogenesis of enteritis caused by this toxinotype, and the role of iota toxin during the disease.

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Case Report

Lipid-Laden Aqueous Humor in a Cat

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Abstract

This case report describes the clinical findings in a 5 years old neutered female cat presented with sudden onset of binocular blindness with cloudy appearance in both eyes. Systematic eye examination was performed after the anamnesis. Only intraocular pressure was increased in right eye. The examination of iris was not properly due to the opacity of humor aquosus. Liver function was checked in terms of diabetes and pancreatitis. Cholesterol and triglyceride levels were increased in blood analysis. The diagnosis was hyperlipidemia and hypertriglyceridemia. Dexamethasone (0.5 mg/kg) was applied to the subconjunctivaly; dexamethasone 0.1% w/v opthalmic solution drops (6 times in a day) and low-fat diet were recommended in the continuation of the treatment. The patient was immediately responded to the treatment within 36 hours. Aqueosus humor reached clear structure in both eyes. A visual activity was improve in the eyes. Post-operatively, the case was followed for 8 months. There were no complications encountered. This report is to contribute to increased awareness regarding the some ocular complications following diet programmes. In addition, the use of steroid ophthalmic solutions along with a diet program in the treatment protocol will accelerate the healing process quickly.

Keywords: *Cat, Hyperlipidemia, Hypertriglyceridemia, Lipid-laden*

Bir Kedide Lipid-Laden Humor Aquosus

Öz

Bu olgu sunumunda, 5 yaşındaki dişi kedinin her iki gözünde ani olarak şekillenen bulutumsu görünüm ile karakterize körlüğe ilişkin bulgular anlatılmaktadır. Anamnez bilgileri alındıktan sonra, olguya gözde sistemik göz muayenesi yapıldı. Göz içi basıncı sağ gözde yükselmişti. İris'in muayenesi, humor aköz daki opasiteden dolayı sağlıklı yapılamadı. Karaciğer fonksiyonlarına bakılarak, diyabet ve pankreatitis yönünden muayene edildi. Kan analizi sonuçlarında kolesterol, trigliserit miktarlarındaki artışın olması, teşhisin hiperlipidemi ve hipertrigliseridemi olduğunu gösterdi. Olguya az yağlı diyet ve subkonjunktival deksametazon (0.5 mg/kg) ve deksametazon %0.1 w/v oftalmik solüsyon damlaları (günde 6 kez), tavsiye edildi. Olgu 36 saat içinde sağaltıma yanıt verdi. Humor aquosus her iki gözde de berrak yapısına ulaştı ve görüş vardı. Post-operatif olarak olgu 8 ay takip edildi. Herhangi bir komplikasyonla karşılaşılmadı. Bu olgu sunumu, kedilerin gözlerinde meydana gelen bazı problemlerde diyet programlarının önemini vurgulamak için paylaşılmıştır. Ayrıca hiperlipidemia olgularında tedavi protokolüne diyet programı ile beraber steroid oftalmik solüsyonların önerilmesi iyileşme sürecini hızlandıracaktır.

Anahtar sözcükler: *Kedi, Hiperlipidemi, Hipertriglyceridemi, Lipid-laden*

INTRODUCTION

Primary lipid disorders are not commonly observed in cats. Lipid disorders secondary to hepatic lipidosis, diabetes mellitus, pancreatitis, hyperadrenocorticism and administration of pregestagens are more likely to account for fasting lipaemia in this species [1]. Lipid and/or mineral accumulation appears as sparkly, crystalline or shiny white areas in the cornea. Lipid metabolism is more affected in obese cats. Plasma triglyceride and cholesterol concentrations were significantly increased in obese cats [2]. As a result of increasing the lipid concentration in cats, lipids pass through the blood-aqueos barrier in the eye and cause the anterior chamber to become opaque or cloudy [3]. The incidence of obesity increases with age and is more frequent in neutered than intact animals. After the neutered cats its important to start special diet for prevent obesity and obesity-related diseases [1]. The aim of this report is to contribute to increased awareness among veterinary practitioners regarding the ocular complications following diet programmes.

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CASE HISTORY

A five-years-old neutered female cat with the complaint of sudden onset of binocular blindness due to a white cloudy appearance in both eyes *(Fig. 1)*. The owner was noted significant weight gain last 4 months after the sterilization procedure. Firstly, the examination is conducted in dim ambient light. Respectively, schirmer tear test and intra-ocular pressure were measured. Intraocular pressures was 15 mmHg in the left eye and 35 mm Hg in the right eyes, The result of schirmer tear test was shown referals. There was no obvious change in size and conformation of both eyes. The surface of cornea was clear and there was no evidence of vascularization, or ulceration during routine examination of both eyes under illumination of light. It was impossible to observe and evaluate the pole components of the anterior segment such as the anterior camera and iris because of the homogeneous blurred white color eyes. Nevertheless, iris has been evaluated for uveitis. Routine hematologic and serum biochemistry profiles were checked for the presence of inflammatory disorders. The bood was taken for complete blood count *(Table 1)*, blood gas *(Table 2)* and serum biochemical profile analysis. Serum Gutamic-Pyruvic Transaminase (SGPT) 178 U/L (normal value 25-97 U/L), Cholesterol 182 mg/dL (normal value 71-156 mg/dL), Triglyceride 117 mg/dL (normal value 10-114 mg/dL), Amylase 2584 U/L (normal value 500-1800 U/L), LDH 135 U/L (normal value 58-120 U/L) [1]. The inflammation was not observed. According to the ocular signs and laboratory findings, a diagnosis of lipemia and subsequent lipid-laden aqueous humor was considered. Treatmet started with subconjunctival dexamethasone sodium phospate injection and Dexamethasone 0.1% w/v Opthalmic Solution Drops (6 times in a day) were prescribed for white colored eye. Eye drops solution that each ml contains 22.26 mg of dorzolamide hydrochloride

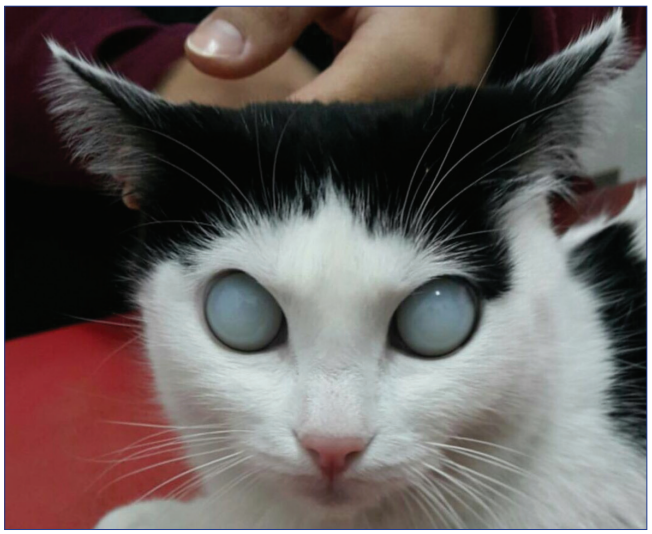

Fig 1. A five-years-old neutered female cat with the complaint of sudden onset of binocular blindness due to a white cloudy appearance in both eyes

Table 1. *Complete blood count results of case*

Parameters		**Values**
Blood gas values	pH	7.327
	pCO_2	30.6 mmHg
	pO_2	41.8 mmHg
Oximetry values	ctHb	13.0 g/dL
	Hctc	400%
	sO_2	62.2%
	FO_2Hb	59.3%
	FCOHb	2.7%
	FHHb	36.1%
	FMetHb	1.9%
	FHbF	45%
Electrolyte values	cK^+	3.4 mmol/L
	cNa^{++}	165 mmol/L
	cCa^{++}	0.81 mmol/L
	cCl^-	127 mmol/L
Metabolite values	cGlu	113 mg/dL
	cLac	1.4 mmol/L
Oxygen status	ctO_2c	10.9 Vol%
	p50c	34.90 mmHg
Acid-base status	cBase (Ecf)c	-10.0 mmol/L
	$cHCO_3$	16.0 mmol/L

Table 2. *Venous blood gas results of case*

Parameters	**Values**
WBC	19.41 m/mm³
Lym.	57.0%
Mon.	6.9%
Gra.	36.1%
Lym#	11.06 m/mm³
Mon#	1.33 m/mm³
Gra#	7.02 m/mm³
RBC	10.83 m/mm³
MCV	43.6 fl
Hct	47.2%
MCH	13.5 pg
MCHC	31.1 g/dL
RDW	10.8
Hb	14.7 g/dL
THR	213 m/mm³
MPV	10.2 fl
Pct	0.22%
PDW	9.0

Fig 2. After the drug administration intraocular components are clearly visible

corresponding to 20 mg dorzolamide and 6.83 mg of timolol maleate corresponding to 5 mg timolol drops were prescribed for high intraocular pressures. Urgently low-fat diet was recommended to patient. On the other hand, it was also assessed in terms of pancreatitis due to the high amylase level.

On the next day ophthalmologic examination, it was observed that the cloudy white color completely disappeared on both eyes and the intraocular components were clearly visible and there was no abnormality in iris shape and conformation *(Fig. 2)*. For this reason, it was emphasized that lipidemia was the main reason. Intraocular pressures were 15 mmHg in the left eye and 37 mmHg in the right eyes for next day. The pupil size and pupillary light response were determined normal in both eyes. On direct ophthalmoscopy, optic disc and tapetal area appeared normal and both eyes seemed to be visual. Retinal examination was not shown any a pale appearance. Thus, no problems were encountered in the follow-up period of 8 months after the treatment and suggestion.

DISCUSSION

Primary lipid disorders are not commonly observed in cats. Lipid disorders secondary to hepatic lipidosis, diabetes mellitus, pancreatitis, hyperadrenocorticism and administraiton or pregestagens are more likely to account for fasting lipaemia in this species [2]. Lipid and/or mineral accumulation appears as sparkly, crystalline or shiny white areas in the cornea. Plasma triglyceride and cholesterol concentrations were significantly increased in obese cats, compared with lean cats [2]. As a result of increasing the lipid concentration in cats, lipids pass through the blood-aqueos barrier in the eye and cause the anterior chamber to become opaque or cloudy [2]. The incidence of obesity increases with age and is more frequent in neutered than intact animals [4]. Special diet programme should be started to prevent obesity and obesity-related diseases in neutered cats [5,6]. The more common ocular consequences of hyperlipidemia in small animals include lipemia retinalis and lipid-laden aqueous humor. In patients with lipemia retinalis, retinal vessels appear white or creamy pink, which is reminiscent of blood taken from an animal that has recently eaten a fatty meal [1]. The blood-aqueous barrier generally prevents leakage of large molecules, like lipoproteins, into the aqueous humor but when barrier has a disorder lipids then enter the eye and cause the aqueous humor to appear turbid, variably cloudy, and, in some cases, completely opaque [1]. Increased triglycerides may result in brain dysfunction, acute pancreatitis, lipid-laden aqueous humor with anterior uveitis, and lipemia retinalis. Increased serum cholesterol can cause lipid keratopathy [6]. The main therapy of primary hyperlipidemia involves feeding a low-fat diet with moderate protein content. Diets low in protein may cause an increase in serum cholesterol concentration and are therefore not recommended unless the presence of other conditions warrant their use.

Although, lipid-Laden or hyperlipidemia is not seen very common in cats, but it should be considered in cats for the wrong diet programme. Cholesterol and triglycerides should be measured for diagnosis and prognosis, after routine eye examination. Uveitis must be assessed before diagnosis of lipid-Laden. Treatment options for hyperlipidemia include treatment of inciting diseases, diet modification, and pharmacologic intervention. Hyperlipidemia secondary to an underlying disorder will probably resolve or improve after the metabolic disturbance is corrected.

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Letter to the Editor

Utility of Transesophageal Echocardiography in the Diagnosis of Tetralogy of Fallot in a Dog
(Bir Köpekte Fallot Tetralojisinin Tanısında Transözefagal Ekokardiyografinin Yararlılığı)

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Dear Editor,

Tetralogy of Fallot (ToF) is an uncommon complex congenital cardiac defect in dogs and cats. Its incidence in dogs has been reported to be 0.0025% [1,2]. Transesophageal echocardiography (TEE) can be valuable in diagnosis and accurate study of complex congenital heart defects in human medicine, but there is limited data on this subject in veterinary medicine [3]. Thus, we reported here utulity of TEE compared with trans-thoracic echocardiography (TTE) for the diagnosis of ToF in a dog.

A 4.5 months-old, male, English Bulldog was presented with a history of poor growth, respiratory stress and exercise intolerance since one-month. Physical examination revealed pale mucous membranes, precordial thrill and murmur (4/6 grade) at right and left cardiac bases suggestive for the presence of congenital cardiac defect. Thoracic radiography showed cardiomegaly (vertebral heart score 12.4; reference <10.5), alveolar oedema, bulging of the main pulmonary artery (1 o'clock position on VD view) and dorsal deviation of caudal vena cava and trachea (LL view). Common causes of pulmonary artery bulge include pulmonary hypertension, pulmonic stenosis, patent ductus arteriosus (PDA) or aortopulmonary window (APW) [4]. In this dog, PDA and APW were not considered during diagnostic work-up because of which continuous machinery murmur was not auscultated, and ECG revealed small complexes of QRS and deep S wave on lead II suggestive for right-sided heart disease [3].

By use of standard TTE techniques, left ventricular (LV) dimensions and interventricular septum and LV free wall thickness at diastole and systole were observed within reference ranges [4], but fractional shortening (16%; reference >25%) and ejection fraction (48%, reference >50%) were lower than their references, incompatible with the poor LV contractility. Wall thickness of right ventricle (RV: 1.71 cm) was nearly equal to that of LV (1.73 cm). Since the RV free wall thickness is normally less than 1/2 of the LV free wall thickness [4], our observation indicated RV hypertrophy due to pulmonary hypertension, pulmonic stenosis, RV outflow tract obstruction or congenital cardiac defect such as ventricular septal defect (VSD) and ToF. TTE had a limitation to show further details in this dog, that is, it was challenging to take the high quality images and video records of diagnostic importance due to anatomical structure of the thorax with dorsoventral compression and narrow intercostal spaces in this breed (English bulldog).

TEE allows imaging of the heart through the oesophagus using a special transducer mounted on a modified endoscope. The proximity of the heart and minimal intervening structures enables the acquisition of high-resolution images that are consistently superior to routine TTE and optimal imaging of the heart base anatomy and related structures [3].

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Thus, in this dog TEE was performed with anesthesia protocol in this dog as reported in our case presentation [5]. Maximal velocities (2.79 m/s; reference <1.5 m/s) and pressure gradients (31.2 mmHg; reference <25 mmHg) of main pulmonary artery indicated the presence of mild pulmonic stenosis. On the parasternal long axis view, VSD (defect size: 0.88 cm) and overriding aorta were detected and thereafter color Doppler imaging of the defect showed high velocity blood flow (Vmax: 4.5 m/s, PG: 82.4 mmHg) from LV into RV and pulmonary artery (left to right shunt). In addition to color Doppler imaging *(Fig. 1)*, micro-bubble study showed the defect localization just below the pulmonary valve and between two ventricular chambers, suggestive for a perimembranous or conoventricular VSD (type-1) in this case. Based on these observations including a VSD, pulmonic stenosis, overriding aorta and right ventricular hypertrophy, ToF was diagnosed in this dog, as suggested [2,4].

Before surgical correction of the defect, general condition of the dog was considered to improve by increasing cardiac contractility and decreasing cardiac volume over load and pulmonary oedema with medical therapy. Thus, diuretic (furosemide 2 mg/kg 2x1 po), ACE-I (enalapril 0.5 mg/kg, 1x1, po) and inodilator (pimobendan 0.5 mg/kg divided twice daily, po) were suggested [4]. Unfortunately, the dog died at home one week after initial of the therapy. Necropsy confirmed the presence of ToF and their diagnostic criteria [2,4].

In conclusion, TEE plays a crucial role in the anatomical, functional, and hemodynamic evaluations of dogs with a wide range of congenital and acquired heart diseases. This case presentation suggests that TEE as compared to traditional TTE is superior to show complex cardiac pathology such as ToF in English bulldog that has unique anatomical structure of thorax. TEE can offer high quality image, however it may be high invasive and critical for some patients, especially with respiratory distress. Poor LV contractility in these cases should also be considered as a poor prognostic indicator despite medical support, as well.

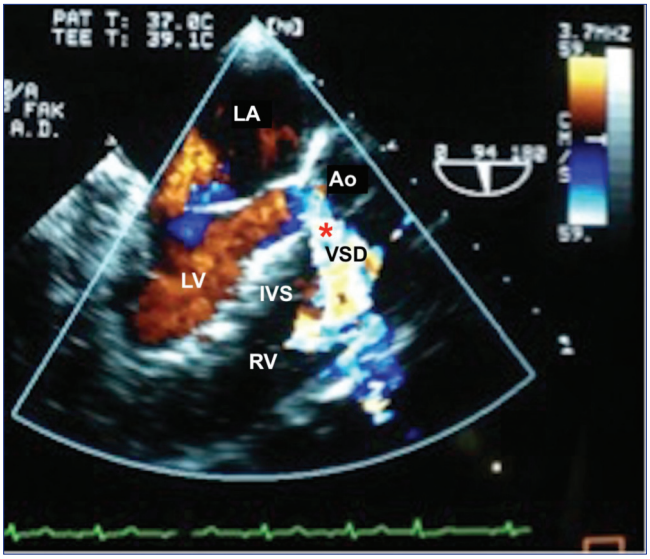

Fig 1. The color flow image of TEE showed the defect with severe turbulence flow on parasternal long axis view

Ao: Aort, LA: Left atrium, RA: Right atrium, IVS: Interventricular septum, VSD: Ventricular septal defect, * defect

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Letter to the Editor

Acute Erosive Gastritis Due to Pine Processionary Caterpillar Setae Ingestion in a Dog
(Bir Köpekte Çam Kese Böceği Setalarının Yenilmesine Bağlı Akut Eroziv Gastritis)

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Dear Editor,

Direct contact with the Pine Processionary Caterpillar (PPC) *Thaumetopoea* species induces severe local allergic and toxic reactions both in man, dogs even in cats in Mediterranean countries, with the necessitate of medical intervention. Caterpillars are the larvae of these insects look like hairy and colored orange-brown with blue bands, have covering of irritant hairs which called as setae; along its whole body except ventral surface [1]. These chitinous spines are rich in thaumetopoein, which makes them an urticating protein [2]. Furthermore, setae of the caterpillar could also keep their potential toxicity in the left nests after chitin changing period [3]. The aim of the present case report is to define first clinical manifestation of an acute erosive gastritis due to PPC setae ingestion in a four months old, male Pembroke Welsh Corgi.

The dog was presented to our Veterinary Teaching Hospital with complaints of acute persistent vomiting in April 2018. The owner informed that the dog had been playing with a pine cone covered with cotton wool just before the persistent vomiting had started. Any abnormalities except persistent vomiting were seen in the physical examination in the dog and initial direct and indirect radiographs were taken for elucidating of a suspicious foreign body. Both radiographs were reflects any problem among the gastrointestinal and other systems. Laboratory findings both whole blood count and serum biochemical profile were also revealed as normal. Later upper gastrointestinal system endoscopy was performed to the dog for a definitive diagnosis. Multiple esophageal and gastric petechial hemorrhages with erosions were started to be seen from the oropharaynx to the pylorus sphincter *(Fig. 1A-H)*. Any foreign body or PPC was seen in both esophagoscopy and gastroscopy. After wakening the effects of sedation procedure the dog starts to vomit again and this time the vomit content was collected and pine pollens were detected during the microscopic examination *(Fig. 1I)*. Finally we confirmed that the "cotton wool" on the pine cone was a nest of the PPC, and acute erosive gastritis due to PPC setae ingestion was diagnosed in the dog based on the endoscopic and microscopic examination of the vomit content with clinical examination. Treatment was started with intravenous antiemetic and fluid supplementation with oral use of gastrointestinal mucosa protectants for several days with restriction of both food and water consumption till the vomiting ended. The dog was being well after the second day of treatment and started to eat.

There have been numerous PPC direct contact reports in animals and man whole around the world. Moreover, those describe the most common findings and treatments in dogs [4,5]. Furthermore, similar reports in dogs were also presented in Turkey [6-8]. The most common clinical findings in such cases are lingual, sublingual and submandibular oedema, lingual necrosis, ptyalism, facial pruritus and vomiting. On the other hand, partial loss of the tongue, following a necrotizing glossitis is the most frequent sequel in dogs with the direct contact of PPC. Contrarily of those reported direct contact animal cases, our event differs with describing an indirect contact of this PPC. Additionally, to the authors' knowledge ingestion of this PPC setae in animals have not been reported before.

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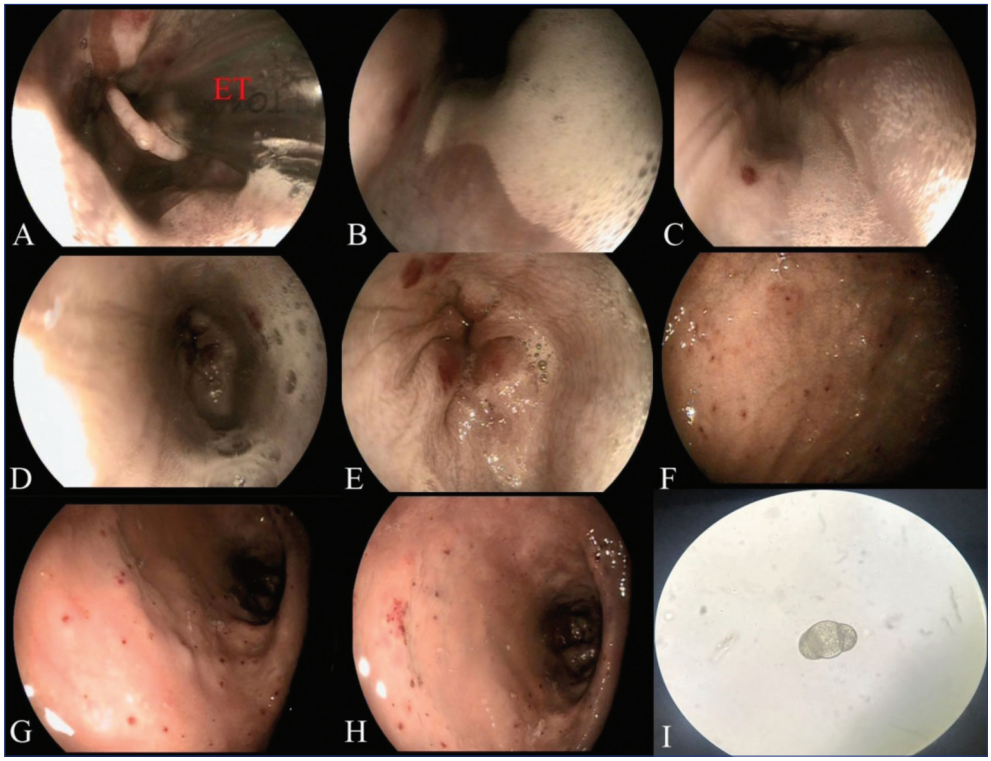

Fig 1. Views of the petechial hemorrhages with erosions in the oropharaynx (A), esophagus (B, C, D), cardiac sphincter (E), corpus of the stomach (F), pylorus sphincter (G, H). View of the pine pollen during the microscopic examination of the vomit content (I); ET: Endotracheal Tube

In conclusion, this case presentation, defining the first clinical manifestation of an acute erosive gastritis due to PPC setae ingestion in a Pembroke Welsh Corgi, could be a useful report for diagnosing unspecified persistent vomiting cases in dogs especially in spring time in the Mediterranean countries.

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Review

Availability, Use and Development of Animal Models for the Assessment of Drug-Drug Interactions and Safety of Direct Oral Anticoagulants

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Abstract

Direct oral anticoagulants (DOACs) are novel, direct acting drugs that are selective for either thrombin or activated factor X. Due to their obvious benefits for patients (broader therapeutic window, are not routinely monitored etc.), they are increasingly used as an alternative to vitamin K antagonists. One of the major indications for the use of DOACs is the stroke prevention in patients with atrial fibrillation (AF). Although, the DOAC use becomes extensive in the clinical area especially cardiology, many drug drug interactions occur when DOACs are used with other drugs. Also, the safety profile of DOACs is still to be investigated. Animal models can be used to investigate the drug drug interactions and safety of DOACs under standart laboratory conditions. Unfortunately, there is not sufficient data that investigates the drug drug interactions and safety of DOACs in animal models. The focus of this review will be the availability, use and development of animal models to assess drug drug interactions and safety profile of DOACs.

Keywords: *DOACs, Animal models, Drug-drug interaction, Safety*

Yeni Nesil Oral Antikoagulanların İlaç İlaç Etkileşimleri ve Güvenliliğinin Değerlendirilebilmesi İçin Hayvan Modellerinin Geliştirilmesi, Kullanımı ve Bulunabilirliği

Öz

Direkt oral antikoagulanlar (DOAK'lar), yeni, thrombine veya aktive faktör Xa'ya selektif olan direkt etkili ilaçlardır. Bu yeni oral antikoagulan ilaçların, klasik antikoagulan ilaçlara göre hastalar açısından çok sayıda avantajları vardır (izleme ihtiyacının olmaması, geniş tedavi penceresi vbg.). Yakın geçmişte, direkt oral antikoagulanlar, tromboembolizmi önleme ve tedavisinde K vitamini antagonistlerine alternatif olarak sunulmuşlardır. DOAKların ana kullanım alanı, atrial fibrilasyonlu hastalarda inmenin önlenmesidir. Ancak, öte yandan, DOAK'ların diğer ilaçlar ile kombine kullanımı sırasında ilaç-ilaç etkileşimleri oluşabilmekte ve güvenlilik profilleri henüz yeterli düzeyde bilinmemektedir. Günümüzde, DOAK'ların ilaç-ilaç etkileşimi ve güvenliliğini araştırmada kullanılan hayvan modelleri henüz yeterli değildir. Bu derlemenin amacı, DOAK'ların ilaç-ilaç etkileşimi ve güvenliliğini araştıran hayvan modellerinin daha da geliştirilmesi, kullanımı ve bulunabilirliğidir.

Anahtar sözcükler: *DOAK, Hayvan modelleri, İlaç-ilaç etkileşim, Güvenlilik*

INTRODUCTION

Vitamin K antagonists (VKAs) were the only available oral anticoagulants for more than 50 years. They are proven effective in preventing stroke. There are already known disadvantages of vitamin K antagonists. These disadavantages can be explained as: they have narrow therapeutic window, there is a need for regular monitoring of the international normalized ratio (INR) and have potential to interact with various drugs and food products [1].

In recent years, the non-vitamin K dependent new direct oral anticoagulants (DOACs) dabigatran, rivoraxaban, apixaban and edoxaban have been introduced into the market worldwide. Due to their clear benefits for patients, they have gained popularity as alternatives to VKAs in

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various clinical areas. In major trials, DOACs have been found effective in preventing stroke. DOACs are increasingly used as an alternative to warfarin, predominantly for indications such as stroke reduction in atrial fibrillation, prevention and treatment of venous thromboembolism and pulmonary embolism [2].

DOACs have been developed in response to the limitations of VKAs. Compared to VKAs, DOACs have several advantages. They are characterized by more defined pharmacokinetic and pharmacodynamic profiles.They have more predictable half-life and elimination. In a lot of indications, DOACs are rapidly replacing vitamin K antagonists due to their ease of use for patients such as without the need for laboratory monitoring. DOACs have improved efficacy and safety ratio and non inferior bleeding risk than VKAs [1].

It is already known that DOACs have fewer drug-drug interactions compared to VKAs. In a recent review about the interactions and safety of DOACs with antiarrhythmic drugs, even though DOACs have fewer interactions with antiarrhythmic drugs than vitamin K antagonists, it has been reported that, required dose adjustments and contraindications are still important to be kept in mind [3].

Despite many favoring properties in clinical trials, the use of DOACs has been relatively modest. In terms of safety, meta analyses of randomized controlled trials suggest that DOACs are noninferior to VKAs for overall risk of bleeding complications. In case of bleeding events, there is a need for an effective anticoagulation reversal strategy by using an antidote [4].

The reversal of DOACs in animal models are already explained in the previous literature. There are suitable animal models for the reversal of DOAC effects. Interestingly, there is not sufficient data that investigates the safety and drug-drug interactions of DOACs in animal models.

This review tries to explain the questions that could be asked in this area:

1. What are the animal models available?

2. What are the perspectives and limitations of the animal models currently in use?

3. How can we investigate safety on animals?

4. Which are the most promising models currently in use? How can they berefined?

5. How could findings generated by the animal models be translated clinically?

The focus of this review will be the availability, use and development of animal models for the critical assessment of drug-drug interactions and safety of DOACs.

DIRECT ORAL ANTICOAGULANTS (DOACs)

a. Direct Thrombin Inhibitor: Dabigatran

Dabigatran etexilate is a potent and direct inhibitor (competetive) of thrombin, both free and bound to fibrin. It is a prodrug with a bioavailability of 6%. After oral administration it is rapidly converted to dabigatran by serum esterases. Dabigatran has a serum half life of 12-17 h and 80% of the drug is excreted by the kidneys [5].

The RE-LY trial showed that dabigatran at a dose of 150 mg BID was superior to warfarin in preventing stroke and systemic embolism with a similar risk of major bleedings [6].

b. Direct Factor Xa Inhibitors: Rivaroxaban, Apixaban and Edoxaban

Rivaroxaban and apixaban are the two direct inhibitors of factor Xa. These agents reversibly inhibit free and clot bound factor Xa. They prevent the conversion of prothrombin to thrombin and subsequent fibrin clot formation. They have a high bioavailability (around 50% in apixaban, 62% in edoxaban to 100% in rivaroxaban when taken with food) [7].

ANIMAL STUDIES IN DOAC RESEARCH

This part of the review will try to explain what are the animal models available and what are the perspectives and limitations of the animal models currently in use?

Research animals that are used in experimental studies are valuble tools for understanding the pathophysiology of a situation or in developing therapeutic interventions for a disease. Feasibility, similarities to human and drug safety are the basic reasons that animals are used in biomedical research. Animal models are relatively easy to manage. The dietary intake and environmental factors (temperature and lighting) can be controlled easily in standard laboratory conditions. Compared to human studies, there is relatively less environmental variation. Many animals are suitable due to their similarity in anatomical basis and physiological functions with humans. Before the compounds are used in humans, preclinical toxicity testing, pharmacodynamics and pharmacokinetics profile of drugs may be investigated on animals. Prior to testing on humans, the effectiveness of a drug as potential treatment needs to be carried out on animals [8].

Animal models can provide a means to investigate “the safety and drug-drug interactions of DOACs” by different strategies under standart laboratory conditions. As DOAC use becomes extensive in the clinical area, the concept of the safety and drug-drug interactions of DOACs becomes more important.

The concept of the safety and drug-drug interactions of DOACs in animal models becomes mandatory in defining appropriate treatment strategies. All relevant research from the pubmed were checked via using the following key search terms: *safety, drug-drug interactions, DOACs, antidotes, animal models*. In the present literature, mouse, rat, rabbit, pig and baboons are the mostly used species in anticoagulation studies.

There are advantages and disadvantages of the species used for the assessment of anticoagulation reversal: In small animal models (for example: rodents and rabbits) high numbers can be used for the selection of dose. Due to lower body weight of small animals, the drug requirements are lower. With rabbits, the sample collection and regional tissue assessment are more simple.

In the use of large animals (pigs) organ size, blood volume and hemodynamic response are comparable to humans. The sample collection is simple and polytrauma can be inflicted. There may be disadvantages of small animal use (rodent and rabbit) such as small animals have low blood volume and might be less suitable for use in trauma studies. The disadvantages of the use of large animals may be the ethical approvals that can be harder to take and standardization of the study may be more difficult [9].

Animal studies have shown that specific antidotes are likely to be effective means of reversing the anticoagulant effects of DOACs. The most informative experiments for studying DOAC anticoagulation and its reversal are those conducted in large animals with relevant trauma and severe bleeding with hemorrhagic shock and animal species with pharmacological relevance. The animal species and the DOACs used in previous studies are summarized in *Table 1*.

In terms of the assessment of anticoagulation and its reversal, in the review paper of Honickel et al.[9], it was considered that "bleeding volume" should be regarded as the gold standard of outcomes. The "comparisons of bleeding volume versus bleeding time" have been found as a more sensitive and reliable outcome.

There are different "animal models" that has been used in experimental studies of DOACs that can be summarized as:

1. Intracerebral hemorrhage (ICH) [10]
2. Tail bleeding [11-13]
3. Carotid artery occlusion [13]
4. Standardized kidney incision [14]
5. Blunt liver injury [15,16]
6. Mesenteric bleeding [17]
7. Liver laceration [18]

Standardized animal models with standardized outcomes are essential to understand the effects of DOAC reversal research and treatments. The models already used in DOAC reversal research can be explained herein after as:

In a murine intracerebral hemorrhage model associated with rivaroxaban, the reversal of rivaroxaban was studied, and prothrombin complex concentrate, Factor VIIa, and fresh frozen plasma prevented excess intracerebral hematoma expansion [10]. CD1 mice were given warfarin or dabigatran by gavage, and the effects on *in vitro* coagulation assays, volume of blood loss and the bleeding time following tail transection injury were evaluated with different reversal agents. Prothrombin complex concentrates (PCC) reduced blood loss in murine coagulopathy induced by warfarin. PCC treatment prevented excess bleeding much more effectively in warfarin-induced coagulopathy than in dabigatran-induced coagulopathy [11].

In the carotid artery occlusion model, it has been shown that γT -S195A-IIa decreased the anticoagulant effects of dabigatran *in vitro* [13]. The reversal of dabigatran anticoagulation by prothrombin complex concentrate (Beriplex P/N) in a rabbit model has been investigated and it has been found that the prothrombin complex concentrate showed potential as an agent for reversing the effects of dabigatran [14].

In the liver trauma model, prothrombin complex concentrate and activated prothrombin complex concentrate were found effective in reducing the anticoagulant effects of dabigatran under different conditions [15].

Table 1. *The animal models, the species and the used Direct Oral Anticoagulant in studies*

Author	Animal Model	Used DOAC	Animal Species Used
Zhou W [10]	Intracerebral hemorrhage	Rivaroxaban 3; 10 or 30 mg/kg	Mouse (C57BL/6)
Lambourne MD [11]	Tail bleeding	Dabigatran 60 mg/kg, oral (1.5 mg)	Mouse (CD1)
Van Ryn J [12]	Tail bleeding	Dabigatran 30 mg/kg, oral	Rat
Sheffield WP [13]	Tail bleeding, Carotid artery occlusion	Dabigatran 13 mg/kg and 60 mg/kg	Mouse
Pragst I [14]	Standardized kidney incision	Dabigatran 0.4 mg/kg, i.v.	Rabbit
Honickel M [16]	Blunt liver injury, Bilateral femur fractures	Dabigatran 30 mg/kg twice daily	Pigs
Perzborn E [17]	Mesenteric bleeding, Incision on forearm	Rivaroxaban 2 mg/kg i.v. 0.6 mg/kg i.v bolus	Rat; Baboon
Lu G [18]	Liver laceration	Rivaroxaban 1 mg/kg i.v. bolus	Rabbit

A novel antithrombotic agent BAY 59-7939--an oral, direct Factor Xa inhibitor has been studied and the bleeding times in rats and rabbits were not significantly affected at antithrombotic doses (3 mg kg(-1) p.o., AV shunt) and based on the studies results, BAY 59-7939 has been selected for clinical development [17].

In the concept of this review it is important to mention that preclinical safety and efficacy of andexanet alfa in animal models were investigated, and it has been found that, andexanet is a promising therapy for the reversal of FXa inhibitor-induced anticoagulation, supporting clinical studies in humans [19].

DEVELOPMENT OF AN ANIMAL MODEL TO INVESTIGATE THE "SAFETY AND DRUG DRUG INTERACTIONS" OF DOACs

From a pharmacological point of view, for a rational selection of an animal model to study a drug-drug interaction, the animal model should be similar to humans in terms of pharmacokinetic parameters (absorption, distribution, metabolism or excretion [ADME]) processes. In previously published animal models, we also studied with different kind of animal species and develop animal models that are closely reflect the disease scenario similar to pathologies seen in humans (diabetes model, ageing, alcoholic animal models, polymicrobial sepsis model). We characterized the effects of different drugs in terms of their effectiveness, safety and tolerability in the Balb/c mouce, Wistar albino rats and rabbit models [20-24].

As it is already known, cytochrome-p450-system and the p-glycoprotein transport system plays a key role in the DDIs of DOACs. To investigate the safety and drug-drug interaction (DDI) potential of a DOAC and understanding of the underlying mechanism for DDI of a DOAC, different animal models can be developed; especially, *in vivo* animal models might be valuable in this situation.

There are *in vitro* studies that has been done previously: the *in vitro* assessment of pharmacokinetic drug-drug interactions of DOACs has been studied and strong *in vitro* inhibition of DOAC efflux by PDE5 has been shown [25]. Animal and *in vitro* studies in a human placental perfusion model indicated that apixaban, dabigatran and rivaroxaban, respectively, exhibit placental transfer [26].

On the other hand, the literature on the safe use of DOACs includes the management in special situations such as renal impairment, overdosage and bleeding risk. Role of renal function and hepatic function in co-administration of DOACs with other drugs should be taken into consideration to study on the effective animal model. It may be also important to investigate the "safety profile of DOACs" first in *naive animals* without a pathology (renal or hepatic impairment) and then develop a pathological situation (such as bleeding, renal impairment, hepatic impairment) especially for safety studies.

NOVEL APPROACHES TO THE REVERSAL OF DIRECT ORAL ANTICOAGULANTS -THE ANTIDOTES

For the reversal of the DOACs, class and drug-specific compounds are currently in development. A class-specific drug, andexanet alfa, is being developed for the reversal of the oral factor Xa inhibitors and has also shown reversal activity against the indirect Xa inhibitor enoxaparin. A drug-specific agent, currently before the FDA for approval based on data from healthy volunteers as well as patients with active bleeding or needing urgent reversal in preparation for surgery is the idarucizumab [27].

A global universal inhibitor ciraparantag, has demonstrable activity against direct oral factor IIa and Xa inhibitors as well as the indirect Xa inhibitor enoxaparin, and UFH.

Three antidotes are currently in development for DOAC reversal. To date idarucizumab has been approved in the USA for the reversal of dabigatran. Andexanet has completed phase 3b/4 study and is pending further presentation of data. A third one is aripazine which is in phase II trial [28]. DOAC specific antidotes and the synthetic antidote is shown in *Table 2*.

TARGETED REVERSAL DRUGS

1. Idarucizumab (BI655075-Dabi-Fab)(Praxbind): Dabigatran Antidote

Idarucizumab (fragment antigen-binding; Fab) was licensed in 2015 as a specific reversal agent for dabigatran. It is approved for the emergent reversal of the anticoagulant effects of dabigatran [28].

It is a humanized mouse monoclonal antibody fragment. It binds dabigatran and reverses its anticoagulant effects. Idarucizumab has an extremely high affinity for dabigatran and is able to reverse dabigatrans anticoagulant effects at a 1:1 stoichiometric ration. It is generated from mouse monoclonal antibody, then humanised and reduced to a Fab fragment. It binds to the thrombin binding site

Table 2. *DOAC antidotes*

DOAC-Specific Antidotes	Synthetic Antidote
Idarucizumab	Aripazine (ciraparantag; PER977)
Andexanet Alfa (PRT 064445)	

DOAC-Specific antidotes: *Idarucizumab, Andexanet Alfa (PRT 064445)*
Synthetic antidote: *Aripazine (ciraparantag; PER977)*

of dabigatran with an affinity that is 350 times as high as thrombin.

Dabigatran almost completely inhibits fibrinopeptide A formation at the wound site and idarucizumab is aimed at restoring systemic blood coagulation and reenabling the formation of this fibrin [29].

Idarucizumab is currently being evaluated for the emergency reversal of dabigatran, in the ongoing RE-VERSE AD phase 3 study. An analyses of interim data from the first 90 patients enrolled in the study showed that idarucizumab completely reversed dabigatran's anticoagulant effect within minutes, paralleled by a profound reduction in unbound dabigatran concentration. The use of idarucizumab may simplify emergency management of dabigatran treated patients with life threatening bleeds and reduce perioperative complications in patients undergoing emergency surgery [30].

Idarucizumab is administered by intravenous infusion. The half life of Idarucizumab is 45 min. It binds both free and also thrombin bound dabigatran with a rapid on rate and slow off rate. The bound complex is eliminated predominantly by renal excretion. Idarucizumab is distributed solely within the intravascular space. It eliminates dabigatran by drawing it from the extravascular spaces into the intravascular space [31].

2. Andexanet Alfa: Factor Xa Inhibitor Antidote

Andexanet alfa is a reversal agent for FXaI and is currently undergoing phase 3b to 4 trials. It binds FXaI in a 1:1 stoichiometric ratio and restores endogenous factor Xa activity via reducing anti-factor Xa activity. It works for rivaroxaban, apixaban and edoxaban [28].

It is a recombinant and an inactivated form of factor Xa engineered as a universal antidot for factor Xa inhibitors. It also binds LMWH and fondaparinux activated antithrombin III, which acts as indirect Xa inhibitors. It is a small (kDa), catalytically inactive, human recombinant modified molecule that is similar to native factor Xa. Acting as a decoy receptor, it binds and sequesters direct factor Xa inhibitors, preventing them from inhibiting the activity of the native factor Xa, thus restoring the normal haemostatic processes [29].

3. Ciraparantag (Aripazine/PER 977)

Ciraparantag (PER 977) is a small synthetic and cationic molecule that binds direct Xa inhibitors, direct thrombin inhibitors, and unfractionated and low molecular weight heparin.

Developed by Perosphere, this is a small (500 Da), synthetic, water soluble, thermally stable, cationic D-arginine compound that has broad activity against various old (heparin, LMWH) and newer anticoagulants (Dabigatran, rivaroxaban, apixaban, edoxaban) [29].

Ciraparantag is another possible option for the reversal of DOACs which seems to be promising in the future, being able to inhibit dabigatran and the factor Xa inhibitors [32].

This is the third antidote aiming to reverse all anti-thrombotic agents including direct thrombin inhibitors and FXaI. It can be difficult to monitor because it binds to drugs, and not coagulation factors. It has not exhibited procoagulant or anticoagulant properties [33].

CLINICAL CONCEPTS THAT SHOULD BE KNOWN-ANTICOAGULATION REVERSAL

The potential candidates for targeted reversal therapy is important in the decision to reverse anticoagulation [4]. It is important to consider reversal in the following patients and clinical settings:

1. Severe bleeding that results in hemodynamic compromise,

2. Organ dysfunction or a need for massive blood transfusion,

3. Patients within 24 h of receiving an anticoagulant who require emergency surgery or an invasive procedure known to be associated with a significant risk of bleeding.

Consider prior to reversal:

Other factors that must be considered prior to reversal include:

1. The time since a last dose of a DOAC,

2. Indication for anticoagulation and risk of thrombosis,

3. Drug interactions,

4. Associated renal or hepatic dysfunction and local or systemic factors,

that increase the risk of poor outcomes with bleeding, for example, advanced age. Idarucizumab, a monoclonal antibody fragment, was developed to reverse the anticoagulant effect of dabigatran. It is a multicenter, prospective, open-label study to determine whether 5 g of intravenous idarucizumab would be able to reverse the anticoagulant effect of dabigatran in patients who had uncontrolled bleeding (group A) or were about to undergo an urgent procedure (group B) in total of 503 patients. It was concluded that idarucizumab rapidly, durably, and safely reversed the anticoagulant effect of dabigatran [30]. Recent results from phase 3/4 studies demonstrate efficacy for idarucizumab (an antidote to dabigatran) and for andexanet alfa. Ciraparantag for many anticoagulants, including the DOACs, shows promise in results from phase 1 and 2 studies [34].

CONCLUSION AND FUTURE PROSPECTS

Systems related to the development of DDIs of DOACs in co-administration with other drugs are cytochrome-P450-system and the p-glycoprotein transport system. These two systems should be taken into consideration for the animal models investigating the DDIs of DOACs.

Altough, there are already known animal models that are used in the reversal of DOACs that may guide the researchers on this area there is not enough data on the animal models for the "safety and drug-drug interactions of DOACs".

Altough some animal models that are used in DOAC research are already summarized there are still questions that exists: which are the most promising models currently in use and how can they be refined? Also there is another discussion that still needs to be highlighted: how could findings generated by the animal models be translated clinically?

It might be concluded that to investigate the safety profile and DDIs of DOACs in co- administration with other drugs, the development and use of standardized invivo animal models should become true in the future.

Lastly, the findings that can be generated by the animal models should be translated clinically. This review might be an introduction to the availability, use and development of animal models interms of safety and drug-drug interactions of DOACs in pharmacological area which is a clearly lacking concept up todate.

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