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Research Article

Effect of Probiotic and Different Sources of Fat on Performance, Carcass Characteristics, Intestinal Morphology and Ghrelin Gene Expression on Broiler Chickens

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Abstract

The effect of Lactofeed probiotic and different sources of fat on performance, carcass characteristics, intestinal morphology and ghrelin gene expression of broiler chickens was studied in an experiment using a total of 240 one-day-old male chickens from commercial strain (Ross 308) in a completely randomized design via 6 treatments with 4 replicates (10 birds per replicate). The experimental diets included: (1) basal diet (control); (2) diet containing 3% animal fat from tallow (fat); (3) diet containing 3% plant oil from soybean (oil); (4) control + probiotic; (5) probiotic + (fat) and (6) probiotic + (oil). The results showed some improvement in performance in the third group ($P<0.05$). A significant difference in the length, width and depth of crypt was observed between the treatments 3 and 4, and the control group ($P<0.05$). There was a significant difference in ghrelin gene expression of the treatments 2 and 4 in comparison with the control group ($P<0.05$). The results generally showed that there were benefits from the separate use of probiotic and soybean oil in the diet of broiler chicken.

Keywords: *Broiler, Fat, Feed intake, Lactofeed, Performance*

Broiler Tavuklarda Probiyotik ve Değişik Kaynaklı Yağların Performans, Karkas Özellikleri, Barsak Morfolojisi ve Grelin Gen Ekspresyonu Üzerine Etkisi

Öz

Toplam 240 adet bir günlük Ross 308 erkek civciv kullanılarak, tamamen rastgele dizaynda 6 uygulama ve 4 tekrar olmak üzere (her tekrarda 10 hayvan) laktofed probiyotik ve farklı kaynaklı yağ ile beslemenin performans, karkas özellikleri, barsak morfolojisi, bağışıklık sistemi, karaciğer enzimleri, kan parametreleri ve grelin gen ekspresyonu üzerine etkisi incelenmiştir. Deneysel diyetler; (1) bazal diyet (kontrol); (2) %3 donyağı kaynaklı hayvansal yağ içeren diyet; (3) %3 soya fasulyesi kaynaklı bitkisel yağ içeren diyet; (4) kontrol + probiyotik; (5) probiyotik + hayvansal yağ ve (6) probiyotik + bitkisel yağ. Elde edilen sonuçlar üçüncü grupta bazı iyileşmelerin oluştuğunu gösterdi ($P<0.05$). 3. ve 4. gruplarda kontrole göre kript uzunluğu, genişiliği ve derinliğinde anlamlı oranda fark gözlemlendi ($P<0.05$). Kontrol grubu ile karşılaştırıldığında 4. grubun bağışıklık sisteminda anlamlı artış belirlendi ($P<0.05$). 4. grup kontrol grubu ile karşılaştırıldığında AST, ALP ve trigliserid konsantrasyonları anlamlı oranda düşüktü. Kontrol grubuna kıyasla 2. ve 4. grupların grelin gen ekspresyonları anlamlı derecede fark gösterdi ($P<0.05$). Sonuçlar genel olarak broiler tavuklarda probiyotik ve soya fasulyesi yağının ayrı kullanımının daha faydalı olduğunu göstermiştir.

Anahtar sözcükler: *Broiler, Yağ, Yem tüketimi, Laktofed, Performans*

INTRODUCTION

In recent years, the use of additives such as growth promoters has been common in poultry nutrition. In earlier times, using different types of antibiotics in order to protect health, prevent diseases and disorders caused

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by environmental pollution, and growth promoters to increase production, was considered acceptable in livestock and poultry industry. Excessive use of antibiotics in livestock and poultry industry has though caused concerns for consumers, due to an increase of bacterial resistance, in-tissue survival and incidence of dangerous diseases. To find a safe additive to replace antibiotics (to stimulate growth and improve the health of farm animals) is a difficult task. According to different reports, the use of probiotics in poultry nutrition increases performance efficiency [1]. Probiotics, as alternatives to antibiotics and additives, are microbial populations that have a positive effect on improving animal performance and strengthening the immune system by balancing the intestinal flora and preventing gastrointestinal infections [2]. Using fat in the diet could have many benefits. One of these benefits is the longer transit time of food, improving the rate of digestion and absorption of nutrients [3]. It is likely that fat, with the effect on transit time of food, increases digestibility and absorption of other nutrients by enhancing enzyme function and more presentation in the places of absorption [4]. Fat contains high energy, so adding fat to the diet will increase metabolizable energy. However, it is necessary for the absorption of fat into the hepatic portal system that causes micelle formation. Micelles of bile salts, fatty acids, monoglycerides and glycerol bind to fatty acids, monoglycerides and facilitate absorption of fat-soluble vitamins [5].

Thus for absorption of fat, the presence of plenty of bile salts and saturated fatty acids and unsaturated balance are essential. However, the composition of bile acids is changed by the action of microbial flora in the digestive tract. Change in bile acids by intestinal microbial flora (deconjugation and dehydroxylation) damages absorption of fats, and their toxic breakdown products can reduce growth [6]. In addition, today the lactic acid-producing bacteria (*Lactobacillus, Bifidobacterium* and *Streptococci)* are used for the construction of probiotics [7]. These bacteria give enzymic hydrolysis of bile acid and with bile acid dissolution, emulsification of fats and construction of micelles will occur as a result of reduced fat absorption. Therefore, with the use of probiotics in the diet, the small intestine bacterial population increases and this may reduce the digestibility of dietary fat. It seems that the effect of probiotics on the absorption of fat is a function of the amount and type of fat in the diet [8]. On the other hand, it is possible that growth hormone secretion is affected by the additives and dietary ingredients. Ghrelin is one of those hormones. This hormone affects appetite regulation and results in body weight gain [9]. Nowadays, there is less attention to hormones and the factors in dietary ingredients which affect hormone secretion, because of the focus of poultry breeders on performance. Therefore, this study was done to compare the efficacy of probiotic and type fat in the diet on performance, carcass characteristics, intestinal morphology and ghrelin gene expression of broiler chicks.

MATERIAL and METHODS

Chickens, Diets and Management

Research on animals was conducted in Rudsar, Iran (37.1378° N, 50.2836° E) and all the procedures used were approved by the Ethics Committee in Animal Use (Approval date: 10/05/2016; No: 10038). The experiment, in a completely randomized design with 6 treatments and 4 replicates using 240 1-day-old male chicks of strain Ross 308, was conducted in the starter period (1-10 day), grower period (11-28) and finisher period (29-42). Each replication included 10 chicks. The experimental diets were formulated by using Ross-308 *(Table 1, Table 2* and *Table 3)* and animal and poultry feed formulation (WUFFDA) software. During the period, all conditions were similar for chickens, and the feeding was ad libitum for the whole period. The basal diet, based on corn and soybean meal, was balanced. Diets used in the experiment were isocaloric and isonitrogenic. Experimental diets included: (1) basal diet (control); (2) diet containing 3% animal fat from tallow; (3) diet containing 3% plant oil from soybean; (4) basal diet + probiotic; (5) probiotic + diet containing 3% animal fat from tallow and (6) probiotic + diet containing 3% plant oil from soybean. The Lactofeed probiotic preparation was declared to contain *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium* and *Enterococcus faecium* ($1x10^{11}$) c.f.u. per kg by the manufacturer.

Performance

The following growth performance variables were evaluated: production index, feed costs per kg live weight, body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR). The birds were weighed on the first day of the experiment, then weighed weekly throughout the remaining experimental period (7 to 42 d of age). Feed was provided weekly and the leftover fed was weighed weekly for calculating the feed conversion ratio. At day 42 of each replicate, a bird was selected and blood samples were collected from the wing veins.

Carcass Characteristics, Intestinal Morphology

At the end of the experimental period, one chicken per replicate (four chickens per treatment) was randomly (close to average weight) selected, and the digestive system was taken out of the carcass after slaughtering. Then, the percentages of different parts including carcass, breast, thigh and abdominal fat were calculated based on live weight. The different parts of small intestine were separated in order to investigate its morphology. Then, one centimeter pieces from the middle parts of duodenum and jejunum were disconnected. The separated pieces were evacuated of the intestinal contents and tissue blocks were prepared from the tissue samples of duodenum and jejunum after stabilization, dewatering, clarification and placement in paraffin [10]. The lams were studied after

Table 1. *Ingredients and calculated analyses of the basal diets in the starter period*

Ingredients (kg)	Starter (0-10 days old)					
	T1	**T2**	**T3**	**T4**	**T5**	**T6**
Corn grain	63.35	53.76	52.30	63.35	53.76	52.30
Soybean meal	22.57	30.5	35.50	22.57	30.5	35.50
Corn gluten meal	9.3	8	4.20	9.3	8	4.20
Tallow	-	3	-	-	3	-
Soybean oil	-	-	3	-	-	3
Dicalcium phosphate	2.25	2.2	2.15	2.25	2.2	2.15
Calcium carbonate	1.02	1.18	1.50	1.02	1.18	1.50
Sodium bicarbonate	0.29	0.27	0.24	0.29	0.27	0.24
Salt	0.22	0.18	0.20	0.22	0.18	0.20
L-lysine	0.28	0.25	0.20	0.28	0.25	0.20
DL-methionine	0.20	0.14	0.19	0.20	0.14	0.19
Vitamin and mineral permix[1]	0.5	0.5	0.5	0.5	0.5	0.5
Probiotic[2]	-	-	-	0.02	0.02	0.02
Filler	0.02	0.02	0.02	-	-	-
Total	100	100	100	100	100	100
Nutrient						
ME (kcal/kg)	3000	3000	3000	3000	3000	3000
CP (%)	22	22	22	22	22	22
Ca (%)	0.95	1.01	0.95	0.95	1.01	0.95
P (%)	0.47	0.46	0.47	0.47	0.46	0.47
Methionine (%)	0.65	0.47	0.50	0.65	0.47	0.50
Lysine (%)	1.18	1.1	1.13	1.18	1.1	1.13
Methionine + Cysteine (%)	0.98	0.79	0.81	0.98	0.79	0.81
Threonine (%)	0.71	0.72	0.73	0.71	0.72	0.73
Tryptophan (%)	0.21	0.20	0.21	0.21	0.20	0.21
Arginine (%)	1.27	1.18	1.27	1.27	1.18	1.27
Valine (%)	0.91	0.92	0.92	0.91	0.92	0.92
Na (%)	0.16	0.16	0.16	0.16	0.16	0.16
K (%)	0.70	0.72	0.81	0.70	0.72	0.81
Cl (%)	0.21	0.21	0.21	0.21	0.21	0.21

[1] Each kg (DM basis) of vitamin and mineral premix contained: vit A: 11.000 IU; vit D_3: 2.000 IU; vit E: 18 IU; vit K: 4 mg; vit B_{12}: 0.015 mg; Thiamine: 1.8 mg; Riboflavin: 6.6 mg; Calcium pantothenic acid: 12.0 mg; Niacin: 30.0 mg; Pyridoxine: 2.9 mg; Folic acid: 1.0 mg; Choline: 260.0 mg; Manganese: 64.5 mg; Zinc: 33.8 mg; Iron: 100.0 mg; Copper: 8.0 mg; Iodine: 1.9 mg; Selenium: 0.25 mg
T1: Basal diet (control); T2: Diet containing 3% animal fat from tallow; T3: Diet containing 3% plant oil from soybean; T4: Basal diet + Probiotic; T5: Probiotic + Diet containing 3% animal fat from tallow; T6: Probiotic + Diet containing 3% plant oil from soybean
[2] 0.02 kg of probiotic was added to starter diet to constitute the probiotic groups
ME: Metabolizable energy; CP: Crude protein

coloring (Alcian blue) by optical microscope and using an Eyepiece Graticule. The length and width of villus and depth of crypt were measured and villus length to crypt depth ratio was determined [10].

Ghrelin Gene Expression

One proventriculus tissue sample was taken from each replicate, washed with 10X phosphate buffered saline solution and transferred to a liquid nitrogen tank. Tissue samples were held at -80ºC until the extraction of RNA. The samples were first homogenized for RNA extraction. For this purpose, some of the tissue was smashed and put in a mortar and a uniform powder prepared using liquid nitrogen. The extraction kit, Rneasy Mini Kit (QIAGEN), was used in order to extract RNA from biologic samplesaccording to the protocol. The sequence of the primers used for investigating ghrelin gene included some primers for Real time PCR and some primers related to

Table 2. *Ingredients and calculated analyses of the basal diets in the grower period*

Ingredients (kg)	Grower (11-28 days old)					
	T1	T2	T3	T4	T5	T6
Corn grain	61.37	55.39	59.31	61.37	55.39	59.31
Soybean meal	19.00	28.80	30.80	19.00	28.80	30.80
Corn gluten meal	15	8.80	2.75	15	8.80	2.75
Tallow	-	3	-	-	3	-
Soybean oil	-	-	3	-	-	3
Dicalcium phosphate	2.05	1.90	1.95	2.05	1.90	1.95
Calcium carbonate	0.96	1.00	0.95	0.96	1.00	0.95
Sodium bicarbonate	0.61	0.20	0.23	0.61	0.20	0.23
Salt	0.25	0.22	0.20	0.25	0.22	0.20
L-lysine	0.18	0.12	0.16	0.18	0.12	0.16
DL-methionine	0.15	0.04	0.12	0.15	0.04	0.12
Vitamin and mineral permix[1]	0.5	0.5	0.5	0.5	0.5	0.5
Probiotic[2]	-	-	-	0.01	0.01	0.01
Filler	0.02	0.02	0.02	0.01	0.01	0.01
Total	100	100	100	100	100	100
Nutrient						
ME (kcal/kg)	3050	3050	3050	3050	3050	3050
CP (%)	21.50	21.50	21.50	21.50	21.50	21.50
Ca (%)	0.87	0.86	0.87	0.87	0.86	0.87
P (%)	0.43	0.43	0.43	0.43	0.43	0.43
Methionine (%)	0.69	0.37	0.44	0.69	0.37	0.44
Lysine (%)	0.70	0.96	1.00	0.70	0.96	1.00
Methionine + Cysteine (%)	1.02	0.69	0.72	1.02	0.69	0.72
Threonine (%)	0.69	0.71	0.65	0.69	0.71	0.65
Tryptophan (%)	0.16	0.19	0.19	0.16	0.19	0.19
Arginine (%)	1.00	1.16	1.12	1.00	1.16	1.12
Valine (%)	1.29	0.91	0.81	1.29	0.91	0.81
Na (%)	0.16	0.16	0.16	0.16	0.16	0.16
K (%)	0.54	0.70	0.74	0.54	0.70	0.74
Cl (%)	0.20	0.20	0.20	0.20	0.20	0.20

[1] Each kg (DM basis) of vitamin and mineral premix contained: vit A: 11.000 IU; vit D_3: 2.000 IU; vit E: 18 IU; vit K: 4 mg; vit B_{12}: 0.015 mg; Thiamine: 1.8 mg; Riboflavin: 6.6 mg; Calcium pantothenic acid: 12.0 mg; Niacin: 30.0 mg; Pyridoxine: 2.9 mg; Folic acid: 1.0 mg; Choline: 260.0 mg; Manganese: 64.5 mg; Zinc: 33.8 mg; Iron: 100.0 mg; Copper: 8.0 mg; Iodine: 1.9 mg; Selenium: 0.25 mg
T1: Basal diet (control); T2: Diet containing 3% animal fat from tallow; T3: Diet containing 3% plant oil from soybean; T4: Basal diet + Probiotic; T5: Probiotic + Diet containing 3% animal fat from tallow and T6: Probiotic + Diet containing 3% plant oil from soybean
[2] 0.01 kg of probiotic was added to grower diet to constitute the probiotic groups
ME: Metabolizable energy; CP: Crude protein

GAPDH gene as internal control for normalization *(Table 4)*. Gene expression in cDNA samples made of tissue was evaluated using Real time PCR primers and Sybergreen. Apparatus software automatically depicted threshold line at the end of PCR reaction in the Real Time PCR apparatus. The data were analyzed using ABI 7300 sequence detection system and SDS Ver. 1.4 software.

Statistical Analysis

Analysis of the obtained data was conducted by SAS software in a completely randomized design [11]. Differences between means were assessed by Duncan's multiple range test at 5% level.

RESULTS

The *Table 5* and *Table 6* show the effects of trial groups on the performance of broiler chickens. Body weights on treatments 2, 3 and 5 were significantly different from the control group during the entire period (P<0.05). Highest

Table 3. *Ingredients and calculated analyses of the basal diets in the finisher period*

Ingredients (kg)	Finisher (29-42 days old)					
	T1	T2	T3	T4	T5	T6
Corn grain	56.49	61.18	62.42	56.49	61.18	62.42
Soybean meal	30.00	23.23	25.61	30.00	23.23	25.61
Corn gluten meal	9.79	9.0	5.01	9.79	9.0	5.01
Tallow	-	3	-	-	3	-
Soybean oil	-	-	3	-	-	3
Dicalcium phosphate	1.74	1.7	1.69	1.74	1.7	1.69
Calcium carbonate	0.85	0.86	0.98	0.85	0.86	0.98
Sodium bicarbonate	0.20	0.15	0.25	0.20	0.15	0.25
Salt	0.22	0.25	0.19	0.22	0.25	0.19
L-lysine	0.12	0.10	0.22	0.12	0.10	0.22
DL-methionine	0.09	0.03	0.13	0.09	0.03	0.13
Vitamin and mineral permix[1]	0.5	0.5	0.5	0.5	0.5	0.5
Probiotic[2]	-	-	-	0.01	0.01	0.01
Filler	0.02	0.02	0.02	0.01	0.01	0.01
Total	100	100	100	100	100	100
Nutrient						
ME (kcal/kg)	3100	3100	3100	3100	3100	3100
CP (%)	19	19	19	19	19	19
Ca (%)	0.76	0.77	0.81	0.76	0.77	0.81
P (%)	0.38	0.38	0.38	0.38	0.38	0.38
Methionine (%)	0.49	0.32	0.41	0.49	0.32	0.41
Lysine (%)	0.60	0.75	0.95	0.60	0.75	0.95
Methionine + Cysteine (%)	0.91	0.61	0.69	0.91	0.61	0.69
Threonine (%)	0.80	0.64	0.61	0.80	0.64	0.61
Tryptophan (%)	0.15	0.17	0.17	0.15	0.17	0.17
Arginine (%)	0.97	1.02	1.02	0.97	1.02	1.02
Valine (%)	1.08	0.83	0.78	1.08	0.83	0.78
Na (%)	0.15	0.15	0.15	0.15	0.15	0.15
K (%)	0.38	0.61	0.65	0.38	0.61	0.65
Cl (%)	0.20	0.20	0.20	0.20	0.20	0.20

[1] Each kg (DM basis) of vitamin and mineral premix contained: vit A: 11.000 IU; vit D_3: 2.000 IU; vit E: 18 IU; vit K: 4 mg; vit B_{12}: 0.015 mg; Thiamine: 1.8 mg; Riboflavin: 6.6 mg; Calcium pantothenic acid: 12.0 mg; Niacin: 30.0 mg; Pyridoxine: 2.9 mg; Folic acid: 1.0 mg; Choline: 260.0 mg; Manganese: 64.5 mg; Zinc: 33.8 mg; Iron: 100.0 mg; Copper: 8.0 mg; Iodine: 1.9 mg; Selenium: 0.25 mg
T1: Basal diet (control); T2: Diet containing 3% animal fat from tallow; T3: Diet containing 3% plant oil from soybean; T4: Basal diet + Probiotic; T5: Probiotic + Diet containing 3% animal fat from tallow and T6: Probiotic + Diet containing 3% plant oil from soybean
[2] 0.01 kg of probiotic was added to grower diet to constitute the probiotic groups
ME: Metabolizable energy; CP: Crude protein

Table 4. *The sequence of primers designed to Real Time PCR*

Real Time PCR Primers	Sequence of the Primers	Product Size
Forward ghrelin	5′-AATTCTCCTTCTCAGCATCCTTGGG-3′	134 pb
Reverseghrelin	5′-CTGTGCCTCGGCGATGTAATCTTG-3′	
GAPDH forward	5′-CTTTGGCATTGTGGAGGGTC-3′	128 pb
GAPDHreverse	5′-ACGCTGGGATGATGTTCTGG-3′	

Table 5. *The effects of Lactofeed probiotic and different sources of fat on body weight (BW) and economical traits*

Treatments	1 to 42 day		
	BW (g)	Production Index	Feed Costs Per kg Live Weight (Rial)
Basal diet (control)	2294.78^{b}	311.63^{cb}	56427.50^{a}
Diet containing 3% animal fat from tallow	2190.60^{c}	288.87^{dc}	58075.00^{a}
Diet containing 3% plant oil from soybean	2431.93^{a}	374.94^{a}	48987.50^{b}
Basal diet + probiotic	2353.25^{ab}	325.83^{b}	55332.50^{a}
Probiotic + diet containing 3% animal fat from tallow	2159.93^{c}	281.00^{d}	57932.50^{a}
Probiotic + diet containing 3% plant oil from soybean	2292.90^{b}	312.40^{bc}	56255.00^{a}
P-value	0.0001	0.0001	0.0001
SEM	21.567	8.126	944.3862

The means within the same column with at least one common letter, do not have significant difference (P>0.05); SEM: standard error of the means

Table 6. *The effects of Lactofeed probiotic and different sources of fat on body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR)*

Period	Treatments	BWG (g)	FI (g/hen/starter)	FCR
Starter Period [g/week] 1 to 10 day	Basal diet (control)	158.35^{b}	229.67^{a}	1.46^{a}
	Diet containing 3% animal fat from tallow	196.29^{a}	219.35^{b}	1.11^{b}
	Diet containing 3% plant oil from soybean	184.12^{a}	212.00^{b}	1.16^{b}
	Basal diet + probiotic	198.62^{a}	211.05^{b}	1.06^{b}
	Probiotic + diet containing 3% animal fat from tallow	202.52^{a}	228.22^{a}	1.12^{b}
	Probiotic + diet containing 3% plant oil from soybean	196.53^{a}	210.07^{b}	1.06^{b}
	P-value	0.0022	0.0001	0.0002
	SEM	3.971	2.104	0.033
Grower Period [g/week] 11 to 28 day	Basal diet (control)	1001.05	1257.93^{c}	1.26
	Diet containing 3% animal fat from tallow	977.15	1245.40^{d}	1.28
	Diet containing 3% plant oil from soybean	1042.90	1242.23^{d}	1.20
	Basal diet + probiotic	1013.48	1289.95^{a}	1.27
	Probiotic + diet containing 3% animal fat from tallow	991.57	1292.13^{a}	1.30
	Probiotic + diet containing 3% plant oil from soybean	1030.68	1271.10^{b}	1.23
	P-value	0.8783	0.0001	0.7899
	SEM	15.801	4.235	0.021
Finisher Period [g/week] 29 to 42 day	Basal diet (control)	1087.15^{ab}	2458.50^{a}	2.27^{a}
	Diet containing 3% animal fat from tallow	968.95^{bc}	2410.38^{ab}	2.49^{a}
	Diet containing 3% plant oil from soybean	1157.20^{a}	2229.00^{c}	1.93^{b}
	Basal diet + probiotic	1093.38^{ab}	2465.98^{a}	2.25^{a}
	Probiotic + diet containing 3% animal fat from tallow	918.27^{c}	2344.85^{b}	2.57^{a}
	Probiotic + diet containing 3% plant oil from soybean	1018.00^{abc}	2445.28^{a}	2.42^{a}
	P-value	0.0122	0.0001	0.0037
	SEM	23.143	19.787	0.056

The means within the same column with at least one common letter, do not have significant difference (P>0.05); SEM: standard error of the means

weight was for treatment 3 and the lowest for treatment 5. Production indexes of the treatments 3 and 4 were significantly different from the control group (P<0.05). Feed costs per kg live weight in treatment 3 were significantly lower than for the the control group (P<0.05).

The body weight gains of all treatments in the starter period were significantly different from the control group (P<0.05). While none of the treatments were significantly different from the control in the grower period, but treatment 3 had the highest body weight gain among

Table 7. *The effects of Lactofeed probiotic and different sources of fat on carcass characteristics*

Treatments	Carcass (%)	Breast (%)	Thigh (%)	Abdominal Fat (%)
Basal diet (control)	66.14	31.29^{ab}	26.95^{ab}	1.08
Diet containing 3% animal fat from tallow	64.73	27.31^{b}	24.20^{bc}	1.10
Diet containing 3% plant oil from soybean	65.61	33.49^{a}	29.85^{a}	1.11
Basal diet + probiotic	64.77	29.60^{ab}	25.42^{bc}	1.08
Probiotic + diet containing 3% animal fat from tallow	61.91	29.23^{ab}	21.87^{c}	1.07
Probiotic + diet containing 3% plant oil from soybean	66.60	32.32^{a}	27.44^{ab}	1.06
P-value	0.7106	0.0114	0.0035	0.1032
SEM	0.848	0.699	0.682	0.0029

The means within the same column with at least one common letter, do not have significant difference (P>0.05); SEM: standard error of the means

Table 8. *The effects of Lactofeed probiotic and different sources of fat on intestinal morphology*

Intestine	Treatments	Villus Height (µm)	Villus Width (µm)	Crypt Depth (µm)	Villus Height/Crypt Depth
Duodenum	Basal diet (control)	522.00^{e}	50.80^{d}	96.90^{d}	5.39^{c}
	Diet containing 3% animal fat from tallow	530.80^{de}	55.00^{dd}	108.50^{c}	4.89^{c}
	Diet containing 3% plant oil from soybean	586.30^{c}	72.50^{b}	160.80^{a}	3.53^{d}
	Basal diet + probiotic	856.40^{a}	123.30^{a}	73.50^{e}	11.65^{a}
	Probiotic + diet containing 3% animal fat from tallow	536.70^{d}	64.60^{c}	136.10^{b}	3.94^{d}
	Probiotic + diet containing 3% plant oil from soybean	751.80^{b}	65.40^{c}	94.20^{d}	7.98^{b}
	P-value	0.0001	0.0001	0.0001	0.0001
	SEM	38.910	7.270	8.745	0.849
Jejunum	Basal diet (control)	664.00^{d}	58.20^{c}	72.00^{d}	9.22^{b}
	Diet containing 3% animal fat from tallow	330.00^{f}	51.20^{d}	101.60^{b}	3.24^{f}
	Diet containing 3% plant oil from soybean	812.50^{b}	73.50^{b}	100.20^{b}	8.10^{c}
	Basal diet + probiotic	849.50^{a}	123.30^{a}	73.50^{d}	11.56^{a}
	Probiotic + diet containing 3% animal fat from tallow	442.00^{e}	60.50^{c}	95.40^{b}	4.63^{e}
	Probiotic + diet containing 3% plant oil from soybean	766.60^{c}	73.50^{b}	95.20^{b}	8.05^{c}
	P-value	0.0001	0.0001	0.0001	0.0001
	SEM	58.450	7.164	6.104	0.810

The means within the same column with at least one common letter, do not have significant difference (P>0.05); SEM: standard error of the means

the treatments (P>0.05). Body weight gain in treatment 3 was significantly different from the control in the finisher period whilst treatment 5 had the lowest body weight gain (P<0.01).

There was a significant decrease of the difference in feed intakes between the control group and all the treatments (except treatment 5) in the starter period (P<0.05). Feed intakes for all the treatments were significantly different from the control group in the grower period. The probiotic included treatments had higher feed intake than the control and the treatment merely with fat had lower feed intake than the control. Lowest feed intake was related to treatment 3 (P<0.05). The feed intakes of treatments 3 and 5 were significantly different from the control in the finisher period (P<0.05).

The feed conversion coefficient for all the treatments was significantly different from the control in the starter period (P<0.05). None of the conversion coefficients in the treatments were significantly different from the control group in the grower period (P<0.05). In the finisher period, only treatment 3 had a significantly different conversion coefficient compared to the control group (P<0.05), with more efficient feed conversion

Table 7 shows the effect of experimental groups on carcass characteristics in the finisher period. There were no significant differences from the control group for the percentages of carcass, breast and ventricular fat. The percentage of thigh in treatment 5 had significant decrease compared to the control group (P<0.05).

Table 8 shows the effect of experimental groups on length, width and depth of crypt and villus height to crypt depth ratio of small intestine (duodenum and jejunum)

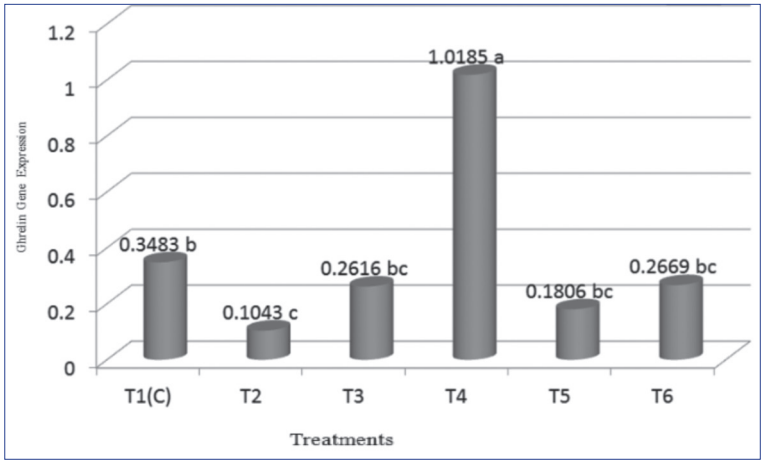

Fig 1. The effects of Lactofeed probiotic and different sources of fat on ghrelin gene expression

in the finisher period. The villi in elemental areas of small intestine had the highest height and the height of villi was lower at the end of intestine. Duodenum villus height and jejunum of all the treatments were significantly different from the control ($P<0.05$). The depths of duodenum villus width and jejunum in treatments 3 and 4 were significantly greater than for the control ($P<0.05$). The length and width of duodenum in treatment 2 was not significantly different from the control. The depth of crypt in duodenum for all the treatments except treatment 6 was significantly different from the control ($P<0.05$). The crypt depth was lowest in treatments of 4 and 6. The depth of crypt in jejunum section for all the treatments except treatment 4 was significantly different from the control group ($P<0.05$).

Villus height to crypt depth ratio of duodenum for all the treatments except treatment 2 was significantly different from the control and the highest value was on treatment 4 ($P<0.05$). Also, villus length to crypt depth ratio in jejunum section for all the treatments differed significantly from the control ($P<0.05$).

Fig. 1 shows the effect treatments on the relative expression of Ghrelin gene at the end of the period. Ghrelin gene expression of treatment 2 was significantly lower than in the control, whilst it was significantly higher in treatment 4 ($P<0.05$).

DISCUSSION

Other studies show that the level of feed intake of broiler chickens in the starter period is lower in fat-included diets [12].

Lower feed intake of fat-included diets, increase in the weight of chickens and improvement in conversion ratio in the starter period, may be because of the decrease in transit speed through digestive system which consequently provides more time for digestive system to absorb nutrients [6].

Leeson and Summers [13] showed that inclusion of fat in the diet causes decrease in feed intake during grower and finisher periods because of decreasing gastric emptying rate. This is consistent with the results of this experiment.

The feed intake with the probiotic-included diet in the grower period (the treatments 4, 5, 6) was increased compared to the control group. Probiotics improve the digestive process via increase of the useful microbial population, enzymatic activity of bacteria and the improvement of intestine microbial balance with consequent effects on food digestion, absorption and intake [14].

Body weight during the entire period, body weight gain and conversion ratio during the finisher period in treatment 3 was improved compared to the control. This is in line with the beneficial effect of fats, specifically soybean oil, on bird body weight gain reported by Shokrollahi et al.[15]. Improvement in weight due to using plant oil-included diets is related to effects on bird feed intake and better use of dietary energy. The better effect of plant oils, such as soybean oil, is due to the high ratio of unsaturated to saturated fatty acids and also better formation of micelle because of creating monoglyceride after its hydrolysis inside intestine results in a better absorption and thus improved performance [15].

Treatment 3 which had the best conversion ratio included only 3% soybean oil. This is related to the effect of fat on feed intake which causes fixed energy absorption of the bird by lower feed intake [16].

Treatment 3 had the best production index and lowest feed cost. These results together with those of den Besten et al.[17] show that soybean oil can improve economc performance, because it is a cheap energy source with beneficial effects on the efficiency of nutrient digestion and absorption resulting from lower rate of transit through the digestive system.

The results of this experiment showed that the effect of dietary fats on performance depends on the type of additive used in the diet; such that there was a lower

performance of birds when both probiotic and fat (tallow or soybean oil) were included in the diets. Intestinal microbial flora *(Lactobacilli, Bifidobacterium* and *Enterococcus)* have been reported to have a role in the decomposition of bile acids. These species are used in the preparation of probiotics and may cause disorder in the bird's fat absorption by creating biologic changes in bile acids and by dehydroxylation and deconjugation. Deconjugation of bile acids by the bacteria of the digestion system was reported by Leeson and Summers [13] to result in less absorption of fats which leadsto decreased absorbed energy and less growth of chickens.

The differences in the weights of carcass, breast and thigh between the treatments and the control were not significant, but the difference in the third treatment was higher. Nobakht et al.[18] stated that the use of soybean oil in the diet increased the weight of the breast and thigh in the poultry, which is consistent with the results of this experiment. The use of soybean oil in the poultry diet, due to the reduced feed transit rate, makes better the digestion and absorption of nutrients, and amino acids are provided in a better position to improve carcass weight.

Also, better absorption of soybean oil than animal fat in the diet results in an increase in carcass weight. The positions of fatty acids in glycerol, as well as the ratio of the fatty acids used in fat formatoin, affect the amount of the metabolizable energy extracted from fat. Non-saturated fatty acids are absorbed more than saturated fatty acids, and thus their metabolizable energy is higher. Since fatty acids used in soybeans were unsaturated, the increase in the absorbed energy led to an increase in carcass weight in the third treatment [19].

Differences in the fat of the abdominal were not significant in any of the treatments compared to the control. The main nutritional factor that can affect the content of abdominal fat is the energy level of the diet and the ratio of diet's energy to protein, and there was no significant difference between the treatments with regard to the energy balance and the energy to protein ratio [20].

Treatments with probiotics and soybean oil alone or in combination had marked effects on the morphology of the intestine. The increase of crypt depth of intestinal wall shows the thickening of intestinal surface. Thickening is due to the body's immune response to the entry of pathogens and toxins. Probiotics prevent thickening of intestinal surface by decreasing intestine's pathogens. Shortening of villi and increase of crypt depth in intestinal surface will decrease absorption from the intestinal wall and decrease of performance [21]. The longer the length of intestinal villus probably results from a lower level of replacing enterocyte cells and renewing intestine tissue. The increase of villus height when probiotics were incluuded is volatile because of their role in increasing fatty acids which are considered as the final product of fermentation by the bacteria used in probiotics *(Lactobacilli* and *Bifidobacteria)*. The aggregation of this material in the intestine decreases intestine's pH and makes the environment inappropriate for Salmonella and Kelly Basil that need pH of about 7. With the decrease of damage to the intestinal wall, the level of renewing intestinal epithelial cells decreases and the length of villi increases [22].

Khatibjoo et al.[23] reported that consuming tallow in the diet instead of soybean oil led to higher values of pH in different parts of the small intestine and increased repelling of bile acids by broiler chickens with increase in intestine pH, higher levels of pathogenic bacteria are expected which result in diarrhea and intestinal tissue destruction. But using unsaturated fatty acids has the opposite effect and causes the decrease of inflammatory responses in the intestine. Therefore, it can be said that using unsaturated fatty acids instead of saturated fatty acid causes the increase in the length and width of villus and also the decrease of intestine's crypt depth with a resulting increase in broiler chicken's performance [24].

As *Fig. 1* shows the lowest level of ghrelin expression in broiler chickens was in the treatment which only had tallow in their diet. Ghazanfari et al.[25] stated in their research that ghrelin plasma concentration decreases when fat is included in the diet indicating that ghrelin secretion is sensitive to diet composition. Salehi et al.[26] found that ghrelin secretion decreased when fat was included in the diet. Cholecystokinin (CCK) is a hormone which is released from intestinal cells during eating fat or protein. This hormone contacts with neural system to announce satiety and at the same time lowers digestion in the digestive system. Since the fat in the diet lowers the rate of feed transit and also digesting saturated fat takes longer than unsaturated fat, the time period of digestion is much slower which causes long-term satiety feeling resulting in less secretion of ghrelin hormone [26].

The level of ghrelin expression in those broiler chickens which only used probiotic in their diet was significantly increased compared with the control. Arosio et al.[27] stated that every factor which increases the capability of digestion and absorption in digestive system and causes faster evacuation of the digestive system, results in the increase of ghrelin secretion. Probiotics keep stomach chymus safe from the damage of pathogenic microorganisms and improve digestion and absorption by removing pathogenic bacteria from the intestine, and consequently increase ghrelin secretion and improve the performance [27]. Probiotics increase ghrelin production via decreasing blood sugar. The bacteria in probiotics use dietary carbohydrates. Therefore, bird's absorption of sugar is reduced which increases the activity of vagus nerve in order to increase the movements of the digestive system. Ghrelin hormone secretion is stimulated by the increase in movements of the digestive system and as the result, feed intake is increased and weight is improved [28].

The results of this work showed that using vegetable fats to supply part of tahe energy in the diet and the separate use of lactofeed probiotic as an additive have beneficial effects in terms of performance of broiler chickens. Separate use of probiotic in the diet of broiler chickens can increase the levels of relative expression of ghrelin gene and this increase improves the weight. This study showed that the energy supplied with 3% animal fat in the diet of broiler chickens and also using the diets of probiotic mixed with (animal and plant) fat did not have adverse effects.

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Research Article

Characteristics of Pastırma Types Produced from Water Buffalo Meat

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Abstract

Pastırma is a traditional Turkish meat product which produced by using whole meat pieces obtained from water buffalo and beef carcasses. Although there are many studies on the general characteristics of the pastırma produced by using beef meat, the number of the studies on the pastırma produced from water buffalo meat is quite limited. In this study, different types of pastırma (sırt, bohça, kuşgömü, şekerpare and kürek) were made from water buffalo meat; and they were investigated in terms of physco-chemical, microbiological, sensorial and textural properties. There were no differences between pastırma types in terms of pH, redness (a*) value and the amount of non-protein nitrogenous substance (P>0.05). However, the lowest mean a_w value (0.84±0.01) was determined in kuşgömü (P<0.05). Şekerpare showed the highest mean lightness (L*) value (P<0.05). While no significant difference was observed between the types of pastırma in terms of *Micrococcus/Staphylococcus* count (P>0.05), the lowest count of lactic acid bacteria was found in kuşgömü (P<0.05). The highest mean odor score was determined in the sırt type of pastırma (P<0.05). Kuşgömü, which dries more quickly, also showed higher values in terms of firmness and chewiness compared to other types of pastırma (P<0.05). Furthermore, textural differences between the types of pastırma were observed more clearly with the principal component analysis (PCA) applied to textural properties.

Keywords: *Pastırma, Water buffalo meat, Lactic acid bacteria, Texture, Principal component analysis*

Manda Etinden Üretilen Pastırma Çeşitlerinin Özellikleri

Öz

Pastırma geleneksel bir Türk et ürünü olup, manda ve sığır karkaslarından elde edilen büyük parça etler kullanılarak üretilmektedir. Sığır eti kullanılarak üretilen pastırmanın genel karakteristik özellikleri üzerine çok sayıda çalışma bulunmasına karşın manda eti ile üretilen pastırma üzerinde araştırma sayısı oldukça sınırlıdır. Bu araştırmada manda etinden farklı pastırma çeşitleri (sırt, bohça, kuşgömü, şekerpare ve kürek) üretilmiş ve fiziko-kimyasal, mikrobiyolojik, duyusal ve tekstürel özellikler açısından incelenmiştir. Pastırma çeşitleri arasında pH, kırmızılık (a*) değeri ve protein tabiatında olmayan azotlu madde miktarı açısından önemli bir farklılık gözlenmemiştir (P>0.05). Buna karşın en düşük ortalama a_w değeri (0.84±0.01) kuşgömünde belirlenmiştir (P<0.05). Şekerpare en yüksek ortalama parlaklık (L*) değerini göstermiştir(P<0.05). *Micrococcus/Staphylococcus* sayısı açısından pastırma çeşitleri arasında önemli bir farklılık gözlenmezken (P>0.05), en düşük laktik asit bakteri sayısı kuşgömü çeşidinde belirlenmiştir (P<0.05). En yüksek ortalama koku değeri sırt çeşidinde tespit edilmiştir (P<0.05). Daha hızlı bir kurumanın gerçekleştiği kuşgömü çeşidi sertlik ve çiğnenebilirlik açısından da diğer pastırma çeşitlerine göre daha yüksek değerler vermiştir (P<0.05). Ayrıca tekstürel özelliklere uygulanan temel bileşen analizi (PCA) ile pastırma çeşitleri arasındaki tekstürel farklılıklar daha açık bir şekilde gözlemlenmiştir.

Anahtar sözcükler: *Pastırma, Manda eti, Laktik asit bakterleri, Tekstür, Temel bileşen analizi*

INTRODUCTION

Pastırma, a traditional Turkish dry-cured meat product, is produced by curing, drying and covering with çemen of meat pieces from certain parts of beef or water buffalo carcasses. This product is included in the intermediate moisture food class [1]. This traditional meat product is widely produced and likely consumed in Turkey [2]. Pastırma is quite different from other dry-cured meat products such as dry cured ham, lacon, country style ham, jambon de Savoie and loin in terms of both process time and conditions and raw material. Beef or water buffalo meat is used as the raw material in pastırma production, and the production period is approximately one month. While *Micrococcus/Staphylococcus* and lactic acid bacteria have an important role in the microflora of this product,

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yeasts are also present at different levels in microflora. The pH value of the product is usually above 5.5. Water activity (a_w) is a significant hurdle effect on the microbiological stability of the product. In addition to this, the extreme drying is undesirable in the product in terms of sensorial properties, and a_w is recommended to be between 0.85 and 0.90 [1]. According to the Turkish Food Codex Meat and Meat Products Communique [3], the pH value and moisture content of pastırma must not exceed 6.00 and 50%, respectively. Çemen paste components and chemical and biochemical reaction products occurred in the pastırma processing have a significant effect on the aroma of pastırma [1].

In pastırma production, 16 and even more types of pastırma can be produced from one carcass. The naming of pastırma types is made according to the part where the muscles and/or muscles are located. The pastırma type called as sırt which is obtained from the posterior rib region and loin of the carcass is the most commonly produced type. Furthermore, the types of pastırma called bohça and şekerpare are produced from the round, kürek and kuşgömü are produced from the chuck and tenderloin, respectively. Moreover, different types of pastırma, which are called by different traditional names, can be produced from the other parts of the carcass. According to the classification made in terms of quality, sırt and kuşgömü are the first class types of pastırma while bohça, şekerpare and kürek are included in the second class types of pastırma [4,5]. Although beef is mostly preferred in pastırma production, water buffalo meat is also used in production.

In particular, the fact that the water buffalo meat includes the higher protein, lower fat and cholesterol levels compared to beef has been increased the interest to water buffalo meat and its products in recent years [6-8]. There are many studies in the literature on the use of water buffalo meat in various meat products [9-18]. However, there is only one study on using water buffalo meat in production of pastırma [19], the effects of the use of starter culture on some quality characteristics of pastırma produced with water buffalo meat had been determined in the study. There is no study on the quality characteristics of different types of pastırma which is produced using water buffalo meat. In this study, different pastırma types (sırt, bohça, kuşgömü, şekerpare and kürek) were produced by using water buffalo meat and these types of pastırma were investigated in terms of textural properties as well as physico-chemical, microbiological and sensory properties.

MATERIAL and METHODS

Material

A 2-year-old male water buffalo was provided for pastırma production, and the carcass was conditioned for 24 h after slaughtering. Then, large pieces of meat for pastırma called sırt, şekerpare, bohça, kürek and kuşgömü were obtained from the carcass. The parts obtained from the left half of the carcass were used as the first replication, and the parts obtained from its right half were used as the second replication. Pastırma production was conducted in a meat processing plant (Kadakçıoğlu A.Ş., Erzurum, Turkey) by a traditional method.

Methods

Pastırma Production

At the beginning of production, muscles were trimmed for visible connective tissue and then shaped. Then, several incisions were made to the trimmed and shaped whole meat pieces. After that, the production was subjected according to the following procedure: strips were rubbed and covered with a curing mixture (50 g saccharose + 10 g KNO_3 + 1 kg NaCl, 100 g curing mixture for 1 kg meat), and kept in a room at about 10°C for 2 days. Thereafter, the meat strips were washed under tap water and air-dried for 6 days at 15-20°C (first drying). After drying, the strips were piled up and pressed with heavy weight (15 kg for weight per kg of meat) for 20 h at about 10°C. After this treatment, the meat strips were dried again for 8 days at 15-20°C (second drying) and pressed again (15 kg for weight per kg of meat) for 7 h at room temperature. After final drying (for 3 days at 15-20°C), strips were put in a bowl of seasoning mixture (çemen containing 500 g flour *(Trigolella foenum graecum* flour), 450 g smashed fresh garlic, 300 g red pepper and 1500 mL water) for 2 days and çemen on the surface of the meat strips was removed to a 3-4 mm layer of paste. And then, paste-covered strips were dried again for 8 days at 15°C.

Determination of Water Activity

The water activity (a_w) device (Novasina TH-500, Switzerland) was used in determining the water activity of the samples. The device was calibrated at 25°C with six different salt solutions before using. The samples were put in plastic sample containers for analysis, they were placed in the measuring cabinet of the device, and a_w value was determined at 25°C [20].

Determination of pH Value

Ten g sample was weighed for analysis and homogenized with Ultra-Turrax for 1 min by adding 100 mL of distilled water. The pH value was determined by the pH meter which was previously standardized with the appropriate buffer solutions [21].

Instrumental Color

The color intensities of the samples were determined by using a Minolta (CR-200, Minolta Co, Osaka, Japan) colorimeter. L*, a* and b* values were measured to the criteria given by the International Commission on

Illumination CIELAB (Commision Internationele de l'E Clairage) based on three-dimensional color measurement. Accordingly, L*; L*=0 indicates the color intensity of black, L*=100 indicates the color intensity of white (darkness/lightness), a*; +a* = indicates the color intensity of red, -a*= indicates the color intensity of green, and b*; +b*= indicates the color intensity of yellow and -b*= indicates the color intensity of blue [20].

Thiobarbituric Acid Reactive Substances Value

Thiobarbituric acid reactive substances (TBARS) values of samples were determined according to the methods of Lemon [22] and were expressed as µmol malondialdehyde (MDA)/kg.

Non-Protein Nitrogenous Substance Content

Non-protein nitrogenous substance content was determined according to Anonymous [23]; 5 g sample was homogenized with 10 mL of dichloromethane and 50 g of 20% trichloroacetic acid, allowed to settle at room temperature for 15 min, centrifuged at 3500 x g for 25 min and filtered. After filtration, total nitrogen in the supernatant was determined by Kjeldahl method. The results were expressed as g/100 g of samples.

Microbiological Analysis

Twenty five gram samples were homogenized in 225 mL of sterile physiological saline (0.85% NaCl) for 1 min by using a Stomacher homogenizer (Lab Stomacher Blander 400-BA 7021, Seward Medical). Serial decimal dilutions were prepared in sterile physiological saline. For the enumeration of *Micrococcus/Staphylococcus*, Mannitol Salt agar (MSA, Merck) was used and plates were incubated at 30°C for 48 h. For lactic acid bacteria and *Enterobacteriaceae*, De Man Rogosa Sharpe agar (MRS, Merck) and Violet Red Bile Agar (VRBD, Merck) were used, respectively and incubation was carried out at 30°C for 48 h anaerobically [24].

Texture Profile Analysis

Pastırma samples were subjected to texture profile analysis (TPA) with triplicate using the Texture Analyzer (TA.XT Plus Stable Micro Systems Ltd., Surrey, England) with Texture Exponent Programs. The test was performed with a 35 mm diameter cylinder probe at 1 mm/s speed and 20% strain, on pastırma samples (2 cm with, 1.5 cm height). A time of 5 s was allowed to elapse between the two compression cycles. Force-time deformation curves were obtained with a 30 kg load cell applied and 10 g trigger force. The following parameters were determined: firmness (N) = maximum force required to compress the sample; springiness = degree to which a product returns to its original shape once it has been compressed (Length 2/Length 1); cohesiveness = extent to which sample could be deformed prior to rupture (A_2/A_1, A_1 being the total energy required for first compression and A_2 the total energy required for the second compression); adhesiveness (Ns) = work necessary to pull the compressing plunger away from sample; chewiness (N) = work to masticate the sample for swallowing (firmness x cohesiveness x Springiness) [25].

Sensory Analysis

Thick slices (1.5 cm) were cut from pastırma samples and used for the sensory analysis. The panellists were selected from the staff of the food engineering department. Ten panellists were asked to evaluate the samples in terms of color, odor, taste, texture and general acceptability parameters at 2 different times and 20 individual results were obtained. The evaluation was performed using a hedonic-type scale (1-9 scales: 1: dislike extremely - 9: like extremely) [21].

Statistical Analyses

The study was established and conducted in accordance with the completely randomized design. The obtained data were subjected to the analysis of variance, and differences between means were evaluated by Duncan's multiple range test using IBM SPSS Statistics 20 package program [26]. A principal components analysis (PCA) [27] was carried out the structure of dependence and correlation among the variables.

RESULTS

The results of the physico-chemical and microbiological properties of different types of pastırma produced from water buffalo meat are presented in *Table 1*. No significant difference was observed between the types of pastırma in terms of pH value ($P>0.05$), and the pH value varied between 5.68-5.78 in the types of pastırma. In the study, the a_w values varying between 0.84-0.92 were determined for all types of pastırma, and it was determined that these values were significantly affected by the type of pastırma ($P<0.01$). The L* and b* values of the colour properties were significantly ($P<0.05$) affected by the type of pastırma factor, whereas there was no difference between the types in terms of the a* value ($P>0.05$). The amount of non-protein nitrogenous substance, which is an indicator of proteolysis, varied between 3.11-3.83 g/100 g for all types of pastırma, and the type factor was not effective on the amount of non-protein nitrogenous substance ($P>0.05$). The TBARS value was determined between 25.39 and 41.33 µmol MDA/kg in the types of pastırma. According to the statistical analyses results, TBARS values were significantly affected by type of pastırma ($P<0.05$).

In the present study, no significant differences were determined between the types of pastırma in terms of *Micrococcus/Staphylococcus* count ($P>0.05$). In contrast, there were significant differences between kuşgömü type and the other types of pastırma in terms of lactic acid bacteria ($P<0.05$).

Table 1. *Physicochemical and microbiological properties of different Pastırma types produced from water buffalo meat*[1]

Properties	Pastırma Types					
	Kuşgömü	Sırt	Bohça	Kürek	Şekerpare	Significance
pH	5.78±0.01^{a}	5.77±0.01^{a}	5.77±0.01^{a}	5.68±0.07^{a}	5.71±0.05^{a}	NS
a_w	0.84±0.01^{c}	0.87±0.01^{b}	0.87±0.01^{b}	0.92±0.00^{a}	0.91±0.03^{a}	**
L*	35.28±1.10^{d}	37.73±0.71^{c}	35.50±0.35^{d}	39.98±0.59^{b}	46.03±1.08^{a}	*
a*	17.12±4.69^{a}	19.08±1.10^{a}	17.08±0.66^{a}	21.55±0.46^{a}	18.66±1.35^{a}	NS
b*	2.85±1.66^{a}	3.54±0.38^{a}	1.43±0.41^{a}	3.60±0.93^{a}	6.96±1.19^{b}	*
Non-protein nitrogenous substance (g/100 g)	3.11±0.10^{a}	3.31±0.30^{a}	3.53±0.17^{a}	3.68±0.19^{a}	3.83±0.00^{a}	NS
TBARS (µmol MDA/kg)	37.11±0.80^{ab}	29.77±5.32^{bc}	41.33±3.53^{a}	25.39±5.79^{c}	27.70±2.33^{bc}	*
Lactic acid bacteria (log CFU/g)	4.74±0.10^{a}	6.55±0.50^{b}	7.15±0.06^{b}	7.09±0.13^{b}	7.30±0.27^{b}	*
Micrococcus/Staphylococcus (log CFU/g)	6.84±0.54^{a}	6.59±0.04^{a}	7.05±0.13^{a}	6.02±0.32^{a}	6.28±0.25^{a}	NS

[1] Presented values are means ±SD; [a-d] any two means in the same row having the same letters are not significantly different (P>0.05), P<0.05, ** P<0.01, **NS:** not significant, **SD:** standard deviation*

Table 2. *Sensory properties of different Pastırma types produced from water buffalo meat*[1]

Sensory Properties	Pastırma Types					
	Kuşgömü	Sırt	Bohça	Kürek	Şekerpare	Significance
Color	6.24±0.75^{a}	7.08±0.54^{a}	6.26±0.05^{a}	6.40±0.42^{a}	6.95±0.28^{a}	NS
Odor	6.77±0.03^{b}	7.59±0.15^{a}	6.77±0.04^{b}	6.91±0.30^{b}	7.10±0.01^{b}	*
Texture	6.59±0.84^{a}	7.60±0.28^{a}	6.50±0.15^{a}	6.85±0.50^{a}	7.33±0.37^{a}	NS
Taste	6.70±0.42^{a}	7.61±0.30^{a}	6.79±0.27^{a}	6.85±0.49^{a}	7.16±0.20^{a}	NS
General Acceptability	6.72±0.74^{ab}	7.61±0.02^{a}	6.45±0.21^{b}	6.84±0.20^{ab}	6.80±0.29^{ab}	*

[1] Presented values are means ±SD; [a-b] any two means in the same row having the same letters are not significantly different (P>0.05), P<0.05, ** P<0.01, **NS:** not significant, **SD:** standard deviation*

Table 3. *Textural properties of different Pastırma types produced from water buffalo meat*[1]

Properties	Pastırma Types					
	Kuşgömü	Sırt	Bohça	Kürek	Şekerpare	Significance
Firmness	63.48±11.72^{c}	38.29±3.95^{b}	31.93±3.31^{ab}	28.35±3.47^{a}	29.18±5.08^{a}	**
Adhesiveness	-0.05±0.01^{b}	-0.04±0.01^{b}	-0.09±0.01^{a}	-0.08±0.04^{a}	-0.04±0.03^{b}	**
Springiness	0.79±0.02^{a}	0.83±0.05^{a}	0.74±0.05^{a}	0.77±0.08^{a}	0.81±0.06^{a}	NS
Cohesiveness	0.68±0.04^{ab}	0.72±0.05^{b}	0.67±0.05^{ab}	0.64±0.02^{a}	0.72±0.05^{b}	**
Chewiness	36.89±6.61^{c}	25.56±2.69^{b}	15.31±1.34^{a}	12.51±2.21^{a}	22.23±5.48^{b}	**

*[1] Presented values are means ±SD; [a-c] any two means in the same row having the same letters are not significantly different (P>0.05), * P<0.05, ** P<0.01, **NS:** not significant, **SD:** standard deviation*

Although no significant differences were determined in terms of color, texture and taste for types of pastırma (P>0.05), different evaluation scores were obtained for odor and general acceptability (P<0.05) *(Table 2)*.

The texture analysis results were given in *Table 3*. The factor of pastırma type had significant effect on firmness, adhesiveness, cohesiveness and chewiness values (P<0.01), while pastırma type did not caused a significant effect on springiness value (P>0.05). However, the highest firmness and chewiness values were found in kuşgömü type of pastırma (P<0.05).

In the research, PCA was applied to assess the relationships between pastırma type and textural parameters. Two principal components were able to explain the 100% (99% PC1 and 1% PC2) of the total variance observed. All of pastırma types as well as chewiness and firmness

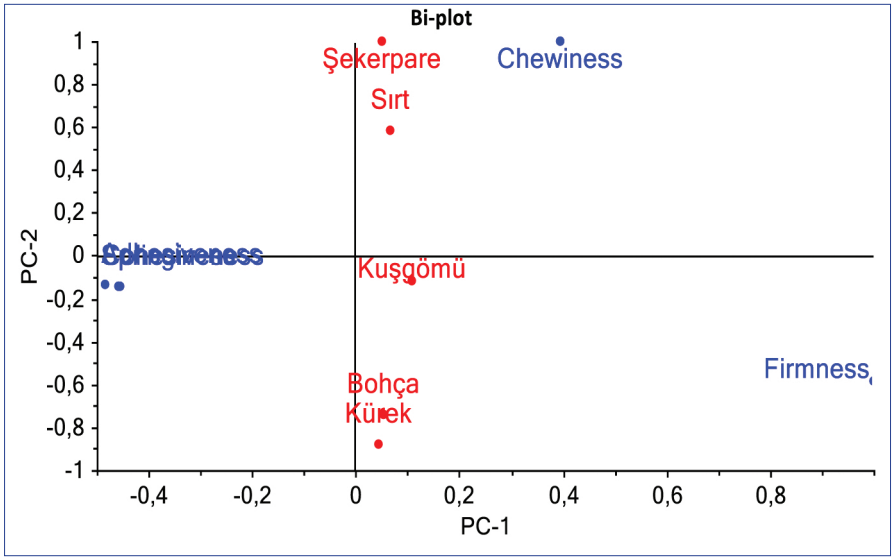

Fig 1. Principal component analysis biplot of the relationships between pastırma types and textural parameters

properties placed to positive side of PC1, while other textural properties located negative side of PC1. On the other hand, scores biplot in *Fig. 1* showed also that textural parameters except chewiness located at negative side on PC2.

DISCUSSION

According to the Turkish Food Codex Meat and Meat Products Communique [3], the pH value in pastırma must be 6.0 at the most. The pH values determined in this study were found to be lower than this limit value specified for all types of pastırma. However, it was determined that the pH value did not fall below 5.5 in the studies on pastırma produced using beef [1,28-31] and water buffalo meat [19] as well as in this study, too. Unlike fermented sausages, no low pH values are observed in pastırma since there is no true lactic acid fermentation [1].

The a_w value is an important parameter which has an effect on various chemical and biochemical reactions and microbiological events. As a result of the production process of pastırma, the a_w value of the product decreases, and because of that this traditional dry-cured meat product could be accepted as shelf stable product. However, Leistner [32] reported that the a_w value should be between 0.85-0.90 for pastırma. In the study, the lowest a_w value was determined in the kuşgömü type of pastırma ($P<0.05$). The a_w value was found to be above 0.90 in the types of şekerpare and kürek which were removed from the round and chuck parts of carcasses, respectively. It is thought that the differences in a_w values were due to the salt diffusion and the drying rate factors affected by the size of the muscles and the fiber structure.

The color is one of the most important quality characteristics for the consumer in accepting food. In the study, the L* value, which is the measure of lightness, was found to be higher in şekerpare, which is a type of pastırma obtained from the round part, compared to other types of pastırma *(Table 1)*. Similarly, Çakıcı *et al.*[33] reported that L* value of şekerpare was significantly higher than the values of sırt and bohça types. On the other hand, there was no difference between the types of pastırma in terms of a* value. According to this result, the desired reddish color formed in all of pastırma types. It is thought that the differences between the L* and b* values determined in the types of pastırma were due to the raw material properties. Indeed, many researchers have reported that the curing mixture and curing process as well as the raw material are effective on the color of pastırma [29,34]. The color pigment formed in pastırma is very sensitive to external factors [4]. Therefore, the product is subjected to a partial heat treatment before çemen-application for color stability, even if just a bit, in industrial production, and a stable color formation is ensured.

Protein degradation products are reported to be highly effective in the development of texture and aroma in meat products [35,36]. The raw material and processing conditions on the level of non-protein nitrogenous substance may be also effective [1]. The levels of non-protein nitrogenous substance obtained in this study are similar to the values determined by Kaban [1] for the pastırma produced from beef meat.

The TBARS value, one of the most important indicators of oxidation in meat products, varied between 25.39-41.33 µmol MDA/kg in the types of pastırma *(Table 1)*. Similar results were also determined in the pastırma samples produced from beef meat by Kaban [1]. The highest TBARS value was determined in bohça type, while the kürek type pastırma showed the lowest TBARS value *(Table 1)*. The raw material properties as well as production and storage conditions are highly effective on TBARS value. It is thought that the properties of raw material were effective on the TBARS value obtained in this study.

It is stated that *Micrococcus/Staphylococcus* and lactic acid bacteria constitute the dominant microflora in pastırma [1,28,37]. While the number of lactic acid bacteria was found to be 4.74 log CFU/g in kuşgömü type of pastırma, high values were obtained in other types of pastırma. Although similar average results were obtained in a study in which the microbiological properties of the types of

pastırma produced from beef meat were determined, the highest counts of *Micrococcus/Staphylococcus* and lactic acid bacteria were determined in sırt type of pastırma [33]. Kaban [1] reported that the numbers of *Micrococcus/Staphylococcus* and lactic acid bacteria increased during pastırma production stages and that the number of lactic acid bacteria was less than 5 log CFU/g and the number of *Micrococcus/Staphylococcus* was higher than 6 log CFU/g in the final product. The number of *Enterobacteriaceae* in pastırma was generally low. In the present study, the number of *Enterobacteriaceae* was also found below the detectable limit (<2 log CFU/g) in all pastırma types.

According to the sensory evaluation, the highest odor and general acceptability scores were determined in the sırt type of pastırma. Tekinşen and Doğruer [5] have reported that the sırt type of pastırma is included in the first-quality pastırma class. While no significant difference was found between the other types of pastırma in terms of odor scores, the lowest evaluation scores were obtained for the bohça type of pastırma in terms of general acceptability.

Texture is one of the factors affecting the quality of meat products and the consumer preferences [38,39]. The product composition and processes applied in production are among the important factors affecting the textural properties of meat products. There are curing, pressing and drying processes in the production of pastırma. These processes can have significant effects both on the composition and the components constituting the composition of the product. As a result of these processes, a significant reduction occurs especially in the water content of the meat and significant changes are also observed in meat proteins [40]. In this study, the highest average firmness and chewiness values were determined in the kuşgömü type of pastırma samples. Because of faster drying, a lower a_w value in this type was observed compared to other types of pastırma. According to these results, a_w was correlated to firmness and chewiness so that decreasing a_w increased firmness and chewiness values of the product. These findings are in agreement with those reported by Lorenzo [41] for dry cured ham. On the other hand, the highest average values in terms of adhesiveness and cohesiveness were found in sırt and şekerpare types, and kuşgömü type followed these types.

According to the PCA results, pastırma types, kürek, bohça, kuşgömü, sırt and şekerpare, were clearly distinguished through PCA in terms of textural properties. Şekerpare and sırt were positively high correlated with chewiness in PC1. Moreover, kuşgömü, bohça and kürek were especially positive correlated with firmness in PC1 *(Fig 1)*.

As a result, there are some differences between the types of pastırma produced from different parts of the water buffalo carcass due to the properties and dimensions of the raw material used. Sırt type gives better results in terms of some textural and sensory properties while şekerpare type shows a better result for L* value, which is an important criterion, when compared to the other pastırma types. Another important finding of the research is that *Micrococcus/Staphylococcus*, which are technologically important, constitute the dominant flora in all types as this is the case for pastırma produced from beef. Moreover, as in pastırma produced by beef, pH of pastırma produced from water buffalo meat is over 5.5.

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Research Article

Molecular Detection of Selected Genetic Polymorphisms in Growth Hormone and Insulin Like Growth Factor 1 Genes in Indigenous Sudanese Baggara Cattle

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Abstract

The study involved 127 bulls of two geographically separate strains of Sudanese Baggara zebu cattle (Nyalawi and Mesairi). The target was to investigate two polymorphisms in growth hormone gene (M57764) and insulin like growth factor1 gene (000162.1). PCR-RFLP was used to genotype DNA samples and DNA sequencing was used to check the accuracy of genotyping results using selected samples. Available sequences were analyzed using BioEdit and MEGA6 softwares. Some population genetic measures in the two strains of Baggara zebu cattle were investigated. PCR-RFLP revealed that the two Baggara cattle strains have high genetic similarity at position 2141C>G of the *GH1* gene (monomorphic showing the ancestral allele C/C). There were no differences between the two strains at position -472C>T of the *IGF-1* gene promoter. The mutant homozygote (TT) was detected in the Mesairi strain only with a frequency of 0.016. The heterozygote (CT) genotype existed in the two strains with low allele frequencies (0. 068 and 0.079 for Nyalawi and Mesairi respectively). Moreover, three mutations were identified in exon5 of the *GH1* sequence including two silent mutations at positions 2230 (C>T) and 2291(A>C) and a third transition mutation at position 2258 (C/T) detected in the sequences of the two strains. In conclusion, the two strains were found to be genetically similar at target positions. The detected mutation at exon 5 of *GH1* (2258 (C/T) should be validated.

Keywords: *Nyalawi, Mesairi, Polymorphisms, IGF-1, GH1*

Yerli Sudan Baggara Sığırında Büyüme Hormonu ve İnsülin Benzeri Büyüme Faktörü 1 Genlerinin Seçili Genetik Polimorfizmlerinin Moleküler Tespiti

Öz

Bu çalışma bölgesel olarak farklı iki (Nyalawi ve Mesairi) Sudan Baggara zebu sığırlarından toplam 127 boğa üzerinde gerçekleştirilmiştir. Çalışmanın amacı bu iki farklı ırk arasında büyüme hormonu geni (M57764) ve insülin benzeri büyüme faktörü 1 genindeki (000162.1) polimorfizmi araştırmaktır. DNA örneklerinde genotiplendirme için PCR-RFLP kullanıldı. Seçili örneklerde genotiplendirmenin doğrulunu kontrol etmek amacıyla DNA sekanslaması kullanıldı. Mevcut sekanslar BioEdit ve MEGA6 yazılımları kullanılarak analiz edildi. Her iki Baggara sığır ırkında bazı popülasyon genetiği ölçümleri araştırıldı. PCR-RFLP sonuçları her iki Baggara sığır ırkında *GH1* geninin 2141C>G pozisyonunda yüksek genetik benzerlik olduğunu ortaya koydu (monomorfik atasal allel C/C görünümü). Her iki ırk arasında *IGF-1* geninin -472C>T pozisyonu bakımından bir fark belirlenmedi. Sadece 0.016 sıklıkta olmak üzere Mesairi ırkında mutat homozigot (TT) belirlendi. Düşük allel sıklıklarında olmak üzere (0. 068 Nyalawi ırkında ve 0.079 Mesairi ırkında) heterozigot (CT) genotip mevcuttu. Ayrıca, *GH1* geninin ekson 5 bölgesinde üç mutasyon tespit edildi. Bunlardan ikisi 2230 (C>T) ve 2291(A>C) pozisyonlarında sessiz mutasyon ve diğeri 2258 (C/T) pozisyonunda geçiş mutasyonuydu. Sonuç olarak, her iki ırkın incelenen hedef poziylonlar bakımından genetik olarak benzer oldukları belirlendi. *GH1* geninin ekson 5 bölgesinde tespit edilen mutasyon (2258 (C/T) ileriki çalışmalarda değerlendirilmelidir.

Anahtar sözcükler: *Nyalawi, Mesairi, Polimorfizm, IGF-1, GH1*

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INTRODUCTION

The Sudan is one of the largest African countries. It is predominantly an agricultural country with a significant development potential, especially in agriculture and livestock production. The local Sudanese cattle breeds belong to the Bos indicus species often referred to as Zebu type. The population of indigenous cattle was estimated at 40 million heads [1]. Kenana and Butana are the main dairy breeds, while western Baggara is the major beef producing cattle breed in Sudan and some neighbouring African countries used for local consumption and export [2]. About 90% of the cattle population is owned by pastoralists mainly concentrated in three major regions, mainly, Western Sudan, the homeland of Baggara cattle. The Baggara cattle and their different strains constitute about 80 percent of the country's total population of cattle.

Nyalawi and Mesairi are two important Baggara cattle strains. The two strains have different characteristic phenotypic morphologies. Both strains are main producers of beef for local consumption and export. However, Nyalawi strain consistently reported as having superior phenotypic beef characteristics [3].

Several genes were putatively controlling growth in animals, in which the somatotropic axis plays a key role. Growth hormone *(GH1)* and insulin-like growth factor 1 *(IGF-1)* genes act and mediate the growth of bones and muscles [4]. The two genes are considered as candidate genes for predicting growth and meat quality traits in animal genetic improvement since they play a key role in growth regulation and development [5]. Growth hormone *(GH),* a single chain polypeptide, was shown to stimulate fatty acid mobilization, amino acid uptake, DNA, RNA and protein synthesis and that it regulates cell division and tissue hypertrophy. It is known that *GH* is the main regulator of postnatal somatic growth, stimulating anabolic processes such as cell division, skeletal growth and protein synthesis and is involved in nutrient partition [6]. The bovine GH gene has several genetic variants including a characterized substitution C>G (leucine/valine) at position 2141 *(rs:4193484)*. The insulin like growth factor 1 *(IGF1)* has many diverse effects; it catalyses skeletal muscle hypertrophy through initiating protein synthesis, and blocking muscle atrophy. The decline in growth hormone secretion and plasma *IGF-1* concentrations is known to occur with age [7]. IGF1 plays an essential role in mammalian reproduction. *IGF* system has a significant relationship with prenatal growth and the growth and differentiation of the mammary gland [8].

The *IGF-1* gene, in comparison to GH1 gene, is extremely conserved among species and few polymorphisms are described including the -472C>T *(rs:109763947)* polymorphism at the 5'region of the gene.

The present study aimed at screening the two cattle strains for the single nucleotide polymorphism 2141C>G in the *GH1* gene and the -472C>T in the *IGF-1* gene. The two mutations were not previously reported in Baggara cattle. The study also used available sequences of the two genes to search for other mutations if they exist and investigate some population genetic measures in the two strains of Baggara zebu cattle (Nyalawi and Mesairi).

MATERIAL and METHODS

Ethics Approval

According to the Animals Use in Research Committee (AURC) of Khartoum University, this study does not require any special approval.

Animals and Sampling

The present investigation was carried out on 127 Baggara bulls that belong to two strains (Nyalawi and Mesairi). All animals were on average 4-5 years old finished in a feedlot near Ganawa commercial abattoir in Omdurman locality, Khartoum State, Sudan. One hundred and twenty seven Baggara blood samples were taken from the jugular vein of each bull using vacutainers containing EDTA as an anticoagulant and held at 4°C. Samples were transferred to the Centre of Excellence, Genetic and Molecular Biology Laboratory at the Department of Zoology Science, Faculty of Science, University of Khartoum where they were kept in a refrigerator pending genotyping and sequencing analysis.

Genomic DNA Extraction

Blood samples (3-5 mL) were collected in EDTA tubes. DNA was extracted following conventional methods [9] procedure with some modifications. Briefly, two ml of lysis buffer were added, followed by 10 µL of proteinase K, 1 mL of guanidine chloride and 300 µL of NH4 acetate. The mixture was incubated over night at 37°C, and then 2 mL of pre-chilled chloroform was added. The upper layer was collected -after centrifugation- to a new tube and DNA was precipitated by absolute ethanol. DNA was washed with 70% Ethanol and the pellet was allowed to dry. The pellet was re-suspended in 200 µL ddH_2O and DNA stock solution was stored at -20°C. The quality of the extracted DNA was measured using Nano-Drop Spectrophotometer (ND 1000) and agarose gel electrophoresis.

Genotyping of GH1 and IGF1 Genes

The growth hormone gene *(GH1)* locus was analyzed targeting a 404 bp fragment covering the sequence containing the 2141C>G missense mutation in exon 5. Similarly, a 249 bp fragment containing the C/T mutation at position -472 in the 5-non coding region of the *IGF-1* gene relative to the start of transcription site was also analyzed. Amplification procedure of the targeted *GH1* [10] was conducted using the forward primer:

F: 5′-TAGGGGAGGGTGGAAAATGGA and the reverse R: 5′ GACACCTACTCAGACAATGCG-3′ and the procedure for the IGF-1 [11] using the primers F: 5′-ATTACAAAGCTGCCTGCC CC-3′ and R: 5′-ACCTTACCCGTATGAAAGGAATATACGT-3′. The resulting PCR fragments were digested using restriction enzymes (Alu1and SnaB1 for the two genes, respectively).

Sequence Analysis and Alignment Procedures

One PCR product of each different genotype of the *GH1* and *IGF-1* genes were sequenced to confirm the detected genotypes using ABI 3730xl DNA Analyzers, with BigDye Terminator v3.1. PCR products were purified prior to sequencing and were sequenced in both directions. Resulted sequences were aligned and compared using BioEdit program in addition to selected available GenBank sequences representing *GH1* (EF592533 and EF592534) and *IGF1* (AF404761, KF202095 and KM111250) genes. The number of base substitutions per site between sequences and standard error estimate (s) analyses were conducted using the Tajima-Nei model [12]. Evolutionary analyses (Molecular Evolutionary Genetic Analysis) were conducted by MEGA6 [13]. The gene and genotype frequencies were calculated using Falconer and Mackay [14] Method.

RESULTS

Genotyping of Growth Hormone1 Gene (GH1)

The missense mutation polymorphism in the *GH1* which was reported to occur in a single nucleotide causing a C/G substitution resulting in the replacement of leucine with valine was not present in the studied populations. The digestion of the 404 bp PCR product *(Fig. 1)* with the *Alu*1(AG/CT) endonuclease enzyme resulted in one restriction pattern which was assigned as homozygous genotype CC that produced four fragments (185-132-51-36 bp) in all animals under study *(Fig. 2)*. There was a complete absence of both homozygous (TT) genotype (236-132-36 bp) and the heterozygous (CT) genotype (236-185-132-51-36 bp) in all animals under study *(Table 1)*.

Bovine GH1 Gene Sequencing

Sequence Comparison: The results of the present study showed that Baggara cattle (Nyalawi and Mesairi) were homozygous for the 2141C allele (monomorphic for the Leucine variant) in the 2141C>G SNP (leucine to valine CTG/GTG) *(Fig. 3* and *Fig. 7-A)*. Similar results were found

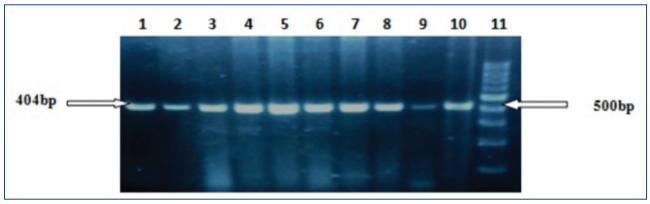

Fig 1. Agarose gel electrophoresis (2%) displaying PCR amplification of exon5 of the Baggara *GH1* Lanes:1-10 PCR products of samples. Lane 11: 100 bp DNA Ladder

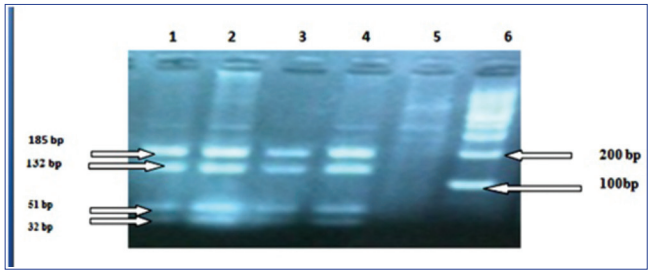

Fig 2. Agarose gel electrophoresis (2%) displaying *Alu*1 restriction digest on an amplified portion of exon 5 of the Baggara *GH1*. Lanes: 1-4 representing CC genotype (185+132+51+36 bp). Lane 5: negative control. Lane 6: 100bp DNA ladder

Table 1. *Genotypic and allelic frequencies, χ2 test values and diversity parameters for the 2141C>G (Leu/Val) substitution of GH1 and the-472 C>T of the IGF-1 gene*

Baggara Strains (Number)	Gene Name	Genotype	*No.	GF	Allele	AF	χ2 /HWE	He
Nyalawi (64)	GH1	CC	64	1	C	1		0
		CG	0	0	G	0		
		GG	0	0				
	IGF-1	CC	51	0.864	C	0.932	0.407 NS	0.1453
		CT	8	0.136	T	0. 068		
		TT	0.0	0.0				
Mesairi (63)	GH1	CC	63	1	C	1		0
		CG	0	0	G	0		
		GG	0	0				
	IGF-1	CC	54	0.857	C	0.921	1.085 NS	0.1455
		CT	8	0.127	T	0.079		
		TT	1	0.016				

** The given numbers are those of DNA samples successfully amplified GF: Genotype frequency, AF: Allelic frequency, χ2 (HWE): Hardy-Weinberg equilibrium χ2 value, Hardy-Weinberg equilibrium (P>.05), He: Gene heterozygosity*

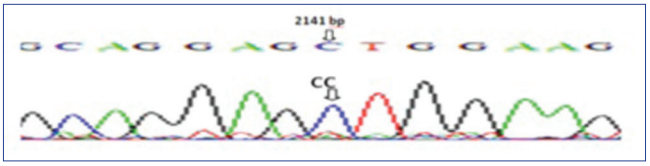

Fig 3. The homozygous (CC) at the 2141 position of the *GH1* detected in both Nyalawi and Mesairi cattle strains

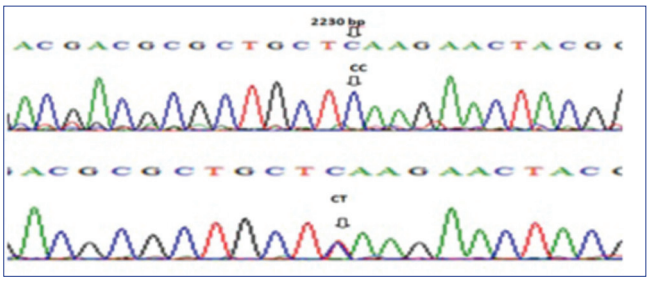

Fig 4. The silent mutation at the position 2230bp (Leucine to leucine, CTC/CTT) of the *GH1* detected in Nyalawi sequence

in Butana and Kenana *GH1* gene according to GenBank (EF592533 and EF592534, respectively). Moreover, three non-targeted mutations were identified in exon 5. The first mutation was a C/T transition at position 2230 in which a C nucleotide turned into T nucleotide (C/T) to produce a silent mutation (Leucine to leucine, CTC/CTT) detected only in Nyalawi sequence *(Fig. 4* and *Fig. 7-D)*. A second transition mutation was detected at 2258 bp (C/T) (Arginine to Tryptophan CGG/TGG), which existed in the sequences of the two Baggara strains *(Fig. 5* and *Fig. 7-D),* but it was not found in Butana and Kenana cattle *GH1* sequence according to GenBank records *(Fig. 7D)*. No mutations were detected in exon 5 of the *GH1* gene sequences at A, B, C, F, H and I *(Fig. 7 A,B,C,F,H,I)*.

The third silent mutation was at 2291A>C (Arginine to Arginine AGG/CGG) of the *GH1* gene and was found in Baggara cattle strains, and in the Sudanese Butana and Kenana cattle *(Fig. 6* and *Fig. 7-E)*.

Alignment of sequences also indicated a nucleotide change (C/T) at position 2346 present in Kenana cattle *(Fig. 7-G)* and absent in all study animals and Butana cattle sequences.

The Phylogenetic analysis of Baggara, Kenana (EF592534.1) and Butana (EF592533.1) cattle *GH1* gene sequences *(Fig. 8)* indicated two major clusters. The Baggara strains in one cluster with two sub-branches containing the two strains (Nyalawi and Mesairi) both in the same major cluster. The Baggara strains were not similar to Kenana *GH1* gene (EF592534.1) and Butana (EF592533.1) cattle at that region of *GH1* gene.

Insulin- Like Growth Factor1 Gene

The missense mutation (on chromosome 5) in the bovine

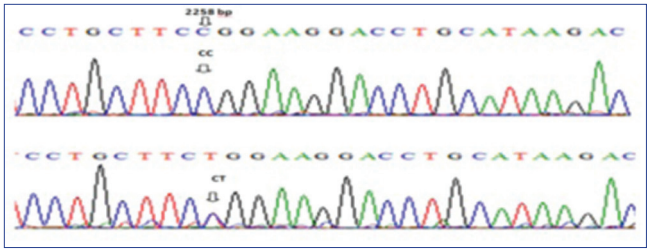

Fig 5. The transition mutation at the position 2258 bp (Arginine to Tryptophan CGG/TGG) GH1detected in both Nyalawi and Mesairi cattle strains

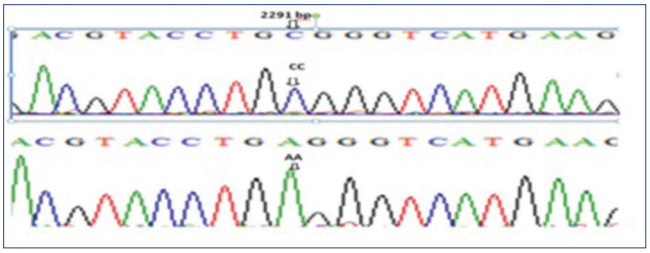

Fig 6. The silent mutation at the position (2291A>C) (Arginine to Arginine AGG/CGG) of the *GH1* gene and was found in Baggara cattle strains, Butana cattle and Kenana cattle

Insulin like growth factor 1 *(IGF1)* gene causes the replacement of cytosine (C) by thymine (T) at position -472 in the 5'noncoding region of the *IGF-1* gene. The digestion of the 249 bp *(Fig. 9)* PCR products was carried out with the restriction endonuclease enzyme SnaBI (TAC/GTA). Three restriction patterns were obtained; the uncut pattern was assigned as the homozygote wild type genotype (CC) (249 bp), the heterozygote genotype (CT) produced three fragments (249, 223 and 26 bp), while the homozygous (TT) produced two fragments (223 - 26 bp) *(Fig. 10)*.

The gene and genotype frequency of the IGF1 gene were as shown in *Table 1*. The wild type C variant frequency was the highest among the two cattle strains. The chi- square test values indicated that the two strains were at Hardy-Weinberg equilibrium *(Table 1)*. The heterozygosity (He) was the same (0.145) in the two cattle strains.

IGF1 Gene Sequencing

In addition to the GenBank *IGF1* gene sequence (AC: 000162.1), the obtained *IGF1* sequences of Baggara strains were aligned and compared with other available *IGF1* gene sequences in the GenBank (AF404761, KF202095 and KM111250) using BioEdit program. The transition mutation in the *IGF1* gene (C>T) was found in the homozygous state (TT) in the Mesairi subtype only, whereas the heterozygote genotype (CT) was detected in both strains *(Fig. 11* and *Fig. 12-C)*. This SNP was also found in Bos taurus according to GenBank (AF404761 and KF202095). Another SNP (-468 bp; T-A; Not registered) was observed in both Bos taurus (AF404761) and Bos indicus (KM111250) and absent in the third sequence *(Bos taurus,*

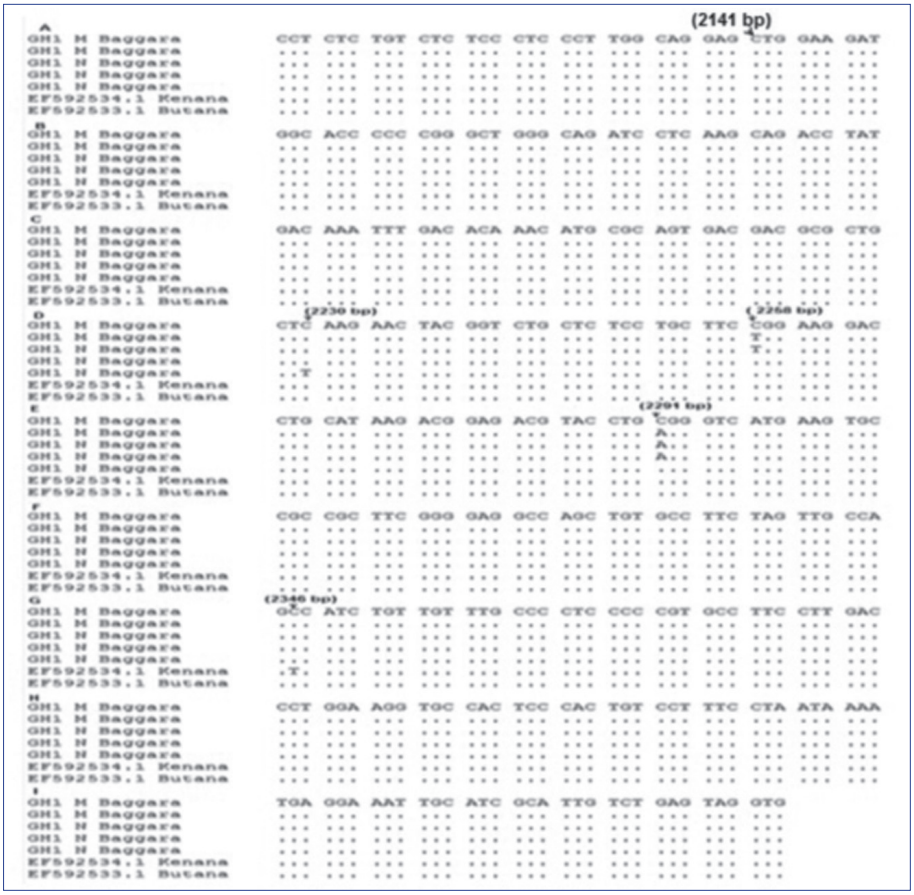

Fig 7. Comparison of the *GH1* (exon5) sequences of Baggara, Kenana and Butana cattle* Accession numbers: EF592533 and EF592534 are the sequences of Butana and Kenana of *GH1* gene in the GenBank. M: Mesairi strain, N: Nyalawi strain. Aligned sequences of complete exon5 and flanking region are presented in 9 patches and given letters for simplicity (A, B, C, D, E, F, G, H & I)

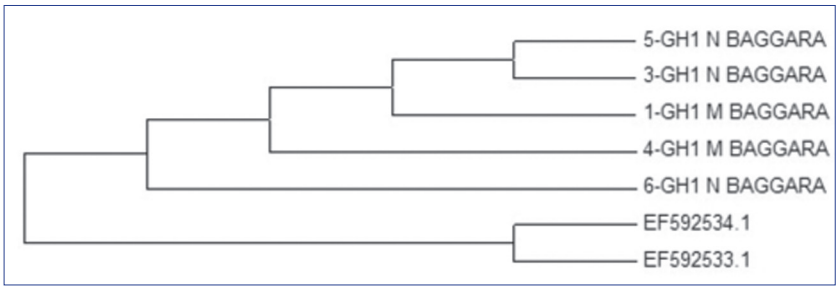

Fig 8. Phylogenetic tree of the *GH1* gene sequences of Baggara, Kenana (EF592534.1) and Butana (EF592533.1) cattle

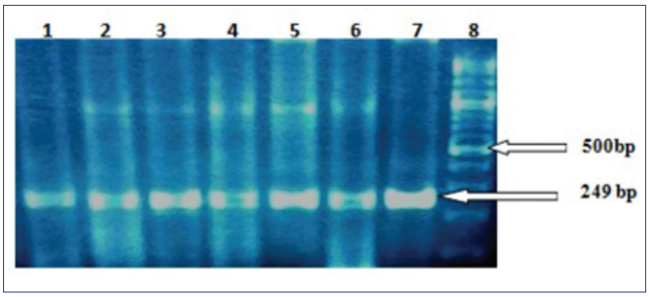

Fig 9. Agarose gel electrophoresis (2%) displaying PCR amplification of Baggara *IGF1* 5'- flanking region. Lanes: 1-7 PCR products of samples. Lane 8: 100 bp DNA Ladder

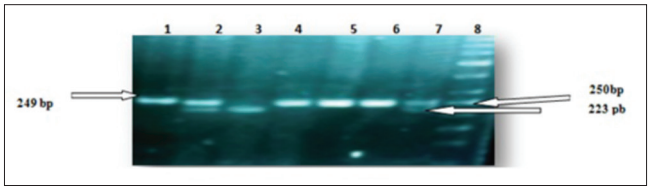

Fig 10. Agarose gel electrophoresis (2%) displaying SnaB1 restriction digest of an amplified portion of Baggara *IGF1* gene in the 5'- flanking region. Lanes: 1, 4, 5 and 6 representing CC genotype (249 bp). Lane 3 representing TT genotype (223+26). Lanes 2 and 7 representing CT genotype (249+223+26). Lane 8: 50 bp DNA ladder

KF202095), while it was presented as homozygote TT in both Baggara cattle strains *(Fig.12 -C)*. No mutations were detected at in the A and B parts of the sequence *(Fig. 12 A,B)*.

In Phylogentic analysis, *IGF1* gene sequence of the Nyalawi and Mesairi clustered within the same main cluster of Bos indicus while the Bos taurus clustered in a different isolated cluster *(Fig. 13)*.

Selected sequences were submitted at the GenBank and

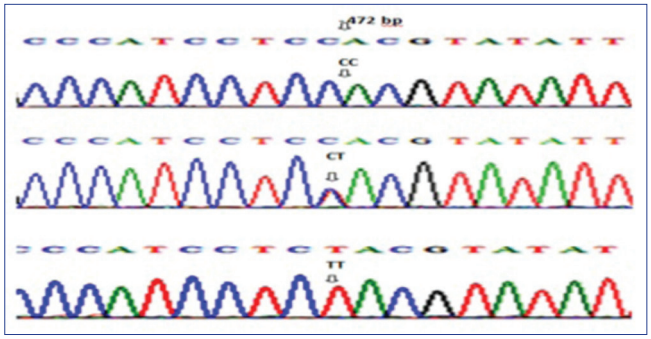

Fig 11. The chromatogram of the sequenced 5'-noncoding region *IGF1* gene showing homozygote (CC and TT) and the heterozygote (CT) genotypes

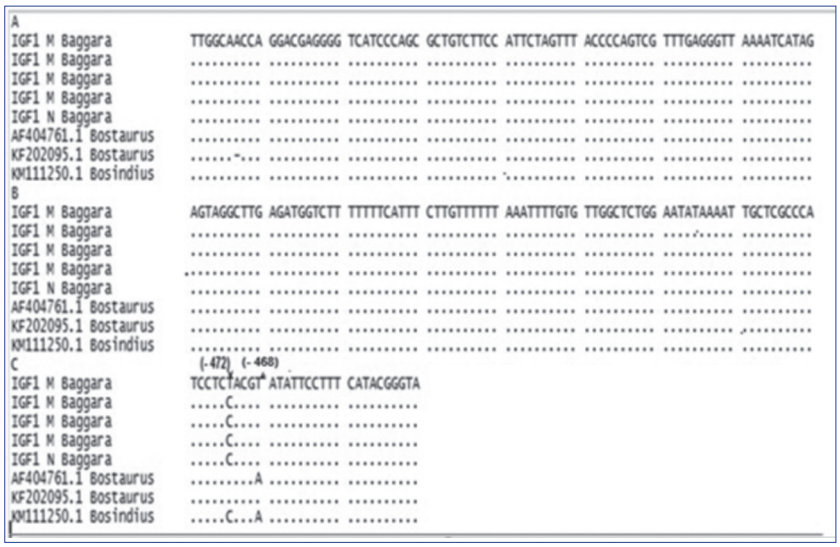

Fig 12. Comparison of the *IGF1* gene sequences of Baggara, Bos taurus (AF404761 and KF202095) and Bos indicus cattle (KM111250). M: Mesairi strain. N: Nyalawi strain. Aligned sequences of partial 5' UTR are presented in 3 patches and given the letters A, B, & C

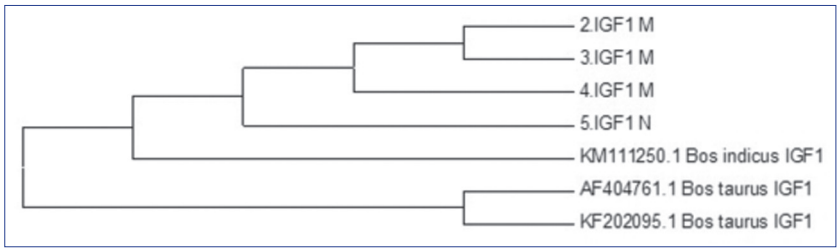

Fig 13. Phylogenetic tree of the *IGF1* gene sequences of Baggara, Bos taurus and Bos indicus cattle. M: Mesairi strain, N: Nyalawi strain

the following accession numbers were given for the *GH1* and *IGF1* respectively:

MG879304, MG879305, MG879306, MG879307, MG879308 and MG879299, MG879300, MG879301, MG879302, MG879303.

DISCUSSION

The implementation of genomics creates many opportunities for beef cattle production through increased genetic progress and the inclusion of new traits of economic importance in the selection programs. The identification of potential SNPs in selected breeds or environments will make the exploitation of novel genomic selection methodologies in farm animals possible. Better genetic characterization of local breeds can help increase selection intensity and decrease generation interval. Local breeds in Sudan are generally poorly characterized and there is the possibility of detecting new mutations. In the current study, two important Sudanese Baggara cattle (Nyalawi and Mesairi) were targeted for genotyping in two important candidate genes in beef production. Sequencing was performed for the purpose of confirmation and to detect new mutations if any. The results of genotyping of the SNP 2141C>G (Leu/Val substitution) showed that all Nyalawi and Mesairi bulls in our study were homozygous for 2141C allele (Leu variant). A previous report [3] mentioned that the genotyping results (using PCR-RFLP) for Nyalawi and Mesairi (Baggara cattle) in exon 5 showed similarity between the strains although they were phenotypically different. Musa et al.[15] reported that the 2141C variant (Leu) appeared to be monomorphic in all studied animals of Kenana and Butana cattle. The mutation was also not found in Bali cattle [16].

Another group [17] found that the substitution at the same position of the *GH1* occurred with allele frequencies of 0.85 and 0.15 for C and G alleles, respectively, in Podolian cattle in Southern Italy. The excess of homozygotes (100% CC) in the current study, which resulted in the disequilibrium, may reflect a series of events such as inbreeding, selection, genetic drift or population subdivision. However, some mutations were detected in the sequenced region the *GH1* sequence of Baggara strains including the 2291A>C SNP. According to GenBank, the mutation was previously reported in Butana, Kenana, Pakistani Dhanni, Red Sindhi, Sahiwal and Kamori cattle breeds *GH1* sequence (accession numbers EF592533, EF592534, DQ307369, DQ307370, EF451794 and EF451795, respectively).

Two other mutations were also detected in Sudanese Baggara *GH1* sequence. The first mutation is 2230 C>T SNP which appeared in the Nyalawi subtype only. A similar result was mentioned by another group in Indonesia [18] who reported the same C>T SNP as a new mutation in

exon five of Aceh cattle (Banda Aceh and Indrapuri). The second mutation (2258 bp C>T) appeared in the two strains of Baggara cattle. The 2258 C>T SNP was previously associated with average daily weight gain (ADG) and carcass weight (CWT) [19]. The high allele frequency of the favorable allele (C) in both European and Asian Bos taurus breeds indicated that selection for genetic improvement of ADG and CWT is associated with the SNP or a locus at Linkage disequilibrium with the SNP [19]. There is a need to further investigate this important mutation in all strains of Baggara cattle. However, according to GenBank records those two SNPs were not reported in Butana and Kenana cattle.

The results of PCR-RFLP also indicated that Baggara cattle strains showed the existence of the targeted SNP of the *IGF1* gene. Genotype frequencies were: 86% (CC) and 14% (CT) in Nyalawi subtype compared to 86% (CC), 13% (CT) and 2% (TT) in Mesairi. Using Chi-square ($\chi 2$) test, both Nyalawi and Mesairi strains were shown to be at Hardy-Weinberg equilibrium ($\chi 2$=0.407and 1.085, respectively) in this locus indicating low exchange of alleles between populations, large population size, and no selective pressure for or against any genotype.

The TT genotype was found only in the Mesairi subtype at a low allele frequency. The reported frequency of the T allele (0.079) was very low in Mesairi subtype in comparison to other reports in which allele frequencies of T and C alleles were 0.64 and 0.36, respectively, in Angus cattle [11], 0.48 and 0.52 in Polish Holstein-Friesian cattle [20], 0.55 and 0.45 in a population of Holstein cattle [21], 0.56 and 0.44 in two commercial lines of dairy cattle [22] and 0.54 and 0.46 in Polish Holstein-Friesian heifers [23].

Our results disagree with those previously published [3] in Sudan using the two Baggara strains in which only the homozygous CC genotype was found. They considered that the reason for the differences in growth rate between Bos taurus and Baggara cattle could be due to nutritional factors. One study [24] proposed that allele B (C allele) is characteristic to indicine populations as it was fixed in a Nellore population.

A potential expression changing SNP was detected at -468 bp (T-A). This SNP was presented in homozygous TT in all sequenced samples and was only observed in both *Bos taurus* (AF404761) and Bos indicus (KM111250). Preliminary analysis indicated changes at putative transcription factor binding sites (PROMO software). The ancestral allele could be any of the detected alleles. Such SNPs might have significant importance in population and evolutionary studies.

The newly detected mutations were not previously reported in Baggara cattle and their frequencies should be validated. The transition mutation in the *GH1* sequence at 2258 bp (C/T) position which existed in the sequences of the two Baggara strains and its association with growth traits should also be investigated. More SNPs need to be screened among Sudanese Baggara cattle to reveal the genetic profile of this important cattle ecotype in Sudan and other African countries using high throughput techniques.

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Research Article

Influence of Feeding *Moringa oleifera* Pods as Phytogenic Feed Additive on Performance, Blood Metabolites, Chemical Composition and Bioactive Compounds of Breast Meat in Broiler

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Abstract

This study was conducted to explore the effect of *Moringa oleifera* pods meal (MPM) on growth performance, blood metabolites, chemical composition, meat quality and immunity of broilers. For the purpose, two hundred (Hubbard classic) broiler birds having weight 40.4±3.02 g, were assigned to four treatments with five replicates and ten birds per replicate in a Completely Randomized Design. Starter and finisher diets were added with four levels (0, 0.5, 1.0 and 1.5% of MPM) over and above. Results of this study showed that growth performance was improved as feed conversion ratio (FCR) and feed intake (FI) were decreased with the increase in supplementation level (P≤0.05). Dressing percentage was lowered, whereas giblet weights were improved with the MPM supplementation levels (P≤0.05). β-carotene, Quercetin and Selenium content of breast meat was linearly increased resulting in higher values of di-phenyl picryl hydrazil (DPPH) radical scavenging and improved shelf life. Serum biochemical compounds like serum glutamic pyruvic transaminase (SGPT), Creatinine, Glucose and meat cholesterol level was significantly decreased and was recorded lowest in 1.5% MPM supplemented diet (P≤0.05). At the end of the trial it was concluded that Moringa pods may positively affect the growth and chemical composition of broiler meat.

Keywords: *Broiler, β-carotene, DPPH, Moringa pods, Quercetin, Selenium*

Gıda Katkısı Olarak *Moringa oleifera* Kabukları İle Beslemenin Broiler Tavuklarda Performans ve Kan Parametreleri İle Göğüs Etinin Kimyasal Kompozisyonu ve Biyoaktif Bileşiklerine Etkisi

Öz

Bu çalışma *Moringa oleifera* kabukları içeren yemin broiler tavuklarda büyüme performansı, kan metobolitleri, etin kimyasal kompozisyonu ve kalitesi ile bağışıklığa olan etkilerini araştırmak amacıyla yapıldı. Bu amaçla, 40.4±3.02 g ağırlığında toplam 200 broiler (Hubbard classic) 4 uygulama grubu ve 5 tekrar olmak üzere (her tekrar için 10 tavuk olacak şekilde) tamamıyla rastgele olarak kullanıldı. Başlangıç ve bitirme diyetlerine %0, 0.5, 1.0 ve 1.5 miktarlarında *Moringa oleifera* kabukları eklendi. Çalışma sonucunda, artan katkı maddesi miktarıyla orantılı olarak büyüme performansının iyileştiği, yem konversiyon oranın ve yem tüketiminin azaldığı tespit edildi (P≤0.05). Artan katkı maddesi miktarıyla orantılı olarak tüy yüzdesi düşerken sakatat ağırlığında artma gözlemlendi (P≤0.05). Göğüs etinde β-karoten, kuersetin ve selenyum miktarı doğrusal olarak artış göstererek daha yüksek değerlerde difenil pikril hidrazil (DPPH) radikal temizleme ve artmış raf ömrü gözlemlenmiştir. Serum glutamik piruvik transaminase, kreatinin, glukoz ve et kolesterol gibi serum biyokimyasal bileşiklerinin seviyeleri anlamlı derecelerde düşmüş ve en düşük olarak %1.5 *Moringa oleifera* kabukları içeren yemde kaydedilmiştir (P≤0.05). Çalışma sonunda Moringa oleifera kabuklarının broiler tavuklarda büyüme ve etin kimyasal kompozisyonuna pozitif etkisinin olduğu sonucuna varılmıştır.

Anahtar sözcükler: *Broiler, β-karoten, DPPH, Moringa kabukları, Kuersetin, Selenyum*

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INTRODUCTION

The broiler meat is one of the most abundant and cheapest sources of quality protein for human consumption. But unfortunately, broiler industry is facing multiple infectious threats caused by different pathogenic microbes. Therefore, safe and healthy chicken production needs proper microbial control for quality meat production [1]. Currently, different synthetic preparations including antibiotics are being used to control the microbial infection. But at the same times, abundant use of antibiotics resulted in hazardous outcomes in the form of emergence of antibiotic resistance in susceptible microbial populations, drug residues in meat and environmental pollution [2,3]. Due to the reason, this irrational use of antibiotics is being discouraged/ banned all over the world [4,5]. Currently, various phytogenic feed additives are encouraged in animal feed industry as a replacement to antibiotic growth promoters [6]. Plants are a rich source of bioactive compounds with diverse biological and pharmacological activities [7]. The functional properties of these bioactive compounds vary with the gradual partitioning of the whole plant starting from leaves, flowers, pods, fruits, stem, bark and roots [7,8]. Bioactive compounds route through modification of pancreatic activity by potentiating hydrolysis and decreasing the cellular damage in the intestine which consequently results in better nutrient utilization by improving metabolism and absorption and thus resulting in better growth performance and feed conversion ratios [9,10]. These compounds also enhance immune status of birds by reducing endotoxins and proliferation of pathogenic microbes [7]. Modern concept of phytogenic feed additives is based on the plant secondary metabolites like carotenoids, flavonoids and essential oils which help in fighting against multiple diseases in human when enriched in animal products through animal feed [11]. These compounds express antioxidant activities by scavenging the free radicals and becomes part of meat and eggs [12-14]. Various plant species including oregano, cinnamaldehyde, *Capsicum oleoresin*, garlic, turmeric and *Moringa (M.) oleifera* are rich in such bioactive compounds [9]. In this regard, *M. oleifera* is easily available in tropical and subtropical countries of the world including Pakistan, Bangladesh, India, Africa, Ethiopia, Kenya and many other countries. Poultry industry in developing countries facing the ingredients shortage, which enhances the importance of non conventional feed resources which could replace the costlier feed ingredients like proteins [15]. The *M. oleifera* pods contain fairly high quantities of essential amino acids enriched proteins, fat, minerals, vitamins and other bioactive compounds [15-17]. Keeping in view the nutritional quality of *M. oleifera* pods, this study was conducted to evaluate the effect of *M. oleifera* pods meal (MPM) in improving the growth performance and antioxidant attributes of broiler meat.

MATERIAL and METHODS

Moringa pods were collected from the fields of central and southern Punjab, Pakistan. After washing, cleaning and grading the pods were shade dried to a moisture level of ≤12% to keep the bioactive compounds intact in the plant material [18]. The dried pods were ground to fine powder, stored in sealed air tight containers at 4°C till further use and subjected to proximate analysis *(Table 1)* for determination of different nutrients by using the methodology described by Association of Official Analytical Chemist [19]. Four iso-caloric and iso-nitrogenous diets A, B, C & D were formulated having crude proteins 20.5 and 19% and ME levels 2850 and 2875 kcal/kg for both starter and finisher phases *(Table 2, Table 3)*, respectively as recommended by National Research Council [20]. Two hundred day old broiler (Hubbard) chicks with authentic hygienic and bio-security standards were randomly assigned to four dietary treatments viz. A (MPM-0%), B (MPM-0.5%), C (MPM-1.0%) and D (MPM-1.5%) with five replicates for every treatment for a duration of six weeks. The birds were reared on floor system in a modern environment controlled poultry house under standard management conditions. All birds were vaccinated according to local vaccination schedule [21]. Experimental diets and water were offered *ad-libitum* to all the birds.

Collection of Data, Serum and Meat Samples

Birds handling and collection of samples was performed according to the procedure approved by advance studies and research board (ASRB) of the University of Veterinary and Animal Sciences, Lahore, Pakistan in the meeting (DAS/1948) held on 23 September 2013. Feed intake and mortality were recorded on daily basis; whereas, cumulative weight gain (CWG) and FCR were calculated

Table 1. *Chemical composition of Moringa oleifera pods meal*

Chemical Composition	Proportion	Unit
Moisture	8.05	g/100 g
Crude Protein	18.98	g/100 g
Ether Extract	2.34	g/100 g
Ash	7.88	g/100 g
Minerals		
Sodium	805	mg/100 g
Potassium	2815	mg/100 g
Calcium	291	mg/100 g
Magnesium	251	mg/100 g
Phosphorus	9456	mg/100 g
Selenium	25.71	mg/100 g
Bioactive Compounds		
Quercetin	114	mg/100 g
β-carotene	2.76	mg/100 g

Table 2. *Experimental broiler starter diets and their chemical compositions*

Ingredients	Proportions in Experimental Diets			
	A	B	C	D
Maize (kg)	50.00	50.00	50.00	50.00
Soybean meal (kg)	22.13	22.13	22.13	22.13
Canola meal (kg)	15.00	15.00	15.00	15.00
Rice polish (kg)	6.54	6.54	6.54	6.54
DCP (kg)	2.34	2.34	2.34	2.34
Soy oil (kg)	1.76	1.76	1.76	1.76
Limestone (kg)	0.60	0.60	0.60	0.60
L-lysine sulphate (kg)	0.55	0.55	0.55	0.55
DL-methionine (kg)	0.25	0.25	0.25	0.25
Soda bicarb (kg)	0.35	0.35	0.35	0.35
(Vit.& Min Premix) (kg)	0.20	0.20	0.20	0.20
Salt (kg)	0.15	0.15	0.15	0.15
L-threonine (kg)	0.12	0.12	0.12	0.12
Total (kg)	100	100	100	100
Moringa pod (%)	0	0.5	1.0	1.5
Chemical Composition	**Nutrients Proportion in Diets**			
Dry matter (%)	90.20	90.20	90.20	90.20
Crude Protein (%)	20.50	20.50	20.50	20.50
ME (kcal/kg)	2850	2850	2850	2850
Fat (%)	5.05	5.05	5.05	5.05
CF (%)	4.74	4.74	4.74	4.74
Ash (%)	6.27	6.27	6.27	6.27
Dig. lysine (%)	1.2	1.2	1.2	1.2
Dig. threonine (%)	0.78	0.78	0.78	0.78
Dig. meth + Cysteine (%)	0.88	0.88	0.88	0.88
Sodium (%)	0.18	0.18	0.18	0.18
Calcium (%)	0.9	0.9	0.9	0.9
Available phosphorus (%)	0.44	0.44	0.44	0.44
Se (mg/kg)	0.13	0.31	0.42	0.65
β-carotene (mg/kg)	0.34	0.57	0.70	0.79
Quercetin (mg/kg)	0.48	7.98	15.81	22.87

Table 3. *Experimental broiler finisher diets and their chemical compositions*

Ingredients	Proportions in Experimental Diets			
	A	B	C	D
Maize (kg)	50.00	50.00	50.00	50.00
Soybean meal (kg)	17.79	17.79	17.79	17.79
Canola meal (Kg)	15.00	15.00	15.00	15.00
Rice polish (kg)	11.29	11.29	11.29	11.29
DCP (kg)	2.22	2.22	2.22	2.22
Soy oil (kg)	1.62	1.62	1.62	1.62
Limestone (kg)	0.62	0.62	0.62	0.62
L-lysine sulphate (kg)	0.38	0.38	0.38	0.38
DL-methionine (kg)	0.24	0.24	0.24	0.24
Soda bicarb (kg)	0.37	0.37	0.37	0.37
Vit & Min Premix (kg)	0.20	0.20	0.20	0.20
Salt (kg)	0.12	0.12	0.12	0.12
L-threonine (kg)	0.14	0.14	0.14	0.14
Total (kg)	100	100	100	100
Moringa pod (%)	0	0.5	1.0	1.5
Chemical Composition	**Nutrients Proportion in Diets**			
Dry matter (%)	90.20	90.20	90.20	90.20
Crude protein (%)	19.18	19.18	19.18	19.18
ME (kcal/kg)	2875	2875	2875	2875
Fat (%)	5.67	5.67	5.67	5.67
CF (%)	5.16	5.16	5.16	5.16
Ash (%)	6.18	6.18	6.18	6.18
Dig. lysine (%)	1.15	1.15	1.15	1.15
Dig. threonine (%)	0.75	0.75	0.75	0.75
Dig. meth + Cysteine (%)	0.84	0.84	0.84	0.84
Sodium (%)	0.18	0.18	0.18	0.18
Calcium (%)	0.84	0.84	0.84	0.84
Available phosphorus (%)	0.43	0.43	0.43	0.43
Se (mg/kg)	0.13	0.31	0.42	0.65
β-carotene (mg/kg)	0.34	0.57	0.70	0.79
Quercetin (mg/kg)	0.48	7.98	15.81	22.87

on weekly basis. Blood samples were collected in EDTA coated vacutainers at 4th and 6th weeks of experiment and stored at -20°C for biochemical (SGPT, Glucose, Cholesterol and Creatinine) tests. The antibody titers against Newcastle disease and Infectious bursal disease vaccines were calculated using haemagglutination inhibition (HI) and enzyme-linked immunosorbent assay kit (Merck Microlab-300, country Germany), respectively. Three birds from each replicate were weighed, slaughtered and examined for dressing percentage and giblet relative weights at termination of experiment. Breast meat samples were taken at termination of experiment and analyzed for the detection and quantification of minerals (Na, K, Ca, Mg, Se, etc.) using atomic absorption spectrophotometry [19].

Breast Meat β-Carotene and Quercetin Analysis

High Performance Liquid Chromatography (HPLC) technique was used for the estimation of carotenoids (β-carotene) and flavonoids with standard methods used in previous studies [22,23]. Briefly, breast meat sample (1 g) was vortexed thrice for 5 min after addition of methanol (0.8 mL) and 1N HCl (0.2 mL) followed by centrifugation at 4000 rpm for 15 min. Supernatant was separated and dried on water bath set at 70°C. Extraction of organic compounds was performed by 0.1 mL mobile phase (70:20:10 v/v/v, Acetonitrile: Dichloromethane: Methanol) addition. The sample was vortexed for 5 min and filtered into HPLC vials by using 0.1 µm filter paper (Whatman No. 40) and

subjected to HPLC analysis for bioactive β-carotene. HPLC system having a diode array detector (DAD) at 450 nm with 5 µm C18 reverse phase column was opted. Samples were injected with a flow rate of 1.0 mL min^{-1} at 30°C and retention time 6.19 min. Standards were used to draw calibration curve, which helped in quantification of β-carotene. For this purpose the standard solution with a serial dilution ranging from 0.01 to 0.08 mg/L were used for the determination of β-carotene [22].

Estimation of quercetin was done with method used by Tokusoglu *et al*.[23]. Standards of quercetin (HPLC grade) were purchased from Sigma chemicals (St Louis MO, USA) through a local supplier. For sample preparation breast meat (1 g) was taken in a glass tube having acidified methanol containing 1% (v/v) HCl and 0.5 mg mL^{-1} TBHQ (Tertiary Butyl Hydroquinone). The temperature of the extract was lowered down to room temperature and centrifugation was done at 1500 g (5000 rpm). The supernatant was removed and sonicated for 5 min to remove air and finally filtered for injection into HPLC. Estimation of Quercetin was conducted with high-performance liquid chromatograph (HPLC) model (LC-10As) Shimadzu, Kyoto, Japan. Sample volume 20 µL was injected with flow rate of 1.0 mL min^{-1} at 30°C for chromatographic separation. Standards were used to draw calibration curve, which helped in quantification of quercetin. For this purpose the standard solution with a serial dilution ranging from 0.01 to 0.08 mgL^{-1} were used for the determination of quercetin.

DPPH- Radical Scavenging Activity

Antioxidant activity of meat samples was quantified by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity as described by Singh *et al*.[24]. A known volume (25 µL) of Butylated hydroxyl anisole (BHA) was added to 50 µL of meat extract in a tube. The final volume was adjusted to 100 µL by adding methanol (MeOH) followed by the addition of methanolic solution of DPPH (5 mL). The tube was shaken vigorously and incubated at 27°C for 20 min. Same method with only difference of meat extract was used for preparation of control along with methanol for baseline correction. Samples were run on UV-visible and absorbance was taken at 517 nm. The parameters for radical scavenging activity was DPPH radical inhibition percentage [24].

Cholesterol Estimation of Breast Meat

Sample was prepared by using the methodology described by AOAC [19]. Briefly, acetone (1 mL) was added in broiler breast meat (1 g) and vortexed for two minutes after vigorous shaking. Acetone was decanted after centrifugation at 10.000 rpm for 10 min. This procedure was repeated thrice and the acetone fractions obtained were pooled down and allowed to evaporate. The acetone extract thus obtained was supposed to have cholesterol and was subjected to UV-visible spectrophotometer to get the absorbance at wavelength of 500 nm. The cholesterol contents were calculated by using the formula given below [25].

Relative standard deviation;

$$\%RSD = \frac{\text{Standard deviation of measurements}}{\text{Mean value of mesarumenets}} \times 100$$

Recovery;

$$\%Recovery = \frac{\text{Amount found}}{\text{Amount added}} \times 100$$

Amount found;

$$\text{Amount found (mg/mL)} = \frac{\text{Mean test absorbance}}{\text{Mean std. absorbance}} \times \text{Std. Conc.}$$

Amount added;

$$\text{Amount added (mg/mL)} = \frac{\text{Weight}}{\text{Volume}}$$

Limit calculation for stability of analytical solution study;

$$\text{Limit} = \frac{\text{Absorbance of Std. initial - Absorbance of Std. (at different time)}}{\text{Absorbance of Std. initial}} \times 100$$

Statistical Analysis

Data thus obtained were analyzed through one-way ANOVA technique using Generaliz Linear Model (Proc Glm, SAS 9.4) [26]. The differences between means were calculated through Duncan's Multiple Range test. The differences were considered significant at $P<0.05$.

RESULTS

Growth Performance

The results of present study showed significant ($P\leq0.05$) growth performance of the broiler birds fed on diet supplemented with *Moringa oleifera* pods meal (MPM). Linear decrease in feed intake was observed and highest feed intake was recorded in the control group whereas, lowest value was recorded in the diet D supplemented with MPM-1.5%. Similarly, carcass traits and organ weights were significantly ($P\leq0.05$) affected with supplementation of MPM. Organs weights like liver, gizzard and heart were significantly ($P\leq0.05$) improved with the MPM supplementation, however, decreased after optimum level of supplementation *(Table 4)*. Body weight and body weight gain showed a quadratic response and was decreased as the supplementation

Table 4. *Growth performance and carcass traits of broilers fed on different levels of Moringa oleifera pod meal at 42 days of age*

Parameter	Control	MPM 0.5 %	MPM 1.0 %	MPM 1.5 %
FI	5068±69.20^{a}	5005±100.19^{ab}	4817±81.48^{b}	4808±44.42^{b}
BW	2500±41.35^{ab}	2525±41.76^{ab}	2575±42.59^{a}	2433±40.24^{b}
BWG	2463±41.49^{ab}	2487±41.26^{ab}	2538±42.63^{a}	2394±39.75^{b}
FCR	2.03±0.04^{a}	1.98±0.04^{ab}	1.87±0.05^{b}	1.98±0.04^{ab}
Liv %	94.00±1.25	94.33±1.13	94.00±1.63	95.56±1.27
Carcass Traits				
Carcass Wt	1895±19.60^{a}	1852±23.54^{ab}	1921±29.00^{a}	1818±22.59^{b}
Dressing %	75.87±0.60^{a}	73.34±0.24^{c}	74.55±0.35^{bc}	74.73±0.49^{ab}
Liver %	52.50±0.58^{ab}	53.03±0.58^{a}	54.09±0.60^{a}	51.10±0.56^{b}
Gizzard %	30.00±0.33^{ab}	30.30±0.33^{a}	30.91±0.34^{a}	29.20±0.32^{b}
Heart %	15.00±0.17^{ab}	15.15±0.17^{a}	15.45±0.17^{a}	14.60±0.16^{b}

*Superscripts on different means within row show significant difference (P≤0.05); FI: Feed Intake (g); **BW:** Body weight (g); **BWG:** Body Weight Gain (g); **FCR:** Feed Conversion Ratio; **Liv:** Liveability %; **Wt:** weight (g)*

Table 5. *Bioactive compounds and selenium experimental feeds and breast meat broilers fed on different levels of Moringa oleifera pod meal*

Parameter	Control	MPM 0.5 %	MPM 1.0 %	MPM 1.5 %
Diet Sample				
β-carotene	0.34±0.01^{d}	0.57±0.01^{c}	0.70±0.02^{b}	0.79±0.01^{a}
Quercetin	0.48±0.02^{d}	7.98±0.04^{c}	15.81±0.09^{b}	22.87±0.10^{a}
Selenium	0.13±0.00^{d}	0.31±0.00^{c}	0.42±0.00^{b}	0.65±0.00^{a}
Breast Meat Sample				
β-carotene	0.00±0.00^{d}	0.06±0.00^{c}	0.07±0.00^{b}	0.08±0.00^{a}
Quercetin	2.47±0.20^{d}	43.58±0.23^{c}	86.34±0.47^{b}	124.89±0.54^{a}
Selenium	19.42±0.26^{d}	45.15±0.26^{c}	71.02±0.26^{b}	95.86±0.26^{a}
Cholesterol	65.50±0.45^{a}	64.85±0.45^{a}	64.20±0.45^{a}	61.63±0.43^{b}
DPPH	20.92±0.41^{d}	25.65±0.45^{c}	29.06±0.43^{b}	32.26±0.48^{a}

Superscripts on different means within row show significant difference (P≤0.05); β-carotene, Quercetin and Selenium in diet: mg/kg; β-carotene, Quercetin and Selenium in breast meat: µg/100 g, Cholesterol: mg/100 g, DPPH: (1, 1-Diphenyl -2-picrylhydrazyl) %

was increased above 1.0% MPM group, offered 1.5% MPM (*P*≤0.05).

Bioactive Compounds and Proximate Profile of Meat

The group supplemented with highest level of Moringa pods was enriched to best levels of bioactive compounds i.e. β-carotene, quercetin, cholesterol, selenium and DPPH contents of broiler breast meat samples were 0.08 µg/100 g, 124.89 µg/100 g, 95.86 mg/100 g, µg/100 g 61.63 and 32.26%, respectively *(Table 5)*. The cholesterol level was also significantly decreased with the increasing supplementation levels. Breast meat samples showed a significant linear decrease (*P*≤0.05) in Ash and EE content with the supplementation *(Table 6)*. Crude protein (CP) content of broiler breast meat samples was significantly increased with the supplementation level and highest value was observed in the diet D supplemented with 1.5% MPM, which increase the lean meat and lower ether extract *(Table 6)*.

Serum Biochemical and Antibody Titers

Blood serum of the broilers fed on *Moringa oleifera* pods meal as a feed additive was analyzed for biochemical indices especially SGPT, glucose, creatinine and cholesterol and significant difference in the treatment groups was recorded (*P*≤0.05). The control group showed highest values of all biochemical indices whereas lowest levels of SGPT, Glucose, and cholesterol were observed in the group fed maximum level of MPM *(Table 7)*. However, the lowest Creatinine was recorded in MPM-1.0% group. Immune response of commercial broiler birds was estimated by evaluating the antibody titers against Newcastle disease (ND) and Infectious bursal disease (IBD). Significantly higher titers of both the viruses ND as well as IBD were recorded in the groups supplemented with *Moringa oleifera* pods meal (MPM) when compare with control groups *(Table 7)*. Highest titers of ND were recorded in the group supplemented with 1.0% MPM during starter phase and 1.5% MPM during finisher phase. Moreover highest

Table 6. *Nutrients and mineral profile of breast meat of broilers fed on different levels of Moringa oleifera pod meal*

Parameter	Control	MPM 0.5 %	MPM 1.0 %	MPM 1.5 %
Proximate[1]				
Moisture	71.21±0.32[a]	63.77±0.16[c]	63.48±0.30[c]	66.41±0.46[b]
Crude Protein	21.89±0.18[b]	21.68±0.26[b]	22.15±0.35[b]	23.52±0.22[a]
Ash	0.46±0.00[a]	0.38±0.01[b]	0.29±0.01[c]	0.20±0.01[d]
Ether Extract	2.32±0.01[a]	2.06±0.02[b]	1.85±0.01[c]	1.69±0.01[d]
Mineral Profile[2]				
Sodium	1588±16.88[a]	1525±3.53[b]	1517±2.26[bc]	1499±3.75[c]
Potassium	3531±15.94[a]	3360±12.08[b]	3222±11.35[c]	3127±10.71[d]
Calcium	83.11±0.27[a]	78.96±0.26[b]	77.96±0.20[c]	75.39±0.18[d]
Magnesium	351.08±1.27[a]	331.13±2.02[b]	314.06±1.74[c]	302.52±0.98[d]
Phosphorus	3233±14.17[a]	3110±13.58[b]	3020±21.63[c]	2896±32.84[d]

Superscripts on different means within row show significant difference (P≤0.05); [1] Parameters for proximate analysis were expressed in g/100 g, [2] Parameters for mineral profile were expressed in mg/100 g

Table 7. *Serum chemistry and antibody response in broilers fed on different levels of Moringa oleifera pod meal*

Parameter	Control	MPM 0.5 %	MPM 1.0 %	MPLM 1.5 %
Blood metabolites and antibody response of serum sample (Starter phase; 0-4 weeks)				
SGPT	26.20±0.38[a]	20.13±0.69[b]	14.45±0.36[c]	12.41±0.33[d]
Glucose	268.07±1.60[a]	250.70±0.81[b]	239.65±0.76[c]	232.57±1.05[d]
Creatinine	1.68±0.01[a]	1.30±0.03[b]	1.12±0.01[d]	1.22±0.01[c]
Cholesterol	166.49±1.44[a]	150.10±1.24[b]	92.22±1.49[c]	86.35±1.20[d]
NDV titres	38.40±5.80[bc]	32.00±4.68[c]	57.60±3.42[a]	51.20±4.19[ab]
IBD titres	1465±110.94[b]	2860±226.12[a]	2842±239.10[a]	2882±106.35[a]
Blood metabolites and antibody response of serum sample (Finisher; 5-6 weeks)				
SGPT	24.72±0.36[a]	18.99±0.65[b]	13.64±0.34[c]	11.71±0.31[d]
Glucose	260.27±1.56[a]	243.40±0.79[b]	232.67±0.73[c]	225.80±1.02[d]
Creatinine	1.59±0.01[a]	1.23±0.03[b]	1.06±0.01[d]	1.16±0.01[c]
Cholesterol	157.07±1.36[a]	87.00±1.41[b]	87.00±1.41[c]	81.47±1.13[d]
NDV titres	44.80±4.19[b]	51.20±4.19[ab]	51.20±4.19[ab]	57.60±3.42[a]
IBD titres	1554±75.12[c]	2296±106.85[b]	2787±72.47[b]	3743±347.59[a]

Superscripts on different means within row show significant difference (P ≤ 0.05); SGPT: U/L; Glucose, Creatinine and cholesterol: mg/dL

values of IBD titers for both starter and finisher phases were recorded in 1.5% supplementation group *(Table 6)*. Moreover lowest values of titers for both ND and IBD were recorded in the control groups in all two phase of rearing.

DISCUSSION

The decrease in the feed intake was due to high density feed on account of essential amino acids, vitamins and minerals present in MPM which meet the body requirement even with smaller intake. During the experimental period best FCR and BWG was recorded in the group C supplemented 1.0% MPM. This might be due to rich availability of essential nutrients like amino acids, vitamins and antioxidant compounds present in *Moringa oleifera* pods which affect the overall health, production and FCR in experimental broilers. The increase in relative giblet weights can be attributed to the bioactive compounds (carotenoids, flavonoids) of Moringa pods meal, which interact with the metabolism and enhance the productive performance by improving digestibility. Some other studies also resulted that *Moringa oleifera* supplementation affect the giblet relative weight [27,28]. Similarly it has been reported in other studies that Moringa supplementation show positive impact on FCR of broiler birds [29-31]. Whereas some other scientist reported that growth performance was not affected by *Moringa oleifera* supplementation in the diet [32,33].

Carotenoids like β-carotene are bioactive chemicals which

are present in sufficient amounts in *Moringa oleifera* supplemented feed *(Table 5)* also reported in different previous studies on *Moringa oleifera* pods meal [34,35]. The flavonoids (quercetin) enrichment in breast meat of broilers fed on MPM also been reported in some studies [31,36]. The significant reduction of cholesterol in broiler meat due to supplementation of *Moringa oleifera* pods meal in feed might be attributed to phytosterols (β-sitosterol) present in the Moringa plant tissues, which decreases the absorption of cholesterol from the intestine with an immediate release in the feces. Similar results haven reported in many other studies where diet was manipulated with antioxidants and plant tissue material and cholesterol level was lowered [37-40]. The linear increase in the selenium level of breast meat samples in commercial broilers may be attributed to the higher selenium content in Moringa pod meal supplemented feed. Similarly it was reported in other studies that breast meat selenium content was increased by offering the birds selenium enriched feed [36,41,42]. The higher DPPH value may be attributed to the antioxidants (Quercetin, β-carotene, and selenium) enriched Moringa pods meal when supplemented in the diet of broilers. Same findings have been reported in some other studies which showed the strong free radical scavenging activity by using phytochemical enriched feeds [30,31].

Decline in moisture content of the breast meat samples could be due to the fact that moisture level is inversely proportional to the lipid content of the body and higher ash content of MLM [24]. Higher energy and protein values may be linked with decreased moisture levels and dense essential nutrients in MLM [43]. Increase in crude protein level may be attributed to higher bioavailability of essential amino acids present in Moringa, resulting in better tissue and muscle growth. Similar results were reported in other studies where chemical composition was significantly affected with *Moringa oleifera* leaf supplementation in the diets [33,38]. Present study showed decreased minerals levels in meat samples with the increase in supplementation level of *Moringa oleifera* pod. This response can be due to some anti-nutritional factor which decreases the feed intake and resulted in poor weight gain.

The bioactive compounds present in Moringa pods meal supplementation in the experimental diets increased efficiency of liver and kidneys which is evident from biochemical indicators showing functionality of kidneys and liver. However lowered cholesterol levels in the treatment groups can be attributed to β-sitosterol a plant sterol present in *Moringa oleifera* pods meal which lowers cholesterol due to its structural similarity to cholesterol, so decreases its absorption from intestine and increased the release in feces [44]. The results of the present study are in line with some previous researches to investigate the effect of Moringa on cholesterol and overall biochemical profile of commercial broilers [27,29,45]. The response of body towards antibody titers is attributed to bioactive compounds (antioxidant) vitamins, minerals and amino acid profile of *Moringa oleifera* pods meal supposed to be responsible for improved immune status of broiler birds. The findings of present study are in line with some earlier experiments which also reported that same response was observed while using *Moringa oleifera* as feed additive [29,46,47].

The *Moringa oleifera* pods meal supplementation in the experimental diets of broilers showed positive impact on the growth, immunity and serum biochemistry. In addition the meat quality of broilers improved due to lowering of cholesterol and enrichment of bioactive compounds in their meat.

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Research Article

Growth Performance, Mucin2 Gene Expression, Morphology of Small Intestine and Intestinal *Lactobacillus* Population of Broiler Chicks Fed with Triticale-Based Diets: Effects of Dietary Physical Form and Dietary Inclusion of Enzyme and Probiotic [1]

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Abstract

This study was conducted to investigate the effects of physical form of diet and dietary inclusion of enzyme and probiotic on growth performance, mucin2 gene expression, morphology of small intestine and intestinal *Lactobacillus* population of broiler chicks fed with triticale-based diets. Six hundred forty 1-d-old broiler chicks were arranged in 8 treatments with 4 replicates (n=20 chicks). This study was done based on a randomized block completely design in a 2×2×2 factorial arrangement with two levels of feed form (pellet and mash), two levels of probiotic (0 and 0.03% diet) and two levels of enzyme (0 and 0.05% diet). Birds fed with pelleted diet had higher weight gain at starter and grower (P<0.001) and also lower feed conversion ratio at starter (P<0.001) and grower periods (P<0.01) than those fed the mash diet. Birds fed with pelleted diets containing enzyme and probiotic consumed the most feed intake at starter period. Mucin2 gene expression was significantly higher in birds fed the pelleted diets containing enzyme or/and probiotic and mash diets containing probiotic than those fed other diets (P<0.01). Intestinal morphology and intestinal *Lactobacillus* population were not influenced by experimental treatments (P>0.05) Thus, physical form of diet plays important role in improving performance and gene expression in birds fed with triticale-based diets.

Keywords: *Broiler chicks, Di-pro probiotic, Mucin2 gene, Pellet, Triticale, Weight gain*

Tritikale Temelli Diyet ile Beslenen Broiler Civcivlerin Büyüme Performansı, Musin2 Gen Ekspresyonu, İnce Bağırsak Morfolojisi ve Bağırsak *Lactobacillus* Popülasyonu: Diyetin Fiziksel Formu ile Enzim ve Probiyotik İlavesinin Etkileri

Öz

Bu çalışma tritikale temelli diyet ile beslenen broiler civcivlerde diyetin fiziksel formu ile diyete enzim ve probiyotik ilavesinin büyüme performansı, musin2 gen ekspresyonu, ince bağırsak morfolojisi ve bağırsak *Lactobacillus* popülasyonu üzerine etkilerini araştırmak amacıyla gerçekleştirilmiştir. Altı yüz kırk adet 1 günlük broiler civciv 8 uygulama ve 4 tekrar olmak üzere kurgulanmıştır (n=20 civciv). Çalışma rastgele dizayn usulünde olmak üzere 2x2x2 faktöriyel düzende planlanmış ve iki yem formu (pelet ve püre), iki probiyotik dozu (0 ve %0.03) ve iki enzim dozu (0 ve %0.05) denenmiştir. Pelet ile beslenen civcivler püre ile beslenenler ile karşılaştırıldığında başlangıç ve büyüme periyotlarında daha yüksek ağırlık kazanımı gösterirken (P<0.001), başlangıç (P<0.001) ve büyüme (P<0.01) periyotlarında daha düşük yem konversiyon oranına sahipti. Enzim ve probiyotik içeren pelet yem ile beslenen civcivler başlangıç periyodunda en yüksek yem tüketimine sahipti. Musin2 gen ekspresyonu enzim ve/veya probiyotik içeren pelet yem ile beslenen civcivler ile probiyotik içeren püre yem ile beslenenlerde diğer yemlemelere göre anlamlı derecede daha yüksekti (P<0.01). Bağırsak morfolojisi ve bağırsak *Lactobacillus* popülasyonu deneme gruplarında fark göstermedi (P>0.05). Sonuç olarak, tritikale temelli diyet ile beslenen civcivlerde yemin fiziksel formu performans ve gen ekspresyonu üzerine önemli rol oynamaktadır.

Anahtar sözcükler: *Broiler civciv, Di-pro probiyotik, Musin2 geni, Pelet, Tritikale, Ağırlık kazanımı*

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INTRODUCTION

Some cereals grains have been commonly used in poultry nutrition at all over world. Triticale is a hybrid cereal which is produced from crossbreeding of wheat and rye and it can be used instead of corn in poultry diets [1]. Triticale and wheat have similar nutrient composition, such as resistance to hard agronomic conditions [2,3]. Triticale is known to have high amounts of soluble non-starch polysaccharides (NSPs) [1]. It is well known that NSPs initially increase digesta viscosity and then reduce digesta passage, feed intake (FI), nutrient digestion and absorption [4], and also increases harmful microbial population [5]. Triticale has more NSP content than wheat and rye. Thus, researchers have used some feeding strategies such as enzyme supplementation to improve the nutrient digestion and absorption in triticale-based diets [6-8].

Physical form of diet (mash, pellet and crumble) can act as key factor in improving growth performance of broiler chicks. Studies have shown that broiler chicks fed with pelleted diets consumed more feed intake and showed higher weight gain (WG) [9,10]. Pelleted diets could improve growth performance via several factors including decreasing feed waste, energy consumption and dustiness of feed and increasing palatability [10]. Earlier studies have reported other advantages of pelleted diets in improving growth performance of animals such as lowering ingredient degradation, increasing digestibility, removing pathogenic microbes and thermal modification of starch and protein [11,12].

It is shown that diet supplementation with enzyme improved feed conversion ratio (FCR) in diets containing high amounts of NSP [6-8]. Dietary inclusion of xylanase to diets containing higher levels of NSP significantly decreased degree of polymerization of NSP and subsequently reduced digesta viscosity and also increased nutritive value of the diet [6,13]. Dietary inclusion of enzyme could increase proliferation of beneficial microflora in the final compartments of the gastrointestinal tract by increasing substrate [14]. Adding xylanase to diet significantly improved growth performance in broiler chicks fed with triticale-based diet [1].

Probiotics are known to have beneficial effects on growth performance of animals [15,16]. Dietary inclusion of probiotics, *Lactobacillus acidophilus* and *L. casei,* to deficient-diets in certain nutrients could increase growth performance in broiler chicks [17]. Probiotics are also known to have positive effects in balancing intestinal microflora [18,19]. In vitro studies have reported different interactions between intestinal mucin2 and intestinal microflora. Studies have also reported adhesion of *Lactobacillus* strains [20] and other bacterial strains [21] to intestinal mucin2 by competition between pathogenic and beneficial bacteria [22].

With regards to adverse effects of NSPs on microflora population and growth performance in triticale-based diet, positive role of physical form of diet, probiotics and enzymes on microbial population and also relation between benefit bacteria and mucin2, it was hypothesized that dietary inclusion of probiotic and enzyme in pelleted diets can improve mucin gene expression, intestinal *Lactobacillus* population and growth performance in broiler chicks fed the triticale-based diets. Thus, the current research was done to investigate the probable interactions among physical form of diet, dietary inclusion of probiotic and enzyme on growth performance, mucin2 gene expression, intestinal *Lactobacillus* population and morphology of small intestine of broiler chicks fed with triticale-based diets.

MATERIAL and METHODS

Birds and Breeding Conditions

All procedures used in this research were approved by the Animal Ethics Committee of the Islamic Azad University, Science and Research Branch, Tehran-Iran. A number of 640 one-day-old Ross-308 broiler chicks (320 males and 320 females), with average body weight 44±2 g, were purchased from a commercial hatchery. Birds had *ad libitum* access to feed and water. Temperature was kept at 33°C in start of trial and then progressively reduced from 33 to 24°C at 21 days of age. Lighting programs were as follows; 23 h light: 1h dark during experiment.

Experimental Design and Diets

This experiment was done in a 2×2×2 factorial arrangement based on a randomized completely block design with 8 treatments consisting of 4 replicates and 20 chicks in each pen or replicate. The experiment was lasted for 42 days. The experimental diets were formulated on the basis Ross 308 catalogue. Nutritional requirements were provided based on the standard recommendations [23]. Analysis of crude protein was done on basis to Association of Official Analytical Chemists, or AOAC [24]. The nutritional requirements were adjusted on basis same catalogue *(Table 1)*. Basal diets were firstly prepared and then 0.03% Di-Pro probiotic (Tak Gen Zist Company product-Iran: containing 1.6×10^9 CFU/g *Bacillus subtilis* and *Bacillus licheniformis)* and 0.05% Rovabio® enzyme (Adiseo Co. product-French; containing 200 IU xylanase and 200 IU β-gluconase) were added to them. The half of diets was prepared in mash form and other part in the pelleted form. Non-probiotic and enzyme diets were prepared before probiotic and enzyme containing feeds. The pelleted diets were prepared by BUHLER (DFCP-65909-S, Bühler AG, Uzwil, Switzerland) pellet press. The pelleted diets were prepared at 78°C and had size 2 mm at starter diet and 3 mm at grower and finisher diets and 1.5 mm length. The triticale-based diets were as follows;

1. Diet prepared in mash form without probiotic and

Table 1. *Ingredients and composition of the basal diets at starter period (1-10 d), growth period (11-24 d) and finisher period (25-42 d)*

Ingredients (%)	Starter (1-10 days)	Grower (11-24 days)	Finisher (25-42 days)
Triticale	58.7	63.08	68.2
Soybean meal	29.9	25.46	20.11
Corn gluten	5.00	5.00	5.00
Soybean oil	1.50	2.00	2.5
Lime stone	1.03	0.91	0.83
Di-calcium phosphate	2.00	1.77	1.60
Salt	0.24	0.24	0.24
Bicarbonate sodium	0.20	0.20	0.20
Vitamin premix[a]	0.25	0.25	0.25
Mineral premix[b]	0.25	0.25	0.25
DL-methionine	0.30	0.26	0.23
L-lysine	0.45	0.43	0.44
L-threonine	0.18	0.15	0.15
Nutrient Composition			
Metabolizable energy (kcal/kg)	3025	3150	3200
Crude protein (%)	23.1	22.00	19.32
Calcium (%)	1.05	0.90	0.85
Available phosphorus (%)	0.50	0.45	0.42
Methionine (%)	0.52	0.45	0.41
Methionine + cysteine (%)	0.91	0.84	0.86
Lysine (%)	1.42	1.24	1.09
Threonine (%)	0.93	0.83	0.75

[a] Vitamin premix provided the following per kilogram of diet: Vit. A, 9.000 IU; Vit. D_3, 2.000 IU; Vit. E, 1.800 IU; Nicotinic acid, 30 mg; Vit. B_{12}, 0.015 mg; Vit. K_3, 4 mg; Biotin, 0.15 mg; Folic acid, 1.0 mg; Niacin, 30.0 mg; Panthotenic acid, 25.0 mg; Pyridoxine, 2.9 mg; Riboflavin, 6.6 mg; Thiamin, 1.18 mg; [b] Mineral premix supplied the following per kilogram of diet: Manganese oxide, 100 mg; $FeSO_4$. $7H_2O$, 50 mg; Zinc oxide, 100 mg; Copper sulfate, 10 mg; I, 1.0 mg; Se, 0.2 mg

enzyme (Treatment 1)

2. Diet prepared in pellet form without probiotic and enzyme (Treatment 2)

3. Diet prepared in mash form containing 500 g enzyme/ per ton diet (Treatment 3)

4. Diet prepared in pellet form containing 500 g enzyme/ per ton diet (Treatment 4)

5. Diet prepared in mash form containing 300 g probiotic/ per ton diet (Treatment 5)

6. Diet prepared in pellet form containing 300 g probiotic/ per ton diet (Treatment 6)

7. Diet prepared in mash form containing 300 g probiotic and 500 g enzyme/per ton diet (Treatment 7) and

8. Diet prepared in pellet form containing 300 g probiotic and 500 g enzyme/per ton diet (Treatment 8)

Performance Parameter

For calculating performance, feed intake (FI) and body weight (BW) were recorded at 10, 24 and 42 days of age. FI was considered as difference between given feed from residue feed. Mortality was daily registered. Any bird that died was weighed and FCR were calculated by dividing FI by WG of live plus dead birds.

Mucin2 mRNA Gene Expression

At the end of trial, 8 broiler chicks (4 males and 4 females) from each treatment (2 birds per replicate) were randomly selected and killed and intestinal segments were removed. The midpoint between the entry of the bile duct and Meckel's diverticulum was considered as jejunum. The jejunum was washed by normal saline and kept at liquid nitrogen (-196°C) and then transferred to lab and stored at-80°C. RNA was extracted from jejunum samples by Fermentas kit (GeneJET RNA Purification Kit-Russia) on the basis manufacturer company instructions. After removing residual DNA, cDNA was synthesized as primary template. RNA was reverse transcribed to cDNA by a Revert Aid First Strand cDNA Synthesis Kit, as recommended by manufacturer's instructions (Fermentas-Russia). The cDNA samples were served at -75°C until analysis. Real-time PCR was performed by PCR master mix for 5 min at 65°C.

The PCR was done in a reaction volume of 25 µL containing reagents at the following final concentrations: 12.5 µL of Maxima® SYBER Green'ROX qPCR Master Mix (2x), forward primer 0.75 µM, reverse primer 0.75 µM, water free nuclease 10 µL and 1 µL of cDNA sample. Quantity and quality of RNA and cDNA were evaluated using spectrophotometry method by Nano drop apparatus (Thermo Company). After determinate of quality and quantity cDNA, all cDNA were diluted. The primers used in present study (Cynagen Company) were as follows;

Forward for GAPDH: 5'GGTGGTGCTAAGCGTGTTAT3'

Reverse for GAPDH: 5' ACCTCTGTCATCTCTCCACA 3'

Forward for Mucin 2: 5'-TCACCCTGCATGGATACTTGCTCA-3'

Reverse for Mucin 2: 5'-TGTCCATCTGCCTGAATGACAGGT-3'

The cycling profiles were as follows: primary denaturation (1 cycle at 95°C for 10 min), denaturation (1 cycle at 95°C for 15 min), annealing (40 cycles at 60°C for 30 min) and final extension (40 cycles at 72°C for 30 min). For per run, a negative control, a calibrator sample, cDNA samples, and endogenous control (GAPDH) were considered. GAPDH samples were evaluated in duplicate and the target genes were analyzed in triplicate. Quality of gene expression was evaluated by using the ΔΔCt method. The difference between the Ct amount of mucin 2 gene and Ct of GAPDH for each sample was considered as ΔCt and ΔΔCt which subsequently calculated as follows;

ΔΔCt = ΔCt of each treatment-ΔCt control

Intestinal Morphology

At the end of trial, tissue samples from jejunum and ileum

broiler chickens (2 males and 2 females each replicate) were excised and placed in (10%) neutral buffer formalin. Formalin-preserved jejunum and ileum tissues were firstly sectioned and then stained with Alcian blue at pH=2.5. The two intestinal segments from per broiler chick were mounted on a glass slides and villus length, villus surface and crypt depth was evaluated. Morphological criteria were as follows; villus height (from the base of the lamina propria to the villus apex); villus width at its midpoint and crypt depth between adjacent villi. Morphological slides were investigated for villus length and crypt depth by using microscope (NOVEX model-Holland country) and the villus length and crypt depth were measured by using micrometer stage for calibration the objective lens (10X) with ocular micrometer.

Lactobacillus Population

Sixty four intestinal contents were collected from all parts of the intestine and stored in a sterile container and refrigerated at 4ºC. Digesta was mixed in a 10 mL pre-reduced salt medium and then diluted as explained by others [25] to examine the count of Lactobacilli (Rogosa, CM 0627, incubated anaerobically 48 h). Gut tissue samples were serially diluted from 10^{-7} to 10^{-3} and then from each dilution, 0.1 mL of the sample was plated into the suitable medium for enumeration of bacteria.

Statistical Analysis

The birds were studied at a 2×2×2 factorial arrangement based on a randomized completely block design. The data were analyzed by SAS software. Duncan's multiple range test was used to detect the differences ($P<0.05$) among different groups ($P\leq0.05$). All of the parameters measured were analyzed as follows:

$$Y_{ijklm}= \mu + R_i + A_j + B_k + C_l + (A*B)_{jk} + [A*Cl)\ jl + (B*C)_{kl} + (A*B*C)_{jkl} + e_{ijklm}$$

Where Y_{ijklm} is the characteristic measured, μ is the overall mean, R_i is block effect (saloon length), A_j is main effect of probiotic, B_k is main effect of enzyme, C_l is main effect of dietary physical form, $(A*B)_{jk}$ is interaction between the probiotic and enzyme, (A*Cl) jl is interaction between the probiotic and dietary physical form, $(B*C)_{kl}$ is interaction between the enzyme and dietary physical form, $(A*B*C_{jkl}$ is interaction among the probiotic, enzyme and dietary physical form and e_{ijklm} is the residual error. Main effect of factors would not be considered when interaction is significant.

RESULTS

Performance

The data for growth performance are presented in *Table 2*. The FI, WG and FCR were affected by experimental treatments. Broiler chicks fed in treatments (2, 4, 6 and 8) significantly showed higher WG than those in treatment 1 (mash diet without additives) at starter period ($P<0.001$) and those in odd treatments (1, 3, 5 and 7) at grower period ($P<0.001$). Treatment 8 (Pellet diet included with probiotic and enzyme) and treatment 6 (pellet diet included with probiotic) significantly had the highest WG at starter period ($P<0.01$). A significant interaction between probiotic and enzyme was seen for FI and FCR at starter period ($P<0.001$). The most feed intake was for broiler chickens receiving the pelleted diets containing enzyme and probiotic (treatment 8) while, those fed the mash diets containing enzyme or probiotic consumed least feed at starter period ($P<0.001$). Birds receiving mash diet without additives also showed higher FCR than those fed pellet diet containing enzyme and probiotic at starter ($P<0.01$) and grower periods ($P<0.01$). As mentioned before, where interaction is significant, main effects would not be discussed.

Mucin 2 Gene Expression

The data for jejunal mucin 2 mRNA expression are presented in *Fig. 1*. Dietary inclusion of probiotic and enzyme to pelleted diets significantly increased mucin 2 mRNA expression. There was higher gene expression in 4, 5, 6 and 8 treatments compared with treatment 1 ($P<0.05$). According some papers, difference in the amount of gene expression is more important than significant different. Therefore treatments 2, 3 and 7 did not have significant different with treatment 1, but there was more gene expression.

Intestine Morphology and Lactobacillus Population

As *Table 3* and *Table 4* shows intestinal morphology parameters and intestinal *Lactobacillus* population were not influenced by dietary form and dietary inclusion of probiotic and enzyme ($P>0.05$). The images for intestinal morphology are presented in *Fig. 2*.

DISCUSSION

Previous studies have shown that broiler chicks receiving the pelleted diets consumed more FI and had higher WG compared with broiler chicks fed with mash diet [9,10,26,27]. In contrast to other studies, triticale-based diets were used in this study. In any case, soluble NSP fraction has negative relation with apparent metabolizable energy, because of increased digesta viscosity [28]. Osek et al.[29] reported that broiler chicks fed the whole with wheat and whole triticale diets exhibited the lowest WG. However, the prepared diets in pellet form improved WG in starter period. In sorghum-based diets, Abdollahi et al.[30] stated that the pelleted diets significantly increased performance rather than mash diets. Increase in WG in broiler chicks fed with pelleted diets can be due to increased FI [31]. In this research, increased FI was seen in broiler chicks fed with pelleted diets in starter period.

Dietary supplementing of probiotic and enzyme significantly

Table 2. *The interaction among probiotic, enzyme and dietary physical form on growth performance at starter period (0-10 d), grower period (11-24 d) and finisher period (25-42 d) of broiler chicks fed with triticale-based diets*

Treatment			WG			FI			FCR		
PF	E (%)	Pro (%)	Starter	Grower	Finisher	Starter	Grower	Finisher	Starter	Grower	Finisher
Mash	0	0	174.08^{d}	584.18^{c}	1449.29	276.39^{c}	985.23	2551.52^{bc}	1.58^{d}	1.68^{c}	1.76
Pellet	0	0	210.39^{abc}	722.21^{ab}	1602.84	293.56^{b}	1097.27	2613.38^{b}	1.40^{c}	1.51^{bc}	1.63
Mash	0.05	0	190.62^{cd}	607.92^{c}	1461.50	257.85^{d}	976.78	2462.53^{c}	1.35^{abc}	1.61^{abc}	1.69
Pellet	0.05	0	222.04^{ab}	730.21^{a}	1511.02	281.19^{bc}	1034.51	2399.73^{c}	1.26^{ab}	1.41^{ab}	1.58
Mash	0	0.03	191.29^{cd}	617.47^{c}	1493.13	258.78^{d}	985.76	2406.47^{c}	1.35^{ab}	1.59^{bc}	1.61
Pellet	0	0.03	228.44^{a}	721.19^{ab}	1535.33	284.26^{bc}	1020.74	2281.00^{d}	1.24^{a}	1.41^{abc}	1.49
Mash	0.05	0.03	197.31^{bcd}	646.88^{c}	1554.23	274.66^{c}	990.77	2290.33^{a}	1.39^{bc}	1.54^{ab}	1.53
Pellet	0.05	0.03	234.57^{a}	711.92^{ab}	1570.07	313.25^{a}	988.71	2298.24^{a}	1.33^{ab}	1.39^{a}	1.46
SEM			1.96	6.07	24.45	1.13	13.12	33.33	0.01	0.024	0.036
P-Value			0.000	0.000	0.753	0.000	0.347	0.149	0.000	0.047	0.453
PF			***	***	NS	***	NS	NS	***	**	NS
E			*	NS	NS	NS	NS	NS	**	NS	NS
Pro			**	NS	NS	*	NS	*	**	NS	NS
PF×E			NS	NS	NS	*	NS	NS	NS	NS	NS
PF×Pro			NS	NS	NS	*	NS	NS	NS	NS	NS
E×Pro			NS	NS	NS	***	NS	NS	***	NS	NS
PF×E×Pro			NS	NS	NS	NS	NS	NS	NS	NS	NS

Superscripts (a-e) show significant different at each column. ***NS:*** *non-significant (P>0.05); * P<0.05; ** P<0.01; *** P<0.001;* ***PF:*** *physical form,* ***E:*** *enzyme,* ***Pro:*** *probiotic.* ***SEM:*** *standard error of means*

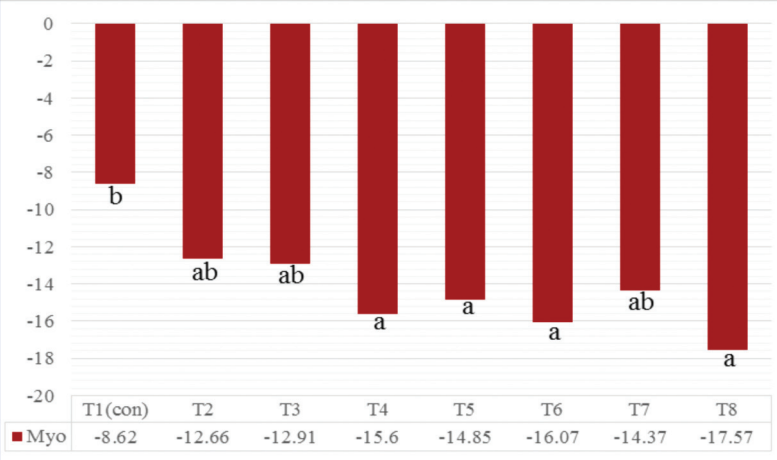

Fig 1. Effect of feed form, probiotic and enzyme on jejunal mucin 2 mRNA expression of broiler chicks fed with triticale-based diets (P=0.002, SEM= 0.4.)

increased WG at starter period (P<0.05). In the current study Rovabio enzyme is contained 200 IU xylanase and 200 IU β-gluconase. Studies have been shown dietary inclusion of xylanase in diets containing higher levels of NSP significantly reduced the degree of polymerization of NSP which subsequently reduced digesta viscosity and finally increased the nutritive value of the diet [7,13]. It was reported that dietary inclusion of enzyme in diets having high levels of NSP increases the efficiency of feed utilization and WG [7]. It is reported that dietary inclusion of xylanase in wheat, rye, or triticale-based diets reduced the degree of polymerization of NSP, digesta viscosity and improved the nutritive value of the diet [7] which may increase WG. Regarding probiotic supplementing, it was reported that supplemnting of probiotic increased WG which may be due to improved absorption of nutrients and reduced harmful bacteria [32]. However, dietary inclusion of probiotic and enzyme and pelleted form increased WG in lower ages. Broiler chicks in lower ages have more physical limitations in digestive and food consumption. Thus, consuming diets having probiotic, enzyme and in pelleted form increases FI in lower ages and thus increases WG. However, birds fed with pelleted diet showed higher WG and lower FCR than birds fed the mash diet, WG (1554 vs 1489) and FCR (1.54 vs 1.65), in finisher period. However, these differences were not significant (P>0.05).

Chickens receiving the pellet diets containing enzyme and probiotic consumed more FI than those fed the mash diet containing enzyme in starter period. This can attributed to synergism interaction effect among pellet, enzyme and probiotic. Studies have shown that NSPs enhance digesta viscosity and decrease FI [4]. It is reported that enzyme supplementing to diet increases the digesta passage rate and subsequently FI [33]. Earlier studies have reported that probiotics act as appetizer supplement [34,35]. Studies have also reported that pelleted diets could increase FI [36].

Table 3. *Interaction among feed form, probiotic and enzyme on ileum and jejunum villus length and crypt depth of 42 d old broilers*

Treatment			Villus Height, µm		Villus Surface Area, mm²		Crypt Depth, µm		Villus Height to Crypt Depth	
PF	E (%)	Pro (%)	I	J	I	J	I	J	I	J
Mash	0	0	0.850	1.286	0.830	0.122	0.082	0.085	10.44	15.133
Pellet	0	0	0.795	1.265	0.775	0.127	0.085	0.088	9.44	14.410
Mash	0.05	0	0.836	1.348	0.851	0.121	0.078	0.086	11.05	15.690
Pellet	0.05	0	0.828	1.198	0.813	0.120	0.086	0.080	9.69	14.935
Mash	0	0.03	0.805	1.330	0.826	0.120	0.083	0.084	9.78	15.972
Pellet	0	0.03	0.831	1.302	0.822	0.121	0.084	0.089	10.09	14.993
Mash	0.05	0.03	0.767	1.287	0.820	0.122	0.080	0.086	9.72	15.084
Pellet	0.05	0.03	0.902	1.310	0.887	0.120	0.082	0.088	11.06	14.858
SEM			0.011	0.014	0.014	0.001	0.001	0.001	0.218	0.269
P-Value*			0.168	0.297	0.718	0.769	0.883	0.688	0.416	0.890

** There was no significant different among groups and NS was deleted.* ***I:*** *ileum, J: jejunum,* ***PF:*** *physical form,* ***E:*** *enzyme,* ***Pro:*** *probiotic.* ***SEM:*** *standard error of means*

Table 4. *Interaction among feed form, probiotic and enzyme on intestinal Lactobacillus (×10⁶ CFU) population of 42 d old broilers*

PF	E (%)	Pro (%)	Mean
Mash	0	0	3.19
Pellet	0	0	3.80
Mash	0.05	0	2.49
Pellet	0.05	0	3.99
Mash	0	0.03	3.44
Pellet	0	0.03	3.89
Mash	0.05	0.03	3.83
Pellet	0.05	0.03	3.69
SEM			0.42
P-Value*			0.169

** There was no significant different among groups and NS was deleted.* ***PF:*** *physical form,* ***E:*** *enzyme,* ***Pro:*** *probiotic,* ***SEM:*** *standard error of means*

Broiler chicks consume FI in pelleted-based diets, because pelleted diets are more density and broiler chicks use lower energy for its consumption.

Broiler chicks fed with pelleted diets containing enzyme and probiotic relatively had lower FCR. Dietary inclusion of *B. subtilis* strain in broilers diet could improve WG at 24 d and reduce FCR at 12-24 days of old in broiler chicks [37]. Improved FCR and the reduced FI in heat-stressed broiler chicks fed with *Lactobacillus* strain have been previously reported [38].

Reduced feed waste, energy consumption and dustiness of feed, the improved palatability [10], the increased digestibility, the removed pathogenic microbes and thermal modification of starch and protein [11,12] can be considered as reasons for the improved FCR in broilers fed the pellet diet. In the present study, the pelleted diet rather than mash diet showed better performance at starter and grower, while it could not improve growth performance in finisher period. Birds receiving mash diet without additive showed higher FCR than other birds. As mentioned NSPs increase harmful bacteria population and the diets prepared in pelleted form especially with enzyme and probiotic at starter and grower period showed lower FCR compared with mash diet without additive. We believed that the decreased FCR could be achieved by the reduced harmful microflora. It can be concluded that the improved performance in starter period cannot be attributed to *Lactobacillus* population and improved intestinal morphology because neither *Lactobacillus* population and nor improved intestinal morphology were not influenced.

In the current study, mucin2 gene expression was increased in pellet diets containing enzyme *(Fig. 1)*. Previous studies reported adhesion of *Lactobacillus* strains [20] and other bacterial strains [21] to intestinal mucin2 through competition between pathogenic and beneficial bacteria [22]. Investigations have been also shown that diet supplementing with probiotic significantly increased mucin2 gene expression [39]. It also seems that microbial balance becomes more complete with increased age. Nutritional modulation cannot improve growth performance in finisher period. It can be stated that the pelleted diet containing enzyme and probiotic improved growth performance by increasing mucin 2 gene expression. The data are conflicting, since studies have reported that increased mucin2 gene expression can increase *Lactobacillus* strains, but such results were not found. It seems that mucin2 gene expression increases *Lactobacillus* population in lower ages. However, studies have reported that dietary inclusion of probiotic significantly increased *Lactobacillus* population by increasing volatile fatty acids [40]. However, it was expected that enzymes increase NSP digestion and help to increasing *Lactobacillus* population. Sampling at higher ages can be reason for non-changing in *Lactobacillus* population.

Feed form and dietary inclusion of probiotic and enzyme could not change intestinal morphology of small intestine.

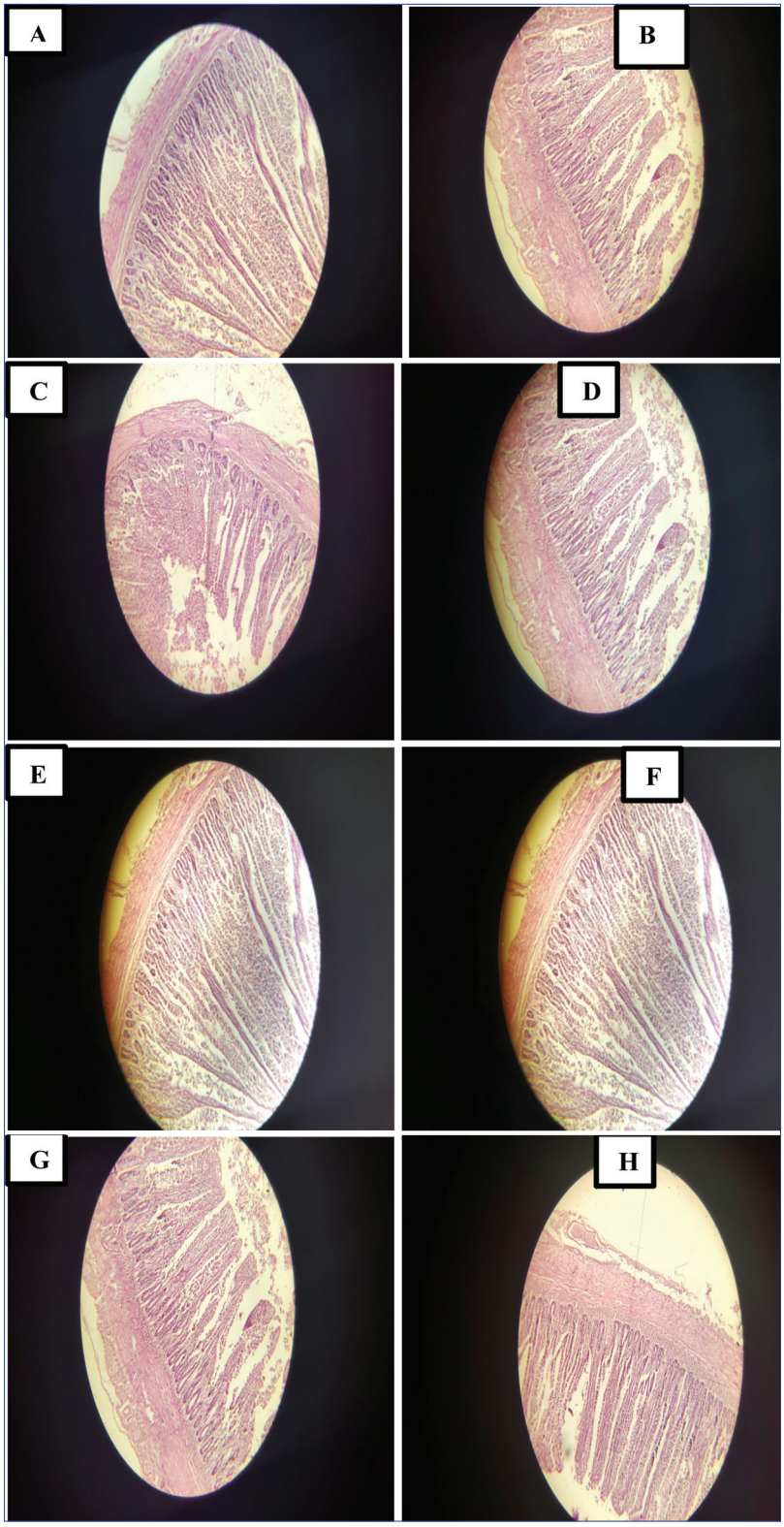

Fig 2. Intestinal morphology of birds, A-H Figures show intestinal morphology in Treatments 1 to 8, respectively

The villus crypt is known as villus factory and deeper crypts are criteria for tissue turnover which allow renewal of the villus and crypt depth is involved in the production of enterokinase that aids to digestion of protein [41]. There are conflicting reports for effect of nutritional modulation on intestine morphology. Some studies have n shown that dietary inclusion of probiotic increases ileum villus height and crypt depth [5,42] while other studies have reported that dietary inclusion of probiotic reduces villus height and crypt depth of the ileum [43,44]. Cereals contain high NSP increase the digesta viscosity and subsequently change bacterial intestinal physiology [45]. It is reported that dietary inclusion of enzymes enhance hydrolysis of NSPs and subsequently increase the height of villi and the proportion height-to-depth of the crypts, and improve the bacterial activity of the intestinal [46].

It can be stated that physical form of diet can efficiently affect growth performance of broiler chicks fed with triticale-based diets. It seems that dietary form improves performance by balancing microbial population at lower ages; however, pelleted diets had higher mucin2 gene expression at 42 d. Measuring nutrient digestibility would be offered in future studies for understanding more mechanisms.

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Research Article

Immunogenicity of Recombinant Adenovirus Co-expressing the L7/L12 and BCSP31 Proteins of *Brucella abortus*

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Abstract

Brucella poses a great threat to animal and human health, and vaccination is a good way of controlling the bacterium. In this study, the immune response and protection ability of a recombinant adenovirus Ad-LL/BP containing L7/L12 and BCSP31 proteins of *Brucella abortus (B. abortus)* were evaluated in BALB/c mice model. Firstly, Adenovirus vector Ad-CMV and the recombinant adenovirus Ad-LL/BP were amplified in HEK 293AD cells. The TCID50 values of Ad-LL/BP and Ad-CMV were $10^{-8.68}$/0.1 mL and $10^{-8.35}$/0.1 mL, respectively. Mice were inoculated with 100 $TCID_{50}$/mouse of Ad-LL/BP or Ad-CMV. Vaccination of mice with Ad-LL/BP vaccine was able to elicit higher IgG, IgG1 and IgG2a antibody levels when compared with Ad-CMV and PBS control animals ($P<0.05$). Splenocytes from Ad-LL/BP-immunized mice significantly proliferated and released Th1 type cytokine IL-12 in comparison with Ad-CMV or PBS-inoculated groups ($P<0.05$). CD^{3+} and CD^{4+} T cell subset in splenocytes from the mice immunized with Ad-LL/BP vaccine were significantly higher compared with those from the mice vaccinated with Ad-CMV or PBS ($P<0.05$), but CD^{8+} T cells had no change in the three groups ($P>0.05$). Ad-LL/BP vaccine was able to reduce significantly the numbers of *B. abortus* in the spleens from the immunized mice. These results indicated that the recombinant Ad-LL/BP vaccine induced mainly cell-mediated immunity and partly humoral immunity, and provided moderately protection against *B. abortus* infection. Therefore, the vaccine could be further developed into a live-vector vaccine against *B. abortus.*

Keywords: *Brucella abortus, L7/L12, BCSP31, Adenovirus, Immunity, BALB/c mice*

Brucella abortus L7/L12 ve BCSP31 Proteinlerini Birlikte Eksprese Eden Rekombinant Adenovirusların İmmunojenitesi

Öz

Brucella insan ve hayvanlarda önemli bir tehdit olup, aşılama bakteriyi kontrol altında tutmak amacıyla iyi bir yöntem olabilir. Bu çalışmada, *Brucella abortus (B. abortus)* L7/L12 ve BCSP31 proteinlerini içeren rekombinant adenovirus Ad-LL/BP'nin immun yanıtı ve koruyuculuğu BALB/c farelerde araştırıldı. Adenovirus vektör Ad-CMV ve rekombinant adenovirus Ad-LL/BP, HEK 293AD hücrelerinde amplifiye edildi. Ad-LL/BP and Ad-CMV'nın TCID50 değerleri sırasıyla $10^{-8.68}$/0.1 mL ve $10^{-8.35}$/0.1 mL olarak tespit edildi. Fareler Ad-LL/BP veya Ad-CMV'nin 100 TCID50/fare ile inoküle edildi. Ad-LL/BP aşısı ile farelerin aşılanması, Ad-CMV ve PBS kontrol hayvanları ile karşılaştırıldığında daha yüksek IgG, IgG1 ve IgG2a antikor seviyelerinin olmasına neden oldu ($P<0.05$). Ad-LL/BP-immunize farelerin şiplenositleri, Ad-CMV veya PBS-inoküle edilmiş gruplardakiler ile karşılaştırıldığında anlamlı derece proliferasyon gösterip Th1 tip sitokin IL-12 salınımında bulundu ($P<0.05$). Ad-LL/BP aşısı ile immunize edilmiş olan farelerden elde edilen şiplenositlerde CD^{3+} ve CD^{4+} T hücre subsetleri Ad-CMV veya PBS ile aşılanan farelerden elde edilenlere göre daha fazla olmasına rağmen ($P<0.05$) CD^{8+} T hücrelerde üç grup arasında fark tespit edilmedi ($P>0.05$). Ad-LL/BP aşısı immunize edilmiş farelerin dalaklarında *B. abortus* miktarını anlamlı derecede azalttı. Bu sonuçlar rekombinant Ad-LL/BP aşısının çoğunlukla hücre-aracılı bağışıklığı ve kımen humoral bağışıklığı indüklediğini ve *B. abortus* enfeksiyonuna karşı orta seviyede koruyuculuk sağladığını göstermiştir. Bu nedenle aşının ileri çalışmalarda *B. abortus*'a karşı canlı-vektör aşı olarak geliştirilebileceği düşünülmektedir.

Anahtar sözcükler: *Brucella abortus, L7/L12, BCSP31, Adenovirus, Bağışıklık, BALB/c fare*

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INTRODUCTION

Brucellosis, a highly infectious disease of socioeconomic and public health importance, is caused by *Brucella*. This pathogen is a Gram-negative facultative intracellular bacteria that affects human, cattle, sheep, goat, swine, rodent, and marine mammals [1,2]. In humans, *Brucella* species are potential biowarfare agents that spread through direct contact with infected animals or the consumption of contaminated food, especially unpasteurized milk products [3]. *Brucella abortus (B. abortus)*, the etiological agent of bovine brucellosis, causes abortion and infertility in cattle and undulant fever, arthritis and endocarditis in humans [4].

Treatment of brucellosis requires a prolonged combination of antibiotic therapy and remains problematic because of potential relapse. The eradication of brucellosis is an important goal of public health programs in affected countries. Ruminant brucellosis may be eradicated by means of adequate testing and slaughtering programs, but in areas with high prevalence of disease, extensive management systems, or low socioeconomic conditions, vaccination is the only practical way to control it [5]. The attenuated strain *B. abortus* S19 is the most widely used vaccine to prevent bovine brucellosis. It can stimulate strong cell-mediated immunity (CMI) and induce reasonable protection against *B. abortus* [6]. In some regions, this vaccine has been replaced with the RB51 strain, a rough mutant that is less virulent for cattle and does not interfere with serological screening [7]. These vaccines are widely available but might trigger adverse reactions; moreover, the use of commercial vaccines interferes with diagnostic testing, precluding eradication programs [8].

Modern biotechnology offers some opportunities for vaccine production. Among the various approaches to develop new vaccines, recombinant or subunit vaccines were the most studied. The selection and use of promising immunodominant proteins or genes have played an important role in effort to develop new candidate *Brucella* vaccines [9]. Some of the immunogenic *Brucella* genes, such as BCSP31, ribosomal L7/L12, ferritin or P39, lumazine synthase, or Cu-Zn superoxide dismutase, have all been tested as vaccines and demonstrated to render protection in mice [10-14].

Adenoviruses (Ads) are non-enveloped DNA viruses that have been extensively studied as recombinant vector vaccines for viral and bacterial disease agents [15-17]. They have many attractive characteristics, including high-titer growth, manufacturability, broad range of infectious cell types, and adequate space in the genome for gene insertions. The replication-incompetent Ad serotype 5 vector (Ad5) is a safe and efficient vaccine vector for humans and animals [18]. In this study, a recombinant Ad comprising two genes encoding L7/L12 and BCSP31 of *B. abortus* was constructed using human replication-incompetent Ad5 as a vector to co-express L7/L12 and BCSP31. BALB/c mice were immunized with the recombinant Ad vaccine, and humoral and cell-mediated immune responses were evaluated. The results of this study would provide information for further research on vaccination against brucellosis in animals.

MATERIALS and METHODS

Mice, Brucella and Cells

SPF-grade female BALB/c mice aged 6-8 weeks old were purchased from the Center of Experimental Animals, Lanzhou Institute of Biological Products (Lanzhou, China). *B. abortus* strain CVCC12 (Biovar II) was obtained from China Veterinary Culture Collection Center (Beijing, China) and proliferated in accordance with the manufacturer's instructions. Bacteria were harvested into phosphate-buffered saline (PBS) (pH: 7.4) to obtain the final concentration needed for each experiment. HEK 293AD cells were purchased from Cell Biolabs, Inc., USA and were cultured as per recommendation.

Amplification of Genes

The coding sequences of the *B. abortus* L7/L12 and BCSP31 genes were amplified by PCR from the CVCC12 strain, respectively. The primers for L7/L12 gene were 5′-AAAGCG GCCGCATGGCTGATCTCGC-3′ and 5′-CGGGATCCTTACTT GAGTTCAACCTTG-3′ and *Not* I and *Bam*H I underlined were restriction sites [19]. The primers for BCSP31 gene were 5′-CGACGCGTATGAAATTCGGAAGCA-3′ and 5′-TTCTCGAGT TATTTCAGCACGCC-3′, and *Mlu* I and *Xho* I underlined were restriction sites. The amplification procedure for BCSP31 gene was as follows: 94°C for 2 min, 35 cycles consisting of denaturation at 94°C (40 s), annealing at 46°C (1 min), primer extension at 72°C (40 s) and a final extension for 10 min at 72°C. The primers and amplification condition for BCSP31 gene were designed in this study.

Construction of Recombinant Plasmid pAd-LL/BP

The PCR products of the L7/L12 and BCSP31 genes were inserted into MCS I using *Not* I and *Bam*H I and MCS II using *Mlu* I and *Xho* I of pQCXIX Retroviral Vector, respectively, generating pQC-LL/BP. The L7/L12-IRES-BCSP31 fragment was cloven by *Not* I/*Xho* I from pQC-LL/BP and cloned into the *Not* I/*Xho* I sites of the transfer vector pShuttle-CMV. The resulting vector was designated pShuttle-LL/BP. The recombinant plasmid was transformed into *E. coli* BJ5183 (Stratagene, CA) competent cells carrying the pAdEasy-1 skeleton vector (Stratagene, CA), generating the pAd-LL/BP plasmid. The recombinants were selected with kanamycin.

Growth of Recombinant Adenovirus Ad-LL/BP

The recombinant adenoviral pAd-LL/BP was cleaved with *Pac* I to expose its inverted terminal repeats and then transfected into HEK 293AD cells using Lipofectamine 2000™ (Invitrogen). The infected cells were incubated for 6-10 days with 5% CO_2 at 37°C and then subjected to

three freeze-thaw cycles. The resulting viral lysates were serial passaged onto fresh monolayers of cells until full cytopathic effect (CPE) was observed. Subsequently, the virus was grown in large quantities by multiple rounds of amplification in HEK 293AD cells and purified by cesium chloride gradient centrifugation [20]. The obtained recombinant Ad was named Ad-LL/BP, and 50% tissue culture infectious dose (TCID50) was used to determine the titer of the virus. The Ad vector Ad-CMV was cultured as the Ad-LL/BP.

Identification of the Co-expressed Product

The L7/L12 and BCSP31 co-expressed by Ad-LL/BP in HEK 293AD cells were detected in a six-well culture plate using indirect immunofluorescence assay (IFA) and Western blot assays.

Immunization and Sample Collection

The study protocol was approved by the Animal Care and Use Committee of Life Science and Engineering College, Northwest University for Nationalities. Four groups of mice (10 per group) were immunized by bilateral intramuscular injection into the gastrocnemius and boosted three times with the same dose with a two-week interval. Group 1 was injected with 100 $TCID_{50}$/mouse of Ad-LL/BP (100 µL). Group 2 was inoculated with A19 vaccine strain (50 million bacteria/mice) as positive control, and the immunization was performed only one time. Group 3 was injected with 100 $TCID_{50}$/mouse of empty Ad serotype 5 (Ad-CMV) as negative control. Group 4 was injected with 100 µL of PBS as blank control.

Blood was collected from the tail vein of each mouse from all groups one day prior to immunization, and sera were stored at −20°C until analysis for specific antibodies. Pre-immune serum samples were used as negative controls. Spleen samples of five mice per group were aseptically collected 2 weeks after the last booster injection. The other mice were continually fed until they were challenged.

Antibody Assays

Analysis of antigen-specific IgG, IgG1, and IgG2a antibodies in serum samples was performed by ELISA as previously described [19]. In brief, 96-well microtiter plates (Costar, Bethesda, MD) were coated with 10 µg/mL ultrasonicated *B. abortus* A19 strain overnight in carbonate buffer (pH 9.6) at 4°C. The plates were blocked with 1% BSA in PBS for 30 min at 37°C. After thorough washing with PBST, the serum samples were added to the plate and allowed to incubate for 30 min at 37°C. The plates were washed again and were reacted with HRP-labeled anti-mouse IgG, IgG1, and IgG2a diluted in PBST at 1:1000 for another 30 min at 37°C. The plates were washed and developed with TMB and kept in the dark for 10 min. Finally, stop solution was added, and optical density (OD) values were immediately measured at 450 nm using an ELISA reader. All samples were run in triplicate.

Lymphoproliferation Assay

Lymphoproliferation assay was performed as previously described [21]. Murine spleens were removed and ground under sterile conditions using a 5 mL syringe plunger, and single-cell suspensions were obtained by filtration through a stainless steel mesh. Splenocytes were isolated with Mouse Lymphoprep (Dakewe, Shenzhen, China) and placed into 96-well plates with 100 µL/well at a density of 5×10^6 cells/mL in complete medium (RPMI 1640 + 10% FBS + 100 U/mL penicillin/streptomycin). The cells were incubated with 5 µg/mL *B. abortus* A19 strain (10 µg/mL) or concanavalin A (ConA, 5 µg/mL) or medium alone (negative control) in a 5% CO_2 humidified incubator at 37°C. Proliferative activity was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL, Sigma) dye assay. The stimulation index (SI) was calculated as the ratio of the average OD_{570} of antigen-stimulated cells to the average OD_{570} of unstimulated cells.

Flow Cytometric Analysis of Surface Markers of Lymphocytes

2 mL splenocyte suspension (5×10^6 cells/mL) was centrifuged for 5 min at 2000 rpm, and the supernatant was discarded. The cells were washed once with 1 mL of fluorescence solution, and the supernatant was discarded. Subsequently, the cells were suspended with 30 µL of fluorescence solution. For each tube, 1 µL of PerCP-CD^{3e}, 1 µL of PE-CD^{8a}, and 0.5 µL of FITC-CD^{4+} were added and mixed except for the control tube. The mixtures were incubated for 45 min in the dark, following three-time washing with fluorescence solution. The cells were suspended with 200 µL fluorescence solution and then analyzed using a flow cytometer.

Cytokine Assay

Splenocyte suspension (5×10^6 cells/mL) was placed into 24-well plates with 2 mL/well in duplicate. The fractions of A19 strain (1×10^8 cells/mL) treated by ultrasonication were placed into the plate with 10 µL/well. The plate was incubated for 120 h at 37°C with 5% CO_2. The supernatant of each well was collected for detection of IL-10 and IL-12.

Protection Experiment

The experiment was performed in a BSL-3 laboratory as previously described [21]. Four weeks after the last vaccination, five mice of each group were challenged by intraperitoneal route with 1×10^5 colony-forming unit (CFU) of *B. abortus* strain CVCC12 in 100 µL of PBS. At 4 weeks post-challenge, spleens of five mice per group were removed and weighed aseptically. Each spleen was homogenized in PBS with 1:10 (g/mL, w/v) and serially diluted 10-fold. Each dilution was applied to *Brucella* agar

to determine CFU. The results were represented using the mean ± standard deviation of Log_{10}CFU per group. Log units of protection were obtained by subtracting the mean Log_{10}CFU of the vaccinated group from the mean Log_{10}CFU of the PBS control group.

Statistical Analysis

One-way analysis of variance was carried out to analyze the differences of the groups using SPSS 20.0. Statistical significance was assumed at $P<0.05$.

RESULTS

By PCR and *Pac* I digestion analysis, pAd-LL/BP recombinant plasmid was successfully constructed. The HEK 293AD cells transfected with linearized recombinant pAd-LL/BP showed CPE. The control cells had no CPE. The TCID50 values of recombinant adenovirus Ad-LL/BP and Ad-CMV were calculated to be $10^{-8.68}$/0.1 mL and $10^{-8.35}$/0.1 mL, respectively. By IFA detection, the HEK 293AD cells infected with Ad-LL/BP showed fluorescence, but the cells infected with Ad-CMV had no fluorescence *(Fig. 1)*. By Western blot, the sample derived from Ad-LL/BP-infected cells had two bands (about 13.8 kD and 36.3 kD), which was consistent with the predicted proteins. However, the Ad-CMV-infected cells had no band *(Fig. 2)*. These results indicated that L7/L12 and BCSP31 proteins were successfully expressed in HEK 293AD cells.

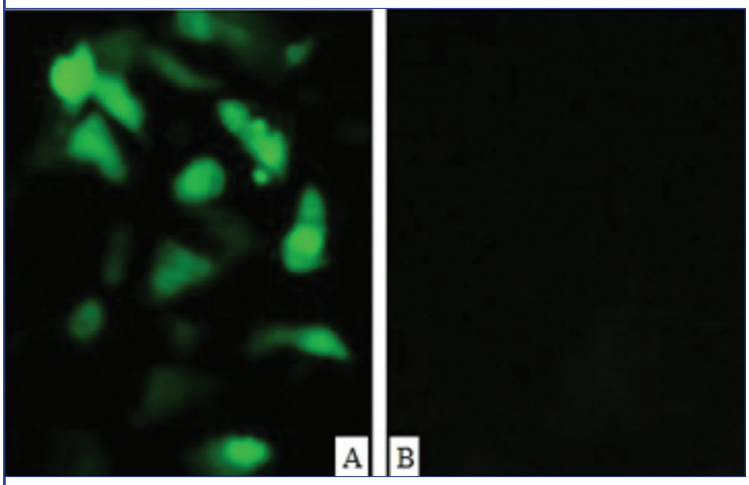

Fig 1. Protein expression of Ad-LL/BP detected by IFA (200×). (A) HEK 293AD cells infected with Ad-LL/BP, (B) HKE 293AD cells infected with Ad-CMV

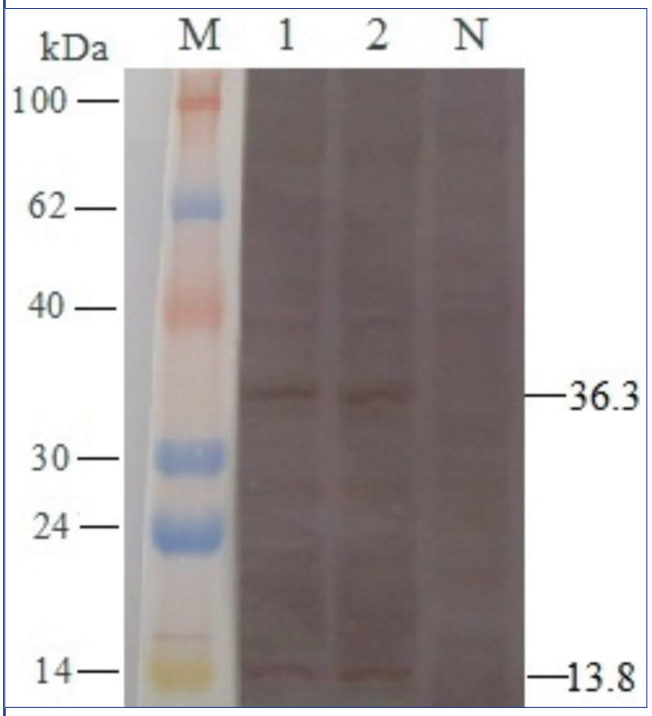

Fig 2. Protein expression of Ad-LL/BP detected by Western blot. Lane M, molecular weight marker; lanes 1 and 2, HEK 293AD cells infected with Ad-LL/BP; lane N, HEK 293AD cells infected with Ad-CMV

For group 1, the levels of IgG, IgG1, and IgG2a antibodies gradually rose following the increase in vaccination times. The IgG, IgG1, and IgG2a levels from groups 1 and 2 were significantly higher compared with those from groups 3 and 4 ($P<0.05$). The antibodies from group 2 were significantly higher than those from group 1 ($P<0.05$). The antibodies from groups 3 and 4 were not changed during the whole process ($P<0.05$). The results are shown in *Fig. 3*. A19 and ConA could promote the T cell proliferation of splenocytes derived from mice in groups 1 and 2. The SIs from groups 1 and 2 were significantly higher compared with those from groups 3 and 4 ($P<0.05$), and the value from group 2 was higher than that from group 1 ($P<0.05$). The SIs showed no difference between groups 3 and 4 ($P>0.05$). The results are shown in *Fig. 4*.

By FCM analysis, the CD^{3+} and CD^{4+} T cells of the mice in groups 1 and 2 were significantly higher compared with those in groups 3 and 4 ($P<0.05$). The numbers of CD^{3+} and CD^{4+} T cells of the mice in groups 1 and 2 showed no difference ($P>0.05$). The CD^{8+} T cells in group 2 were significantly higher compared with those in groups 1, 3, and 4 ($P<0.05$). For CD^{8+} T cells, no statistical differences were observed among groups 1, 3, and 4 ($P>0.05$). The results are shown in *Fig. 5*.

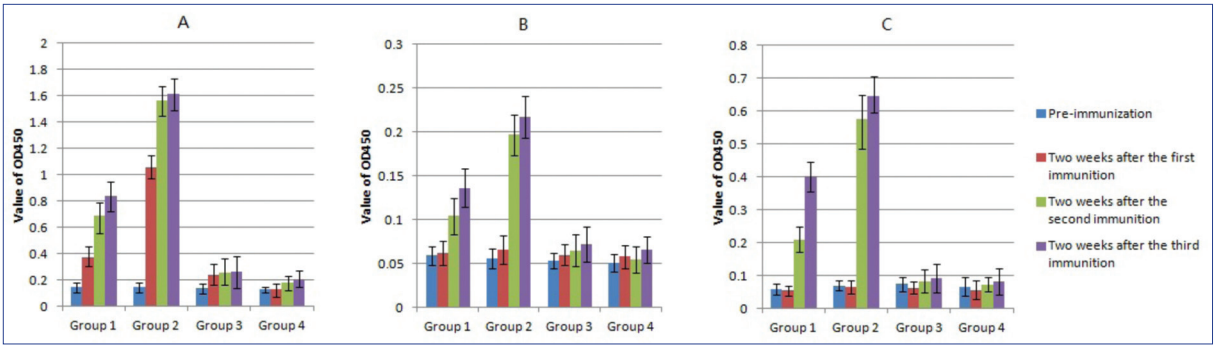

Fig 3. IgG (A), IgG1 (B), and IgG2a levels detected by ELISA

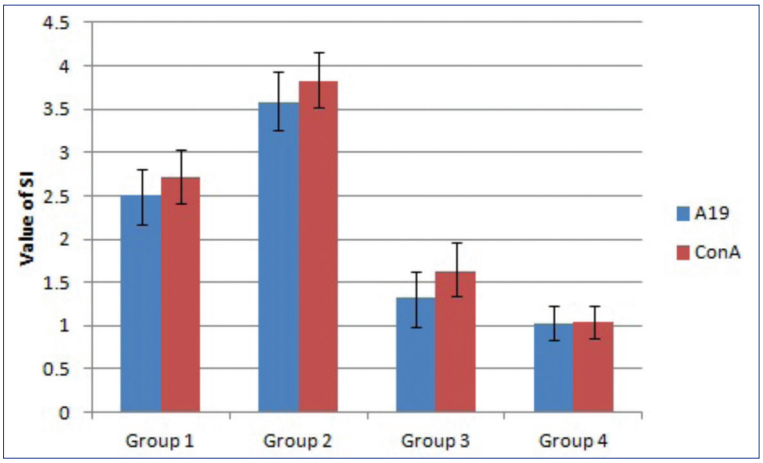

Fig 4. Proliferative responses of lympho-splenocytes derived from immunized mice against A19 antigen and ConA stimulation

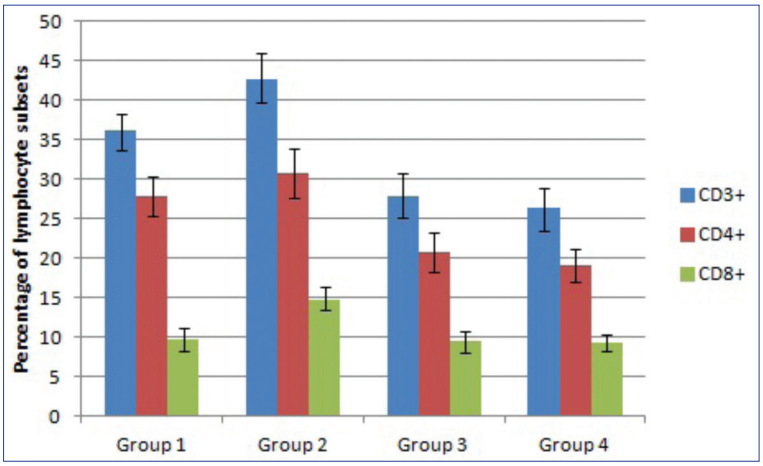

Fig 5. Analysis of spleen T lymphocyte subsets by FCM

The levels of IL-10 in groups 1, 3, and 4 showed no statistical difference ($P>0.05$). IL-10 was higher in group 2 than in groups 1, 3, and 4. The levels of IL-12 in groups 1 and 2 were 38.7±5.15 and 85.3±8.2 pg/mL, respectively. Therefore, the level of IL-12 in group 2 was significantly higher than that in group 1 ($P<0.05$). However, in group 1, it was higher compared with those in groups 3 and 4. The cytokine level between groups 3 and 4 showed no difference ($P>0.05$). The results are shown in *Fig. 6*.

The numbers of *B. abortus* significantly reduced in the spleen of mice immunized with Ad-LL/BP in comparison with Ad-CMV and PBS control animals. Ad-LL/BP conferred a significant protection ability against *B. abortus* infection with 0.8 log unit when comparable to Ad-CMV or PBS controls *(Table 1)* ($P<0.01$). The values from Ad-CMV and PBS control groups were only 0.1 and 0, respectively. A19 vaccine is used as positive control in this study because A19 strain is widely used to protect against *B. abortus* infection in cow in China. However, the unit of protection from Ad-LL/BP vaccine was significantly lower in comparison with that from A19 strain ($P<0.05$).

DISCUSSION

The animals infected with *Brucella* mainly induced cellular immunity, so excellent vaccines against *Brucella* should induce Th1-dominant immune response based on T cell antigen [22]. Some studies about recombinant Ad vaccine have been reported, and the causative agents consisted of *Streptococcus pneumonia*, *Chlamydophila psittaci*, *Bacillus anthracis*, and *Mycobacterium tuberculosis* [15,16,23]. However, the genetic vaccine based on adenovirus as vector has not yet been reported for *Brucella*. The L7/L12 protein can elicit strong CMI, mixed Th1 and Th2 immune responses, and protection from *Brucella* infection in immunized animals [20,24]. Another promising immunodominant protein specific to *Brucella* is a 31 kDa protein, BCSP31. Its immune response-inducing properties are attributable to the presence of an immunogenic and protective BCSP fraction, possibly lipopolysaccharide [25]. A study indicated that the recombinant L7/L12-TOmp31 protein elicited stronger humoral and cellular immune responses and provided significant protection level against *B. melitensis* and *B.*

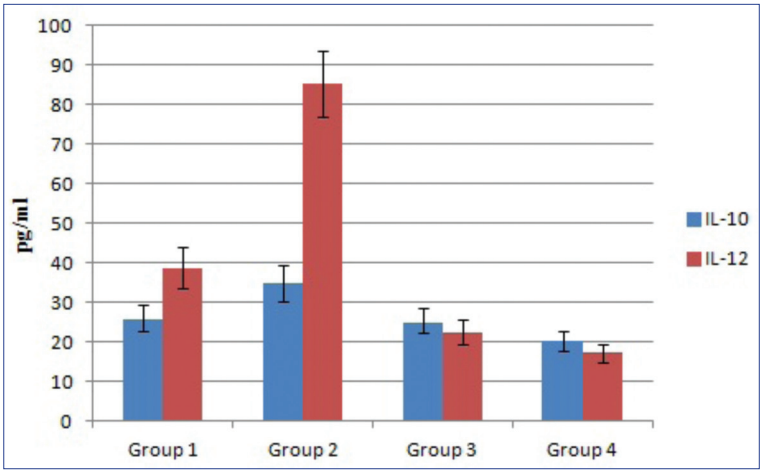

Fig 6. Analysis of IL-10 and IL-12 levels

Table 1. *Protection against B. abortus CVCC12 infection induced by vaccination*

Groups (n=10)	Vaccine	log_{10} CFU of CVCC12 in Spleen (mean±SD)	Units of Protection in Spleen
1	rAd-LL/BP	5.1±0.15*	0.8
2	A19	4.4±0.13*	1.5
3	Ad-CMV	5.8±0.15	0.1
4	PBS	5.9±0.12	0

** Significantly different compared to control group (P <0.05)*

abortus challenge [19]. A divalent DNA vaccine encoding *Brucella* L7/L12-truncated Omp31 fusion protein was able to improve protection against *B. abortus* or *B. melitensis* infection [26]. A divalent genetic vaccine based on the L7/L12-Omp16 or L7/L12-P39 could elicit a stronger cellular immune response and better immunoprotection against *B. abortus* in comparison to single protein [27]. A combined DNA vaccine encoding BCSP31, SOD, and L7/L12 induced specific CTL responses and conferred high protection against *B. abortus* 2308 strain [14]. Based on these works, we want to evaluate the immunogenicity and protection ability of a recombinant adenovirus vaccine co-expressing L7/L12 and BCSP31 proteins of *B. abortus* in BALB/c mice model.

In the present work, the replication-incompetent Ad5 was used as a vector to construct recombinant Ad, which expressed L7/L12 and BCSP31 proteins of *B. abortus*. BALB/c mice were immunized with the recombinant Ad vaccine, and cell-mediated and humoral immune responses were evaluated. The results of this study would provide information for further research on vaccination against brucellosis in animals.

Th1 cells mainly mediate cellular immunity to accelerate IgG2a antibody, and Th2 cells mediate humoral immunity to promote IgG1 antibody [26]. Therefore, the IgG, IgG1, and IgG2a levels of the mice were detected. For groups 1 and 2, the IgG, IgG1, and IG2a levels gradually rose following the increase in vaccination, with the rise in IgG2a level being the fastest, followed by IgG and IgG1. The antibody levels from groups 1 and 2 were significantly higher compared with those from groups 3 and 4. These results indicate that the recombinant vaccine could induce cellular immune response mainly and humoral response partly in mice. Lymphoproliferation assay showed that the SIs were higher in groups 1 and 2 than in groups 3 and 4, which was consistent with the results of antibodies. FCM analysis indicated that the recombinant vaccine could induce T cell response.

IL-12 (Th1-type cytokine) and IL-10 (Th2 type cytokine) were tested. The level of IL-12 from group 2 was significantly higher compared with that from group 1. Moreover, the cytokine level from group 1 was higher than that from groups 3 and 4. IL-10 was high in group 2 but had no difference in groups 1, 3, and 4. These results showed that the recombinant vaccine mainly induced Th1 immune response.

In conclusion, a recombinant Ad co-expressing the L7 L12 and BCSP31 proteins of *B. abortus* was constructed, and the immune response and efficacy of the vaccine were evaluated as the log unit of protection in mice. The results indicated that Ad-LL/BP conferred a significant protection against *B. abortus* CVCC12. Although the efficacy of this vaccine was weaker than that of live A19 vaccine, it could be further developed into a live-vector vaccine against *B. abortus*. Further work should focus on the development of an excellent adjuvant to improve the efficacy of the vaccine.

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Research Article

Physical, Haematological and Biochemical Responses of Arabian Horses to Jereed (Javelin Swarm) Competition [1]

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Abstract

The aim of this study is to evaluate physical, haematological and biochemical changes in horses after acute exercise in a jereed game. Jereed is a traditional Turkish equestrian team sport. A total of 14 horses were included in this study. All horses were Arabian stallions aged between 4-14 years old. Respiration rate, heart rate, capillary refill time, mucous membrane and skin turgor (as an indicator for dehydration) were measured and blood samples were collected before the exercise (T0), immediately after first period (T1) and second period (T2), after 30 min (T3) and 60 min of recovery time (T4). Blood samples were analyzed for total cell counts and for determination of lactate, glucose, urea, total protein, albumin, globulin, calcium, magnesium, phosphorous, chloride, sodium, potassium, creatinine kinase, lactate dehydrogenase, aspartate aminotransferase and alkaline phospahatase. All parameters were analyzed using Linear Mixed effect model to evaluate changes of repeated measurements at 5 different time points and LSD was used as post-hoc test. Exercise caused significant increases in heart rate ($P<0.001$), respiratory rate ($P<0.001$), capillary refill time ($P<0.001$), mucous membrane colour ($P<0.001$), dehydration ($P<0.001$), red blood cells ($P<0.001$), hemoglobin ($P<0.001$), hematocrit ($P<0.001$), red cell dispersion width ($P<0.001$), total white blood cells ($P<0.001$), neutrophils ($P<0.001$), and basophils ($P<0.05$). It also caused significant increases in lactate ($P<0.01$), glucose ($P<0.001$), urea ($P<0.001$), total protein ($P<0.001$), albumin ($P<0.001$), globulin ($P<0.001$), sodium ($P<0.001$), potassium ($P<0.001$), creatinine kinase ($P<0.05$), lactate dehydrogenase ($P<0.001$), aspartate aminotransferase ($P<0.001$) and alkaline phosphatase ($P<0.001$) but a decrease in calcium ($P<0.01$), magnesium ($P<0.01$), phosphorous ($P<0.05$), and chloride ($P<0.001$). Although majority did not return to the baseline 30 or 60 minutes after competition ($P<0.05$) most were within or near to the reference range values.

Keywords: *Jeered, Horse, Exercise, Haematology, Biochemistry*

Cirit Müsabakalarının Arap Atlarında Oluşturduğu Fiziksel, Hematolojik ve Biyokimyasal Değişiklikler

Öz

Bu çalışmanın amacı cirit müsabakalarının atlarda oluşturduğu fiziksel, hematolojik ve biyokimyasal değişiklikleri incelemektir. Cirit, Türklerin oynadığı geleneksel bir atlı spor müsabakasıdır. Bu çalışmada, yaşları 4-14 arasında değişen, 14 adet Arap aygırı kullanıldı. İstirahat halinde (T0), müsabakanın hemen ilk (T1) ve ikinci devrelerinin sonunda (T2) ve müsabaka tamamlandıktan 30 dak. (T3) ve 60 dak. (T4) sonra atların kalp atım ve soluk sayıları, kapillar dolum zamanı, mukoz membran rengi, deri elastikiyeti ölçüldü ve intravenöz yolla kan örnekleri toplandı. Kan örneklerinden tam kan sayımı ve biyokimyasal olarak laktat, glikoz, üre, total protein, albumin, globulin, kalsiyum, magnezyum fosfor, klor, sodium, potasyum, kreatin kinaz, laktat dehidrogenaz, aspartat aminotransferaz, alkalin fosfataz analizleri yapıldı. 5 farklı zamanda tekrarlanan ölçümleri değerlendirmek için Linear Karışık etki modeli ve post-hoc test olarakta LSD kullanıldı. Atlı cirit müsabakası atların kalp atım ve soluk sayılarında ($P<0.001$), kapillar dolum zamanında ($P<0.001$), mukoz membran rengi ($P<0.001$), dehidrasyon ($P<0.001$), eritrosit sayısı ($P<0.001$), hemoglobin ($P<0.001$), hematokrit ($P<0.001$), toplam lökosit sayısı ($P<0.001$), nötrofil ($P<0.001$) ve bazofil ($P<0.05$) sayılarında artışa sebep olmuştur. Ayrıca, atların laktat ($P<0.01$), glikoz ($P<0.001$), üre ($P<0.001$), total protein ($P<0.001$), albumin ($P<0.001$), globulin ($P<0.001$), sodium ($P<0.001$), potasyum ($P<0.001$), kreatin kinaz ($P<0.05$), laktat dehidrogenaz ($P<0.001$), aspartate aminotransferaz ($P<0.001$), alkalin fosfataz ($P<0.001$) değerlerinde artış olmuştur. Kalsiyum ($P<0.01$), magnezyum ($P<0.01$), fosfor ($P<0.05$), klor ($P<0.001$) değerlerinde ise azalma oluşmuştur. Ölçülen parametrelerin büyük bir kısmı müsabaka bittikten 30 veya 60 dak. sonra başlangıç değerlerine geri dönemese de ($P<0.05$) referans aralığına çok yakın değerlere ulaştığı tespit edilmiştir.

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INTRODUCTION

Physiological responses to exercise differ according to the type of exercise being performed. Equestrian sports vary in physiological demand depending on the speed and duration of the exercise bout. The physiological responses associated with exercise within equestrian sports also vary.

Exercise has many variable effects on physical, haematological and biochemical parameters of horses [1]. Some of these changes have been studied in equestrian sports such as flat racing [2-4], endurance [5-7], show jumping [8,9], eventing [10], reining [11], marcha [12], cavalcade [13], and polo [14,15]. However, to the best to our knowledge, there is no such a study focused on jereed horses.

Jereed is a traditional Turkish equestrian team sport played outdoors on horseback. It has been played by Turks for many centuries, dating back to the days of the Turkish states. The game were used to be played with native horse breeds of Anatolian Native, Kolu Kisa of Hinis, Turkish Arab, and East Anatolia which were raised for jereed game in old times [16,17]. Arabian horses, especially male ones which have not been involved in flat racing, have been preferred since the 1980s [18]. Apart from Turkey, jereed is also played in many Asian, Arabian, and Eastern European countries such as Iran, Afghanistan, Turkmenistan, *Kyrgyzstan*, Indonesia, and Hungary [19].

The game is held outdoors on a sandy pitch that is 140±10 m long by 40±5 m wide and played by two teams of 7 players. Each team has 3 reserve horses and players. The game lasts around one and half hours and is divided into 2 periods of 40 min with an interval of 10 min between them. All horses and players play in both periods if not injured or excluded. The jereed game requires an effort of constantly alternating intensities between high and low exercise. Horses do hard galloping with sudden pauses, high speed startups, and sudden changes of directions during the game. The speed and maneuver is determinative factor of game [20,21].

The aim of this study is to evaluate the response of physical, haematological and biochemical parameters after each period and during the recovery times. This study will be the first report of physiological and biochemical changes observed in horses during jereed competition. This study is noteworthy since it will serve as a basis of development of national training of jereed horses as well as jereed competition. Furthermore, these findings will make reference values for horses in jereed competition and help veterinarians to better judge the metabolic and electrolyte disturbances of horses during this competition.

MATERIAL and METHODS

The study received ethical approval from the local ethic committee of animal experiments in Kocaeli University (No: 2017-22). The study was performed in Turkey (Çayırova, Kocaeli, N40° 47′ 59′, E 29° 25′ 0′) in July 2017 during official national jeered competition for Kocaeli provience held by Turkish Federation of Traditional Sports. Games were played according to the national jereed rules. The mean temperature, humidity and wind were 29°C, 31%, 9 km/sn (from North West), respectively (Data supplied from Turkish Regional Meterological Service website). All seven horses on both teams were studied. A total of 14 horses were included in this study. All horses were Arabian stallions, aged between 4-14 years and weighed approximately 330 kg. Horses were clinically examined in the morning before the game (T0). Thereafter, they were examined immediately after first period (T1), immediately after second period (T2) and after 30 min (T3) and 60 min of recovery period (T4). Heart rate, respiratory rate, capillary refill time, mucous membane colour, and signs of deydration were examined. Mucose membrane were described as normal (1), hyperemic (2) and congested (3). Capillay refill time was tested by pressing one's thumb firmly into the gum, blanching colour out, removing quickly, and counting seconds for gum to turn to normal, pink color. Skin turgor test (dehydration) was measured by pinching the skin on the neck into a tent, then counting seconds until it returned to normal contour.

In all time points, blood samples were collected by jugular venipuncture into 2 mL ethylenediaminetetraacetic acid vacutainer tubes (K3EDTA, Greiner Bio-one GmbH, USA) for haematological evaluation. Blood samples were collected into 2 mL sodium floride-ethylenediaminetetraacetic acid vacutaner tubes (FE Sodium Floride/K3EDTA, Greiner Bio-one GmbH, USA) for lactate measurement and 8.5 mL serum separator tube (Vacutainer SST Plus; BD) for all other biochemical analyses. Serum was separated by centrifugation at 4000 rpm for 5 min (Eppendorf Centrifuge 5702 R, Germany) and refrigerated until the next day. All analysis were carried out in the Equine Hospital of the Turkish Jockey Club. Red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell dispersion width (RDW), white blood cells (WBC), neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS), basophils (BASO) and platelets (PLT) were analysed using Abott Cell-Dyn 3500 Hematology analyser (Abbott Diagnostics, Santa Clara, CA). Lactate, glucose, urea, total protein, albumin, globulin, calcium (Ca), magnesium (Mg), phosphorous (P), sodium (Na), potassium (K), chloride (Cl), creatinine kinase (CK), lactate dehydrogenase (LDH) aspartate aminotransferase (AST), alkaline phosphatase (ALP) were measured using Dimension Xpand biochemistry analyser (Dimension Xpand plus, Siemens, USA).

Statistical Analyses

Collected variables were analyzed by using Linear Mixed effect model to evaluate changes of repeated measure-

ments at 5 different time points and LSD was used as post-hoc test. Due to the fact that variances at each time point were different and correlations between measurements were different for each time pairing, unstructured covariance type was selected for modeling. All statistical analyses were performed with SPSS for Windows version 24.0 and a P value <0.05 was accepted as statistically significant.

RESULTS

All results are shown as means±standard error of mean (SEM) in *Table 1, Table 2* and *Table 3*. All animals were examined clinically before game at T0. At T0, mean values of respiratory rate, heart rate, capillary refill time and mucous membrane colour were near to reference range. Mean value for dehydration were mildly high *(Table 1)*. At T1, respiratory rate (P<0.001), heart rate (P<0.001), capillary refill time (P<0.001), skin turgor time (P<0.001), and mucous membrane colour (P<0.01) were significantly increased. There was no significant difference in these parameters between T1 and T2 whereas all values at T2 were significantly higher than T0 (P<0.001). At T3, respiratory rate (P<0.01), heart rate (P<0.001), capillary refill time (P<0.05) and mucous membrane (P<0.001) were significantly higher compared to T0. Respiratory rate, heart rate and mucous membrane colour were decreased significantly from T2 to T4 (P<0.05, P<0.001, P<0.05; respectively). At T4, heart rate (P<0.01), capillary refill time (P<0.001), mucous membrane (P<0.05) and skin turgor (P<0.01) were significantly higher than T0 while there was no significant difference in respiratory rate between T4 and T0.

RBC, HGB and HCT were significantly higher at T1 and T2 (each, P<0.001) than T0. Their values at T3 (each, P<0.05) and T4 (each, P<0.001) were significantly lower than T2 but significantly higher than T0 (each, P<0.001) *(Table 2)*.

RDW was significantly higher both at T1 (P<0.01) and T2 (P<0.001) than T0. Their values both at T3 and T4 were significantly higher than T0 (each, P<0.001). MCV did not show any significant difference at T1 but decreased significantly at T2 (P<0.05) and its value at T2 significantly lower than T0 (P<0.001). MCV value at T4 was significantly lower than T0 (P<0.05).

WBC was increased significantly at T1 (P<0.01) because of increases in the numbers of neutrophils and monocytes (P<0.001 and P<0.01, respectively). At T2, the numbers of WBC, NEU and BASO were higher than T0 (P<0.001, P<0.001, P<0.05; respectively). The number of WBC was significantly higher at T4 than T0 (P<0.001) because of significant increases in NEU (P<0.01), MONO (P<0.01) and BASO (P<0.05) numbers. There was no significant difference in the number of PLT among all periods (P>0.05).

Ca, Mg and Cl concentrations decreased significantly from T0 to T1 (P<0.001, P<0.01, P<0.001; respectively) while Na was increased significantly (P<0.001*) (Table 3)*. Ca, Mg, Cl and P were significantly lower at T2 than T0 (P<0.01, P<0.01, P<0.001, P<0.05; respectively) while Na and K were higher (each, P<0.001). Ca, Mg, Cl increased significantly from T2 to T4 (P<0.001, P<0.05, P<0.001; respectively) and there was no significant difference in these parameters between T4 and T0 (each, P>0.05). Na and K were significantly higher (both, P<0.05) and P were significantly lower (P<0.001) at T4 than T0.

Total protein, albumin and globulin increased significantly from T0 to T2 (each, P<0.001) and were significantly higher at T3 (each, P<0.001) and T4 (each, P<0.001) than T0.

Lactate, glucose and urea were increased significantly at T1 (P<0.001, P<0.001, P<0.01; recpectively) and were significantly higher at T2 (P<0.01, P<0.001, P<0.001; respectively) than T0. Lactate and glucose significantly decreased from T2 to T4 (for each, P<0.01) while urea increased (P<0.05). Lactate, glucose and urea were significantly higher at T4 than T0 (P<0.01, P<0.01, P<0.001; respectively).

Table 1. *Physical parameters at rest (T0), immediately after first (T1) and second period (T2), after 30 min (T3) and 60 min of recovery time (T4). Comparisons between eight time points are shown (T1 vs. T0, T1 vs. T2, T2 vs. T0, T2 vs. T3, T3 vs. T0, T3 vs. T4, T2 vs. T4, and T4 vs. T0)*

Parameters (with reference range)[Δ]	T0 X_0±SEM	T1 X_1±SEM	T2 X_2±SEM	T3 X_3±SEM	T4 X_4±SEM	Statistical Difference (P)							
						T1 vs T0	T1 vs T2	T2 vs T0	T2 vs T3	T3 vs T0	T3 vs T4	T2vsT4	T4vsT0
Respiratory rate (10-16 respiration/min)	24±2	63±6	71±7	94±6	39±4	***	Ns	***	***	**	Ns	*	Ns
Heart rate (28-40 beats/min)	40±2	83±4	79±6	62±5	54±3	***	Ns	***	**	***	*	***	**
Capillary refill time (1-2 sec)	2.0±0.2	3.3±0.4	3.1±0.3	2.7±0.2	3.1±0.2	***	Ns	***	Ns	*	Ns	Ns	***
Mucous membrane	1±0.00	1.5±0.2	1.7±0.2	1.7±0.2	1.4±0.1	**	Ns	***	Ns	***	*	*	*
Skin turgor test/ dehydration (1-2 sec)	2.6±0.4	4.1±0.4	4.4±0.3	3.1±0.2	3.7±0.3	***	Ns	***	***	Ns	Ns	Ns	**

** P<0.05, ** P<0.01, *** P<0.001,* ***Ns:*** *Not significant,* ***Δ:*** *Reference ranges are referred* [22]

Table 2. *Haematological parameters at rest (T0), immediately after first (T1) and second period (T2), after 30 minutes (T3) and 60 minutes of recovery time (T4). Comparisons between eight time points are shown (T1 vs. T0, T1 vs. T2, T2 vs. T0, T2 vs. T3, T3 vs. T0, T3 vs. T4, T2 vs. T4 and T4 vs. T0)*

Parameters (with reference range)[Δ]	T0 Xo±SEM	T1 X_1±SEM	T2 X_2±SEM	T3 X_3±SEM	T4 X_4±SEM	Statistical Difference (P)							
						T1 vs T0	T1 vs T2	T2 vs T0	T2 vs T3	T3 vs T0	T3 vs T4	T2vsT4	T4vsT0
RBC (6.0-10.4x10^6/µL)	8.09±0.40	10.13±0.45	10.89±0.46	10.14±0.56	10.01±0.57	***	*	***	*	***	Ns	**	***
HGB (10.1-16.1 g/dL)	13±0.6	17±0.7	18±0.7	17±0.9	16±0.9	***	Ns	***	*	***	Ns	**	***
HCT (32%-43%)	39±2	50±2	52±2	48±2	48±2	***	Ns	***	*	***	Ns	**	***
MCV (37-49 fL)	48.4±0.8	48.3±0.8	47.7±0.8	48.1±0.8	47.8±0.8	Ns	*	***	Ns	Ns	Ns	Ns	*
MCH (13.7-18.2 pg)	16.6±0.2	16.4±0.2	16.4±0.2	16.5±0.2	16.4±0.2	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
MCHC (35.3-39.3 g/dL)	34.3±0.2	34.3±0.3	34.6±0.2	34.4±0.2	34.4±0.3	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
RDW (17%-27%)	24.7±0.5	26.4±0.6	27.5±0.6	26.9±0.6	26.7±0.8	**	*	***	Ns	***	Ns	Ns	***
WBC(5.6-12.1x10^3/µL)	7.8±0.4	9.1±0.3	11.6±1.0	11.5±1.2	12.1±1.0	**	*	***	Ns	**	Ns	Ns	***
NEU(2.9-8.5 x10^3/µL)	5.9±0.4	7.2±0.4	9.6±0.9	7.9±0.9	8.6±0.9	***	*	***	*	*	Ns	Ns	**
LYM(1.2-5.1 x10^3/µL))	1.7±0.1	1.7±0.2	1.6±0.2	2.6±0.5	2.05±0.2	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
MONO (0.0-0.7 x10^3/µL)	0.15±0,02	0.09±0.01	0.24±0.06	0.80±0.23	0.90±0.21	**	*	Ns	*	*	Ns	*	**
EOS (0.0-0.8 x10^3/µL)	0.17±0.03	0.13±0.02	0.18±0.04	0.18±0.06	0.34±0.10	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
BASO (0.0-0.3 x10^3/µL)	0.02±0.00	0.02±0.00	0.02±0.00	0.03±0.01	0.10±0.03	Ns	Ns	*	Ns	Ns	*	**	*
PLT (117-256x10^3/µL)	133.8±11.2	149.8±25.9	162.8±22.6	182.3±28.4	161.1±27.1	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns

** P<0.05, ** P<0.01, *** P<0.001,* ***Ns:*** *Not significant,* ***Δ:*** *Reference ranges are referred* [22]

CK, LDH, AST and ALP increased significantly from T0 to T1 (P<0.001, P<0.001, P<0.01, P<0.001; respectively), and to T2 (P<0.05, P<0.001, P<0.001, P<0.001; respectively). CK, LDH, AST and ALP were significantly higher at T3 (P<0.05, P<0.001, P<0.001, P<0.001; respectively) and at T4 (P<0.05, P<0.05, P<0.001, P<0.001; respectively) than T0.

DISCUSSION

To the knowledge of literature review, the evaluation of the physical, hematological and biochemical responses of horses to jeered competition is the first.

In this study, respiratory rate at T4 (39±4) were significantly lower than T2 (71±7) and reached the baseline (T0). Heart rate at T4 (54±3) were significantly lower than T2 (79±6) but did not reach the baseline (40±2). Heart rate recovery might be a good indicator for assessing fitness level of warm blood sport horses [23]. However, ambient temperature and humidity also affect the heart rate. Higher heart rate elevation in the hot conditions increases cardiac output to provide skin blood for heat dissipation [24]. The present study was performed in July at 29°C temperature and 31% humidity. This might explain that heart rate at 60 min after exercise did not reach the resting value at T0. Actually, in endurance competition, horses having 64 beats/min heat rate within 30 min at the final inspection are considered as fit [25]. In polo, horses with 48 beats/min of heart rate at 30 min after exercise were considered as fit although not reached the resting value [14]. The mean capillary refill time and skin turgor time at T0 (2.0±0.2, 2.6±0.4; respectively) observed in the present study shows that horses might be mildly dehydrated. According to personel communications with horse owners, the last time of horses to consume water was 10 h before the competition. This might explain the mild dehydration observed in the horses before competition. Thereafter, capillary refill time was increased and skin turgor was decreased (as time increased for skin to return to normal contour) at T1 and T2. These might be attributed to the fact that more dehydration occured after exercise. When the exercise is performed in hot conditions, body temperature rises excessively, the demands of skin blood flow for heat dissipation arise resulting in dehydration [26]. Furthermore, the condition did not improve after 60 min of rest. This might be explained by the fact that horses were

Table 3. *Biochemical parameters at rest (T0), immediately after first (T1) and second period (T2), after 30 minutes (T3) and 60 minutes of recovery time (T4). Comparisons between eight time points are shown (T1 vs. T0, T1vs. T2, T2 vs. T0, T2 vs. T3, T3 vs. T0, T3 vs. T4, T2 vs. T4 and T4 vs. T0)*

Parameters (with reference range)Δ	**T0 Xo±SEM**	**T1 X_1±SEM**	**T2 X_2±SEM**	**T3 X_3±SEM**	**T4 X_4±SEM**	**Statistical Difference (P)**							
						T1 vs T0	**T1 vs T2**	**T2 vs T0**	**T2 vs T3**	**T3 vs T0**	**T3 vs T4**	**T2 vs T4**	**T4 vs T0**
Lactate (0-2 mmol/L)	0.69±0.08	14.67±1.98	9.23±2.54	4.79±1.41	2.79±0.69	***	Ns	**	**	*	*	**	**
Glucose (62-134 mg/dL)	92.07±2.76	158.08±8.68	180.86±17.56	158.29±19.49	143.50±17.32	***	Ns	***	Ns	**	Ns	**	**
Urea (24-48 mg/dL)	27.86±1.61	30.17±1.79	36.89±2.06	37.81±1.95	39.18±2.09	**	***	***	Ns	***	*	*	***
TotalProtein (5.6-7.6 g/dL)	6.71±0.13	7.31±0.22	7.74±0.20	7.63±0.23	7.73±023	***	**	***	Ns	***	Ns	Ns	***
Albumin (2.6-4.1 g/dL)	3.49±011	3.92±0.16	4.03±0.19	4.01±0.19	4.09±0.19	***	Ns	***	Ns	***	*	Ns	***
Globulin (2.6-4.0 g/dL)	3.21±0.14	3.39±0.15	3.71±0.14	3.62±0.14	3.64±0.14	Ns	**	***	Ns	***	Ns	Ns	***
Calcium (10.2-13.4 mg/dL)	11.80±0.15	11.18±0.21	11.09±0.27	11.40±0.29	11.69±0.24	***	Ns	**	***	Ns	*	***	Ns
Magnesium (1.4-2.3 mg/dL)	2.06±0.06	1.86±0.06	1.84±0.08	1.86±0.09	1.97±0.10	**	Ns	**	Ns	*	**	*	Ns
Phosphorous (1.5-4.7 mg/dL)	3.09±0.15	2.94±0.20	2.48±0.25	2.24±0.21	2.34±0.22	Ns	Ns	*	Ns	***	Ns	Ns	***
Sodium (128-142 mmol/L)	136.07±1.13	139.00±1.07	138.79±1.04	138.93±1.32	138.57±1.44	***	Ns	***	Ns	**	Ns	Ns	*
Potassium (2.9-4.6 mmol/L)	3.99±0.14	4.52±0.28	5.66±0.45	5.35±0.67	5.62±0.72	Ns	**	***	Ns	*	Ns	Ns	*
Chloride (98-109 mmol/L)	98.29±0.95	93.92±1.34	94.36±1.36	95.93±1.39	97.00±1.49	***	Ns	***	***	*	**	***	Ns
CK (60-330 U/L)	220.00±23.05	342.38±32.27	520.86±108.08	602.57±139.23	638.14±162.27	***	Ns	*	*	*	Ns	Ns	*
ALP (138-251 U/L)	136.21±7.01	164.08±8.85	174.50±11.12	173.64±10.32	175.36±11.54	***	Ns	***	Ns	***	Ns	Ns	***
LDH (112-456 U/L)	335.43±11.49	440.00±20.15	479.86±28.83	478.00±33.66	444.50±38.45	***	Ns	***	Ns	***	*	*	*
AST (160-412 U/L)	381.500±63.549	424.846±57.133	460.357±60.498	464.929±64.433	474.500±65.508	**	*	***	Ns	***	**	Ns	***

** P<0.05, ** P<0.01, *** P<0.001,* ***Ns:*** *Not significant, Δ: Reference ranges are referred* [27]

not given water within two hours after competition in jeered competition since most horse owners traditionally believe that it could be dangerous for horses.

Total protein, albumin and globulin concentrations were increased at T2. These parameters did not reach the resting values after 30 and 60 min of recovery but were within the reference range. Increases in plasma proteins with an increased capillary refill time and a decreased skin turgor might be attributed to the acute dehydration. The increase in serum protein concentrations after exercise has been observed in other equestrian competitions such as polo [14,15], flat racing [26], cross country [10] and endurance [24]. It has been reported that hyperproteinemia occurs in athletic horse as a result of dehydration [1]. It has been also reported that there is an increase in total protein and albumin concentration as a result of intercompartmental fluid shifts or real fluid loss [24]. Total protein concentration returns to baseline by 30 min after high intensity and short duration of exercises while total protein recovery requires more time in prolonged exercises or exercises with excessive sweating [1]. The fact that jeered competition lasts approximately for 90 min and horses usually are not given water within two hours after exercise might explain the reason for total protein not to be recovered 30 or 60 min after competition in the present study.

Urea concentration was increased after exercise and did not reach the baseline after 30 or 60 min of recovery but were within reference range. Transient increase in urea concentration with exercise has been found normal responses to high and low intensity exercises as a result of reduction in renal blood flow or real fluid loss [1].

The RBC, HGB and HCT were increased after exercise. It has been reported that erythrocytes from spleen reservoir are realeased to the circulation under the influence of catecholamine during exercise [1]. Apart from spleen contraction, the increase in HCT might be attributed to intercompartmental fluid shifts or real fluid loss by sweating. RBC, HGB and HCT at T4 (10.01±0.57, 16±0.9, 48±2; respectively) were significantly lower than T2 (10.89±0.46, 18±0.7, 52±2; respectively). These parameters at T4 did not reach the baseline but were near to the reference range. Circulating erythrocytes return to spleen over a period of 1-2 h to reach preexercise values [1].

MCV were decreased but RDW were increased at T2. Both of the parameters did not return to the baseline 60 min after competition but were within reference range. Both conditions observed in the present study may be attributed to the fluid shifts from erythrocytes to the extracellular compartment because of dehydration. Actually an increase in RDW has been observed in human athletes after acute exhaustive exercise [28].

Increased leukocytosis was observed because of a significant rise in the neutrophil count at T1 and T2, with rises in monocytes at T1 and basophils at T2. Lymphocyte count was not affected but the increase in neutrophil: lymphocyte ratio (NEU/LYM T0 = 3.47, NEU/LYM T1 = 4.24 and NEU/LYM T2 = 6.00) was observed in the present study. It has been suggested that exercise causes the mobilization of marginated leucocytes sequestered in the spleen and capillary beds to the circulation [1,2,29]. It has been reported that neutrophil : lymphocyte ratio has been decreased after polo competition [14] while neutrophil: lymphocyte has been increased after endurance [1]. In high intensity and shorter duration of exercises, relatively more lymphocytes are realesed from spleen to the circulation under the effect of catecholamines [2,14]. Low to modeate intensity but longer duration exercises produces a marked leukocytosis due to neutrophilia and lymphopenia under the effect of cortisol [1,30]. The values at T3 and T4 did not reach to the baseline but were in the reference range 30 and 60 min after exercise. The increased leucocyte count after exercise returns to baseline values witin 6 h [31].

Lactate and glucose levels were significantly increased at T1 (14.67±1.98, 158.08±8.68; respectively) and T2 (9.23±2.79, 180.86±17.56; respectively). It has been reported that lactate concentrations immediately after exercise are less than 2 mmol/L in endurance race, 10.24 mmol/L in polo competition, 20-25 mmol/L in throughbred racing [14,26,32]. Lactate increases have been found to be associated with anaerobic metabolism of pyruvate and the stimulaton of glycogenolysis [1,33]. It has been reported that glucose level increases in all form of exercise because of hepatic glycogenolysis [26]. In high intensity with short duration exercises, glucose concentrations have peak value of 180-206 [1]. Lactate and glucose levels at T4 (2.79±0.69, 143.50±17.32; respectively) were significantly lower than T2 (9.23±2.79, 180.86±17.56; respectively). These parameters did not reach the baseline at T4 but were near to the reference range.

Ca, Mg, P and Cl concentrations were decreased at T2. The decrease observed in Ca, Mg, P concentrations could have been caused by intracellular movement, which is necessary for their use in muscle function and/or their loss with sweating [14]. Ca and Mg concentration reached the baseline after 30 and 60 min of recovery; respectively. P concentration did not return to the baseline at T4 but were in the reference range. The decrease in chloride concentration after exercise could have been the result of sweating. Chloride has been considered as the principle anion lost in sweat [1,13]. However, chloride concentration reached the baseline at T4. It has been reported that when plasma chloride concentration falls through sweating, renal reabsorption of Cl occurs for compensation [1].

The increase in Na concentration after exercise was observed in this study. Na concentration did not return to the baseline 60 min after exercise but were in reference values. It has been reported that the increase in Na concentration occurs following high intensity exercise

such as flat racing [1]. Na concentrations have been reported to be unchanged, decreased, increased, depending on the environmental conditions and duration of endurance competition [1,34]. It has been suggested that the increase in sodium concentration could have been the result of fluid movement out of extracellular space [35]. It has been also reported that the increase in Na concentration might be because of renal reabsorption of Na to compensate dehydration [36,37]. The decrease in Na concentration after calcavede competition might be associated with the loss of Na from sweating [38]. The intensity and duration of exercise as well as environmental conditions might all affect the amount of fluid lost by sweating and hence, Na concentration in blood.

The increase in K concentration was observed at T2. It has been reported that the increase in potassium concentration after high intensity exercise might be due to the impairment of Na-K pump by increased hydrogen ions in muscle cell [1]. On the contrary, the decrease of K concentration has been detected in endurance competition in which horses have slower speed but longer durations [13]. The decrease of K in that study has been indicated to be associated with the loss of this element in sweat [13].

LDH, CK, AST and ALP concentrations were increased at T1 and T2 and did not reach to the baseline at T4 but were near to reference range except CK. These enzyme activities have been shown to be increased following racing in galloping but still remain within normal reference ranges [1]. Similiary, moderate increases in CK and AST have been observed following endurance exercises [1]. The reason for CK concentration to be high and not to reach near reference range values at T4 might be due to small muscle damage since potassium concentration was also high at T4 compared to reference values.

Physical, haematological and biochemical changes observed in this study were the physiological responses of horses to jereed competition which has a characteristic of mixed aerobic/anaerobic pathway metabolism. Most of the parameters changed significantly after exercise. Although majority did not return to the baseline 30 or 60 min after competition most were within or near to the reference range values.

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Research Article

Molecular Characterization of Tick-Borne Blood Protozoa in Stray Dogs from Central Anatolia Region of Turkey with a High-Rate *Hepatozoon* Infection

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Abstract

Tick-borne blood protozoa play an important role in canine health, especially, *Babesia* and *Hepatozoon* species. In dogs, these species lead to clinical symptoms ranging from mild to severe. This study aimed to investigate the presence of tick-borne blood protozoa in stray dogs using microscopy and molecular methods. While none of the blood smears showed any *Babesia* spp. piroplasms, *Hepatozoon* spp. gametocytes were detected in 3.8% of examined samples. PCR analyses revealed the presence of a *Hepatozoon* infection in 49.5% of dogs. However, the presence of *Babesia* spp. was not found in any dogs. Sequence and phylogenetic analyses revealed that 86% of the positive cases were *Hepatozoon canis* and 13.7% were *Hepatozoon* sp. MF. The positivity rate obtained in this study was higher than the reported rates in many regions in Turkey. This suggests that *Hepatozoon* infections present a risk to dog health in this region. In addition, *Hepatozoon* sp. MF (a new genotype of undetermined pathogenicity) is reported in dogs from this region and detailed pathogenicity and epidemiological studies are thus required for this genotype. This study, therefore, suggests that *H. canis* is common in stray dogs in Ankara. Canine hepatozoonosis should be taken into consideration in pet clinics and the differential diagnosis should not be overlooked.

Keywords: *Dog, Hepatozoon, Babesia, PCR, Molecular phylogeny*

İç Anadolu Bölgesindeki Sokak Köpeklerinde Kene-Kaynaklı Kan Protozoonları Varlığının Araştırılması ve Yüksek Orandaki *Hepatozoon* Enfeksiyonunun Moleküler Karakterizasyonu

Öz

Kene kaynaklı kan protozoonları köpek sağlığında önemli bir yer tutmaktadır. Bu protozoonların başında *Babesia* ve *Hepatozoon* türleri yer almaktadır. Mevcut türler köpeklerde ılımlı hastalık tablosundan şiddetli enfeksiyona varan derecede klinik belirtilere neden olmaktadırlar. Bu çalışmada, sokak köpeklerindeki kene kaynaklı kan protozoonlarının varlığı mikroskobik ve moleküler olarak araştırılmıştır. İncelenen kan preparatlarının hiçbirinde *Babesia* spp.'nin piroplazmik formuna rastlanmamıştır. Ancak örneklerin %3.8'inde *Hepatozoon* spp.'nin gametositleri tespit edilmiştir. Yapılan PCR analizleri sonucunda köpeklerde yüksek oranda (%49.5) *Hepatozoon* enfeksiyonu tespit edilmiştir. Buna karşın hiçbir köpekte *Babesia* spp. varlığına rastlanmamıştır. Sekans ve filogenetik analizler sonucunda pozitiflerin %86'sının *Hepatozoon canis,* %13.7'sinin ise *Hepatozoon* sp. MF olduğu belirlenmiştir. Bu çalışmada elde edilen pozitiflik oranın, Türkiye'deki birçok bölgeden rapor edilen oranlardan daha yüksek olduğu görülmüştür. Bu durum bölgedeki köpekler için *Hepatozoon* enfeksiyonu açısından risk teşkil etmektedir. Ayrıca henüz patojenitesi hakkında net bir veri bulunmayan ve yeni bir genotip olan *Hepatozoon* sp. MF varlığı da bölgedeki köpeklerden bildirilmiş olup bu genotip ile ilgili detaylı patojenite ve epidemiyolojik çalışmalara ihtiyaç duyulduğu görülmüştür. Sonuç olarak Ankara'daki sokak köpeklerinde *H. canis*'in yaygın olarak bulunduğu ortaya konmuştur. Bu nedenle, kliniklerde hepatozoonosis enfeksiyonları dikkate alınmalı ve ayırıcı tanıda göz ardı edilmemelidir.

Anahtar sözcükler: *Köpek, Hepatozoon, Babesia, PCR, Moleküler filogeni*

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INTRODUCTION

In recent years, worldwide interest in canine vector-borne diseases has been increasing due to the social role of dogs in human life and as the cause of zoonotic diseases. Tick-borne diseases are at the top of the list for both animal and public health [1,2]. In addition, tick-borne diseases also take an important place in the "one medicine, one health" concept, which has featured in the news in recent years and has become increasingly popular [3]. Due to this importance, studies on tick-borne pathogens are being carried out, revealing new ecological, epidemiological, and taxonomic data [2]. The primary protozoan tick-borne diseases in dogs are babesiosis and hepatozoonosis [2,4]. Canine babesiosis is an important tick-borne disease that is common worldwide and caused by intra-erythrocytic protozoan species of the *Babesia* genus [4,5]. These *Babesia* species classically occur in two forms, small *(B. gibsoni, B. condrae, B. vulpes)* and large *(B. canis, B. rossi, B. vogeli)* based on intraerythrocytic morphology [5-7]. The clinical picture of babesiosis in dogs ranges from sub-clinical to multi-organ failure and may eventually lead to death [4,5].

Canine hepatozoonosis is a tick-borne disease caused by the introduction of *Hepatozoon* protozoan species into dogs. It is known that two hepatozoan species *(H. canis and H. americanum)* cause this infection [8]. However, the existence of new genotypes has emerged in recent years, thanks to developing molecular methods [9]. The disease is widespread in Europe, Asia, Africa, and South America [2,5,10]. *H. canis* infection is mainly transmitted by *Rhipicephalus sanguineus* ticks. *H. canis*, which usually induces mild clinical symptoms, causes anemia and lethargy by infecting hemolymphatic tissues in dogs. However, in case of high parasitemia, it has also been reported to cause severe clinical outcomes [5,10].

Both *H. canis* and *H. americanum* are transmitted by hard (Ixodidae) ticks. However, the *Babesia* species are transmitted by ticks during feeding [4], while the *Hepatozoon* species are transmitted by ingestion of ticks [8]. The distribution of these diseases is determined by their vector ticks [5,10].

Published studies of babesiosis in Turkey have identified *B. canis* [11-16], *B. vogeli* [13,15,17-19], *B. gibsoni* [20], and an unnamed *Babesia* spp. [15] in dogs. In addition, the presence of *B. rossi* has been reported in *Haemaphysalis parva* ticks collected from a human and wild boars [21,22].

Printed articles regarding the existence of *Hepatozoon* spp. in Turkey show that the disease was first reported in a dog in 1933 [23]. Subsequently, the presence of *H. canis* was determined morphologically [24-29], molecularly [9,13,15,16,19,25,26,28-30], and serologically [26] in dogs. *Hepatozoon* spp. were also identified in unfed and semi-fed *Rh. sanguineus* ticks obtained from dogs [28,31] and *Rh. sanguineus, Dermacentor marginatus, Haemaphysalis sulcata, Haemaphysalis* spp. (nymph), *Ixodes ricinus* and *Ixodes* spp. (nymph) collected from humans [32]. In addition, a new *Hepatozoon* spp. genotype has been reported in dogs from Central Turkey [9].

In this study, we aimed to investigate the presence of tick-borne blood protozoa and focus on high-rate hepatozoonosis infections that were confirmed in stray dogs in Ankara.

MATERIAL and METHODS

Blood samples were collected from 103 stray dogs living in a shelter in Ankara. Study materials were obtained from blood taken from dogs during routine examinations by the veterinarian in charge of the shelter. It has been recorded that the examined dogs are asymptomatic as clinical appearance. Blood samples were collected in EDTA-containing tubes. One half of the blood sample was set aside for examination by microscopy and the rest was stored at -20°C until PCR analysis. Blood smears were immediately (1-2 min) prepared for microscopic examination, fixed with methanol for 5 min, and stained with 5% Giemsa for 45 min. After Giemsa staining, slides were examined for the presence of piroplasmic forms of *Babesia* spp. and gamonts of *Hepatozoon* spp. by light microscopy at 100x magnification. A Nikon Eclipse 80*i* (Nikon, Tokyo, Japan) light microscope equipped with a Leica MC170 HD (Leica, Heerbrugg, Switzerland) digital camera and LAS (V4.11.0) software was used for microscopic examination.

Genomic DNA was extracted from blood samples using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified DNA samples were stored at -20°C until PCR analysis was performed. To determine the presence of *Babesia* spp. in blood samples, genus-specific PCR analyses were performed using BJ1 and BN2 primers [33], which amplify the partial 18S ribosomal RNA *(18S rRNA)* gene of *Babesia* spp. In addition, genus-specific PCR analyses were performed on all blood samples using HepF and HepR primers [34], which amplify the partial *18S rRNA* region of *Hepatozoon* spp. DNase-RNase-free sterile water was added to each reaction as a negative control and DNA samples from *B. bigemina* (isolated from naturally infected cattle) and *H. canis* (isolated from naturally infected dog) were included as positive controls. Positive PCRs were purified using a QIAquick Extraction Kit (Qiagen) and sequenced using the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA).

Sequence analysis was performed bidirectionally using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Obtained nucleotide sequences were compared to sequences registered in the GenBank database using BLAST (www.ncbi.nlmn.nih.gov/BLAST). The sequences were then edited and aligned using the BioEdit program [35]. We used jModeltest version 0.1.1 [36] to determine the most

appropriate phylogenetic model for our sequences. Phylogenetic and molecular evolutionary analyses were carried out using the MEGA version 7.0 [37] with the maximum likelihood method based on the general-time reversible model (GTR + I + G) and a phylogenetic tree was constructed. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The nucleotide sequences obtained in this study were stored in the GenBank database with accession numbers MG254573-MG254623.

RESULTS

Blood samples were collected from 103 dogs in total. While none of the smears obtained from the blood samples showed any *Babesia* spp. piroplasms, the presence of *Hepatozoon* spp. gametocytes was detected in 4 (3.8%) samples *(Fig. 1)*. PCR analysis of the DNA collected from the blood samples confirmed the absence of *Babesia* spp. However, *Hepatozoon* spp. were identified in 51 (49.5%) blood samples. Sequence analysis revealed that 44 (86.2%) of the positive samples were *H. canis* and 7 (13.7%) were *Hepatozoon* sp. MF. BLAST analyses showed that the *H. canis* sequences were 99.2-100% similar to *H. canis* isolates obtained from a dog (accession number FJ497022) and a fox (accession number HM212626) in Croatia, from dogs (accession numbers KU360326, KU360328, and KX880506) in Iran, from a dog (accession number KX588232) in Samsun, from foxes (accession numbers KX879135 and KX887327) in Slovakia, and a Golden Jackal (accession number KX712124) in the Czech Republic. The *Hepatozoon* spp. sequences were also determined to be 100% identical to *Hepatozoon* sp. MF identified in dogs from Karaman (accession number KF439864) and Konya (accession number KF439865). Phylogenetic trees were constructed using *Hepatozoon* spp. sequences obtained in this study and published *Hepatozoon* spp. sequences. The data were registered in GenBank and the phylogenetic tree is illustrated in *Fig. 2*.

DISCUSSION

Canine vector-borne diseases directly threaten the health of dogs. In addition, there are zoonotic vector-borne disease agents that affect human health, for which dogs are known reservoir hosts [1]. The life cycle of these microorganisms is maintained between host, pathogen, and vector. It is well known that ticks are one of the most important vector arthropods. Ticks are responsible for the transmission of a number of pathogens to dogs; therefore, the distribution of these pathogens is determined by the distribution of their vector ticks [2]. Given that dogs have an important place in human life, both their vector-borne diseases and their zoonotic pathogens are an issue [1]. *Babesia* and *Hepatozoon* species are the leading blood protozoa that cause infections in dogs. These species can lead to clinical symptoms ranging from mild to severe and even death. Both diseases are globally distributed [2,5,10] and wild canids are known reservoir host for some of these pathogens [5,10].

The aim of this study was to determine the presence of tick-borne blood protozoa in stray dogs. *Hepatozoon* infections were detected at high levels in 49.5% dogs by PCR, while 3.8% positivity was detected by microscopy. Additionally, we detected very low parasitemia level in blood smears of 4 microscopy-positive dogs. This situation indicated that PCR is much more sensitive than microscopy and all positive dogs are chronically infected with *Hepatozoon* spp. However, *Babesia* spp. were not identified in any dogs. Sequence analysis of the 51 *Hepatozoon* spp., positive samples revealed that 44 (86.2%) were *H. canis* and 7 (13.7%) were *Hepatozoon* sp. MF. *H. canis* isolates obtained in this study were similar to *H. canis* isolates reported in

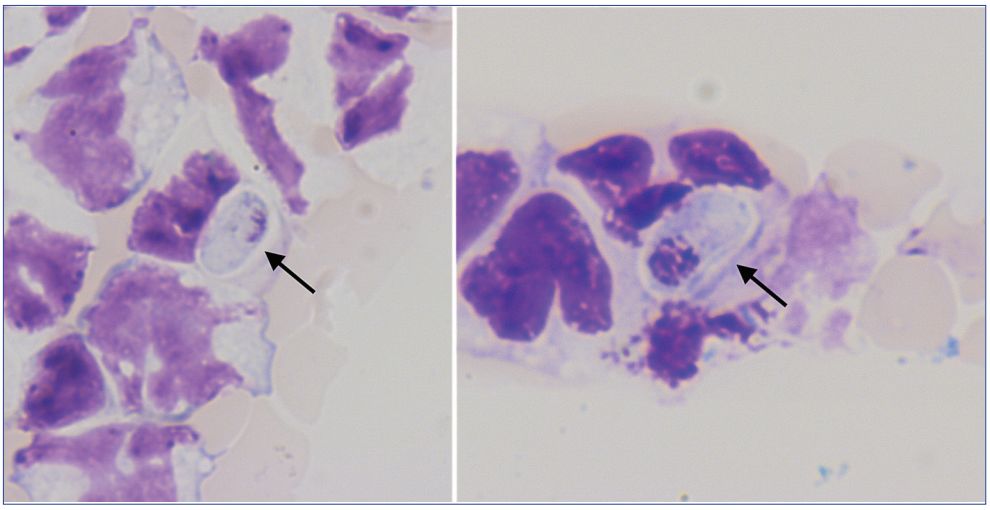

Fig 1. Ellipsoidal-shaped gamonts of *Hepatozoon* spp. (arrows) in peripheral blood from naturally infected dogs

Fig 2. Phylogenetic tree based on aligned sequences of *18S rRNA* of *Hepatozoon* spp. with *Babesia vulpes* as outgroup and constructed by using Maximum Likelihood method calculated under the GTR+I+G substitution model. The *Hepatozoon* sequences obtained in this study are shown in bold. GenBank accession numbers of sequences and names of lineages are given before species names

dogs, foxes and a jackal. In addition, *Hepatozoon* sp. MF isolates were identical to isolates obtained from dogs in the Central Anatolia region.

A number of publications have documented the presence of *Hepatozoon* spp. in dogs in Turkey. Karagenc et al.[26] took samples from 349 dogs in the Aegean region and found *Hepatozoon* spp. in 10.6% of samples by microscopy and 25.8% of samples by PCR. All of the sequenced samples were *H. canis*. In addition, they reported that 36.8% of the blood samples obtained from these dogs had antibodies against this disease. A study carried out in Diyarbakır Province identified the presence of *H. canis* infection in 15.87% of the 63 dogs sampled [28]. In a study including a total of 694 dogs from Sakarya, Kocaeli, Mersin, Giresun, İzmir, Elazığ, Erzurum, Ankara and Nevşehir, 3 (1.05%) of the 285 dogs examined were determined to be positive by microscopy and 155 (22.3%) of the 694 dogs were positive for *H. canis* by PCR. From Ankara, 49 dogs were tested and 2 dogs (4%) were *H. canis*-positive [29]. In Erzurum Province, 43 (32.3%) of 133 asymptomatic dogs were positive for *Hepatozoon* spp. by PCR. Seven of the positive samples were sequenced and were all confirmed as *H. canis* [16]. In Kayseri Province, 21 (5.3%) of 400 dogs were reported as positive for *H. canis* by real-time PCR [13]. In Samsun Province, only one dog (0.5%) of 200 dogs examined was reported as positive for *H. canis* by PCR [30]. By comparing these reports from many parts of Turkey with our results, it is evident that the hepatozoan infection rate is higher in stray dogs in Ankara. Moreover, the molecular method of identification is much more sensitive than microscopy. In addition, Aktas et al.[29] found a 4% positive rate in dogs in Ankara, which is much lower than our rate (49.5%). We believe that this is due to the limited number of samples in their study.

In a study conducted in Diyarbakır, samples from 219 street dogs were examined by microscopy and PCR for the presence of *Hepatozoon* spp. No samples were positive by microscopy, but 54.3% of the samples were determined to be infected with *H. canis* by PCR [15]. Thus, the data obtained in Diyarbakır are compatible with our study and the infection rate is very similar in both regions.

In a study in the Central Anatolia region, Aydin et al.[9] took blood samples from 221 dogs in Konya and Karaman Provinces and tested for *Hepatozoon* spp. by PCR. *Hepatozoon* spp. were found in eight dogs (3.61%). Of these, *H. canis* was found in 6 dogs (2.71%) in Konya Province and an unclassified *Hepatozoon* spp. *(Hepatozoon* sp. MF) was reported in 2 dogs in both Konya and Karaman Provinces. Two species/genotypes were identified in our study. *Hepatozoon* sp. MF was detected more frequently than in previous reports and is only the third such report of this genotype to-date. Sequence and phylogenetic analyses show that these isolates are identical to isolates obtained in this study. This is the first identification of this hepatozoan genotype (of unknown pathogenicity) in dogs in Ankara. In addition, the existence of *H. canis* infection was much higher in our work than in Konya and Karaman Provinces. In another study conducted in Konya Province, blood samples were taken from 192 dogs and *Hepatozoon* spp. were identified in only 8 dogs (4.2%). Sequence analysis revealed that 7 samples were *H. canis* and one sample was *Hepatozoon* sp. MF [19]. This study is consistent with the other study in the same region [9]

but the infection rate is very low compared to our study. Thus, the hepatozoonosis infection rate in dogs in the Central Anatolia region is very different and the infection is more common in the Ankara than other Provinces. We speculate that the reason is related to the distribution of vector ticks. Moreover, studies show that *Hepatozoon* sp. MF is circulating in dogs in the Central Anatolia region. Therefore, more studies of this genotype are needed.

To our knowledge, there are no reports of *Babesia* infection in dogs in Ankara Province. However, *B. rossi* was observed in *Ha. parva* collected from a human and wild boars in Ankara [21,22]. In this study, *Babesia* spp. were not found in any dogs. However, more epidemiological studies are needed to determine the prevalence of babesiosis in dogs in this region. *B. rossi*, causes severe clinical manifestations in dogs [6], is circulating in ticks and thus poses a considerable risk to the dogs of this region.

A high rate of *Hepatozoon* infection (49.5%) was detected in stray dogs in Ankara. In positive dogs, *H. canis* (a cosmopolitan species) and *Hepatozoon* sp. MF genotypes were identified. Detailed pathogenicity and epidemiological studies are required for *Hepatozoon* sp. MF because this genotype is circulating in dogs and the pathogenicity is not clear. Together, these results show that hepatozoonosis is common in dogs in Ankara and this infection should be taken into account in clinical differential diagnoses.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

Anesthetic Efficiency of 2-Phenoxyethanol on Broodstock of *Salmo munzuricus*, a New Trout Species Originating from Munzur Stream [1]

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Abstract

Salmo munzuricus is a newly-named species of trout that spreads through rivers flowing along the Munzur Valley National Park. The efficacy of anaesthetic 2-phenoxyethanol was evaluated in indigenous brown trout in the present study. Fish were divided into three weight classes: 100-200 g (W1), 200-400 g (W2) and 400-600 g (W3) and they were exposed to four concentrations of anaesthetic (0.2, 0.3, 0.4 and 0.5 mL L^{-1}). The lowest induction time was identified in the group W3 while the highest one was in the group W1. The lowest recovery time occurred after 73.2±3.83 sec in the group W3 and the highest one was 136±31.46 sec in the group W1. The recovery times were increased with increasing the concentration of 2-phenoxyethanol in the group W1. However, recovery times in the groups W2 and W3 were significantly longer than observed in W1, generally. Besides, it was indicated that fish size affected induction times and recovery times. As a result of the present study, the most appropriate concentrations were determined as 0.4 mL L^{-1} at weights of 100-400 g while 0.5 mL L^{-1} at weights of 400-600 g for 2-phenoxyethanol in *Salmo munzuricus* broodstock.

Keywords: *Fish anesthesia, Recovery, Brown trout, Salmo munzuricus, Broodstock, 2-phenoxyethanol, Anesthetic, Effective concentration, Fish size*

Munzur Akarsuyu Orijinli Yeni Bir Alabalık Türü Olan *Salmo munzuricus*'un Anaç Bireyleri Üzerinde 2-Fenoksietanol'ün Anestezik Etkinliği

Öz

Salmo munzuricus Munzur Vadisi Milli Parkı boyunca akan akarsularda yayılım gösteren yeni isimlendirilmiş bir alabalık türüdür. Bu çalışmada, anestetik madde olarak kullanılan 2-fenoksietanolün etkinliği Munzur akarsuyundan yakalanan yerli kahverengi alabalık üzerinde değerlendirilmiştir. Balıklar, 100-200 g (W1), 200-400 g (W2) ve 400-600 g (W3) olmak üzere üç ağırlık sınıfına ayrılmıştır ve çalışma 0.2, 0.3, 0.4 ve 0.5 mL L^{-1} olmak üzere 4 farklı anestetik konsantrasyonda yürütülmüştür. En kısa indüksiyon zamanı W3 grubunda, en uzun indüksiyon zamanı W1 grubunda bulunmuştur. En kısa anesteziden uyanma süresi W3 grubunda 73.2±3.83 sn sonra ortaya çıkmış ve en uzun uyanma süresi W1 grubunda 136±31.46 sn olarak tespit edilmiştir. W1 grubundaki 2-fenoksietanol konsantrasyonunun artması ile iyileşme süreleri artış göstermiştir. Bununla birlikte, W2 ve W3 gruplarındaki uyanma sürelerinin, W1 grubunda gözlemlenenden genellikle anlamlı derecede daha uzun olduğu belirlenmiştir. Ayrıca balık büyüklüğünün indüksiyon sürelerini ve uyanma sürelerini etkilediği de tespit edilmiştir. Bu çalışmanın sonucu olarak, 2-fenoksietanol için *Salmo munzuricus* anaçlarında en uygun konsantrasyonlar 100-400 g ağırlık aralığında 0.4 mL L^{-1}, 400-600 g aralığında ise 0.5 mL L^{-1} olarak belirlenmiştir.

Anahtar sözcükler: *Balık anestezisi, Uyanma, Kahverengi alabalık, Salmo munzuricus, Anaç balık, 2-phenoxyethanol, Anestetik, Etkili konsantrasyon, Balık boyutu*

INTRODUCTION

Anesthetics, first applied in the medical field in the 1840 [1] are also widely used in aquatic and terrestrial animals [2-6]. Tricaine methanesulfonate (MS222), benzocaine and 2-phenoxyethanol (2-PE) are the most widely used anesthetics in aquaculture [7-9], with anesthesia usually being induced by immersing the fish in a solution of a given concentration. After MS222, 2-PE is the most commonly used anesthetic in aquaculture. Permissible anesthetic agents are very limited in fishes which are consumed as food, despite the variety of anesthesia applications. Optimum anesthetic

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concentrations can minimize the negative impact and thus reduce stress in fish. Optimum anesthetic concentrations are usually expected to induce anesthesia within 3 min and recovery within 10 min [10-12]. An ideal anesthetic agent should induce anesthesia or recovery rapidly with minimum hyperactivity or stress, should be easy to use and inexpensive, and should be effective at low concentrations with being safe for users and consumers [13,14].

The mass-specific basal metabolic rate -oxygen consumption divided by body mass- decreases with increase in body mass of an animal and thus larger animals have lower oxygen consumption relative to body size than do smaller animals [3,15]. Results of the numerous studies on anesthetic agents revealed that the effective concentrations for anesthesia vary with fish body size and water temperature [3,7] with the smaller fish being more responsive than the larger one [14].

Trouts of the genus Salmo are present in almost all cold streams and rivers of Anatolia where they have a high diversity. The Munzur Stream arises from the Munzur Mountains in the north of Ovacik and joins the Pülümür Stream and flows into the Keban Dam Lake. *Salmo munzuricus,* their identity was not clear up to now, newly described by Davut et al.[16], commonly known as red spotted trout is distinguished from other Anatolian Salmo species by having a large adipose fin in male (almost as large as dorsal or anal fins in older males), with a very narrow white margin, then red submarginal band, then a white stripe or spots, then red again in males. It is an endangered salmonid species with high economic value in Munzur Stream which is an important water supply in Turkey. Developing the breeding program is so important to sustain natural population and introduction of the species to aquaculture and to protect its natural population, as well. Red spotted trout breeding studies are ongoing by catching them from the wild. Because this is a new species there is lack of data referring to anesthesia concentrations which are important topic for future studies. Handling stress can cause bad quality of eggs and also some mortality after stripping in trout. Sedation of fish before manipulating is an important issue in aquaculture studies. The objective of this study is to investigate the effects of 2-PE at different concentrations on broodstock specimens of *Salmo munzuricus* at different body weights.

MATERIAL and METHODS

Fish Holding and Experimental Design

The trout broodstock used in the study were caught from the Munzur stream and stocked in 300 L of stock tanks before the treatments. After 3 days of each fish stocking they were randomly taken from the tanks and transferred immediately into the aquariums of 40 L tap water which contains 2-phenoxyethanol at different concentrations, one by one. Surveys of total lengths and weights were performed as quickly as possible on anaesthetized fish which lost reflex to external stimuli. The study was approved from the Fırat University Animal Experiments Local Ethics Committee (FÜ-HADYEK/2016-35, Desicion Number: 47).

In the experiment, fish were sorted by size into three groups as W1, W2 and W3 of the average weight of 150±70.71 g, 300±141.42 g, 500±141.42 and length of 22.68±0.60 cm, 31.87±0.32 cm and 37.98±0.17 cm of, respectively. Twenty animals were used in each group of weight in the study. The experiments were conducted independently for each group, using a completely randomized design, with four concentrations (0.2, 0.3, 0.4 and 0.5 mL L^{-1}) of 2-PE (with %99 purity, BASF, Germany). Each anesthetic treatment consisted of 5 repetitions (each fish was considered a replicate) in a total of 60 animals. All of the fish were starved for 48 h prior to experiment.

The tap water used in the aquariums was aged for a day before in the large tanks while fixing the water temperature using 100 watt power heater at 15°C with continuous aeration. Then it was transferred to the aquariums just before the anesthesia treatments. Temperature was measured by 0.1°C sensitivity digital thermometer while dissolved oxygen was measured by hand-held oxygen meter (YSI Professional Plus). Firstly, 2-PE was diluted with the water from the aquarium by mixing with glass stripe at calculated portions until it was fully dissolved and then added to the treatment aquarium.

Anesthetic bath was constantly mechanically aerated and the oxygen concentration was maintained at 7.25±2.5 mg L^{-1} during the treatments by this way. pH level was measured 8.05±1.5. After anesthetic assessments, fish from each treatment were placed in a large tank and monitored for 24 h before feeding was resumed. The specified stages of anesthesia were recorded as the second by chronometer [17].

Anesthesia Phases and Fish Behavior Characteristics

The criteria to identify each particular stage are presented in *Table 1*. Determination of induction (sedation = S) and recovery (R) stages modified from Keene et al.[18] .

Statistical Analysis

Analysis of data was carried out using SPSS 15.0. In comparison of the groups, nonparametric Kruskal-Wallis variance analysis was applied. The Mann-Whitney U test was used to compare differences between two independent groups. Statistically significant differences were expressed as $P<0.05$.

RESULTS

Fig. 1, Fig. 2 and *Fig. 3* present the results of induction and recovery of *Salmo munzuricus (Fig. 4)*, exposed to different concentrations of the 2- phenoxyethanol for three different

Table 1. Anesthesia phases and fish behavior characteristics			
Main Stages	**Definite Stages**	**Fish Behavior Characteristics**	**Code**
Induction	*Light sedation*	Equilibrium normal, opercular rate slightly decreased,	*S1*
	Deep sedation	Equilibrium normal, slight decrease in opercular rate no respond to weak external stimulus,	*S2*
	Partial loss of equilibrium	Swimming erratic, opercular movements fast, no respond to strong external stimulus,	*S3*
	Total loss of equilibrium	Total loss of equilibrium, regular opercular movement but slow	*S4*
	Loss of reflex	No reflex, opercular movements irregular and slow	*S5*
Recovery	*Partial recovery of equilibrium*	Partial equilibrium and swimming, opercular movements starting,	*R1*
	Total recovery of equilibrium	Total recovery of equilibrium, swimming erratic starting	*R2*
	Total behavioral recovery	Normal swimming starting	*R3*

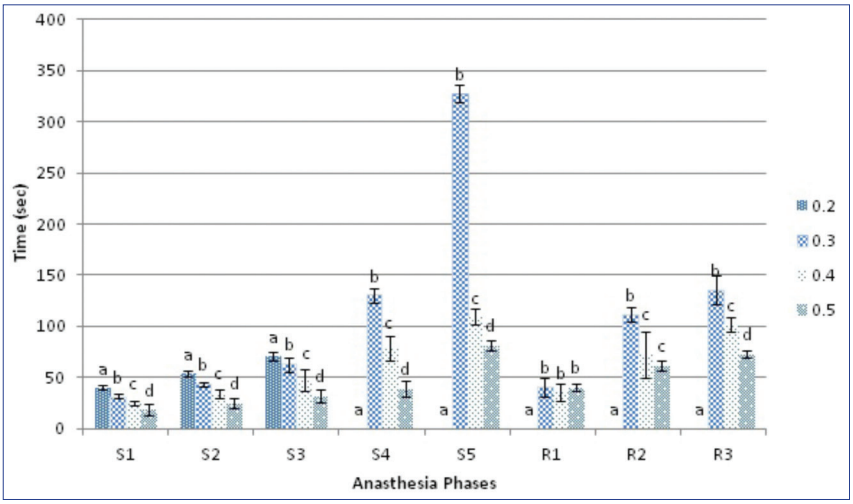
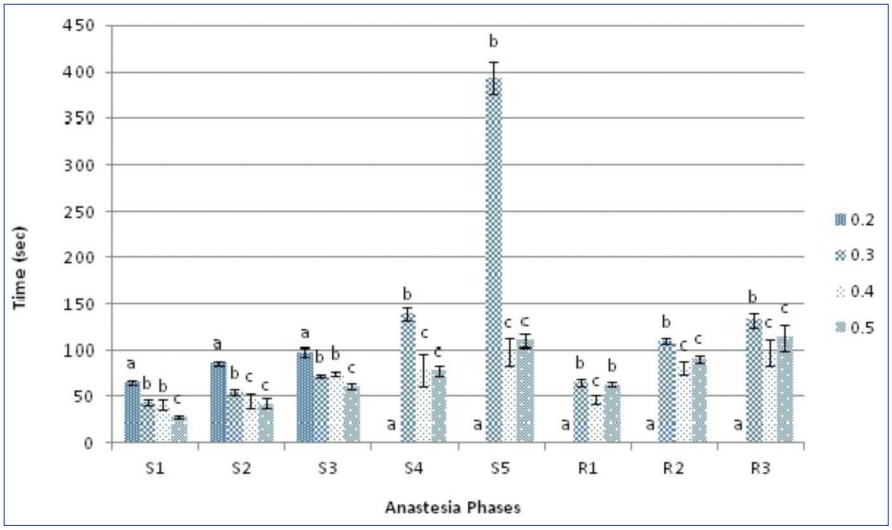


Fig 1. The timing of anesthesia phases at different concentrations of 2-phenoxyethanol for the group of W1 (100-200 g); * Values (mean± SE) with different superscripts within the same phase are significantly different (P<0.05)

Fig 2. The timing of anesthesia phases at different concentrations of 2-phenoxyethanol for the group of W2 (200-400 g); * Values (mean± SE) with different superscripts within the same phase are significantly different (P<0.05)



size classes. No died fish was found during the observation of 24 h post exposure.

The induction times of anesthesia varied with anesthetic concentrations, decreasing with the increase of 2-phenoxyethanol concentrations. On the other hand, the recovery times increased with decreasing of 2- phenoxyethanol concentrations (P<0.05). During the study, at the concentrations of 0.2 mL L^{-1} the total loss of equilibrium was not observed at all groups. Loss of reflex activity (S5) was induced faster at higher concentrations of anesthetic than 0.2 mL L^{-1} at all weights (P<0.05) *(Fig. 1, Fig. 2, Fig. 3)*. Loss of reflex activity (S5) was induced faster at anesthetic concentrations higher than 0.2 mL L^{-1} at all weights. S5 was between 73.00±3.16 sec and 476.80±28.09 sec in W3 and W1 group, respectively. On the other hand, the lowest time value of total behavioral recovery (R3) was determined as 73.2±3.83 sec at the

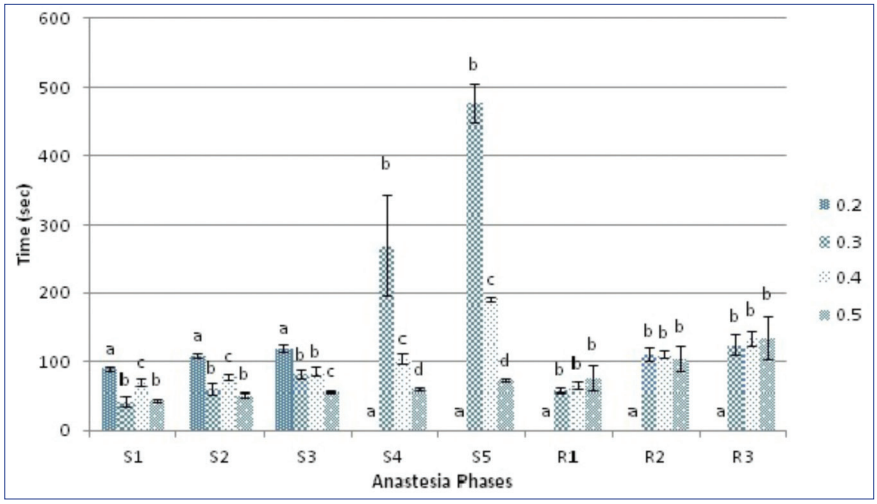


Fig 3. The timing of anesthesia phases at different concentrations of 2-phenoxyethanol for the group of W3 (400-600 g); * Values (mean± SE) with different superscripts within the same phase are significantly different (P<0.05)

Fig 4. *Salmo munzuricus* during the anesthesia treatment

group of W3 and the highest one was 136±31.46 sec at the W1 group.

DISCUSSION

Animal welfare is compromised by stress and has become an increasing concern in the operation of capture, rearing and research operations [19,20]. In aquaculture, sedative and anesthetic agents are very useful for reducing the stress caused by handling, sorting, transportation, artificial reproduction, tagging, administration of vaccines and surgical procedures [7,8,21].

Water quality needs to be carefully controlled during an anesthesia procedure, the main problems involved being those faced by all aquatic animals: control of temperature, dissolved oxygen concentration, ammonia levels and other solids in the baths [22,23].

In this study, the effective anesthetic concentrations of 2-phenoxyethanol for *Salmo munzuricus,* a fish species new to aquaculture, were determined. Effective concentration of 2- phenoxyethanol ranged from about 0.2 to 0.5 mL L^{-1} at different size class. An ideal anesthetic should produce anesthesia rapidly (e.g., less than 3 min), allow a speedy recovery, not be toxic to fish and users, leave low tissue residues, and be inexpensive [24]. 2-phenoxethanol was found to be effective anesthetic for *Salmo munzuricus* broodstock in the present study. However, total loss of equilibrium was not observed despite 10 min of waiting at all groups at 0.2 mL L^{-1} concentration. After this period, the exposure to this concentration was finished assuming that the concentration of phenoxyethanol in the bath was too low. However, the loss of reflex was occurred at all concentrations between 73.00±3.16 and 476.80±28.09 sec in W3 and W1 group, respectively.

Recovery times were observed in the range of 73.2±3.83 and 136±31.46 sec at all concentrations except for 0.2 mL L^{-1}. According to Weyl et al.[4] recovery time positively correlated with concentration of anesthetics in goldfish. In the present study, recovery times were increased with increasing the concentration of 2- phenoxyethanol at the group of W1, as reported in previous studies [4,7,25] although some researchers determined that increasing the concentration did not affect the recovery time [2,26]. However, the group W2 and W3 was different from the W1. However there is actually an insignificant difference between recovery times of W1 and W2. This finding suggests that the fish size may be related to the anesthetic activity. So, the group of W3 was the biggest one. It is because the rate of oxygen consumption, ratio of body volume to gill surface area and rate of gill perfusion are important factors affecting uptake and elimination of anesthetic agents [27], and gill surface area decreases in relation to increased body weight [27,28]. Fish size had an effect on induction times of 2-P phenoxyethanol in other fish [8,17,29], and in the present study, as well.

According to the results of the study with rainbow trout *(Oncorhynchus mykiss)* the authors reported that anaesthetic concentration of 0.3 mL.L^{-1} 2-phenoxyethanol is safe for rainbow trout [30]. Our optimum effective concentrations are higher than theirs although they were lower than the concentration of 0.8 mL.L^{-1} in common carp

(Cyprinus carpio) [28]. This difference may also be due to the effect of anesthetic is species specific since anesthetic absorption through gills can be varied in different species.

Additionally, it was determined that induction and recovery times are related to the anesthetic concentration. However, it was observed that it is difficult to precisely distinguish the anesthesia phases from each other. This leads to confusion among the evaluations of various researchers.

Consequently, our results indicated that 2-phenoxyethanol could be used to anesthetize indigenous brown trout *(Salmo munzuricus)* broodstock, it is important for the future studies of this species, at the concentrations of 0.4 mL L^{-1} at weights of 100-400 g, and 0.5 mL L^{-1} concentrations at weights of 400-600 g, safely.

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Research Article

Determination of the Tumor Virus B Locus in Turkish Native Chicken Breeds [1][2]

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Abstract

Avian leukosis viruses (ALV) are retroviruses that can induce tumors in chicken. Typically, ALV is controlled through husbandry. However, genetic improvement and/or, selective breeding techniques, offer the unique possibility of true resistance. The tumor virus B (TVB) locus transcribes the cellular receptor that mediates an infection through B, D, and E subgroups of avian leukosis virus. Two single nucleotide polymorphisms (SNPs) at nucleotide positions 172 and 184 of the TVB locus account for the three major alleles, TVB*S1, TVB*S3, and TVB*R. The receptor encoded by TVB*R allele prevents viral entry into the cell of ALVB, ALVD, or ALVE subgroups. In this study, both SNPs at the TVB locus of Turkish native chicken breeds were investigated using a PCR-RFLP technique to detect. In both Gerze and Denizli breeds, the TVB*S1 allele was common and TVB*S3 was rare, respectively. The TVB*R allele was rare in the Gerze population and absent in the Denizli population. Allele frequencies of TVB*S1, TVB*S3, and TVB*R were evaluated as 0.96, 0.02, and 0.02 in Gerze chickens and 0.98, 0.02, and 0.00, in Denizli chickens, respectively.

Keywords: *Denizli cocks, Gerze fowls, PCR-RFLP, Tumor virus B locus*

Türkiye Yerli Tavuk Irklarında Tümör Viral B Lokusunun Belirlenmesi

Öz

Avian leukosis virusleri (ALV), tavuklarda tümör oluşturan retrovirüslerdir. Tipik olarak, ALV hayvan yetiştirme yöntemleri ile kontrol edilir. Bununla birlikte, genetik gelişme ve/veya selektif ıslah teknikleri, hastalığa direnç için benzersiz olanaklar sunmaktadır. Tümör viral B (TVB) lokusu ALV üç alt grubunun (B, D ve E) viral girişine ortam sağlayan/engelleyen veya aracı olan gruplara özgü yüzey reseptörlerini kodlar. Bu lokusun 172. ve 184. bazlarındaki iki adet tek nükleotit polimorfizmleri TVB*S1, TVB*S3 ve TVB*R allellerini ayırt edilmesine imkan sağlar. TVB*R'nin kodladığı reseptör ALVB, ALVD veya ALVE alt gruplarının hücreye viral girişini engeller. PCR-RFLP yöntemi kullanılarak Türkiye yerli tavuk ırkları olan Denizli ve Gerze tavuklarındaki TVB genotipleri belirlenmiştir. Hem Gerze hem de Denizli ırkında, TVB*S1 alleli yaygın ve TVB*S3 alleli nadir olarak görülmüştür. TVB*R alleli, Gerze popülasyonunda seyrek olarak görülürken Denizli horozu popülasyonlarında tespit edilememiştir. TVB*S1, TVB*S3 ve TVB*R'nin allel frekansları sırasıyla Gerze tavuklarında 0.96, 0.02 ve 0.02, Denizli horozlarında sırasıyla 0.98, 0.02 ve 0.00 olarak hesaplanmıştır.

Anahtar sözcükler: *Denizli horozu, Gerze tavuğu, PCR-RFLP, Tümör viral B lokus*

INTRODUCTION

Avian Leukosis Viruses (ALVs) affect poultry production and cause economic losses through increased tumor mortality and reduced productivity. ALV is a retrovirus and is classified into six major viral subgroups based on virus and cell receptor interaction patterns. One ALV subgroup (subgroup E, or ALVE) is endogenous and encoded by genes within the chicken genome, whereas all other variations, ALVA, B, C, D, and J are exogenous [1]. Three autosomal tumor viral (TV) loci determine subgroup-specific surface receptors on host cells that either mediate

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or block the entry of all ALV viral subgroups. The TV-A, TV-C, and TV-J loci encode receptors for the ALVA, ALVC, and ALVJ subgroups, respectively [2]. The most complex locus is TV-B, or TVB, which encodes receptors for the ALVB, ALVD, and ALVE subgroups. TVB encodes a tumor necrosis factor receptor (TNFR)-related death receptor, and its three alleles determine which ALV subgroups can infect expressing cells [3]. Two SNPs at nucleotide positions 172 (C/T) and 184 (T/A) of the TVB gene cDNA sequence (GenBank accession number AF507016.1) diversify the allelic transcripts and produce TVB*S1, TVB*S3, and TVB*R. A host cell receptor encoded by the TVB*S1 allele mediates viral entry of ALVB, ALVD, and ALVE subgroups. The TVB*S3 allele encodes receptors promoting viral infection with both ALVB and ALVD, but not ALVE. The TVB*R allele, termed a resistant allele, transcribes an abnormal and truncated receptor that does not allow ALVB, ALVD, or ALVE subgroups to enter the cell and cause infection. The TVB*R allele is recessive to TVB*S1 and TVB*S3, and the TVB*S3 allele is recessive to TVB*S1 [4].

Using TVB sequence polymorphisms, PCR-RFLP [4] and pyrosequencing methods [5] were developed and validated to distinguish TVB genotypes. Additionally, a new single nucleotide mutation at TVB cDNA position 184 (G→T) of the TVB gene was identified using direct DNA sequencing methods in Chinese native chicken breeds [6].

The Denizli and Gerze are the only two Turkish native chicken breeds. They are named after the cities from which they originate and are conserved as genetic resources. Denizli cocks are well known for their long crowing and Denizli and Gerze breeds are reared for hobby purposes [7]. To the best of our knowledge, there are some published study about genetic diversity of Turkish native chicken populations, Denizli and Gerze, at molecular level but there are no reports about ALV infection events or the TV locus status of Turkish native chicken populations [7-9]. The aim of this study was to determine the genotype of the TVB locus in Turkish native chicken breeds using the PCR RFLP method.

MATERIAL and METHODS

All experimental techniques, including animal handling and sample collection, were approved by the Medical and Surgical Experimental Research Center Committee (TICAM) of Eskisehir Osmangazi University with the Decision No: 2014/419-1. The study was carried out in the Molecular Genetics Laboratory of the Agricultural Biotechnology Department at Osmangazi University, Eskisehir, Turkey.

Animals

In this study, a total of 175 chickens were genotyped from original populations of two Turkish native chicken breeds (Denizli, n = 148; and Gerze, n = 27).

DNA Extraction and Genotyping

Genomic DNA was purified from whole blood samples using a phenol-chloroform method and stored at -20°C until analysis. TVB locus genotypes were identified using the PCR-RFLP method described by Zhang et al.[4]. PCR was performed to amplify the polymorphic TVB locus regions. Each 25 µL PCR reaction mixture contained: 50-100 ng DNA template, 10 X *Taq* polymerase buffer, 1.5 mM $MgCl_2$, 2.5 mM dNTPs, 0.5 U *Taq* DNA polymerase, and 5 pmol of each primer. Reaction mixtures (15 µL) containing 10 µl of the PCR product and 5 U of restriction enzyme (RE) were incubated for 6 h at the appropriate temperature *(Table 1)*. The resulting restriction fragments were separated by agarose gel electrophoresis and stained using RedSafe to determine the corresponding genotypes. All oligonucleotides, PCR profiles, PCR amplicon lengths, and REs used for digestion of PCR amplicons are shown in *Table 1*. TVB locus allele frequencies were calculated using the PopGene version 1.31 computer software package [10].

Table 1. *The oligonucleotides, PCR profile and product lengths, and restriction endonuclease (RE) for digestion of PCR products [4]*

Mutation	Primers	PCR				RE and Incubation Temperature
		Profile			Product Size bp	
TVB202 172. bp	F: 5′ GGT AAG GCA GTC ACAAGC ATC ACT C 3′ R: 5′TAC TCG TCT TTC TTA CAT GGG AGG CTC T 3′	94°C	05 min	30 cycle	202	*XbaI* 65°C
		94°C	60 sec			
		56°C	60 sec			
		72°C	45 sec			
		72°C	05 min			
TVB303 184. bp	F: 5′ ACC CCT TCT TGC AGG CAC CTA TGA 3′ R: 5′ -GGA TGC TGT GCT GCG TGG AGA 3′	94°C	03 min	30 cycle	303	*NlaIII* 65°C
		94°C	60 sec			
		60°C	60 sec			
		72°C	60 sec			
		72°C	05 min			

RESULTS

In this study, we used PCR-RFLP to examine the TVB genotypes of the only two Turkish native chicken breeds, Denizli and Gerze. The TVB PCR-RFLP assay comprises two different PCR reactions (TVB 202 and TVB 303) followed by two independent endonuclease reactions and electrophoresis. We successfully amplified DNA fragments of 202 and 303 bp from partial TVB genomic sequences *(Table 1)*. After PCR amplicons were digested with *XbaI* and *NlaIII*, allelic haplotypes were detected based on electrophoretic patterns reflecting the presence or absence of the SNP at positions 172 or 184 in TVB202 or TVB303, respectively. Nucleotide substitutions in the SNPs at TVB locus positions 172 *(Fig. 1a)* and 184 *(Fig. 1b)* were examined in all chickens based on the criteria established by Zhang et al.[4].

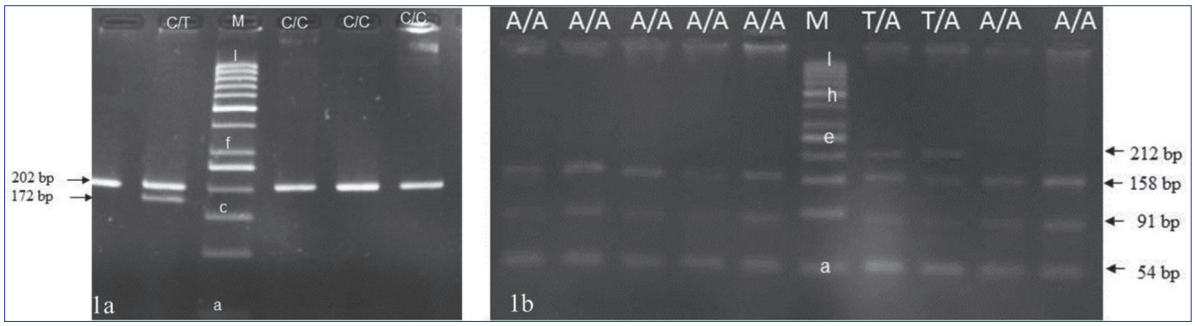

Fig 1. a- The gel-image of 202 bp PCR products after treatment with *XbaI* restriction enzyme and **b-** 303 bp PCR products after treatment with *NlaIII* restriction enzyme (M, 50 bp Fermentas® GeneRuler DNA ladder, a:50 bp, c: 150 bp, e:250 bp, f:300 bp h:500 bp and l:1000 bp)

Among the 175 chickens investigated, only one Gerze breed chicken, and no Denizli breed chickens, had the TVB*R allele. Gerze chickens are conserved in the city of Gerze, where they originated, and the entire population of this flock was genotyped (n = 27). The common allele at the TVB locus in both chicken populations was the TVB*S1 allele. The TVB*S3 allele was rare in both Turkish native chicken breeds and the TVB*R allele was not detected in Denizli chicken populations. Moreover, among the 141 birds of the Denizli population, five cocks of the Pekmez Kefi variety and only two hens were found to have the TVB*S1/S3 genotype, whereas all others were homozygous for the TVB*S1 allele. In Gerze breed hens, only one of 27 chickens had the TVB*S3/R genotype. The frequency of TVB*S1, TVB*S3, and TVB*R alleles were evaluated as 0.96, 0.02, and 0.02 in Gerze and 0.98, 0.02, and 0.00 in Denizli chickens, respectively.

DISCUSSION

For the first time, TVB locus genetic polymorphisms were determined and allele frequencies calculated for Turkish native chicken breeds. Molecular information revealing whether the Turkish native chicken breeds examined are or are not genetically resistant to any of ALVB, ALVD, and ALVE infections will aid conservation efforts.

In addition to PCR-RFLP techniques [11], other studies have used sequencing [6] and pyrosequencing methods [5] to examine the TVB locus polymorphism. Our findings are consistent with those of studies performed in other countries.

A study conducted in India, using the PCR-RFLP technique, detected TVB*S1 alleles in the White Leghorn (WL) line and in native Kadaknath hens, whereas the TVB*R allele was not detected in either breed. Furthermore, Kadaknath chickens were clearly homozygous for the TVB*S1 allele [11].

Zhang et al.[5] researched the genetic diversity at the TVB locus in 36 broilers and 16 laying chicken lines in the USA using the pyrosequencing method. They found that, in broiler lines, the frequency of TVB*S1/S1, TVB*S1/R, and TVB*R/R genotypes were 83%, 14% and 3%, respectively. On the other hand, the frequency of TVB*S1/S1 and TVB*R/R genotypes in laying lines was 44% and 15%, respectively.

Using the pyrosequencing method, a Chinese study of 258 chickens, consisting of two domestic chicken breeds and two WL populations, found the frequency of the TVB*R/R genotype in one of the WL populations to be 0.53, and one of the native breeds was found to be homozygous for TVB*S1/S1. Among domestic breeds, only one chicken was found to have the TVB*S1/R genotype [12]. Another study, conducted with a total of 1428 chickens from ten domestic Chinese breeds and 15 commercial broiler flocks, the frequencies of TVB*S1, TVB*S3, and TVB*R alleles varied between 0.71 and 0.91, 0.00 and 0.09, and 0.04 and 0.29, respectively. Eleven of the 25 breeds were homozygous for the TVB*S1 allele and five breeds of those had the TVB*R/R resistant genotype at varying frequencies (ranging from 0.03 to 0.15) [13].

Yu et al.[6] used the sequencing method to examine the genetic variation at the TVB locus in a total of 459 chickens from nine domestic and WL breeds in China. They detected the TVB*R allele in only two of the nine breeds. The TVB*R

allele frequency was determined to be 0.44 in WL and 0.11 in Tibetan chicken breeds and the common allele was TVB*S1. Additionally, a new G→T mutation was detected at the TVB locus and called TVB*S'. Furthermore, the TVB*S1/S' genotype was observed in other native Chinese breeds with the exception of Tibetan chicken breeds [6].

In general, our results presented here are in agreement with those of similar studies around the world. Taken together, these studies show that the TVB*S1 allele is the common allele and that the TVB*R resistance allele is seldom observed in native chickens. Consistent with the results of analyses in Chinese [6,12,13] and Indian [11] domestic chicken breeds, the TVB*R allele was rarely observed in Turkish native breeds. These results are parallel to the observation that the TVB*R allele frequency in native chicken breeds is lower than that observed in WL populations [6].

In this study, all animals of Gerze breed were genotyped in Gerze city. The existing number of animals in the Gerze population is very low, resulting in a restricted study population size. Yang et al.[12] reported that the frequency of the TVB*R allele might be higher given a larger sample size. Here, the TVB*R allele was rarely detected in the Gerze breed, yet it is possible that chickens genetically resistant to ALV would have been identified with a larger sample size. While there are no vaccines for ALV diseases, genetic resistance is very important to protect chickens [14]. Additionally, Liao et al.[13] noted that the TVB*R allele could be used to improve laying performance and reduce lymphoid leukosis in chickens. Therefore, selection of chickens with TVB*R alleles could improve lifespan as well as egg and meat production in native breeds.

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Research Article

Antibiotic Resistance Gene Profiles of *Staphylococcus aureus* Isolated From Foods of Animal Origin [1]

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Abstract

In this study, the investigation of the antibiotic resistance gene profiles of *Staphylococcus aureus* isolates from foods of animal origin was aimed. Totally, 95 *S. aureus* strains, obtained during a period between 2009 and 2012, from culture collection of the Food Hygiene and Technology Laboratory, were examined. The isolates were confirmed by phenotypic tests and PCR. The antibiotic susceptibilities of the isolates were analyzed by disc diffusion method and the minimal inhibition concentrations of the antibiotics were determined by E test. PCR were also utilized for determining the presence of resistance genes including *bla*Z, *erm*A, *erm*C, *tet*K, *tet*M, *mec*A, *Van*A, *Van*B, *Vat*A, *Vat*B and *aacA-aphD*. Resistance to penicillin, tetracycline, vancomycin, erythromycin, cefoxitin, gentamycin and quinupristin-dalfopristin were evident as 81.1%, 28.4%, 18.9%, 17.9%, 9.4%, 9.4% and 3.2% respectively. E test results were compatible with the disc diffusion method. Multidrug resistance was observed from 29.5% of *S. aureus* isolates. Positive compatibility was observed between conventional methods and PCR for the resistance of the isolates, except for vancomycin. In addition, all of the tested isolates found to include a resistance gene for at least one antibiotic. In conclusion, more efficient interventions must be followed to control the redundant use of antibiotics in veterinary practice. Furthermore, appropriate control measures are needed to be implemented to reduce contamination and the spread of multiresistant *S. aureus* strains.

Keywords: *Antibiotic resistance, Animal origin foods, Multidrug resistance, Resistance genes, Staphylococcus aureus*

Hayvansal Gıdalardan İzole Edilen *Staphylococcus aureus*'ların Antibiyotik Dirençlilik Gen Profilleri

Öz

Bu çalışmada hayvansal gıdalardan izole edilen *Staphylococcus aureus* izolatlarının antibiyotik dirençlilik ve ilgili gen profillerinin araştırılması amaçlanmıştır. Besin Hijyeni ve Teknolojisi laboravuarımızda yer alan kültür koleksiyonunda, 2009 ve 2012 yılları arasında toplanan 95 *S. aureus* izolatları, fenotipik testler ve PCR ile doğrulanmıştır. İzolatların antibiyotiklere duyarlılıkları disk difüzyon testi ile, minimal inhibitor konsantrasyonları ise E test ile incelenmiştir. Ayrıca çalışmada PCR, *bla*Z, *erm*A, *erm*C, *tet*K, *tet*M, *mec*A, *Van*A, *Van*B, *Vat*A, *Vat*B ve *aacA-aphD* genlerinin varlığını tespit etmek amacıyla kullanılmıştır. Testler sonunda penisilin, tetrasiklin, vankomisin, eritromisin, sefoksitin, gentamisin ve quinupristin-dalfopristin antibiyotiklerine karşı direnç oranları sırasıyla %81.1, %28.4, %18.9, %17.9, %9.4, %9.4 ve %3.2 olarak bulunmuştur. E test sonuçları ile disk difüzyon bulguları birbiri ile uyumlu bulunmuştur. *S. aureus* izolatlarının çoklu ilaç direnci %29.5 bulunmuştur. Vankomisin dışında izolatların antibiyotiklere dirençlilikleri hususunda PCR ile konvansiyonel metotlar arasında uyum tespit edilmiştir. Ayrıca test edilen tüm izolatların en az bir antibiyotik için gen bulundurduğu gözlenmiştir. Sonuç olarak, veteriner pratikte gereksiz antibiyotik kullanımını kontrol etmek için daha etkin uygulamalar izlenmelidir ve çoklu ilaç direncine sahip *S. aureus* izolatlarının yayılımını ve bulaşını azaltmak için uygun kontrol önlemlerinin uygulanması gerekmektedir.

Anahtar sözcükler: *Antibiyotik direnci, Çoklu ilaç direnci, Direnç genleri, Hayvansal gıdalar, Staphylococcus aureus*

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INTRODUCTION

Staphylococcus aureus is a pathogen which is usually incriminated for a various kind of diseases ranging from skin infections to serious diseases, such as cellulitis, endocarditis and bacteremia [1-3]. Being commonly found on the skin and mucosae of food producing animal reservoirs, *S. aureus* is one of the most important worldwide food poisoning agents [1].

S. aureus developed resistance shortly after the introduction of penicillin in 1940s, followed by methicillin resistance in 1961. Hitherto, numbers of publication have been reported concerning the resistance of *S. aureus* isolates to diverse spectrum of antibiotics [3,4]. *S. aureus* is causing a concern due to its ability to become resistant to antibiotics via acquired by horizontal transfer of genes and chromosomal mutation [5]. Moreover, enzymatic drug modifications, changes in the target sites and membrane bound efflux pumps are additional mechanisms for the bacteria to combat against antimicrobial agents [6].

Multidrug-resistant (MDR) *S. aureus* isolates are of great public concern as it is possible that these resistant organisms can be transferred to humans via the food chain which in turn leads a limited choice for their control [7]. MDR *S. aureus* strains have frequently been reported from meat, dairy, fishery, poultry, eggs and salads [8-13].

The aim of this study was to characterize the recovered *S. aureus* isolates obtained from various foods of animal origin for their antimicrobial resistance by conventional and molecular methods.

MATERIAL and METHODS

Bacterial Strains

A total of 95 *S. aureus* strains, collected during a period between 2009 and 2012, were obtained from culture collection of the Food Hygiene and Technology Laboratory, Faculty of Veterinary Medicine, Erciyes University, Kayseri, Turkey. These isolates were recovered from raw milk (n=12), sheep cheese (n=12), dairy dessert (n=11), chicken meat (n=12), pastrami (n=12), sausage (n=12), salami (n=12) and soudjouk (n=12). All isolates were confirmed as *S. aureus* by Gram staining, catalase activity, tube coagulase test and *nuc* gene amplification [14,15].

Antibiotic Susceptibility Test

In this study, antibiotic susceptibility testing was performed by disc diffusion method. The antibiotics investigated were (Oxoid, UK) gentamycin (CN, 10 µg), erythromycin (E, 15 µg), tetracycline (TE, 30 µg), penicillin G (P, 10 IU), cefoxitin (FOX, 30 µg), vancomycin (VA, 30 µg) and quinupristin-dalfopristin (QD, 15 µg). The minimal inhibition concentrations (MICs) of all above mentioned antibiotics for each isolates were determined by the E test (Oxoid, UK; Biomerieux, France and Liofil chem, Italy). The disc diffusion test results and MICs were interpreted using the criteria published by Clinical and Laboratory Standards Institute [16]. *Escherichia coli* ATCC 25922, *S. aureus* ATCC 29213 (for microdilution method) and *S. aureus* ATCC 25923 (for disc difusion method) were included as quality control strains in each run. The multidrug resistance was reported whether the single isolate is resistant to three or more unique antimicrobial classes.

DNA Extraction

Total genomic DNA was extracted from overnight-grown at 35°C *S. aureus* cultures in Brain Heart Infusion Broth (Acumedia, 7116A, USA) with the Genomic DNA Purification Kit (InstaGene™ Matrix, BIO-RAD, USA) as specified by the manufacturer.

Amplification of Nuc Gene

PCR assay conditions were used according to Cremonesi et al.[14] and carried out in a reaction mixture of 50 µL final volume containing, 5 µL of template DNA, 5 mL of 10x PCR buffer (Vivantis, Chino, CA), 2 U Taq polymerase (Vivantis), 2 mM dNTP mix (Vivantis), 1.5 mM $MgCl_2$ (Vivantis) and 30 mM of the primer pairs of each primer (NUC-F166 and NUC-R565). PCR conditions were: 5 min at 94°C for initial denaturation, 30 cycles of 1 min at 94°C, 1 min at 56°C for annealing and 1 min at 68°C for extension. The final extension was achieved 7 min at 72°C (Techne TC-512, Keison Products, Chelmsford, UK).

Detection of Selected Resistance Genes by PCR

The *bla*Z, *mec*A, *aac*A-*aph*D, *erm*A, *erm*C, *van*A, *van*B, *tet*K, *tet*M, *vat*A and *vat*B specific primer pairs were used for the amplifications of antibiotic resistance genes *(Table 1)*. PCR amplifications were performed with 50 µL PCR reaction mixture containing 5 µL of template DNA, 1x PCR Buffer (200 mM TrisHCl (pH 8.4), 500 mM KCl), 10 pmol of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$ and 2.5 U Taq polymerase. The thermal cycling protocol for PCR was comprised initial denaturation at 94°C for 3 min, followed by 30 cycles of amplification with 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s with a final extension of 4 min at 72°C (Techne TC-512, UK) for *aac*A-*aph*D, *erm*A, *erm*C, *tet*K, *tet*M, *vat*A and *vat*B genes [17].

For *bla*Z gene, an initial step of 5 min at 94°C was followed by 35 cycles of 1 min at 94°C, 1 min at 54°C and 1 min at 72°C, and a final step at 72°C for 10 min [18]. For the amplification of *mecA* gene, PCR reaction included an initial step at 94°C for 5 min followed by 35 cycles of 92°C for 2 min, 55°C for 2 min, and 72°C for 1 min with a final extension step at 72°C for 7 min [19]. For *vanA* and *vanB* genes an initial denaturation step at 94°C for 2 min; followed by 30 cycles of 94°C for 1, 54°C for 1 min

Table 1. *The oligonucleotide sequence and predicted sizes used in the PCR*

Primer	Target Gen	Name	Sequence (5'-3')	Product Size (bp)	Anneling Temperature	Reference
Nuc-F166	*nuc*		AGT TCA GCA AAT GCA TCA CA	400	56°C	[14]
Nuc-R565			TAG CCA AGC CTT GAC GAA CT			
aacA-aphD-1	*aac*A-*aph*D	Gentamycin	TAA TCC AAG AGC AAT AAG GGC	227	55°C	[17]
aacA-aphD-2			GCC ACA CTA TCA TAA CCA CTA			
tetK-1	*tet*K	Tetracycline	GTA GCG ACA ATA GGT AAT AGT	360	55°C	[17]
tetK-2			GTA GTG ACA ATA AAC CTC CTA			
tetM-1	*tet*M		AGT GGA GCG ATT ACA GAA	158	55°C	
tetM-2			CAT ATG TCC TGG CGT GTC TA			
vatA-1	*vat*A	Quinupristin -Dalfopristin	TGG TCC CGG AAC AAC ATT TAT	268	55°C	[17]
vatA-2			TCC ACC GAC AAT AGA ATA GGG			
vatB-1	*vat*B		GCT GCG AAT TCA GTT GTT ACA	136	55°C	
vatB-2			CTG ACC AAT CCC ACC ATT TTA			
blaZ-1	*bla*Z	Penicillin G	TTA AAG TCT TAC CGA AAG CAG	377	54°C	[18]
blaZ-2			TAA GAG ATT TGC CTA TGC TT			
ermA-1	*erm*A	Erythromycin	AAG CGG TAA ACC CCT CTG A	190	55°C	[17]
ermA-2			TTC GCA AAT CCC TTC TCA AC			
ermC-1	*erm*C		AAT CGT CAA TTC CTG CAT GT	299	55°C	
ermC-2			TAA TCG TGG AAT ACG GGT TTG			
mecA-1	*mec*A	Cefoxitin	ACTGCTATCCACCCTCAAA	163	55°C	[19]
mecA-2			CTGGTGAAGTTGTAATCTGG			
vanA-1	*van*A	Vancomycin	GTAGGCTGCGATATTCAAAGC	231	54°C	[20]
vanA-2			CGATTCAATTGCGTAGTCCAA			
vanB-1	*van*B		GTAGGCTGCGATATTCAAAGC	330	54°C	
vanB-2			GCCGACAATCAAATCATCCTC			

and 72°C for 1 min and final extension at 72°C for10 min were done [20]. Amplification products were visualized by electrophoresis in 1.5% agarose gel (100 V for 40 min, EC250-90, Thermo, USA).

RESULTS

In the antibiotic susceptibility test, resistance to penicillin G (P), tetracycline (TE), vancomycin (VA) and erythromycin (E) was evident in 81.1%, 28.4%, 18.9% and 17.9% of the isolates used in this study, respectively. A small percentage of isolates demonstrated resistance to gentamycin (CN), cefoxitin (FOX) and quinupristin-dalfopristin (QD) with the rate of 9.4%, 9.4% and 3.2% respectively *(Table 2)*. The results of E test were compatible with that of disc diffusion method. In the E test, the ranges of MIC values were; gentamycin 0.06-256 µg/mL, erythromycin 0.03-128 µg/mL, cefoxitin 0.5-128 µg/mL, vancomycin 0.03-256 µg/mL, tetracycline 0.03-256 µg/mL, penicillin G 0.015-16 µg/mL and quinupristin-dalfopristin 0.008-32 µg/mL.

Multidrug Resistance

In this study, 28 (29.5%) of *S. aureus* isolates were resistant to three or more antibacterial classes. The majority of MDR *S. aureus* isolates (22.1%) were found resistant to three antimicrobials whereas resistances to four and six antimicrobials were observed from 5.2% and 2.1% of the isolates respectively *(Table 3)*.

Relationship Between Antibiotic Susceptibility Testing and PCRs

In this study, the antibiotic susceptibility test results and the presence of antibiotic resistance genes of 95 *S. aureus* isolates were compared *(Table 4)*. All nine gentamycin and cefoxitin resistant isolates (100%) were carried *aac*A-*aph*D and *mec*A genes, respectively. Nine (53%) and eight (47%) of 17 erythromycin resistant isolates were shown to have *erm*A and *erm*C genes, respectively. Seventy of 77 (91%) penicillin G resistant isolates carried *bla*Z gene. Although none of the isolates were found having *van*A gene, two of 18 vancomycin resistant isolates were carried *van*B gene. Twelve (44%) of 27 tetracycline resistant isolates had only *tet*K gene, 10 (37%) had only *tet*M gene while five (19%) had both *tet*K and *tet*M genes. All of three quinupristin-dalfopristin resistant isolates were found to harbour *vat*B gene *(Table 4)*.

Table 2. *Antibiotic resistance profiles of S. aureus isolates*

Antibiotics	S		I		R		S (%)		I (%)		R (%)	
	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC
Gentamycin	≥15	≤4	13-14	8	≤12	≥16	79 (83.1)	79 (83.1)	7 (7.4)	7 (7.4)	9 (9.4)	9 (9.4)
Erythromycin	≥23	≤0.5	14-22	1-4	≤13	≥8	32 (33.7)	32 (33.7)	46 (48.4)	46 (48.4)	17 (17.9)	17 (17.9)
Penicillin G	≥29	≤0.12	-	-	≤28	≥0.25	18 (18.9)	18 (18.9)	-	-	77 (81.1)	77 (81.1)
Cefoxitin	≥22	≤4	-	-	≤21	≥8	86 (90.5)	86 (90.5)	-	-	9 (9.4)	9 (9.4)
Vancomycin	≥15	≤2	-	4-8	-	≥16	73 (76.8)	73 (76.8)	4 (4.2)	4 (4.2)	18 (18.9)	18 (18.9)
Tetracycline	≥19	≤4	15-18	8	≤14	≥16	68 (71.6)	53 (55.8)	-	15 (15.8)	27 (28.4)	27 (28.4)
Quinupristin-Dalfopristin	≥19	≤1	16-18	2	≤15	≥4	82 (86.3)	82 (86.3)	10 (10.5)	10 (10.5)	3 (3.2)	3 (3.2)

S: Susceptile; I: Intermediate; R: Resistance, DD: Disc Diffusion Test

Table 3. *Multidrug resistances of S. aureus isolates*

Number of Resistances	Resistance Patterns	Number of Resistant Isolates
3	CN, P, VA	2
3	CN, E, VA	1
3	E, P, TE	3
3	E, P, VA	2
3	P, VA, TE	8
3	CN, E, P	1
3	P, VA, FOX	4
4	CN, E, P, TE	2
4	CN, P, VA, TE	1
4	CN, P, VA, FOX	1
4	P, VA, FOX, QD	1
6	CN, E, P, FOX, VA, QD	1
6	E, P, VA, FOX, TE, QD	1

CN: Gentamicin; E: Erythromicin, P: Penicillin; OX: Oxacillin; VA: Vancomycin; TE: Tetracycline, FOX: Cefoxitin; QD: Quinupristin-Dalfopristin

Table 4. *Relationship between antibiotic susceptibility testing and PCRs*

Antibiotics	Target Gen	Resistance (%)	Gene Frequency (%)
Gentamycin	*aac*A- *aph*D	9 (9.4)	9/9 (100)
Tetracycline	*tet*K	27 (28.4)	12/27 (44)
	*tet*M		10/27 (37)
	*tet*K + *tet*M		5/27 (19)
Quinupristin-Dalfopristin	*vat*A	3 (3.2)	-
	*vat*B		3/3 (100)
Penicillin G	*bla*Z	77 (81.1)	70/77 (91)
Erythromicin	*erm*A	17 (17.9)	9/17 (53)
	*erm*C		8/17 (47)
Cefoxitin	*mec*A	9(9.4)	9/9 (100)
Vancomycin	*van*A	18 (18.9)	-
	*van*B		2/18 (11)

DISCUSSION

S. aureus is increasingly developing resistance to formerly effective antimicrobial agents [5,21]. Food is an important vehicle for the transfer of resistant *S. aureus* strains from animals to humans and antimicrobial resistant strains are emerging as a global problem. MDR *S. aureus* isolated from different foods exhibited a various distribution throughout the world [22,23].

In this study, remarkable levels (3.2-81.1%) of resistance to antibiotics which were all broad-spectrum, P, VA, TE, FOX, E, CN and QD *(Table 2)* were found. These results are not surprising for the antibiotics mentioned above as they are commonly used in both veterinary and human medicine.

In the present study, the resistance of gentamycin was found to be 9.4%. On the contrary, Groves et al.[24] and Gomes et al.[25] found higher results at rates of 83.4% and 26.8% from human isolates than that of ours. All gentamycin-resistant strains had the *aac*A-*aph*D gene similar to Strommenger et al.[17] (in Germany), Adwan et al.[26] (in Palestine) and Groves et al.[24] (in Australia) from clinical human isolates. In addition, Gomes et al.[25] (in Brazil) and Oksuz et al.[27] (in Turkey) reported that *aac*A-*aph*D gene was present in 43% and 95% of gentamycin-resistant isolates from clinical samples, respectively.

Erythromycin resistance was determined at rate of 17.9%. Moreover, we found nine (53%) *erm*A and eight (47%) *erm*C positive strains out of 17 erythromycin resistant isolates. In contrast, the frequency of erythromycin resistance (40%) was reported relatively high and related genes (*erm*A; 22.8% and *erm*C; 17.1%) was reported relatively low by Zmantar et al.[28] in *S. aureus* strains isolated from auricular infections in Tunisia. Moreover, Gao et al.[29] have also reported that erythromycin-resistant was evaluated as 44% and all erythromycin-resistant isolates to carry *erm*A and *erm*C genes from milk samples. In addition, Adwan et al.[26] stated that the presence of *erm*A and *erm*C genes among MRSA isolates were 30.9% and 74.5% from different clinical samples in Palestine. These inagreements

might be due to the source of isolates and mutation in the genes located in coding or promotor region of the PCR-detected genes or genes in small plasmids, seldomly lost. Moreover, the *erm*C gene encoding for ERM resistance is located on a small plasmid was reported by Fluit et al.[30]. Some reports indicated *erm*A to be more dominant factor in *S. aureus* infections [31,32].

In our study, the rate of penicillin G resistance was detected at 81.1%, the *bla*Z gene was present in 70 (91%) of 77 in resistant strains. However, Goa et al.[29] have reported penicillin resistance was 29%, the *bla*Z gene was present in 81% of them. Moreover, Yang et al.[33] reported that *bla*Z gene was detected in 94.6% of 37 penicillin resistant *S. aureus* strains isolated from bovine mastitis.

Regarding the methicillin-resistance and related gene (*mec*A), was detected in 9.4% and 100% of *S. aureus* isolates in this study, respectively. MRSA strains were detected by Pehlivanoglu and Yardimci [34], by Türütoğlu et al.[35] and by Sareyyupoglu et al.[36] in Turkey, by Kumar et al.[37] in India, by Moon et al.[38] in Korea, by Fessler et al.[39] in German. The rates of having *mec*A gene in milk samples have been reported as 77%,61.9%, 57%,16.7 % and 3.1% by Kumar et al.[37] in India, by Moon et al.[38] in Korea, by Pehlivanoglu and Yardimci [34], by Türütoğlu et al.[35] and by Sareyyupoglu et al.[36] in Turkey. Fessler et al.[39] have also reported that presence of *mec*A gene that has been observed is 37.2% in food and food products of poultry origin in German. The high rate of *mec*A gene obtained in this study, might be due to the horizontal transmission of this gene between the strains found together in food processing environment [40].

In our study, about 18.9% of the isolates were found to be resistant to vancomycin and 11% of the vancomycin resistant isolates harbored *van*B gene. Our results demonstrated that the presence of vancomycin resistance gene *(vanB)* in *S. aureus* strains isolated from food in Kayseri is of utmost importance. Therefore, further studies are necessary to keep the emergence and spread of these isolates. Similarly, Abulreesh [41] detected in 14% of 51 *S. aureus* isolates were resistant to vancomycin. Moreover, Pehlivanoglu and Yardimci [34], Turkyılmaz et al.[42] and McMillan et al.[43] reported that in milk samples, all strains were susceptible to vancomycin by disk diffusion test. Contrary to our study, Simeoni et al.[44] (from swine meat commodities in Italy), Pehlivanoglu and Yardimci [34] (from milk samples in Turkey), McMillan et al.[43] (from raw milk sources in Australia), and Abulreesh [41] (from potable water samples in Saudi Arabia) reported that none of the *S. aureus* isolates were found to be positive for *van*A/*van*B gene by PCR. The reasons for the different results might be explained with changes in biosynthesis of cell wall of the resistant strains [34,45,46].

The rate of quinupristin-dalfopristin resistant isolates were 3.2% and all resistant isolates had *vat*B gene in this study. Contrary, Adwan et al.[26] noted the prevalence of *vat*A was 1.8% and *vat*B gene was not found among MRSA isolates respectively. Fessler et al.[39] reported that from 86 samples originated from food and food products of poultry origin, four MRSA isolates were found to be resistant to quinupristin-dalfopristin, however 11 isolates were detected as intermediate. However, they detected that all isolates tested were negative for *vat*A or *vat*B gene.

In our study, no resistance genes were detected in some resistant isolates, which agreed with the findings of Gao et al.[47]. The phenotypic resistance may be caused by other resistance mechanisms, including biofilm formation, rather than gene acquisition [48]. The resistance mechanisms to antibiotics are so complicated that the presence or absence of a particular resistance gene cannot be regarded as a certain evidence for the isolate to be resistant or sensitive to the related antimicrobial agents [49].

In this study, a positive relation was observed between phenotypic and PCR results for the determination of antimicrobial resistance which is in agreement with Gao et al.[29] and Saadat et al.[50]. However, Zmantar et al.[28] and Salih et al.[51] reported no correlation between phenotypic and PCR methods.

According to MDR, 28 (29.5%) of *S. aureus* isolates were found resistant to three or more antimicrobial agents *(Table 3)*. Attention should be given to the fact that all of the isolates demonstrated resistance to more than one antibiotic. MDR has also been reported by Elbargisy et al.[52], Fan et al.[11], and Waters [53], at the rate of 17.1%, 66.3% and 52%, respectively. These findings might be due to the abundant use of antimicrobials for farm animals especially in countries where antibiotic use is not well regulated [11]. Large presence of MDR *S. aureus* in foods causes high risk of infections and a possible transmission of resistances to other pathogens which could lead to failure of antibiotic treatments.

In conclusion, spreading of MDR *S. aureus* via foodstuffs is a potential hazard for public health and might result in difficulties to treat MDR-related diseases. These results suggest that the incidence of MDR *S. aureus* are steadily increasing and attention needs to be paid to decrease or eliminate the contamination of MDR *S. aureus*. Specified education programs should be supported to define the prudent antibiotic use besides clinical guidelines should be developed and put into practice.

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Research Article

Molecular Typing of *Clostridium perfringens* Toxins (α, β, ε, ι) and Type 'A' Multidrug Resistance Profile in Diarrheic Goats in Pakistan

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Abstract

Clostridium perfringens (*C. perfringens*) causes disease, generally, named as enterotoxemia in the animals. This bacterium is a normal inhabitant in gastro-intestinal tract (GIT) of animals and become harmful by increasing its colony counts as well as toxin liberation whenever gets an opportunity of favorable conditions. This study focused molecular typing of *C. perfringens* (α, β, ε, ι toxins) and type 'A' multidrug resistance profile in diarrheic goats in Pakistan. Diarrheic fecal samples (n=192) were collected from goats and 80.73% (155/192) of the samples were found positive for *C. perfringens* on the basis of culture growth and PCR. Elevated *C. perfringens* counts (>10^7 CFU/g) were recorded in 33.55% (52/155) of positive samples, while, 66.45% (103/155) of the positive sample appeared in normal range of bacterial counts (10^4-10^7 CFU/g). Molecular detection was carried out by targeting specific toxin genes i.e. *cpa* (α), *cpb* (β), *etx* (ε) and *iap* (ι) of *C. perfringens* using PCR. Among the positive samples, 78.06% of the isolates were found as type 'A', 5.16% isolates were type 'B', 3.23% isolates were type 'C' while, 13.55% of the positive samples were type 'D' *C. perfringens*. None of the isolates was found positive for *iap* toxin gene (type 'E'). *C. perfringens* type 'A' was subjected to in-vitro antibiotic sensitivity test. Penicillin, ciprofloxacin and ceftriaxone were found sensitive while bacitracin, amoxicillin and ampicillin were found least sensitive antibiotics. This study concludes that *C. perfringens* type 'A' is highly prevalent among goats in Pakistan and clinical cases of enterotoxemia can be effectively dealt with penicillin, ciprofloxacin and ceftriaxone antibiotics.

Keywords: *Clostridium perfringens, Toxino-typing, Antibiotic sensitivity, Goat*

Pakistan'da İshalli Keçilerde *Clostridium perfringens* Toksinlerinin (α, β, ε, ι) Moleküler Tiplendirilmesi ve Tip 'A' Çoklu İlaç Direnç Profili

Öz

Clostridium perfringens (C. perfringens) hayvanlarda enterotoksemi olarak adlandırılan hastalığına neden olur. Bu bakteri hayvanların gastrointestinal sisteminin normal florasında yer alır ve uygun şartlar oluştuğunda fırsat bularak koloni sayısını artırarak ve aynı zamanda toksinlerini salarak zararlı hale gelir. Bu çalışma Pakistan'da ishalli keçilerde *Clostridium perfringens*'in (α, β, ε, ι) moleküler tiplendirilmesini ve tip 'A' çoklu ilaç direnç profilinin belirlenmesini amaçlamaktadır. İshalli dışkı örnekleri (n=192) keçilerden toplandı ve örneklerin %80.73'ü (155/192) kültürde üreme ve PCR analizleri ile *C. perfringens* pozitif olarak belirlendi. Pozitif örneklerin %33.55'inde (52/155) artmış *C. perfringens* miktarları (>10^7 CFU/g) tespit edildi. Pozitif örneklerin %66.45'inde (103/155) bakteri miktarları normal aralıklarda (10^4-10^7 CFU/g) gözlemlendi. Moleküler tespit amacıyla PCR kullanılarak *C. perfringens*'in *cpa* (α), *cpb* (β), *etx* (ε) ve *iap* (ι) toksin genleri hedef alındı. Pozitif örnekler arasında izolatların %78.06'sı tip 'A', %5.16'sı tip 'B', %3.23'ü tip 'C' ve %13.55'i tip 'D' *C. perfringens* olarak belirlendi. İzolatların hiçbiri iap toksin geni (tip 'E') için pozitif değildi. *C. perfringens* tip 'A'ya *in-vitro* antibiyotik sensitivite testi uygulandı. Bakterilerin penisilin, siprofloksasin ve seftriaksona karşı duyarlı oldukları, basitrasin, amoksisilin ve ampilisine karşı ise en az duyarlı oldukları tespi edildi. Bu çalışma, Pakistan'da *C. perfringens* tip 'A'nın keçiler arasında oldukça yaygın olduğunu ve klinik enterotoksemi vakalarında penisilin, siprofloksasin ve seftriaksonun etkili bir şekilde kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Clostridium perfringens, Toksin tiplendirmesi, Antibiyotik sensitivitesi, Keçi*

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INTRODUCTION

Clostridium perfringens (C. perfringens) is a Gram's positive anaerobe bacterium. It is normally present in animals and humans intestinal contents but sometimes causes infection and proves highly pathogenic regarding intestinal diseases [1,2]. Different toxino-types of *C. perfringens* cause different intestinal pathologic conditions in animals that's why typing of this bacteria have gained importance. *C. perfringens* has 5 major types (A-E) based on the types of toxins it produces. Alpha (α) toxin is produced by all toxino-types of *C. perfringens*; beta (β) toxin is produce by 'B' and 'C' types, epsilon (ε) toxin is produced by 'B' and 'D' type of *C. perfringens,* while, iota (ι) toxin is produced only by type 'E' along with alpha (α) toxin [3,4]. *C. perfringens* can also produce some minor toxins like β2 and enterotoxins but their presence is not associated with the 'typing' of bacteria as these toxins are not linked permanently with some specific types, all the times [5,6]. There is a new toxin named as 'perfingolysin O' (PFO) also identified in the *C. perfringens* that is considered as having potential of causing disease [7]. Enterotoxemia is the name generally used for the disease, caused by all toxino-types of *C. perfringens*. This disease is sometimes named in accordance with pathological conditions or with involvement of some specific strains (toxino-type) of *C. perfringens*. *C. perfringens* type 'A' causes food poisoning and gas gangrene in humans. In a recent report, *C. perfringens* is found with the necrotic pancreatitis in the human beings [8]. Type 'D' is associated with 'pulpy kidney disease' and dysentery in sheep, while, type 'C' causes necrotic enteritis in animals and a condition named as struck [3,4]. *C. perfringens* type 'A' is also involved in the acute enterotoxemia in the goat kids [9]. Epsilon toxin present in the type B and D of *C. perfringens* is mostly involved in the disease pathogenesis. Epsilon toxin in goats causes enterocolitis, but in sheep it is also seen in systemic effects on the brain and lungs. The difference in the pathogenesis in two species is lies in the fact that epsilon toxin modify water and ion transport in the intestines of sheep and goats with different mechanism [10].

In spite of vaccination, some sporadic outbreaks of entero-toxemia are observed over the study area in recent years that are usually not reported. Hence, it is important to know about the types of *C. perfringens* prevailing in the animals that will give assistance for improving vaccines. *C. perfringens* toxino-typing can be done either by detection of toxins itself or its gene. Traditionally, typing has been done by toxin neutralization test in the mice that is difficult, time consuming and having ethical issues. PCR is reliable and convenient method for the typing of *C. perfringens,* as each of the toxins has a specific toxin gene in the genome. Alpha (α) toxin gene *(cpa)* is located on the bacterial chromosome, while, β *(cpb),* β2 *(cpb2)*, ε *(etx)*, and ι *(iap)* toxin genes are considered to be plasmid based. Enterotoxin *(cpe)* gene can be either chromosomal or plasmid borne [4]. Presence of toxin's gene does not give guarantee for the presence of toxin but in 99% of the cases, genotype matches with the phenotype of bacteria [11].

In the recent years, anaerobe bacteria have shown resistance against antimicrobial, globally, even against universally active antibiotics like carbapenems and imidazole [12,13]. In Pakistan, there is irrational practice regarding use of antibiotics in animal and it is important to know about the current resistance profile of *C. perfringens* against commonly available antibiotics in the field conditions. Keeping in view the importance of this disease, this project was designed with the following objectives; assessment of *C. perfringens* load in the fecal samples of diarrheic goats, toxino-typing of *C. perfringens* local isolates and antimicrobial resistance profile of *C. perfringens*.

MATERIAL and METHODS

Study Design

This study was conducted in Sargodha division of Punjab province in Pakistan. Small goat farms and household animals were targeted and a total of n=192 fecal samples were collected from diarrheic goats irrespective of age, sex and breed. Samples were collected from all 04 districts of Sargodha division and from each district, 48 diarrheic animals were selected.

Sample Culturing and Quantification of Bacteria

Collected samples were transported to the Microbiology laboratory maintaining the cold chain. The samples were cultured anaerobically on Tryptose Sulphite Cycloserine (TSC) media (Himedia Labs, Mumbai, India) using AnaeroGen™ Sachets (AN35, Oxoid®, Hampshire, UK) to produce anaerobic conditions. TSC is a selective media for the growth of *C. perfringens.* Serial dilutions of fecal samples were made by taking 1 g of fecal material in the Phosphate Buffer Saline (PBS) and then it was cultured on TSC media plates. After 48 hours of incubation at 37°C typical black colonies suspected for the *C. perfringens* appeared on the media plates. Colonies were enumerated using colony counter and the dilution which produced 30-300 colonies was considered for the CFU count of *C. perfringens*.

PCR for Toxin Genes

After purification of primary culture by sub-culturing on the TSC media, 4-5 black colonies were picked for the DNA extraction using DNA extraction kit TIANGEN® (TIANamp Genomic DNA Kit, Catalogue no. DP302). The extracted DNA quantity and purification were checked using Nano-drop 260/280nm wavelength and DNA was stored at -20°C. PCR was performed for the amplification of 04 toxin genes of *C. perfringens*, α *(cpa)*, β *(cpb)*, ε *(etx)*, and ι *(iap)* using specific primers [14] *(Table 1)*.

Concentration of each forward and reverse primer was 5 pmoles. PCR reaction mixture was consisting of 12.5 µL of

Table 1. *C. perfringens toxin genes with respective primer sequences*

Toxin Gene	Primer	Sequence (5'-3')	Product Size
cpa (α-toxin)	CPAlphaF	GCTAATGTTACTGCCGTTGA	324bp
	CPAlphaR	CCTCTGATACATCGTGTAAG	
cpb (β-toxin)	CPBetaF3	GCGAATATGCTGAATCATCTA	195bp
	CPBetaR3	GCAGGAACATTAGTATATCTTC	
etx (ε-toxin)	CPEpsilonF	TGGGAACTTCGATACAAGCA	376bp
	CPEpsilonR2	AACTGCACTATAATTTCCTTTTCC	
iap (ι-toxin)	CPIotaF2	AATGGTCCTTTAAATAATCC	272bp
	CplotaR	TTAGCAAATGCACTCATATT	

master mix (2X Ampmaster™Aq, GeneAll®), 2 µL of DNA sample, 1 µL of each primer (5 pmol) and 8.5 µL of distilled water. PCR programming on the thermocycler for all the toxin genes was initial denaturation at 94°C (10 min), then 40 cycles of denaturation at 94°C (1 min), annealing at 53°C (45 s), and extension at 72°C (1 min). Final extension was done at 72°C (10 min). PCR products were electrophoresed on ethidium bromide stained 2% agarose gel (Invitrogen®). A 100bp molecular weight marker was used as a ladder for the determination of the sizes of PCR products *(Fig. 1)*.

Antibiotic Sensitivity

In-vitro antibiotic sensitivity was checked for 06 different *C. perfringens* type 'A' isolates using Kirby-Bauer antibiotic sensitivity test method. Ten antibiotics selected for sensitivity test were tetracycline (30 µg), metronidazole (5 µg), penicillin (10U), ampicillin (10 µg), amoxicillin (30 µg), erythromycin (15 µg), vancomycin (30 µg), ciprofloxacin (10U), bacitracin (10 µg), and ceftriaxone (30 µg).

RESULTS

Isolation and Identification of C. perfringens Toxinotypes

This study revealed 80.73% (155/192) fecal samples positive for *C. perfringens,* appeared as typical black colonies on TSC selective media. The normal ranges (10^4-10^7) CFU/g of bacterial count was recorded in 66.45% (103/155) of positive samples, while, the rest of the positive samples 33.54% (52/155) had shown elevated level of CFU/g (>10^7) for the *C. perfringens.* As the *C. perfringens* is normal inhabitant of intestinal tract of animals, 10^4-10^7 CFU/g is considered normal range of bacterial concentration in fecal samples [12]. PCR results showed that all the *C. perfringens* isolates were positive for alpha (α) toxin gene *(cpa)*. Presence of *cpa* gene in the genome of bacteria is confirmatory for the *C. perfringens* presence. None of the isolates was positive for the iota *(ι)* toxin gene *(iap)*. *C. perfringens* type 'A' which contains only *cpa* gene was dominant strain in all the toxinotype and it was found 78.06% (121/155) of all positive samples. As the type 'A' was the most prevalent toxino-type of *C. perfringens* in the samples, it was selected for the antibiotic sensitivity. *C. perfringens* type 'B' containing *cpa, cpb* and *etx* gene was only 5.16% (8/155) of all positive isolates. Type 'C' *C. perfringens* contributed 3.23% (5/155) having *cpa* and *cpb* genes, while, *C. perfringens* type 'D' was found 13.55% (21/155) with *cpa* and *etx* gene combination. None of the isolates was positive for type 'E' *(iap gene)* *(Table 2)*.

Table 2. *Distribution of C. perfringens different toxin genes in goats diarrheic samples*

Distribution *of C. perfringens* Toxin Gene	Number of Animals	Type of *C. perfringens*
cpa (α)	121	A (78.06%)
cpa (α), *cpb* (β), *etx* (ε)	08	B (05.16%)
cpa (α), *cpb* (β)	05	C (03.23%)
cpa (α), *etx* (ε)	21	D (13.55%)
cpa (α), iap (ι)	00	E (00.00%)
Total samples positive for *C. perfringens*	155	
Total negative	037	
Total animal tested	192	

Table 3. *Antibiotic Sensitivity against Type A, C. perfringens*

Antibiotic Discs	No of Isolates	Antibiotic Sensitivity		
		Resistant (%)	Intermediate (%)	Sensitive (%)
Tetracycline (30 µg)	6	0 (00.00)	2 (33.33)	4 (66.67)
Metronidazole (5 µg)	6	0 (00.00)	1 (16.67)	5 (83.33)
Penicillin (10U)	6	0 (00.00)	0 (00.00)	6 (100.0)
Ampicillin (10 µg)	6	4 (66.67)	2 (33.33)	0 (00.00)
Amoxicillin (30 µg)	6	1 (16.67)	5 (83.33)	0 (00.00)
Erythromycin (15 µg)	6	1 (16.67)	3 (50.00)	2 (33.33)
Vancomycin (30 µg)	6	0 (00.00)	3 (50.00)	3 (50.00)
Ciprofloxacin (10U)	6	0 (00.00)	0 (00.00)	6 (100.0)
Bacitracin (10 µg)	6	3 (50.00)	3 (50.00)	0 (00.00)
Ceftriaxone (30 µg)	6	0 (00.00)	0 (00.00)	6 (100.0)

Antibiotic Sensitivity of C. perfringens Type 'A'

Antibiotic sensitivity test was applied on 06 different isolates of *C. perfringens* type 'A'. Ten antibiotics *(Table 3)* were tested by Kirby Bauer antibiotic sensitivity test method against *C. perfringens* type 'A'. Penicillin, ciprofloxacin and ceftriaxone were the most sensitive antibiotic according to the results based on the zone of inhibitions they produced.

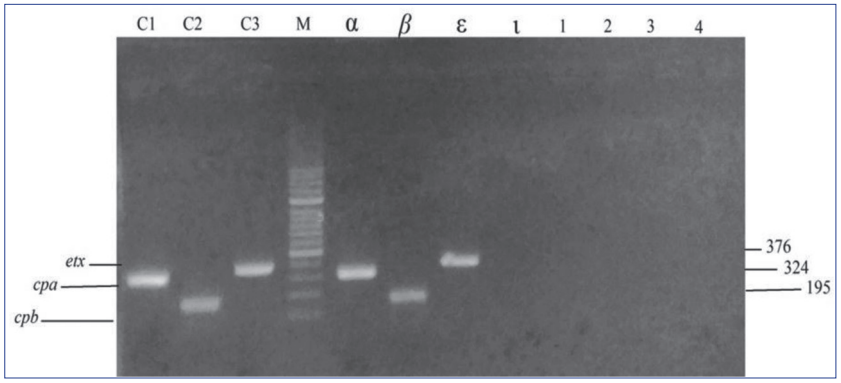

Fig 1. Showing 2% agarose gel PCR products. C1, C2, C3: positive control of *cpa*, *cpb* and *etx* gene of *C. perfringens*. M; (marker 100 bp) DNA Ladder. α, β, and ε are gene products from sample isolated *C. perfringens.1,2,3,4* are the negative control

Ampicillin, amoxicillin and bacitracin were found least sensitive against *C. perfringens* type 'A'.

DISCUSSION

C. perfringens is a normal inhabitant of GIT of animals and 10^4-10^7 CFU/g level in feces is considered as normal range of bacterial count in sheep and goats. In the disease conditions bacterial count elevates $>10^7$ CFU/g [15]. In this study, (n=192) diarrheic goats fecal samples were processed to determine *C. perfringens* bacterial count on TSC selective media. It was found that 80.72% (155/192) of the samples were positive for *C. perfringens.* Out of 155 positive samples, 33.54% (52/155) samples had elevated bacterial count, while, 66.45% (103/155) of the samples had normal range of bacterial load for *C. perfringens*. Kumar *et al.*[16] studied the prevalence of *C. perfringens* in enterotoxemia suspected sheep and found 69.29% of the sheep positive for the *C. perfringens.* In the current study, prevalence was found 80.73% in the diarrheic goats. Vaikosen & Ikhatua [17] found 91 samples positive for the lecithinase enzyme of the *C. perfringens* out of 342 fecal samples of the sheep and goats in Nigeria. Goekce *et al.*[18] declared prevalence of *C. perfringens* 84.61% in a study based on ELISA, while on IAT test bases, it was 58.46% in the sheep. Presence and bacterial count of *C. perfringens* fluctuate in the intestinal contents. Its presence or absence in animal fecal content cannot be declared absolute. In Iran, Ahsania *et al.*[19] found prevalence of *C. perfringens* 2.2% and 54.0% in vaccinated and non-vaccinated local sheep breeds, respectively. A variation pattern in the prevalence of *C. perfringens* is reviewed in different regions of the world. It varies even from herd to herd in the same region. Such variations are obvious from the fact that enterotoxemia is a risk factors oriented disease. Micro and macro environmental changes (determinants) like crowding status, carbohydrate richness in diet, sudden change in diet, deworming status and even season can affect the growth rate of bacteria in the intestine.

Albini *et al.*[20] used simple conventional PCR and Real time multiplex PCR technique for the evaluation of different toxin genes of *C. perfringens* in ten different animal's fecal samples. He found both techniques in agreement for the detection of toxin genes of *C. perfringens.* Aras & Hadimli [21] found 95 samples positive for *C. perfringens* from 300 meat samples of beef, chicken and turkeys. Confirmation was done through detection of alpha toxin gene in all isolates. *C. perfringens* type A, B, C, and D were found 88.33%, 0.0%, 6.4%, and 3.2% in all the meat samples, respectively. In two of the isolates of turkey meat samples type 'E' was also found. These results percentages of *C. perfringens* types come in accordance with the current study in which type 'A' (78.06%), 'B' (5.16%), 'C' (3.23%), 'D' (13.55) and 'E' (0.0%) prevalence was found. Most of the studies showed type 'A' as dominant strain of the *C. perfringens* that was also found in this study. Hashimoto *et al.*[22] studied 804 *C. perfringens* different strains isolated from different sewage water system of humans and animals (chicken, pig, cattle). It was revealed in the study that the *C. perfringens* isolated from human's sewage water had enterotoxin gene, while, animals sewage *C. perfringens* isolates did not have enterotoxin gene. So it was concluded that if the *C. perfringens* enterotoxin gene found positive in aquatic system, it might be considered that it was polluted with human fecal contents. Interestingly, Gkioutzidis *et al.*[23] found 5.13% prevalence of *C. perfringens* type 'A' (*cpa*) in diseased lambs, while, prevalence of type 'B' *(cpa, cpb, etx)*, type 'C' *(cpa, cpb)* and type 'D' *(cpa, etx)* was 46.15%, 20.51%, and 28.20%, respectively (minor toxins detail not present here). In a recent study, the prevalence of *C. perfringens* was studied age wise in the lambs. The prevalence was highest (100%) in the lambs up to 1 month of age, then it decreased to (67%) in the age of two months. Older than this age, the prevalence was found in between 7 to 36%. Even *C. perfringens* was found in one day old lamb fecal sample. Based upon multiplex PCR, *C. perfringens* type A was present in highest ratio. Type C and D were also present but in low prevalence [24].

C. perfringens type A was the predominant strain (78.06%) in all the toxinotypes, so this strain was selected for the *in-vitro* antibiotic sensitivity test. Un-judicial use of antimicrobial on the animals is making the bacteria resistant against commonly available antibiotics. Anaerobes are also getting resistance against antimicrobials. In a hospital study of antimicrobial against clostridial species, penicillin was found 100% sensitive, while, Clindamycin was only

50% sensitive against clostridia isolates [25]. In the current study, penicillin, ciprofloxacin, and ceftriaxone appeared 100% sensitive against *C. perfringens* type 'A' strain, while bacitracin, amoxicillin and ampicillin were the least sensitive antibiotics. It is observed that ampicillin and amoxicillin are commonly being used in the animals in field conditions. Khan *et al*.[26] in Pakistan tested different antibiotics for resistance profile of *C. perfringens* and found amoxicillin resistant against *C. perfringens,* isolated from different meat samples. Bacteria use three fundamental mechanisms for developing resistance against antibiotics. These are (1) enzymatic degradation of antibiotics (2) alteration in the proteins structure which is target for antibiotics (3) changes in the permeability of cell member. These adaptions are directed by specific genes located either on plasmid or chromosomes [27].

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Authors' Contributions

KH, MI, AAA, designed and executed the study, KI, SHF did study sampling, KH, ASA processed the samples, SHF, ASA, AIA arranged and analyzed the statistical data, KH wrote the manuscript. AZD, MI reviewed and approved the manuscript for submission.

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Research Article

Immunohistochemical Studies on Infectious Laryngotracheitis in the Respiratory Tract Lesions in Naturally Infected Laying Hens [1]

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Abstract

In this study, naturally infected by Gallid Herpesvirus type-1 in laying hens to be diagnosed by pathological and PCR methods. Sixty pieces of hens were collected in coops from Central Anatolia region. After necropsy, routine pathological processes were applied to the trachea/larynx, sinuses, lungs and air sacs. All organs were also stained by immunoperoxidase method, and PCR methods were applied to formalin fixed paraffin embedded (FFPE) tissues. Immunohistochemically, the positivities were seen in trachea/larynx (78.3%), sinuses (61.6%), lungs (45%) and air sacs (50%). Positive reactions were observed, in mucous and gland epithelia especially located at intracytoplasmic and rarely intranuclear. PCR positivity was observed in the trachea/larynx in 15 (25%) cases, in infraorbital sinus in 11 (18.3%) cases, in lungs in 8 (13.3%) cases and in air sacs in 6 (10%) cases following the tests performed. Following these results, it is easily concluded that histopathology and immunoperoxidase method can usable for diagnosing of the ILT. However, PCR results made by FFPE tissues showed that this method is not adequate to diagnose the ILT alone.

Keywords: *Histopathology, ILT, Immunohistochemistry, Laying hens, PCR*

Enfeksiyöz Laringotraheitis İle Doğal Enfekte Yumurta Tavuklarında Solunum Kanalı Lezyonları Üzerine İmmunohistokimyasal Çalışmalar

Öz

Bu çalışmada Gallid Herpesvirus tip-1 ile doğal enfekte yumurta tavuklarında patolojik yöntemlerle teşhis konularak immunohistokimyasal ve PCR yöntemlerinin teşhiste kullanılabilirliği araştırıldı. Bu amaçla Orta Anadolu'da bulunan bazı illerdeki kümeslerden toplam 60 adet enfekte tavuk toplandı. Yapılan nekropsilerin sonrasında trake, larinks, infraorbital sinus, akciğer ve hava kesesi parçaları alınarak rutin patolojik işlemler uygulandı. Alınan tüm organlar ayrıca indirekt immunperoksidaz yöntemi ile boyandı ve organlara ait formolle fikse edilmiş parafine gömülü (FFPG) dokulara PZR testi yapıldı. İmmunohistokimyasal boyamalar sonucu trake/larinkste %78.3, sinuslarda %61.6, akciğerlerde %45 ve hava keselerinde %50 oranında pozitiflik gözlendi. Pozitif boyanmalar özellikle mukoza ve bez epitellerinde intrasitoplazmik nadiren de intranüklear olarak gözlendi. Aynı zamanda lümene dökülmüş eksudattaki hücre ve sinsityal dev hücrelerinin sitoplazmalarında da boyanmalar tipikti. Yapılan PCR testlerinin ardından trake/larinkste %25, infraorbital sinuslarda %18.3, akciğerlerde %13.3, hava keselerinde %10 oranında pozitiflik gözlendi. Bu sonuçların ardından hastalığın teşhisinde histopatolojik ve immunperoksidaz yönteminin rahatlıkla kullanılabileceği ortaya konmuştur. Ancak, FFPG dokulardan yapılan PCR yönteminin tek başına İLT'yi teşhis etmek için yeterli olmadığı gösterildi.

Anahtar sözcükler: *Histopatoloji, İLT, İmmunohistokimya, Yumurta tavukları, PCR*

INTRODUCTION

Infectious laryngotracheitis (ILT) is a viral disease characterized by breathing difficulty, wheezing and bloody exudate accumulation especially in the larynx, trachea, and upper respiratory tract in chickens, turkeys and pheasants [1]. The causing agent is a Gallid alphaherpesvirus-1 (GaHV-1) belonging to *Iltovirus* genus in the *Alphaherpesvirinae* subfamily, which is in the *Herpesviridae* family [2]. This disease appeared firstly in the United States in 1925 [3,4].

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Although it is reported that the disease is seen in chickens of all age groups, hatching chickens are more affected [5].

In the pathogenesis of the disease, the virus is primarily affinity to tracheal and larynx epithelium. Also has been emphasized that the virus is replicating in conjunctiva, sinus, air sacs and lungs. It has been reported that virus has high cytolytic activity in these tissues, as well as bleeding and damage to the epithelium [1]. In experimental studies, tracheal tissues and secretions are present on days 6-8 of virus inoculation has been reported [6]. On the other hand, there is no evidence that *Infectious Laryngotracheitis Virus* (ILTV) causes viremia [7]. It has been noted that the virus may be found in trigeminal ganglia, especially in subacute and latent infection period [8].

Histopathologic findings can vary according to the stage of the disease [4]. The first changes are goblet cell loss and inflammatory cell infiltrates in the tracheal mucosa. Findings such as bloating, ciliate loss and oedema can be observed in the epithelial cells. Mononuclear cell infiltrations and syncytial giant cells start to be seen in the days 2 between 3 in the beginning of the infection. In this phase, Cowdry A type intranuclear eosinophilic inclusion body may be found in the desquamated epithelial cells. These inclusion bodies can also be found in syncytial giant cells. It was emphasized that the inclusion bodies were encountered within the first 5 days of the disease and that disappeared with the progression of the sickness [9,10].

Immunohistochemically, Preis et al.[11] reported positive reactions in the cytoplasm of syncytial giant cells and epithelial layer in trachea, larynx, lung, and paranasal sinuses during field studies.

In this study, it was aimed to determine the spread of viral antigens in the respiratory system organs by using immunohistochemical and PCR methods in natural ILT infection in laying hens. In addition, it was aimed to compare macroscopic, histopathologic, immunohistochemical and PCR findings and usability of these methods.

MATERIAL and METHODS

Material

The material of the study comprised 10-90 weeks-old hens that were clinically demonstrating ILT. Hens were collected from the coops that perform egg laying and serologically ILTV positive in certain regions of the cities of Aksaray, Afyonkarahisar and Konya in Turkey. All hens were collected from September to December 2014. A total of 60 animals were used from ten different commercial hen coops. All layers were Leghorn strain. The necropsy of the hens were performed and samples were taken for histopathologic and immunohistochemical examinations. After the pathologic examinations, PCR method applied to samples which taken from paraffin blocks of each case.

Histopathological Method

Following necropsy, respiratory tract organs (Infra-orbital sinuses, trachea, larynx, lungs and air sacs) were examined grossly and fixed in 10% formalin solution. After the fixation, the tissues were subjected to alcohol, xylene and paraffin blocks, respectively. Subsequently, they were cut at 5 µm thickness of sections by microtome, glued to slides and examined under light microscope (Olympus BX51, Tokyo, Japan), after stained with Haematoxylin-Eosin (HE) [12].

The changes observed in larynx/trachea of HE-stained sections were scored as those of Guy et al.[13]. According to these, histopathologic changes were evaluated as normal (0), minimal (+1), mild (+2), moderate (+3), severe (+4) and very severe changes (+5).

Immunohistochemical Method

For immunohistochemistry, Totally 60 formalin fixed paraffin embedded (FFPE) the Infra orbital sinus, trachea, larynx, lung and air sac tissues were used. Polymer-based indirect immunoperoxidase method was applied for immuno histochemical (IHC) staining. Firstly, Proteinase-K solution was instilled for 10 minutes. Then, 3% H_2O_2 peroxidase block solution was dropped for 10 min. After that, the protein block was instilled and incubated for 5 min. Following this procedure, Rabbit polyclonal anti Infectious Laryngotracheitis Virus antibody (Biorbyt, orb10560) was instilled and incubated for 2 h at room temperature. Then, post-primer block solution was added to the slides and incubated for 30 min and the Polymer solution was dropped for 30 min. Slides stained by DAB (3,3'- diaminobenzidine tetrahydrochloride) for 5 min. After counter-staining with hematoxylin, slides were closed by coverslips and evaluated under a light microscope. The negative control slides were also stained according to the same procedure. However, TBS was used instead of the primer antibody.

PCR Analysis

Deparaffinization of Samples: For deparaffinization, FFPE tissues were cut at 20 µm for 4 times then the samples were placed into the Eppendorf tubes. Eppendorf tubes were filled with 1 mL of xylol. Tubes were mixed for 2 min in the vortex and incubated in the heat block at 56°C for 5 min. Then tubes vortexed again for 2 min and centrifuged for 2 min at 13.200 rpm. Then supernatant fluid was drained out. This process was repeated twice. The steps described above were repeated. However, ethanol was used instead of xylol. The liquid remaining in the bottom of the tubes were taken with a micropipette into another tube and then these tubes were subjected to DNA extraction procedures.

DNA Extraction: DNA extraction from the samples was done by the Vivantis Tissue DNA Extraction Kit (GF TD-50) as specified by the manufacturer's instructions. 250 µL

of lysis solution and 20 µL of proteinase K were added to the samples and incubated at 65°C for 3 h. At the end of the period, 560 µL Buffer TB was added to the tube and incubated at 65°C for 10 min. Then, 200 µL of ethanol was added, to the filter, and centrifuged at 5.000 rpm for 1 min. The obtained DNA samples were stored at -20°C until use.

PCR Method: Polymerase chain reaction analysis of the ICP4 gene was performed using the primers ICP4-1F (5'-CCT TGG TTC GGG AT¬G AAA CC-3') and ICP4-1R (5'-TTC ATT ACC TCC AGC GGT TCC-3') described by Preis et al.[14]. These primers showed a single amplicon, the size of which was 237 bp.

The extracted DNA was amplified in a total volume of 25 µL (2.5 µL 10x PCR buffer, 170 mM from each dNTPs (Vivantis), 10 µmol each of the primers (IDT), 1.5 mM $MgCl_2$, 1.25 U Taq polymerase (Vivantis), and 2.5 µL extracted DNA). The cycling conditions with the Biorad gradient (T100) were the initiation step at 94°C for 3 min, followed by 40 cycles coupling 94°C for 30 s, 54°C for 30 s and 72°C for 2 min and a final extension at 72°C for 15 min. The PCR products (10 µL) were analyzed by electrophoresis on 1.6% agarose gel, and the gel was stained with ethidium bromide (1.5 g/mL) and photographed [15].

RESULTS

Gross Results

Lesions that seen in microscopic examinations of trachea, larynx, lung, infraorbital sinuses and air sacs are given in *Table 1*. Also, the number and percentages of the gross pathology results are given in *Table 2*. The most frequent lesions were observed in trachea and larynx. Bloody, mucopurulent, fibrinous exudate deposits and diphtheroid lesions were observed in the lumen of trachea and larynx *(Fig. 1A-C)*. In 7 cases, both mucopurulent and fibrinous lesions were observed together *(Fig. 1D)*. Mucopurulent exudate deposits were observed in 16 cases in the infraorbital sinus. In these cases, swelling of the sinuses was also noticed on the gross examination. The lesions observed in the lungs consisted of congestion, and findings related to pneumonia were not observed. The air sacs had whitish colored, thickened and opaque.

Histopathological Results

The numbers and percentages of histopathological results and inclusion bodies are given in *Table 2*. Distribution of histopathological lesions in the organs is given in *Table 3*. Pathological changes were mostly detected in the trachea and larynx. Various degrees of thickening was noted due to oedema, mononuclear cell infiltrates, hyperemia, and heterophil granulocyte accumulation in the mucosa of the trachea and larynx *(Fig. 2A-E)*. In some cases, erythrocytes and desquamated epithelial cells, as well as intranuclear inclusion bodies in syncytial giant cells were found in the lumen of the trachea *(Fig. 2F)*. These inclusions were often determined in the nuclei of giant cells. In infraorbital sinuses, oedema, mononuclear cells, and heterophil granulocytes infiltrations were observed in the subendothelial layer, whereas in some cases only epithelial desquamation was noticed. Lymphoid hyperplasia was observed around the parabronchus in the lungs. In few cases, inclusion bodies were determined in giant cells in the exudate of bronchial lumens. In the air sacs, the propria was found to thickened due to lymphoid cells, mononuclear cells, and oedema.

Immunohistochemical Results

IHC staining results are given in *Table 2*. Larynx and trachea were the organs showed most positivity (47 (78.3%))

Table 1. *Gross results in the respiratory tract organs*

Gross Changes (n=60)	Organs									
	Trachea and Larynx				Infraorbital Sinus		Lung		Air Sac	
	No Lesion Observed	Bloody and Mucopurulent Lesions	Diphtheroid Lesions	Bloody, Mucopurulent and Diphtheroid Lesions	No Lesion Observed	Exudate Accumulation	No Lesion Observed	Congestion	No Lesion Observed	Opaque
Numbers of lesions	10	38	5	7	44	16	56	4	41	19

Table 2. *The numbers and percentages of gross, histopathology, inclusion bodies, immunohistochemistry and PCR results*

Results According to Methods and Findings (n=60)	Larynx/ Trachea	Infraorbital Sinus	Lung	Air Sac
Number and percentage of gross findings (+)	50 (83.3%)	16 (26.6%)	4 (6.6%)	19 (31.6%)
Number and percentage of histopathology results (+)	60 (100%)	32 (53.3%)	32 (53.3%)	17 (28.3%)
Number and percentage of inclusion bodies (+)	30 (50%)	2 (3.3%)	4 (6.6%)	1 (1.6%)
Number and percentage of IHC results (+)	47 (78.3%)	37 (61.6%)	27 (45%)	30 (50%)
Number and percentage of PCR results (+)	15 (25%)	11 (18.3%)	8 (13.3%)	6 (10%)

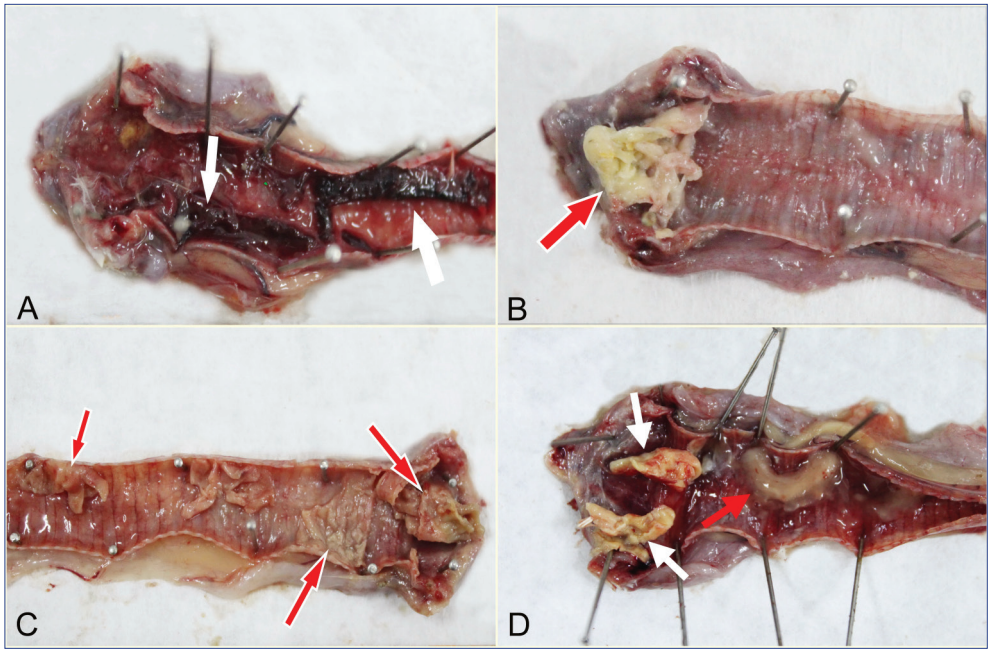

Fig 1. A. Trachea. Hyperemic appearance in the mucosa of the trachea, and bloody exudate accumulation in the lumen *(arrows),* **B.** Larynx. Fibrinous exudate deposits in the cavum larynx *(arrow),* **C.** Larynx and Trachea. Diphtheroid lesions on the surface of the larynx and trachea *(arrows),* **D.** Larynx and Trachea. Fibrin mass accumulation in the cavum larynx *(white arrows)* and mucopurulent exudate deposits in the lumen of the trachea *(red arrow)*

Table 3. *Distribution of histopathological lesions in the organs*

Organs (N=60)	Histopathological Changes							
	Score of Lesions/ Number of Lesions		Degeneration, Necrosis, and Desquamation in the Epithelia	Thickening of the Propria (Hyperemia, Edema, MNC Infiltration)	Inclusion Bodies	Syncytial Giant Cells	Heterophil Granulocyte Infiltrations	Lymphoid Cell Infiltrations
Trachea and Larynx	0	0	0	0	0	0	0	0
	+1	5	0	4	0	0	0	2
	+2	9	5	8	3	3	0	6
	+3	19	15	18	18	15	0	14
	+4	18	16	11	9	8	5	7
	+5	9	9	2	0	0	2	2
Infraorbital Sinus	+	32	5	31	2	2	3	1
Lungs	+	32	4	21	4	4	9	19
Air Sac	+	17	2	17	1	0	0	2

against anti-Infectious Laryngotracheitis Virus antibody (Anti-ILTV antibody). In 37 cases (61.6%) in the infraorbital sinus, in 27 cases (45%) in lungs and in 30 cases (50%) in air sacs, positive immunoreactivities were observed. Immunopositive staining was seen in the cytoplasm and rarely in the nuclei of macrophages, mucosal epithelial cells, gland epithelia and mononuclear cells in lamina propria and submucosa in the larynx and trachea *(Fig. 3A-C)*. Immunopositive reactions were also detected in the infraorbital sinuses in the mucosal epithelia and luminal desquamated epithelial cells. Immunohistochemical positivity was seen in the cell debris in the lumen of the parabronchus in the lungs *(Fig. 3D)*. Positive stainings were noted in inflammatory cells in the epithelium and subepithelial layer in the air sacs *(Fig. 3E)*. In addition, intracytoplasmic immunopositivity was also observed in the giant cells in all examined organs *(Fig. 3F)*.

PCR Results

PCR results from FFPE tissues are given in *Table 2*. The most positive reaction was observed in 15 (25%) cases in trachea and larynx. This was followed by infraorbital sinuses in 11 (18.3%) cases, in lungs in 8 (13.3%) cases and air sacs in 6 (10%) cases.

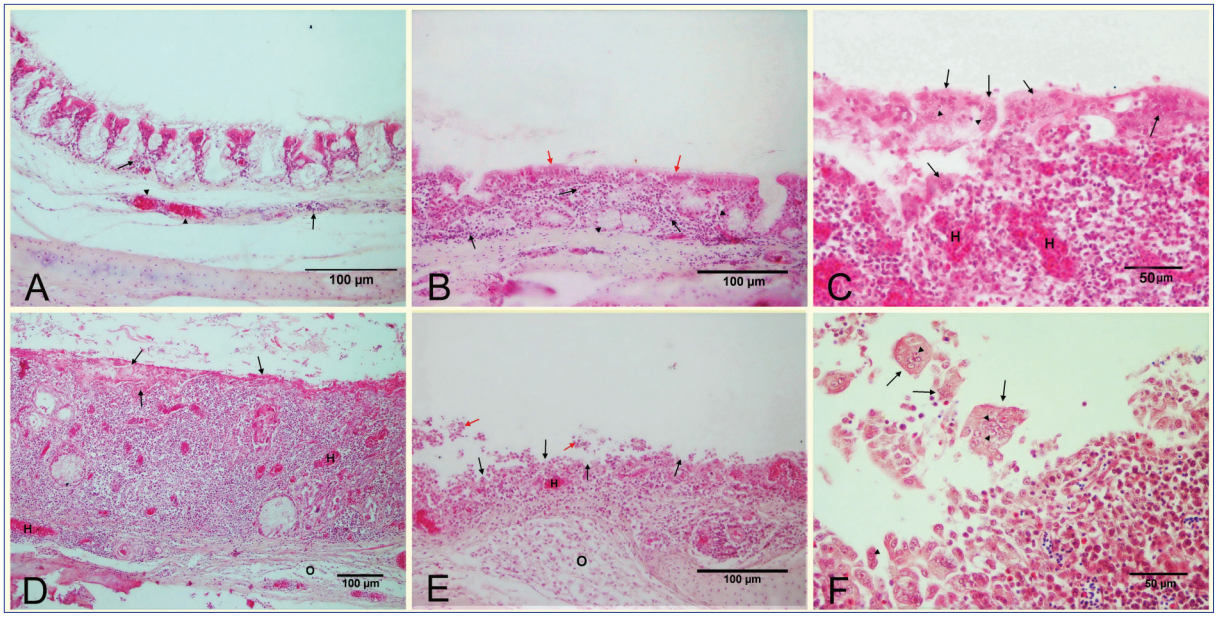

Fig 2. A. Trachea. +1 (minimal) lesions. Mild mononuclear cell infiltrates *(arrows)* in lamina propria and hyperemia in blood vessels *(arrowheads),* HxE, **B.** Trachea. +2 (mild) lesions. Mononuclear cell infiltrations *(arrows),* normal mucous glands *(arrowheads)* in the lamina propria, and normal mucosal epithelia *(red arrows),* HxE, **C.** Trachea. +3 (moderate) lesions. Epithelial degeneration, necrosis and desquamation. Numerous giant cells and Cowdry A type intranuclear eosinophilic inclusion bodies *(arrowheads)* in these cells. Hyperemia in blood vessels (H), HxE, **D.** Trachea. +4 (severe) lesions. Mucosa thickened due to mononuclear cell infiltrates, oedema (O) and hyperemia (H). Mucosal epithelia are flattened *(arrows)* due to fibrinonecrotic masses, HxE, **E.** Trachea. +5 (Very serious) lesions. The epithelial layer is completely separated from the mucosa (black arrows) and desquamated in the lumen *(red arrows).* Hyperemia in the blood vessels (H) and oedema in the lamina propria (O), HxE, **F.** Larynx. Lumen has numerous giant cells *(arrows),* and eosinophilic inclusion bodies *(arrowheads)* in the nuclei of these cells, HxE

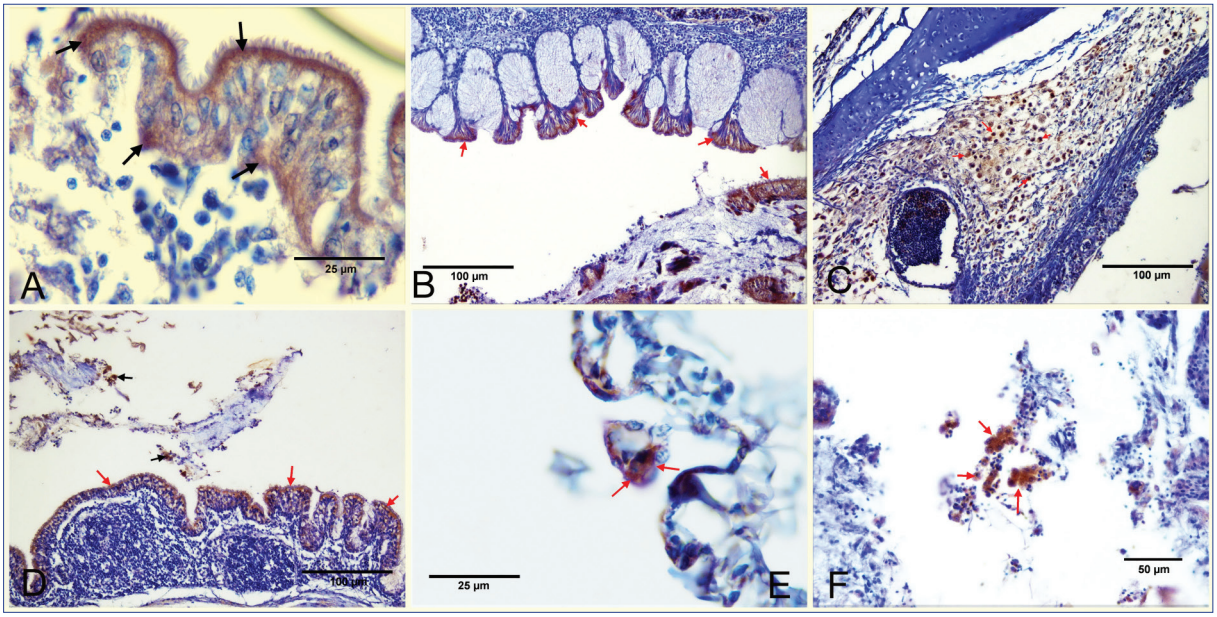

Fig 3. A. Trachea. Positive immunostaining in the mucosal epithelia *(arrows),* IHC, **B.** Larynx. Positive immunoreactions in the mucosal epithelia *(arrows),* IHC, **C.** Trachea. Positive reactions *(arrows)* in the cytoplasm of macrophages in the submucosa layer, IHC, **D.** Lungs. Positive reactions *(red arrows)* in parabronchial epithelia and desquamated epithelial cells *(black arrows)* in the lumen, IHC, **E.** Air sac. Positive staining in the epithelium *(arrows),* IHC, **F.** Trachea. Positive reactions *(arrows)* in the cytoplasm of macrophages and epithelial cells that desquamated in the lumen, IHC

DISCUSSION

ILT is a viral disease that infects the respiratory tract of the chickens and, rarely pheasants and turkeys. It has been reported to be diagnosed by clinical, necropsy and laboratory examinations [4,15,16]. Laboratory methods include serological methods, histopathological methods, fluorescent antibodies (FA), IHC methods, electron microscopy, virus

isolation, and PCR [1,16]. In a previous study, histopathological methods have been reported to be a more reliable method than virus isolation. However, the PCR method is more sensitive and to reduces false positives [17]. On the other hand, FA test was found to be as reliable as histopathological methods [18]. However, it needs for fluorescence microscopy has been reported as a negative aspect [9]. Williams et al.[19] compared virus isolation, electron microscopy, and PCR methods and they found that most sensitive methods were PCR, virus isolation and electron microscopy, respectively. They have also emphasized that the PCR was an advantageous method because of the other methods more costly. Abbas et al.[20] compared immunohistochemistry, virus isolation, histopathology, and PCR methods and they found that the most sensitive method is immunohistochemistry.

In this study, it was aimed to investigate the pathologic, immunohistochemical and PCR findings in naturally infected laying hens, and which method could be used to diagnose the disease faster and more reliably.

Although it is easy to recognize the disease by necropsy findings, it is emphasized that the advanced laboratory methods should be used, because of similar findings to some other respiratory tract infections such as Infectious Bronchitis [1,20-22]. Macroscopically; lesions are frequently seen in the laryngeal and tracheal lumen, sinuses, air sacs, and lungs [1,23-25]. The larynx, trachea, sinuses, air sacs and lungs were examined macroscopically in this study. Macroscopic changes ranged from bloody and mucopurulent contents to fibrinous and diphtheroid lesions in the larynx and trachea. Tracheal and laryngeal lesions were observed in 50 of 60 hens by grossly. Blood-mucopurulent contents were observed in thirty-eight cases, while fibrinous and diphtheroid lesions were observed in 5 cases. In seven cases, both bloody-mucopurulent and diphtheroid exudate were noted. In particular, the exudate accumulation in the tracheal lumen was similar to the findings reported by researchers [14,17,21,26]. In infraorbital sinus, mucous exudate was detected in 16 cases. Congestion was detected in only 4 cases in the lung, while thickening was observed in 19 cases at the air sacs. Mucous exudate accumulation in the sinuses and the thickening of air sacs are suggest to be that the disease is in the acute stage, as mentioned in the other studies [23,27]. Previous studies have reported that the lung is rarely affected by the disease [1]. However, only the congestions were noted in this study, while no macroscopic finding of pneumonia was found.

Histopathologically, The presence of intranuclear eosinophilic inclusion bodies and giant cells in respiratory organs such as the larynx, trachea, sinus and lung are defined as a characteristic finding for the ILT [3]. The diagnosis based on histopathologic findings may be inadequate due to the inclusion bodies are observed between the 3rd and 5th days from the beginning of the disease [1,10]. In histopathological examinations; it has been stated that ILT mainly affects upper respiratory tracts, such as larynx and trachea, as well as lesions can be seen in organs such as air sacs and lungs [28-30]. In this study, it was revealed that the larynx, trachea, infraorbital sinus, lung and air sacs were affected at various grades from the disease. It has been found that moderate and severe changes are observed more frequently. It was noted that intranuclear inclusion bodies in epithelial cells and syncytial giant cells that are typical signs of the disease were observed in exudates, both mucosa and luminal desquamation. In addition, degeneration, necrosis and desquamation of the epithelium, thickening due to mononuclear cell infiltrates and hyperemia and oedema in lamina propria were similar to the findings previously reported by others [1,31]. Thickening due to oedema and mononuclear cell infiltrate in the subepithelial layer of infraorbital sinuses and air sacs were quite remarkable. Giant cells and inclusion bodies within the exudate and desquamated epithelial cells were also seen in these organs. Hyperplasia of the lymphoid tissue was frequently observed in the lungs. But in some cases, intranuclear inclusions in giant cells within the parabronchial lumens were similar to those reported previously [1,4]. In only 4 cases, typical inclusion bodies and giant cell accumulations were detected in the lungs. It was noticed that the lungs were less affected than the trachea, larynx and sinus.

Immunohistochemical methods have recently been used to diagnose ILT by many researchers [8,9,11,32-34]. IHC methods have been shown to be helpful for diagnosing ILT when macroscopical and histopathological findings are not possible [35]. In this study, 78.3% positive reactions observed in the larynx and trachea. In the lungs, the immunopositive staining rate was 45%. Tadese et al.[33] found 18.18% immunoactive reactions against anti-ILTV antibody in the trachea. Preis et al.[11] detected 70% positivity in the larynx and trachea tissues, and 53.8% positive results in the lungs in the field study immunohistochemically. They reported that these positive reactions were intracytoplasmic located in the mucosa and desquamated epithelial cells in the larynx and trachea, and parabronchial epithelium in the lung. At the same time, they stated that cytoplasms of giant cells found in these tissues had immunopositive reactions against anti-ILTV antibody. In an experimental study, immunopositive stainings were observed in laryngeal and tracheal tissues, especially in the cytoplasm of epithelial desquamated and unciliated cells such as goblet cells and mucous gland epithelia [36]. Guy et al.[9] reported that in their experimental study, they found positive reactions in tracheal mucosal epithelia and desquamated epithelial cells. In this study, immunopositive reactions were observed in the larynx and tracheal epithelium, luminal epithelial cells and gland epithelium. In this study, strongly positive staining was detected in the cytoplasm of the giant cells located in the lumen. These reactions were similarly observed in the sinus and lung. Immunoactive staining was observed in the cytoplasm of the macrophages in the

submucosal layer of the trachea and larynx. This suggests that the virus tends to spread to deeper layers rather than mucosa. Kirkpatrick et al.[36] support this view that different virus strains may have different tropism in the tissues.

Preis et al.[11] applied PCR method in fresh larynx, trachea, paranasal sinus, and lung from ILT suspected chickens, and they found positive reactions in 63.2% of larynx/trachea, 56% in sinus and 57.6% in the lung. However, they found FFPE positivity was 25% in tracheal tissues. In this study, which was made from FFPE tissues the rate of positive cases were 25% in the larynx/trachea tissues. The positivity of FFPE tissues is lower than fresh tissues. They explained that the long-term fixation by formalin causes DNA damage [37]. Kleter et al.[38] emphasized that liquid paraffin at high temperature during paraffin block preparation may cause damage to DNA. Sivaseelan et al.[24] obtained positive results only in the larynx, trachea and conjunctiva as a result of the PCR tests performed on the tissue specimens. In this study, positive reactions were also observed in the lungs (13.3%), infraorbital sinuses (18.3%) and air sacs (10%). This result suggests that the virus may be present in various tissues and organs at different stages of the disease. But the viral DNA is mostly found in trachea and larynx, and less in sinuses, lungs and air sacs.

In gross examination of larynx and trachea, in 50 (83.3%) cases were diagnosed as ILT. However, histopathologically 60 (100%) cases, immunohistochemically 47 (78.3%) cases were positive for ILT. Although findings were observed in all (100%) cases in histopathology, ILT specific inclusion bodies were detected in 30 (50%) cases in the larynx and trachea. Preis et al.[14] found inclusion bodies in the trachea (70%), larynx (50%), and lung (10%). This suggests that reduces the reliability of the histopathological diagnosis. Immunohistochemically, the reaction against Anti-ILTV antibody can easily be demonstrated in the tissues. However, in histopathological examinations, ILT-like findings can also be observed in other respiratory system diseases. It can be said that the IHC results are more precise than histopathologic results. In PCR analyses, there were only 15 (25%) cases positive in the trachea and larynx. The superiority of IHC over PCR, viral structures can easily be demonstrated in the tissues. In contrast, virus localization cannot be predicted by PCR. Although FFPE tissues can be stored for many years, the positive rate of PCR is lower than other methods such as histopathology and immunohistochemistry, so it cannot be used to the diagnostic tool. In macroscopic examinations of other tissues such as infraorbital sinus and lung, there were 16 cases in the sinuses and 4 cases in the lungs, suggesting ILT findings. Thirty-two cases in the sinuses and lungs were positive in histopathology. It suggests histopathology is more sensitive against gross pathology. Histopathological (28.3%) and macroscopic (31.6%) positivity rates of air sacs were close to each other, but immunohistochemically, this ratio was 50%. Grossly, opaque appearance of the air sacs may be regarded as a helpful finding in the diagnosis. However, IHC analyses needed to definitive diagnose.

In conclusion, lesions were evaluated by macroscopic, histopathologic, immunohistochemical and PCR methods in naturally infected with Infectious Laryngotracheitis Virus in laying hens. The larynx, trachea, infraorbital sinuses and air sacs should be carefully examined by gross examinations. In histopathological examinations of the larynx and trachea, all cases were found positive for ILT, however, a positivity rate of 78.3% was determined by IHC examinations, and it was thought that this method could be used for definite diagnosis. On the other hand, positive immunostaining was obtained in 61.6% in the sinus, 45% in the lung and 50% in the air sacs. This suggests that it may be useful to examine trachea and larynx as well as the infraorbital sinuses, lungs and air sacs to evaluate the severity/prevalence of the ILT. The location of viral antigens not only in the epithelium and lamina propria but also in the submucosa layer reveals that deeper layers should be taken into account along with mucosal layers by IHC staining, and it is also an indicator of virulence of the agents.

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Research Article

In-vitro Evaluation of the Fermentation Characters of Maize Stover and Rice Straw with Different Level from *Bacillus coagulans*

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Abstract

The study was carried out to investigate the impact of *Bacillus coagulans* supplementation with different concenteration (0, 0.25×10^7, 0.50×10^7, and 0.75×10^7 cfu/mL) on *in vitro* parameters as methane (CH_4) parameters, nutrients digestibility and the fermentation character of rumen of fibrous agriculture by-products (maize stover and rice straw) using probiotics preparations in ruminants. The results showed that, maximum gas production (V_f), gas production fraction (k) and the time when half of the maximum gas production was achieved ($t_{0.5}$) of maize stover were significantly increased when compared to group of the rice straw. Additionally, *in vitro* dry matter disappearance; (IVDMD), *in vitro* neutral detergent fiber disappearance (IVNDFD), individual volatile fatty acids (VFAs) and total VFA (TVFA) of maize stover were significantly in related to the rice straw group. Also, the gas production rate at the early incubation stage (FRD_0), pH value of rumine and the acetate: propionate ratio of maize stover is significantly lower than the rice straw. V_f of rice straw was quadratic ($P<0.05$) increased when adding *Bacillus coagulans*. These results indicate that, *in vitro* gas production (IVGP) was numerically increased when maize stover and rice straw were fermented using *Bacillus coagulans* at a level of 0.75×10^7 cfu/mL. Additionally, maize stover (CP 0.053, NDF 0.636, ADF 0.386) can be used as a superior roughage for ruminants compared to rice straw. The present *in vitro* positive results should be further testified using in vivo experiments in future.

Keywords: *Bacillus coagulans, Maize strover, In vitro gas production, CH4, Volatile fatty acids*

Farklı Miktarlarda *Bacillus coagulans*'ın Mısır Hasat Kalıntısı ve Pirinç Samanının Fermantasyon Özelliklerine Etkisinin *In Vitro* Değerlendirilmesi

Öz

Bu çalışma, farklı konsantrasyonlarda (0, 0.25×10^7, 0.50×10^7 ve 0.75×10^7 cfu/mL) *Bacillus coagulans*'ın ruminantlarda metan (CH_4) parametresi, besinlerin sindirilebilirliği ve fibröz yapılı zirai yan ürünlerin (mısır hasat kalıntısı ve pirinç samanı) rumen fermantasyon karakteri üzerine etkisini araştırmak amacıyla yapılmıştır. Elde edilen bulgular, pirinç samanı ile karşılaştırıldığında mısır hasat kalıntısı ile maksimum gaz üretimi (V_f), gaz üretim fraksiyonu (k) ve maksimum gazın yarısının oluştuğu zamanın ($t_{0.5}$) anlamlı oranda arttığını gösterdi. Ayrıca, pirinç samanı ile karşılaştırıldığında mısır hasat kalıntısı grubunda *in vitro* kuru madde kaybolması (IVDMD), *in vitro* nötral deterjan lif kaybolması (IVNDFD), bireysel uçucu yağ asitleri (VFAs) ve total VFA (TVFA) daha yüksek olarak bulundu. Pirinç samanı ile karşılaştırıldığında mısır hasat kalıntısının erken inkübasyon döneminde gaz üretim oranı (FRD_0), rumen pH'sı ve asetat:propiyonat oranı daha düşük olarak gözlemlendi. Pirinç samanının V_f değeri *Bacillus coagulans* ilavesi ile kuadratik ($P<0.05$) artım gösterdi. Elde edilen sonuçlar, mısır hasat kalıntısı ve pirinç samanının 0.75×10^7 cfu/mL oranında *Bacillus coagulans* ile fermente edildiğinde *in vitro* gaz üretimini (IVGP) numerik olarak artırdığını göstermektedir. Mısır hasat kalıntısının (CP 0.053, NDF 0.636, ADF 0.386) pirinç samanı ile karşılaştırıldığında ruminantlar için kaba yem olarak tercihen kullanılabileceği düşünüldü. Bu *in vitro* bulgular *in vivo* çalışmalar ile desteklenerek ileriki çalışmalarla teyit edilmelidir.

Anahtar sözcükler: *Bacillus coagulans, Mısır hasat kalıntısı, In vitro gaz üretimi, CH_4, Uçucu yağ asitleri*

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INTRODUCTION

Fibrous agricultural by-products such as maize stover, and crop straws of rice and wheat are abundantly available in many countries [1]. However, they are rich sources of crude fiber with high lignin contents, but their protein content is low, which decreases the dry matter and nutrient degradations in the animal rumen [2]. Different methods have been examined such as chemical and physical treatments to increase the nutritive value of such by-products. Although these methods were effective to improve feed intake and/or digestibility of these fibrous feedstuffs [1-3], they are expensive and harmful to both users and environment [4]. Furthermore, biological methods such as probiotics (microorganism preparations) were found to be a good economic and safe alternative to increse the digestibility of such fibrous by-products [5]. Probiotics are last recent biological methods by using alive and suitable microorganisms that can beneficial effects to the host health when consumed in appropriate and regular quantities [6]. This microorganisms have been used to improve the *in vitro* fermentation characteristics of the roughages with low-quality [7] The scientific interest to several *bacillus* strains have been screened for their potential probiotic functionalities with special regard to *Bacillus natto* and *Bacillus subtilis* due to their beneficial effects on ruminants [8]. However, these species were firstly used as probiotics product (Enterogermina®) in Italy since 1958 [9]. So, the data is limited regarding the effect of *Bacillus coagulans* on the improvement of low quality feeds in ruminant nutrition manipulation.

Therefore, this study was a first detailed report to identify the effects of *Bacillus coagulans* supplementation to fibrous agricultural by-products (maize stover and rice straw) on *in vitro* rumen fermentation parameters and nutrients degradation using gas production technique for further understanding their mode of action in the rumen, and providing more knowledge about their application to ruminants' nutrition.

MATERIAL and METHODS

Animal Care Committee, Institute of Subtropical Agriculture (ISA), the Chinese Academy of Sciences (CAS), Changsha, China was approved this experiment.

Fermented Substrates Probiotics and Experimental Design

Maize stover from Kexiangtian 1 (bred by ISA) and rice straw from Xiang 125s (a local popular breed) were selected as the fibrous agricultural by-products. They were oven dried (at 65ºC for 24 h), ground (filterated through a 1 mm sieve) and stored for further analysis. They were analyzed on DM basis for crude protein (CP) (5.3% and 6.2%), NDF (63.6% and 63.2%) and ADF (38.6% and 43.4%) respectively. *Bacillus coagulans* (NO. 20138) was purchased, reactivated and amplified to contain 1×10^{11} cfu/g viable bacteria using the spread plate method by the China Center of Industrial Culture Collection (CICC). *Bacillus coagulans* preserved at 4°C after culture amplification and counting. The study was induced in completely block experimental design, and *Bacillus coagulans* was supplemented at four levels (0×10^{7} cfu/mL, 0.25×10^{7} cfu/mL, 0.50×10^{7} cfu/mL and 0.75×10^{7}cfu/mL, respectively).

In vitro Gas Production and Sampling

The modified anaerobic (continuous CO_2 pumping for 2 h) artificial saliva was used for *in vitro* fermentation according to [10]. Around three Holstein dairy cows were housed individually and fed a rice straw based completely mixed ration with adlibtum water. Rumen content was collected through the ruminal fistula before the morning feeding and placed into thermos flasks pre-heated at 39°C and quickly transferred to the laboratory. After collection, the inoculum was strained through 4 layers of cheesecloth maintained anaerobic condition with CO_2 flux.

Approximately 0.5 g of each feedstuff was transferred into fermentation bottles 100 mL. Every sample was measured in triplicates at each point of incubation time. *Bacillus coagulans* was added with graded levels to the tested feedstuffs altogether with 45 mL artificial saliva and 5 mL rumen content as previously mentioned at the start of *in vitro* fermentation. All bottle required for fermentation process were sealed and incubated at 39°C. During the *in vitro* fermentation process, the pressure in the bottle was recorded at 0, 1, 2, 4, 6, 12, 24, 36 and 48 h. And after 12, 24 or 48 h of incubation the fermentation was interrupted due to presence of undegraded residues which were filtered through two layers of nylon cloth. Around 5 mL from gas sample was collected by plastic syringe into the vacuum flask for detect CH_4. Finally, at 12, 24 and 48 h from the incubation, the incubated sample from each treatment was used to calculate NH_3-N level and VFAs concentrations respectively

Chemical Analysis

Dry matter (DM) (method 930.15) of the tested substrate and filtered residues were analyzed by drying at 105°C for 12 h and weighed for *in vitro* DM degradation (IVDMD) and they ground for further chemical analysis. CP was detected according to [11]. NDF and ADF values were estimated as described by [12] and all triplicate samples were analyzed. Alpha amylase (Sigma A-3306, Sigma, Aldrich, China), and sodium sulphite were added for NDF determination. The NDF of the dried residues was analyzed for *in vitro* NDF digestibility (IVNDFD). 2 mL of the fermented solutions were centrifuged at 10000 × g and 4°C for 15 min, then 1.5 mL of the supernatant were mixed with 0.15 mL meta-phosphoric acid. Then another centrifugation at 10.000 × g and 4°C for 15 min, and the supernatant was taken to

analyze VFA content with a gas chromatograph (HP5890, Agilent 5890; Agilent Technologies Co. Ltd, USA). The peak of VFA was calculated using their standard concentration curve, which was prepared using 10 samples for each treatment. Total molar concentration was determined by the sum of individual VFA as 100% [13]. pH meter was used to mesure pH of the fermented fluids immediately. For ammonia level determination, around 5 mL of fermented solutions were centrifuged at 4000 ×g at 4°C for 10 min, and 2 mL of the supernatant were mixed 8 ml 0.2 M HCl. 0.4 mL from mixed solution was subsequently mixed with 2 mL of sodium nitroprusside solutions (0.08 g sodium nitroprusside dissolved in 100 mL of 0.14 natrium salicylicum) and 2 mL of prepared solutions (2 mL sodium hypochlorite solution mixed with 100 mL 0.3 M sodium hydroxide solution), then homogenized at room temperature for 10 min. The absorption was determined at 700 nm using spectrophotometer. The NH_4Cl standard solutions were prepared as follows: 0.382 g of NH_4Cl was diluted with 0.2 M HCl to 100 mL as the preservation solutions kept at 4°C. After that, 10 mL of preservation solutions were diluted to 100 mL with distilled water as the working solution in which the concentration of N was 10 mg/dL. Subsequently, 0, 1, 2, 4, and 6 mL of working solutions were separately mixed with 10, 9, 8, 6, and 4 mL of distilled water and then all diluted with 0.2 M HCl to 50 mL as the NH_4Cl standard solutions in which the concentrations of N were 0, 0.2, 0.4, 0.8 and 1.2 mg/dL, respectively. Finally, 0.4 mL of the NH_4Cl standard solution of each concentration was treated to obtain a standard curve as previously mentioned. CH_4 was analyzed using gas chromatography equipped with a Hayesep Q packing column (2.44 M × 1/8 in. × 2.0 mm ID). The microbial crude protein production in rumen liquor was analyzed using the trichloroacetic acid. Firstly, 0.5 g ground dry sample was weighted into a 125 Erlenmeyer flask then around 50 mL from distilled water was added and wait for 30 min. 10 mL from 10% trichloroacetic acid was add to the mix and incubate the solution for 20-30 min then was filtrated on whatman #54 or 541 paper by gravity. Finally, the filter paper was washed twice with trichloroacetic acid solution followed by transfer the paper to Kjeldahl apparatus to calculate nitrogen percent. NPN was calculated by subtracting residual nitrogen from total nitrogen. NPN value may be expressed as crude protein value which equal to (N X 6.25) or percent of total feed nitrogen [14].

Statistical Analysis

The experimental data were analyzed separately using the PROC MIXED procedure of SAS (SAS Institute, 2001) Orthogonal polynomial contrasts was used for detect linear and quadratic effect. Cubic effects of dose were not analyzed due to the inexplicability in biology. The significance was detected at $P<0.05$ and the least squares means are reported throughout the text.

RESULTS

Effects of *Bacillus coagulans* supplementation levels on gas production parameters of fibrous agriculture byproducts are shown in *Table 1*. The supplementation of *Bacillus coagulans* had significantly lowered ($P<0.01$) the maximum gas production (V_f) of rice straw, while it had no ($P>0.05$) effect among the other three supplemental treatments. The supplementation levels of *Bacillus coagulans* had no significant effect ($P>0.05$) V_f on maize stover, gas production fraction (k), the initial fractional rate of degradation (FRD_0) and the time when half of the maximum gas production was achieved ($t_{0.5}$) for both fermentation substrates. The value of V_f, k and $t_{0.5}$ of maize stover was significant ($P<0.05$) higher than that of rice straw, which was increased by 20.76, 7.86 and 35.48%, respectively. While FRD_0 of maize stover was significant ($P<0.0001$) lower than that of rice straw, which was decreased by 117.16%. Both the fermentation substrate and supplementation level had no significant effect ($P>0.05$) onCH_4 production. The combination between fibrous - by product and *bacillus coagulans* supplementation level had significant ($P<0.05$) effects on gas fermentation characteristics except V_f and CH_4 for both maize stover and rice straw.

Effect of *Bacillus coagulans* adding with different levels on IVDMD, IVNDFD and MCP production of both maize stover and rice straw are shown in *Table 2*. IVDMD, IVNDFD and MCP production of maize stover were significant ($P<0.05$) higher than that of rice straw, which was higher by 16.75, 40.04 and 1.50%, respectively while, this combination had no significant effect on IVDMD, IVNDFD and MCP production for the two fermentation substrates.

The impact of *Bacillus coagulans* supplementation at different levels on ruminal acidity and ammonia level of fermented substrates are recorded in *Table 3*. The pH value of maize stover was significantly ($P<0.05$) decreased when compared to that of rice straw, which was decreased by 0.89%. While the supplementation levels and the combination between substrate level have no statically affect ($P>0.05$) of ruminal NH_3-N concentration of maize stover and rice straw.

The effects of different supplementation levels of *Bacillus coagulans* on *in vitro* ruminal VFA contents of maize stover and rice straw are shown in *Table 4*. VFA of maize stover was significantly ($P<0.01$) elevated in compared to the rice straw, which was increased by 28.86, 31.61, 60.79, 52.50, 89.23 and 76.28%, respectively. While A:P of maize stover was significant ($P<0.05$) lower than that of rice straw, which was decreased by 25.45%. The TVFA content was not significantly affected ($P>0.05$) by substrates, supplementation levels and the interaction between substrate and supplementation level. The supplementation levels and the combination between substrate and *bacillus coagulans* supplementation level not significantly effect ($P>0.05$) on ruminal individual

Table 1. *Effects of different supplementation levels of Bacillus coagulans on in vitro gas production kinetic parameters and CH_4 production of maize stover and rice straw*

Item	Substrate	Supplementation Levels (× 10^7 cfu/mL)					SEM[2]	Significance[3]		
		Mean[1]	0	0.25	0.50	0.75		Substrate	Level	S×L
[1]V_f (mL)	Maize stover	67.43[e]	68.72	51.53	72.63	76.84	6.63	<0.05	NS	NS
	Rice straw	55.84[f]	55.89[a]	53.93[b]	56.86[b]	56.68[b]			Q (P<0.01)	
	SEM[4]	3.32								
[2]$k(10^{-2})$	Maize stover	9.47[e]	9.31	9.34	9.38	9.84	0.87	<0.01	NS	<0.05
	Rice straw	8.78[f]	8.58	11.20	8.05	7.29			NS	
	SEM[4]	0.44								
[3]FRD_0 (10^{-2}) (mL/h)	Maize stover	1.34[e]	1.50	1.29	1.25	1.32	0.10	<0.0001	NS	<0.05
	Rice straw	2.91[f]	2.75	2.65	3.08	3.16			NS	
	SEM[4]	0.05								
[4]$t_{0.5}$ (h)	Maize stover	22.11[e]	21.25	22.61	22.82	21.76	0.34	<0.0001	Q(P<0.01)	<0.05
	Rice straw	16.32[f]	16.53	16.46	15.99	16.32			NS	
	SEM[4]	0.18								
[5]CH_4 (mL/g)	Maize stover	9.02	8.98	8.93	11.47	6.69	1.50	NS	NS	NS
	Rice straw	8.86	10.12	9.69	6.66	8.99			NS	
	SEM[4]	0.76								

[a,b] Means within a row for supplementation levels do not have a common superscript differ (P<0.05); [e,f] Means within a column for Bacillus coagulans do not have a common superscript differ (P<0.05); [1] Mean = mean for individual Bacillus coagulans across supplementation levels including the level of 0; [2] SEM for supplementation level×substrate; [3] NS = not significant (P>0.05); S×L = interaction between substrate and supplementation level; Q = quadratic effect of supplementation levels; [4] SEM for pooled mean of substrate including the level of 0; During the initial stages of this work, the correlativity between the pressure in bottle and gas volume was measured at 39°C, and the regression equation was then established: y = 1.506x (n = 20, R^2 = 0.999, P<0.0001); Where y represents gas volume (mL), x is the pressure in bottle (kPa), 1.506 is a constant; Measured pressure was then converted to gas production (mL). In vitro gas production at 0, 1, 2, 4, 6, 12, 24 and 48 h were fitted to Logistic-Exponential [31]: GP = Vf (1 - exp (d - t × k))/(1 + exp(b - k × t)) (2); Where GP represents gas production at t time, Vf means the maximum gas production (mL), k represents gas production fraction (/h), b and d represent the shapes of the gas production curve. The following equation: $t_{0.5}$ = ln (exp (b) + 2 exp (d))/k [34] was used to calculate the time ($t_{0.5}$, h) when half of the maximum gas production was achieved. FRD_0 = k/(1 + exp(b)) was used to calculate the initial fractional rate of degradation (/h)

Table 2. *Effects of different supplementation levels of Bacillus Coagulans on IVDMD, IVNDFD and MCP production of maize stover and rice straw*

Item	Substrate	Supplementation Levels (× 10^7 cfu/mL)					SEM[2]	Significance[3]		
		Means[1]	0.00	0.25	0.50	0.75		Substrate	Level	S×L
IVDMD (%)	Maize stover	50.18[e]	49.64	52.11	49.58	49.38	3.82	<0.05	NS	NS
	Rice straw	42.98[f]	42.91	43.34	42.30	43.36			NS	
	SEM[4]	1.91								
IVNDFD (%)	Maize stover	38.58[e]	35.90	45.78	37.31	35.32	5.87	<0.05	NS	NS
	Rice straw	27.55[f]	28.14	27.32	27.84	26.91			NS	
	SEM[4]	2.98								
MCP (mg/mL)	Maize stover	3.39[e]	3.38	3.41	3.39	3.38	0.02	<0.001	NS	NS
	Rice straw	3.33[f]	3.33	3.33	3.34	3.31			NS	
	SEM[4]	0.01								

[e,f] Means within a column for Bacillus coagulans do not have a common superscript differ (P<0.05); [1] Mean = mean for individual Bacillus coagulans across supplementation levels including the level of 0; [2] SEM for supplementation level×substrate; [3] NS = not significant (P>0.05); S×L = interaction between substrate and supplementation level; [4] SEM for pooled mean of substrate including the level of 0

VFA content and A:P for both fermentation substrates.

DISCUSSION

The *in vitro* fermentation cumulative gas production technique widely used [15]. It is an important technique which used to evaluate rumen fermentation for ruminants, which provide valuable information for the kinetics of feed digestion in rumen, reflect the utilization efficiency of fermentation substrates [16]. The results showed that, V_f of maize stover was significantly elevated than the rice straw under different supplementation levels of *Bacillus*

Table 3. *Effects of different supplementation levels of Bacillus coagulans on in vitro ruminal pH value and NH_3-N concentration of maize stover and rice straw*

Items	Substrates	Supplementation Levels (× 10^7 cfu/mL)					SEM[2]	Significance[3]		
		Means[1]	0.00	0.25	0.50	0.75		Substrate	Level	S×L
pH	Maize stover	6.75[f]	6.78	6.74	6.75	6.73	0.04	<0.05	NS	NS
	Rice straw	6.81[e]	6.82	6.84	6.82	6.77			NS	
	SEM[4]	0.02								
NH_3-N (mg/dL)	Maize stover	6.12	6.28	6.13	6.08	6.01	0.55	NS	NS	NS
	Rice straw	6.39	6.16	6.36	6.53	6.53			NS	
	SEM[4]	0.28								

[e,f] Means within a column for Bacillus coagulans do not have a common superscript differ (P<0.05); [1] Mean = mean for individual Bacillus Coagulans across supplementation levels including the level of 0; [2] SEM for supplementation level×substrate; [3] NS = not significant (P>0.05); S×L = interaction between substrate and supplementation level; [4] SEM for pooled mean of substrate including the level of 0

Table 4. *Effects of different supplementation levels of Bacillus coagulans on in vitro ruminal VFA contents of maize stover and rice straw*

Items	Substrates	Supplementation Levels (× 10^7 cfu/mL)					SEM[2]	Significance[3]		
		Mean[1]	0.00	0.25	0.50	0.75		Substrates	Level	S×L
Acetate (mmol/L)	Maize stover	21.21[e]	21.0	22.28	21.16	20.33	2.14	<0.01	NS	NS
	Rice straw	16.46[f]	16.1	16.54	16.48	16.66			NS	
	SEM[4]	1.08								
Propionate (mmol/L)	Maize stover	7.87[e]	7.68	8.19	7.71	7.88	0.87	<0.01	NS	NS
	Rice straw	5.98[f]	5.84	6.03	5.98	6.04			NS	
	SEM[4]	0.44								
Isobutyrate (mmol/L) (10^{-2})	Maize stover	23.99[e]	24.00	25.16	23.94	22.88	2.82	<0.0001	NS	NS
	Rice straw	14.92[f]	14.51	14.86	14.95	15.35			NS	
	SEM[4]	1.42								
Butyrate (mmol/L)	Maize stover	2.44[e]	2.42	2.54	2.45	2.36	0.19	<0.0001	NS	NS
	Rice straw	1.60[f]	1.56	1.61	1.59	1.62			NS	
	SEM[4]	0.10								
Isovalerate (mmol/L) (10^{-2})	Maize stover	38.64[e]	38.82	39.94	37.96	37.82	6.14	<0.0001	NS	NS
	Rice straw	20.42[f]	21.91	18.97	20.08	20.72			NS	
	SEM[4]	3.11								
Valerate (mmol/L) (10^{-2})	Maize stover	26.83[e]	26.77	27.74	26.79	26.01	2.26	<0.0001	NS	NS
	Rice straw	15.22[f]	14.94	15.18	14.87	15.89			NS	
	SEM[4]	1.14								
TVFA (mmol/L)	Maize stover	32.61[e]	32.07	33.94	32.20	32.22	3.34	<0.01	NS	NS
	Rice straw	24.54[f]	24.09	24.67	24.56	24.85			NS	
	SEM[4]	1.68								
A:P	Maize stover	2.75[f]	2.75	2.73	2.77	2.75	0.07	<0.05	NS	NS
	Rice straw	2.85[e]	2.87	2.83	2.85	2.86			NS	
	SEM[4]	0.04								

[e,f] Means within a column for Bacillus coagulans do not have a common superscript differ (P<0.05); [1] Mean = mean for individual Bacillus coagulans across supplementation levels including the level of 0; [2] SEM for supplementation level×substrate; [3] NS = not significant (P>0.05); S×L = interaction between substrate and supplementation level; [4] SEM for pooled mean of substrate including the level of 0

coagulans, it might result from the outcome that IVDMD of maize stover was significantly higher than that of rice straw *(Table 2)*. The maximum gas production was positively related to readily fermentable substrates [17], hemicellulose and crude protein (CP) contents, and negatively related to the ADF and NDF contents, while other studies observed an adverse relationship between the production of gas and nitrogen content [18,19].

The indices of FRD_0 and $t_{0.5}$ generally means the rate of deterioration in early incubation stages "<12 h" and the incubation time to reach half of the maximum gas

production, respectively. In general, the faster FRD_0 the shorter $t_{0.5}$ become [20]. FRD_0 value of maize stover was significantly higher than that of rice straw, while it was reverse for $t_{0.5}$. It specified that the rate of degradation at early incubation period of maize stover was significantly higher than that of rice straw because of the supplementation of *Bacillus coagulans*. The reason might cause by the difference between maize stover and rice straw, because maize stover was C4 plant while rice straw was C3 plant, C4 plant could synthesize more carbohydrates than C3 plant during the process of photosynthesis and then resulted in faster fermentation rate for maize stover.

CH_4 is an inevitable product generated from dietary carbohydrates during anaerobic fermentation in the rumen, and methanogenesis possesses specific biological regulatory mechanism. Many researchers focused on ruminant CH_4 formation in recent years, due to its contribution to global climatic change [21]. During the ruminant metabolism process, Methane generation in the rumen is the main reason of energy loss in the rumen fermentation, about 6%-15% of the feed energy is loss by the form of methane [22]. CH_4 production may be affected by the composition of fermented carbohydrates, such as cellulose, hemicellulose, soluble residues and digestible ADF in the diets are also important fiber fractions enhancing CH_4 production [23]. CH_4 production had a stronger relationship with digestible NDF, ADF and cellulose intake [24,25]. However, in the current study, CH_4 production was not affected when supplemented with *Bacillus coagulans*, it might be caused by differences between *in vitro* and *in vivo* experiments, and different fermentation substrates (single fermented cell wall substrates VS total mixed ration substrates).

This study showed that IVDMD, IVNDFD and MCP of maize stover were significant higher than those of rice straw, this result might be associated with differences of CP contents and components (especially for rumen degradable protein) between two fermentation substrates. The ruminal microbial population might be another key reason caused this difference, because rumen is a very complex ecosystem, in which numerous microorganisms and factors play an important role and *Bacillus coagulans* do not possess any enzymatic capability to hydrolyze cell-wall constituents, and the activity of cellulolytic bacteria might be not affected by their supplementation administered [26]. Further investigations are needed to evaluate the mechanism of *Bacillus coagulans* supplementation on the activity of amylolytic, proteolytic and cellulolytic microorganisms in *in vitro* rumen fermentation. Our results also showed that IVDMD and IVNDFD were not affected by the supplementation of *Bacillus coagulans*, which was in line with the previous reported results [27].

As acidity is an important indicator for ruminal homeostasis, therefore maintenance of ruminal pH within a physiological range (about 5.5-7.0) is a key factor for efficient fermentation [28]. The results of the study showed that *in vitro* ruminal pH value was kept at 6.73-6.84, which was suitable for fermentation, microbial activity, and fiber digestion in the rumen [29]. Our results also showed that the pH value of maize stover was significantly lower than that of rice straw under different supplementation levels of *Bacillus coagulans*, it might result from the outcome that TVFA contents of maize stover was significantly increased than rice straw *(Table 4)*. There was no significant effect on *in vitro* ruminal pH value for both fermentation substrates after adding *Bacillus coagulans*, it indicated that the different supplementation levels of *Bacillus coagulans* possessed positive significance for ruminal stability manipulation.

Ruminal NH_3-N concentration reflects the equilibrium state for protein degradation and synthesis under specific dietary condition in a certain extent. It is consider an important nitrogen source for microbial growth and protein synthesis, ruminal NH_3-N had a low efficiency for milk protein synthesis partially due to NH_3-N losses in the rumen [30,31] stated that the optimum level of ruminal ammonia concentration should be above 5 mg/dL in order to maintain the microbial growth as well as the microbial protein synthesis, but excessive ammonia could adversely affect its microbial utilization [32]. The results showed that NH_3-N concentration was not affected across four supplemented levels of *Bacillus coagulans*, and it ranged from 6.01 to 6.53 $mg \cdot dL^{-1}$, indicating that the microbial activity was not affected when supplemented with *Bacillus coagulans*.

Ruminal VFAs are the main source of energy for ruminants, both its content and composition are important physiological indexes to reflect rumen digestion and metabolism. Ruminal microorganisms could transform carbohydrates (e.g. crude fiber, starch and soluble sugar) to pyruvic acid, which could be transferred into different VFAs by metabolic pathways. Many researches have been conducted to verify that VFAs produced from the rumen could provide 50-80% energy needed by ruminants [33]. Our result showed that individual VFA and TVFA contents of maize stover were significantly higher than that of rice straw, it might result from the differences of fiber content and its continents, starch content and other carbohydrates between two fermentation substrates, and then affected the microorganism activities or the activation of microbial enzyme to alter fermentation model. Wang et al.[34] reported that dietary supplementation of *B. subtillis natto* to lactating cows trended to decrease ruminal A:P. In this study, the A:P was not affected by different supplementation levels of *Bacillus coagulans* for both fermentation substrates, it might cause by the utilization of various bacterial stain and differences between *in vivo* and *in vitro* experiments. Additionally, the significant differences of A:P between two fermentation substrates when supplemented with *Bacillus coagulans* might result form or FROM difference in composition of carbohydrate between maize stover and rice straw.

The results concluded that, *Bacillus coagulans* numerically increased IVGP when crop straws were used as fermented substrates, and the optimal dose might be 0.75×10^7 cfu·mL^{-1}. Additionally, maize stover increased IVGP, the rate of gas production at early incubation stage, *in vitro* dry matter digestibility, *in vitro* neutral detergent fiber degradation and TVFA were increased when maize stover was supplemented with *Bacillus coagulans* compared to the results of rice straw. Finally, the present *in vitro* study should be repeated using *in vivo* experiments with different period in the future study.

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Research Article

Effect of Corn Distillers Dried Grains With Soluble With or Without Xylanase Supplementation in Laying Hen Diets on Performance, Egg Quality and Intestinal Viscosity

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Abstract

A 14-week experiment was conducted to evaluate the effect of Corn Distillers Dried Grains with soluble (DDGS) with or without xylanase on layers' performance and egg quality traits as well as intestinal viscosity. Four hundred and eighty Atak-S Brown laying hens (32-week-old) were randomly distributed among the eight dietary treatments with six replicates of ten birds each. There was no significant (P>0.05) interaction between DDGS levels and enzyme supplementation for any of the studied parameters (P>0.05). The addition of either 0.0, 10.0, 20.0 or 30.0% DDGS to the diet had no significant (P>0.05) effect on egg production, egg weight, feed intake, feed conversion ratio, body weight gain, livability, eggshell braking strength, eggshell thickness, eggshell ratio, egg albumen height and haugh unit. The addition of any level of DDGS positively affected egg yolk color. On the other hand, 30% of DDGS in the diet caused a significant (P<0.05) decrease in egg mass. The supplementation of enzyme to diets significantly (P<0.05) increased the percentage of egg production. In addition, enzyme supplementation resulted an overall reduction in intestinal viscosity. So, it can be concluded that up to 20% DDGS can be used in laying hen nutrition without adversely affecting any of performance and egg quality parameters, besides xylanase based enzyme supplementation could improve egg production and decrease intestinal viscosity regardless of the inclusion rate of DDGS.

Keywords: *Laying hens, DDGS, Xylanase, Egg production, Egg quality, Viscosity*

Kurutulmuş Damıtma Çözünürlü Taneleri İçeren Yumurta Tavuğu Yemlerine Ksilanaz Enzim İlavesinin Performans Yumurta Kalitesi ve Bağırsak Viskozitesi Üzerine Etkisi

Öz

Ondört hafta yürütülen çalışmada farklı oranlarda mısır damıtma çözünürleri kurusu (DDGS) içeren yumurtacı tavuk yemlerine ksilanaz enzim ilavesinin performans, yumurta kalitesi ve bağırsak viskozitesi üzerine etkisi değerlendirilmiştir. Çalışma 32 haftalık yaşta 480 adet ATAK-S kahverengi yumurtacı tavuk kullanılarak 6 tekerrürlü ve her bir alt grupta 10 adet tavuk olmak üzere 8 grupta tesadüf parselleri deneme düzeninde yürütülmüştür. Araştırma sonucunda yumurta tavuğu yemlerinde DDGS kullanım seviyeleri ile ksilanaz enzimi ilavesi arasında araştırmada incelenen performans yumurta kalitesi ve incebağırsak viskozitesi parametrelerinin hiçbirinde önemli bir interaksiyon tespit edilmemiştir (P>0.05). Ayrıca yemlerde farklı oranlarda DDGS bulunmasının yumurta verimi, yem tüketimi, yem değerlendirme sayısı, yumurta ağırlığı, yaşama gücü, canlı ağırlık yanında yumurta kabuk kırılma direnci, kabuk kalınlığı, kabuk oranı, albumen yüksekliği ve haugh birimi gibi yumurta kalite kriterleri üzerine de önemli bir olumsuz etkisi bulunmamışken (P>0.05), DDGS varlığı yumurta sarı rengini önemli oranda iyileştirmiştir (P<0.05). Ancak %30 DDGS seviyesi yumurta kütlesinin önemli düzeyde azalmasına sebep olmuştur (P<0.05). Ksilanaz enzimi uygulaması yumurta verimini önemli oranda artırırken (P<0.05), ince bağırsak viskozitesini de düşmüştür (P<0.05). Araştırmada yumurta tavuğu yemlerinde %20 seviyesine kadadr DDGS kullanımının yumurta tavuklarının beslenmesinde verim ve yumurta kalitesini olumsuz etkilemeksizin kullanılabileceği ve ksilanaz esaslı enzimin DDGS düzeyinden bağımsız olarak yumurta verimini önemli düzeyde artırabileceği, bağırsak viskozitesini ise düşüreceği sonucuna varılmıştır.

Anahtar sözcükler: *Yumurta tavuğu, DDGS, Ksilanaz, Yumurta verimi, Yumurta kalitesi, Viskozite*

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INTRODUCTION

Nowadays renewable energy has gained great importance as the population of the world and the energy demand increase today. Kyoto Protocols and the most recent Paris Protocols are calling for the use of clean, green and renewable transportation fuels to replace gasoline, diesel and jet fuel [1]. The production of biofuels is important as an alternative energy source to fossil fuels and having much concern because of low and competitive production costs and, being harmless to environment. Biofuels can be produced from dry distillers grains with solubles (DDGS), that are leftover from corn consumed in ethanol production, using a chemical process.

One of the main purpose of nutritionals researches is to minimize the cost of poultry production and increase profit by enhancing the utilization of nutrients in feeds. The rising trends in grain and soybean meal prices are putting pressure on poultry producers, thus the feed industry is looking for ways to reduce feed costs using new feed ingredients [2]. With growing demand for food, feed and short resources, the animal livestock industry does not have the luxury of letting anything go to waste. Maize-based dried distillers' grain with solubles (DDGS) is a byproduct obtained during the dry-milling process of maize to produce ethanol after the fermentation of maize-starch by selected yeasts [3]. As well as being potentially cost effective [4,5], maize DDGS are also a good source of energy, protein, vitamins and minerals [6,7]. There have been a number of recent studies done on the use of high quality maize DDGS in layer diets supporting that it is an excellent partial substitution for maize and soybean meal and supports high layer performance and egg quality [8]. On the other hand, there are certain anti-nutritional factors including high level of phytate and insoluble fibers such as arabinoxylans in DDGS which limit its use in poultry nutrition [9,10]. Insoluble fibres hold water building more bulk in the animal's gut, which can reduce feed intake and subsequent production [11]. As a result of the high amount of non starch polysaccharides contetnt, mainly arabinoxylans, the optimal inclusion level of DDGS in poultry diets for maximum production is controversial [12]. The supplementation of exogenous enzymes to animal feeds in order to improve nutrient digestion is not a new idea [13,14]. Most of the commercial enzyme products have been targeted for poultry [15,16] and are typically added to diets containing barley, wheat, oats, rye, or peas [11]. Nevertheless there are not many studiesevaluating xylanase based enzymes at the high level of DDGS inclusion in layin hen diets [17]. Therefore, the objective of this study was to examine if increasing the level of DDGS in diet with and without the addition of an enzyme complex would affect production parameters, egg quality and intestinal viscosity.

MATERIAL and METHODS

The animal care protocol used in this study was reviewed and approved by the Ethics Committee of the Poultry Research Institute Ankara, Turkey (21.01.09-2009.05)

The experiment was carried out at Ankara Poultry Research Institute. Four hundred and eighty ATAK-S laying birds of thirty two weeks of age were used. The experiment lasted for 14 weeks. ATAK-S is a commercial brown egg heavy hybrid bred in the Turkish Republic. The birds were placed in individually cages in the experimental house of the capacity of 480 cages. The house's temperature control system was set to maintain a daily minimum of 21°C at the middle tier level of the cages by controlling the ventilation rate. The house was windowless with artificial light (16 h light and 8 h dark; from 05:00 to 21:00 h light) by 40 Watt tungsten bulbs. The experimental cages were 25 cm wide, 47 cm high and 55 cm deep. Prior to the experiment, wheat, DDGS, corn, sunflower seed meal, soybean meal were analyzed for crude protein, ether extract and crude fiber using standard AOAC procedures [18], and diets were formulated based upon the obtained values. The results of the analysis showed that DDGS was composed of 92.4% dry matter, 22.80% crude protein, 11.5% ether extract, 12.3% starch, 1.7% sugar and 4.9% ash. The ingredients used and the calculated nutrient content of the diet formulations used in this study are shown in *Table 1*. The normal daily feed intake would be approximately 120 g/day for ATAK-S [19]. Based on the expected average feed intake, four diets were formulated. The diets were formulated as isocaloric (2750.00, 2751.00, 2750.00 and 2750.50 kcal/kg of ME) and isonitrogenous (16.42, 16.42, 16.43 and 16.41 CP). For all diets, nutrient specifications were set to meet or exceed National Research Council (NRC) nutrient requirements [9].

Before starting the experiment, a two-week period was allowed for the birds to adapt to the control diet (without enzyme supplementation). After that, egg production and egg weight were measured. Birds of similar body weight and egg production were then equally distributed to each replicate and treatment.

All diets contained 5% sunflower seed meal and 5% wheat. The experiment was designed according to the 4 × 2 factorial design, four inclusion levels (0, 10, 20 and 30%) of DDGS, without or with enzyme supplementation (0 and 400 g/tonne). Each of the eight treatments (60 birds per group) were randomly assigned to six replicates (10 birds per replicate). According to the manufacturer, the enzyme complex (Hostazym X250, Huvepharma, location) contained xylanase activity of 6500-7500 endo-pentosanase units/g with side-activities of cellulase, hemicellulase, amylase and protease. Diets, in mash form, were offered *ad- libitum* and water was freely available.

Table 1. *Composition of the experimental diets*

Ingredients Composition	Diets (g/kg)			
	Control	10% DDGS	20% DDGS	30% DDGS
Wheat	50.00	50.00	50.00	50.00
Sunflower seed meal, 28% CP	50.00	50.00	50.00	50.00
DDGS	0.00	100.00	200.00	300.00
Yellow corn	511.85	458.50	404.25	350.25
Soybean meal, 48% CP	243.50	199.50	155.50	111.00
DCP[1]	14.00	11.00	8.00	5.00
Limestone	95.00	96.35	98.00	100.00
Salt (NaCl)	4.00	4.00	4.00	4.00
Vegetable oil	27.00	25.50	24.50	23.50
Lysine HCL	0.20	1.00	1.90	2.70
DL-methionine	1.25	0.95	0.65	0.35
Vitamin/Mineral premix[2]	1.70	1.70	1.70	1.70
Salmonella inhibitor	1.50	1.50	1.50	1.50
Nutrient Composition				
Metabolizable energy (Kcal/kg)[3]	2750.00	2751.00	2750.00	2750.50
Crude protein, %[4]	16.42	16.42	16.43	16.41
Ether extract, %[4]	5.46	6.04	6.67	7.31
Crude fiber, %[4]	4.03	4.43	4.83	5.23
Calcium%[3]	3.90	3.90	3.90	3.92
Available phosphorus, %[3]	0.36	0.36	0.36	0.36
Metionine + Cystine, %[3]	0.66	0.66	0.66	0.66
Lysine, %[3]	0.88	0.88	0.88	0.88
Ash, %	13.84	13.83	13.85	13.89

[1] The composition of dicalcium phosphate provided the following amounts per kilogram of diet: Ca 23% and P20%
[2] Vitamin-mineral premix provided per kg of diet; vitamin A, 15.000 IU; vitamin D_3, 5.000 IU; vitamin E, 50 mg; vitamin K_3, 10 mg; thiamine, 4 mg; riboflavin, 8 mg; pyridoxine, 5 mg; vitamin B_{12}, 0.025 mg; niacin, 50 mg; Ca-pantothenate, 20 mg; folic acid, 2 mg; biotin, 0.25 mg; ascorbic acid, 75 mg; choline, 175 mg; Mg, 35 mg; Mn, 56 mg; Zn, 140 mg; Fe, 56 mg; Cu, 10.5 mg; I, 1 mg; Co, 0.28 mg; Se, 0.28 mg; Mo, 0.7 mg
[3] Based on NRC 1994 values for wheat, DDGS, corn, sunflower seed meal, soybean meal and vegetable oil
[4] Based on analysis of wheat, DDGS, corn, sunlower seed meal and soybean meal

The number of eggs laid by each bird was recorded daily. Also, eggshell defects including broken, cracked, leaking, soft-shelled eggs and misshapen eggs were determined daily for each cage throughout the experiment. Average daily feed consumption was determined by weighing feed in the morning of the first day, and weighing back in the next morning for each replicate every two weeks, considering the number of birds.

Similarly to feed consumption, egg weights were measured gravimetrically and recorded for each bird every two weeks. Egg mass (percentage egg production x average egg weight) and feed conversion ratio (average daily feed intake/egg mass) were also calculated to better evaluate overall hen performance. In addition, for the internal egg quality, twenty four randomly selected eggs from each treatment groups were collected every 4 weeks, and egg quality characteristics were determined 24 hours after collection of the eggs. Eggshell thickness was measured after peeling off the membrane under the shell with Mitutoyo digital micrometer gauge (digital 395 series with 0.001 mm sensitivity, Kawasaki, Japan) on three locations (broad, equator and sharp end) from the equatorial region of each egg, and calculated as an average value. Eggshell breaking strength, egg albumen height and haugh unit were measured by using Futura 3/A egg quality measuring system (Futura, Lohne, Germany). Yellowness of egg yolk was determined by CR-10 Konica Minolta Color Reader (Osaka, Japan). Body weight of the birds was measured individually at the beginning and at the end of the experiment, and then body weight gain was calculated. Moreover, mortality of each replicate was daily determined during the study for each cage.

For the viscosity analysis, the intestinal content of 4 hens from each group was taken into tubes at the end of the

experiment. While awaiting analysis, samples were placed on ice blocks within ice buckets. The samples were centrifuged at 3500 rpm when they reached room temperature. The liquid accumulating on top was taken by pipette, and the viscosity values were determined as centipoise (cPs) by Brookfield Viscosimeter (Model LVDVII + CP) with spindle No 42 at 40°C [20]. Crude fiber intakes were calculated from feed consumptions and from values obtained from analysis of raw materials (wheat, DDGS, corn, sunflower seed meal, soybean meal).

Statistical analyses of data were performed as a randomized block design, with a factorial arrangement of 4×2, taking into consideration main effects of DDGS levels (0, 10, 20 and 30%) and enzyme supplementation (with or without) with an equal number of 6 replicates for each treatment by using statistical software Minitab R Release 16.1.0. Significant differences were tested further using a Tukey's Honestly Significant Difference multiple range tests to determine the differences among treatments.

RESULTS

The results for egg production, egg mass, egg weight, feed intake, feed conversion ratio, body weight gain and livability of birds fed different levels of DDGS in diet with or without enzyme supplementation during the experiment are shown in *Table 2*. Significant interaction was not found between DDGS and the enzyme supplementation (P>0.05), therefore, primary attention was directed to the main effects of DDGS and the enzyme supplementation on egg production, egg mass, egg weight, feed intake, feed conversion ratio, body weight gain and livability.

The results for eggshell breaking strength, eggshell thickness, eggshell ratio, egg albumen height, haugh unit and egg yolk color of birds consuming different level of DDGS in diet with or without enzyme supplementation during the experiment are shown in *Table 3*. There was no significant interaction between DDGS and the enzyme supplementation, therefore main attention was directed to the effects of DDGS and the enzyme supplementation on eggshell breaking strength, eggshell thickness, eggshell ratio, egg albumen height, haugh unit and egg yolk color.

The results for gut viscosity of birds is shown in *Table 4*. There was no significant interaction between DDGS and enzyme supplementation for gut viscosity.

Table 2. *Effect of enzyme supplementation in laying hens diets including different levels DDGS on performance*

14 Weeks Period	Enzyme		SEM	DDGS Levels				SEM	P		
	Without Enzyme	With Enzyme		0	10%	20%	30%		E	D	ExD
Egg production (%)	86.0[a]	87.3[b]	0.4	87.1	87.2	86.3	86	0.6	0.0	0.1	0.5
Egg weight (g)	63.0	62.9	0.3	63.6	62.9	62.7	62.7	0.4	0.8	0.1	0.5
Egg mass (g/hen/day)	54.2	54.9	0.4	55.4[a]	54.8[ab]	54.2[ab]	53.9[b]	0.5	0.2	0.0	0.7
Feed intake (g/hen/day)	119.9	120.7	0.6	120.3	121.7	120.6	118.7	0.7	0.3	0.1	0.2
Feed conversion ratio (g feed/g egg)	2.2	2.2	0.0	2.2	2.2	2.2	2.2	0.0	0.6	0.3	0.8
Body weight gain (g)	249	262	15	272	224	287	240	20.8	0.5	0.8	0.1
Livability (%)	90.1	90.2	0.1	90.1	90.2	90.2	90.1	0.1	0.3	1.0	0.8

SEM values are pooled standard errors of mean for enzyme (n=24 replicate) and dietary (n=12 replicate) treatments
[a,b] Means in a row within the same treatment with different superscripts differ significantly (P<0.05)

Table 3. *Effect of enzyme supplementation in laying hens diets including different levels DDGS on eggs quality characteristics*

14 Weeks Period	Enzyme		SEM	DDGS Levels				SEM	P		
	Without Enzyme	With Enzyme		0%	10%	20%	30%		E	D	ExD
Eggshell breaking strength (Newton)	38.5	38.9	0.48	40.1	38.0	38.1	38.7	0.66	0.45	0.17	0.14
Eggshell thickness (10^{-2} mm)	319.4	319.1	1.58	322.3	316.3	318.6	319.7	2.18	0.87	0.51	0.07
Eggshell ratio %	8.9	9.0	0.03	9.0	9.1	8.9	8.9	0.05	0.98	0.85	0.40
Egg albumen height	7.5	7.5	0.07	7.4	7.6	7.4	7.5	0.09	0.83	0.72	0.49
Haugh Unit	85.4	85.3	0.42	84.8	85.9	85.0	85.6	0.59	0.93	0.52	0.70
Egg yolk color	12.2	12.1	0.06	12.0[a]	12.2[b]	12.3[b]	12.2[b]	0.06	0.36	0.01	0.11

SEM values are pooled standard errors of mean for enzyme (n=24 replicate) and dietary (n=12 replicate) treatments
[a,b] Means in a row within the same treatment with different superscripts differ significantly (P<0.05)

Table 4. *Effect of enzyme supplementation in laying hens diets including different levels DDGS on intestinal viscosity, crude fiber intake*

14 Weeks Period	Enzyme		SEM	DDGS Levels				SEM	P		
	Without Enzyme	With Enzyme		0%	10%	20%	30%		E	D	ExD
Intestinal viscosity cPs	1.53	1.38	0.05	1.53	1.44	1.40	1.46	0.07	0.04	0.42	0.81
Crude fiber intake (g/hen/day)	5.85	5.89	0.11	5.34^{a}	5.73^{b}	5.68^{b}	6.73^{c}	0.04	0.82	0.00	0.17

SEM values are pooled standard errors of mean for enzyme (n=24 replicate) and dietary (n=12 replicate) treatments
^{a,b} Means in a row within the same treatment with different superscripts differ significantly (P<0.05)

DISCUSSION

Our study showed that there were no significant effects of adding 0, 10, 20 or 30% DDGS into laying hen diets on egg production during the 14-week of the experimental period. This result is in agreement with earlier findings. Pineda et al.[21] examined the effect of increasing DDGS level (0, 23, 46, or 69%) on egg production, and reported that laying hens could be fed DDGS levels as high as 69% without adverse effects on egg production, but advised that all nutrients (e.g. amino acids) should be considered when formulating diets containing DDGS. Similar results were obtained by Masa'deh [22] who found that increasing DDGS level up to 25% did not affect negatively egg production. In contrast, Deniz et al.[23] found that the inclusion of 20% DDGS significantly (P<0.05) depressed egg production of layers. More recent studies showed that rising DDGS level up to 22% decreased egg production [12,17].

The overall egg production of birds with and without enzyme supplementation was 87.3% and 86.0%, respectively, representing a significant (P<0.05) difference of 1.3%, a result independent from DDGS levels. These results agree with the findings of Nelson [24] who stated that laying performance was improved by adding enzyme preparations containing a variety of enzyme. In our study, the beneficial effect of enzyme which has the high level of xylanase and low amounts of hemicellulase, α-amylase and protease activity- may have no effect on the added DDGS but work to improve the nutritive value of the constant ingredients (5% wheat and 5% sunflower seed) of the diet for laying hens. In addition, its cellulase content may be improve fiber digestibility.

Differences in egg weight in association with the different levels of DDGS content with or without enzyme supplementation to diet were not significant.

Egg mass production is defined as hen-day production multiplied by average egg weight for each replicate. In contrast to egg weight, the significant difference observed in egg mass between the groups fed the control or 30% DDGS diet was due to the numeric reduction in egg weight and rate of lay. However, there were no significant differences in egg mass between the groups with or without enzyme supplementation. This result is partly in agreement with the observation by Lumpkins et al.[25] who found that 15% DDGS in the basal diet has no significant impact on egg weight. Similarly, Roberson et al.[26] claimed that DDGS has no effect on egg weight and egg mass. In contrast, some studies found significant decrease in egg weight and egg mass due to the inclusion of 20% DDGS into the diet [8,27].

The results of the current study *(Table 2)* indicate that increasing DDGS level up to 30% and supplementing enzyme has no significant effect on feed intake and feed conversion ratio. Similarly to these findings, earlier publications showed that the inclusion of various levels (15%, 20% or 25%) of DDGS in diet causes no significant difference in feed intake of hens [8,23,25,26]. In contrast, Deniz et al.[22] reported that the addition of 20% DDGS into the layers' diet significantly depresses feed intake (P<0.05). Some studies suggest that feed conversion is also not affected negatively by DDGS [26] while contradictive results were also reported showing reduced feed conversion (P<0.05) by the inclusion of 20% of DDGS compared to lower levels [22]. The authors attributed this reduction to the decreased percentage of laying rate and egg weight at this inclusion level of DDGS. However, in the present study differences observed in egg production and egg weight in association with the different levels of DDGS in diets were not significant among the groups. In addition, enzyme supplementation increased overall egg production (P<0.05). Body weight changes were calculated from values measured before and after the experiment. The results did not demonstrate a pattern with respect to increasing DDGS level and enzyme supplementation. Mean changes in body weight were positive but the relatively high standard error of means at the same time suggests that the body weight of many of the birds responded differently in each treatment. Wide variations in body weight change are, however, not surprising for mature laying hens [28]. Findings in this experiment on body weight change are in accord with the literature [17,23,25,29-31]. More recently noticed that inclusion of various levels of DDGS in the diet did not exert any detrimental (P>0.05) effect on final body weight and body weight change [8]. In addition, there were no significant differences of livability as a result of the inclusion of DDGS with or without enzyme supplementation.

Both the findings of this study and the literature suggest that DDGS does not affect shell strength. Differences observed in eggshell breaking strength, eggshell thickness, eggshell ratio in association with the increasing level of DDGS and with or without enzyme supplementation were not significant among the DDGS and enzyme groups. Several authors reported similar results [25,27,32,33], although some reported increased shell thickness [34], while others observed negative effect on shell thickness [35] in association with increasing DDGS level in the diet.

Similar to eggshell quality parameters, no significant differences were observed in egg albumen height and haugh unit in association with the increasing level of DDGS and with or without enzyme supplemented diet consumed by birds. Masa'deh [23] suggested that high inclusion levels of DDGS caused no significant differences in haugh units among the different treatments. Furthermore, Deniz et al.[22] stated that feeding laying hens on DDGS up to 15% with or without enzyme supplementation had no adverse effects on both exterior and interior egg quality criteria.

In contrast to eggshell breaking strength, eggshell thickness, eggshell ratio, egg albumen height and haugh unit, there were significant differences in egg yolk color between the control and the DDGS groups regardless to enzyme supplementation. Intensified yolk color was expected. Because DDGS contains high concentrations of xanthophylls, which is responsible for the yellow color of yolk. However, the increase in yolk color was not linear and there were no significant differences among the DDGS groups. This finding was partly in agreement with the finding of Masa'deh [23] who explained that egg yolk color was linearly increased (P<0.001) as dietary level of DDGS increased throughout the study. Also, El-Hack and, Mahgoub [17] pointed out that yolk color density increased as the increasing DDGS level.(basal diet or diets including 5%, 10% and 15% DDGS). In contrast, Roberts et al.[29] noted that 10% DDGS had no effect on yolk color, and Lumpkins et al.[25] found no improvement in yolk color when 15% DDGS was used in the diet. Xanthophyll pigments are susceptible to light and heat damage, thus the observed different effects on yolk color from different studies might have been related to the different xanthophyll content in DDGS sources. Xanthophyll content can vary in DDGS because of heat demolition during drying [36].

Differences observed in gut viscosity in association with the increasing level of DDGS and with or without enzyme supplemented diet consumed by birds were not significant among the DDGS (P>0.05) but in enzyme groups (P<0.05). Viscosity reducing effects of xylanase based enzymes in the gut have been well documented in laying hens. Increased nutrient absorbtion because of decreased gut viscosity by enzyme supplementation could be possible reason for the improvement in the egg production (P<0.05) in the present study *(Table 2)*. This study showed that inclusion of 20% DDGS in a diet could be the upper feeding limit for laying hens. As most of the starch is removed from corn during ethanol production, the resultant co-product, dried distillers grains with solubles (DDGS), contains concentrated levels of protein, minerals, and fiber [37-39]. DDGS is linked to the high level of indigestible fiber components present in corn DDGS. Our results *(Table 4)* show that when DDGS inclusion reaches 30% in the feeds, the amount of calculated fiber intake increased by 26% (6.73 g/hen/day) compared to the (P<0.05) hens received the diets without DDGS (5.34 g/hen/day). The increase in fiber intake is also accompanied with a decrease in egg mass. It appears that the use of specific enzyme preparations in this study containing mainly xylanase to target the DDGS diet and its NSP (non-starch polysaccharides) components has been partly successful. This could be due to the fact that in ethanol plants "viscosity-reducing" enzymes are used to facilitate the fermentation process. In this context, the "viscosity-reducing" enzyme preparation would contain appreciative amounts of xylanase, glucanase and cellulase activities which would contribute to a significant NSP depolymerization during the fermentation process. Thus the clear effects of enzyme supplementation in DDGS diets for all studied parameters other than gut viscosity and egg production could be hidden by presence of some enzyme activities originated from the fermentation process in ethanol production. As DDGS is a corn co-product and, as mentioned earlier, the majority of commercial enzyme products are typically added to diets containing barley or wheat, to date there is little indication of success from the development of enzyme preparations specific to corn/soybean diets [40]. Therefore, targeting the indigestible components specific to DDGS with the correct blend of supplemental carbohydrase enzymes may allow for greater inclusion of DDGS into poultry diets and thus overall profitability is improved significantly.

This experiment investigated the effects of increasing level of DDGS in diet with and without the addition of an enzyme complex on production parameters, egg quality and intestine viscosity. The potential effects of enzyme and maximum inclusion level of DDGS for laying hen diets have been demonstrated. The data suggest that the use of DDGS up to a level of 20% in the diet of brown egg hens is possible without causing any problem on the performance and egg quality parameters, and additional contribution can be arises from the use of enzyme. The results of the present study showed that xylanase based enzyme supplementation could improve egg production and decrease intestinal viscosity regardless of the inclusion level of DDGS. However, layers' performance can be negatively affected (reduction in egg mass) with inclusion levels of 30% DDGS in diets due to the high fiber

content. In addition, the use of DDGS in the diets of laying hens offers the possibility of improvement in yolk colour at even the lowest level applied in this experiment. Therefore, the inclusion of DDGS in layers' diets might be cost saver to producers if attention is given to nutrient balance.

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Research Article

Immunohistochemical and Molecular Detection of *Mannheimia* spp. and *Pasteurella* spp. in Sheep with Pneumonia in Kars Province - Turkey [1] [2]

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Abstract

In this investigation, presence of *Mannheimia haemolytica* and *Pasteurella multocida* in sheep brought to Kafkas University Faculty of Veterinary Medicine between 2011 and 2013 with a suspicion of pneumonia was studied by immunohistochemical and bacteriological methods. Study materials were 100 sheep lungs. After routine histopathological investigations lungs were immunohistochemically stained with antibodies prepared against both of the agents. In bacteriological investigations, tissue samples were inoculated into 7% sheep blood agar and Mc Conkey agar and incubated for 24-48 h in aerobic conditions, and the suspected colonies were evaluated for *Mannheimia* and *Pasteurella* species. In histological investigations, pneumonias were classified as 28% fibrinous bronchopneumonia, 21% prulent bronchopneumonia, 20% acute-catharal bronchopneumonia, 18% interstitial pneumonia, 11% verminous pneumonia and 2% adenomatous pneumonia. In bacteriological investigations *Mannheimia haemolytica* and *Pasteurella multocida* was detected in 19 and 3 cases, respectively, these numbers were 17 and 2, respectively in immunohistochemical investigations. The results of PCR analysis for both *Mannheimia haemolytica* and *Pasteurella multocida* paralleled with the findings of microbiological culture. The results of the study showed that *Mannheimia haemolytica* is an important cause of pneumonia in sheep in Kars, and *Pasteurella multocida,* although with lesser importance can cause pneumonia in this species.

Keywords: *Mannheimia haemolytica, Pasteurella multocida, Pneumonia, Sheep, Immunohistochemistry*

Kars İlinde Pnömonili Koyunlarda *Mannheimia* spp. ve *Pasteurella* spp.'nin İmmunohistokimyasal ve Moleküler Tanımlanması

Öz

Bu çalışmada Kafkas Üniversitesi Veteriner Fakültesine 2011-2013 yılları arasında pnömoni şüphesi ile getirilen koyunların akciğerlerinde *Mannheimia haemolytica* ve *Pasteurella multocida* etkenlerinin varlığı immunohistokimyasal ve bakteriyolojik yöntemlerle araştırılmıştır. Çalışma materyalini pnömoni şüpheli 100 adet koyun akciğeri oluşturdu. Akciğer doku örnekleri rutin histopatolojik incelemelerin ardından her iki etkene karşı hazırlanan antikorlar kullanılarak immunohistokimyasal olarak boyandı. Bakteriyolojik incelemeler için alınan örneklerin %7 koyun kanlı agar ve Mc Conkey agara ekimleri yapıldı. Aerobik koşullarda 24-48 saat inkübasyonu takiben şüpheli koloniler *Pasteurella* ve *Mannheimia* türleri açısından incelendi. Histopatolojik incelemeler sonucunda pnömonilerin %28'i fibrinli bronkopnömoni, %21'i prulent bronkopnömoni, %20'si akut-kataral bronkopnömoni, %18'i intersitisyel pnömoni, %11'i verminöz pnömoni ve %2'si adenomatöz pnömoni olarak sınıflandırıldı. Bakteriyolojik incelemeler sonucunda 19 koyunda *Mannheimia haemolytica* ile 3 vakada *Pasteurella multocida* tespit edilirken bu oranlar immunohistokimyasal incelemelerde sırasıyla 17 ve 2 olarak belirlendi. PZR analizleri hem *Mannheimia haemolytica* hem de *Pasteurella multocida* için mikrobiyolojik analizler ile paralellik gösterirdi. Çalışmanın sonucunda Kars ilindeki koyun pnömoni vakalarında *Mannheimia haemolytica*'nın önemli derece yer aldığı buna karşın *Pasteurella multocida*'nın da az sayıda olmakla beraber pnömoni etkeni olarak görev yaptığı belirlendi.

Anahtar sözcükler: *Mannheimia haemolytica, Pasteurella multocida, Pnömoni, Koyun, İmmunohistokimya*

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INTRODUCTION

Respiratory system diseases are common in sheep, and cause important economic losses due to excessive treatment costs, drop in yield, and death [1-4]. Besides various bacterial and viral infectious agents, various stress factors causing hygiene and sanitation problems such as insufficient forage and housing conditions, changes in climate, early weaning, and improper transportation play roles in development of pneumonia [5-8]. Respiratoric mannheimiosis, also known as pneumonic pasteurellosis, is caused primarily by *Mannheimia haemolytica* and *Pasteurella multocida,* and commonly seen in ruminants [9]. *M. haemolytica* is also known as the causative agent of enzootic pneumonia and septicemia in lambs and kids [10,11]. These infectious agents are within the normal flora of ora-nasopharynx, and with the help of predisposing factors and some viral agents can cause pneumonia [12-14]. It is referred that deadly fibrinous lobar or bronchopneumonia is caused chiefly by *M. haemolytica* while *P. multocida* is responsible from the less severe fibrinopurulent bronchopneumonia, though this is not always true [12].

In epidemiologic studies conducted on sheep in Turkey, *M. haemolytica* was detected at 37.3% in Kars [15], 12.5% in Şanlıurfa [16], 11.3-12.7% in Van [17,18], 56.1% in Konya [19], and 2.3% in Elazığ [8]. *P. multocida* was also detected in sheep that showed pneumonia symptoms at 11.2% in Kars [20], 31.6% in Şanlıurfa [16], 10.52% in Konya [19], and 4.3% in Elazığ [8].

The results of the studies in worldwide have been reported different infection rate for *M. haemolytica* as 13% in Norway [21], 21.9-98.7% in Ethiopia [22,23], 4% in Western United States [24] and 25-66.9% in Ethiopia for *P. multocida* [22,23].

In diagnosis of pneumonic pasteurellosis, microbiological (culture and serology) and immunohistochemical methods are commonly used. PCR, which can be applicable on both isolates and tissue samples, is also now often used to detect bacteriological agents. Microbiologic culture technique is quite time consuming and bears the risk of sample contamination [25] while serological tests shows high cross-reaction. On the other hand PCR was suggested to be more sensitive, specific and faster method compared to the other conventional techniques [8,24]. Immunohistochemical detection of the bacterial agents in sections prepared from paraffin tissue blocks was also suggested to be quite successful [25-29].

The purpose of this study is to detect the bacterial agents of *M. haemolytica* and *P. multocida* by the methods of PCR and immunohistochemistry in sheep that show signs of pneumonia. It is also aimed to investigate the extent of pathological changes takes place, the localization of the bacterial agents in tissues, and the correlation between the bacterial presence and the lesion severity.

MATERIAL and METHODS

The study material was 100 lung samples collected from sheep that showed signs of pneumonia at gross examination. All tissue samples were collected at Kafkas University Faculty of Veterinary Medicine. Ethics of the study was confirmed by Kafkas University Laboratory Animals Local Ethics Committee (KAÜ-HADYEK 2011-43).

Polyclonal Antibody Production

For antibody production 10 week old female New Zealand rabbits weighing approximately 2.5 kg were used. Pure colonies of *P. multocida* and *M. haemolytica* were grown in nutrient broth. Pure cells of both bacteria provided by Kafkas University Department of Microbiology were washed several times with sterile water (4500 rpm for 10 min) and suspended in phosphate saline buffer (PBS, pH 7.2). The inoculum containing 10^9 cfu/mL was treated for 30 min at 90°C in a water bath. The immunization was performed subcutaneously (200 µL for per injection site) using 1.0 mL of fresh bacterial cells introduced at five different locations of the rabbit's body. The immunization scheme was shown in *Table 1*. The sera were centrifuged to eliminate the red blood cells and heated at 56°C for 1 h in a water bath. The sera were stored frozen at -20°C in 1.5 mL Eppendorf tubes. The obtained antiserums were purified using a protein-A column (ABICAP, Merck, Germany) to extract the IgG fraction. After purification, the antibodies were neutralized using NaOH (0.5 M) to achieve pH 7.0.

Bacteriological Investigations

Lung tissue samples collected from sheep with pneumonia were inoculated onto sheep blood agar (Oxoid, CM0271) and Mc Conkey agar (Merck, 105465). Samples were incubated at aerobic environment for 24-48 h, and the suspected samples were further investigated for *Pasteurella* and *Mannheimia* species based on the characteristics of colony morphology, hemolysis, oxidase, catalase and indole activities and growth capabilities on Mc Conkey agar plates [30,31].

Pathological Investigations

Lung tissue samples collected at systemic necropsy were fixed in 10% neutral buffered formaldehyde solution and

Table 1. *Immunization and application scheme of rabbits for polyclonal antibody production*

Day Intervals	Applications
Day 0	Pre-immunisation bleed and initial antigen injection
Day 14-21	First antigen booster
Day 28-35	Second antigen booster
Day 35-42	Test bleed
Day 42-56	Third antigen booster
Day 49-66	Blood collection

embedded in paraffin. Following routine procedures, tissue sections at 5 µm were stained with hematoxylin and eosin and investigated under light microscope.

Immunohistochemical Investigations

Avidin-biotin-peroxidase method (Histastain® Plus Broad Spectrum, Invitrogen Cat No: 859043) with diaminobenzidine substrate (DAB-Plus Substrate Kit, Invitrogen Cat No: 002020) color development with hematoxylin background staining was used for immunohistochemical staining (IHC) in lung tissue sections. Antigen retrieval was accomplished with microwave treatment in Citrate solution. Primer antibodies were diluted at 1:50 dilution with phosphate buffer saline and the sections were incubated with them at room temperature for 1 h. Other procedures were completed routinely and the sections were observed under a light microscope for presence of positive immunoreactivity and the extent of it.

PCR Analysis

Primers designed for PCR amplification of *P. multocida toxA* and *M. haemolytica sodA* genes were used in molecular detection of the bacteria. Total DNA was isolated from the suspected lung tissue samples as follow: tissue samples were homogenized with a homogenizer and then the homogenates were suspended with Tris EDTA (TE) buffer solution and centrifuged at 9000 g for 10 min. Pellets were suspended with TE buffer, and then incubated with lysis buffer (10 mg/mL Proteinase K, 1 M Tris-HCl, 0.5 M EDTA, 10% SDS) at 55°C overnight. DNA isolation was accomplished with phenol-chloroform-isoamyl alcohol. To precipitate DNA, 3M sodium acetate and cold absolute ethanol was added onto the samples and let overnight at -20°C. Finally the samples were centrifuged at 5400 g for 30 min and the pellets were suspended with distilled water. DNA concentration in samples was measured spectrophotometrically at 260 nm.

PCR amplification of *P. multocida toxA* gene was accomplished according to the method described by Kamp et al.[32] with some minor modifications. A PCR sample size of 50 µL contained 100 ng DNA sample, 10 mM Tris:HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 M forward (5′-GGTCAGATGATGCTAGATACTCC-3′) and reverse (5′-CCAAACAGGGTTATATTCTGGAC-3′) primers, and 10 U/mL Taq DNA polymerase. PCR conditions for *P. multocida toxA* gene was accomplished in PCR conditions of initial denaturation at 95°C for 5 min followed by 32 cycles of denaturation at 95°C for 30 sec, hybridization at 65°C for 1 min, and synthesis at 72°C for 2.5 min. A final was extension at 72°C for 20 min was applied.

M. haemolytica sodA gene amplification was performed according to the method described by Guenther et al.[33] with some modifications. The PCR sample size of 50 µL was composed of 100 ng DNA sample, 10 mM Tris:HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 M forward (5′-AGCAGCGACTACTCGTGTTGGTTCAG-3′) and reverse (5′-AAGACTAAAATCGGATAGCCTGAAACGCCTG-3′) primers, and 10 U/mL Taq DNA polymerase. PCR cycling procedure were as follow; initial denaturation at 94ºC for 5 min, 30 cycles of denaturation at 94ºC for 1 min, annealing at 56ºC for 1 min, and elongation at 72ºC for 2 min. A final elongation step was performed at 72ºC for 5 min.

All of the PCR products were run in 2% agarose gels containing ethidium bromide and the PCR product bands of 338 bps for *P. multocida toxA* gene and 327 bps for *M. haemolytica sodA* gene were visualized with ultraviolet transilluminator.

RESULTS

Bacteriological Findings

In bacteriological examination, suspicious 20 colonies on blood agar plates that were small, glistening, mucoid and dew-drop like and appeared as Gram negative cocobacilli when stained with Gram stain were further investigated. Out of these colonies 3 (3%) were identified as *P. multocida* and 19 (19%) were *M. haemolytica,* based on the phenotypic characteristics (hemolysis, oxidase, catalase and indole reactivity and growth capabilities on Mc Conkey agar plates).

Gross Necropsy Findings

Total of 100 lung tissue samples that were recognized as pneumonic were investigated in the study. In most of the cases, dark red to gray consolidated firm areas were recognized in the cranioventral lobes of lungs. In some cases, lobar pneumonia, which included the great portion of the lobes with white-gray necrotic areas, was recognized. In verminous pneumonia cases, lesions were seen in the caudal lobes. In acute catarrhal bronchopneumonia cases the lesions were accompanied by hyperemia and edema in the lungs and exudate in the bronchi.

The lungs were not collapsed and the rib traces were found on the lung surface in the cases of interstitial pneumonia. In adenomatous pneumonia cases, the lungs were observed enlarged several folds normally and became heavier and a white marble appearance was detected in the lung pleura. In the cases of verminous pneumonia, caudal lobes were gray colored and had thick nodules. In addition, red colored, atelectatic areas and interstitial emphysema were also seen.

Histopathological Findings

In microscopic examination, the cases were categorized as acute catarrhal bronchopneumonia, purulent bronchopneumonia, fibrinous pneumonia, interstitial pneumonia, verminous pneumonia, and adenomatous pneumonia. Numbers of cases according to the histopathological evaluation were shown in *Table 2*.

Table 2. *Numbers of cattle according to the type of pneumonia, and the cases with M. haemolytica and P. multocida detected with immunohistochemical staining, bacteriological culture methods and PCR*

Pneumonia	Total Number of Cases	Bacteriology		IHC		PCR	
		P. multocida	*M. haemolytica*	*P. multocida*	*M. haemolytica*	*P. multocida*	*M. haemolytica*
Acute Catarrhal Bp	20	-	-	-	-	-	-
Purulent Bp	21	1	1	-	2	1	1
Fibrinous Bp	28	2	16	2	15	2	16
Interstitial Pneumonia	18	-	2	-	-	-	2
Verminous Pneumonia	11	-	-	-	-	-	-
Adenomatous Pneumonia	2	-	-	-	-	-	-

Bp: *Bronchopneumonia,* ***IHC:*** *immunohistochemistry*

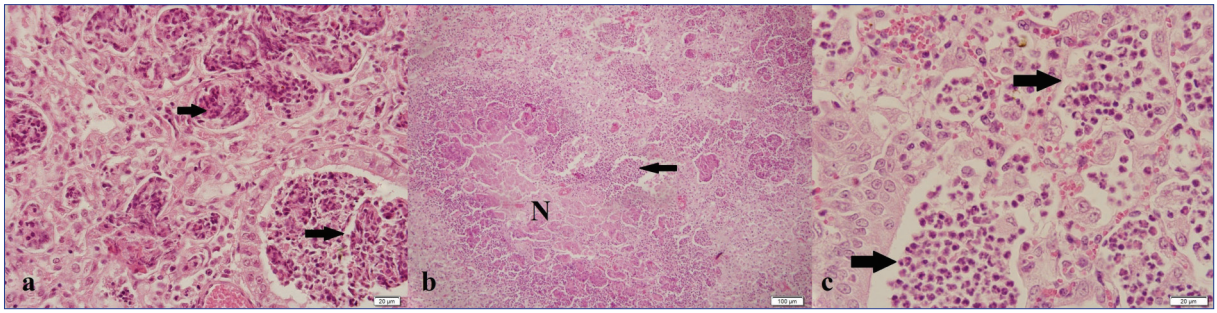


Fig 1. a) Oat cells *(arrows)* in the alveolar and bronchiolar lumens, bar = 50 µm, **b)** Necrosis (N) with numerous mononuclear cellular infiltration *(arrow)* in a case, bar = 100 µm, **c)** A large number of neutrophil granulocytes *(arrows)* in the alveolar and bronchiolar lumen in a case of purulent bronchopneumonia, bar = 20 µm, Hematoxylin-eosin

In fibrinous pneumonia cases, classical red and gray hepatisation phases were observed. Leucocyte infiltration in alveolar and bronchial lumens, hyperemia in alveolar capillaries, fibrin exudation in alveolar lumens and interlobular septa were seen in these cases. In 7 cases of fibrinous bronchopneumonia, spindle shaped leucocytes were also noted in the lumens of alveoli *(Fig. 1a)*. In 10 cases necrotic changes accompanied fibrinous lesions and were named as fibrinonecrotic bronchopneumonia. In these cases, foci of coagulation necrosis that were surrounded by inflammatory cellular infiltration were recognized *(Fig. 1b)*. In some cases, multifocal necrotic bronchitis and bronchiolitis were determined.

Purulent bronchopneumonia cases were characterized by predominant neutrophil infiltration in alveolar lumens *(Fig. 1c)*. In 6 cases, multifocal necrosis accompanied purulent lesions. In interstitial pneumonias, widening in interalveolar septa due to mononuclear cellular infiltration with occasional lymphocyte infiltration around the bronchia and bronchioles were seen.

Capillary hyperemia and neutrophil infiltrations in the alveolar lumen were detected in acute catarrhal bronchopneumonia cases. In the cases of interstitial pneumonia, thickness of the interalveolar septa and hyperplasia of bronchus, bronchiole and lymphoid tissue around the veins were striking. In adenomatous pneumonia cases, alveoli were placed with cubic epithelium and the papillary projections extending to the lumen was determined. In verminous pneumonia cases, hyperplasia in the smooth muscle layer of parenchymal pneumonia and bronchiolitis were seen. Granulomatous structures around the dead larvae and eggs have been identified.

Immunohistochemical Findings

Number of cases showing immunoreactivity for bacterial antigens was given in *Table 2*. Immunoreactivity for *M. haemolytica* antigens was detected in 17 out of 100 cases (17%). 15 animals with fibrinous bronchopneumonia showed positive immunoreactivity including all of the fibrinonecrotic bronchopneumonia cases. The ratio of animals with positive reaction within the total number of 28 fibrinous bronchopneumonia cases was 53.5%. Other 2 cases with positive immunoreactivity were seen in animals having purulent necrotic bronchopneumonia. This yielded a ratio of 9.5% within the purulent bronchopneumonia cases. Immunoreactivity against *M. haemolytica* antigens was observed in the cytoplasm of pneumocytes and the epithelial cells of bronchi and bronchioles, between the oat cells located in the alveoli, cytoplasm of macrophages, and the peribronchiolar glands *(Fig. 2a,b)*. Positive immunoreactivity was also noted around the foci of coagulation necrosis. In cases with necrotic purulent bronchopneumonia, positive immunoreactivity against *M. haemolytica* antigens were observed in the cytoplasm of leucocytes located in the lumens of alveoli, epithelial cells of bronchi and

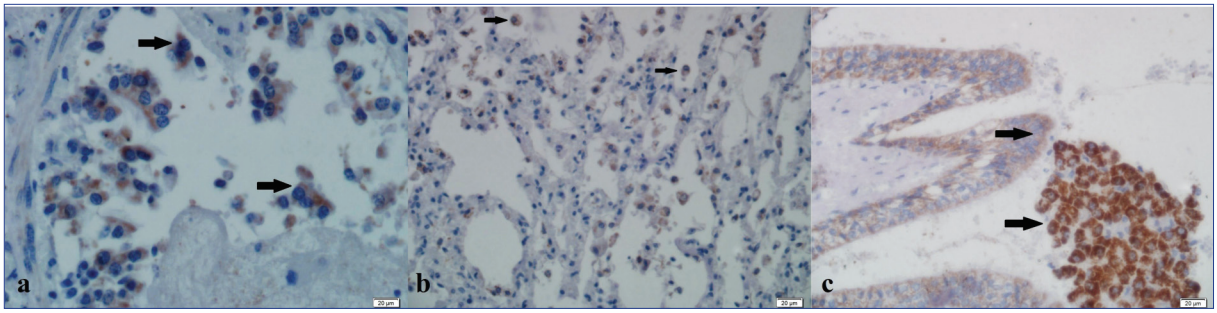


Fig 2. a) Immunopositivity against *M. haemolytica* antigens in spilled bronchiol epithelial cell cytoplasm (arrows), bar = 20 µm, **b)** Immunopositivity against *M. haemolytica* antigens in alveolar macrophage cytoplasm *(arrows)*, bar = 20 µm, **c)** Immunopositivity against *P. multocida* antigens *(arrows)* in spilled and intact bronchial epithelial cell cytoplasm, bar = 20 µm, Immunohistochemistry

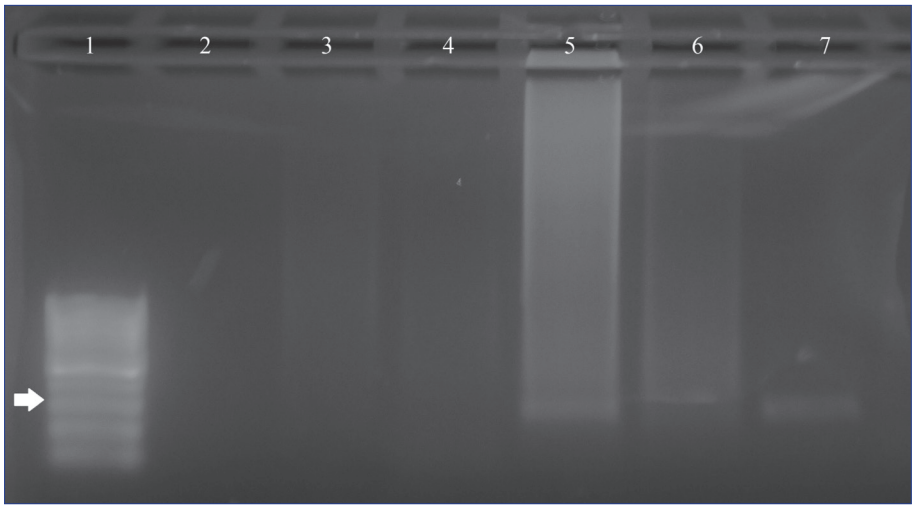
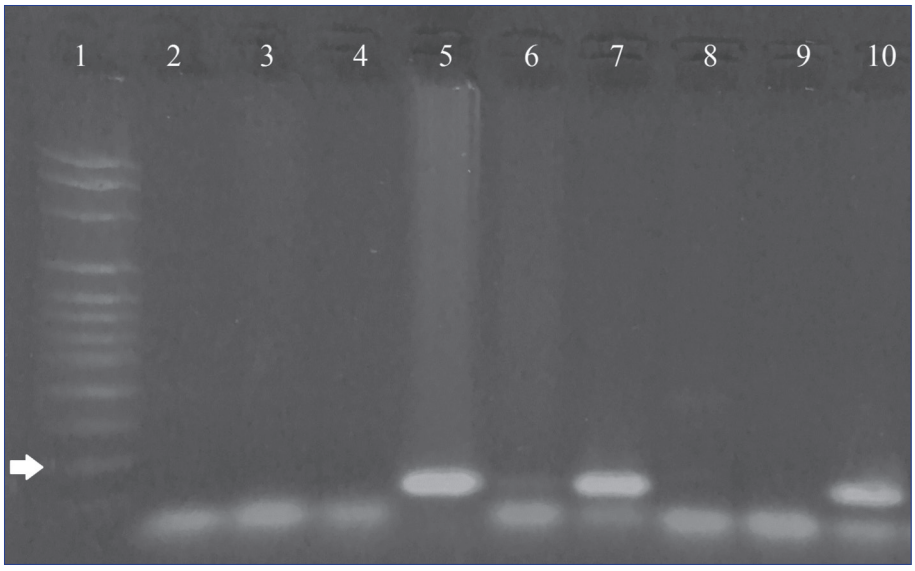


Fig 3. PCR analysis of *P. multocida* isolates from sheep showing 338 bp lanes for *toxA gene*. Lane 1: 100 bp DNA ladder, Lane 2: Negative control, Lane 3-6: Samples, Lane 7: Positive control

Fig 4. PCR analysis of *M. haemolytica* isolates from sheep showing 143 bp lanes for *sodA gene*. Lane 1: 100 bp DNA ladder, Lane 2: Negative control, Lane 3-9: Samples, Lane 10: Positive control



bronchioles, and around the necrotic foci. Positive immunoreactivity against *P. multocida* antigens was detected only in 2 cases with fibrinous bronchopneumonia. The ratio of positive immunoreactivity among the purulent bronchopneumonia cases was detected to be 7.1%. The immunostaining pattern for *P. multocida* antigens *(Fig. 2c)*, in general, resembled to that of *M. haemolytica* antigens.

PCR Analysis

PCR amplification of *P. multocida toxA* and *M. haemolytica sodA* genes yielded DNA product bands of 338 bps and 143 bps, respectively. Out of 100 cases, 19 were detected to be positive for *M. haemolytica (Fig. 3)* and 3 for *P. multocida (Fig. 4)* with PCR analysis.

DISCUSSION

Pneumonia in sheep and lamb is one of the most important problems in breeding, and pneumonic pasteurellosis is the commonly recognized cause of pneumonia in small ruminants. *P. multocida* and *M. haemolytica* are the bacterial agents of the disease, and cause drop in yield and death, resulting significant economic losses [25,27]. These bacterial agents are also often reported in pneumonia cases in Turkey [8,15,18,19]. *P. multocida* and *M. haemolytica* are normally found in the nasopharynx and the upper respiratory system, and as the immune barrier of the respiratory system fails become active causing pneumonia. *M. haemolytica* induced pulmonary infections are mostly recognized with peracute-acute fibrinosuppurative and necrotic inflammatory response [1].

In studies performed in Turkey aiming the detection of pasteurella species in sheep pneumonia, *M. haemolytica* was recorded between 2.3% and 51.6% [7,17,34,35] while *P. multocida* was seen between 2.9% and 31.6% [7,16,35]. These ratios were seen to change greatly with the ratios of these bacteria detected around the world. Using different detection techniques, *M. haemolytica* was recorded to be between 4% and 98.7% [21-24], and *P. multocida* between 25% and 66.9% [22,23]. A variety of different detection tools, sample collection methods, geographical locations etc. might be in effect for different results besides the actual presence in a location. In the present study, *M. haemolytica* was detected in 19% while *P. multocida* was seen in only 3% of the cases. As compared to the previous investigations, isolation rate of the bacterial agents were in the low end of the scale, probably reasoning to the differences in housing conditions, climate, transportation factors, age, breed and the season studied.

Bacterial culture technique is still the golden standard for detection of *Pasteurella* species though some problems in the process of the technique such as long time requirement in isolation and identification and the need for some special transport media [36] are present. In the current investigation, out of 100 cases 19 were detected to have *M. haemolytica*, and 3 to *P. multocida*, by bacteriological culture technique. In comparison with the results of immunohistochemical investigation, in 1 case no bacterial agents were detected where immunopositive immunoreactivity was seen against *M. haemolytica* antigens in 2 cases of purulent necrotic bronchopneumonia. Sampling from the different areas of the lung samples might be the cause of this difference in the results.

PCR is a commonly used technique in molecular detection of infectious agents. It has high specificity and sensitivity in general compared to the other detection techniques. It has also been reported that PCR is more rapid and specific in determination of *P. multocida* and *M. haemolytica* directly from specimens compared to the standard technique of bacteriological culture [37]. In the current investigation, the bacterial agents were detected in total of 22 cases, from which the bacterial agents were also detected to be positive by bacteriological culture technique. Therefore the results of two detection methods were in complete agreement with each other.

In the present investigation, lung lesions in *M. haemolytica* caused pneumonia mostly showed cranioventral localization. Less frequently, both cranial and middle localization presenting lobar or lobular distribution was seen. In few cases, whole cranial and/or middle lobe or 1/3 of the caudal lobe was affected. This pattern of distribution in *M. haemolytica* caused pneumonia was in accordance with the previous reports [25,28,35].

The results of the immunohistochemical staining and the bacteriological culture technique were in great agreement, having 17 immunopositivity as compared to the 19 with bacteriological culture. The remaining two culture positive samples that were found IHC negative might be explained by the different sampling area and the focal accumulation of bacteria in the lungs. In the present study, *M. haemolytica* positive immunoreactivity was detected in 15 cases with fibrinous bronchopneumonia out of 28 cases. This result paralleled the results of previous investigations, indicating that *M. haemolytica* causes mostly fibrinous or fibrinonecrotic types of pneumonia [1,25]. It has also been noted that the localization of the bacterial antigens correlated the histopathological changes. In tissue sections, positive immunoreactivity against bacterial antigens was observed in the cytoplasm of leucocytes, pneumocytes, bronchial and bronchiolar epithelial cells, epithelial cells fallen off into the lumens of bronchia and bronchioles, around the necrotic areas, and among the oat cells. This immunolocalization pattern was in accordance with the previous investigations [25,27-29].

Immunohistochemical detection technique was reported to be quite useful in showing the bacterial agents of *M. haemolytica* and *P. multocida* [25-28]. In a study conducted by Hazıroğlu et al.[27], *M. haemolytica* was detected by immunohistochemistry in 68.3% of the lambs that showed signs of pneumonia. In a similar study performed by Bemani et al.[29] in Iran, 63.7% positivity was determined in sheep with bronchopneumonia. In this study, positive immunoreactivity against the bacterial antigens was reported in the surface and cytoplasm of epithelial cells, and the cytoplasm of macrophages, leucocytes, and the bronchial exudate. Özyıldız et al.[28] was also reported 31.8% *M. haemolytica* and 27.2% *P. multocida* immunopositivity in 110 sheep with pneumonia. They have indicated that the immunolocalization of both agents were similar; the lumen of alveoli, bronchia, and bronchioles, cytoplasm of leucocytes and the epithelial cells of bronchia and bronchioles, interstitium, lumen of vena, and peribronchial glands. In the current investigation lower rates of immuno-

positivity was detected as compared to the previous studies. On the other hand immunolocalization of the antigens were noted to be similar to those investigations.

Oat cells are commonly described in pneumonias induced by *M. haemolytica*, and was also seen in 7 cases showing fibrinous bronchopneumonia in the current investigation. These cells are known to be due to leucotoxins produced by the bacteria. Widespread foci of necrosis were also seen in 10 cases in the present study. These necrotic changes and the associated exudate are known to be caused by leucotoxins, lipopolysaccharides, and polysaccharides produced by the bacteria as well as the inflammatory substances produced by neutrophils and the other inflammatory cells [1,11,38].

In this study *M. haemolytica* and *P. multocida* were detected in sheep showing signs of pneumonia, and the findings were compared to the previous investigations. It was concluded that *M. haemolytica* is an important infectious agent causing lobar bronchopneumonia and even death in lambs and sheep. The agent is also involved in purulent pneumonia. It was also seen that bacteriological culture, immunohistochemical staining, and PCR techniques could be used in detection of the bacteria almost with same safety. However, immunohistochemistry could give the chance of determining the tissue distribution of the bacterial agents, and hence could be used in routine diagnosis.

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Research Article

Improved Lactational Performance in Dairy Cows Supplemented with Methionine or Rumen-Protected Choline During the Transition Period [1]

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Abstract

The current study was aimed to determine the effects of dietary supplementation with rumen-protected choline and methionine on dry matter intake, milk yield, milk composition and body condition score of transition cows. In this study, 32 Holstein dairy cows which made one parturition at least was used as the animal material. The cows were randomly assigned to four groups according to lactation number, previous lactation milk yield and body condition score before close-up. Cows within each group was fed a basal control (CON) diet (n=8). Cows in treatment group methionine (MET, n=8) were supplemented with 42 g/d of methionine (Metasmart^{R}). Cows in treatment group choline (CHOL, n=8) were supplemented with 75 g/d of rumen protected choline (RPC) source (CholiPEARL^{TM}). Cows in treatment group Mix (MIX, n=8) were supplemented with 75 g/d RPC and 42 g/d methionine. Milk yields and dry matter intakes were determined during the trial as daily. At the results of analyses that were detected; milk yield and composition the highest level in MET group ($P<0.028$). In conclusion of this study that was determined; the supplementation of methionine to the rations of dairy cows affected milk yield and composition positively.

Keywords: *Methionine, Rumen-protected choline, Transition periods, Dairy cows*

Geçiş Dönemindeki Süt İneklerinde Metiyonin veya Korunmuş Kolin İlavesinin Laktasyon Performansını Geliştirmesi

Öz

Bu çalışma, geçiş dönemindeki süt sığırlarında, rasyonlarına ilave edilen korunmuş kolin ve metiyoninin, kuru madde tüketimi, süt verimi, süt kompozisyonu ve vücut kondisyon skoru üzerine etkilerini değerlendirmek amacıyla yapıldı. Çalışmada, en az bir doğum yapmış 32 adet Holştayn inek kullanıldı. İnekler, laktasyon sayısı, önceki süt verimi ve geç kuru dönem öncesindeki kondisyon skorlarına göre rastgele dört gruba ayrıldı. Bütün gruplardaki inekler bazal kontrol rasyonu ile beslendi. Metiyonin grubundaki ineklere günlük 42 g metiyonin ilave edildi. Kolin grubundaki ineklere günlük 75 g korunmuş kolin ilave edildi. Miks grubundaki ineklere günlük 75 g korunmuş kolin ve 42 g metiyonin ilave edildi. Süt verimleri ve kuru madde tüketimleri günlük belirlendi. Yapılan analizler sonucunda süt verimi ve süt bileşenlerinin MET grubunda en yüksek düzeyde olduğu tespit edilmiştir ($P<0.028$). Sonuç olarak, yüksek verimli süt sığırlarının rasyonlarına korunmuş metiyonin ilavesinin, süt verimi ve bileşenlerini olumlu yönde etkilediği belirlenmiştir.

Anahtar sözcükler: *Metiyonin, Korunmuş kolin, Geçiş dönemi, Süt sığırı*

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INTRODUCTION

The periparturient period (transition period), defined as 3 weeks before calving and 3-4 weeks postpartum, dairy cattle enter into a period of negative energy and metabolizable protein balance as a result of increased metabolic demand by the mammary gland and the low dry matter intake (DMI) [1,2]. Methyl donors are needed for the synthesis of important compounds such as phosphatidylcholine and carnitine [3].

Choline and methionine are key methyl donors in mammals and their availability is important for various biological functions. Methionine is an essential amino acid and building block for protein and typically is considered one of the two most limiting amino acids for the production of milk and milk protein in lactating dairy cows [4,5]. 6% of the available choline in the body is derived from methionine and 28% of the body's methionine is used for choline synthesis [6]. The protected choline in the rations contributes to the backup of methionine [7]. Protected choline in rations can be caused to waste the methionine for synthesis of daily choline requirements, and this methionine can be used for milk production [8]. Moreover, the addition of protected choline to the rations leads to the release of more of the methionine for milk protein synthesis and affects the milk protein level positively [8-10]. Some of the studies have detected a significant effect of methionine [2,11,12] or choline [13,14] supplementation though other studies did not determine beneficial improvements on peripartal production performance with methionine [15,16] or choline [8].

The objectives of this study were to evaluate the effects of feeding methionine products or rumen-protected choline, and both (methionine + choline) on DMI, body condition score (BCS), milk yield and composition during the transitional period of high yield milk cows.

MATERIAL and METHODS

Experimental Design and Dietary Treatments

The experimental protocols were applied by the Animal Care and Use Committee of Uludag University and are in accordance with the National Institue of Health Guide for the care and use of Laboratory Animals. The study was carried out with the permission of Uludag University Animal Experimentation Local Ethics Committee (Approval No: 2013-14/04)

In this study, a total of 32 high-yielding Holstein breed milk cows with at least one birth were used. The cows were randomly assigned to four groups according to lactation number, previous lactation milk yield and BCS before close-up. Cows within each group was fed a basal control (CON) diet (n=8). Cows in treatment group methionine (MET, n=8) were supplemented with 42 g of methionine (Metasmart^{R}). Cows in treatment group choline (CHOL, n=8) were supplemented with 75 g of rumen protected choline (RPC) source (CholiPEARL^{TM}). Cows in treatment group mix (MIX, n=8) were supplemented with 75 g RPC and 42 g methionine. The dosage of methionine and choline were supplied by the manufacturer's recommendations. All cows fed the same close-up diet from -21±2 day to calving and the same lactation diet from calving to 70 days in milk (DIM). Ingredients and chemical compositions of the diets were determined according to National Research Council [4] recommendations *(Table 1, Table 2)*.

Methionine and RPC were supplemented as a topdressed from -21±2 day to 21 DIM once daily at the a.m. feeding. The Metasmart^{R} used in this study contains 57% of 2-hydroxy-4-methylthio butanoic acid isopropyl ester. The CholiPEARl^{TM} supplement contains 24% choline chloride and is protected by spray freezing technology.

Animal Management

Experimental studies were carried out at the Omer Matli Animal Production Training and Research Center in Bursa, Turkey from November 2013 to June 2014, with an average temperature of 11.3±10.7ºC. The animals used in the research were housed in a semi-open free-standing stall and automatic feeders were used to determine the individual dry matter intake. Dry cows were fed individually the close up diet once daily (07:00 h). After calving, cows were individually fed a common lactation diet once daily (09:00) to allow for ad libitum consumption. Close-up and lactation diets were mixed daily and fed as a total mixed ration. DMI was determined daily for each animal from 21

Table 1. *Ingredients and chemical composition of close-up diet*

Ingredients	**%DM[1]**
Wheat straw	21.75
Alfalfa hay	18.10
Corn silage	22.22
Commercial concentrate mixture[2]	37.26
Ammonium chloride	0.67
Chemical Composition	**%DM[1]**
Neutral detergent fibre	48.15
Acid detergent fibre	28.60
Crude protein	13.29
Ether extract	4.14
Ash	7.82
Non-fibre carbonhydrates[3]	26.6
Calcium	1.02
Phosphorus	0.28

[1] Dry Matter; [2] Commercial Concentrate Mixture, Matli Feed Industry, Karacabey/Turkey; [3] Non-fibre carbonhydrates, 100 – (% NDF + % CP + % EE + % Ash)

Table 2. *Ingredients and chemical composition of early lactation diet*

Ingredients	%DM[1]
Wheat straw	7.26
Alfalfa hay	21.28
Corn silage	25.98
Commercial concentrate mixture[2]	43.98
Corn gluten	0.71
Sodium bicarbonate	0.54
Magnesium oxide	0.25
Chemical composition	**%DM[1]**
Neutral detergent fibre	43.76
Acid detergent fibre	24.43
Crude protein	16.43
Ether extract	5.62
Ash	7.99
Non-fibre carbonhydrates[3]	26.2
Calcium	0.92
Phosphorus	0.63

[1] Dry Matter; [2] Commercial Concentrate Mixture, Matli Feed Industry, Karacabey/Turkey (Ingredients: Corn DDGS, corn, soybean meal,48% CP, wheat bran, rice bran, full fat soybean, limestone, sunflower meal, salt, vitamin-mineral premixes); [3] Non-fibre carbonhydrates, 100 – (% NDF + % CP + % EE + % Ash)

days before the expected parturition to the first 70 days of lactation. Cows were milked 3 times at nearly 06:00, 14:00 and 22:00 h. Milk yield was recorded daily at 70 DIM. At the end of the research, the cows returned to the farm herds at 70 DIM.

BCS were determined by the same observer for 21 days before calving (-21 day), time of calving (0 day), 21 days after calving (+21 day) and 70 days after calving (+70 day). The determination of the BCS was based on five-point scale with 0.25 unit intervals (scale 1 = thin, to 5 = obese) [17].

Feed and Milk Samples

Weekly samples were frozen at -20°C and then composited montly for analyses. Chemical analysis (dry matter, crude protein, ether extract, ash, calcium, phosphorus) of diets were performed according to AOAC [18], neutral detergent fiber and acid detergent fiber analyses were performed according to Van Soest et al.[19].

Homogenous milk samples were collected individually from all cows for two consecutive days each week starting from the 8th day to the 70th day of lactation following the calving by means of the sampling equipment of the milking equipment. On the same day, the milk samples were analyzed with the milk analyzer (MilkoScan™ FT1 User Manual 6004 5478/Rev 1) for fat, protein, lactose, solid non-fat, total solids and milk urea nitrogen.

Statistical Analysis

BCS, DMI and milk yields were evaluated using the 'General Linear Model'. Model; The numbers of subjects (cows) were entered as random effect, duration and group as a fixed effect. One-way analysis of variance was used to compare milk components. Significance was declared at $P<0.05$. Statistical analyses of the data were performed using SPSS [20] (version 20.0, SPSS Inc, USA) program.

RESULTS

This study was conducted to determine the effect of feeding methionine products or rumen-protected choline and both (methionine + choline) on DMI, BCS, milk yield and composition during the transitional period of high yield milk cows.

DMI during the experiment are presented for all groups and periods in *Table 3*. There were no statistical differences among the experimental groups for the close-up period DMI ($P>0.05$). Significant differences were determined among the treatment groups in terms of lactation DMI ($P<0.028$). The DMI during the early lactation period averaged 21.17 kg/d for CON, 21.89 kg/d for MET, 19.90 kg/d for CHOL and 22.01 kg/d for MIX. The effects of methionine and RPC supplementation on milk yield and milk composition were shown in *Table 3*. Milk yield and milk composition were significantly affected by dietary treatments ($P<0.028$). However, dietary treatments did not any significant effect on milk urea nitrogen. Methionine supplementation significantly increased milk yield, milk fat, milk protein, milk lactose, solid non-fat and total solids at the early lactation period ($P<0.028$).

There were no statistical differences for body condition scores among treatments. But body condition variation was significantly affected by the addition of methionine and RPC. Cows lost 0.97, 0.72, 0.81 and 0.53 body score unit between close-up period and week 10 of lactation for CON, MET, CHOL and MIX, respectively *(Table 4)*. Body condition variation was the lowest in MIX.

DISCUSSION

The effects of rumen-protected methionine supplementation on DMI in previous studies have been conflicted by some researchers [2,11,12,16]. For example, Zhou et al.[2] observed an increase in prepartal and postpartal DMI with Smartamine M. Although Ordway et al.[16] determined an increase in postpartal DMI with Metasmart supplementation, there is no differences in groups fed by Smartamine M. On the other hand, Socha et al.[15] detected that there was no effect of feeding Smartamine M or Smartamine ML during the prepartum period on prepartum DMI during the last week of gestation. In the present study, differences in mean prepartum DMI were not determined; but postpartum DMI for MET and MIX treatments were significantly higher

Table 3. *Effect of supplementing Holstein cows during the peripartal period with methionine (Metasmart®), rumen-protected choline (CholiPEARL™) or both on DMI, milk yield and milk composition*

Parameters	CON X±Sx	MET X±Sx	CHOL X±Sx	MIX X±Sx
Close-up DMI (kg/d)	14.70±2.66	14.79±3.24	13.99±2.82	13.92±3.55
Lactation DMI (kg/d)	21.17±4.4^{b}	21.89±4.12^{a}	19.90±4.27^{c}	22.01±4.13^{a}
Milk yield (kg/d)	40.85±5.5^{c}	42.88±4.72^{a}	40.05±5.93^{c}	42.00±5.09^{b}
Milk fat (%)	3.80±1.07^{b}	4.02±1.20^{a}	3.49±1.13^{c}	3.73±1.05^{b}
Milk protein (%)	3.06±0.37^{b}	3.17±0.35^{a}	2.93±0.43^{c}	3.04±0.32^{b}
Milk lactose (%)	4.61±0.16^{b}	4.74±0.20^{a}	4.63±0.22^{b}	4.63±0.17^{b}
Solids non fat(%)	8.51±0.49^{b}	8.76±0.48^{a}	8.38±0.49^{c}	8.51±0.40^{b}
Total solids (%)	12.42±1.3^{b}	12.93±1.46^{a}	11.93±1,42^{c}	12.35±1.30^{b}
Milk urea nitrogen (mg/dL)	14.45±3.73	14.30±3.54	14.27±2.94	14.26±3.29

Different superscripts indicate stastical differences ^{a,c} P<0.028

Table 4. *Effect of supplementing Holstein cows during the peripartal period with methionine (Metasmart®), rumen-protected choline (CholiPEARL™) or both on body condition score*

Weeks	CON X±Sx	MET X±Sx	CHOL X±Sx	MIX X±Sx
-3. week	3.53±0.16	3.66±0.38	3.47±0.28	3.53±0.36
Calving	3.56±0.18	3.59±0.35	3.47±0.28	3.59±0.38
3. week	2.87±0.30	3.19±0.37	2.87±0.46	3.16±0.42
10. week	2.64±0.32	2.93±0.28	2.64±0.43	3.00±0.38
Variation*	0.97±0.21^{a}	0.72±0.31^{ab}	0.81±0.35^{ab}	0.53±0.16^{b}

** -3. week body condition score and lactation 10. week body condition score difference; Different superscripts indicate stastical differences ^{a,b} P<0.046*

than for the CON treatment. In accordance with previous reports, rumen-protected choline supplementation did not affect DMI [8,21,22]. As a result of many scientific studies have shown that supplemented with methionine and rumen-protected choline to transition rations in dairy cattle may be caused different DMI. It has been suggested that the contradictory results both in our and other studies may be due to the differences in the level of methionine and choline supplementation, length of feeding, stage of lactation, or combination of these.

Methionine or its analogue supplements have an important role in milk yield production [2,12,23]. In our study, methionine supplementation resulted in an increased milk yield and milk components whereas milk urea nitrogen was not significantly affected. Some other studies there were no differences defined by rumen-protected methionine [15] or methionine analog supplement [16]. The methionine has been identified as one of the two most limiting amino acid for lactating dairy cows and this is a greater DMI which increase the daily protein intake [4]. Thereby, milk yield was being optimum as expected due to the optimum Lys:Met ratio. Many studies have shown that supplementing with rumen-protected methionine has improved milk protein synthesis [2,12,16]. It was not a surprise increase in milk protein due to increased milk yield in MET group. Moreover, the significant increase in milk composition were due to the increase in milk yield. High percentages of total solids in the MET group was probably associated with over milk protein and milk fat percentage in those cows. Hartwel et al.[24] reported that milk yield and composition were not affected by supplementing with choline chloride. Also, Guretzky et al.[8] observed that supplementing choline from -21 days to 42 days or longer did not affect the milk yield and composition. The result of our study was in agreement with Hartwel et al.[23] and Guretzky et al.[8]. Although significant effects on milk yield were reported in studies when choline was supplemented during the peripartal period [13,25,26], in the present study, the milk yield is lower in the CON and CHOL groups than in the other treatment groups, suggesting that lactation DMI is less in these groups. Zahra et al.[25] determined greater milk yield with supplemental choline in dairy cows with a BCS of 4 beginning the close-up period that was driven primarily by greater DMI. So, our results from the present study do not seem to support the suggestion by Zahra et al.[25]. Nevertheless, there were no statistical differences in BCS among treatments. In the previous studies, methionine or choline supplementation did not effect of BCS were reported [2,16]. However, in the present study, the differences in body condition variations were significant which would suggest that fat mobilization was the highest in CON group.

In conclusion, current study results suggested that supplementation of methionine to the rations of dairy cows affected milk yield and composition positively. Given the change in body condition variations, the control group was observed more loss of condition than the supplemented with methionine, rumen protected-choline or both. More studies must be conducted to evaluate effects of supplemented with methionine, rumen-protected choline or both during the transition period on dairy cow performance.

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Research Article

Management of Humeral Fractures in Dogs By Using Semicircular External Fixator and Intramedullary Pin Tie-in Combination

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Abstract

The purpose of present study was to report our experience with use of semicircular external skeletal fixator-intramedullary pin tie-in configurations for management of various types of humeral fractures in 33 dogs. Fracture description, history, frame configuration, complications, limb use, fixator removal time, and functional outcome were evaluated. All of the fractures except one with sufficient follow up healed. Twenty-six dogs started to use the operated limb in 1 to 3 days after the operation whereas initial limb use was 4 to 9 days in the other 7 dogs. Destabilization of the frame was performed between 21 to 42 days (median, 32 days) by removal of the IM pin. Time to fixator removal ranged from 42 to 96 days (mean, 56 days). Functional outcome was excellent in 26 cases, good in 6, and poor in 1 dog. As a result of this study, in which all the fractures except one healed, we believe that the unilateral semicircular ESF-IM pin tie-in configurations can be used as an alternative fixation method for the management of humeral fractures in dogs. The patients used the operated limb early in the postoperative period and limb use was improved throughout the healing period.

Keywords: *Dog, External skeletal fixation, Fracture, Humerus, Intramedullary pin*

Köpeklerin Humerus Kırıklarının Semisirküler Eksternal Fiksatör ve İntramedullar Bağlamalı Pin Kombinasyonu İle Sağaltımı

Öz

Bu çalışmanın amacı değişik tipteki köpek humerus kırıklarının sağaltımında semisirküler eksternal fiksatör intramedullar bağlamalı pin kombinasyonu ile ilgili tecrübelerin aktarılmasıdır. Kırığın tipi, anamnez, çerçeve konfigürasyonu, komplikasyonlar, bacağın kullanımı, fiksatörün çıkarılma zamanı ve sonuçlar değerlendirildi. Bir olgu haricindeki tüm kırıklarda kaynama şekillendi. Yirmi altı olgu operasyondan sonraki 1-3 gün arasında ilgili bacağını kullanmaya başladı. Geriye kalan 7 olguda bu sürenin 4- 9 gün arasında değiştiği gözlendi. Postoperatif 21-42. günlerde IM pin uzaklaştırılarak sistemde bilinçli zayıflatma uygulandı. Fiksatörün uzaklaştırılma süresi 42 ila 96 gün (ortalama 56 gün) arasında değişim gösterdi. Fonsiyonel değerlendirmede, olguların 26'sında çok iyi, 6'sında iyi ve 1'inde ise zayıf olarak değerlendirildi. Sonuç olarak, bir olgu haricinde tüm kırıkların iyileştiği göz önünde bulundurulduğunda, bize göre unilateral semisirküler eksternal fiksatör ve intramedullar bağlamalı pin kombinasyonu köpeklerin humerus kırıklarının sağaltımında alternatif bir yöntem olarak kullanılabilir. Hastalar operasyonlardan kısa zaman sonra ilgili ekstremitelerini kullanmaya başlamış ve iyileşme sürecinde ilgili eksremitenin kullanımı daha da iyi hale gelmiştir.

Anahtar sözcükler: *Köpek, Eksternal fiksasyon, Kırık, Humerus, İntramedullar pin*

INTRODUCTION

The humerus is the least commonly fractured long bone in the dog. The fracture incidence ranges from 5 to 13% and most of the fractures involve the middle and distal one third of the bone [1,2]. Because humerus has an S-shape from the lateral perspective -a cranial bow proximally and a caudal bow distally- with large surrounding muscle mass, management of this bone's fractures are challenging [3,4]. This shape can cause difficulty in application of dense intramedullary pins and contouring of the plates. Humerus is in relation to the thorax, so concurrent injuries such as pneumothorax, pulmonary contusions, hemothorax, diaphragmatic hernia, rib fractures, and brachial plexus injuries may be encountered in patients with humeral fractures [4,5]. Reported treatment options include intra-

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medullary (IM) pins and cerclage wires, bone plates, interlocking nails, tie-in IM-external skeletal fixator (ESF) combinations, and IM rod/bone plate combination [2-9]. External skeletal fixators can be used alone or in combination with intramedullary pins in the repair of humeral fractures of dogs and have several advantages compared with other fixation methods. Versatility and requiring minimal soft tissue damage are two featured benefits of ESF [7].

The purpose of this paper was to describe the repair of various types of humeral fractures using semicircular external skeletal fixator and IM pin tie-in combination and to report the outcome in 33 medium/large breed dogs.

MATERIAL and METHODS

Inclusion Criteria and Medications

Between January 2009 and September 2016, dogs (>15 kg) with humeral metaphyseal or diaphyseal fractures (n = 33) with complete clinical and radiographic follow up of at least 24 weeks duration were included in the study. Signalment and history, description of the fracture, frame configuration, concomitant injuries, complications, time to first use of the limb, time to fixator removal and functional outcome was recorded.

Cefazolin (20 mg/kg intravenously, Cefozin 500 mg, Bilim, Istanbul, Turkey) was administered at anaesthetic induction and every 2 hours throughout the surgical procedure. Dogs were premedicated with diazepam (0.1 mg/kg IV, Diazem, Deva, Istanbul, Turkey), induced with propofol (6 mg/kg IV, Propofol 1%, Fresenius Kabi, Upsala, Sweden), and anaesthesia maintained with isoflurane (Isoflurane-Usp, Adeka, Samsun, Turkey) in oxygen. Transdermal fentanyl patch (25-50 µg/h, Durogesic transdermal patch, Janssen-Cilag, Beerse, Belgium) was adhered to the inguinal area 2 h before the surgery and stayed in place for 48 h. Meloxicam (0.2 mg/kg/day subcutaneously, Maxicam, Sanovel, Istanbul, Turkey) was administered for 3 days after surgery.

Frame Design and Features

The ESF system used in this study was composed of 7-holed 60° (180 mm inside Ø, 1/6 ring arch, 7x20x95 mm) carbon-fiber arches, 6 mm Ø threaded rods, cannulated pin and wire fixation bolts, and 4 mm Ø negative profile end-threaded half pins. 3 or 4 mm Ø Steinmann pins were used for intramedullary pinning. Depending on fracture type and length of fractured bone, either 3 or 4 arched frame configurations were used. Size of the IM pins were adjusted to a diameter not exceeding 30% of the medullary canal of the distal humerus [10]. The edge holes of the arches were used to secure to the threaded rods and the other holes for the half pin fixation bolts. Caudal rod was adjusted 3-4 cm longer than the cranial rod in order to attach the IM pin to the ESF frame in "tie-in" fashion. Another carbon-fiber arch was used as a connector for the linkage of the two fixation systems *(Fig. 1)*.

Surgical Technique

Fracture reduction was accomplished using an open or limited open approach from the cranio-lateral border of the humerus [11]. Following exposition of the fracture, a 3 or 4 mm Ø Steinmann pin was inserted to the medullary cavity via retrograde technique. After achieving axial reduction, half pins were perpendicularly and bicortically placed with a low speed (<150 rpm) power-drill. The proximal pin was placed at the level of the base of the greater tuberculum while the distal pin was placed from cranio-distal to the lateral epicondyle to exit the bone medially at a similar point halfway between the epicondyle and the articular surface [5]. Once acceptable reduction of the fracture was achieved, the construct was completed by inserting additional fixation pins (depending of the fracture type and localization) to the humeral shaft by avoiding muscles and radial nerve *(Fig. 2)*. Before skin closure half pin fixation bolts were firmly secured to the carbon fiber arches and then IM pin was tied-in to the caudal rod.

Postoperative Period and Evaluation of Outcome

Clients were informed regarding wound care and strongly adviced to clean the pin-skin interface with 10% povidone iodine solution (Batticon 10% sol: Adeka, Samsun, Turkey) every day until the removal of the fixator. Recheck clinical and radiographic evaluations were performed for two/

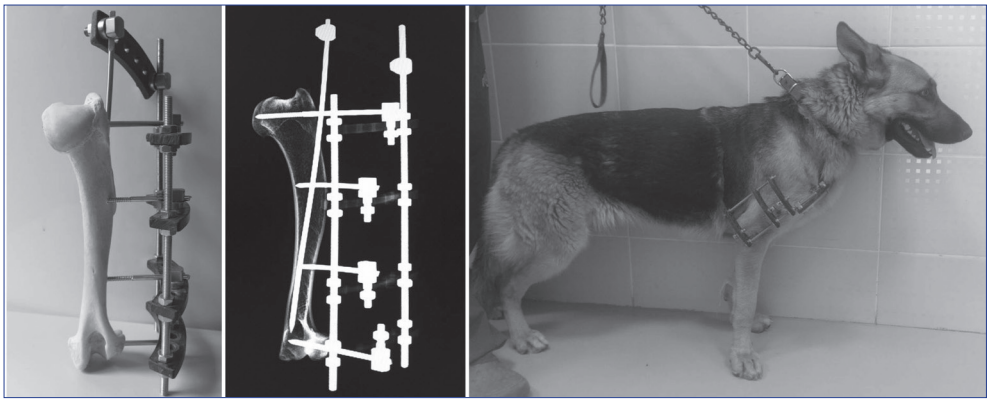


Fig 1. A four arched semicircular external fixator - IM pin tie-in combination and its clinical view on a standing dog

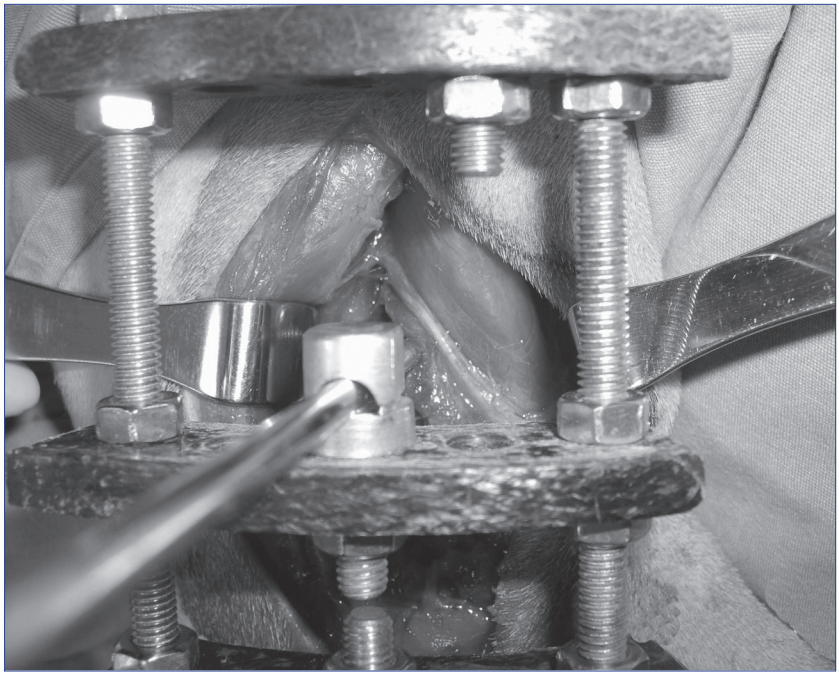


Fig 2. The most important advantage of the multiplanar feature of a semicircular arch is possibility of avoidance of the muscles, vessels and nerve injury during pin insertion. Note that the avoidance of the radial nerve with brachial and triceps muscles in a distal humeral fracture

three weeks intervals. Postoperative complications were classified as "*major*" which were defined as morbidities that require further medical or surgical interventions or "*minor*" which were defined as no need for an additional surgical or medical intervention to resolve. The frame was removed in two stages; first destabilization by removal of the IM pin and then removal of the ESF frame. Time to remove the IM pins varied depended on the presence of radiographically visible active bridging callus tissue. Clinical union was defined as the radiographic evidence of a bony callus bridging on at least one cortex on both medio-lateral and cranio-caudal views [12]; at this stage, EFSs were entirely removed. Treatment success was based on clinical evaluation of the operated limb, including willingness to use the limb in daily activities. Final clinical outcomes were graded as: *excellent*; willingness to use the limb at a walk and run, no evidence of obvious lameness, no pain on palpation - *good*; willingness to use the limb at all times but slight lameness evident when running, no pain on palpation - *fair*; reluctance to use the operated limb, moderate lameness evident, obvious pain on palpation - and *poor*; unwilling to use the limb, constant non-weight-bearing lameness.

RESULTS

Ages and bodyweights of the dogs ranged from 6 months to 10 years (mean, 24 months) and 15 to 39 kg (mean, 24 kg) respectively. Of the 33 dogs 21 were male and 12 were female. Fracture types were; oblique (n=15), transverse (n=11), and comminuted (n=7) and involved at the distal (n=18), diaphyseal (n=13), and proximal (n=2) part of the humerus. Fractures were caused by road accidents (n=28), gunshot injury (n=2), and unknown causes (3). Frame configuratios were IM pin + 3 arched ESF in 12 dogs and IM pin + 4 arched ESF in 21 dogs. Depending on the diameter of the distal humeral medullary canal 3 (n=10) or 4 mm Ø (n=23) IM pins were used. In all dogs - especially in dogs with early active limb use - mild serosanguineous discharge was observed at the point where IM pin protruded from the skin. Following removal of the IM pin, this minor complication was completely resolved. As seen in all ESF cases, discharge from the pin-skin interfaces during the convalescence period was seen in most of the dogs. Discharge was more apparent in proximal pins which were surrounded by bulky muscle groups. Serosanguineous or serohemorrhagic discharge responded to improved cleaning of the pin-skin interfaces with povidone iodine solutions, but in 6 cases with purulent discharge oral 12.5 mg/kg amoxicillin-clavulonate (Synulox 250 mg tablet, Pfizer, Italy) was administered for 10 days. In one case with extensive soft tissue loss after gunshot injury, purulent pin tract discharge due to osteomyelitis was observed. Despite the aggressive broad spectrum antibiotic use we could not resolve the infection and amputation was performed.

Pin breakage was seen in one dog in the early post-operative period. According to the information given by the client, the dog could use the affected limb but after running away from the house in the 3rd day and being found on the postoperative 4th day it had a non-weight bearing lameness. The radiographic examination performed after this revealed a broken half pin on the proximal fragment *(Fig. 3a-b)*. Fortunately, the fragments were still in alignment so the insertion of a half pin percutaneously salvaged the complication *(Fig. 3c)*. After-this application, the patient started using the afore-mentioned limb without showing any obvious signs

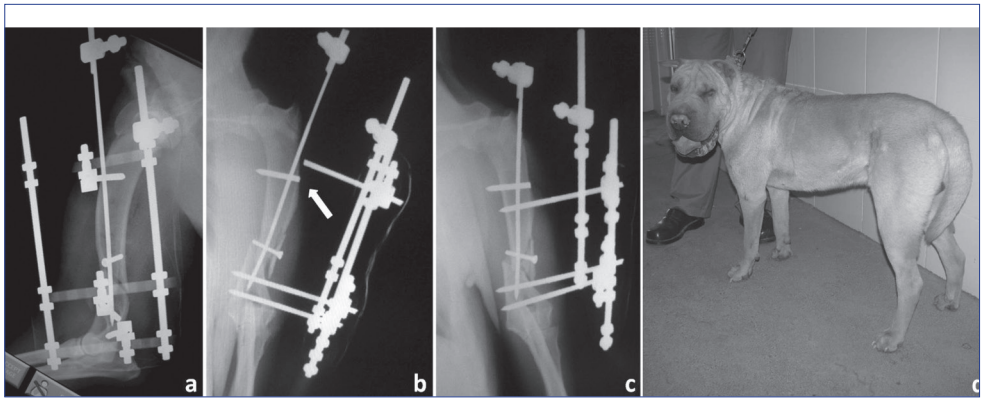
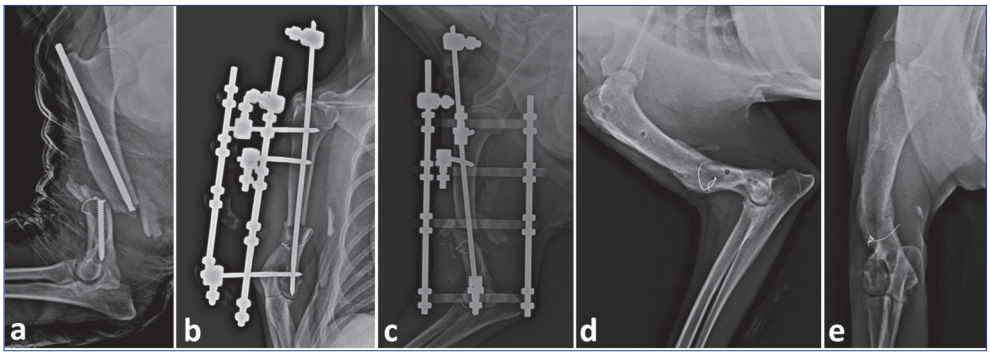


Fig 3. Immediate postoperative radiograph of a dog with a fixed comminuted distal diaphyseal humeral fracture (a). Note the broken half pin *(white arrow)* in the proximal fragment (b) in the early postoperative period. Because alignment of the fragments was not disturbed, fixation was performed with a new half pin inserted below the broken pin (c). Clinical view of the dog 4 weeks after fixator removal (d)

Fig 4. Radiographic view of the refracture of a diaphyseal humeral fracture due to early pin breakage (a). Stabilization with IM pin - ESF combination provided a rigid fixation (b-c) but fracture healing was longer compared to other dogs. Note that pin holes were still apparent despite the passed 36 weeks following removal of the fixator (d-e). This dog was a typical delayed union case



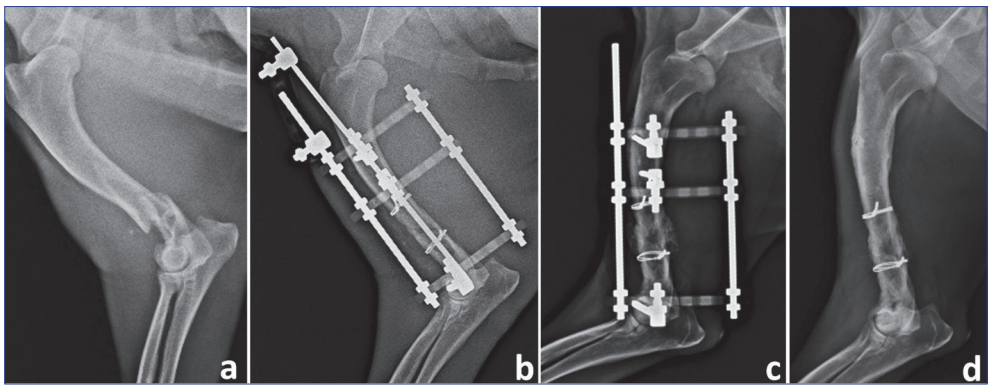
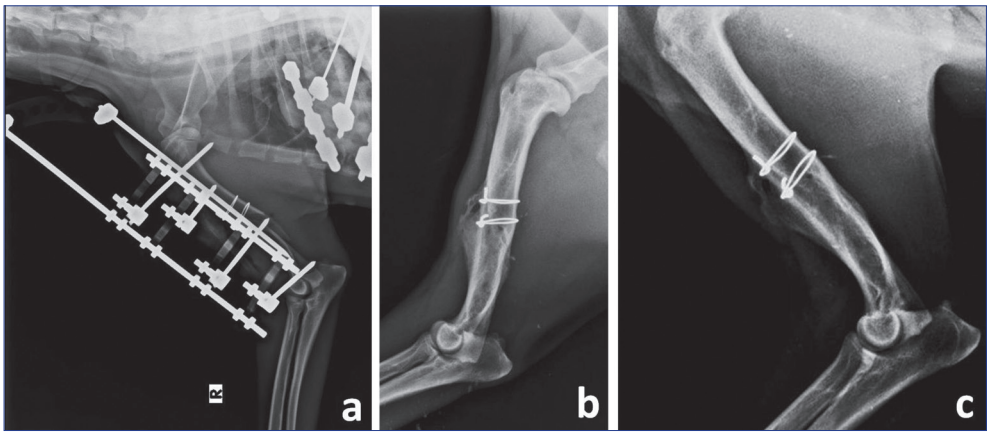


Fig 5. Preoperative (a) and immediate postoperative (b) radiographs of a comminuted distal humeral fracture. System was destabilized by removal of the IM pin 4 weeks after the operation (c). Bridging callus tissue was seen to be resorbed related to the remodelling process at the radiographs taken 15 weeks following removal of the fixator

Fig 6. Postoperative immediate radio-graph of a mid-diaphyseal long oblique humeral fracture after fixation (a). Diminution of the bridging callus and reorganization of the medullary cavity can be seen on the radiographs at 5th (b) and 12th (c) weeks following removal of the implants



of pain and lameness. The dog was able to bear full weight to the limb following removal of the fixator *(Fig. 3d)*.

One of the dog was operated due to early breakage of the intramedullary pin which was operated in a private veterinary clinic *(Fig. 4a)*. After removal of the cast, draining

operation wound was observed. After controlling of the wound infection, fixation was performed with ESF and IM pin combination *(Fig. 4b-c)*. Following the operation we performed, bone infection was recurred due to the necrotic bone fragments. Bone loss after removal of these necrotic fragments formed a large fracture gap and resulted with delayed union of the case. In this case, time for bone healing was longer compared with other cases. Fixator was removed on 96th day postoperatively and restricted activity for three months was advised to the clients. At long term radiographic controls -36th week after removal of the fixator- although remodeling was not properly completed *(Fig. 4d-e)*, dog was able to use the limb without signs of obvious lameness.

In all dogs, semicircular ESF-IM pin combination provided a stable fixation and all of the cases tolerated this scaffold (weighing about 0.5 kg) well. In the dogs with purulent pin tract discharge volcano chimney image was observed in radiographs at the point where half pins protrudes from the bone. All of these periosteal reactions were improved after completion of remodelling process. Destabilization of the frame was performed between 21 to 42 days (median, 32 days) by removal of the IM pin *(Fig. 5)*. In most of the dogs limb use was significantly improved following removal of the IM pin.

All of the fractures but one with sufficient follow-up healed *(Fig. 6)*. Twenty six of the dogs bore weight on the operated limb within three days (median, 2 days) following the operation. In the other seven dogs time to first use of the limb ranged between four to nine days. These prolonged cases were the dogs with comminuted fractures and moderate to severe soft tissue loss. Time to ESF removal ranged from 42 to 96 days (mean, 56 days). All clinical evaluations were done by the same operators. Final clinical outcome was excellent in 26, good in six, and poor in one dog.

DISCUSSION

Intramedullary pinning is mainly used to treat simple fractures as a primary fixation method or as an ancillary fixation for plates or external skeletal fixators. But in humerus, medullary canal ends far proximal to the distal part of the bone limiting Steinmann pin purchase in the medial condyle of the bone which restricts the use of IM pins in communitued or juxta-articular fractures primarily. Steinmann pin serves to assist in fracture alignment and also protects the ancillary fixation from catastrophic fractures [4]. Although each technique has its advantages and disadvantages, plate fixation is the most commonly used method today [3,13-15]. Both reduction and fixation of the humerus pose a challenge due to its anatomical location and shape, being surrounded by bulky muscle groups, the radial nerve traversing close to the skin in the distal part and having a natural "S" shape because of its proximal and distal curvatures [4,5]. In this study, favourable results were obtained in 33 medium/large breed dogs treated for different types of humeral fractures, considering biological fixation criteria, by semicircular external fixation/ intramedullary pin combination using a limited open approach. Adequate stabilization was achieved with this technique and patients were able to use their affected limbs functionally in the early post-operative period.

The main purpose of fracture fixation is to achieve the fastest possible bone healing and encourge the patient for functional limb usage by promoting postoperative early ambulation [16]. The golden standart for a perfect ESF procedure is to construct frames that allow full weight-bearing without interfering with the use of the limb. Early ambulation accelerates bone healing by stimulating axial micromotion at the fracture line and also prevents fracture disease such as disuse atrophy and muscle contractures [17,18]. This is critical in large breed dogs with concomitant injuries. In present study 26 of the dogs bore weight on the operated limb within three days (median, 2 days) following the operation. According to the data received during the convalescence period, the dogs tolerated the frame well and this frame did not impede the daily activities of the dogs.

There are several advantages of external skeletal fixation over internal fixation techniques; they can be applied with minimal surgical exposure, limiting soft tissue, bone and periosteal damage. They are safer to apply on infected or compound fractures, the frames may be reinforced or destabilized after the initial surgery and they do not require bandages on the recovery period [19-21]. The semicircular arches offer the option of inserting half pins unilaterally, but in a multiplanar fashion owing to its novel design which enables half pin insertion up to five different planes. The semicircular arches allow the insertion of two pins with a single arch, one from a bolt placed over the arch and the other placed below it. This versatility makes the semicircular arch superior to linear fixators. This property allows preserving the muscles and the radial nerve depending on the shape of the fracture, unlike linear external fixators. This is particularly important in the fixation of distal fractures that may endanger the radial nerve. Optimal and safe fixation can be achieved by preserving muscle and nervous tissue with the semicircular arch, owing to its multiplanar pin insertion options. The distal region fractures in this study (n=18) healed without any major complications with the exception of one osteomyelitis case, which required amputation. Also, the pin breakage complication that was seen in one case could be easily corrected with the insertion of a half pin percutaneously in about 15 min duration. This property of the external frame which allows recovery of such complications should not be discounted.

There were several reports of complications due to mishaps or failed post operative maintenance of external

fixators [22,23]. These are pin tract lysis, haemorrhage, drainage, infection, pin loosening and breakage, osteomyelitis, malunion, malalignment and non-union. Pin tract drainage is the most common complication seen in almost every case and nature of the discharge is important. While serosanguinous or serohaemorrhagic discharge may be prevented with daily cleansing of the pin tract, discharge with purulent characteristics require the use of antibiotics to prevent possible bone infections. Pin tract discharge was seen in each of our cases and this finding correlated with previous studies. The intramedullary pin protruding from the dorsal trochanteric region enlarged the pin tract considerably due to repetitive cranio-caudal movement. The pin exiting this location provides direct access to the medullary tract from the outside, so the potential for bone infection should not be discounted if pin tract maintenance is not adequately performed. We did not encounter any such complication in this study, because pin tract maintenance was performed with utmost care with patient collaboration.

As a result of this study, in which all the fractures except one healed, we believe that the unilateral semicircular ESF-IM pin tie-in configurations can be used as an alternative fixation method for the management of humeral fractures in dogs. The patients used the operated limb early in the postoperative period and limb use was improved throughout the healing period.

Conflict of Interest

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript. The authors declare that there is no conflict of interest regarding the publication of this article.

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Research Article

Effects of Ovary Transport and Storage Temperature on *In Vitro* Maturation and Cumulus Cell Apoptosis Rates in Cat Oocytes [1]

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Abstract

The objective of the present study was to examine the effects of two different transport temperature (37°C vs 4°C) and cold storage of ovaries for 24 h on cumulus cell apoptosis and maturation rates of cat oocytes *in vitro.* Ovaries were collected from 15 ovariohysterectomized domestic cats and maintained and transported to the laboratory in phosphate buffer saline at 37°C and 4°C. In order to determine the effects of storing time, some ovaries transported at 4°C were stored at the same temperature for 24 h. Selected cumulus oocyte complexes (COCs) were matured for 48 h at 38°C in four-well petri dishes containing 500 µL of modified oviduct medium (mSOF) under mineral oil in a 5% CO_2 incubator with nearly 100% humidified. The morphological features of apoptosis were analysed in the cumulus cells at the beginning of *in vitro* maturation in both transporting temperature groups and after 24 h of cold stored group. The degree of apoptosis in cumulus cells were measured by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL). The IVM rates of oocytes were determined using Hoechst (33342) staining. Although the apoptotic morphological features were seen rarely and in similar rates in 37 and 4°C transporting groups (19.40 and 21.55%, P>0.001), it was seen more intensely in the 24 h cold stored group (34.80%, P<0.001). The IVM findings were similar (49.77, 44.55%) at 37°C and 4°C groups (P>0.05), and importantly lower at 4°C transporting and 24 h cold stored groups (18.90%, P<0.05). In conclusion, the results of this study suggest that (I) cumulus cells of cat oocytes are partially exposed to apoptosis during transportation at warm or cold temperature, (II) storing of ovaries for 24 h at 4°C causes apoptosis of the cumulus cells at much higher rates and (III) storing of ovaries for 24 h at 4°C affects negatively IVM rate of oocytes.

Keywords: *Cat, Ovary, Transport temperature, Oocyte, Cumulus, Apoptosis*

Kedi Oositlerinin *In Vitro* Olgunlaştırılması ve Kumulus Hücrelerinin Apoptoz Oranları Üzerine, Ovaryum Taşıma ve Saklama Sıcaklığının Etkisi

Öz

Çalışmanın amacı, ovaryumların iki farklı sıcaklıkta (37°C ve 4°C) taşınma ve soğukta 24 saat bekletilmenin kumulus hücrelerindeki apoptoza ve kedi oositlerinin *in vitro* olgunlaşma oranları (IVM) üzerine etkilerini incelemektir. Kısırlaştırılmış 15 kediden ovaryumlar alındı ve yarısı 37°C, diğer yarısı da 4°C'de olmak üzere, fosfat tampon tuzlu solüsyonunda (PBS) laboratuara taşındı. Soğukta bekletmenin etkilerini belirlemek içinse, 4°C 'de taşınan ovaryumların yarısı, aynı sıcaklıkta olmak üzere 24 saat bekletildi. Seçilen kumulus oosit kompleksleri (COCs), %100'e yakın nemin sağlandığı %5 CO_2'li inkübatörde mineral yağ altındaki 500 µL modifiye Sentetik Ovidukt Medyumu (mSOF) içeren dört gözlü petrilerde olmak üzere 38°C'de 48 saat süreyle olgunlaştırıldı. Apoptozun etkileri, hem iki farklı taşıma grubunda, hem de soğukta 24 saat bekletilen grupta olmak üzere *in vitro* olgunlaşmanın hemen öncesinde kumulus hücrelerinde test edildi. Apoptosis derecesi, deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) test ile ölçüldü. Oositlerin *in vitro* olgunlaşma düzeyleri Hoechst (33342) boyama ile belirlendi. Apoptotik hücre oranları, 37°C ve 4°C taşıma gruplarında sırasıyla; %19.40 ve %21.55 olarak belirlendi (P>0.001). Aynı değer, 24 saatlik soğukta bekletilen grupta ise daha yoğun olarak (%34.80) görüldü (P<0.001). IVM bulguları da sırasıyla 37°C ve 4 °C taşıma gruplarında %49.77, %44.55 (P>0.05) iken, 24 saat soğukta bekletme grubunda ise %18.90 olarak bulundu (P<0.05). Çalışma sonuçları, (I) kedi ovaryumlarının sıcak veya soğukta taşıma sırasında kumulus hücrelerinin, apoptoza kısmen maruz kaldıklarını, (II) ovaryumların 4°C'de 24 saat bekletilmesinin kumulus hücrelerinin apoptozunu önemli derecede artırdığını ve (III) 4°C'de 24 saat bekletmenin, oositlerin IVM oranlarını olumsuz etkilediğini göstermiştir.

Anahtar sözcükler: *Kedi, Ovaryum, Taşıma sıcaklığı, Oosit, Kumulus, Apoptoz*

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INTRODUCTION

Domestic cat is a valuable model for researching human genetic diseases and for developing assisted reproduction of endangered felids [1]. Long term storage of ovaries can provide opportunity to rescue oocytes from ovaries of endangered felids recently dead in the field or to rescue ovaries that are ovariohysterectomized for medical reasons [2]. Cumulus cells surround the oocyte and provide nutrients and signals regulating oocyte growth and maturation [3]. Cumulus cells also keep the oocyte under meiotic arrest, transmit the LH signal to the oocyte and participate in meiosis induction and are responsible for cytoplasmic maturation of oocytes [4]. The quality of the oocyte and cumulus cells are not only important for oocyte maturation or fertilization but also affects the cleavage and further embryonic developments. Therefore, the oocytes enclosed within the follicles must remain metabolically active while they are transported to the laboratory in order to preserve their maturation ability *in vitro* [5]. The oocyte cytoplasmic maturation is also crucial for the developmental potential of embryos after fertilization [3]. Transportation of ovaries to the laboratory may result in long transport time which causes some possible post mortem changes in the tissues. Cell metabolism linked to enzymatic activity which depends on the temperature and duration of storage, and the temperature of the ovaries kept could affect the apoptosis and consequently maturation rates of the oocytes *in vitro* [6]. It was shown that oxidative stress during *in vitro* culture leads to changes in maturation and developmental disruption in bovine oocytes [7]. It is also known that reactive oxygen species (ROS) can cause DNA damage and induce apoptosis in human and porcine oocytes during *in vitro* culture period [8].

Programmed cell death (apoptosis) is cell self-destruction under physiological control and is regulated by genes [9]. Apoptosis plays some physiological roles on the ovarian cycle, the growth and the atresia of the follicles, the selection of the dominant follicle and regression of the corpus luteum [10]. The ability to promote apoptosis is always present within the cell nucleus and could be initiated by maintaining the cells under suboptimal conditions [11]. Apoptosis is characterized by a loss of cell volume, nuclear pyknosis and margination of the chromatin and its redistribution against the nuclear envelope. As a biochemical perspective, one of the most hallmark features of apoptotic cells is the loss of DNA integrity by endonuclease-mediated fragmentation of the genomic pool [12]. The terminal deoxyribonucleotidyltransferase (TDT)- mediated dUTP-digoxigenin nick end labeling (TUNEL) assay is a widely used approach to label and recognize DNA fragmentation by using the terminal deoxyribonucleotidyl transferase [13].

The aims of the present study were to (I) examine the effect of two ovary transport temperatures (37℃ vs 4℃) and cold storage for 24 h on cumulus cell apoptosis and maturation rate of cat oocytes *in vitro* and based on the results of the study, (II) suggest new practices of transporting cat ovaries.

MATERIAL and METHODS

Ethics Statement

The study was performed in accordance with guidelines for animal research from Istanbul University Ethics Committee on Animal Research (2017/303104).

Collection and Storing of Ovaries and Oocyte Recovery

Ovaries were collected from 15 domestic cats at various stages of the estrus cycle by routine ovariohysterectomy at different local veterinary clinics, and maintained in physiological saline at 37℃ or 4℃ until oocyte recovery for approximately two hours. Some of the ovaries transported at 4℃ were stored at the same temperature for 24 h. Ovaries were sliced and rinsed by washing medium (heparin supplemented HEPES modified TCM 199) at room temperature in order to obtain cumulus oocytes complexes (COCs). The COCs were washed three times with modified synthetic oviduct fluid medium. Large oocytes with darkly pigmented ooplasm and completely surrounded by at least one layer of cumulus cells were selected for *in vitro* maturation (IVM) [14].

In vitro Maturation (IVM)

In vitro maturation was performed as described in our previous study [15]. In brief, the IVM medium was modified Synthetic Oviduct Fluid, supplemented with 10 μg/mL follicle stimulating hormone (FSH), 10 μg/mL luteinizing hormone (LH), 4% bovine serum albumin (BSA, Fraction V) and antibiotics. The COCs were selected and maturated at 38℃ for 48 h in four-well petri dishes (NUNCR, Denmark) including 500 μL maturation medium under mineral oil. *In vitro* maturation was performed at 38℃ in a humidified atmosphere with 5% CO_2 for 48 h.

Assessment of the Nuclear Maturation

At the end of IVM, oocytes were transferred into hSOF medium containing 0.2% (w/v) hyaluronidase and vortexed for 30 secs and then denuded by gentle pipetting. In order to dispersal the chromatins, the oocytes were placed in KCl solution (0.7%, w/v) for 3-5 min and the nuclear maturation rates were examined under a fluorescent microscope after 20-30 min Hoechst (33342) staining.

TUNEL Assay (Tdt-Mediated dUTP Nick-end Labelling)

In order to determine the effects of transporting temperature and storage time on apoptosis rate, 5-10 COCs were randomly selected from each transport temperature and stored groups before *in vitro* maturation procedure. Apoptotic cells in the sections were visualized by TUNEL

(terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) using ApopTag® Plus Peroxidase In Situ Apoptosis Kit (Millipore, S7101, Darmstadt, Germany). Staining was performed according to the manufacturer's instructions. Briefly, the samples (COCs) were fixed in methanol, and then washed in phosphate buffered saline solution (PBS) containing 50% Tween-20 for 15 min. The samples were then treated with 3% H_2O_2 (Merck, 1.08597) in PBS in a dark room to inhibit endogen peroxidase activity. In order to remove H_2O_2 solution, sections were washed three times for two minutes in PBS solution with Tween-20. Then, 50 µL balancing buffer was applied to per each section and waited for 30 min at room temperature. Balancing buffer was wiped off from sections with a napkin, and TdT enzyme was applied to the tissues, and then sections were covered and were allowed to incubate for one hour. To stop TdT enzyme reaction, stop/wash buffer in the kit was applied for 10 min at room temperature, after this sections were washed in PBS with Tween-20 for 15 min (3 times x 5 min). Afterwards, 50 µL of anti-digoxigenin peroxidase enzyme was applied for 30 min at room temperature. After sections were washed in PBS with Tween-20 for 15 min (3 times x 5 min) 3,3'-diaminobenzidine (DAB) substrate was applied as a chromogen. After 20 min of application, the reaction was stopped with distilled water when observed the brown color. Sections were painted with methyl green used as a contrasting paint, then quickly were passed through butanol. Slides were clarified with a total of 15 min (3 times x 5 min) xylol application, closed with entallan and prepared for examination under light microscope.

Calculation of Apoptotic Index

Apoptotic index percentage were determined independently by two observers by counting apoptotic and non-apoptotic cumulus cells in randomly selected five different areas in three different groups at 400x magnification. After cell counting, apoptotic index was calculated using 100x (number of TUNEL-positive cell nuclei/total number of cell nuclei) formula [16]. All sections were photographed by a DM4000 B (Leica) microscope *(Fig. 1)*.

RESULTS

In Vitro Maturation (IVM) Results

The findings of the *in vitro* maturation rates of the study are summarized in *Table 1*. According to the oocytes matured to MII stage, there was no difference between 37℃ and 4°C transport temperature groups (P>0.05) However, the rate of oocytes reached to MII stage in cold stored group was significantly lower than the other two groups (P<0.05).

Findings of TUNEL Method

While apoptotic cells in oocyte sections containing cumulus oophorus were examined by TUNEL method, it was determined that the apoptotic morphological features were seen rarely and in similar rates in 37℃ and 4°C transporting groups (19.40 and 21.55%) *(Fig. 2* and *Fig. 3)* but, it was seen more intensely in the cold stored group (34.80%) (P<0.001). Although apoptotic index percentage of 4°C transportation group was higher than 37°C group, this increase was not statistically significant (P>0.05) *(Fig. 4)*.

Statistical Analysis

The experiments were replicated 5 times. Statistical analysis of IVM rates of oocytes and apoptotic activity rates of cumulus cells were performed using by "Mann Whitney U" test by SPSS for Windows version 13.0.

DISCUSSION

This study is performed to determine the effects of two ovary transport temperature (37℃ vs 4°C) and long term cold storage of ovaries on cumulus cell apoptosis and *in vitro* maturation rates of cat oocytes. The effects of the transporting and storage temperature and time on cat ovaries are controversial. It is known that the modification of the physiological composition of the follicular fluid due to the transport of mammalian ovaries may induce changes in oocyte quality, causing a negative impact on

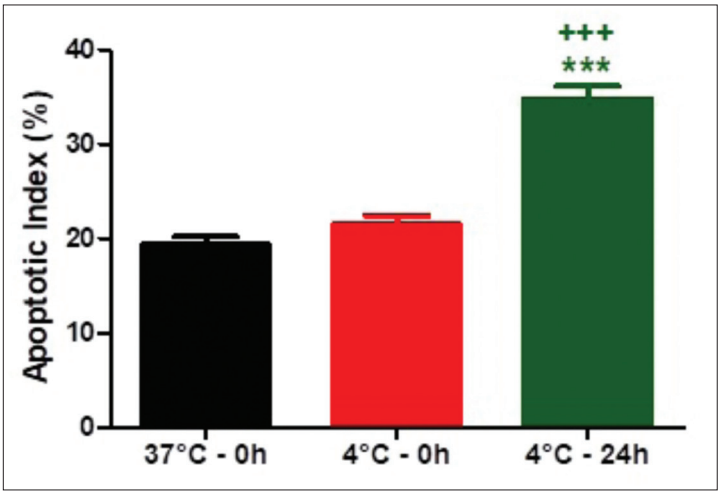

Fig 1. Apoptotic index (%) in all groups (*** P<0.01, compared to 37°C - 0 h group; (+++ P<0.001, compared to 4°C - 0 h group)

Table 1. *In vitro maturation rates of oocytes obtained from ovaries transported in two different temperatures and cold stored for 24 h*

Groups	n	GV n (%)	GVBD n (%)	MI n (%)	MII n (%)	UDNM n (%)
Transported in 37°C	217	3 (1.38)[a]	18 (8.30)[a]	69 (31.80)[a]	108 (49.77)[a]	19 (8.75)[a]
Transported in 4°C	202	10 (4.95)[b]	28 (13.86)[a]	60 (29.70)[a]	90 (44.55)[a]	14 (6.93)[a]
Transported and stored 24h in 4°C	201	32 (15.92)[c]	48 (23.88)[b]	23 (11.44)[b]	38 (18.90)[b]	60 (29.80)[b]

[a,b,c] Values with different superscripts in the same column are significantly different (P<0.05); ***GV:*** *Germinal Vesicle,* ***GVBD:*** *Germinal Vesicle Breakdown,* ***MI:*** *Metaphase I,* ***MII:*** *Metaphase II,* ***UDNM:*** *Undetermined Nuclear Material*

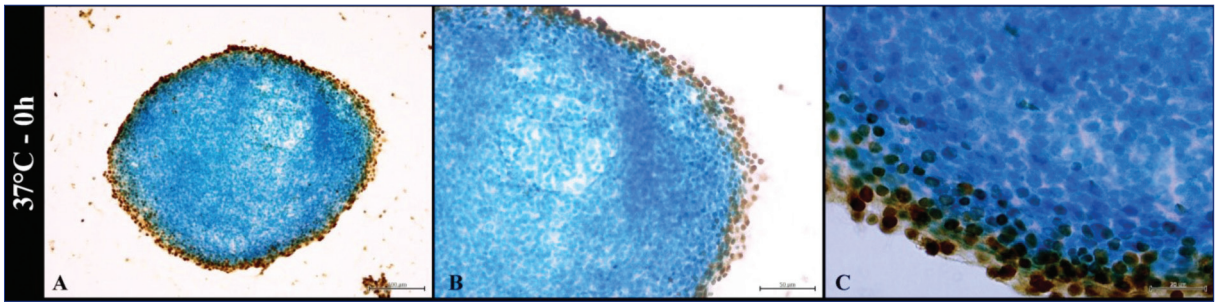

Fig 2. Representative TUNEL assay images of COCs in 37°C transporting group. Scale Bar=100 µm (A), 50 µm (B) and 20 µm (C); **A, B, C:** Apoptotic cumulus cells show intense brown fluorescence, whereas normal cells appear blue stained with methyl green

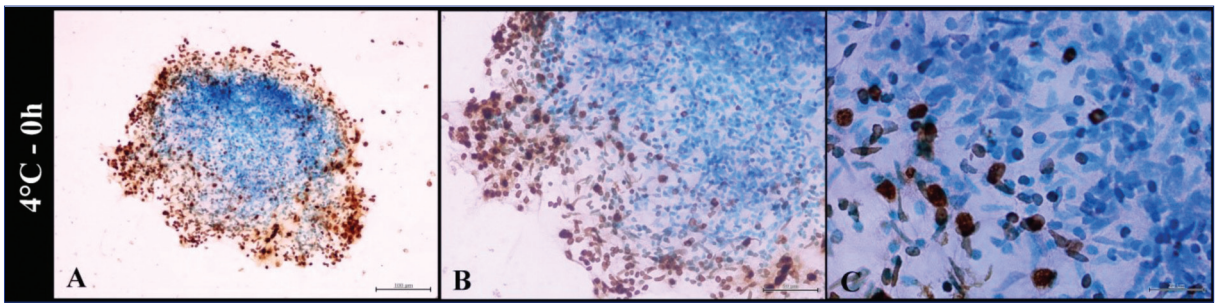

Fig 3. Representative TUNEL assay images of COCs in 4°C transporting group. Scale Bar=100 µm (A), 50 µm (B) and 20 µm (C); **A, B, C:** Apoptotic cumulus cells show intense brown fluorescence, whereas normal cells appear blue stained with methyl green

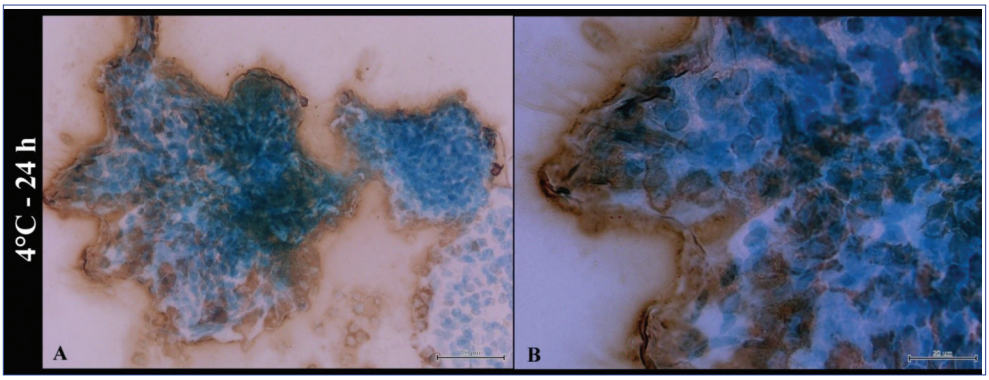

Fig 4. Representative TUNEL assay images of COCs in 4°C 24h storing group. Scale Bar=50 µm (A) and 20 µm (B); **A, B:** Apoptotic cumulus cells show intense brown fluorescence, whereas normal cells appear blue stained with methyl green

maturation [17]. It is indicated that storage of cat ovaries at high temperature (>23°C) for 24 h reduced the meiotic capacity of oocytes and the length of storage at room temperature affected the quality and developmental competence of oocytes [2]. It is also indicated that storage of domestic cat ovaries at room temperature even for a short time can negatively influence the competence of oocytes to undergo nuclear maturation *in vitro* [18]. The

effect of transport temperature on oocytes also vary depending on species. Some researchers [19] stated that warm storage (25°C) of bovine oocytes for up to 11 h resulted in fertilization and development to blastocyst stage, but longer warm storage or storage for any time at 4°C resulted in a few fertilizable oocytes**.** It has been demonstrated that unlike other species cat oocytes have a unique ability to mature *in vitro* after storage of ovaries for 24 h at 4°C [1]. Parallel to this finding, our previous study demonstrated that domestic cat oocytes have the ability to mature successfully *in vitro* after storage of ovaries for 24 h at 4°C [14]. However, it is stated that granulosa cell apoptosis in feline ovaries was increased after 12 h of storage at 4°C and consequently affected IVM results negatively [10]. Although it has been reached to high maturation ratios after the storage of ovaries for 24 h at 4°C, cat oocytes began to lose the fertilization and cleavage ability *in vitro* after the storage [1]. In our study, although the apoptotic index percentage of 4°C transportation group was found higher than 37°C group, this increase was not statistically significant. This result consistent with the researcher's [10] result that transporting ovaries both 37°C and 4°C temperatures have not harmful effect on cumulus cells of cat oocytes. It is stated that to avoid changes in cumulus oophorus morphology the ovaries should be held at 35-37°C and for less than 2 h before processing, and to avoid oocyte chromatin configuration changes ovaries should be stored for less than 6 h [11]. However, it is proposed that storing of bovine ovaries at 10°C for 24 h, did not affect oocyte maturation rates compared with controls [20]. Since the enzymes in animal blood are known to work most efficiently at body temperature, the transport temperature lower than 35-37°C may be expected to delay apoptosis [21]. It is stated that most of the glucose in the follicular fluid is consumed by the granulosa and cumulus cells in the glycolytic pathway and lactate accumulates in the first two hours of the mammalian oocytes in warm temperatures [17]. It is expected that the low temperature decreases the metabolism and slows down the activities of the enzymes. Same researchers showed that transportation ovaries at warm temperatures (25-35°C) for more than two hours decreased follicular pH, increased ROS levels and thus reduced *in vitro* maturation rates [17]. Parallel to this knowledge, it is indicated that storage of porcine ovaries in 35°C for 6 h was efficient to support the developmental competence of oocytes but, these results were lower than those stored at the same temperature for three hours [22]. However, some researchers unexpectedly reported that although the transport temperature of ovaries did not influence the equine oocyte chromatin, it affected cumulus morphology and lower storage temperature resulted in more denuded and expanded COCs [11]. It is possible that due to protein degradation or loss of certain functions in the cumulus cells might cause apoptosis during cold storage. It is stated that long-term storage or transportation of porcine ovaries increased the number of oocytes with DNA fragmented nuclei by inducing acidosis in follicular fluids [22]. In this study, we have found that the rate of oocytes having undetermined nuclear material (UDNM) in cold stored group was significantly higher (29.80%) than the other two groups ($P<0.05$). This finding is inconsistent with our previous results [14] and the researchers claiming that cat oocytes have a unique ability to mature *in vitro* after storage of ovaries for 24 h at 4°C [1]. Some researchers supplemented superoxide dismutase (SOD) to the ovary transport and storage medium to prevent the damage of reactive oxygen species (ROS), and they found beneficial effects of SOD such as lower cellular apoptosis and higher COC survival and *in vitro* embryo production rates. In the same study, researchers obtained blastocysts from *in vitro* fertilized oocytes from cat ovaries stored at 4°C for up to 72 h in transport media supplemented with SOD [23]. These results suggested that different approaches are needed with further studies to identify species-specific factors that would allow feline ovaries to be stored for longer periods.

In conclusion, this study has shown that there is a distinctive relationship between the long storage at cold temperature and COCs quality and IVM rates of the cat ovaries. According to our results, it can be concluded that (I) transporting of the cat ovaries at 37°C or 4°C for a period not longer than two hours provides the appropriate conditions to maintain the quality of COCs and meiotic development *in vitro*, (II) the cold (4°C) storage of cat ovaries for 24 h has deleterious effects on COCs quality and IVM rate of oocytes and (III), cumulus cell apoptosis rate would be a valuable marker for the ability of IVM of cat oocytes.

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Short Communication

Brucella melitensis Isolated from Aborted Cow and Sheep Fetuses in Northwest of China

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Abstract

This study aimed to investigate the causes of abortion in cows that were mixed fed with sheep and/or goats, and the presence of *B. melitensis* infection in cows. PCR of 34 (28%) specimens out of 120 samples were identified as *B. melitensis*. The traditional bacteriological tests identified all of the isolates from sheep/cow aborted fetuses and milk as *B. melitensis* biovar 3. This is the first study to demonstrate *B. melitensis* as the main etiological agent for cows mixed fed with sheep and/or goats in XUAR, northwest of China. This may cause severe infection in the local population, and pose a potential public health risk, especially when eating or drinking the products of contaminated milk.

Keywords: *Brucella melitensis, Cow, Abortion, China*

Kuzey Batı Çin'de Atık İnek ve Koyun Fetüslerinden *Brucella melitensis* İzolasyonu

Öz

Bu çalışmanın amacı koyun ve/veya keçilerle birlikte yetiştirilen ineklerde atıkların sebeplerini araştırmak ve *B. melitensis* enfeksiyonunun mevcudiyetini belirlemektir. Toplam 120 örneğin 34'ünde (%28) PCR ile *B. melitensis* tespit edildi. Klasik bakteriyolojik testler, koyun ve inek atık fetüsleri ile sütten elde edilen izolatların tümünde etkenin *B. melitensis* biovar 3 olduğunu belirledi. Bu çalışma, Çin'in Sincan Uygur Özerk Bölgesi'nde koyun ve/veya keçilerle birlikte yetiştirilen ineklerde *B. melitensis*'in atıklarda ana etiyolojik ajan olduğunu göstermektedir. Bu durum bölge popülasyonda ciddi enfeksiyona neden olabilir ve bu suretle özellikle kontamine süt ürünleri tüketiminde potansiyel halk sağlığı riski oluşturabilir.

Anahtar sözcükler: *Brucella melitensis, İnek, Abort, Çin*

INTRODUCTION

Brucellosis, a zoonosis of public health and economic importance worldwide, leads to great loss to domestic animals, principally in cows, sheep and goats. In some countries, particularly in southern Europe and Western Asia, where cattle are kept in close contact with sheep and goats, infection can also be caused by *B. melitensis* [1], but the symptom of abortion in cows is not as severe as in sheep or goats. Mixed farming is also adopted by smallholder farmers in China.

Six classical species of *Brucella* have been identified, including *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae*. Although *B. abortus* is considered as the main etiological agent of infected dairy cows, *B. melitensis*

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results in the greatest loss to domestic animal industry and public health [2,3]. Therefore, investigating the infection of *B. melitensis*, as a nonspecific and heterogeneous agent in dairy herds, is crucial.

Xinjiang Uygur Autonomous Region (XUAR) in the northwest of China, is the largest province in China, and the livestock industry is the main source for its economic growth [4]. Very few studies have been conducted on prevalence and distribution of brucellosis in remote areas. The aim of this study was to investigate the etiological agents responsible for abortions in sheep or cows in endemic areas of brucellosis, and the presence of *B. melitensis* as a heterogeneous agent in dairy cows.

MATERIAL and METHODS

Bacterial Strains

Reference strains of *Brucella melitensis* 16M, *B. abortus* 2308 and *Toxoplasma gondii* as well as *Campylobacter fetus spp*, *Theileria sergenti* and *Tritrichomonas fetus* were provided by Anthropozoonosis laboratory in Shihezi University.

Sample Collection and DNA Extraction

The samples including aborted fetuses (n = 120) and raw milk (n = 1) were collected from Ili region (northwest of XUAR) in some sheep and cow mix feeding farms between April and May in 2016. Samples of spleen, liver and lung tissues and stomach contents were collected aseptically from sheep or cow aborted fetuses and raw milk from a cow with clinical signs of joint swelling and abortion history. Then the DNA extraction from tissue samples was performed using the TIANamp Genomic DNA Kit (TIANGEN BIOTECH CO., LTD) according to the manufacturer's instructions. The nucleic acid extraction from raw milk was performed as previously described [5]. DNA concentrations were determined by measuring the A_{260}, and the samples were stored at -20°C until further processing.

Synthetic Oligonucleotide Design

Oligonucleotide species-specific primers for *Brucella* genus [6], *Toxoplasma gondii* [7], *Campylobacter fetus* [8], *T. buffeli* [9], and *Tritrichomonas fetus* [10] are listed in *Table 1*.

PCR Amplification and Sequence Analysis

All samples were examined by PCR in a total volume of 30 µL, with 12.5 µL ddH_2O, 15 µL mix, 0.5 µL of each primer and 1.5 µL DNA template. The reaction was performed in a DNA thermal cycler (Perkin-Elmer) and 2 µL of the product was fractionated in a 1.5% or 2% agarose gel, stained with 0.5 mg/mL ethidium bromide solution, and visualized under UV light [11]. The positive amplication products were purified using the TIAN-gel Midi Purification Kit (TIANGEN, Beijing, China) and then subjected to sequencing. All of these data was analyzed using SPSS version 17.0 software.

Bacterial Isolation

Brucella was isolated from raw milk sample as previously described [12]. The tissue samples were homogenized before plating on the Brucella-selective agar. Then, 100 µL of the homogenized suspension was inoculated onto Brucella-selective agar plates. The suspension was spread with a loop producing a depot followed by single colonies. All cultures were incubated at 37°C with 5% CO_2 for five days. *Brucella* identification and species differentiation were accomplished using PCR protocols [13]. Furthermore,

Table 1. *PCR primers used for screening abortion-inducing pathogens in sheep*

Primer	Primer Sequences (5'-3')	Size (bp)
Brucella-omp22- primer -F	TGATGGGAGGGACCGACTA	526
Brucella-omp22- primer -R	TGGTTCTTCAGGTTGTTACGC	
B. abortus-IS711- primer -F	GACGAACGGAATTTTTCCAATCCC	526
B. abortus- IS711- primer -R	TGCCGATCACTTAAGGGCCTTCAT	
B. melitensis- IS711- primer -F	AAATCGCGTCCTTGCTGGTCTGA	731
B. melitensis- IS711- primer -R	TGCCGATCACTTAAGGGCCTTCAT	
C. fetus- sapB2- prime r-F	GCAAATATAAATGTAAGCGGAGAG	435
C. fetus- sapB2- prime r-R	TGCAGCGGCCCCACCTAT	
T. buffeli-18S rRNA- primer -F	AAACTGCGAATGGCTCAT	816
T. buffeli-18S rRNA- primer -R	ACATCCTTGGCAAATGCT	
T. fetus-TFITS- primer - F	CTGCCGTTGGATCAGTTTCG	208
T. fetus-TFITS- primer - R	GCAATGTGCATTCAAAGATCG	
Toxoplasma gondii-specific-primer -F	CGCTGCAGGGAGGAAGACGAAAGTTG	529
Toxoplasma gondii-specific-primer-R	CGCTGCAGACACAGTGCATCTGGATT	

[a] The pair of primers of Brucella omp22 were used to screen Brucella spp. in the first round identification; [b] The pair of primers of Brucella IS711 were used to differentiate the species of Brucella

biotyping of the *Brucella* was based on conventional bacteriological and typing methods [14]. This process was completed at the Center for Disease Prevention and Control (CDC) of China.

RESULTS

Molecular Detection

In the present study, molecularly positive products for *Brucella* genus were found but not for *T. gondii*, *C. fetus spp*, *T. sergenti* and *Tr. fetus*. Thirty-four (28.09%) samples, including 13 aborted sheep fetuses, 20 cow fetuses and one raw milk sample, were positive and further identified as *B. melitensis* by targeting *IS711* gene and only part of the positive samples were presented in *Fig.1*, the rest of data was not shown. *B. abortus* and other species were not detected in the organs of aborted sheep, cow fetuses and raw milk. The nucleotide sequences from our study have been deposited in the GeneBank database (accession number: KY312521). There were no differences in DNA sequences as compared to that of *B. melitensis* biovar 3 NI strain (accession number: CP002931) [15].

Isolation of Brucella spp. from Organs

Bacteria were isolated from 34 samples and positively detected by *IS711* gene. The *Brucella* wild strains were isolated from 13 aborted sheep fetuses, 20 aborted cow fetuses and one raw milk sample. The detailed information is shown in *Table 2*. Furthermore, the culture isolates were identified as *B. melitensis* biovar 3 by conventional bacterial tests.

DISCUSSION

In XUAR, brucellosis has prevailed for decades [16], where the seropositive rates for cows and sheep were 0.66% and 3.25%, respectively, during 2013-2014 [17], and there are many pathogens could induce abortions in pregnant animals such as *Coxiella burnetii*, *Chlamydophila abortus*, *Salmonella enterica Serovar Abortusovis*, *T. gondii*, and *Neospora caninum* [18]. But, in the present study, *Brucella* was found to be the main pathogen responsible for livestock abortion and the rest of pathogens listed in *Table 1* were not found in these aborted fetuses, the result suggests that the *Brucella* pose the biggest threat to local livestock and people due to the infected cow could spread the disease through milk or contaminated dairy products. Interestingly, all of the isolates were identified as *B. melitensis* biovar 3 by conventional bacteriological and typing methods [14].

In Turkey, *B. melitensis* biovar 3 was first isolated from bovine aborted fetus [1]. In China, It was isolated in raw milk from an aborted cow at a farm that had about 300 sheep and 40 cows in Inner Mongolia, north of China [15]. The phenomenon of a host shift (i.e., the ability of a pathogen to colonize or infect a new host) is rare and appears in

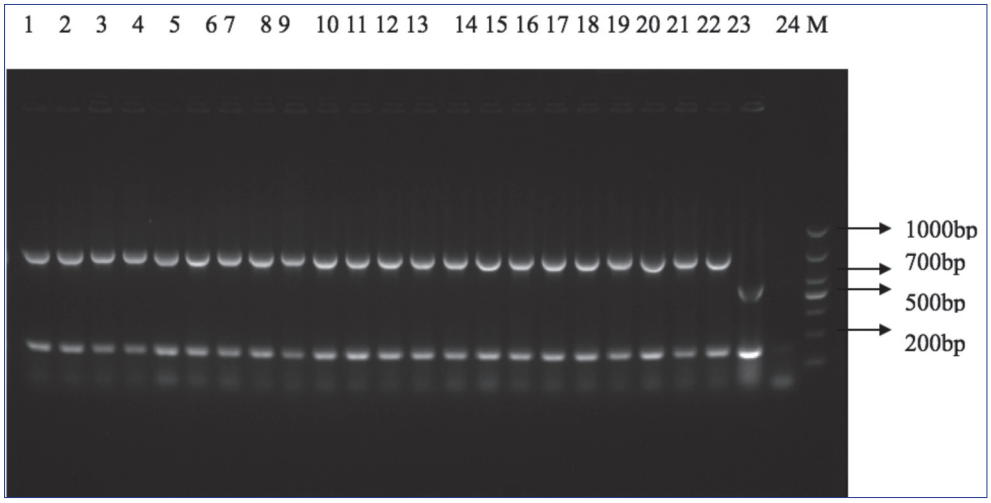

Fig 1. PCR product of *IS711* gene amplification
Lane 1-10: sheep aborted fetuses; Lane 11: milk sample; Lane 12-21: cow aborted fetuses; Lane 22: *B. melitensis* 16M; Lane 23: *B. abortus* 2308; Lane 24: Negative control; Lane M: DM1000

Table 2. *Detection B. melitensis in individual tissue or milk samples by bacterial isolation*

Animal No	Organs	Host	Result
1	Spleen Liver Lung	Cow	+ + -
2	Spleen Liver Lung	Sheep	+ - -
3	Spleen Liver Stomach contents	Sheep	- + +
4	Spleen Liver Milk	Cow	+ - +
5-15	Splen Liver	Sheep	+ +
16-34	Splen Liver Stomach contents	Cow	+ + -

resource-poor communities in China due to the mixed feeding of cows with infected sheep and/or goats and ignoring brucellosis quarantine. This study described *B. melitensis* isolates from aborted cow fetuses and raw milk. The result suggests that *B. melitensis* infection in cows is an emerging livestock industry and public health issue in China. As demonstrated in this study, *B. melitensis* can be shed in raw milk from infected cows. In addition, infection might spread to farm workers, slaughterers, and veterinarians through handling infected animals or organs after slaughter [19]. *B. melitensis* infection in cows may become more common in the future, although no data is available on brucellosis patients due to *B. melitensis* infection transmitted by raw milk or its products in China.

The problem of cows infected by *B. melitensis* has potentially important implications for the control programs of brucellosis in China. The clinical symptoms of *B. melitensis* infection in cows is not apparent as compared to that of *B. abortus* infection [20]. The infected cow, as a reservoir, is susceptible to disseminating contaminated milk to the local or neighboring population. This study recommends: i) avoiding intermixed feeding model of cows, sheep or/and goats in the same yard in endemic areas, and ii) increasing regular quarantine of brucellosis, and timely elimination of the infected sheep, goats and cows from the herd.

Acknowledgments

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Case Report

Pathomorphological and Immunohistochemical Findings of Subacute Lobullary Calcifying Panniculitis in Two Cats [1]

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Abstract

This report aimed to reveal the characteristics of calcifying panniculitis in two cats. Two biopsies taken from the lumbosacral region of an 8-month old and a 1-year old, male, mixed breed cats were evaluated. Macroscopically, there were masses of different sizes varying from 1.5 to 2.5 cm in diameter. The masses had a generally firmness and were grayish-white in colour. Histopathology revealed necrotic and degenerative lipocytes in lobules of subcutaenous fat tissue. In some areas, there were lymphocyte, macrophage and neutrophil leukocytes infiltrations and connective tissue proliferation. There were also large calcifying areas at the centre of degenerated-necrotic fat lobules. Alizarin Red S detected the calcifying areas and Masson's trichrome differentiated connective tissue proliferation. In ABC-P, CD3 slightly reacted with lymphocytes and lymphoblasts. Vimentin moderately reacted with connective tissue proliferation at the periphery of the necrotic areas and the septum. A1AC reacted in the cytoplasm of macrophages and peripheral necrotic areas. No reaction was determined with A1AT. In conclusion, such cases have not been documented in veterinary pathology. It is believed that the two cats possibly had a renal deficiency problem or nephrotic syndrome in the pathogenetic mechanism. It was also considered that A1AC (serine proteinase inhibitor) expressions could have a role in such cases despite there being no A1AT (another serine proteinase inhibitor) expression.

Keywords: *Calcifying panniculitis, Cat, Immunohistochemistry, Pathomorphology*

İki Kedide Karşılaşılan Subakut Lobüller Kalsifiye Panniküliti̇sin Patomorfolojik ve İmmunohistokimyasal Bulguları

Öz

Sunulan bu iki olguda kedilerdeki kalsifiye panniküliti̇sin karakteristik özelliklerinin açığa çıkarılması amaçlandı. Sekiz aylık ve bir yaştaki iki erkek, tekir kedide lumbosakral bölgeden alınan biyopsiler değerlendirildi. Makroskopik olarak çapları 1.5-2.5 arasında değişen, sert kıvamda beyazımsı kitleler mevcuttu. Histopatolojik incelemede deri altı yağ dokusundaki lobüllerde dejeneratif ve nekrotik lipositler gözlendi. Bazı alanlarda lenfosit, makrofaj ve nötrofil lökosit infiltrasyonları ile bağ doku proliferasyonu vardı. Ayrıca dejenere-nekrotik yağ lobüllerinin merkezinde geniş kalsifiye alanlar da gözlendi. Alizarin Red S boyaması ile kalsifiye alanların, Masson'un trikrom boyamasıylada bağ doku proliferasyonunun ayrımını yapıldı. ABC-P yönteminde, CD3 lenfosit ve lenfoblastlarla hafif reaksiyonlar verdi. Vimentin nekrotik alanlarda ve septumda yer alan bağ doku proliferasyonlarıyla orta şiddette reaksiyon verdi. A1AC makrofajların sitoplazmasında ve perifer nekrotik alanlarda reaksiyon verdi. Sonuç olarak bu türden vakalar veteriner patolojide bildirilmemiştir. Her iki kedide de gelişen lezyonların patogenetik mekanizmasında böbrek yetmezliği ya da böbrek kökenli bir sendrom olabileceği düşünülmektedir. Ayrıca, serin proteinaz inhibitörü olan A1AT ekspresyonu olmamasına karşın başka bir serin proteinaz inhibitörü olan A1AC ekspresyonlarının bu tür olgularda rolü olduğuna inanılmaktadır.

Anahtar sözcükler: *İmmunohistokimya, Kalsifiye panniküli̇ti̇s, Kedi, Patomorfoloji*

INTRODUCTION

A panniculitis is a group of heterogeneous disease whose hallmark is inflammation of subcutaneous adipose tissue or panniculus adiposus [1]. The inflammation frequently affects the deep dermis. In the etiology, there are generally infectious agents, foreign bodies, vitamin E deficiency, trauma, pancreatic disease, vasculitis, drug eruption and

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lupus erythematosus in human beings. The disease may sometimes develop after steroid application, exposure to cold air or ice and α1-antichymotrypsin (A1AC), α1-antitrypsin (A1AT) deficiency, especially in humans. It has also been documented that the etiology of Weber Christian disease in humans remains unknown. The clinical classification of panniculitis has always been a problem in human dermatology, so classification has been performed on the basis of histological appearance. In medicine, these inflammatory changes of subcutaneous fat tissue can be classified in several types, although lobular (fat lobules), septal (interlobular septa including connective tissue) and diffuse (both fat lobules and septa) forms of panniculitis are found according to distribution in veterinary medicine [2]. The classification can be diversified into many types related to the etiology. Four main types have been defined as: 1) lobular panniculitis without vasculitis, 2) lobular panniculitis with vasculitis 3) septal panniculitis without vasculitis, 4) septal panniculitis with vasculitis [3]. Although calcifying panniculitis has been known since the 1980s, it is seen in the classification as a special topic [4-10]. That type of panniculitis has been generally reported in humans with renal failure and has primarily involved anticoagulant-like heparin injection sites [4,9]. The place of calcifying panniculitis has not been conclusive despite being known as a sub-class of lobular panniculitis without vasculitis [11]. It is a peculiar form of calcinosis cutis which belongs to the spectrum of calciphylaxis [4]. The most possible reason is the disturbance of the calcium-phosphate balance [12]. Macroscopically, lesions are seen as symmetrical violaceous to black patches or plaques. They frequently develop on the legs, and sometimes on the upper extremities and trunk. The lesions can be enlarged with necrotic and black eschar tissues. Over time, ulcers may open from those areas of subcutaneous necrosis. Microscopically, there is mucinous degeneration of fat tissue, lymphocytic vasculitis and adventitia and medial calcium deposits within adipose lobules of the interstitial connective region [4,7,10,11]. The aim of this report was to determine the different characteristics of calcifying panniculitis in two cats.

CASE HISTORY

Two brother cats, aged 8 months and 12 months old were brought to a private veterinary clinic with complaints of inappetence, depression and occasional jaundice. In clinical examination, there was seen to be cachexia and tenderness to abdominal palpation. The left lumbosacral region of the dorsal trunk in both cats was seen to be painful and partly ulcerated with eschar masses. Blood samples were collected from the cats and sent to a private veterinary laboratory for examination of blood parameters. It was then decided to remove the masses surgically and they were sent for diagnosis to the Pathology Department of the Veterinary Medicine Faculty of Ankara University. After macroscopic examination, tissue samples were taken from the lesions for histopathology examination. The samples were fixed in 10% formalin, processed routinely and embedded in paraffin. Sections were cut from the paraffin blocks of 5 µm thickness and stained with haematoxylin-eosin (H&E), Alizarin Red S and Masson's trichrome methods. After the histochemical staining, the indirect immunoperoxidase method (ABC-P) was applied using CD3, CD4, vimentin, alpha-1 antitrypsin and alpha-1 antichymotrypsin antibodies. The tissue sections were deparaffinized with xylene and rehydrated in decreasing dilutions of alcohol. Then, the sections were washed with phosphate buffered saline (PBS; pH 7.4). Endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 20 min. The sections were processed with 0.1% trypsin at 37°C for 30 min. After the PBS washes, sections were incubated with protein blocking sera (Peroxidase Detection System, Novocastra, RE7110-K, Leica Biosystems). The sections were treated with primary antibodies (polyclonal rabbit alpha-1 antitrypsin-1/100, Abcam, monoclonal mouse anti-vimentin, clone V9, 1/100, Dako, polyclonal rabbit anti alpha-1 antichymotrypsin- 1/50, Abcam, polyclonal rabbit anti CD3 1/50, antibodiesonline, monoclonal mouse anti CD4, 1/100, Dako) in a humidified chamber for 1 h at room temperature.. Then, biotinylated secondary antibody and streptavidin peroxidase complex (Peroxidase Detection System, RE7110-K, Novocastra, Leica Biosystems) were consecutively applied for 30 min each, and the section was washed 3 times with PBS between the applications. Control samples were treated with PBS instead of primary antibodies. As the chromogen, 3-amino- 9-ethylcarbazole (AEC) (Santa Cruz Biotechnology Inc.) was selected. Counterstaining was performed with Gill's haematoxylin. Sections were mounted with glycergel and examined under a light microscope (Leica).

Macroscopically, the mass had focal features of elastic consistency and was grayish-yellow in colour in the 8-month old cat. The mass weighed 1.5 g and measured 1.5x1.3x0.5 cm. In the elder cat, there were three masses of the same appearance as the first one. The masses weighed a total of 7 g and measured 2.5x1.5x1.3 cm, 1.5x2x1.2 cm, and 1.5x1x0.7 cm.

Microscopic examination revealed necrotic and degenerated fat cells in lobules of subcutaneous fat tissue *(Fig. 1A-B)*. In some areas, lymphocyte, macrophage and neutrophile leukocyte infiltrations and connective tissue proliferation were observed. There were also large calcifying areas at the centre of the degenerated-necrotic fat lobules. Calcifying areas were detected with Alizarin Red S stain and connective tissue proliferation was detected with Masson's trichrome stain *(Fig. 1C)*.

Immunohistochemical staining for CD3 slightly reacted with lymphocytes and lymphoblasts *(Fig. 1D)*. Vimentin moderately reacted with connective tissue proliferation at the periphery of necrotic areas and the septum *(Fig. 1E)*. A1AC reacted in the cytoplasm of macrophages and

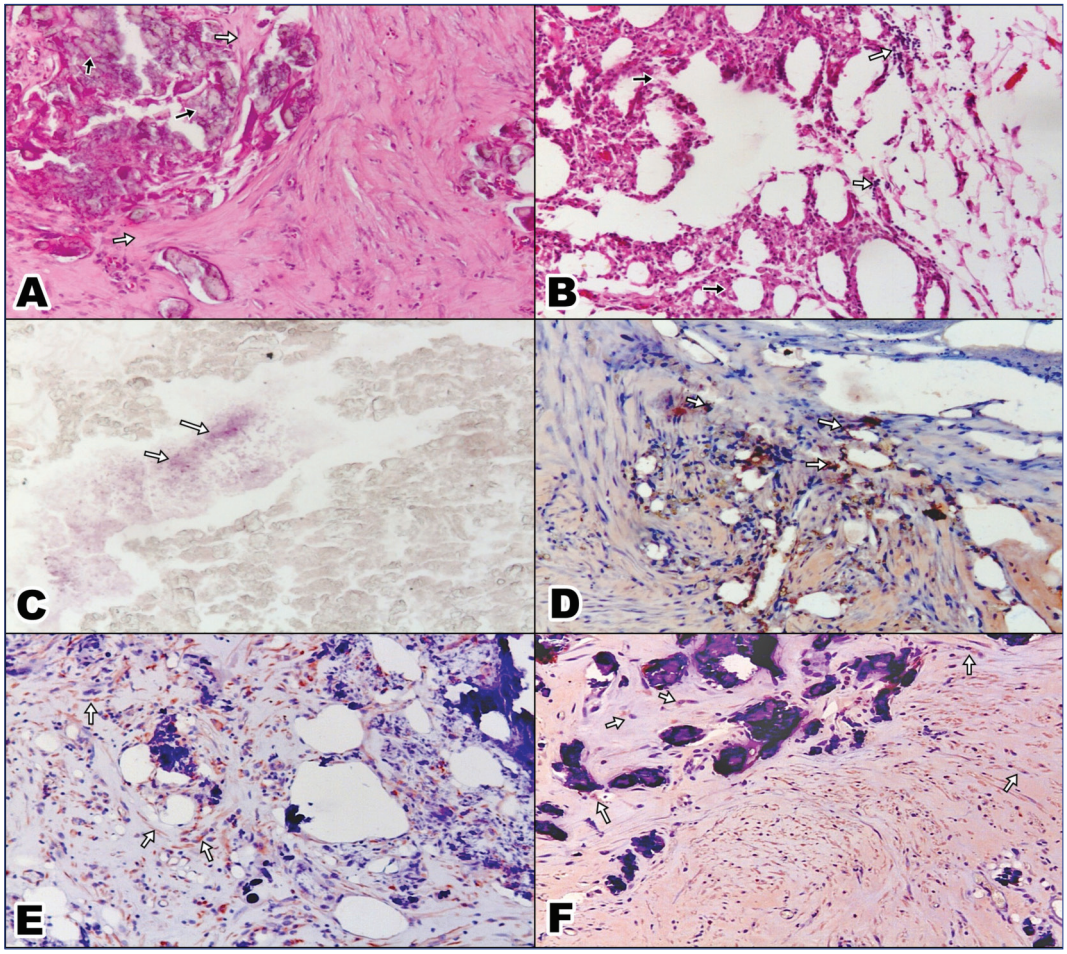

Fig 1. A- Degenerated and necrotic fat *(black arrow)* and collagen *(white arrows)* tissue, x100, H&E; **B-** Degenerated fat cells *(black arrows)* and inflammatory cell infiltration *(arrows)*, x100, H&E; **C-** Calcium deposits in fat tissue *(arrows)*, x100, Alizarin Red S Stain; **D-** CD3 positive T lymphocytes *(arrows)*, x100, ABC-P; **E-** Vimentin-positive fibrocytes *(arrows)*, x100, ABC-P; **F-** A1AC positivity in macrophages *(arrows)*, x100, ABC-P

peripheral necrotic areas *(Fig. 1F)*. CD4 and A1AT were negative.

DISCUSSION

In these two cases, the clinical examination together with the results from the blood parameters suggested an inflammatory skin disease which may have been acute or sublinical in a disease connected to one of the internal organs. The anamnesis was useful for the clinician as vaccinations and parasite therapy had been administered to both cats 2.5 months and 1 month ago, respectively. However, the blood parameters demonstrated different results with low RBC, haemoglobin and high creatinine, calcium, phosphorus and WBC. From these results it was considered that a renal problem and anaemia may be related to eritropoetin dys-synthesis m. Patho-morphologically, lobular evaded necrotic adipocytes with severe inflammation in H&E staining and calcium deposits in Alizarin Red S staining and fibrocytic and fibroblastic proliferation in Masson's trichrome staining revealed calcification in all the masses. Calcifying panniculitis has been reported in literature together with fatigue and a severe condition when considering calciphylaxis and nephrotic syndrome or renal failure in human counterparts [5,7,8,10]. However, despite the lack of appetite and mild cachexia, both cats appeared healthy and not in a serious condition. Histopathologically however, calcium deposits have not been reported to be dense on small or large vessels except on the interstitium although there have been a few reports [4,5]. Unlike other reports in literature and in humans, connective tissue proliferation was noticeable in this study and there was seen to be a tendency to granulomatous inflammation in one of the masses. Immunohistochemically, CD3 confirmed T-lymphocytes even though CD4 was negative for B-lymphocytes. Vimentin showed fibrocytic and fibroblastic proliferation developing at the periphery of necrotic areas. On the other hand, AAT was negative. ACT reacted in macrophages on the periphery of necrotic areas and between adipocytes and also in collagen bundles. In a few reports in recent years, emphasis has been placed on the relationship between alpha-1 antitrypsin (AAT) and alpha-1 antichymotrypsin (ACT) and panniculitis [13,14].

ACT and AAT are members of the serine protease inhibitor (serpin) superfamily of proteins [15,16]. AAT plays a critical role by blocking the proinflammatory effect of neutrophil leukocytes [15]. ACT also inhibits chymotrypsin-like serine proteases, with neutrophil cathepsin G thought to be the main target. It can also inhibit mast cell chymases and angiotensin-converting enzyme proteases which have a role in active vasoconstriction. Rajpara et al.[13] reported that there is a relationship between overactivity of membrane bound serine proteases and neutrophil elastases in subcutaneous fat tissue. However, no immunohistochemical study was applied in terms of localization of positivities. In the current cases, AAT was applied and found to be negative, suggesting that there was no relationship between them, although it was considered that ACT might have a role in the pathogenesis of panniculitis. The ACT positivities from the cases can be considered to support that there is a connection between patients with renal failure and calciphylaxis.

In conclusion, this report is the first in respect of panniculitis classification in veterinary medicine in Turkey. It can be considered of guidance for researchers studying the role of serine protease inhibitors for other types of panniculitis cases in kidney-related disorders.

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Case Report

Concomitant Mammary Tuberculosis and Malignant Mixed Tumor in a Dog [1]

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Abstract

Tuberculosis and tumors are two major health problems in both humans and animals; and there are still many question marks in the association between these two important diseases. In this case, malignant mixed tumor and productive tuberculosis were observed simultaneously in 8 years old, terrier, and female dog. After mastectomy of the left mammary chain; removed tissue was brought for pathological examination. For the histopathology examination, specimens were fixed in 10% neutral buffered formalin. Tissues were processed routinely and stained with hematoxylin-eosin and Ziehl-Neelsen for acid-fast bacilli detection. Microscopically, atypical neoplastic cells and caseation necrosis located in the center of tubercle were seen. After these findings, *Mycobacterium* spp. was investigated in the same sample and was confirmed by PCR amplification. Concomitant tuberculosis and neoplasm, previously reported in only a goat, is a rare occurrence in domestic animals.

Keywords: *Dog, Histopathology, Malignant mixed tumor, Mycobacterium spp.*

Bir Köpekte Memede Beraber Seyreden Tüberküloz ve Malign Mikst Tümör

Öz

Tüberküloz ve tümörler hem insanlarda hem de hayvanlarda gözlenen önemli birer sağlık problemi olup bu iki hastalık arasındaki ilişki hakkında halen çok soru işaretleri mevcuttur. Bu olguda 8 yaşlı, dişi, terrier bir köpekte malign miks tümör ve prodüktif tüberkülozün birlikte seyrettiği gözlendi. Klinikte operasyonla alınan sol meme zinciri patolojik yönden incelenmesi için getirildi. Histopatolojik inceleme için doku %10'luk tamponlu formalin solüsyonunda tespit edildi. Rutin doku takibinin ardından hazırlanan parafin bloklardan kesitler alınarak hematoksilen eozin ve asid fast tüberküloz basili tespiti için Ziehl- Neelsen boyamaları yapıldı. Mikroskobik incelemede atipik neoplazik bez epitel hücreleri ile merkezinde kazeifikasyon nekrozu bulunan tüberküllere rastlandı. Ayrıca *Mycobacterium* spp. PCR amplifikasyonu ile teyit edildi. Tüberküloz ve tümörün bir arada bulunması daha önce sadece bir keçide bildirilmiş olup evcil hayvanlarda nadir gözlenen bir olgudur.

Anahtar sözcükler: *Histopatoloji, Köpek, Malign mikst tümör, Mycobacterium spp.*

INTRODUCTION

Tuberculosis is one of the major zoonotic diseases. *Mycobacterium tuberculosis* and *Mycobacterium bovis* are important pathogenic agents, which are able to infect many animals and humans [1-3]. Dog to dog transmission is rare and also most cases of tuberculosis are transmitted from human reservoirs to dogs [4].

Tuberculosis lesions in carnivores are different from the other species. Typical tubercles are not always observed; and when observed, caseous necrosis of the tubercles are not macroscopically significant. Microscopically, typical granulomas are composed of mononuclear cell infiltration and epithelioid histiocytes surrounded by fibrous tissue. Also, necrosis is present in the center of the granulomas. Giant cells are rare or not seen [5].

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Mammary tumors are neoplasms that are commonly seen in domestic animals such as dogs and cats. The mammary malignant mixed tumor is the most frequent neoplasm in female dogs. This tumor is composed of two cell components: epithelial and connective tissue components, both having malignant character [6,7].

Co-existence of tuberculosis and neoplasm has been found to be important for many years. Previously, concomitant tuberculosis and different types of neoplasms, such as meningioma, renal carcinoma and lymphoma were reported in humans [8-10]. In animals, concomitant mammary carcinoma and tuberculosis was reported only in a goat [11].

The purpose of the case is to describe concomitant mammary tuberculosis and malignant mixed tumor for the first time in a dog.

CASE HISTORY

An 8-year-old terrier, female dog was brought to a private clinic with tumor suspicion in mammary glands and surgical operation was performed. Left mammary chain was removed and sent to Ankara University, Faculty of Veterinary Medicine, Department of Pathology for pathological examinations.

Macroscopically, multifocal areas, smaller than 1 cm in diameter, with yellowish/white caseous appearance were seen on mammary glands. For the histopathological examination, specimens were fixed in 10% neutral-buffered formaldehyde. The tissue samples subjected to routine tissue processing, were embedded in paraffin and cut at a thickness of 4-6 µm. First, all sections were stained with the routine haematoxylin-eosin. In addition to routine staining, Ziehl- Neelsen staining was done for detection of acid-fast bacilli.

With haematoxylin-eosin staining, neoplastic mammary gland cells that had differences in size and shape, variations in the cytoplasm/nucleus ratio, pale cytoplasm with unobvious border with mitotic figures were observed *(Fig. 1)*. Metaplastic changes in the mesenchymal tissue accompanied these cells. Also, a few areas of hyalinization and necrosis were recognized *(Fig. 1)*. In the same areas, chronic granulomatous inflammation resembling tubercles was seen. At the center of the tubercles caseous necrosis was present, surrounded by lymphocytes and epithelioid cells infiltration and a fibrous tissue capsule *(Fig. 2)*. With Ziehl- Neelsen staining, acid-fast bacilli were freely scattered in the granulomas.

After these findings, frozen tissue sample suspected to be *Mycobacterium* spp. was sent to Microbiology Department of Ankara University, Faculty of Veterinary Medicine, for the investigation of *Mycobacterium* spp. The confirmation of the histopathological examination was performed by Mycobacterium genus specific PCR *(Fig. 3)*. DNA was extracted from the tissue sample with a commercial kit (Genomic DNA Purification, Catalog No: K0512, Thermo Fisher Scientific, U.S.A.) following the manufacturer's recommended protocol. PCR was performed using specific primers (Forward- B16F 5'-GGG ACGAAGTCGTAACAAGG-3', Reverse- B16R 5'-TGATGCTC GCAACCACTATC-3'), which were designed and tested in-silico for this study to amplify a 270 bp product. The PCR reaction was performed containing 0.2 µM of each primer, 0.2 mM dNTPs (10 mM dNTP mix; (Thermo Fisher Scientific, USA), 3 mM of $MgCl_2$ (Thermo Fisher Scientific, USA), 2.5 µL PCR reaction buffer, 2U of Taq DNA polymerase (Thermo Fisher Scientific; EP0402), and nuclease-free water to a final volume of 25 µL. In the reaction, 1 µL of DNA was used as template. The amplification was performed as follows: strand separation at 95°C for

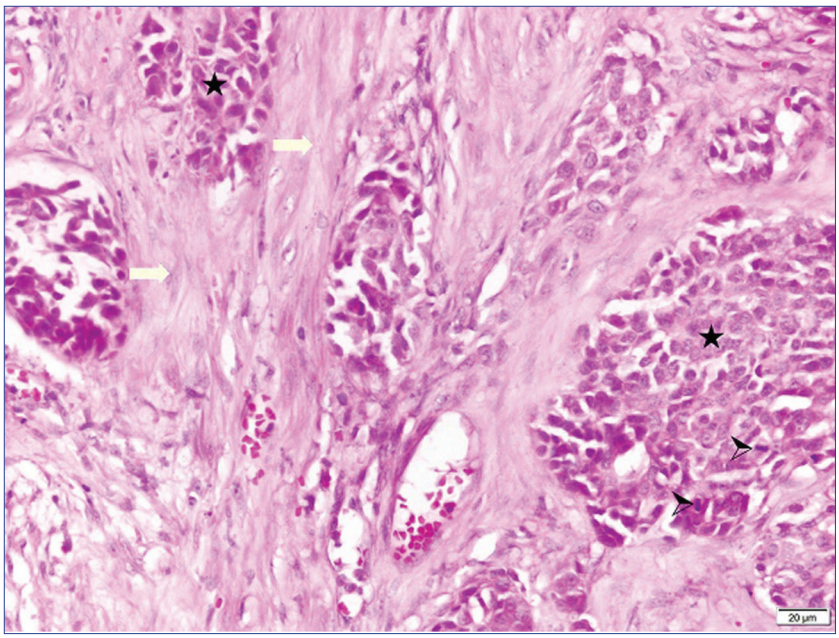

Fig 1. Anaplastic mammary gland cells *(stars)*, mitotic figures *(arrow heads)*, hyalinization of fibrous connective tissue *(white arrows)*, HxE, bar: 20 µm

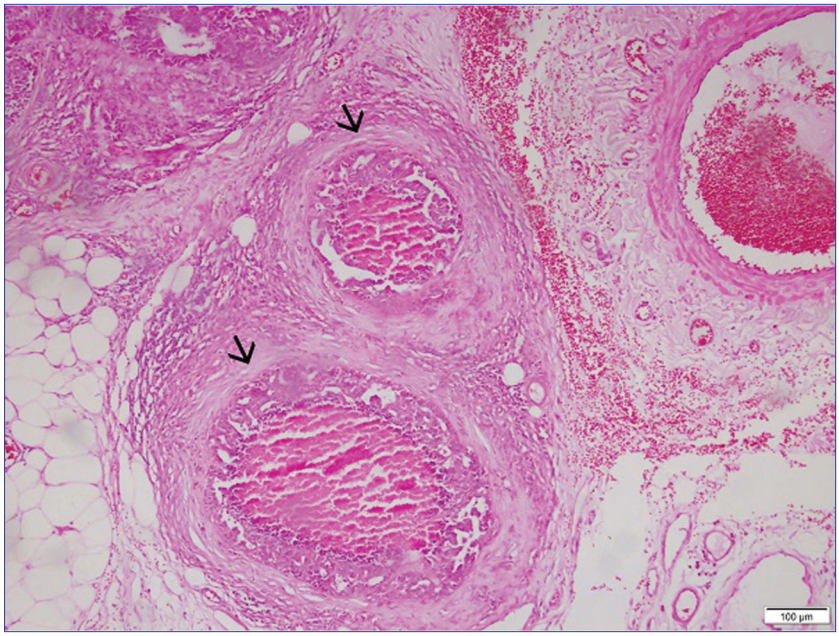

Fig 2. Microscopic structures of tubercle *(arrows):* caseous necrosis at its centre, mononuclear cell infiltrations and fibrous capsule at periphery, HxE, bar: 100 µm

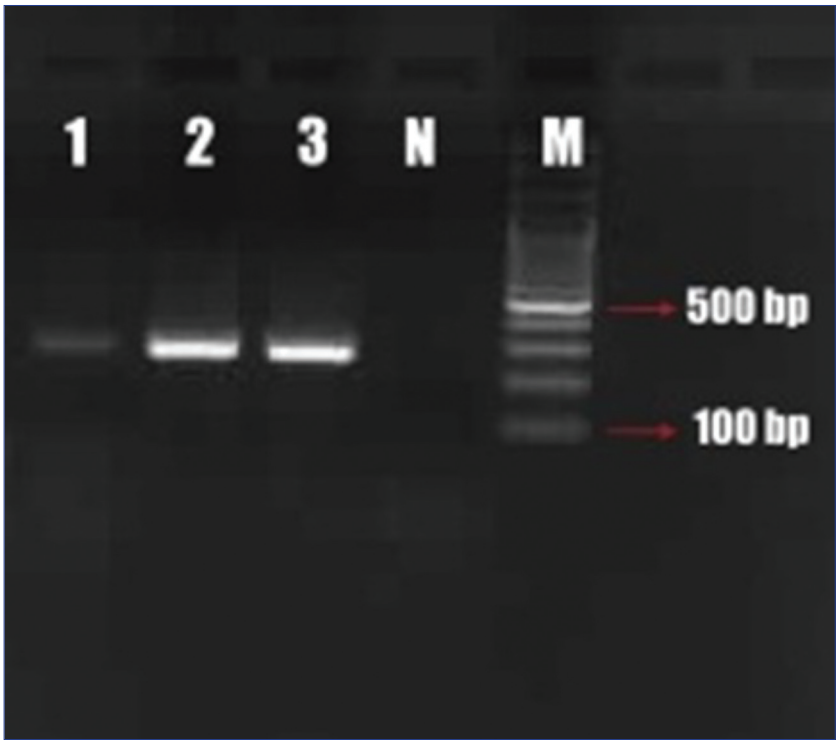

Fig 3. Result of DNA Gel Electrophoresis. 1: sample (270 bp); 2: *Mycobacterium tuberculosis* (positive control); 3: *Mycobacterium bovis* (positive control); N: negative control; M: 100 bp plus DNA Ladder (Thermo Fisher Scientific, USA)

7 min, followed by 40 cycles of 95°C for 45 sec, 60°C for 45 sec, and 72°C for 45 sec. Finally, there was a 7 min at 72°C for further strand extension. Ten microliters of the amplified PCR product was analyzed by electrophoresis on 1.5% agarose gel (Promega Corporation, USA) with 4 µL of SafeView Classic (Applied Biological Materials, Canada) in Gel Electrophoresis Apparatus with 90v for 45 min. *Mycobacterium tuberculosis* and *Mycobacterium bovis* strains which were selected from the collection of Microbiology Department were used as a positive control in all reactions.

DISCUSSION

Inflammation of the mammary gland is known to be rare and nonspecific in dogs [12]. Conversely, in this case, a specific agent such as mycobacterium has been diagnosed in mammary gland. Additionally, malignant mixed tumor was also detected histopathologically in the same mammary gland. In this direction, appearance of tumor was similar to other literature in terms of age, breed and gender susceptibility [6]. The appearance of tuberculosis and malignant mixed tumor together in the same tissue is

an unusual condition. So, tuberculosis should also be also considered if there is a tumor in a dog by veterinarians.

The mechanism of concomitant tuberculosis and neoplasm has been unclear and keep its uncertainty [13]. In humans, observing tumors and tuberculosis at the same time and the same organ causes difficulties in diagnosis and treatment [14]. It was confirmed in experimental studies that prolonged chronic infection, scar tissue formation and irritation lead to the carcinogenesis in time [15]. Also, a study performed in mice reported that chronic tuberculosis infection is enough to cause multi-step transformation of cells like dysplasia, metaplasia and finally carcinomas [16]. Lungs with tuberculosis infection in humans, compared with those without tuberculosis infection were found to be 11 times more sensitive to lung cancer [17].

On the other hand, it is a well-known fact that due to the local and systematic effects (causing malnutrition and immunodeficiency) of the neoplastic disease, susceptibility to tuberculosis infection increases [18,19].

Although there are various opinions; currently the relationship has remained controversial. So, more research is needed to enlighten the association between tumor and tuberculosis.

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Review

Geriatric Cardiology in Dogs - Part 2: Challenge with HF Therapy in Geriatric Dogs: Adverse Drug Reactions and Comorbidities

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Abstract

In geriatric dogs, aging-specific changes in cardiovascular physiology, drug metabolism, drug pharmacokinetics and drug tolerance contribute to adverse drug reactions (ADRs). Comorbidities can also cause polypharmacy and thus drug-drug interactions. Adverse drug reactions for ACE inhibitors, β–blockers, furosemide, aldosterone antagonists and digoxin are common. Geriatric cardiology epitomizes the principle that cardiovascular disease is only 1 component of a larger, multidimensional disease state with concomitant geriatric syndromes. Comorbidities (cardiorenal syndrome, hypertension, diabetes mellitus, atherosclerotic disease, metabolic syndrome, obesity, chronic obstructive pulmonary disease and frailty and cognitive dysfunction) are common, aggravate HF, complicate therapy and increase the total heart failure burden.

Keywords: *Geriatric cardiology, Adverse drug reactions, Comorbidities, Dog*

Köpeklerde Geriatrik Kardiyoloji - Bölüm 2: Geriatrik Köpeklerde Kalp Yetmezliği Tedavisinde Zorluklar: İlaç Reaksiyonları ve Komorbitler

Öz

Geriatrik köpeklerde yaşa bağlı kardiyovasküler fizyoloji, ilaç metabolizması, ilaç farmakokinetiği ve ilaç töleransındaki değişiklikler ilaç yan etki reaksiyonlarına yol açar. Komorbitler de polifarmasiye ve böylece ilaç-ilaç etkileşimlerine neden olabilir. ACE inhibitörleri, β-blokörler, furosemid, aldosteron antagonistleri ve digoxin'e karşı ilaç reaksiyonları yaygındır. Geriatrik kardiyoloji, kardiyovasküler hastalığın geriatrik sendromlarla birlikte çok boyutlu bir oluşumun bir komponentini temsil eder. Komorbidler (kardiyorenal sendrom, hipertansiyon, diabetes mellitus, atherosklerotik hastalık, metabolik sendrom, obesite, kronik obstrüktif pulmoner hastalık ve güçsüzlük ve kognitif disfonksiyon) yaygındır, kalp yetmezliğini şiddetlendirirler ve tedaviyi komplike hale getirirler.

Anahtar sözcükler: *Geriatrik kardiyoloji, İlaç yan etkileri, Komorbitler, Köpek*

INTRODUCTION

Whereas cardiovascular (CV) guidelines and standards of care are oriented toward younger adults, most clinicians devise individual strategies to optimize care for their geriatric patients. Indeed, many cardiologists are adept at integrating patient-centered priorities with existing medical science. Nonetheless, the principles of geriatric medicine combined with management and process for older CV patients are not standardized, and core quality metrics for measuring patient-centered outcomes are not sufficiently delineated to teach, implement, or monitor [1]. Since elderly patients differ from non-elderly patients and develop changes in cardiovascular physiology and metabolism, pharmacotherapy must consider aging-specific changes in cardiovascular physiology, drug metabolism, drug pharmacokinetics and drug tolerance, as well as comorbidities, polypharmacy and drug-drug interactions that contribute to adverse effects [1,2].

ADVERSE DRUG REACTIONS

Treatment of the geriatric dogs with heart failure (HF) is not optimal. Polypharmacy is common and can lead to drug interactions, raising issues of efficacy and safety [3].

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Adverse Drug Reactions for ACE Inhibitors

In general, ACE inhibitors should be discontinued if life-threatening adverse drug reactions (ADRs) develop, including angio-edema and anuric renal failure. Caution is advisable in geriatric dogs with low blood pressure (<90 mmHg), high serum creatinine (>3 mg/dL) or high serum potassium (>5 mEq/L) levels. Renal function and serum potassium should be monitored at frequent intervals [2,4].

ACE inhibitors are thought to minimize the hypertrophic process in blood vessels and the myocardium and to retard the process of abnormal collagen deposition. It is important to note that much of the beneficial effect of ACE inhibitors stems from their ability to block bradykininase, thus increasing the beneficial effects of bradykinin. Because nonsteroidal anti-inflammatory drugs (NSAIDs) block the bradykininase inhibition of ACE inhibitors, the use of NSAIDs with ACE inhibitors is contentious [3].

Since fluid retention blunts the therapeutic effects of ACE inhibitors and fluid depletion potentiates ADRs, fluid balance should be monitored and a diuretic given prior to and during ACE inhibitor therapy [1].

Persistent cough, anuric renal failure and angioedema warrant discontinuation of the ACE inhibitor and replacement by an ARB. However, this requires caution since ARBs can also lead to angioedema [5,6].

Adverse Drug Reactions for β-Blockers

ADRs include reactive airways disease (asthma), fluid retention, fatigue, bradycardia (sick sinus syndrome, and second- or third-degree heart block) and hypotension [2]. Rather than discontinue the β-blocker, fluid retention should be treated with a diuretic with concurrent monitoring for excessive weight gain (>2 kg/day) [1].

Fatigue usually resolves within several weeks but may require dose adjustment. In the very elderly, fatigue may be due to comorbidities (e.g., anemia) or concurrent medications [1].

Treatment of HF symptoms is complicated by orthostasis. Evidence of organ hypoperfusion or worsening of renal function may require withdrawal of the β-blocker [1].

Bradycardia and hypotension are more common with carvedilol owing to (α_1-blockade, resulting in light-headedness, dizziness or blurred vision that alarm the very elderly and may lead to falls. Volume depletion may exacerbate hypotension. Care should be taken not to use beta-blockers until the geriatric dog is stable, because these agents can exacerbate the signs of overt heart failure [2].

Adverse Drug Reactions for Furosemide

Reduced circulating blood volume and/or blood pressure will stimulate the RAAS, which is the argument for adding an ACE inhibitor or an aldosterone antagonist to dogs treated with furosemide [2].

Adverse Drug Reactions for Aldosterone Antagonists

Close monitoring of renal function and serum potassium should be performed when using aldosterone antagonists, especially in the geriatric dogs [2]. Since the use of aldosterone antagonists in patients with renal dysfunction increases the risk of hyperkalemia and this risk is greater in the elderly and those receiving an ACE inhibitor or ARB concurrently, spironolactone or eplerenone should be used at a low dose under those circumstances and avoided in those with a creatinine clearance under 30 mL/min. Gynecomastia can occur in 10% and hyperkalemia in 2% of treated human patients [1].

Adverse Drug Reactions for Digoxin

Common ADRs are nausea and vomiting (10%), visual disturbances (15%), cardiac arrhythmias and heart block (3%), in humans [1]. The geriatric dogs should be closely monitored for these ADRs as well as drug-drug interactions, and the dose of digoxin reduced if amiodarone or verapamil are used [2].

COMORBIDITIES

Geriatric cardiology epitomizes the principle that CVD is only 1 component of a larger, multidimensional disease state with concomitant geriatric syndromes. Selection of assessments and therapies is best accomplished in the context of the aggregate circumstances [1].

Comorbidities are common, aggravate HF, complicate therapy and increase the total HF burden. Cardiorenal syndrome, hypertension, diabetes mellitus, atherosclerotic disease, metabolic syndrome, obesity, chronic obstructive pulmonary disease and frailty and cognitive dysfunction need to be targeted early and aggressively with appropriate measures to prevent progression to HF stage D [3,7].

Cardiorenal Syndrome

Cardiorenal syndrome (CRS) occurs when worsening renal function limits diuresis despite clinical volume overload associated with HF. In dogs being treated for chronic HF, declining renal function should be anticipated. The diagnostic marker for chronic kidney disease (CKD), isosthenuria, cannot be relied upon in dogs being treated with diuretics. Monitoring of creatinine especially should be used to discern trends in renal function. A progressive rise even within the normal range should alert the practitioner, along with clinical signs: PU/PD, hyporexia, anorexia, weight loss and vomiting. Goals of treatment are to recognize CRS, reverse it as much as possible and deal with the renal consequences of HF and the complex relationship between HF and renal injury. The difficult balance is to “dry out” the HF and hydrate the kidneys.

Different therapeutic strategies are based upon the degree of compromise of each organ [2].

ACE inhibitors are the mainstay of therapy for CRS especially in the presence of hypertension or proteinuria. Dogs with CRS should be hydrated before starting therapy. Low dose benazepril or enalapril 0.25 mg/kg q 24 h can be increased to proved better control for HF. Benazepril is metabolized in the liver, Enalapril in the kidneys. Therefore, dogs with CRS may need a lower dose of enalapril than benazepril. Initiation of therapy may show a transient increase in BUN/creatinine concentrations. If persistent, lowering the dose is usually sufficient [3].

If azotemia is becoming a concern, the first step is to lower the dose of diuretics. The goal is to find the lowest effective dose that controls HF. The dose must be continuously reassessed. The ideal dose for an individual patient achieves the threshold rate of drug excretion. Adequate natriuresis can be grossly assessed by observation of increased urine volume and decreased specific gravity. Periodic drainage of pleural fluid or ascites can be used to avoid excessive diuretic use [3].

In the event that diuretic resistance occurs, several options are available to correct fluid balance. A CRI of furosemide (0.3-0.6 mg/kg/h IV inhibits sodium resorbtion more effectively than oral or IV boluses. Once the volume overload has resolved, most cats will again respond to oral therapy. Another loop diuretic, torsemide has superior diuretic action and long half-life (0.2 mg/kg PO q 12 h). It appears to be 10 times more potent than furosemide. Dual-diuretic therapy can be considered when furosemide dose needs to be decrease. Spironolactone (1-2 mg/kg q 12 h) may cause severe facial pruritus and must be used with caution [3].

Systemic hypertension is common in CKD and by increasing afterload increases the cardiac workload. Hypertension worsens both CKD and HF. If present, amlodipine (0.0625-0.25 mg/cat PO q 24 h) should be added. Blood pressure monitoring is critical to avoid the effects of iatrogenic hypotension [2,8].

In advanced CRS, a positive inotrope (pimobendan) may improve azotemia, demeanor and appetite and allow reduction in diuretic dose [2].

Dietary modification should consider both conditions. Sodium restriction and Lower phosphorus diets may be helpful in managing kidney disease but may result in the loss of lean body condition. High quality protein should be given to the level that it does not worsen azotemia. Omega-3 polyunsaturated fatty acids have been shown to be beneficial in both cardiac and renal conditions [2,3].

Fluid administration is a balance between improving renal blood flow without precipitating congestive HF. Fluids should be given slowly to correct azotemia, tailored to the individual's ability to tolerate. Abrupt changes in weight, a new gallop heart sound and/or heart rate may indicate impending congestive event and justify fluid rate reduction. Sometimes a low-dose CRI of furosemide will be indicated concurrently in dogs with end-stage CRS. SQ fluids may be less likely to trigger a congestive event and can be given every 24-48 h via a balanced electrolyte solution and adjusted to the individual patient's ability to tolerate. In fragile patients, a smaller volume of fluids may be necessary, titrating slowly upward if the expected effect on uremia is not evident. Electrolytes should be monitored closely, especially potassium, as hypokalemia can trigger arrhythmia. Correction can take place through fluid therapy or oral means [2,8]. Although renal function may remain stable for a period of time in dogs with HF, when CRS occurs it leads to frequent hospitalization, difficulty maintaining good quality of life and eventually euthanasia [2,3].

Hypertension and Heart Failure

Hypertension increases the cardiac workload by increasing afterload. There is a critical role of hypertension in the pathogenesis of HF. Elevated levels of diastolic and especially systolic blood pressure are major risk factors for the development of HF [2,9,10]. Also, hypertension is frequently accompanied by metabolic risk factors and obesite, which themselves increase the risk of HF. On the basis of the 44-year follow-up data of the Framingham Heart Study, 75% of patients with HF have antecedent hypertension [11]. In humans, both acute and chronic hypertension have been linked to the risk of HF. Sudden elevation of blood pressure (such as in hypertensive emergencies) can lead to acute left ventricular strain and acute HF [12] and is a common precipitating cause for decompensation in a patient with chronic HF [8]. Progression from chronic hypertension to structural ventricular changes and then to asymptomatic diastolic and systolic ventricular dysfunction is well established by longitudinal epidemiological studies, such as the Framingham Heart Study [9].

Elevated blood pressure places greater hemodynamic burden on the myocardium and leads to left ventricular hypertrophy. Left ventricular hypertrophy is associated with increased myocardial stiffness and decreased compliance, initially during exercise and subsequently at rest [2,13,14]. The initial concentric hypertrophy (thick wall, normal chamber volume, and high mass-to-volume ratio) helps keep wall tension normal despite high intraventricular pressure. Because systolic stress (afterload) is a major determinant of ejection performance, normalization of systolic stress helps maintain a normal stroke volume despite the need to generate high levels of systolic pressure [2,15].

Aggressive control of blood pressure is the most effective approach to reduce the incidence of HF in a hypertensive population. Primary prevention trials have demonstrated up to a 50% reduction in the incidence of HF in patients with hypertension who are treated with blood pressure-

lowering agents [16]. The Hypertension in the Very Elderly trial achieved a 64% relative risk reduction in heart failure with the diuretic indapamide, with or without the angiotensin-converting enzyme (ACE) inhibitor perindopril [17].

Inhibition of the renin-angiotensin system with ACE inhibitors or angiotensin receptor blockers (ARBs) appears to exert a greater benefit on left ventricular hypertrophy and remodeling than would be predicted from their pressure-lowering effect. Their effectiveness in reducing morbid events in nonhypertensive patients with atherosclerotic disease may involve pressure-independent as well as pressure-dependent mechanisms [18].

β-Blockers are also effective in preventing HF in hypertensive geriatric dogs, partly through pressure reduction and partly through inhibition of structural remodeling of the left ventricle. Diuretic therapy also is effective in preventing heart failure, not only through blood pressure reduction but also by intravascular volume contraction, which reduces the risk of congestion. Diuretics are not known to affect remodeling directly. Calcium channel antagonists, especially amlodipine, contribute to prevention of heart failure by their powerful vascular effects that reduce blood pressure and diminish reflected waves [2].

Diabetes Mellitus and Heart Failure

Diabetes and insulin resistance are important risk factors for the development of HF [19]. The presence of clinical diabetes mellitus markedly increases the likelihood of HF in patients without structural heart disease and adversely affects the outcomes of patients with established HF [20,21]. The Framingham Heart Study showed that the prevalence of heart failure was twice as high among diabetic men and five times as high among diabetic women aged between 45 and 74 years as in age-matched nondiabetic controls [22]. After the age of 65 years, the association became even stronger, with a fourfold higher prevalence in diabetic men and an eightfold higher prevalence in diabetic women [22]. HF is the most common admission diagnosis for diabetic patients, and more than one third of patients with type 2 diabetes die of heart failure [23,24]. There is no date on this concern in dogs.

The basic reason for the increased prevalence of HF among diabetic patients is the presence of a distinct diabetic cardiomyopathy that is structurally characterized by cardiomyocyte hypertrophy, microangiopathy, endothelial dysfunction, and myocardial fibrosis [25]. At the cellular level, diabetic cardiomyopathy is associated with defects in subcellular organelles and downregulation of catecholamine receptors as a result of chronically elevated catecholamine levels [26]. Also, in animal models with the onset of hyperglycemia, changes in myocardial calcium transportation and alterations in contractile proteins occur, both of which lead to systolic and diastolic dysfunction that worsens as the collagen content of the myocardium increases [27]. Doppler imaging studies have been used to provide load-independent assessments of cardiac relaxation. These studies not only have confirmed evidence of diastolic dysfunction in asymptomatic patients with diabetes but also have demonstrated a direct relationship between the extent of diastolic dysfunction and glycemic control [28]. Although diastolic dysfunction is the hallmark of diabetic cardiomyopathy, concomitant subtle systolic dysfunction is present even at earlier stages of the disease [23].

Fasting glucose levels are predictive of hospitalizations for congestive heart failure, with a 10% increase in the risk of heart failure-related hospitalization for each 18 mg/dL increase in fasting glucose level [29]. The choice of oral hypoglycemic agent that may be used is restricted. For instance, metformin is contraindicated in the presence of either HF or renal impairment, and precautions also apply to the use of the thiazolidinediones [30,31]. In addition to treating hyperglycemia, it is crucial to control all other cardiovascular and metabolic risks and to prevent complications in patients with diabetes. ACE inhibitors or ARBs can prevent the development of end-organ disease and the occurrence of clinical events in diabetic patients, even in those who do not have hypertension [32]. Long-term treatment with several ACE inhibitors or ARBs has been shown to decrease the risk of renal disease in diabetic patients [33,34], and prolonged therapy with the ACE inhibitor ramipril has been shown to lower the likelihood of heart failure, myocardial infarction, and cardiovascular death [32]. Likewise, the use of ARBs in patients with diabetes mellitus and hypertension or left ventricular hypertrophy has been shown to reduce the incidence of first hospitalization for heart failure, in addition to having other beneficial effects on renal function [35,36].

Atherosclerotic Disease and Heart Failure

Patients with known atherosclerotic disease (e.g., of the coronary, cerebral, or peripheral blood vessels) are at increased risk of developing heart failure. Although it is less common in dogs, coronary atherosclerotic disease can lead to acute or chronic ischemia, thereby predisposing to left ventricular dysfunction and symptomatic heart failure [2].

ACE inhibitors reduce incidence of heart failure by 23% among patients who have coronary artery disease and normal systolic function and by 37% among patients who have reduced left ventricular systolic function, in humans. Observational studies and small clinical investigations have suggested that hydroxymethylglutaryl-coenzyme A reductase inhibitors (statins) may be beneficial in patients with ischemic and nonischemic heart failure [1].

Metabolic Syndrome and Heart Failure

The term "metabolic syndrome" refers to a cluster of risk factors for cardiovascular disease and type 2 diabetes mellitus. According to the National Cholesterol Education Program Adult Treatment Panel III, metabolic syndrome

is diagnosed when three or more of the following five risk factors are present [36]: (1) fasting plasma glucose of 100 mg/dL or higher; (2) high-density lipoprotein (HDL) cholesterol level lower than 40 mg/dL in men or lower than 50 mg/dL in women; (3) triglyceride levels of 150 mg/dL or higher; (4) waist circumference of 102 cm or more in men or 88 cm or more in women; and (5) systolic blood pressure of 130 mmHg or higher or diastolic blood pressure of 85 mm Hg or higher, or the presence of drug treatment for hypertension.

The prevalence of metabolic syndrome reaches epidemic levels and ranges from 6.7% among people 20 to 29 years of age to 43.5% for people 60 to 69 years of age and 42.0% for those 70 years of age or older [37,38].

Mechanisms underlying elevated cardiovascular risk associated with metabolic syndrome appear to involve subclinical organ damage [39]. Among patients with hypertension but without diabetes, those with metabolic syndrome seem more likely to have microalbuminuria, left ventricular hypertrophy, and increased carotid intima thickness than did those without metabolic syndrome [39]. In addition, the greater the number of metabolic syndrome components present, the more severe were the microalbuminuria and left ventricular hypertrophy [39].

Treatment of metabolic syndrome consists of aggressive management of each of its individual components, including impaired fasting glucose concentration, dyslipidemia, and hypertension. Drugs targeting nuclear peroxisome proliferator-activated receptors (PPARs) PPAR-α (e.g., fenofibrate and gemfibrozil) are used in the treatment of metabolic syndrome. Fibrates decrease triglyceride level, increase HDL cholesterol, and may have some anti-inflammatory effects; however, their effect on cardiovascular disease outcomes continues to be evaluated [40]. Among patients with impaired glucose tolerance and cardiovascular disease or risk factors, the use of the ARB valsartan along with lifestyle modification led to a relative reduction of 14% in the incidence of diabetes but did not reduce the rate of cardiovascular events [41].

Obesity and Heart Failure

In several studies, obesity has been associated consistently with left ventricular hypertrophy and dilation ,which are known precursors of heart failure [42-44]. In the Framingham Heart Study population, overweight, lesser degrees of obesity and extreme obesity were associated with an increased risk of HF [45,46].

There are several plausible mechanisms for the association between obesity and heart failure. Obesity is a risk factor for hypertension [47] diabetes mellitus [47,48], and dyslipidemia, all of which augment the risk of myocardial infarction [22,49], an important antecedent of HF, in humans. Adipose tissue acts as an endocrine organ, secreting hormones and other substances that create a proinflammatory state and promote formation of atherosclerotic plaques [50].

In humans, a number of strategies have been used to treat obesity, including diet, exercise, behavior therapy, medications, and surgery. To select among these treatments, clinicians must evaluate the obesity-related risks to the individual patient and balance those risks against any possible problems with the treatment. Because all medications inherently carry more risks than do diet and exercise, medications should be chosen only for people in whom the benefit justifies the risk [51,52].

Chronic Obstructive Pulmonary Disease and Heart Failure

The importance of COPD as a cause of death is probably underestimated, in as much as COPD is probably a contributor to other common causes of death, in humans [53]. The risk ratio of developing heart failure in patients with COPD is 4.5, in comparison with age-matched controls without COPD, after adjustments for cardiovascular risk factors [54]. Among the comorbid conditions commonly associated with HF, COPD is the one that most delays the diagnosis of heart failure and is most often blamed for nonadherence to therapeutic guidelines, especially β-blockade [55].

A working hypothesis to account for the high prevalence of left ventricular systolic dysfunction in patients with COPD is that low-grade systemic inflammation in COPD accelerates progression of coronary atherosclerosis, which ultimately results in ischemic cardiomyopathy. Such a hypothesis is based on the fact that patients with COPD have higher elevation of inflammatory markers [56,57], and it fits the clinical observation of a higher incidence of troponin elevation [56] and left ventricular wall motion abnormalities [58] noted in patients with COPD and left ventricular dysfunction. Patients with HF and concomitant COPD have higher activation of neurohormones, particularly norepinephrine and plasma rennin [56]. COPD may also lead to right ventricular failure from pulmonary hypertension, a common complication of COPD [59]. The cause of pulmonary hypertension in COPD is generally assumed to be hypoxic pulmonary vasoconstriction [2,59].

Bronchodilators, corticosteroids, and antibiotics in the treatment of acute exacerbations constitute the mainstay of current drug therapy for COPD [60]. Long-term oxygen therapy was also shown to reduce mortality and improve quality of life in patients with severe COPD and chronic hypoxemia (partial pressure of arterial oxygen <55 mm Hg) [61]. The presence of COPD affects the treatment of HF, in as much as COPD is still viewed as a contraindication to β-blockade. Therefore, patients with HF caused by left ventricular systolic dysfunction who also have COPD are often deprived of the most beneficial pharmacological intervention. A large body of data indicates that patients with COPD may tolerate selective β-blockade, and these

medications should not necessarily be denied to patients with heart failure and concomitant COPD [19,62].

Frailty and Cognitive Dysfunction and Heart Failure

Other problems such as frailty and cognitive dysfunction common in the elderly HF patient can also complicate management [4]. Frailty and cognitive impairment are common and lead to reduced compliance. Response to diuretics, ACE inhibitors, β-blockers and/or positive inotropes may be diminished [4].

Cognitive dysfunction (CD) lead to polypharmacy. The most common signs of CD are disorientation in time and space, altered learning, house soiling, altered interactions (e.g. attention seeking, anxiety, irritability), changes in activity (wandering or pacing), changes in sleep patterns, decreased appetite and increased vocalization [63].

Therapies extrapolated from studies in humans and dogs include anti-oxidant enriched diets, supplements phosphatidyserine, omega-3 fatty acids, Vitamins E and C, L-carnitine. SAMe improved activity and awareness in dogs. Selegiline (Anipryl), which has been anecdotally reported to be proven beneficial in dogs for CD. Environmental enrichment have often been recommended. Any changes should take place slowly [2,63].

CONCLUSION

In geriatric dogs, aging-specific changes in cardiovascular physiology, drug metabolism, drug pharmacokinetics and drug tolerance contribute to ADRs. Comorbidities can also cause polypharmacy and thus drug-drug interactions.

Comorbidities (cardiorenal syndrome, hypertension, diabetes mellitus, atherosclerotic disease, metabolic syndrome, obesity, chronic obstructive pulmonary disease and frailty and cognitive dysfunction) are common, aggravate HF, complicate therapy and increase the total HF burden.

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