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Research Article

Effects of Stocking Density on Performance and Immunity in Ross 308 Broiler Chickens

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Abstract

To investigate the effect of stocking density on productive traits, blood parameters and immunity, 1305 Ross 308 male broiler chicks (initial weight: 45±1.5 g) were used as a completely randomized design with 2 treatments and 15 replications. Treatments included 1) 12 chicks per pen 2) 18 chicks per pen (1.2x1.2 m^2). The results were recorded in three periods of 0-24, 25-42 and 0-42 d. From 0 to 24 d of age, the stocking density had no significant effect on feed intake and body weight gain (P>0.05), but feed conversion ratio significantly decreased in 12 chicks density group (P<0.05). Feed conversion ratio, body weight gain and feed intake were improved significantly in 12 chicks compared to 18 chicks density for 25-42 and 0-42 periods (P<0.05). The production per m^2 was significantly higher in 18 chicks density group at 0-42 d period (P<0.05). High stocking density caused to increase the moisture content of bedding in 4-6th weeks of age (P<0.05). Stocking density had no significant effect on mortality percentage (P>0.05). Also the relative weights of spleen, bursa of fabricius, abdominal fat, thigh and breast were not affected by experimental treatments (P>0.05). Increasing the number of chicks per m^2 caused to increase heterophile count and decrease lymphocyte count and increase heterophile to lymphocyte ratio (P<0.05). Newcastle's titer was not affected by treatments (P>0.05). Given that performance indices were better under low stocking density, but live body weight per m^2 was significantly higher in 18 chicks density group (32%), it's seems economically 18 chicks per m^2 is the best stocking density.

Keywords: *Broiler chickens, Immune system, Performance traits, Stocking density*

Ross 308 Broiler Tavuklarda Yerleşim Sıklığının Performans ve Bağışıklığa Etkileri

Öz

Yerleşim sıklığının üretim özellikleri, kan parametreleri ve bağışıklığa etkilerini incelemek amacıyla, 1305 adet Ross 308 erkek broiler civciv (ön ağırlık: 45±1.5 g) rastgele dizaynda, 2 uygulama ve 15 tekrar olmak üzere kullanıldı. Uygulamalar 1: her 2 kafes için 12 civciv ve 2: her kafes (1.2x1.2 m^2) için 18 civciv olarak gerçekleştirildi. Sonuçlar 0-24, 25-42 ve 0-42 gün olmak üzere üç periyotta incelendi. 0-24 günlük periyotta yerleşim sıklığı yem tüketiminde ve vücut ağırlık kazanımında anlamlı bir etkiye neden olmazken (P>0.05) yem konversiyon oranı 12 civciv sıklık grubunda anlamlı derecede düştü (P<0.05). Yem konversiyon oranı, vücut ağırlık kazanımı ve yem tüketimi 12 civciv grubunda 18 civciv grubu ile karşılaştırıldığında 25-42 ve 0-42 gün periyotlarında anlamlı derecede iyileşme gösterdi (P<0.05). Her m^2 için üretim 18 civciv sıklık grubunda 0-42 gün periyotta anlamlı derecede daha yüksekti (P<0.05). Yüksek yerleşim sıklığı 4-6. haftalarda altlığın nem miktarının artmasına neden oldu (P<0.05). Yerleşim sıklığı mortalite yüzdesinde anlamlı bir etkiye neden olmadı (P>0.05). Dalak, bursa Fabricius, abdominal yağ, but ve göğüs görece ağırlıkları deneysel uygulamalardan etkilenmedi (P>0.05). Civciv sayısını m^2 başına artırmak heterofil sayısında artmaya ve lenfosit sayısında azalmaya neden olurken heterofil lenfosit oranı artma gösterdi (P<0.05). Newcastle titresi uygulamalardan etkilenmedi (P>0.05). Bu performans endekslerine göre; düşük yerleşim sıklığı daha iyi sonuçlar verirken, 18 civciv sıklık grubunda m^2 başına canlı vücut ağırlık daha yüksek olması sebebiyle ekonomik olarak her m^2'ye 18 civciv en iyi yerleşim sıklığı olarak gözükmektedir.

Anahtar sözcükler: *Broiler tavuk, Bağışıklık sistemi, Performans özellikleri, Yerleşim sıklığı*

INTRODUCTION

One of the common subjects in poultry breeding is the proper stocking density. The excessive increase of broiler chick's density may reduce welfare, health, body weight, feed intake, feed efficiency, flock's uniformity and increase

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skeletal disorders and mortality [1]. On the other hand, increasing density will reduce fixed costs and increase production per area unit, and by increasing density to reach the critical point, profitability will also increase. It is difficult to determine a proper stocking density because of different environmental conditions and equipment, chick comfort criteria and the used strain genetic in experiments [2]. Reducing the air quality of poultry houses, in particular increasing ammonia, and reducing access to water and feed can reduce performance under high density condition. The reduction of performance by increasing stocking density can be due to reduced feed intake and thus Decrease received nutrients for maximum growth [3]. By increasing density, the number of birds per unit of floor space increases thus higher efficiency can be obtained, but this economic profit may be at the expense of reduced performance, hygiene and comfort of the bird [4]. In a research [5] investigated the effects of stocking density, chick's sexuality and the amount of metabolizable energy on performance and leg problems in broiler chickens, they stated that the stocking density have severe effects on the growth rate of male broiler chickens before 35 d of age, and male chickens need more space than females to reach marketing age. Based on the findings of this study, there was a significant interaction between stocking density, sexuality and age of chicks on performance, as high density decreased body weight gain and increased feed conversion ratio, and male chicks had a significant increase in feed intake, weight gain and reduced feed conversion ratio in comparison with female chicks. Also they expressed that the prevalence and severity of leg problems are related to stocking density, chick's sexuality and type of the diet.

In a similar study two levels of density (10 and 16 chicks per m^2) were examined, lower density increased body weight, improved feed conversion ratio and the Newcastle titer significantly [6]. Ventura *et al*.[7] studied the effect of stocking density and sleeping wood on leg health, fear and performance of broiler chickens. They stated that increasing stocking density has negative effect on leg health and chicks reared under high density (18 chicks) had severe lesions in their legs and rabbit joint. The aim of this study was to investigate the effect of stocking density on performance and immunity traits of broiler chickens and compare the density of 18 and 12 chicks per m^2.

MATERIAL and METHODS

Birds and Treatments

A total of 1305 male broilers (Ross 308) were used as a completely randomized design with 2 treatments and 15 replications. Treatments included 2 levels of density (12 and 18 chicks per m^2). The experimental pen dimensions were 1.2 in 2.4 meters, therefore 35 chicks (for 12 density) and 52 chicks (for 18 density) were reared in each pen. All birds had freely access to feed and water throughout the experiment and bird management was according to Ross strain standards and was the same for all treatments. Feed intake and body weight gain were recorded for 1-25, 25-42 and 1-42 d of age and also mortality was recorded by hen day method.

Diets

Corn and soybean based diets were formulated according to Ross 308 nutritional recommendation *(Table 1)*.

Feed ingredients and diets were analyzed for dry matter [8], protein [8], digestible amino acids [9], crude fiber [9], calcium [10], phosphorus [10] and crude fat [10]. The chemical analysis of feeds is presented in *Table 1*.

Performance Traits

Performance traits including body weight gain, feed intake and feed conversion ratio were recorded for 1-24, 25-42 and 1-42 d of age. Then feed intake and feed conversion ratio data were corrected based on mortality and hen day.

Slaughtering and Carcass Analysis

At the end of experiment, 2 birds close to average weight from each experimental unit were selected, weighed and were slaughtered according to the regulations approved by the Animal Protection Committee of Islamic Azad University, Science and Research Branch protocol [11]. Then the relative weights (% of BW) of bursa of fabricius, abdominal fat, breast, thighs and spleen were recorded.

Bedding Moisture Measurement

To evaluate bedding moisture, 1 kg combined sample was formed by mixing collected samples from 6 areas of each pen (around the plate, the drinkers and end of the pens) weekly [12]. Each sample was weighed accurately and then dried in an oven at 105°C for 24 h. Then, the moisture content was calculated from the subtraction of initial weight from dried sample weight and expressed as a percentage of the initial weight [10].

Evaluation of Heterophile to Lymphocyte Ratio and Bursa and Spleen Organs

At 41 d of age, two chickens were selected from each replicate and 2 mL blood were taken from each chicken to evaluate the heterophile to lymphocytes ratio, and the number of heterophile to lymphocyte was determined by staining on a slide. After slaughter, the spleen and bursa were separated, weighed and expressed as a percentage of carcass weight [1].

Newcastle Antibody Titer by HI Method

At 41 d of age, blood sampling was carried out from wing vein of two chickens of each replicate and the titer of Newcastle antibody was determined in them [13].

Table 1. *Ingredients of basal diets (%)*

Ingredients %	Starter (1-10 d)	Grower (11-24 d)	Finisher (25-42 d)
Ground corn	47.43	54.27	59.27
Soybean meal (45%)	39.50	32.75	28.16
Soybean oil	1.09	1.00	1.00
Wheat	7.00	7.00	7.00
DL-methionine	0.35	0.27	0.25
L-lysine hydrochloride	0.27	0.20	0.16
Threonine	0.14	0.09	0.07
Choline chloride 60%	0.02	0.02	0.02
D-calcium phosphate	1.99	1.57	1.35
Calcium carbonate	1.16	1.12	1.06
Sodium bicarbonate	0.27	0.17	0.22
NaCl	0.22	0.17	0.16
Mineral supplement[1]	0.25	0.23	0.20
Vitamin supplement[2]	0.25	0.23	0.20
Bentonite	-	0.85	0.80
Phytase5000 (Biochem,Germany)	0.00	0.01	0.01
Salinomycin	0.05	0.05	0.05
Total	100	100	100
Nutrient			
Crude protein	22.64	20.00	18.8
Energy (kcal/kg)	2877	2936	2995
Calcium	1.03	0.91	0.83
Available phosphorus	0.51	0.44	0.39
Sodium	0.18	0.14	0.14
Crude fiber	3.08	3.84	3.68
Digestible methionine	0.65	0.54	0.50
Digestible methionine + Cysteine	0.95	0.82	0.76
Digestible lysine	1.3	0.95	0.98
Digestible threonine	0.89	0.75	0.67

Vitamin and mineral supplements per kilogram of diet provide the following amounts: [1] Mineral: I, 0.43 mg; Cu, 13.56 mg; Zn, 29.3 mg; Se, 6.57 mg; Mn, 88.51 mg; Fe, 17.28 mg; [2] Vitamins: vitamin A, 15600 IU; vitamin D_3,6750 IU; vitamin E, 120 IU; vitamin K_3, 4.8 mg; vitamin B_1, 3.84 mg; vitamin B_2, 10.32 mg; vitamin B_3, 72 mg; vitamin B_5, 20.4 mg; vitamin B_6, 6.48 mg; vitamin B_{12}, 0.021 mg; vitamin B_9, 2.75 mg; Biotin 0.36 mg

Statistical Analysis

Data were subjected to the GLM procedure for ANOVA in a completely randomized design. Differences among means were separated with the LSMEANS option of SAS (2002) [14]. Statistical significance was considered at $P \leq 0.05$. The statistical model was as follows:

$$Y_{ij} = \mu + A_i + e_{ij}$$

The components of this model are:

Y_{ij} = The measured value of each observation

μ = mean

A_i = Effect of treatment

e_{ij} = Experimental error

RESULTS

Table 2 show the effect of two levels of density (12 and 18 chicks) on the performance of broiler chickens in different periods. Increasing the density from 12 chicks to 18 per m^2 significantly reduced the weight gain and feed intake, over 25-42 d and 1-42 d of age, also significantly increased feed conversion ratio for all periods ($P<0.05$).

As shown in *Table 3*, increasing density caused to a significant increase in production per area unit ($P<0.05$).

In this study, carcass parameters did not affected by increasing the number of chicks per area unit ($P>0.05$) *(Table 4)*.

Table 2. *Effect of stocking density on body weight gain, feed intake and feed conversion ratio of broiler chickens in different growth periods (g)*

Period	Treatments	Body Weight Gain	Feed Intake	Feed Conversion Ratio
(1-25 d)	12 chicks density	1299.70	1884.40	1.45[b]
	18 chicks density	1269.90	1867.20	1.47[a]
	SEM	11.61	13.60	0.005
	P value	0.0801	0.379	0.0203
(25-42 d)	12 chicks density	1552.1[a]	3082.80[a]	1.99[b]
	18 chicks density	1273.7[b]	2760.00[b]	2.17[a]
	SEM	26.74	33.01	0.036
	P value	0.0001	0.0001	0.0001
(1-42 d)	12 chicks density	2851.9[a]	4967.20[a]	1.74[b]
	18 chicks density	2543.6[b]	4627.20[b]	1.81[a]
	SEM	23.93	37.65	0.018
	P value	0.0001	0.0001	0.0135

Table 3. *Effect of stocking density on live weight of broiler chickens per m² at the end of experiment (kg/m²)*

Treatments	Production per m²
12 chicks density	34.70[b]
18 chicks density	45.90[a]
SEM	0.39
P value	0.0001

Table 4. *Effect of stocking density on some carcass parameters (% of BW)*

Treatments	Spleen	Bursa	Abdominal Fat	Thigh	Breast
12 chicks density	0.13	0.06	1.08	19.00	26.50
18 chicks density	0.12	0.05	1.15	19.30	25.90
SEM	0.0056	0.0049	0.059	0.1500	0.3400
P value	0.544	0.673	0.389	0.062	0.228

Table 5. *Effect of stocking density on some blood parameters and mortality percentage in the intervals of 1-25 and 25-42 d*

Treatments	Heterophile/Lymphocyte	Heterophile (%)	Lymphocyte (%)
12 chicks density	0.52[b]	33.10[b]	63.60[a]
18 chicks density	0.71[a]	40.60[a]	57.00[b]
SEM	0.044	1.703	1.610
P value	0.007	0.0034	0.0082
Treatments	**Mortality Percent (1-25)**	**Mortality Percent (25-42)**	
12 chicks density	1.52	1.90	
18 chicks density	1.91	1.67	
SEM	0.64	0.79	
P value	0.676	0.427	

According to the data presented in *Table 5*, increasing stocking density, significantly increased heterophile percentage, heterophile to lymphocyte ratio and decreased lymphocyte percentage (P>0.05). Results also show that stocking density has not any effect on mortality in 1-25 and 25-42 d of age (P>0.05).

According to *Fig. 1*, increasing the stocking density increased the moisture content of the bedding material in the 4th, 5th and 6th weeks of breeding (P<0.05). At the third week, the difference in moisture between two groups was close to significant (P=0.0621) *(Fig. 1)*.

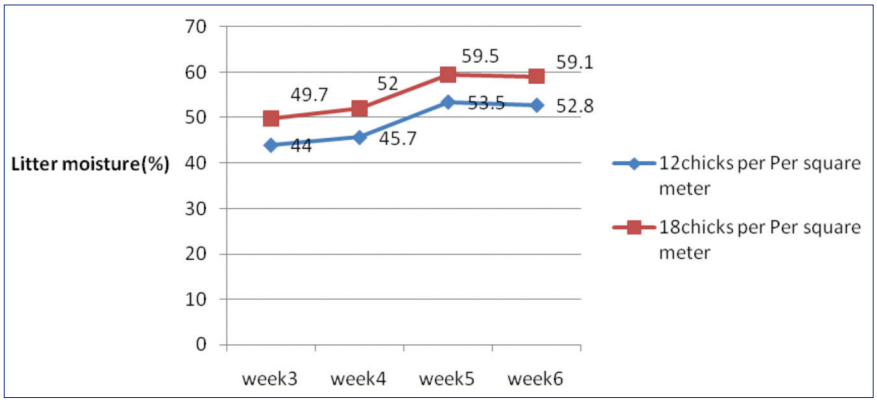

Fig 1. Effects of stocking density on litter moisture in different weeks

DISCUSSION

In this experiment, the performance traits including body weight gain, feed intake and feed conversion ratio, in 12 chicks per m^2 group were significantly better than 18 chicks group. Low density (12 chicks) provided the best environmental conditions for chicks and allowed the chicks to have the best feed consumption without any competition, followed by the best body weight gain and feed conversion ratio. Considering that about 32% in live body weight was obtained in 18 chicks density and according to some researchers however, at higher densities, the performance per bird decreases, but the kilogram of produced meat per m^2 increases and leads to more economic profit [15,16]. In another study Puron *et al.*[2] showed that increasing stocking density caused to gain more amount of meat produced per m^2. However, the relationship between density and economic efficiency is not a linear relationship. According to their statement this relationship is valid only to a certain extent, because under high density condition, the performance of the chick is reduced, and if the density exceeds from 17 male and 19 female chicks per m^2, finally the weight of the produced meat will be the same in both densities. These results indicate that if the stocking density exceeds the above limit, flock profitability and bird welfare will disturb [17]. A number of researchers have reported that the lower stocking density (11.9) compared to higher density (23.8) significantly improved body weight gain, but the production per m^2 was significantly higher for high density groups [18]. A number of studies have shown the improvement of performance traits in lower stocking density in broiler chickens [19-21], while others reported no effect [22] or even negative effects of density on the performance of broiler chickens [18]. In the present study, decreasing daily weight gain and feed intake is consistent with some studies [23-25]. The results of performance characteristics are consistent with some studies, including the study of Nahashon *et al.*[26] that they were investigated the effect of stocking density on performance and carcass characteristics of broiler chicks in Guinea. For this purpose, they used four treatments of 10.7, 12, 13.6 and 15.6 chicks per area unit. Feed intake in 10.7 density group was significantly higher than other groups also the average weight gain of chickens in 15.6 chicks density was significantly lower than other treatments. In total, there was a significant difference in feed conversion ratio and high differences for carcass efficiency of chicks in 12 and 13.6 treatments compared to other treatments. Therefore, according to the findings of this research, broiler chickens in Guinea [27] showed the best performance in 13.6 and 12 density groups. One of the factors affecting the reduction of performance in high stocking density is reducing feed intake and thus reducing nutrient intake for maximum growth [3]. Also Sekerogla *et al.*[27] expressed that 13 chicks per area unit density compared to 17 and 19 has led to the best performance conditions. In the present experiment the stocking density had no significant effect on carcass parameters, which is consistent with [19], they reported that stocking density was ineffective on mortality, breast muscle size and carcass quality. The percentage of carcass, thigh, breast and abdominal fat was not affected by stocking density, which is consistent with the study of other researchers [4,21,28,29].

The evaluation of blood parameters in this experiment showed that the heterophil to lymphocyte ratio in 18 chicks density was higher than 12 chicks density, which was consistent with Kuan *et al.*[30], they reported that with 10.5, 14, 17.5 and 20.8 chicks per m^2 densities, from 2-6 weeks of age, the treatment of 20.8 chicks per m^2 decreased feed intake and improved feed conversion ratio without affecting the growth efficiency. After the sixth week, the highest daily weight gain and feed intake and the lowest feed conversion ratio were observed in the lowest stocking density group, whereas the highest stocking density showed the lowest body weight gain and feed intake and the highest feed conversion ratio and after 4 weeks, also the heterophile to lymphocyte ratio was increased due to stress.

In another study Dozier *et al.*[17] examined the effect of stocking density on growth rate and stress indices in male broiler chickens up to 1.8 kg weight and showed that by increasing stocking density up to 35 d of age, body weight gain, feed intake and feed conversion ratio decreased significantly and the bedding moisture content increased,

which caused to an increase in foot injuries. Also, the results of their study indicated that increasing the stocking density to more than 30 kg body weight per m^2 until the chick's weight reached 1.8 kg had a negative effect on the growth and production rate of poultry meat, while physiological stress indicators (such as plasma corticosterone, glucose, cholesterol, heterophile and lymphocyte) have not changed. Previous studies have evaluated the immunity response and blood parameters of broiler chickens at 3 stocking density (15, 20 and 25 chicks/m^2) in summer [13] and they reported that stocking density had no significant effect on measured blood parameters such as heterophile to lymphocyte ratio and Newcastle titer.

Thaxton *et al*.[30] investigated the stocking density, adaptation and physiological conformity of broiler chickens to environmental conditions through three experiments. They measured stress creator indices including plasma corticosterone, glucose, cholesterol and heterophile to lymphocyte ratio on 49th d of breeding period. In the first experiment, the stocking density consisted of 20, 25, 30, 35, 40, 45, 50 and 55 kg of live weight per m^2, and in the second and third experiments were 30, 35, 40 and 45. The stocking density was calculated based on the final weight of 3.3 kg and finally, linear analysis was used to evaluate the role of stocking density on each physiological parameter. The results showed that stocking density had no effect on adaptation and physiological stress indicators of chickens. In line with this research, Uzum *et al*.[31] stated that when stocking density was 12 and 18 chickens per m^2 under heat stress condition (32-35°C), overall, body weight gain and feed intake were decreased significantly in 18 chicks density, and the heterophile to lymphocyte ratio was lower in 12 chicks density. In the present study, the effect of density on blood parameters, carcass efficiency and internal organs percentage such as spleen, bursa of fabricius, abdominal fat, thigh and breast were not significant. These results were consistent with Zuowei *et al*.[5] and Petek *et al*.[32]. Various factors can effect on the reduction of performance under high stocking density, such as reducing air quality, increasing the ammonia gas and decreasing access to water and feed.

Under the condition of this experiment, the negative effects of stocking density on growth performance traits were quite obvious and the greatest negative effect of stocking density was on reducing feed intake and increasing the moisture content of bedding, although the heterophile to lymphocyte ratio increased in 18 chicks density. The results were obtained when all birds were kept in pens until the end of experimental period. Production per m^2 is one of the important factors in choosing the best stocking density. In high stocking density (18 chicks) the live weight of chick was over 45 kg/m^2 and 32% more than low density (12 chicks). On the other hand, in this experiment, density did not have a significant effect on mortality. Therefore, due to the amount of production per m^2 and the expiring price of chicken, high density had an economic explanation. It seems that if at 30-35 d of age we took a part of chickens out of the pens (decreasing density), by increasing feed intake and compensatory growth, it could also have a positive effect on the results of study. Of course, these assumptions can be the foundation of other researches.

REFERENCES

1. Engberg RM, Hedemann MS, Jensen BB: The influence of grinding and pelleting of feed on the microbial composition and activity in the digestive tract of broiler chickens. *Br Poult Sci*, 43, 569-579, 2002. DOI: 10.1080/0007166022000004480

2. Puron D, Santamaria R, Segaura JC, Alamilla JL: Broiler performance at different stocking densities. *J Appl Poult Res,* 4, 55-60, 1995. DOI: 10.1093/japr/4.1.55

3. Dozier WA, Thaxton JP, Branton SL, Morgan GW, Miles DM, Roush WB, Lott BD, Vizzier-Thaxton Y: Stocking density effects on growth performance and processing yields of heavy broilers. *Poult Sci,* 84, 1332-1338, 2005. DOI: 10.1093/ps/84.8.1332

4. Adeyemo GO, Fashola OO, Ademulegun TI: Effect of stocking density on the performance, carcass yield and meat composition of broiler chickens. *Br Biotechnol J*, 14 (1): 1-7, 2016. DOI: 10.9734/BBJ/2016/24372

5. Zuowei S, Yan L, Yuan L, Jiao H, Song Z, Guo Y, Lin H: Stocking density affects the growth performance of broilers in a sex-dependent fashion. *Poult Sci*, 90, 1406-1415, 2011. DOI: 10.3382/ps.2010-01230

6. Houshmand M, Azhar K, Zulkifli I, Bejo MH, Kamyab A: Effects of prebiotic, protein level, and stocking density on performance, immunity, and stress indicators of broilers. *Poult Sci*, 91 (2): 393-401, 2012. DOI: 10.3382/ps.2010-01050

7. Ventura BA, Siewerdt F, Estevez I: Effects of barrier perches and density on broiler leg health, fear, and performance. *Poult Sci,* 89, 1574-1583, 2010. DOI: 10.3382/ps.2009-00576

8. AOAC: Official Methods of Analysis of AOAC International. 18th ed., 2006.

9. AOAC: Official Methods of Analysis of AOAC International. 17th ed., 2000.

10. Animal Protection Committee of Islamic Azad University, Science and Research Branch Protocol (17-16-5-10938; 90-11-15).

11. AOAC: Official Methods of Analysis. 15th ed., AOAC, Arlington, VA, 1990.

12. Barker KJ, Coufal CD, Purswell JL, Davis JD, Parker HM, Kidd MT, McDaniel CD, Kiess AS: In-house windrowing of a commercial broiler farm during early spring and its effect on litter composition1. *J Appl Poult Res,* 22, 551-558, 2013. DOI: 10.3382/japr.2013-00744

13. Turkyilmaz MK: The effect of stocking density on stress reaction in broiler chickens during summer. *Turk J Vet Anim Sci*, 32 (1): 31-36, 2008.

14. SAS. SAS User Guide: Release 9.2. SAS Istitute; Cary, North Carolina, USA, 2010.

15. Proudfoot FG, Hulan HW, Ramey, DR: Effect of four stocking densities on broiler carcass grade, the incidence of breast blisters, and other performance trials. *Poult Sci,* 58, 791-793, 1979. DOI: 10.3382/ps.0580791

16. Shanawany MM: Broiler performance under high stocking densities. *Br Poult Sci*, 29 (1): 43-52, 1988. DOI: 10.1080/00071668808417025

17. Dozier WA, Thaxton JP, Purswell JL, Olanrewaju HA, Branton SL, Roush WB: Stocking density effects on male broilers grown to 1.8 kilograms of body weight. *Poult Sci*, 85, 344-351, 2006. DOI: 10.1093/ps/85.2.344

18. Feddes JJ, Emmanuel EJ, Zuidhof MJ: Broiler performance, bodyweight variance, feed and water intake, and carcass quality at different stocking densities. *Poult Sci*, 81, 774-779, 2002. DOI: 10.1093/ps/81.6.774

19. Biligili SF, Hess JB: Placement density influences broiler carcass

grade and meat yields. *J Appl Poult Res,* 4, 384-389, 1995. DOI: 10.1093/japr/4.4.384

20. Chmelničná L, Solčianska L: Relationship between cage area and yield of the main elements of chicken carcasses. *Pol J Food Nutr Sci*. 57, 81-83, 2007.

21. Mtileni BJ, Nephawe KA, Nesamvuni AE, Benyi K: The influence of stocking density on body weight, egg weight, and feed intake of adult broiler breeder hens. *Poult Sci*, 86, 1615-1619, 2007. DOI: 10.1093/ps/86.8.1615

22. Thomas DG, Ravindran V, Thomas DV, Camden BJ, Cottam, YH, Morel PCH, Cook CJ: Influence of stocking density on the performance, carcass characteristics and selected welfare indicators of broiler chickens. *N Z Vet J*, 52, 76-81, 2004. DOI: 10.1080/00480169.2004.36408

23. Beloor J, Kang HK, Kim YJ, Subramani VK, Jang IS, Sohn SH, Moon YS: The effect of stocking density on stress related genes and telomeric length in broiler chickens. *Asian-Australas J Anim Sci,* 23 (4): 437-443, 2010. DOI: 10.5713/ajas.2010.90400

24. Farhadi D, Hosseini SM: Evaluation of growth performance, carcass characteristics, litter quality and foot lesions of broilers reared under high stocking densities. *Iran J Appl Anim Sci,* 6 (1): 187-194, 2016.

25. Gonzalez-Esquerra R, Leeson S: Physiological and metabolic responses of broilers to heat stress-implications for protein and amino acid nutrition. *Worlds Poult Sci J*, 62, 282-295, 2006. DOI: 10.1079/WPS200597

26. Nahashon SN, Adefope N, Amenyenu A, Tyus J, Wright D: The effect of floor density on growth performance and carcass characteristics of French guinea broilers. *Poult Sci*, 88, 2461-2467, 2009. DOI: 10.3382/ps.2008-00514

27. Sekeroglu A, Sarica M, Sukru Gulay M, Duman M: Effects of stocking density on chick performance, internal organ weights and blood parameters in broiler. *J Anim Vet Adv,* 10 (2): 246-250, 2011. DOI: 10.3923/javaa.2011.246.250

28. Ravindran V, Thomas DV, Thomas DG, Morel PCH: Performance and welfare of broiler as affected by stocking density and zinc bacitracin supplementation. *Anim Sci J*, 77, 110-116, 2006. DOI: 10.1111/j.1740-0929.2006.00327.x

29. Kuan, KK, Adnan S, Ramlah H: Effect of increasing stocking density on performance and heterophil/lymphocyte ratios in broilers. *Pertanika*, 13 (2): 171-175, 1990.

30. Thaxton JP, Dozier WA, Branton SL, Morgan GW, Miles DW, Roush WB, Lott BD, Vizzier-Thaxton Y: Stocking density and physiological adaptive responses of broilers. *Poult Sci*, 85, 819-824, 2006. DOI: 10.1093/ps/85.5.819

31. Uzum MH, Oral Toplu HD: Effects of stocking density and feed restriction on performance, carccas, meat quality characteristics and some stress parameters in broiler under heat stress. *Revue Méd Vét*, 164, 546-554, 2013.

32. Petek M, Ustuner H, Yesilbag D: Effects of stocking density and litter type on litter quality and growth performance of broiler chicken. *Kafkas Univ Vet Fak Derg,* 20 (5): 743-748, 2014. DOI: 10.9775/kvfd.2014.11016

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Research Article

The Efficacy of Alone or Combined Treatment of Aglepristone and Cabergoline on Termination of Mid-term Pregnancy in Cats [1][2]

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Abstract

This study determined the efficacy of a combination of aglepristone and cabergoline on termination of mid-term cat pregnancies. Twenty cats with unwanted pregnancies between 30-40 days were included in the study. Aglepristone (10 mg/kg, sc) was given to the AGL group (n=6) twice in a 24-h interval. Cabergoline (5 µg/kg, peros) was administered to the CBG group (n=7) once daily until abortion started or for 8 days. AGL+CBG (n=7) received a combined treatment with both drugs. Abortion occurred in 50% of cats in the AGL group, 71.4% in the CBG group, and 100% in the AGL+CBG. However, the completion of pregnancy termination rate was 85.7% because of fetal retention in one cat from the AGL+CBG group. The interval between treatment-start of abortion (T-SA) was shorter in the AGL+CBG group (3.6±0.3 days) than in the AGL (6.5±0.0) and CBG (6.2±0.2) groups (P<0.01). Similarly, the interval between treatment-end of abortion (T-EA) was shorter in the combined group (4.3±0.5 days) than the others (7.3±0.3 and 6.9±0.9 days, respectively) (P<0.01). Decreasing in progesterone concentration was non-significant in the AGL group from the start of treatment to abortion completion day (dA/d8), but significant in the others (P<0.001). On dA/d8, it was significantly lower in the CBG group (P<0.01) and combined group (P<0.01) than in the AGL group. Only slight diarrhea was observed in 15.4% of the AGL+CBG group. In conclusion, the AGL+CBG combination increased the rate of abortion induction and significantly shortened T-SA and T-EA with negligible side effects.

Keywords: *Aglepristone, Dopamine agonist, Pregnancy termination, Queen*

Kedilerde Orta Dönem Gebeliklerin Sonlandırılmasında Aglepriston ve Cabergolinin Tek Başlarına veya Birlikte Uygulanmasının Etkinliği

Öz

Bu çalışmada kedilerde orta dönem gebeliklerin sonlandırılmasında aglepriston ve cabergolin kombinasyonunun etkinliği belirlendi. Çalışmada istenmeyen gebeliği olan ve gebeliğin 30-40. günleri arasında olan 20 kedi kullanıldı. AGL grubuna (n=6) aglepriston (10 mg/kg, sc) 24 saat arayla iki defa uygulandı. CBG grubuna (n=7) cabergolin (5 µg/kg, peros) abortuslar başlayana kadar veya 8 gün süreyle günlük olarak uygulandı. AGL+CBG (n=7) grubuna ise her iki ilaç birlikte verildi. AGL grubunda kedilerin %50'sinde, CBG grubunda %71.4'ünde ve AGL+CBG grubunda ise %100'ünde abortuslar şekillendi. Bununla birlikte AGL+CBG grubundaki bir kedide fetal retensiyondan dolayı gebelik sonlanma oranı %85.7 olarak şekillendi. Tedavi-abortus başlama aralığı (T-SA) AGL+CBG grubunda (3.6±0.3 gün) AGL (6.5±0.0) ve CBG (6.2±0.2) gruplarından daha kısa bulundu (P<0.01). Benzer şekilde, tedavi-abortus tamamlanma aralığı (T-EA) kombine grupta (4.3±0.5 gün) diğer gruplardan (sırasıyla, 7.3±0.3 ve 6.9±0.9 gün) daha kısa olarak belirlendi (P<0.01). Tedavi başlangıcından abortus tamamlanma gününe (dA/d8) arasında progesterone konsantrasyonundaki azalma AGL grubunda önemsizken CBG ve kombine grupta önemliydi (P<0.001). Progesteron düzeyi dA/d8 gününde CBG ve kombine grupta AGL'den önemli derecede düşüktü (P<0.01). AGL+CBG grubunda 15.4% oranında sadece hafif derecede ishal görüldü. Sonuç olarak, AGL+CBG kombinasyonun abortusları uyarma oranını artırdığı, T-SA ve T-EA'yı göz ardı edilebilecek düzeyde yan etki ile önemli oranda kısalttığı belirlendi.

Anahtar sözcükler: *Aglepriston, Dopamin agonisti, Gebelik sonlandırma, Dişi kedi*

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INTRODUCTION

Corpora lutea are the main progesterone source in cats like most other domestic species. However, as in dogs, after 25 to 30 days of gestation, prolactin is secreted as a luteotrophic hormone [1,2] and its plasma concentration reaches a plateau around 50 days of gestation [1]. Thus, corpora lutea may continue to produce progesterone. At the same time, placental progesterone begins to appear during the second half of pregnancy in cats. Recently two studies at the molecular level by Braun et al.[3] and Siemieniuch et al.[4] demonstrated that the feline placenta is capable of progesterone secretion. Therefore, high plasma progesterone concentrations are maintained, which is of vital importance for the continuation of pregnancy after 30 days of gestation. Thus, difficulties could be encountered in termination of mid-term pregnancies in cats.

Because of the critical changes to support progesterone secretion in mid-term pregnancy, the hormones $PGF_2\alpha$, dopamine agonists, and progesterone receptor blockers or their combinations could be used for pregnancy termination in cats. Frequent and repetitive applications of $PGF_2\alpha$ as a luteolytic causes serious side effects: vomiting, diarrhea, hyperpnea, hypersalivation, etc.[5]. Therefore, it is not preferable to use it alone. In addition, $PGF_2\alpha$ is ineffective after the 38^{th}-40^{th} days of gestation [6,7].

Prolactin is secreted from the pituitary by simultaneously increasing plasma serotonin and decreasing dopamine concentrations. Therefore, the dopamine agonists bromocriptine and cabergoline (CBG) act as prolactin inhibitors. Thus, luteotrophic concentrations of prolactin decrease or disappear [2,8,9]. Both agents act as strong dopamine D2-receptor agonists and stimulate the chemoreceptive trigger zone. However, CBG is preferred in clinical practice because it has a more specific D2-receptor activity, which induces less severe side effects than bromocriptine [10]. Although its abortifacient feature is known [2], CBG has not been studied in cats as much as dogs. A drawback of CBG use is prolonged fetal expulsion arising from insufficient uterine smooth muscle contraction [9]. It is also more effective after 40 days of gestation [8]. In addition, the duration of pregnancy termination in CBG treatment is quite wide [11].

Aglepristone (AGL) is licensed as a progesterone receptor blocker in veterinary practice and its use in cats [7,12-14] with 9.26-times higher affinity than native progesterone. It prevents pregnancy in bitches when applied early diestrus even at the lower than standard doses [15]. After binding to the progesterone receptor, it abolishes the biological effects of progesterone [16,17] and leads to fetal death and fetal expulsion [17]. Data from studies with AGL show the abortifacient effect varies between 66.7% and 100%, depending on gestational age [12,14,18]. The abortifacient effect of AGL decreases as the gestational age increases [16].

Combined hormone treatments for termination of pregnancy such as AGL+PGE1 [19], AGL+$PGF_2\alpha$ [20], or CBG+$PGF_2\alpha$ [8] have been used with satisfying results in dogs. Although reports of combined hormone treatments in cats are not as prevalent as in dogs, a few studies use combined treatments to terminate the pregnancy in cats. In these studies, CBG was combined with $PGF_2\alpha$ analogs [11,21,22]. Unfortunately, in these regimens, the disadvantages of $PGF_2\alpha$ remain. Therefore, new methods or combinations are needed for termination of mid-term pregnancies in cats with a higher abortion induction rate and lower side-effect ratio in parallel with clinical rationales for combination therapy. To the best of our knowledge, there is no study using a combination of a progesterone receptor blocker and dopamine agonist to terminate the pregnancy in cats. Therefore we designed an AGL and CBG combination as a new, effective, and reliable method for termination of mid-term pregnancies in cats.

MATERIAL and METHODS

Twenty healthy mix breed cats with unwanted pregnancies between 30 and 40 days (mean 35.8±3.6 days), mean aged 17.5±6.3 months (range 9 to 30 months), and body weight 3.4±0.2 kg (range 2.4 to 4.7 kg) were used in a randomized design. Animals were hospitalized indoors, fed a commercial dry cat food once daily, and given water *ad-libitum* during the study. The study was approved by the animal experiments local committee of Kafkas University, Turkey (KAÜ-HADYEK/2017-052).

Assessment for gestational age was made using the internal dimensions of the gestational sac, fetal occipital-sacral length, fetal parietal diameter, and thoracic diameter with ultrasound (5-7.5 MHz probe; MyLab™Five VET, Esaote) described by Zambelli [23]. Those between 30-40 days were included in the study and assigned to three groups. Aglepristone (10 mg/kg, subcutaneously, Alizin®, Virbac) was administered twice in 24 h intervals in the AGL group (n=6). Cabergoline (5 µg/kg, peros, Galastop®, Ceva) was administered to the CBG group (n=7) once daily until abortion started. The last group, AGL+CBG (n=7), was treated with an AGL and CBG combination using the same dose and route as the single treatments. After treatments, monitoring occurred every 12 h using clinical and ultrasonographic examinations. The start of abortion was marked with the start of bloody-dark vaginal discharge. A total absence of any fetal structures in ultrasonographic examination marked the end of abortion. Animals were followed-up for 8 days and those that did not start abortions during this period were considered abortion negative. At the request of a patient's owner, one animal in the CBG group was followed-up for a longer period.

Blood samples were collected at the start of treatment (d0), 4 days later (d4), and when abortion was completed or at the end of treatment day 8 (dA/d8). Sera were stored at -20°C until analyzed. Concentrations of progesterone

were measured using an electrochemiluminescence immunoassay with Cobas Modular E170 Analyzer (Roche Diagnostics, Germany) by an accredited laboratory (Düzen Laboratories Group, Ankara, Turkey: TÜRKAK, TS EN ISO/IEC 17025:2005).

Generalized linear models (PROC GENMOD) for T-SA, T-EA, and D-A were analyzed according to functions corrected as normal probability functions. Intergroup comparisons were made by analyzing the contrast structure in orthogonal polynomials. The comparisons were completed by chi square test in accordance with nxc contingency table with one sided hypothesis for the abortion rates. Progesterone analyses were made by Kruskal-Wallis test. Differences were considered significant at P<0.05.

RESULTS

There were no significant differences in age, body weight, and gestational age between the groups at the beginning of the study (P>0.05).

Abortions were successfully induced in all animals (100%, 7/7) administered the AGL and CBG combination, whereas only 3 of 6 (50%) cats treated with AGL (P<0.05) and 5 of 7 cats (71.4%) treated with CBG were successfully induced. Fetal death was observed in one cat treated with the AGL and CBG combination 4 days after treatment started. Ovariohysterectomy was performed in this cat since there was no evidence of fetal expulsion 24 h after detected fetal death (at day 45 of gestation). Therefore, abortion was successfully induced, but not completed, in this cat. During statistical evaluation of the interval between treatment-end to abortion interval and duration of abortion, parameters were analyzed without the values from this cat. Thus, while all animals that started abortions in AGL and CBG group terminated pregnancies, in the combined group this rate was decreased to 85.7% (6/7) (P>0.05).

The interval between treatment-start of abortion (T-SA), the interval between treatment-end of abortion (T-EA), and duration of abortion (D-A) are presented in *Table 1*. The combined treatment reduced the T-SA (3.6±0.3 days) approximately 44.6% and 41.9% compared with the AGL (6.5±0 days) and CBG (6.2±0.2 days) groups, respectively *(Table 1, Fig. 1)*. Similarly, the T-EA value (4.3±0.5 days) in the combined group was approximately 41% and 37.6% shorter than in the AGL (7.3±0.3 days) and CBG (6.9±0.9 days) groups, respectively (P<0.01) *(Table 1, Fig. 2)*. No significant difference (P>0.05) was detected in the duration of abortion (interval between T-SA and T-EA) between groups *(Table 1)*.

Table 1. *The interval between treatment-start/end of abortion (day, mean± stderr)*

Groups	n	T-SA	T-EA	D-A
AGL	6	6.5±0.0[a]	7.3±0.3[a]	0.8±0.3
CBG	7	6.2±0.2[a]	6.9±0.9[a]	0.7±0.1
AGL+CBG	7	3.6±0.3[b]	4.3±0.5[b]	0.7±0.1
P value		P<0.01	P<0.01	P>0.05

Different superscripts [a,b] in the same colon means statistically different
T-SA: The interval between treatment-start of abortion; T-EA: The interval between treatment-end of abortion; D-A: The interval between T-SA and T-EA

All pregnancies (100%, 6/6) were terminated on the 5th days post-treatment in the combined group and 66.67% (4/6) of these pregnancies were terminated on the 4th day post-treatment. In the AGL group, none of the pregnancies were terminated during the first 5 days post-treatment (6/6 vs 0/3, P<0.01). In the AGL group, pregnancies were terminated on the 7th (2/3, 66.67%) and 8th (1/3, 33.33%) days post-treatment. Pregnancies were terminated in a wide range between day 4 and 9 post-treatment in the CBG group *(Fig. 3)*.

In the AGL group, a non-significant decrease in progesterone concentration was found from d0 to dA/d8 (P>0.05). In both the CBG and combined groups, a significant difference was found in the progesterone concentration from d0 to d4 and from d4 to dA/d8 (P<0.001) *(Fig. 4)*.

At the start of treatment (d0), progesterone concentrations

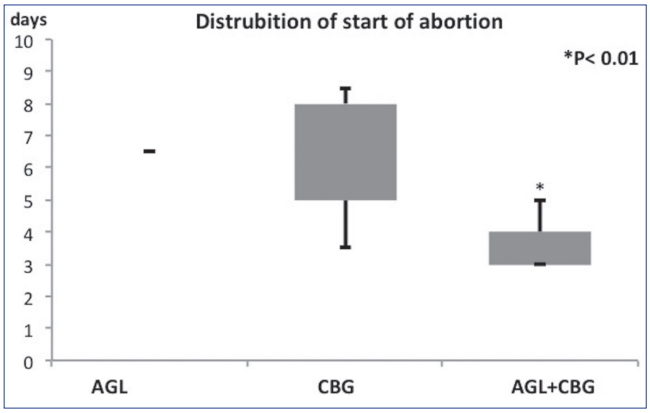

Fig 1. Distribution of interval between treatment-start of abortion

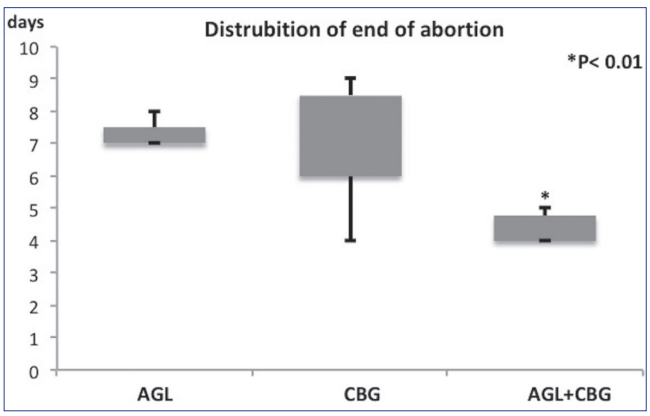

Fig 2. Distribution of interval between treatment-end of abortion

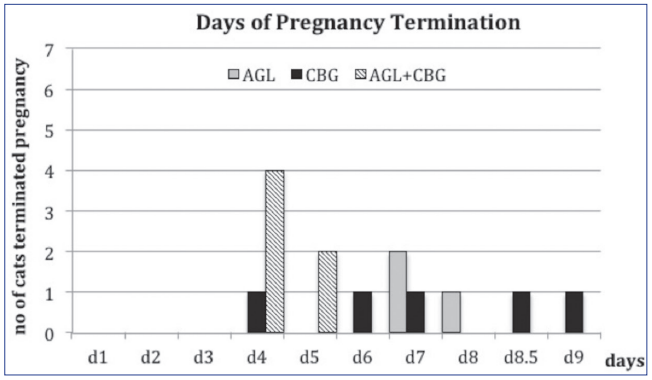

Fig 3. The number of cats terminated pregnancies

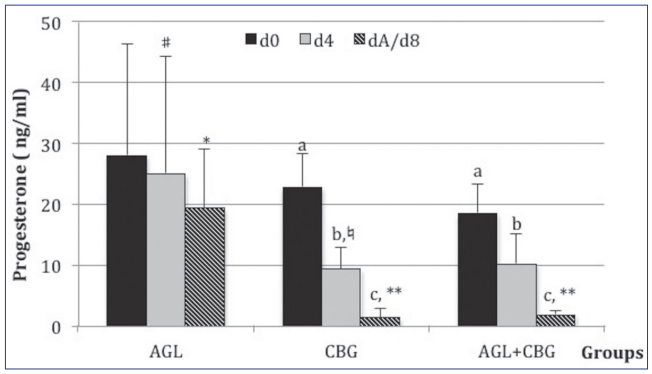

Fig 4. Progesterone concentrations in groups (day, mean± stderr)
In same groups, different superscripts [a,b,c] are significantly different (a,b and b,c: < 0.01, a,c <0.001). In different groups asterix (#, ǂ,*,**) are significantly different (#, ǂ <0.05 and *, ** <0.01)

in all groups were not different (P>0.05). However, progesterone concentrations in the CBG group were significantly lower on d4 compared with the AGL group (P<0.05) and continually increased to the dA/d8 (P<0.01). In the combined group, progesterone concentrations on dA/d8 were significantly lower than in the AGL group (P<0.01) *(Fig. 4)*.

Only diarrhea was observed in 4 of 26 applications (15.4%) in the AGL+CBG group during the study. No other side effects were seen in all groups. Observed diarrhea was slight and lasted for 1 or 2 days.

DISCUSSION

In the present study, the most satisfactory results were obtained in the combination of AGL and CBG group in accordance with our hypothesis. Contributing factors in achieving this result may be hormone doses, gestational age of cats, and duration of follow-up

Fienni et al.[14] reported that 88.5% of pregnancies are terminated using a dose of 15 mg/kg AGL at approximately 31 d of gestation with 90% success [24]. In addition, 10 mg/kg AGL terminated the pregnancies in 87% of cats at 25-26 days of gestation [18]. Verstegen et al.[21] terminated 80% of pregnancies in cats at 30 days of gestation using the dose 1.65 µg/kg CBG orally. Erünal-Maral et al.[11] achieved 100% pregnancy termination on 34-42 days of gestation using 15 µg/kg CBG.

In this study, the doses of AGL and CBG were determined according to manufacturer recommendations and the literature. The results obtained from the AGL and CBG studies are satisfactory. Based on the above data, we can speculate that satisfactory results will be obtained even if both AGL and CBG are administered at different doses than those found in the literature. Therefore, we postulate that our results are related to the gestational ages of cats and duration of follow-up, not the dose used in the study.

In the cat, the mid-term of pregnancy is a physiological transition period due to the reasons mentioned in the introduction. After this stage, AGL becomes less effective and CBG more effective (>40 days) [8] for pregnancy termination in cats. These data may explain why we obtained lower pregnancy termination rates in the AGL and CBG groups than previous studies.

Abortifacient results of the CBG+$PGF_2\alpha$ combinations in previous cat studies were satisfactory, but side effects from $PGF_2\alpha$ were very intense. Using these combinations, pregnancies were terminated within 8-10 days [22] and 3-10 days [11] from the beginning of treatment.

In our study, abortions were successfully induced (7 of 7 cats) in all animals in the combined group. Although the rate of terminated pregnancies was 87.7% due to fetal retention in one cat (1 of 7 cats), the result is consistent with previous studies in cats and dogs using combined applications.

After a successful abortion attempt, pregnancies are terminated by the way of fetal expulsion (in late-term pregnancy) or embryonic/fetal resorption. In accordance with this study, Erünal-Maral et al.[11] observed embryonic/fetal resorption or fetal expulsion between 25 to 35 days of gestation and fetal expulsion higher than 35 days of gestation in cats administered CBG for pregnancy termination. Verstegen et al.[21] reported 75% of pregnancies treated with CBG induced fetal resorption without any clinical symptoms except vaginal discharge. However, there is always the possibility of treatment failure (viable birth of kittens/puppies) and fetal retention during termination of pregnancies following treatment with AGL [14,18,25,26] or CBG [11] in dogs and cats.

In the literature, AGL treatment failures have been reported. Partial abortion resulted in physiologic parturition at mid-term in 2 of 69 dogs [26] and 1 of 21 cats [18], fetal retention in 4 of 61 cats [14], endometritis in two cats [25], physiologic parturition with viable kittens in 1 of 21 cats, and fetal maceration in 1 of 21 cats [18]. Similarly, three cats had CBG treatment failure in the Erünal-Maral et al.[11] study, resulting

in physiologic parturition for two cats and premature birth for one cat. Therefore, follow-up of the pregnancy termination process with ultrasonography is important for early intervention and protection of maternal health.

Embryonic/fetal deaths start one day after the first AGL application and end 4-7 days later (range 3.6-14 days) [16]. In our case, fetal deaths were determined on day 44 of gestation after 4 days of treatment (40 days of gestation) in accordance with the literature. Our expectation was fetal expulsion in all cats. However, one cat was ovario-hysterectomized in the request of its owner day 45 of gestation because fetal expulsion was not achieved within 24 h after the kittens died.

According to previous studies, pregnancies are terminated within 1 to 7 days [14] or 5-9 days [18] after AGL treatment and in these studies, the follow-up period was 14 and 20 days, respectively. Cabergoline applications terminate pregnancies within 3-10 days [11]. In CBG and $PGF_2\alpha$ combined applications, pregnancies are terminated within 3-10 days. Our follow-up period (8 days) was shorter than in previous studies and this could explain our low pregnancy termination rate in the AGL and CBG groups. However, our data show that abortions were started earlier in the combined group than AGL and CBG ($P<0.01$). This reduction is also reflected in T-SA and abortions were ended 41% and 37.6% earlier in the combined group than in the AGL and CBG groups, respectively ($P<0.01$). In our opinion, this data is the most important result of the study. Up to now, only a few studies were performed to reduce side effects, increase the abortifacient effect of hormones [8], and reduce the treatment period [19] in dogs. According to our results, the AGL+CBG combination does not reduce the duration of abortion, similar to Agaoglu et al.[19] Two studies demonstrated that the combination of AGL and PGE1 reduces the completion to abortion interval [19,27] during mid-term dog pregnancies, but not the combinations of AGL+$PGF_2\alpha$ (cloprostenol) and AGL+CBG+PGE1. As clearly seen in this study, the combined treatment of AGL and CBG significantly reduced the T-SA and T-EA intervals. To the best of our knowledge, this is the first study reporting the combined treatment caused an approximately 40% reduction in the treatment period in cats.

The efficiency of abortifacient treatment can be monitored by blood serum progesterone measurements. In cats, blood serum progesterone concentrations should drop under 2 mg/mL within 24 h to provide normal parturition [28]. On the other hand, parturition and abortion could be induced by AGL without any significant effect on serum progesterone concentration as opposed to other methods ($PGF_2\alpha$ or CBG). In previous studies of abortion induced with AGL, serum progesterone concentrations are higher than basal levels in dogs [19,27] and cats [18]. In this study, the progesterone concentration in the AGL group did not significantly decrease throughout the study, similar to previous studies.

However, CBG caused significant decreases in progesterone concentration in both CBG groups, similar to other studies. Onclin et al.[29] reported under 2 ng/mL progesterone concentration five days after the start of CBG treatment is an indicator of successful pregnancy termination in dogs. CBG and $PGF_2\alpha$ (alphaprostol, cloprostenol) treatment on day 30 of gestation cause progesterone concentration to drop under 1 ng/mL within 8 days in cats [21,22].

While AGL has genotoxic and cytotoxic effects on bone marrow when used to terminate mid-term pregnancies in rabbits [30], there is no evidence of such side effects in cats or dogs; both AGL and CBG have been reported to be safe in dogs and cats aside from some mild side effects (diarrhea, anorexia/depression, inflammation at injection site, lethargy for AGL) [13,14,20] and (vomiting for CBG) [1]. The side effects from combined use were related to $PGF_2\alpha$ [11]. In this study, no side effects were observed in the AGL and CBG groups. In the combined group, observed diarrheas were so mild that we considered that stress from the abortion process, hospital environment, or feed changes may have caused diarrhea.

Pregnancy termination in cats is not as well studied as in dogs. Safe methods are still needed that have a high abortion induction rate and induce a quick-response. Our results clearly show that combined treatment with aglepristone and cabergoline is capable of meeting these requirements and supports our hypothesis. As expected from the combined treatment, aglepristone and cabergoline synergistically increase the rate of abortion induction and significantly shorten interval between treatment-start of abortion and interval between treatment-end of abortion with negligible side effects.

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REFERENCES

1. Johnston SD, Kustritz MVR, Olson PS: Feline pregnancy. **In,** Canine and Feline Theriogenology. 414-430, Saunders, Philadelphia, 2001.

2. Eilts BE: Pregnancy termination in the bitch and queen. *Clin Tech Small Anim Pract,* 17 (3): 116-123, 2002. DOI: 10.1053/svms.2002.34325

3. Braun BC, Zschockelt L, Dehnhard M, Jewgenow K: Progesterone and estradiol in cat placenta-biosynthesis and tissue concentration. *J Steroid Biochem Mol Biol,* 132 (3-5): 295-302, 2012. DOI: 10.1016/j.jsbmb.2012.07.005

4. Siemieniuch MJ, Jursza E, Szostek AZ, Zschockelt L, Boos A, Kowalewski MP: Placental origin of prostaglandin F2α in the domestic cat. *Mediators Inflamm,* 2014, 364787, 2014. DOI: 10.1155/2014/364787

5. Romagnoli SE, Camillo F, Cela M, Johnston SD, Grassi F, Ferdeghini M, Aria G: Clinical use of prostaglandin F_2 alpha to induce early abortion in bitches: Serum progesterone, treatment outcome and interval to

subsequent oestrus. *J Reprod Fertil Suppl,* 47, 425-431, 1993.

6. Nachreiner RF, Marple DN: Termination of pregnancy in cats with prostaglandin F_2 alpha. *Prostaglandins,* 7 (4): 303-308, 1974.

7. Garcia Mitacek MC, Stornelli MC, Praderio R, Stornelli MA, de la Sota RL: Efficacy of cloprostenol or aglepristone at 21-22 and 35-38 days of gestation for pregnancy termination in queens. *Reprod Domest Anim,* 47 (Suppl.-6): 200-203, 2012. DOI: 10.1111/rda.12023

8. Onclin K, Silva LDM, Verstegen JP: Termination of unwanted pregnancy in dogs with the dopamine agonist, cabergoline, in combination with a synthetic analog of PGF_2alpha, either cloprostenol or alphaprostol. *Theriogenology,* 43 (4): 813-822, 1995. DOI: 10.1016/0093-691X(95)00024-3

9. Post K, Evans LE, Jöchle W: Effects of prolactin suppression with cabergoline on the pregnancy of the bitch. *Theriogenology,* 29 (6): 1233-1243, 1988. DOI: 10.1016/0093-691X(88)90003-9

10. Romagnoli S: Practical use of hormones in small animal reproduction. *Rev Bras Reprod Anim, Belo Horizonte,* 41 (1): 59-67, 2017.

11. Erünal-Maral N, Aslan S, Findik M, Yuksel N, Handler J, Arbeiter K: Induction of abortion in queens by administration of cabergoline (Galastop™) solely or in combination with the PGF_2alpha analogue Alfaprostol (Gabbrostim™). *Theriogenology,* 61 (7-8): 1471-1475, 2004. DOI: 10.1016/j.theriogenology.2003.08.014

12. Georgiev P, Bostedt H, Goericke-Pesch S, Dimitrov M, Petkov P, Stojanthev K, Tsoneva V, Wehrend A: Induction of abortion with aglepristone in cats on day 45 and 46 after mating. *Reprod Domest Anim,* 45 (5): e161-e167, 2010. DOI: 10.1111/j.1439-0531.2009.01540.x

13. Goericke-Pesch S, Georgiev P, Wehrend A: Prevention of pregnancy in cats using aglepristone on days 5 and 6 after mating. *Theriogenology,* 74 (2): 304-310, 2010. DOI: 10.1016/j.theriogenology.2010.02.014

14. Fieni F, Martal J, Marnet PG, Siliart B, Guittot F: Clinical, biological and hormonal study of mid-pregnancy termination in cats with aglepristone. *Theriogenology,* 66 (6-7): 1721-1728, 2006. DOI: 10.1016/j.theriogenology.2006.02.026

15. Kanca H, Karakaş K: Effectiveness of aglepristone at lower-than-standard doses in prevention of pregnancy in mismated bitches. *Kafkas Univ Vet Fak Derg,* 18 (3): 517-521, 2012. DOI: 10.9775/kvfd.2012.5936

16. Gogny A, Fieni F: Aglepristone: A review on its clinical use in animals. *Theriogenology,* 85 (4): 555-566, 2016. DOI: 10.1016/j.theriogenology.2015.10.010

17. Galac S, Kooistra HS, Butinar J, Bevers MM, Dieleman SJ, Voorhout G, Okkens AC: Termination of mid-gestation pregnancy in bitches with aglepristone, a progesterone receptor antagonist. *Theriogenology,* 53 (4): 941-950, 2000. DOI: 10.1016/s0093-691x(00)00241-7

18. Georgiev P, Wehrend A: Mid-gestation pregnancy termination by the progesterone antagonist aglepristone in queens. *Theriogenology,* 65 (7): 1401-1406, 2006. DOI: 10.1016/j.theriogenology.2005.08.011

19. Agaoglu AR, Schafer-Somi S, Kaya D, Kucukaslan I, Emre B, Gultiken N, Mulazimoglu BS, Colak A, Aslan S: The intravaginal application of misoprostol improves induction of abortion with aglepristone. *Theriogenology,* 76 (1): 74-82, 2011. DOI: 10.1016/j.theriogenology.2011. 01.019

20. Kaya D, Kucukaslan I, Agaoglu AR, Ay SS, Schafer-Somi S, Emre B, Bal Y, Einspanier A, Gurcan IS, Gultiken N, Aslan S: The effects of aglepristone alone and in combination with cloprostenol on hormonal values during termination of mid-term pregnancy in bitches. *Anim Reprod Sci,* 146 (3-4): 210-217, 2014. DOI: 10.1016/j.anireprosci.2014.03.002

21. Verstegen JP, Onclin K, Silva LD, Donnay I: Abortion induction in the cat using prostaglandin F_2 alpha and a new anti-prolactinic agent, cabergoline. *J Reprod Fertil Suppl,* 47, 411-417, 1993.

22. Onclin K, Verstegen J: Termination of pregnancy in cats using a combination of cabergoline, a new dopamine agonist, and a synthetic PGF_2 alpha, cloprostenol. *J Reprod Fertil Suppl,* 51, 259-263, 1997.

23. Zambelli D, Castagnetti C, Belluzzi S, Bassi S: Correlation between the age of the conceptus and various ultrasonographic measurements during the first 30 days of pregnancy in domestic cats *(Felis catus). Theriogenology,* 57 (8): 1981-1987, 2002. DOI: 10.1016/S0093-691X(01)00686-0

24. Fiéni FBJ, Tainturier D: Interruption de la gestation chez la chatte par administration d'Alizine. *Vet Repro,* 1, 11, 2000.

25. Gorlinger S, Kooistra HS, van den Broek A, Okkens AC: Treatment of fibroadenomatous hyperplasia in cats with aglepristone. *J Vet Intern Med,* 16 (6): 710-713, 2002. DOI: 10.1111/J.1939-1676.2002.tb02412.x

26. Fiéni F TD, Bruyas JF, Badinand F, Berhelot X, Ronsin P, Racihal L, Lefay MP: E'tude clinique d'une antihormone pour provoquer l'avortement chez la chienne: l'agépristone. *Rec Med Vet,* 172, 359-367, 1996.

27. Agaoglu AR, Aslan S, Emre B, Korkmaz O, Ozdemir Salci ES, Kocamuftuoglu M, Seyrek-Intas K, Schafer-Somi S: Clinical evaluation of different applications of misoprostol and aglepristone for induction of abortion in bitches. *Theriogenology,* 81 (7): 947-951, 2014. DOI: 10.1016/j.theriogenology.2014.01.017

28. Johnson SD, Kustritz MVR, Olson PNS: Feline parturition. **In,** Canine and Feline Theriogenology. 431-437, Saunders, Philadelphia, 2001.

29. Onclin K, Silva LD, Donnay I, Verstegen JP: Luteotrophic action of prolactin in dogs and the effects of a dopamine agonist, cabergoline. *J Reprod Fertil Suppl,* 47, 403-409, 1993.

30. Vatan Ö, Bagdas D, Cinkilic N, Wehrend A, Özalp GR: Genotoxic and cytotoxic effects of the aglepristone, a progesteron antagonist, in mid-gestation pregnancy termination in rabbits. *Kafkas Univ Vet Fak Derg,* 21 (2): 241-246, 2015. DOI: 10.9775/kvfd.2014.12210

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Research Article

Comparison of Bovine Pregnancy Associated Glycoproteins (bPAG-Serum and Milk), Bovine Pregnancy Specific Protein B (bPSP-B) Tests with Each Other and with Transrectal Ultrasonographic Findings for Early Pregnancy Diagnosis [1] [2]

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Abstract

The aim of this study was to compare the accuracy of bovine pregnancy-associated glycoproteins (bPAG) and bovine pregnancy-specific protein B (bPSP-B) tests with transrectal ultrasonography (TRUS) for early pregnancy diagnosis in dairy cows. The study was carried out on 58 Holstein cows. Blood and milk samples were collected on days 20, 25, 28, 30, 32 and 40 following artificial insemination (AI). TRUS was performed on all cows on days 30 and 40 following AI. The positive predictive values for bPAG-serum and bPAG-milk tests were 100% on days 28 and 30. For bPSP-B tests, positive predictive values were 93.7% and 100% on days 28 and 30, respectively. The Kappa test revealed a high agreement between TRUS and the three tests on days 28 and 30 with the values of 0.954 and 0.946 (P<0.001), respectively. Specificity was found to be 100% for bPAG-serum and bPAG-milk, and 96.7% for bPSP-B on day 28. Sensitivity values were calculated as 94.1% and 93.8% for bPAG-serum and bPSP-B, respectively. The area under ROC curve was between 0.967 and 0.991 for on day 28 following AI. In conclusion, all three tests were found to be reliable methods for pregnancy diagnosis in cows on days 28 and 30 following AI. However, test results may give false positive values in case of embryonic deaths.

Keywords: *Pregnancy diagnosis, Bovine, Pregnancy-associated glycoprotein, Pregnancy-specific protein-B, ELISA*

Erken Gebelik Teşhisi Amacıyla Sığır Gebelik İlişkili Glikoprotein (bPAG-Serum ve Süt) ve Sığır Gebelik Spesifik Protein B (bPSP-B) Testlerinin Birbirileriyle ve Transrektal Ultrasonografik Bulgularla Karşılaştırılması

Öz

Bu çalışmanın amacı, süt ineklerinin erken gebelik tanısında sığır gebelik ilişkili glikoproteinlerin (bPAG) ve sığır gebelik spesifik protein B (bPSP-B) testlerinin transrektal ultrasonografiyle (TRUS) doğruluğunun karşılaştırılmasıdır. Araştırma 58 adet Holstein ineğinde gerçekleştirildi. Suni tohumlamayı (AI) takiben 20, 25, 28, 30, 32 ve 40. günlerde kan ve süt örnekleri toplandı. TRUS, AI'yi takiben 30. ve 40. günlerde tüm ineklerde yapıldı. bPAG-serum ve bPAG-süt testleri için pozitif prediktif değerler, 28 ve 30. Günlerde %100 idi. bPSP-B testleri için, pozitif prediktif değerler sırasıyla 28 ve 30. Günlerde %93.7 ve %100 idi. Kappa testi TRUS ile 28 ve 30. günlerde yapılan üç test arasında sırasıyla 0.954 ve 0.946 (P<0.001) değerlerinde yüksek bir mutabakat olduğunu ortaya koymuştur. Spesifiklik, bPAG-serum ve bPAG-sütü için %100 ve bpsp-B için 28. Günde %96.7 olarak bulundu. Duyarlılık değerleri, bPAG-serum ve bPSP-B için sırasıyla %94.1 ve %93.8 olarak hesaplandı. ROC eğrisi altındaki alan AI'yi takip eden 28. günde 0.967 ile 0.991 arasında idi. Sonuç olarak, üç testinde AI sonrası 28 ve 30. günlerde ineklerde gebelik tanısında güvenilir yöntemler olarak kullanılabileceği tespit edildi. Bununla birlikte, test sonuçları embriyonik ölüm durumunda yanlış pozitif değerler verebilir.

Anahtar sözcükler: *Gebelik tanısı, Sığır, Gebelik ilişkili glikoprotein, Gebelik spesifik protein-B, ELISA*

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INTRODUCTION

In cows, it is important to diagnose early pregnancy, especially on farms where oestrus is not well-monitored [1,2]. In general, even though transrectal palpation and ultrasonographical methods are applied for the detection of pregnancy, ultrasonographical methods have been shown to be more successful [3] the main diagnostic methods used for reproductive control in cattle included rectal palpation, inspection of vaginal discharge and vaginoscopy. Since the 1990 s, the use of ultrasound (US. In one study, it was determined that when ultrasonographic controls were compared with progesterone (P_4), bovine pregnancy-specific protein B (bPSP-B) and bovine pregnancy-associated glycoprotein 1 (bPAG-1), there was indeed no difference between those three methods. When heart rate was used as a parameter during ultrasonography, it was determined that significantly fewer pregnancies were verified by ultrasonography than with the two tests [4].

Trophoblast binuclear cells synthesize proteins during early pregnancy. bPAG or bPAG-1 and bPSP-B participate in the circulatory system. bPSP-B is identified as a foetal protein while PAG is identified as placenta specific protein. This molecule is present in the maternal circulation and can reliably be determined on days 29 and 30 following insemination. It was reported that both factors (bPAG 1; bPSP-B) could be determined in the serum of some pregnant cows on days 15 to 22 following insemination, but more reliable results were determined on days 28 to 30 following conception [4].

It was determined based on the results obtained from the comparison of transrectal ultrasonography (TRUS) with both RIA [4] and ELISA [5] tests, that the bPSP-B test can be used for the diagnosis of pregnancy. There is also evidence that the bPSP-B can be measured until 70-100 days after parturition due to the persistence of postpartum high peripheral concentrations [4].

The aim of this study was to determine, during the here observed period, on which day highest levels of the bPAG-Serum, bPAG-Milk and bPSP-B serum tests can be measured in pregnant and non-pregnant cows. Furthermore, which of these three tests is the most accurate and suitable for determination of early pregnancy. We also aimed to determine the statistical reliability of the cut-off points (ideal test values) determined in the tests according to Youden J index in the early stages of pregnancy.

MATERIAL and METHODS

Animals

In the study, 58 clinically healthy Holstein cows, from five different commercial dairy farms were used. The study was performed with blood and milk samples sent from these farms. The average age of the animals studied was between 3 and 7 years and the average milk yield ranged from 6000 to 8000 L in one lactation. On all farms where the study was conducted, the animals were housed in a free system and all were fed with the same food (Corn silage, hay, green grass, TMR, admixture of minerals and vitamins). Cows were housed in free stall barns and had ad libitum access to feed and water.

Transrectal Ultrasonography (TRUS) and Test Groups

Transrectal ultrasonography was carried out in order to diagnose pregnancy on days 30 and 40 after insemination by using a portable scanner (B-Mode, 5.0 to 7.5 MHz, linear probe, ECM IMAGO®Veterinary; Angouleme/France). Animals were furthermore determined to be pregnant or non-pregnant on day 40 by using the ELISA test kits for the bPAG-milk tests and for measurement of bPAG, bPSP-B samples. The samples collected until the 28th day were compared with ultrasonography results on day 30. Comparisons on day 40 were made to determine whether the pregnancy was continued and to reaffirm the diagnosis in cows previously determined to be non-pregnant.

When pregnancies where assessed by ELISA on day 30 but not determined by ultrasonography on day 40, animals were allocated to the group of animals diagnosed as pregnant on day 30.

When cows were examined as not being pregnant on day 40, but pregnancy had been determined by using TRUS on day 30, this was diagnosed as embryonic death. These animals (n=9) were not taken into the statistical evaluation.

The following groups were formed after evaluation of the obtained blood and milk samples (N=49): A=PAG-Serum (PAG-S; N=49); B=PAG-Milk (PAG-M; N=49); C=PSPB-Serum (PSPB-S; N=49)

Collecting Blood and Milk Samples

Blood and milk samples were collected (*v. jugularis*) from each animal on days 20, 25, 28, 30, 32 and 40 following insemination (day 0).

Blood and milk samples were then brought to laboratory within 2 h under cold chain Serum was obtained by centrifugation of blood samples at 1500 X g for 10 min. Milk samples were centrifuged at 4800 X g for 20 min to obtain skimmed milk and were stored at -20ºC until analyses. The laboratory staff was blinded to the results of the TRUS exams.

Bovine Serum PAG, Milk PAG and Serum PSP-B ELISA Analysis

Sandwich ELISA test (IDEXX Bovine Pregnancy Test Ref 99-41169 Lot. E171, One IDEXX Drive, Westbrook, ME, USA; IDEXX Milk Pregnancy Test Ref 99-41209 Lot. E881, One IDEXX Drive, Westbrook, ME, USA; BioPRYN test Lot No: 5P106, Biotracking LLC, Moscow, ID, USA) was performed

for the measurement of serum bPAG; milk bPAG; and serum bPSP-B. The tests were carried out in accordance with the directives of the manufacturers. The washing steps were performed using an automated microtiter washer (MW-12A Microplate washer, Mindray, Shenzhen, China); analysis results (as optical density) of the samples were obtained using a microtiter plate reader (MR-96A Microplate reader, Mindray, Shenzhen, China). Test results were calculated according to the manufacturer's instructions and expressed as sample-negative (S-N) (all optical densities of the animals were subtracted from the optical density of the negative control). For bPAG serum samples ≥0.300 optical density was assessed as pregnancy positive (pregnant), and <0.300 optical density as pregnancy negative (not pregnant). The intra- and inter-assay coefficients of variation (CV) were 4.2% and 8.8%, respectively. For bPAG milk samples ≥0.150 OD was evaluated as pregnancy positive (pregnant), and <0.100 OD as pregnancy negative (not pregnant). The results, which ranged between 0.100 and 0.150, were considered uncertain. The intra- and inter-assay coefficients of variation (CV) were 3.2% and 9.6%, respectively. And for PSP-B, the test results were evaluated as >0.210 OD pregnancy positive (pregnant); OD between 0.135 and 0.210, uncertain; and <0.135 OD pregnancy negative (not pregnant). The intra- and inter-assay coefficients of variation (CV) were 2.6% and 5.4%, respectively.

Statistical Analyses

Statistical evaluations were performed using SPSS (version 17 for Windows, SPSS Ltd, Hong Kong). Crosstabs were used for specificity, sensitivity, false negative and false positive calculations. The Chi-Square test was used to compare percentages. The significance of the intersection value, which was obtained by defining Roc-Curve and the Area under Roc-Curve, was demonstrated. Normality and homogeneity of groups were determined by the Shapiro-Wilk test. The Kruskal-Wallis test was used to identify potential differences. Due to the relatively small sample numbers, a non-parametric Mann-Whitney U test was performed for comparisons between groups (i.e., treatment vs. control). Mean values and standard deviation were determined by descriptive calculation. Estimates of the agreement between TRUS and PSP-B results were determined by Kappa values and 95% confidence intervals (95% CI) were also determined. Differences were considered statistically significant when P<0.05.

RESULTS

Transrectal ultrasonography examinations performed on the days 30th and 40th; cows (n=9) who were found to be pregnant on the 30th day but not on the 40th day were evaluated as embryonic death was occurred.

In bPAG-Serum test, positive values were obtained in 8 cows (88.9%) whereas negative values were obtained in 1 cow (11.1%) on day 28. In bPAG-Milk positive values in 7 cows, and negative values in two animals were obtained whereas in bPSP-B test, positive values in 7 cows, uncertain values in 1 cow and negative values in 1 cow were obtained. TRUS findings obtained on day 30 were mostly found to be in agreement with bPAG-serum findings (positive in 8 cows, negative in one cow). After ultrasonographical detection of embryonic deaths in 9 cows, both positive and negative results could be obtained from all three tests on day 40; in 44.4% of cases the bPSP-B test results were false positive, in 77.7% the bPAG-Serum test and in 44.4% the bPAG-Milk test.

On day 25 of pregnancy, positive results obtained by TRUS could be confirmed by the bPSP-B-serum test in 42.4% of cases, by the bPAG-Serum test in 90.6% of cases and by using the PAG-Milk test in 78.1% of cases. On days 28 and 30, bPAG-serum and bPAG-milk tests showed a 93.7% and 100% accordance with TRUS, respectively. From day 40 on, more than 93% of results were in accordance with the TRUS results, in all tests evaluated.

Negative test results obtained by measurement of bPAG in serum and milk on day 20, could be confirmed by TRUS in 100%. On day 25 and 28, the accordance with TRUS was 94.1%, and increased to 100% later on with the bPAG-serum test. In bPAG-milk test, the accordance remained the same with 94.1% from day 25 to 40. With the bPSP-B-serum test, the accordance was 94.1% on day 20, 88.2% on day 25 and 30, 94.1% on day 32 and 100% on day 40 *(Table 1)*.

Similarly, the measure of agreement calculations made with the Kappa statistical test showed that the accordance with TRUS was highest with the bPAG-Serum and -Milk test on day 28 (both 0.954; P<0.001). With the bPSP-B Test, the highest accordance was found on day 30 (0.946; P<0.001) *(Table 2)*.

With bPAG-serum and -milk tests, a specificity of 94.1% and 100 % could already be determined on day 25 and 28, respectively; whereas with bPSP-B test a specificity of >90% was only detectable from day 28 *(Table 3)*.

As to the bPAG-serum test, the ideal cut-off points according to Youden J index of the statistical test results calculated with ROC-Curve was determined to be 0.865 on day 28, with a 100% of positive results. Sensitivity of the test was 1.0 and the specificity value was 0.059. The area under the curve value of 0.974 indicated that the test was reliable (P<0.001). Likewise, the ideal cut-off point for bPAG-milk was 0.2695 and the sensitivity of the test was 1.0; and the specificity value was 0.059 (P<0.001). The area under the curve value was 0.991, indicating that the test was reliable (P<0.001). The bPSP-B test was also found reliable on day 28 (cut-off point: 0.21; sensitivity: 0.938; specificity value: 0.059; Area under the curve: 0,967; P<0.001) *(Table 4)*.

Table 1. *Positive and negative pregnancy detection rates with the ELISA test (bPAG-Milk, bPAG-Serum, bPSPB-Serum)*

Day	Positive (bPAG/M) (n/x)	Positive (bPAG/S) (n/x)	Positive (bPSPB/S) (n/x)	Negative (bPAG/M) (n/x)	Negative (bPAG/S) (n/x)	Negative (bPSPB/S) (n/x)	Uncertain (bPAG/M) (n/x)	Uncertain (bPAG/S) (n/x)	Uncertain (bPSPB/S) (n/x)
D20	-	3.1%a (32/1)	6.3%a (32/2)	100% (17/17)	100% (17/17)	94.1% (17/16)	-	-	-
D25	78.1%a (32/25)	90.6%b (32/29)	42.4%b (32/14)	94.1% (17/16)	5.9% (17/1)	88.2% (17/15)	9.4% (32/3)	-	24.2% (33/8)
D28	100%b (32/32)	100%b (32/32)	93.7%c (32/30)	94.1% (17/16)	94.1% (17/16)	88.2% (17/15)	-	-	3.1% (32/1)
D30	100%b (32/32)	100%b (32/32)	100%c (32/32)	94.1% (17/16)	76.5% (17/13)	88.2% (17/15)	-	-	-
D32	93.6% (32/30)	93.7% (32/30)	93.9% (32/31)	94.1% (17/16)	94.1% (17/16)	94.1% (17/16)	-	-	-
D40	100% (32/32)	96.9% (32/31)	93.5% (32/29)	94.1% (17/16)	100% (15/15)	100% (15/15)	-	-	3.2% (31/1)

Table 2. *Measure of Agreement (Test/TRUS. Kappa) Test*

Test	Days	Value	SE	P
bPAG - Serum (N=49)	25	0.825	0.084	0.001
	28	0.954	0.045	0.001
	30	0.809	0.090	0.001
	32	0.867	0.074	0.001
	40	0.958	0.042	0.001
bPAG - Milk (N=49)	25	0.811	0.081	0.001
	28	0.954	0.045	0.001
	30	0.910	0.062	0.001
	32	0.867	0.074	0.001
	40	0.954	0.045	0.001
bPSP-B - Serum (N=49)	25	0.630	0.089	0.001
	28	0.920	0.044	0.001
	30	0.946	0.037	0.001
	32	0.921	0.044	0.001
	40	0.895	0.049	0.001

Kappa value as a measure of agreement between TRUS and ELISA

DISCUSSION

It has great importance to diagnose early pregnancy in order to increase the production volume of livestock sector. This will also lead to increased profitability and rural development [6]. bPAG produced in the ruminant trophoblast cells can be detected in the blood of pregnant cows [7]. Similarly, serum concentration of bPSP-B can be measured to determine early bovine pregnancy by using an ELISA test [5] and a bPAG-milk test has been developed to assess early pregnancy by using milk, which can be collected more easily than serum [8].

In this study, three tests (bPAG-Serum, bPAG-Milk and bPSP-B-Serum) were performed in order to determine early pregnancy in cows. The aim was to assess the rate of positive and negative test results between days 20 and 40 of pregnancy, and to determine the earliest day, on which each test can be used with sufficient reliability.

Embryonic deaths were detected in 9 cows during TRUS examinations performed on day 40; although pregnancy was detected in these cows by TRUS on day 30, this could not be confirmed on day 40. When all early results of the three ELISA tests were compared with the laboratory test results obtained on day 28 and TRUS findings obtained on day 30, the bPAG-Serum test was 100% compatible with

Table 3. *The specificity and sensitivity values of all tests examined (by days)*

Test	Specificity (%)					Sensitivity (%)				
	Days					Days				
	25	28	30	32	40	25	28	30	32	40
bPAG-Serum	94.1 (n=32)	100 (n=32)	100 (n=32)	93.7 (n=30)	96.9 (n=31)	90.6 (n=17)	94.1 (n=16)	76.5 (n=13)	94.1 (n=16)	100 (n=15)
bPAG- Milk	94.1 (n=32)	100 (n=32)	100 (n=32)	93.7 (n=30)	100 (n=32)	86.2 (n=17)	94.1 (n=16)	94.1 (n=16)	94.1 (n=16)	94.1 (n=16)
bPSPB- Serum	55.1 (n=33)	96.7 (n=30)	100 (n=32)	93.7 (n=30)	96.7 (n=30)	52 (n=17)	93.8 (n=15)	88.2 (n=15)	94.1 (n=16)	100 (n=15)

Table 4. The Cut-off Values calculated by ROC curve results, sensitivity, specificity and significance (AUC) of obtained values

Test	Cut-off Point (J index)	Sensitivity	Specificity	AUC*
bPAG-Milk	0.2695	1.0	0.059	0.991
bPAG-Serum	0.865	1.0	0.059	0.974
bPSPB-Serum	0.21	0.938	0.059	0.967

** Area under the Curve*

the TRUS findings. The other tests were found to be 88.9% compatible with TRUS on day 28. In cows, embryonic deaths is frequently seen until day 45 of pregnancy [4]. It is known that uncertain or negative test results can be caused by embryonic death. It has also been reported that false positive findings can be obtained due to embryonic mortality [9]. In the present study, in case of embryonic death, reliability of TRUS was greater. Even though no embryo was detected in TRUS controls performed on day 40, false positive results were obtained in bPSP-B test, bPAG-serum test and bPAG-milk in 44.4%, 77.7% and 44.4% of cases, respectively.

In this study, it was determined that in case of normal pregnancies, positive results obtained with bPSP-B serum ELISA on day 25 correlated with positive TRUS findings in 42.4% of cases; this percentage was 90.6 with the bPAG-serum test and 78.1 with the bPAG-milk test, which increased on days 28 and 30 to 100%; whereas with the bPSP-B test a 100% was not reached before day 30.

In case of negative pregnancies on day 20, compliance between bPAG serum and milk ELISA results, and TRUS findings was 100%, and 94.1% on the other days. With bPSP-B serum ELISA, this percentage only ranged between 94.1% and 88.2% in the early pregnancy period. Uncertain negative/positive test results were detected on some days in the other tests, however, no uncertain test results were obtained with the bPAG-serum ELISA.

Therefore, based on these findings, all the tests in this study were able to detect positive and negative pregnancies at high rates on day 28 and 30; however, with the bPAG-serum and milk tests only, compatibility with TRUS was 100% on days 28 and 30 of the pregnancy. In another study, the bPAG serum test was used on day 27 of pregnancy, and the compliance with TRUS findings was 93.7% and 97.8% [10]. With the bPAG-Milk test, results were found to be more variable than with the serum test, which was supposed to be mainly due to milk storage [11]. In other studies, it was suggested that milk amount [12], milk composition, contamination of milk samples [13] or lactation period [14]. In this study, no difference was found between bPAG-Serum and bPAG-Milk in the detection of pregnancy on day 28 and 30 following insemination.

Compliance between ultrasonography and these tests can be calculated using the Kappa test [5]. Considering all positive and negative comparisons, the results obtained with the Kappa test were found to be significant at the level of 0.954 ($P<0.001$) on day 28 with the bPAG-serum and milk ELISA, and 0.9446 ($P<0.001$) on day 30 with the bPSP-B test. In one study, comparison of results of transrectal palpation with the bPSP-B test by using the kappa test, resulted in 0.83 [15]. In another study, the kappa data of the agreement between bPAG ELISA test and determination of pregnancy increased from 0.87 (first insemination) up to 0.94 (third insemination) depending on the number of inseminations [10].

In bovine plasma, bPSP-B was detected between days 15 and 22 of pregnancy after insemination. It has been determined that because this glycoprotein is synthesized in the binuclear trophoblastic cells of placenta, good results can be obtained when the test is applied after day 30 post-conception [4]. In this study, with the bPSP-B test, compliance with TRUS on day 28 was 93.7% whereas 100% compliance was found on day 30. Others reported a 90% positive and a 99.5% negative compatibility with PSPB test on the same day [4,16].

In most studies, bPAG and bPSP-B tests were found to be similarly useful for the detection of pregnancy [4]. On the other hand, on days 28, 30 and 35 of pregnancy, uncertain bPSP-B sample rates were found to have changed through the rates of 8.5%, 4.8% and 3.3%, respectively [5]. In our study, with bPSP-B-Serum ELISA, uncertain results were found on days 25 (24.2%), 28 (3.1%) and 40 (3.2%) of pregnancy, with bPAG-Milk test, on day 25 (9.4%) only, whereas no uncertain results were found in bPAG serum test.

In bPAG serum and milk, the negative pregnancy rate, which was determined to be compatible with TRUS, was 100% on day 20 following insemination, and was determined as 94.1% on the consecutive days, including day 32. This ratio was determined to be 94.1% in bPSP-B test on day 20, and between 88.2% and 100% (on day 40) on the consecutive days. When the test results were examined, in bPAG-serum test, the specificity was 100% on days 28 and 30; in bPAG Milk, 100% on day 28 only; and in bPSP-B test, 100% on day 30 (96.7% on day 28). On the contrary, sensitivity in bPAG Serum was 94.1% on day 28; and in bPAG milk, 94.1% on both days 28 and 30. Sensitivity was determined to be 93.8% on day 28 in bPSP-B Serum test. Silva et al.[10] tested cows on day 27 for bPAG by ELISA and compared those results with TRUS 32 days after AI. They reported sensitivity ranging between 93.5% and 96.3% and specificity between

91.7% and 96.8%. In another study, bPAG analysis correctly diagnosed pregnancy on day 28 post-AI [17]; however only samples from females that delivered live calves were analysed. Finally, when bPAG-1 was analysed by RIA on day 35 and compared with transrectal palpation on day 45, the sensitivity and negative predictive values were 98.8% and 97.9%, respectively [16]. In this study, the fact that sensitivity was achieved at 94.1% rate on day 28 in bPAG Serum and milk test shows that if samples are examined at this stage of pregnancy, reliable pregnancy results can be obtained.

Szenci et al.[4], testing for bPSP-B by RIA, reported a sensitivity of 92% from samples collected on both days 29 and 30, and 98.1% on days 33 and 34. Howard et al.[18], using the same bPSP-B ELISA test, reported a sensitivity of 100% and a specificity of 87.8% for cows between days 30 and 36 compared with US performed between days 37 and 43. Interestingly, they found no uncertain results in 336 samples. In bPSP-B test, both sensitivity and specificity were high, again, on day 28. It is necessary to bear in mind that the specificity was calculated as 100% (specificity as 88.2%) on day 30.

The numerical Cut-off values obtained by the Area under the ROC curve on day 28 ranged from 0.967 to 0.991 and even approached 1, which means high specificity and sensitivity as well as reliability of the test.

In conclusion, the data obtained show that all of these tests are reliable and suitable for determination of pregnancy, with highest accuracy on days 28 and 30, when both specificity and sensitivity are considered. In some animals, when pregnancy was detectable by ELISA on days 28 and 30, positive results were obtained later, on the day when embryonic deaths was detected by TRUS.

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REFERENCES

1. Walsh SW, Williams EJ, Evans ACO: A review of the causes of poor fertility in high milk producing dairy cows. *Anim Reprod Sci*, 123 (3-4): 127-138, 2011. DOI: 10.1016/j.anireprosci.2010.12.001

2. Holman A, Thompson J, Routly JE, Cameron J, Jones DN, Grove-White D, Smith RF, Dobson H: Comparison of oestrus detection methods in dairy cattle. *Vet Rec*, 169 (2): 47, 2011. DOI: 10.1136/vr.d2344

3. Quintela LA, Barrio M, Peña AI, Becerra JJ, Cainzos J, Herradón PG, Díaz C: Use of ultrasound in the reproductive management of dairy cattle. *Reprod Domest Anim*, 47 (Suppl 3): 34-44, 2012. DOI: 10.1111/j.1439-0531.2012.02032.x

4. Szenci O, Beckers JF, Humblot P, Sulon J, Sasser G, Taverne MAM, Varga J, Baltusen R, Schekk G: Comparison of ultrasonography, bovine pregnancy-specific protein B and bovine pregnancy-associated glycoprotein 1 tests for pregnancy detection in dairy cows. *Theriogenology*, 50 (1): 77-88, 1998. DOI: 10.1016/S0093-691X(98)00115-0

5. Romano JE, Larson JE: Accuracy of pregnancy specific protein-B test for early pregnancy diagnosis in dairy cattle. *Theriogenology*, 74 (6): 932-939, 2010. DOI: 10.1016/j.theriogenology.2010.04.018

6. Buragohain L, Nanda T, Ghosh A, Ghosh M, Kumar R, Kumar S, Gupta SS, Bharali A, Mohanty AK, Singh I, Balhara AK: Identification of serum protein markers for early diagnosis of pregnancy in buffalo. *Anim Sci J*, 88 (8): 1189-1197, 2017. DOI: 10.1111/asj.12754

7. Green JC, Volkmann DH, Poock SE, McGrath MF, Ehrhardt M, Moseley AE, Lucy MC: Technical note: A rapid enzyme-linked immuno-sorbent assay blood test for pregnancy in dairy and beef cattle. *J Dairy Sci*, 92 (8): 3819-3824, 2009. DOI: 10.3168/jds.2009-2120

8. Friedrich M, Holtz W: Establishment of an ELISA for measuring bovine pregnancy-associated glycoprotein in serum or milk and its application for early pregnancy detection. *Reprod Domest Anim*, 45 (1): 142-146, 2010. DOI: 10.1111/j.1439-0531.2008.01287.x

9. Romano JE, Thompson JA, Kraemer DC, Westhusin ME, Forrest DW, Tomaszweski MA: Early pregnancy diagnosis by palpation per rectum: Influence on embryo/fetal viability in dairy cattle. *Theriogenology*, 67 (3): 486-493, 2007. DOI: 10.1016/j.theriogenology.2006.08.011

10. Silva E, Sterry RA, Kolb D, Mathialagan N, McGrath MF, Ballam JM, Fricke PM: Accuracy of a pregnancy-associated glycoprotein elısa to determine pregnancy status of lactating dairy cows twenty-seven days after timed artificial ınsemination. *J Dairy Sci*, 90 (10): 4612-4622, 2007. DOI: 10.3168/jds.2007-0276

11. Le Roux Y, Colin O, Laurent F: Proteolysis in samples of quarter milk with varying somatic cell counts. 1. Comparison of some ındicators of endogenous proteolysis in milk. *J Dairy Sci*, 78, 1289-1297, 1995. DOI: 10.3168/jds.S0022-0302(95)76749-2

12. López-Gatius F, Garbayo JM, Santolaria P, Yániz J, Ayad A, de Sousa NM, Beckers JF: Milk production correlates negatively with plasma levels of pregnancy-associated glycoprotein (PAG) during the early fetal period in high producing dairy cows with live fetuses. *Domest Anim Endocrinol*, 32 (1): 29-42, 2007. DOI: 10.1016/j.domaniend.2005.12.007

13. Friedrich M, Kalscheuer E, Hoedemaker M, Holtz W: Pregnancy detection in dairy cows by measuring pregnancy-associated glycoprotein (PAG) in milk. *Reprod Domest Anim*, 42, 70, 2007.

14. Gáspárdy A, Schwartz Z, Zöldág L, Veresegyházy T, Fekete S: Changes in daily energy amounts of main milk components (lactose, protein and fat) during the lactation of high-yielding dairy cows. *Acta Vet Hung*, 52 (4): 457-467, 2004. DOI: 10.1556/AVet.52.2004.4.8

15. Breed MW, Guard CL, White ME, Smith MC, Warnick LD: Comparison of pregnancy diagnosis in dairy cattle by use of a commercial ELISA and palpation per rectum. *J Am Vet Med Assoc*, 235 (3): 292-298, 2009. DOI: 10.2460/javma.235.3.292

16. Zoli AP, Guilbault LA, Delahaut P, Ortiz WB, Beckers JF: Radio-immunoassay of a bovine pregnancy-associated glycoprotein in serum - Its application for pregnancy diagnosis. *Biol Reprod*, 46 (1): 83-92, 1992. DOI: 10.1095/biolreprod46.1.83

17. Green JA, Parks TE, Avalle MP, Telugu BP, McLain AL, Peterson AJ, McMillan W, Mathialagan N, Hook RR, Xie S, Roberts RM: The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. *Theriogenology*, 63 (5): 1481-1503, 2005. DOI: 10.1016/j.theriogenology.2004.07.011

18. Howard J, Gabor G, Gray T, Passavant C, Ahmadzadeh A, Sasser N, Pals D, Sasser G: BioPRYN, a blood-based pregnancy test for managing breeding and pregnancy in cattle. *Proc West Sect Am Soc Anim Sci*, 58, 295-298, 2007.

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Research Article

Impact of Various Carbohydrate Sources on Functional Attributes, Colony Population, Feed Intake, and Quality of Honey Produced by the Honeybee

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Abstract

The effect of various carbohydrate sources on bee function was investigated in this study. To conduct the experiment, 12 treatments in 6 replications in a completely randomized design carried out on *(Apis mellifera meda)*. The treatments are consisted of control treatment (honey, white sugar, brown sugar) and sweet dough treatments containing corn, potato, wheat starch and corn liquid fructose with ratios of 15 and 30%.population size, feeding rate and egg, larvae and pupae amount as well as honey production and honey analysis were investigated. The results of supplying the colonies with diverse starch targeted groups show that there is a significant difference between the highest and the lowest tested treatments (P<0.05). In conclusion starch content at 15% increased the performance of the honey bee in comparison to the 30% level. Thus, along with the starch sources it is recommended to use a small amount of 1:1 sugar syrup one day in between due to performance improvement, especially in the seasons when the flow of nectar is low. Sweet dough containing 15 and 30% corn liquid fructose improves honeybee efficiency. The purpose of this study is to select the appropriate alternative for white sugar in terms of economic, ease of supply and nutritional health for honey bee.

Keywords: *Bee, Carbohydrate sources, Attributes, Starch, Sweet dough*

Çeşitli Karbonhidrat Kaynaklarının Balarılarında Fonksiyonel Nitelik, Koloni Popülasyonu, Yem Tüketimi İle Üretilen Bal Üzerine Etkisi

Öz

Bu çalışmada çeşitli karbonhidrat kaynaklarının arı fonksiyonları üzerine etkisi araştırıldı. Araştırmayı yürütmek amacıyla tamamıyla rastgele dizaynda, 6 tekrar olmak üzere 12 deneysel uygulama *Apis mellifera meda* üzerinde gerçekleştirildi. Deneysel uygulamalar; kontrol uygulaması (bal, beyaz şeker veya kahverengi şeker) ile %15 ve %30 olmak üzere mısır, patates, buğday nişastası veya mısır sıvı fruktoz içeren tatlı hamurdan oluşmaktaydı. Popülasyon büyüklüğü, besleme oranı, yumurta, larva ve pupa miktarları, bal üretimi ve bal analizi araştırıldı. Farklı nişasta ile hedeflenen kolonilerde en yüksek ve en düşük uygulamalar arasında anlamlı farkların oluştuğu belirlendi (P<0.05). Sonuç olarak %30 ile karşılaştırıldığında %15 nişasta içeriği bal arısı performansını artırdı. Bu nedenle nişasta kaynakları ile birlikte aralarında bir gün olacak şekilde düşük miktar 1:1 şeker şurubu verilmesi özellikle nektar akışının düşük olduğu sezonlarda performansı geliştirebilir. %15 ve %30 mısır sıvı fruktoz içeren tatlı hamur balarısı etkinliğini iyileştirir. Bu çalışmanın amacı ekonomik, sağlama kolaylığı ve gıda sağlığı bakımından bal arılarında kullanılabilecek beyaz şekere bir alternatif belirlemektir.

Anahtar sözcükler: *Arı, Karbonhidrat kaynakları, Nitelik, Nişasta, Tatlı hamur*

INTRODUCTION

Researchers, scientists and severely beekeepers have a significant consideration of nutrition of honeybees to solve the upcoming challenges that affect their ability to stay healthy and improve the efficiency of production [1-4]. This condition gets intense in commercial bee operations that include a diverse management style regarding the colonies movement, quality and amounts of food. An intelligent decision on how to keep a honeybee is possible only when the fundamental demands for feeding bees correctly and in perfect detail has been investigated. In these insects,

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nutrition should be considered in a completely two independent manners, since the larval period is usually different from the adult insects. However, the process of feed intake of larva and adult bee are relatively close since a matured insect should actively and gradually feed larvae [5]. Nutrition involves all the operations by which an organism converts various nutrients, minerals, water, vitamins and other substances into body parts or acquired energy for various vital processes [5].

Barker and Lehner [6] reported that 4 mg of sugars is essential for an adult honeybee worker to survive, while Rortais et al.[7] calculated one worker larva needs 59.4 mg of carbohydrates.

Pollen, produced by flowers, is the major source of protein for honeybees [8], though it does not provide energy for the bee. Pollen grain supplies all the requirements of the colonies in terms of protein that plays a vital role in the growth of the body and is essential for the restoration of tissues and other body functions. The bulk of the contents of the honey is made up of glucose, fructose and sucrose, while the additional sugars content found in nectar, have less nutritive value. Considering the incapability of honeybees to break the additional content down, the utilized percentage has toxic effect during the ingestion [9]. In an emergency sugar and carbohydrates sources feeding can be used as a supplementary material or substitutes when the colony is running short of stored honey, especially in winters. For this purpose, dense phase of material is most probably suitable for feeding to bees. Attention to the protein components of the diet has to be taken into account to increase the population numbers [10].

To store the carbohydrates as honey disaccharides, decompose into monosaccharides to prepare an appropriate form of material for cell usage. Simultaneous to this transformation, honeybee synthesizes micro-organisms and diminish the volume of water. As much the produced material gets dense, the percentage of fermentation reduced. It helps to produce a stable honey with normal content [11] Fraudulent and adulterated honey production is a problem all over the world [12]. Above all, we do not know how to distinguish adulterated honeys taken from the colonies that were [12].

Starch is the main form of storage of carbohydrates in the tubers and endosperm of the plant seeds. It is a cluster of linear polymers that in which some of the alpha chain are linked with glucose units and is stored in the molecule as energy. Starch consists of two types of carbohydrate polymers called amylose and amylopectin Biochemically [13]. Amylose is a polymeric carbohydrate consisting of a large number of glucose units joined by glycoside bonds. Amylopectin is a relatively larger molecule in comparison with amylose, which is associated with heavy branches of 95% alpha 1-4 and 5% alpha 1-6. Due to the difficulty of complete separation of natural amylose from amylopectin existing in starch sources a pure component is not available in the market, although individually, phosphorylase-catalyzed enzymatic polymerization is the appropriate method [14].

Most starch amylose has a very small amount of lipids that, along with amylose, form free lipid and fatty complexes. In between all types of starch groups, wheat has a higher level of lipid and glycolipid [15]. The composition of lipid and amylose affects starch granules, both in terms of structure [16] and performance. Starch molecules arrange themselves in the plant in semi-crystalline granules. Each plant species has a unique starch granular size. For example, corn granular forms a multidimensional shape, wheat and potato starch creates convex and ovoid shape, respectively. Potato starch with 110≥ micrometer is positioned prior than wheat 30≥ and corn 25≥ in comparing the granular diameter of the starch sources [17].

The aim of this study was to investigate the sources and levels of carbohydrates in feeding honey bee and replacing these sources with white sugar to enhance performance, increase the quality of honey as well as affordable prices, ease of supply and usability for honeybees.

MATERIAL and METHODS

The experiment was carried out in 12 treatments in 6 replicates in a completely randomized design on *Apis mellifera meda* at the Agricultural Research Center of Isfahan for a period of 6 months. (March to September 2017). In this study, the tendency of feeding honeybee from sweet dough treatments was investigated. Controlling treatments of honey, white sugar, brown sugar accompanied by sweet dough with diverse percentages have been exerted as sweet dough containing 25% honey and 75% white sugar, sweet dough containing 15% potato starch, sweet dough containing 30% potato starch, sweet dough containing 15% wheat starch, sweet dough containing 30% wheat starch, sweet dough containing 15% corn starch, sweet dough containing 30% corn starch, sweet dough containing 15% corn liquid fructose and sweet dough containing 30% corn fluid fructose *(Table 1)*. The measurable attributes of feed intake, colony population, amount of egg, larva and pupae, honey production and honey analysis with respect to the diet were evaluated by the controlling treatments.

To conduct the study, 72 colonies were selected and based on the identification of each hive a balance was established. Each hive includes 5 frame; 2 of eggs, larvae and pupae (brood), 2 honey frames and 1 empty comb.

Moreover, it was attempt to provide a similar condition with respect to the flower pollen storage. Before the experiment, homogenization was performed to minimize differences in colony count in terms of population, infant and honey stock. Thus, homogenization began with the creation of a balance between strong colonies

Table 1. *The amount of nutrients in the starch used in this study*

Nutrients	Corn Starch (%)	Wheat Starch (%)	Potato Starch (%)
Fat	0.8	1.2	0.1
Protein	0.5	0.2	0.1
Ash	0.1	0.2	0.3
P	0.08	0.05	0.09

from the point of population and larva to weak colonies and continued by adding the honeycomb to honey-lacked colonies to harmonize the conditions. Hives with sister queens were used to coordinate the apiary and finally, the colonies were randomly divided into treatments. During this 6-month period, records were taken every 21 d.

The amount of feed consumed by the bees was calculated from the weight of the sweet dough given to the hive when they were eaten during the test. The brood breeding and population growth was calculated by measuring the level of brood (eggs, larvae and pupae) by a brood chamber. Tabulated empty frame in square centimeter was placed on the surface of the brood and the number of brood chamber was counted. Therefore, the brood growth rate and development of the population were calculated in each colony per square centimeter. Since there is no possibility of extracting all the honey contained in a hive, a number of clean frame, which were previously collected from hives, were weighed and the average weight of an empty frame was obtained. Afterwards, all honey frames contained in each hive were weighed and recorded. By subtracting the weight of empty hulls, the net weight of honey was obtained for each hive. At the end of the experiment, at least 150-100 gm of produced honey in the hive is sampled from each replicate of the controlling treatments. Samples transferred to the laboratory for qualitative control (physicochemical) experiments that to determine the amount of sucrose in honey, the Ferreling experiment was used by the Sucramat apparatus and the method of Polarized light was used to determine the ratio of fructose to glucose.

Statistical Analysis

Derived data were recorded by excel software and then analyzed for variance analysis using SAS (Statistical Analysis System) software which is developed by SAS [18] Institute for advanced analytics applying GLM procedure. Applying Duncan's multiple range tests, the average was compared at a probability level of 5%. All of parameters were examined as follows:

$$Yij = \mu + Ti + eij$$

Where Yij is individual observation, μ is the overall mean, Ti is the effect of treatment, and eij shows the random error.

RESULTS

The results showed that in the control treatments (honey, white sugar, brown sugar), the highest amount was related to honey treatment and the lowest amount of feed intake was related to brown sugar treatments, which showed a significant difference ($P<0.05$) the amount of feed consumed by honey bees was lower in sweet dough compared to the control group ($P<0.05$) *(Table 2)*.

The highest amount of feed in the treatments containing sweet dough was related to sweet dough containing 15% corn liquid fructose. Among starch containing treatments, the highest consumption was found in sweet dough containing 15% corn starch and the lowest amount was for sweet dough it contains 30% of wheat starch, that these two treatments showed a significant difference ($P<0.05$) Also, among the control treatments the highest population *(Table 3)* was for brown sugar treatment and the lowest amount for honey treatment, which showed significant difference ($P<0.05$). Among the treatments, the highest amount of population was found to contain 30% corn liquid fructose, which showed a significant difference with all treatments. In total, sweet dough treatments containing 15% starch were more than 30% starch in comparison with sweet dough treatments.

The amount of brood fertility (eggs, larvae and pupae) showed the highest levels of brown sugar and the lowest amount of white sugar in the control treatments *(Table 4)*, which showed a significant difference ($P<0.05$) and sweet dough treatment containing 30% potato starch had a higher numerical value than sweet dough treatment

Table 2. *The effect of different sources of carbohydrates on the average amount of feed intake*

Controlling Treatments		Average of Feeding Rate (g)
Honey		15750^{a}
White sugar		15000^{b}
Brown sugar		14250^{c}
Sweet dough containing (%)	SD (25 honey & 75 white sugar)	3948.0^{d}
	SD (15 potato starch)	1075.0^{g}
	SD (30 potato starch)	645.0^{h}
	SD (15 wheat starch)	2037.5^{f}
	SD (30 wheat starch)	500.0^{h}
	SD (15 corn starch)	2212.5^{f}
	SD (30 corn starch)	636.0^{h}
	SD (15 corn liquid fructose)	4137.5^{d}
	SD (30 corn liquid fructose)	2750.0^{e}
(P-value)		P<0.001
SEM		2385

SEM: *Standard error of means. Footnotes (a-h) show significant differences each column (P<0.05)*

Table 3. *Effect of various carbohydrate sources on the average population of the colony*

Controlling Treatments		Average of Hive Population (per/cm²)
Honey		5880^{cd}
White sugar		6022^{cd}
Brown sugar		6777^{ab}
Sweet dough containing (%)	SD (25 honey & 75 white sugar)	6110^{c}
	SD (15 potato starch)	5666^{cde}
	SD (30 potato starch)	5347^{ef}
	SD (15 wheat starch)	5570de
	SD (30 wheat starch)	5125^{fg}
	SD (15 corn starch)	5990^{cd}
	SD (30 corn starch)	4890^{g}
	SD (15 corn liquid fructose)	6675^{b}
	SD (30 corn liquid fructose)	7120^{a}
P-value		P<0.001
SEM		86

SEM: *Standard error of means. Footnotes (a-g) show significant differences each column (P<0.05)*

Table 4. *Effects of various carbohydrate sources on the average of brood*

Controlling Treatments		Average of Brood (per/cm²)
Honey		4320^{a}
White sugar		2612^{d}
Brown sugar		4410^{a}
Sweet dough containing (%)	SD (25 honey & 75 white sugar)	4232^{a}
	SD (15 potato starch)	3210^{c}
	SD (30 potato starch)	3407^{c}
	SD (15 wheat starch)	3210^{c}
	SD (30 wheat starch)	3763^{b}
	SD (15 corn starch)	3200^{c}
	SD (30 corn starch)	3130^{c}
	SD (15 corn liquid fructose)	3382^{c}
	SD (30 corn liquid fructose)	4176^{a}
P-value		P<0.001
SEM		242

SEM: standard error of means. Footnotes (a-d) show significant differences each column (P<0.05)

containing 15% potato starch, but no significant difference was observed between the two treatments (P>0.05). Sweet dough treatments contained 30% of wheat starch compared to sweet dough containing 15% wheat starch was higher in number and there was a significant difference between two treatments (P<0.05).

The amount of honey production and its quality *(Table 5* and *Table 6)* show that the control treatments did not show any significant difference in terms of honey production

Table 5. *Effect of various carbohydrate sources on the average production of honey*

Controlling Treatments		Average of Honey Production (g)
Honey		3125^{a}
White sugar		3175^{a}
Brown sugar		3075^{a}
Sweet dough containing (%)	SD (25 honey & 75 white sugar)	1825^{b}
	SD (15 potato starch)	1450^{bc}
	SD (30 potato starch)	967^{cd}
	SD (15 wheat starch)	1335^{bcd}
	SD (30 wheat starch)	1140^{cd}
	SD (15 corn starch)	1500^{bc}
	SD (30 corn starch)	775^{d}
	SD (15 corn liquid fructose)	1600^{bc}
	SD (30 corn liquidfructose)	2750^{a}
P-value		P<0.001
SEM		380

SEM: *Standard error of means. Footnotes (a-d) show significant differences each column (P<0.05)*

(P>0.05) but the highest amount was related to white sugar treatment and sweet dough containing 30% corn liquid fructose had no significant difference with control treatments, but there was a significant difference with other sweet dough treatments (P<0.05).

The highest amount of Hydroxyl methyl furfuran (HMF) is related to sweet dough containing 30% corn liquid fructose and the lowest amount is related to brown sugar treatment, which showed a significant difference and among the control treatments the highest amount of HMF was in white sugar and the lowest in brown sugar which showed a significant difference (P<0.05). The highest amount of sucrose was found in 30% corn starch containing sweet dough and the lowest amount of honey treatment showed a significant difference (P<0.05). The ratio of fructose to glucose in sweet dough treatments 30% corn starch and 15% corn fluid fructose and 30% corn fluid fructose did not show any significant difference. Also, there was no significant difference between the control group. Regarding the presence of artificial sugar, all treatments were negative and the starch content in honey was observed only in treatments containing 15% wheat starch and 15% corn starch.

DISCUSSION

The results showed that the highest amount of feed intake is related to honey treatment and the lowest amount is for sweet dough containing 30% wheat starch. Statistical analysis showed that there is a significant difference (P<0.05) between the highest and the lowest amount of starch sources used as nutrition *(Table 2)*. Conversely, Rate

Table 6. *Analysis of honey produced by hives fed with various sources of carbohydrates*

Controlling Treatments		HMF	Starch	Fructose/ Glucose (%)	Synthetic Sugar	Sucrose (%)
Limit of allowance		< 40 (mL/kg)	Negative	> 9	Negative	< 5
Honey		3.16^{de}	Negative	1.4^{b}	Negative	2.93^{i}
White sugar		3.8^{c}	Negative	1.4^{b}	Negative	15^{a}
Brown sugar		3.1^{e}	Negative	1.39^{b}	Negative	12^{b}
Sweet dough containing (%)	SD (25 honey & 75 white sugar)	5^{b}	Negative	1.14^{c}	Negative	6.14^{h}
	SD (15 potato starch)	5.2^{b}	Negative	1.15^{c}	Negative	7.8^{f}
	SD (30 potato starch)	3.7^{cd}	Negative	0.94^{c}	Negative	9.06^{d}
	SD (15 wheat starch)	4.1^{c}	Positive	1.14^{c}	Negative	7.83^{f}
	SD (30 wheat starch)	3.9^{c}	Negative	1.15^{c}	Negative	8^{ef}
	SD (15 corn starch)	5.2^{b}	Positive	1.7^{a}	Negative	9^{d}
	SD (30 corn starch)	3.5^{cde}	Negative	1.6^{ab}	Negative	9.38^{c}
	SD (15 corn liquid fructose)	3.8^{c}	Negative	1.5^{ab}	Negative	8.06^{e}
	SD (30 corn liquid fructose)	13.5^{a}	Negative	1.5^{ab}	Negative	7.5^{g}
(P-value)		P<0.001		P<0.001		P<0.001
SEM		1.71		0.10		1.13

SEM: *standard error of means. Footnotes (a-i) show significant differences each column (P<0.05)*

of feeding 30% potato starch with 30% corn starch and 30% wheat starch treatment did not differ significantly (P>0.05). Amid the sweet dough containing starch, the highest feed intake of it was for 15% corn starch that did not show any significant difference with 15% wheat starch (P>0.05). However, there was a significant difference with sweet dough containing 15% potato starch that estimated to be due to starch diameter size. Respectively, Potato starch, wheat and corn are placed in the next position. As Cornell and Wallace [19] and later Kim and Huber [20] reported that digestion of various starches depend on the structure and the shape. Taking into consideration, the botanical origin plays an important role to form starch of various sources as oval, rounded and truncated with diverse range of diameter.

In comparison with other treatments, consumption of white sugar was higher subsequently than honey treatment, which is corresponded with the results of Ruiz-Matute et al.[21] and Weis [22] indicating the amount of white sugar was higher than the rest of the treatments. Of the total sugars consumed by bees, sucrose is most important in terms of acceptance and nutritional value demonstrating by Vogel et al.[23]; furthermore, it is in line with the consequences of this study. In control treatments, the highest feed intake was related to honey, white sugar and brown sugar, respectively which showed a significant difference (P<0.05). Also, sweet dough containing 15% fructose after control group had the highest consumption. In addition, it is consistent with outcomes of Barker and Lehner [6], the consumption of corn syrup containing fructose was utilized further by bees. This rate is due to the similarity of sugars inside the corn liquid fructose with honey.

In this research, the highest population size was for sweet dough contains 30% corn liquid fructose while the lowest amount obtained for the treatment containing 30% corn starch *(Table 3)*. The evaluation of the two treatments showed a significant difference. LeBlanc et al.[24] revealed the result of their study as corn fructose has no toxic effect on the bee and increases the population that is one of the substantial resultants of this research.

Long-term accumulation at high temperature and abundance of HMF shift the condition in which the corn fluid fructose can initiate toxicity and reduce the life span of the bee [21]. The highest population of starchy treatments is related to the sweet dough comprising 15% corn starch. To specify the correlation of this trail with sweet dough treatments contain 30% potato, 30% wheat and 30% corn starch (P<0.05). This is because corn starch possesses more proteins than other starches [25]. Haydak [26] reported that protein is undoubtedly a major contributor to the feeding of larvae and adult bees and expand the lifetime of the bees.

Considering the observations the population declined through supplying the feed by sweet dough containing 30% starch rather than 15%. It addresses that reducing the population is directly related to the amount of food consumed. Furthermore, Burenside and Vvansell [27] expressed objectivity of a direct link between the amount of sugar intake and the life of the bees while the relevance of low sugar reception with death was not yet determined. They presented that there is no clue for the conclusion of the exact reason of death concerning the toxicity due to the consumption of some sugars or low sugar reception.

The lowest colony population was found in sweet dough treatments containing 30% corn starch and 30% wheat starch due to high level of amylose [28]. Therefore, this factor decreases the digestibility and the same reduced the population size.

Among the control treatments, the highest amount of the population was for brown sugar, while the lowest amount was for honey and white sugar respectively (P<0.05).

Brouwers [29] in a study of the effect of glucose and fructose levels on bee honey diet on larval stages showed that glucose is the main source of glucose in the larval diet, as well as in all stages of the larvae of the bees The worker and the male are glucose, and fructose is a major source of growth in the next stages of larvae. Therefore, the presence of these sugars in honey and sugar can increase the population of bees' population that matches the results.

In this research, the maximum number of egg, larvae and pupae produced by feeding the colonies with treatment of the brown sugar. This is probably due to molasses in brown sugar containing proteins, energy and minerals, which can increase larvae and pupae. Also, all treatments containing sweet dough and control group with white sugar had a significant difference (P<0.05).This study was inconsistent with the results of Sammataro and Weiss et al.[30], which states that the use of sucrose syrup rather than corn liquid fructose treatment increases egg and larvae.

Also, no significant difference was observed between controlling treatments of (honey, brown sugar) and sweet dough containing 30% corn fluid fructose *(Table 4)* but with other treatments there was a significant difference between sweet dough containing starches.

According to observations, utility of sweet dough treatments containing 15 and 30% starch showed better spawning than white sugar. Correlation of the two treatments was over 0.05 resulting to a significant difference. Likewise, the same relevance authenticated between sweet dough containing 30 and 15% liquid fructose and White sugar. This research was consistent with the results of Sammataro and Weiss [30] that the use of sucrose syrup does not increase the egg and larvae in comparison with corn-fructose treatment. Similarly, the results are consistent with Schneider and Blewis [31], which showed that starch, increases the population and health of the colony. Adaptation of the consequences of this study with Woodring et al.[32] was zero, as they reported that sucrose increases the spawning rate. Probably the reason for the decrease in egg, larvae and pupae in white sugar treatment compared to the rest of the treatments is because of some protein and minerals component of starch treatments [25] that nursing bees need them, especially protein materials, to more care the brood and handle the condition of the egg, larvae and pupae.

Christy et al.[33] found in the comprehensive study of more than 20 types of sugar in honey, many of which are not found in nectar, and are produced by the honey enzymes and acids in the process of getting honey and in the reservoir. Simple sugars, dextrose volvulus, are the dominant sugar content of honey and have the property of absorbing moisture and energy production for honeybees, and since honey is rich in other nutrients and can provide all the needs of the brood and increase the emerged rate and increasing the generation that is being matched by research.

The results showed that the highest amount of honey production was related to white sugar treatment and the lowest amount was for sweet dough containing 30% corn starch. Investigating the relationship between these two treatments demonstrated a significant difference at 5% level. Woodring et al.[32] reported that consumption of sucrose increased honey. Also there was no significant difference between white sugar, honey and brown sugar as the control group with sweet dough treatments containing 30% liquid fructose. Likewise, sweet dough containing 15% corn liquid fructose, corn starch, potato starch and wheat starch did not show any significant difference (P>0.05).

White sugar treatments had the highest amount of honey production than all treatments, and no significant difference was observed between sweet dough containing 30% corn liquid fructose with control group (P>0.05). In total sweet dough products containing starch were 15% higher than the 30% level.

Following supplementary studies, a direct relation between the rate of honey production and population was obtained [26]. Factors that increase population concurrent raise the number of nectars in the hives. No significant differences were observed between the control treatments (white sugar, honey, brown sugar), while the control treatments with the remaining showed a significant difference at 5% level. In the observations, sweet dough treatments containing 15% starch had more population, compared to sweet dough containing 30% starch that collected more honey *(Table 5)*.

In the analysis of honey *(Table 6)*, the highest amount of sucrose was related to white sugar with a rate of 15% and the lowest was for honey treatment, which showed a significant difference. Also, the result of sweet dough containing 15% of potato starch was significantly different from sweet dough containing 30% of potato starch. However, sweet dough containing 15% of wheat starch and sweet dough containing 30% of wheat starch did not show any significant difference and the amount of sucrose in white sugar treatment is higher than sweet dough containing corn liquid fructose that is consistent with the studies that were reported by Guler et al.[34], the amount of sucrose in white sugar treatment was higher than in corn liquid fructose treatment and had a significant difference

(P<0.05). In the observations, the highest amount of HMF was observed in sweet dough containing 30% corn fluid fructose and the lowest amount was for brown sugar. There was a significant difference between white sugar, honey and brown sugar as a control controlling treatments. HMF levels in sweet dough containing 15% corn liquid fructose and white sugar were the same which was consistent with studies by Guler et al.[34] that there was no significant difference between white sugar treatment and corn liquid fructose treatment (P>0.05). The highest amount of (HMF) observed in sweet dough contains 30% corn liquid fructose. Weiss et al.[22] and Ruiz-Matute et al.[21] reported that corn liquid fructose syrup would increase the amount of HMF in the production process if not maintained in proper condition.

The ratio of fructose to glucose in sweet dough containing 15% corn starch was highest and the lowest value was for sweet dough containing 30% of potato starch, which showed a significant difference. White sugar, brown sugar and honey did not differ significantly (P>0.05) but there was a significant difference between them and sweet dough (P<0.05). The ratio of fructose to glucose in sweet dough containing 30% potato starch was lower than all treatments, which probably made it easier for bees to alter potato starch to become glucose. Gomand et al.[28] reported that the highest level of amylose is related to wheat starch and the least associated with potato starch, and the degree of amylose and amylopectin polymerization of potato starch is higher than the rest of the starch sources. Fructose-Glucose ratio did not show significant difference in sweet dough composition containing corn liquid fructose with white sugar which is inconsistent with the results of Guler et al.[34].

No synthetic sugar was observed in any of the treatments, and starch content of honey in two treatments of sweet dough containing 15% wheat starch and sweet dough containing 15% corn starch were positive. This is probably due to the higher consumption of these treatments compared to sweet dough treatments containing 30% starch. Gomand et al.[35] reported that amylose content of wheat and corn starch was more than potatoes, as well as the number of amylose in corn and wheat starch that is more than potato. The solubility of potato starch is most. The total of factors in terms of the status of amylose and amylopectin, as mentioned above, are effective in digestibility of bees. Due to the high consumption of sweet dough containing 15% corn starch and sweet dough containing 15% wheat starch along with the low digestibility of bees, because of the high level of amylose, starch cannot break down in digestion system of the honeybee and thus enters into honey.

In conclusion, the nutritional effects of a food must be tested in different ways. Only accepting and consuming that substance by honey bees cannot be a precise criterion for judging its quality. In addition to its intake, other factors such as the effect of that substance on colony population, brood population and production honey should be investigated. Starch sources can be used in feeding bees, but the research showed that due to the different starch structure, the effect of each starch source on the performance of honey bees is different, and the level of 15% of starch in the total yield of honey bees compared to level increased by 30%. In this study, corn liquid fructose, considering the economic cost of white sugar, as well as increased yield, could be a good alternative to sugar and it is also recommended that starchy sweet potatoes can be used in the treatment of 15% starch content because with increasing starch content, we are faced with a decrease in bee function such as population decline and honey production.

In this research, other studies, such as heating, and the amount and duration of heating of various starch sources, can be studied, and their impact on consumption and performance increase.

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REFERENCES

1. Keller I, Fluri P, Imdorf A: Pollen nutrition and colony development in honey bees: Part 1. *Bee World*, 86 (1): 3-10, 2005. DOI: 10.1080/0005772X.2005.11099641

2. Toth AL, Robinson GE: Worker nutrition and division of labor in honeybees. *Anim Behav*, 69 (2): 427-435, 2005. DOI: 10.1016/j.anbehav.2004.03.017

3. Alaux C, Ducloz F, Crauser D, Le Conte Y: Diet effects on honeybee immune competence. *Biol Lett*, 6 (4): 562-565, 2010. DOI: 10.1098/rsbl.2009.0986

4. Brodschneider R, Crailsheim K: Nutrition and health in honey bees. *Apidology*, 41 (3): 278-294, 2010. DOI: 10.1051/apido/2010012

5. Hrassnigg N, Crailsheim K: Differences in drone and worker Physiology in honeybees *(Apis mellifera L.)*. *Apidology*, 36 (2): 255-277, 2005. DOI: 10.1051/apido:2005015

6. Barker RJ, Lehner Y: Acceptance and sustenance value of naturally occurring sugars fed to newly emerged adult workers of honey bees *(Apis mellifera L.)*. *J Exp Zool*, 187 (2): 277-285, 1974. DOI: 10.1002/jez.1401870211

7. Rortais A, Arnold G, Halm MP, Touffet-Briens F: Modes of honeybee's exposure to systemic insecticides: Estimated amounts of contaminated pollen and nectar consumed by different categories of bees. *Apidology*, 36 (1): 71-83, 2005. DOI: 10.1051/apido:2004071

8. Roulston TH, Cane JH: Pollen nutritional content and digestibility for animals. *Plant Syst Evol*, 222, 187-209, 2000. DOI: 10.1007/BF00984102

9. Johansson TSK, Johansson MP: Feeding sugar to bees. *Bee World,* 58 (2): 137-143, 1976.

10. Hendriksma HP, Shafir S: Honey bee foragers balance colony nutritional deficiencies. *Behav Ecol Sociobiol*, 70 (4): 509-517, 2016. DOI: 10.1007/s00265-016-2067-5

11. Hendriksma HP, Oxman KL, Shafir S: Amino acid and carbohydrate tradeoffs by honey bee nectar foragers and their implications for plant-

pollinator interactions. *J Insect Physiol,* 69, 56-64, 2014. DOI: 10.1016/j.jinsphys.2014.05.025

12. Önder H, Güler A, Biyik S, Ekinci D, Garipoğlu AV, Kocaokutgen H: Origin estimation of honey samples by using constant and discriminative function coefficients of pure honey and honey produced by colonies feeding with different sugars. *Kafkas Univ Vet Fak Derg,* 22, 21-28, 2016. DOI: 10.9775/kvfd.2015.13586

13. Kadokawa JI: Preparation and applications of amylose supramolecular by means of phosphorylase-catalyzed enzymatic polymerization. *Polymers*, 4, 116-133, 2012. DOI:10.3390/polym4010116

14. Kitamura S: Starch, polymers, natural and synthetic. **In,** The Polymeric Materials Encyclopedia, Synthesis Properties and Applications. 7915-7922. New York, NY, USA, CRC Press, 1996.

15. Larsen NG: Glycolipid analysis in wheat grains. **In**, Modern Methods of Plant Analysis. 214-261. Seed Analysis, 1992.

16. Ao Z, Jane JL: Characterization and modelling of the A- and B-granule starches of wheat, triticale, and barley. *Carbohydr Polym*, 67 (1-2): 46-55, 2007. DOI: 10.1016/j.carbpol.2006.04.013

17. Wilson JD, Bechtel DB, Todd TC, Seib PA: Measurement of wheat starch granule size distribution using image analysis and laser diffraction technology. *Cereal Chem*, 83 (3): 259-268, 2006. DOI: 10.1094/CC-83-0259

18. SAS Institue: SAS SQL Procedure User Guide, Version 9. SAS, Cary, ISBN: 13:978-158025599X, 576. 2000.

19. Cornell H: Starch in food: Structure, function and applications. *J Starch,* 57 (3-4): 211-214, 2004.

20. Kim HS, Huber KC: Channels within soft wheat starch A- and B-type granules. *J Cereal Sci*, 48 (1): 159-172, 2008. DOI: 10.1016/j.jcs.2007.09.002

21. Ruiz-Matute AI, Weiss M, Sammataro D, Fnely J, Sanz ML: Carbohydrate composition of high-fructose corn syrups (HFCS) used for bee feeding: Effect on honey composition. *J Agric Food Chem*, 58 (12): 7317-7322, 2010. DOI: 10.1021/jf100758x

22. Weiss M: Supplemental carbohydrates in apiculture: effects upon honey bee *(Apis mellifera L.)* health and productivity, M.S. Thesis, Department of Entomology, University of Arizona, Tucson, 2009.

23. Vogel B: Uber die beziehung switchmen sussgesch mark and honey biene. *Z Vergl Phisip,* 14, 273-347, 2000.

24. LeBlanc BW, Eggleston G, Sammataro D, Cornett C, Dufault R, Deeby T, Cyr ES: Formation of hydroxymethylfurfural in domestic high-fructose corn syrup and its toxicity to the honey bee *(Apis mellifera)*. *J Agric Food Chem*, 57 (16): 7369-7376, 2009. DOI: 10.1021/jf9014526

25. Waterschoot J, Gomand SV, Ellen Fierens, Jan A. Delcour JA: Laboratory of Food Chemistry and Biochemistry. *Leuven Food Science and Nutrition Research Centre,* 66, 1-16, 2014.

26. Haydak MH. Honey bee nutrition. *Annu Rev Entomol*; 15, 143-156, 1970. DOI: 10.1146/annurev.en.15.010170.001043

27. Burenside CE, Vvansell GH: Plant poisoning of bee. U.S. Department of Agriculture, Bureau of Entomology and Plant Quarantine, No. E–398, 1936.

28. Gomand SV, Verwimp T, Goesaert H, Delcour JA: Structural and physicochemical characterization of rye starch. *Carbohydr Res*, 346 (17): 2727-2735, 2011. DOI: 10.1016/j.carres.2011.09.024

29. Brouwers EVM: The utilization of carohydrates as food by honey bee. *J Apis Res*, 23 (3): 94-101, 1984.

30. Sammataro D, Weiss M: Comparison of productivity of colonies of honey bees, *Apis mellifera*, supplemented with sucrose or high fructose corn syrup. *J Insect Sci*, 13, 1-13, 2013. DOI: 10.1673/031.013.1901

31. Schneider SS, Blewis LA: The vibration signal modulatory communication and the organization of labor in honey bees, *Apis mellifera*. *Apidology*, 35, 117-131,2004. DOI: 10.1051/apido:2004006

32. Woodring J, Boulden M, Das S, Gade G: Studies on blood sugar homeostasia in the honey bee *(Apis mellifera L.)*. *J Insect Physiol*, 39 (1): 89-97, 1993. DOI: 10.1016/0022-1910(93)90022-J

33. Manyi-Loh CE, Ndip RN, Clarke AM: Volatile compounds in honey: A review on their involvement in aroma,botanical origin determination and potential biomedical activities. *Int J Mol Sci,* 12 (12): 9514-9532. 2011. DOI: 10.3390/ijms12129514

34. Guler A, Garipoglu AV, Onder H, Biyik S, Kocaokutgen H, Ekinci D: Comparing biochemical properties of pure and adulterated honeys produced by feeding honeybees (*Apis mellifera L.*) colonies with different levels of industrial commercial sugars. *Kafkas Univ Vet Fak Derg*, 23 (2): 259-268, 2017. DOI: 10.9775/kvfd.2016.16373

35. Gomand SV, Lamberts L, Derde LJ, Goesaert H, Vandeputte GE, Goderis B, Visser RGF, Delcour JA: Structural properties and gelatinization characteristics of potato and cassava starches and mutants thereof. *Food Hydrocoll*, 24 (4): 307-317, 2010. DOI: 10.1016/j.foodhyd.2009.10.008

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Research Article

Effect of Grape Seed Extract on the Oxidative and Proliferative Status of Porcine Intestinal Epithelial Cells

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Abstract

The aim of this study was to investigate the oxidative and proliferative effects of grape seed extract (GSE). Piglet intestinal epithelial cells (IPEC1) were selected as an unstressed cell model, or they were exposed to 400 μM H_2O_2 to establish a H_2O_2-stimulated cell model. The glutathione (GSH) and total antioxidant capacity in response to GSE addition were tested in the unstressed and H2O2-stimulated cell models. The relative mRNA levels of antioxidant or antioxidant enzymes and apoptosis-related genes were measured by Real-Time RT-PCR. In the unstressed status, the cell survival ratio and GSH increased with the addition of GSE at 1 and 10 μg/mL but diminished at 60 μg GSE/mL. The addition of 1 μg/mL GSE upregulated the mRNA expression levels of B-Cell Lymphoma protein-2 (Bcl-2), cysteine aspartases-3 (Caspase-3), cysteine aspartases-8 (Caspase-8) and glutathione peroxidase-1(GPx-1), while it downregulated that of Bcl2-associated X protein (Bax), copper-zinc superoxide dismutase, glutathione S-transferase (GST), thioredoxin, thioltransferase and thioredoxin reductase. As GSE reached 60 μg/mL, the tumor protein p53 (p53) and caspase-8 gene expressions were upregulated. In stressed status, 1 and 10 μg GSE/mL promoted the increase of GSH. H2O2-induced increases in Bax, p53, and Caspase-3 mRNA expressions were attenuated by the subsequent addition of 1 μg GSE/mL and promoted the gene expression of tumor necrosis factor-α, GPx-1 and thioltransferase (Ttas). Treatment with 60 μg GSE/ml resulted in a significant reduction in Bax, p53, manganese superoxide dismutase and GST mRNA expressions. These results indicate that GSE exhibits antioxidative and proliferative functions on unstressed IPEC1 cells at low and medium levels and oxidative and antiproliferative functions at high levels.

Keywords: *Grape seed extract, Antioxidation, Proliferation, Intestinal epithelial cells*

Üzüm Çekirdeği Ekstraktının Domuz Barsak Epitel Hücreleri Üzerine Oksidatif ve Proliferatif Etkileri

Öz

Bu çalışmanın amacı üzüm çekirdeği ekstraktının (ÜÇE) oksidatif ve proliferatif etkilerini araştırmaktır. Domuz barsak epitel hücreleri (IPEC1) strese maruz kalmamış veya 400 μM H_2O_2 uygulanarak H_2O_2 ile uyarılmış modeller olarak kullanıldı. ÜÇE ilavesinde strese maruz kalmayan ve H_2O_2 ile uyarılmış modellerde glutatyon (GSH) ve total antioksidan kapasite tespit edildi. Antioksidanlar veya antioksidan enzimler ile apoptozisle ilişkili genlerin orantısal mRNA seviyesi gerçek zamanlı RT-PCR ile ölçüldü. Strese maruz kalmayanlarda hücre hayatta kalma oranı ve GSH seviyesi 1 ve 10 μg/mL miktarlarında ÜÇE ilavesi ile artarken 60 μg/mL ile azalma gösterdi. 1 μg/mL ÜÇE ilavesi; B-hücre Lenfoma protein-2 (Bcl-2), sistein aspartaz-3 (Kaspaz-3), sistein aspartaz-8 (Kaspaz-8) ve glutatyon peroksidaz-1 (GPx-1) mRNA ekspresyonlarında upregulasyona neden olurken Bcl2-alakalı X protein (Bax), Bakır-çinko süperoksit dismutaz, glutatyon S-transferaz (GST), thioredoksin, thioltransferaz ve thioredoksin reduktaz mRNA ekspresyonlarında downregulasyona neden oldu. 60 μg/mL ÜÇE; tümör protein p53 (p53) ve kaspaz-8 gen ekspresyonunda upregulasyona yol açtı. Strese maruz kalınmada 1 ve 10 μg/mL miktarlarında ÜÇE; GSH miktarındaki artmayı destekledi. H2O2 ile oluşturulan Bax, p53 ve kaspaz-3 mRNA ekspresyonlarındaki artmalar 1 μg/mL ÜÇE ilavesi ile düşürülürken tümör nekroz faktör-α, GPx-1 ve thioltransferaz (Ttas) gen ekspresyonları arttı. 60 μg/mL ÜÇE uygulaması Bax, p53, manganez süperoksit dismutaz ve GST mRNA ekspresyonlarında anlamlı oranda azalmaya neden oldu. Bu sonuçlar; strese maruz kalmamış olan IPEC1 hücrelerinde düşük ve orta seviyede dozlarda ÜÇE uygulamasının antioksidatif ve proliferatif, yüksek dozda ise oksidatif ve antiproliferatif etkileri olduğunu göstermektedir.

Anahtar sözcükler: *Üzüm çekirdeği ekstraktı, Antioksidasyon, Proliferasyon, Bağırsak epitel hücreleri*

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INTRODUCTION

Grape seed extract (GSE) comprises diverse types of bioactive phenolic substances, such as anthocyanin, flavanol and resveratrol, and can effectively scavenge the reactive oxidative species (ROS) of mammalian cells [1]. Moreover, relevant evidence suggests that a series of enzymes are altered at the gene and/or protein levels in response to GSE in certain cells; this alteration brings about a wider interest in how GSE removes excessive ROS in organic systems [1].

In practical applications, GSE has been used extensively to alleviate damage from harmful chemical substances and fatty foods, particularly in terms of antioxidation and antiapoptosis. The exact mechanisms of the damaging effects of a wide range of chemical substances, such as cisplatin, cadmium, doxorubicin and cyclosporine A, as well as high-fat diets, are not fully understood; however, the formation of free radicals, which lead to oxidative stress, is primarily responsible, and the generation of ROS, depletion of glutathione (GSH), inhibition of antioxidant enzyme activity and lipid peroxidation are common signs of these damaging processes [2,3]. The generation of massive ROS is a strong signal of the initiation and stimulation of cell apoptosis, further inducing serious damage, whereas the experimental co-administration of GSE successfully counteracts the apoptotic effect, with a significant decrease in the apoptotic index and improvement in antioxidant capacity [4,5].

Grape seed extract, however, shows oxidative and apoptotic effects in some cases. GSE has been widely utilized to deter the growth of diverse types of cancers, such as breast cancer [6] and liver cancer [7], primarily by facilitating apoptosis in these cancer cells. Although the mechanisms by which GSE causes apoptosis are still controversial, increasing evidence points to the excessive generation of ROS that is induced by GSE. Some studies have found that GSE-mediated apoptosis is remarkably reversed by antioxidants.

Therefore, the objective of this study was to investigate the effect of different concentrations of GSE on the *in vitro* oxidative and proliferative status of porcine intestinal epithelial cells under unstressed and H_2O_2-stressed conditions.

MATERIAL and METHODS

Materials

Commercially available, dried and powdered GSE obtained from Tarac Technologies (Nuriootpa, South Australia) contained 5.01% (+)-catechin, 4.78% (2)-epicatechin, 2.35% (2)-epigallo-catechin, 14.1% dimericproanthocyanidin, 11.60% trimericproanthocyanidins, 7.69% tetramericproanthocyanidins and 40.0% polymeric proanthocyanidins.

Cell Culture and Treatment

Piglet intestinal epithelial cells were kindly provided by Dr. Bi-e Tan from the Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China. Cells were grown in DMEM/F-12 (1:1) medium supplemented with 5% fetal calf serum, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium, 5 ng/mL epidermal growth factor and 1% penicillin-streptomycin under standard culture conditions. The growth medium was changed every other day until nearly 80% confluence was reached. Subsequently, the cells were harvested by treatment with 0.25% trypsin and 0.53 mM EDTA. Without a specific indication, IPECs were used between passages 42 and 48 and starved of FBS for 24 h before any experimental treatment. For all studies, GSE was dissolved in dimethyl sulfoxide (DMSO) as a 200 mg/mL stock solution and diluted as desired directly in the medium.

To characterize the effect of GSE on IPEC under unstressed and stressed conditions, two cell models were prepared as follows: one group of cells was separately cultured in the medium with 1, 10 and 60 µg GSE/mL for 24 h as the unstressed cell model, and the other group of cells was subjected to oxidative stress insult through incubation with 400 µM H_2O_2 for 1 h before being treated with GSE, as with the former group. Notably, after being incubated for 1 h with 400 µM H_2O_2, the stressed cells were washed twice with PBS to remove the H_2O_2 to avoid a reaction between the H_2O_2 and the GSE. Untreated cells that served as the control were also run in parallel and subjected to the same changes in the medium but were not supplemented with either GSE or H_2O_2.

Cell Survival Ratio Test

After treatment, the cells were washed twice with PBS to eliminate interference in the subsequent assay and were seeded in a 96-well plate (1×10^4 cells/well). The cell survival ratio was assessed by using a CCK-8 commercial kit (Dojindo, China) according to the manufacturer's instructions.

Total Antioxidant Capacity and GSH Measurement

The total antioxidant capacity (TAOC) was measured using the azino-diethyl-benzthiazoline sulfate (ABTS) method. In this assay, the incubation of ABTS with H_2O_2 and a peroxidase (metmyoglobin) resulted in the production of the blue-green radical cation $ABTS^+$, whereas the antioxidants in the tested samples suppressed the production of this color, which was proportional to their concentrations [8]. The system was standardized using Trolox, a water-soluble vitamin E analog. The results were expressed as mmol Trolox equivalents/protein concentration of plasma supernatants of cells lysed [9]. The measurement of GSH was based on a Glutathione Quantification Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

RNA Isolation and Real-Time RT-PCR

These studies were performed as previously described [10] *(Table 1)*.

Statistical Analysis

Data are presented as the mean ± SEM for analysis. Statistical differences were analyzed by one-way ANOVA or Mann-Whitney U tests, as appropriate. $P<0.05$ was considered to indicate statistically significant differences [11].

Table 1. *Oligonucleotide primer sequences for the real-time PCR reactions*

Target Gene	Orientation	Primer (5′-3′)
Catalase	Forward	CAGCTTTAGTGCTCCCGAAC
	Reverse	AGATGACCCGCAATGTTCTC
Mn-SOD	Forward	TGCAGGCCCTCACTTTAATC
	Reverse	CTGCCCAAGTCATCTGGTTT
CuZn-SOD	Forward	CAGGTCCTCACTTCAATCC
	Reverse	CCAAACGACTTCCASCAT
GPx1	Forward	TGGGGAGATCCTGAATTG
	Reverse	GATAAACTTGGGGTCGGT
GPx2	Forward	GACATCAAGCGCCTCCTC
	Reverse	AGACCAGAAAGGCAAGGTTC
GPx4	Forward	GATTCTGGCCTTCCCTTGC
	Reverse	TCCCCTTGGGCTGGACTTT
GST	Forward	TTTTTGCCAACCCAGAAGAC
	Reverse	GGGGTGTCAAATACGCAATC
Trx	Forward	GCTGCCAAGATGGTGAAGCAGATT
	Reverse	GCAACATCCTGACAGTCATCCACA
TR	Forward	GCTTTGGAGTGCGCTGGATTTCTT
	Reverse	CGTGAAAGCCCACAACACGTTCAT
Ttas	Forward	CCTGTCAGCATGGCTCAAGCATTT
	Reverse	ATCCACCAGGAAGCGCTGTCATTA
Bax	Forward	CTACTTTGCCAGTAAACTGG
	Reverse	TCCCAAAGTAGGAGAGGA
Bcl-2	Forward	GGAGCTGGTGGTTGACTTTC
	Reverse	CTAGGTGGTCATTCAGGTAAG
TNF-α	Forward	CGTTGTAGCCAATGTCA
	Reverse	TAGGAGACGGCGATGC
p53	Forward	GTCACGAACTGGCTGGATG
	Reverse	GAAGGGACAAAGGACGACAG
Caspase-3	Forward	TCTAACTGGCAAACCCAAACTT
	Reverse	AGTCCCACTGTCCGTCTCAAT
Caspase-8	Forward	TCCCAGGATTTGCCTC
	Reverse	AAGCCAGGTCATCACTGTC
β-Actin	Forward	CTGCGGCATCCACGAAACT
	Reverse	AGGGCCGTGATCTCCTTCTG

RESULTS

Cell survival ratios are presented in *Fig. 1*. Cells were actively growing and had the greatest proportions of viable cells under the unstressed status with the addition of 1 and 10 μg GSE/mL. However, instead of continuing this growing trend, the addition of 60 μg GSE/mL notably inhibited ($P<0.01$) the cell survival ratio compared to the control. The treatment with H_2O_2 resulted in marked decreases ($P<0.01$) in cell survival ratios in comparison with the control, and the low cell survival ratio rebounded after the addition of 1 and 10 μg GSE/mL, even though this ratio decreased with the addition of 60 μg/mL GSE.

Cell anti/oxidant status was evaluated by TAOC assay. As shown in *Fig. 2*, the TAOC numerically increased after cells were incubated with 1 or 10 μg GSE/mL under the unstressed status, but this increase did not reach statistical significance ($P>0.05$). In contrast, the TAOC level was found to decrease to less ($P<0.05$) than half that of the control after the 60 μg GSE/mL treatment. Exposure to H_2O_2 was associated with a nonsignificant decline in the TAOC. The subsequent addition of GSE, regardless of the doses applied in our study, also did not show a significant difference ($P>0.05$) in TAOC, although a marginal elevation was shown at 10 μg GSE/mL compared to the control.

In the unstressed cells, GSH increased with GSE doses from 0 to 10 μg/mL, as shown in *Fig. 3*, but was followed by a pronounced depletion ($P<0.01$) of GSH, which was approximately 30% of that in the control cells. The level of GSH in the H_2O_2-treated IPEC1 was also less than the control, but this difference was unremarkable. Following treatment with 1 and 10 μg GSE/mL, the intracellular GSH level was notably enhanced ($P<0.05$) compared with the H_2O_2-treated IPEC1, but little or no effect on GSH level was observed when GSE was added at 60 μg/mL.

The alterations in the gene expression related to anti-oxidation and apoptosis in response to GSE addition in

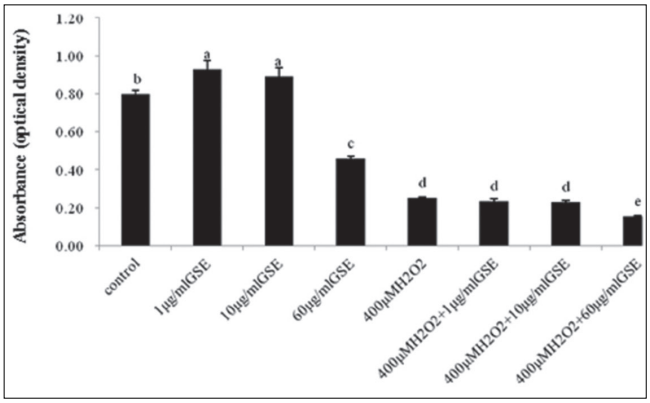

Fig 1. Alteration of the cell survival ratio after IPEC1 cells were incubated with different grape seed extract (GSE) concentrations for 24 h with or without H_2O_2 pretreatment. Bars represented mean ± SEM (n = 6). Bars not sharing a common letter differ *(P*<0.05)

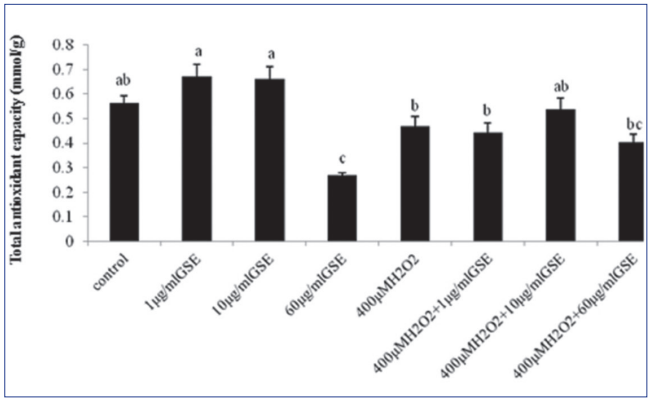

Fig 2. Alteration of total antioxidant capacity (TAOC) after IPEC1 cells were incubated with different grape seed extract (GSE) concentrations for 24 h with or without H_2O_2 pretreatment. Bars represented mean ± SEM (n = 3). Bars not sharing a common letter differ (*P*<0.05)

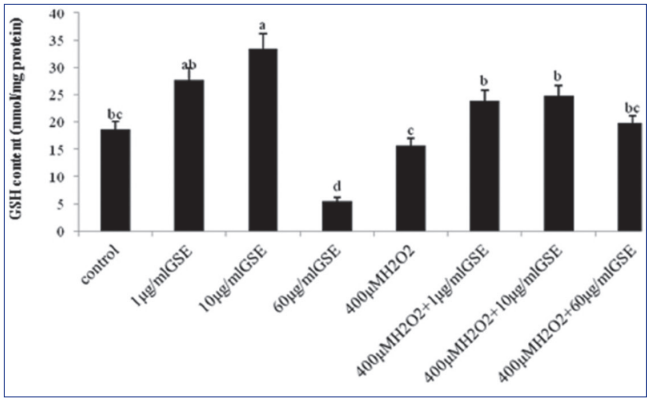

Fig 3. Alteration of glutathione (GSH) after IPEC1 cells were incubated with different grape seed extract (GSE) concentrations for 24 h with or without H_2O_2 pretreatment. Bars represented mean ± SEM (n = 3). Bars not sharing a common letter differ (*P*<0.05)

Table 2. *Effects of grape seed extract (GSE) (μg GSE/mL) on the mRNA expression of antioxidant or antioxidant enzymes in IPEC1 pretreated with or without H_2O_2*

Item	Control	Unstressed IEPC1			400 μM H_2O_2-stressed IPEC1				SEM	*P* Value
		1	10	60	H_2O_2-stressed	1	10	60		
Catalase	1.01^{ab}	0.83^{bc}	1.25^{a}	0.70^{bcd}	0.46^{cd}	0.34^{d}	0.58^{cd}	0.50^{cd}	0.11	<0.005
GuZn-SOD	1.01^{a}	0.37^{bc}	0.57^{b}	0.21^{c}	0.15^{c}	0.26^{c}	0.45^{bc}	0.21^{c}	0.07	<0.001
Mn-SOD	1.02^{b}	0.87^{b}	0.84^{b}	0.40^{c}	1.44^{a}	1.69^{a}	0.92^{b}	0.43^{c}	0.37	<0.005
GPx-1	1.04^{b}	1.60^{a}	1.33^{ab}	0.32^{c}	0.59^{c}	1.60^{a}	0.89^{bc}	0.48^{c}	0.20	<0.005
GPx-2	1.07^{b}	0.70^{b}	0.97^{b}	0.86^{b}	1.11^{b}	1.58^{ab}	2.02^{a}	1.30^{ab}	0.26	<0.005
GPx-4	1.00^{a}	1.20^{a}	0.91^{a}	0.37^{b}	0.35^{b}	0.44^{b}	0.93^{a}	0.37^{b}	0.14	<0.005
GST	1.01^{a}	0.36^{c}	0.68^{ab}	0.47^{c}	0.66^{ab}	0.34^{c}	0.41^{c}	0.47^{c}	0.11	<0.005
Trx	1.01^{a}	0.42^{b}	0.20^{c}	0.40^{b}	0.13^{c}	0.23^{bc}	0.10^{c}	0.23^{bc}	0.05	<0.001
Ttas	1.03^{a}	0.36^{bc}	0.26^{c}	0.15^{c}	0.16^{c}	0.63^{b}	0.13^{c}	0.18^{c}	0.07	<0.001
TR	1.00^{b}	0.73^{c}	0.51^{cd}	0.24^{d}	1.22^{b}	1.10^{b}	0.40^{d}	1.68^{a}	0.10	<0.005

$^{a-d}$ Means within a row with different superscripts differ (P<0.05)

Table 3. *Effects of grape seed extract (GSE) (μg GSE/mL) on the mRNA expression of apoptosis-related genes in IPEC1 with or without H_2O_2 pretreatment*

Item[1]	Control	Unstressed IEPC1			400 μM H_2O_2-stressed IPEC1				SEM	*P* Value
		1	10	60	H_2O_2-stressed	1	10	60		
Bax	1.01^{b}	0.59^{c}	0.23^{d}	0.58^{c}	2.65^{a}	0.79^{bc}	0.11^{d}	0.17^{d}	0.24	<0.001
Bcl-2	1.03^{c}	2.73^{a}	1.88^{b}	0.70^{c}	1.42^{bc}	2.38^{ab}	2.10^{ab}	1.34^{bc}	0.43	<0.005
p53	1.12^{c}	6.97^{c}	11.53^{bc}	22.84^{b}	35.53^{a}	6.30^{c}	7.16^{c}	13.29^{bc}	4.01	<0.001
TNF-α	1.08^{b}	1.89^{ab}	1.48^{b}	1.03^{b}	1.71^{b}	3.07^{a}	2.76^{a}	1.59^{b}	0.65	<0.005
Caspase-3	1.10^{c}	4.01^{a}	3.44^{ab}	1.64^{bc}	5.01^{a}	2.46^{b}	2.89^{b}	4.34^{a}	0.66	<0.005
Caspase-8	1.08^{d}	4.19^{b}	2.24^{cd}	6.53^{a}	3.14^{bc}	2.92^{bc}	2.81^{bc}	1.63^{cd}	0.47	<0.005

$^{a-d}$ Means within a row with different superscripts differ (P< 0.05)

IPEC1 are listed in *Table 2* and *Table 3*. In the unstressed status, the addition of a low level of GSE (1 μg/mL) facilitated (P<0.05) the mRNA expression levels of glutathione peroxidase-1 (GPx-1), B-Cell Lymphoma protein-2 (Bcl-2), cysteine aspartases-3 (caspase-3) and cysteine aspartases-8 (caspase-8) genes, while suppressing (P<0.05) the mRNA expression levels of copper-zinc superoxide dismutase (CuZn-SOD), glutathione S-transferase (GST), thioredoxin (Trx), thioltransferase (Ttas), and thioredoxin reductase (TR), Bcl2-associated X protein (Bax) genes. A relatively high level of GSE at 10 μg/mL also increased Bcl-2 and caspase-3 but not caspase-8 and GPx-1 expression,

whereas it decreased the expression of the Bax, CuZn-SOD, Trx, Ttas and TR genes. As the GSE dose reached 60 μg/mL, a markedly positive effect ($P<0.05$) on the mRNA expression levels of p53 and caspase-8 genes was observed, but the Bax gene, as well as the antioxidant genes CuZn-SOD, manganese superoxide dismutase (Mn-SOD), GPx-1, GPx-4, GST, Trx, Ttas, and TR, were inhibited ($P<0.05$).

In H_2O_2-stressed cells, increases in the mRNA expression levels of p53 and caspase-3 were dramatically attenuated by the subsequent addition of 1 μg GSE /mL, but this addition of 1 μg GSE/mL also increased the mRNA expression level of tumor necrosis factor α (TNF-α). Meanwhile, 1 μg GSE/mL promoted ($P<0.05$) the GPx-1 and TtasmRNA expression that was suppressed by stress with H_2O_2 but reduced ($P<0.05$) the mRNA expression level of GST. The addition of 10 μg GSE/mL also alleviated the Bax, p53, caspase-3 and GST expression but enhanced TNF-α mRNA expression. Differences between the addition of 1 μg and 10 μg GSE/mL were noted with the addition of 10 μg GSE/mL, which increased GPx-2 and GPx-4 mRNA expression instead of increasing GPx-1 and TtasmRNA expression, and decreased Mn-SOD mRNA expression. Incubation with 60 μg GSE/mL caused a significant reduction in the mRNA expression levels of the Bax, p53, Mn-SOD and GST, in addition to a significant increase in the mRNA expression level of TR.

DISCUSSION

In this study, the contribution of GSE to the TAOC at low and medium levels (1 and 10 μg/mL, respectively) was moderate in unstressed or stressed IPEC1, but GSH was increased at 10 μg GSE/mL in unstressed cells, as well as at 1 and 10 μg/mL in the stressed cells. These results may represent the first direct evidence that a certain level of GSE can elevate the GSH contents in intestinal epithelial cells, although a few related studies have reported that dietary supplementation with GSE promotes an increase in GSH in the rat liver, the hippocampus and human serum [12,13]. Evidence indicates that some compositions of GSE (such as Quercetin, Epicatechin and Epicatechingallate) have the ability to stimulate GRed and γ-GCS activity, which act as the rate-limiting and key enzymes, respectively, in two major processes of GSH synthesis [14], suggesting that GSE accelerated the synthesis of GSH.

Glutathione exerts great influence on the regulation of the apoptosis/survival balance of cells exposed to damaging stimuli. Cells that undergo apoptosis actively extrude GSH, abruptly creating a redox imbalance that favors the formation of ROS and protein disulfides [15], and antioxidants do not hamper apoptotic GSH efflux but instead protect GSH-depleted cells from apoptosis [16], indicating the importance of GSH serving as antioxidant. Further investigation has revealed more details on the GSH's role in maintaining critical protein sulfhydryls and donating hydrogens that are necessary for DNA repair, synthesis and expression [17]. GSH is a cofactor for anti-oxidative enzymes such as GPx and GST [17] and regulates relative transcriptional factors, such as nuclear factor kappa B and NF-E2-related factor 2, which take over cell survival/apoptosis [18]. Therefore, GSE's promotion of GSH presumably could explain why GSE exposure at the medium level promoted cell proliferation in unstressed cells.

As the addition of GSE reached 60 μg/mL, a decrease in the cell survival ratio was accompanied by a sharp decline in the TAOC and GSH, which was observed in the unstressed IPEC1 cells. Oxidative stress is well known to be the consequence of an imbalance between the formation of ROS and TAOC [19]. The low-level TAOC suggested that the antioxidant barrier was unable to fend off the amount of ROS present, and cells were prone to accumulate a mass of ROS. Moreover, the GSH-depleted cells could passively allow oxidative stress to take place, contributing to the formation of protein (S-) glutathionylation, which is thought to be an early molecular event in apoptosis induction [20]. Additionally, GSH depletion induces increased mitochondrial ROS exposure and cytochrome c release, representing another mechanism behind cells' commitment to apoptosis [21]. Therefore, a combination of a reduced cell survival ratio and the increase in ROS observed herein reinforced the previous suggestion that GSE exerts the ability to inhibit cell proliferative in IPEC1 cells by a ROS-mediated mechanism. Meanwhile, it is reasonable to believe that GSE at a high dose switches the cell from proliferation to antiproliferation, thereby partly accounting for the transformation of the role of the GSE from antioxidant to oxidant.

Interestingly, unstressed IPEC1 cells treated with 60 μg GSE/mL, compared to IPEC1 cells directly exposed to 400 μM H_2O_2 only, showed less TAOC and GSH but a higher cell survival ratio. One rational explanation is that the ROS generated by the excess GSE was different from that generated in the H_2O_2-stressed cells, and comparatively, H_2O_2 was presumably much more potent for inducing apoptosis, thereby resulting in a lower cell survival ratio. H_2O_2 is recognized as a strong oxidant and mediator of apoptosis, and millimolar concentrations of H_2O_2 can directly induce apoptosis [22]. The types of ROS generated by GSE and how they are generated remain elusive.

In our previous work, GSE was shown to have the capability to regulate a series of antioxidant enzymes that impacted the anti/oxidative status of lamb muscle cells in a chemical manner [1]. Similarly, the direct exposure of IPEC1 cells to 1 μg GSE/mL led to the downregulated mRNA expression of genes such as CuZn-SOD, GST, Trx, Ttas, and TR, with only GPx-1 being upregulated. With the addition of GSE at 10 μg/mL, the CuZn-SOD, Trx, Ttas and TR genes remained suppressed, and even the upregulation of GPx-1 was aborted. CuZn-SOD, GST, Trx,

Ttas and TR are critical antioxidant enzymes or proteins in organic systems that control the reduced intracellular redox environment. CuZn-SOD and GST are responsible for converting free radical superoxide to H_2O_2 and providing protection against electrophiles and products of oxidative stress, respectively [14,23]. The thioredoxin system comprises Trx and TR plus NADPH and functions in thiol-dependent thiol-disulfide exchange reactions, namely, the reduction of protein disulfides [24]. Ttas represents a pivotal partner with the Trx system in redox regulation, especially for ribonucleotide reduction [25]. Thus, the inhibition of these antioxidant enzymes and proteins seemingly run counter to our former conclusion that GSE at low and medium levels exerted primarily an antioxidant effect. It may still be premature to discuss how GSE regulated these genes, mainly due to the conflict between the sophisticated transcriptional mechanism of these genes and the relatively few studies in this field. Glutathione (GST) is regulated by at least 100 structurally diverse xenobiotics and chemicals. The ROS is the regulator of GST, and GST shows an adaptive upregulation response to ROS [26], which presumably explains, in part, why GST was suppressed by low levels of GSE that was supposed to create an antioxidant environment.

As the dose of GSE reached 60 μg/mL, several antioxidant genes, such as CuZn-SOD, Mn-SOD, GPx-1, GPx-4, GST, Trx, Ttas and TR, were inhibited, which, combined with the abovementioned evidence (remarkably decreased TAOC and GSH), suggested that the cells were subjected to strong oxidative stress. These results also suggested that GSE at high concentrations induced the generation of ROS, not only through the depletion of GSH but also through the downregulation of a number of crucial genes of antioxidant enzymes and proteins, further restraining the antioxidant function of these enzymes and proteins and the elimination of ROS.

In the stressed cells, 1 μg/mL GSE also reduced GST mRNA expression but promoted GPx-1 and Ttas mRNA expression that were suppressed by H_2O_2. In the group receiving 10 μg/mL GSE, instead of GPx-1 being upregulated, two isoforms of GP-x (GPx-2 and GPx-4) were dramatically upregulated. Enhanced mRNA expression of GPx-2 at the medium level of GSE in stressed cells was demonstrated in our previous study of the effects of GSE on lamb primary muscle cells as well [1]. Unfortunately, the mRNA expression of catalase in our work was not influenced by GSE in the stressed cells, and the treatment with 400 μmol H_2O_2 was speculatively beyond the handling capacity of the GPxs. Therefore, this may explain why the cell survival ratio barely recovered with GSE at 1 and 10 μg GSE/mL doses in stressed cells, even though the mRNA expression level of GPx was upregulated.

Apoptosis negatively affects the cell survival ratio, and several genes that modulate apoptosis varied differently in response to the GSE in our work. In unstressed cells, the addition of 1 μg and 10 μg GSE/mL promoted the mRNA expression level of Bcl-2 and mitigated that of Bax in the IPEC1 cells, thus leading to the downregulation of Bax/Bcl-2 ratio. The Bcl-2 and Bax belong to the Bcl-2 protein family, which is important in mitochondrial-mediated apoptotic events having either a proapoptotic or antiapoptotic function. In prior studies, Bax has been found to play an important role in GSE-induced apoptosis, which is blocked by the knockdown of Bax through Bax siRNA transfection to 4T1 cells [27]. A previous study verified that the ratio of Bax to Bcl-2 proteins is the determining factor in the transmission of the apoptotic signal [28]. The present study demonstrated that the proliferative effects of low and moderate levels of GSE on unstressed IPEC1 cells may be mediated by modulating the Bax/Bcl-2 ratio.

The most noteworthy variation at 60 μg GSE/mL in the unstressed IPEC1 cells was the exponential increase in the mRNA expression of p53. p53 uses various cellular inputs to regulate apoptosis and proliferation [29]. The ROS-mediated DNA damage and ROS-activated signaling pathways, such as the MAPK-mediated pathways, are able to activate p53 [30]. In our work, cellular exposure to H_2O_2 showed remarkable upregulation of the mRNA expression of p53. The results indicated that the addition of 60 μg GSE/mL inhibited the proliferation of the unstressed IPEC1 cells by increasing the p53.

In stressed cells, low and medium levels of GSE also exhibited antiapoptosis potential with the downregulation of the mRNA expression levels of Bax, p53, and caspase-3, but an increase in the TNF gene expression also suggested the apoptosis potential of GSE. The apoptosis mediated by TNF occurs through the extrinsic apoptosis pathway, unlike the intrinsic apoptosis pathway, which relies primarily on the Bcl-2 protein family and involves the acceptance of extracellular ligand-binding stimulation to initiate apoptosis [31]. The present results indicated that GSE regulates apoptosis-related genes differently in different circumstances.

In summary, the GSE primarily exerted an antioxidant and proliferative effect on unstressed IPEC1 cells at low and medium levels and an oxidative and antiproliferative effect at the high levels. Studies are needed to further verify the effect of GSE *in vivo*, possibly expanding the use of GSE supplementation to animal studies.

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REFERENCES

1. Yang T, Li XM, Zhu W, Chen C, Sun ZH, Tan ZL, Kang JH: Alteration of antioxidant enzymes and associated genes induced by grape seed extracts in the primary muscle cells of goats *in vitro*. *PLoS One*, 9, e107670, 2014. DOI: 10.1371/journal.pone.0107670

2. Cedo L, Castell-Auvi A, Pallares V, Blay M, Ardevol A, Arola L, Pinent M: Grape seed procyanidin extract modulates proliferation and apoptosis of pancreatic beta-cells. *Food Chem*, 138, 524-530, 2013. DOI: 10.1016/j.foodchem.2012.10.088

3. Li XL, Li BY, Cheng M, Yu F, Yin WB, Cai Q, Zhang Z, Zhang JH, Wang JF, Zhou RH, Gao HQ: PIMT prevents the apoptosis of endothelial cells in response to glycated low density lipoproteins and protective effects of grape seed procyanidin B2. *PLoS One*, 8, e69979, 2013. DOI: 10.1371/journal.pone.0069979

4. Ozkan G, Ulusoy S, Alkanat M, Orem A, Akcan B, Ersoz S, Yulug E, Kaynar K, Al S: Antiapoptotic and antioxidant effects of GSPE in preventing cyclosporine A-induced cardiotoxicity. *Ren Fail*, 34, 460-466, 2012. DOI: 10.3109/0886022X.2012.656563

5. Chen Q, Zhang R, Li WM, Niu YJ, Guo HC, Liu XH, Hou YC, Zhao LJ: The protective effect of grape seed procyanidin extract against cadmium-induced renal oxidative damage in mice. *Environ Toxicol Pharmacol*, 36, 759-768, 2013. DOI: 10.1016/j.etap.2013.07.006

6. Dinicola S, Pasqualato A, Cucina A, Coluccia P, Ferranti F, Canipari R, Catizone A, Proietti S, D'Anselmi F, Ricci G, Palombo A, Bizzarri M: Grape seed extract suppresses MDA-MB231 breast cancer cell migration and invasion. *Eur J Nutr*, 53, 421-431, 2014. DOI: 10.1007/s00394-013-0542-6

7. Hamza AA, Heeba GH, Elwy HM, Murali C, El-Awady R, Amin A: Molecular characterization of the grape seeds extract's effect against chemically induced liver cancer: *In vivo* and *in vitro* analyses. *Sci Rep*, 8, 2018. DOI: 10.1038/s41598-018-19492-x

8. Li XC, Ding XM, Peng X, Chi XF, Cui HM, Zuo ZC, Fang J: Effect of chitosan oligosaccharides on antioxidant function, lymphocyte cycle and apoptosis in ileum mucosa of broiler. *Kafkas Univ Vet Fak Derg*, 23, 571-577, 2017. DOI: 10.9775/kvfd.2016.17329

9. Locato V, Gadaleta C, De Gara L, De Pinto MC: Production of reactive species and modulation of antioxidant network in response to heat shock: A critical balance for cell fate. *Plant Cell Environ*, 31, 1606-1619, 2008. DOI: 10.1111/j.1365-3040.2008.01867.x

10. Zhong RZ, Zhou DW, Tan CY, Tan ZL, Han XF, Zhou CS, Tang SX: Effect of tea catechins on regulation of antioxidant enzyme expression in H_2O_2-induced skeletal muscle cells of goat *in vitro*. *J Agric Food Chem*, 59, 11338-11343, 2011. DOI: 10.1021/jf202839t

11. Sun J, Ye YR, Liao HR, Zhang L, Yang H: Effects of steroid hormones on epithelial and stromal cells culture and proliferation of laying hens, *in vitro*. *Kafkas Univ Vet Fak Derg*, 24, 401-407, 2018. DOI: 10.9775/kvfd.2017.19055

12. Fernandez-Iglesias A, Quesada H, Diaz S, Pajuelo D, Blade C, Arola L, Salvado MJ, Mulero M: Combination of grape seed proanthocyanidin extract and docosahexaenoic acid-rich oil increases the hepatic detoxification by GST mediated GSH conjugation in a lipidic postprandial state. *Food Chem*, 165, 14-20, 2014. DOI: 10.1016/j.foodchem.2014.05.057

13. Zhen JL, Qu ZZ, Fang HB, Fu L, Wu YP, Wang HC, Zang HM, Wang WP: Effects of grape seed proanthocyanidin extract on pentylenetetrazole-induced kindling and associated cognitive impairment in rats. *Int J Mol Med*, 34, 391-398, 2014. DOI: 10.3892/ijmm.2014.1796

14. Lopez-Oliva ME, Agis-Torres A, Goni I, Munoz-Martinez E: Grape antioxidant dietary fibre reduced apoptosis and induced a pro-reducing shift in the glutathione redox state of the rat proximal colonic mucosa. *Br J Nutr*, 103, 1110-1117, 2010. DOI: 10.1017/S0007114509992996

15. Ghibelli L, Coppola S, Rotilio G, Lafavia E, Maresca V, Ciriolo MR: Non-oxidative loss of glutathione in apoptosis via GSH extrusion. *Biochem Biophys Res Commun*, 216, 313-320, 1995. DOI: 10.1006/bbrc.1995.2626

16. Liuzzi F, Fanelli C, Ciriolo MR, Cerella C, D'Alessio M, Denicola M, Magrini A, Bergamaschi A, Ghibelli L: Rescue of cells from apoptosis by antioxidants occurs downstream from GSH extrusion. *Ann N Y Acad Sci*, 1010, 441-445, 2003. DOI: 10.1196/annals.1299.080

17. Mari M, Morales A, Colell A, Garcia-Ruiz C, Fernandez-Checa JC: Mitochondrial glutathione, a key survival antioxidant. *Antioxid Redox Signal*, 11, 2685-2700, 2009. DOI: 10.1089/ARS.2009.2695

18. De Nicola M, Ghibelli L: Glutathione depletion in survival and apoptotic pathways. *Front Pharmacol*, 5, 267, 2014. DOI: 10.3389/fphar.2014.00267

19. Campise M, Bamonti F, Novembrino C, Ippolito S, Tarantino A, Cornelli U, Lonati S, Cesana BM, Ponticelli C: Oxidative stress in kidney transplant patients. *Transplantation*, 76, 1474-1478, 2003. DOI: 10.1097/01.TP.0000090344.61975.F0

20. Di Stefano A, Frosali S, Leonini A, Ettorre A, Priora R, Di Simplicio FC, Di Simplicio P: GSH depletion, protein S-glutathionylation and mitochondrial transmembrane potential hyperpolarization are early events in initiation of cell death induced by a mixture of isothiazolinones in HL60 cells. *BBA-Mol Cell Res*, 1763, 214-225, 2006. DOI: 10.1016/j.bbamcr.2005.12.012

21. Yuan L, Kaplowitz N: Glutathione in liver diseases and hepatotoxicity. *Mol Aspects Med*, 30, 29-41, 2009. DOI: 10.1016/j.mam.2008.08.003

22. Perez-Cruz I, Carcamo JM, Golde DW: Caspase-8 dependent trail-induced apoptosis in cancer cell lines is inhibited by vitamin C and catalase. *Apoptosis*, 12, 225-234, 2007. DOI: 10.1007/s10495-006-0475-0

23. Townsend DM, Tew KD: The role of glutathione-S-transferase in anti-cancer drug resistance. *Oncogene*, 22, 7369-7375, 2003. DOI: 10.1038/sj.onc.1206940

24. Nilsonne G, Sun X, Nystrom C, Rundlof AK, Fernandes AP, Bjornstedt M, Dobra K: Selenite induces apoptosis in sarcomatoid malignant mesothelioma cells through oxidative stress. *Free Radic Biol Med*, 41, 874-885, 2006. DOI: 10.1016/j.freeradbiomed.2006.04.031

25. Circu ML, Aw TY: Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med*, 48, 749-762, 2010. DOI: 10.1016/j.freeradbiomed.2009.12.022

26. Hayes JD, Pulford DJ: The glutathione S-Transferase supergene family: Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Part I. *Crit Rev Biochem Mol Biol*, 30, 445-600, 1995. DOI: 10.3109/10409239509083491

27. Mantena SK, Baliga MS, Katiyar SK: Grape seed proanthocyanidins induce apoptosis and inhibit metastasis of highly metastatic breast carcinoma cells. *Carcinogenesis*, 34, 1941, 2006. DOI: 10.1093/carcin/bgs215

28. Kroemer G: The proto-oncogene Bcl-2 and its role in regulating apoptosis. *Nat Med*, 3, 614-620, 1997. DOI: 10.1038/nm0697-614

29. Speidel D: Transcription-independent p53 apoptosis: An alternative route to death. *Trends Cell Biol*, 20, 14-24, 2010. DOI: 10.1016/j.tcb.2009.10.002

30. Janicke RU, Sohn D, Schulze-Osthoff K: The dark side of a tumor suppressor: Anti-apoptotic p53. *Cell Death Differ*, 15, 959-976, 2008. DOI: 10.1038/cdd.2008.33

31. Derry M, Raina K, Agarwal R, Agarwal C: Differential effects of grape seed extract against human colorectal cancer cell lines: The intricate role of death receptors and mitochondria. *Cancer Lett*, 334, 69-78, 2013. DOI: 10.1016/j.canlet.2012.12.015

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Research Article

Discrimination of Early Pregnancy and Endometrial Cyst by Ultrasonographic Assessment of Uterine Echotexture in Mares

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Abstract

The present study was performed to demonstrate the tissue differentiation of uterine ultrasonographic image by computer assisted analysis programs in mares with endometrial cyst and early pregnancy. A total of 124 thoroughbred Arabian mares were used in the study, with early pregnancy and endometrial cyst. Mares were divided into six groups according to stage of early pregnancy (Group I, n=12 (13th day pregnancy); Group II, n=15 (14th day pregnancy); Group III, n=29 (15th day pregnancy); Group IV, n=17 (16th day pregnancy); Group V, n=12 (17th day pregnancy) and as endometrial cysts (Group VI, n=39). The mean gray value (MGV), heterogeneity (HET) and contrast (CON) of the uterine images that were recorded during ultrasound scan were measured through computer assisted program. The MGV values of the echotexture parameters were significantly higher in the pregnant mare (55.20±4.95, 58.31±3.88, 56.23±1.88, 54.15±3.47 and 56.08±2.94, respectively) than the endometrial cyst mares (42.71±0.71) (P<0.001). The HET values were significantly higher in the pregnant mare (20.75±1.53, 19.23±1.39, 21.42±0.88, 22.37±1.32 and 22.20±1.09, respectively) than the endometrial cyst mares (15.06±0.44) (P<0.001). CON values were lower in pregnant mare (152.83±7.02, 144.02±8.18, 141.03±4.83, 145.93±4.70 and 154.65±4.86 respectively) than endometrial cyst mare (191.35±3.97) (P<0.001). As a result; it has been concluded that the measurement values of echotexture parameters (MGV, HET, CON) may be important reference criteria when early pregnancy and endometrial cyst are distinguished in mare, which can often cause misconception.

Keywords: *Early pregnancy, Endometrial cyst, Echotexture, Mare*

Kısraklarda Ultrasonografik Uterus Ekotekstür Değerlendirmesinde Erken Gebe ve Endometriyal Kist Ayrımı

Öz

Sunulan çalışma kısraklarda erken gebelik ile endometriyal kistli uterusun ultrasonografik görüntüsünün bilgisayar destekli analiz programları ile ayrımını ortaya koymak amacıyla yapıldı. Çalışmada erken gebe ve endometriyal kistli olmak üzere toplam 124 safkan Arap kısrak kullanıldı. Kısraklar erken gebelik dönemlerine (Grup I, n=12 (13 günlük gebelik); Grup II, n=15 (14 günlük gebelik); Grup III, n=29 (15 günlük gebelik); Grup IV, n=17 (16 günlük gebelik); Grup V, n=12 (17 günlük gebelik) ve endometriyal kist (Grup VI, n=39) olmak üzere altı gruba ayrıldı. Ultrasonografik muayene sırasında kaydedilen ve daha sonra bilgisayara aktarılan uterus görüntülerinin ortalama gri değeri (MGV), heterojenitesi (HET) ve kontrastı (CON) ölçüldü. Ekotekstür parametrelerinden MGV değeri gebe kısraklarda (sırasıyla; 55.20±4.95, 58.31±3.88, 56.23±1.88, 54.15±3.47 ve 56.08±2.94), endometrial kistli kısraklardan (42.71±0.71) daha yüksekti (P<0.001). Diğer ekotekstür parametresi olan HET değerleri gebe kısrakta (sırasıyla; 20.75±1.53, 19.23±1.39, 21.42±0.88, 22.37±1.32 ve 22.20±1.09), endometriyal kistli kısraklardan (15.06±0.44) daha yüksekti (P<0.001). CON değerleri ise gebe kısraklarda (sırasıyla; 152.83±7.02, 144.02±8.18, 141.03±4.83, 145.93±4.70 ve 154.65±4.86), endometriyal kistli kısraklardan (191.35±3.97) düşüktü (P<0.001). Sonuç olarak; kısraklarda sıklıkla yanılgıya sebep olabilecek erken gebelik ile endometriyal kistin ayırt edilmesinde, ekotekstür parametrelerinin (MGV, HET ve CON) ölçümsel değerlerinin önemli referans kriterleri olabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Erken gebelik, Endometriyal kist, Ekotekstür, Kısrak*

INTRODUCTION

Real-time B-mode ultrasonography is one of the most widely used diagnostic methods for monitoring reproduction in farm animals, which was first used in mares [1] in 1980 and later in cows [2,3]. Ultrasonography is also an important

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diagnostic tool used routinely in the physiological and pathological detection of early pregnancy, follicular changes in the ovaries and ovarian cysts and reproductive areas such as tumors, cysts, infections in the genital organs [4,5].

Ultrasonographic imaging is based on the ability of tissues to reflect high-frequency sound waves and this reflection depends on tissue density [6,7]. The images are displayed in a two-dimensional, gray scale based on the location and strength of the echoes from the tissue interfaces [8]. A two-dimensional ultrasonographic image is a matrix of square picture elements (pixels) varying in gray scale values ranging from 0 (absolute black) to 255 (absolute white) [6,7,9]. The ultrasonographic image of a tissue varies depending on the histological structure of the tissue and is called echotexture. Computer-assisted analysis (computer algorithm) allows an object to be evaluated objectively and provides determination of visual analysis subjectivities, allowing quantitative assessment of the intensity of each pixel in an image [10]. In recent years, computer-assisted ultrasonographic image analysis techniques have been used in the evaluation of genital organs including testicles [11], ovarian follicles [12], corpus luteum [12-14] and uterus [15]. The echotextural changes in the endometrium are used in the field of reproduction in veterinary medicine [16-18]. Computer assisted image analysis removes individual observation bias by allowing quantitative evaluation. The mean gray value (MGV), heterogeneity (HET) and contrast (CON) of the parameters obtained from the ultrasonographic images define the uterine echogenic image changes [19]. In computer-assisted analysis, images are obtained in three ways; point measurement, linear-time series analysis, and regional surface analysis [6]. When the images obtained in the studies were measured in computer environment, the target tissue was divided into four equal quadrants according to the point measurement and a small circular area was selected from these regions and average numerical data was formed [16,19-24].

In mares, endometrial cysts are fluid-filled formations that can be seen anywhere on the normal or chronically inflamed endometrium [25]. They have glandular (endometrial) or lymphatic structure, depending on their characteristics. Glandular cysts are found in the lamina propria and are typically smaller (1-10 mm) in size and exhibit multifocal distribution in one or more areas of the uterus. Lymphatic cysts are typically larger (>10 mm) and are usually isolated at one or two loci. Ultrasonography or hysteroscopy should be used to identify both types of cysts [26]. These types of cysts in the mare uterus can easily be confused with early or twin pregnancies in ultrasonographic examination and pregnancies may even be accidentally terminated during manipulation of uterine cyst in singleton pregnant mares that are mistaken for twin pregnancy. Furthermore, the inability of the embryonic vesicle to move freely in the uterine horns in the presence of cysts cannot reduce the secretion of prostaglandin F2α and reveal the failure of maternal diagnosis in pregnancy [27], impaired implantation can lead to insufficient blood flow to the conceptus and inadequate availability of nutrients resulting in early loss of embryo [27]. For this reason, early identification of uterine cysts and their discrimination from pregnancy are important in terms of fertility. Despite the fact that literature [25-27] studies conducted in this respect have differentiated early pregnancy (13-17 days) and endometrial cysts with periodic clinical and ultrasonographic examinations, the measurement and discrimination of these images by computer-assisted analysis programs has not done previously which manifest deficiency in this topic.

This study aimed to analyze the echotexture changes of uterine tissue by computer analysis program and to inform the veterinarians as an adjunct clinical modality in distinguishing early pregnancy in mares from endometrial cysts that can be confused with the former condition.

MATERIAL and METHODS

Ethics Statement

This study was approved by the Animal Ethics Committee of the University of Harran (Number: 2018/001/01-03).

Selection of the Animals

The present study was carried out on the mares brought to the Veterinary Clinics of Faculty of Veterinary Medicine, Harran University, for pregnancy and genital organ examination during breeding season. This area is located at 37°10'N - 39°03'E and 518 m higher from sea level. In this research, a total of 124 thoroughbred Arabian mares clinically healthy, with age interval of 7.6 and 14.69 years were used. Mares with early pregnancy and endometrial cysts are included in the study. Mares were divided into six groups according to stage of early pregnancy as Group I, n=12, (13^{th} day pregnancy); Group II, n=15, (14^{th} day pregnancy); Group III, n=29, (15^{th} day pregnancy); Group IV, n=17, (16^{th} day pregnancy); Group V, n=12, (17^{th} day pregnancy) and (Group VI, n=39) with endometrial cyst.

Ultrasound Examination

The examination of genital organs or early pregnancy in mares brought to the clinic was performed with Portable B-mode 5 MHz linear probe transrectal ultrasonograph (SonoSite Edge II Vet®, Providian Medical Equipment LLC, Highland Heights, Ohio, United States). During examinations, images of mares with endometrial cyst and early pregnancy were recorded in the memory sticks. Pregnancy and endometrial cyst images were recorded for evaluation during the breeding season and 13-17 days after the natural mating date in these mares (examination-1). Mares included in this study were then taken to the second examination on the 30^{th} day, and those found to have embryo and fetal heartbeat were considered pregnant (examination-2). In the evaluation of recorded image

analysis, records of mares with early pregnancy findings and endometrial cyst obtained from the first examination (examination-1) were transferred to the computer. Ultrasonographic settings (depth, gain, focus, brightness) were kept unchanged throughout the entire study while images were taken. All ultrasonographic examinations were performed by the same person.

Computer-Assisted Image Analysis

The obtained images were transferred to the computer for evaluation of the digital echotexture. The images were saved as PNG file type with a resolution of 640x480 pixels. Computer analysis was generated using a special software (ImajeJ 1.42q; NIH, USA-Image Processing and Analysis Java). The relevant program gives a qualitative value between 0-255. When measurement analysis was performed, polygonal boundaries were created covering every region of the uterus as 'relevant region' by avoiding fluid accumulation in uterus and myometrium, and MGV, HET, CON measurements were made *(Fig. 1, Fig. 2)*. Our study was done according to regional surface analysis which was [28] previously performed to determine the echotexture in dog ovaries. Edematous regions of myometrium were avoided during analysis. All uterine tissues were selected in polygonal style and the mean numerical data were obtained. It is thought that this method would give more reliable results compared to the analysis of whole tissue.

Statistical Analysis

Statistical analyses were performed using the SPSS software version 22. The variables were investigated using visual (histograms, probability plots) and analytical methods (Kolmogorov-Simirnov/Shapiro-Wilk's test) to determine whether they are normally distributed or not. Descriptive analyses were presented using means and standard deviations for normally distributed MGV, HET and CON variables. One-way ANOVA was used to compare these parameters among the early pregnancy and endometrial cyst groups. Levene test was used to assess the homogeneity of the variances. An overall P-value of less than 0.05 was considered to show a statistically significant result. When an overall significance was observed, pairwise post-hoc test was performed using Tukey's test. The data in the section of table, graph and results are expressed as mean±standard deviation.

RESULTS

The MGV, HET and CON values were analyzed with 95% confidence in evaluating echotexture from images taken from uterine tissue. As a result of the analysis, a significant difference was found between the all group (P<0.001) *(Table 1)*. The MGV values of the echotexture parameters were significantly different between the endometrial cyst group (Group VI) and the 13 days pregnancy group (Group I) (P<0.05). This difference was found to be more significant (P<0.01) between 14 days (Group II), 16 days (Group IV) and 17 days (Group V) pregnancy and even more significant difference was found in the 15 days pregnancy (Group III) (P<0.001). There was no difference when the according to stage of pregnant groups were evaluated among themselves (P>0.05) *(Fig. 3A)*. HET was statistically significant between the endometrial cyst group and the 13 days pregnancy group (P<0.01). This difference was found to be more significant in 15, 16 and 17 days of pregnancy (P<0.001). However, the difference between the 14 days pregnancy and the cyst group was not significant (P>0.05). There was no difference when the pregnant groups were

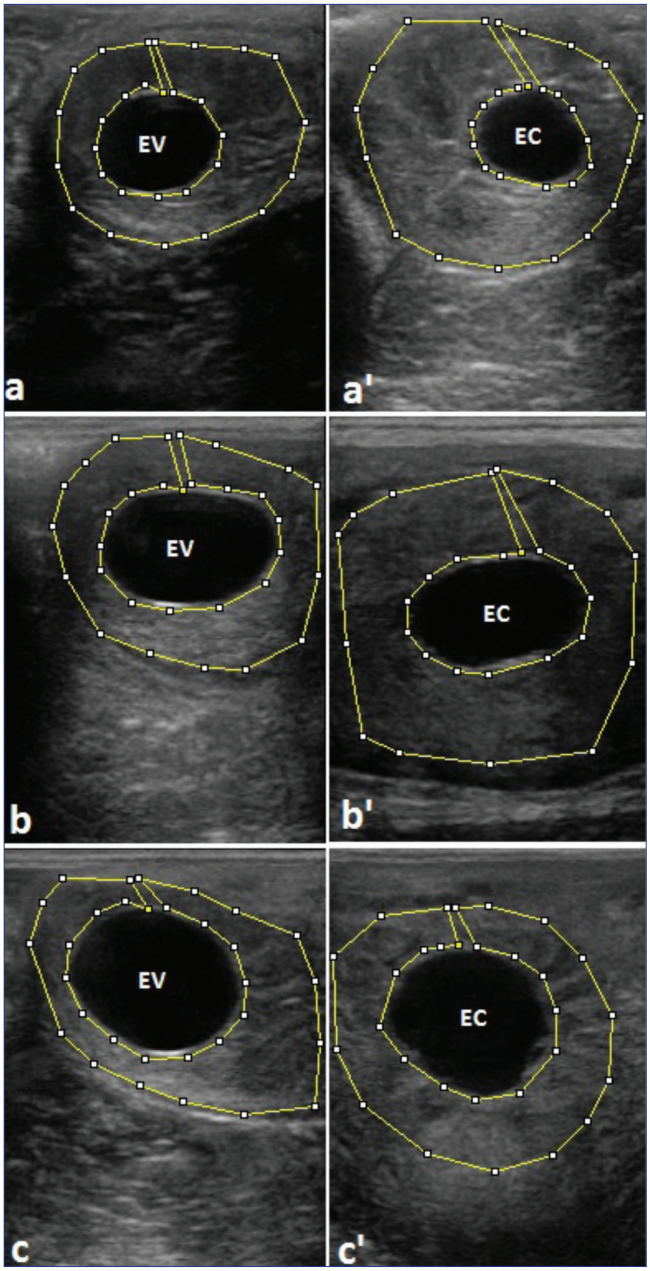

Fig 1. 13 days pregnancy (a), endometrial cyst (a'), 14 days pregnancy (b), endometrial cyst (b'), 15 days pregnancy (c), endometrial cyst (c'), EV: embryonic vesicle. EC: endometrial cyst

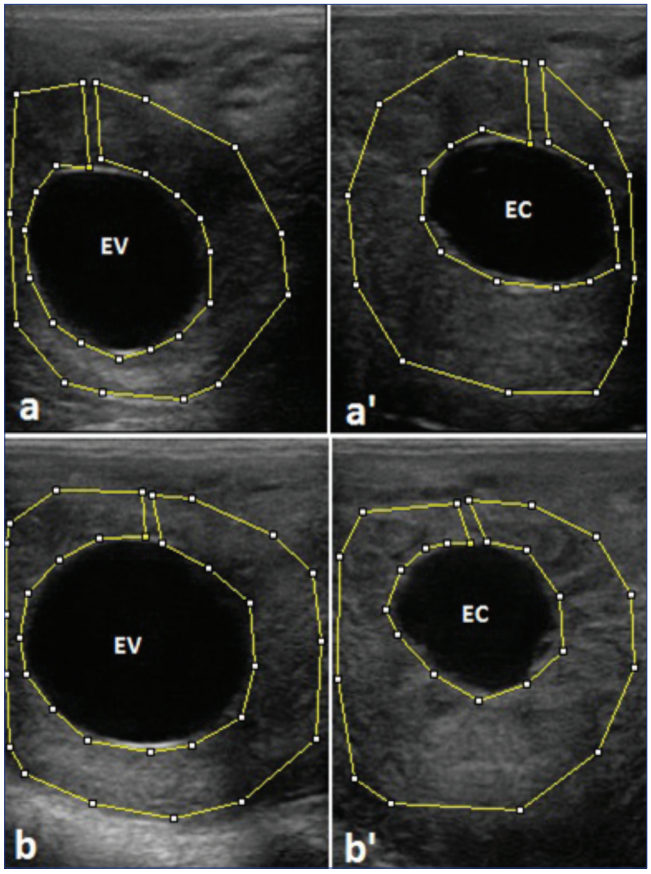

Fig 2. 16 days pregnancy (a), endometrial cyst (a'), 17 days pregnancy (b), endometrial cyst (b'), EV: embryonic vesicle. EC: endometrial cyst

evaluated among themselves (P<0.05) *(Fig. 3B)*. The CON value was found statistically significant among all the other pregnant group in the endometrial cyst group (P<0.001). There was no difference when the pregnant groups were evaluated among themselves (P>0.05) *(Fig. 3C)*.

DISCUSSION

The precise diagnosis of early pregnancy in mares during breeding season is critical for breeders. Although rectal palpation is widely used for diagnosis of pregnancy, it can be confused with endometrial cyst since it is not possible to palpate the embryonic vesicle [29]. It is important to monitor the embryonic vesicle without inner cell mass and embryo in the management of clinical diagnosis of early pregnancy in mares. Although the diagnosis of a product of conception is routinely detected by transrectal ultrasonography between 14-18 days after ovulation in mares, it is not possible to diagnose an abnormal vesicle because ultrasonographic appearance of the inner cell mass or embryo development cannot be obtained at this stage. By transrectal ultrasonography, embryo in the embryonic vesicle can be detected earliest at day 20 ovulation and the embryonic heart beat can be seen between 24-25 days [29]. Examination was done in mares between days 13 and 18 and embryonic heartbeats were observed parallel to the growth of the embryonal sac in the repeated examination. However, there was no change in vesicle size and appearance in the mares with endometrial cyst. In studies conducted on endometrial cysts, the incidence of cysts increased with age; the most commonly affected mares were reported to be over 10 years old [30,31]. The incidence of endometrial cysts in the fertile and subfertile mares has been reported to be between 13 and 22% [32]. In some studies, encountered 55.5% of endometrial cysts in ultrasonographic examinations routinely performed on 310 mares, ranging in age from 8 to 20 years [33]. In another study, a total of 95 cysts were found in 58 of 259 healthy mares, ranging in age from 3 to 22 years. While 73.1% of these cysts belonged to the mares older than 14 years, 29.1% belonged to mares between 7-14 years of age [32]. The number of cystic structure in mares varies from 1 to

Table 1. *The difference between the average MGV, heterogeneity and contrast values of the uterus of endometrial cystic and early pregnant mares*

Groups	N	MGV	Heterogeneity	Contrast
		X±Sx	X±Sx	X±Sx
Group I (13th day pregnancy)	12	55.20±4.95[a]	20.75±1.53[a]	152.83±7.02[a]
Group II (14th day pregnancy)	15	58.31±3.88[a]	19.23±1.39[b]	144.02±8.18[a]
Group III (15th day pregnancy)	29	56.23±1.88[a]	21.42±0.88[a]	141.03±4.83[a]
Group IV (16th day pregnancy)	17	54.15±3.47[a]	22.37±1.32[a]	145.93±4.70[a]
Group V (17th day pregnancy)	12	56.08±2.94[a]	22.20±1.09[a]	154.65±4.86[a]
Group VI (Endometrial cyst)	39	42.71±0.71[b]	15.06±0.44[b]	191.35±3.97[b]
P value (one way ANOVA)		P=0.000 ***	P=0.000 ***	P= 0.000 ***

[a,b]The letters on the table are statistically different. A significant difference was found between the six group averages for the related parameter variables (P<0.001), ANOVA: Analysis of variance. Values in the table mean the mean±SEM

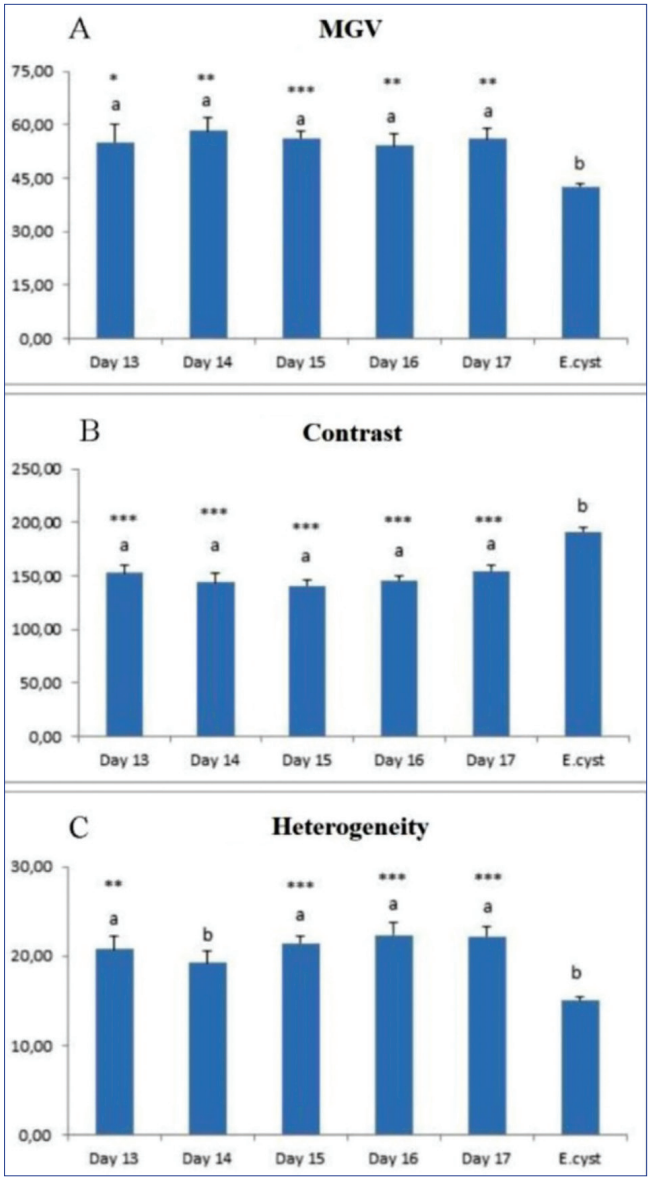

Fig 3. Mean gray value (MGV) (3A), heterogeneity (3B) and contrast (3C) in pregnant and endometrial cyst groups. Values in the column values the mean±SEM

A: [a,b] The letters on the graph are statistically different. [*,**,***] Differentiation of endometrial cysts compared to early pregnancy periods, * P<0.05, ** P<0.01, *** P<0.001; **B:** [a,b] The letters on the graph are statistically different. [***] Differentiation of endometrial cysts compared to early pregnancy periods, *** P<0.001; **C:** [a,b] The letters on the graph are statistically different. [**,***] Differentiation of endometrial cysts compared to early pregnancy periods, ** P<0.01, *** P<0.001

7 per mare with 3 to 48 mm in diameter which increases with age [32,34]. In this study, mares between 5 and 18 years of age were used and both fertility and predisposition to cyst development were achieved, and the prevalence of endometrial cysts (localization to a single uterine region) by ultrasonographic examinations and localization of cysts was found to be consistent with the literature data. While endometrial cysts can be found anywhere within the uterus or cervix, they are more common near the bifurcation area of the horns and in the uterus [35]. In a retrospective study of 55 mares treated for cysts, 33.8% of the cysts were found endometrium in the uterine tissue, and 16.4% were found in the endometrium in the conjunctional region of the uterine horns [36]. Two other studies conducted on 48 and 259 mares, respectively, reported that the most common location of cysts is the junction of the uterine horns and body of the uterus [30,32]. In our study, the vast majority of cysts were found in the endometrium and only one of them was in the junction of the horns. Twins have also been encountered during the study, but they have not been evaluated.

B-mode ultrasonography computer-assisted image analysis has been used for evaluation of uterus [37] and ovaries [20,23,38-40]. In order to determine the relationship of embryonic fixation with echotexture parameters, a significant increase in uterine echotexture was demonstrated in the MGV measurements on day 21 after mating in cattle [16,41], on day 15-16 in mares [42], on day 16 in goats [19] and in pigs [17]. In the mares [43] and in cows [16], echotextural changes in the endometrial tissue were reported to be associated with peripheral concentrations of ovarian hormones. It has been reported that uterine echotexture values increased during the pre-estrus and pre-ovulation period [41] in the heifers and decreased in the mares [42]. In the goats, computer-assisted analysis measurements for reproductive organs and luteal function were established [19,44,45]. Studies on echotextures in farm animals have been carried out on sexual cycle follow-up, pregnancy, evaluation of genital organs and monitoring ovarian hormones. Increased echotexture parameters in early pregnancy were reported in mares, but endometrial cyst and early pregnancy, which constitute significant problems, have not been evaluated for echotextural changes. In the present study, increased uterus echotexture in early pregnancy was compatible with the previous studies and uterus echotexture was found to be statistically significantly higher in pregnancies between 13-17 days compared to mares with endometrial cysts. This has led to the conclusion that the uterine echotexture may have been increased in pregnancy due to endometrial folding, which begins to increase during early pregnancy and to the effect of the incoming blood flow on vascular permeability [29].

As a result, endometrial cysts that may lead to confusion in mares during early pregnancy can be distinguished from early pregnancy by analyzing their MGV, HET and CON of echotexture parameters.

REFERENCES

1. Palmer E, Driancourt MA: Use of ultrasonic echography in equine gynecology. *Theriogenology*, 13, 203-216, 1980. DOI: 10.1016/0093-691X(80)90082-5

2. Pierson RA, Ginther OJ: Ultrasonography of the bovine ovary. *Theriogenology*, 21, 495-504, 1984. DOI: 10.1016/0093-691X(84)90411-4

3. Reeves JJ, Rantanen NW, Hauser M: Transrectal real-time ultrasound scanning of the cow reproductive tract. *Theriogenology*, 21, 485-494, 1984. DOI: 10.1016/0093-691X(84)90410-2

4. Fissore RA, Edmondson AJ, Pashen RL, Bondurant RH: The use of

ultrasonography for the study of the bovine reproductive tract. II. Non-pregnant, pregnant and pathological conditions of the uterus. *Anim Reprod Sci*, 12, 167-177, 1986. DOI: 10.1016/0378-4320(86)90037-0

5. Pierson RA, Ginther OJ: Ultrasonographic appearance of the bovine uterus during the estrous cycle. *J Am Vet Med Assoc*, 190, 995-1001, 1987.

6. Pierson RA, Adams GP: Computer-assisted image analysis, diagnostic ultrasonography and ovulation induction: Strange bedfellows. *Theriogenology*, 43, 105-112, 1995. DOI: 10.1016/0093-691X(94)00014-L

7. Singh J, Pierson RA, Adams GP: Ultrasound image attributes of the bovine corpus luteum: Structural and functional correlates. *J Reprod Fertil,* 109, 35-44, 1997. DOI: 10.1530/jrf.0.1090035

8. Zagzebski JA: Physics of diagnostic ultrasound. **In,** Zagzebski JA (Ed): Essentials of Ultrasound Physics. 1-19, Mosby Year Book Inc, St. Louis, 1996.

9. Griffin PG, Ginther OJ: Research application of ultrasonic imaging in reproductive biology. *J Anim Sci,* 70, 953-972, 1992. DOI: 10.2527/1992.703953x

10. Singh J, Adams GP, Pierson RA: Promise of new imaging technologies for assessing ovarian function. *Anim Reprod Sci,* 78, 371-399, 2003. DOI: 10.1016/S0378-4320(03)00100-3

11. Gabor G, Sasser RG, Kastelic JP, Mezes M, Falkay G, Bozo S, Csik JV, Barany I, Hidas A, Jr Szasz F, Boros G: Computer analysis of video and ultrasonographic images for evaluation of bull testes. *Theriogenology*, 50, 223-228, 1998. DOI: 10.1016/S0093-691X(98)00129-0

12. Vassena R, Adams GP, Mapletoft RJ, Pierson RA, Singh J: Ultrasound image characteristics of ovarian follicles in relation to oocyte competence and follicular status in cattle. *Anim Reprod Sci,* 76, 25-41, 2003. DOI: 10.1016/S0378-4320(02)00234-8

13.Davies KL, Bartlewski PM, Pierson RA, Rawlings NC: Computer assisted image analyses of corpora lutea in relation to peripheral concentrations of progesterone: A comparison between breeds ofsheep with different ovulation rates. *Anim Reprod Sci,* 96, 165-175, 2006. DOI: 10.1016/j.anireprosci.2005.12.003

14. Liu X, Dai Q, Hart EJ, Barrett DMW, Rawlings NC, Pierson RA, Bartlewski PM: Ultrasonographic characteristics of ovulatory follicles and associated endocrine changes in cyclic ewes treated with medroxyprogesterone acetate (MAP)-releasing intravaginal spongesand equine chorionic gonadotropin (eCG). *Reprod Domest Anim,* 42, 393-401, 2007. DOI: 10.1111/j.1439-0531.2006.00798.x

15. Ginther OJ: How ultrasound technologies have expanded and revolutionized research in reproduction in large animals. *Theriogenology*, 81, 112-125, 2014. DOI: 10.1016/j.theriogenology.2013.09.007

16. Schmauder S, Weber F, Kiossis E, Bollwein H: Cyclic changes in endometrial echotexture of cows using a computer-assisted program for the analysis of first-and second-order grey level statistics of B Mode ultrasound images. *Anim Reprod Sci*, 106, 153-161, 2008. DOI: 10.1016/j.anireprosci.2007.12.022

17. Kauffold J, Von Dem Bussche B, Failing K, Wehrend A, Wendt M: Use of B-mode ultrasound and grey-scale analysis to study uterine echogenicity in the pig. *J Reprod Dev*, 56, 444-448, 2010. DOI: 10.1262/jrd.09-220T

18. Kucukaslan I: Evaluation of endometrial echostructure following Lotagen® administiration in cows with chronic endometritis. *PhD Thesis,* Institute of Health Sciences, Ankara University, 2010.

19. Cengiz M, Kanca H, Salar S, Bastan A, Kucukaslan I, Alkan H, Karakas K, Yuksel O, Hayirli A: Endometrial echotexture parameters in Turkish Saanen Goats (Akkeci) during oestrus and early pregnancy. *Anim Reprod Sci,* 146, 27-33, 2014. DOI: 10.1016/j.anireprosci.2014.02.004

20. Herzog K, Kiossis E, Bollwein H: Examination of cyclic changes in bovine luteal echotexture using computer-assisted statistical pattern recognition techniques. *Anim Reprod Sci,* 106, 289-297, 2008. DOI: 10.1016/j.anireprosci.2007.05.004

21. Noseir WMB, Sosa GAM: Treatment of ovarian cysts in Buffaloes with emphasis to echotexture analysis. *J Dairy Vet Anim Res*, 2 (2): 1-7, 2015. DOI: 10.15406/jdvar.2015.02.00030

22. Polat B, Cengiz M, Cannazik O, Çolak A, Oruç E, Altun S, Salar S, Baştan A: Endometrial echotexture variables in postpartum cows with subclinical endometritis. *Anim Reprod Sci,* 155, 50-55, 2015. DOI: 10.1016/j.anireprosci.2015.01.015

23. Scully S, Evans ACO, Carter F, Duffy P, Lonergan P, Crowe MA: Ultrasound monitoring of blood flow and echotexture of the corpus luteum and uterus during early pregnancy of beef heifers. *Theriogenology*, 83, 449-458, 2015. DOI: 10.1016/j.theriogenology.2014.10.009

24. Cengiz M, Çolak A, Hayırlı A, Cannazik O: Optical density changes in ultrasonographic images of the endometrium and corpus luteum in pregnant and cyclic cows. *Turk J Vet Anim Sci,* 41, 18-24, 2017. DOI: 10.3906/vet-1604-41

25. Kenney RM, Ganjam VK: Selected pathological changes of the mare uterus and ovary. *J Reprod Fertil Suppl*, 23, 335-339, 1975.

26. Ley WB, Higbee RG, Holyoak GR: Laser ablation of endometrial and lymphatic cysts. *Clin Tec Equine Pract*, 1 (1): 28-31, 2002. DOI: 10.1053/ctep.2002.33174

27. McDowell KJ, Sharp DC, Grubaugh W, Thatcher WW, Wilcox CJ: Restricted conceptus mobility results in failure of pregnancy maintenance in mares. *Biol Reprod*, 39, 340-348, 1988. DOI: 10.1095/biolreprod39.2.340

28. Erdoğan G, Küçük N, Kanca H, Aksoy M: *In vivo* and *in vitro* assessment of ovarian echotexture through computer assisted real time ultrasonography in bitches. *Ankara Üniv Vet Fak Derg,* 64, 171-176, 2017.

29. Ginther OJ: The single embryo. Ultrasonic imaging and reproductive events in the mare. Cross Plains, Wis: Equiservices, 212-213, 1986.

30. Bracher V, Mathias S, Allen WR: Videoendoscopic evaluation of the mare's uterus: II. Findings in subfertile mares. *Equine Vet J*, 24, 279-284, 1992. DOI: 10.1111/j.2042-3306.1992.tb02835.x

31. Bilkslager AT, Tate LP, Weinstock D: Effects of neodymium:yttrium aluminum garnet laser irradiation on endometrium and on endometrial cysts in six mares. *Vet Surg*, 22, 351-356, 1993. DOI: 10.1111/j.1532-950X.1993.tb00411.x

32. Tannus RJ, Thun R: Influence of endometrial cysts on conception rate of mares. *Zentralbl Veterinarmed A*, 42, 275-283, 1995. DOI: 10.1111/j.1439-0442.1995.tb00378.x

33. Wilson GL: Equine hysteroscopy: equipment for diagnostic endoscopy and photography. *Vet Med*, 80, 76-88, 1985.

34. Adams GP, Kastelic JP, Bergfelt DR, Ginther OJ: Effect of uterine inflammation and ultrasonically-detected uterine pathology on fertility in the mare. *J Reprod Fertil Suppl*, 35, 445-454, 1987.

35. Ragon AC: Diagnosis and management of uterine cysts. *Proceedings American College of Theriogenologists and Society for Theriogenology Mare Reproduction Symposium.* August, Hastings, Neb, 21-26, 1996.

36. Griffin RL, Bennett SD: Nd:YAG laser photoablation of endometrial cysts: A review of 55 cases (2000-2001). *Proceedings AAEP,* Orlando, Fla, 48, 58-60, 2002.

37. Bertmann J, Diaz L, Weber F, Stolla R, Bollwein H: Changes inuterine blood flow and endometrial echostructure during the earlypregnancy of cows. *Wiener Tierärzt Monat,* 91, 11, 2004.

38. Pierson RA, Ginther OJ: Ultrasonic evaluation of the preovulatory follicle in the mare. *Theriogenology*, 24, 359-368, 1985. DOI: 10.1016/0093-691X(85)90228-6

39. Tom JW, Pierson RA, Adams GP: Quantitative echotexture analysis of bovine corpora lutea. *Theriogenology*, 49, 1345-1352, 1998. DOI: 10.1016/S0093-691X(98)00081-8

40. Siqueira LGB, Torres CAA, Amorim LS, Souza ED, Camargo LSA, Fernandes CAC, Viana JHM: Interrelationships among morphology, echotexture, and function of the bovine corpus luteum during the estrous cycle. *Anim Reprod Sci,* 115 (1-4): 18-28, 2009. DOI: 10.1016/j.anireprosci.2008.11.009

41. Bonafos LD, Kot K, Ginther OJ: Physical characteristics of the uterus during the bovine estrous cycle and early pregnancy. *Theriogenology*, 43, 713-721, 1995. DOI: 10.1016/0093-691X(95)00014-Y

42. Griffin PG, Ginther OJ: Dynamics of uterine diameter and endometrial morphology during the estrous cycle and early pregnancy in mares. *Anim Reprod Sci,* 25, 133-142, 1991. DOI: 10.1016/0378-4320(91)90038-2

43. Ginther OJ: Tubular genitalia. **In,** Ginther OJ (Ed): Ultrasonic Imaging and Animal Reproduction. 105-128, Equiservices Publishing, Cross Plains, USA, 1998.

44. Simoes J, Almeida JC, Baril G, Azevedo J, Fontes P, Mascarenhas R: Assessment of luteal function by ultrasonographic appearance and measurement of corpora lutea in goats. *Anim Reprod Sci,* 97, 36-46, 2007. DOI: 10.1016/j.anireprosci.2006.01.006

45. Arashiro EK, Fonseca JF, Siqueira LGB, Fernandes CA, Brandao FZ, Oba E, Viana JH: Assessment of luteal function in goats by ultrasonographic image attribute analysis. *Small Ruminant Res,* 94, 176-179, 2010. DOI: 10.1016/j.smallrumres.2010.07.007

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Research Article

Identification and Molecular Characterization of *Hysterothylacium* (Nematoda: Raphidascarididae) Larvae in Bogue *(Boops boops* L.) from the Aegean Sea, Turkey [1]

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Abstract

Hysterothylacium species are the most prevalent ascaridoid nematode reported from various freshwater, estuarial and marine fish species. However, there are very few studies about molecular identification and characterization of *Hysterothylacium* species in marine fish species from Turkish waters. The aim of the present study was to molecular identification and characterization of Hysterothylacium larvae from *Boops boops* caught off Aegean Sea, Turkey. *Hysterothylacium* larvae were found in 10 of 109 *B. boops.* The prevalence of *Hysterothylacium* spp. larvae was 9.2%. A total of 10 larvae of *Hysterothylacium* spp. were collected from all infected fish. *Hysterothylacium* larvae were genetically identified as *H. aduncum, H. fabri* and *H. reliquens* from Turkish waters by using sequence analyses of rDNA ITS regions in *B. boops* for the first time. In addition, *B. boops* was reported as a new host for *H. reliquens.* Moreover, *H. reliquens* was characterized for the first time by sequencing of the ITS regions from the Turkish waters with the present study.

Keywords: *Hysterothylacium larvae, Boops boops, Molecular identification, rDNA ITS, Aegean Sea, Turkey*

Türkiye'nin Ege Denizi'nden Yakalanan Kupes *(Boops boops)* Balıklarında *Hysterothylacium* Larvalarının İdentifikasyonu ve Moleküler Karakterizasyonu

Öz

Hysterothylacium türleri çeşitli tatlı, acı su ve deniz balığı türlerinden bildirilen en yaygın ascaridoid nematodlardır. Ancak Türkiye sularındaki deniz balıklarında *Hysterothylacium* türlerinin moleküler tanımlanması ve karakterizasyonu konusunda çok az çalışma bulunmaktadır. Bu çalışmada Türkiye'nin Ege Denizi'nden yakalanan *Boops boops*'larda *Hysterothylacium* larvalarının moleküler tanımlanması ve karakterizasyonlarının belirlenmesi amaçlanmıştır. *Hysterothylacium* larvaları 109 *B. boops*'un 10'unda tespit edilmiş olup enfeksiyon oranı %9.2 olarak belirlenmiştir. Tüm enfekte balıklardan toplamda 10 adet *Hysterothylacium* larvası toplanmıştır. Türkiye sularından *B. boops*'larda ilk defa *Hysterothylacium* larvalarından rDNA ITS gen bölgesinin dizi analizleri ile *H. aduncum, H. fabri* ve *H. reliquens* türleri genetik olarak tanımlanmıştır. Ek olarak *B. boops* türü *H. reliquens* için yeni bir konak olarak rapor edilmiştir. Ayrıca, *H. reliquens* ITS gen bölgesinin sekans analizi ile Türkiye sularından ilk kez karakterize edilmiştir.

Anahtar sözcükler: *Hysterothylacium larva, Boops boops, Moleküler tanımlama, rDNA ITS, Ege Denizi, Türkiye*

INTRODUCTION

Hysterothylacium species belonging to the Raphidascarididae family are the most cosmopolitan marine ascaridoid reported as larvae in various fish species [1,2]. At present, there are approximately 70 recognizable *Hysterothylacium* species around the world [3]; however, only two species, *H. aduncum* and *H. fabri*, have been morphologically or

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molecularly described in different fish species from Turkish waters [4-10].

The bogue, *B. boops* is a demersal to semi-pelagic, non-migratory and economically important species of the Mediterranean Sea. The species is prevalent in the Eastern Atlantic, Mediterranean and Black Sea. It is gregarious, and it is found on the shelf or coastal pelagic on different bottoms at a depth range 0-350 m. *B. boops* is omnivorous, feeding mainly on benthic copepods and plants but is also planktonophagous (www.fishbase.org, version 06/2017). *Hysterothylacium* species have been already reported in *B. boops* from different regions of the world [6,11,12].

Morphologic definitions of species in Anisakidae and Raphidascarididae are still complicated and limited. Because of the similarity between the organs morphology and the existence of sibling or cryptic species of ascaridoid larvae, it is often difficult to morphologically identify the *Hysterothylacium* larvae at species level. The absence of many distinctive morphological features and lack of systematic and biological data on *Hysterothylacium* species, are the other important factors limiting the definitive species identification [13-17]. Therefore, genera of *Contracaecum* and *Hysterothylacium* had been often confused in the past [14]. Recently, using PCR and sequencing of ITS (ITS-1 and ITS-2) and 5.8S region have overcome above mentioned problems and these techniques have become absolutely necessary for the accurate or precise identification of ascaridoid nematodes [7,8,15,18-22].

Molecular data on *Hysterothylacium* genus infecting fishes from Turkish waters is still not sufficient. Therefore, with the present study, it has been aimed to specifically determine the existence of different *Hysterothylacium* species in the bogue, *B. boops* (L.) from Turkish waters, and to reveal the genetic characterization of the isolates belonging to determined species.

MATERIAL and METHODS

Fish Collection and Parasitological Examination

Parasitological examinations of *Hysterothylacium* larvae were performed on *B. boops*. A total of fresh and dead 109 samples were purchased from local fishermen between October 2016 and February 2017 caught from the Aegean Sea, Turkey (FAO zone 37.3.1). Fishes were dissected under stereomicroscope (Olympus SZX10 Tokyo, Japan) and examined for the presence of *Hysterothylacium* larvae. Nematodes were recovered from the digestive tract of *B. boops* and then washed in physiological saline and placed in 70% ethanol for molecular investigation. The morphological identification of the collected larvae specimens as *Hysterothylacium* spp. was performed according to the position of the excretory pore, the digestive systems and morphology of the tail [2,23].

PCR Amplification and Sequencing of DNA

All *Hysterothylacium* larvae from *B. boops* were analysed by molecular methods. Genomic DNA (gDNA) was isolated individually by using DNA purification kit (GeneJET Genomic DNA Purification Kit, Thermo Scientific, Waltham, MA, USA) following to the manufacturer's instructions. PCR was conducted to amplify the ITS regions using the NC5/NC2 primers [18] and cycling conditions were modified as follows: at 95°C (5 min), then 30 cycles of at 95°C (1 min), at 55°C (1 min) and at 72°C (1 min) followed by a final extension step at 72°C (5 min). Amplicons were checked on SafeView™-stained 1.5% agarose gel (Applied Biological Materials, Richmond, BC, Canada) and visualized by UV illumination (Quantum CX5, Vilber Lourmat, France). Positive PCR products were purified by using the commercial kit (High Pure PCR Product Purification Kit, Roche, Germany) and sequenced in both directions with using the same primers (NC5/NC2), (Macrogen, Amsterdam, The Netherlands).

Phylogenetic Analyses

Sequences were assembled and edited by Geneious 11.0.2 [24]. Nucleotide sequences were aligned with formerly submitted sequences of *Hysterothylacium* species in GenBank to make species-based identification using the BLASTn algorithm. Genetic distances were analyzed by using the Kimura two-parameter model (Kimura, 1980) with pairwise deletion in Mega 6.0 [25]. The aligned sequences were tested with Mega 6.0 model test to determine the most suitable DNA model according to the correct Akaike's Information Criterion (AIC) to infer the phylogenetic trees [25]. Phylogenetic analysis was conducted by using Maximum-Likelihood (ML) analysis based on Hasegawa-Kishino-Yano (HKY) +G model in PhyML [26] over the South of France Bioinformatics Platform (http://www.atgc-montpellier.fr/phyml/) with 1000 bootstrap replicates [27]. *Raphidascaris acus* (ERURacus) was used as an out group.

RESULTS

Hysterothylacium larvae were isolated from 10 *B. boops* among the total of 109 specimens with a mean prevalence of 9.2%. A total of 10 larvae belonging to *Hysterothylacium* spp. were collected from all infected fish. Each infected fish specimen carried one larva in their digestive tract. gDNAs from all collected larvae were subjected to the PCR analyses. gDNAs from the six larvae showed amplification on agarose gel electrophoresis while the gDNAs from the remaining four larvae were negative in PCR probably due to primer mismatches especially in the 3' end. The six amplified PCR products were sent to DNA sequencing for identification of *Hysterothylacium* species. The BLASTn analysis revealed the presence of three *Hysterothylacium* species. Four larvae were identified as *H. reliquens*. In addition, one was identified as *H. aduncum* and the other

was *H. fabri*. The ITS sequences of *H. reliquens*, *H. aduncum* and *H. fabri* were deposited in GenBank with the accession numbers of MF062506-09, MF062510 and MF062511, respectively.

Phylogenetic tree clearly indicated that all observed *Hysterothylacium* isolates clustered together with same species in monophyletic groups supporting of high bootstrap values over 92% *(Fig. 1)*. *H. aduncum* ERU-H. adun isolate (MF062510) was clustered together with the different geographical isolates of *H. aduncum*. ERU-H. adun isolate from the Aegean Sea, Turkey showed 100% similarity with the isolates of *H. aduncum* reported from Turkey (JX413596-97), Croatia (JQ934882-83), Denmark (JX845135-KU306719), Greenland (KT852549) in GenBank, while it showed 99.2% to 99.5% similarity with some other isolates reported from Eastern Mediterranean Sea, Turkey (KJ748530-31-32). *H. fabri* ERU-H.fab isolate determined as 100% similar with MS003, MS023, MS036 recovered from Italy (KU948632-35-36) and it showed 99.1% similarity with

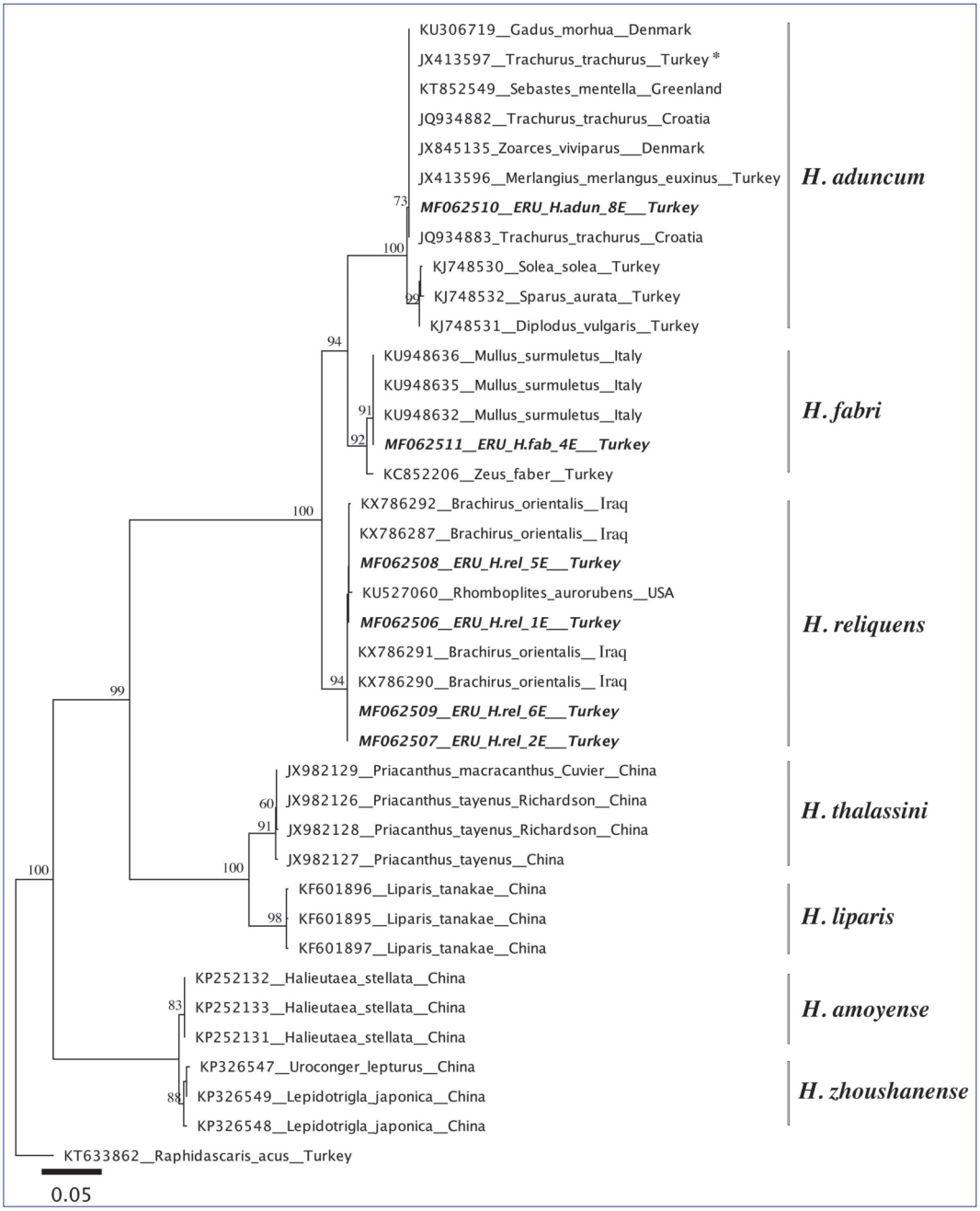

Fig 1. Phylogenetic relationships between *H. aduncum, H. fabri, H. reliquens* and other previously recorded species of *Hysterothylacium* in GenBank as inferred by maximum-likelihood (ML) analysis of ITS regions and 5.8S. *Raphidascaris acus* was used as outgroup taxa. The isolates were given with GenBank accession numbers, hosts and countries. The isolates obtained with the study were shown as bold italic character and other isolates from Turkey were labelled with asterisk. The scale bar represents 0.05% divergence

a recorded isolate (KC852206) from Mediterranean Sea, Turkey. Moreover, the ITS gene sequences of *H. reliquens* ERU-H.rel (MF062506-09) have been obtained for the first time from Turkish waters and no intraspecific nucleotide diversity was observed in the ITS region. ERU-H.rel isolates also showed 99.9% to 100% identity with Arabian Gulf off Basrah, Southern Iraq (KX786287-90-91-92) and Gulf of Mexico, USA (KU527060) isolates. Interspecific genetic differences were determined as 3.8% to 27.2% among the species indicated in *Fig. 1*. Pairwise genetic distance between ERU-H.adun (MF062510) and Sa/Dv/Ss isolates (KJ748530-31-32) from the Eastern Mediterranean Sea, Turkey displayed variation ranged from 0.5% to 0.8%. Similarly, ERU-H.fab isolate (MF062511) indicated 0.9% genetic differences isolate registered in GenBank from Mediterranean Sea, Turkey (KC852206). Pairwise comparison between the *H. reliquens* (ERU-H.rel) and our other species *H. fabri* and *H. aduncum* (ERU-H.fab and ERU-H.adun) displayed 4.3-6.2% interspecific nucleotide differences, respectively.

DISCUSSION

There has been limited knowledge on the *Hysterothylacium* infections in fishes from Turkish waters. To date, only two *Hysterothylacium* species have been morphologically and molecularly reported from different fish species from Turkish waters. *H. aduncum* was found in *Merlangius merlangus euxinus, Trachurus trachurus, Gadus* sp. *Oncorhynchus mykiss, Sparus aurata, Solea solea, Diplodus vulgaris* [4-7,9,10,28-31]. *H. fabri* was identified from *Phycis phycis, Alosa fallax, Coris julis, Trachinus draco, Mullus surmeletus, B. boops, T. mediterraneus, Pagellus acerna, Squalus blainvillei, Symphodus* sp. and *Diplodus annularis* (as *Contracaecum fabri*) [6] and *Zeus faber* [8]. However, there are no molecular studies that confirm the distribution of the *H. reliquens* larvae of *B. boops* in Turkey. In the present study, larvae of *H. reliquens* infecting *B. boops* caught off the Aegean Sea, Turkey were characterized for the first time by sequencing of the rDNA ITS and *H. reliquens* is the first record for the Turkish fish parasite fauna. Moreover, *B. boops* is the new host record for larvae of *H. reliquens*.

In the present study, two previously known *Hysterothylacium* species were characterized by molecular approaches namely *H. aduncum* and *H. fabri*. The obtained sequence from *H. aduncum* (ERU-H.adun) isolate demonstrated 100% identity with previously submitted data in GenBank from (JX413596-JX413597), Croatia (JQ934882-JQ934883), Denmark (JX845135), Greenland (KT852549), Denmark (KU306719). However, ERU-H.adun isolate displayed ITS sequence variation with Sa/Dv/Ss (KJ748530-31-32) isolated from the Eastern Mediterranean Sea, Turkey (range from 0.5 to 0.8%). Similarly, *H. fabri* (ERU-H.fab) isolate showed 100% identity with MS003, MS023, MS036 isolate from Italy (KU948632-35-36), while this isolate indicated 0.9% nucleotide differences with recorded isolate from Mediterranean Sea, Turkey (KC852206). Species belonging to the family of Anisakidae and Raphidascarididae have low host specificity and large intermediate/definitive host populations. This situation can cause spread across a wide area of the world and genetic similarities to be high in these species, while host variability and minimal environmental changes may cause intraspecific genetic differences [9,22,32-34]. *H. reliquens* (ERU-H.rel) isolate showed 99.9% to 100% identity with isolates reported from Arabian Gulf of southern Iraq (KX786287-90-91-92) and Gulf of Mexico, USA (KU527060) isolates. Our isolates were compared with two isolates which only registered in GenBank based on the entire ITS fragment including (ITS-1 and ITS-2) and 5.8S sequences.

The phylogenetic analyses of the ribosomal ITS and 5.8S sequence data set indicated the monophyletic future of the all examined *Hysterothylacium* species with high bootstrap values. Genetic distance analyses also revealed no or low intraspecific genetic distance among the isolates from all examined *Hysterothylacium* species including the isolates obtained in this study. However, Pontaja et al.[17] reported insufficiency of nuclear genomic regions such as rDNA ITS1 and ITS2 in the discrimination of possible interspecific patterns of some *Hysterothylacium* larval types. Our result on *H. reliquens* indicated no intraspecific genetic distance among the obtained isolates with 100% identity to each other. The corresponding isolates also exhibited very low genetic distance to the *H. reliquens* isolates from different countries. This could be attributed to the spreading of the species belonging to the family of Anisakidae and Raphidascarididae across a wide area around the world which might lead high genetic similarities within species. On the other hand, host variability and minimal environmental changes may cause intraspecific genetic differences [9,22,32-34]. In accordance to this inference, phylogenetic tree clearly showed that the *Hysterothylacium* species identified in different fish species *(S. solea*, *S. aurata* and *D. vulgaris)* caught from different waters of Turkey [9] were grouped under different cluster and they exhibited a much more genetic difference to our ERU-H.adun isolate and some other isolates from different countries although they clustered into a monophyletic clade. We also concluded that genetic characterization based nuclear and mitochondrial gene regions with sufficient phylogenetic signal should be conducted to obtain the true identification of the same larval stages of *Hysterothylacium* species from similar geographical areas and to explore the species diversity. In accordance to this inference Shamsi et al.[16] also indicated that, even in the genetic characterization based on the ITS-1 and ITS-2 sequences of the same larval stage, some morphotypes could contain different genotypes.

In conclusion, species of *Hysterothylacium* that are difficult to describe morphologically have been genetically characterized, and three species *(H. aduncum, H. fabri,*

H. reliquens) have been identified in the present study. Findings on *H. reliquens* infecting *B. boops* have been original data contributing the Turkish fish parasite fauna and molecular epidemiology of this parasite. *B. boops* was also characterized as a new host for *H. reliquens* with this study although it has been reported from 25 fish species belonging to eight different orders [22]. Further researches using different host species from various geographical areas are necessary to understand population genetic structure of *Hysterothylacium* species from Turkish waters.

Conflict of Interest

The authors do not have any potential conflicts of interest to declare.

REFERENCES

1. Deardorff TL, Overstreet RM: Larval *Hysterothylacium* (=*Thynnascaris*) (Nematoda: Anisakidae) from fishes and invertebrates in the Gulf of Mexico. *Proc Helminthol Soc Wash*, 48, 113-126, 1981.

2. Moravec F: Parasitic Nematodes of Freshwater Fishes of Europe. 473, Academia and Kluwer, Academic Publishers, Praha and Dordrecht, Boston, London, 1994.

3. Moravec F, Justine J: Anisakid nematodes (Nematoda: Anisakidae) from the marine fishes *Plectropomus laevis* Lacépède (Serranidae) and *Sphyraena qenie* Klunzinger (Sphyraenidae) off New Caledonia, including two new species of *Hysterothylacium* Ward & Magath, 1917. *Syst Parasitol*, 92, 181-195, 2015. DOI: 10.1007/s11230-015-9597-5

4. Doganay A: A record of *Hysterothylacium aduncum* (Rudolphi, 1802) in codfish (*Gadus* sp.) from Black Sea. *Vet J Ankara Univ*, 41, 208-217, 1994.

5. Saglam N: Infection of *Hysterothylacium aduncum* (Namatoda: Anisakidae) in farmed rainbow trout *(Oncorhynchus mykiss* Walbaum, 1792). *Afr J Agric Res*, 8 (47): 5953-5957, 2013.

6. Akmirza A: Parasitic nematodes of fish in the coastal waters of Gökceada. *Türkiye Parazitol Derg*, 37, 199-202, 2013. DOI: 10.5152/tpd.2013.44

7. Pekmezci GZ, Bolukbas CS, Gurler AT, Onuk EE: Occurrence and molecular characterization of *Hysterothylacium aduncum* (Nematoda: Anisakidae) from *Merlangius merlangus euxinus* and *Trachurus trachurus* off the Turkish coast of Black Sea. *Parasitol Res*, 112, 1031-1037, 2013. DOI: 10.1007/s00436-012-3227-9

8. Pekmezci GZ, Yardimci B, Onuk EE, Umur S: Molecular characterization of *Hysterothylacium fabri* (Nematoda: Anisakidae) from *Zeus faber* (Pisces: Zeidae) caught off the Mediterranean coasts of Turkey based on nuclear ribosomal and mitochondrial DNA sequences. *Parasitol Int*, 63, 127-131, 2014. DOI: 10.1016/j.parint.2013.10.006

9. Keskin E, Koyuncu CE, Genc E: Molecular identification of *Hysterothylacium aduncum* specimens isolated from commercially important fish species of Eastern Mediterranean Sea using mtDNA cox1 and ITS rDNA gene sequences. *Parasitol Int*, 64, 222-228, 2015. DOI: 10.1016/j.parint.2014.12.008

10. Ozer A, Kornyychuk YM, Yurakhno V, Öztürk T: Seasonality and host-parasite interrelationship of *Hysterothylacium aduncum* (Nematoda) in whiting *Merlangius merlangus* off the southern and northern coasts of the Black Sea. *Helminthologia*, 53 (3): 248-256, 2016. DOI: 10.1515/helmin-2016-0025

11. Ichalal K, Ramdane Z, Ider D, Kacher M, Iguerouada M, Trilles JP, Courcot L, Amara R: Nematodes parasitizing *Trachurus trachurus* (L.) and *Boops boops* (L.) from Algeria. *Parasitol Res,* 114 (11): 4059-4068, 2015. DOI: 10.1007/s00436-015-4633-6

12. Benhamou F, Marzoug D, Boutiba Z, Kostadinova A, Perez-Del-Olmo A: Parasite communities in two sparid fishes from the western Mediterranean: A comparative analysis based on samples from three localities off the Algerian coast. *Helminthologia*, 54 (1): 26-35, 2017. DOI: 10.1515/helm-2017-0003

13. Moravec F: Experimental studies on the development of *Contracaecum rudolphii* (Nematoda: Anisakidae) in copepod and fish paratenic hosts. *Folia Parasitol*, 56, 185-193, 2009. DOI: 10.14411/fp.2009.023

14. Lopes LPC, Pimpão DM, Takemoto RM, Malta JCO, Varella AMB: *Hysterothylacium* larvae (Nematoda, Anisakidae) in the freshwater mussel *Diplodon suavidicus* (Lea, 1856) (Mollusca, Unioniformes, Hyriidae) in Aripuanã River, Amazon, Brazil. *J Invertebr Pathol*, 106, 357-359, 2011. DOI: 10.1016/j.jip.2010.12.002

15. Li L, Liu YY, Zhang LP: Morphological and molecular identification of *Hysterothylacium longilabrum* sp. nov. (Nematoda: Anisakidae) and larvae of different stages from marine fishes in the South China Sea. *Parasitol Res*, 111, 767-777, 2012. DOI: 10.1007/s00436-012-2897-7

16. Shamsi S, Gasser R, Beveridge I: Description and genetic characterisation of *Hysterothylacium* (Nematoda: Raphidascarididae) larvae parasitic in Australian marine fishes. *Parasitol Int*, 62, 320-328, 2013. DOI: 10.1016/j.parint.2012.10.001

17. Pantoja CS, Pereira FB, Santos CP, Luque JL: Morphology and molecular characterization hold hands: Clarifying the taxonomy of *Hysterothylacium* (Nematoda: Anisakidae) larval forms. *Parasitol Res*, 115, 4353-4364, 2016. DOI: 10.1007/s00436-016-5221-0

18. Zhu XQ, Gasser RB, Podolska M, Chilton NB: Characterization of anisakid nematodes with zoonotic potential by nuclear ribosomal DNA sequences. *Int J Parasitol*, 28, 1911-1921, 1998. DOI: 10.1016/S0020-7519(98)00150-7

19. Jahantab M, Haseli M, Salehi Z: Morphological and genetic characteristics of the anisakid nematode *Raphidascaris acus* from the Southwest Caspian Sea: Evidence for the existence of sibling species within a species complex. *Parasitol Res*, 113, 3419-3425, 2014. DOI: 10.1007/s00436-014-4007-5

20. Abdel-Ghaffar F, Abdel-Gaber R, Bashtar AR, Morsy K, Mehlhorn H, Al Quraishy S, Saleh R: *Hysterothylacium aduncum* (Nematoda, Anisakidae) with a new host record from the common sole *Solea solea* (Soleidae) and its role as a biological indicator of pollution. *Parasitol Res,* 114, 513-522, 2015. DOI: 10.1007/s00436-014-4213-1

21. Simsek E, Ciloglu A, Aypak S, Yildirim A, Aldemir OS, Unlu AH, Pekmezci GZ: First molecular characterization of *Raphidascaris acus* Bloch, 1779 (Nematoda: Anisakidae) from European eels *(Anguilla anguilla* Linnaeus, 1758) caught off the Aegean Region streams, Turkey. *Kafkas Univ Vet Fak Derg*, 22 (4): 529-532, 2016. DOI: 10.9775/kvfd.2016.14980

22. Zhao JY, Zhao WT, Ali AH, Chen HX, Li L: Morphological variability, ultrastructure and molecular characterisation of *Hysterothylacium reliquens* (Norris & Overstreet, 1975) (Nematoda: Raphidascarididae) from the oriental sole *Brachirus orientalis* (Bloch & Schneider) (Pleuronectiformes: Soleidae). *Parasitol Int*, 66, 831-838, 2017. DOI: 10.1016/j.parint.2016.09.012

23. Petter AJ, Maillard C: Larves d'ascarides parasites de poissons en Méditerranée 416 occidentale. *Bull Mus Natl Hist Nat Paris 4 Sér*, 10 [A]: 347-369, 1988.

24. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A: Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics,* 28, 1647-1649, 2012. DOI: 10.1093/bioinformatics/bts199

25. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S: MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol*, 30, 2725-2729, 2013. DOI: 10.1093/molbev/mst197

26. Guindon S, Delsuc F, Dufayard JF, Gascuel O: Estimating maximum likelihood phylogenies with PhyML. *Methods Mol Biol*, 537, 113-137, 2009. DOI: 10.1007/978-1-59745-251-9_6

27. Felsenstein J: Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39, 783-791, 1985. DOI: 10.1111/j.1558-

5646.1985.tb00420.x

28. Ismen A, Bingel F: Nematode infection in the whiting *Merlangius merlangus euxinus* off Turkish coast of the Black Sea. *Fish Res*, 42, 183-189, 1999. DOI: 10.1016/S0165-7836(99)00022-3

29. Keser R, Bray RA, Oguz MC, Celen S, Erdogan S, Doguturk S, Aklanoğlu G, Marti B: Helminth parasites of digestive tract of some teleost fish caught in the Dardanelles at Canakkale, Turkey. *Helminthologia*, 44, 217-221, 2007. DOI: 10.2478/s11687-007-0035-3

30. Kalay M, Donmez AE, Koyuncu CE, Genc E, Sahin G: Seasonal variation of *Hysterothylacium aduncum* (Nematoda: Raphidascarididae) infestation in sparid fishes in the Northeast Mediterranean Sea. *Turk J Vet Anim Sci*, 33, 517-523, 2009. DOI: 10.3906/vet-0703-12

31. Pekmezci GZ: Metazoan parasite fauna of rainbow trout (*Oncorhynchus mykiss*) reared in Samsun Province, Turkey. *PhD Thesis,* University of Ondokuz Mayis, Samsun, Turkey, 2010.

32. Kellermanns E, Klimpel S, Palm HW: Molecular identification of ascaridoid nematodes from the deep-sea onion-eye grenadier *(Macrourus berglax)* from the East Greenland Sea. *Deep-Sea Res Pt I*, 54, 2194-2202, 2007. DOI: 10.1016/j.dsr.2007.09.001

33. Mattiucci S, Abaunza P, Damiano S, Garcia A, Santos MN, Nascetti G: Distribution of *Anisakis* larvae identified by genetic markers and their use for stock characterization of demersal and pelagic fish from European waters: An update. *J Helminthol*, 81, 117-127, 2007. DOI: 10.1017/S0022149X07754718

34. Mattiucci S, Nascetti G: Advances and trends in the molecular systematics of anisakid nematodes, with implications for their evolutionary ecology and host-parasite co-evolutionary processes. *Adv Parasitol*, 66, 47-148, 2008. DOI: 10.1016/S0065-308X(08)00202-9

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Research Article

[^{68}Ga]Ga-AntiCAD1: Radiosynthesis and First Imaging Study on Rats

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Abstract

Cadherins are cell adhesion and cell signaling molecules that provide the molecular link between each adjacent cells and have critical importance for the initiation and continuation of adhesion mechanism. N-cadherin expression provides a correlation between upregulation of N-cadherin and inflammation of the lesions. In this paper, we concentrated on the radiolabeling and evaluation of [^{68}Ga]Ga-AntiCAD1 agent as a potential candidate for *in vivo* PET/CT imaging of adhesions. The synthetic N-Ac-CHAVC-NH_2 cyclic peptide sequence designed as (ADH-1)c containing the selective binding His-Ala-Val (HAV) motif based on the chimeric antigen receptor sequence acts as the N-cadherin antagonist. In our previous study, AntiCAD1 conjugate has been studied in detail which is in the process of publication. In this study, the conjugate was radiolabelled with the [^{68}Ga]Ga radionuclide eluted from the ^{68}Ge/^{68}Ga generator (IDB Holland). Radiochemical purity of [^{68}Ga]Ga-AntiCAD1 agent was analysed with TLC methods. The 'shake-flask' method was applied to determine lipophilicity of the agent by calculating the P distribution coefficient (logP=-2.69±0.54). The biodistribution of the agent was investigated using PET/CT on Wistar Albino rats. Significant uptake was found in liver, kidneys, spleen, salivary gland and targeted region with $SUV_{max-mean}$ of 1.36, 1.96, 1.38, 1.16 and 2.14 respectively. The Pearson Factorial method is used to test the relationship between the targeted region and other body tissues, to measure the degree of this relationship (R=0.73). Radiolabelled agent was demonstrated to react specifically with N-cadherin in targeting of rat tissues.

Keywords: *AntiCAD1 agent, Peptide radiopharmaceuticals, PET/CT imaging, N-cadherin expression*

[^{68}Ga]Ga-AntiCAD1: Radyosentez ve Sıçanlar Üzerinde İlk Görüntüleme Çalışması

Öz

Kadherinler, bitişik hücreler arasındaki moleküler bağlantıyı sağlayan, yapışma mekanizmasının başlatılması ve devamı için kritik öneme sahip olan hücre adezyon ve hücre sinyal molekülleridir. N-kadherin ekspresyonu, N-kadherinin artışı ve lezyonların inflamasyonu arasında bir korelasyon sağlar. Bu makalede, adezyonların *in vivo* PET/CT görüntülenmesi için potansiyel bir aday olarak [^{68}Ga]Ga-AntiCAD1 ajanının radyoişaretlenmesi ve değerlendirmesine odaklanılmıştır. Kimerik antijen reseptörü dizisine dayanan seçici bağlayıcı His-Ala-Val (HAV) motifini içeren (ADH-1)c olarak tasarlanan sentetik N-Ac-CHAVC-NH_2 siklik peptid dizisi, N-kadherin antagonisti olarak işlev görür. Yayın aşamasındaki önceki çalışmamızda AntiCAD1 konjugatı ayrıntılı olarak incelenmiştir. Bu çalışmamızda, konjugat, ^{68}Ge/^{68}Ga jeneratöründen (IDB Holland) elde edilen [^{68}Ga]Ga radyonüklidi ile radyoaktif olarak işaretlendi. [^{68}Ga]Ga-AntiCAD1 ajanının radyokimyasal saflığı TLC metodları ile analiz edildi. Ajanın lipofilisitesini belirlemek için P dağılım katsayısı hesaplanarak 'çalkalama şişesi' yöntemi uygulandı (logP=-2.69±0.54). Ajanın biyodağılımı, Wistar Albino sıçanları üzerinde PET/CT kullanılarak araştırıldı. Karaciğer, böbrekler, dalak, tükürük bezi ve hedef bölgede sırasıyla ortalama 1.36, 1.96, 1.38, 1.16 ve 2.14 SUV_{max} değerleri ile kayda değer oranda tutulum tespit edildi. Hedeflenen bölge ile diğer vücut dokuları arasında olan ilişkini test etmek ve bu ilişkinin derecesini ölçmek için istatistiksel Pearson Faktörü hesaplama yöntemi kullanılmıştır (R=0.73). Sıçan dokularının hedeflenmesinde radyoişaretli ajanın N-kadherin ile spesifik olarak etkileşime girdiği gösterildi.

Anahtar sözcükler: *AntiCAD1 ajanı, Peptid radyofarmasötikleri, PET/CT görüntüleme, N-kadherin ekspresyonu*

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INTRODUCTION

Cadherins are cell surface glycoproteins responsible for morphogenesis in the embryo, selective cell recognition in the adult organism, and lifelong normal tissue architecture. Adherens junctions which are essential for the formation and maintenance of functional cellular junctions are composed of cadherins [1]. Cadherins are cell adhesion and cell signaling molecules that provide the molecular link between adjacent cells and have critical importance for the initiation and continuation of adhesion mechanism. It differs from the other members of the cadherin group compounds with a large extracellular N-terminal consisting of repeating domains and important Ca^{++} binding, and a single transmembrane region linked to the cytoplasmic region. N (Neural)-cadherin is presented in mesenchymal tissues, in endothelial cells and found in adhesive complexes that cover them between endothelial cells and pericytes [2-4]. N-cadherin antagonists which are expressed in some tumor cells and blood vessels target N-cadherin. The re-expression of N-cadherin in endothelial cells is accompanied by neovascularization. There was a correlation between upregulation of N-cadherin and inflammation events by indicating it in the enlarged blood vessels of the inflamed tissue of the lesions [5]. It has been shown that the synthetic N-Ac-CHAVC-NH_2 cyclic peptide sequence designed as ADH-1 which contains the selective binding HAV (His-Ala-Val) motif based on the CAR(Chimeric Antigen Receptors)sequence acts as the N-cadherin antagonist [6]. Cyclic (ADH-1)c peptide analogue has the ability to disrupt many processes through N-cadherin, as well as the linear peptide [7]. There are studies on targeting of N-cadherin expression in order to understand the mechanism of many different cancer types and the functions of proximal renal tubular epithelial cells [8-10]. In this study, AntiCAD1 agent was targeted to the N-cadherin receptor in epithelial tissue adhesion. The data based on the positron emission tomographic imaging-computed tomography (PET/CT) which is the 'gold standard' in imaging was obtained by the combination of metabolic chemical activity of the agent and anatomic information in rat organism. PET imaging provides detailed information about the results of metabolic activities and cellular functions. Peptides labelled with a positron-emitting radionuclide can be used to make an accurate diagnosis, determine staging and quantify the radiation dose to tumors and critical organs, thus allowing dose planning and dose monitoring for successful radiotherapy, and to follow target response to therapy, thus providing personalized patient management [11-13]. The compatibility of [^{68}Ga]Ga with peptides for targeted imaging of such receptors as somatostatin, bombesin, human epidermal growth factor, integrin, vascular endothelial growth factor, cholecystokinin-2, gastrin-releasing peptide, melanocyte stimulation hormone, glucagon-like peptide 1, gonadotropin-releasing hormone, folate, neurotensin, and neuropeptide Y receptors has been demonstrated [12,13]. The corresponding ligands commonly have satisfactory pharmacokinetics with fast blood clearance, relatively low hepatobiliary excretion, and mostly renal elimination as well as excellent tissue penetration, minimal side-effects, and no or low antigenicity.Imaging of vascular endothelial growth factor (VEGF) receptors overexpressed in tumors, adhesive and violated tissues(by injury or surgical intervention) that have extensive formation of new capillaries is an important means not only for diagnosis and monitoring response therapy but also for antiangiogenic drug development.

MATERIAL and METHODS

The cyclic (ADH-1)c peptide analogue was obtained in our laboratory with using CEM Liberty microwave assisted automatic peptide synthesis system and applying cyclization procedure, respectively. EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride], EDTA (Ethylenediaminetetraacetic acid) and all other chemicals purchased from Sigma Aldrich Chemicals (St. Louis), USA. Ultra-pure water was obtained from Millipore Milli-Q system.

Koc University National Ethics Committee for Animal Experiments gave approval for the animal experiments (Protocol no: 2016.HADYEK.018, 02/06/2016 dated).

Preparation of Cyclic (ADH-1)c Peptide-EDTA Conjugate

Peptide synthesis and conjugation with EDTA have been studied in detail in our previous study, which is in the process of publication. Briefly, the linear ADH-1 peptide sequence (Ac-Cys-His-Ala-Val-Cys-$CONH_2$) was synthesized using the Fmoc chemistry in the CEM Liberty Microwave Assisted Solid Phase Peptide Synthesis System. The peptide precipitated as a white powder was stored at -18°C for cyclization [14-16]. The cyclization process is based on the procedure described by Dubey et al.[17]. A disulfide bridge was formed between the two cysteines by oxidation of the thiol groups of the cysteine residues.After the process was complete, the cyclic peptide was lyophilized [17]. Conjugate of the cyclic (ADH-1)c peptide with EDTA chelator was synthesized by the water-soluble carbodiimide procedure in the presence of the EDC crosslinker [18-20]. LC-MS m/z for cyclic(ADH-1)c peptide$[M+H^+]^+$=572.85 and EDTA-(ADH-1) c conjugate (AntiCAD1 conjugate) $[M+H^+]^+$=867.

Elution of ^{68}Ge/^{68}Ga Generator

The ^{68}Ga radionuclide ($t_{1/2}$ = 67.7 min., β^+ = 89%, E β^+maks, 1.9 MeV, EC: 11%, Eγmaks: 4.0 MeV) was manually eluted from the SnO_2-based ^{68}Ge/^{68}Ga generator (IDB Holland iThemba Lab. Generator, 30mCi) using a disposable syringe with 15 mL of 0.6 M HCl acid and collected into a vial containing 1 mL of9.5 MHCl acid. The resulting ^{68}Ga solution was subjected to cationic pre-purification to bring it to levels that would allow labeling with

peptides. Approximately 1.5 mL of 15 mCi [^{68}Ga]Ga was obtained.

Radiolabelling of AntiCAD1 Conjugatewith [^{68}Ga]Ga Radionuclide

To carry out the labeling with the [^{68}Ga]Ga radionuclide eluted from the ^{68}Ge/^{68}Ga generator, after transferring the 30 µL AntiCAD1 conjugate to the reaction vial, 1.5 µM HEPES buffer was added and pHadjusted to 4. Immediately thereafter, 1.5 mL of eluted [^{68}Ga]Ga was added and the reaction was carried out in a microwave oven under nitrogen and dry air atmosphere at 125 degrees centigrade for 6 min. At the end of the reaction, the radiopharmaceutical labeling rate of the product *(Fig. 1)* was evaluated by adding 15 mL of PBS buffer to the reaction vial.

TLC for Radiochemical Purity Analysis of [^{68}Ga]Ga-AntiCAD1 Agent

Product purity was analyzed by TLC on iTLC-SG plates: Labeling rate of [^{68}Ga]Ga-AntiCAD1 agent were studied with three different mobile phase solutions [Solution A; Isopropil alcohol:n-butanol:0.2 N amonnium hydroksit (2:1:1 (v:v:v)), Solution B; n-butanol:water:acetic acid(4:2:1 (v:v:v)) and Solution C; TFA 0.1%:MeOH (30:70 (v:v))]. Solution A, B and C were added to the chromatography tanks. Ten µL of the synthesized product was dropped onto the sample spot in the start line of each chromatographic strip. The solutions were run to solvent line. Each strip was allowed to dry at room temperature. The drying strips were cut to equal lengths. The activity value of each piece was read in a gamma counter (Biodex, Atom Lab 500).

Rf and dose values were calculated for each parts. Quantitative analysis of radioactive spots were done to determine the amount of radiolabelled agent and unchelated radiometal [21,22].

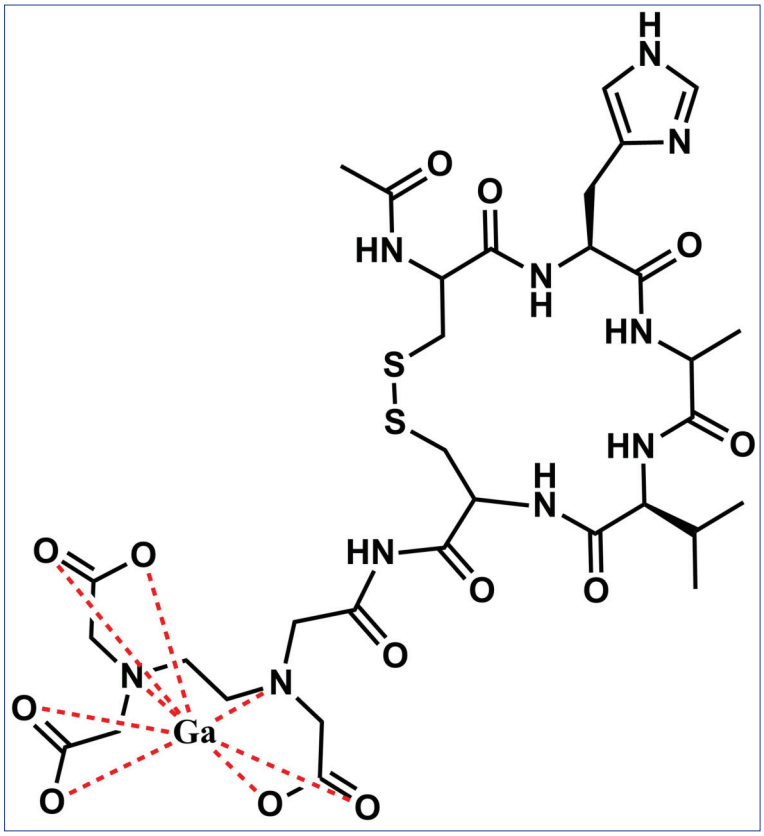


Fig 1. Molecular structure of [^{68}Ga]Ga-AntiCAD1 agent

Lipophilicity

Lipophilicity to drug development is an important early indicator of potential *in vivo* pharmacokinetic and dynamic behavior. Lipophilicity measurements provide information on non-specific binding, metabolic stability, drug distribution and excretion [23,24]. Because of this diversity, lipophilicity is one of the most important physicochemical properties and most frequently analyzed and published parameters [23,25]. The'shake-flask' method is the 'gold standard' for determining the logP value [25-28]. While lipophilic peptides are excreted through the liver and intestines (hepatobiliary excretion), hydrophilic peptides are primarily excreted through the kidneys (renal excretion). Radiolabeled peptides are usually highly hydrophilic and therefore do not show significant accumulation in the hepatobiliary system. However, peptide lipophilicity is increased by labeling through prosthetic groups [29]. In our study, the shake-flask method was applied to determine lipophilicity. Lipophilicity values were determined by calculating the P distribution coefficient ($[A]_o/[A]_w$). One hundred µL of the radiolabelled compound was added to the tube containing 3 mL of n-octanol and 3 mL of water. The mixture was stirred with magnetic stirrer for 1 h. It was centrifuged at 3000 rpm for 5 min to completely separate the phases. Activity values were measured in the gama counter by pipetting 500 µL from both phases. LogPvalue was calculated by the logarithm of the ratio between the radioactive counts in the octanol and water fractions.

Animal Studies

Metabolic functions of the agent applied on Wistar Albino rats were studied withPET/CT (General Electric Healthcare/Discovery 710) and Gama Kamera/SPECT(Siemens) equipped with a low energy, high-resolution collimator under a protocol approved by Koç University Animal Experiments Local Ethics Committee. Ratswerefasted for four hours before the each intervention. All interventional and imaging studies were performed under anesthetize by using isoflurane without a vaporizer. The liquid anesthetic was applied to cotton which was

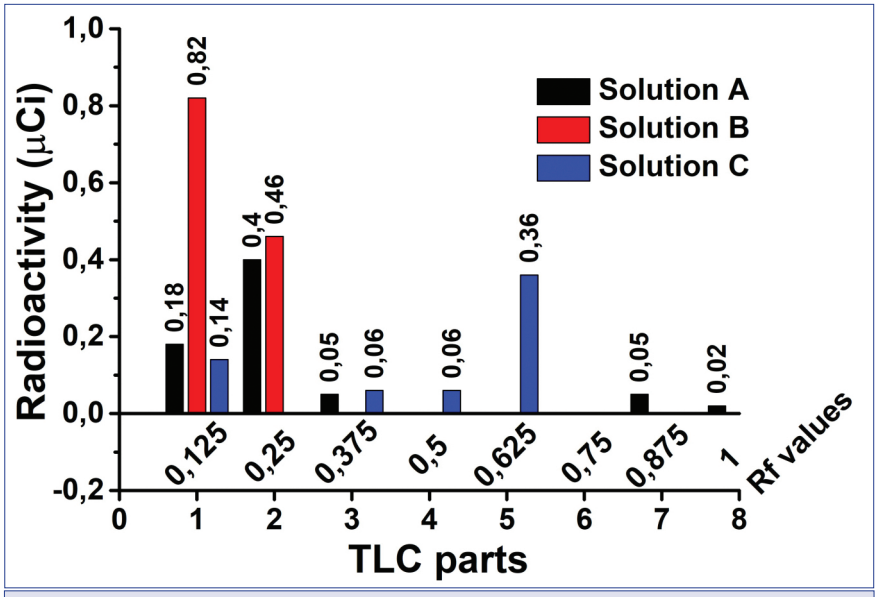


Fig 2. Rf values of relevant complex region

Table 1. *The binding ratios of the radioactive compounds on the TLC*

Substance	Solvent A	Solvent B	Solvent C
[^{68}Ga]Ga-AntiCAD1	57.14%	64.06%	58.06%
[^{68}Ga]Ga-compounds	25.71%	35.94%	22.58%

placed in a conical tube for maintenance of anesthesia. Because of the high risk of waste anesthetic gas exposure, this method was performed in a special biosafety cabinet with a carbon filter.

RESULTS

Radionuclide Purity Assay with Radio-TLC

Product purity was analyzed by iTLC-SG plates using TLC with three different mobile phases.The Rf values calculated for each activity value read in the gamma counter are shown in *Fig. 2*. The un-chelated metalalso seen in the first part (Rf=0.125) for the three solution types, the second part for solution A and solution B, the fifth part for the solution C and a higher Rf value. The impurities in the solution are migrated to the upper region due to the chemical structure (solubility and polarity) of solution A. The relatively high radioactivity value of the first part in Solution B is due to the fact that solution B has activity in the same part as the un-chelated metal without dragging impurities. The un-chelated metal Rf values and doses measured in the first part in solution A and solution C support each other. The ratios of labelled analytes and unchelated metals are shown in *Table 1*.

Scintigraphic and Tomographic Studies

[^{99}Tc^{m}]pertechnetate and[^{177}Lu]Lu-PSMAPre-Imaging Studies with Gamma Camera: Pre-imaging study was carried out for the purpose of reference imaging. The studies were performed on Wistar Albino rat by using agents which are routinely administered in Nuclear Medicine and Molecular Imaging Department except our new agent. Biodistribution of the new agent in rat tissues and organs can be investigated more sensitively and accurate by using reference imaging. In addition, the amount of dose to be administered to the Wistar Albino rat is determined in advance so that the animal is prevented from overdosing.

Scintigraphic images were obtained from Siemens Gamma Camera/ SPECT. Prior to the injection of the radio-pharmaceutical agent, [^{99}Tc^{m}] pertechnetate and [^{177}Lu]Lu-PSMA injections were performed at physiological pH at different times. Many preimages were obtained by the application of intravenous injection of radiolabeled agentsto the Wistar Albinoratwhichuptakeandaccumulation regions in physiological tissue is previously known.

Gamma camera images were examined at 5th, 10th, 15th, 30th, 60th, 120th, 180th, 240th min after intraperitonal injection by using 0.1 mCi pertechnetate. In the first 10 min, stomachuptake, that is the injection site and thyroid uptake, a selective target for the pertechnetate were displayed. At the 15th min, the [^{99}Tc^{m}] pertechnetate accumulation in bladder started and after the 120 min the intestinal accumulation was viewed. In this way, thyroid, intestine, stomach and bladder regions were determined in the ratas shown in *Fig. 3*.

Gamma camera images were investigated at 10th, 60th, 120th, 180th min and 24th h after intravenous injection from the tail vein with 0.1 mCi [^{177}Lu]Lu-PSMA at physiological pH. *Fig. 4* indicates that as the injection region tail vein and epididymis-testis region accumulation, right-left kidney uptake and bladder-prostate region accumulation were displayed at the 10th min imaging. It is understood from the first hour image that the accumulation in the injection site decreases while uptake increases in the other regions. At the second and third hour's of imaging, left-right kidney uptake which is selective to the prostate-specific membrane agent, and bladder-prostate area accumulation were displayed. At the 24th h, right and left kidney uptake was displayed intensely. After all; right-left kidney, urethral continence zone-prostate, testis-epididymis and tail vein regions were determined with [^{177}Lu]Lu-PSMA injection.

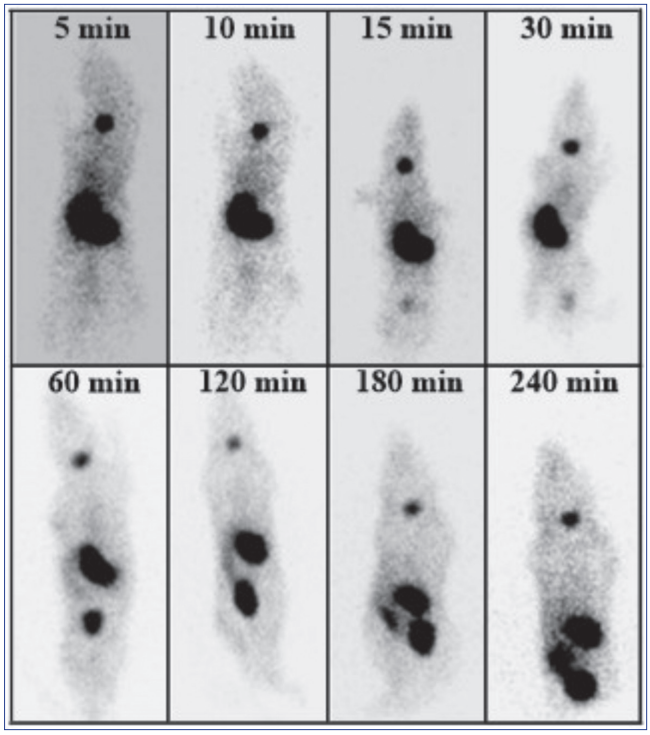


Fig 3. Gamma camera images at 5th, 10th, 15th, 30th, 60th, 120th, 180th, 240th min after intraperitoneal injection of rat with using[$^{99}Tc^{m}$] pertechnetate

PET/CT Adhesion Imaging Studies of [^{68}Ga]Ga-AntiCAD1 Agent: [^{68}Ga]Ga-AntiCAD1 consisting of five amino acids was applied to display adhesion molecules in violated tissue via specific binding of bioconjugate to the N-cadherin. The distribution of this agent was studied inthe Wistar Albino rat organism by using PET/CT. Tomographic images given in *Fig. 5* were obtained from GE Healthcare/Discovery 710 PET/CT. In the study with left hand injuried rat as shown in *Fig. 6*, administration of 0.1 mCi[^{68}Ga]Ga-AntiCAD1 at physiological pH was performed by intravenous tailinjection. Increased uptake of the [^{68}Ga] Ga-AntiCAD1agent in the injuried adhesive left hand was detected. When PET/CT images of the 1st and 3rd h were examined besides the intense kidney and bladder uptake as well as synthesized radiopharmaceutical agent there was an expected selective uptake in the targeted region.

Correlation of PET/CT Standardized Uptake Mesaurement Values: The binding specificity was detected with the increased uptake of the [^{68}Ga]Ga-AntiCAD1 agent in the injuried adhesive left hand.In other tissues the uptake of the agent was low. The statistical method of Pearson Factor was used to test the relationship betweenthe targeted region and other body tissues, to measure the degree of this relationship and to predict similar outcomes. In this way, the degree of linear relationship for all body uptake of two continuous or parametric variables of uptake regions can be calculated. Mean standardized uptake values $(SUV)_{max\text{-}mean}$ in liver, kidneys, spleen, salivary gland and targeted region were found 1.36, 1.96, 1.38, 1.16 and 2.14 respectively. The R value was calculated to be 0.7331. This value is in the range of 0.70-0.89, indicating a strong positive correlation.

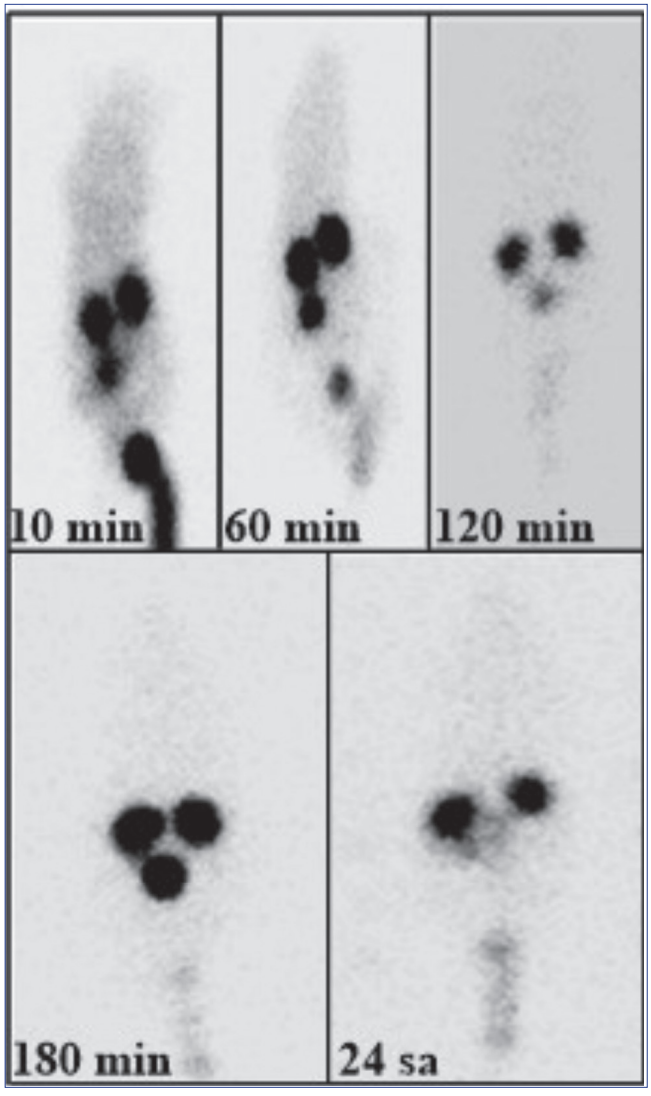


Fig 4. Gamma camera images at 10th, 60th, 120th, 180th minutes and 24 h after intravenous injection of rat with using[^{177}Lu]Lu-PSMA

Determination of Lipophilicity

The measured lipophilicity value (log P) of [^{68}Ga]Ga-AntiCAD1was calculated at pH 7 as -2.69±0.54 (n=3) by the 'shake-flask method'.Minus logP values indicate that the concentration of the labelled compounds in water is higher than its concentration in octanol. This value indicates that the targeted agent is primarily excreted by renal excretion [21,22,30].

DISCUSSION

The synthesized conjugate was labeled with [^{68}Ga] Ga radionuclide whichwas eluted from the ^{68}Ge/^{68}Ga generator and the product purity was checked by TLC on iTLC-SG plates. The results were found appropriate for

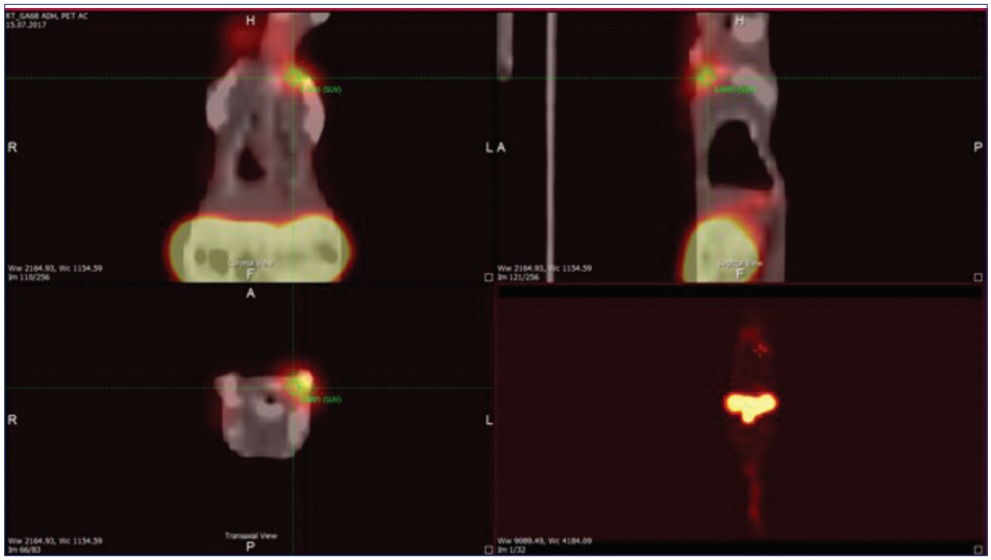
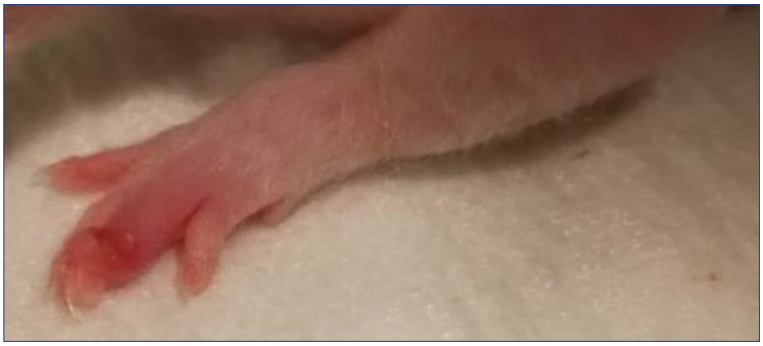


Fig 5. PET/CT adhesion imageat 60th min after intravenous tail injection of rat with using [68Ga]Ga-AntiCAD1 agent

Fig 6. Left hand injuried ratmodel



animal studies, but the labeling yield could be increased by developing different methods.

Lipophilicity study was performed for *in vitro* evaluation of radiolabelled agent. logP values emphasize that excretion is primarily eliminated through the renal clearance. Because of the low lipophilicity of the radiolabelled agent, SUV_{max} value of the kidney uptakesis higher in comparison with liver, spleen, and salivary gland uptakes. As a result, theoretical lipophilicity value (-2.69±0.54) was supported with SUV_{max} values [31,32].

Determination of the anatomical location of organs which is essential for animal imaging studies in nuclear medicine is very important in terms of evaluation of the images. The accumulation regions of [$^{99}Tc^{m}$] and [^{177}Lu]Lu-PSMA are thyroid, intestine, stomach, bladder and salivary gland, kidneys, prostate and liver respectively. The critical organ locations of the animal were fully identified by the uptake of these regions by scintigraphy studies.

PET/CT images were obtained via tail injection of the radiolabelled AntiCAD1 agent. The first and third hours of PET/CT images were analyzed bycalculating SUV_{max}within the scope of tomographic study. In this study, we investigated injury-induced disruption of the N-cadherin in adhesive tissue and it was sufficient to maintenance of functional cellular junctions. The uptakes of the [68Ga]Ga-AntiCAD1 agent in the liver, spleen, kidney, salivary gland and adhesive tissue were compared on the basis of SUV_{max} values and the uptake in the targeted region was found to be higher. This result is important for the protection of non-target healthy organs. Radiolabeled agent was demonstrated to react specifically with N-cadherin in targeting of rat tissues or selectively targeted in the injured adhesive tissue. The degree of the linear relationship between the uptake regions and all body uptake was calculated by the Pearson factorial method. The R value (0.7331) indicates that the uptake in the target region correlates with organ uptakes.

Although N-cadherin expression and targeting have been studied, there is no information available about the metabolic chemical activity that results from the specific targeting of this receptor.In this context, PET/CT provides detailed information on metabolic activity and the results of cellular functions. In addition, at this moment injured tissues and focus of infection can not be detected with PET/CT and there is not any common specific PET agent used in the imaging of all solid tumor types. The targeted agent may be a new strong candidate as radiopharmaceutical agent because of its less hepatic uptake than prostate-specific new generation peptidic

agent DKFZ-1007-PSMA, less urine excretion than DCFPyL and through protection feature of healthy tissues [31]. As a result, radiolabeled agent derived from the cyclic (ADH-1)c peptide analogue which is an adhesion molecule from cancer epithelial mesenchymal transformation markers may be a potential compound that can be used to discrimination of damaged mesenchymal tissues, determination of metastatic solid tumors anddetection of focus of infection by using PET/CT imaging technique.

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Conflicts of Interest

The authorsdeclare that they have no conflict of interest.

REFERENCES

1. Citi S: The molecular organization of tight junctions. *J Cell Biol,* 121 (3): 485-489, 1993. DOI: 10.1083/jcb.121.3.485

2. Gerhardt H, Wolburg H, Redies C: N-cadherin mediates pericytic-endothelial interaction during brain angiogenesis in the chicken. *Dev Dyn,* 218 (3): 472-479, 2000. DOI: 10.1002/1097-0177(200007)218:3<472::AID-DVDY1008>3.0.CO;2-#

3. Blaschuk OW, Devemy E: Cadherins as novel targets for anti-cancer therapy. *Eur J Pharmacol,* 625 (1-3): 195-198, 2009. DOI: 10.1016/j.ejphar.2009.05.033

4. Fosgerau K, Hoffmann T: Peptide therapeutics: Current status and future directions. *Drug Discov Today,* 20 (1): 122-128, 2015. DOI: 10.1016/j.drudis.2014.10.003

5. Bobryshev YV, Cherian SM, Inder SJ, Lord RSA: Neovascular expression of VE-cadherin in human atherosclerotic arteries and its relation to intimal inflammation. *Cardiovasc Res,* 43 (4): 1003-1017, 1999. DOI: 10.1016/S0008-6363(99)00125-X

6. Blaschuk OW, Gour BJ: McGill University. Compounds and methods for modulating cell adhesion. Canada US6169071B1. 2006.

7. Blaschuk OW, Gour BJ, Farookhi R, Ali A, George S: Adherex Technologies Inc. Compounds and methods for modulating endothelial cell adhesion. CanadaUS20060183884A1. 2003.

8. Mariotti A, Perotti A, Sessa C, Rüegg C: N-cadherin as a therapeutic target in cancer. *Expert Opin Investig Drugs,* 16 (4): 451-465, 2007. DOI: 10.1517/13543784.16.4.451

9. Keller C, Kroening S, Zuehlke J, Kunath F, Krueger B, Goppelt-Struebe M: Distinct mesenchymal alterations in N-cadherin and E-cadherin positive primary renal epithelial cells. *PLoS One,* 7 (8): e43584, 2012. DOI: 10.1371/journal.pone.0043584

10. Nieman MT, Prudoff RS, Johnson KR, Wheelock MJ: N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. *J Cell Biol,* 147 (3): 631-644, 1999. DOI: 10.1083/jcb.147.3.631

11. Lundqvist H, Tolmachev V: Targeting peptides and positron emission tomography. *Biopolymers,* 66 (6): 381-392, 2002. DOI: 10.1002/bip.10348

12. Varshney R, Hazari PP, Fernandez P, Schulz J, Allard M, Mishra AK: ^{68}Ga-labeled bombesin analogs for receptor-mediated imaging. **In,** Baum RP, Rösch F (Eds): Theranostics, Gallium-68, and Other Radionuclides: A Pathway to Personalized Diagnosis and Treatment. 211-256, Springer, Berlin, 2013.

13. Velikyan I: The diversity of 68Ga-based imaging agents. **In,** Baum RP, Rösch F (Eds): Theranostics, Gallium-68, and Other Radionuclides: A Pathway to Personalized Diagnosis and Treatment. 101-131, Springer, Berlin, 2013.

14. Ozdemir ZO, Karahan M, Karabulut E, Mustafaeva Z: Characterization of foot-and-mouth disease virus's viral peptides with LC-ESI-MS. *J Chem Soc Pakistan,* 32 (4): 531-536, 2010.

15. Ozdemir ZO, Topuzogulları M, Karabulut E, Akdeste ZM: Characterization and purification of viral peptides synthesized with microwave assisted solid phase method. *IJNES,* 3 (2): 41-44, 2009.

16. Palasek SA, Cox ZJ, Collins JM: Limiting racemization and aspartimide formation in microwave-enhanced Fmoc solid phase peptide synthesis. *J Pept Sci,* 13 (3): 143-148, 2007. DOI: 10.1002/psc.804

17. Dubey N, Varshney R, Shukla J, Ganeshpurkar A, Hazari PP, Bandopadhaya GP, Mishra AK, Trivedi P: Synthesis and evaluation of biodegradable PCL/PEG nanoparticles for neuroendocrine tumor targeted delivery of somatostatin analog. *Drug Deliv,* 19 (3): 132-142, 2012. DOI: 10.3109/10717544.2012.657718

18. Mustafaeva Z: Synthesis and characterization of antigenic peptide of sheep pox disease. *Kimya Problemleri,* 3, 255-263, 2017.

19. Deliloglu Gurhan SI, Akdeste MM, Akdeste Mustafaeva Z, Aynagoz G, Unver G, Unal N, Celik N: Peparation of synthetic peptide FMD vaccine with newly developed antigen- polymere conjugates be used as immonogen and vaccine in veterinary medicine. *Report of the Europ Comm Cont FMD, Sess Res Gr Stand Tech Com, Çeşme, Izmir, Turkey, FAO/UN Rome*, 17-20 September 2002.www.fao.org/ag/againfo/commissions/docs/research_group/izmir/App41.pdf

20. Kızılbey K, Mansuroglu B, Derman S, Battal YB, Mustafaeva Z: Conjugation of BSA protein and VP/AA Copolymers. *IJNES,* 3 (2): 36-40, 2009.

21. Sarikaya M, Enginar H: Radioabeling of L-tyrosine with ^{131}I and investigation of radiopharmaceutical potantial. *AKU J Sci Eng,*12, 1-9, 2012.

22. Baur B, Solbach C, Andreolli E, Winter G, Machulla HJ, Reske SN: Synthesis, radiolabelling and *in vitro*characterization of the Gallium-68-, Yttrium-90-and Lutetium-177-Labelled PSMA Ligand, CHX-A″-DTPA-DUPA-Pep. *Pharmaceuticals,* 7 (5): 517-529, 2014. DOI: 10.3390/ph7050517

23. Pike VW: PET radiotracers: Crossing the blood-brain barrier and surviving metabolism. *Trends Pharmacol Sci,* 30 (8): 431-440, 2009. DOI: 10.1016/j.tips.2009.05.005

24. Waterhouse RN: Determination of lipophilicity and its use as a predictor of blood-brain barrier penetration of molecular imaging agents. *Mol Imaging Biol,* 5 (6): 376-389, 2003. DOI: 10.1016/j.mibio.2003.09.014

25. Wilson AA, Jin L, Garcia A, DaSilva JN, Houle S: An admonition when measuring the lipophilicity of radiotracers using counting techniques. *Appl Radiat Isot,* 54 (2): 203-208, 2001. DOI: 10.1016/S0969-8043(00)00269-4

26. Berthod A, Carda-Broch S: Determination of liquid-liquid partition coefficients by separation methods. *J Chromatogr A,* 1037 (1-2): 3-14, 2004. DOI: 10.1016/j.chroma.2004.01.001

27. Rubas W, Cromwell MEM: The effect of chemical modifications on octanol/water partition (log D) and permeabilities across Caco-2 monolayers. *Adv Drug Deliv Rev,* 23 (1-3): 157-162, 1997. DOI: 10.1016/S0169-409X(96)00433-4

28. Leo A, Hansch C, Elkins D: Partition coefficients and their uses. *Chem Rev,* 71 (6): 525-616, 1971. DOI: 10.1021/cr60274a001

29. Wester HJ: Pharmaceutical radiochemistry (I) - (Munich Molecular Imaging Handbook Series. Vol. 1. 198-200, Scintomics, Munich, 2010.

30. Ertay T, Unak P, Sencan Eren M, Biber Muftuler FZ, Ozdoğan O, Ulker O, Yesilagac R, Durak H: A new ^{99m}Tc labeled peptide: ^{99m}Tc β-casomorphin 6, biodistribution and imaging studies on rats. *Kafkas*

Univ Vet Fak Derg, 23 (1): 15-22, 2017. DOI: 10.9775/kvfd.2016.15691

31. Umbricht CA, Benešová M, Schmid RM, Turler A, Schibli R, van der Meulen NP, Müller C: ^{44}Sc-PSMA-617 for radiotheragnostics in tandem with ^{177}Lu-PSMA-617-preclinical investigations in comparison with ^{68}Ga-PSMA-11 and ^{68}Ga-PSMA-617. *EJNMMI Res,* 7 (9): 1-10, 2017. DOI: 10.1186/s13550-017-0257-4

32. Klein W, Kördel W, Weiss M, Poremski HJ: Updating of the OECD test guideline 107 "partition coefficient n-octanol/water": OECD laboratory intercomparison test on the HPLC method. *Chemosphere,* 17 (2): 361-386, 1988. DOI: 10.1016/0045-6535(88)90227-5

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Research Article

Prevalence and Molecular Characterization of *Trypanosoma* spp. in Domestic Geese *(Anser anser domesticus)* from the North-East Anatolia Region of Turkey [1] [2]

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Abstract

Avian trypanosomiosis is a protozoan disease transmitted by blood-sucking arthropods belonging to the Simuliidae, Culicidae, Ceratopogonidae, Hippoboscidae, and Dermanyssidae. *Trypanosoma* spp. are diagnosed by using microscopic and/or molecular methods. In this study, a total of 400 domestic geese blood samples were examined using nested PCR with the primers that amplify a gene fragment of 18S rRNA of *Trypanosoma* spp. The molecular prevalence of *Trypanosoma* spp. was determined as 50.25%. 18S rRNA amplicons from the two positive isolates were sequenced with the PCR primers. The obtained sequences from the two isolates exhibited 99.7% identity to each other. Sequence and blastn analyses revealed that two isolates were highly identical (99.7-100%) to the published 18S rRNA sequences of *T. thomasbancrofti, T. gallinarum* and *T. avium* from *Xanthomyza phrygia* (regent honeyeater) and *Gallus gallus* (the red junglefowl). This study provides the first molecular data on *Trypanosoma* generations in poultry from Turkey. The presence of *Trypanosoma* spp. was also firstly identified in domestic geese *(Anser anser domesticus)* in the world by molecular tools and genetic characterization of a short fragment of the 18S rRNA gene region has been achieved.

Keywords: *Domestic geese, Kars, Molecular characterization, Trypanosoma spp.*

Türkiye'nin Kuzeydoğu Anadolu Bölgesinde Kazlarda *(Anser anser domesticus) Trypanosoma* spp. Prevalansı ve Moleküler Karakterizasyonu

Öz

Kanatlı trypanosomiosisi, Simuliidae, Culicidae, Ceratopogonidae, Hippoboscidae ve Dermanyssidae ailelerinde bulunan kan emen artropodlar tarafından bulaştırılan bir protozoan enfeksiyonudur. *Trypanosoma* türleri mikroskobik ve/veya moleküler yöntemlerle teşhis edilebilir. Bu çalışmada, *Trypanosoma* türlerinin 18S rRNA gen fragmanını çoğaltan primerler kullanılarak nested PCR ile toplam 400 kazın kan örneği incelenmiştir. *Trypanosoma* spp. prevalansı %50.25 olarak tespit edilmiştir. İki pozitif izolattan elde edilen 18S rRNA amplikonlarının PCR primerleri ile sekans analizi yaptırılmıştır. İki izolattan elde edilen sekanslar birbirleri ile %99.7 oranında identiklik göstermiştir. Sekans ve blastn analizleri, bu iki izolatın *Xanthomyza phrygia* (regent honeyeater) ve *Gallus gallus*'tan (kırmızı junglefowl) izole edilen *T. thomasbancrofti, T. gallinarum* ve *T. avium*'un 18S rRNA dizilimlerine çok benzer olduğunu (%99.7-100) ortaya koymuştur. Bu çalışma Türkiye'de kanatlılarda yayılış gösteren *Trypanosoma* türleriyle ilgili ilk moleküler verileri sağlamaktadır. Ayrıca *Trypanosoma* türlerinin evcil kazlarda *(Anser anser domesticus)* varlığı dünyada ilk olarak tespit edilmiş ve 18S rRNA gen bölgesinin kısa bir parçasının genetik karakterizasyonu sağlanmıştır.

Anahtar sözcükler: *Evcil kaz, Kars, Moleküler karakterizasyon, Trypanosoma spp.*

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INTRODUCTION

Avian trypanosomes (Trypanosomatidae, Kinetoplastidae) are widespread worldwide and transmitted by blood-sucking arthropods belonging to the Simuliidae, Culicidae, Ceratopogonidae, Hippoboscidae, and Dermanyssidae [1-5]. *Trypanosoma* spp. are well adapted for various ecosystems from oceanic islands to the countries with cold climates where avian hosts and vectors are present [6-8].

To date, in spite of poorly taxonomical development, more than 100 avian *Trypanosoma* species have been described and named [6-8]. Trypanosomiosis is caused by the species of *T. avium* Danilewsky, 1885, *T. calmettei* Mathis and Leger, 1909 and *T. gallinarum* Bruce, Hamerton, Bateman, Mackie and Bruce, 1911 in geese. *T. avium* and *T. gallinarum* species are common in Canadian geese, many avian species in Europe and chickens in Africa, and also *T. calmettei* is seen in ducks in Southeast Asia. But there are no clinical signs in domestic geese. *Trypanosoma* spp. can be diagnosed by detecting trypomastigote forms in blood and/or bone marrow smears and using PCR based methods [1,8-12]. However, due to nucleated erythrocytes of avian species DNA may contain primarily host DNA leading to reduce efficiency of PCR amplification of the target parasite gene. Therefore, in order to optimize DNA amplification by using ribosomal genes, a nested PCR protocol was developed [12].

Due to the broad host range and wide geographical distribution, many studies have been focused on [5,8,9,12-14] the prevalence of trypanosomiasis in wild birds but the epidemiological parameters of avian trypanosomiasis has not yet been investigated in Turkey.

The aim of this study was therefore to determine the prevalence and molecular characterization of *Trypanosoma* spp. by nested PCR in domestic geese *(Anser anser domesticus)*.

MATERIAL and METHODS

Study Area and Collection of Blood Sample

Having obtained ethics approval from Kafkas University-Animal Experiments Local Ethics Committee (Aproval No: KAÜ-HADYEK: 2014-052) and the approval of the farmers, a total of 400 blood samples were collected, using an insulin syringe, from domestic geese (*Anser anser domesticus*) which are grown for nutrition purposes when they were slaughtered in November 2015 in Kars province (40°36'04.82"N, 43°05'50.83"E), Northeastern Turkey.

Extraction of gDNA and PCR

Blood samples were extracted according to the commercial DNeasy kit protocol (Zymo Research, Quick-gDNA Blood Mini Prep, USA). The extracted gDNA was stored at -20°C until analysis. Concentrations of DNA samples were measured (Qubit Fluorometric Quantitation, Invitrogen, Life Technologies) to optimize the amount of gDNA used in the PCR master mix. Genomic DNA was used in nested PCR reactions to amplify 18S rRNA fragments. The primers [S762 (5'-GACTTTTGCTTCCTCTAWTG-3') and S763 (5'-CATATGCTTGTTTCAAGGAC-3')] were used in the first step of nested PCR. The cycling profile conditions were as follows: initial denaturation at 95°C for 5 min, followed by 5 cycles of denaturation at 95°C for 1 min, annealing at 45°C for 30 sec, and extension at 65°C for 1 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 30 sec, extension at 72°C for 1 min, and then a final extension at 65°C for 10 min. A total volume of 25 µL reaction mixture containing 8.5 µL nuclease-free water, 12.5 µL master miks (Mytaq, Bioline), 1 µL of each primer and 2 µL of template DNA was used. Two mL of the first PCR reaction products were used as the template for the second PCR. The primers [S755 (5'-CTACGAACCCTTTAACAGCA-3') and S823 (5'-CGAAYAACTGCYCTATCAGC-3')] were used in the second step of nested PCR. The reaction conditions were as follows: initial denaturation at 96°C for 3 min, followed by 35 cycles of denaturation at 96°C for 30 sec, annealing at 58°C for 1 min, extension at 72°C for 30 sec, and then a final extension at 72°C for 7 min [12]. Positive control blood samples were supplied from Prof. Dr. Gediminas VALKUINAS (Institute of Ecology, Nature Research Centre, Vilnius, Lithuania) and nuclease free water was used as negative controls to check for contamination in each PCR. PCR products were run out on a 1.5% agarose gel using 0.5X TBE and visualized by an ethidium bromide stain under ultraviolet light. A 326-bp fragment of PCR products were accepted positive.

Sequence and Phylogenetic Analysis

In order to confirm PCR results and to explore the phylogenetic relationships, two PCR products were chosen and purified by excising from agarose gel, using a commercial kit (High Pure PCR Product Purification Kit, Roche Life Science). The amplified 18S rRNA target fragments were sequenced in both directions, using S-755 and S-823 primers for *Trypanosoma* spp. (Sentegen, Turkey). Sequences were oriented, edited, and aligned with Geneious 10.2.3 software [10] to produce a single consensus. Intra- and interspecific genetic diversity was determined with MEGA version 7 by using the Kimura two-parameter (K2P) distance model [15,16].

The obtained consensus sequences compared at 301 bp with sequences in the GenBank database using the Basic Local Alignment Search Tool (NCBI website) [17]. The characterized isolates were deposited in GenBank with the accessions MG593843 and MG593844. Phylogenetic reconstructions were performed by Bayesian (BA) inference. The best-fit DNA-substitution model for BA analyses based on the Akaike information criterion (AIC) algorithm was selected by using jModel test v.0.1.1 [18]. The BA analyses were run in MrBayes version 3.2.6 [19] and

PhyML [20] through the plugin available with Geneious 10.2.3 software [21].

RESULTS

Among the 400 examined domestic geese blood samples, 201 were found positive for *Trypanosoma* spp. by Nested PCR and the mean prevalence of *Trypanosoma* spp. was determined as 50.25%.

The partial 18S rRNA gene region (301 bp) of two isolates (KAU-Gtryp1, KAU-Gtryp2) from the positive samples were sequenced and the final consensus of the isolates were deposited in GenBank with accessions MG593843 and MG593844. The characterized isolates within *Trypanosoma* genus provides the first genetic data from domestic geese. Pairwise analyses of the KAU-Gtryp1 and KAU-Gtryp2 genotypes revealed %0.03 genetic difference between the isolates. The phylogenetic analyses of the isolates were shown on BA tree in *Fig. 1*. Blastn and multiple alignment analyses of the obtained *Trypanosoma* spp. KAU-Gtryp2 isolate indicated that this isolate was identical (100%) with the isolates *T. thomasbancrofti* and *T. avium* reported from *Xanthomyza phrygia* in Australia (KT728396, KT728395, KT728394, KT728402) and *T. gallinarum* reported from *Gallus gallus* in Uganda (DQ676828, DQ676827, DQ676826). *Trypanosoma* spp. KAU-Gtryp1 isolate was also determined to close (99.7%) with the isolates indicated above and presented a novel haplotype.

DISCUSSION

Goose breeding is one of the source of income with the highest number of domestic geese from Kars province in Northeastern Region of Turkey. Not to use any medicines for preventive purposes, domestic geese breeding is performed by traditional methods instead of scientific methods in Kars province [22,23].

Trypanosoma spp. are found in some species of birds

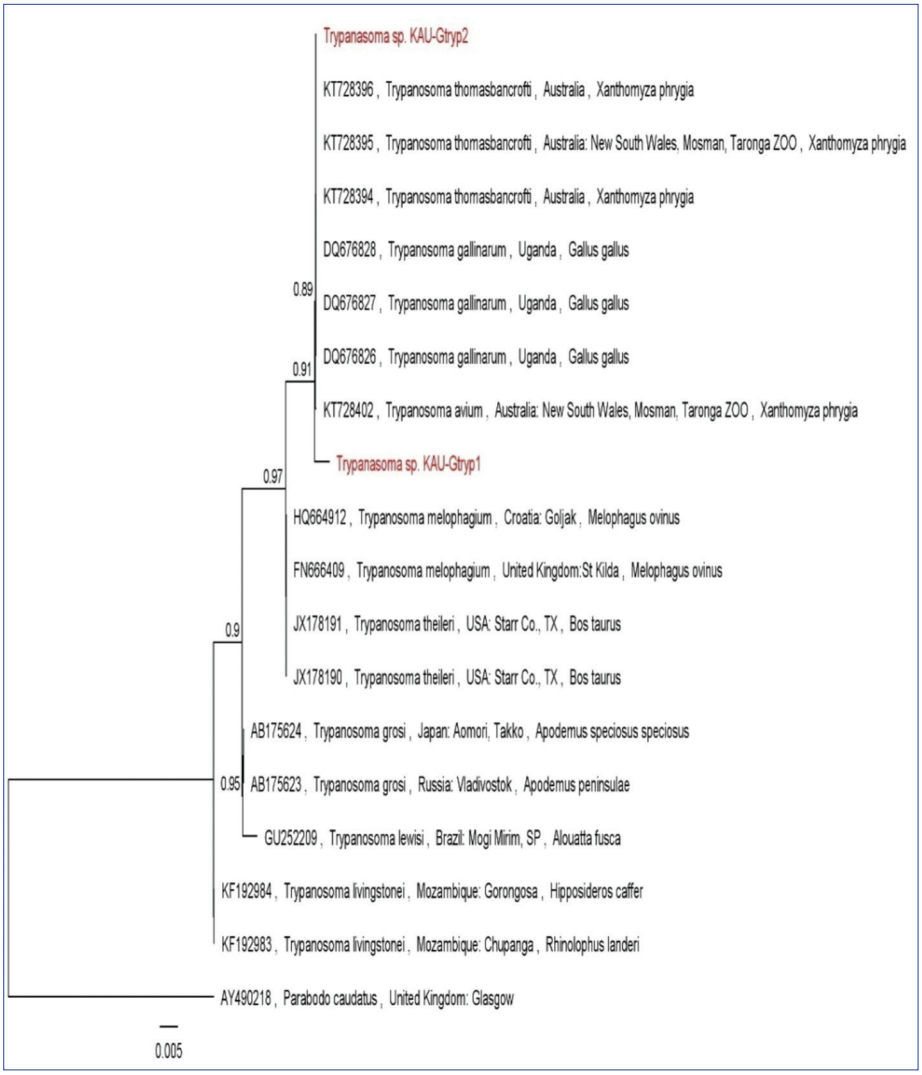

Fig 1. BA tree based on partial 18S rRNA sequences deposited in GenBank and our original data (red character) for *Trypanosoma* species. BA posterior probability values are shown before the nodes. *Parabodo caudatus* was used as outgroup taxa. Bars represent 0.1 substitutions per site

occasionally [6,12,14,24]. But there is no data about the prevalence of *Trypanosoma* spp. in domestic geese around the world. This is the first study to determine the prevalence of trypanosomes in domestic geese.

Limited studies on *Trypanosoma* infections in avian species have generally focused on passerines or raptor birds. In a study, the overall prevalence was determined as 31% [12]. Using microscopy, the prevalence of *Trypanosoma* spp. was found as 7.3% (consisting of 121 species from 21 families and 8 orders) in West African Nations of Cameroon, Equatorial Guinea and Ivory Coast [25], and (68 species from 15 families and 4 orders) 11.4% in Uganda [26], 36.4% in blood samples collected from Europe, Africa, and North America [27], 18.0% of American robins and 16.9% of house sparrows in Chicago, USA [14], 60.0% in Northern Goshawk [28]. In another study, 26.1% (40/153) of yellow-breasted chats were found infected with trypanosomes [29]. *Trypanosoma* spp. prevalence was ranged between 1.9 and 87.2% in Eurasian sparrow hawks *(Accipiter nisus)* and common buzzards *(Buteo buteo)* [24]. The prevalence of avian trypanosomes was 51.3% in Cameroon and Ghana [13].

In our study, since the parasitemia in host peripheral blood and the sensitivity of microscopy are very low [6,28], we did not use microscopical examination of blood smears. Among the 400 examined gDNA from the domestic geese, 201 samples were found positive for *Trypanosoma* spp. by nested PCR. It is expected that due to prolonged exposure and seasonally increasing occurrence of vectors, birds have a greater probability and higher parasite prevalence of infection [24]. In the direction of this knowledge, this may be the reason why we found a high rate of infection in domestic geese.

For the molecular characterization of *Trypanosoma* lineages found in the research area, sequence analyses were conducted on partial 18S rRNA sequences from two positive isolates. Two lineages were determined by sequence analyses and a mean of 0.03% genetic distance was found between the two isolates under these lineages. *Trypanosoma* spp. KAU-Gtryp2 isolate was found to be identical (100%) with the isolates *T. thomasbancrofti* and *T. avium* reported from *Xanthomyza phrygia* in Australia and *T. gallinarum* reported from *Gallus gallus* in Uganda. *Trypanosoma* spp. KAU-Gtryp1 isolate was also highly identical (99.7%) to the above-mentioned isolates. This isolate also designated as a new haplotype. While Sehgal *et al*.[12] found eight trypanosome lineages in African rainforests, only *T. avium* was detected by Oakgrove *et al*.[30]. Although the phylogenetic resolution of the targeted 326 bp region of 18S rRNA successfully explore the lineage diversity trypanosome lineages in African rainforests [12], our findings revealed that this region is not enough to resolve species delimitation of trypanosomes in order anseriformes.

In this study, the first molecular data has been provided on *Trypanosoma* generations spreading in poultry in Turkey, the presence of *Trypanosoma* spp. was firstly identified in domestic geese (*Anser anser domesticus*) by molecular tools in Turkey and genetic characterization of a short fragment of the 18S rRNA gene region has been achieved. We concluded that sequence characterization with 18S rRNA nested primers amplifying 301 bp region was insufficient to resolve genetically based generations. In order to be able to establish the phylogenetic structures of the isolates we determined in the outbreaks, it has been found that there is a need for sequence characterization and phylogenetic analysis with a longer fragment of the gene region of interest or different gene regions such as mitochondrial genes (eg COX1).

Most trypanosomes, especially *T. avium*, which is transmitted by blackflies (Diptera, Simuliidae), *T. bennetti*, whose vector is unknown, and *T. corvi*, which is transmitted by hippoboscid flies (Diptera, Hippoboscidae) were isolated from raptor birds [4,24,31-34]. Therefore, more detailed studies on avian trypanosomiasis, especially on the condition of the disease in the vectors, will solve questions about avian host-parasite relationships.

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Conflict of Interests Statement

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. Karaer Z, Çiçek H: Kanatlılarda Dolaşım Sisteminde Görülen Protozoon Hastalıkları. **In,** MA Özcel (Ed): Veteriner Hekimliğinde Parazit Hastalıkları. 515-531, Meta Basim Matbaacılık Hizmetleri, Turkiye Parazitoloji Dernegi Yayin No: 24, İzmir, 2013.

2. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S: MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol,* 30 (12): 2725-2729, 2013. DOI: 10.1093/molbev/mst197

3. Votýpka J, Lukeš J, Oborník M: Phylogenetic relationship of *Trypanosoma corvi* with other avian trypanosomes. *Acta Protozool,* 43, 225-231, 2004.

4. Baker JR: Studies on *Trypanosoma avium* Danilewsky, 1885 II. *Transmission by Ornithomyia avicularia* L. *Parasitol,* 46, 321-334, 1956. DOI: 10.1017/S0031182000026536

5. Sehgal RNM, Iezhova TA, Marzec T, Valkiūnas G: *Trypanosoma naviformis* sp. *nov.* (Kinetoplastidae: Trypanosomatidae) from widespread African songbirds, the Olive sunbird *(Cyanomitra olivacea)* and Yellow-whiskered greenbul *(Andropadus latirostris)*. *Zootaxa,* 4034 (2): 342-350, 2015. DOI: 10.11646/zootaxa.4034.2.6

6. Valkiunas G: Avian malaria parasites and other haemosporidia. CRC Press, Florida, 2004.

7. Baker JR: Biology of the Trypanosomes of Birds. **In,** WHR Lumsden, DA Evans (Eds): Biology of the Kinetoplastida. Vol. 1., 131-174, Academic Press, London, UK, 1976.

8. Valkiunas G, Iezhova TA, Carlson JS, Sehgal RNM: Two new *Trypanosoma* species from African birds, with notes on the taxonomy of avian trypanosomes. *J Parasitol,* 97 (5): 924-930, 2011. DOI: 10.1645/GE-2796.1

9. Sehgal RNM, Valkiūnas G, Iezhova TA, Smith TB: Blood parasites of chickens in Uganda and Cameroon with molecular descriptions of *Leucocytozoon schoutedeni* and *Trypanosoma gallinarum*. *J Parasitol,* 92 (6): 1336-1343, 2006. DOI: 10.1645/GE-927R.1

10. Permin A, Esmann JB, Hoj CH, Hove T, Mukaratirwa S: Ecto-, endo- and haemoparasites in free-range chickens in the Goromonzi District in Zimbabwe. *Prev Vet Med,* 54 (3): 213-224, 2002. DOI: 10.1016/S0167-5877(02)00024-7

11. Soulsby EJL: Helminths, Arthropods and Protozoa of Domesticated Animals. 7th ed., Bailliere, Tindall, London, 1986.

12. Sehgal RNM, Jones HI, Smith TB: Host specificity and incidence of *Trypanosoma* in some African rainforest birds: A molecular approach. *Mol Ecol,* 10 (9): 2319-2327, 2001. DOI: 10.1046/j.1365-294X.2001.01339.x

13. Valkiunas G, Iezhova TA, Sehgal RNM: Deforestation does not affect the prevalence of a common trypanosome in African birds. *Acta Trop,* 162, 222-228, 2016. DOI: 10.1016/j.actatropica.2016.07.003

14. Hamer GL, Anderson TK, Berry GE, Makohon-Moore AP, Crafton JC, Brawn JD, Dolinski AC, Krebs BL, Ruiz MO, Muzzall PM, Goldberg TL, Walker ED: Prevalence of filarioid nematodes and trypanosomes in American robins and house sparrows, Chicago USA. *Int J Parasitol Parasites Wildl,* 2, 42-49, 2013. DOI: 10.1016/j.ijppaw.2012.11.005

15. Nei M, Kumar S: Molecular Evolution and Phylogenetics. Oxford University Press, 2000.

16. Kimura M: A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol,* 16 (2): 111-120, 1980.

17. Anonymous: BLAST. http://www.ncbi.nlm.nih.gov/BLAST/; *Accessed:* 01.12.2017.

18. Posada D: jModelTest: Phylogenetic model averaging. *Mol Biol Evol,* 25 (7): 1253-1256, 2008. DOI: 10.1093/molbev/msn083

19. Huelsenbeck JP, Ronquist F: MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics,* 17 (8): 754-755, 2001. DOI: 10.1093/bioinformatics/17.8.754

20. Guindon S, Gascuel O: A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol,* 52 (5): 696-704, 2003. DOI: 10.1080/10635150390235520

21. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A: Geneious basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics,* 28 (12): 1647-1649, 2012. DOI: 10.1093/bioinformatics/bts199

22. Kırmızıbayrak T, Önk K, Ekiz B, Yalçıntan H, Yılmaz A, Yazıcı K, Altınel A: Effects of age and sex on meat quality of Turkish native geese raised under a free-range system. *Kafkas Univ Vet Fak Derg*, 17 (5): 817-823, 2011. DOI: 10.9775/kvfd.2011.4587

23. Kalaycı S, Yılmaz Ö: Effect of cereal grains on the total lipid, cholesterol content and fatty acid composition of liver and muscle tissues in native geese. *Kafkas Univ Vet Fak Derg*, 20 (1): 27-34, 2014. DOI: 10.9775/kvfd.2013.9394

24. Svobodova M, Weidinger K, Peske L, Volf P, Votypka J, Vorisek P: Trypanosomes and haemosporidia in the buzzard (*Buteo buteo*) and sparrowhawk *(Accipiter nisus)*: Factors affecting the prevalence of parasites. *Parasitol Res,* 114, 551-560, 2015. DOI: 10.1007/s00436-014-4217-x

25. Sehgal RNM, Jones HI, Smith TB: Blood parasites of some West African rainforest birds. *J Vet Med Sci,* 67 (3): 295-301, 2005. DOI: 10.1292/jvms.67.295

26. Valkiūnas G, Sehgal RNM, Iezhova TA, Smith TB: Further observations on the blood parasites of birds in Uganda. *J Wildl Dis,* 41 (3): 580-587, 2005. DOI: 10.7589/0090-3558-41.3.580

27. Valkiūnas G, Iezhova TA, Križanauskienė A, Palinauskas V, Sehgal RNM, Bensch S: A comparative analysis of microscopy and PCR-based detection methods for blood parasites. *J Parasitol,* 94 (6): 1395-1401, 2008. DOI: 10.1645/GE-1570.1

28. Hanel J, Doležalová J, Stehlíková Š, Modrý D, Chudoba J, Synek P, Votýpka J: Blood parasites in northern goshawk (*Accipiter gentilis*) with an emphasis to *Leucocytozoon toddi. Parasitol Res,* 115 (1): 263-270, 2016. DOI: 10.1007/s00436-015-4743-1

29. Soares L, Ellis VA, Ricklefs RE: Co-infections of haemosporidian and trypanosome parasites in a North American songbird. *Parasitology,* 143 (14): 1930-1938, 2016. DOI: 10.1017/S0031182016001384

30. Oakgrove KS, Harrigan RJ, Loiseau C, Guers S, Seppi B, Sehgal RNM: Distribution, diversity and drivers of blood-borne parasite co-infections in Alaskan bird populations. *Int J Parasitol,* 44 (10): 717-727, 2014. DOI: 10.1016/j.ijpara.2014.04.011

31. Kirkpatrick CE, Terway-Thompson CA, Iyengar MR: Biochemical characterization of some raptor trypanosomes. II. Enzyme studies, with a description of *Trypanosoma bennetti* n. sp. *Can J Zool,* 64 (1): 195-203, 1986. DOI: 10.1139/z86-031

32. Zidkova L, Cepicka I, Szabova J, Svobodova M: Biodiversity of avian trypanosomes. *Infect Genet Evol,* 12 (1): 102-112, 2012. DOI: 10.1016/j.meegid.2011.10.022

33. Bennett GF: On the specificity and transmission of some avian trypanosomes. *Can J Zool,* 39 (1): 17-33, 1961. DOI: 10.1139/z61-003

34. Votýpka J, Obornik M, Volf P, Svobodová M, Lukeš J: *Trypanosoma avium* of raptors (Falconiformes): Phylogeny and identification of vectors. *Parasitology,* 125 (3): 253-263, 2002. DOI: 10.1017/S0031182002002093

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Research Article

The Correlations Between Mean Echogenicity and Laboratory Findings of Superficial Swellings in 50 Dairy Cows

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Abstract

Ultrasonography, as a non-invasive diagnostic tool, can provide valuable information about the type, nature and duration of certain musculoskeletal inflammatory processes in animals. The aim of this study was to investigate the correlation between mean echogenicity (ME) of effusion content and its laboratory finding values. Fifty dairy cows with superficial effusions on their legs were examined in this study. Ultrasound images were analysed by software processing and ME was determined for every case of effusion. Samples of effusion contents were collected and sent to the laboratory. Investigated parameters were: Concentration of total proteins, concentration of glucose, concentration of uric acid, number of leukocytes, percentage of neutrophils, number of erythrocytes, specific weight, viscosity, colour, clarity and Gram stain. High correlation values between ME and concentration of total proteins (R2=0.7313; P<0.001), concentration of uric acid (R2=0.7427; P<0.001), percentage of neutrophils (R2=0.6923; P<0.001) and specific weight (R2=0.6963; P<0.001) showed that these laboratory parameters could have some impact on echogenicity of effusion content.

Keywords: *Ultrasonography, Musculoskeletal disorders, Echogenicity, Laboratory findings, Cattle*

Sütçü 50 İnekte Gözlenen Yüzeysel Ekstremite Şişliğin Ortalama Ekojeniteleri İle Laboratuvar Bulguları Arasındaki İlişki

Öz

Ultrasonografik muayene, sığırlarda kas iskelet sisteminin belirli yangısal bozukluklarında lezyonun tipi, yapısı ve kronolojisi hakkında değerli bilgiler sunan noninvazif bir tanı yöntemidir. Bu çalışma, yangısal şişliklerde, şişliğin ortalama ekojenitesi (ME) ile şişlik içeriğinin laboratuvar bulguları arasında olası bir korelasyonun varlığını araştırmak amacıyla yapıldı. Çalışmada, ekstremitelerinde yüzeysel şişlik izlenen 50 süt sığırı kullanıldı. Şişliklerden elde edilen ultrasonografik görüntüler, görüntü çözümleme programı ile analiz edilerek, her bir olgu için ME belirlendi. Şişliklerin punksiyonundan elde edilen içeriklerin laboratuvar analizleri yapılarak total protein konsantrasyonu, glikoz konsantrasyonu, ürik asit miktarı, lökosit sayısı, nötrofil yüzdesi, eritrosit sayısı, özgül ağırlığı, vizkositesi, rengi, berraklığı ve Gram boyama sonucu kaydedildi. İşlemciden elde edilen ME değerleri ile total protein konsantrasyonu (R2=0.7313; P<0.001), ürik asit konsantrasyonu (R2=0.7427; P<0.001), nötrofil yüzdesi (R2=0.6923; P<0.001) ve özgül ağırlık (R2=0.6963; P<0.001) arasında yüksek bir korelasyon bulunduğu tespit edildi. Bu bulgular, söz konusu laboratuvar parametrelerin, yangısal içeriğin ekojenitesi üzerinde etkili olabileceği şeklinde değerlendirildi.

Anahtar sözcükler: *Ultrasonografi, Kas-iskelet sistemi bozuklukları, Ekojenisite, Laboratuvar bulgular, Sığır*

INTRODUCTION

Depending on the type and intensity, some disorders and diseases of locomotor system in cattle such as bursitis, hygromas, abscesses, tenosynovitis and arthritis, are characterized by extensive soft tissue swelling and presence of inflammatory exudation [1,2].

Ultrasonography is one of the most useful diagnostic tools for their evaluation. Ultrasound allows us to evaluate echogenicity of the content, extent of effusion, type of the border of the lesion, cavity and swelling, presence of flow phenomena and presence of ultrasound artefacts (acoustic enhancement or acoustic shadowing) [1-4]. Depending on these criteria, ultrasonography provides

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certain information about the nature of the content in the affected area (fluid and semisolid masses), but it cannot definitively characterize the composition of the content and the type of effusion by itself [1,2].

Definitive answer about the type of effusion and its characteristics is provided by obtaining samples by centesis and laboratory analysis of aspirated fluid [1,2,5-8].

The aim of this study was to establish the correlation between the echogenicity of fluid content of certain superficial swellings on limbs in dairy cattle and parameters obtained by laboratory analysis of aspirated fluid samples (biochemical, cytological and microbiological findings).

MATERIAL and METHODS

During our mobile clinic work, in the period between 2014 and 2016, 50 cows with superficial swellings on their limbs were observed. Clinical and orthopaedic examinations were performed to identify "the region of interest" for ultrasound examination. According to clinical examination, it was found that 4 of 50 cows had swellings on their hind limbs at the femoral region, and 46 of 50 cows had swellings at the carpal or tarsal region. Before ultrasound examination was conducted, the skin over the affected area was clipped and shaved. Ultrasound examination was conducted with an ultrasound machine (Esaote Pie Medical®, Netherlands) using an 8 MHz linear transducer, in real-time. Echogenicity of the content and presence of flow phenomena were evaluated. Four ultrasound images were taken for every case of swelling and analysed by using image analyser software ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Values of pixel distribution within the grayscale (0 = black, 255 = white), which present values of mean echogenicity (ME), were determined for every ultrasound image in that part which present a liquid content of effusion. These values were quantified by randomly selecting ten circles with a size of 15 pixels on every ultrasound image *(Fig. 1)*. Average values of ME were determined for every case of swelling.

After clinical and ultrasound examination, fluid sampling was performed. Before centesis, the skin was disinfected with 10% povidone-iodine solution. The fluids were obtained using a syringe and EDTA vacutainers, stored in a portable fridge and analyzed in the laboratory within 4 h. Next laboratory findings were measured and analysed: Concentration of total proteins (Tp), concentration of glucose (Glc), concentration of uric acid, number of leukocytes (Le), percentage of neutrophils (Ne), number of erythrocytes (Er), specific weight, viscosity, colour, clarity and Gram stain. Concentration of Tp, Glc and uric acid were measured using Analyser A15 (Biosystems S.A., Barcelona, Spain). Number of Le and Er were analysed using ADVIA 120 haematology system (Siemens, Germany).

Descriptive statistic values based on average ME values and distribution of frequency for average ME values were calculated. Correlation between average ME values and laboratory findings of effusions was examined. Pearson's correlation was used to detect correlations between average ME values and concentrations of Tp, Glc and uric acid, specific weight, number of Er and percentage of Ne. Correlation between average ME values and number of Le was investigated by logistic regression. For the purpose of logistic regression, cows were divided into two groups according to median value determined by descriptive statistics. Then, significance of differences between the values of laboratory findings in cows above the median value and in cows under the median value were determined by t-test. The correlation between average ME values and descriptive characteristics of the effusions such as viscosity (very viscous, viscous, sero-viscous, serous), colour (milky yellow, yellow, red), clarity (blurry, blurred, slightly cloudy, clear) and Gram stain (presence of bacteria, absence of bacteria), was tested using χ^2 test and $2 \times k$ contingency tables, so the significance of difference in the proportion

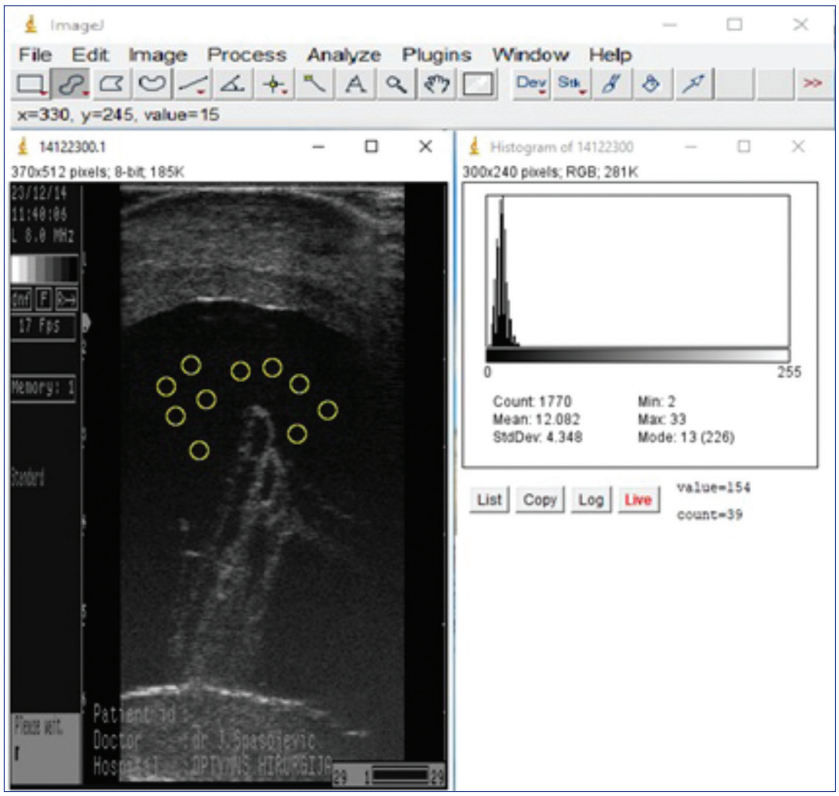

Fig 1. Determination of mean echogenicity (ME)

of cows that have certain characteristics of effusions in the groups based on average ME values above and under the median value determined by descriptive statistics has been investigated. All statistical analyses were performed with Statistica (TIBCO Software Inc., USA) and Microsoft Office Excel 2007 (Microsoft Corporation, USA).

RESULTS

Descriptive statistic values based on average ME values and distribution of frequency for average ME values are presented in *Table 1* and *Fig. 2*.

Average ME values were between 5.6 and 34.62. Skewness and kurtosis indicate that average ME values had normal frequency distribution.

Average ME values were correlated with different laboratory findings of the effusions *(Fig. 3-9, Table 2)*. Positive correlation was found between average ME values and concentrations of Tp and uric acid, specific weight, number of Er and percentage of Ne. Negative correlation was found between average ME values and concentration of Glc. There was found significant correlation between the number of Le and average ME which is corresponding to positive correlation. Another valuable indicator of the usability of average ME in evaluation of effusions is the difference between laboratory finding values of effusions in groups of cows above and under the median value. Results showed that there were higher concentrations of Tp and uric acid, higher specific weight, higher number of Le, higher percentage of Ne and lower concentration of Glc in the group of cows with average ME values above the median value. A significant difference in number of Er between groups of cows above and under the median value was not found *(Table 2)*.

Table 1. *Descriptive statistic values based on average ME values*

Variable	Descriptive Statistics									
	N	Mean	Median	Min	Max	Lower Quartile	Upper Quartile	Std. Dev.	Skewness	Kurtosis
Mean Echogenicity (ME)	50	13.02	9	5.6	34.62	7	14	8.95	1.51	0.86

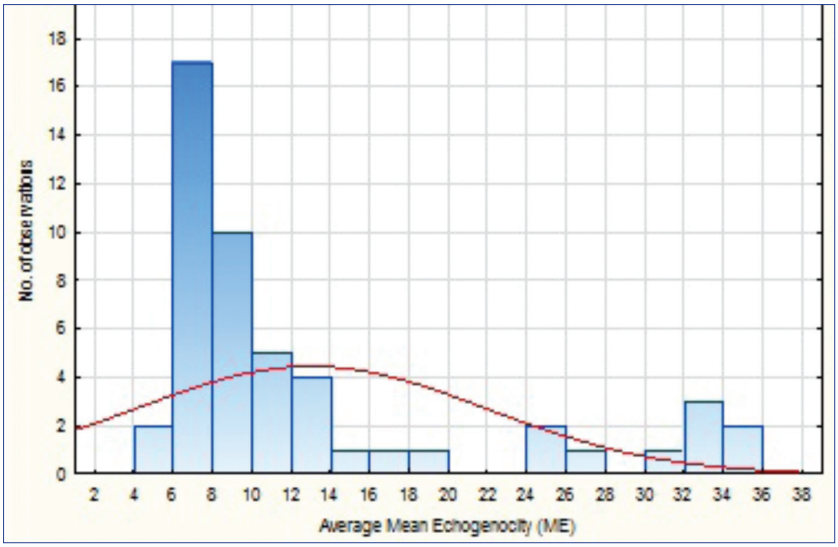

Fig 2. Distribution of frequency for average ME values

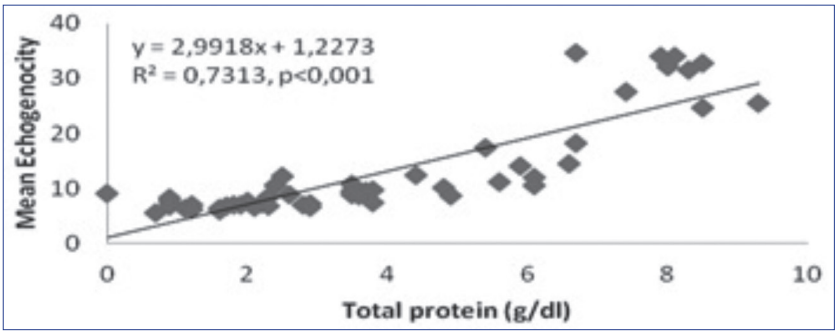

Fig 3. Linear correlation and regression between average mean echogenicity (ME) values and concentration of total proteins

The results in *Table 3* show that there is a significant difference in the proportion of cows with certain characteristics of effusions in function of that whether the average ME values were high or low. In cows, in which higher average ME values were found, very viscous and viscous consistency, milky yellow colour, and poor transparency (blurry) of the effusions dominated and in a large proportion of the samples there was presence of bacteria. On the other hand, in cows with lower average ME values of ultrasound findings, serous, yellow colour, and cloudy or clear effusions dominated, and also absence of bacteria in a large portion of the samples.

DISCUSSION

Ultrasonography is an ideal, non-invasive diagnostic tool for the examination and evaluation of musculoskeletal disorders and diseases such as arthritis, tenosynovitis, bursitis, hygromas and abscesses, because these diseases are

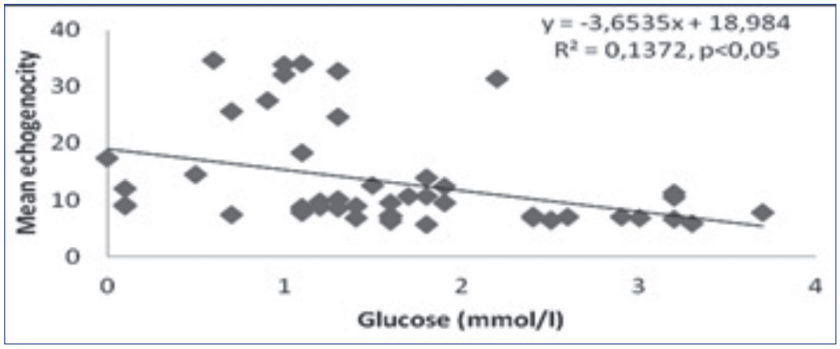

Fig 4. Linear correlation and regression between average mean echogenicity (ME) values and concentration of glucose

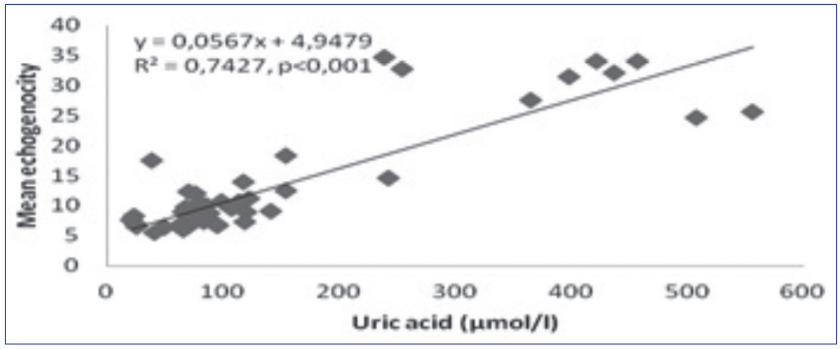

Fig 5. Linear correlation and regression between average mean echogenicity (ME) values and concentration of uric acid

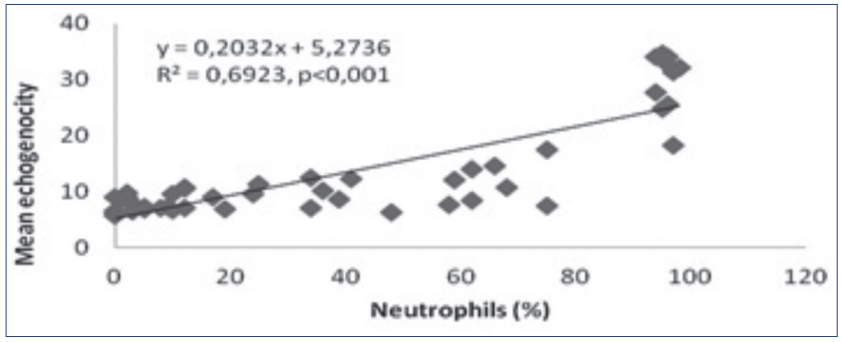

Fig 6. Linear correlation and regression between average mean echogenicity (ME) values and percentage of Ne

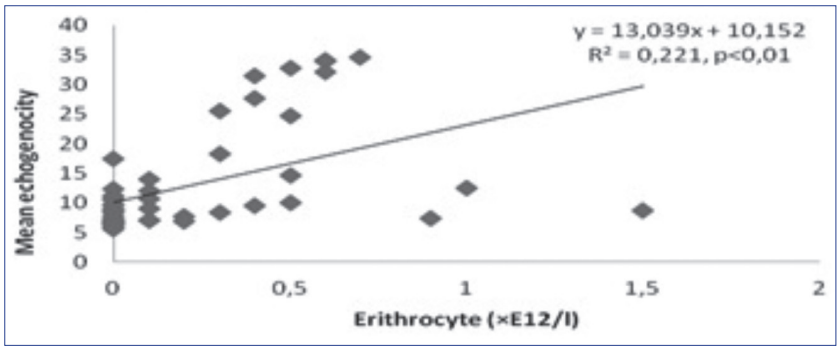

Fig 7. Linear correlation and regression between average mean echogenicity (ME) values and number of Er

frequently associated with extensive soft tissue swelling and inflammatory exudation [1,2].

Ultrasonographic examination can povide valuable information about the type, nature, and duration of some inflammatory processes, and these information can be helpful in planning proper therapy protocols for mentioned diseases [1,2,9,10].

In recent studies [1,2,6-8,11], it was reported that evaluation of the echogenicity of effusions and flow phenomena in some musculoskeletal disorders and diseases, which ranges from anechoic to echoic, depend on the type and nature of its inflammatory content (serous, serofibrinous, fibrinous, purulent). In the study with horses [12], the relationship between utrasonographic examination and the degree of effusion of the synovial cavity, the degree of synovial membrane thickening and the time of the beginning of inflammatory process was found. In the study with cows [10], it was reported that capsule echogenicity instead of capsule thickness is a more reliable ultrasonographic parameter for determination of the duration of the inflammatory process.

It has been also mentioned that ultrasonography cannot definitely characterize the composition of the content and the type of effusion by itself. In that purpose, it was recommended to perform centesis of the inflammatory process and laboratory analysis of aspirated fluid samples [1,2,5-8].

In our study we investigated correlations between echogenicity of effusions on ultrasound images and laboratory finding values obtained from aspirated fluid samples. In order to investigate these correlations, we used medical image analyser and quantified echogenicity of effusions. Up to our knowledge, it could not be found that researchers quantified the echogenicity of effusions in their studies about musculoskeletal disorders in cattle such as arthritis, tenosynovitis, bursitis, hygromas and abscesses, and only descriptive methods were used for their evaluation.

Rohde et al.[13], defined some laboratory

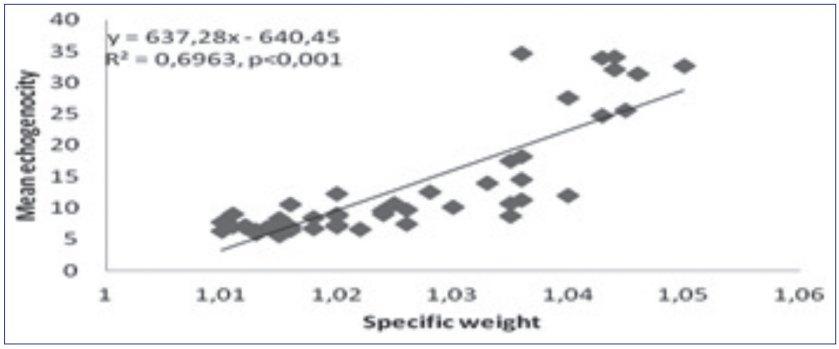

Fig 8. Linear correlation and regression between average mean echogenicity (ME) values and specific weight

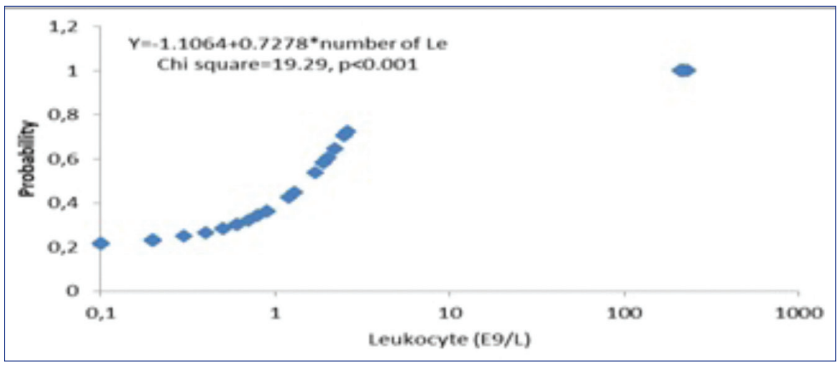

Fig 9. Correlation and regression between average mean echogenicity (ME) values and number of Le

skeletal disorders and diseases in cattle which are associated with extensive soft tissue swelling and inflammatory exudation. It could be possible to correlate ME values with laboratory parameters such as concentrations of Tp and uric acid, specific weight and percentage of Ne.

Our results could be used for confirmation of clinical cases of effusions in cattle and evaluation of therapy effects. That means that those patients with higher ME values could be in higher risks to have effusions with septic process in it, and according to that, it could define a therapy protocol, evaluation and prognosis of a diagnosed diseases.

In future studies, the limits of ME that allow detection of different types of effusions should be examined and validated.

REFERENCES

1. Kofler J: Ultrasonography as a diagnostic aid in bovine musculoskeletal disorders. *Vet Clin North Am Food Anim Pract*, 25 (3): 687-731, 2009. DOI: 10.1016/j.cvfa.2009.07.011

Table 2. *Laboratory parameters of effusions in two groups classified as above or below median of average ME values*

Parameter	Average ME Values		P
	Above Median	Below Median	
Total protein (g/dL)	5.75±2.33	2.14±1.05	<0.000001
Glucose (mmol/L)	1.32±0.82	1.94±0.9	<0.05
Uric acid (μmol/L)	216.44±160.18	68.46±27.28	<0.00005
Leukocyte (10^9/L)	87.05±107.57	0.62±0.8	<0.0005
Neutrophils (%)	59.36±36.15	16.88±22.23	<0.00005
Erythrocyte (10^{12}/L)	0.31±0.28	0.13±0.34	>0.05
Specific weight	1.03±0.01	1.02±0.006	<0.000005

parameters in their study, and gave some recommendations about their values in synovial fluid which distinguish infectious from non-infectious processes. These recommendations helped us to define which laboratory parameters we should use in our study.

In the study with horses [12], relations between ultrasonographic findings and white blood cell count have been described. The authors reported that ultrasonographic findings were not conditioned by white blood cell count.

In conclusion, according to our results, evaluation of echogenicity of effusions and determination of ME values could be useful in the purpose of evaluation of musculo-

2. Kofler J, Geissbühler U, Steiner A: Diagnostic imaging in bovine orthopedics. *Vet Clin North Am: Food Anim Pract*, 30 (1): 11-53, 2014. DOI: 10.1016/j.cvfa.2013.11.003

3. Kofler J: Ultrasonographic examination of the carpal region in cattle-normal appearance. *Vet J*, 159 (1): 85-96, 2000. DOI: 10.1053/tvjl.1998.0339

4. Kofler J, Edinger HK: Diagnostic ultrasound imaging of soft tissues in the bovine distal limb. *Vet Radiol Ultrasound*, 36 (3): 246-252, 1995. DOI: 10.1111/j.1740-8261.1995.tb00255.x

5. Heppelmann M, Rehage J, Kofler J, Starke A: Ultrasonographic diagnosis of septic arthritis of the distal interphalangeal joint in cattle. *Vet J*, 179 (3): 407-416, 2009. DOI: 10.1016/j.tvjl.2007.10.017

6. Kofler J: Arthrosonography - The use of diagnostic ultrasound in septic and traumatic arthritis in cattle - A retrospective study of 25 patients. *Br Vet J*, 152 (6): 683-698, 1996. DOI: 10.1016/S0007-1935(96)80122-9

Table 3. *Connection between average ME values and physical characteristic and presence of bacterial cell in effusions*

Characteristics of Effusions	Category	Number of Cows According to Average ME Values		χ^2 Test
		Above the Median Value	Under the Median Value	
Viscosity	very viscous	7	0	P<0.0005
	viscous	7	1	
	sero-viscous	6	7	
	serous	5	17	
Colour	milky yellow	9	0	P<0.005
	yellow	10	18	
	red	6	7	
Clarity	blurry	16	4	P<0.005
	blurred	7	10	
	slightly cloudy	2	3	
	clear	0	8	
Presence of bacteria (Gram stain)	Presence of bacteria	16	3	P<0.0005
	Absence of bacteria	9	22	

7. Kofler J, Altenbrunner-Martinek B: Ultrasonographic findings of disorders of the tarsal region in 97 cattle-arthritis, bursitis, tenosynovitis, periarticular abscess and vein thrombosis. *Berl Munch Tierarztl Wochenschr*, 121 (3-4): 145-158, 2007.

8. Kofler J, Martinek B: Ultrasonographic imaging of disorders of the carpal region in 42 cattle-arthritis, tenosynovitis, precarpal hygroma, periarticular abscess. *Tierarztl Prax*, 32 (2): 61-74, 2004.

9. Abouelnasr K, El-Shafaey ES, Mosbah E, El-Khodery S: Utility of ultrasonography for diagnosis of superficial swellings in buffalo *(Bubalus bubalis). J Vet Med Sci*, 78 (8): 1303-1309, 2016. DOI: 10.1292/jvms.15-0629

10. Seyrek-Intas D, Celimli N, Gorgul OS, Cecen G: Comparison of clinical, ultrasonographic, and postoperative macroscopic findings in cows with bursitis. *Vet Radiol Ultrasound*, 46 (2): 143-145, 2005. DOI: 10.1111/j.1740-8261.2005.00028.x

11. Magda MA, Abdel-Hakiem MAH: Ultrasonographic differential diagnosis of superficial swellings in farm animals. *J Adv Vet Res*, 2 (4): 292-298, 2012.

12. Beccati F, Gialletti R, Passamonti F, Nannarone S, Di Meo A, Pepe M: Ultrasonographic findings in 38 horses with septic arthritis/tenosynovitis. *Vet Radiol Ultrasound*, 56 (1): 68-76, 2015. DOI: 10.1111/vru.12183

13. Rohde C, Anderson DE, Desrochers A, St-Jean G, Hull BL, Rings DM: Synovial fluid analysis in cattle: A review of 130 cases. *Vet Surg*, 29 (4): 341-346, 2000. DOI: 10.1053/jvet.2000.5605

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Research Article

Moraxella ovis and *Mycoplasma conjunctivae* Isolation from an Ovine Infectious Keratoconjunctivitis Outbreak and Fortified Treatment Approaches

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Abstract

The aim of this study was to determine the etiology of naturally occurring infectious keratoconjunctivitis (IKC) outbreak observed in a Tuj sheep herd rearing semi-intensively and to try fortified treatment options in symptomatic sheeps. Conjunctival samples from 42 sheep with keratoconjunctivitis were collected aseptically using sterile cotton swabs. All swab were cultivated for bacterial isolation. To test treatment options symptomatic animals were randomly divided into 3 groups each were composed of adult ewes. The first group (n=13) was treated with the subconjunctival administration of penicillin G potassium (250.000 IU/animal), the second group (n=14) was treated with oxytetracycline and polymyxin B sulfate, and the third group (n=15) was treated with amikacin sulfate (diluted with isotonic NaCl to 25 mg per mL), ceftriaxone disodium and fluconazole (2 mg/mL) combination. As the results of the bacteriological examination, 36 (85.71%) of 42 conjunctival samples were found positive for *Moraxella ovis.* Also, *Mycoplasma conjunctivae* was identified in 3 (7.14%) conjunctival samples positive for *M. ovis*. After treatment, 84.61% (11/13) of the first group; 71.42% (10/14) of the second group and all animals in the third group were observed to be healed. This study demonstrated that *M. ovis* was the primary pathogen causing IKC in Tuj sheeps and *M. conjunctivae* was the second rank. The combination of amikacin sulfate, ceftriaxone disodium and fluconazole have been identified as the most effective treatment option of IKC in sheep.

Keywords: *Keratoconjunctivitis, Sheep, Moraxella ovis, Mycoplasma conjunctivae*

Bir İnfeksiyöz Koyun Keratokonjonktivit Salgınından *Moraxella ovis* ve *Mycoplasma conjunctivae* İzolasyonu ve Güçlendirilmiş Tedavi Yaklaşımı

Öz

Bu çalışmada, semi-entansif olarak yetiştirilen bir Tuj koyun sürüsünde doğal olarak gözlenen bir infeksiyöz keratokonjonktivit (IKC) salgınının etyolojisinin belirlenmesi ve semptom gösteren koyunlarda kuvvetlendirilmiş tedavi seçeneklerinin denenmesi amaçlanmıştır. Klinik keratokonjunktivitis belirtisi olan 42 koyunun konjunktival örnekleri, steril sıvap kullanılarak aseptik koşullarda toplandı. Tüm örnekler bakteri izolasyonu için kültüre edildi. Tedavi seçeneklerini test etmek için ergin koyunlardan oluşan semptomatik hayvanlar rastgele 3 gruba ayrıldı. Birinci grupta (n=13) penisilin G potasyum'un (250.000 IU/hayvan) subkonjunktival uygulanarak, ikinci grupta (n=14) oksitetrasiklin ve polimiksin B sülfat'ın gözkapağı içine mercimek tanesi büyüklüğünde konularak ve üçüncü grupta (n=15) ise amikasin sülfat, seftriakson disodyum (0.5 g) ve flukonazol (2 mg/mL) kombinasyonu damlatma şeklinde uygulanarak tedavi denemeleri yapıldı. Bakteriyolojik muayene sonucunda, 42 konjunktival sıvap örneğinin 36'sında (%85.71) *Moraxella ovis* ve bu pozitif örneklerin 3'ünde (%7.14) *Mycoplasma conjunctivae* izole edildi. Tedavi sonrası, birinci grubun %84.61'sı (11/13); ikinci grubun %71.42'si (10/14) ve üçüncü grubun ise tümünde iyileşme görüldü. Bu çalışma, *M. ovis*'in Tuj koyunlarında IKC'ye neden olan primer patojen, *M. conjunctivae*'nın ise sekonder etken olduğunu göstermiştir. Amikasin sülfat, seftriakson disodyum ve flukonazol kombinasyonu, koyunlarda IKC'nin en etkili tedavi seçeneği olarak tanımlanmıştır.

Anahtar sözcükler: *Keratokonjunktivit, Koyun, Moraxella ovis, Mycoplasma conjunctivae*

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INTRODUCTION

Infectious keratoconjunctivitis (IKC), also known as pink eye disease of cattle, sheep and goats, is characterized by blepharospasm, conjunctivitis, lacrimation, and varying degrees of corneal opacity and ulceration [1]. In sheep and goats, naturally occurring conjunctivitis or kerato-conjunctivitis can be associated with *Chlamydia pecorum, Chlamydophila psittaci*, *Mycoplasma* spp. (notably *Mycoplasma conjunctivae*), *Moraxella ovis*, *Colesiota conjunctivae, Listeria monocytogenes, Acholeplasma oculi*, *Staphylococcus aureus, Corynebacterium* spp., *Escherichia coli* and *Thelazia* spp.[2,3]. *M. ovis* has been implicated in epizootics of IKC in domestic sheep and goats [4]. Dagnall [5] reported that *M. ovis* could be isolated from both healthy sheep and those with IKC, but isolation occurred at a higher rate in sick animals. *M. conjunctivae* has been associated with most of the IKC outbreaks in small domestic ruminants [4] and wild caprinae worldwide [6] and is considered the primary pathogen of this infection. However, *M. conjunctivae* is commonly detected in the eyes of asymptomatic sheep and is eventually endemic in sheep herds throughout Europe [4]. IKC is a highly contagious disease and it is influenced by host and environmental factors. Predisposing factors such as age, breed, immune response and eye pigmentation, season (fly season), mechanical irritation (dust, grass, weeds, etc.), and concurrent presence of the disease-involved pathogenic bacteria in the environment is influences the prevalence of this disease [7]. There is usually no mortality reported associated with IKC however, the morbidity rate can reach as high as 80% [1]. Loss of productions are compounded by the cost of keratoconjunctivitis for producers in terms of incurring additional labour and treatment costs [8]. If the control and treat of the disease are not taken, it may spread in the flock and blindness may result and blind animals on range may subsequently die [9].

The effective antibiotic selection is important for the treatment of keratoconjunctivitis cases in animals. The fortified antibiotic treatments are reported to be extremely effective and reliable in the treatment of acute bacterial keratitis in human medicine [10-12]. Fortified preparations of ophthalmic antibiotics are made from antibiotics found in the market. These fortified eye drops have two main advantages, which are an increase in antibiotic concentration in the corneal stroma and a wide range option of availability. Given the potential for synergistic effects on corneal penetration, strong antibiotic combinations continue to be used in treatment for severe bacterial keratitis [13]. Fluconazole is a drug that is soluble in water and is suitable for topical application of epithelial defect. This drug with corneal penetration is effective against *Candida* sp. and *Aspergillus* sp in both human and animals [14-17]. Polymyxins have similar effect on fungi and can also be used against bacterial keratitis [18]. The combination of cephalosporins (ceftriaxone, cephazol, ceftazidime-50 mg/mL) and aminoglycosides (tobramycin, gentamycin or amikacin) gives good results when used topically against polybacterial keratitis [19]. Penicillin and tetracycline susceptibility was reported to control *M. ovis* infection, especially to avoid exacerbation of lesions primarily caused by *M. conjunctivae* [20]. Additionally, animals with *M. conjunctivae* require antibiotic combination of oxytetra-cycline and polymyxin B, but not to penicillin [21].

In this study, it was aimed to determine the etiology of naturally occurring infectious keratoconjunctivitis and to try different treatment approaches in a Tuj sheep herd semi-intensively farming in Veterinary Faculty Farm of the Kafkas University in Kars, Turkey.

MATERIAL and METHODS

Ethical Approval

The experiment was carried out with the approval of Kafkas University Local Ethical Committee for Animal Experiments (KAÜ-HADYEK/2018-016).

Case Definition

The study was carried out on a naturally occurring infectious keratoconjunctivitis outbreak observed in a Tuj sheep herd rearing semi-intensively in the farm of the Faculty of Veterinary Medicine, Kafkas University, in July 2017. Out of 150 animals, 42 (28%) adult (>3 years) sheep were affected by the disease and thus subjected to the study. While all affected sheep had clinical complaints such as lacrimation and conjunctivitis, some had ulceration and corneal opacity in generally one eye in addition. But no subclassification was carried out on the severity of clinical presentation of animals. The fluorescent staining test was used in the detection of corneal ulcer together with clinical examination, and defects were controlled under the daylight.

Sampling

The conjunctival samples of each of 42 sheep with clinical complaints were collected aseptically using two sterile cotton swabs, and the samples was transferred within 1 h for process at the Microbiology Laboratory of Veterinary Faculty, Kafkas University, Turkey.

Bacterial Isolation

For the pre-enrichment progress of *Mycoplasma* sp., one of conjunctival swab samples was transferred in a 3 mL Mycoplasma broth (Oxoid, England) enriched with 10% fresh yeast extract (Oxoid, England), 20% heat-inactivated horse serum (Sigma, USA), 0.025% thallium acetate (Fluka, Belgium), and 100 IU of penicillin G (IE, Istanbul) and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 3 days. One hundred µL of pre-enriched content was then sub-cultured on Mycoplasma agar plates (Oxoid,

England) enriched with 10% fresh yeast extract, 20% heat-inactivated horse serum, 0.025% thallium acetate, and 100 U of penicillin G, and incubated again at 37°C with 5% CO_2 in a moist environment. These agar plates were incubated for up to 21 days and monitored every second day for signs of *Mycoplasma* growth [22]. The second samples were also inoculated on 7% sheep blood agar (Oxoid, England) plates and MacConkey agar (Oxoid, England) plates in order to determine the presence of other bacterial agents and incubated at 37°C, aerobically for two days. Identification of *Mycoplasma* isolates were carried out by conventional bacteriological methods such as catabolism of glucose, hydrolysis of arginine, phosphatase activity, tetrazolium reduction, serum digestion, digitonin sensitivity, film and spot formation [23]. The other causative agents were identified by using tests as catalase, oxidase, indole, methyl red, voges proskauer, citrate, nitrate reduction which were carried out as described earlier [24].

Clinical Trials

Affected animals were randomly distributed into three treatment groups. The subconjunctival penicillin G potassium (Ibrahim Ethem®) was administered three times (every other day) at the daily dose of 250.000 IU/animal to animals in the first group (n=13). The ocular ointment (Terramycin, Pfizer®) containing oxytetracycline and polymyxin B sulfate were applied to the animals in the second group (n=14) once per day in the lentil size area on the inside of the eyelid and treatment was continued for 1 week. Amikacin sulphate (Amikosit, Zentiva®) (diluted with isotonic NaCl to 25 mg per mL), ceftriaxone sodium (Unacefin 0.5 g, Avis®) and fluconazole (2 mg/mL) (Triflukan, Pfizer®) [19,25-27], for fungal infection probability was administered in combination with 2 drops three times per day for 1 weeks to animals in third group.

RESULTS

Culture Results

As the results of bacteriological examination, *M. ovis* was isolated and identified in 36 (85.71%) of 42 conjunctival samples. In addition, *M. conjunctivae* was identified in 3 (7.14%) conjunctival samples of ewe together with *M. ovis*. Six conjunctival swab samples were found bacterial culture negative. The other bacteria could not isolate from samples. All the treatment groups were included two culture negative animals, as well. The results of the cultivated samples and fortified treatment options were showed in *Table 1*.

Clinical Trial Results

The treatment groups were randomly adjusted as three groups. All the groups were included animals suffered from *M. ovis* and *M. conjunctivae*, except the first group which is infected only with *M. ovis*. After treatment, it was detected that 84.61% (11/13) of the animals in first group; 71.42% (10/14) of the animals in the second group and 100% (15/15) of the animals in the third group were observed to be healed *(Fig. 1)*.

Table 1. *The summary of the cultivated samples and fortified treatment options*

Total Number of Samples	Culture Results	Treatment Options (Healing Rate)			
	Positive	Groups	Penicillin G	Oxytetracycline/ Polymyxin B	Amikacin/Ceftriaxone/ Fluconazole
42	36 (36 *M. ovis* and 2 *M. conjunctivae*)	1st group n: 13	11/13 (84.61%)	NA	NA
		2nd group n: 14	NA	10/14 (71.42%)	NA
		3rd group n: 15	NA	NA	15/15 (100%)

NA: *Not applicated*

Fig 1. While affected sheeps were exhibited corneal opacity, remarkable healing was observed after treatment

DISCUSSION

The etiology of infectious keratoconjunctivitis involves many predisposing factors such as age, race, daylight, dust irritations which facilitate the colonization of the pond are important in the formation of the disease [27]. Bacteria such as *C. pecorum, Mycoplasma* spp. (notably *M. conjunctivae*), *Moraxella* spp., *C. conjunctivae, L. monocytogenes, A. oculi* are often blamed as causative agents of IKC in animals [2]. *Moraxella bovis* is reported to be the most common agent of infectious bovine keratoconjunctivitis in the world [8,20]. In contrast, the main microorganisms isolated from IKC of sheep lesions are *M. conjunctivae*, *C. psittaci* and *M. ovis* [5,28]. Karthik et al.[29] reported 2 *Moraxella* spp. were isolated from the eye swabs of 5 animals with keratoconjunctivitis in a sheep herd consist of 100 animals in India. Also, they found to be 99% compatible with *M. bovis* by PCR-based sequence analysis. The researchers showed that the isolates were susceptible to all antimicrobials as gamyoxyclav, enrofloxacin, ciprofloxacin, cefixime, gentamicin, tetracycline and norfloxacin. Åkerstedt and Hofshagen [3], conducted a study on Norwegian Sheep in which they took conjunctival swab from 43 sick and 42 healthy animals in 15 farms with ovine keratoconjunctivitis and 50 conjunctival swabs from sheep in 17 farms without disease and cultivated for the presence of *Mycoplasma* and other bacterial species. They reported that *M. (Branhamella) ovis* was isolated in 28% of the samples taken from the affected herds and 10% of the samples from healthy animals. In addition, *M. conjunctivae* was isolated in 16 (37%) animals with keratoconjunctivitis and 3 (7%) non-clinical animals from the farms where keratoconjunctivitis was observed, whereas *M. conjunctivae* was isolated in only 4 (8%) samples from farms without evidence of keratoconjunctivitis. *L. monocytogenes* (1%), *S. aureus* (5%), *Corynebacterium* spp. (2%) and *E. coli* (4%) were isolated in herd. The researchers has reported that it is the first time that *M. conjunctivae* was isolated from a conjunctivitis case in Norway and the other predisposing agents can be isolated such as *M. (Branhamella) ovis* and *L. monocytogenes.* They claimed that different agents may play a role in the formation of keratoconjunctivitis in the sheep, and that some are seconder invaders.

Shahzad et al.[30] have reported an IKC infection in a few Lohi lambs showing clinical symptoms as conjunctivitis, keratitis, severe lacrimation and varying degree of blindness in Livestock Experiment Station (LES), Bahadurnagar, Okara, Pakistan. Thirty six conjunctival swabs sampled from the animals were evaluated by cultural and molecular methods. Sixteen (44.44%) out of 36 samples showed turbidity in PPLO broth. Twelve (75%) out of 16 broth samples showed colony growth on PPLO agar and this isolates identified as *M. conjunctivae*. Twenty one (59%) out of 36 conjunctival direct swab samples were found positive for *M. conjunctivae* by polymerase chain reaction (PCR). Topical application of 0.5% sterile solution of gentamycin (100 mg/mL) (Gentafar 10%, FARVET, Netherlands) proved suitable for the treatment of IKC in Lohi lambs as all clinical signs of IKC disappeared after 5 days of treatment with this antibiotic. Taghavi Razavizadeh and Razmyar [31] have reported an IKC infection in sheep herds consisting of 300 ewes with ocular lesions in Mashhad, Iran. About 10% of the flock was affected by the disease and 6 animals with severe clinical symptoms were sampled for cultural process. Following inoculation of the ocular swab samples in PPLO broth and agar, turbidity and growth of colonies were observed in them, respectively. The causative agent of the outbreak has identified to be *M. conjunctivae*. This was also the first documented report of isolation and molecular characterization of *M. conjunctivae* in Iran. The affected sheep were succesfully treated with oxytetracycline 20%, IM (20 mg/kg) twice, with interval of 72 h, and flunixin meglumin, IM (2.2 mg/kg) three times, with interval of 24 h. In addition to the listed drugs, tylosin 20%, IM (17 mg/kg), was administrated three times for sheep with pulmonary involvement. Motha et al.[32] have performed direct PCR for examination of *M. conjunctivae* in samples taken form 5 sheep herds with infectious keratoconjunktivitis. In addition, the researchers have run ELISA for screening antibody produced against *M. agalactiae* and *M. conjunctivae* and CFT for antibody of *M. capricolum,* and *Chlamydophila* spp. *M. conjuntivae* was detected specifically from conjunctival swab samples from 3 herds. Antibodies only against to *M. conjunctivae* antibodies were also detected in blood sera of animals (60/75) from all farms. *M. (Branhemella) ovis* was isolated from the remaining 2 herds. The researchers have reported that *M. conjuctivae* is the primary pathogen of IKC in sheep and that this is the first time that the agent has been reported in New Zealand. Fernandez-Aguilar et al.[4] have reported a study of IKC on sheep and goat population in Pakistan with quantitative PCR (qPCR) to investigate the presence of *M. conjunctivae* and *Chlamydiaceae*. The infection rate for *M. conjunctivae* has been reported as 19.3% for sheep and 9.5% for goat. In India, Vaid et al.[33] reported an outbreak of IKC in nomadic sheep herds and 3 *M. ovis* were isolated out of 6 cases of IKC in sheep.

In Turkey, Erdeğer et al.[34] repoted that *M. bovis* was isolated from 41 (19.7%) of 208 conjunctival swab that were obtained from 168 cattle with IBK suspects. Samsar et al.[35] reported that *M. bovis* was isolated from the all of 51 (100%) symptomatic cattle with IBK. *M. bovis* was isolated from 26 (17.9%) of 145 cattle with IBK suspected cattle in Erzurum and central villages [36].

In this study, conjunctival swab samples from 42 (28%) animals with an epidemic keratoconjunctivitis in Tuj sheep herd were collected and cultured for bacteriological examination. *M. ovis* was isolated from 36 (85.71%) of the samples, while *M. conjunctivae* (with *M. ovis*) was detected from 3 (7.14%). In this study, *M. ovis* was isolated from IKC infection similar to the results obtained in other

studies [3,32,33] and identified as the primary agent of the cases. The bacteria such as *L. monocytogenes*, *E. coli*, *Corynebacterium* spp. have been reported previously [2,3] from IKC in sheep but they did not isolated from the samples in this study except the bacteria *M. ovis* and *M. conjunctivae*. The researches have predominatly shown the presence of *M. conjunctivae* as causative agent of IKC in sheep [30-32]. But in this study a substantial amount of *M. ovis* has been reported and this could indicate an outbreak of IKC caused by a monopolise agent, *M. ovis*. This study has also an importance as being the first ovine infectious keratoconjunctivitis epidemic caused by *M. ovis* in Turkey. It was believed this epidemic has developed and evolved due to the fact that the sheep have been exposed to more dust irritations during the grazing season at poor pastures resulting from the last years drought and that some environmental inducers such as the failure of the herd management, farm conditions and insect control. It was reported that these factors were effective in the emergence of the infection [7,31]. In addition, considering the variety of agents isolated from cases of infectious keratoconjunctivitis, this shows that not only the eyes of the animals can be exposed to many obligate pathogens by grazing, feeding or insect exposure periods, but also the involvement of the opportunistic pathogens present in the eye flora with the acceleration of the predisposing factors.

Fortified therapy in the case of bacterial keratitis in human medicine is widely used with different antimicrobial combinations [11,13]. Fortified drugs are made from antibiotics (parenteral or lyophilized preparations) on the market. The first advantage of these drugs is the increased antibiotic concentration in the corneal stroma when applied and the second advantage is being a wide selection chance [13]. Given the potential for synergistic and combined effects of corneal penetration and antibiotic association, fortified antibiotic drops for severe keratitis remain standard therapy [13]. Animal studies with fortified applicaitons especially in ocular infections are widely reported in cattle [37-39] but ovine cases are quite rare [32,40]. Kibar et al.[38] have reported mean time for healing of corneal ulcers and amelioration of clinical signs was significantly less for calves that received enrofloxacin or penicillin + streptomycin than for the untreated controls. Gokce et al.[37] reported that 30 *M. bovis* isolates were obtained from IBK outbreaks of calves and found that animals treated with florfenicol recovered more rapidly than the animals treated with oxytetracycline. However, studies addressing the effectiveness of clinical application of antimicrobials on animals infected with *Moraxella* spp. and *Mycoplasma* spp. are scarce in the literatures [28,41]. Most of antimicrobials are limited with *in vitro* susceptibility of isolated microorganisms and are lack of the clinical applications on existing cases. Nevertheless, susceptibility profile were individually reported both *Moraxella* spp. and *Mycoplasma* spp., as *Moraxella* species show susceptibility to ampicillin, ceftiofur, enrofloxacin, florfenicol and gentamicin [20,42] and *Mycoplasma* species to tetracyclines, kanamycin, danofloxacin, tylosin and linco-spectin [39].

As the results of the treatment options in this study, it was found that the healing rates were higher in the first and third group, in which penicillin G and amikacin/ceftriaxone/fluconazole combination were administered, respectively. When considering the cultivated agents (completely *M. ovis,* except 2 culture negative ewes) of the first group animals the penicillin effectiveness is expectable on *Moraxella* spp. However, the penicillin resistance is likely as reported before for *M. ovis* [20,28] and was encountered in the first group (11/13) of this study. Combinated antibiotic therapy is widely practised in polyfactorial infectious disease and in that in order to limit the spread of antimicrobial resistance. The trilateral combination antimicrobials in the third group of this study yielded a maximum (15/15) healing of animals infected with *M. ovis* and *M. conjunctivae*. This can support a concomitant synergistic interaction between cephalosporins and aminoglycosides [43]. An antifungal (fluconazole) supplementation into the treatment may have contributed to the healing since the remaining two animals found as bacterial culture negative and a fungal infections are included in the probability. Oxytetracycline is usually the first choice for antimicrobial treatment of IKC [44]. Chapman et al.[40] has gained the greatest clinical improvement when sheep with clinical signs of IKC were treated with long-acting oxytetracycline with intramuscularly. In the present study, oxytetracyline and polymyxin B combination was administered in the second treatment group animals infected *M. ovis* and *M. conjunctivae* and 71.42% (10/14) of healing was obtained. Resistance of *Moraxella* spp. to oxytetracycline is scarcely reported [20], but the indiscriminate use of oxytetracycline (most typically is terramycin) in Kars province over the years can be related with the bacterial agent resistant to this drug. Furthermore, polymyxin B as a cationic decapeptide cell membrane destroyer of Gram-negative bacteria may be inadequate in treatment of Gram positive *M. ovis*.

As the result, determining the etiology of infectious keratoconjunctivitis in animals with appropriate microbiological methods and developing an ideal fortified treatment approach to implementation will reduce the size of estimated damages of disease and contribute to animal walfare.

REFERENCES

1. Radostits OM, Gay C, Hinchcliff KW, Constable PD: A textbook of the diseases of cattle, horses, sheep, pigs and goats. Veterinary Medicine. 10th ed., 1548-1551, London: Saunders, 2007.

2. Angelos JA: Overview of infectious keratoconjunctivitis. https://www.msdvetmanual.com/eye-and-ear/infectious-keratoconjunctivitis/overview-of-infectious-keratoconjunctivitis; *Accessed:* 25.10.2017.

3. Åkerstedt J, Hofshagen M: Bacteriological investigation of infectious keratoconjunctivitis in Norwegian Sheep. *Acta Vet Scand*, 45 (1): 19-26, 2004. DOI: 10.1186/1751-0147-45-19

4. Fernandez-Aguilar X, Rossi L, Cabezón O, Giorgino A, Llopis IV, Frey J, López-Olvera JR: Infectious keratoconjunctivitis and occurrence of *Mycoplasma conjunctivae* and *Chlamydiaceae* in small domestic ruminants from Central Karakoram, Pakistan. *Vet Rec*,181 (9): 1-7, 2017. DOI: 10.1136/vr.103948

5. Dagnall GJR: The role of *Branhamella ovis*, *Mycoplasma conjunctivae* and *Chlamydia psittaci* in conjunctivitis of sheep. *Br Vet J,* 150 (1): 65-71, 1994. DOI: 10.1016/S0007-1935(05)80097-1

6. Giacometti M, Janovsky M, Belloy L, Frey J: Infectious keratoconjunctivitis of ibex, chamois and other Caprinae. *Rev Sci Tech*, 21, 335-345, 2002.

7. Bath GF, Van Wyk JA, Pettey KP: Control measures for some important and unusual goat diseases in southern Africa. *Small Rumin Res*, 60 (1-2): 127-140, 2005. DOI: 10.1016/j.smallrumres.2005.06.007

8. O'Connor AM, Shen HG, Wang C, Opriessnig T: Descriptive epidemiology of *Moraxella bovis*, *Moraxella bovoculi* and *Moraxella ovis* in beef calves with naturally occurring infectious bovine keratoconjunctivitis (Pinkeye). *Vet Microbiol*, 155 (2-4): 374-380, 2012. DOI: 10.1016/j.vetmic.2011.09.011

9. Smith MC, Sherman DM: Goat Medicine, 2nd ed., June 2009, Wiley-Blackwell, 2011.

10. Hyndiuk RA, Eiferman RA, Caldwell DR, Rosenwasser GO, Santos CI, Katz HR, Badrinath SS, Reddy MK, Adenis JP, Klauss V: Comparison of ciprofloxacin ophthalmic solution 0.3% to fortified tobramycin cefazolin in treating bacterial corneal ulcers. *Ophthalmology*, 103 (11): 1854-1863, 1996. DOI: 10.1016/S0161-6420(96)30416-8

11. O'Brien TP, Maguire MG, Fink NE, Alfonso E, McDonnell P: Efficacy of ofloxacin vs cefazolin and tobramycin in the therapy for bacterial keratitis. *Arch Ophthalmol*, 113 (10): 1257-1265, 1995. DOI: 10.1001 archopht.1995.01100100045026

12. Panda A, Ahuja R, Sastry SS: Comparison of topical 0.3% ofloxacin with fortified tobramycin plus cefazolin in the treatment of bacterial keratitis. *Eye*, 13, 744-747, 1999. DOI: 10.1038/eye.1999.220

13. Chiquet C, Romanet JP: Prescribing fortified eye drops. *J Fr Ophtalmo,* 30 (4): 423-430, 2007.

14. Behrens-Baumann W, Klinge B, Rüshel R: Topical fluconazole for experimental Candida keratitis in rabbits. *Br J Ophthalmol*, 74, 40-42, 1990. DOI: 10.1136/bjo.74.1.40

15. Köse S, Pamukçu K, Akkın C, Yağcı A, Tümbay E: Fungal keratitlerde sistemik flukonazol tedavisinin etkinliği. *T Oft Gaz,* 23, 380-3, 1993.

16. Savani DV, Perfect JR, Cobo LM, Durack DT: Penetration of new azole compounds into the eye and efficacy in experimental Candida endophthalmitis. *Antimicrob Agents Chemother*, 31, 6-10, 1987. DOI: 10.1128/AAC.31.1.6

17. Tümbay E: Sistemik etkili yeni bir antifungal kemoterapötik: Flukonazol. *Turk J Infect,* 3 (1): 1989.

18. Shiun XW, Lo XJ, Ogawa-Lin: Effectiveness of povidone–iodine to treat infectious conjunctivitis in dogs. *RECVET*, 3 (1): 1-5, 2008.

19. O'Brien TP: Management of bacterial keratitis: Beyond exorcism towards consideration of organism and host factors. *Eye*, 17 (8): 957-974, 2003. DOI: 10.1038/sj.eye.6700635

20. Maboni G, Gressler LT, Espindola JP, Schwab M, Tasca C, Potter L, Vargas AC: Differences in the antimicrobial susceptibility profiles of *Moraxella bovis*, *M. bovoculi* and *M. ovis*. *Braz J Microbiol*, 46 (2): 545-549, 2015. DOI: 10.1590/S1517-838246220140058

21. Baas EJ, Trotter SL, Franklin RM, Barile MF: Epidemic caprine keratoconjunctivitis: Recovery of *Mycoplasma conjunctivae* and its possible role in pathogenesis. *Infect Immun*, 18 (3): 806-815, 1977.

22. Gupta B: *Mycoplasma capricolum*: Isolation and molecular diagnosis. *MSc. Thesis* submitted to the U. P. Pt. Deen Dayal Upadhyaya Pashu-chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura (U. P.). pp. 12-44, 2005.

23. Barile MF, Del Giudice RA, Tully JG: Isolation and characterization of *Mycoplasma conjunctivae* sp. *n.* from sheep and goats with keratoconjunctivitis. *Infect Immun*, 5 (1): 70-76, 1972.

24. Quinn PJ, Markey BK, Carter ME, Donnelly WJ, Leonard FC: Veterinary Microbiology and Microbial Disease. 536, Iowa State University Press, Ames, 2002.

25. Hu FR, Huang WJ, Huang SF: Clinicopathologic study of satellite lesions in nontuberculous mycobacterial keratitis. *Jpn J Ophthalmol,* 42 (2): 115-118, 1998. DOI: 10.1016/S0021-5155(97)00117-2

26. Kalkan Akçay E, Açıkgöz ZC, Can ME, Çelikbilek N, Dereli Can G, Çağıl N: Fungal keratitis caused by *Seedosporium apiospermum*: First report from Turkey. *Mikrobiyol Bul*, 47 (4): 727-733, 2013. DOI: 10.5578/mb.5262

27. Scott PR: Sheep Medicine. 265-270, London, UK, The Veterinary Press, Manson Publishing, 2007.

28. Elad D, Yeruham I, Bernstein M: *Moraxella ovis* in cases of infectious bovine keratoconjunctivitis (IBK) in Israel. *Zentralbl Veterinarmed B*, 35, 431-434, 1988. DOI: 10.1111/j.1439-0450.1988.tb00516.x

29. Karthik K, Manimaran K, Mahaprabhu R, Shoba K: Isolation of *Moraxella* sp. from cases of keratoconjunctivitis in an organized sheep farm of India. *Open J Vet Med,* 7, 138-143, 2017. DOI: 10.4236/ojvm.2017.710014

30. Shahzad W, Munir R, Rana MY, Ahmad R, Khan MS, Akbar G, Ijaz M, Mehmood F: Prevalence, molecular diagnosis and treatment of *Mycoplasma conjunctivae* isolated from infectious keratoconjunctivitis affected Lohi sheep maintained at Livestock Experiment Station, Bahadurnagar, Okara, Pakistan. *Trop Anim Health Prod*, 45 (3): 737-742, 2013. DOI: 10.1007/s11250-012-0282-2

31. Taghavi Razavizadeh AR, Razmyar J: Molecular diagnosis of *Mycoplasma conjunctivae* in an outbreak of infectious keratoconjunctivitis in sheep. *IJVR*, 15 (1): 72-74, 2014. DOI: 10.22099/IJVR.2014.1988

32. Motha MXJ, Frey J, Hansen MF, Jamaludin R, Tham KM: Detection of *Mycoplasma conjunctivae* in sheep affected with conjunctivitis and infectious keratoconjunctivitis. *N Z Vet J,* 51 (4): 186-190, 2003. DOI: 10.1080/00480169.2003.36362

33. Vaid RK, Anand T, Bera BC, Shukla BN, Nagar DK, Singh G, Virmani N, Barua S, Singh K, Singh NK: Isolation and molecular identification of *Moraxella ovis* and *Moraxella* spp. from Infectious Keratoconjunctivitis in sheep in India. *J Veterinar Sci Technol,* DOI: 10.4172/2157-7579.S1.006

34. Erdeğer J, Aydın N: Sığırlarda izole edilen *Moraxella bovis* suşlarının çeşitli özelliklerinin araştırılması. *Turk J Vet Anim Sci,* 15, 140-147, 1991.

35. Samsar E, Akın F, Gökçe P, Bilir B: Sığırların İnfeksiyöz Keratokonjuktivitisinde subkonjuktival antibiyotik ve alfakimotripsin uygulamaları. *Ankara Üniv Vet Fak Derg*, 40 (4): 453-474, 1993.

36. Işık N: Investigation on isolation and sensitivity of antibiotic on *Morexella bovis* in cattle with infectious bovine keratoconjuctivitis. *Dicle Üniv Vet Fak Derg,* 1, 17-22, 2008.

37. Gökçe I, Çitil M, Genç O, Erdoğan HM, Güneş V, Kankavi O: Comparision of the efficacy of florfenicol and oxytetracycline in the treatment of naturally occurring infectious bovine keratoconjunctivitis. *Ir Vet J,* 55 (11): 573-576, 2002.

38. Kibar M, Gümüşsoy KS, Öztürk A: Evaluation of various antibiotic treatments in calves with ınfectious bovine keratoconjunctivitis. *Turk J Vet Anim Sci,* 30, 553-559, 2006.

39. Abdou NEMI, Abdou MY: Bacterial conjunctivitis in cattle and antibiotic sensitivity of the isolates. *Res J Anim Vet Sci*, 5, 38-42, 2010.

40. Chapman HM, Murdoch FR, Robertson ID, Beatty DT: Detection, identification and treatment of infectious ovine keratoconjunctivitis (Pink Eye) in sheep from a Western Australian pre-export feedlot. Meat & Livestock Australia, North Sydney, Australia, 2010.

41. Egwu GO: *In vitro* antibiotic sensitivity of *Mycoplasma conjunctivae* and some bacterial species causing ovine infectious kerato-conjunctivitis. *Small Rumin Res*, 7, 85-92, 1992. DOI: 10.1016/0921-4488(92)90071-B

42. Loy JD, Brodersen BW: *Moraxella* spp. isolated from field outbreaks of infectious bovine keratoconjunctivitis: A retrospective study of case submissions from 2010 to 2013. *J Vet Diagn Invest*, 26 (6): 761-768, 2014. DOI: 10.1177/1040638714551403

43. Rankin GO, Sutherland CH: Nephrotoxicity of aminoglycosides and cephalosporins in combination. *Adverse Drug React Acute Poisoning Rev,* 8 (2): 73-88, 1989.

44. Alexander D: Infectious bovine keratoconjunctivitis: A review of cases in clinical practice. *Vet Clin North Am: Food Anim Pract*, 26, 487-503, 2010. DOI: 10.1016/j.cvfa.2010.09.006

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Research Article

Anaplasma sp., *Ehrlichia* sp., and *Rickettsia* sp. in Ticks: A High Risk for Public Health in Ibagué, Colombia

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Abstract

The Order Rickettsiales comprises intracellular bacteria, including Rickettsiaceae and Anaplasmataceae; members of these families cause zoonotic diseases transmitted by ticks. The aim of this study was to make a detection of *Anaplasma, Ehrlichia* and *Rickettsia* in ticks of the Ixodidae (Acari: Ixodidae) family collected from domestic animals in Ibagué, Colombia. Ticks were collected from dogs, horses and cattle, classified taxonomically, and then subjected to DNA extraction. To detect *Anaplasma* sp., *Ehrlichia* sp., and *Rickettsia* sp. we carried out a conventional PCR to detect the *gltA* gene for *Rickettsia*, 16S rRNA for *Anaplasma* and *Ehrlichia*, and the *dsb* gene for *Ehrlichia*. The phylogenetic trees were constructed with the Neighbor-Joining method. A total of 1.247 ticks, mainly *R. microplus, R. sanguineus* and *D. nitens*, were collected. *Anaplasma* and *Ehrlichia* were detected in thirteen samples. The sequences showed a genetic similarity with *E. canis, E. mineirensis, A. phagocytophilum,* and *A. marginale*. No *Rickettsia* was found. This is the first time that active circulation of *Anaplasma* and *Ehrlichia* is demonstrated in Ibagué. Both pathogens are important because can produce economic losses in animals and humans disease. This finding will contribute to the implementation of early epidemiological alerts, as well as the design of measures to prevent and control diseases in the region.

Keywords: *Domestic animal, Environment and public health, Infectious disease vectors, Ixodidae, Molecular phylogeny, Tick*

Kenelerde *Anaplasma, Ehrlichia ve Rickettsia* Türleri: Ibagué, Kolombiya'da Halk Sağlığı Yönünden Yüksek Risk Faktörleri

Öz

Rickettsiales takımı, Rickettsiaceae ve Anaplasmataceae'nın da dahil olduğu hücre içi bakterileri içerir; bu ailelerin üyeleri keneler tarafından bulaştırılan zoonotik hastalıklara neden olurlar. Bu çalışmanın amacı, Colombia, Ibagué'de evcil hayvanlardan toplanan Ixodidae (Acari: Ixodidae) familyasına ait kenelerde *Anaplasma, Ehrlichia* ve *Rickettsia* türlerinin varlığını belirlemektir Köpek, at ve sığırlardan toplanan keneler taksonomik olarak sınıflandırıldı ve ardından DNA ekstraksiyonuna tabi tutuldu. *Anaplasma, Ehrlichia* ve *Rickettsia* türlerini belirlemek amacıyla, *Rickettsia* için *gltA* geni, *Anaplasma* ve *Ehrlichia* için 16S rRNA geni ve *Ehrlichia* için *dsb* genini tespit edecek konvansiyonel PCR yöntemi kullanıldı. Filogenetik yapılandırma için NJ (Neighbour-Joining) analizi kullanıldı. *R. microplus, R. sanguineus* ve *D. nitens* başta olmak üzere toplam 1.247 kene toplandı. On üç örnekte *Anaplasma* ve *Ehrlicha* tespit edildi. Sekanslar *E. canis, E. mineirensis, A. phagocytophilum* ve *A. marginale* ile genetik bir benzerlik gösterdi. *Rickettsia* bulunamadı. Bu çalışma ile Ibagué'de *Anaplasma* ve *Ehrlichia*'nın aktif sirkülasyonu ilk kez ortaya konuldu. Hayvan ve insan hastalıklarında ekonomik kayıplara neden olabileceğinden dolayı, her iki patojen de önemlidir. Bu bulgu, bölgedeki hastalıkların önlenmesi ve kontrol altına alınmasına yönelik önlemlerin oluşturulmasının yanı sıra, erken epidemiyolojik uyarıların uygulanmasına da katkıda bulunacaktır.

Anahtar sözcükler: *Çevre ve halk sağlığı, Enfeksiyöz hastalık vektörleri, Evcil hayvan, Ixodidae, Kene, Moleküler filogeni*

INTRODUCTION

Ticks are important vectors that may transmit pathogens such as viruses, bacteria, and protozoa and they may pose a significant problem in human and animal public health [1]. The Colombian tropic, climate, lush vegetation, high temperature, and the rich fauna in the area, promote the development of tick populations. In addition to these

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conditions, the animal production systems and human activities can aid in the risk of tick bites, which leads to the transmission of pathogenic agents in animals as well as in humans [2,3].

Genus *Anaplasma, Ehrlichia*, and *Rickettsia*, are Gram-negative coccobacilli of obligatory intracellular growth, and cannot survive outside a vector [4]; they infect endothelial cells, as well as cells in the immune system, monocytes or granulocytes, destroy the phagosome, multiply by binary fission in the cytoplasm, and some in the nucleus of infected cells [5].

The presence of *Ehrlichia, Anaplasma,* and *Rickettsia* in different ticks is closely related to reports of human [6,7] and animal disease cases throughout the world. Bovine anaplasmosis is one of the diseases that causes significant economic losses due to the high morbidity and mortality rates in tropical reported through serological tests in a Colombian army soldier who had been in contact with companion animals [8]. *Anaplasma phagocitophylum* was reported for the first time in serum of rural workers from Córdoba and Sucre [9]. In Colombia, the rickettsiosis is a disease that appears intermittently in tropical areas with some outbreaks [10]. In Latin America, during the last decades, has seen an increase in new species of *Rickettsia* of unknown pathogenicity, many of them detected in ticks at first, some were considered nonpathogenic, but later, their human pathogenicity was demonstrated, such as *Rickettsia massiliae* and *Rickettsia parkeri* [11].

There is no epidemiological information of circulation of *Anaplasma*, *Ehrlichia,* and *Rickettsia* in the city of Ibagué, located at the South-West of Colombia. This lack of epidemiological knowledge sets the ground for confusions, with wrong diagnoses, and therefore, wrong treatment. The purpose of this study was to detect *Anaplasma*, *Ehrlichia,* and *Rickettsia* in ticks collected in the province of Ibagué (Tolima).

MATERIAL and METHODS

Type of Study and Location

The province of Ibagué comprises 3.261 km^2 and eleven municipalities. The province is dedicated to agricultural and livestock activities. The sampled zones corresponded to the municipalities of Ibagué, located at 4°26´10¨N and 75°12´10¨W, Alvarado, El Espinal, Piedras, and Rovira *(Fig. 1)*. The mean annual temperature ranges between 18 and 22°C, and its altitude ranges between 800 and 1000 meters above sea level.

Tick Gathering

Ticks were collected from dogs, cattle, and horses between August and November 2014. The dogs came from veterinary clinics and cattle and horses came from farms in the Ibagué area. The ticks were preserved in ethanol and identified through the taxonomic keys [12,13]. The ticks were arranged in groups of 2 to 5 ticks of the same genus, species, and host.

DNA Extraction

The protocols described by Miranda et al.[14] were followed with slight modifications. In the DNA extraction, we selected a 200 mL volume of the homogenized material to extract DNA from the commercial QIAamp™ DNA Mini kit (Qiagen, Chatsworth, CA). The extracted DNA was stored at -20°C until its PCR analysis.

Molecular Identification of Anaplasma, Ehrlichia and Rickettsia

For *Anaplasma* and *Ehrlichia,* we performed a conventional PCR using primers GE2` F2` (5´-GTT AGT GGC AGA CGG GTG AGT-3´, forward) and HE3 (5´-TAT AGG TAC CGT CAT TAT CTT CCC TAT-3´, reverse) to amplify a 360 bp fragment of the *16S rRNA* gene of the Anaplasmataceae family, and dsb-330 (5´-GAT GAT GTC TGA AGA TAT GAA ACA AAT-3´, forward) and dsb-728 (5´-CTG CTC GTC TAT TTT ACT TCT TAA AGT-3, reverse) to amplify a portion of 409 bp of gene *dsb*, for *Ehrlichia* sp.[15].

For *Rickettsia* sp., we used primers CS-78 (5´-GCA AGT ATC GGT GAG GAT GTA AT-3´, forward) and CS-323 (5´-GCT TCC TTA AAA TTC AAT AAA TCA GGA T-3´, reverse) to amplify a 401 bp fragment of gene *gltA* and 120.2788 (5´-AAA CAA TAA TCA AGG TAC TGT-3´, forward) and 120.3599 (5´-TAC TTC CGG TTA CAG CAA AGT-3´, reverse) for the 812 bp portion of the *ompB* gene [16]. PCR products were visualized in a 1.5% agarose gel, stained with ethidium bromide.

Sequence Analysis

The amplified products were sequenced with the dideoxy method in an automatic MegaBACE 750 (Amersham, Biosciences, Piscataway, NJ, USA) DNA sequencer. The nucleotide sequences of the positive samples underwent a BLAST to determine their similarity with other species of *Anaplasma, Ehrlichia,* and *Rickettsia*. The obtained sequences were aligned through the Geneious R9.1.2 and MEGA 7.0 software, the phylogenetic relations were inferred using the Molecular Evolutionary Genetics Analysis (MEGA 7.0) software [17]. For each analyzed gene, we built a phylogenetic tree with the Neighbor-Joining method, using the Kimura parameter as a nucleotide substitution model. The reliability of the phylogenetic tree analysis was determined by bootstrap with 1000 replications.

RESULTS

A total 1.247 adults ticks were collected from bovines, equines, and canines, and three species were identified: *Rhipicephalus sanguineus* (50.8%), *Dermacentor nitens* (27.3%) and *Rhipicephalus microplus* (21.9%). We found 685

ticks (54.9%) on 50 canines, 371 (29.7%) on 13 equines, and 191 ticks (15.3%) on 16 bovines. A total 119 groups were formed to extract DNA.

The 16S rRNA 4.2% (5/119) and *dsb* 6.7% (8/119) genes were amplified. Amplifications of gene 16S rRNA were from *R. microplus* collected from bovines; *dsb* gene amplifications were from *R. microplus* and *R. sanguineus* collected from canines in the areas of Alvarado and Ibagué. The conventional PCR was negative for *gltA* and *ompB* gene amplification in *Rickettsia*.

Sequence analysis with samples who amplified the two genes 16S rRNA and *dsb* showed 99% and 100% similarities with *Ehrlichia mineirensis* (KF621013.1, JX629805.1, and DQ379966 for 16S rRNA and KT314243.1, KM015219.1, JX629808.1 for *dsb*) in three bovine samples. Those for gene 16S rRNA in *R. microplus* DNA samples showed 99% similarity with *Anaplasma marginale* (KP877314.1, AB916498.1 and LC007100.1) and 99% with *A. phagocitophylum* (EU287434.1, KJ195692.1 and GU111741.1), collected from bovines in Alvarado and Ibagué, respectively.

The *dsb* gene analysis showed that, in four groups of canines, sequences with 99% and 100% similarity with *Ehrlichia canis* (KR732921.1, AF403710.1 and KU323869.1) were found; these groups were amplified from *R. sanguineus*. The phylogenetic trees with genes 16S rRNA and *dsb* show the various positions of the partial sequences of the genes with those available in GenBank *(Fig. 1, Fig. 2)*.

DISCUSSION

Anaplasma marginale and *E. canis* were detected in *R. microplus* and *R. sanguineus*; this is an important finding, since it is the first time they are detected in Ibagué, an area in the Colombian southwest. Finding *E. mineirensis* in Ibagué for the first time is also important, since it had only been reported in Córdoba (Colombia) [18].

In Colombia, many species of ticks have been described [12]. However, the diversity of ticks species collected in the present study could be explained by the number of canine, bovine, and equine hosts from where the ectoparasite samples were collected. On the other hand, despite finding *R. sanguineus* in two host species, it may also develop in rodents and other mammals, but the dog is the primary host and plays an important role in the development of high populations of this ectoparasite [19]. *R. microplus* is a primary pathogen in bovines [20,21]; however, it may appear in several hosts among which we can highlight buffalo, horses, donkeys, goats, sheep, deer, pigs, dogs, and some wild animals [22]. In this study, *R. microplus* was found in the three types of host vertebrates but mainly in bovines, which demonstrates its adaptability.

In this study, 119 groups of adult ticks were analyzed. Having only adults ticks limit the association of other stages with possible infection processes with *Anaplasma* and *Ehrlichia* and their vectorial capacity, since it has been identified

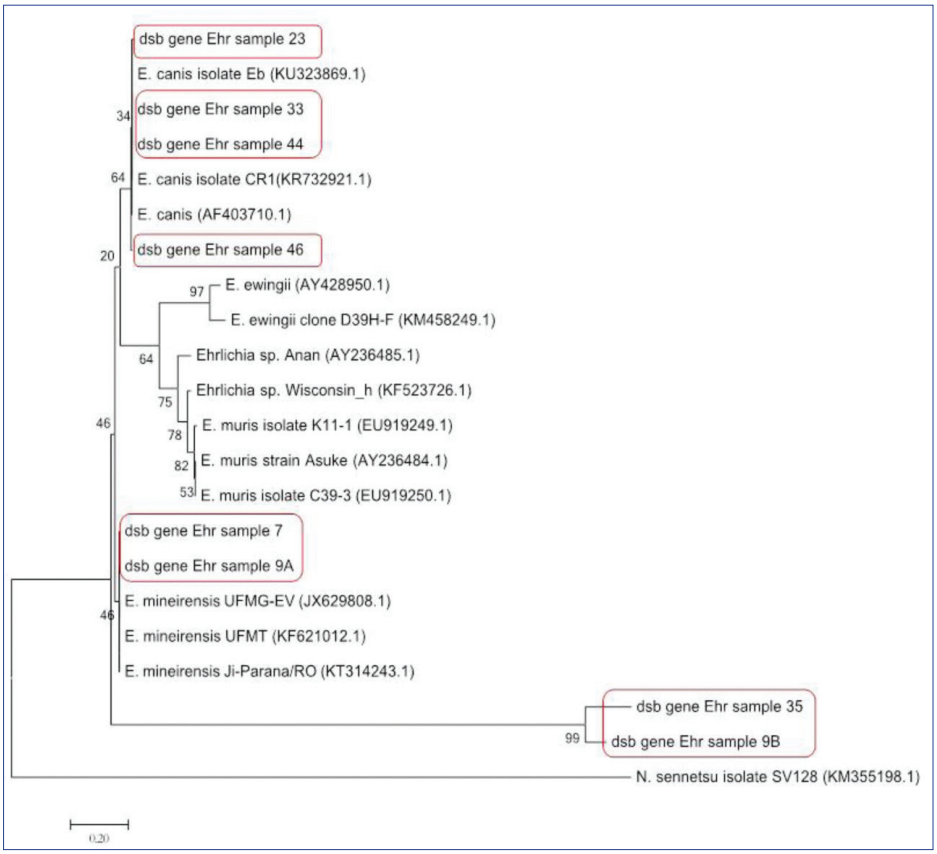

Fig 1. Phylogenetic tree showing samples positions, obtained by comparing the partial sequences of ribosomal 16S RNA gene (*16S rRNA*) with *Anaplasma* sp. and *Ehrlichia* sp. sequences available at GenBank (access number in parenthesis). The Neighbor-Joining method was used, based on the Kimura parameter model. The beginning of the branches show the bootstrap values (1000 replications). The analysis was carried out using the MEGA 7 program. The sample groups of the study are framed in a rectangle. *Neorickettsia sennetsu* was used as an external group

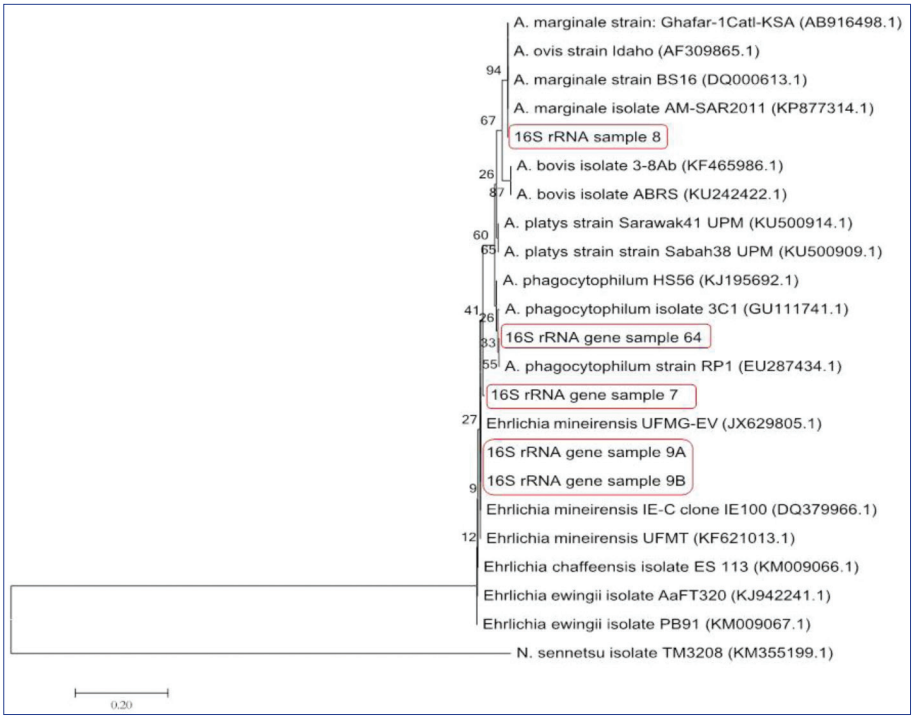

Fig 2. Phylogenetic tree showing samples positions, obtained by comparing the partial sequences of the *dsb* gene available at GenBank (access number in parenthesis). The Neighbor-Joining method was used, based on the Kimura parameter model. The beginning of the branches show the bootstrap values (1000 replications). The analysis was carried out using the MEGA 7 program. The sample groups of the study are framed in a rectangle. *N. sennetsu* was used as an external group

that larvae awaiting a host show more aggressive behavior in mass attacks to susceptible hosts; this description has been documented on human infestations of *Amblyomma cajennense* [23].

This study detected a group of ticks with a sequence 99% similar to *A. marginale* in a group of *R. microplus* ticks. The results of this study match with the research made by Wen et al.[24] in Tibet, who detected *Ehrlichia* and *Anaplasma* in *R. microplus* collected in cows. In Mongolia, Ybanez et al.[25] made the molecular characterization of *A. marginale* species detected in 8 (13.3% positivity) *R. microplus* from bovine cattle. *R. microplus* is important in veterinary medicine because it transmits *A. marginale*, which causes great economic losses to livestock farming in many subtropical and tropical countries [18]. *R. microplus* is not considered an ectoparasite that transmits pathogens to humans [20], however, *R. microplus* has been found bitten humans [19].

Argas persicus, *Rhiphicephalus annulatus*, *Rhiphicephalus decoloratus*, *R. microplus*, *Dermacentor albipictus*, *Dermacentor andersoni*, *Dermacentor occidentalis*, *Dermacentor variabilis*, *Ixodes ricinus*, *R. sanguineus,* and *Rhiphicephalus simus* are among the vector ticks that mechanically or biologically transmit *A. marginale*. Intra- or interspecies transmission is common in single-host *Rhiphicephalus* species; also, some authors have describe them as significant vectors of anaplasmosis in regions such as Central America, Latin America, the Caribbean, Australia, and South Africa [26]. *A. marginale* has been identified in differents regions in Colombia, farms animals in the Colombian Caribbean, found 31.7% (923 of 2909 animals) of positives to *A. marginale* in 101 of 104 farms studied [27]. Researchers in the Department of Córdoba report sequences with 99-100% identity with *A. marginale*, in the DNA of ticks collected from dogs, horses, and cows [18]. In Purificacion (Tolima), a 20% of *A. marginale* was reported with Giemsa stain on bovine coccygeal vein peripheral blood bovine [28]. This results in different regions of Colombia, confirm ours findings where the sequence detected showed a high genetic identity with *A. marginale*.

Anaplasma phagocytophilum has been confirmed in Asia and Europe and some countries in South America [29,30]. Serological studies in dogs from three cities of Colombia during 2011, reported *A. phagocytophilum* in 51%, 40% and 12% in Cartagena, Barranquilla and Medellin cities respectively [31]. Seroepidemiological data suggest that many human infections go unrecognized in Sucre and Cordoba (north of Colombia), where a prospective study in people with occupational risk factors (farmers and day workers) found a seroprevalence rate of 20% (15/75) for *A. phagocytophilum* [9]. There are a few reports of *A. phagocytophilum* in ticks from Colombia. This work has detected a sequence 99% identity with *A. phagocytophilum* in DNA of *R. microplus* collected in bovine from Ibagué area.

Five species of *Ehrlichia* have been identified, out of which three are known to cause human ehrlichiosis *(E. canis*, *E. chaffeensis* and *E. ewingii)* [32]. The phylogenetic characterization of *Ehrlichia* species isolated from ticks has identified *E. mineirensis* as a new species that has been reported in some parts of Latin America and Brazil [33]. It has been reported in Colombia by Miranda & Mattar [18]. New studies aimed at identifying this bacteria

are indispensable, in order to confirm the adaptation evidence just with *R. microplus* or with some other type of vectors. Our results agree with other studies, four ours sequences detected in *R. microplus* collected from bovine were 99 to 100% identical to *E. mineirensis*, further work to do a better characterization and to establish the current status of this strain are necessary, as well as needed to clarify the pathogenic potential, geographic distribution or host range of this agent.

Ehrlichia is one of the most reported species in South American vertebrates, and has been proven to circulate in canines in Colombian cities such as Cali and Medellín [34-36]. Dogs are the final hosts of ticks that transmit ehrlichioses, and therefore this disease must be considered as a public health issue due to its zoonotic potential [37].

Vargas-Hernandez et al.[36], detected *E. canis* in *R. sanguineus* ticks collected from dogs in Bogotá, Bucaramanga, and Villavicencio. *E. canis* is nowadays considered as a species that causes ehrlichiosis; not only has it been reported in canines, but also in felines and humans, and many of these cases have been detected from the transmission by *R. sanguineus* bites [38]. In this study, partial sequences 99-100% similar to *E. canis* were identified from the extraction of DNA from *R. sanguineus* captured on canines.

In the present work, the samples were amplified using conventional PCR, but yielded no positive results for *Rickettsia*. Studies conducted in Colombia have been identified the presence of *Rickettsia belli* [39], *Rickettsia rickettsii* [40], *Rickettsia felis* [41], *Candidatus Rickettsia colombianensis* [14] and *Rickettsia* sp. *Atlantic Rainforest strain* [42]. More ticks, especially from the *Amblyomma* genus, must be collected, and from other hosts different from those in this study, in order to detect species of *Rickettsia* in the zone.

In conclusion, the bacteria species reported in this study had not been previously reported in the department of Tolima, on the south-west of Colombia, and are of great interest for the region, since they are pathogens that cause diseases which entail economic losses in animals and humans. Detection of *Anaplasma* and *Ehrlichia* is fundamental in order to implement early prevention and control alerts by the epidemiological surveillance authorities in the region. All the cases evidenced in previous research and in this study represent a reappearance of such diseases, and must alert the epidemiological surveillance authorities of the country.

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Conflicts of Interest

The authors hereby declare that we do not have any conflict of interest in regards to the information provided in this study.

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REFERENCES

1. Alberdi MP, Nijhof AM, Jongejan F, Bell-Sakyi L: Tick cell culture isolation and growth of *Rickettsia raoultii* from Dutch *Dermacentor reticulatus* ticks. *Ticks Tick Borne Dis,* 3 (5-6): 349-354, 2012. DOI: 10.1016/j.ttbdis.2012.10.020

2. Maegli A, Loy JD, Cortinas R: Note on *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and "*Borrelia lonestari*" infection in lone star ticks (Acari: Ixodidae), Nebraska, USA. *Ticks Tick Borne Dis,* 7 (1): 154-158, 2016. DOI: 10.1016/j.ttbdis.2015.10.008

3. Oteo JA, Portillo A: Tick-borne rickettsioses in Europe. *Ticks Tick Borne Dis,* 3 (5-6): 271-278, 2012. DOI: 10.1016/j.ttbdis.2012.10.035

4. Biggs HM, Behravesh CB, Bradley KK, Dahlgren FS, Drexler NA, Dumler JS, Folk SM, Kato CY, Lash RR, Levin ML, Massung RF, Nadelman RB, Nicholson WL, Paddock CD, Pritt BS, Traeger MS: Diagnosis and management of tickborne rickettsial diseases: rocky mountain spotted fever and other spotted fever group rickettsioses, ehrlichioses, and anaplasmosis - United States. *MMWR Recomm Rep,* 65 (2): 1-44, 2016. DOI: 10.15585/mmwr.rr6502a1

5. Dagnone AS, Souza AI, Andre MR, Machado RZ: Molecular diagnosis of Anaplasmataceae organisms in dogs with clinical and microscopical signs of ehrlichiosis. *Rev Bras Parasitol Vet,* 18 (4): 20-25, 2009. DOI:10.4322/rbpv.01804004

6. Dahmani M, Loudahi A, Mediannikov O, Fenollar F, Raoult D, Davoust B: Molecular detection of *Anaplasma platys* and *Ehrlichia canis* in dogs from Kabylie, Algeria. *Ticks Tick Borne Dis,* 6 (2): 198-203, 2015. DOI: 10.1016/j.ttbdis.2014.12.007

7. da Silva GC, Benitez Ado N, Girotto A, Taroda A, Vidotto MC, Garcia JL, de Freitas JC, Arlington SH, Vidotto O: Occurrence of *Ehrlichia canis* and *Anaplasma platys* in household dogs from northern Parana. *Rev Bras Parasitol Vet*, 21, 379-385, 2012. DOI: 10.1590/S1984-29612012005000009

8. Botero AH, Muñoz Ramirez F, Vega Miranda J: Primer caso de ehrlichiosis monocítica humana reportado en Colombia. *Infect,* 18 (4): 162-166, 2014. DOI: 10.1016/j.infect.2014.04.001

9. Máttar S, Parra M: Detection of antibodies to *Anaplasma, Bartonella* and *Coxiella* in rural inhabitants of the Caribbean area of Colombia. *Rev MVZ Córdoba,* 11, 781-789, 2006.

10. Hidalgo M, Faccini-Martinez AA, Valbuena G: Tick-borne rickettsioses in the Americas: Clinical and epidemiological advances, and diagnostic challenges. *Biomedica,* 33 (Suppl.1): 161-178, 2013. DOI: 10.7705/biomedica.v33i0.1466

11. Labruna MB, Salim Mattar V, Nava S, Bermudez S, Venzal JM, Dolz G, Katia A, Romero L, de Sousa R, Oteo J, Zavala-Castro J: Rickettsioses in Latin America, Caribbean, Spain and Portugal. *Rev MVZ Córdoba,* 16, 2435-2457, 2011.

12. Osorno-Mesa E: Ticks of the Republic of Colombia. 1940. *Biomedica,* 26 (3): 317-336, 2006.

13. Benavides Ortiz E, Lopez Valencia G: Clave Pictórica para la Identificación de Garrapatas en Colombia y Norte de Sudamérica. *CORPOICA Bogotá, Colombia*, 2007.

14. Miranda J, Portillo A, Oteo JA, Mattar S: *Rickettsia* sp. strain colombianensi (Rickettsiales: Rickettsiaceae): A new proposed Rickettsia detected in *Amblyomma dissimile* (Acari: Ixodidae) from iguanas and free-living larvae ticks from vegetation. *J Med Entomol,* 49 (4): 960-965, 2012. DOI: 10.1603/ME11195

15. Doyle CK, Labruna MB, Breitschwerdt EB, Tang YW, Corstvet RE, Hegarty BC, Bloch KC, Li P, Walker DH, McBride JW: Detection of medically important *Ehrlichia* by quantitative multicolor TaqMan real-time polymerase chain reaction of the *dsb* gene. *J Mol Diagn,* 7 (4): 504-510, 2005. DOI: 10.1016/S1525-1578(10)60581-8

16. Labruna MB, Whitworth T, Horta MC, Bouyer DH, McBride JW, Pinter A, Popov V, Gennari SM, Walker DH: Rickettsia species infecting *Amblyomma cooperi* ticks from an area in the state of Sao Paulo, Brazil, where Brazilian spotted fever is endemic. *J Clin Microbiol,* 42 (1): 90-98, 2004. DOI: 10.1128/JCM.42.1.90-98.2004

17. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S: MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol,* 30 (12): 2725-2729, 2013. DOI: 10.1093/molbev/mst197

18. Miranda J, Mattar S: Molecular detection of *Anaplasma* sp. and *Ehrlichia* sp. in ticks collected in domestical animals, Colombia. *Trop Biomed,* 32 (4): 726-735, 2015.

19. Guglielmone AA, Beati L, Barros-Battesti DM, Labruna MB, Nava S, Venzal JM, Mangold AJ, Szabo MP, Martins JR, Gonzalez-Acuna D, Estrada-Pena A: Ticks (Ixodidae) on humans in South America. *Exp Appl Acarol,* 40 (2): 83-100, 2006. DOI: 10.1007/s10493-006-9027-0

20. Guerrero FD, Lovis L, Martins JR: Acaricide resistance mechanisms in *Rhipicephalus (Boophilus) microplus*. *Rev Bras Parasitol Vet*, 21 (1): 1-6, 2012. DOI:10.1590/S1984-29612012000100002

21. Bustillos R, Carrillo J, Jacho G, Enríquez S, Rodríguez R: Comportamiento Poblacional de la garrapata *Rhipicephalus* (Boophilus) *microplus* en bovinos en dos areas geograficas del Ecuador. *RTE,* 28 (4): 68, 2015.

22. Estrada-Pena A, Bouattour A, Camicas JL, Guglielmone A, Horak I, Jongejan F, Latif A, Pegram R, Walker AR: The known distribution and ecological preferences of the tick subgenus *Boophilus* (Acari: Ixodidae) in Africa and Latin America. *Exp Appl Acarol,* 38 (2-3): 219-235, 2006. DOI: 10.1007/s10493-006-0003-5

23. Labruna MB: Ecology of rickettsia in South America. *Ann N Y Acad Sci,* 1166, 156-166, 2009. DOI: 10.1111/j.1749-6632.2009.04516.x

24. Wen B, Jian R, Zhang Y, Chen R: Simultaneous detection of *Anaplasma marginale* and a new *Ehrlichia* species closely related to *Ehrlichia chaffeensis* by sequence analyses of 16S ribosomal DNA in *Boophilus microplus* ticks from Tibet. *J Clin Microbiol,* 40 (9): 3286-3290, 2002. DOI: 10.1128/JCM.40.9.3286-3290.2002

25. Ybanez AP, Sivakumar T, Battsetseg B, Battur B, Altangerel K, Matsumoto K, Yokoyama N, Inokuma H: Specific molecular detection and characterization of *Anaplasma marginale* in Mongolian cattle. *J Vet Med Sci,* 75 (4): 399-406, 2013. DOI: 10.1292/jvms.12-0361

26. Ruybal P, Moretta R, Perez A, Petrigh R, Zimmer P, Alcaraz E, Echaide I, Torioni de Echaide S, Kocan KM, de la Fuente J, Farber M: Genetic diversity of *Anaplasma marginale* in Argentina. *Vet Parasitol,* 162 (1-2): 176-180, 2009. DOI: 10.1016/j.vetpar.2009.02.006

27. Orjuela J, Navarrete M, Betancourt A, Roqueme L, Cortez E, Morrison R: Salud y productividad en bovinos de la costa norte de Colombia. *World Anim Rev,* 69 (4): 7-14, 1991.

28. Useche J: Prevalencia de hemoparásitos en bovinos de seis veredas del municipio de purificación-Tolima. [Trabajo de grado, Facultad de Ciencias Agropecuarias, Programa de Medicina Veterinaria]. Bogotá: Universidad de la Salle Colombia. 2010.

29. Koh FX, Panchadcharam C, Tay ST: Vector-Borne Diseases in stray dogs in peninsular malaysia and molecular detection of *Anaplasma* and *Ehrlichia* spp. from *Rhipicephalus sanguineus* (Acari: Ixodidae) ticks. *J Med Entomol,* 53 (1): 183-187, 2016. DOI: 10.1093/jme/tjv153

30. Santos HA, Thome SMG, Baldani CD, Silva CB, Peixoto MP, Pires MS, Vitari GLV, Costa RL, Santos TM, Angelo IC, Santos LA, Faccini JLH, Massard CL: Molecular epidemiology of the emerging zoonosis agent *Anaplasma phagocytophilum* (Foggie, 1949) in dogs and ixodid ticks in Brazil. *Parasit Vectors,* 6, 348, 2013. DOI: 10.1186/1756-3305-6-348

31. McCown ME, Monterroso VH, Cardona W: Surveillance for *Ehrlichia canis*, *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, and *Dirofilaria immitis* in dogs from three cities in Colombia. *J Spec Oper Med,* 14 (1): 86-90, 2014.

32. Thomas RJ, Dumler JS, Carlyon JA: Current management of human granulocytic anaplasmosis, human monocytic ehrlichiosis and *Ehrlichia ewingii* ehrlichiosis. *Expert Rev Anti Infect Ther,* 7 (6): 709-722, 2009. DOI: 10.1586/eri.09.44

33. Cabezas-Cruz A, Vancova M, Zweygarth E, Ribeiro MFB, Grubhoffer L, Passos LMF: Ultrastructure of *Ehrlichia mineirensis*, a new member of the *Ehrlichia* genus. *Vet Microbiol,* 167 (3-4): 455-458, 2013. DOI: 10.1016/j.vetmic.2013.08.001

34. Silva-Molano RF, Sánchez-Ucrós N, Loaiza-Echeverri AM: Recurrence report of *Ehrlichia canis* in dog blood samples in Cali, Colombia. *Vet Zootec,* 2 (1): 27-31, 2008.

35. Yarce LMMC, Osorio LAR, Arias JAC: Seroprevalencia de *Ehrlichia canis* en perros con sospecha de infección por patógenos transmitidos por garrapatas en Medellín, 2012-2014. *Rev Med Vet*, 29, 51-62, 2015. DOI: 10.19052/mv.3446

36. Vargas-Hernandez G, Andre MR, Faria JL, Munhoz TD, Hernandez-Rodriguez M, Machado RZ, Tinucci-Costa M: Molecular and serological detection of *Ehrlichia canis* and *Babesia vogeli* in dogs in Colombia. *Vet Parasitol,* 186 (3-4): 254-260, 2012. DOI: 10.1016/j.vetpar.2011.11.011

37. Perez M, Bodor M, Zhang C, Xiong Q, Rikihisa Y: Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. *Ann N Y Acad Sci,* 1078, 110-117, 2006. DOI: 10.1196/annals.1374.016

38. Bowman D, Little SE, Lorentzen L, Shields J, Sullivan MP, Carlin EP: Prevalence and geographic distribution of *Dirofilaria immitis*, *Borrelia burgdorferi*, *Ehrlichia canis*, and *Anaplasma phagocytophilum* in dogs in the United States: Results of a national clinic-based serologic survey. *Vet Parasitol,* 160 (1-2): 138-148, 2009. DOI: 10.1016/j.vetpar.2008.10.093

39. Miranda J, Mattar S: Molecular detection of *Rickettsia bellii* and *Rickettsia* sp. strain Colombianensi in ticks from Cordoba, Colombia. *Ticks Tick Borne Dis,* 5 (2): 208-212, 2014. DOI: 10.1016/j.ttbdis.2013.10.008

40. Faccini-Martinez AA, Costa FB, Hayama-Ueno TE, Ramirez-Hernandez A, Cortes-Vecino JA, Labruna MB, Hidalgo M: *Rickettsia rickettsii* in *Amblyomma patinoi* ticks, Colombia. *Emerg Infect Dis,* 21 (3): 537-539, 2015. DOI: 10.3201/eid2013.140721

41. Ramirez-Hernandez A, Montoya V, Martinez A, Perez JE, Mercado M, de la Ossa A, Velez C, Estrada G, Correa MI, Duque L, Ariza JS, Henao C, Valbuena G, Hidalgo M: Molecular detection of *Rickettsia felis* in different flea species from Caldas, Colombia. *Am J Trop Med Hyg,* 89 (3): 453-459, 2013. DOI: 10.4269/ajtmh.12-0698

42. Londoño AF, Diaz FJ, Valbuena G, Gazi M, Labruna MB, Hidalgo M, Mattar S, Contreras V, Rodas JD: Infection of *Amblyomma ovale* by *Rickettsia* sp. strain Atlantic rainforest, Colombia. *Ticks Tick Borne Dis,* 5 (6): 672-675, 2014. DOI: 10.1016/j.ttbdis.2014.04.018

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Research Article

The Role of JAK2/STAT3 Signaling Pathway Regulation in Macrophage Apoptosis During *Brucella* M5-90 Infection

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Abstract

Brucellosis is serious zoonotic disease affecting both animals and humans. *Brucella* inhibits the apoptosis of host macrophages. And the JAK2/STAT3 pathway regulates various cellular physiological activities. However, the association between *Brucella*-mediated inhibition of macrophage apoptosis and regulation of the JAK2/STAT3 pathway is unclear. In the current study, We tested the the activation of JAK2/STAT3 pathway and evaluated its function during *Brucella* M5-90 infection in cells. The result was found that infection with *Brucella* M5-90 activated the JAK2/STAT3 pathway and induced phosphorylation of both JAK2 and STAT3 in a time-dependent manner. JAK2 and STAT3 phosphorylation were inhibited by AG490 in a dose-dependent manner. Inhibition of the JAK2/STAT3 pathway with AG490 significantly induced proinflammatory responses, macrophage apoptosis at the transcriptional and protein levels, as well as intracellular survival and replication of *Brucella* M5-90. This study indicates that the JAK2/STAT3 pathway plays a major role in macrophage apoptosis induced by infection with *Brucella* M5-90. In addition, TNF-α plays a major role in the regulation of the JAK2/STAT6 pathway during *Brucella* M5-90 infection. The above information may help to unravel the pathogenic mechanism of Brucella infection.

Keywords: *Brucella, JAK2/STAT3 pathway, Apoptosis, Infection, AG490*

Brucella M5-90 Enfeksiyonu Süresince Makrofaj Apoptozisinde JAK2/STAT3 Uyarı Yolağının Rolü

Öz

Brusellozis hem hayvanları hem de insanları etkileyen ciddi zoonotik bir hastalıktır. *Brucella* konakçı makrofajlarının apoptozisini inhibe eder. JAK2/STAT3 yolağı çeşitli hücresel fizyolojik aktiviteleri düzenler. Ancak *Brucella* aracılı makrofaj apoptozisinin inhibisyonu ile JAK2/STAT3 yolağının düzenlenmesi arasındaki ilişki belirsizdir. Bu çalışmada; JAK2/STAT3 yolağının aktivasyonu çalışılarak hücrelerde *Brucella* M5-90 enfeksiyonu boyunca fonksiyonu değerlendirildi. *Brucella* M5-90 ile enfeksiyonun JAK2/STAT3 yolağını active ettiği ve hem JAK2 hem de STAT3 fosforilasyonunu zamana bağlı olarak uyardığı tespit edildi. JAK2 ve STAT3 fosforilasyonu doza bağlı olarak AG490 ile inhibe edildi. JAK2/STAT3 yolağının AG490 ile inhibe edilmesi anlamlı derecede proinflamatory yanıtı, transkripsiyon ve protein seviyesinde makrofaj apoptozisini ve *Brucella* M5-90'nın hücre içi hayatta kalma ve replikasyonunu uyardı. Bu çalışma *Brucella* M5-90 enfeksiyonu ile oluşturulan makrofaj apoptozisinde JAK2/STAT3 yolağının önemli bir rol oynadığını göstermiştir. Ayrıca, *Brucella* M5-90 enfeksiyonu süresince JAK2/STAT6 yolağının regülasyonunda TNF-α önemli rol oynar. Yukarıda ifade edilen bulgular *Brucella* enfeksiyonunun patogenezini açığa çıkarmada faydalı olabilir..

Anahtar sözcükler: *Brucella, JAK2/STAT3 yolağı, Apoptozis, Enfeksiyon, AG490*

INTRODUCTION

Brucella abortus are Gram-negative and facultative intracellular pathogens affecting humans and animals and can enter various cell types during infection [1]. *Brucella* penetrates the nasal cavity, oral cavity, and/or pharynx mucous membrane and is phagocytosed by host macrophages, in which they can survive and replicate.

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Brucellosis is a zoonotic disease, which is difficult to diagnose. Infection in humans causes fever, hyperhidrosis and arthralgia along with other symptoms. During *Brucella* infection, bacteria preferentially invade host macrophages or trophoblast cells. Although bacterial invasion generally activates the body's specific immunity and natural immunity to promote the lethality of immune cells, *Brucella* strains have developed strategies for evading these cells [2]. *Brucella* resides and replicates within the endoplasmic reticulum of cells, even regulating the apoptosis of these host cells, thus weakening the killing ability and phagocytosis of immune cells. Studies have shown that Tumor Necrosis Factor (TNF-α) is important for inducing specific immune responses against a series of intracellular infections [3]. Furthermore, it has been proven that TNF-α-mediated apoptosis plays a role in pathologies related to chronic inflammation and autoimmune diseases [4]. Therefore, understanding the pathogenesis, survival and replication of *Brucella* in macrophages, as well as the immune mechanisms involved in combating bacterial infection, are vitally important for treating chronic infection with *Brucella*.

The JAK2/STAT3 signal transduction pathway is an important intracellular signal transduction pathway and is a common pathway for a variety of cytokines and growth factors. The JAK2/STAT3 pathway plays an important role in cell proliferation, differentiation, apoptosis and immune regulation [5]. Additionally, research [6] has shown that at present, there are few studies of the regulatory mechanisms involved in host cell immune pathways after intracellular infection. The current study examines whether the JAK2/STAT3 pathway plays a role in the regulation of host cell apoptosis and survival of intracellular bacteria survival during *Brucella* infection.

The inhibitor, AG490, was used to inhibit JAK2 activity [7] in macrophage model of mice infected with *Brucella* M5-90 to determine the relationship between the JAK2/STAT3 signal pathway and apoptosis of infected macrophages. Western blot, ELISA, RT-PCR and CFU assays were used to detect JAK2 and STAT3 phosphorylation, cytokine secretion, changes in apoptosis and intracellular survival of *Brucella*, respectively. The aim of this study was to explore the regulatory role of the JAK2/STAT3 signal pathway in macrophage apoptosis induced by *Brucella* and to determine whether the JAK2/STAT3 signal pathway may be useful in developing treatments for *Brucella* infection.

MATERIAL and METHODS

Cell Culture

RAW264.7 macrophages were obtained from the China Academy Typical Culture Preservation Committee Cell Library (Shanghai, China). RAW264.7 cells were cultured in 12-well plates in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at a concentration of 1×10^6 cells per well and cultured overnight at 37°C in humidified 5% CO_2.

Brucella Infection

To determine whether AG490 (JAK2/STAT3 pathway inhibitor) affects the activity of the JAK2/STAT3 signaling pathway and the expression of apoptosis related proteins in macrophages, RAW264.7 cells were pre-treated with different concentrations of AG490; control cells were not treated with inhibitor. RAW264.7 macrophages were infected with *Brucella* M5-90 at a multiplicity of infection (MOI) of 100:1. The cells were incubated for 1 h at 37°C, washed twice with phosphate-buffered saline (PBS), and then incubated in DMED, supplemented with 25 μg/mL gentamicin, for 45 min to kill remaining extracellular and adherent *Brucella* M5-90. The infected cells were washed three times with ice cold PBS and new culture medium was added. At 2, 4, 8, 12 and 24 h post-infection, one group of cells were lysed with RIPA Lysis Buffer (Beyotime, China), centrifuged at 12000 rpm for 20 min at 4°C and the protein supernatant used for analysis of JAK2, STAT3 phosphorylation by Western blot, another group of cells was lysed with 0.1% tritonX-100 for mRNA transcriptome analysis of JAK2, STAT3, Bax, Bcl-2 and a third group of cells were used to detect the TNF-α level in the supernatant using an ELISA Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). All samples were stored at -80°C.

MTT Assay

RAW264.7 cells were cultured in 96-well plates at 37°C under humidified 5% (vol/vol) CO_2 and then treated with 10, 20, 40, or 60 μM AG490 (treated groups) or 0.1% dimethyl sulfoxide (DMSO; Thermo Fisher Scientific, Inc.) (control group). Cells were incubated in humidified 5% CO_2 at 37°C for 16-48 h to assess cell viability by MTT assay. A 20 μL volume of MTT solution (0.5% MTT) was added to each well and the cells were incubated for 4 h. The culture supernatants were removed. DMSO (150 μL) was then added to each well and the wells were shaken at low speed for 10 min. The optical density (OD) of each well was measured at 490 nm by the ELISA reader (or a microplate reader).

Western Blot Assay

Extracted protein was boiled for 10 min at 100°C and 20 μL samples were subjected to 15% SDS-PAGE. The protein samples were electrophoretically transferred onto a NitroBind nitrocellulose membranes (Bio-Rad; 0.45-μm) at 200mA for 1 h. The membranes were then blocked with Non-protein blocking solution (Shenggong, China) for 1 h and washed three times with TBST buffer (100mM trus-HCl; 150mM NaCl; 0.05% Tween 20, pH 7.2). The membranes were then incubated overnight at 4°C with a 1/1500 dilution of specific primary anti-rabbit pJAK2 (sc-34479), pSTAT3 (sc-56747), Bax(sc-20067), or Bcl-2 (sc-56015), caspase-3(sc-136219) (Santa Cruz Biotechnology, USA) antibodies.

The membranes were subsequently incubated with goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (EarthOx Life Sciences, USA) for 1 h at 37°C and the bound conjugate was visualized using HRP-3, 3′-Diaminobenzidine (DAB) Substrate Solution (Thermo Fisher Scientific, USA).

Quantitative Real-Time PCR Analysis

As above, RAW264.7 cells were treated with AG490 (20 μM) for 1 h and then infected with *Brucella* M5-90. Cells were then washed three times with PBS at 4, 8, 12, or 24 h post-infection. TRIzol (1 mL) was added to each well, and passing the cell lysate several times. Total RNA was extracted with AMV (Avian Myeloblastosis Virus) reverse transcriptase (Takara, Tokyo, Japan) following the manufacturer's instructions. Real time quantitative PCR was used to validate cDNA using a Light-Cycler 480 (Roche, Switzerland). The PCR reaction system were as follows: Total 20 μL, SYBR premix E_xTaq 10 μL, cDNA model 2.0 μL, Up Primer 0.4 μL, Down-Primer 0.4 μL, dH_2O 7.2 μL. And the reaction conditions were as follows: 5 min at 95°C followed by 40 cycles at 95°C for 30 sec, 55°C for 30 min, and 72°C for 5 min. GAPDH was used as the reference. The primers for Bax were sense 5′-GACTTCUCUCGTCGCTACCG-3′ and anti-sense 5′-ACAATCCTCCCCCAGTTCAC-3′, the primers for Bcl-2 were sense 5′-TTCTTTGAGTTCGGTGGGGTC-3′ and anti-sense 5′-TGCATATTTGTTTGGGGCA

GG-3′ and the primers for GAPDH were sense 5′ CTGCCC AGAACATCATCCCT3′,and anti-sense 5′ GACACATTGGGGG TAGGAAC-3′.

Apoptosis Assay

Brucella M5-90-infected RAW264.7 cells were incubated with Annexin V and propidium iodide (PI) at 37°C for 20 min using an Annexin V-FLUOS staining kit (BB-410; Bestbio, Shanghai). The rate of apoptosis in the RAW264.7 cells was then detected immediately following incubationvia flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Intracellular Survival Assay

RAW264.7 cells were pre-treated with AG490 (20 μmol/L) for 1 h and then infected with *Brucella* M5-90 at a 100:1 MOI, as described above. The infected cells were then lysed at 4, 8, 12, 24, or 48 h post-infection using 1%Triton X-100. The number of bacteria was enumerated by plating different dilutions of the lysates on TSA plates after several days.

Statistical Analysis

Differences among of experimental groups were analyzed by student's t-test. A P-value of <0.05 was considered statistically significant and a P-value of <0.01 was considered greatly significant.

RESULTS

Inhibitor AG490 Affects Cell Viability in a Concentration-Dependent Manner

Cell viability was determined via MTT assay after RAW264.7 cells were incubated for 48 h with a range of AG490 (JAK2/STAT3 inhibitor) concentrations. AG490 affected RAW264.7 cells viability in a concentration-dependent manner. As shown in *Fig. 1*, there was no obvious effect on RAW264.7 cell viability after treatment with 20 μM AG490.

Brucella M5-90 Activated the JAK2 /STAT3 Signaling Pathway

A Western blot was performed to detect the phosphorylation levels of JAK2 and STAT3 at 2, 4, 8, 12 and 24 h post-infection in order whether *Brucella* M5-90 regulates the activity of the JAK2 /STAT3 signaling pathway. As shown in *Fig. 2A*, *Brucella* M5-90 induced JAK2 and STAT3 phosphorylation from 4 h to 24 h post-infection, but the degree of activation was different; phosphorylation peaked at 12 h post-infection. The difference in phosphorylation (12 h and 24 h) was extremely significant compared with 0 h post-infection ($P<0.01$). The phosphorylation level increased at first and then decreased indicating that infection with *Brucella* M5-90 resulted in improved transient phosphorylation of JAK2 and STAT3 proteins.

Whether AG490 affects phosphorylation of the JAK2/STAT3 signaling pathway in AG490-treated RAW264.7 cells infected with *Brucella* M5-90 was also tested. AG490 inhibited JAK2, STAT3 phosphorylation in a concentration-dependent manner. Phosphorylation of the signaling pathway was almost completely blocked after treatment with 20 μM AG490 *(Fig. 2B)*, further confirming that

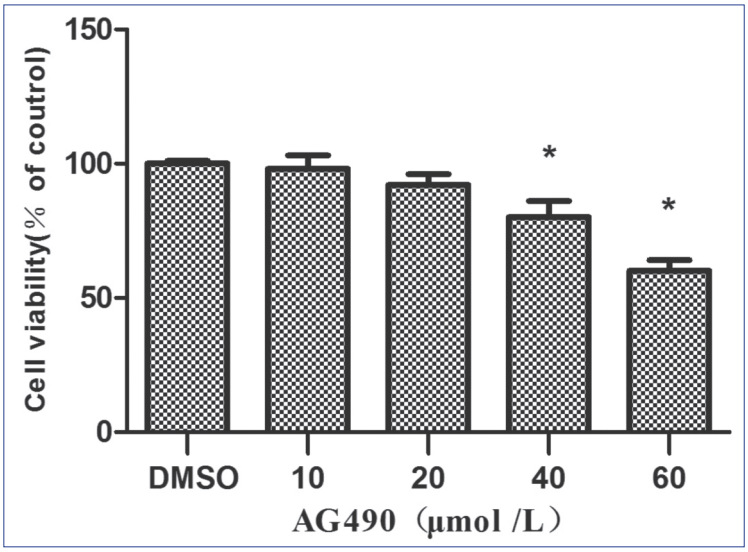


Fig 1. RAW264.7 cell viability was inhibited following AG490 treatment in a concentration-dependent manner. Viability of RAW264.7 cells treated with 40 and 60 μmol/L AG490 was significantly different compared to control cells treated with DMSO. *P<0.05

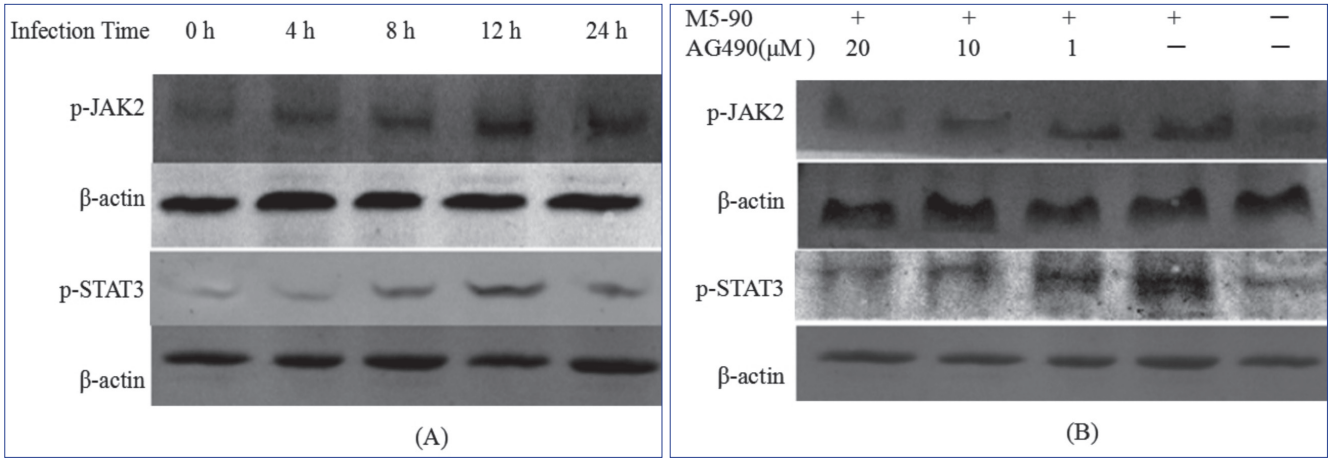


Fig 2. *Brucella* M5-90 activated the JAK2 /STAT3 signaling pathway. (A) Western blot analysis of JAK2 and STAT3 phosphorylation levels from *Brucella*-infected RAW264.7 cells at different time points. (B) Cells were treated with different concentrations of AG490 inhibitor and then infected with M5-90 for 12 h. JAK2 and STAT3 phosphorylation levels were detected by Western blot analysis. β-actin was used as the reference

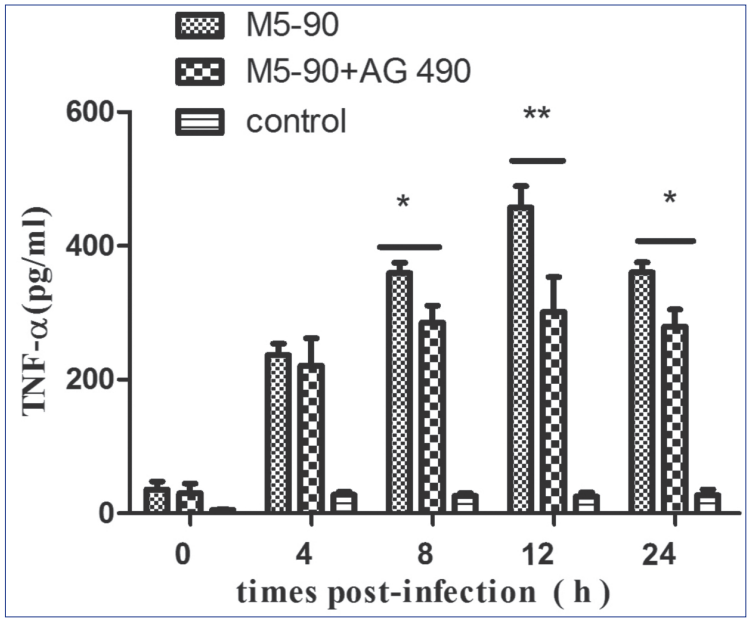


Fig 3. TNF-α levels in AG490-treated RAW264.7 cells and in control cells infected with *Brucella* M5-90. *P<0.05; **P<0.01

Brucella M5-90 activates the JAK2/STAT3 pathway.

The JAK2 /STAT3 Signaling Pathway Regulates the Level of TNF-α in M5-90-Infected Cells

To determine whether the JAK2 /STAT3 signaling pathway regulates the level of TNF-α in M5-90-infected cells, RAW264.7 cells were pre-treated with 20 μM AG490 for 1 h and then infected with *Brucella* M5-90. TNF- α levels were then measured at 0, 4, 8, 12, and 24 h post-infection. The expression of TNF-α gradually increased from 0 h to 12 h and then decreased from 12 h to 24 h. AG490 inhibitor-treated cells produced significantly higher levels of TNF-α compared to control cells (PBS) at 12 h ($P<0.01$); the difference was also significant difference at 8 h and 24 h (*Fig. 3;* $P<0.05$). This indicates that AG490 reduces the expression of TNF-α via inhibiting the activation of the JAK2 /STAT3 signaling pathway.

Analysis of Apoptosis-Related Protein (caspase-3, Bax) Expression in Brucella M5-90-Infected RAW264.7 Cells Treated with AG490

Activation of TNF receptor-1 by TNF-α can induce cell apoptosis and involves consecutive activation of both the NF-κB and caspase pathways [8,9]. Thus, the expressions of Bax and caspase-3 proteins were determined at different time points post-infection to investigate whether the JAK2 /STAT3 signaling pathway is involved in the apoptosis of *Brucella*-infected RAW264.7 cells. Bax is an apoptosis-promoting protein belonging to the B-cell lymphoma 2 (Bcl-2) protein family [10]. Caspase-3 is the most important terminal shear enzyme in the process of apoptosis and can induce apoptosis after caspase-activation [11]. In this study, the expressions of caspase-3 and Bax increased gradually, reaching their highest levels at 12 h, and the expressions of the Bax and caspase 3 proteins were induced after 12

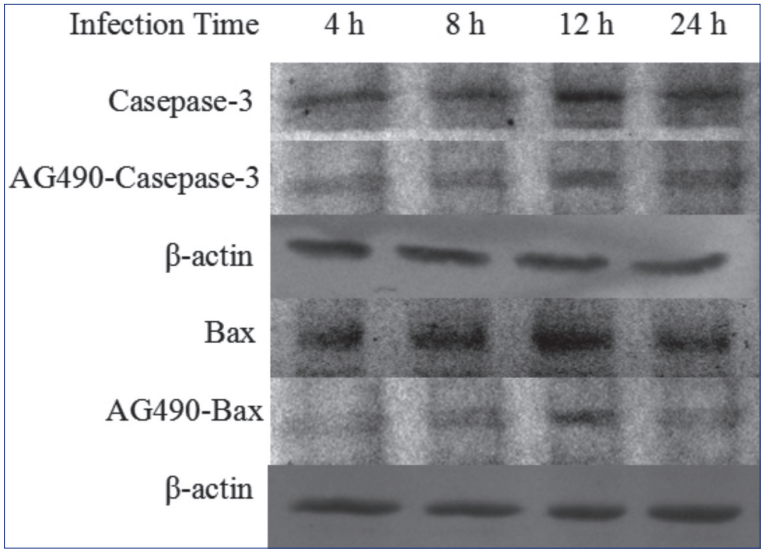


Fig 4. Western blot analysis of apoptosis promoting protein (caspase-3 and Bax) expression in *Brucella* M5-90-infected RAW264.7 cells at different time points

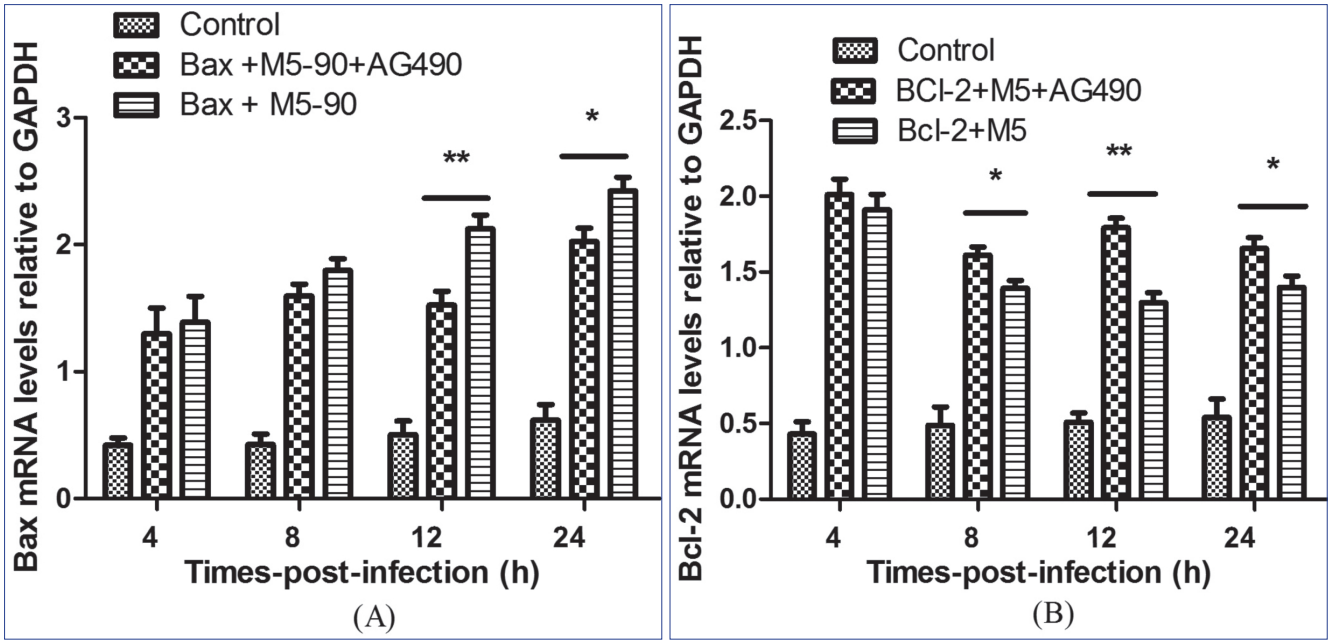


Fig 5. The JAK2/STAT6 pathway significantly affects the mRNA expressions of apoptosis-related genes Bax and Bcl-2 at the transcriptional level. (A) Bax mRNA levels and (B) Bcl-2 mRNA levels at different time points. *P<0.05; **P<0.01

h *(Fig. 4)*. However, exposure of cells to AG490 (20 μM) resulted in a gradual decrease of both Bax and caspase-3 expressions from 4 h to 24 h compared with control cells. The difference in Bax and caspase-3 expression between AG490-treated and control cells was particularly significant at 12 h. These results suggest that AG490 affects secretion of apoptosis related proteins (Bax, caspase-3) in a time-dependent manner indicating a role of the JAK2 /STAT3 signaling pathway in the apoptosis of M5-90-infected cells.

AG490 Affects the mRNA Expressions of Apoptosis-Related Genes Bax and Bcl-2

Bax is involved in the regulation of the apoptosis, Bcl-2 enhances cell activity and survival via the production of Bax- induced cytochrome (Cyt) c, and Cyt c activates the caspase gene in the cytosol, thereby causing cell death or apoptosis [12,13]. Thus, to detect whether the JAK2 /STAT3 signaling pathway affected the expression of apoptosis-related genes on an mRNA level, real-time polymerase chain reaction (RT- PCR) was used to analyze Bax and Bcl-2 mRNA expression levels. As shown in *Fig 5A*, the M5-infected AG490-treated cells transcribed significantly higher mRNA levels of Bax gene than the M5-90-infected cells at 12 h (P<0.01) and 24 h (P<0.05) post infection. In contrast, the result for Bcl-2 shows that the M5-infected AG490-treated cells produced significantly lower mRNA levels of Bcl-2 gene in the M5-infected cells at 8 h (P<0.05), 12 h (P<0.01) and 24 h (P<0.05) post infection *(Fig. 5B)*. These results indicate that AG490 inhibits Bax mRNA levels

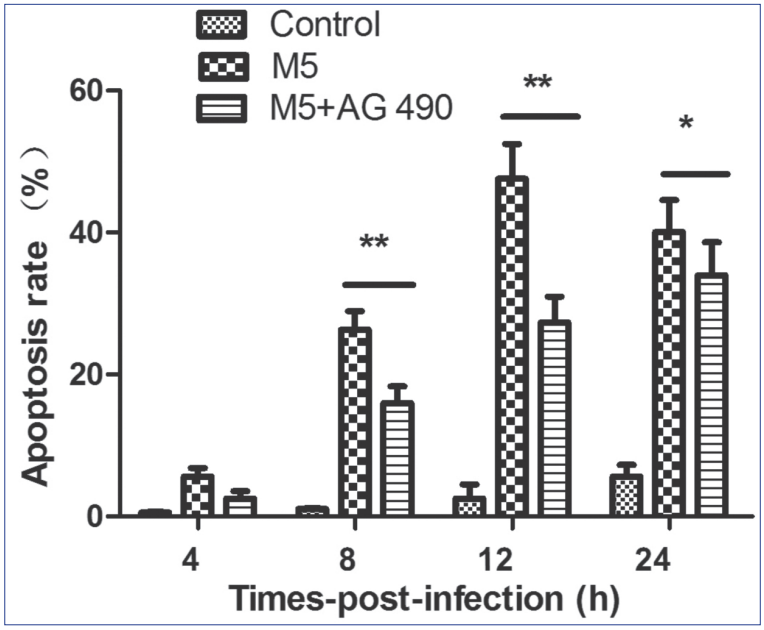
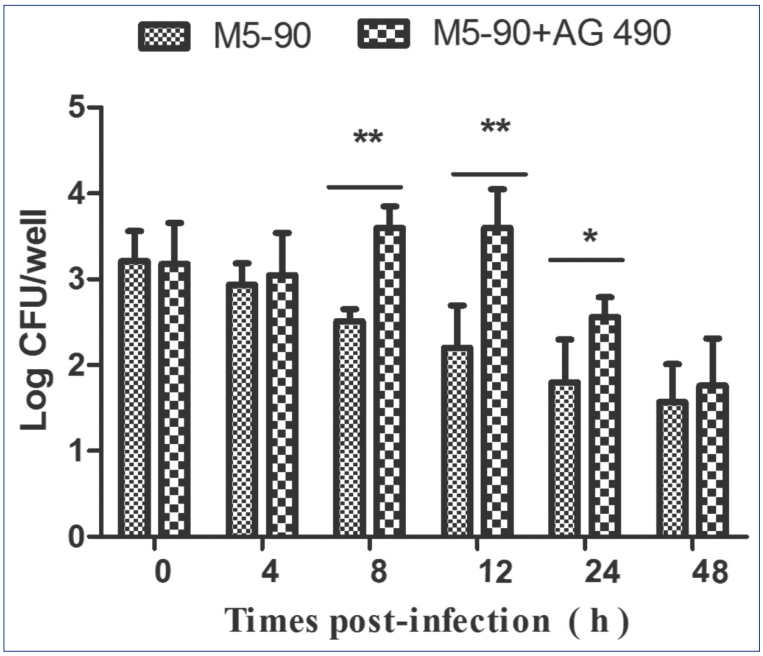


Fig 6. Flow cytometry analysis of apoptosis at different time points using Annexin FITC/PI in RAW264.7 cells infected with *Brucella* M5-90 in the absence or presence of AG490, compared with M5-90-infected untreated control cells.*P<0.05; **P<0.01

Fig 7. Increased *Brucella* M5-90 survival inside RAW264.7 cells with the use of AG490. *P<0.05; **P<0.01



and increases Bcl2 mRNA levels and confirms that the JAK2 /STAT3 signaling pathway regulates apoptosis in M5-90-infected RAW264.7 cells at the transcriptional level.

AG490 Inhibitor of JAK2 /STAT3 Signaling Pathway Induces Apoptotic Rates in M5-90-Infected RAW264.7 Cells

Cells were preincubated with AG490 for 1 h prior to infection with M5-90 for 4 h, 8 h, 12 h, or 24 h; PBS was used as the control. The M5-dependent apoptosis rates were then analyzed using flow cytometry. As shown in *Fig 6,* the apoptotic rate ranged from 2.55% to 33.95% in M5-90-infected AG490-treated cells from 5.64% to 40.09% in M5-90 infected cells and from 0.97% to 5.64% in control cells treated with PBS. The apoptosis rate in M5-90-infected RAW264.7 cells was significantly high than in M5-90 infected cells treated with AG490, particularly at 8 and 12 h (P<0.01). In addition, there was no difference at 4 h post-infection (P>0.05). This result confirmed that inhibition of the JAK2 /STAT3 signaling pathway by AG490 decreased apoptotic rates in M5-90-infected RAW264.7 cells.

The JAK2/STAT3 Signaling Pathway Regulates the Intracellular Survival and Replication of Brucella M5-90

The intracellular survival and replication of *Brucella* M5-90 was measured at six time points in macrophages after a 100:1 MOI. There was no significant difference in the amount of *Brucella* M5-90 intracellular survival inside

AG490-treated macrophages at 0, 4, or 48 h post-infection compared to untreated macroghages. However, bacterial number was higher in AG490-treated macrophages at 8, 12, and 24 h post compared to untreated control cells (1.08-log,1.4-log, 0.76-log; $P<0.05$; *Fig. 7*). These results show that AG490 significantly increased intracellular growth and replication of *Brucella* M5-90 in macrophages, indicating that the JAK2/STAT3 signaling pathway regulates the intracellular survival of *Brucella*. In addition, the results of the above experiment confirmed that the intracellular survival of *Brucella* was inversely proportional to the apoptosis of M5-90-infected cells.

DISCUSSION

JAK2 belongs to the family of protein tyrosine kinases, which mediate the cascade activation reaction of signal protein molecules. JAK2 is activated after cytokines and growth factors bind to its corresponding receptor, thereby phosphorylating STAT3 [14]. STAT3 is an important protein that can be activated by multifunctional cytokines and growth factors. The phosphorylated STAT3 protein regulates various physiological and biological activities [15]. Previous studies have found that the JAK2/STAT3 pathway not only closely affects cell proliferation, differentiation and apoptosis, but also more importantly, it is also involved in mediating the inflammatory and immune responses during the process of disease development [16,17]. AG490, an inhibitor of JAK2, can directly inhibit Osteopontin (OPN)-induced nuclear localization and DNA binding activity of STAT3 indicating that the JAK2 protein is involved in this process [18]. Mycobacterium tuberculosis is an intracellular bacterium and activation of the JAK2/STAT3 pathway can regulate expression of immune factors after human cells are infected. Therefore, the JAK2/STAT3 pathway may regulate cell apoptosis and affect the survival of *Brucella* in macrophages similar to tuberculosis, since *Brucella* is also an intracellular bacterium. In this study, it was confirmed that *Brucella* M5-90 infection activates the JAK2/STAT3 signaling pathway and that the activation of this signaling pathway regulates the apoptosis of the RAW264.7 cells and proinflammatory reactions, thereby affecting the intracellular survival of *Brucella* M5-90.

Brucella initially infects macrophages, which are a first line of defense in the immune system. Macrophages, as immunomodulatory cells, can rapidly kill pathogenic bacteria that invade the body [19]. However, *Brucella* developed a strategy to escape being killed by macrophages [20]. Recent research has indicated that the JAK2/STAT3 pathway plays a crucial role in immune regulation and apoptosis [21]. In this study, the phosphorylation levels of JAK2 and STAT3 increased in RAW264.7 cells infected with *Brucella* M5-90. Therefore, inhibition of the JAK2/STAT3 pathway may affect the transcription of related immune factors when *Brucella* invades the macrophages. To test this hypothesis, cells were treated with AG490 (an inhibitor as described above). It was found that different concentrations of AG490 affected activation of the pathway to varying degrees and that AG490 substantially reduced the expression of TNF-α. TNF-α induces cells apoptosis after engaging TNF-α receptor-1 by activating caspases in the death receptor pathway [8] suggesting that the JAK2/STAT3 pathway triggers macrophage apoptosis via TNF-α. Other studies have also confirmed that Bcl-2 affects TNF-α-induced activation of caspase-8, caspase-3 and apoptosis [22]. It has been reported that TNF-α induces phosphorylation of signaling pathways, such as PI3K/Akt. Thus, the JAK2/STAT3 pathway may play a major role in apoptosis in *Brucella*-infected cells via TNF-α-induction.

To further verify the above results, caspase-3, Bcl-2 and Bax were analyzed by RT-PCR or Western blot. Caspase-3 is one of the most important executors of apoptosis and is the main effector in the process of apoptosis [23]. Bcl-2 is an antiapoptotic gene belonging to the Bcl-2 family of proteins; Bax is a member of the Bcl-2 family, which can promote apoptosis [24]. The results of this study indicated that the expression of apoptosis-related proteins (caspase-3, Bax) were reduced in *Brucella* M5-90-infected RAW264.7 cells treated with AG490, however the results of Bc12 expression were opposite. Taken together, the results obtained in the current study demonstrate that the JAK2/STAT3 signaling pathway regulates apoptosis in M5-90-infected RAW264.7 cells at the transcriptional level and protein level.

Notably, Previous studies have indicated that the regulation of macrophage apoptosis is also one of the mechanisms by which *Brucella* escapes the immune system [25]. In this study, flow cytometry experiments showed that AG490 contributes to the inhibition of apoptosis, and the inhibition of the JAK2/STAT3 signaling pathway by AG490 decreased apoptotic rates in M5-90-infected RAW264.7 cells, other research show that *Brucella* infection-induced down-regulation of apoptotic level is an essential factor for the intracellular survival of *Brucella* within macrophages [25], this conclusion was also confirmed by this study. Thus, the JAK2/STAT3 signaling pathway indeed plays an important role in apoptosis of RAW264.7 cells infected with *Brucella* M5-90, thereby it is beneficial for survival and replication of *Brucella* M5-90. In addition, this study also found that secretion of TNF-α was positively related to transcriptional levels of caspase-3, Bax, and apoptosis rates. AG490 also affected the secretion of TNF-α. Thus, activation of the JAK2/STAT3 pathway could regulate *Brucella* M5-90 survival via induction of TNF-α.

In summary, the JAK2/STAT3 pathway is activated by *Brucella* M5-90 and plays an important role in regulating apoptosis of TNF-α-induced anti-*Brucella* activity. Inhibition of the JAK2/STAT3 pathway can inhibit Th1 immune responses, apoptosis rates, and intracellular survival of *Brucella*. This study may provide a preliminary theoretical basis for the study of the immune escape mechanism of

Brucella. However, the specific molecular mechanisms and the interactions between each signaling pathway needs to be examined in depth.

Acknowledgment

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REFERENCES

1. O'Callaghan D: Novel replication profiles of *Brucella* in human trophoblasts give insights into the pathogenesis of infectious abortion. *J Infect Dis,* 207 (7): 1034-1036, 2013. DOI: 10.1093/infdis/jit010

2. De Figueiredo P, Ficht TA, Rice-Ficht A, Rossetti CA, Adams LG: Pathogenesis and immunobiology of brucellosis: Review of *Brucella*-host interactions. *Am J Pathol,* 185 (6): 1505-1517, 2015. DOI: 10.1016/j.ajpath.2015.03.003

3. Aggarwal BB: Signalling pathways of the TNF superfamily: A double-edged sword. *Nat Rev Immunol,* 3 (9): 745-756, 2003. DOI: 10.1038/nri1184

4. Wang P, Qiu W, Dudgeon C, Liu H, Huang C, Zambetti GP, Yu J, Zhang L: PUMA is directly activated by NF-kappaB and contributes to TNF-alpha-induced apoptosis. *Cell Death Differ,* 16 (9): 1192-1202, 2009. DOI: 10.1038/cdd.2009.51

5. Nobori K, Munehisa Y: Intracellular signaling pathways for cardiac hypertrophy: ERK, JAK-STAT, S6 kinase. *Nihon Rinsho,* 65 Suppl 4: 196-200, 2007.

6. Rojas M, Olivier M, García LF: Activation of JAK2/STAT1-alpha-dependent signaling events during *Mycobacterium tuberculosis*-induced macrophage apoptosis. *Cell Immunol,* 217 (1-2): 58-66, 2002. DOI: 10.1016/S0008-8749(02)00515-4

7. Luo C, Laaja P: Inhibitors of JAKs/STATs and the kinases: A possible new cluster of drugs. *Drug Discov Today,* 9 (6): 268-275, 2004. DOI: 10.1016/S1359-6446(03)03014-9

8. Ashkenazi A: Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer*, 2 (6): 420-430, 2002. DOI: 10.1038/nrc821

9. Micheau O, Tschopp J: Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell,* 114 (2): 181-190, 2003. DOI: 10.1016/S0092-8674(03)00521-X

10. Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ: Movement of bax from the cytosol to mitochondria during apoptosis. *J Cell Biol,* 139 (5): 1281-1292, 1997. DOI: 10.1083/jcb.139.5.1281

11. Monney L, Olivier R, Otter I, Jansen B, Poirier GG, Borner C: Role of an acidic compartment in tumor-necrosis-factor-alpha-induced production of ceramide, activation of caspase-3 and apoptosis. *Febs J,* 251 (1-2): 295-303, 1998. DOI: 10.1046/j.1432-1327.1998.2510295.x

12. Antonsson B: Bax and other pro-apoptotic Bcl-2 family "killer-proteins" and their victim the mitochondrion. *Cell Tissue Res,* 306 (3): 347-361, 2001. DOI: 10.1007/s00441-001-0472-0

13. Parone PA, James D, Martinou JC: Mitochondria: Regulating the inevitable. *Biochimie,* 84 (2-3): 105-111, 2002. DOI: 10.1016/S0300-9084(02)01380-9

14. Proia DA, Foley KP, Korbut T, Sang J, Smith D, Bates RC, Liu Y, Rosenberg AF, Zhou D, Koya K, Barsoum J, Blackman RK: Multifaceted intervention by the Hsp90 inhibitor ganetespib (STA-9090) in cancer cells with activated JAK/STAT signaling. *PloS One,* 6 (4): e18552, 2011. DOI: 10.1371/journal.pone.0018552

15. Levy DE, Inghirami G: STAT3: A multifaceted oncogene. *Proc Natl Acad Sci USA,* 103 (27): 10151-10152, 2006. DOI: 10.1073/pnas.0604042103

16. Imada K, Leonard WJ: The Jak-STAT pathway. *Mol Immunol,* 37 (1-2): 1-11, 2000. DOI: 10.1016/S0161-5890(00)00018-3

17. Lee C, Lim HK, Sakong J, Lee YS, Kim JR, Baek SH: Janus kinase-signal transducer and activator of transcription mediates phosphatidic acid-induced interleukin (IL)-1beta and IL-6 production. *Mol Pharmacol,* 69 (3): 1041-1047, 2006. DOI: 10.1124/mol.105.018481

18. Behera R, Kumar V, Lohite K, Karnik S, Kundu GC: Activation of JAK2/STAT3 signaling by osteopontin promotes tumor growth in human breast cancer cells. *Carcinogenesis,* 31 (2): 192-200, 2010. DOI: 10.1093/carcin/bgp289

19. Pei J, Kahl McDonagh M, Ficht TA: *Brucella* dissociation is essential for macrophage egress and bacterial dissemination. *Front Cell Infect Microbiol,* 4 (2): 23, 2014. DOI: 10.3389/fcimb.2014.00023

20. Martirosyan A, Moreno E, Gorvel JP: An evolutionary strategy for a stealthy intracellular *Brucella* pathogen. *Immunol Rev,* 240 (1): 211-234, 2011. DOI: 10.1111/j.1600-065X.2010.00982.x

21. Ahmed-Choudhury J, Williams KT, Young LS, Adams DH, Afford SC: CD40 mediated human cholangiocyte apoptosis requires JAK2 dependent activation of STAT3 in addition to activation of JNK1/2 and ERK1/2. *Cell Signal,* 18 (4): 456-468, 2006. DOI: 10.1016/j.cellsig.2005.05.015

22. Dbaibo GS, Perry DK, Gamard CJ, Platt R, Poirier GG, Obeid LM, Hannun YA: Cytokine response modifier A (CrmA) inhibits ceramide formation in response to tumor necrosis factor (TNF)-α: CrmA and Bcl-2 Target distinct components in the apoptotic pathway. *J Exp Med,* 185 (3): 481-490, 1997. DOI: 10.1084/jem.185.3.481

23. Cryns V, Yuan J: Proteases to die for. *Gene Dev*, 12, 1551-1570, 1998. DOI:10.1101/gad.12.11.1551

24. Karabay AZ, Aktan F, Sunguroğlu A, Buyukbingol Z: Methylsulfonylmethane modulates apoptosis of LPS/IFN-γ-activated RAW 264.7 macrophage-like cells by targeting p53, Bax, Bcl-2, cytochrome c and PARP proteins. *Immunopharmacol Immunotoxicol,* 36 (6): 379-389, 2014. DOI: 10.3109/08923973.2014.956752

25. Cui G, Wei P, Zhao Y, Guan Z, Yang L, Sun W, Wang S, Peng Q: *Brucella* infection inhibits macrophages apoptosis via Nedd4-dependent degradation of calpain2. *Vet Microbiol,* 174 (1-2): 195-205, 2014. DOI: 10.1016/j.vetmic.2014.08.033

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Research Article

Helminths That Are Detected by Necropsy in Wrestling Camels [1]

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Abstract

The present study aims to carry out a helminthological examination and epizootiological determination of the parasites that were detected by the parasitological examination in the carcasses and internal organs of wrestling camels that were slaughtered due to miscellaneous reasons. The material of the study comprised wrestling camels that were sent to the slaughterhouse for various reasons including sickness, old age, and underperformance due to an unknown cause. The present study was conducted on 24 male wrestling camels of ages ranging between 5 to 20 years. After slaughtering, the carcasses and internal organs were examined parasitologically. All internal organs, except for abomasum and intestines, were macroscopically analyzed in the slaughterhouse to determine whether any helminth larvae or adult helminths were present and those that bore traces of a parasitic period were brought to the laboratory for further analysis. For the examination of the digestive system, abomasa, and intestines, along with their contents, were brought to the laboratory to perform macroscopic and microscopic analyses by following appropriate methods. At least one helminth infection was seen in 14 (58%) of 24 camels in necropsy. The analysis of the organs revealed hydatid cysts in 12 (50%) animals, which were localized in the lungs of 7 animals, livers of 3 animals, both in the lung and liver of one animal and in the spleen of another animal. By examining the stomachs and intestines of the animals, Stilesia globipunctata was detected in the small intestines of 3 camels (12.5%), while Nematodirus filicollis was detected in the small intestine of one animal (4.1%), and Oesophagostomum venulosum was detected in the large intestine of the same animal (4.1%). Neither helminth larvae nor adult helminths were observed in other organs. The presence of hydatid cysts in over fifty percent of the examined camels is noteworthy. As a disease of great importance for public health, its prevalence in camels brings forth the absolute requirement for slaughtering animals in slaughterhouses or under the control of a veterinary physician. This study is the first one to report the occurrence of Stilesia globipunctata, Nematodirus filicollis and Oesophagostomum venulosum in camels at species level.

Keywords: *Camel, Helmint, Hydatid cyst, Necropsy, Turkey*

Güreş Develerinde Nekropsi İle Tespit Edilen Helmintler

Öz

Bu çalışmada, güreş develerinin çeşitli nedenlerle kesime sevk edilenlerinde karkas ve tüm iç organların helmintolojik açıdan muayenesi ve tespit edilen parazitlerle bu hayvanlarda epizootiyolojik bir durum tespiti yapılması amaçlanmıştır. Çalışmanın materyalini hastalık, ileri yaş, nedeni bulunamamış performans düşüklüğü gibi değişik sebeplerle mezbahada kesilen güreş develeri oluşturmuştur. Çalışma yaşları 5-20 arasında değişen tamamı erkek 24 güreş devesinde gerçekleştirilmiştir. Kesim sonrası karkas ve tüm iç organlar parazitolojik açıdan incelenmiştir. Abomasum ve bağırsaklar dışındaki tüm iç organlar helmintlere ait larva ya da erişkin dönemleri açısından mezbahada makroskobik olarak incelenmiş, her hangi bir paraziter döneme rastlananlar sonraki incelemeler için laboratuvara getirilmiştir. Sindirim sistemine ilişkin incelemeler için abomasum ve bağırsaklar içerikleri ile birlikte laboratuvara getirilerek makroskobik ve mikroskobik incelemeleri uygun yöntemlerle burada tamamlanmıştır. Nekropsisi yapılan 24 devenin 14'ünde (%58) en az bir helmint enfeksiyonu görülmüştür. Yapılan organ muayenelerinde 7'si akciğer, 3'ü karaciğer, 1'i akciğer karaciğer birlikte ve 1'i dalak yerleşimli olmak üzere toplam 12 (%50) devede hidatik kiste rastlanmıştır. Mide ve bağırsaklarda yapılan incelemelerde ise 3 devenin ince bağırsaklarında Stilesia globipunctata (%12.5), bir devede Nematodirus filicollis (%4.1) ve aynı devenin kalın bağırsaklarında Oesophagostomum venulosum (%4.1) tespit edilmiştir. Bunlar dışında kalan organlarda herhangi bir helminte ait larva ya da erişkin döneme rastlanmamıştır. İncelemesi yapılan develerin yarısından fazlasında hidatik kiste rastlanması dikkat çekicidir. Halk sağlığı açısından son derece önemli olan bu hastalığın develerdeki yaygınlığı kesimlerin mutlaka mezbahalarda ya da veteriner hekim kontrolünde yapılması gerekliliğini ortaya çıkarmıştır. Bu çalışmayla Stilesia globipunctata, Nematodirus filicollis ve Oesophagostomum venulosum develerden tür düzeyinde ilk kez bildirilmiştir.

Anahtar sözcükler: *Deve, Helmint, Hidatid kist, Nekropsi, Türkiye*

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INTRODUCTION

Competitive and struggling behaviors that male camels display in mating period due to hormonal alterations were colloquially considered similar to wrestling. This competition became apparent in camel owners as well, which turned into annual festival organizations and became traditional. Camel wrestling dates back to the fifteenth century, and it is still performed in Turkey [1]. Camel wrestling events are held annually between December and mid-March in almost 100 towns in Marmara, Mediterranean, and mostly Aegean region [2]. Although the relation between these animals and people is very long considering their use for transportation and traveling, there are very few scientific studies on the diseases in these animals. Studies on parasitic diseases, which may be an issue for almost any kind of animal breeding, are also limited to the detections based on fecal examination [1,3,4].

The presence of parasites living in the digestive system and related organs can be revealed at a certain level in living animals by the detection of helminth eggs and larvae in their feces. Accordingly, a recent study performed in Turkey identified these parasites mainly at the genus level through fecal cultivation [1]. These worms, most of which were reported for the first time, also contributed significantly to the parasite fauna. In this study, 74% (81/109) of the camels were infected with one or more parasites. Some species, such as *Trichostrongylus* spp. *Ostertagia* spp., *Haemonchus* spp., *Nematodirus* spp., *Chabertia ovina*, *Oesophagostomum* spp., *Trichuris* spp., *Capillaria* spp., *Cooperia* spp., *Cooperia oncophora*, *Fasciola* spp., *Dicrocoelium* spp., *Paramphistomum* spp. *Dictyocaulus viviparus*, *Eimeria cameli*, *Eimeria* spp. have been reported at different rates [1]. However, organ examinations that use necropsy techniques are more valuable because they allow species-level identification and a morphological examination of the larval stages of cestodes; the parasites in organs not related to the digestive system, and all of the parasites found as well.

In this study, it was aimed to make a parasitological examination of the wrestling camels' carcass and all the internal organs that were subjected to slaughtering due to various reasons, and to determine the epizootiological condition of these animals based on the parasites detected.

MATERIAL and METHODS

The subjects of this study were wrestling camels slaughtered in slaughterhouses due to various reasons such as illness, old age, or lack of performance with no detected reason. This study was conducted between November 2016 and October 2017 in Integrated Meat Facilities located in Umurlu district of Aydın on 24 male wrestling camels aged between 5 and 20 years. Because wrestling camels frequently change owners and some camels are raised in Turkey whereas others are brought from abroad (generally Iran), we were unable to determine the origin of the camels we assessed.

After slaughtering, the carcass and all internal organs were examined in the slaughterhouse and the laboratories at the Department of Parasitology, Veterinary Medicine, Adnan Menderes University. All internal organs except abomasum and intestines were macroscopically examined in the slaughterhouse for larvae or adult helminths, and the ones in any parasitic stage were brought to the laboratory for further examination.

A double ligature was placed at the end and beginning of small and large intestines, and they brought with their content to the laboratory for the examination related to the digestive system. Initially, macroscopic examinations were performed and parasites were collected after removing the mesenteric and adipose tissues found in these sections and cutting them lengthwise in a bathtub. The contents were then washed using a suitable porous sieve under mild running water. The residue that stayed on the sieve was moved to another container for microscopy. The collected content was transferred to Petri dishes in small quantities and examined under a stereomicroscope. The parasites were precleaned in physiological saline, and stored in a 70% ethyl alcohol solution that is close to its boiling temperature until detailed examinations were made. They were kept in Lactophenol solution for transparency during the period they were diagnosed and examined between a slide and a cover slide [5]. The detection was performed using a microscope (Olympus BX51) that was capable of making digital measurement and visualization (Olympus DP70) based on the relevant literature [6-9].

RESULTS

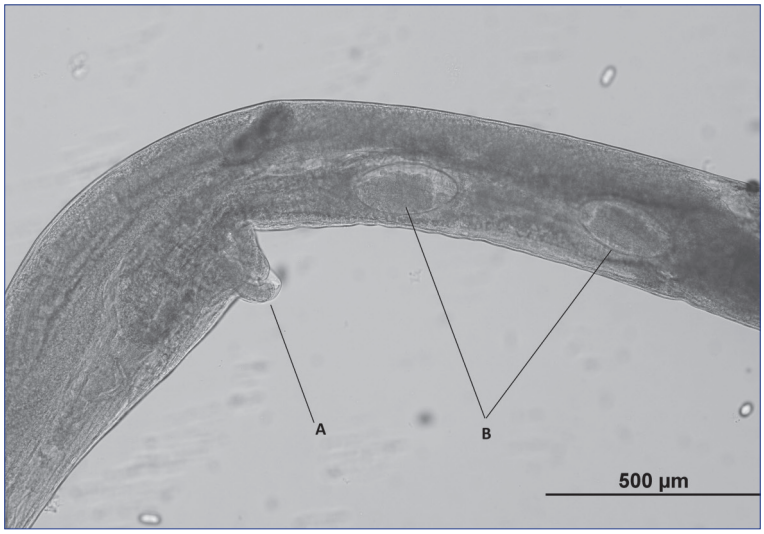
At least one helminth infection was observed in 14 of 24 (58%) of the animals on which necropsy was performed. A total of 12 (50%) hydatid cysts were found in organ examinations. Of them, 7 were in the lung, 3 were in the liver, 1 was in the lung and liver together, and 1 was in the spleen. According to the examinations made in the stomach and intestine of the animals, there was *Stilesia globipunctata* (camel 1; 7, camel 2; 23, camel 3; 121 parasites) in the small intestine of three (12.5%) camels, *Nematodirus filicollis* (Total 31 parasites; 21 female, 10 male) in one (4.1%) of the camels *(Fig. 1, 2, 3)*, and also *Oesophagostomum venulosum* (Total 13 parasites; 9 female, 4 male) in the large intestine of the same (4.1%) animal *(Fig. 4, 5, 6)*. Neither helminth larvae nor adult forms were found in the other organs than these ones.

DISCUSSION

In live animals, eggs, oocytes, or larvae of parasites can

Fig 1. *Nematodirus filicollis* anterior end

Fig 2. Female *Nematodirus filicollis*; A: Vulva, B: Eggs



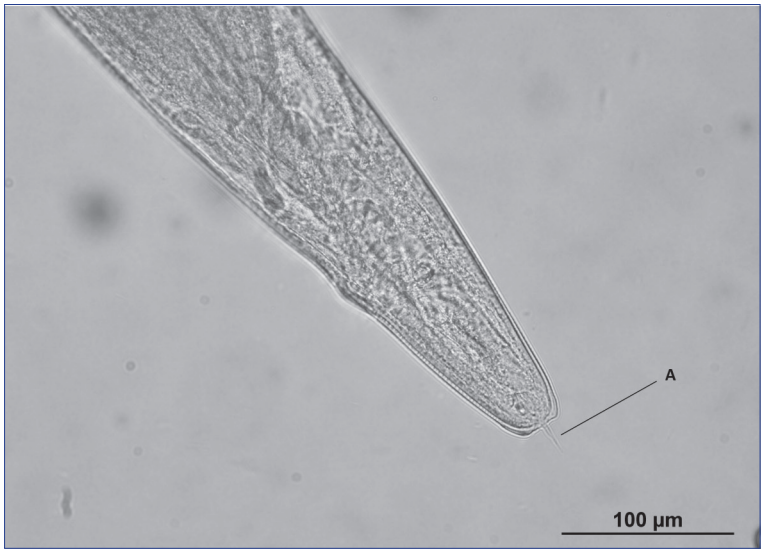


Fig 3. Female *Nematodirus filicollis* posterior end, A: Spin

be detected in stool samples or antigen/antibody in blood serum using a serologic test. However, they may not always reveal the parasitic load precisely. Parasitic infections can be diagnosed accurately during necropsy. Macroscopic or microscopic detection of the parasite itself or its larvae in relevant organs is the way to demonstrate

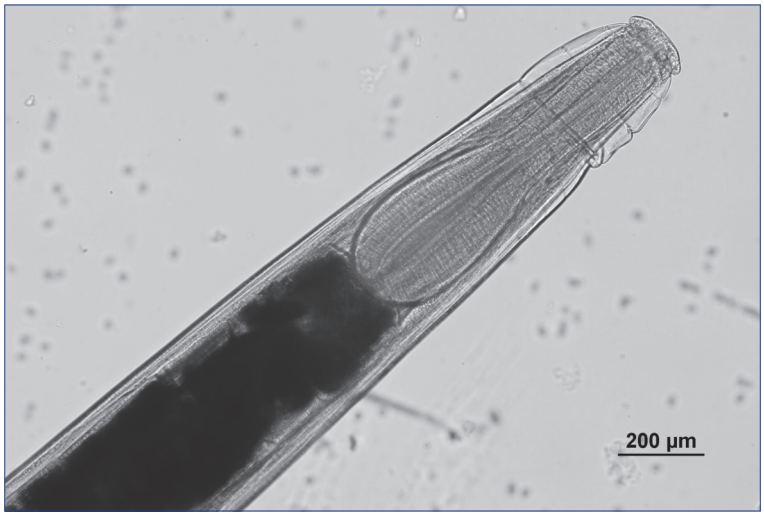
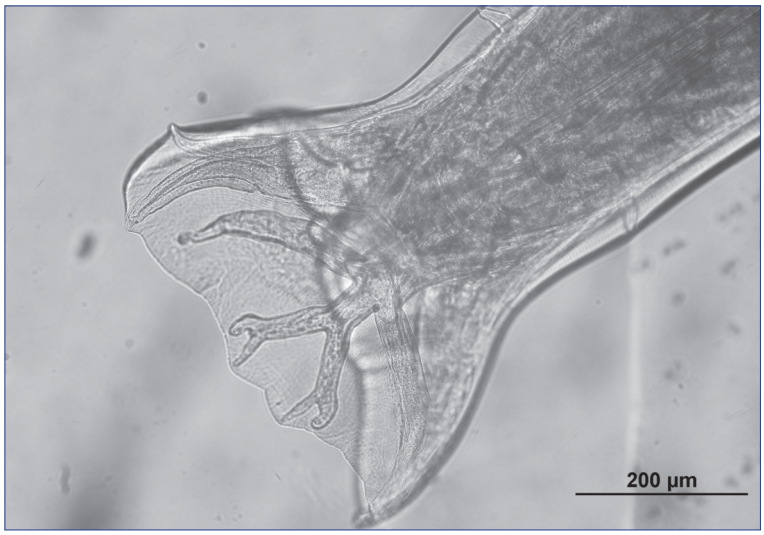


Fig 4. *Oesophagostomum venulosum* anterior end

Fig 5. Male *Oesophagostomum venulosum* posterior end



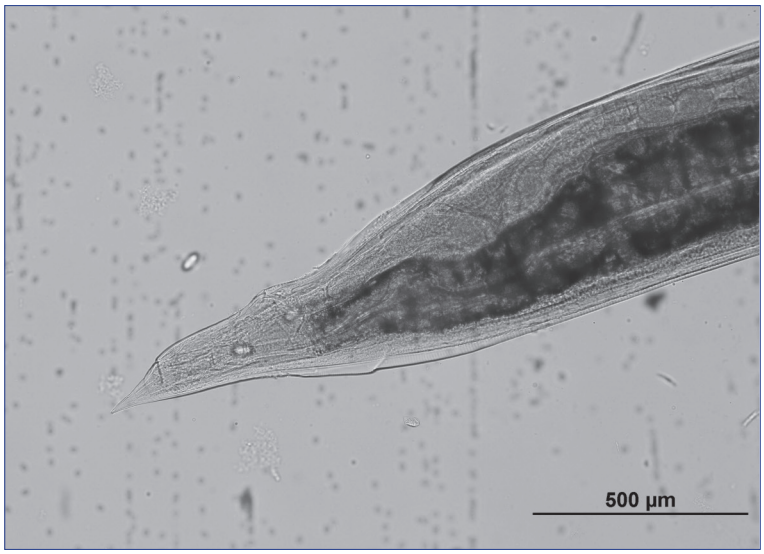


Fig 6. Female *Oesophagostomum venulosum* posterior end

the presence and density of the parasites most clearly. This method was used in this study, and hydatic cysts were found which are not possible to detect by using fecal examination method, and also cestodes and nematodes which were detected in a much easier and more accurate way.

The hydatid cyst, the larval form of *Echinococcus granulosus*, infects canidae such as dogs, wolfs, and coyotes. The larvae develop in the internal organs of humans and other mammals, including sheep, goats, cattle, and pigs. This parasite, which causes great economic losses, is one of the most common parasitic zoonoses worldwide [10]. Hydatidosis in the final and intermediate hosts is highly endemic worldwide, especially in the rural populations of South America, coasts of Mediterranean Sea, Eastern Europe, the Near and Middle East, East Africa, Central Asia, China, and Russia [11,12]. In countries with large numbers of camels, hydatidosis is also reported in these animals. According to these results, the prevalence of hydatidosis was found to be 7.45-70% in Iran [13], 5.3% in Oman [14], 2.53% in Egypt [15], 6.86% in Saudi Arabia [16], 8.8% in Jordan [17], and 6.94% in Kenya [18]. In Central Asian camels, hydatic cysts have high viability and fertility rates [12]. In Turkey, parasites exist in 1-40% of final hosts and 3.5-58.6% of intermediate hosts [12]. In a study conducted in Aydin [19], 1% of owned dogs were positive for *E. granulosus*. In addition, hydatid cysts were detected in two of six camels slaughtered in Aydin [4]. In our study, hydatic cysts were highly prevalent in the lung, liver, and spleen of camels. This parasite was seen in 50% of camels, which is indicative of a high transmission risk from camels.

Stilesia globipunctata can cause growth retardation, especially in young animals, due to enteritis in ruminants where it is located. In addition, this parasite can cause septic peritonitis and related incidences of death since it is capable of perforating the small intestine actively [20]. The prevalence of this species was reported 19% and 5.5% in Ethiopia [21] and Pakistan [22], respectively. In addition, *S. vittata* species may be found in camels as well [23,24]. In a study conducted in Syria, *S. globipunctata* and *S. vittata* were found together [25]. *Stilesia globipunctata*, which was detected in three camels (12.5%) in the present study, is a parasite known to exist in sheep and goats in Turkey [20]. It is reported to be one of the most common cestodes in Iran [13]. The researcher believes that this high prevalence may be related to the large number of camels brought from Iran, Turkey's neighbor.

Nematodirus spp., a thin-neck or thread-neck worm with approximately 30 species, infects the small intestine of rodents and other mammals. Since the egg-laying period of *N. filicollis* is relatively long, the chance for the parasite to increase in numbers reduces during the spring season when the conditions are more suitable for the parasite. Hence, its pathogeny is relatively lower than that of other species. The pathogenicity of the intestinal mucosa and villi during development varies depending on the number of parasites. The development of enteritis during intensive infections may lead to different clinical results including different types of yield loss and even death [9]. A study in Syria [25], it was reported that *N. oiratianus* as one of the most common species (57%), while *N. helvetianus* and *N. dromedarii* species were also identified. Reviews for camel parasites in Saudi Arabia [26] and India [27] have shown that the infections were caused by *Nematodirus* spp. The existence of *N. oiratianus, N. abnormalis, N. dromedarii, N. helvetianus, N. mauritanicus*, and *N. spathiger* species in Iran has been reported with reference to various investigators [13]. *Nematodirus mauretanicus* ve *N. dromedarii* are parasites that are unique in camels and have not yet been reported in any other ruminants. Other *Nematodirus* spp., except the aforementioned two species, were also observed in other ruminants [24]. *Nematodirus filicollis*, which was detected in a camel in the present study (4.5%), was also seen in camels in Egypt [28]. In Turkey, *N. spathiger, N. filicollis, N. helvetianus,* and *N. abnormalis* were reported in ruminants and *N. lanceolatum* was detected in sheep and goats [9]. *N. battus* and *N. spathiger* species were reported in the same study as well. In studies conducted in Turkey, Çırak et al.[3] found *Nematodirus* spp. eggs in the stool of one of 10 camels and Aypak et al.[1] in four of 109 camels.

Pathogenic effects of *Oesophagostomum venulosum* may be indicated in the larval, adult, and nodal stages of the parasite that are included in the final host. They feed on blood in larval and adult stages. Intestinal wall edema, hemorrhage, and protein loss that emerge in relation to this situation and many other clinical results are particularly remarkable in animals that encounter this infection for the first time. The nodular plate in recurrent infections can cause severe absorption disorders depending on the intensity of the infection. If the nodule is torn to the peritoneum, peritonitis and also death can occur [9]. This parasite was seen at a prevalence of 2.5% in a study conducted in Pakistan [22]. The presence of Oesophagostomum spp. have been reported in several studies in Saudi Arabia [23], *O. venulosum* and *O. radiatum* species were reported in Iran [13]. In Turkey, Aypak et al.[1] detected *Oesophagostomum* spp. at a prevalence of 4.5% (at the genus level) in wrestling camels through egg cultivation from the feces. *Oesophagostomum venulosum* was detected in the large intestine of the camel (4.5%) with *N. filicollis*. In addition, the presence of *O. columbianum* and *O. venulosum* in ruminants and *O. radiatum* in cattle and buffaloes are known in Turkey [9].

According to the studies based on fecal examination in wrestling camels in Turkey, the rate of gastrointestinal helminth was between 44-74% in different studies [1,4]. In the present study, the prevalence of gastrointestinal helminth was found to be 16%, which is much lower than these two studies [1,4]. The researcher thinks that the application of antiparasitic drugs along with many other ways of treatment in animals with unexplained disease or poor performance might have abolished the existing parasites. In addition, high parasitemia detected in wrestling camels in previous studies [1,4] will probably create a high awareness in animal owners and veterinarians.

There are very few studies on damages that are caused by the parasites found in camels [1,13,23]. The damages caused by parasites and the yield losses can be estimated from studies conducted in other ruminants. Especially, their effect on wrestling performance is open to investigation.

In Turkey, camel breeding is mainly practiced in the Aegean, Mediterranean and Marmara regions. Although the use of camel meat is not the main aim of the breeding, the meat is offered for consumption when they are slaughtered. Infection of dogs with organisms at dangerous larval stages such as hydatic cysts in the improper slaughtering places can lead to serious public health issues. This high prevalence of hydatidosis, which has an important place among zoonotic infections, emphasized the importance of slaughtering these animals in slaughterhouses or under the supervision of a veterinarian.

This study is the first one to report the occurrence of *Stilesia globipunctata*, *Nematodirus filicollis* and *Oesophagostomum venulosum* at species level in camels.

REFERENCES

1. Aypak S, Eren H, Bakırcı S, Uner S, Sımsek E, Boga B, Guler AG, Unlu AH: Parasites detected by examination of fecal samples in wrestling camels. *Kafkas Univ Vet Fak Derg*, 19 (3): 371-374, 2013. DOI: 10.9775/kvfd.2012.7484

2. Çalışkan V: Bir Dünya Kültür Mirası: Anadolu Devecilik Kültürü ve Deve Güreşleri. 4-5, İncirliova Belediyesi Kültür Yayınları, Pozitif Matbaa, Ankara, 2016.

3. Cirak VY, Senlik B, Gulegen E: Gastrontestinal parasites of camels *(Camelus dromedarius)* from Turkey and efficacy of doramectin against *Trichostrongyles*. *J Camel Pract Res*, 18 (2): 283-285, 2011.

4. Eren H, Aypak S, Selek N: Aydın yöresinde Deve *(Camelus dromedurius)*'lerde dışkı bakılarına göre saptanan parazitler. *YYÜ Vet Fak Derg*, 14 (1): 59-60, 2003.

5. Senlik B: Teşhis yöntemleri. **In,** Tınar R (Ed): Helmintoloji. 463-535, Nobel Yayın Dağıtım, Ankara, 2006.

6. Güralp N: Helmintoloji. 2. Baskı, 392-402, Ankara Üniversitesi Veteriner Fakültesi Yayınları, 368, Ankara, 1981.

7. Soulsby EJL: Helminths, Arthropods and Protozoa of Domesticated Animals. 7th ed., 87-346, Bailliere Tindall, England, 1986.

8. Toparlak M, Tüzer E: Veteriner Helmintoloji. Ders Notu No: 102., 137, İstanbul Üniversitesi Veteriner Fakültesi Yayını, İstanbul, 1999.

9. Umur Ş, Köroğlu E, Güçlü F, Tınar R: Nematoda. **In,** Tınar R (Ed): Helmintoloji. 213-536, Nobel Yayın Dağıtım, Ankara, 2006.

10. Ayaz E, Tınar R: Cestoda. **In,** Tınar R (Ed): Helmintoloji. 167-180, Nobel Yayın Dağıtım, Ankara, 2006.

11. Brunetti E, Kern P, Vuitton DA: Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. *Acta Trop,* 114, 1-16, 2010. DOI: 10.1016/j.actatropica.2009.11.001

12. Deplazes P, Rinaldi L, Alvarez Rojas CA, Torgerson PR, Harandi MF, Romig T, Antolova D, Schurer JM, Lahmar S, Cringoli G, Magambo J, Thompson RCA, Jenkins EJ: Global distribution of alveolar and cystic echinococcosis. *Adv Parasitol,* 95, 315-493, 2017. DOI: 10.1016/bs.apar.2016.11.001

13. Sazmand A, Joachim A: Parasitic diseases of camels in Iran (1931-2017) - A literature review. *Parasite*, 24, 2017. DOI: 10.1051/parasite/2017024

14. Al Kitani FA, Al Riyami S, Al Yahyai S, Al Rawahi AH, Al Maawali M, Hussain MH: Abattoir based surveillance of cystic echinococcosis (CE) in the Sultanate of Oman during 2010-2013. *Vet Parasitol*, 211 (3-4): 208-215, 2015. DOI: 10.1016/j.vetpar.2015.06.011

15. Haridy FM, Ibrahim BB, Elshazly AM, Awad SE, Sultan DM, El-Sherbini GT, Morsy TA: Hydatidosis granulosus in Egyptian slaughtered animals in the years 2000-2005. *J Egypt Soc Parasitol,* 6 (3): 1087-1100, 2006.

16. Toulah FH, El Shafei AA, Alsolami MN: Prevalence of hydatidosis among slaughtered animals in Jeddah, Kingdom of Saudi Arabia. *J Egypt Soc Parasitol*, 42 (3): 563-572, 2012. DOI: 10.12816/0006341

17. Al-Yaman FM, Assaf L, Hailat N, Abdel-Hafez SK: Prevalence of hydatidosis in slaughtered animals from North Jordan. *Ann Trop Med Parasitol*, 79 (5): 501-506, 1985.

18. Mbaya H, Magambo J, Njenga S, Zeyhle E, Mbae C, Mulinge E, Wassermann M, Kern P, Romig T: *Echinococcus* spp. in central Kenya: A different story. *Parasitol Res*, 113 (10): 3789-3794, 2014. DOI: 10.1007/s00436-014-4045-z

19. Boğa Kuru B, Aypak S, Aysul N: Aydın yöresindeki köpeklerde *Echinococcus granulosus* yaygınlığının polimeraz zincir reaksiyonu ile belirlenmesi. *Türkiye Parazitol Derg*, 37, 78-83, 2013. DOI: 10.5152/tpd.2013.20

20. Gökçen A, Adanır R: Koyun ve Keçilerde Parazit Hastalıkları. **In,** Yukarı BA, Özcel MA (Eds): Veteriner Hekimliğinde Parazit Hastalıkları. 907-911, Türkiye Parazitoloji Derneği Yayın No: 24, Meta Basım, İzmir, 2013.

21. Bekele T: Epidemiological studies on gastrointestinal helminths of dromedary *(Camelus dromedarius)* in semi-arid lands of eastern Ethiopia. *Vet Parasitol,* 105 (2): 139-152, 2002. DOI: 10.1016/S0304-4017(01)00583-0

22. Anwar M, Hayat CS: Gastrointestinal parasitic fauna of camel *(Camelus dromedarius)* slaughtered at Faisalabad abattoir Pakistan. *Pak J Biol Sci,* 2 (1): 209-210, 1999. DOI: 10.3923/pjbs.1999.209.210

23. El Bihari S: Helminths of the camel: A review. *Br Vet J,* 141 (3): 315-326, 1985. DOI: 10.1016/0007-1935(85)90070-3

24. Aypak S: Develerin helmint enfeksiyonları. *Türkiye Parazitol Derg*, 31 (3): 225-228, 2007.

25. Kassouha MA: Prevalence of Gastro-Intestinal Helminthes of Camels in Syria. Thesis. Syrian Arab Republic, Al-Baath University Faculty of Veterinary Medicine Department of Microbiology, Syria, 2011.

26. Banaja AA, Ghandour AM: A review of parasites of camels *(Camelus dromadorius)* in Saudi Arabia. *JKSUS,* 6, 75-86, 1994.

27. Parsani HR, Singh V, Momin RR: Common parasitic diseases of camel. *Vet World,* 1 (10): 317-318, 2008.

28. Abd El-Wahed MM: Incidence of Nematodirus species and their differentiation through the infective third stage larvae among Egyptian camels. *J Egypt Soc Parasitol*, 35 (2): 447-450, 2005.

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Research Article

Ultrasonografical Monitoring as Diagnostic Tool for Reproductive Management in Female Buffaloes *(Bubalus bubalis)*

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Abstract

The aim of this study was to estimate the frequency of physiological and pathological reproductive conditions in female Italian Mediterranean Buffaloes. Female animals (n=444), ranging from 3 to 14 years of age were monitored by ultrasonography (USG). A total of 7319 USG images were collected and analyzed for pregnancy status, fetal sexing, gynecological measurements and pathologies. While pregnancy rate was determined as 38.75% and ovarium-genital canal disorders as 11.71%; the most common pathologies in nonpregnant animals are defined as ovarian cystic degeneration (OCD), hydrosalpinx and salpingitis (SALP), metritis, pyometra (METR) and pneumovagina (PVAG). OCD represented 4.27% of pathological conditions during long day season (February-June) with a higher frequency above 5 years of age ($P<0.05$); and characterized by the presence of rounded anechoic structures with a 3.12±0.54 cm outer and 2.5±0.22 cm an internal diameter. While SALP frequency was determined as 6.75% and more intense over 7 years; METR and PVAG frequencies were 0.67% in total. In conclusion, these results demonstrated that ultrasonographic monitoring is a useful diagnostic tool to optimize the reproductive management through pregnancy and delivery time determination in addition to certain pathological conditions of the reproductive tract in buffaloes.

Keywords: *Buffalo, Reproductive pathologies, Reproductive management, Ultrasonography*

Dişi Mandalarda *(Bubalus bubalis)* Reprodüksiyon Yönetiminde Tanı Yöntemi Olarak Ultrasonografik Görüntüleme

Öz

Bu çalışmanın amacı, dişi İtalyan Akdeniz Mandalarında fizyolojik ve patolojik reprodüktif durumların frekansının belirlenmesidir. 3-14 yaş aralığındaki dişi hayvanlar (n=444) ultrasonografi (USG) ile görüntülenerek, toplam 7319 USG görüntüsü ile gebelik durumu, fötal cinsiyet, jinekolojik ölçümler ve patolojiler açısından analiz edildi. Gebelik oranı %38.75 ve ovaryum-genital kanal bozuklukları %11.71 olarak belirlenirken; gebe olmayan hayvanlarda en sık görülen patolojiler ovaryumun kistik dejenerasyonu (OKD), hidrosalpinks ve salpingitis (SALP), metritis, pyometra (METR) ve pnömovagina (PVAG) olarak tanımlanmıştır. OKD, uzun gün mevsimi süresince (Şubat-Haziran) ve 5 yaş üstü dişilerde ($P<0.05$) patolojik koşulların %4.27'sini temsil ederken; ortalama 3.12±0.54 cm dış ve 2.5±0.22 cm iç çapa sahip yuvarlak anekoik yapıların varlığı ile karakterizeydi. SALP sıklığı %6.75 ve 7 yaş üzeri mandalarda daha yoğun tespit edilirken; METR ve PVAG frekansları toplamda %0.67 olarak belirlenmiştir. Sonuç olarak, reprodüktif kanalın bazı patolojik koşullarının belirlenmesine ek olarak gebelik ve doğum tarihinin ultrasonografik görüntüleme ile saptanmasının mandalarda reprodüktif bir yönetim aracı olarak kullanılabilirliği ortaya konulmuştur.

Anahtar sözcükler: *Manda, Reprodüktif patoloji, Reprodüksiyon yönetimi, Ultrasonografi*

INTRODUCTION

Ultrasonography (USG) monitoring holds a great potential for the reproductive management of dairy buffalo industry. Initially, it was an "elite" technique that was used only in a limited way by some veterinarians and solely in horse and cattle rearing [1-3]. Nowadays, USG is a widely used technique not only in research but also in the management of the reproduction in farm animals. As for Buffalo, as well as on cattle, it was used to diagnose pregnancy and detection

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of some pathological conditions in reproductive tract and ovaries, but now the use is considered as a diagnostic method to evaluate the reproductive performance, as well as a method of support to plan and to implement assisted reproduction techniques and farm management [4,5]. USG of the genital tract and ovaries allows to perform artificial insemination on more accurate time, pregnancy diagnosis following the implementation of the Ov-synch protocol in postpartum in order to implement the re-synchronization of those non-pregnant buffaloes. This protocol also allows to synchronize and inseminate a group of buffaloes without identifying the signs of estrus and to manage the postpartum period. USG monitoring allows identifying the pregnant and non-pregnant buffaloes through the present appearance of follicles and corpora lutea after insemination not only in an early manner, also simultaneously creates an opportunity for a management tool. Indeed, USG system is very useful for pregnancy diagnosis, since it allows performing diagnosis of pregnancy around 26-27 d, approximately 7 d anticipating the diagnosis by rectal palpation. The pregnancy diagnosis by rectal palpation, in fact, has an accuracy of 60% when performed between 31 and 40 d from the coupling, 92% when performed between 41 and 50 d and 100% between 51 and 55 d [6]. Recently some studies performed to demonstrate that corpus luteum (CL) can be considered to distinguish between pregnant and non-pregnant buffaloes [7,8]. These studies have shown, between 5 and 10 d after artificial insemination, an increase in the area and the diameter of CL can be detected in pregnant buffaloes, while not evident in those where conception has failed. CL functionality is also crucial in the early days of pregnancy which can be assessed by color Doppler and shows off the blood flow to his load. In fact, this flow tends to be higher in pregnant buffaloes 18 d after the insemination, compared to those which do not become pregnant. Reproductive evaluation also allows programming the additional synchronization (Re-synch) of the non-pregnant animals as soon as possible, where there is evidence for more than a centimeter-sized follicle, in the presence of a CL in regression. Studies in this regard have shown that there are no significant differences in conception rates according to the period of post-partum. Although the ideal to get a 12-month calving interval should be inseminating the buffaloes within 60 d after delivery [9]. In this regard, it can be suggested that real-time US monitoring is essential as a reproductive management tool to decrease the service period.

In buffalo breeding, anestrus (absence of estrus signs) and prolonged postpartum acyclicity (absence of ovarian cyclic activity) are the main reasons of economic loss. In dairy buffaloes first ovulation as detected by progesterone analysis and rectal palpation occurred between 24-55 d and 28-71, respectively, after calving and postpartum estrus occurred between 44 and 87 d [10]. In abattoir studies, oviductal pathologies have been largely mentioned in buffalo and include pyosalpinx (0.6%-11.9%), hydrosalpinx (0.7%-14.2%), salpingitis (0.2%-14.2%), blockage of the oviduct (1.2%-37.8%) congenital defects (0.2%) and adhesions (1.5%-1.7%) [11]. Apart from above mentioned problems, fimbriae cover a greater area than in the bovine, which may give rise to adhesions causing the onset of acute salpingitis with consequent hydrosalpinx in Buffalo. However, a linear probe with frequencies higher than 7.5 MHz presents a good resolution and allows to evaluate anatomical structures as well as detecting the presence of follicles and CL, pregnancy diagnosis and to perform fetal sexing.

Through the evaluation of the pregnancy, fetal measurements, vitality, and pathological conditions of the reproductive tract with the US reproductive efficiency improves thus the interval between artificial insemination and service period decreases. Therefore, we aimed to report USG findings in the reproductive tract and ovaries of female buffaloes during long term monitoring activity and concurrently to define the frequency of physiological and pathological condition diagnosed with USG in a buffalo farm during reproduction management.

MATERIAL and METHODS

Ultrasonography monitoring was carried out with 444 buffaloes in a single farm, located in the northeast Italian territory (Venice) raising mainly Italian Mediterranean Buffalo. The US, with adjoining image collection, has been done in the period between February of 2013 and July of 2015 during the breeding season. The USG examination carried out transrectally with linear array probe (10 MHz) (MyLab Vet-One, Esaoete, Italy) within the scope of the entire reproductive system; vagina, through the scanning of a body, both uterine horns up to the ovaries respectively. This study was approved by the Animal Care Ethics Committee of Ankara University and all experiments were conducted according to ethical principles and laws (2013-15-114).

Ovarian and Uterine Ultrasonography and Image Evaluation

The US images were classified considering the structures during the monitoring (gestational sac, vaginal-uterine and salpinx pathological conditions, follicles, CL and OCD) and defined remotely. OCD was characterized by the presence of rounded anechoic structures bigger than 2.7 cm and in absence of a functional CL [12]. OCD measurements considered: total diameter of the cyst; diameter of the internal cavity; thickness of the cyst wall. A follicular cyst is a structure which characterized by spherical shape; with a thin wall and regular border, less than 3.5 mm thickness; cavities homogeneously anechoic. The distinction of the luteal cyst from the follicular cyst is based on the wall thickness greater than 3 mm; irregular border; internal cavity with inhomogeneous content [13].

In fact, characteristic of the luteal cyst is the ability to see, inside the cavity of the cyst of the hyper-echogenic septa [14]. SALP condition was characterized by the presence of abundant anechoic areas within the oviduct. METR is determined according to the presence of anechoic fluid in cases of endometritis along with snowy echogenic particles [15]. PVAG is characterized by the presence of anechoic air within the vagina or both vagina and uterus [16]. Dimensional image measurements were done with ImageJ software.

Ultrasonography in Pregnancy

Ultrasound image classification was carried out according to Pieterse et al.[17], Pawshe and Purohit [18] *(Table 1)*.

Within the same scope, determination of gestational age and predicted delivery time, embryo-fetus measurements were made and following fetal parameters [19]: Crown-rump length (CRL), biparietal diameter (BPD) and placentome diameter (PLD) were recorded. Fetal sexing (FS) were performed considering the genital tubercle (lobed structure, two hyper-echogenic parallel lines) close to the umbilical cord or close to the tail base. As a further aid, FS was examined between the hind legs to the scrotum or bladder [19]. Male FS was recorded when the genital tubercle was identified caudal to the point of abdominal insertion of the umbilical cord or detected in between the hind legs of the scrotum. Female FS was recorded when genital tubercle was identified close to the tail base or rudimentary teats [20,21].

Table 1. *The timetable for pregnancy related-structures*

Day	Structure	Reference
19th	Embryonic vesicles	Pieterse et al., 1990 [17]
40th	Umbilical cord	Pawshe and Purohit, 2013 [18]
42th	Head	Pawshe and Purohit, 2013 [18]
44th	Trunk and limb buds	Pawshe and Purohit, 2013 [18]
50th	Cotyledons	Pawshe and Purohit, 2013 [18]
55th	Fetal ribs	Pawshe and Purohit, 2013 [18]
62th	Brain ventricles eye patch, the front and rear paws	Pawshe and Purohit, 2013 [18]

Fetal Measurements and Gestational Age Estimation with Ultrasonography

Measurements regarding the fetal parameters were then inserted into the equations identified by the Ali and Fahmy [19] expressing the relationship between the different fetal parameters. The fetal parameters were expressed in centimeters and the gestational age was calculated in weeks.

The equations used are: $y = 0.0282x^2 + 0.1589x - 0.1427$ where y = CRL, cm; x = Gestational Age, weeks (R^2 = 0.9451); $y = 0.0179x^2 - 0.1222x + 1.0688$ where y = BPD, cm; x = Gestational Age, weeks (R^2 =0.8999); $y = - 0.0031x^2 + 0.2712x - 0.9265$ where y = PLD, cm; x = Gestational Age, weeks (R^2 = 0.6792). Gestational age of the fetus was calculated through these equations, which were expressed in weeks. They were then calculated the missing weeks at the end of gestation whereas the duration of pregnancy in Italian Mediterranean Buffalo is 315 d, which corresponds to 45 weeks. Thus, the obtained results and FS were compared with the actual dates of birth and sex of the calf.

Data Analysis

On-farm and remotely collected data were analyzed using Excel datasheet and Image-J. The frequencies of parameters were calculated. Significant statistical differences between a month of monitoring in terms of frequency and estimated (embryo-fetal measurements) and actual delivery time were tested using Sigmastat 2.03.

RESULTS

Four hundred and forty-four buffalos were examined by a total of 7319 US images. According to results with USG, 38.75% of buffalos were detected as pregnant and, 11.71% of animals had genital tract disorders while the rest of the animals were identified as cyclic, non-pregnant animals *(Fig. 1)*.

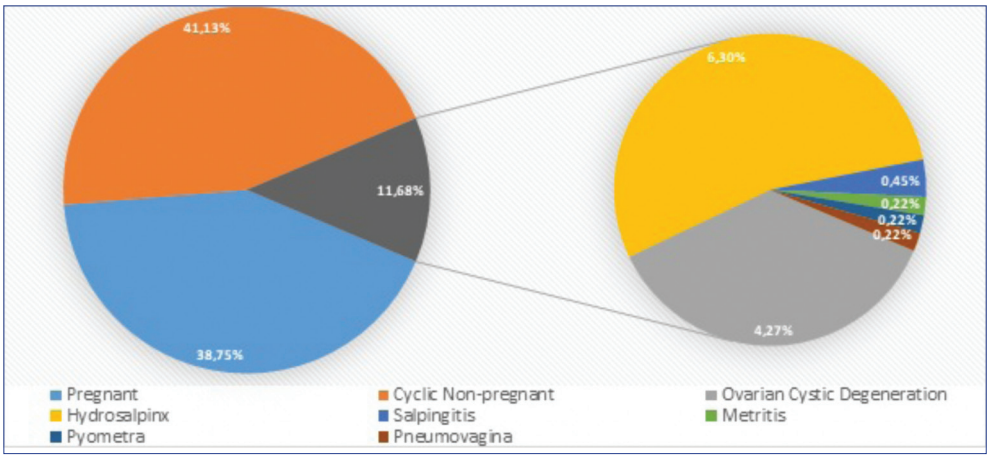

Fig 1. Graphic representation of the absolute and relative frequencies of the ultrasonographic findings in buffalo cows

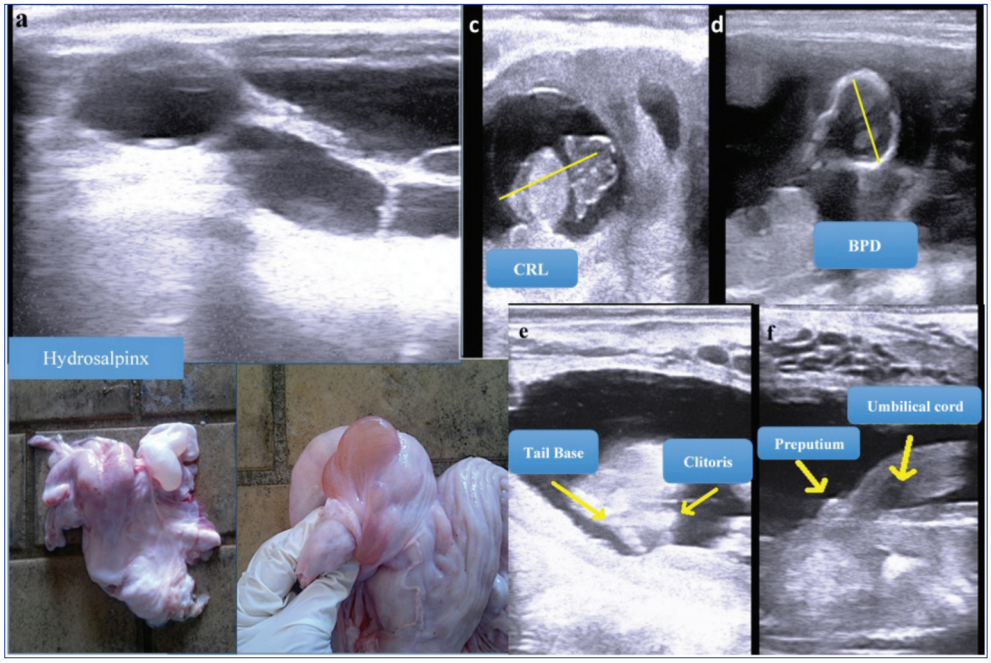

Fig 2. Some of the reproductive pathological findings and fetal measurement in buffaloes

Ultrasound appearance (a) and slaughterhouse finding (b) of hydrosalpinx in buffaloes, embryo and fetal measurements (c) CRL: Crow-rump length; (d) BPD: Biparietal Diameter, fetal sexing starting from 55 days of pregnancy (e: female; f: male)

It was possible to identify the presence of the fetus on images of 66 buffaloes, and the measurements of fetal parameters in 46 buffaloes for a total of 49 parameters. These 14 are the measurement of the CRL, 15 of BPD and 20 of PLD. Fetal measurements were calculated to estimate the delivery time equations reported by Ali and Fahmy [19]. It was possible to compare the estimated delivery date with the actual one and were calculated the differences in between. CRL gives an estimation with an error varying from 2 to 12 d; BPD gives a difference from 2 to 14 d, while in the case of PLD varies between 14 and 99 d. In 66 buffaloes in which is highlighted by the presence of the fetus, the fetal sexing was performed in 9 animals. USG fetal sexing was possible to compare with the gender of neonates (n=5) resulting in a 4 of 5 right determinations. It should also be noted that fetal sexing, comparing the date of execution of USG and the birth date, was performed between 8 and 15 weeks of gestation.

The evaluation of ultrasound images made it possible to carry out the diagnosis of female genital tract disorders in 11.71% of the buffaloes. Diseases identified as: ovarian cystic degeneration (4.27%), hydrosalpinx (6.30%), salpingitis (0.45%), metritis (0.22%), pyometra (0.22%) and pneumovagina (0.22%) *(Fig. 2)*. The cystic degeneration diagnosis was made on 19 subjects of total herd. The total measurements made highlights such as the cystic structures shown in the ultrasound image level on average, a total diameter of 3.29±0.54 cm, a diameter of the internal cavity of 2.56±0.22 cm and a wall thickness equal to 0.45±0.14 cm. Cysts were found mainly in the period between February and June and in subjects over the age of five years ($P<0.05$). The diseases diagnosed other than cystic degeneration have been reported in subjects aged between 4 and 14 years, with a higher incidence in those with more than 7 years.

Ultrasound appearance (a) and slaughterhouse finding (b) of hydrosalpinx in buffaloes, embryo and fetal measurements (c) CRL: Crow-rump length; (d) BPD: Biparietal Diameter, fetal sexing starting from 55 days of pregnancy (e: female; f: male)

DISCUSSION

This study presented the results from the ultrasonographical monitoring of the buffaloes' genital tract and ovaries. It has been shown that the ultrasound monitoring has enabled the assessment of the state of the ovaries, making a diagnosis of pregnancy and to detect the presence of reproductive anomalies.

In buffalo reproduction management, natural service is still widespread due to the certain seasonality, expression of estrus sign, high variability of ovulation time and thus reduced reproductive efficiency. This means that the exact date of fertilization generally is not known. However, owing to USG, it is possible to estimate the gestational age and the birth date by measuring these fetal parameters. The substitution in this equations according to Ali and Fahmy [19] shows that fetal parameters that allow a more accurate estimate of the date of birth are the CRL and BPD, considering as acceptable with a maximum of 25 d difference between the estimated date and the actual date of birth. However, Placentomes diameter (PLD) is found to be an unreliable parameter for estimating the date of confinement [19]. This outcome is also in line with the present study as PLD gave an estimation with an error varying from 14 to 99 d.

During the estimation of the delivery date, it was also possible to sex the fetus with an accuracy of 80%. This result

can be considered admissible comparing to the accuracy deviates highlighted in the study by Ali and Fahmy [19], equal to 97.1%, (in general the accuracy of fetal sexing by ultrasound examination is between 95% and 100%). Our lower accuracy can be related to the fact that the number of fetal sexing performed in this case is reduced, in addition to the fact that a more accurate diagnosis for a single image, as has occurred in some of these cases, the abdomen of the fetus in level of the umbilical cord or of the area between the limbs is not sufficient. However, it would take the more accurate diagnosis of the images continuously at the passage of the probe on the ventral abdomen, the umbilical cord to the tail of the fetus. The fetal sexing was performed between 8 and 15 weeks of gestation, a result that is consistent with that observed in the study by Ali and Fahmy [19], in which sexing was possible from the eighth week of pregnancy. Thus, compared to the number of pregnant buffaloes that, were detected with the presence of the fetus, the low number of fetal parameters measurements and fetal sexing can be linked to the gestational age, in which the diagnosis has been made, considering that the genital tubercle begins to migrate from the 45th d of intrauterine life and only reaches the final position starting from the 55th d of pregnancy.

The ultrasound examinations and the remote image analysis has also allowed the detection of genital tract diseases. Consequently, the reproductive tract disorders are covering a considerable percentage of the problems in buffalo breeding. In fact, these problems result in economic losses due to reduced fertility with a calving interval extension, treatment costs and loss in milk production. With an early detection of the problem enables early intervention thus managing to minimize the economic losses. Ultrasound visits over the years on the farm and the subsequent evaluation of images showed the most frequent diseases were ovarian cystic degeneration (4.27%) and hydrosalpinx (6.30%). The incidence of ovarian cystic degeneration in buffaloes is very low; in clinical trials, it is variable from 0.07% [22] to 1.48% [23]. Several studies have been made against ovarian disease in buffaloes, and specifically of cystic degeneration, but a perfect clinical description of the latter, in buffaloes, does not yet exist. In fact, it can be said that there is a lack of information about biometrics of ovarian cysts in buffaloes as well as limited information on the physical, biochemical, hormonal and mineral composition of this disease [24]. Aiumlamai et al.[25] argue that ovarian cyst, in buffaloes, presenting as a follicular structure with a diameter greater than 2.5 cm. Other authors [26] argue that ovarian cysts are always anovulatory structures with a diameter larger than 2 cm and which, persist for a variable period in the absence of the CL. The use of ultrasound for the diagnosis of cystic degeneration in buffaloes turns out to be an advantage since it allows to highlight, with greater accuracy and speed to assess the size of the cyst allows evaluating the absence of the CL, which is a necessity to classify ovarian cystic degeneration. Relying solely on behavioral changes linked to ovarian cystic degeneration and rectal palpation is not ideal in buffaloes since the behavioral alterations in buffalo, unlike the bovine, are not very noticeable. Rectal palpation can be run into an incorrect diagnosis, as in buffalo ovarian structures appear to be located deeper in the ovarian stroma. It is possible to detect the presence of cysts in an early manner, i.e. before the onset of symptoms via ultrasound, thus allowing timely intervention on the problem for reducing the calving interval in buffaloes. Generally, although the incidence of this condition is not very high, the early diagnoses allow the breeder to solve the problem where the buffaloes are reduced their reproductive efficiency with cystic degeneration more than cows [27].

The identification of such problems like hydrosalpinx, salpingitis and pyometra in the reproductive tract is very important since it affects fertility and reproduction and, consequently, reproductive efficiency. A load abnormality in any of the cervix, uterus or uterine horns can reduce the fertility in buffaloes [28]. Studies carried out at the slaughterhouse showed an incidence of diseases borne by the oviduct between 10% and 29% [29], with greater frequency of salpingitis. However, the hydrosalpinx compared to salpingitis are more frequent. Similarly, in the present study, the frequency of hydrosalpinx was 6.30% whereas salpingitis frequency was 0.45%. Hydrosalpinx is ultrasonographically notable for the presence of small anechoic areas, dates from the accumulation of fluid in the oviductal lumen, up to the complete relaxation of the oviductal wall in severe cases. It is manifested through the amber fluid accumulation, resulting in oviductal distension based on the severity of the injury. In this study, the percentage of buffaloes suffering from hydrosalpinx is found to be in line with the variable incidence between 0.7% and 14.3% reported in other studies [30,31]. Consequently, an early identification of pathological conditions of the oviduct is essential for conception and embryo survival in the days before the descent into the uterus [28]. These pathologies can lead to infertility and sterility in buffaloes, where the obstruction of the oviductal lumen prevent the encounter of gametes or by creating an environment unsuitable for embryonic development. Thus, it can be clearly suggested that USG is essential for the diagnosis of hydrosalpinx where there are not noticeable clinical symptoms or behavioral changes comparing to cystic degeneration.

In conclusion, this study has shown that the use of ultrasound can be used for different purposes in order to optimize the management of the reproduction consequently with pregnancy diagnosis. Primarily, the implementation of the re-synchronization program after insemination at postpartum where pregnancy fails. In the case of pregnant animals, the measurement of specific fetal parameters. Secondly, remote image analysis has allowed the detection

of genital tract diseases and an early identification of diseases to increase the reproductive efficiency. In conclusion, overall results show that ultrasound has a very important role in buffalo reproduction management.

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REFERENCES

1. Ginther OJ: How ultrasound technologies have expanded and revolutioned research in reproduction in large animals. *Theriogenology.* 81, 112-125, 2014. DOI: 10.1016/j.theriogenology.2013.09.007

2. Pancarci SM, Ari UC, Atakisi O, Gungor O, Cigremis Y, Bollwein H: Nitric oxide concentrations, estradiol-17β progesterone ratio in follicular fluid, and COC quality with respect to perifollicular blood flow in cows. *Anim Reprod Sci,* 130 (1-2): 9-15, 2012. DOI: 10.1016/j.anireprosci.2011.12.013

3. Pancarci SM, Gungor O, Atakisi O, Cigremis Y, Ari UC, Bollwein H: Changes in follicular blood flow and nitric oxide levels in follicular fluid during follicular deviation in cows. *Anim Reprod Sci,* 123 (3-4): 149-156, 2011. DOI: 10.1016/j.anireprosci.2011.01.001

4. Terzano MG: Ultrasonography and reproduction in buffalo. *J Buffalo Sci,* 1, 163-273, 2012. DOI: 10.6000/1927-520X.2012.01.02.06

5. Piotrowska-Tomala KK, Jonczyk AW, Bah MM, Warmowski P, Skarzynski DJ: Comparison of ultrasonographic images retrieved using two different probes (mechanical sector and linear ones) and macroscopic features of bovine reproductive organs: Biometric studies. *Kafkas Univ Vet Fak Derg*, 23 (3): 487-490, 2017. DOI: 10.9775/kvfd.2016.16562

6. Karen AM, Darwish S, Ramoun A, Tawfeek K, Hanh NV, de Sousa NM, Sulon J, Szenci O, Beckers JF: Accuracy of transrectal palpation for early pregnancy diagnosis in Egyptian buffaloes. *Trop Anim Health Prod,* 43, 5-7, 2011, DOI: 10.1007/s11250-010-9675-2

7. Vecchio D, Neglia G, Gasparrini B, Russo M, Pacelli C, Prandi A, D'Occhio MJ, Campanile G: Corpus luteum development and function and relationship to pregnancy during the breeding season in the Mediterranean buffalo. *Theriogenology*, 77 (9): 1811-1815, 2012. DOI: 10.1016/j.theriogenology.2011.12.025

8. Scully S, Evans ACO, Carter F, Duffy P, Lonergan P, Crowe MA: Ultrasound monitoring of blood flow and echotexture of the corpus luteum and uterus during early pregnancy of beef heifers. *Theriogenology*, 83, 449-458, 2015. DOI: 10.1016/j.theriogenology.2014.10.009

9. Baruselli PS, Barnabe VH, Barnabe RC, Visintin JA, Molero-Filho JR, Porto R: Effect of body condition score at calving on post partum reproductive performance in buffalo. *Buffalo J,* 17, 53-65, 2001.

10. El-Wishy AB: The postpartum buffalo: II. Acyclicity and anestrus. *Anim Reprod Sci*, 97 (3-4): 216-236, 2007. DOI: 10.1016/j.anireprosci.2006.03.003

11. Purohit GN: Ovarian and oviductal pathologies in the buffalo: Occurrence, diagnostic and therapeutic approaches. *Asian Pac J Reprod*, 3 (2): 156-168, 2014. DOI: 10.1016/S2305-0500(14)60020-8

12. Lopez-Diaz MC, Bosu WTK: A review and update of cystic ovarian degeneration in ruminants. *Theriogenology,* 37, 1163-1183, 1992. DOI: 10.1016/0093-691X(92)90173-O

13. Garverick HA: Ovarian follicular cyst in dairy cows. *J Dairy Sci,* 8, 995-1004, 1997. DOI: 10.3168/jds.S0022-0302(97)76025-9

14. Carroll DL, Pierson RA, Hauser ER, Grummer RR, Combs DK: Variability of ovarian structures and plasma progesterone profiles in dairy cow with ovarian cyst. *Theriogenology.* 34, 349-370, 1990. DOI: 10.1016/0093-691X(90)90528-2

15. Kumar V, Purohit GN: Ultrasonographic diagnosis of the bovine genital tract disorders. *VetScan*, 4 (2): 43, 2009.

16. Goncagul G, Intas KS, Kumru IH, Intas DS: Prevalence and accompanying signs of pneumovagina and urovagina in dairy cows in the Southern Marmara region. *Tierarztl Prax Ausg G Grosstiere Nutztiere*, 40 (6): 359-366, 2012.

17. Pieterse MC, Szenci O, Willemse AH, Bajcsy CSA, Dielman SJ, Taverne MAM: Early pregnancy diagnosis in cattle by means of linear erray real-time ultrasound scanning of uterus and qualitative and quantitative milk progesterone test. *Theriogenology*, 33, 697-707, 1990. DOI: 10.1016/0093-691X(90)90547-7

18. Pawshe CH, Purohit GN: Approaches for diagnosis of pregnancy in female buffaloes. **In,** Purohit GN (Ed): Bubaline Theriogenology. IVIS, Ithaca NY, A5708.0613, 2013.

19. Ali A, Fahmy S: Ultrasonographic fetometry and determination of fetal sex in buffaloes (*Bubalus bubalis*). *Anim Reprod Sci,* 106, 90-99, 2008. DOI: 10.1016/j.anireprosci.2007.04.010

20. Yotov S, Atanasov A, Georgiev P: Determination of foetal sex in buffaloes through a single sonographic examination. *Bulgarian J Vet Med,* 14, 39-44, 2011.

21. Bürstel D, Meinecke-Tillman S, Meinecke B: Ultrasonographic diagnosis of fetal sex in small ruminants bearing multiples fetuses. *Vet Rec,* 23, 635-636, 2002. DOI: 10.1136/vr.151.21.635

22. Modi LC, Patel PA, Patel SP, Patel GG, Joshi AH, Suthar DN: Prevalence of reproductive problems in buffaloes in Mehsana milk-shed area of Gujarat. *IJAVMS,* 5, 424-428, 2011. DOI: 10.5455/ijavms.20110801094631

23. Raju KGS, Naidu KV, Rao KS: Incidence of reproductive disorders in buffaloes under field conditions of Andhra Pradesh. *Indian J Anim Reprod*, 28, 46-48, 2007.

24. Khan FA, Nabi SU, Pande M, Das GK, Sarkar M: Bilateral follicular cyst in a water buffalo. *Trop Anim Health Prod,* 43, 539-541, 2011. DOI: 10.1007/s11250-010-9738-4

25. Aiumlamai S, Jarassaeng C, Sangkaew A, Ratanaphorn V: Ovarian cysts in swamp buffalo. *KKU Vet J,* 14, 49-58, 2004.

26. Braw-Tal R, Pen S, Roth Z: Ovarian cysts in high-yielding dairy cows. *Theriogenology,* 72, 690-698, 2009. DOI: 10.1016/j.theriogenology.2009.04.027

27. Peter AT: Managing post-partum anoestrus in bovines. **In,** *Proceedings Eighteenth Annual Western Canadian Dairy Seminar: Advances in Dairy Technology.* 85-99, Alberta, Canada, 2000.

28. Tandle MK, Purohit GN: Genital Tract Affections in the Female Buffalo. **In,** Purohit GN (Ed): Bubaline Theriogenology. IVIS, Ithaca NY, A5724.0713, 2013.

29. Azawi OI, Ali AJ, Lazim EM: Pathological and anatomical abnormalities affecting buffalo cows reproductive tract in Mosul. *Iraqi J Vet Sci,* 22, 59-67, 2008

30. Mittal D, Garg UK, Jatav GP, Shukla S, Sharda R: Prevalence of different pathological affections of ovaries in buffaloes (Bubalus bubalis) in Malwa Region of Madha Pradesh. *Buffalo Bull,* 29, 39-42, 2010.

31. Rahawy MA: Pathological affection of reproductive tract of Iraqi buffalo cow suffering from repeat breeding in Ninavaha province. *Iraqi J Vet Sci,* 23 (Suppl 1): Ar155-Ar161, 2009.

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Research Article

Determination of Telomerase Enzyme Level in Enzootic Intranasal Tumors of Goats

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Abstract

Enzootic intranasal adenocarcinoma (ENA) of goats are contagious tumoral disease that characterized occurrence of tumoral masses in nasal cavity of the animals. The etiological agent of the disease is retrovirus and the disease cause high economical loses in goat industry. Retroviruses, respectively inducing lung or nasal adenocarcinomas. The true economic impact of ENA is not known because affected animals are given insights from the herd before actual diagnosis, and suspected disease incidence is rarely reported. Telomerase regulates the proliferative capacity of cells and this enzyme has critical role in tumor progression. The aim of this study is to evaluate telomerase activity in serum and tumoral tissue in goat with ENA. For this aim 11 goat's serum were analyses for telomerase activity. Tumoral tissue sections of totally 14 death goats were examined by immunohistochemically for telomerase expression. In addition, 10 normal goat's serum and nasal tissues that belong slaughtered healthy animals used as control. This study showed that increased serum and tissue telomerase activity in goats with ENA comparing to controls. Also our results showed that telomerase activity becomes a useful prediction marker for tumor development in infected but clinically healthy goats in infected flocks.

Keywords: *Enzootic intranasal tumor, ELISA, Immunohistochemistry, Telomerase*

Keçilerin Enzootik Intranazal Tümörlerinde Telomeraz Enzim Düzeyinin Belirlenmesi

Öz

Keçilerin enzootik intranazal adenokarsinomu (ENA), hayvanların burun boşluğunda tümoral kitlelerin şekillenmesi ile karakterize bulaşıcı bir hastalıktır. Hastalığın etkeni retrovirüs olup, keçi endüstrisinde ekonomik kayıplara neden olmaktadır. Retroviruslar sırasıyla akciğer veya nazal adenokarsinomlara yol açar. Hastalıktaki ekonomik kayıp tam olarak bilinmemektedir. Çoğu zaman hasta hayvanlar teşhisten önce sürüden çıkarıldıkları için hastalığın gerçek insidansı ile ilgili bildirimler nadirdir. Telomeraz, hücrelerin proliferatif kapasitesini düzenler ve bu enzim tümörün yayılmasında kritik bir role sahiptir. Bu çalışmanın amacı ENA'lı keçilerin kan serumu ve tümöral dokularında telomeraz aktivitesini değerlendirmektir. Bu amaçla, 11 ENA'lı keçi serumunda telomeraz aktivitesi için analizler yapıldı. Ayrıca toplam 14 ölü keçinin tümoral doku kesitleri, telomeraz ekspresyonu için immunohistokimyasal olarak incelendi. Kontrol olarak, kesimi yapılan 10 sağlıklı keçinin serumu ve burun dokusu kullanıldı. Bu çalışmada ENA'lı keçilerde serum ve tümör dokularında telomeraz aktivitesinin kontrol grubundaki keçilere kıyasla arttığı tespit edildi. Enfekte olmuş fakat klinik olarak sağlıklı görünen keçilerde telomeraz aktivitesinin tümör gelişimi için yararlı bir belirteç olabileceği görüldü.

Anahtar sözcükler: *Enzootik intranazal tümör, ELISA, İmmunohistokimya, Telomeraz*

INTRODUCTION

Telomerase activity is an important determinant of telomere length in mammalian cells, in which lack of telomerase activity could exacerbate cell senescence, especially in highly proliferative tissues [1,2]. Telomerase activity has been shown to be specifically expressed in immortal cells, cancer and germ cells where it compensates for telomere shortening during DNA replication and thus stabilizes telomere length [3].

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Telomere shortening during the cell division disturbed tumor progression. Because of the continuous cell growth in malign tumors telomerase activity correlated by tumor behavior [4]. Telomerase is a cellular reverse transcriptase and adds new DNA onto the telomeres that are located at the ends of chromosomes [4-6]. Expression of telomerase is associated with the stage of differentiation and inhibition or absence of telomerase may result in cell crisis in cancer cells and cause tumor regression [7]. In addition, telomerase may express in permanently renewing epithelia such as gastrointestinal tract [8]. Shortening of telomeres may contribute to the control of the proliferative capacity in normal cells, and telomerase may be essential for unlimited cell proliferation [9]. An ideal cancer treatment would specifically target cancerous cells and have little or no effect on normal cells. Because of the compelling correlation that most normal cells not expressed telomerase and activity is detected in almost all cancer types, it may be a universal target for cancer therapeutics. Telomerase-based therapies should possess greater specificity, lower toxicity, and reduced side effects compared to conventional chemotherapeutic approaches [10,11].

Many viruses have been shown to be able to increase telomerase activity, for example the LMP1 gene of Epstein Barr virus (EBV) [12], Kaposi's sarcoma herpesvirus [13], Marek's disease virus [14], herpes simplex virus type-1 (HSV-1) [15] and Bovine Herpesvirus type1 -all up-regulate telomerase activity. On the other hands some viruses such as human immunodeficiency virus [16], the LMP2A gene of Epstein Barr virus [17] and Hepatitis B virus [18] down-regulate telomerase activity.

Sheep and goats are widely infected by the non-oncogenic Small Ruminant LentiViruses, related to Human Immunodeficiency Virus-1 and responsible for slowly evolving inflammatory and/or degenerative diseases, and by oncogenic retroviruses, and Enzootic Nasal Tumour Virus (ENTV), respectively inducing lung or nasal adeno-carcinomas [19,20]. Enzootic nasal adenocarcinoma (ENA) is an economically important contagious tumor of the nasal mucosa in sheep and goats [21]. The true economic impact of ENA is not known because affected animals are given insights from the herd before actual diagnosis, and suspected disease incidence is rarely reported [22]. Although the exact prevalence of ENA is unknown [23] and often is not diagnosed.

The agent of ENA is a simple retrovirus and it induces unilateral or bilateral neoplastic growth in the mucosal nasal glands of the ethmoidal area [21]. No metastasis has been reported in ENA cases, but disruption of the nasal septum structure as well as erosion of the cribiform plate has been reported [23,24].

Clinical signs of ENA include seromucosal nasal discharge leading to a 'washed nose' appearance, accompanied by snoring, coughing, wheezing and dyspnea. The duration of disease, from the appearance of clinical signs to the time of death, varies from 3 weeks to 1 year or more.

Based on these findings, we investigated the relationship between telomerase value in serum and immuno expression in tissue in enzootic nasal adenocarcinoma in goats.

MATERIAL and METHODS

This study was carried out on a total of eleven (5 males and 6 females) alive and 3 died (all of female) goats *(Capra hircus)* from a herd numbering 225 goats with a 10-year history of respiratory nasal problems and death with respiratory distress. The goats kept in a small shelter together and they ages were different between 2 to 5 years old.

The farm was visited and blood samples from 11 clinically ill animals (infected group=11) collected for evaluate the serum levels of telomerase. Clinically, signs of dyspnea and mucous nasal discharge were observed in many of the animals. Mild to severe emaciation was noted in all of animals. According to the history that taken from owner, more than 120 goats died from same illness with dyspnea and in some cases nasal bone perforation in recent years. Blood samples were collected in tubes without anticoagulant and were centrifuged at 3000 rpm, at 4°C for 10 min. Furthermore, ten healthy goats (control group=10) were used from another healthy flock. Serum samples were carefully harvested and stored at -20°C until used. These sera were then used to establish the values of telomerase levels using an ELISA commercial kits [Goat Telomerase (TE) ELISA KIT].

The 11 alive goats with clinical symptoms (blood samples collected from) were euthanatized, in addition 3 death goats were underwent to necropsy. At the gross examination of nasal cavity, unilateral intranasal tumors were found in all 14 goats. During necropsy, tissue samples were taken from tumoral masses for histopathological examinations. Samples were fixed in 10% neutral formalin. Using standard methods, tissues were stained with Hematoxylin-Eosin (HE), and examined microscopically.

Selected tumor sections were stained immunohisto-chemically in order to demonstrate telomerase activity in tumor tissue [Anti-Telomerase reverse transcriptase anti-body -C-terminal ab183105, (1/100 dilution)] using a routine streptavidin biotin peroxidase technique. For immunohistochemical examination sections were routinely processed according the manufacturer's instructions.

Student t test were used for to evaluate the differences of serum telomerase activity between the goats with and without ENA Variables were presented as, "mean±standard deviation" deviations. Calculations were made using the SPSS 15.0 program pack (SPSS Inc., Chicago, IL, USA). $P<0.05$ was set as the value for significance.

RESULTS

Clinical Signs

The age of the affected goats ranged from 2 to 5 years old (mean, 3.5 years old), and the flock had a history of 10 years of disease. Clinically, displayed clinical sings of ENA, including nasal discharge and noisy breathing, respiratory distress were the most prominent findings in the eleven affected animals, and also among most of the animals in the farm.

The mean values of serum telomerase value in ten healthy and 11 ENA-affected goat are shown in *Table 1*.

Table 1. *Statistical analysis results of serum telomerase activity in infected and control groups*

Control Group (ng/mL)	Infected Group (ng/mL)	P Value
18.97±5.58	49.52±5.39	<0.001

Necropsy Findings

At necropsy, unilateral intranasal tumors were found in nasal cavity of all animals *(Fig. 1)*. Distortion and blockage of the nasal canal were the common findings. Tumor tissues generally were soft but has hard areas in cut surface of the some tumoral. Seromucous exudate covered the tumor tissues in most of the cases. Pinkish white masses include hemorrhagic areas in rare cases. Although no metastasis was observed any cases, invasion to the nasal sinuses were seen in two cases.

Histopathological Findings

Histopathological examination revealed acinar, tubular and papillary patterns. Tumors contained well-differentiated, epithelial cells and scant fibrovascular stroma. The tumoral cells were in cuboidal to columnar shapes with distinct cell borders and eosinophilic cytoplasm. The nuclei were generally round or oval and centrally located. No indication

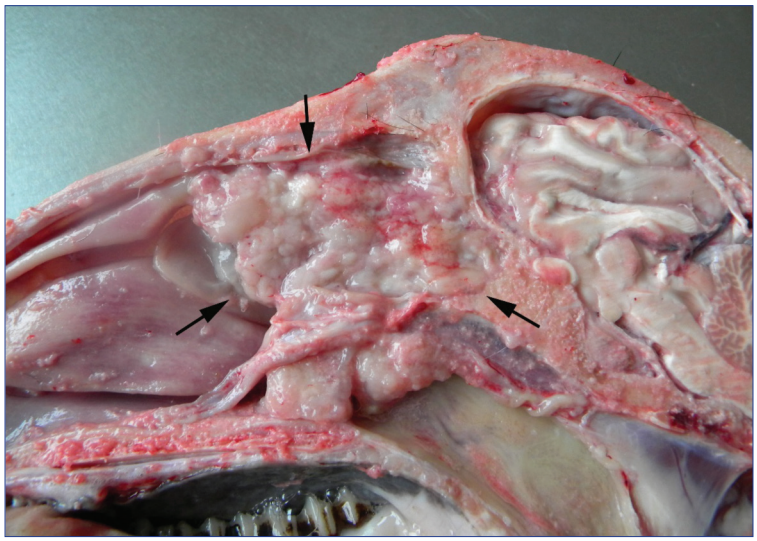

Fig 1. Gross appearance of ENA *(arrows)* localized in nasal cavity

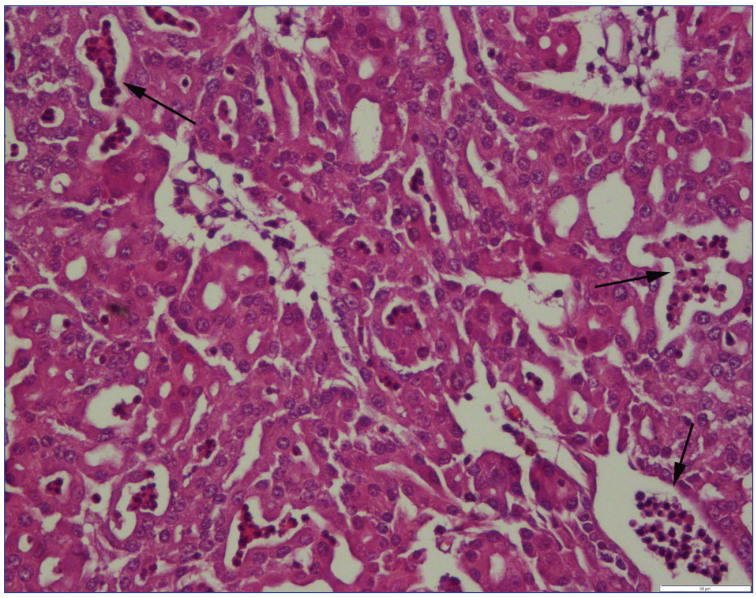

Fig 2. Histopathology of the tumor, uniform tumoral cells, acinar patterns and infiltrations *(arrows)*, HE, Bar = 100 µm

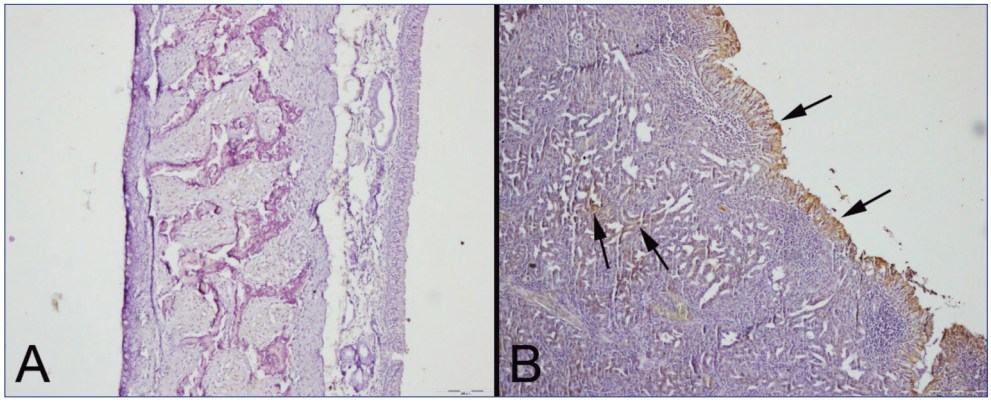

Fig 3. A- Negative telomerase activity of the control nasal tissue, **B-** Marked increase of the telomerase immuonoreaction *(arrows)* in tumoral mass, Streptavidin biotin peroxidase method, Bar = 100 µm

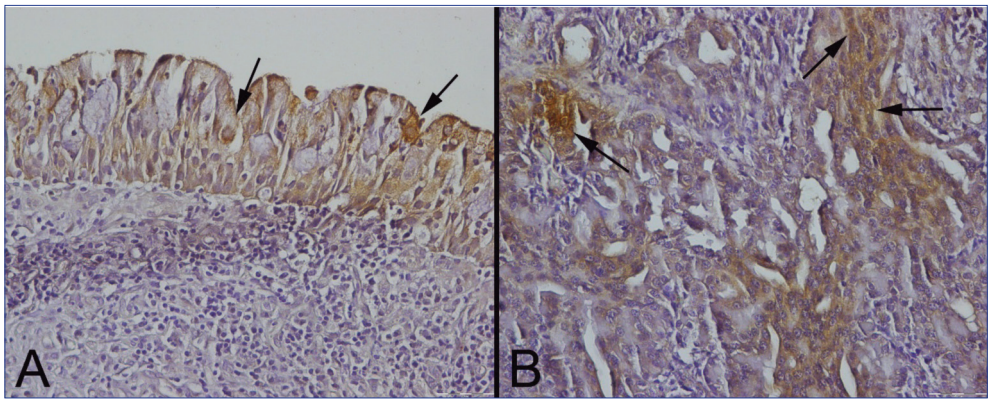

Fig 4. A- Higher magnification of increased telomerase immunoreaction *(arrows)* in nasal epithelial cells, **B-** Increased telomerase immunoreaction *(arrows)* in tumoral cells. Streptavidin biotin peroxidase method, Bar = 50 µm

of pleomorphism and cellular atypia were observed but, slight necrosis, inflammatory infiltrations and mitoses were seen *(Fig. 2)*.

At the immunohistochemistry, increase in telomerase activity in the nucleus and cytoplasm of the tumoral cells was observed in granular appearance *(Fig. 3, 4)*. The severity of the telomerase expression was not uniform and changing slight to severe in the tumoral mass.

DISCUSSION

Enzootic nasal adenocarcinoma is a contagious neoplasm of the secretory epithelial cells of the nasal mucosa of small ruminants and the causative agents of the tumor is retrovirus. ENA is commonly spread horizontally, most likely by the respiratory route. Although clinical symptoms and chronic behavior suspected the diseases, the easiest way for to diagnosis is the examination of nasal cavity of dead animals. Histopathological and/or ultrastructural examinations are necessary for definitive diagnosis [25]. In this study diagnosis of ENA was made base on the clinical, macroscopically, microscopically and ultrastructural findings. Only weld differentiated and characteristic cases included this study.

The telomerase activity plays an important role in maintaining chromosome stability, cellular immortality and oncogenesis. Telomerase is highly active in 90% of human cancers and transformed cells possess longer telomeres at their chromosomes [26]. Beyond its role in telomere maintenance, telomerase provides additional functions in tumorigenesis, DNA repair and cell survival. Telomerase protects cells from apoptosis and necrosis, and stimulates growth in adverse conditions [27].

The telomerase enzyme is ribonucleoprotein reverse transcriptase enzyme that adds telomeric repeats into end of chromosomes [28]. This activity protects the integrity of chromosomes from digestion by exonucleases, fusion to the neighboring chromosomes and occurring the chromosomal defects [29,30]. Possible relation between carcinogenesis and telomere dysfunctions was reported [31,32]. Previous studies cataloging TERT expression and telomerase activity reported potent suppression in human somatic tissues, while robust expression and activity in germ cells and cancer cells. Progressive telomere shortening from cell division provides a barrier for tumor progression. However, one of the hallmarks of advanced malignancies is continuous cell growth and this almost

universally correlates with the reactivation of telomerase [32]. Many authors suggested that determination of telomerase activity may serve as a diagnostic and prognostic tool in oncology. The same results were also observed in tumor cells in animals [33,34]. Telomerase activity was also determined in malignancies of feline tissues and in canine mammary tumors. Results showed that measurement of telomerase activity may be an effective method for detecting malignancy in animals [34,35]. Because it is a viral and tumoral disease, in this study telomerase activity of ENA examined by immunohistochemically method in tissue and by ELISA in serum. Marked increases in serum and tissue telomerase activity observed in both serum and tissue.

Recent studies reported that telomerase activation has an important role in normal somatic cells, and that failure to activate sufficient telomerase also promotes disease. Similarly, some viruses modulate the telomerase activity in animal cells [36,37]. Result of the present study showed that telomerase activity is effected by the disease. This results also indicated that telomerase activity has an important role in ENA. Detection of the telomerase activity in tissue by immunohistochemical methods is recent and reliable method in human tumors. Telomerase expressed in cytoplasm and nucleus of the tumoral cells [38-40]. In this study we used this technique in ENA and our results showed that telomerase expression in tumor tissue was increased compared to normal nasal tissue. The expression was observed both intracytoplasmic and intranuclear localisations.

Effect of virus on telomerase activity in animals previously was reported [36,37] but there is no report about ENA virus and telomerase activity in goats. This study showed that ENA virus up-regulate telomerase activity of infected cells and serum level. Understanding the mechanisms by which ENA virus exerts this further studies are needed.

In recent times, the incidence of ENA has dramatically increased throughout the word. Thus, the prediction of tumor development may require a decrease in the economic damage incurred. Our results showed that telomerase activity becomes a useful prediction marker for tumor development in infected but clinically healthy goats in infected flocks. In this study we have shown that telomerase activity increase in serum and tumoral tissue in ENA cases in goats.

Conflicts of Interest

The authors declare no conflict of interest

REFERENCES

1. Al-Ajmi N, Saretzki G, Miles C, Spyridopoulos I: Dietary restriction ameliorates haematopoietic ageing independent of telomerase, whilst lack of telomerase and short telomeres exacerbate the ageing phenotype. *Exp Gerontol*, 58, 113-119, 2014. DOI: 10.1016/j.exger.2014.07.010

2. Richardson RB, Allan DS, Le Y: Greater organ involution in highly proliferative tissues associated with the early onset and acceleration of ageing in humans. *Exp Gerontol,* 55, 80-91, 2014. DOI: 10.1016/j.exger.2014.03.015

3. Chiu CP, Harley CB: Replicative senescence and cell immortality: The role of telomeres and telomerase. *Proc Soc Exp Biol Med,* 214, 99-106, 1997.

4. Collins K, Mitchell JR: Telomerase in the human organism. *Oncogene*, 21, 564-579, 2002. DOI: 10.1038/sj.onc.1205083

5. Dikmen G, Doğan P: Telomeraz ve Kanser. *T Klin Tıp Bilimleri,* 23, 334-341, 2003.

6. Yıldız MG, Aras S, Duman DC: Telomerlerin yaşlanma ve kanser ilişkisindeki rolü. *Türk Hij Den Biyol Derg,* 66, 187-195, 2009.

7. Kim NW: Clinical implication of telomerase in cancer. *Eur J Cancer*, 33, 781-786, 1997. DOI: 10.1016/S0959-8049(97)00057-9

8. Bachor C, Bachor OA, Boukamp P: Telomerase is active in normal gastrointestinal mucosa and not up-regulated in precancerous lesions. *J Cancer Res Clin Oncol,* 125, 453-460, 1999. DOI: 10.1007/s004320050302

9. Chadeneau C, Hay K, Hirte HW, Gallinger S, Bacchetti S: Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Res*, 55, 2533-2536, 1995.

10. Dikmen GZ, Dikmen E, Doğan P: Kanserde telomeraza yönelik tedavi stratejileri. *Hacettepe Tıp Der,* 37, 49-55, 2006.

11. Shay JW, Wright WE: Role of telomeres and telomerase in cancer. *Semin Cancer Biol,* 21, 349-353, 2011. DOI: 10.1016/j.semcancer.2011.10.001

12. Yang J, Deng X, Deng L, Gu H, Fan W, Cao Y: Telomerase activation by Epstein-Barr virus latent membrane protein 1 is associated with c-Myc expression in human nasopharyngeal epithelial cells. *J Exp Clin Cancer Res,* 23, 495-506, 2004.

13. Verma SC, Borah S, Robertson ES: Latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus upregulates transcription of human telomerase reverse transcriptase promoter through interaction with transcription factor Sp1. *J Virol,* 78, 10348-10359, 2004. DOI: 10.1128/JVI.78.19.10348-10359.2004

14. Djeraba-AitLounis A, Soubieux D, Klapper W, Rasschaert D: Induction of telomerase activity in avian lymphoblastoid cell line transformed by Marek's disease virus, MDCC-MSB1. *Vet Pathol,* 41, 405-407, 2004. DOI: 10.1354/vp.41-4-405

15. Yang CT, Song J, Bu X, Cong YS, Bacchetti S, Rennie P, Jia WW: Herpes simplex virus type-1 infection upregulates cellular promoters and telomerase activity in both tumor and nontumor human cells. *Gene Ther,* 10, 1494-1502, 2003. DOI: 10.1038/sj.gt.3302005

16. Franzese O, Comandini A, Adamo R, Sgadari C, Ensoli B, Bonmassar E: HIV-Tat down-regulates telomerase activity in the nucleus of human CD4+ T cells. *Cell Death Differ,* 11, 782-784, 2004. DOI: 10.1038/sj.cdd.4401346

17. Chen F, Liu C, Lindvall C, Xu D, Ernberg I: Epstein-Barr virus latent membrane 2A (LMP2A) down-regulates telomerase reverse transcriptase (hTERT) in epithelial cell lines. *Int J Cancer,* 113, 284-289, 2005. DOI: 10.1002/ijc.20594

18. Fan XG, Huang Y, Tang FQ, Yi H: Telomerase activity of peripheral blood lymphocytes in patients with chronic hepatitis B. *Immunol Lett,* 73, 7-11, 2000. DOI: 10.1016/S0165-2478(00)00187-5

19. Leroux C, Cruz JC, Mornex JF: SRLVs: A genetic continuum of lentiviral species in sheep and goats with cumulative evidence of cross species transmission. *Curr HIV Res,* 8, 94-100, 2010. DOI: 10.2174/157016210790416415

20. Leroux C, Mornex JF: Retroviral infections in sheep and the associated diseases. *Small Ruminant Res,* 76, 68-76, 2008. DOI: 10.1016/J.SMALLRUMRES.2007.12.010

21. De las Heras M, Ortin A, Cousens C, Minguijon E, Sharp JM: Enzootic nasal adenocarcinoma of sheep and goats. *Curr Top Microbiol Immunol,* 275, 201-223, 2003.

22. Caswell J, Williams K: Enzootic nasal tumour of sheep. **In**, Maxie M (Ed): Jubb, Kennedy & Palmer's Pathology of Domestic Animals. 5th ed.,

640-643, Philadelphia, Elsevier Saunders, 2007.

23. Walsh SR, Linnerth-Petrik NM, Laporte AN, Menzies PI, Foster RA, Wootton SK: Full-length genome sequence analysis of enzootic nasal tumor virus reveals an unusually high degree of genetic stability. *Virus Res,* 151, 74-87, 2010. DOI: 10.1016/j.virusres.2010.04.002

24. McKinnon AO, Thorsen J, Hayes MA, Misener CR: Enzootic nasal adenocarcinoma of sheep in Canada. *Can Vet J,* 23, 88-94, 1982.

25. Ozmen O, Sahinduran S, Haligur M, Demir N: Clinical, pathological, immunohistochemical and ultrastructural observations on enzootic nasal adenocarcinoma in five goats. *Kafkas Univ Vet Fak Derg,* 16, 633-639, 2010. DOI: 10.9775/kvfd.2009.1340

26. Shay JW, Wright WE: Hallmarks of telomeres in ageing research. *J Pathol,* 211, 114-123, 2007. DOI: 10.1002/path.2090

27. Gorbunova V, Seluanov A: Telomerase as a growth-promoting factor. *Cell Cycle,* 2, 534-537, 2003. DOI: 10.4161/cc.2.6.515

28. Blackburn EH: Structure and function of telomeres. *Nature,* 350, 569-573, 1991. DOI: 10.1038/350569a0

29. Nasir L, Devlin P, Mckevitt T, Rutteman G, Argyle DJ: Telomere lengths and telomerase activity in dog tissues: A potential model system to study human telomere and telomerase biology. *Neoplasia,* 3, 351-359, 2001. DOI: 10.1038/sj.neo.7900173

30. Artandi SE, DePinho RA: Telomeres and telomerase in cancer. *Carcinogenesis,* 31, 9-18, 2010. DOI: 10.1093/carcin/bgp268

31. Kim NW, Pietyszek MA, Prowse KR, Harley CB, West MD, Ho PLC, Coviello GM, Wright WE, Weinrich SL, Shay JW: Specific association of human telomerase activity with immortal cells and cancer. *Science,* 266, 2011-2015, 1994. DOI: 10.1126/science.7605428

32. Argyle DJ, Nasir L: Telomerase: A potential diagnosis and therapeutic tool in canine oncology. *Vet Pathol,* 40, 1-7, 2003. DOI: 10.1354/vp.40-1-1

33. Varon D, Jiang C, Hedican C, Dome JS, Umbricht CB, Carey LA, Thompson HJ, Sukumar S: Telomerase activity in the normal and neoplastic rat mammary gland. *Cancer Res,* 57, 5605-5609, 1997.

34. Cadile CD, Kitchell BE, Biller BJ, Hetler ER, Balkin RG: Telomerase activity as marker for malignancy in feline tissues. *Am J Vet Res*, 62, 1578-1581, 2001. DOI: 10.2460/ajvr.2001.62.1578

35. Yazawa M, Okuda M, Setoguchi A, Iwabuchi S, Nishimura R, Sasaki N, Masuda K, Ohno K, Tsujimoto H: Telomere length and telomerase activity in canine mammary gland tumors. *Am J Vet Res,* 62, 1539-1543, 2001. DOI: 10.2460/ajvr.2001.62.1539

36. Pagnini U, De Martino L, Montagnaro S, Diodato A, Longo M, Pacelli F, Pisanelli G, Iovane G: Bovine herpesvirus type 1 (BHV-1) up-regulates telomerase activity in MDBK cells. *Vet Microbiol,* 113, 231-236, 2006. DOI: 10.1016/j.vetmic.2005.11.006

37. Suzuki K, Shuto S, Miura Y, Sentsui H: Measurement of telomerase activity in bovine leukaemia virus infected cows. *Vet Microbiol,* 127, 142-146, 2008. DOI: 10.1016/j.vetmic.2007.08.016

38. Palani J, Lakshminarayanan V, Kannan R: Immunohistochemical detection of human telomerase reverse transcriptase in oral cancer and pre-cancer. *Indian J Dent Res,* 22, 362-363, 2011. DOI: 10.4103/0970-9290.84281

39. Raghunandan BN, Sanjai K, Kumaraswamy J, Papaiah L, Pandey B, Jyothi BM: Expression of human telomerase reverse transcriptase protein in oral epithelial dysplasia and oral squamous cell carcinoma: An immunohistochemical study. *J Oral Maxillofac Pathol*, 20, 96-101, 2016. DOI: 10.4103/0973-029X.180953

40. Hiyama E, Hiyama K, Yokoyama T, Shay JW: Immunohistochemical detection of telomerase (hTERT) protein in human cancer tissues and a subset of cells in normal tissues. *Neoplasia*, 3, 17-26, 2001. DOI: 10.1038/sj.neo.7900134

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Research Article

Investigation of the Calculus Microbiome in Canines and Felines Using Next-Generation Sequencing

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Abstract

The oral cavity of dogs and cats is colonized by hundreds of bacterial species. Here, we describe the bacterial composition in the dental calculus of dogs and cats. Dental calculus samples from 43 dogs and 4 cats were pooled into four different groups. Dogs were categorized into three groups: non-small breed dogs (NSB), non-brachycephalic small breed dogs (SB) and brachycephalic small breed dogs (SBb). The fourth group included cats. Bacterial communities were identified based on 16S rRNA sequencing (V3 and V4 hypervariable regions) with the Illumina platform. The numbers of operational taxonomic units (OTUs) identified in the three groups of dogs were 180, 190 and 150 and in NSB, SBb and SB, respectively, while in cats there were 111 OTUs. In dental calculus from both dogs and cats, the phylum Firmicutes had the highest proportion of read number, especially the class Clostridia. PCoA and UPGMA analysis revealed differences in the microbiomes of canine and feline calculus. Our findings demonstrated that the bacterial communities in calculus seemed to differ from those in other sites of the oral cavity. Calculus may serve as a potential habitßat for the growth of bacteria linked to canine and feline periodontal disease.

Keywords: *Bacteria, Cat, Dog, 16S rRNA analysis, Dental calculus*

Köpek ve Kedi Diş Taşı Mikrobiyomunun İleri Jenerasyon Sekanslama Kullanılarak Araştırılması

Öz

Köpek ve kedilerin ağız boşluğu yüzlerce bakteri türü tarafından kolonize edilmiştir. Bu çalışmada; köpek ve kedilerin diş taşlarının bakteriyal kompozisyonu tanımlanmıştır. 43 köpek ve 4 kediye ait diş taşı örnekleri dört faklı grupta toplandı. Köpekler üç grupta kategorize edildi: küçük olmayan ırk köpekler, brakisefalik olmayan küçük ırk köpekler ve brakisefalik küçük ırk köpekler. Dördüncü grup ise kedileri içermekteydi. Bakteriler Illumina platform kullanılarak 16S rRNA sekanslama (V3 ve V4 çokdeğişken bölgeler) temeline göre belirlendi. Üç grup köpekte operasyonel taksonomik birimlerin sayısı küçük olmayan ırk köpekler, brakisefalik küçük ırk köpekler ve brakisefalik olmayan küçük ırk köpekler için sırasıyla 180, 190 ve 150 olarak tespit edilirken kedilerde 111 olarak belirlendi. Hem köpek hem de kedi diş taşlarında, Firmicutes filumu, özellikle de Clostridia sınıfı, en fazla okuma sayısına sahipti. PCoA ve UPGMA analizi köpek ve kedi diş taşlarının mikrobiyomu arasında farklılık olduğunu gösterdi. Elde edilen sonuçlar diş taşlarındaki bakteriyal topluluklarının ağız boşluğunun diğer taraflarından belirlenenlerden farklı olduğunu gösterdi. Diş taşları köpek ve kedilerde periodontal hastalıklar ile ilişkili bakterilerin büyümesi için uygun bir ortam oluşturabilir.

Anahtar sözcükler: *Bakteri, Kedi, Köpek, 16S rRNA analizi, Diş taşı*

INTRODUCTION

The oral microbiome is closely associated with many diseases, both oral and systemic. Researchers in worldwide have reported that the oral microbiome is associated with periodontal disease, which is prevalent in dogs [1,2] and

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cats [3], as well as humans [4]. Periodontal disease not only causes localized disease but also affects organs in other systems, e.g. the cardiovascular [5], renal and respiratory systems [6]. There is evidence that periodontal disease is related to histological changes in the heart and other internal organs in dogs [7]. A study in 2016 [8] found interconnections between periodontal disease and the pathogenesis of coronary heart disease (CHD), the greatest cause of death in humans worldwide. Moreover, periodontal disease is also related to chronic kidney disease (CKD), as increasing severity of periodontal disease is significantly associated with increasing blood urea nitrogen and serum creatinine concentration [9].

The formation of dental calculus, or calcified dental plaque, is always preceded by plaque formation. Plaque accumulations serve as the organic matrix for subsequent mineralization of the deposit [10]. Initially, small crystals appear in the intermicrobial matrix in close apposition to bacteria. Gradually, the matrix between the microorganisms becomes calcified and then the bacteria become mineralized [10]. Coignoul and Chevilie [11] studied the histological structure of canine dental calculus using transmission electron microscopy. Intact bacterial populations were found to constitute the superficial layers. Central zones consisted of masses of minerals, mucosubstances, and cellular and bacterial debris. Deep layers, adjacent to tooth surfaces, were dominated by leukocytes, desquamated epithelial cells, and intact bacteria.

Dental calculus is calcified dental plaque covered by an unmineralized bacterial layer. Supragingival calculus formation is common to tooth surfaces adjacent to the salivary duct opening, while subgingival calculus is distributed randomly around the mouth. Dental calculus is considered to be an etiologic factor in the initiation and progression of periodontal disease. Due to its porous structure, it can absorb substances from saliva, gingival exudates and bacterial endotoxins that damage the periodontal tissue. Dental calculus is associated with gingival recession and localized attachment loss. Moreover, it affects pocket bleeding status and also expands the radius of damage associated with plaque.

Oral microbiomes have been investigated increasingly for various reasons: for example, to characterize the features of the bacterial community for different conditions. Previous studies have reported on the bacterial microbiome of biofilms in dogs [12] and cats [13]. To date, reports on the bacteria in dental calculus in animals and humans are limited due to the technical limitations of collecting bacteria from calculus. In a 1984 study [11], bacterial cultures of ground calculus material revealed large numbers of streptococci and actinomycetes. Other bacteria commonly present include *Acinetobacter calcoaceticus*, *Corynebacterium xerosis*, *Eikenella corrodens*, *Moraxella* spp., *Pseudomonas* spp. and *Staphylococcus* spp.

Nowadays, new technology has provided an opportunity for study which was impossible in the past. The analysis of 16S rRNA using next-generation sequencing (NGS) is a technique whose main objective is to determine the microbial population that can be found in a particular environment, studied in the context of its community [12,14-16]. Here we identified a broad range of bacteria in samples by a DNA-based method. The purpose of this study was to determine the bacterial population in dental calculus in dogs and cats. The results of this study contribute to knowledge on bacterial communities. Moreover, the presence of certain bacteria in dental calculus in dogs and cats might be related to dental calculus formation.

MATERIAL and METHODS

Sample Collection

Dental calculus was collected from 43 dogs and 4 cats *(Table 1)*. Dogs were categorized into three groups: non-small breed dogs (NSB), n=8; brachycephalic small breed dogs (SBb), n=5; and non-brachycephalic small breed dogs (SB), n=30. In this study, the dogs was assigned into three groups in relation to the breed's size because the previous studies have reported the small breeds had more prevalence of calculus formation compared to large breeds [2,17,18]. Brachycephalic breeds has been reported to be vulnerable to developing the advanced stages of the disease [2]. These animals were referred to the dental unit, Small Animal Teaching Hospital, Faculty of Veterinary Medicine, Chiang Mai University, for professional dental cleaning. This study was approved by the Animal Use Committee of the Faculty of Veterinary Medicine, Chiang Mai University, Thailand, in 2016 (R23/2559). All methods were performed in accordance with the relevant guidelines and regulations.

Collection of dental calculus samples was performed under general anesthesia using a standard protocol. Oral cleaning with chlorhexidine gluconate 0.12% (Virbac, Fort Worth, TX, USA) was performed. The surface of calculus was polished to remove biofilm mechanically; dental calculus was then removed with sterilized tartar removing forceps and an ultrasonic scaler (iM3, New South Wales, Australia). Dental calculus samples were washed five times in sterile saline and then placed into individual 1.5 mL tubes containing sterile saline. All samples were kept at -20°C until the DNA extraction process.

Decalcification and DNA Extraction Process

Calculus samples were immediately washed five times with sterile saline at the time of collection and then exposed to UV irradiation for 2 min to eliminate surface bacteria.

For the decalcification process, dental calculus samples were placed in individual 1.5 mL tubes containing sterile saline with 500 µL of 0.5 M EDTA, then ground with a

Table 1. *Animal information in each group*

No.	Age (years)	Sex	Breed	Weight (kg)
Non-small breed dogs (NSB)				
1	15	M	Thai Bangkaew	13.7
2	6	F	Beagle	15.0
3	3	F	Cocker Spaniel	15.0
4	12	M	Golden Retriever	30.0
5	9	M	Siberian Husky	25.0
6	10	F	Mongrel	14.0
7	5	F	Mongrel	18.0
8	9	M	Mongrel	26.0
mean±sd	8.6±3.9			19.6±6.4
Brachycephalic small breed dogs (SBb)				
1	10	F	Shih Tzu	5.6
2	9	M	Shih Tzu	7.1
3	11	M	Shih Tzu	4.5
4	10	M	Shih Tzu	4.2
5	11	M	Shih Tzu	8.0
mean±sd	10.2±0.8			5.9±1.6
Non-brachycephalic small breed dogs (SB)				
1	8	F	Pomeranian	2.0
2	4	M	Pomeranian	2.0
3	7	M	Pomeranian	9.5
4	7	M	Pomeranian	3.3
5	6	M	Pomeranian	4.0
6	5	F	Pomeranian	5.5
7	15	M	Pomeranian	5.5
8	5	M	Pomeranian	2.9
9	7	M	Pomeranian	2.2
10	9	F	Pomeranian	5.0
11	11	M	Poodle	5.2
12	6	M	Poodle	2.1
13	12	F	Poodle	2.1
14	12	F	Poodle	4.6
15	9	M	Poodle	4.8
16	6	M	Poodle	7.8
17	6	M	Poodle	3.9
18	11	F	Poodle	5.0
19	10	M	Poodle	4.5
20	6	F	Poodle	6.3
21	11	F	Poodle	7.8
22	8	F	Poodle	4.8
23	5	M	Chihuahua	3.6
24	10	F	Chihuahua	2.4
25	7	M	Yorkshire Terrier	2.8
26	11	M	Jack Russell	10.6
27	16	M	Mongrel	5.5
28	15	M	Mongrel	6.9
29	5	F	Mongrel	4.0
30	11	M	Mongrel	4.7
mean±sd	8.2±3.0			4.3±2.0
Cats				
1	4	M	Mongrel	5.0
2	7	F	Mongrel	4.4
3	5	F	Mongrel	3.6
4	2	F	Mongrel	2.9
mean±sd	4.5±2.1			4.0±0.9

micropestle until the calculus turned to power. Next, 10% SDS (Vivantis, Selangor, Malaysia) and proteinase K (20 mg/mL) (Vivantis) were added, followed by overnight lysis at 55°C. Genomic DNA in dental calculus was extracted using a RealGenomics DNA extraction kit (RBC Bioscience, New Taipei City, Taiwan). DNA samples were quantified using a NanoDrop spectrophotometer (BioDrop, Cambridge, UK).

Next-generation Sequencing and 16S rRNA Analysis

Extracted DNA from each sample in the same quantity as 100 ng was pooled into four different groups-NSB, SBb, SB and cats given as a final concentration of 20, 20, 30 and 5 ng/μL respectively. Subsequently, the pooled DNA of 10 ng of each group were used as template for 16S rRNA amplication.

Amplification of 16S rRNA

Bacterial communities were barcoded and identified based on ribosomal RNA (16S rRNA) sequencing. The sequencing libraries were prepared according to the 16S rRNA Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA) to amplify the V3 and V4 hypervariable regions. DNA concentration was measured with PicoGreen reagent and input gDNA (10 ng) was amplified by polymerase chain reaction (PCR). The barcoded fusion primer sequences used for amplification were as follows:

V3-F:5′-CGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′

V4-R:5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′

Library Preparation

The final purified product was then quantified by real-time PCR (qPCR) according to the technical guidelines for KAPA Library Quantification Kits for Illumina platforms (KAPA Biosystems, Boston, MA, USA) and qualified using a TapeStation system and Genomic DNA ScreenTape assay (Agilent Technologies, Waldbronn, Germany). Paired-end sequencing (2 × 300 bp) was performed by Macrogen on the MiSeq platform (Illumina).

Sequence Process and Analysis

The short reads of the four pooled calculus samples (NSB, SBb, SB and cats) obtained from the MiSeq platform were assembled using fast length adjustment of short reads (FLASH) [19], after which the poor quality reads were filtered out. The filtered reads were denoised and clustered at 100% identity using the CD-HIT-OTU clustering program [20]. The remaining representative reads after removing the identified chimeric reads were clustered into operational taxonomic units (OTUs) using a greedy algorithm with a cutoff of >97% identity at the species level. In this study, OTUs were given the code name "denovo", ranging from denovo0 to denovo205.

A multiple sequence alignment of total OTUs was performed in MEGA 7.0 and then converted into a NEXUS file with an online conversion tool (http://www.bugaco.com). The NEXUS file was used to acquire the best model of DNA sequence evolution of 16S rRNA (V3 and V4 regions) in MrModeltest v2.3 [21]. The appropriate model of nucleotide substitution, TVM+I+G, was selected to generate phylogenetic trees using Bayesian inference (BI) implemented in MrBayes 3.1.2 [22]. For BI, two independent searches were performed with random starting trees for 200.000 generations while sampling over 1,000 generations and compared using four Markov chain Monte Carlo chains (temp = 0.2). The log-likelihood scores were used to plot the convergence in Tracer v1.6 [23] and a consensus tree was generated after removing the first 25% of the generations from each run. Maximum likelihood analysis was carried out with RAxML 7.0.4 [24] using the TVM+I+G model of nucleotide substitution, the same as for BI analysis, with 1,000 bootstrap replicates.

Statistical Analysis

The number of OTUs and their relative abundance were used to obtain the taxonomic composition at the phylum level and the rarefaction curve (QIIME script: alpha_rarefaction.py), and for principal coordinates analysis (PCoA; QIIME script: make_2d_plots.py), unweighted pair-group method with arithmetic mean (UPGMA; QIIME script: upgma_cluster.py), and measures of species diversity (QIIME script: alpha_diversity.py) including Shannon and Simpson indices, Chao1 (species richness) and Good's coverage (using QIIME) [25]. Information from the heatmap of abundance and UPGMA of calculus microbiota of the different hosts was supplemented for all members of each taxon and displayed as a phylogenetic tree using a web-based tool, the Interactive Tree of Life [26] (iTOL). In addition, to determine the host-specific taxa of calculus microbiota, the selective indexes were calculated according to the following formulas:

(i) canine-specific taxa = the average abundance of taxa in dogs/the average abundance of taxa in cats

(ii) feline-specific taxa = the average abundance of taxa in cats/the average abundance of taxa in dogs

RESULTS

Sequence Quality

The four pools of calculus samples, consisting of three groups of dogs (NSB, SBb and SB) and one group of cats, were analyzed by the MiSeq system. A total of 738,408 reads that passed read quality assessment by FLASH were processed by the sequencing provider's initial sequence quality filter (short reads were filtered and long reads were trimmed). The filtered data was clustered with a cutoff at 97% identity, and chimeras and noisy sequences were removed using CD-HIT-OTU. A total of 575,657 reads were removed, consisting of 11,280 low-quality reads, 26,354 chimeric reads and 538,023 others. The final number of sequence reads was reduced to 162,751, with a mean among the four groups of 40,687 reads, including 36,333

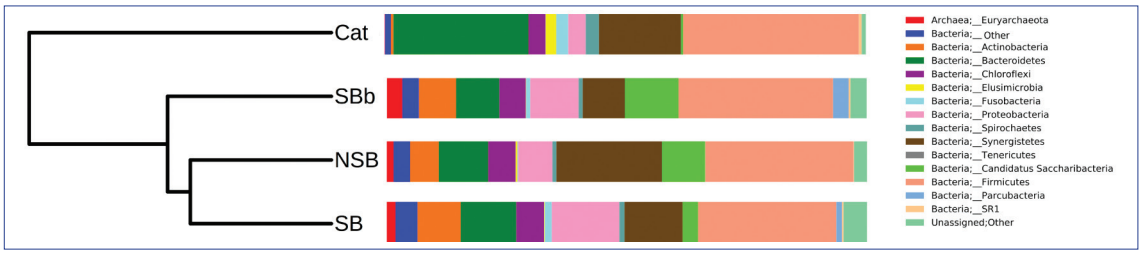

Fig 1. The relative distribution of bacteria at the phylum level in four pooled calculus samples - from cats, brachycephalic small breed dogs (SBb), non-small breed dogs (NSB) and non-brachycephalic small breed dogs (SB) - and their cluster, using UPGMA based on information on bacterial communities

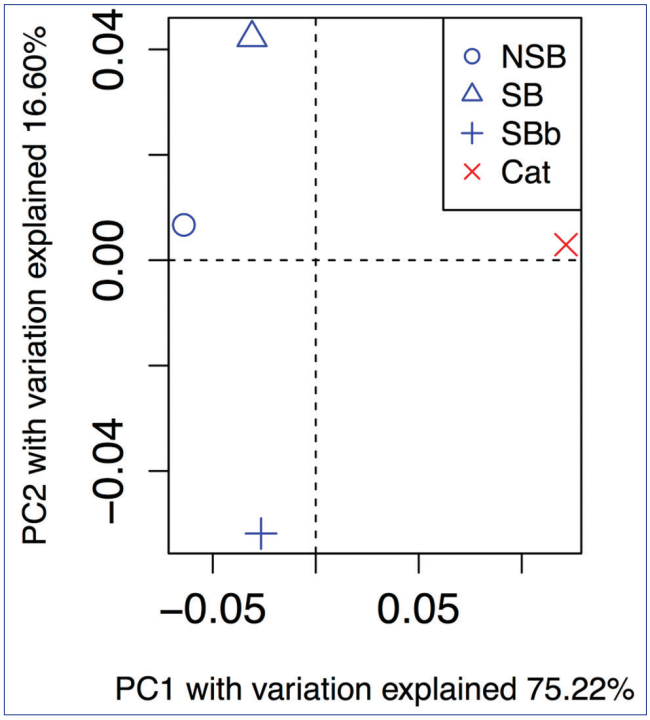

Fig 2. Classification of the microbiome in tartar samples collected from two different species: dogs - including non-small breed dogs (NSB), brachycephalic small breed dogs (SBb) and non-brachycephalic small breed dogs (SB) - and cats (red), using PCoA

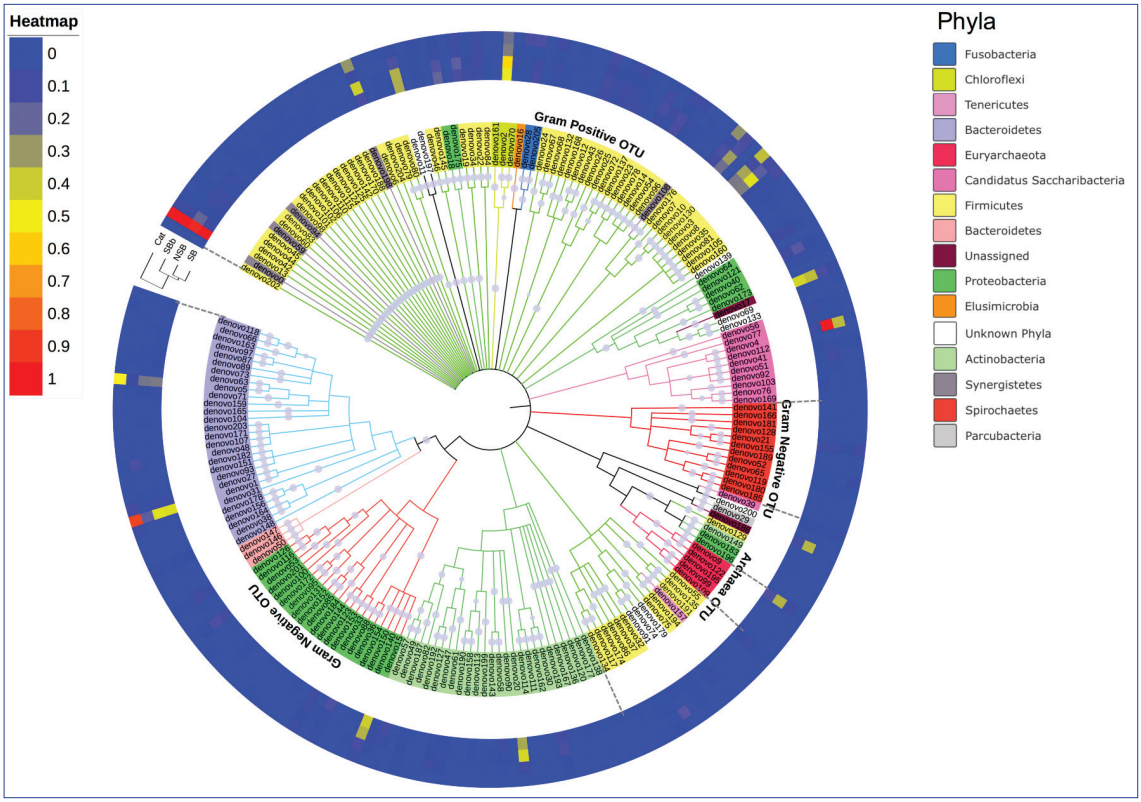

Fig 3. Phylogenetic tree of each OTU observed in calculus samples, with their proportion in a heatmap. The small blue circles represent Bayesian posterior probability >95%. NSB: non-small breed dogs; SBb: brachycephalic small breed dogs; SB: non-brachycephalic small breed dogs

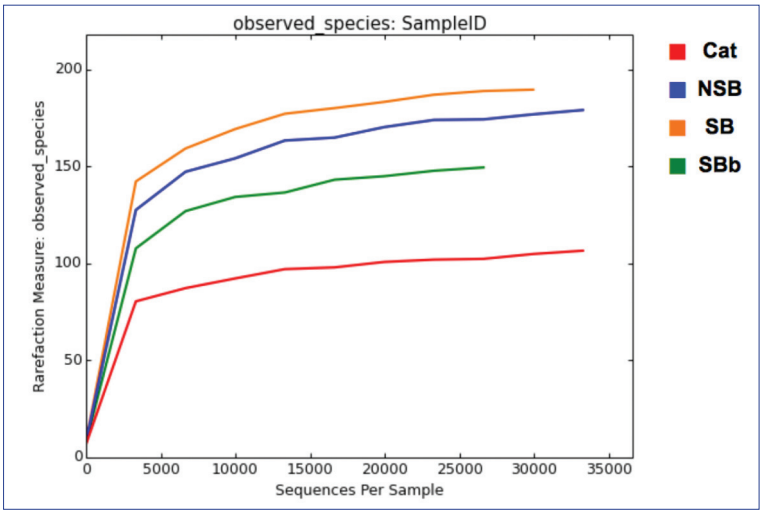

Fig 4. Rarefaction plots of each tartar group, including NSB (non-small breed dogs), SBb (brachycephalic small breed dogs), SB (non-brachycephalic small breed dogs) and cats

(NSB), 28,613 (SBb), 30,169 (SB) and 67,636 reads (cats).

Bacterial Community in Dogs and Cats, Using OTU-based Analysis

The consensus taxonomy was assessed using CD-HIT-OTU and rDnaTools, resulting in 206 unique OTUs. Pooled samples from the NSB, SBb, SB and cat groups showed different numbers of OTUs: 180, 150, 190 and 111 OTUs, respectively *(Fig. 1)*. The relative distribution of bacterial phyla in each sample revealed a significant difference between canine and feline groups, whereas that of intra-species samples (dogs) was similar *(Fig. 1)*. This corresponded to the results of UPGMA *(Fig. 1)* and PCoA *(Fig. 2)* based on the composition of OTUs in each sample, exhibiting two separate groups, i.e. dogs and cats *(Fig. 1* and *Fig. 2)*. In the cat group the phylum Bacteroidetes was the second highest in abundance, yet in the dog groups the second highest was either Synergistetes, Proteobacteria or Candidatus Saccharibacteria (TM7), likely depending on the type of dog *(Fig. 1)*. These

Table 2. *Relative abundance of OTUs at least 75% from different calculus groups, and selective index*[a]

OTU	Phylum	Genus	Relative % of total				Selective index (SI)	
			NSB	SBb	SB	Cat	Dog/Cat	Cat/Dog
denovo0	Synergistetes	*Fretibacterium*	8.51%	21.59%	11.77%	16.61%	0.84	1.19
denovo4	Candidatus Saccharibacteria	Saccharibacteria genera incertae sedis	9.02%	8.03%	0.66%	0.00%	1,331.87	0.00
denovo2	Chloroflexi	Uncultured Anaerolineae	5.15%	5.31%	5.63%	3.58%	1.50	0.67
denovo8	Firmicutes	*Parvimonas*	4.02%	4.03%	2.89%	1.76%	2.07	0.48
denovo1	Bacteroidetes	*Tannerella*	4.00%	4.02%	5.09%	14.59%	0.30	3.34
denovo32	Firmicutes	*Schwartzia*	0.01%	3.72%	0.35%	0.00%	NA	0.00
denovo11	Unclassified (WS6)	Unclassified	3.13%	3.25%	4.06%	0.79%	4.43	0.23
denovo10	Firmicutes	Uncult. Clostridiales	2.80%	3.15%	2.58%	2.17%	1.31	0.77
denovo48	Bacteroidetes	*Bacteroides*	0.00%	2.96%	0.03%	0.21%	4.83	0.21
denovo15	Proteobacteria	*Brachymonas*	3.18%	2.82%	4.78%	0.64%	5.61	0.18
denovo23	Firmicutes	*Peptostreptococcus*	1.13%	2.47%	0.68%	0.35%	4.10	0.24
denovo3	Firmicutes	*Peptostreptococcus*	2.97%	2.21%	2.82%	6.25%	0.43	2.34
denovo20	Actinobacteria	*Corynebacterium*	3.81%	2.09%	3.92%	0.00%	2,215.29	0.00
denovo22	Firmicutes	*Peptostreptococcus*	0.79%	1.93%	0.75%	1.22%	0.95	1.06
denovo5	Bacteroidetes	*Petrimonas*	2.09%	1.72%	2.87%	8.65%	0.26	3.89
denovo6	Firmicutes	Uncult. Clostridiales	3.69%	1.67%	1.58%	4.94%	0.47	2.13
denovo13	Firmicutes	*Anaerovorax*	1.81%	1.52%	0.91%	0.99%	1.43	0.70
denovo19	Firmicutes	Peptostreptococcaceae (Family)	1.69%	1.46%	1.61%	0.95%	1.67	0.60
denovo47	Actinobacteria	*Actinomyces*	0.27%	1.46%	0.20%	0.00%	NA	0.00
denovo9	Euryarchaeota	*Methanobrevibacter*	3.17%	1.44%	1.77%	0.11%	18.69	0.05
denovo12	Firmicutes	*Saccharofermentans*	1.11%	1.43%	1.14%	1.88%	0.65	1.53
denovo7	Firmicutes	Uncult. Clostridiales	1.22%	1.10%	1.30%	4.79%	0.25	3.97
denovo18	Proteobacteria	*Campylobacter*	1.48%	0.78%	2.36%	0.46%	3.35	0.30
denovo61	Actinobacteria	*Actinomyces*	0.44%	0.71%	0.31%	0.07%	6.85	0.15
denovo30	Actinobacteria	*Euzebya*	1.26%	0.70%	1.24%	0.15%	7.28	0.14
denovo26	Firmicutes	Lachnospiraceae (Family)	0.73%	0.70%	0.78%	0.87%	0.85	1.18
denovo43	Firmicutes	*Saccharofermentans*	0.58%	0.67%	0.62%	0.00%	NA	0.00
denovo53	Proteobacteria	*Suttonella*	1.35%	0.67%	1.20%	0.05%	21.35	0.05
denovo33	Proteobacteria	*Propionivibrio*	0.53%	0.62%	0.96%	1.01%	0.70	1.43
denovo36	Proteobacteria	*Xenophilus*	0.39%	0.58%	1.77%	0.66%	1.38	0.73
denovo40	Proteobacteria	*Desulfovibrio*	1.56%	0.58%	1.17%	0.13%	8.65	0.12
denovo21	Spirochaetes	*Treponema*	0.51%	0.57%	0.66%	1.66%	0.35	2.85
denovo25	Firmicutes	Uncult. Lachnospiraceae	0.50%	0.57%	0.73%	1.33%	0.45	2.21
denovo14	Firmicutes	*Acetoanaerobium*	1.24%	0.57%	1.58%	1.47%	0.77	1.30
denovo73	Bacteroidetes	*Porphyromonas*	0.08%	0.51%	0.47%	0.03%	12.67	0.08
denovo70	Chloroflexi	Uncult. Anaerolineae	0.36%	0.42%	0.13%	0.00%	NA	0.00
denovo34	Firmicutes	*Fusibacter*	0.40%	0.41%	0.85%	0.46%	1.21	0.83
denovo72	Firmicutes	Uncult. Lachnospiraceae	0.31%	0.41%	0.75%	0.26%	1.88	0.53
denovo64	Proteobacteria	*Desulfobulbus*	0.63%	0.40%	0.84%	0.08%	7.98	0.13
denovo57	Actinobacteria	*Actinomyces*	0.46%	0.35%	0.35%	0.01%	32.53	0.03
denovo16	Elusimicrobia	*Atopobium* sp.	0.07%	0.35%	0.15%	2.20%	0.09	11.60
denovo77	Candidatus Saccharibacteria	Saccharibacteria genera incertae sedis	0.06%	0.34%	0.19%	0.00%	NA	0.00
denovo35	Firmicutes	Peptostreptococcaceae bacterium	0.32%	0.29%	0.61%	2.01%	0.20	4.98

[a] *Selective index represents the ratio of relative abundance of dogs to cats or cats to dogs, indicating the species-specific OTUs;* ***NA:*** *not available;* ***NSB:*** *non-small breed dogs; SBb: brachycephalic small breed dogs; SB: non-brachycephalic small breed dogs*

Table 3. *Species richness, diversity indices and coverage used*

Sample Name	OTUs	Richness	Shannon	Simpson	Good's Coverage
NSB	180	187	5.5813	0.9655	0.9996
SBb	150	159.75	4.9925	0.9306	0.9995
SB	190	195.5	5.7011	0.9648	0.9996
Cat	111	114	4.7005	0.9286	0.9999
Mean	157.75	164.0625	5.2439	0.9474	0.9997

NSB: *non-small breed dogs; SBb: brachycephalic small breed dogs;* ***SB:*** *non-brachycephalic small breed dogs*

results may indicate microbiome divergence among species.

In this study, the microbiome of tartar samples embraced 14 phyla among the 206 OTUs, with seven phyla showing a relative sequence abundance greater than 5%: Firmicutes (32.1%), Synergistetes (15.0%), Bacteroidetes (14.7%), Proteobacteria (8.8%), Candidatus Saccharibacteria (5.9%), Actinobacteria (5.8%) and Chloroflexi (5.2%). The other phyla had a relative abundance of less than 5%: Euryarchaeota (1.7%), Spirochaetes (1.4%), Fusobacteria (1.2%), Parcubacteria (1.1%), Elusimicrobia (0.7%), SR1 (0.4%) and Tenericutes (0.01%) *(Fig. 1)*. Approximately 6.1% of sequences were unable to be classified. When considering bacteria at the genus level, it was noted that denovo0, similar to *Fretibacterium* sp. with 99% genus identity and belonging to Synergistetes, showed the highest proportion, with a mean of 15% in all sample groups except NSB in which it was the second highest *(Fig. 3)*. Although in this study the phylum Firmicutes was treated as having the largest number of members in the bacterial community of calculus, the genus level in this phylum showed a relative abundance equal to the second highest number of members, accounting for 11%. The major members of the phylum Firmicutes, observed in high abundance, were the class Clostridia. Interestingly, we found the presence of archaea (phylum Euryarchaeota) in both canine and feline calculus. Most members of this phylum were found to be the genus *Methanobrevibacter* (denovo9), with an average of 1.6%; the highest proportion was observed in NSB (3.17%), followed by SB (1.77%), SBb (1.44%) and cats (0.11%).

Phylogenetic analyses using Bayesian inference showed the clades of bacteria species, most of which were related to their phyla. However, the phyla Firmicutes and Proteobacteria appeared to possess high complexity, leading to the existence of two clades, as shown in *Fig. 3*. Furthermore, the clade of archaea exhibited a closer relatedness to Gram-negative bacteria.

Species-specific OTUs

The species-specific OTUs *(Table 2)* were investigated using selective indexes (SI) and the ratio of the relative abundance of dogs to cats as well as cats to dogs. It was evident that two OTUs, denovo4 and denovo20, exhibited the highest SI for canine-specific OTUs, 1,331.87 and 2.215.29, respectively. Denovo4 was a member of the TM7 phylum, whose most abundant sequence reads were observed in NSB, with 9.02%, followed by SBb (8.03%), SB (0.66%) and cats (0.00%). Denovo20, with SI of 2.215.29, was putative *Corynebacterium canis* with 99% identity in the phylum Actinobacteria for which the highest relative abundance was observed in SB (3.92%), followed by NSB (3.81%), SBb (2.09%) and cats (0.00%). Besides these, other OTUs having moderate selective indexes (10 <SI <1.000) were denovo57 (phylum Actinobacteria: *Actinomyces cardiffensis* with 98% identity, SI = 32.53), denovo53 (phylum Proteobacteria: *Cardiobacterium* sp. with 99% identity, SI = 21.35) and denovo9 (phylum Euryarchaeota: *Methanobrevibacter oralis* with 99% identity, SI = 18.69). In cats, five OTUs, including denovo1, denovo3, denovo7, denovo16 and denovo35, showed SI greater than 3. The highest SI (11.60) was noted in denovo16, which was assigned to be *Atopobium* sp., followed by denovo35 (SI = 4.98, a Peptostreptococcaceae bacterium with 99% identity), denovo7 (SI = 3.97, a Clostridiales bacterium with 100% identity), denovo5 (SI = 3.89, *Petrimonas* sp. with 99% identity) and denovo1 (SI = 3.34, *Tannerella forsythia* with 100% identity).

Diversity Analysis

The species richness, diversity indices and coverage are shown in *Table 3*. There was an initial steep increase in OTU identification, which appeared to flatten after approximately 10,000 sequence reads *(Fig. 4)*. Differences of species richness in each group (NSB, SBb, SB and cats) were observed, given as 187, 159.75, 195.5 and 114, respectively, which were close to the actual number of OTUs observed in each group. Shannon (mean, range: 5.24, 4.70-5.70) and Simpson indices (mean, range: 0.95, 0.92-0.96) were used as indicators for the level of microbiota diversity in calculus as a result of a little variability among the samples, in addition to Good's coverage of 99.9 for all samples.

DISCUSSION

By targeted 16S rRNA deep sequencing approaches, several previous studies revealed the complex community membership in saliva and plaque samples from humans,

dogs and even cats, leading to insight into the connection between the oral microbiome and the host's health status. Little information was known about the bacterial community in dental calculus. This study was the first report to explore the bacterial communities in canine and feline calculus based on 16S rRNA sequencing (V3 and V4 hypervariable regions) with the Illumina platform. The number of OTUs identified in the calculus of dogs and cats averaged 173 and 111, respectively. The number of OTUs in each species corresponded to the diversity indices (richness, Shannon and Simpson), which were higher in dogs than in cats.

The dog oral microbiome has been the subject of several studies using pyrosequencing and cloning, which exhibited a difference in the most abundant phyla between oral samples (oral cavity, buccal site, palatal site and the subgingival pouch) and plaque [12,27-29]. Studies by Sturgeon et al.[12] and Oh et al.[29] demonstrated that the most abundant bacteria in oral samples were members of the phyla Bacteroidetes or Proteobacteria, respectively, whereas Dewhirst et al.[27] and Davis et al.[28] found that the phylum Firmicutes was the most prevalent in canine plaque, similar to canine calculus. However, the second most abundant phylum in dog calculus was Synergistetes [12,27-29]. This indicated a significant discrepancy in the bacterial communities in dog oral samples, plaque and calculus, where both of the former had a low representation of the phylum Synergistetes. Furthermore, from the phylogenetic tree, the members of the phylum Synergistetes were grouped with a clade of the phylum Firmicutes, because organisms from the phylum Synergistetes have previously been mistakenly included in the phylum Firmicutes [30]. For the cat oral microbiome, there were a few previous reports which collected oral samples (from the oral cavity, gums, teeth and buccal mucosa) [13] and plaque [31], and then determined the bacterial composition by cloning and next-generation sequencing, respectively. The phylum Proteobacteria was the most prevalent in oral samples, but in plaque the phylum Firmicutes had the largest number of taxa, similar to cat calculus [13,31].

In calculus of both dogs and cats, Firmicutes was the most abundant phylum, especially the class Clostridia, which had the largest number of OTUs (58) compared to other classes. This class is recognized as anaerobes, which prosper in non-oxygen conditions, likely similar to calculus. In addition to the class Clostridia, other facultative and obligate anaerobic species can be found in both canine and feline calculus, including *Tannerella*, *Parvimonas*, *Peptostreptococcus*, *Actinomyces* and *Desulfovibrio*. When considering at the genus level, the most abundant genus in dog and cat oral samples and plaque was *Porphyromonas* [12,27-29]. while that in calculus was *Fretibacterium* which belongs to the phylum Synergistetes. Members of this genus are strict anaerobes, motile, Gram-stain-negative, curved bacilli, and can be found in the human oral cavity as *Fretibacterium fastidiosum*, producing hydrogen sulfide [32]. Taken together, differences in the colonization of microbiota within the mouth can be observed in the saliva, tongue, tonsils, throat, and supra- and subgingival plaque, while the buccal mucosa, gingivae and hard palate have similar microbiota [33]. Also, the calculus samples appeared to have distinct bacterial communities compared to other sites within the mouth.

PCA and UPGMA of the microbiota of dog and cat calculus samples revealed an obvious discrepancy. This disclosed that the host species may have a significant influence. For this reason, the distinctive form of endogenous salivary and gingival crevicular fluid of each species, such as pH, glycoprotein, ion content and saliva flow rate, is the primary determinant of colonization of bacterial species, due to the fact that the primary nutrients of oral microbiota are saliva and gingival crevicular fluid [34]. The salivary pH of dogs (pH=8) and cats (pH=7.5) is quite different [35], presumably leading to distinctive calculus microbiota. In the formation of calculus, basic condition, calcium, inorganic phosphate, alkaline phosphatase and protease are the relevant factors which stimulate calculus origin [36], and these factors may vary across various species leading to altered oral microbiota. Perhaps surprisingly, an interesting report on 120 human individuals from across the globe showed no significant geographical differences in their salivary microbiota [37]. Here, the different types of dogs (NSB, SBb and SB) appeared to have a similar pattern of relative distribution. This phenomenon indicated that the variation of food intake in diverse individuals has little effect on the bacterial composition in the mouth [34].

Moreover, we found a remarkably species-specific taxon for canine calculus, *Corynebacterium canis* (denovo20), whose proportion was more than 2,000 times higher than in cats. For feline calculus, *Atopobium* sp. is a specific taxon, with SI of about 11. These species may grow in specific conditions, depending on their capability of binding to the host's adhesion molecules and co-aggregating with other bacterial species [38]. According to a survey of companion animals in Australia [39], periodontal disease was more prevalent in dogs than in cats. The dissimilarity of the bacterial communities in calculus may involve the occurrence of dental diseases such as gingivitis and periodontitis. Nonetheless, only a limited number of cat calculus samples were analyzed in this study. Further investigations should be performed with an increased number of specimens.

Interestingly, the archaeal community, all of which are methanogens, was observed in both canine and feline calculus, with five OTUs *(Fig. 3)*, especially in dogs which showed a higher prevalence than cats. The most prevalent taxon in the phylum Euryarchaeota was the genus *Methanobrevibacter*, not withstanding that several previous studies did not report the presence of archaea in oral samples and plaque from both dogs and cats [12,27-29].

However, archaeal species such as *Methanobrevibacter oralis* can be detected in human dental plaque [40]. An increased abundance of archaea as a *Methanobrevibacter oralis*-like phylotype was found to be related to the severity of periodontal disease within a cohort of patients [41]. In addition, in the present study we noted a negative relationship between *Methanobrevibacter* sp. and *Treponema* sp. Lepp et al.[41] reported that the relative abundance of *Treponema* rDNA was reduced at sites with a high abundance of archaeal rDNA because *Treponema* is a potential hydrogen competitor.

Despite an abundance of data obtained from a recently developed molecular method, next-generation sequencing still does not provide confident identification at the species level, since the genetic exchange of the 16S rRNA gene between oral genera such as *Treponema*, *Streptococcus* and *Neisseria* leads to difficulty in interpretation of phylogenetic relationships [42,43]. Therefore, some OTUs do not fall into the correct or appropriate clades, as seen in *Fig. 3*.

In conclusion, this approach served as a powerful method for generating a massive amount of data on bacterial communities and commensal colonization. It is known that in animals, multiple bacterial species interact at various sites in the mouth. In canine and feline calculus there is a high diversity of microbiota, which gives rise to the distinctive composition of the microbiome. Differences in the bacterial community of each species might bring us to a better understanding of the interaction between microorganisms and host specificity, leading to insight into the commensal microbiota in healthy individuals which prevent dental diseases.

Ethics Approval and Consent to Participate

This study was approved by the Animal Use Committee of the Faculty of Veterinary Medicine, Chiang Mai University, Thailand, in 2016 (R23/2559). All methods were performed in accordance with the relevant guidelines and regulations. All owners gave written consent prior to including in the study.

Consent to Publish

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

T.R. wrote the proposal and performed sample collection, DNA extraction and analysis. W.P. and S.C. assisted with DNA extraction. K.T. and K.N. designed and conducted all experiments. K.B. performed data and statistical analysis and support of information for discussion. K.N., K.B. and K.T. assisted in discussions and in writing of the manuscript. All authors have read and approved the final manuscript.

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REFERENCES

1. O Neill DG, Church DB, McGreevy PD, Thomson PC, Brodbelt DC: Prevalence of disorders recorded in dogs attending primary-care veterinary practices in England. *PLoS One*, 9, e90501, 2014. DOI: 10.1371/journal.pone.0090501

2. Kyllar M, Witter K: Prevalent of dental disorders in pet dogs. *Vet Med - Czech*, 50, 496-505, 2005.

3. Booij-Vrieling HE, van der Reijden WA, Houwers DJ, de Wit WEAJ, Bosch-Tijhof CJ, Penning LC, van Winkelhoff AJ, Hazewinkel HAW: Comparison of periodontal pathogens between cats and their owners. *Vet Microbiol*, 144, 147-152, 2010. DOI: 10.1016/j.vetmic.2009.12.046

4. Pihlstrom BL, Michalowicz BS, Johnson NW: Periodontal diseases. *Lancet*, 366, 1809-1820, 2005. DOI: 10.1016/S0140-6736(05)67728-8

5. Meyer DH, Fives-Taylor PM: Oral pathogens: From dental plaque to cardiac disease. *Curr Opin Microbiol*, 1, 88-95, 1998. DOI: 10.1016/S1369-5274(98)80147-1

6. Xu P, Gunsolley J: Application of metagenomics in understanding oral health and disease. *Virulence,* 5, 424-432, 2014. DOI: 10.4161/viru.28532

7. Pavlica Z, Petelin M, Juntes P, Erzen D, Crossley DA, Skaleric U: Periodontal disease burden and pathological changes in organs of dogs. *J Vet Dent*, 25, 97-105, 2008. DOI: 10.1177/089875640802500210

8. Mathews MJ, Mathews EH, Mathews GE: Oral health and coronary heart disease. *BMC Oral Health*, 16, 122, 2016. DOI: 10.1186/s12903-016-0316-7

9. Glickman LT, Glickman NW, Moore GE, Lund EM, Lantz GC, Pressler BM: Association between chronic azotemic kidney disease and the severity of periodontal disease in dogs. *Prev Vet Med,* 99, 193-200, 2011. DOI: 10.1016/j.prevetmed.2011.01.011

10. Roberts-Harry EA, Clerehugh V: Subgingival calculus: Where are we now? A comparative review. *J Dent*, 28, 93-102, 2000. DOI: 10.1016/S0300-5712(99)00056-1

11. Coignoul F, Chevilie N: Calcified microbial plaque. Dental calculus of dog. *Am J Pathol,* 117, 499-501, 1984.

12. Sturgeon A, Stull JW, Costa MC, Weese JS: Metagenomic analysis of the canine oral cavity as revealed by high-throughput pyrosequencing of the 16S rRNA gene. *Vet Microbiol,* 162, 891-898, 2013. DOI: 10.1016/j.vetmic.2012.11.018

13. Sturgeon A, Pinder SL, Costa MC, Weese JS: Characterization of the oral microbiota of healthy cats using next-generation sequencing. *Vet J*, 201, 223-229, 2014. DOI: 10.1016/j.tvjl.2014.01.024

14. Garrido-Cardenas JA, Manzano-Agugliaro F: The metagenomics worldwide research. *Curr Genet,* 63, 819-829, 2017. DOI: 10.1007/s00294-017-0693-8

15. Ghurye JS, Cepeda-Espinoza V, Pop M: Metagenomic assembly: Overview, challenges and applications. *Yale J Biol Med,* 89, 353-362, 2016.

16. König LM, Klopfleisch R, Höper D, Gruber AD: Next generation sequencing analysis of biofilms from three dogs with postoperative surgical site infection. *Int Sch Res Notices,* 2014, 282971, 2014. DOI: 10.1155/2014/282971

17. Hamp S, Olsson S, Farso-Madsen K, Viklands P, Fornell J: A macroscopic and radiologic investigation of dental diseases of the dog. *Vet Radiol Ultrasound,* 25, 86-92, 1984. DOI: 10.1111/j.1740-8261.1984.tb01916.x

18. Harvey CE, Shofer FS, Laster L: Association of age and body weight with periodontal disease in North American dogs. *J Vet Dent,* 11, 94-105, 1994.

19. Magoč T, Salzberg SL: FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics,* 27, 2957-2963, 2011. DOI: 10.1093/bioinformatics/btr507

20. Li W, Fu L, Niu B, Wu S, Wooley J: Ultrafast clustering algorithms for metagenomic sequence analysis. *Brief Bioinform,* 13, 656-668, 2012. DOI: 10.1093/bib/bbs035

21. Nylander JAA: MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre. *Uppsala University*, 2004.

22. Ronquist F, Huelsenbeck JP: MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics,* 19, 1572-1574, 2003.

23. Rambaut A, Suchard MA, Xie D, Drummond AJ: Tracer v1.5. 2013. Accessed: 22 June 2017. http://beast.bio.ed.ac.uk/Tracer

24. Stamatakis A, Hoover P, Rougemont J: A rapid bootstrap algorithm for the RAxML web servers. *Syst Biol,* 57, 758-771, 2008. DOI: 10.1080/10635150802429642

25. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R: QIIME allows analysis of high-throughput community sequencing data. *Nat Methods,* 7, 335-336, 2010. DOI: 10.1038/nmeth.f.303

26. Letunic I, Bork P: Interactive tree of life (iTOL) v3: An online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res,* 44, W242-W245, 2016. DOI: 10.1093/nar/gkw290

27. Dewhirst FE, Klein EA, Thompson EC, Blanton JM, Chen T, Milella L, Buckley CM, Davis IJ, Bennett ML, Marshall-Jones ZV: The canine oral microbiome. *PloS One,* 7, e36067, 2012. DOI: 10.1371/journal.pone.0036067

28. Davis IJ, Wallis C, Deusch O, Colyer A, Milella L, Loman N, Harris S: A cross-sectional survey of bacterial species in plaque from client owned dogs with healthy gingiva, gingivitis or mild periodontitis. *PLoS One,* 8, e83158, 2013. DOI: 10.1371/journal.pone.0083158

29. Oh C, Lee K, Cheong Y, Lee SW, Park SY, Song CS, Choi IS, Lee JB: Comparison of the oral microbiomes of canines and their owners using next-generation sequencing. *PLoS One,* 10, e0131468, 2015. DOI: 10.1371/journal.pone.0131468

30. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE: Bacterial diversity in human subgingival plaque. *J Bacteriol,* 183, 3770-3783, 2001. DOI: 10.1128/JB.183.12.3770-3783.2001

31. Dewhirst FE, Klein EA, Bennett ML, Croft JM, Harris SJ, Marshall-Jones ZV: The feline oral microbiome: A provisional 16S rRNA gene based taxonomy with full-length reference sequences. *Vet Microbiol,* 175, 294-303, 2015. DOI: 10.1016/j.vetmic.2014.11.019

32. Vartoukian SR, Downes J, Palmer RM, Wade WG: Fretibacterium fastidiosum gen. nov., sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol,* 63, 458-463, 2013. DOI: 10.1099/ijs.0.041038-0

33. Segata N, Haake SK, Mannon P, Lemon KP, Waldron L, Gevers D, Huttenhower C, Izard J: Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. *Genome Biol,* 13, R42, 2012. DOI: 10.1186/gb-2012-13-6-r42

34. Wade WG: The oral microbiome in health and disease. *Pharmacol Res,* 69, 137-143, 2013. DOI: 10.1016/j.phrs.2012.11.006

35. Driessens FCM, Borggreven JMPM, Verbeeck RMH, van Dijk JWE, Feagin FF: On the physicochemistry of plaque calcification and the phase composition of dental calculus. *J Periodontal Res,* 20, 329-336, 1985. DOI: 10.1111/j.1600-0765.1985.tb00442.x

36. Duckworth RM: The teeth and their environment: physical, chemical and biochemical influences. 154, Karger Medical and Scientific Publishers, 2006.

37. Nasidze I, Li J, Quinque D, Tang K, Stoneking M: Global diversity in the human salivary microbiome. *Genome Res,* 19, 636-643, 2009. DOI: 10.1101/gr.084616.108

38. Kolenbrander PE, Palmer JRJ, Periasamy S, Jakubovics NS: Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol,* 8, 471-480, 2010. DOI: 10.1038/nrmicro2381

39. Watson A: *Diet and disease in companion animals. Australian Veterinary Association,* 29, 1994.

40. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner ACR, Yu WH, Lakshmanan A, Wade WG: The human oral microbiome. *J Bacteriol,* 192, 5002-5017, 2010. DOI: 10.1128/JB.00542-10

41. Lepp PW, Brinig MM, Ouverney CC, Palm K, Armitage GC, Relman DA. Methanogenic *Archaea* and human periodontal disease. *Proc Natl Acad Sci U S A,* 101, 6176-6181, 2004. DOI: 10.1073/pnas.0308766101

42. Wang BY, Chi B, Kuramitsu, HK: Genetic exchange between *Treponema denticola* and *Streptococcus gordonii* in biofilms. *Oral Microbiol Immunol,* 17, 108-112, 2002. DOI: 10.1046/j.0902-0055.2001.00001.x

43. Hanage WP, Fraser C, Spratt BG: Fuzzy species among recombinogenic bacteria. *BMC Biol,* 3, 6, 2005. DOI: 10.1186/1741-7007-3-6

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Short Communication

Genetic Properties of Copy Number Variations in Some Pakistani Cattle Breeds

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Abstract

A copy number variation (CNV) is information of DNA segment containing deletion, duplication or insertion exist within diverse populations. This study detected 53 CNVs regions that overlapped with immune response, body size and parasitic resistance traits in Pakistani cattle breeds. This study characterized genetic diversity and provides lineage differentiated CNVs in these indigenous cattle breeds.

Keywords: *Copy number variation, Diversity, Traits, Breed, Pakistan*

Pakistan'da Bazı Sığır Irklarında Kopya Sayısı Değişikliklerinin Genetik Özellikleri

Öz

Kopya sayısı değişikliği (CNV) farklı popülasyonlar içerisinde delesyon, duplikasyon ve insersiyonları içeren DNA segmentlerinin bilgisidir. Bu çalışma ile Pakistan sığır ırkları arasında bağışıklık yanıtı, vücut boyutu ve parazit direnci özelliklerini içeren 53 CNV tespit edildi. Bu çalışma; belirtilen yerel sığır ırkları arasında genetik çeşitliliği göstermiş ve hat farklı CNV'leri belirlemiştir.

Anahtar sözcükler: *Kopya sayısı değişikliği, Çeşitlilik, Özellikler, Irk, Pakistan*

INTRODUCTION

Copy number variations (CNV) are DNA segments containing deletions, insertions and duplications existing as complex allelic variants within the diverse population [1]. CNVs markers are more effective as compared to SNPs markers, due to more genomic sequences, including gene structure changing and dosage, changing gene regulation, and recessive alleles exposing [2]. Recently, a number of studies confirmed CNVs regions in bovine genome that cover a number of genes related to environment adaptation [3]. CNVs regions have tendency to demonstrate breed`s history and their formation patterns [4]. There is very little information about population genetic properties of CNVs in cattle, unlike microsatellites and SNPs markers [1]. The characterization of diversity and origin of CNVs, population genetic properties and functional impacts of CNVs is still a very dynamic research area in animal sciences. This study reported first comprehensive CNVs regions based on population genetic properties, focusing diversity pattern and population structure, within indigenous Pakistani cattle breeds.

MATERIAL and METHODS

Selected animal`s blood sampling and DNA extraction is already described in a previously study [5]. Additional, Angus (n=10) and Holstein (n=10) samples were added for this analysis using the USDA available dataset. Log R Ratio file was imported into SNP and variation suite (SVS

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8.0) and 440 370 SNPs were successfully mapped onto UMD 3.1 genome assembly. Default GC correlation file was used to normalize Log R Ratio and copy number module (CNM) to segment pairwise chromosomes permutations (n=1000). Three state covariates (0.3) was used across the all selected samples for CNVs genotyping as three levels (loss, neutral and gain). All 154 deletion CNVs recoded manually, converting loss event into "11" and neutral into "12". Multidimensional scaling (MDS) analysis was used to identify individual`s clustering. PLINK (9.0 version) was used to determine pairwise relationship of 154 CNVs deletion. The cluster analysis was also performed to verify separation based on the mean Log R Ratio values using R version 13.1. We recovered ENSEMBL and REFRSEQ genes overlapping CNVs regions by at least 1 bp including the 5` and 3`untranslated regions from the available UCSC genome browser and annotated CNVs regions using custom software. Enrichment analysis was performed using PANTHER classification system and only clusters with enrichment scores more than 1 (P value <0.05) were considered. VST was calculated for each CNV by using the following equation: (VT-VS)/VT, to detect lineage differentiated CNVs. where, VT is the total variance in mean Log R Ratio across all individuals and VS is the average variance in cattle within each breed.

RESULTS

A total 156 individuals from twelve different breeds were used for CNVs investigation. Total 100,500 CNVs segments were extracted using the default multivariate option of Golden Helix SVS (version 8.0). Above 1% segment frequency were retained for further analysis, where multi-variance option in SVS was actually developed to detect moderate to high CNVs frequency. After filtration total 210 CNVs (total length of 10221 kb) with an average length of 45.2 kb were retained and samples were categorized three type events (loss, neutral and gain). The mean LR ratio ± 0.3 threshold level was used as three state models, there was 154 CNVs deletion and stored as CNV1 to CNV210 list with descending frequency. However, SVS results of this study indicate low concordant. This may be due the absence of these breeds during the bovine high density SNP bead chip designing or due to low sample size and breed undistinguished in this data set. Two hundred and ten CNVs of this data set were used for VST and frequency calculations.

Multidimensional Scaling (MDS) analysis was performed based on CNVs to investigate the population structure based on 154 CNVs deletion. Three clusters were found, which clearly separate these breeds into three groups. The two distinct groups B (All indigenous Pakistan breeds except Tharparkar) and C (Angus and Holstein) both need more clearance of diversion from other breeds *(Fig. 1)*. The CNVs results based on MDS analysis are reliable with similar results based on SNPs analysis (The Bovine HapMap Consortium, 2009) and suggest that CNVs can be used as genetic markers to separate cattle individuals into distinct groups.

The results of this study revealed that CNVs can also be used effectively as genetic markers in population genetic studies. However, CNVs markers as compared with SNPs have some little resolution issues, may be due to small size sampling. Population diversity and differentiated CNVs were estimated using 210 CNVs. CNVs frequencies were used across all 3 groups (A, B & C). Top four high frequency deletions were on chromosomes 9, 21, 1 and 12 that are

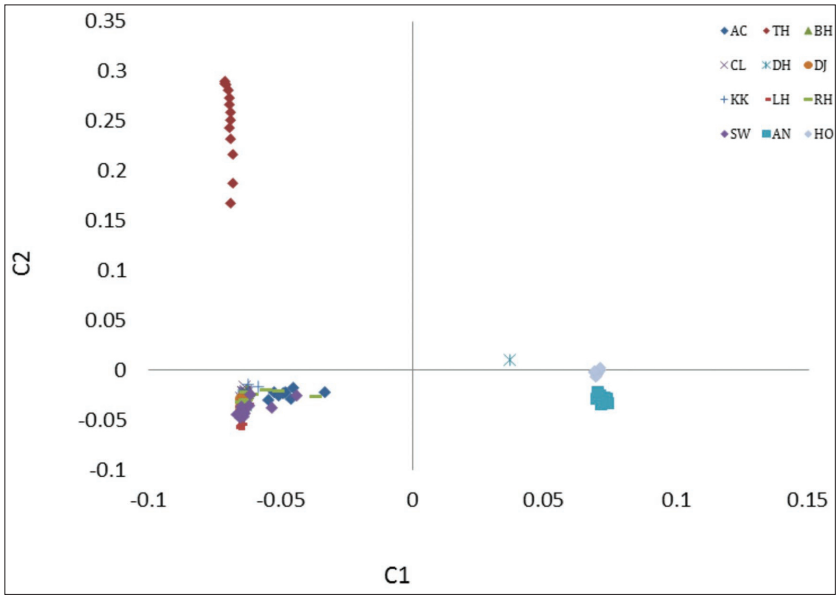

Fig 1. MSD analysis based on 154 deletion CNVs and plotted according to their first two components

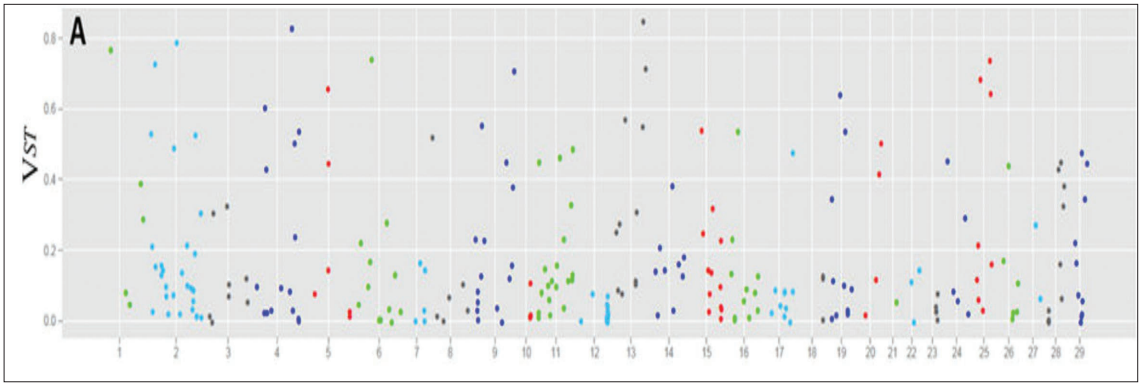

Fig 2. Population differentiations estimated by Vst plotted along each chromosome for all breed comparison (All Pakistan cattle vs Holstein and Angus)

0.90, 0.89, 0.83 and 0.81, respectively and were used to estimate CNVs with differentiated frequencies.

Comparing CNVs regions with the UMD 3.1, UCSC annotation and found 82 CNVs partially overlapping with genes. Gene ontology (GO) was also performed to detect genes enrichments. Response to interferon gamma and response to stimulus were the most enriched biological process. One hundred seventy two CNVs were not encompassed any UCSC genes, but only 38 CNVs overlapped. CNVs without genes were shorter (mean 22170±5251, n=172 vs. 77200±0.0182, n=38, t-test, P<0.0001) and at higher frequency (0.3234±0.0173, n=172 vs. 0.3121±0.0182, n=38, P=0.0112), when compared CNVs overlapped with genes and not overlapped with genes. The lack of common deletion CNVs overlapping with genes is positive indicator that population is under selection and deleterious variants removes from the population and already.

Pairwise VST (0-1) was investigated, to test CNVs association with population specific selection *(Fig. 2)*. High VST values indicate regions under increased selection pressure or evolutionary forces (bottlenecks or founder effects). A total of 12, 1 and 14 CNVs were identified at VST>0.6 threshold and at 0.4 CNVs were found 40, 09 and 41, respectively. The higher VST differential detected in these CNVs regions may suggest the variability of their genomic sequence that could be further involved in phenotypic diversity across these cattle breeds. Seven genes (HIATL1, MRPL48, PRAME, PLCB1, TSPY, ROBO4, and ZNF280B), which overlapped with CNVs regions and potentially impact their functions and evolution.

DISCUSSION

This study is first comprehensive attempt to explore CNVs as potential genetic markers based on population genetic properties in some indigenous cattle breeds in Pakistan. Previous studies in some species, including Human, Dog, Zebra fish, Stickleback fish and some worldwide cattle breeds have identified CNVs, based on population genetic properties, within and between population [6-8]. Confidence deletion CNVs was used because; it was difficult to detect accurately genotypes complex CNVs events, due to non-biallelic duplication [8]. The CNVs deletion events have some advantage over duplication, due to its bi-allelic nature and compatibility with genetic analysis procedures designed for SNPs markers [9]. In this study, CNVs was used to explore the population genetic properties in some indigenous cattle breeds in Pakistan with moderate intra population frequencies as reported by Bickhart et al.[6]. SVS method, although it reported limited amount of CNVs calls (71.6% deletion and 28.4 duplications). The results of this study revealed that different cattle breeds in different agro-ecological zones clustered roughly and two indicus Lohani and Tharparkar were genetically distinct from other selected cattle populations *(Fig. 1)* which is similar as reported by previously SNPs based studies in different cattle population worldwide, including taurine, indicus or sanga etc.[7]. Seventy two percent of the CNVs were identified, which were marked by flanking SNPs as reported by Xu et al.[8]. However, the results of this investigation were not efficiently capable to distinguish recent divergent populations of common geographic origin, such as Cholistani and Bhagnari cattle breeds and also reported by Silva et al.[7] and Xu et al.[8] in Holstein and Angus cattle breeds. Human published data also revealed that the CNVs based on population genetic properties can only be investigated with large data set of continent population level [10]. The satisfactory level of population structure identified by SNPs based methods may depends on genotyping accuracy and sample size [11]. Therefore, it is highly recommended that studies based on high throughput SNPs technology will defiantly enhance to make accurately genotype CNVs [12].

Furthermore, estimated CNVs based population differentiation and detected some potential candidate CNVs in these breeds. VST values were calculated and scanned gene enrichments among CNVs regions and found 54 distinctive CNVs with threshold VST value 0.4 as reported in a previous study by Liu et al.[13]. It is

important here that selection pressure, the amount divergence between breeds (divergence time, effective population size and gene flow) can affect VST value and overall differentiation among selected populations [13,14]. At this stage a hypotheses can be developed that high VST value between populations does not necessarily involve in divergent (selection for different alleles in both populations) or can occur in the absenteeism of selection by bottlenecks or founder effects by drift [9]. All these hypotheses needs further research using large population samples size of these breeds as similar observed by Xu et al.[8].

In this study, only a small amount of lineage differentiated CNVs were detected in all breeds comparison especially Cholistani and Bhagnari and similar was reported in Holstein and Angus Xu et al.[8]. These results suggest that these breeds might not have had sufficient time to diversify their CNVs deletion contents and assume that CNVs occurred before split of these breeds and deletion content should eventually fix in these breeds except Tharparkar. However, there are also some evidence of fewer lineage differentiated CNVs in laboratory mouse, some zebra fish strains and cattle breeds which compared with their wild populations [6]. This may suggest CNVs differently fixed as large effective population size or indicate inbreeding effects of wild populations [9]. There is still very little information about genetic landscape and exact domestication events of these breeds.

In conclusion, CNVs as genetic markers in indigenous cattle breeds in Pakistan raised some important questions about the domestication and divergence of these cattle breeds especially Tharparkar cattle. However, this study provided substantial evidence to support CNVs as potential genetic markers that can be used across population diversity and capture subspecies relationships. Additionally, this study suggest that a comprehensive study of population genetic differentiation in these cattle breeds along with other worldwide cattle breeds will provide a clear picture of diversity pattern and population structure. Finally, some more powerful analytical software tools can maximize the prediction level of population genetics in livestock species and expand the scope with next generation sequencing (NGS) data.

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Conflict of Interest Statement

The authors declare no conflict of interest

REFERENCES

1. Keel BN, Lindholm-Perry AK, Snelling WM: Evolutionary and functional features of copy number variation in the cattle genome. *Front Genet*, 7, 207, 2016. DOI: 10.3389/fgene.2016.00207

2. Sasaki S, Watanabe T, Nishimura S, Sugimoto Y: Genome-wide identification of copy number variation using high-density single-nucleotide polymorphism array in Japanese Black cattle. *BMC Genet*, 17, 26, 2016. DOI: 10.1186/s12863-016-0335-z

3. Zhang L, Jia S, Plath M, Huang Y, Li C, Lei C, Zhao X, Chen H: Impact of parental *Bos taurus* and *Bos indicus* origins on copy number variation in traditional Chinese cattle breeds. *Genome Biol Evol,* 7, 2352-2361, 2015. DOI: 10.1093/gbe/evv151

4. Wu ZJ, Jin W: Copy-number variation: A new pattern of structural diversity in genome. *Yi Chuan,* 31, 339-347, 2009.

5. Mustafa H, Euisoo K, Heather HJ, Ajmal A, Riley D, Pasha TN, Ali A, Javed K, Sonstegard TS: Genome-wide SNPs analysis of indigenous zebu breeds in Pakistan. *Biotechnol Anim Husb,* 33, 13-25, 2017. DOI: 10.2298/BAH1701013M

6. Bickhart DM, Xu L, Hutchison JL, Cole JB, Null DJ, Schroeder SG, Song J, Garcia JF, Sonstegard TS, Van Tassell CP, Schnabel RD, Taylor JF, Lewin HA, Liu GE: Diversity and population-genetic properties of copy number variations and multi-copy genes in cattle. *DNA Res,* 23, 253-262, 2016. DOI: 10.1093/dnares/dsw013

7. da Silva VH, de Almeida Regitano LC, Geistlinger L, Pertille F, Giachetto PF, Brassaloti RA, Morosini NS, Zimmer R, Coutinho LL: Genome-Wide detection of CNVs and their association with meat tenderness in Nelore cattle. *PLoS One,* 11, e0157711, 2016. DOI: 10.1371/journal.pone.0157711

8. Xu L, Hou Y, Bickhart DM, Zhou Y, Hay el HA, Song J, Sonstegard TS, Van Tassell CP, Liu GE: Population-genetic properties of differentiated copy number variations in cattle. *Sci Rep*, 6, 23161, 2016. DOI: 10.1038/srep23161

9. Zhang X, Wang K, Wang L, Yang Y, Ni Z, Xie X, Shao X, Han J, Wan D, Qiu Q: Genome-wide patterns of copy number variation in the Chinese yak genome. *BMC Genomics,* 17, 379, 2016. DOI: 10.1186/s12864-016-2702-6

10. Zhang Q, Ma Y, Wang X, Zhang Y, Zhao X: Identification of copy number variations in Qinchuan cattle using BovineHD Genotyping Beadchip array. *Mol Genet Genomics*, 290, 319-327, 2015. DOI: 10.1007/s00438-014-0923-4

11. Jiang L, Jiang J, Yang J, Liu X, Wang J, Wang H, Ding X, Liu J, Zhang Q: Genome-wide detection of copy number variations using high-density SNP genotyping platforms in Holsteins. *BMC Genomics,* 14, 131, 2013. DOI: 10.1186/1471-2164-14-131

12. Salomon-Torres R, Gonzalez-Vizcarra VM, Medina-Basulto GE, Montano-Gomez MF, Mahadevan P, Yaurima-Basaldua VH, Villa-Angulo C, Villa-Angulo R: Genome-wide identification of copy number variations in Holstein cattle from Baja California, Mexico, using high-density SNP genotyping arrays. *Genet Mol Res*, 14, 11848-11859, 2015. DOI: 10.4238/2015.October.2.18

13. Liu GE, Hou Y, Zhu B, Cardone MF, Jiang L, Cellamare A, Mitra A, Alexander LJ, Coutinho LL, Dell'Aquila ME, Gasbarre LC, Lacalandra G, Li RW, Matukumalli LK, Nonneman D, Regitano LC, Smith TP, Song J, Sonstegard TS, Van Tassell CP, Ventura M, Eichler EE, McDaneld TG, Keele JW: Analysis of copy number variations among diverse cattle breeds. *Genome Res*, 20, 693-703, 2010. DOI: 10.1101/gr.105403.110

14. Öner Y, Yılmaz O, Okut H, Ata N, Yılmazbaş-Mecitoğlu G, Keskin A: Associations between GH, PRL, STAT5A, OPN, PIT-1, LEP and FGF2 polymorphisms and fertility in Holstein-Friesian heifers. *Kafkas Univ Vet Fak Derg*, 23 (4): 527-534, 2017. DOI: 10.9775/kvfd.2016.17192

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Short Communication

Bio-stimulatory Effect of Bull on Postpartum Estrus Interval in Nili-Ravi Buffaloes

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Abstract

The objective of this study was to determine the effect of bull exposure after calving on postpartum interval to estrus, in Nili-Ravi buffalo. Buffaloes were divided into 4 groups; BEC & BEI; exposed continuously or intermittently to the physical presence of bull, EPB & NE; exposed or not exposed to discharge waste (urine & feces) of bull. Buffaloes were exposed on d 5 after parturition. Postpartum interval to first behavioural estrus was shorter in BEC than BEI, EPB and NE treatments. The mean serum progesterone concentration did not differ significantly between BEC and other (BEI, EPB and NE) treatments. It was concluded that presence of bull has positive effect in reducing calving interval in Nili Ravi buffalo.

Keywords: *Calving interval, Biostimulation, Buffalo, Bull exposure*

Nili Ravi Mandalarında Postpartum Östrus Aralıkları Üzerine Boğanın Biyo-uyarıcı Etkisi

Öz

Bu çalışmanın amacı Nili Ravi mandalarında buzağılama sonrası boğaya maruz kalmanın postpartum östrus aralıkları üzerine etkisini belirlemektir. Mandalar 4 gruba ayrıldı; BEC: Sürekli olarak boğayla fiziki olarak beraber bulunduruldu, BEI: Aralıklı olarak boğayla fiziki olarak beraber bulunduruldu, EPB: Boğanın atıkları (idrar ve dışkı) ile beraber bırakıldı NE: Boğanın atıkları (idrar ve dışkı) ile beraber bırakılmadı. Boğaya veya atıklarına maruz bırakma doğum sonrası 5. günde gerçekleşti. İlk davranışsal östrus postpartum aralık BEC'de BEI, EPB ve NE gruplarından daha kısaydı. Ortalama progesteron konsantrasyonu BEC ile diğer gruplar (BEI, EPB ve NE) arasında anlamlı bir fark göstermedi. Nili Ravi mandalarında boğa mevcudiyetinin buzağılama aralıklarına olumlu etkisinin olduğu sonucuna varıldı.

Anahtar sözcükler: *Buzağılama aralığı, Biyouyarı, Manda, Boğa mevcudiyeti*

INTRODUCTION

Reproductive efficiency is hampered in female buffaloes due to their inherent vulnerability to environmental stress, distinct seasonal reproductive patterns, delayed puberty, poor estrus expression, delayed age at first calving and prolonged inter calving intervals. Fertility is affected by poor body condition which is resulted from poor nutrition at calving, characterized by reduced conception rates, more services per conception and prolonged postpartum interval. As in postpartum buffaloes, the resumption of ovarian cyclicity after calving plays a major role in obtaining a satisfactory reproductive performance, the extremely long postpartum interval (400 to over 600 d) in this species results in extensive economic loss [1].

Bio-stimulation or bull impact can be characterized as the stimulus incited by the nearness of male, which actuate estrus and ovulation through genital incitement, pheromones, or other outer signals [2]. Male existence

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provoked stimulus which can effect on estrus cycle. These signals include olfactory, optical, and acoustic signs. In cattle, presence of male enhance ovarian activity after parturition through discharging wastes and cervical secretion of females having estrus also take part in post-partum ovarian function [3]. Bio-stimulation can apply intense effects on reproductive function involving hypo-thalamic process. The bio-stimulatory effect is considered to be facilitated through pheromones, which are airborne chemical materials secreted externally in the excretory product or feces or integumentary secretions and detected by the olfactory sense provide basis for behavioural and hormonal responses in the animal of same species [4].

Bio-stimulatory effect of bull is a low-cost and appropriate technique and it can be described as "control system aimed to improve or manage propagation of animal" [5]. In small ruminants, the part of bio-stimulation is well explained. It has extreme importance that in a herd of pre mature heifers the existence of a teaser bull did not change the ovaries or accelerate the adolescence [6].

The phenomenon of the male influence on postpartum recommencement of ovarian action is well documented in cattle [7], sheep and goats. Continuous contact of post-partum cows to a vasectomized bull restrains the occurrence of silent ovulation and improves the conception rate [8]. In Pakistan, since no such study has been undertaken, so presently, there is scanty information in literature regarding effects of bull exposure or its excretory products in buffalo and on physiological mechanisms by which bull may influence postpartum reproductive functions. The objective of this study was to determine the effect of bull exposure continuously or intermittently or its excretory products after calving on postpartum interval to estrus in Nili-Ravi buffalo.

MATERIAL and METHODS

The current study was performed at a livestock farm located between 31.0167° N latitude and 73.8500° E longitudes. The experimental period was extended from June 2014 to November 2015. Forty-eight Nili-Ravi breed buffaloes were used in this experiment. All buffalo were allocated one of the four treatments in a totally randomized plan using a 4 x 1 factorial design. Influences were exposure form 1) exposed continuously to the physical presence of a bull (BEC; n=12); 2) exposed intermittently to the physical presence of bull (BEI; n=12); 3) exposed to discharge waste (urine & feces) of bull (EPB; n=12) and 4) not exposed to a bull or discharge waste of bulls (NE; n=12). Buffaloes were exposed on d 5 after parturition. Day 5 postpartum represented d 0 for each treatment.

Buffalo exposed continuously or intermittently to bulls and its waste material do not have interaction with bulls during pregnancy and later giving birth till they were sited into treatments approximately 5 d (d 0) after calving. Postpartum detection of estrus was observed five d later in each treatments group. Estrus was detected two times every day (07.00 and 18.00) in the course of research. A buffalo stood for mounting considered to be in estrus.

BEC and BEI treatments, the buffalo which were exposed to continuous physical presence of bull (BEC) treatment, the bull was present in the herd all time. The ratio of buffalo to bull (12:1) was maintained throughout the study period. The buffalo that was exposed to bull intermittently (BEI) treatment, the bull was introduced for 2 h daily.

EPB and NE treatments, the buffalo exposed to the excretory (EPB) products of bulls, Buffalo in this treatment were sited into the shed at nearly 07.00 and were expelled at 20.30 (approximately 13.5 h). Bulls (n=10) were then moved into the shed overnight between 20.30 and 07.00 (approximately 10.5 h) each d throughout the experiment. The sheds were cleaned daily. Buffalo in the EPB treatment have no visual contact with these bulls. Buffalo assigned to the NE treatment considered as control group and have no interaction with bull and its excretory products.

Buffaloes were given free access to good-quality ration (green fodder and Vanda) according to their requirement that was available in their respective treatment sheds. The criteria used to determine interval from calving to estrus includes, behavioural estrus and existence of corpus luteum. Blood samples were collected from buffalo of each treatment by puncturing jugular vein starting on d 0 and calculations were done after every four d till the end of experiment. For the assessment of progesterone, serum was collected and saved at -20°C. In order to decide the interval from calving to estrus, serum progesterone value was calculated by using ELISA. The sandwich type of ELISA was used in this experiment. Once an ascent of progesterone over 1 ng/mL was measured and a corpus luteum was detected by using rectal palpation technique, interval from calving to the comparing d of the progesterone rise was computed. The bioethical committee of Bahauddin Zakariya University approved the methodology and procedures used in present study. The data regarding interval from calving to estrus was determined by Statistix 8.1 (Tukey HSD All-pair wise comparison test of days by group) and SPSS was used to find out (Mean ± SE) of serum progesterone concentration.

RESULTS

The postpartum interval from calving to first behavioural estrus was 55.2±0.78 d in BEC, 66.71±0.93 d in BEI, 68.25±0.87 d in EPB and 68.57±0.93 d in NE treatments. Significant difference was seen between BEC and other treatments (BEI, EPB and NE) for interval from parturition to first behavioural estrus. Postpartum interval to first behavioural estrus was shorter in BEC than BEI, EPB and

Table1. *Interval from Calving to Estrus and Serum Progesterone Concentration in BEC, BEI, EPB and NE Buffaloes (Mean ± SE)*

Parameter	BEC (n=12)	BEI (n=12)	EPB (n=12)	NE (n=12)
Percentage of buffaloes showing estrus per treatment	83.33% (10/12)	58.33% (07/12)	66.66% (08/12)	58.33% (07/12)
Interval from calving to first behavioural estrus (days)	55.2±0.78 [a]	66.71±0.93 [b]	68.25±0.87 [b]	68.57±0.93 [b]
Progesterone concentration from calving to first behavioural estrus	0.88±0.1 [a]	0.86±0.06 [a]	0.84±0.08 [a]	0.82±0.06 [a]

Values lacking common superscripts letters differ significantly (P≤0.05)

NE treatments. The buffalo assigned BEC treatment showed estrus 13 d earlier than EPB and NE treatments and 11 d earlier than EPI treatment. There was no significant difference in postpartum interval to estrus between BEI, EPB and NE treatments *(Table1)*. In present study, the percentage of buffaloes showing estrus during experimental period was 83.33%, 58.33%, 66.66% and 58.33% in BEC, BEI, EPB and NE treatments, respectively. The mean serum progesterone concentration did not differ significantly between BEC and other (BEI, EPB and NE) treatments *(Table1)*. Progesterone concentration was measured from d-1 (d 0 = 5 after calving) to first rise in progesterone above 1 ng/mL.

DISCUSSION

In the present study we determined whether the proportion of buffalo and postpartum interval to estrus could be changed in continuously bull exposed buffalo (BEC), the buffalo that was exposed to bull intermittently (BEI), the buffalo exposed to excretory product of the bull (EPB) and the buffalo treated as control have no interaction with bull and its excretory materials.

Exposing Nili-Ravi buffalo on d 5 after parturition to mature bull continuously (BEC) increased the percentage of buffalo showing estrus and reduced the postpartum interval to estrus than buffalo that have no visual contact with bull (NE). This result agrees with the result of Custer et al.[9] and Fernandez et al.[10]. In present study buffalos exposed continuously to bull return to estrus 13 d prior than buffalo without exposure to bull which is agreed with Naasz and Miller [11]. Postpartum interval to estrus curtail in bull exposed buffalo as related to isolated from bulls. Postpartum interval to estrus did not alter in buffalo exposed intermittently to bull (BEI) for 2 h daily. Postpartum estrus interval differed non-significantly (P>0.05) among BEI, EPB and NE buffaloes. Petropavlovskii and Rykova [12] reported that postpartum interval to conception decreased in intermittently exposed animal than isolated from bull exposure, this is because the duration of exposure was 3 to 4 h two times daily. The bio-stimulatory effect of bull can be obtained when the duration of exposure was greater than 2 h daily [13]. In present study the result of postpartum interval to estrus in EPB treatment is very close to control NE treatment. There was non-significant difference (P>0.05) of postpartum estrus interval between EPB and NE buffaloes. The results of present study disagree with Berardinelli and Joshi [3]. Berardinelli and Joshi [3] reported that postpartum interval to recommencement of luteal function was decreased significantly in cows exposed to excretory products in comparison with not exposed cows. Experimental difference is responsible of this contrast in results. In our study the place of buffalo for EPB treatment cleaned daily while in Berardinelli and Joshi [3] experiment, enclosure of EPB animals was cleaned every 4 to 5 d.

The proportion of buffalo displayed estrus in BEC was more than BEI, EPB and NE. Exposing postpartum Nili-Ravi buffalo continuously to bull increased the proportion of buffalo that showed estrus. The results of present study show compatibility agreement with other studies [10]. The present work also agrees with Rekwot [4]. The proportion of buffalo exposed intermittently to bull in BEI was equivalent to the proportion of buffalo not exposed to bull in NE *(Table 1)*. This result agrees with the result of Fernandez et al.[13] and in contrast with Petropavlovskii and Rykova [12] due to the difference of duration and period of exposure. The buffalo exposed to excretory product of bull (EPB) show greater proportion (66.66%) of displaying estrus than NE. This result also supported the result of Berardinelli and Joshi [3].

The mechanism exactly how bulls hasten resumption of ovarian activity and estrus interval is unknown. The bio-stimulatory effect of bull alters the LH secretion that reached at highest point in ovulation [9,13]. Bio-stimulatory effect of bull on estrus interval is associated with pheromonally activated signals that stimulate release of GnRH through hypothalamus and increased the release of LH. Continuous presence of bull involves in signaling trigger as well as other cues that enhance estrus [8]. Bio-stimulatory pheromones are present in excretory product of bull reported by [3]. Pheromonal activity in the bio-stimulatory effect is most likely to have in urine of the bull [7].

The progesterone concentrations (ng/mL) were apparently higher in the buffalo of BEC treatment than buffalo not exposed to bull (NE) treatment. Several researches observed transient increase in progesterone concentration from postpartum to estrus [14]. Increase in progesterone concentration enhance conception rate and in turn curtails the postpartum interval to estrus [15].

It was concluded that buffalo exposed to the bull continuously showed reduced postpartum interval to estrus. Presence of bull has positive effect in reducing calving interval in Nili Ravi buffalo. Although bulls are considered burdens by some farmers due to the cost of nutrition and management, it is clear that presence of bull is beneficial for improving the reproductive efficiency of buffalo.

REFERENCES

1. Ramesh V, Vanan TT, Varadhrajan A: Improvement of reproductive performances of buffaloes. *Pashudhan,*17, 1-4, 2002.

2. Wright IA, Rhind SM, Whyte TK, Smith AJ: Effects of body condition at calving and feeding level after calving on LH profiles and the duration of the post-partum anoestrous period in beef cows. *Anim Sci*, 55, 41-46, 1992. DOI: 10.1017/S0003356100037259

3. Berardinelli JG, Joshi PS: Initiation of postpartum luteal function in primiparous restricted-suckled beef cows exposed to a bull or excretory products of bulls or cows. *J Anim Sci*, 83, 2495-2500, 2005. DOI: 10.2527/2005.83112495x

4. Rekwot PI, Ogwu D, Oyedipe EO, Sekoni VO: The role of pheromones and bio-stimulation in animal reproduction. *Anim Reprod Sci*, 65, 157-170, 2001. DOI: 10.1016/S0378-4320(00)00223-2

5. Martin GB: Reproductive research on farm animals for Australia--some long-distance goals. *Reprod Fertil Dev*, 7, 967-982, 1995. DOI: 10.1071/RD9950967

6. Roberson MS, Ansotegui RP, Berardinelli JG, Whitman RW, McInerney MJ: Influence of biostimulation by mature bulls on occurrence of puberty in beef heifers. *J Anim Sci*, 64, 1601-1605, 1987.

7. Tauck SA, Berardinelli JG: Putative urinary pheromone of bulls involved with breeding performance of primiparous beef cows in a progestin-based estrous synchronization protocol. *J Anim Sci*, 85, 1669-1674, 2007. DOI: 10.2527/jas.2006-849

8. Barman P, Yadav MC, Kumar H, Meur SK: Effect of bull exposure on ovarian cyclicity in postpartum buffaloes. *Buff Bull*, 30, 240-249, 2011.

9. Custer EE, Berardinelli JG, Short RE, Wehrman M, Adair R: Postpartum interval to estrus and patterns of LH and progesterone in first-calf suckled beef cows exposed to mature bulls. *J Anim Sci,* 68, 1370-1377, 1990. DOI: 10.2527/1990.6851370x

10. Fernandez D, Berardinelli JG, Short RE, Adair R: The time required for the presence of bulls to alter the interval from parturition to resumption of ovarian activity and reproductive performance in first-calf suckled beef cows. *Theriogenology,* 39, 411-419, 1993. DOI: 10.1016/0093-691X(93)90384-H

11. Naasz CD, Miller HL: Effects of bull exposure on postpartum interval and reproductive performance in beef cows. *Can J Anim Sci*, 70, 537-542, 1990. DOI: 10.4141/cjas90-065

12. Petropavlovskii VV, Rykova AI: The stimulation of sexual functions in cows. In *Anim Breed Abstracts,* 29, 168, 1961.

13. Fernandez DL, Berardinelli JG, Short RE, Adair R: Acute and chronic changes in luteinizing hormone secretion and postpartum interval to estrus in first-calf suckled beef cows exposed continuously or intermittently to mature bulls. *J Anim Sci,* 74, 1098-1103, 1996. DOI: 10.2527/1996.7451098x

14. Shah RG, Dhami AJ, Kharadi VB, Desai PM, Kavani FS: Hormonal profile in fertile and infertile postpartum Surti buffaloes. *Buffalo J*, 20, 29-42, 2004.

15. Noseir W: Changes in serum progesterone patterns during the short luteal cycles initiated after calving and relationship to conception in buffalo-cows. *Assiut Vet Med J*, 42, 330-336, 1999.

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Short Communication

The First Molecular Detection and Genotyping of *Encephalitozoon cuniculi* in Rabbit's Eye in Turkey

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Abstract

Encephalitozoon cuniculi was first recognized as the disease agent in rabbits in 1922. The genotype of *E. cuniculi* isolated from laboratory rabbits with the neurologic disease was described as genotype I. In the eye, this parasite causes damage to the lens, causing phacoclastic uveitis and cataracts. Intraocular infection often occurs in cases of transplacental transmission. There has been no report on the molecular diagnosis of the parasite in Turkey. The current study is the first report on the detection of *E. cuniculi* spores using the molecular method in Turkey. In our previous study, a rabbit breeding facility was determined seropositive for *E. cuniculi* infection monitored for five years in terms of clinical symptoms. An autopsy was performed for a definite diagnosis of the infection. Samples were stained according to the hematoxylin-eosin (H&E) staining after tissue processing procedure and histopathologic analysis was performed. In addition to, the samples for DNA extraction were also taken during the autopsy. ECUNF and ECUNR species-specific primer pairs were used for amplification and genotyping of *E. cuniculi*. The animals were observed no clinical symptoms except ocular lesion (n=9). Therefore, one of these rabbits was used in the autopsy to definite diagnosis and determination of the damage to the eye. As histopathological, the lesions in the eye were found in the initial or middle stage of progressive infection. The DNA sequence showed that *E. cuniculi* examined in the present study were genotype I. Possible cause of the visible white mass in the rabbit's eye may be the parasite infection. Therefore, clinicians may consider *E. cuniculi* as one of the possible causes of ocular lesions in rabbits during daily inspection or ophthalmological examination.

Keywords: *Rabbit, Eye, Molecular diagnosis, Encephalitozoon cuniculi, Phylogenetic analysis, Turkey*

Türkiye'de Tavşan Gözünde *Encephalitozoon cuniculi*'nin İlk Moleküler Tayini ve Genotiplemesi

Öz

Encephalitozoon cuniculi tavşanlarda hastalık ajanı olarak ilk kez 1922 yılında tanınmıştır. Nörolojik hastalıklı laboratuvar tavşanlarından izole edilen *E. cuniculi* genotipi, genotip I olarak tanımlanmıştır. Bu parazit, gözde lens hasarına, bu da fakoklastik üveite ve katarakta neden olur. Intraokuler enfeksiyon sıklıkla transplasental bulaşma vakalarında ortaya çıkar. Türkiye'de parazitin moleküler tanısı hakkında herhangi bir rapor bulunmamaktadır. Bu çalışma, Türkiye'de moleküler yöntem kullanılarak *E. cuniculi* sporlarının saptanmasıyla ilgili ilk rapordur. Önceki çalışmamızda *E. cuniculi* enfeksiyonu seropozitif belirlenen bir yetiştirme tesisindeki tavşanlar, klinik semptomlar açısından beş yıl boyunca izlenildi. Enfeksiyonun kesin tanısı için otopsi yapıldı. Doku işleme prosedüründen sonra, doku örnekleri hematoksilen-eozin (H & E) boyamasına göre boyandı ve histopatolojik analiz yapıldı. Bununla birlikte, DNA ekstraksiyonu için otopsi sırasında örnekler alındı. E. cuniculi'nin amplifikasyonu ve genotiplemesi için ECUNF ve ECUNR türe özgü primer çifti kullanıldı. Hayvanlarda oküler lezyon (n=9) dışında klinik semptom görülmedi. Bu nedenle, bu tavşanlardan biri, kesin tanı koymak ve göze verilen zararı belirlemek için otopside kullanıldı. Histopatolojik olarak, gözdeki lezyonlar enfeksiyonun ilerleyici ilk veya orta evresinde bulundu. Bu çalışmada sunulan *E. cuniculi*'nin DNA dizisi, genotip I olduğunu göstermiştir. Tavşan gözündeki görünür beyaz kitlenin olası bir nedeni parazit enfeksiyonu olabilir. Bu nedenle klinisyenler, *E. cuniculi*'yi, günlük muayenede veya oftalmolojik muayenede tavşanlardaki göz lezyonlarının olası nedenlerinden biri olarak görebilirler.

Anahtar sözcükler: *Tavşan, Göz, Moleküler tanı, Encephalitozoon cuniculi, Filogenetik analiz, Türkiye*

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INTRODUCTION

Encephalitozoon cuniculi is an obligatory spore-forming, intracellular parasite belonging to the phylum Microsporidia. *E. cuniculi* was identified as a pathogen in rabbits for the first time by Wright and Craighead [1]. Presently, various reports of *E. cuniculi* infestation have been increasing in the fields of human and veterinary medicine. Therefore, the disease is well-documented, particularly in Lagomorphs. Moreover, molecular analysis has shown that *E. cuniculi* is divided into four genotypes according to the number of GTTT repeats in the internal transcribed spacer of the rDNA gene. Consequently, hitherto, these strains have been termed as the rabbit (genotype I), mouse (genotype II), dog (genotype III) and human (genotype IV) strains [2,3].

Diagnosis of encephalitozoonosis is difficult because most rabbits do not have clinical signs. Nonetheless, the infection has been diagnosed using different methodologies, such as microscopic identification, antibody detection or polymerase chain reaction (PCR). In pet, farm and laboratory rabbits, encephalitozoonosis is a problem affecting their health status. An opportunistic and zoonotic pathogen can have a significant impact on the results of experiments [4].

In Turkey, *E. cuniculi* infection in rabbits has been first reported histopathologically and later serologically using the carbon immunoassay test and histopathological test as diagnostic tools [5-8]. In addition, to the best of our knowledge, there has been no report on the molecular diagnosis of the parasite in Turkey. Therefore, this is the first molecular study on genotyping of *E. cuniculi* in Turkey.

MATERIAL and METHODS

Animals and Clinical Evaluation

The present study has been assessed and approved by the National Animal Experiment Central Ethics Board in accordance with the ethical regulation (2018/111334). According to our study [8] previously serologically reported the infection in the rabbit breeding facility licensed by the Ministry of Food, Agriculture and Livestock, therefore, the facility clinically monitored to prevent *E. cuniculi* infection throughout five years. The rabbits aged 6 to 28 months with a body weight of 1.5-2.5 kg in the colony were examined in terms of body condition, daily food, and water intake, appetite, and behaviour, in addition, was observed to the most common clinical symptoms such as torticollis, ataxia, aggressiveness, seizures, swaying, paralysis, ocular lesion. The animals were housed in the individual cages in standard room conditions. They were given ad libitum commercial diet and water.

Pathological Evaluation

In the case of any clinical symptoms in rabbits, as a further analysis, histopathological examination was performed to determine definitive diagnosis of the infection. For this reason, autopsy was performed to show the definite diagnosis of a rabbit infection according to clinical symptoms. Therefore, the rabbit was autopsied and organs were marcoscopically examined after euthanasia with the high dose anesthetic protocol (xylazine hydrochloride 60 mg/kg and ketamine hydrochloride 100 mg/kg via intramuscular injection). Tissues fixed in buffered 10% formalin for 24 h. Following routine procedures, the tissue samples were embedded in paraffin blocks. The sections 4 µm in thickness and were stained according to the Hematoxylin-Eosin (H&E) staining method and were examined under digital light microscope (Euromex). The slides were photographed.

DNA Extraction and PCR Amplification

The protozoan spores for positive control were obtained from the Department of Biology and Genetics, University of Veterinary Medicine in Kosice, Slovakia. The DNA extraction procedures for the spore and sample were run in parallel. Samples of aqueous humor and lens material were taken from the affected rabbit's eye during postmortem examination. Next, suitable specimens were homogenised with a ceramic bead in phosphate buffered saline (PBS) solution under sterile conditions. Subsequently, this suspension of PBS (200 µL) was exposed to mechanical microwave (600 W) disruption three times for 20 sec each. At this time, for the DNA extraction, tissue lysis buffer (Qiagen, Hilden, Germany) and 25 µL proteinase K (25 mg/mL) were used, and the specimens were incubated for one h at 56°C. Then, the specimens were processed in accordance with the manufacturer's instructions. In the last step of DNA purification, water was used to resuspend the DNA to use in PCR. The specific primer pairs ECUNF (5'-ATG AGA AGT GAT GTG TGT GCG-3) and ECUNR (5-TGC CAT GCA CTC ACA GGC ATC-3') were used for the amplification of 550-base pair (bp) small subunit ribosomal RNA product [9]. The standard PCR protocol was performed as described previously by Valencakova et al.[9]. The DNA yield obtained in a PCR reaction was analysed by electrophoresis in 1% agarose gel and stained with ethidium bromide.

DNA Sequence and Phylogenetic Tree

In accordance with the manufacturer's instructions, PCR amplicons were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) on the ABI PRISM 3700 Genetic Analyzer (Applied Biosystems). The phylogenetic tree was constructed using the neighbour-joining method with MEGA 4.0 software with the sequences chosen from GenBank records. The result of sequence analysis also verified DNA sequence data of the protozoan parasite by Basic Local Alignment Search Tool software (BLAST) analysis.

RESULTS

Animals and Clinical Evaluation

Feed and water consumption of the animals and general health conditions were normal. No neurological clinical signs and no mortalities were seen because of the parasite, however, ocular lesions with a visible white mass in nine rabbits was observed during clinical examination.

Histopathological Evaluation

One of nine animals showing ocular lesions was examined histopathologically to determine the damage in the eye and make a definite diagnosis of the infection. For this reason, the routine method was used to open the skull. Then, eye globes were removed from the orbital fossa based on techniques used for the species and cleaned from peripheral ligaments and other tissues. The changes in the outside and inside of eye globes were examined according to general macroscopic evaluation criteria. The sagittal sections of whole eyes were cut into sections and mounted with a nonaqueous mounting medium. According to result of histopathological examination, ocular discharge, shown by a yellowish colour and corneal opacity, was predominant in the left eye. The contralateral eye was observed to be normal in terms of opacity. In gross examination, corneal opacity was predominant in the left eye, in which the iris could not be detected due to opacity in contrast to the contralateral eye *(Fig. 1A)*. In this animal, after sagittally sectioning the right eye globe, iridocorneal sites appeared normal, although corneal thickness was increased in the left eye. The lens was also opaque in appearance and partially emulsified from the central side *(Fig. 1B)*. Other lesions were increased in corneal thickness in the left eye section. However, there were no other findings except corneal and lens lesions in both eye globes. Histopathologically, the anterior capsules of the ocular lens partially lost their lens epithelial cells (LECs) and were surrounded by a thick capsule. In particular, the central LECs were degenerated and disorganized. Many *E. cuniculi* parasites were attached to the anterior lens capsule and the capsule was ruptured or thinned in some areas *(Fig. 1C)*. The change in the left eye was diagnosed as cataract. Another prominent lesion was subacute keratitis.

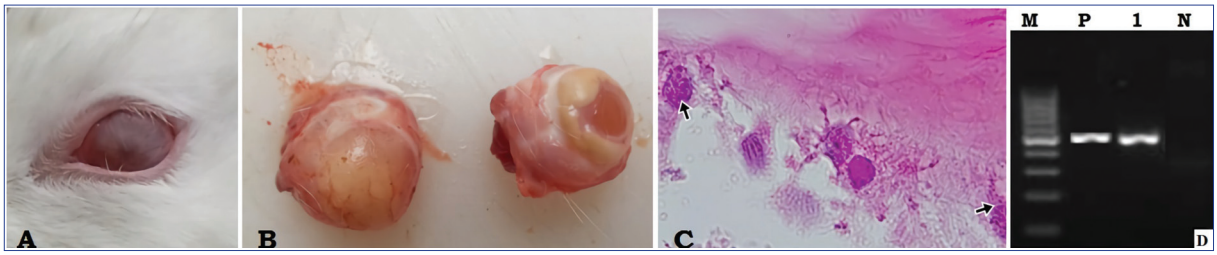

Fig 1. A- Clinical view of corneal opacity in left eye, **B**- After removing of eye bulbs, fully covered corneal opacity in the left eye, **C**- *Encephalitozoon cuniculi* spores (arrows) and rupture in fibrous lens capsule, **D**- Polymerase chain reaction was conducted on eye specimen of the rabbit using specific primer pairs. The sample was generated amplicons approximately 550 bp. In lanes 1, DNA sequences of amplicons produced and verified as *E. cuniculi* genotype I based on BLASTn analysis. Molecular weight (100bp repeat) ladder (M); Positive *E. cuniculi* control (P); Negative control (N)

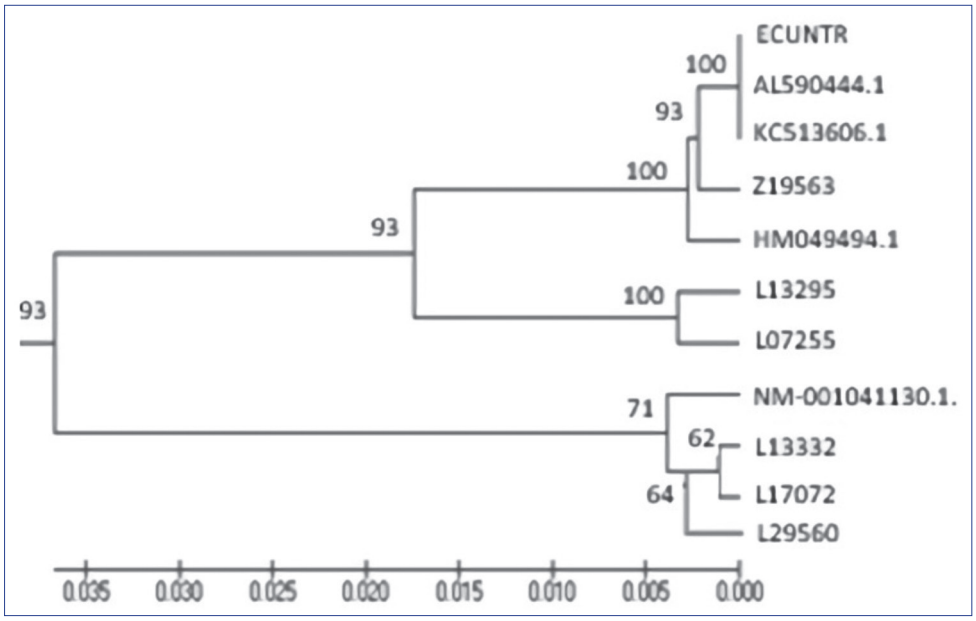

Fig 2. The phylogenetic tree was constructed with our sequence data (ECUNTR) from the NCBI database (L13295; L29560; L17072; L13332;L07255;Z19563;KC513606.1; HM049494.1; AL590444.1 and NM-001041130.1)

Bowman's capsule and Descement's membranes had lost their epithelial integrities. The stroma showed numerous neutrophils and mononuclear cell infiltrations, including macrophage and lymphocyte infiltrations. However, there were no parasitic infestations here.

Molecular Evaluation

In the present study, ECUNF and ECUNR species-specific primer pairs were used for amplification and genotyping of *E. cuniculi*. The sizes of fragments of the PCR products were compared using the standard 100 bp DNA ladder. Electrophoresis showed that the amplified products had 550 bp in *E. cuniculi (Fig. 1D)*. Database homology searching was performed with BLAST software (available in the public domain at https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Furthermore, the partial DNA sequence was sequenced to BLASTn analysis, and blasted with *E. cuniculi* (GenBank accession numbers L13295; L29560; L17072;L13332;L07255;Z19563;KC513606.1;HM049494.1; AL590444.1 and NM-001041130.1) and identified as genotype I. The phylogenetic tree was constructed with DNA sequences from the NCBI database. The phylogenetic tree was constructed using the neighbour-joining method and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown *Fig. 2*. Our sequence data (ECUNTR-550 bp) was blasted with *E. cuniculi* (GenBank accession numbers KC513606.1, HM049494.1 and NM-001041129.1). In addition to the sequence data blasted 99% homology with accession numbers AL590444.1 and NM-001041130.1 *(Fig. 2)*.

DISCUSSION

Four decades ago, the first record related to *E. cuniculi* was reported accidentally and characterised by focal granulomas in the brain of rabbits in Turkey [7]. In later years, the infection was identified serologically and histopathologically using diagnostic methods in rabbits [10]. This eukaryotic organism can be found in renal, eye and central nervous tissues of infected rabbits [6,12]. Transmission of the parasite occurs through the ingestion or inhalation of spores present in secretions or body exudates and transplacentally [11]. In the kidney, infection is usually asymptomatic. In the eye, this agent causes damage to the lens, causing phacoclastic uveitis, loss of vision, hypopyon and cataracts, particularly in cases of transplacental transmission [10,12-14].

In this study, the prominent lesions are seen in lens tissue, but how parasites access and infest the tissue could not fully be elucidated. In fact, an adult lens tissue comprises lens LECs and a thick fibrous capsule. Therefore, this avascular tissue does not permit infections in older animals. However, animals can be infected in utero (during the course of lens development) and in the early period of life, and parasites can settle on the thinner capsule with a richer vascular structure on the lens. As the animal grows (getting) older, the lesions can develop progressively in the lens. As a result of the infestation, cataract, uveitis and hypopyon can occur. Uveitis usually occurs as a result of spontaneous rupture of the anterior lens capsule after lens material, including proteins, is released into the anterior chamber. Cataracts also can develop as a result of disruption of the lens fibres. This situation frequently leads to granulomatous uveitis and a posterior synechia [11-14]. In particular, phacoclastic uveitis progresses in the intraocular lesions secondary to lens rupture in animals infested with *E. cuniculi* [12-14]. However, in our study, phacoclastic uveitis was not seen although lens fibre disorganisation and parasitic attachments on the thinned lens capsule. There were only focal lens ruptures in relation to disorganized fibrous structure and degenerated or necrotic LECs.

This situation was commented as progression of the infestation in the eye and being found initial or middle stage during ocular infestation. It was reported that ongoing stages of parasitic infestation, the lens materials in fibres lost their integrities. On the other hand, granulomatous inflammation in the affected ruptured area in the lens and uvea could be seen easily. Mainly, granulocytes, macrophages and foreign body giant cells cause the rupture of the lens capsule. In addition, plasma cells and lymphocyte infiltrations can develop in the iris and ciliary body. Diffuse lens fibre necrosis and degeneration and posterior lens epithelial cell migration can occur [11-13].

In our study, there was no granulomatous reaction in lens but only some granulocytes and macrophage infiltrations in the ruptured area. Sometimes, granulocytes and macrophages infiltrate fully into the posterior chamber and cause a series of results, including anterior iris displacement, anterior lens luxation, anterior synechia, and secondary corneal lesions in relation to endothelial-epithelial necrosis or ulceration and stromal thinning and focal corneal perforation in some cases [13,14].

In the present study, endothelial and epithelial integrities were lost in some areas. In addition, Bowman's capsule and Descement's membranes lost their epithelial integrities. The stroma showed numerous neutrophils and mononuclear cell infiltrations, including macrophage and lymphocyte infiltration. Neither stromal thinning nor any perforation in the cornea was encountered. In contrast to this finding, there was focal thickness in some areas due to inflammatory infiltration. Another outstanding finding is the lack of any parasitic infestation in the cornea. Therefore, we believe that corneal lesions can be resourced from secondary lesions after lens lesions. And, parasitic infestation was not evaded to cornea although lesions in the eye were found in initial or middle stage of progressive infestation.

Encephalitozoon cuniculi may be a possible reason for the ocular lesions, such as a visible white mass in the eye,

cataract and uveitis. Therefore, a clinician can observe ocular lesion formations in rabbits during daily inspection or ophthalmological examination. Presently, PCR has been accepted as a highly specific and sensitive molecular diagnostic tool for the detection of *E. cuniculi* spores [4,10]. Even though our previous study (unpublished data) reported on ocular lesions due to the infection, to our knowledge there has been no report on the molecular diagnosis of the parasite to date in Turkey. Therefore, this is the first report on the the detection of *E. cuniculi* spores using PCR in Turkey. The partial DNA sequence (ECUNTR-550 bp) was matched 100% homology with *E. cuniculi* (GenBank accession numbers KC513606.1, HM049494.1 and NM-001041129.1) whereas the ECUNTR sequence blasted 99% homology with accession numbers AL590444.1 and NM-001041130.1 *(Fig. 2)*. The homology between our sequence data (ECUNTR) and the GeneBank sequences confirmed the presence of *E. cuniculi* and verified it as genotype I.

In conclusion, the present study is the first report on the molecular diagnosis of the parasite in Turkey. Particularly eye affected from the parasite, therefore, clinicians are considered *Encephalitozoon cuniculi* as one of the possible causes of ocular lesion formation in rabbits.

Conflict of Interest

We declare that we have no conflict of interest.

Acknowledgments

We would like to thank Prof. Valenckova for providing *E.cuniculi* spore samples.

REFERENCES

1. Pelin A, Moteshareie H, Sak B, Selman M, Naor A, Eyahpaise MÈ, Farinelli L, Golshani A, Kvac M, Corradi N: The genome of an *Encephalitozoon cuniculi* type III strain reveals insights into the genetic diversity and mode of reproduction of a ubiquitous vertebrate pathogen. *Heredity,* 116 (5): 458-465, 2016. DOI: 10.1038/hdy.2016.4

2. Reetz J, Nöckler K, Reckinger S, Vargas MM, Weiske W, Broglia A: Identification of *Encephalitozoon cuniculi* genotype III and two novel genotypes of *Enterocytozoon bieneusi* in swine. *Parasitol Int,* 58 (3): 285-292, 2009. DOI: 10.1016/j.parint.2009.03.002

3. Talabani H, Sarfati C, Pillebout E, Van Gool T, Derouin F, Menotti J: Disseminated infection with a new genovar of *Encephalitozoon cuniculi* in a renal transplant recipient. *J Clin Microbiol*, 48, 2651-2653, 2010. DOI: 10.1128/JCM.02539-09

4. Csokai J, Joachim A, Gruber A, Tichy A, Pakozdy A, Künzel F: Diagnostic markers for encephalitozoonosis in pet rabbits. *Vet Parasitol,* 163 (1-2): 18-26, 2009. DOI: 10.1016/j.vetpar.2009.03.057

5. Berkin S, Kahraman MM: Türkiye'de tavşanlarda *Encephalitozoon (Nosema) cuniculi* enfeksiyonu. *Ankara Univ Vet Fak Derg,* 30, 397-406, 1983. DOI: 10.1501/Vetfak_0000000181

6. Eroksuz Y, Eroksuz H, Metin N, Ozer H: Morphologic examinations of cases of naturally acquired encephalitozoonosis in a rabbit colony. *Turk J Vet Anim Sci,* 23, 191-195, 1999.

7. Eroksuz H, Eroksuz Y, Ozer H, Cevik A, Unver O: A survey of *Encephalitozoon cuniculi* infection in rabbit colonies in Elazıg, Turkey: pathomorphologic and serologic (Carbonimmunoassay test) studies. *Isr J Vet Med,* 54 (3): 73-77, 1999.

8. Ozkan O, Ozkan AT, Karaer Z: Encephalitozoonosis in New Zealand rabbits and potential transmission risk. *Vet Parasitol,* 179 (1-3): 234-237, 2011. DOI: 10.1016/j.vetpar.2011.02.007

9. Valencáková A, Bálent P, Novotný F, Cisláková L: Application of specific primers in the diagnosis of Encephalitozoon spp. *Ann Agric Environ Med,* 12 (2): 321-323, 2005.

10. Künzel F, Fisher PG: Clinical signs, diagnosis, and treatment of *Encephalitozoon cuniculi* infection in rabbits. *Vet Clin North Am: Exot Anim Pract,* 21 (1): 69-82, 2018. DOI: 10.1016/j.cvex.2017.08.002

11. Baneux PJR, Pognan F: In utero transmission of *Encephalitozoon cuniculi* strain type I in rabbits. *Lab Anim,* 37, 132-138, 2003. DOI: 10.1258/00236770360563778

12. Williams D: Rabbit and rodent ophthalmology. *EJCAP,* 17 (3): 242-252, 2007.

13. Giordano C, Weigt A, Vercelli A, Grilli M, Giudice C: Immunohistochemical identification of *Encephalitozoon cuniculi* in phacoclastic uveitis in four rabbits. *Vet Ophthalmol,* 8, 271-275, 2005. DOI: 10.1111/j.1463-5224.2005.00394.x

14. Jeklova E, Leva L, Kovarcik K, Matiasovic J, Kummer V, Maskova J, Skoric M, Faldyna M: Experimental oral and ocular *Encephalitozoon cuniculi* infection in rabbits. *Parasitology,* 137 (12): 1749-1757, 2010. DOI: 10.1017/S0031182010000648

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Short Communication

Isolation and Molecular Characterization of Extended Spectrum Beta-Lactamase (ESBL) Producing *Escherichia coli* from Cage Birds in Adana Region, Turkey

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Abstract

In this study, it was aimed to investigate the presence of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* in caged birds. A total of 139 fecal swab samples were collected from cage birds from 14 different aviaries in Adana. ESBL producing *E. coli* was detected in 4.3% (n=6) of the fecal samples and these isolates harbored $bla_{CTX\text{-}M\text{-}15}$ gene. ERIC-PCR analysis revealed three different band patterns among the isolates. These results indicate that cage birds are carrier of ESBL-producing *E. coli*. Therefore, further epidemiological studies are needed to determine the presence of resistant bacteria including ESBL producing *E. coli* in various animal species.

Keywords: *Cage birds, Extended spectrum beta-lactamase (ESBL), Escherichia coli, ERIC-PCR*

Adana Yöresinde Kafes Kuşlarından Geniş Spektrumlu Beta-Laktamaz (GSBL) Sentezleyen *Escherichia coli* İzolasyonu ve Moleküler Karakterizasyonu

Öz

Bu çalışmada, kafes kuşlarında geniş spektrumlu beta-laktamaz (ESBL) sentezleyen *Escherichia coli* varlığının araştırılması amaçlandı. Bu amaçla Adana'daki 14 farklı kafes kuşu satışı yapan işyerinden toplam 139 fekal svab örneği toplandı. İncelenen örneklerin %4.3'ünden (n=6) GSBL sentezleyen *E. coli* saptandı ve izolatların tamamında $bla_{CTX\text{-}M\text{-}15}$ geni belirlendi. ERIC-PCR analizi izolatlar arasında üç farklı band patterni gösterdi. Bu sonuçlar kafes kuşlarının ESBL sentezleyen *E. coli* için taşıyıcı olduğunu göstermektedir. Bu nedenle, çeşitli hayvan türlerinde ve ortamlarda ESBL sentezleyen *E. coli* dahil dirençli bakterilerin varlığını belirlemek için daha fazla epidemiyolojik çalışmalara ihtiyaç vardır.

Anahtar sözcükler: *Kafes kuşları, Geniş spektrumlu beta laktamaz (GSBL), Escherichia coli, ERIC-PCR*

INTRODUCTION

Antimicrobial resistant microorganisms are globally major concern for animal and public health due to limited therapy options [1]. One of the important resistance mechanism encountered in the members of Enterobactericae family is resistance to extended-spectrum cephalosporins mediated by extended-spectrum beta-lactamase (ESBL) synthesis. ESBLs have high hydrolytic activity against the majority of beta-lactams, including cephalosporins but not against carbapenems (e.g. meropenem, imipenem, ertapenem) and cephamycins (e.g. cefoxitin, cefotetan). Their activities are completely or partially inhibited by beta-lactamase inhibitors such as clavulanic acid, tazobactam, sulbactam [2]. ESBL are mainly mediated by CTX-M, SHV and TEM enzymes. The genes encoding these enzymes are frequently localized on plasmids, leading these genes to spread easily within species as well as within genera. Prior to 2000, TEM and SHV were dominant ESBLs types, but after this date CTX-M became dominant ESBL type all over

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the world [3]. In recent years, presence of ESBL-producing bacteria have started to receive more attention in different bird species (e.g. seagulls, migratory birds, birds of prey, pigeon) and it has been shown that these bacteria were found in these animals with a various prevalence rates [4-7]. However, the data has been very scarce on the presence of these bacteria in cage birds [8].

The aim of this study was (i) to investigate the potential role of cage birds as carriers of ESBL producing *E. coli* and (ii) to determine antimicrobial susceptibilities and clonal relationship of the isolates.

MATERIAL and METHODS

Sample Collection

Sampling was randomly performed in 14 different aviaries in Adana, Turkey between March 2017 and June 2017. The birds were frequently cage-bred alone or/and in groups. The aviaries also housed imported birds. A total of 139 fresh faecal samples were collected from each cage using Stuart Transport Medium (LP Italiana, 118898, Italy). The samples were taken from fresh stool droplets of the same type of birds held in each cage and were considered as a single sample. Distribution of cage birds species was given in *Table 1*.

Selective Isolation and Phenotypic Confirmation of ESBL/pAmpC Producing E. Coli

Faecal swab samples were incubated in buffered peptone water (BPW) (Merck, Germany, 107228) at 37°C for overnight under aerobic conditions. Then, 100 µL of culture was inoculated onto Eosin Methylen Blue (EMB) agar (Merck, Germany, 101347) supplemented with 2 µl/mL cefotaxime (Sigma, Germany) and incubated 37°C for 24 h under aerobic conditions. One colony per plate with typical metallic sheen appearance was randomly selected and subcultured onto Blood agar (Merck, Germany, 110886) supplemented with 7% defibrinated sheep blood in order to obtain pure culture. Identification was performed based on biochemical tests (Gram staining, IMVIC tests, oxidase, catalase) [9] and confirmed by polymerase chain reaction (PCR) for amplification of *E. coli* specific 16S RNA using E16Sa-CCCCCTGGACGAAGACTGAC and E16Sb-ACCGCTGGCAACAAAGGATA primers [10]. Phenotypic confirmation of ESBL producers was done by disc combination method [11] and double disc synergy method [12].

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of ESBL producing *E. coli* isolates was determined by disc diffusion method as per Clinical Laboratory Standards Institute (CLSI) guidelines [11]. The antimicrobials (Bioanalyse, Turkey) tested were: ampicillin (AM, 10 µg), amoxicillin-clavulanic acid (AMC, 10/20 µg), nalidixic acid (NA, 30 µg), ciprofloxacin (CIP, 5 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg) cefepime (FEB, 30 µg), cefoxitin (FOX, 30 µg), aztreonam (ATM, 30 µg), imipenem (IMP, 10 µg), chloramphenicol (30 µg), streptomycin (S, 10 µg), gentamicin (CN, 10 µg), tobramycin (TOB, 10 µg), amikacin (AK, 10 µg), kanamycin (K, 30 µg), tetracycline (TE, 30 µg) and sulfametoxazole-trimethoprim (SXT, 1.25/23.75 µg). *E. coli*

Table 1. *Distribution of cage birds sampled in the study*

Aviary Code	Number of Collected Samples	Species of Cage Birds (Sample Number)
A	25	*Melopsittacus undulatus* (11), *Taeniopygia guttata* (5), *Erythrura gouldiae* (4), *Nymphicus hollandicus* (3), *Agapornis roseicollis* (1), *Serinus canaria* (1)
B	7	*Melopsittacus undulatus* (2), *Hippolais icterina* (1), *Psittacus erithacus* (1), *Cacatua galerita* (1), *Serinus canaria* (1), *Agapornis roseicollis* (1)
C	3	*Melopsittacus undulatus* (2), *Fringilla coelebs* (1)
D	8	*Melopsittacus undulatus* (7), *Serinus canaria* (1)
E	45	*Serinus canaria* (45)
F	3	*Melopsittacus undulatus* (3)
G	3	*Melopsittacus undulatus* (3)
H	2	*Melopsittacus undulatus* (1), *Serinus canaria* (1)
I	5	*Melopsittacus undulatus* (3), *Serinus canaria* (1), *Agapornis roseicollis* (1)
J	4	*Melopsittacus undulatus* (2), *Agapornis roseicollis* (1), *Nymphicus hollandicus* (1)
K	10	*Melopsittacus undulatus* (4), *Taeniopygia guttata* (1), *Psittacus erithacus* (1), *Serinus canaria* (1), *Poicephalus senegalus* (1), *Agapornis roseicollis* (1), *Fringilla coelebs* (1)
L	6	*Melopsittacus undulatus* (4), *Nymphicus hollandicus* (2)
M	3	*Melopsittacus undulatus* (1), *Psittacus erithacus* (1), *Taeniopygia guttata* (1)
N	15	*Melopsittacus undulatus* (7), *Psittacus erithacus* (1), *Fringilla coelebs* (1), *Serinus canaria* (1), *Platycercus Flaveolus* (1), *Taeniopygia guttata* (1), *Geopelia cuneata* (1), *Psittacus erithacus* (1), *Nymphicus hollandicus* (1)
Total	139	

ATCC 25922 was used as quality control strain. The isolates showing resistance to three or more different class of antimicrobials were considered as multi-drug resistant (MDR).

DNA Isolation

DNA isolation was done using boiling method as previously reported by Ahmed *et al.*[13].

PCR Detection of ESBL Genes and Sequencing

Presence of ESBL genes (bla_{TEM}, bla_{OXA}, bla_{SHV} and bla_{CTX-M}) were screened by PCR as previously described [13]. Amplified PCR products in ESBL positive isolates were sequenced from both ends using sequencing primers for the determination of the exact subtypes of beta-lactamase genes (Medsantek, İstanbul, Turkey) and compared with deposited sequences in the GenBank database (NCBI) (https://www.ncbi.nlm.nih.gov).

Determination of Plasmid Mediated Quinolone Resistance (PMQR) Genes

PMQR genes *qnr*A, *qnr*B and *qnr*C genes were investigated as previously suggested by Kim *et al.*[14].

Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

The clonal relationship among ESBL producing *E. coli* isolates was determined as described previously by Versalovic *et al.*[15], using hsdR+2758-5′-CAGCCATGAACAACTGGTGGCG-3′ and hsdR+3235R-5′-TGCTTTGCGCAGGGAAGATTCC-3′ primers.

Computer-Assisted ERIC-PCR Fingerprint Analysis

ERIC-PCR data were recorded to generate a binary matrix as either (1) for the presence of a band and (0) for its absence and analyzed using NTSYS-pc software (version 2.02 K, Applied Biostatistics, Inc., NY, USA). The similarity between the strains was determined on the basis of the Jaccard coefficients. The dendrogram was constructed using the UPGMA (Unweighted Pair Group Method Arithmetic Average) method.

RESULTS

ESBL producing *E. coli* was isolated from six (4.3%) of 139 fecal samples. ESBL producing *E. coli* was isolated from three *Melopsittacus undulatus*, one in aviary D and two in aviary L, two *Serinus canaria* in aviary E, and one *Fringilla coelebs* in aviary K. Isolated isolates were also confirmed to be as *E. coli* by PCR amplification of 16S rRNA gene *(Fig. 1)*.

All isolates were positive for $bla_{CTX-M-15}$. *qnr* genes were detected in 5 (83.3%) isolates and of which three isolates carried *qnr*S, one isolate carried *qnr*B and one isolate carried both *qnr*B and *qnr*S *(Fig. 2)*.

Antimicrobial susceptibility testing results revealed that all isolates were resistant to ampicillin, amoxicillin-clavulanic acid, cefotaxime, ceftazidime, but susceptible to imipenem, nalidixic acid, ciprofloxacin, cefepime, amikacin. Various resistance rates to sulfametoxazole-trimethoprim (5; 83.3%), streptomycin (5; 83.3%), tetracycline (5; 83.3%), aztreonam

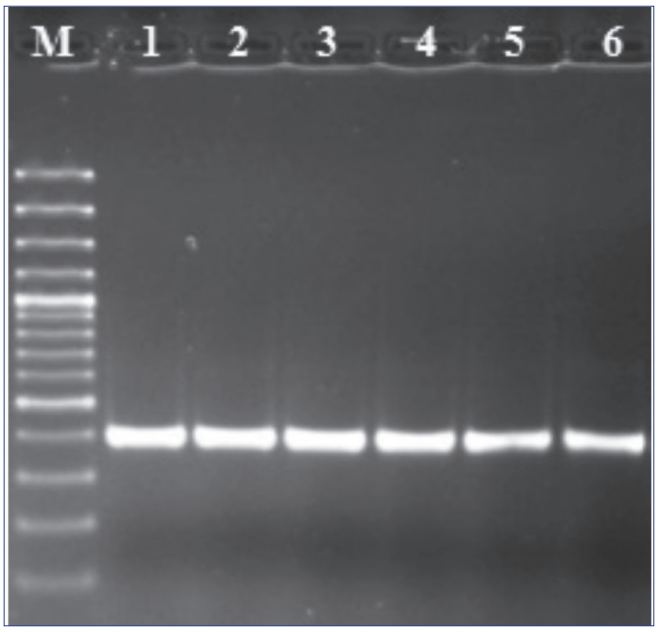


Fig 1. PCR amplification of *E. coli* spesific 16S rRNA gene (401 bp). Lane M: 100 bp plus molecular marker, Lane 1-6: 16S rRNA positive isolates

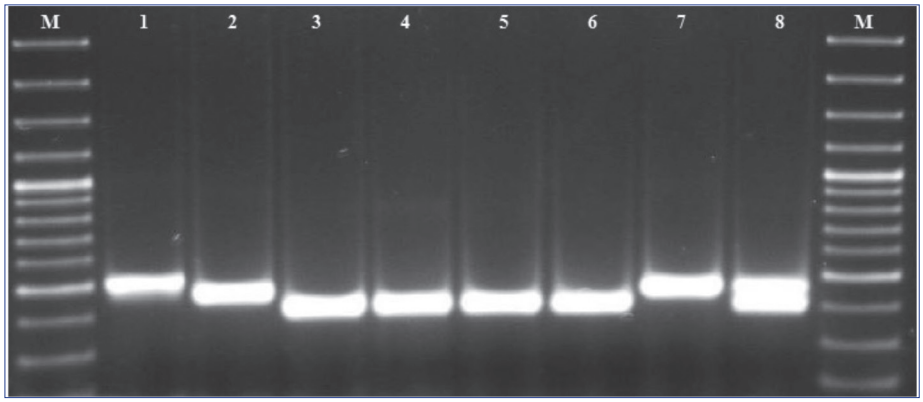
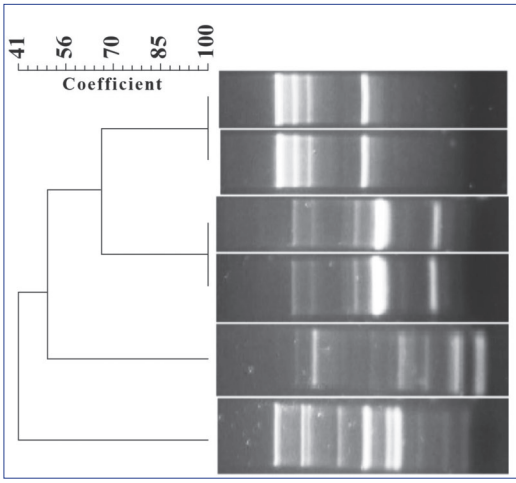


Fig 2. Agarose gel electrophoresis of PMQR genes. Lane M: 100 bp plus molecular marker, Lane 1: *qnr*A (516 bp) positive control, Lane 2: *qnr*B (476 bp) positive control, Lane 3: *qnr*S (428 bp) positive control, Lane 4-6: *qnr*S positive samples, Lane 7: *qnr*B positive sample and Lane 8: *qnr*B and *qnr*S positive sample



Isolate ID (Aviary)	ERIC-PCR Profile	ESBL Type	PMQR Genes	Resistance to non-β-lactams
26 (K)	A	CTX-M-15	*qnr*S	SXT, TE, S
27 (L)	A	CTX-M-15	*qnr*S	SXT, TE, S
29 (E)	B	CTX-M-15	*qnr*S	TOB, SXT, S, CN
41 (E)	B	CTX-M-15	*qnr*B	TOB, SXT, TE, C, S, CN, K
17 (L)	C	CTX-M-15	*qnr*S, *qnr*B	SXT, TE, C, S, K
14 (D)	D	CTX-M-15	-	TE, C

Fig 3. Dendogram showing the results of ERIC-PCR of six ESBL producing *E. coli* isolates. ERIC-PCR profiles are indicated as capital letters. ERIC-PCR groups A, B, C and D consisted of the isolates having a similarity coefficient ≥85%. The scale bar given on the top indicates similarity percentages detected for ERIC-PCR types

(4; 66.7%), chloramphenicol (3; 50%), cefoxitin (3; 50%), gentamicin (2; 33.3%), tobramycin (2; 33.3%), and kanamycin (2; 33.3%) were determined *(Fig. 3)*. According to ERIC-PCR analysis, four different band patterns were detected among the isolates *(Fig. 3)*.

DISCUSSION

In previous studies [4-7], carriage of ESBL producing *E. coli* has been well documented in various wild avian species including migratory birds, birds of prey and waterfowl. However, there is a paucity of the carriage of ESBL producing *E. coli* in cage birds both in the world and in Turkey. We therefore investigated the occurence of ESBL producing *E. coli* in cage birds. The present study revealed low carriage rate 4.3% (6/139) ESBL producing *E. coli* in cage birds. Recently, a low carriage rate of 2.7% (4/148) in cage birds was also reported by Yılmaz and Dolar [8]. In contrast, higher carrige rates have been reported in migratory birds from Pakistan (17.3%) [6], in birds of prey from Portugal 26.9% [7], and in waterbirds from Poland (10.5%) [5]. However, in Germany, Guenther *et al.*[4] reported a low carriage rate (2.3%) in wild birds. Various prevalence rates of ESBL producing *E. coli* in different bird population might be explained by different ecological niches, human influence and antibiotic exposure.

CTX-M-15 was only the ESBL genotype detected among six ESBL producing *E. coli* isolates in the study. This finding is in agreement with previous study carried out by Yılmaz and Dolar [8], who detected $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-1}}$ together with $bla_{\text{TEM-1}}$ among the isolates. Similarly, CTX-M genotype has been reported to be the dominant genotype in ESBL producing *E. coli* from different wild avian species [4-7]. These data are also in coherence with human cases in Turkey, where CTX-M-15 is the most common genotype reported in ESBL producing *E. coli* isolates from various clinical settings [16,17]. Despite the lack of spesific molecular data, it might be speculated that current carriage status of ESBL producing *E. coli* in cage birds is of anthropogenic nature. Worldwide distribution of CTX-M is explained by localization these genes on plasmid, which facilitates the dissemination of these genes within species and among genera [3].

Another important cause of concern encountered in ESBL strains is that these isolates are frequently resistant to different class of antimicrobials. Because ESBL genes are localized on plasmids and these plasmids also carry other resistance genes to other antimicrobials such as aminoglycosides, trimethoprim-sulfamethoxazole, chloramphenicol, tetracycline and quinolone. Infectious diseases that may arise from multiresistant ESBL strains is a major cause of concern for both human and animal health because due to limited treatment options. Indeed, all isolates showed a MDR phenotype. Similarly, Yılmaz and Dolar [8] found all ESBL producing *E. coli* isolates as MDR. The authors also stressed that usage of non-beta lactam antibiotics may lead selection of ESBL genes.

Plasmid-mediated resistance is of growing clinical concern due to transfer resistance genes to other species via horizontal gene transfer, conferring resistance quinolones. In addition, co-existence of ESBL and qnr genes on the same plasmid has been reported [18]. The *qnr* proteins are capable to protect DNA gyrase from quinolones by binding this gene. Although all isolates were found to be susceptible to ciprofloxacin and nalidixic acid, *qnr* genes were detected in 83.3% of the isolates in the study. The *qnr* genes have been shown to be responsible for *in vitro* low-level quinolone resistance, but facilitate the emergence of high-level resistance in the presence of quinolones in therapeutic levels [19].

ERIC-PCR has been shown to be a useful method for investigating clonal relationship among multi-resistant

Enterobactericeae isolates [20]. In the study, ERIC-PCR analysis revealed four different pattern among the isolates. In a previous study, Yılmaz and Dolar [8] found two identical pattern among four ESBL isolates using pulsed field gel electrophoresis (PFGE) analysis.

In conclusion, this study shows that different cage bird species are colonized with ESBL-producing *E. coli* strains, even at low rates. Since cage birds are close contact with humans, these bacteria might be transmitted from birds to humans or vice versa. Therefore, there is a need to further epidemiologic studies to gain better understanding of transmission route of this bacterium among birds, humans and environment.

Conflicts of Interest

The authors declare no conflict of interest.

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REFERENCES

1. World Health Organization (WHO): Antimicrobial resistance: global report on surveillance. 2014. http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf?ua=1; *Accessed:* 13.03.2018.

2. Bradford PA: Extended-spectrum beta-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev*, 14 (4): 933-951, 2001. DOI: 10.1128/CMR.14.4.933-951.2001

3. von Wintersdorff CJH, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB, Savelkoul PHM, Wolffs PFG: Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Front Microbiol*, 7, 173, 2016. DOI: 10.3389/fmicb.2016.00173

4. Guenther S, Grobbel M, Beutlich J, Bethe A, Friedrich ND, Goedecke A, Lübke-Becker A, Guerra B, Wieler LH, Ewers C: CTX-M-15-type extended-spectrum beta-lactamases-producing *Escherichia coli* from wild birds in Germany. *Environ Microbiol Rep*, 2 (5): 641-645, 2010. DOI: 10.1111/j.1758-2229.2010.00148.x

5. Literak I, Dolejska M, Janoszowska D, Hrusakova J, Meissner W, Rzyska H, Bzoma S, Cizek A: Antibiotic-resistant *Escherichia coli* bacteria, including strains with genes encoding the extended-spectrum beta-lactamase and QnrS, in waterbirds on the Baltic Sea Coast of Poland. *Appl Environ Microbiol*, 76 (24): 8126-8134, 2010. DOI: 10.1128/AEM.01446-10

6. Mohsin M, Raza S, Schaufler K, Roschanski N, Sarwar F, Semmler T, Schierack P, Guenther S: High prevalence of CTX-M-15-Type ESBL-producing *E. coli* from migratory avian species in Pakistan. *Front Microbiol*, 8, 2476, 2017. DOI: 10.3389/fmicb.2017.02476

7. Pinto L, Radhouani H, Coelho C, Martins da Costa P, Simões R, Brandão RML, Torres C, Igrejas G, Poeta P: Genetic detection of extended-spectrum beta-lactamase-containing *Escherichia coli* isolates from birds of prey from Serra da Estrela Natural Reserve in Portugal. *Appl Environ Microbiol*, 76 (12): 4118-4120, 2010. DOI: 10.1128/AEM.02761-09

8. Yılmaz EŞ, Dolar A: Detection of extended-spectrum β-lactamases in *Escherichia coli* from cage birds. *J Exot Pet Med*, 26 (1): 13-18, 2017. DOI: 10.1053/j.jepm.2016.10.008

9. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn JrWC: Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed., 171-241, Lippincott Williams & Wilkins, Philadelphia, 1997.

10. Wang G, Clark CG, Rodgers FG: Detection in *Escherichia coli* of the genes encoding the major virulence factors, the genes defining the O157:H7 serotype, and components of the type 2 Shiga toxin family by multiplex PCR. *J Clin Microbiol*, 40 (10): 3613-3619, 2002. DOI: 10.1128/JCM.40.10.3613-3619.2002

11. CLSI: Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement, *CLSI document M100-S22*, Wayne, PA: Clinical and Laboratory Standart Institute; 2012.

12. Jarlier V, Nicolas MH, Fournier G, Philippon A: Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: Hospital prevalence and susceptibility patterns. *Rev Infect Dis*, 10 (4): 867-878, 1988. DOI: 10.1093/clinids/10.4.867

13. Ahmed AM, Motoi Y, Sato M, Maruyama A, Watanabe H, Fukumoto Y, Shimamoto, T: Zoo animals as a reservoir of Gram negative bacteria harboring integrons and antimicrobial resistance genes. *Appl Environ Microbiol*, 73, 6686-6690, 2007. DOI: 10.1128/AEM.01054-07

14. Kim HB, Park CH, Kim CJ, Kim EC, Jacoby GA, Hooper DC: Prevalence of plasmid-mediated quinolone resistance determinants over a 9-year period. *Antimicrob Agents Chemother,* 53 (2): 639-645, 2009. DOI: 10.1128/AAC.01051-08

15. Versalovic J, Koeuth T, Lupski JR: Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res*, 19 (24): 6823-6831, 1991. DOI: 10.1093/nar/19.24.6823

16. Gür D, Gülay Z, Arkan Akan O, Aktaş Z, Bal Kayacan C, Cakici O, Eraç B, Gültekin M, Oğünç D, Söyletir G, Unal N, Uysal S: Resistance to newer beta-lactams and related ESBL types in gram-negative nosocomial isolates in Turkish hospitals: Results of the multicentre HITIT study. *Mikrobiyol Bul*, 42 (4): 537-544, 2008.

17. Yumuk Z, Afacan G, Nicolas-Chanoine MH, Sotto A, Lavigne JP: Turkey: A further country concerned by community-acquired *Escherichia coli* clone O25-ST131 producing CTX-M-15. *J Antimicrob Chemother*, 62 (2): 284-288, 2008. DOI: 10.1093/jac/dkn181

18. Rawat D, Nair D: Extended-spectrum β-lactamases in Gram negative bacteria. *J Glob Infect Dis*, 2 (3): 263-274, 2010. DOI: 10.4103/0974-777X.68531

19. Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A: Plasmid-mediated quinolone resistance: A multifaceted threat. *Clin Microbiol Rev*, 22 (4): 664-689, 2009. DOI: 10.1128/CMR.00016-09

20. Eckert C, Gautier V, Saladin-Allard M, Hidri N, Verdet C, Ould-Hocine Z, Barnaud G, Delisle F, Rossier A, Lambert T, Philippon A, Arlet G: Dissemination of CTX-M-type beta-lactamases among clinical isolates of *Enterobacteriaceae* in Paris, France. *Antimicrob Agents Chemother*, 48 (4): 1249-1255, 2004. DOI: 10.1128/AAC.48.4.1249-1255.2004

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Case Report

Oophorectomy with Plastronotomy in a Red-Eared Slider

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Abstract

An 8-year-old female red-eared slider *(Trachemys scripta elegans)* was presented to our clinics with appettite loss for 3 months, dyspnea and weight loss. After clinical examination, complete blood counts, radiography, ultrasonography and computed tomography has been used for diagnosis. All diagnostic tests with detailed clinical examination clearly showed that the patient had follicular stasis. Plastronotomy was performed to enter the coelomic cavity under general anesthesia. At least 20 Follicles were removed with oophorectomy and the plastronotomy gap was closed. The patient was hospitalized for a week and then discharged with full recovery. The patient has died 10 days after discharging.

Keywords: *Plastronotomy, Oophorectomy, Turtle, Follicular stasis*

Bir Kızıl Yanaklı Su Kaplumbağasında Plastronotomi İle Ooforektomi

Öz

Sekiz yaşındaki dişi kızıl yanaklı su kaplumbağası *(Trachemys scripta elegans)* 3 aydır iştahsızlık, solunum güçlüğü ve zayıflama şikayeti ile kliniğimize getirildi. Hastanın detaylı klinik muayenesi, kan tahlilleri, radyolojik, ultrasonografik ve tomografik değerlendirmeleri sonucunda hastada foliküler staz olduğu tespit edildi. Genel anestezi altında plastronotomi uygulanarak solöm boşluğuna girildi ve ooforektomi ile en az 20 folikül uzaklaştırıldı ve plastronotomi açıklığı kapatıldı. Hasta bir hafta kliniğimizde hospitalize edildi ve ardından sağlıklı bir şekilde taburcu edildi. Hasta taburcu edildikten 10 gün sonra kaybedildi.

Anahtar sözcükler: *Plastronotomi, Ooforektomi, Kaplumbağa, Foliküler staz*

INTRODUCTION

Red-eared slider is a semi aquatic turtle that belongs to the family Emydidae. In captive, female red-eared terrapins reproductive disorders are relatively common. These reproductive disorders include oophoritis, salpingitis, dystocia, ectopic eggs and follicular stasis. Follicular stasis is the condition in which the follicle remains in the ovary and has not been able to progress to ovulation. Follicles produce anorexia and blockage effects and in some cases follicles are weigh up to 15% of body weight [1,2].

Follicular stasis is mostly seen in female turtles that had a recent exposure to a male prior to isolation [3]. Follicles that neither ovulate nor regress can become calcific or necrotic. Clinical signs includes anorexia, lethargy and appetite loss. Non-ovulated follicules can be seen with different imaging techniques such as radiography, ultrasonography, computed tomography (CT) and endoscopy. Also, blood tests (complete blood cell count (CBC) and blood biochemistry) can be helpful for diagnosis [2].

Proligestone may given for the treatment of the follicular stasis however has not been successful in most cases [6]. For this reason, surgical removal of follicules (oophorectomy) is accepted as more suitable treatment option. An oophorectomy has two major approaches; transplastron and pre-femoral approach to coelom [2-5].

CASE HISTORY

An 8-year-old, female red-eared slider was presented to

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our clinics with 3 months of appetite loss, dyspnea and anorexia. Patient's husbandry conditions and nutrition status were not optimal. The weight of the turtle was 1300 g. Blood for CBC is collected from subcarapacial sinus. The blood smear was made for the blood cell counts. Complete blood cell values were within normal limits *(Table 1)*. Only generalised pulmonary oedema was seen on dorso-ventral, cranio-caudal and latero-lateral radiographic views *(Fig. 1)*. Ultrasonographic examinations (Terason 2000, Samsung, Chine) were performed within right and left prefemoral spaces. 10 MHz multi-convex transducer was used for this

Table 1. *CBC parameters of the red-eared slider (Trachemys scripta elegans)*

Parameters	Patient	Normal Values
WBC (x 10^3/µL)	10	3.2-25.5
RBC (x 10^6/µL)	0.5	0.3-0.8
Hct (%)	30	25-33
Hgb (g/dL)	9	8
Heterophil (%)	35	36
Lmphocyte (%)	43	24

premedication, butorphanol (Butomidor-Richterfarma Austria) 0.4 mg/kg IM and meloxicam (Melox -Nobel Turkey) 0.2 mg/kg IM were administered. After that, medetomidine 0.1 mg/kg IM (Domitor Pfizer USA) was used for induction. Following adequate sedation, patient was intubated with a number 2 non-cuffed intubation tube and anaesthesia continued with 2% isoflurane (Forane-Abbott England). During the surgery, anaesthesia was maintained with assisted breathing.

For the surgical procedure the patient was positioned in dorsal recumbency and surgical area was prepared with povidone iodine (Batticon antiseptic ADEKA Turkey). The area was limited with sterile covers. A sterile orthopedic saw with a fine tip was used for central plastron osteotomy. The area was soaked with isotonic serum to reduce heat necrosis that may occur during the incision. To expose coelomic cavity, three sides of abdominal scute were cut exactly, the fourth side was half cut and considered to work as a hinge. The flap connections were separated using an osteotome and the fourth edge was not touched. The follicles seen in the coelomic space were carefully removed from the incision area. Fine needle aspiration was used for bigger

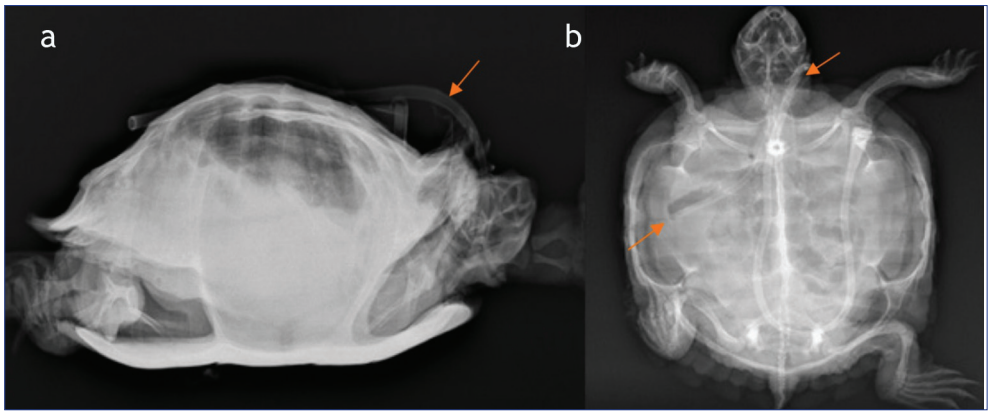

Fig 1. a- LL views of the turtle. Pneumonia was detected and nutrional probe can be seen, **b-** VD view of the turtle

purpose. 1.21x1.47 cm and 1.02x1.26 cm follicles were detected in front of the urinary bladder in ultrasonography *(Fig. 2)*. Computed tomography (Shimadzu AX-180G-2004 Kyoto) also showed multiple follicules and collapsed left lung lobe *(Fig. 3)*.

Because of the anorexia and to minimise the stress of repeated assist feeding, an esophagostomy tube was placed. The patient was fed 2% of its weight each day with a special diet (Oxbow Critical Care). Marbofloxacin (Marbocyl-Novakim Turkey) 2 mg/kg/SID IM, for 5 d and meloxicam (Melox -Nobel Turkey) 0.2 mg/kg/SID IM for 10 d were started initially for the treatment of the pneumonia.

One week later, after the patients clinicial condition has gotten better, the follicles were decided to removed with plastronotomy. For

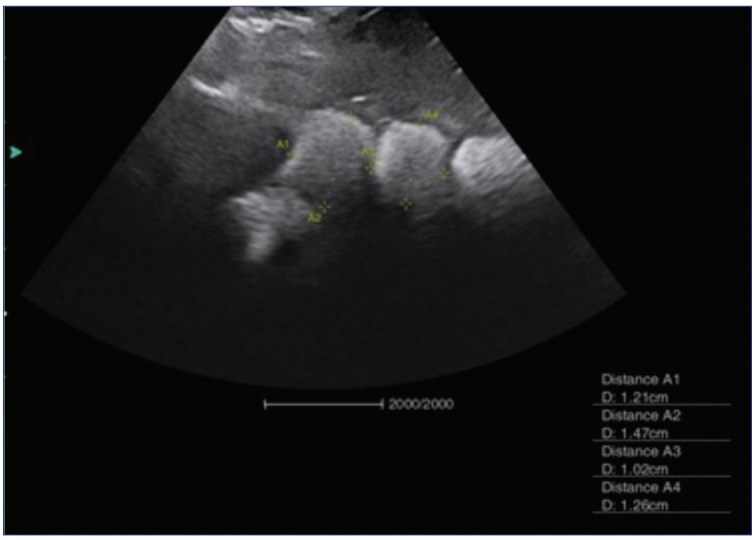

Fig 2. Follicules were seen in USG from prefemoral area. The sizes of the follicules were on the right corner of the figure

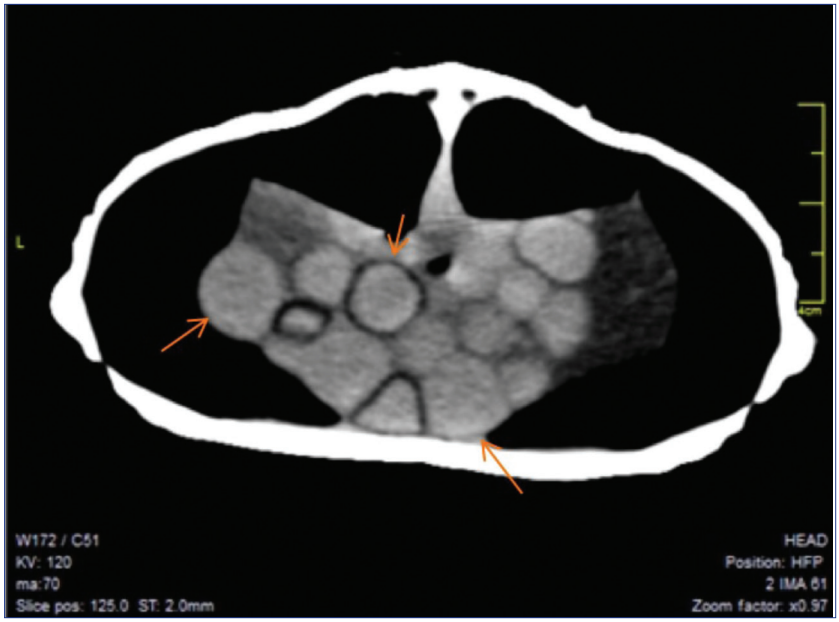

Fig 3. CT view of the turtle. Multiple folliculles were detected

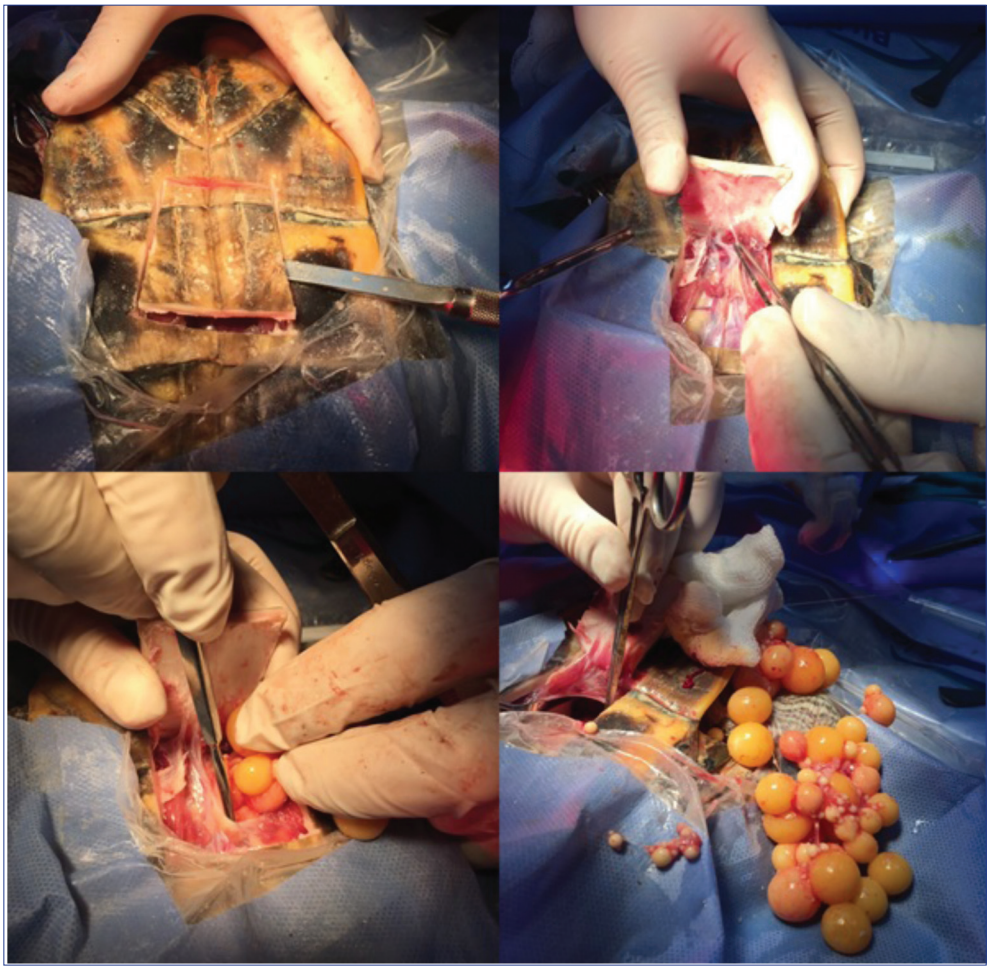

Fig 4. Central plastronotomy, oophrectomy and flap detection

follicules for easier removal. The coelomic membrane was sutured with a absorbable monofilament 4/0 polyglactin 910 (Vicryl Ethicon). The flap was then closed and the plastron was fixed by applying mini-plates and screws on the three opened sides. The plastronotomy area on the three sides was covered with manuka honey and covered with a gauze soaked with isotonic saline *(Fig. 4)*. Intracoelomic 0.9% saline (İE.Ulugay Turkey) was administered preoperatively and postoperatively at a dose of 20 mg/kg. After the anaesthesia, the

patient was extubated and kept in intensive care unit. The postoperative weight of the patient was determined as 1050 g. The patient was hospitalized for postoperative care for five d, and postoperative antibiotics (Enrofloxacin 5 mg/kg for 1 week) and nonsteroidal anti-inflammatory drugs (Meloxicam 0.2 mg/kg for 1 week) were administered and discharged during the period when the patient was fully recovered. However, 10 d after discharging, it was learned from the owner that the patient had died, and necropsy could not be done due to the owner's request.

DISCUSSION

Although the causes of retained follicles are unclear, previously discussed reasons may involved like the absence of stimules from a male. Especially because of this reason in addition with the poor husbandry conditions, reproductive disorders such as oophoritis, salpingitis, dystocia, retained or ectopic eggs and follicular stasis are common in captive turtles [1,5,6]. The accumulation of follicles in the animal can cause anorexia. In some cases, the follicles comprise 10-15% of the body weight [3,4]. In this case report, it was determined that the weight of follicles removed from the turtle constituted approximately 19% (250 g) of body weight.

Although endoscopy is the preferred method; ultrasonography and computed tomography are also an effective methods for diagnosing follicular stasis. However radiography is not a very effective option unless the eggs are calcified [5].

In the case of follicular stasis, the increase in circulating lipid and protein is indicated as the cause of loss of appetite [6]. For the closure of the flap, adhesives such as polymethylmethacrylate, epoxy resin, fiberglass etc. can be used [1,2]. Also, for the stabilization, mini-plate and screw application was better than other methods and there are not any toxic effects when compared to acrylic-like chemicals. In this pathology, the most effective treatment methods are the removal of follicles by prefemoral coelotomy and central plastronotomy. Prefemoral approach, compared to plastronotomy, is considered to be less invasive but its inadequate and requires advanced experience when using in small-sized tortoises. For this reason central plastronotomy was preferred in the treatment of this case [1,2].

REFERENCES

1. Chitly A, Raftery A: Follicular stasis. **In,** Chitly A, Raftery A (Eds): essentials of tortoise medicine and surgery. 1st edn., 200-204, Wiley Blackwell, United Kingdom, 2013.

2. Mader DR: Surgery. **In,** Mader DR (Ed): Reptile Medicine and Surgery. 2nd edn., 787-792, Saunders-Elsevier, Canada, 2006.

3. Innis CJ, Boyer TH: Chelonian reproductive disorders. *Vet Clin North Am Exot Anim Pract,* 5, 555-578, 2002. DOI: 10.1016/S1094-9194(02)00013-0

4. Sykes JM: Updates and practical approaches to reproductive disorders in reptiles. *Vet Clin North Am Exot Anim Pract,* 13, 349-373, 2010. DOI: 10.1016/j.cvex.2010.05.013

5. McArthur S, Wilkinson R, Meyer J: Surgery. **In,** McArthur S, Wilkonson R, Meyer J (Eds): Medicine and surgery of tortoises and turtles. 1st edn., 403-464, Blackwell, United Kingdom, 2004.

6. Mans C, Sladky KK: Diagnosis and management of oviductal disease in three red-eared slider turtles (Trachemys scripta elegans). *J Small Anim Pract,* 53 (4): 234-239, 2012. DOI: 10.1111/j.1748-5827.2011.01172.x

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Case Report

First Isolation of *Pseudomonas aeruginosa* from Ear Abscess of a Red-Eared Slider *(Trachemys scripta elegans)*

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Abstract

This case report is the first of the isolation and identification of *Pseudomonas aeruginosa* in a female, one year old red-eared slider hospitalized at the Surgery Clinics of Veterinary Faculty, Bingol University, Turkey, for unilateral ear abscess. Susceptibility of the agent to several antibiotics was also tested.

Keywords: *Red-eared slider, Ear abscess, Pseudomonas aeruginosa*

Kırmızı Yanaklı Bir Su Kaplumbağasının Kulak Apsesinden *Pseudomonas aeruginosa*'nın İlk İzolasyonu

Öz

Bu olgu sunumunda tek taraflı kulak apsesi şikayeti ile Bingöl Üniversitesi Veteriner Fakültesi Cerrahi Kliniğine getirilen bir yaşlı dişi kırmızı yanaklı bir su kaplumbağasından ilk kez *Pseudomonas aeruginosa*'nın izolasyonu ve identifikasyonu yapıldı. Ayrca bu etkenin antibiyotiklere olan duyarlılılıkları araştırıldı.

Anahtar sözcükler: *Kırmızı yanaklı su kaplumbağası, Kulak apsesi, Pseudomonas aeruginosa*

INTRODUCTION

In Red-Eared Sliders, unilateral or bilateral ear abscesses may have a mild course as well as reach the severity that restricts the movement of the animal. Although many predisposing factors such as poor hygienic conditions, stress, vitamin A deficiency and malnutrition play a role in the formation of ear abscess, the etiology of the disease has not been fully elucidated [1,2]. The opportunistic pathogens found in water are thought to play an important role in the abscess formation by passing through the auditory canal and colonizing in the ear [1]. Although in a limited number of studies, various Gram positive and Gram negative bacteria are suggested to get involved in the etiology of ear abscesses of turtles [3,4], no data are available in the literature indicating possible role of *P. aeruginosa* as a primary factor in ear abscesses of Red-Eared Sliders. Therefore, this case report presents novel information toward the etiology of ear abscesses in turtles by detecting *P. aeruginosa* for the first time. One of the interesting properties of *P. aeruginosa* is its progressively increasing resistance to many antibiotics in the market in recent years. Hence, antibiotic resistance levels of the agent isolated in the present study were also investigated.

CASE HISTORY

The material of this case report was a female, one-year old Red-Eared Slider admitted to the Surgery Clinics of Veterinary Faculty, Bingol University, Turkey, with the complaint of swelling on one side of its head that developed over a period of one month. In clinical examination, the swelling was observed to be a unilateral ear (auricular) abscess *(Fig. 1)* and, it was decided to clean the content with surgical procedure. General anesthesia was provided by intramuscular administration of 20 mg/

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Fig 1. Clinical outlook of unilateral ear abscess

kg of Ketamine HCl (Alfamine, Egevet, Turkey) to the patient. A 3-4 mm linear skin incision covering the upper part of the swelling in the ear area was made, carried up to the abscess pouch, which was then opened. Its content was caseified and removed under sterile conditions. For microbiological examination, samples were taken from both abscess content and by applying a sterile swab to the inner part of the abscess pouch. Next, the abscess pouch was washed with isotonic saline solution.

Samples taken from abscess pouch by sterile swabs were inoculated onto Tryptone Soy Agar (Oxoid, CM0131) and incubated at 37°C in aerobic conditions for 24 h. The growing colonies were identified as *P. aeruginosa* according to Gram staining, motility, colony morphology, growth at 42°C, pigment production, catalase, oxidase, indole and citrate tests. The agent was confirmed as *P. aeruginosa* (99.9%) following the analyses performed with VITEK 2 automated system. Antibiotic susceptibilities of the isolate were investigated by using disc-diffusion method [5]. For this purpose, the susceptibility of the isolate against a total number of nine antibacterial agents, including ceftazidime (10 µg), piperacillin-tazobactam (30-6 µg), gentamicin (10 µg), amikacin (30 µg), imipenem (10 µg), meropenem (10 µg), cefepime (30 µg), ciprofloxacin (5 µg) and colistin (10 µg), which are frequently used in human medicine was tested. The results showed that *P. aeruginosa* isolate was sensitive to the majority of the antibacterial substances at various levels.

DISCUSSION

The morbidity and mortality of tympanic infections in turtle species are rather high. Several factors have been linked to the etiology of ear abscesses which have significant place among these infections [1,2]. A number of bacteria which are common in the environment and water and, mostly comprised of opportunistic pathogens have been put forward as leading factors. In a study on box turtles, Joyner *et al*.[3] isolated Gram negative bacteria, including *Citrobacter, Morganella, Pasteurella* and *Proteus* and Gram positive bacteria, including *Corynebacterium, Listeria, Staphylococcus* and *Streptococcus* from ear abscesses. Similar results have also been reported in previous studies [1,4,6]. Therefore, this is the first study reporting the involvement of *P. aeruginosa* in the etiology of ear abscesses of Red-Eared Sliders. *P. aeruginosa* can be found extensively in the environment (particularly in soil and water), as well as in the mouth cavity and intestines of reptiles commensally [7,8]. The agent can also cause stomatitis, pneumonia, keratoconjunctivitis, dermatitis, septicemia and death in the immunosuppressed reptiles [9]. The interesting information obtained from anamnesis of the patient examined in the present study was the formation of conjunctivitis prior to ear abscess. Bearing this information in mind, it was concluded that *P. aeruginosa* might have caused abscess formation by colonizing the ear cavity following conjunctivitis or, alternatively, the infection by this opportunist agent might be the result of the immunosuppression.

P. aeruginosa should also be considered in terms of public health because it causes serious infections in people with cystic fibrosis, immunosuppression and chronic diseases [10]. In particular, contact with pet turtles is considered as an important factor increasing this risk. Therefore, people who keep such animals as pets should strictly observe hygiene and sanitation rules.

Following intensive use of antibiotics, as in the case of other bacteria, the level of antibiotic resistance in *P. aeruginosa* appears to be progressively increasing. It has been reported that multidrug resistance in *P. aeruginosa* against medicines used in both human and veterinary medicine has reached such a level that threatens public health and it is very difficult to control the infections caused by this agent [10,11]. Wendt *et al*.[12] reported that 17 *P. aeruginosa* isolates originated from pet turtles were resistant to amoxicillin, colistin sulphate, streptomycin, cephalothin, trimethoprim, chloramphenicol, imipenem, cefoxitin and nalidixic acid, but sensitive to ciprofloxacin and ofloxacin. The isolate obtained in this study was found to be sensitive to all the antibiotics tested, in contrast to other studies. It was commented that drug resistance has not been developed in the animal used in the current study due probably to the application of good sanitation conditions and no use of antibiotics treatment, so far.

In conclusion, the present study was the first to report the isolation and identification of *P. aeruginosa* as primary agent in the ear abscess of a Red-Eared Slider.

REFERENCES

1. Murray MJ: Aural abscesses. **In,** Mader DR (Ed): Reptile medicine and surgery. 3rd ed., WB Saunders Co, Philadelphia, Pennsylvania, 349-352, 1996.

2. Holladay SD, Wolf JC, Smith SA, Jones DE, Robertson JL: Aural abscesses in wild-caught box turtles *(Terrapene carolina)*: Possible role of organochlorine-induced hypovitaminosis A. *Ecotoxicol Environ Saf,* 48, 99-106, 2001. DOI: 10.1006/eesa.2000.1993

3. Joyner PH, Brown JD, Holladay S, Sleeman JM: Characterization of the bacterial microflora of the tympanic cavity of eastern box turtles with and without aural abscesses. *J Wildl Dis,* 42 (4): 859-864, 2006. DOI: 10.7589/0090-3558-42.4.859

4. Yardımcı B, Yardımcı C, Ural K, Seçer S: Auricular abscessation in Red-Eared Sliders *(Trachemys scripta elegans)*. *Kafkas Univ Vet Fak Derg,* 16 (5): 879-881, 2010. DOI: 10.9775/kvfd.2009.1076

5. The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Disk Diffusion Method for Antimicrobial Susceptibility Testing. v 6.0, January, 2017.

6. Stewart JS: Anaerobic bacterial infections in reptiles. *J Zoo Wildl Med,* 21,180-184, 1990.

7. Botzenhart K, Döring G: Ecology and epidemiology of *Pseudomonas aeruginosa*. **In,** Campa M, Bendinelli M, Friedman H (Eds): *Pseudomonas aeruginosa* as an Opportunistic Pathogen. 1-18, Plenum Press, New York 1993.

8. Warwick C, Arena PC, Steedman C, Jessop M: A review of captive exotic animal-linked zoonoses. *J Environ Health Res* 12 (1): 9-24, 2012.

9. Denardo D: Dystocias. **In,** Mader DR, Divers SJ (Eds): Reptile Medicine and Surgery. 2nd ed., St Louis (MO), Elsevier, 787-792, 2006.

10. Stefani S, Campana S, Cariani L, Carnovale V, Colombo C, Lleo MM, Iula VD, Minicucci L, Morelli P, Pizzamiglio G, Taccetti G: Relevance of multidrug-resistant *Pseudomonas aeruginosa* infections in cystic fibrosis. *Int J Med Microbiol,* 307 (6): 353-362, 2017. DOI: 10.1016/j.ijmm.2017.07.004

11. Ruiz-Garbajosa P, Cantón R: Epidemiology of antibiotic resistance in Pseudomonas aeruginosa. Implications for empiric and definitive therapy. *Rev Esp Quimioter,* 1, 8-12, 2017.

12. Wendt M, De Silva BCJ, Heo GJ: Virulence factors and antimicrobial resistance of *Pseudomonas aeruginosa* isolated from pet turtles. *Asian J Anim Vet Adv,* 12 (4): 205-211, 2017. DOI: 10.3923/ajava.2017.205.211

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24 (4): 627-630, 2018
DOI: 10.9775/kvfd.2018.19663

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Case Report

Treatment of Traumatic Thoracal Instability with Pedicle Screw-Rod Fixation System in a Dog

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Abstract

In this paper, clinical and neurological results of stabilization of a thoracic fracture and luxation with a pedicle screw-rod fixation system (PSRFS) in a dog have been reported. A physical and neurological examination revealed non-ambulatory paraparasis, upper motor neuron lesions of hind limbs which localized the lesion to thoracolumbal vertebra. On the radiographic survey; lateral views of the thoracolumbal region revealed luxation between Thoracal 11-12 (Th11-12) and fracture of end plate of Th11. After laminectomy and discectomy, two Ø 3.5 mm polyaxial pedicle screws were placed to each side of Th11-12 corpus and connected with a rod. The dog was able to stand without any support and was ambulatory with assistance after 6 weeks postoperatively. Full recovery was seen and normal alignment of the luxated vertebrae and fusion was seen on radiographic views on postoperative 6th month. As a conclusion PSRFS can be successfully used in fractures/luxations of vertebras in large breed dogs. The main disadvantages of the PSRFS are higher cost, limited screw dimensions and variability of pedicle safe corridors.

Keywords: *Dog, Pedicle screw, Vertebral fracture*

Bir Köpekte Travmatik Torakal İnstabilitenin Pediküllü Vida Rod Fiksasyon Sistemiyle Tedavisi

Öz

Bu yazıda, bir köpekte torakal kırık ve luksasyonun pediküllü vida-rod fiksasyon sistemiyle stabilizasyonunun klinik ve nörolojik sonuçları bildirilmiştir. Fiziksel ve nörolojik muayenede, ambulatorik olmayan paraparazis ve arka bacaklarda torakolumbal vertebrada lokalize olan yukarı motor nöron lezyonu belirlendi. Radyografik görüntülemede; Torakal 11-12 (Th11-12) vertebra arasında luksasyon ve Th11 vertebranın son plağında kırık belirlendi. Laminektomi ve diskektomi sonrasında, iki adet Ø 3.5 mm poliaksiyal pedikül vidası, Th11-12 korpusunun her iki yanına yerleştirildi ve rod ile bağlandı. Köpek, operasyondan sonraki 6. ayda herhangi bir destek almadan ayakta durabiliyor ve yardımla gezebiliyordu, radyografide, lukse olan vertebranın hizasının normal olduğu, vertebrada füzyon ve tamamen iyileşme görüldü. Sonuç olarak, poliaksiyal pedikül vidası rod sistemi büyük ırk köpeklerde vertebra kırık ve luksasyonlarında başarılı bir şekilde kullanılabilir. Bu sistemin temel dezavantajları, maliyetinin yüksek, vida boyutlarının kısıtlı ve pedikül güvenli koridorlarının değişkenlik göstermesidir.

Anahtar sözcükler: *Köpek, Pedikül vida, Vertebra kırığı*

INTRODUCTION

Fractures, luxation and/or subluxation of vertebral column are seen frequently in companion animals [1,2]. The common causes of vertebral trauma are vehicle accident, falls, bite wounds and gun shots. In general, surgical treatment is required for these kind of traumas and several surgical techniques have been described including vertebral body plating, external splinting, spinal process plating in combination with application of a Kirschner-Ehmer apparatus, composite fixation with pins or screws and polymethylmethacrylate (PMMA), stabilization with external fixation, spinal stapling, modified segmental spinal instrumentation and tension band stabilization [3-6].

In human medicine, spinal fracture and luxation, or instability related to disk disease and spinal stenosis have been treated with surgical decompression combined with pedicle screw-rod fixation system (PSRFS) [7,8]. The primary goal of PSRFS is stabilization of decompressed spinal segments and thereby achieving bony fusion of spinal segment [7,9]. In veterinary practice PSRFS has been used

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for stabilization of lumbosacral junction in degenerative lumbosacral stenosis [10,11].

The purpose of this paper is to report the clinical and neurological outcomes of stabilization of thoracal fracture and luxation with PSRFS, which has been used in traumatic instability of vertebra.

CASE HISTORY

Ten months-old, male Labrador Retriever was admitted to the Department of Surgery, following a motor vehicle accident. According to the owner, following the accident the dog was ambulatory and medical treatment including corticosteroid, analgesic and B complex vitamin was performed in referral clinic, however, it soon deteriorated and became non-ambulatory. According to neurological examination, non-ambulatory paraparasis, upper motor neuron lesions of hind limbs which localized the lesion to thoracolumbal vertebra. And also presence of deep pain sensation was detected. Direct radiography was carried out under anesthesia which was provided by diazepam (0.28 mg/kg, IV, Diazem 10 mg, Deva, Istanbul, Turkey) and propofol (3 mg/kg, IV, Propofol 1% 20 mL, Fresenius, Istanbul, Turkey). On the radiograpic survey; lateral views of the thoracolumbal region revealed luxation between Thoracal 11-12 (Th 11-12) and fracture of end plate of Th 11 vertebra *(Fig. 1)*. Computed tomography views were also obtained *(Fig. 2)*. The dog received methylprednisolone (20 mg/kg, IV, Prednol-L 250 mg amp., Mustafa Nevzat, İstanbul, Turkey) and cefazolin (20 mg/kg, IV, Cefozin 500 mg, Bilim, Istanbul, Turkey) and morphine (0.01-0.02 mg/kg, IV, Morfin HCl 0.01 g/mL, Osel, Turkey) before induction of anesthesia. The dog was premedicated with acepromazine (0.025 mg/kg, IV, Calmivet Solution 5 mg/mL, Vetaquinol, Paris, France). Anesthesia was induced with propofol (3 mg/kg, IV, Propofol 1% 20 mL, Fresenius, Istanbul, Turkey) given intravenously until the desired level of induction was achieved. A size 7-cuffed endotracheal tube was inserted, and isoflurane anesthesia was maintained at 1-2% via low flow, semi-closed-circuit technique at an oxygen flow rate of 1 L/min. The dog was aseptically prepared and placed in the ventral recumbence and a dorsal approach was performed as previously described [12].

Laminectomy had been performed between Th 11-12 vertebrae and the luxation was reduced by traction of vertebral bodies with hooks. Discectomy and removal of fractured end plate of Th 11 were done. Two Ø 3.5 mm polyaxial pedicle screws were placed to each side of Th 11-12 corpus and connected with a rod *(Fig. 3)*. Cancellous bone graft, maintained from wings of ileum was placed to the intervertebral disc space.

A cast including PVC (polyvinylchloride) as a support material, which was extended from interscapular region to lumbar 5 was used for external support. The normal aligment of the vertebra was detected after the operation *(Fig. 4)*. Fentanyl (10-20 µg/kg, IV, Fantanyl 50 µg/mL, Hameln, London, United Kingdom) was applied for post-operative analgesia. Cefazolin was continued every 12 h for one week after the operation. The dog was hospitalized

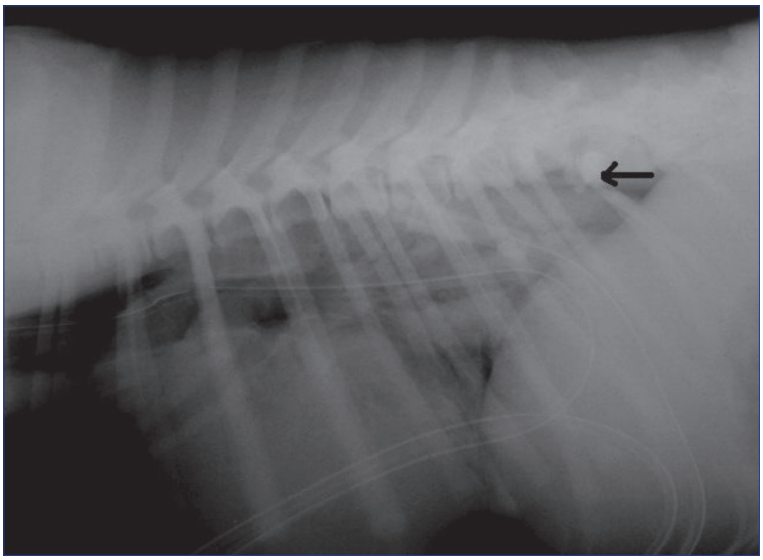

Fig 1. Lateral radiographic view of thoracolumbal vertebra. Notice the fracture and luxation of Th 11-12 *(black arrow)*

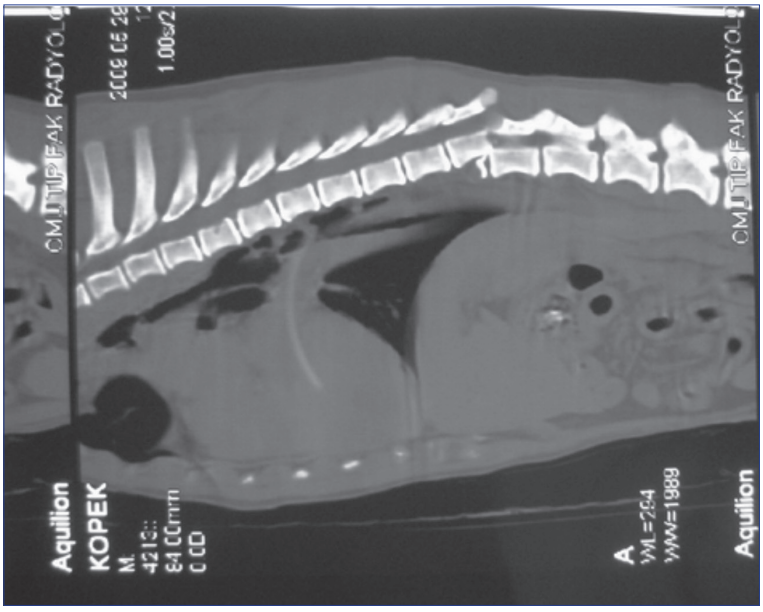

Fig 2. Sagittal computed tomography view of thoracolumbal vertebra. The luxation and fracture of end plate of Th11 is seen more obviously

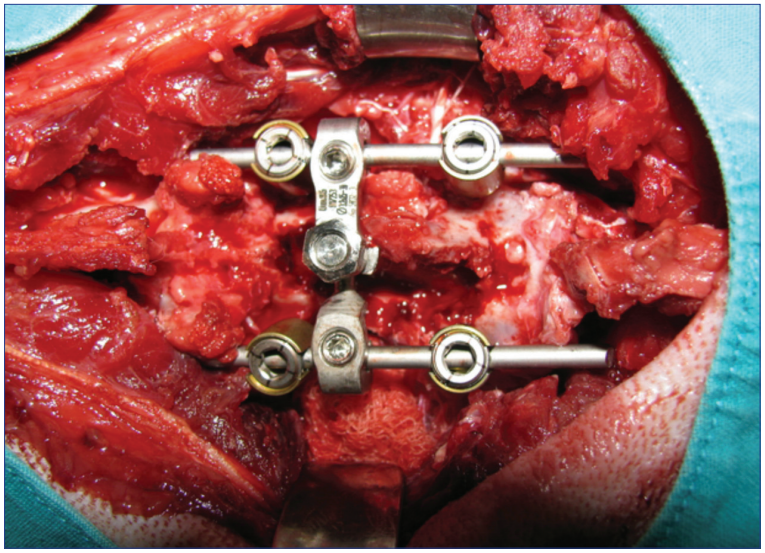

Fig 3. Dorsal view of PSRFS after it was applied

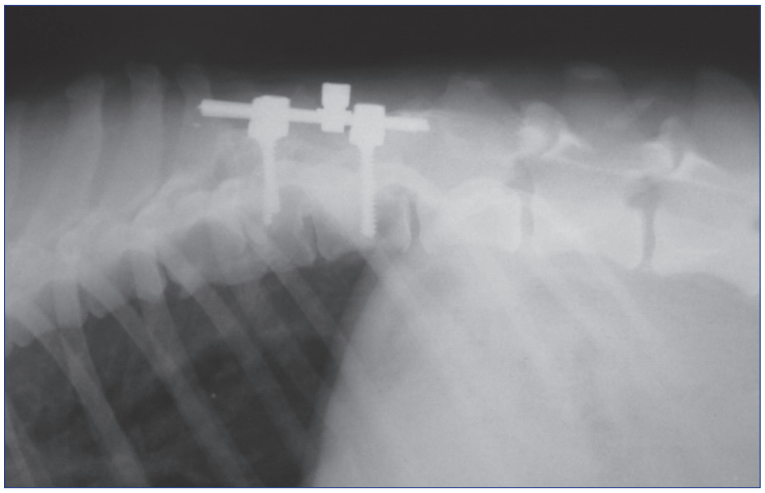

Fig 4. Lateral radiographic view of operation site after the PSRFS application

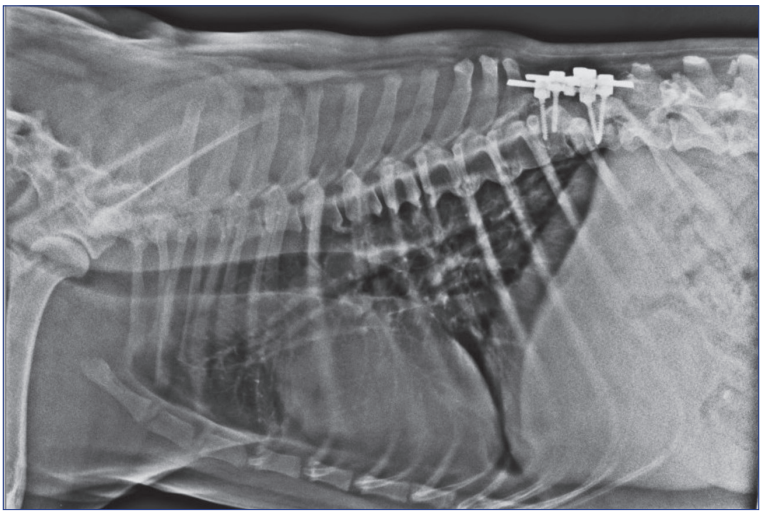

Fig 5. Lateral radiographic view of operation site on postopartive 6th month. The normal alignment of vertebra and the fusion were detected

and examined daily for 6 weeks. During hospitalization, physical therapy was carried out by placing the patient into a custom made cart and each joint of both limbs were extended and flexed through their full range of motion following a thorough hydrotherapy massage every day. This therapy was carried out on a custom made designed cart. The hind limbs were supported with a sling. The cast was changed at 10 day intervals and removed 4 weeks after the operation.

The dog was able to stand without any support and was ambulatory with assistance after 6 weeks postoperatively. However, the gait of the pelvic limbs was moderately uncoordinated. Adequate fracture healing and stable implant was detected by radiography at the end of hospitalization. Clinical, neurological and radiographic examinations were done on the third and sixth month postoperatively. On reexamination three months postoperatively, the gait had almost returned to normal, however, slight proprioceptive deficit of effect leg was detected. However, on postoperative 6th month postoperatively, full recovery and normal alignment of the luxated vertebrae and fusion was seen on radiographic views *(Fig. 5)*.

DISCUSSION

The fractures and/or luxations are generally seen at thoracolumbar region in dogs [1,5,13]. Involvement of end plate to the fracture or luxation is frequently seen in cats and dogs younger than one year. The closure of end plates in dogs is completed after 11-14 months [1]. In our case the localization of the luxation and end plate physeal fracture, and the age of the case were compatible with the reports.

In human medicine PSRFS has been used for treatment of spinal stenosis, spinal fractures/luxations, and degenerative disk disease [7,8]. The aim of the PSRFS is to improve deformity and stabilize the spine in its natural position until fusion has occurred and prevent further degenerative changes [7,9]. In veterinary literatures, PSRFS has been only used to stabilize lumbosacral region after laminectomy for treatment of degenerative lumbosacral stenosis in dogs [10,11,14,15]. Furthermore, the system was used to investigate fusion in lumbar vertebrae [16]. According to authors' knowledge PSRFS has not been used to stabilize the fracture and luxation of spine in dogs.

Several methods have been described to stabilize

the fracture and luxation of spine [3-6]. Generally at least two cranial and caudal vertebras of the fracture should be stabilized [5,6,13]. Transpedicular screw fixation maintains a rigid anchorage of the screw to the vertebral body. This anchorage enables a secure three-dimensional positional control between the screw and the longitudinal elements, which can provide a restoration of normal stiffness even in short segmental (i.e. one or two-level) instrumentation [7]. The screws were applied to the corpus vertebra of Th 11-12. According to the perioperative observation rigid fixation was achieved, even though one level instrumentation. Meij et al.[14] reported that dorsal laminectomy and partial discectomy had changed the stiffness characteristics of the spine specimen in neutral zone without changing range of motion, however, in natural cases with degenerative lumbosacral stenosis laminectomy and discectomy could decrease stiffness significantly in vivo. In the case the stiffness of the vertebral region was markedly decreased after discectomy and removal of fractured end plate. According to our perioperative observation it was determined that stiffness of fractured region has been obtained after application of PSRFS.

PSRFS has been designed for human vertebras, so the dimensions of the screws are not suitable even for large breed dogs. The use of pedicle screws for adult humans led to fracture of the lateral and the medial wall of the pedicle. Pedicle screws for pediatric patients better fit for adult canine vertebral pedicle [14]. The pediatric pedicle screws which is Ø 3.5 mm diameter were successfully used for the dog in this case and it was determined that pediatric PSPFS systems are suitable for large breed dogs

The dimensions and shapes of the vertebras are differed even though in same breed animals. This is the main problem for safe screw insertions and prediction of the screw dimensions [17]. Smolders et all indicated that CT- and/or MRI- based evaluation of the vertebral dimensions of both L7 and S1 is necessary for optimal screw insertion corridors for individual dogs [15]. Before the surgery CT images had been evaluated for pedicle dimensions to predict the dimension of screws dimensions. Because, in human PSRFS the dimensions of the screw are limited for animals and eventually, Ø 3.5 mm diameter screws which are pediatric for human patients were used according to evaluation of CT images.

The fracture healing was detected at the end of hospitalization by radiographic examination which is consisted with spinal fractures can heal in as short as a time as 4 weeks [4].

As a conclusion PSRFS can be successfully used in fractures/luxations of vertebras in large breed dogs. The main disadvantages of the PSRFS are higher cost, limited screw dimensions and variability of pedicle safe corridors. Further studies with large amount of cases with fractures/luxations of vertebras will give more successful and supportive information for the usage of PSRFS in veterinary neurosurgery.

REFERENCES

1. Bali MS, Lang J, Jaggy A, Spreng D, Doherr MG, Forterre F: Comparative study of vertebral fractures and luxations in dogs and cats. *Vet Comp Orthop Traumatol,* 22 (1): 47-53, 2009.

2. Bruce CW, Brisson BA, Gyselinck K: Spinal fracture and luxation in dogs and cats. *Vet Comp Orthop Traumatol,* 21 (3): 280-284, 2008. DOI: 10.1055/s-0037-1618862

3. Bruecker KA: Principles of vertebral fracture management. *Semin Vet Med Surg (Small Anim),* 11 (4): 259-272, 1996.

4. Shores A, Nichols C, Rochat M, Fox SM, Burt GJ, Fox WR: Combined Kirschner-Ehmer device and dorsal spinal plate fixation technique for caudal lumbar vertebral fractures in dogs. *J Am Vet Med Assoc,* 195 (3): 335-339, 1989.

5. Voss K, Montavon PM: Tension band stabilization of fractures and luxations of the thoracolumbar vertebrae in dogs and cats: 38 cases (1993-2002). *J Am Vet Med Assoc,* 225 (1): 78-83, 2004. DOI: 10.2460/javma.2004.225.78

6. Wheeler JL, Lewis DD, Cross AR, Sereda CW: Closed fluoroscopic-assisted spinal arch external skeletal fixation for the stabilization of vertebral column injuries in five dogs. *Vet Surg,* 36 (5): 442-448, 2007. DOI: 10.1111/j.1532-950X.2007.00290.x

7. Boos N, Webb JK: Pedicle screw fixation in spinal disorders: A European view. *Eur Spine J,* 6 (1): 2-18, 1997. DOI: 10.1007/BF01676569

8. Yahiro MA: Comprehensive literature review: Pedicle screw fixation devices. *Spine,* 19 (20-Suppl.): 2274S-2278S, 1994.

9. Davne SH, Myers DL: Complications of lumbar spinal fusion with transpedicular instrumentation. *Spine,* 17 (6-Suppl.): S184-S189, 1992. DOI: 10.1097/00007632-199206001-00021

10. Méheust P: Une nouvelle technique de stabilisation lombosacrée: l'arthrodèse par vissage pédiculaire, étude clinique de 5 cas. *Prat Med Chir Anim Comp,* 35, 201-207, 2000.

11. Méheust P, Mallet C, Marouze C: Une nouvelle technique de stabilisation lombosacrée: L'arthrodèse par vissage pédiculaire. Considérations anatomiques. *Prat Med Chir Anim Comp,* 35, 193-199, 2000.

12. Piermattei DL, Johnson KA: The Vertebral Column. **In,** Piermattei DL, Johnson KA (Eds): An Atlas of Surgical Approaches to the Bones and Joints of the Dog and Cat. 4th ed., 78-83, Saunders Philadelphia, New York, 2004.

13. Krauss MW, Theyse LFH, Tryfonidou MA, Hazewinkel HAW, Meij BP: Treatment of spinal fractures using Lubra plates. A retrospective clinical and radiological evaluation of 15 cases. *Vet Comp Orthop Traumatol,* 25 (4): 326-331, 2012.

14. Meij BP, Suwankong N, Van der Veen AJ, Hazewinkel HAW: Biomechanical flexion-extension forces in normal canine lumbosacral cadaver specimens before and after dorsal laminectomy-discectomy and pedicle screw-rod fixation. *Vet Surg,* 36 (8): 742-751, 2007. DOI: 10.1111/j.1532-950X.2007.00331.x

15. Smolders LA, Voorhout G, van de Ven R, Bergknut N, Grinwis GCM, Hazewinkel HAW, Meij BP: Pedicle screw-rod fixation of the canine lumbosacral junction. *Vet Surg,* 41 (6): 720-732, 2012. DOI: 10.1111/j.1532-950X.2012.00989.x

16. Mutlu Z, Devecioglu Y, Demirutku A, Aktas M, Eravci E, Duzgun O: The use of polyaxial pedicle screw for the fusion of lumbar vertebra in sheeps. *J Anim Vet Adv,* 10 (9): 1202-1204, 2011. DOI: 10.3923/javaa.2011.1202.1204

17. Watine S, Cabassu JP, Catheland S, Brochier L, Ivanoff S: Computed tomography study of implantation corridors in canine vertebrae. *J Small Anim Pract,* 47 (11): 651-657, 2006. DOI: 10.1111/j.1748-5827.2006.00070.x

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Letter to the Editor

Diagnostic Steps of a Cat with Patent Ductus Arteriosus (Patent Ductus Arteriosuslu Bir Kedide Diagnostik Basamaklar)

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Dear Editor,

Patent ductus arteriosus (PDA), a fetal blood vessel between pulmonary artery and aorta that has to be closed in hours right after birth triggered by first breath of life, may result life threatening heart failure or even sudden death depend on size of the ductus [1]. PDA has been reported as a most common congenital heart defect in dogs and cats, however there is limited information on diagnostic procedure of PDA by use of three-dimensional computed tomography angiography (3D-CTA) in cats [1,2]. Thus, we report here the use of 3D-CTA to confirm the clinical diagnosis of PDA in a kitten.

A British shorthair cat (3 months, female, and 1.3 kg) was presented to Animal Hospital (Dep. of Internal Medicine, Faculty of Veterinary Medicine, Uludag University, Bursa-Turkey) with a history of lethargy, poor growth, exercise intolerance, panting and fatigue for one month. The cat was standing with open mouth breathing in sternal position and tachypneic (76 breaths/min). Cardiac auscultation revealed a tachycardia (250 bpm) with a loud continuous cardiac murmur (grade 3/6). ECG examination was consistent with the left ventricular enlargement (increased R wave amplitude, 2.4 mV/DII) and a sinus tachycardia. Thoracic radiography showed a mild bronchial pattern, perihilar pulmonary oedema and cardiomegaly as well as pulmonary artery and aortic bulging. Serum cardiac troponin I (cTnI) was slightly higher (0.19 ng/mL) than cut-off value (<0.16 ng/mL). All other parameters including complete blood cell count and serum biochemistry profile (Comprehensive Profile, VetScan®, Abaxis, UK) were within reference ranges. Cat was clean for enteric parasites checked by an easy faecal smear examination, as well.

Based on these clinical observations, congenital cardiac pathologies such as PDA were suspected. Echocardiographic examination was performed by standard techniques with a 7.5-10 MHz phased-array transducer in this cat without sedation (CarisPlus®, Esoate, Florence, Italy), as suggested [3]. Two-dimensional and M-mode echocardiography showed left atrial (2.03 cm, reference <1.0 cm) and ventricular enlargements (2.12 cm, reference <1.5 cm) with preserved fractional shortening (46%, reference 30-59%). Colour Doppler examination revealed severe turbulence in pulmonary artery trunk and its right and left branches *(Fig. 1)*. A connection was observed by colour Doppler between main pulmonary artery and ascending aorta *(Fig. 1)*, suggesting the presence of PDA. Peak systolic pulmonary artery flow velocity and pressure gradient were 1.24 m/s and 6.1 mmHg (reference <1.2 m/s and <10 mmHg, respectively), indicating left to right ductal shunt in this cat. Three-dimensional volume-rendered CT images (3D-CTA) were used to assess the malformations and to plan surgery for the treatment of the vascular anomalies. 3D-CTA was run with the contrast medium (Omnipaque™, 10 mL, iv) under general anaesthesia (1). 3D anatomy of the kitten confirmed the diagnosis of a large PDA with its size (9.1 mm) and localisation *(Fig. 2)*.

The cat was treated symptomatically with an ACE-i drug (enalapril, 0.5 mg/kg 2x1, po), diuretic (furosemide, 2 mg/kg, 2x1, po) and salt restriction, in order to control pulmonary oedema and reduce left sided volume overload for one week till the surgical correction, as suggested [4]. One week after initial of the treatment, clinical signs were improved and cat was undergone to surgery as reported in our previous study [5].

In summary, echocardiographic examination is clinically essential in diagnosis of cats with PDA. However presence

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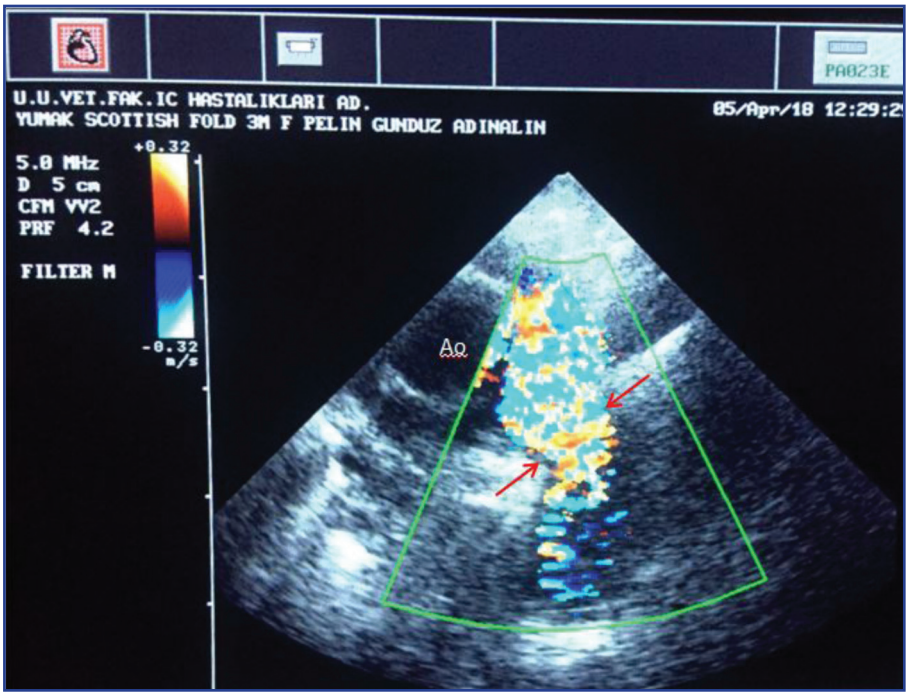

Fig 1. Presence of a severe regurgitant jet of the PDA *(red arrows)* is seen on colour flow Doppler echocardiography of the pulmonary artery of the cat (Ao: Aorta)

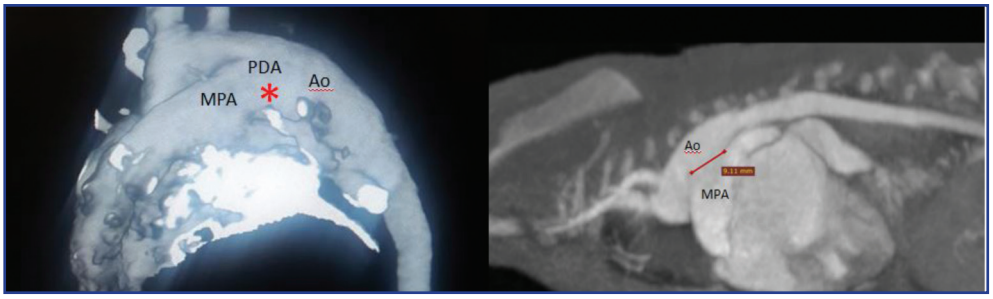

Fig 2. Three-dimensional modelling of the PDA *(arrow* and *asterix)* between mean pulmonary artery and aorta is shown by computed tomography angiography (MPA: Mean pulmonary artery, Ao: Aorta, PDA: Patent ductus arteriosus)

of PDA is not always detected by echocardiography clearly, high technological diagnostic imaging systems like 3D-CTA is needed in small sized patients like kittens. Clinician should be careful auscultating patients with/without symptoms to follow the steps to the correct PDA diagnose and to confirm the diagnosis with CTA.

REFERENCES

1. Bascuñán A, Thieman Mankin KM, Saunders AB, Bright JM, Scharf V, Singh A, O'Sullivan L, Brisson B, Estrada AH, Tou SP, Ruoff C: Patent ductus arteriosus in cats *(Felis catus):* 50 cases (2000-2015). *J Vet Cardiol,* 19 (1): 35-43, 2017. DOI: 10.1016/j.jvc.2016.10.002

2. Saunders AB, Birch SA: Three-dimensional modeling of a patent ductus arteriosus in a cat. *J Vet Cardiol,* 17 (Suppl.-1): S349-S353, 2015. DOI: 10.1016/j.jvc.2015.08.006

3. Boon JA: Veterinary Echocardiography. 2nd ed., Wiley-Blackwell, USA, 2010.

4. Wustefeld-Janssens BG, Burrow R, Mõtsküla P, Martin M, Dukes-McEwan J: Clinical findings and treatment outcomes for cats diagnosed with patent ductus arteriosus in the UK: A retrospective study of 19 cases (2004-2012). *Vet Rec*, 179 (1): 17, 2016. DOI: 10.1136/vr.103647

5. Yilmaz Z, Kocatürk M, Salci H, Levent P, Canatan U, Sag S, Güllülü S: Diagnostic importance of transesophageal echocardiography for detecting patent ductus arteriosus (PDA) in a puppy. *Kafkas Univ Vet Fak Derg*, 22 (5): 807-808, 2016. DOI: 10.9775/kvfd.2016.16081

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