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RESEARCH ARTICLE

Türk Toplumunun Hayvan Hakları Kavramına Yaklaşımının Belirlenmesine Yönelik Bir Araştırma: III. Hayvanları Korumaya, Hayvan Hakları Sorunlarına ve Bölgesel Farklılıklara Yönelik Tutum Analizi [1]

Türel ÖZKUL [1] Taner SARIBAŞ [2] Ender UZABACI [3] Erhan YÜKSEL [4]

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Özet

Bu çalışma, Türk toplumunun hayvan hakları konusunda bilinç düzeyi ve algısına yönelik saptamalar yapmak amacıyla gerçekleştirildi. Bu amaçla hazırlanan anket, Türkiye'nin coğrafi bölgelerini temsil ettiği kabul edilen yedi ilde 2016 kişiye yüz yüze görüşme tekniği ile uygulandı. Anket verilerinin değerlendirilmesinde; hayvanları korumaya, hayvan hakları sorunlarına ve bölgesel farklılıklara yönelik sorulara verilen yanıtlar kendi içlerinde gruplandırılarak SPSS istatistik programı ile analiz edildi. Türkiye'de hayvan haklarına yeterli önem verilmediğini düşünenlerin aksi yönde düşünenlere göre, kısırlaştırmayı doğru bulmayanların bulanlara göre ve hayvanları koruma veya benzer amaçlarla kurulan derneklere üye olanların olmayanlara göre hayvan hakları konusunda daha pozitif tutum sergiledikleri saptandı (P<0.001). Ayrıca, hayvan haklarına yönelik gösterilen tutumun yaşanılan coğrafi bölgeye göre değiştiği de çalışma sonucu ortaya konuldu.

Anahtar sözcükler: *Türk toplumu, Tutum, Hayvanları koruma, Hayvan hakları sorunları, Bölgesel farklılık*

A Survey to Identify the Turkish People's Approach on Animal Rights Concept: III. Attitude Analysis Related to Animal Protection, Animal Rights Issues and Regional Differences

Summary

The present study was undertaken to determine the Turkish people's cognitive level and perception on the concept of animal rights. For this aim, a questionnaire was developed for collecting data and was applied to participants in seven provinces which are considered to represent geographical regions of Turkey. The survey was applied by interviewing with 2016 participants individually. The responses to the questions of animal protection, animal rights issues and regional differences are grouped themselves after the evaluation of survey data and analysed statistically. The results are given as: Those who think animal rights is not sufficient consideration in Turkey, members of animal protection societies and disapproving sterilization are showed more positive attitude towards animal rights concept than the others (P<0.001). Furthermore as a result of the study it was revealed that the attitude shown towards animal rights varies by geographic region of residence.

Keywords: *Turkish society, Attitude, Animal protection, Animal rights issues, Regional differences*

INTRODUCTION

Türkiyede hayvanları korumaya yönelik düzenlemeleri padişah fermannamelerinde, ihtisab (belediye) kanunlarında ve yabancıların, Osmanlı Dönemine ilişkin kaleme aldıkları seyahatnamelerde görmek mümkünse de hayvan-

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ları koruma hareketinin ilk sistemli adımları Robert Kolejinin öğretmen ve öğrencileri tarafından oluşturulan "*Şefkat Kolları*" aracılığı ile atılarak, 1912 yılında Türk tarihinin ilk hayvanları koruma derneği olan "*İstanbul Himâye-i Hayvânât Cemiyeti*" kurulmuştur. Birinci Dünya Savaşı nedeniyle faaliyetleri durdurulan Dernek, Cumhuriyetin ilanını takiben 1924 yılında tekrar faaliyetlerine başlayarak "*Türkiye Hayvanları Koruma Derneği*" adıyla günümüze kadar gelmiştir. Hayvanların korunmasına ilişkin tüm bu çabalara rağmen, sokak hayvanlarının korunmasına yönelik ilk kapsamlı Kanunun çıkartılması için 2004 yılına kadar beklenmiştir [1-3].

Kanunun esas uygulayıcıları olması beklenen toplumun, hayvan haklarına yönelik tutumu tarih boyunca; muhtaç olunan durumlar, çıkarlar, dini inançlar, insan merkezci yaklaşımlar ve çevre merkezci yaklaşımlara bağlı olarak değişiklik göstermiş, sosyolojik bir kavram olarak sosyo-kültürel ortamı tanımlayan "*çevresel değişkenler*", insanların hayvanlara yönelik davranışlarını belirlemiştir [4,5].

Türkiye içinde ve dışında yürütülen çalışmalarda [6-11] hayvanları koruma derneklerine üye olmanın, hayvanların kısırlaştırılması ve reklamlarda kullanılmasına ilişkin yaklaşımların, bölgesel ve kültürel farklılıkların hayvanlara yönelik gösterilen tutumu etkilediği ifade edilmiştir. Türk toplumunun konuya bakışına ve bilinçlilik düzeyine yönelik, toplumun genelini kapsayan bir çalışma saptanamamıştır. Çalışmada, hayvanları koruma ve hayvan hakları konusunda Türk toplumunun tutum ve yaklaşımlarını ortaya koymak amaçlanmaktadır.

MATERYAL ve METOT

Bu çalışmanın örneklemi ve veri toplama aracına ilişkin ayrıntılı bilgi "*Türk Toplumunun Hayvan Hakları Kavramına Yaklaşımının Belirlenmesine Yönelik Bir Araştırma: I. Demografik Özelliklere Göre Tutum Analizi*" başlıklı makalede sunuldu [12]. Bu makalede, veri toplama aracı olarak geliştirilen anketin hayvanları koruma, hayvan hakları sorunları ve bölgesel farklılıklarla ile ilgili parametreleri kullanıldı.

Yüz yüze görüşme yöntemiyle yürütülen çalışma sonucunda toplam 2016 kişiyle anket yapılarak, elde edilen veriler SPSS 13.0 for Windows (Chicago, IL) istatistik paket programı ile analiz edildi. Verilere ait geçerli frekans (sıklık) ve yüzde değerleri metin içerisinde ve tablolarda gösterilmiştir.

Hayvan haklarına yönelik sergilenen tutum düzeyleri için elde edilen puanların, bağımsız değişkenlerle karşılaştırılmasında, ikiden çok alt değişken grupları arasındaki karşılaştırmalarda Kruskal Wallis Testi, iki grup için karşılaştırmalarda ise Mann Whitney U testi uygulandı. Tüm analizlerde anlamlılık düzeyi $P<0.05$ olarak kabul edildi. Tutum ölçeğinden elde edilen puanların değerlendirilmesinin yanısıra, çalışmadaki sorular ile demografik veriler arasındaki ilişkileri belirleyebilmek için Pearson χ^2 (kikare) testi yapıldı.

Anket uygulamaları, 2009 Eylül - 2010 Mart tarihleri arasında gerçekleştirildi.

BULGULAR

Araştırmada katılımcılara, *"Hayvanları koruma veya benzer amaçlarla kurulmuş bir derneğe üye misiniz?* sorusu yöneltildi ve katılımcıların büyük çoğunluğu (%95.9) *"hayır"* cevabını verirken; %4.1'lik bölümü "*evet*" cevabını verdi. Hayvanları koruma derneğine üyelik değişkenine göre yapılan tutum değerlendirilmesinde hayvanları koruma derneğine üye olanların olmayanlara göre daha pozitif tutum sergiledikleri gözlendi ($P<0.001$) *(Tablo 1)*.

Tablo 1. *Katılımcıların hayvanları koruma derneğine üye olma, hayvan haklarına verilen önem, kedi-köpek gibi evcil hayvanların kısırlaştırılması ve hayvanların reklamlarda kullanılmasına yönelik görüşlerine göre tutum değerlendirmesi*

Table 1. *Assessment of participants' attitude according to the variables of membership to the animal protection association, the views on the importance given to animal rights, sterilization of domestic animals such as cat and dog and animals used for advertising*

Soru	Seçenekler	N	%	X±Sx	Medyan	P	Minimum Değer	Maksimum Değer
S1	Evet	83	4.1	4.75±0.90	4.61	<0.001	2.58	6.90
	Hayır	1923	95.9	4.19±0.78	4.20		0.00	6,69
S2	Evet	339	17.0	3.54±0.71	3.64	<0.001	1.10	6.41
	Hayır	1658	83.0	4.35±0.84	4.33		0.00	6.90
S3	Evet	686	34.3	4.03±0.86	4.08	<0.001	0.47	6.46
	Hayır	779	38.9	4.33±0.77	4.32		0.00	6.90
	Emin değilim	537	26.8	4.25±0.68	4.22		2.15	6.66
S4	Evet	280	13.9	4.54±0.93	4.54	<0.001	0.47	6.46
	Hayır	1527	76.0	4.12±0.75	4.15		0.00	6.90
	Emin değilim	203	10.1	4.42±0.71	4.42		1.92	6.66

n: *Frekans;* ***X±Sx:*** *Ortalama±Standart Hata;* ***S1:****"Hayvanları koruma veya benzer amaçlarla kurulmuş bir derneğe üye misiniz?";* ***S2:*** *"Size göre Türkiye'de hayvan haklarına yeterli önem veriliyor mu?";* ***S3:****"Kedi-köpek gibi evcil hayvanların kısırlaştırılmasını doğru buluyor musunuz?";* ***S4:*** *"Hayvanların reklamlarda kullanılmasından rahatsız oluyor musunuz?"*

"Size göre Türkiye'de hayvan haklarına yeterli önem veriliyor mu?" sorusuna, katılımcıların büyük çoğunluğunun (%83) "*hayır*" yanıtını, %17'lik diliminin de "*evet*" yanıtını verdiği görüldü. Hayvan haklarına verilen önem üzerine yapılan tutum değerlendirilmesinde; sözü edilen soruya "*hayır*" yanıtını verenlerin pozitif "*evet*" yanıtını verenlerin ise negatif tutum sergiledikleri belirlendi (P<0.001) *(Tablo 1)*.

"Kedi-köpek gibi evcil hayvanların kısırlaştırılmasını doğru buluyor musunuz?" sorusuna katılımcıların %34.3'ü "*evet*", %38.9'u "*hayır*", %26.8'i "*emin değilim*" yanıtını verdi. Kedi-köpek gibi evcil hayvanların kısırlaştırılmasının doğruluğu üzerine yapılan tutum değerlendirilmesinde, kısırlaştırmayı doğru bulmayanların ve emin olmayanların kısırlaştırmayı doğru bulanlara göre daha pozitif tutum sergiledikleri belirlendi (P<0.001) *(Tablo 1)*.

"Hayvanların reklamlarda kullanılmasından rahatsız oluyor musunuz?" sorusuna katılımcıların %13.9'u "*evet*", %76'sı "*hayır*", %10.1'i "*emin değilim*" yanıtını verdi. Hayvanların reklamlarda kullanılması üzerine yapılan tutum değerlendirilmesinde *"evet"* ve *"emin değilim"* cevabını verenlerin *"hayır"* cevabını verenlere göre daha pozitif tutum sergiledikleri belirlendi (P<0.001) *(Tablo 1)*.

Katılımcıların bölgelere göre dağılımı incelendiğinde; %31'inin Bursa'dan (Marmara Bölgesi), %11'inin Samsun'dan (Karadeniz Bölgesi), %13'ünün Antalya'dan (Akdeniz Bölgesi), %14'ünün İzmir'den (Ege Bölgesi), %7'sinin Elazığ' dan (Doğu Anadolu Bölgesi), %7'sinin Urfa'dan (Güneydoğu Anadolu Bölgesi) ve %17'sinin Ankara'dan (İç Anadolu Bölgesi) araştırmaya dahil olduğu görüldü. Bölgelere göre yapılan tutum değerlendirilmesinde *(Tablo 2)* sırasıyla: Ankara'nın, İzmir ve Samsun'un, Bursa'nın, Urfa'nın daha pozitif tutum sergilediği; Antalya'nın ve Elazığ'ın negatif tutum sergiledikleri belirlendi (P<0.001) *(Tablo 2)*.

"Hayvanları koruma veya benzer amaçlarla kurulmuş bir derneğe üye misiniz? sorusuna verilen cevaplarda eğitim düzeyi (P=0.005), gelir grubu (P<0.001) ve meslek grubu (P<0.001) değişkenleri arasında; *"Size göre Türkiye'de hayvan haklarına yeterli önem veriliyor mu?"* sorusuna verilen cevaplarda cinsiyet (P<0.001), eğitim düzeyi (P<0.001), gelir grubu (P=0.034) ve meslek grubu (P<0.001) değişkenleri arasında; *"Kedi-köpek gibi evcil hayvanların kısırlaştırılmasını doğru buluyor musunuz?"* sorusuna verilen cevaplarda da cinsiyet (P=0.002), eğitim düzeyi (P<0.001) ve meslek grubu (P<0.001) değişkenleri arasında anlamlı farklılıklar olduğu saptandı *(Tablo 3)*.

TARTIŞMA ve SONUÇ

Araştırmada, hayvanları koruma derneğine üye olanların olmayanlara göre daha pozitif tutum sergilemesi *(Tablo 1)* Signal ve Taylor [8] tarafından yürütülen çalışma ile uyum göstermektedir. Söz konusu çalışmada, hayvanları koruma derneğine üye olanların toplumun diğer kesimindekilere göre hayvanlara yönelik tutum, hayvan hakları ve empati konularında daha pozitif tutum sergiledikleri vurgulanmaktadır. Bununla birlikte lisansüstü eğitim seviyesine sahip olanların, geliri 4001 ve üstü olanlar ile sağlık personelinin *"Hayvanları koruma veya benzer amaçlarla kurulmuş bir derneğe üye misiniz?* sorusuna daha fazla olumlu yanıt verdikleri görülmektedir *(Tablo 3)*. Elde edilen verilerin, adı geçen grupların "*Türk Toplumunun Hayvan Hakları Kavramına Yaklaşımının Belirlenmesine Yönelik Bir Araştırma: I. Demografik Özelliklere Göre Tutum Analizi*" başlıklı araştırmada [12] hayvan hakları konusuna yönelik sergiledikleri pozitif tutumu destekler nitelikte olduğu ileri sürülebilir. Söz konusu grupların hayvan haklarına yönelik gösterdikleri pozitif tutumun ve konuyla ilgili farkındalık seviyelerinin, hayvanları korumaya yönelik açılan derneklere üyeliklerini tetiklediği söylenebilir.

"Size göre Türkiye'de hayvan haklarına yeterli önem veriliyor mu?" sorusuna katılımcıların büyük çoğunluğu "*hayır*" cevabını vermiştir *(Tablo 3)*. Bu soruya bayanların, lisansüstü eğitim alanların, öğretmenlerin daha yüksek düzeyde "*hayır*" cevabını vermesi *(Tablo 3)* ve hayvan haklarına yeterince önem verilmediğini düşünen katılımcıların hayvan haklarına yönelik pozitif; aksi görüş bildirenlerin ise negatif tutum sergilemesi *(Tablo 1)* Özkul ve ark.[12] tarafından yürütülen araştırmanın verileri ile uyum göstermektedir. Bu durum eğitim parametresi ile hayvan hakları konusundaki bilinç düzeyi arasındaki güçlü bağın altını çizmektedir. Katılımcıların büyük çoğunluğunun Türkiye genelinde hayvan haklarına yeterli önem verilmediğini düşünmesi hayvan

Tablo 2. *Katılımcıların bölgelere göre tutum değerlendirmesi*
Table 2. *Assessment of attitudes of the participants by region*

Bölgeleri Temsil Eden İller	N	%	X±Sx	Medyan	Minimum Değer	Maksimum Değer
Ankara	340	17	4.44±0.65	4.38	2.58	6.39
Antalya	253	13	3.92±1.17	4.22	1.51	6.90
İzmir	288	14	4.34±0.71	4.32	1.92	6.42
Elazığ	147	7	3.86±0.83	3.96	0.00	5.39
Samsun	220	11	4.34±0.64	4.36	2.68	6.41
Urfa	143	7	4.05±0.59	4.06	2.07	5.75
Bursa	625	31	4.19±0.69	4.11	2.15	6.69

n: *Frekans;* ***X±Sx:*** *Ortalama±Standart Hata*

Tablo 3. *Hayvanları koruma derneğine üye olma, hayvan haklarına verilen önem, kedi-köpek gibi evcil hayvanların kısırlaştırılmasına ilişkin soruların bazı demografik özelliklere göre dağılımı*

Table 3. *Distribution of questions relating to membership to the animal protection association, the views on the importance given to animal rights, sterilization of domestic animals such as cat and dog according to some demographic traits*

Demografik Özellikler		N %	Soru 1			Soru 2			Soru 3			
			Evet	Hayır	P	Evet	Hayır	P	Evet	Hayır	Emin Değilim	P
Cinsiyet	Erkek	n	44	1128	= 0.283	250	917	< 0.001	434	445	291	= 0.002
		%	3.8	96.2		21.4	78.6		37.1	38.0	24.9	
	Kadın	n	39	786		86	735		246	331	246	
		%	4.7	95.3		10.5	89.5		29.9	40.2	29.9	
Eğitim Düzeyi	Okuryazar Değil	n	0	3	= 0.005	2	1	< 0.001	0	3	0	< 0.001
		%	0.0	100		66.7	33.3		0.0	100	0.0	
	Okuryazar	n	0	7		1	6		3	1	3	
		%	0.0	100		14.3	85.7		42.9	14.2	42.9	
	İlkokul	n	6	134		33	104		40	67	33	
		%	4.3	95.7		24.1	75.9		28.6	45.9	25.5	
	Ortaokul	n	4	158		40	120		52	71	37	
		%	2.5	97.5		25.0	75.0		32.5	44.4	23.1	
	Lise	n	15	477		109	382		161	207	124	
		%	3.0	97.0		22.2	77.8		32.7	42.1	25.2	
	Üniversite	n	41	963		128	837		334	370	298	
		%	4.1	95.9		13.3	86.7		33.3	36.9	29.8	
	Lisansüstü	n	17	149		17	149		82	45	39	
		%	10.2	89.8		10.2	89.8		49.4	27.1	23.5	
Gelir Grubu	500 ve altı	n	2	24	< 0.001	4	23	= 0.034	5	15	6	= 0.462
		%	7.7	92.3		14.8	85.2		19.2	57.7	23.1	
	501-1000	n	13	308		75	241		108	122	89	
		%	4.0	96.0		23.7	76.3		33.9	38.2	27.9	
	1001-2000	n	17	655		107	564		226	275	170	
		%	2.5	97.5		15.9	84.1		33.7	41.0	25.3	
	2001-3000	n	11	402		71	338		141	160	113	
		%	2.7	97.3		17.4	82.6		34.1	38.6	27.3	
	3001-4000	n	14	156		23	146		68	58	43	
		%	8.2	91.8		13.6	86.4		40.2	34.3	25.5	
	4001 ve üst	n	21	211		37	198		89	91	55	
		%	9.1	90.9		15.7	84.3		37.9	38.7	23.4	
Meslek	Sağlık Personeli	n	22	180	< 0.001	28	173	< 0.001	97	61	43	< 0.001
		%	10.9	89.1		13.9	86.1		48.3	30.3	21.4	
	Mühendis	n	6	148		21	133		60	50	44	
		%	3.9	96.1		13.6	86.4		39.0	32.5	28.5	
	Öğretmen	n	5	184		12	176		53	76	59	
		%	2.6	97.4		6.4	93.6		28.2	40.4	31.4	
	İşçi	n	8	261		75	194		90	110	70	
		%	3.0	97.0		27.9	72.1		33.3	40.8	25.9	
	Çiftçi	n	4	108		19	91		29	53	29	
		%	3.6	96.4		17.3	82.7		26.1	47.7	26.2	
	Hukukçu	n	7	72		13	67		29	32	19	
		%	8.9	91.1		16.2	83.8		36.2	40.0	23.8	
	Memur	n	6	192		32	166		73	64	61	
		%	3.0	97.0		16.2	83.8		36.9	32.3	30.8	
	Serbest Meslek	n	12	360		84	286		122	167	79	
		%	3.2	96.8		22.7	77.3		33.2	45.4	21.4	
	Öğrenci	n	7	244		39	210		74	101	76	
		%	2.8	97.2		15.7	84.3		29.5	40.2	30.3	
	Diğer *	n	3	136		9	128		40	51	49	
		%	2.2	97.8		6.6	93.4		28.5	36.5	35.0	

n: *Frekans,* ***Soru 1:*** *"Hayvanları koruma veya benzer amaçlarla kurulmuş bir derneğe üye misiniz?";* ***Soru2:*** *"Size göre Türkiye'de hayvan haklarına yeterli önem veriliyor mu?";* ***Soru 3:*** *"Kedi-köpek gibi evcil hayvanların kısırlaştırılmasını doğru buluyor musunuz?",* ********* *Arkeolog, Bankacı (15), Bilgi İşlemci, Bilişim Teknolojileri Uzmanı, Ekonometri, Ekonomist (2), Ev Hanımı (53), Futbolcu, Finasman Uzmanı (2), Haberleşme, Hostes, İktisatçı (3), İşletmeci (6), Kimyacı, Mimar (2), Muhasebeci (7), Müzisyen (3), Özel Güvenlik (2), Psikolog, Radyo-Tv, Satış Yöneticisi, Sekreter (5), Sosyolog, Sporcu, Tasarımcı (2), Tiyatro Yönetmeni (2), Turizm (22), Uluslarası İlişkiler Uzmanı (2)*

haklarına yönelik bir sorunun varlığına işaret sayılabilir.

Hayvanların kısırlaştırılmasına ilişkin katılımcı görüşleri incelendiğinde kısırlaştırmayı doğru bulmayanların ve emin olmayanların, kısırlaştırmayı doğru bulanlara göre daha pozitif tutum sergiledikleri görülmektedir *(Tablo 1)*. Bu konuda Lupus [13], kısırlaştırma gibi hayvanların onamı dışında yapılan müdahalelerin doğanın kurallarına karşı işlenen bir suç olduğunu ve bu türden işlemler sonucu kaybedilen biyolojik ve davranışsal vasıfların hayvanların bünyesinde yürüttüğü diğer işlevleri ve değerliliği azalttığını ileri sürmektedir. Bu çerçeveden bakıldığında kısırlaştırmayı doğru bulmayanların doğayı merkeze alan bir yaklaşımla bu soruyu değerlendirdikleri söylenebilir. Benzer bir yaklaşım hayvanların reklamlarda kullanılması konusundan rahatsız olanların ve emin olmayanların, rahatsız olmayanlara göre daha pozitif tutum sergilemesinde de gözlendi *(Tablo 1)*. Hayvanları Koruma Kanunu [14] ile hayvanların ticarî amaçla reklam için kullanılmasının izne tâbi tutulması, hayvanların; acı, ıstırap ya da zarar görecek şekilde reklam ve benzeri işler için kullanılamayacağının vurgulanması, hayvanların reklamlarda kullanılmasından rahatsız olanların tutumuna hukuki bir dayanak kazandırmaktadır. Kısırlaştırma ve hayvanların reklamlarda kullanılması konularına, hayvanların doğasına uygun olan ve olmayan müdahaleler çerçevesinden bakmak gerekmektedir. Ayrıca, kısırlaştırmanın hayvan populasyonunun orantısız artışı ve bu artışı takiben gündeme gelen halk sağlığı, itlaf ve ötenazi konuları ile beraber değerlendirilmesi uygun olacaktır.

Bölgeler arası farklılıklar incelendiğinde *(Tablo 2)*; katılımcıların verdikleri cevaplardan; Ankara'da yaşayan katılımcıların hayvan haklarına yönelik en pozitif; Elazığ'da yaşayan katılımcıların ise en negatif tutumu sergiledikleri belirlenmiştir. Bu durum bölgeler arası eğitim düzeyindeki farklılıklar ile ilişkili olarak açıklanabileceği gibi Paul ve Podberscek'in [15] veteriner fakültesi öğrencilerinin hayvan refahına ilişkin tutum değişikliklerini açıklarken ifade ettiği şekilde bölgesel ve alt kültürel farklılıklardan da kaynaklanmış olabilir. Öte yandan bölgeler arası ortaya çıkan bu farklılığın; araştırma çerçevesinde seçilen ve pozitif tutum sergileyen Ankara, İzmir, Samsun ve Bursa'nın büyükşehir kapsamında olmasından ve çeşitli araştırmalarda [9,10,16] vurgulandığı gibi burada yaşayanların doğa ve içinde barındırdığı hayvanlara yönelik özleminden kaynaklandığı da ileri sürülebilir. Bu çerçeveden değerlendirildiğinde Antalya'da yaşayan katılımcıların negatif tutumu anlaşılamamaktadır. Urfa İlindeki katılımcıların pozitif tutum sergilemesinin ise adı geçen ildeki yaygın hayvancılık faaliyetleri ve hayvanlarla temasın yoğun yaşanması ile açıklanabileceği söylenebilir.

"Kedi-köpek gibi evcil hayvanların kısırlaştırılmasını doğru buluyor musunuz?" sorusuna meslek gruplarından en fazla evet cevabını sağlık personeli olanlar verirken en fazla hayır cevabını çiftçiler vermiştir. Özkul ve ark.[12] tarafından yürütülen araştırmada hayvan haklarına yönelik en pozitif tutumu gösteren ve hayvan hakları perspektifinden bakıldığında kısırlaştırmayı desteklemeyeceği düşünülen sağlık personelinin bu soruya evet cevabı vermesi, bu grupta yer alanların soruyu, sahipsiz hayvan populasyonunun artışı ve halk sağlığı konuları ile beraber değerlendirmesinden kaynaklanmış olabilir. Söz konusu araştırmada [12], hayvan haklarına yönelik daha az pozitif tutum sergileyen çiftçilerin tutumu, konuyu kedi köpek özelinde değil ekonomik değeri olan hayvanlar penceresinden değerlendirdiklerini düşündürmektedir.

Sonuç olarak; katılımcıların büyük çoğunluğunun Türkiye' de hayvan haklarına yeterli önem verilmediğine ilişkin görüşleri araştırma verileri ile ortaya konulmuştur. Araştırma verilerinden hareketle, Türkiye genelinde hayvanları koruma derneklerine üyeliklerin teşvik edilmesi ve bölgesel farklılık, öncelikler dikkate alınarak hayvan haklarına yönelik bilgilendirmeler yapılması ile hayvanlara ve haklarına ilişkin pozitif tutumun artacağı ileri sürülebilir.

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RESEARCH ARTICLE

Parasites Detected by Examination of Fecal Samples in Wrestling Camels

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Summary

First historical findings on camel wrestling, which is now practiced as a festival in Turkey, particularly in certain regions (Marmara, Aegean, Mediterranean) date back to the 15th century. In terms of animal husbandry, parasitic diseases may result in negative outcomes ranging from loss of performance to death for camels. In the present study, annual camel wrestling arenas were visited between December and March (2010-2011), and stool samples were collected from camels from different cities for parasitological analysis. Stool samples of 109 camels from 7 different cities (Aydin, Izmir, Manisa, Denizli, Mugla, Balikesir, and Canakkale) were examined using Baermann-Wetzel stool culture, flotation, and sedimentation techniques for the parasites that live in gastrointestinal tract. The analyses revealed that 74% of the camels (81 of 109) were infected with one or more parasites: *Trichostrongylus* spp. (47.7%), *Ostertagia* spp. (27.5%), *Dicrocoelium* spp. (24.7%), *Trichuris* spp. (11.9%), *Eimeria cameli* (11.9%), *Capillaria* spp. (6.4%), *Fasciola* spp. (6.4%), *Dictyocaulus viviparous* (5.5%), *Haemonchus* spp. (4.5%), *Oesophagostomum* spp. (4.5%), *Cooperia* spp. (4.5%), *Cooperia oncophora* (3.6%), *Nematodirus* spp. (3.6%), *Chabertia ovina* (2.7%), *Eimeria* spp. (1.8%), and *Paramphistomum* spp. (0.9%). 16 different parasites, at the level of species and genus, were found, of which 14 were helminth (11 nematodes, 3 trematodes), and 2 were protozoans. The present study was the first to report *Ostertagia* spp., *Fasciola* spp. *Dictyocaulus viviparus, Haemonchus* spp., *Oesophagostomum* spp., *Cooperia* spp., *Cooperia oncophora, Chabertia ovina* and *Paramphistomum* spp. in camels in Turkey. As high as 74 percent of the incidence of parasitic diseases and the wide variety of parasites found in the present study suggest that parasitic infections may be overlooked entity in wrestling camels that are meticulously brought up.

Keywords: *Camel, Helminth, Protozoon, Turkey*

Güreş Develerinde Dışkı Bakılarına Göre Saptanan Parazitler

Özet

Deve güreşlerinin tarihine ilişkin ilk bulgular 15. yüzyıla ait olup, günümüzde Türkiye'nin özellikle belli bölgelerinde (Marmara, Ege ve Akdeniz Bölgeleri) festival havasında yapılan etkinlikler şeklindedir. Yetiştirilme hedefleri doğrultusunda paraziter hastalıklar, bu hayvanlarda performans kayıplarından başlayıp ölüme kadar gidebilen değişik derecelerde olumsuzluklara neden olabilmektedir. Bu çalışmada Aralık-Mart ayları arasındaki dönemlerde her yıl düzenlenen deve güreşi alanlarına gidilmiş (2010-2011), değişik illerden gelen develerden dışkı örnekleri alınmış ve parazitolojik açıdan incelenmiştir. 7 ayrı ilden (Aydın, İzmir, Manisa, Denizli, Muğla, Balıkesir, Çanakkale) gelen toplam 109 hayvandan alınan dışkı örnekleri Baerman Wetzel, dışkı kültürü, flotasyon ve sedimentasyon metotları uygulanarak sindirim sistemi ve ilişkili organlarda bulunan parazitler açısından muayeneleri yapılmıştır. Yapılan incelemelerde develerin %74'ü (81/109) bir ya da daha fazla parazitle enfekte bulunmuş olup; *Trichostrongylus* spp. %47.7, *Ostertagia* spp. %27.5, *Dicrocoelium* spp. %24.7, *Trichuris* spp. %11.9, *Eimeria cameli* %11.9, *Capillaria* spp. %6.4, *Fasciola* spp. %6.4, *Dictyocaulus viviparus* %5.5, *Haemonchus* spp. %4.5, *Oesophagostomum* spp. %4.5, *Cooperia* spp. %4.5, *Cooperia oncophora* %3.6, *Nematodirus* spp. %3.6, *Chabertia ovina* %2.7, *Eimeria* spp. %1.8, *Paramphistomum* spp. %0.9 oranlarında tespit edilmiştir. Tür ya da cins düzeyinde 16 farklı parazit varlığı tespit edilmiş olup bunlardan 14'ü helmint (11 nematod, 3 trematod) 2'si protozoondur. Bu çalışmayla develerde *Ostertagia* spp., *Fasciola* spp. *Dictyocaulus viviparus, Haemonchus* spp., *Oesophagostomum* spp., *Cooperia* spp., *Cooperia oncophora, Chabertia ovina* ve *Paramphistomum* spp. Türkiye'den ilk kez bildirilmiştir. Bu sonuçlara göre %74'lük enfeksiyon oranı ve parazit çeşitliliği, yetiştiriciliği özenle yapılan güreş develerinde paraziter enfeksiyonların göz ardı edildiğini düşündürmektedir.

Anahtar sözcükler: *Deve, Helmint, Protozoon, Türkiye*

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INTRODUCTION

The fact that these outdoor games previously held by cameleers among themselves as challenging winter entertainment have been adopted by the people has led to the development of camel wrestlings [1]. First historical findings on camel wrestling, which are now held in the form of festival activities in Turkey, particularly in certain regions (Marmara, and Akdeniz Regions), date back to the 15th century [2]. In many countries, camels are utilized for carrying people or goods, and for their meat. However, camels that are bred in our country are primarily used in camel wrestling, and utilized for their meat when necessary, as well (especially in the production of sujuk, a spicy sausage).

Wrestling camels are hybrids of female Dromedary (Arabian) camels *(Camelus dromedaries)* and male Bactrian (Asian) camels *(Camelus bactrianus)*. The hybrid male camels, which are bigger and heftier than their parents, are better at carrying loads and speed [2]. In regards to the upbringing goals, parasitic diseases may have negative effects in degrees varying from loss of performance to death cases on camels.

Camel wrestling and cameleering have an important position in local cultures in certain regions of Turkey (Ege, Marmara, and Akdeniz Regions), and attract an increasing attention, but few scientific studies on the diseases of this animal group utilized for various purposes for centuries are available both in Turkey and in the world. Therefore, the current study was aimed at determining the parasitic variety in these animals by identifying eggs, larvae, and oocytes that might lead to the growth of parasites found in stool.

MATERIAL and METHODS

Camels and the Collection of Stool Samples

For convenience in gathering materials, stool samples were collected from the camels in the wrestling arenas in the months of December, January, February, and March (2010-2011), which are estrous period of the camels, and which also correspond to the wrestling months. Stool samples were collected from camels from different cities taking part in the events, primarily in the city of Aydın and those in its vicinity, and İzmir and Muğla. Since the camels were dangerous and the procedure were considered by the owners so delicate, stool samples were collected fresh from the ground where camels were standing apart from each other, instead of collecting them directly from the rectums of the animals, taking extra caution and making sure that the samples were clean. It was also made sure that the camels from which the stool samples were collected did not receive any anthelmintic drugs within the last six months. The stool samples collected from 109 animals (all from males between the ages of 7 and 18) from 7 different cities (Aydin, 54; Mugla, 21; Izmir, 16; Denizli, 5; Canakkale, 5; Manisa, 4; Balikesir, 4), whose data were recorded and enclosed individually (age, sex, city, and contact information of owner), were transported to the laboratory in big containers .

Examination of Stool Samples

Stool samples from each camel were individually separated into three parts. The Baermann-Wetzel technique was immediately employed for the first part to investigate the presence of lungworms [3].

The second part of stool samples were separately cultivated to identify at the level of genus or species gastro-intestinal nematodes, known as *Trichostrongylidae*. Stool samples were blended with fine tree sawdust and water to form a slurry (3 parts stool, 1 part sawdust), then placed in plastic containers and incubated in an incubator at 26-28°C for one week. During the incubation period, stool samples were taken out of the incubator, stirred for aeration, and water was added to the containers with lower amounts of water [3,4]. The larvae developed at the end of the period were collected using the Baermann-Wetzel technique, and morphologically identified in accordance with the relevant literature [5,6].

Third-part stool samples were analyzed using Fülleborn's saturated saltwater flotation and Benedek's sedimentation techniques [3,4].

RESULTS

Co-evaluation of findings from lungworm analyses, egg and oocyte determination analyses, and cultivation results revealed that 74% of the camels (81 out of 109) were infected with one or more parasites.

Sixteen different parasites were determined at the level of genus or species, of which 14 were helminth (11 nematodes, 3 trematodes) and 2 were prootozoans *(Table 1)*. The most common parasite among all was *Trichostrongylus* spp. by 47.7%, followed by *Dicrocoelium* spp. among trematodes by 24.7%, and *Eimeria cameli* among protozoans by 11.9%. The least detected parasite was *Paramphistomum* spp. with a rate of 0.9%, being identified in only one animal.

DISCUSSION

In studies on camel diseases, it has been observed that parasitic infections are the major cause of reduced nutrient utilization, as well as decreased meat and milk yield, reduced growth rate in youngsters, and reproductive deficiency [7,8]. In Sudan, which has the second largest camel population in the world, the most common diseases in camels are associated with parasitism [9]. Much of the information on the gastrointestinal helminthes of camels was obtained from those studies conducted in Northern African countries. These studies have reported that camels

Table. *Identified parasites and diffusion rates in camels whose stools were inspected*
Tablo. *Dışkı bakısı yapılan develerde, bulunan parazitler ve yayılış oranları*

Species of Parasite	General Situation		Aydın	Muğla	İzmir	Denizli	Çanakkale	Manisa	Balıkesir
	NIA (n=109)	PIA (%)	NIA (n=54)	NIA (n=21)	NIA (n=16)	NIA (n=5)	NIA (n=5)	NIA (n=4)	NIA (n=4)
Trichostrongylus spp.	52	47.7	33	6	7	4	1	1	-
Ostertagia spp.	30	27.5	16	7	3	4	-	-	-
Trichuris spp.	23	11.9	9	6	4	-	2	-	-
Capillaria spp.	10	6.4	2	4	2	1	-	-	1
Dictyocaulus viviparus	6	5.5					-	-	-
Haemonchus spp.	5	4.5	4	1	-	-	-	-	-
Oesophagostomum spp.	5	4.5	5	-	1	-	-	-	-
Cooperia spp.	5	4.5	2	1	-	1	1	-	-
Cooperia oncophora	4	3.6	3	1	-	-	-	-	-
Nematodirus spp.	4	3.6	3	-	-	1	-	-	-
Chabertia ovina	3	2.7	2	-	-	-	1	-	-
Dicrocoelium spp.	27	24.7	15	2	5	2	-	2	-
Fasciola spp.	7	6.4	3	-	1	-	-	-	-
Paramphistomum spp	1	0.9	-	-	1	-	-	-	-
Eimeria cameli	13	11.9	10	2	2	-	-	-	-
Eimeria spp.	2	1.8	-	-	-	1	-	-	-

NIA: *Number of infected animal,* ***PIA:*** *Percentage of infected animals*

are susceptible to more than 60 types of helminthes. It should be surprising that little is known about the endoparasites in camels that in the Arabian Peninsula, are the most important source of meat and milk for the nomads, as well as used as means of transportation [7].

There are few helminthological studies on camels in Turkey [10,11]. Merdivenci [12] and Turkutanit [13] have come across *Dipetalonoma evansi* in the testicular connective tissue and testicular (spermatic) arteries of camels. In a study by Eren *et al.*[14] on the analyses of stool samples from 150 camels, Trichostrongylidae eggs (26%), *Nematodirus* spp. eggs (12%), *Trichuris* spp. eggs (10%), *Dicrocoelium* spp. eggs (7%), and *Eimeria* spp. oocytes (4.6%) were identified. In the same study, six cases of "hydatid cysts" were identified in organ examinations of 6 camels that were cut in the slaughterhouse, 2 of which had hydatid cysts both in their lungs and liver. Despite similarities with the current study, in the above-mentioned study [14], helminths were identified by only morphologically identifying larvae in the stool samples, and because cultivation was not performed, parasite identifications were only at family or genus levels. In the current study, Trichostrongylidae, which can affect yield characteristics, were identified at genus and species levels. The latest Turkish study we were able to obtain was the study by Cirak *et al.*[15] on the efficacy of Doramectin conducted with a total of 10 camels. Cirak *et al*.[15] have reported the presence of *Trichostrongylus* spp., *Teladorsagia* spp., *Nematodirus* spp., *Trichuris* spp., *Capillaria* spp., Anoplocephalidae, *Dicrocoelium dendriticum*, *Eimeria cameli* and *E. rajasthani*, with Trichostrongylus spp. being the most common species, as in the present study.

While no *Dictyocaulus filaria*, the most common nematode found in the lungs of those in Africa and Asia [10,16], were detected in the current study, the less frequently seen, *D. viviparous,* was detected by 5.5%, which is thought to be due to the fact that camels share the meadows with other animals in their specific geographic locations, certainly affecting the diversity of other parasites found in camels. Lungworms that cause symptoms associated with the respiratory system, as well as general depression and rapid loss of fitness are particularly important in camels from which performance is expected [17].

The most common coccidiosis factor in camels, *Eimeria cameli* [16-19], was detected by 11.9% in the current study. Although the animals in the current study were between the ages of 7 and 18 and coccidiosis would not expected to occur, it is especially crucial for animal owners to take necessary precautions considering the fact that infected animals are carriers for young camels.

This study was the first to report *Ostertagia* spp., *Fasciola* spp. *Dictyocaulus viviparus*, *Haemonchus* spp., *Oesophagostomum* spp., *Cooperia* spp., *Cooperia oncophora*,

Chabertia ovina and *Paramphistomum* spp., in camels in Turkey. Camels, like cattle or sheep, have a broad spectrum of helminths. The fact that the present study has found the incidence of the infection as high as 74% and the wide variety of parasites suggest that parasitic infections may be overlooked factor among wrestling camels that are meticulously brought up. We hope that the results of the present study will raise awareness among camel owners and veterinarians of the risk factors of the parasitic diseases that progress without symptoms.

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RESEARCH ARTICLE

Phenotypic, Genotypic Characterisation and Antimicrobial Susceptibility Determination of *Lactococcus garvieae* Strains [1]

Soner ALTUN * Ertan Emek ONUK ** Alper ÇİFTÇİ ***
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Summary

In this study, phenotypic and genotypic features of 10 *L. garvieae* strains isolated from rainbow trout farms were examined with 3 reference strains (Spain, England and ATCC 43921) comperativly. Rapid 32 STREP and conventional microbiologic tests were used for determining phenotipic features of *L. garvieae* strains. Altough there are differences, in Rapid 32 STREP system, between strains in terms of β-Glucuronidase, D-ribose, sorbitol, lactose, raffinose, alanyl-phenylalanyl-proline arylamidase, pyrrolidonyl arylamidase, hippurate hydrolysis, urease tests, all strains have been confirmed as *L. garvieae* by API web. In RAPD PCR analysis, in which ERIC 2 primer is used, *L. garvieae* isolates were genotyped within 3 separate clusters according to similarity coefficient index of 70%, and it was detected that a vast majority of isolates with Turkey-origin (8 isolates) belongs to predominant type LG1 genotype. In addition to this, antimicrobial tests of *L. garvieae* strains shows that there are resistance against gentamycin, neomycin, lincomycin, sulfamethoxazole-trimethoprim, oxytetracycline, erythromycin, amoxicillin, florfenicol and doxycycline, which are frequently used on fish in our country.

Keywords: *Oncorhynchus mykiss, Lactococcus garvieae, Rapid32 STREP, RAPD PCR, Antimicrobial sensitivity*

Lactococcus garvieae Suşlarının Fenotipik, Genotipik Karakterizasyonu ve Antimikrobiyal Duyarlılıklarının Belirlenmesi

Özet

Araştırmada, gökkuşağı alabalığı işletmelerinden izole edilmiş olan 10 adet *Lactococcus garvieae* suşunun 3 adet referans suşla (İspanya, İngiltere ve ATCC 43921) karşılaştırmalı olarak fenotipik ve genotipik özellikleri incelenmiştir. *L. garvieae* suşlarının fenotipik özelliklerinin belirlenmesinde konvansiyonel mikrobiyolojik ve Rapid 32 STREP testleri kullanılmıştır. Rapid 32 STREP sistemde suşlar arasında β-Glucuronidase, D-ribose, sorbitol, lactose, raffinose, alanyl-phenylalanyl-proline arylamidase, pyrrolidonyl arylamidase, hippurate hydrolysis, urease testleri yönüyle farklılıklar olmasına rağmen API Web'de tüm suşlar *L. garvieae* olarak doğrulanmıştır. ERIC2 primerinin kullanıldığı RAPD PCR analizinde *L. garveae* izolatları %70 benzerlik katsayısına göre 3 farklı genotipe ayrılmış ve Türkiye kökenli izolatların büyük bir bölümü (8 izolat) predominant tip olan LG1 genotipine dahil olduğu belirlenmiştir. Ayrıca bu çalışmada *L. garvieae* suşlarının antibiyotik duyarlılık testlerinde ülkemizde balıklarda sıklıkla kullanılan gentamisin, neomycin, lincomycin, sulphamethoxazole-trimetoprim, oksitetrasiklin, eritromycin, amoxycillin, florfenikol ve doksisiklin'e karşı direnç geliştirmiş oldukları belirlenmiştir.

Anahtar sözcükler: *Oncorhynchus mykiss, Lactococcus garvieae, Rapid32 STREP, RAPD PCR, Antimikrobiyal duyarlılık*

INTRODUCTION

Lactococcus garvieae is the etiological agent of lactococcosis, causes significant economic losses both in marine and freshwater aquaculture all over the world [1]. The first isolation of agent from fish was originally made

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in Japan in 1991 and referred to *Enterococcus seriolicida* [2]. From that time to today, Lactococcosis was progressively spread in aquatic organisms and the agent has been isolated from most areas of the world such as Bulgaria, Brazil, Greece, England, France, Italy, Israel, Portugal and Spain [3-10]. In Turkey first isolation was performed in 2001 from farmed rainbow trout [11]. Since then, such infections have been reoccurred, especially during the warm summer months. Therefore, *L. garvieae* is now considered one of the most important pathogens in the rainbow trout industry in Turkey [11,12].

The identification of *L. garvieae* can be performed by conventionally microbiological methods and API diagnostic kits. However, in these methods clindamycin sensitivity test and PCR method are recommended for the exact identification of the agent, because different results are obtained depending on the culture mediums used, and a great majority of phenotypic characteristics of *L. garvieae* and *L. lactis* subs. l*actis* which is the most associated species, is similar [1,13,14]. In addition, it is stated that the presence of clindamycin resistant *L. lactis* strains may lead to wrong diagnosis. Therefore, PCR technique in identification of *L. garvieae* strains comes to the forefront [13].

Molecular characterization of isolates plays a very important role in detecting transmission ways, as well as in describing genetic relationships among strains isolated from different ecologic regions [6]. Ribotyping, pulsed-field gel electrophoresis, random amplified polymorphic DNA (RAPD) PCR, sau-PCR and amplified fragment length polymorphism methods have been used for detecting epidemiologic features of *L. garvieae* isolates so far. Numerous studies, while there was very low genetic relations among isolates isolated from different hosts or environmental sources, there was genetic variety among fish isolates isolated from different countries [6,15,16].

The aim of the present study was to determine morphologic, physiologic, biochemical features and anti-microbial sensitivity profiles of *L. garvieae* strains isolated from rainbow trout farms in Turkey. In addition to these, it is aimed to create RAPD patterns of *L. garvieae* strains isolated from different geographic regions in Turkey by PCR and to determine genetic relationship among isolates, and thus to introduce an epidemiological data source for our country.

MATERIAL and METHODS

Bacterial Isolates and Growth Conditions

We examined 12 *L. garvieae* isolates, comprising 10 isolates from different cities of Turkey, one isolate from Spain, and one from England. The type strain *L. garvieae* ATCC 43921 was included in analyses for comparative purposes *(Table 1)*. For all experiments, the strains were routinely grown on trypticase soy agar (TSA) and Columbia blood agar (CA) plates and incubated aerobically at 22°C for 24-48 h.

Table 1. *L. garvieae strains investigated in the study*
Table 1. *Çalışmada kullanılan L. garvieae suşları*

Isolate No	Origin	Source
1	Muğla/Turkey	Rainbow trout
2	Muğla/Turkey	Rainbow trout
3	England	Rainbow trout
4	Spain	Rainbow trout
5	Antalya/Turkey	Rainbow trout
6	Kütahya/Turkey	Rainbow trout
7	Bilecik/Turkey	Rainbow trout
8	Isparta/Turkey	Rainbow trout
9	Bursa/Turkey	Rainbow trout
10	Samsun/Turkey	Rainbow trout
11	Samsun/Turkey	Rainbow trout
12	Samsun/Turkey	Rainbow trout
13	ATCC 43921	Bovine mastitis

Phenotypic Characterization

All isolates were characterized using the following classical phenotypic tests: Gram staining reaction, motility, oxidation-fermentation (O/F), oxidase, catalase, gelatinase, Simmons citrate, indole production, methyl red (MR), reduction of nitrate, starch hydrolysis, O/129, H_2S, growth in MacConkey agar (MA), Bile esculin azide agar, eosin methylene blue (EMB) agar, nutrient agar (NA), TSA and brain heart infusion agar (BHIA), hemolysis in 5% sheep blood agar, ability to growth in pH 9.6, 1%, 2.5%, 6.5% and 8% NaCl [17,18]. These isolates were further characterized biochemically by using the rapid ID 32 STREP (Biomerieux) according to the manufacturer's instructions, except for the temperature of incubation which was set at 35°C 4 h, and results were compared with the manufacturer database.

Molecular Confirmation by PCR

PCR reaction with *L. garvieae* specific primer pairs (pLG-1/pLG-2) was performed for genetic confirmation of isolates as described by Zlotkin et al.[13]. Isolates giving a predicted amplification product of 1100 bp were identified as *L. garvieae*.

Genotyping by RAPD Analysis

The RAPD technique was performed to investigate clonal relatedness among 13 *L. garvieae* isolates, using the universal primer ERIC 2 primer (5'-AAGTAAGTGACTGG GGTGAGCG-3') as described with some modification [19]. PCR amplifications were performed in a total reaction volume of 25 µl. The reaction mixture contained, 200 µM of each dNTP, 2.5 mM $MgCl_2$, 25 pmol primer, 2.5 unit of Taq DNA polymerase, 5 µl of template DNA. The amplification program was one cycle at 95°C for 1 min; 30 cycles at 94°C for 1 min, at 40°C for 1 min, at 72°C for 1 min; followed by one cycle at 72°C for 5 min. The PCR products were separated on

1.5% agarose gel stained with 2 mg/ml of ethidium bromide.

The DNA profiles were analyzed with CHEF-DR® III, Quantity One® software (Bio-Rad Laboratories, Hercules, CA). Dendrogram was constructed using the unweighted-pair group method (UPGMA). To determine reproducibility of RAPD analysis, 5 isolates were selected randomly and RAPD analysis was repeated 3 times subsequently.

Antimicrobial Susceptibility Patterns

L. garvieae isolates were tested for antimicrobial susceptibility by the disc diffusion method on Mueller-Hinton agar. The antimicrobial agents (Oxoid) were tested including neomycin (10 µg), gentamycin (120 µg), oxytetracycline (30 µg), florfenicol (30 µg), erythromycin (15 µg), sulfamethoxazole + trimethoprim (1.25 µg/ 23.75 µg), doxycycline (30 µg), lincomycin (2 µg) and amoxicillin (25 µg). Both at 24th and 48th h of the incubation, incubation zone diameters were measured and evaluated. The isolates were classified as sensitive (S), intermediary sensitive (I), or resistant (R), on the basis of the size of the zone of bacterial growth inhibition, according to the Clinical and Laboratory Standards Institute [20].

RESULTS

Phenotypic Characterization

There were phenotypic differences among *L. garvieae* isolates in β-Glucuronidase, D-ribose, sorbitol, lactose, raffinose, voges proskauer (VP), alanyl-phenylalanyl-proline arylamidase (APPA), pyrrolidonyl arylamidase (PyrA) and

Table 2. *Phenotypic characteristic of L. garvieae strain with convansionally tests*

Tablo 2. *L. garvieae suşlarında konvansiyonel testler kullanılarak belirlenen fenotipik özellikleri*

Phenotypic Characters	**Strain No**												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Colony morphology	S*	S*	S*	S*	S*	S*	S*	S*	S*	S*	S*	S*	S*
Gram staining	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-
O-F	F	F	F	F	F	F	F	F	F	F	F	F	F
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate Reduction	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-
O/129 (10 µg)	S	S	S	S	S	S	S	S	S	S	S	S	S
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on													
MacConkey agar	-	-	-	-	-	-	-	-	-	-	-	-	-
Bile esculine azide agar	+	+	+	+	+	+	+	+	+	+	+	+	+
Eozin methylen blue agar	-	-	-	-	-	-	-	-	-	-	-	-	-
Nutrient agar	+	+	+	+	+	+	+	+	+	+	+	+	+
Trypticase soy agar	+	+	+	+	+	+	+	+	+	+	+	+	+
Brain Heart infusion agar	+	+	+	+	+	+	+	+	+	+	+	+	+
Hemolysis in blood agar	α	α	α	α	α	α	α	α	α	α	α	α	A
Growth in													
pH 9.6	+	+	+	+	+	+	+	+	+	+	+	+	+
%1 NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
%2.5 NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
%6.5 NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
%8 NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-

S: Smooth colony, **S:** Sensitive*

urease tests. Only reference strain in 13 isolates hydrolyized hippurate. Despite differences among isolates, all isolates were identified as *L. garvieae* in API web. Results obtained from conventional microbiologic tests and Rapid ID 32 STREP test kits are given in *Table 2* and *3*.

Molecular Identification by PCR

All isolates used in the study were confirmed by specific PCR, giving the expected amplification product of 1100 bp belonging to the 16S rRNA gene *(Fig. 1)*.

Genotyping

In RAPD method, *L. garvieae* isolates were grouped within three seperate genotype clusters according to 70% similarity coefficient index. It was detected that 8 of the isolates (66.6%) belong to predominant type LG 1 genotype, 3 other isolates (25%) to LG3 genotype, and one isolate (8.3%) to LG2 *(Fig. 2)*. Furthermore, it was detected that isolates belonging to LG2 and LG3 genotypes have high similarity. It was found that reproducibility of RAPD PCR, in which M13 primer was used, is 100%.

Table 3. *Phenotypic characteristic of L. garvieae strains with Rapid 32 Strep tests*
Tablo 3. *L. garvieae suşlarında Rapid 32 Strep testi kullanılarak belirlenen fenotipik özellikler*

Phenotypic Characters	Strain No												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Adh	+	+	+	+	+	+	+	+	+	+	+	+	+
β-glucosidase	+	+	+	+	+	+	+	+	+	+	+	+	+
β-galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-
β-glucuronidase	+	+	-	-	-	-	+	+	+	+	+	+	-
α-galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-
Pal	-	-	-	-	-	-	-	-	-	-	-	-	-
D-ribose	-	-	-	-	-	-	+	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	+	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	+	-	-	-	-	-	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	+	-	-	-	-	-	-
Voges proskauer	-	+	-	-	-	-	-	+	-	+	+	+	-
Appa	+	+	-	-	-	-	+	+	+	+	+	+	+
β-galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-
Pyra	+	+	-	-	-	-	-	+	+	+	+	+	+
β –Nag	-	-	-	-	-	-	-	-	-	-	-	-	-
Gta	-	-	-	-	-	-	-	-	-	-	-	-	-
Hip	-	-	-	-	-	-	-	-	-	-	-	-	+
Glycogen	-	-	-	-	-	-	-	-	-	-	-	-	-
Pullulan	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-
Saccharose	+	+	+	+	+	+	+	+	+	+	+	+	+
L-arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
M-βdg	+	+	+	+	+	+	+	+	+	+	+	+	+
Tagatose	+	+	+	+	+	+	+	+	+	+	+	+	+
β-mannosidase	-	-	-	-	-	-	-	-	-	-	-	-	-
Cyclodextrin	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	+	-	+	+	+	-	+	+	+	-	-	+	+

Adh: *arginine dihydrolase,* ***Pal:*** *alkaline phosphatase,* ***Appa:*** *alanyl-phenylalanyl-proline arylamidase,* ***Pyra:*** *pyrrolidonyl arylamidase,* ***ß-Nag:*** *N-acetyl-β-glucosaminidase,* ***Gta:*** *glycyl-tryptophan-arylamidase,* ***Hip:*** *hippurate hydrolysis,* ***M-βdg:*** *methyl-β-D-glucopyranoside acidification*

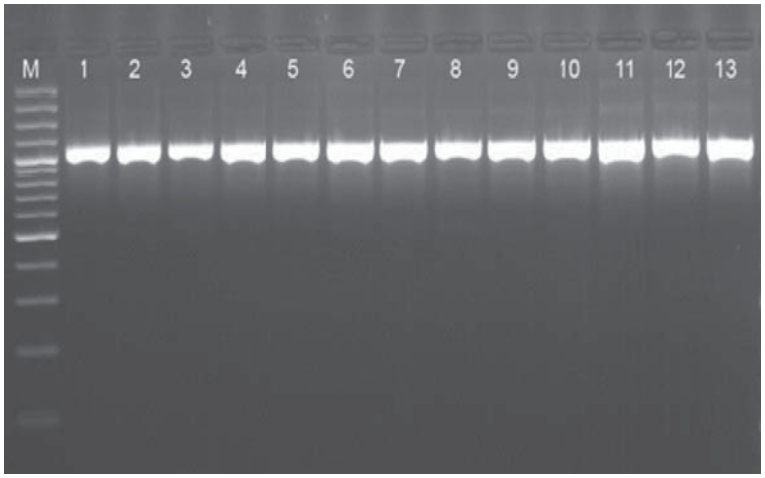

Fig 1. *L. garvieae* specific PCR, 1100 bp. molecular weight standard (100-3000 bp), 1-13; *L. garvieae* field ve referance strains (see *Table 1*).

Şekil 1. *L. garvieae* spesifik PCR, 1100 bp. M; 3000 bp moleküler marker 1-13; *L. garvieae* saha ve referans suşları (bknz. *Tablo 1*)

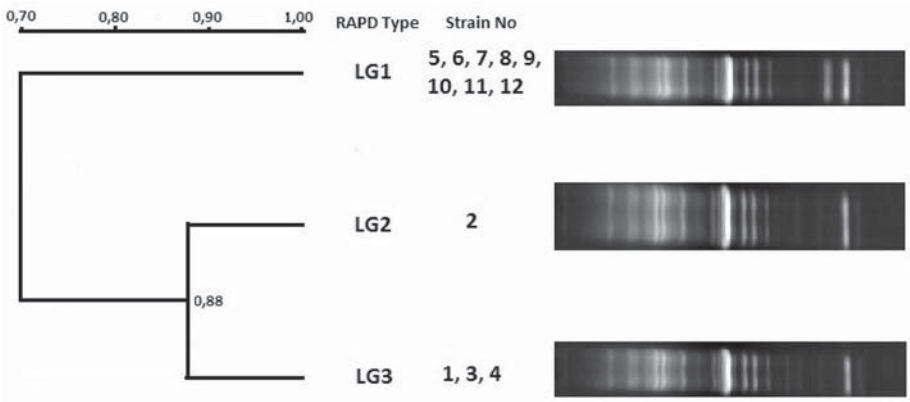

Fig 2. RAPD-PCR profiles of *L. garvieae* strains

Şekil 2. *L. garvieae* suşlarının RAPD PCR profilleri

Table 4. *Antimicrobial susceptibility profiles of L. garvieae strains*
Tablo 4. *L. garvieae suşlarının antibiyotik duyarlılık profilleri*

Antimicrobial Disc	**Strain No**												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Gentamycin	R	R	R	R	R	R	R	R	R	R	R	R	R
Neomycin	R	R	R	R	R	R	R	R	R	R	R	R	R
Lincomycin	R	R	R	R	R	R	R	R	R	R	R	R	R
Oxytetracycline	R	R	R	R	R	I	R	R	R	S	R	R	R
Amoxicillin	R	R	R	I	R	R	R	R	R	I	R	I	R
Florfenicol	R	R	R	R	R	R	R	I	I	I	I	I	I
Erythromycin	R	R	R	R	R	I	R	R	R	R	R	R	R
Doxycycline	R	R	R	R	R	I	I	I	R	I	R	I	R
Sulphamethoxazole- trimethoprim	R	R	R	R	R	R	R	R	R	R	R	R	R

R: *Resistant,* ***I:*** *Intermediate,* ***S:*** *Sensitive*

Antimicrobial Susceptibility Patterns

Thirteen *L. garvieae* isolates were examined with nine different antimicrobial in terms of antimicrobial sensitivity and results were given in *Table 4*. All *L. garvieae* isolates used in the study were resistant to neomycin, lincomycin, sulfamethoxazole-trimethoprim, oxytetracycline (except for 6th isolate) and erythromycin (except for 6th isolate); besides, 10, 7 and 8 isolates were resistant to amoxicillin, florfenicol and doxycycline, respectively.

DISCUSSION

Lactococcosis has become increasingly widespread after epizooties occurred in Turkey, as is the case all around the world, causing significant economic losses in trout farms in particular [21]. Knowing genotypic and phenotypic features of the agent is very important to understand epidemiology of infections and to reduce economic losses caused by infection.

Miniaturizing systems, as well as conventional tests, are used for identification of *L. garvieae* isolates and for determination of phenotypic features [1,7]. In phenotypic characterization studies which are done with conventional methods and miniaturizing systems such as API 20 strep, API 50CH, *L. garvieae* strains isolated different regions without caring host range have a lot of common biochemical features and phenotypic homogeneity [4,15,22]. At the same time some studies shows that there may be different results in miniaturizing systems [16]. It is suggested that there is huge diversity among *L. garvieae* isolates in characterization studies carried out with miniaturizing systems (Rapid ID 32 Strep and API 50CH). API 50 CH and Rapid ID 32 strep systems give different results about ribose acidification; thus, this test must be considered in routine identification with Rapid ID 32 Strep of clinic isolates of *L. garvieae* [14]. Conventional methods and miniaturizing systems were evaluated comparatively and *L. garvieae* isolates used in this study exhibited very high level of phenotypic similarity regardless of geographic origins and sources thereof. Researchers got different results among strains about utilization of hip, ß-alactosidase, ß-Nag, ß-mannosidase and D-arabitol, melezitose and pululan (acid from). In our study, it has been established with conventional microbiologic tests that strains isolated from different geographic regions *(Table 1)* (ATCC 43921) have common phenotypic features. There are differences among strains in β-glucuronidase (8 positive), D-ribose (1 positive), sorbitol (1 positive), lactose (1 positive), raffinose (1 positive), VP (5 positive), Appa (9 positive), Pyra (8 positive) and urease (9 positive) tests, when Rapid ID 32 Strep system is applied. While some of our findings obtained from Rapid 32 Strep system are consistent with those of Vela et al.[16] (acification of pullatan, M-βdg and β-mannosidase and presence of enzyme Gta), some other results are not (presence of enzyme Appa and urease and acidification of cyclodextrin). Results of biochemical parameters in our study are not consistent with those of Ravelo et al.[14]. This difference is most probably linked to the incubation temperature and duration used. In our experiments we have employed the instructions of manufacturer (35°C for 4 h) where the others incubated at 25°C or 30°C for 24 h (Ravelo et al.[14], Vela et al.[16]).

RAPD PCR method, which has been commonly used for detecting genetic relationships of bacterial fish pathogens recently [22-28], is a simple, sensitive, reproducible, and easy to apply technique with high differentiating rate [6,29,30]. However, this method is not so common in molecular typing of *L .garvieae* isolates, being limited to only a few studies [6,10,29]. Ravelo et al.[10] used RAPD PCR method to determine genetic similarity of strains isolated from different geographic regions and fish type. Same authors; Random primers used in test were determined comparatively and P5 and P6 primers gave reproducible pattern and isolates were divided into 3 genogroups according to analysis of similarity among different profiles. Spain, Portugal, England and Turkey isolates isolated from rainbow trouts were grouped into group 1, France and Italy isolates isolated from rainbow trouts were grouped into group 2, and Japan isolates isolated from yellowtails and *L. garvieae* NCDO 2155-reference strain were grouped into group 3. In another study, P5 primers were used and Spain, England and Turkey trout isolates were grouped into same genotype. Isolates isolated from Israel has unique RAPD profile. Foschino et al.[6] used RAPD method, in which M13 and P5 primers are used, and it was suggested that M13 primers had the best differentiating rate. Eighty one isolates collected from Italian fish and dairy samples were divided into 52 RAPD genotype in 5 group. Isolates isolated from fish were classified into three different groups. In conclusion, researchers suggested that there was a low genetic relation between dairy and fish isolates, and that Spanish and English isolates were generally included in the same group in terms of geographic origin. ERIC 2 was used as a random primer in our study and *L. garvieae* gave different band patterns with this primer, thereby suggesting that it can be used in epidemiologic studies. *L. garvieae* isolates were grouped into group which had 3 different genotypes with ERIC 2 primer and some of the isolates isolated from our country (isolates 3 and 4) had high similarity with Spain and England isolates, consistent with the study of Ravelo et al.[10]. According to RAPD PCR results, isolates 5-12 belong to predominant LG1 genotype by %66.6 and these isolates shows genetic heterogeneity with Spain and England isolates and isolates isolated from outbreak in 2001. Accordingly, isolates in LG2 and LG3 genotypes have very close relationship. Altun et al.[21] reported that *L. garvieae* strains isolated from Turkey, Spain and England with a different method (16s rDNA sequence analysis) were genetically similar 99-100%. However, *L. garvieae* strains isolated from Turkey in 2001 (isolates 1 and 2) are not related to the other strains in our study. That local isolates are included in dominant group suggests that *L. garvieae* isolates obtained from rainbow trout farms, except for those in Muğla, are associated with a single epidemiologic strain, in Turkey. Researches show that strains originated from the same root can be infective for a long time [31].

The disease can be treated with chemotherapeutics, but choosing sensitive antimicrobial agents is very important due to the resistant strains. *L. garvieae* isolates in our study are totally resistant to gentamycin, neomycin, lincomycin, sulfamethoxazole-trimethoprim, which are very common in our country; moreover, resistance is developed against oxytetracycline, erythromycin, amoxicillin, florfenicol and doxycycline to a great extent. Although, in previous studies [11,20,32,33] carried out in our country on this topic, *L. garvieae* was sensitive to oxytetracycline, erythromycin, amoxicillin, florfenicol and doxycycline, strains tested in this study developed resistance to these antimicrobial agents.

In conclusion, it is required that isolation of agent and antimicrobial sensitivity tests in fish diseases treatment should be performed sooner, that the treatment should be started with sensitive antimicrobial agents from the onset

of diseases, and that the use of any antimicrobial to develop resistance against drugs should be prevented. This situation suggests that bacteriostatic and bactericidal effect of antimicrobial drugs to be used in diseases either for protective, or therapeutic purposes against specific disease agent should be carefully determined, and that sensitive antimicrobial agents should be selected and administered in enough dosage and for sufficient time. RAPD PCR method, in which ERIC primer 2 is used, can be used to determine genetic differences among *L. garvieae* strains and it provides rapid results with high differentiating rate. In addition to this, knowing distribution to different geographic regions of genetic groups of *L. garvieae* can help to prepare effective vaccine formulation and take protective measures against lactococcosis.

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RESEARCH ARTICLE

Analysis of Genetic Diversity in Indigenous Çine Çaparı Sheep under Conservation by Microsatellite Markers [1]

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Summary

In this study, 123 animals from 3 different flocks (ADU-ÇÇKP conservation flock and two breeders' flocks) were genotyped with 10 microsatellite markers to investigate genetic diversity in the endangered native Çine Çaparı sheep breed. The number of alleles per microsatellite marker ranged from 7 (OarCP34) to 17 (OarFCB193), with an average of 11.5 alleles per locus. Total number of alleles for the investigated 10 microsatellite markers was found to be 104, 72 and 45 for ADÜ-ÇÇKP, EA and MV flocks, respectively. The considerable difference in the allele numbers of the flocks indicates high genetic variability in the ADÜ-ÇÇKP flock versus the other two breeders' flocks. The observed alleles have different size from other shows the existence of private alleles for flocks. According to the polymorphism information content (PIC) values, the highest and lowest polymorphism was detected to be 0.875 and 0.699 for OarFCB193 and OarFCB304 markers, respectively. The mean expected heterozygosity (He) and the mean observed heterozygosity (Ho) for the whole Çine Çaparı population under conservation were 0.727 and 0.789, respectively. The highest genetic similarity (0.8262) was observed between the ADU-ÇÇKP conservation flock and Erdoğan Aktürk's flock. The results obtained in the present study will help to interpret the genetic structure of indigenous Çine Çaparı sheep and will be of benefit to the efforts for conservation of this breed.

Keywords: *Genetic resources, Sheep, Çine Çaparı, Diversity, Microsatellites*

Koruma Altındaki Çine Çaparı Koyunlarda Genetik Çeşitliliğin Mikrosatellit İşaretleyiciler İle Analizi

Özet

Çalışmada, Adnan Menderes Üniversitesi Çine Çaparı Koruma Programı (ADÜ-ÇÇKP) kapsamında oluşturulan koruma sürüsü ile 2 yetiştiricide bulunan toplam 123 baş hayvanda 10 mikrosatellit lokusu kullanılarak genotipleme yapılmıştır. Sürüler arası genetik benzerlik ve uzaklıklar incelenmiştir. Mikrosatellit işaretleyici başına elde edilen allel sayısı 7 (OarCP34) ile 17 (OarFCB193) arasında değişmekle birlikte lokus başına ortalama allel sayısı 11.5'tur. Populasyon bütününü oluşturan ADÜ-ÇÇKP, EA ve MV sürülerinde belirlenen toplam allel sayıları sırasıyla 104, 72 ve 45 olarak belirlenmiştir. Allel sayıları bakımından sürüler arası gözlenen bu ciddi fark iki yetiştirici sürüsüne oranla ADÜ-ÇÇKP koruma sürüsünde yüksek genetik çeşitliliğe işaret etmektedir. Diğer allelere oranla farklı büyüklükte allelerin gözlenmesi sürülere özgün özel allellerin varlığına işaret etmektedir. Polimorfik bilgi içeriği (PIC) değerleri dikkate alındığında en yüksek ve en düşük polimorfizm değerleri OarFCB193 ve OarFCB304 lokusları için sırasıyla 0.875 and 0.699 olarak belirlenmiştir. Ele alınan tüm lokuslara dayalı değerlendirme sonucunda gözlenen (Ho) ve beklenen (He) heterozigotluk ortalamaları sırasıyla 0.727 ve 0.789 olarak elde edilmiştir. En yüksek genetik benzerlik (0.8262) ADÜ-ÇÇKP koruma sürüsü ile Erdoğan Aktürk'e ait sürü arasında ortaya çıkmıştır. Bu çalışmadan elde edilen sonuçlar yerli Çine Çaparı koyunlarda genetik yapının tanımlanmasına yardımcı olmakla birlikte gelecekte bu ırkın korunmasına yönelik çabalara fayda sağlayacaktır.

Anahtar sözcükler: *Genetik kaynaklar, Koyun, Çine Çaparı, Çeşitlilik, Mikrosatellit*

INTRODUCTION

Agricultural biodiversity includes the diversity of the cultivated plants and domestic animals utilized by humankind for the production of food and other goods and services. The livestock species (over 40 species) contributing

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to today's agriculture and food production are shaped by a long history of domestication and development. The term animal genetic resources (AnGR) is used to include all animal species, breeds and strains (and their wild relatives) that are of economic, scientific and cultural interest to humankind in terms of food and agricultural production for the present or in the future [1-3].

Being part of the Fertile Crescent, Turkey has an important position for domestication. Therefore, native breeds of sheep, goat and cattle have an important level of diversity. Sheep in Turkey's animal husbandry have great genetic potential together with high numbers and with wide breeds and local type diversity which is formed according to different ecological conditions. The large genetic changes observed in sheep populations in Western Anatolia with the intensification of agriculture have threatened the existence of native Turkish sheep breeds in last decades [4]. In a short period of time including the last two to three decades, some breeds have become extinct (the Ödemiş breed) or endangered (the Dağlıç, Çine Çaparı, Sakız, Kıvırcık breeds).

The fat-tailed Çine Çaparı is a local native sheep breed originating in the mountains (especially in mountain Madran) of the Aydın province in Turkey. In the last 20 to 30 years, the number of purebred Çine Çaparı sheep has declined very much due to the backcrossing of the breed with rams of the Kıvırcık and prolific Sakız (Chios) breeds. Consequently, this process has put them in danger of extinction. A conservation program termed the "Adnan Menderes University - Çine Çaparı Conservation Program (ADÜ-ÇÇKP)" was established in 1996 to characterize and conserve this endangered breed. Studies to establish a conservation flock at Adnan Menderes University were also initiated in the same year. In mountainous villages only two flocks including purebred animals remained. Members of all other flocks were turned to a synthetic form, namely Karya. The main reasons for breeders to keep this breed are the easiness of management and its resistance to diseases and high temperatures.

Studies for the characterization and conservation of indigenous breeds in Turkey in the last decade are encouraging. The efforts made to characterize and conserve the Çine Çaparı sheep breed are a good example of conservation activities in Turkey. The trend of a decrease in the animal number was changed to a trend of an increase with the efforts of researchers at Adnan Menderes University and the support of the Ministry of Food, Agriculture and Animal Husbandry. The ongoing studies have stopped the process of extinction of the breed and have guaranteed the future of the breed.

Microsatellites are valuable genetic markers due to their dense distribution in the genome, great variation, codominant inheritance and easy genotyping. In recent years, they have been extensively used in parentage testing, linkage analyses, population genetics and other genetic studies [5,6].

The aim of this study was to determine the within-breed genetic diversity in endangered Çine Çaparı sheep using 10 microsatellite markers and to obtain the genetic similarities and distances among animals within and between flocks.

MATERIAL and METHODS

DNA samples of 123 animals from 3 existing flocks were genotyped with 10 microsatellite markers (OarCP34, OarFCB193, OarFCB304, OarJMP29, OarFCB128, BM8125, OarJMP58, OarVH72, MAF65, and DYMS1) that selected from the list recommended by FAO [7]. Forward markers were marked with three fluorescent dyes (D2, D3, D4). Three multiplex groups were formed with 8 out of the 10 microsatellites. Annealing temperatures of MAF65 and DYMS1 were not appropriate for the other 3 multiplex groups. Therefore, these two microsatellites were amplified by Polymerase Chain Reaction (PCR) separately. *Table 1* shows details for the considered microsatellites.

DNA was isolated from blood samples using a DNA extraction kit. Specific genomic regions were amplified by Polymerase Chain Reaction (PCR) in accordance with the touchdown PCR technique. The thermal cycling conditions are given in the *Table 2*.

For every microsatellite locus, the amplification reaction took place in a total volume of 25 µl and contained the following constituents in the final concentrations indicated in brackets; dNTP's (0·2 mM for each one), $MgCl_2$ (2.0 mM), primers (0.25 mM for each one), and *Taq* DNA polymerase (1 unit reaction^{-1}). Approximately 100 ng of genomic DNA was used as template for each of PCR amplification. Fragment analysis was achieved using the automatic laser-induced fluorescence DNA sequencer (Beckman Coulter CEQ 8000 Genetic Analysis System). Obtained data was analyzed by the Beckman Coulter CEQ Fragment Analysis Software.

The frequency of particular alleles of the microsatellite sequences were used to calculate the number of alleles (n_A), the number of effective alleles (n_E), the polymorphic information content (PIC), the observed heterozygosity (Ho), the expected heterozygosity (He) and average heterozygosity ($\hat{H}$) [8-11]. Nei's genetic similarities and genetic distances between flocks [12] and F statistics [8] were also estimated. A dendrogram based on Nei's genetic distances was obtained using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method. We used the microsatellite loci analyzed to find out if the examined herd of Çine Caparı was in a genetic equilibrium conformable to the Hardy-Weinberg principle. Propriety of allele frequencies Hardy-Weinberg Equilibrium (HWE) was checked by χ^2 test ($P<0.01$), which estimated observed genotype frequencies and expected numeric values of the same genotypes. These parameters were calculated using GenAlEx [13], PowerStatsV12 [14], MEGA 4 [15], Arlequin 3.5 [16] and POPGENE [17] softwares.

Table 1. *Details of considered microsatellites*
Tablo 1. *Çalışmada kullanılan mikrosatelitlere ait bilgiler*

Locus Name	Primers	Base Pair (bp)	Multiplex Group
OarCP34	**F:** GCTGAACAATGTGATATGTTCAGG **R:** GGGACAATACTGTCTTAGATGCTGC	112-130	1
OarFCB304	**F:** CCCTAGGAGCTTTCAATAAAGAATCGG **R:** CGCTGCTGTCAACTGGGTCAGGG	150-188	
OarFCB193	**F:** TTCATCTCAGACTGGGATTCAGAAAGGC **R:** GCTTGGAAATAACCCTCCTGCATCCC	96-136	
OarJMP29	**F:** GTATACACGTGGACACCGCTTTGTAC **R:** GAAGTGGCAAGATTCAGAGGGGAAG	96-150	2
OarFCB128	**F:** ATTAAAGCATCTTCTCTTTATTTCCTCGC **R:** CAGCTGAGCAACTAAGACATACATGCG	96-130	
BM8125	**F:** CTCTATCTGTGGAAAAGGTGGG **R:** GGGGGTTAGACTTCAACATACG	116-122	
OarJMP58	**F:** GAAGTCATTGAGGGGTCGCTAACC **R:** CTTCATGTTCACAGGACTTTCTCTG	145-169	3
OarVH72	**F:** GGCCTCTCAAGGGGCAAGAGCAGG **R:** CTCTAGAGGATCTGGAATGCAAAGCTC	121-135	
DYMS1	**F:** AACAACATCAAACAGTAAGAG **R:** CATAGTAACAGATCTTCCTACA	159-211	Individual
MAF65	**F:** AAAGGCCAGAGTATGCAATTAGGAG **R:** CCACTCCTCCTGAGAATATAACATG	123-135	Individual

Table 2. *Thermal cycling conditions according to Touchdown PCR*
Tablo 2. *Touchdown PZR koşulları*

Loci	Denaturation (°C)	Time (sec)	Annealing (°C)	Time (sec)	Extension (°C)	Time (sec)
OarCP34	95	45	60-58	45	72	45
OarFCB193						
OarFCB304						
OarJMP29	95	45	61-57	45	72	45
OarFCB128						
BM8125						
OarJMP58	95	45	60-56	45	72	45
OarVH72						
MAF65	95	45	59-57	45	72	45
DYMS1	95	45	52-50	45	72	45

RESULTS

One hundred and fifteen alleles were determined from 10 microsatellites markers. Total number of alleles for the investigated 10 microsatellite markers was found to be 104, 72 and 45 for ADÜ-ÇÇKP, EA and MV flocks, respectively. In the all loci, the number of alleles ranged from 7 (OarCP34) to 17 (OarFCB193), with an average of 11.5 alleles per locus. The frequency of alleles varied according to locus; the allele size range (ASR), number of allele (n_A), number of effective allele (n_E), the polymorphic information content (PIC), the observed heterozygosity (Ho), the expected heterozygosity (He) and average heterozygosity (Ĥ) are given in *Table 3* according to all flocks.

Among the ten microsatellite loci studied, the highest observed heterozygosity value was 0.950 for OarFCB193, and the lowest value was 0.450 for DYMS1. The number of alleles for the OarFCB193, DYMS1, OarJMP29, BM8125, OarJMP58, OarFCB304, OarFCB128, MAF65, OarVH72 and OarCP34 loci were found to be 7, 14, 13, 13, 11, 10, 9, 8 and 7, respectively.

Loci in all flocks were examined in terms of polymorphic information content (PIC) and the results showed that OarFCB193 (0.875), OarFCB128 (0.765) and BM8125 (0.765) loci take highest values.

The number of alleles (n_A), number of effective alleles (n_E), the polymorphism information content (PIC), the observed heterozygosity (Ho), the expected heterozygosity (He) and average heterozygosity (Ĥ) according to all three flocks are given in *Table 4*.

Considering the number of alleles, OarFCB193 showed the highest polymorphism of the three flocks. Ho and He

Table 3. *Allele size range (ASR) (bp), n_A, n_E, PIC, Ho, He and Ĥ values in considered microsatellites in all flocks*
Tablo 3. *Tüm sürüler için kullanılan mikrosatellitlere ait genel gözlenen boyut aralığı (GBA, bp), örnek sayısı (N), n_A, n_E, PIC, Ho, He ve Ĥ değerleri*

Loci	N	ASR (bp)	n_A	n_E	Ho	He	Ĥ	PIC
OarCP34	242	112/124	7	4.479	0.843	0.78	0.777	0.744
OarFCB193	242	96/136	17	8.668	0.950	0.888	0.885	0.875
OarFCB304	242	160/190	11	3.798	0.727	0.740	0.737	0.699
OarJMP29	244	116/156	13	4.289	0.820	0.770	0.767	0.738
OarFCB128	234	100/128	10	4.859	0.692	0.798	0.794	0.765
BM8125	238	108/138	13	4.775	0.782	0.794	0.791	0.765
OarJMP58	244	141/171	13	4.325	0.713	0.772	0.769	0.740
OarVH72	244	123/143	8	3.876	0.730	0.745	0.742	0.708
MAF65	222	125/141	9	4.380	0.568	0.775	0.772	0.738
DYMS1	240	169/201	14	4.312	0.450	0.771	0.768	0.739
Mean	**239**		**11.5**	**4.776**	**0.727**	**0.783**	**0.78**	
St. dev.			**3.064**	**1.407**	**0.141**	**0.041**	**0.041**	

ASR: *allele size range,* ***n_A:*** *number of alleles,* ***n_E:*** *number of effective allele,* ***PIC:*** *polymorphic information content,* ***Ho:*** *observed heterozygosity,* ***He:*** *expected heterozygosity* ***Ĥ:*** *average heterozygosity*

Table 4. *n_A, n_E, PIC, Ho, He and Ĥ values in considered microsatellites according to flocks*
Tablo 4. *Sürülere göre kullanılan mikrosatellitlere ait n_A, n_E, PIC, Ho, He ve Ĥ değerleri*

Loci	ADU-CCKP							EA Flock							MV Flock						
	N	n_A	n_E	Ho	He	Ĥ	PIC	N	n_A	n_E	Ho	He	Ĥ	PIC	N	n_A	n_E	Ho	He	Ĥ	PIC
OarCP34	154	7	3.80	0.857	0.741	0.737	0.69	62	6	4.23	0.839	0.776	0.764	0.730	26	3	2.30	0.769	0.588	0.565	0.471
OarFCB193	154	15	6.84	0.935	0.859	0.854	0.84	62	10	5.70	0.968	0.838	0.825	0.802	26	7	4.28	1.000	0.797	0.766	0.732
OarFCB304	154	11	4.69	0.766	0.792	0.787	0.76	62	6	2.50	0.581	0.609	0.599	0.520	26	4	2.50	0.846	0.625	0.601	0.534
OarJMP29	154	9	4.01	0.805	0.756	0.751	0.72	62	7	3.62	0.807	0.736	0.724	0.691	28	6	3.32	0.929	0.725	0.699	0.649
OarFCB128	150	9	4.82	0.653	0.798	0.793	0.77	62	8	3.59	0.742	0.734	0.722	0.680	22	3	2.31	0.818	0.593	0.566	0.504
BM8125	152	10	4.71	0.790	0.793	0.788	0.77	62	9	4.65	0.839	0.798	0.785	0.752	24	4	2.97	0.583	0.692	0.663	0.600
OarJMP58	154	13	3.98	0.753	0.754	0.749	0.71	62	6	2.86	0.677	0.661	0.650	0.618	28	7	4.78	0.571	0.82	0.791	0.760
OarVH72	154	7	4.60	0.753	0.788	0.782	0.75	62	7	2.43	0.677	0.598	0.588	0.536	28	4	2.78	0.714	0.664	0.640	0.595
MAF65	142	9	4.84	0.563	0.799	0.794	0.77	62	6	3.59	0.645	0.733	0.721	0.679	18	3	2.16	0.333	0.569	0.537	0.468
DYMS1	148	14	4.42	0.514	0.779	0.774	0.75	64	7	4.50	0.438	0.790	0.778	0.746	28	4	2.82	0.143	0.669	0.645	0.577
Mean	152	10.4	4.67	0.739	0.786	0.781		62	7.2	3.77	0.721	0.727	0.716		25	4.5	3.02	0.671	0.674	0.647	
St.dev.		2.797	0.851	0.129	0.033	0.033			1.398	1.03	0.151	0.081	0.08			1.58	0.876	0.269	0.086	0.085	

n_A: *number of alleles,* ***n_E:*** *number of effective allele,* ***PIC:*** *polymorphic information content,* ***Ho:*** *observed heterozygosity,* ***He:*** *expected heterozygosity* ***Ĥ:*** *average heterozygosity*

values obtained from the study were seen to vary between 0.950 and 0.740 with 0.450 and 0.788, respectively in all populations. The average heterozygosity value was 0.780 for all loci. The observed alleles with different sizes indicate the existence of unique or private alleles for flocks within this breed. 44 out of the 115 alleles were observed in only one of three flocks as private alleles. Determined private alleles are given in *Table 5*. In general, these private alleles are found at either end of the allelic range with a low frequency (between 0.06 and 0.143), were observed to be 33 in ADÜ-ÇÇKP, 7 in EA and 4 in MV flock.

In particular, null allele frequencies were found to be remarkable for OarFCB128, OarJMP58, MAF65 and DYMS1 loci *(Table 6)*.

The high (>0.05) null allele frequencies observed for OARFCB128, OARJMP58, MAF65 and DYMS1 loci indicated the presence of null alleles and loss of heterozygosity. In spite of higher null allele frequencies of MAF65 and DYMS1 loci, the frequencies very close to 0.05 for OarFCB128 and OarJMP58 loci could be tolerated.

The Hardy-Weinberg equilibrium (HWE) was tested for all populations and results are given in *Table 7*.

The assessment of genetic equilibrium in the examined herd, based on the analysis of observed and expected frequencies of individual genotypes, showed highly significant differences between the expected and observed values for the OarFCB304, OarFCB128, OarJMP58, MAF65 and DYMS1

Table 5. *Number and frequency of common alleles in whole population and private alleles in three flocks*
Tablo 5. *Ortak allelerin tüm populasyondaki ve özgün allellerin sürülerdeki sayı ve frekansları*

Loci	n_A	Common Alleles		Private Alleles					
		No	Frequency (Min-Max)	ADÜ-ÇÇKP Flock		EA Flock		MV Flock	
				No	Size: Freqeency	No	Size: Freqeency	No	Size: Freqeency
OarCP34	7	6	0.016-0.500	1	**124:**0.013	-	-	-	-
OarFCB193	17	11	0.006-0.346	4	**96:**0.143	-	-	2	**126:**0.038
					102:0.013				**128:**0.038
					112:0.013				
					120:0.071				
OarFCB304	11	6	0.013-0.538	5	**160:**0.013	-	-	-	-
					166:0.104				
					184:0.019				
					188:0.045				
					190:0.032				
OarJMP29	13	6	0.019-0.452	3	**116:**0.019	2	**122:**0.016	2	**142:**0.036
					146:0.032		**132:**0.016		**154:**0.107
					148:0.026				
BM8125	13	6	0.016-0.417	4	**108:**0.039	3	**110:**0.016	-	-
					114:0.020		**124:**0.032		
					130:0.033		**134:**0.016		
					138:0.007				
OarJMP58	13	9	0.006-0.548	4	**153:**0.026	-	-	-	-
					155:0.006				
					169:0.006				
					171:0.006				
OarVH72	8	6	0.006-0.581	1	**137:**0.019	1	**143:**0.016	-	-
MAF65	9	6	0.016-0.611	3	**133:**0.056	-	-	-	-
					135:0.014				
					139:0.042				
DYMS1	14	8	0.007-0.429	6	**169:**0.007	-	-	-	-
					189:0.034				
					193:0.020				
					195:0.014				
					199:0.041				
					201:0.014				
OarFCB128	10	7	0.013-0.591	2	**100:**0.053	1	**108:**0.016	-	-
					120:0.007				

n_A: *number of alleles*

(P<0.001) in the ADÜ-ÇÇKP flock; OarJM29, OarFCB128, BM8125 and DYMS1 (P<0.001) in the EA flock; OarJMP58, DYMS1 (P<0.001) and MAF65 (P<0.05). The other markers were in the Hardy-Weinberg (HW) equilibrium in all 3 flocks.

Genetic similarity and genetic distance values are presented in *Table 8*. A dendrogram of genetic distances between the three flocks is given in *Fig. 1*.

Both *Table 1* and *Fig. 1* show that the highest and lowest genetic similarities were found between ADÜ-ÇÇKP and EA (0.826) and EA and MV flocks (0.710), respectively. When the drawn dendrogram was examined, it was found that the MV flock was in a separate group from ADÜ-ÇÇKP and EA flocks.

A Factorial Correspondence Analysis (FCA) chart was drawn in order to demonstrate how much individuals in the three flocks were separated. Factorial correspondence analysis of the Çine Caparı sheep in the three flocks based on the 10 microsatellite loci is given in *Fig. 2*.

Table 6. *Null allele frequencies obtained from 10 STR loci*
Tablo 6. *İncelenen 10 mikrosatellit lokusuna ait null allel frekansları*

Loci	Null Allele Frequency
OARCP34	0.0000
OARFCB193	0.0000
OARFCB304	0.0290
OARJMP29	0.0000
OARFCB128	0.0532*
BM8125	0.0262
OARJMP58	0.0503*
OARVH72	0.0178
MAF65	0.1375*
DYMS1	0.1731*

** null alleles with high frequency*

F statistics are widely used for defining population structure. Fis, Fit and Fst values of between the flocks averaged over ten microsatellites are shown in *Table 9*.

Fis values were found to be between -0.194 and 0.502 in general. High values of Fis for all loci in the MV flock indicate for the existence of high inbreeding. It is mainly stem from small size of this flock. The results *(Table 9)* indicated that using the microsatellites in this population was useful for the planned objectives. When ADÜ-ÇÇKP, EA and MV flocks are evaluated for this parameter, five loci in the ADÜ-ÇÇKP flock, seven loci in the EA flock and six loci in the MV flock showed high heterozigosity rates. Low Fst values imply that flocks are similar due to some gene flow between them. This result is strongly supported with efforts are given within ADÜ-ÇÇKP at past years to change rams between flocks to limit inbreeding.

Table 7. *Chi-Square test values belong to 10 microsatellites in all population*
Tablo 7. *Tüm sürülerde incelenen 10 mikrosatellit lokusuna ait Ki-kare test değerleri*

Loci	ADÜ-ÇÇKP				EA				MV			
	DF	χ^2	Prob	Sig.	DF	χ^2	Prob	Sig.	DF	χ^2	Prob	Sig.
OarCP34	21	18.690	0.605	NS	15	5.985	0.980	NS	3	2.479	0.479	NS
OarFCB193	105	80.181	0.966	NS	45	55.314	0.139	NS	21	19.810	0.533	NS
OarFCB304	55	125.283	0.000	P<0.001	15	8.900	0.883	NS	6	8.258	0.220	NS
OarJMP29	36	36.581	0.442	NS	21	72.412	0.000	P<0.001	15	8.226	0.914	NS
OarFCB128	36	91.898	0.000	P<0.001	28	88.696	0.000	P<0.001	3	5.272	0.153	NS
BM8125	45	51.902	0.223	NS	36	104.634	0.000	P<0.001	6	4.113	0.661	NS
OarJMP58	78	158.569	0.000	P<0.001	15	10.073	0.815	NS	21	48.351	0.001	P<0.001
OarVH72	21	16.749	0.726	NS	21	7.223	0.998	NS	6	4.542	0.604	NS
MAF65	36	140.760	0.000	P<0.001	15	11.218	0.737	NS	3	9.146	0.027	P<0.05
DYMS1	91	204.811	0.000	P<0.001	21	55.388	0.000	P<0.001	6	28.505	0.000	P<0.001

DF: *Degree of freedom,* ***NS:*** *Non-significance*

Table 8. *Genetic similarity (above diagonal) and genetic distance (below diagonal) in three flocks*
Tablo 8. *Üç sürüdeki genetik benzerlik (diyagonal üzeri) ve genetik mesafe (diyagonal altı)*

Flocks	ADÜ-ÇÇKP	EA	MV
ADÜ-ÇÇKP	****	0.8262	0.7612
EA	0.1909	****	0.7107
MV	0.2729	0.3415	****

DISCUSSION

The considerable differences in allele numbers in flocks indicate high genetic variability in the ADÜ-ÇÇKP flock versus the other two breeders' flocks (EA and MV). The observed allele numbers show the existence of unique alleles for flocks.

The results show that number of alleles, which was observed in three small Çine Çaparı sheep flocks, was higher than some reported studies [18,19] and relatively lower than

Fig 1. Dendrogram based on Nei's genetic distances among three flocks
Şekil 1. Üç sürü arası Nei'nin genetik uzaklıklarına dayalı elde edilen dendrogram

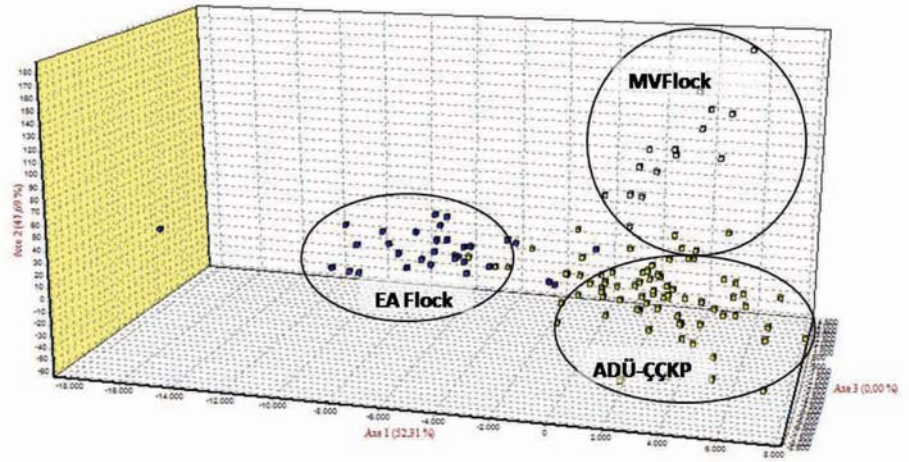

Fig 2. Factorial correspondence analysis of Cine Capari sheep in three flocks

Şekil 2. Üç sürüdeki Çine Çaparı koyunlara ait faktöriyel birleştirici analizine ait grafik

Table 9. *Fis, Fit and Fst values for whole Çine Çaparı population and for each flock*
Tablo 9. *Çine Çaparı populasyonunun tamamı ile her biri sürüye ait Fis, Fit ve Fst değerleri*

Loci	General				ADÜ-ÇÇKP			EA			MV		
	N	Fis	Fit	Fst	N	Fis	Fit	N	Fis	Fit	N	Fis	Fit
OarCP34	242	-0.194	-0.075	0.100	154	-0.164	-0.164	62	-0.098	-0.098	26	-0.361	-0.361
OarFCB193	242	-0.187	-0.084	0.087	154	-0.095	-0.095	62	-0.174	-0.174	26	-0.305	-0.305
OarFCB304	242	-0.104	-0.068	0.033	154	0.026	0.026	62	0.031	0.031	26	-0.409	-0.409
OarJMP29	244	-0.169	-0.109	0.051	154	-0.072	-0.072	62	-0.114	-0.114	28	-0.329	-0.329
OarFCB128	234	-0.064	0.031	0.089	150	0.176	0.176	62	-0.028	-0.028	22	-0.445	-0.445
BM8125	238	0.011	0.040	0.029	152	-0.002	-0.002	62	-0.068	-0.068	24	0.120	0.120
OarJMP58	244	0.086	0.173	0.096	154	-0.006	-0.006	62	-0.042	-0.042	28	0.277	0.277
OarVH72	244	-0.067	-0.026	0.038	154	0.037	0.037	62	-0.152	-0.152	28	-0.116	-0.116
MAF65	222	0.249	0.301	0.070	142	0.290	0.290	62	0.105	0.105	18	0.379	0.379
DYMS1	240	0.502	0.511	0.018	148	0.336	0.336	64	0.438	0.438	28	0.779	0.779
Mean	239	0.007	0.068	0.062	152	0.053	0.053	62	-0.008	-0.008	25	-0.036	-0.036

some of the other researches [20,21]. The size of Çine Çaparı population is very small, so it has led to a low number of alleles. In addition, differences between the literature and the present study are natural due to the use of different breeds and using a number of microsatellites.

In conservation studies, the most important criterion to decide the priority of the breed is the high level of heterozygosity that it exhibits. The average heterozygosity values (Ĥ) (ranging between 0.737-0.885) were higher than reported studies in other sheep breeds [22-26]. These results occurred due to high heterozygosity levels in the studied loci. In addition to the high genetic diversity in this breed is one of the supporting observations for the proximity of the breeds to the centre of domestication.

Genetic distance and genetic similarity values obtained from the study were similar to the values in a previous study conducted for the same genotype [27].

It is understood from *Fig. 2* that among all the individuals in every flock a group formed between themselves. Individuals of the MV flock seem to have a relatively higher level of decomposition of the other flocks. Although ADÜ-ÇÇKP and EA flocks show dense clustering, some of the animals involved themselves between the two clusters, some others entered into other clusters depending on the brood transfers among the flocks. The results obtained from factorial corresponding analysis indicated that clustering will become clearer if we use a large number of microsatellites for identification.

Four loci in the ADÜ-ÇÇKP flock, 4 loci in the EA flock and 3 loci in the MV flock were not in the Hardy-Weinberg (HW) equilibrium in the studied population. This situation may have occurred as a result of a controlled reproduction program for many years to prevent inbreeding in this population.

All Fis values obtained from the studies showed that pure breeding did not apply in these flocks. Although Fst values diverged from 1, this value was 0 at the basis of the flocks. This result points out that individuals came from a common ancestor in terms of these loci.

The minimum allele number was observed in the MV flock. This result is natural due to the fact that the population number has decreased dramatically in the MV flock.

When *Table 6* is examined, the MAF65 and DYMS1 loci, which had high null allele frequency, should not be used in genetic diversity studies in this population to prevent incorrect interpretation of results.

The spread of specific alleles in all populations can be ensured by the transfer of rams among flocks. In this way, unique or private alleles will be spread over all populations.

The present study results indicate that although the Çine Çaparı sheep population size is very small, genetic diversity was significantly high in the gene pool. Results show that the conservation flock founded at Adnan Menderes University has higher genetic diversity than the other 2 farmers' flocks according the results of 10 loci studied. These results stem from the establishment of the conservation flock with animals from different flocks and from implementation of planning mating for many years according to relationships between members of the flock. Maintenance of controlled breeding practices considering pedigree and molecular genetic data and transferring studs between flocks will maintain genetic diversity in this breed in the future.

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RESEARCH ARTICLE

The Evaluation of Affection of *Methylobacterium extorquens* - Modified Silica Fume for Adsorption Cadmium (II) Ions from Aqueous Solutions Affection

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Makale Kodu (Article Code): KVFD-2012-7865

Summary

In this present study, it was investigated for adsorbtive removal of cadmium (Cd) ions from aqueous solutions using *Methylobacterium extorquens* - modified silica fume waste material. Batch adsorption experiments have been performed as a function of pH, contact time, temperature and adsorbent dosage. The optimum results were obtained at pH 5.0, contact time of 60 min, temperature of 25°C and an adsorbent dose of 1 mg/mL. The adsorption data was correlated with Langmuir and Freundlich adsorption models. The maximum adsorption capacity obtained from Langmuir adsorption model was 166.67 mg/g. The results show that the bacteria - modified silica fume could be used for the treatment of aqueous solutions containing Cd as an alternative low cost adsorbent.

Keywords: *Wastewater, Bacteria modified silica fume, Cadmium pollution, Environmental pollution, Adsorption kinetics*

Sulu Çözeltilerden Cadmiyum (II) İyonlarının Adsorpsiyonu için *Methylobacterium extorquens - Modifiye Silis Dumanı* Etkinliğinin Değerlendirilmesi

Özet

Bu çalışmada, *Methylobacterium extorquens* ile modifiye edilmiş atık malzeme silis dumanı kullanılarak sulu çözeltilerden kadmiyumun uzaklaştırılması araştırılmıştır. Adsorpsiyon deneyleri pH, temas süresi, sıcaklık ve adsorban dozajının bir fonksiyonu olarak yapılmıştır. Optimum sonuçlar pH 5.0'de, 60 dk temas süresinde, 25°C sıcaklıkta ve 1 mg/mL adsorban dozunda elde edilmiştir. Adsorpsiyon verileri Langmuir ve Freundlich adsorpsiyon modelleri ile korelasyonu göstermektedir. Langmuir adsorpsiyon modelinden elde edilen maksimum adsorpsiyon kapasitesi 166.67 mg/g'dır. Elde edilen sonuçlar bakteri modifiye - silis dumanının Cd (II) içeren sulu çözeltilerin iyileştirilmesinde alternatif düşük maliyetli adsorban olarak kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Atık su, Bakteri modifiye silis dumanı, Kadmiyum kirliliği, Çevre kirliliği, Adsorpsiyon kinetikleri*

INTRODUCTION

Heavy metals pollutions are considered to be a serious threat, non-biodegradable and have great environmental, public health and economic impacts. The presence of heavy metals in the environment is one of the major concerns because of their toxicity and threat to human life. They accumulate in living tissues throughout the food chain which has humans at its top [1-3].

One such heavy metal, cadmium, along with its compounds, is widely used in pigments, as heat stabilizers for plastics, for corrosion resistance of steel and cast iron, metal plating, phosphate fertilizer, mining, pigments, alloy industries, in soldering and brazing and in the battery industry (Ni-Cd batteries). Cadmium is highly toxic and there is some evidence that it is carcinogenic [3]. Cadmium

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poisoning may occur that cadmium or cadmium compounds enters the body through the digestive or respiratory [4-6].

Removal, separation and enrichment of heavy metal ions in aqueous solutions play an important role for the environmental remediation of wastewater. Different treatment techniques such as chemical precipitation, chemical reduction, ion exchange, membrane separation, evaporation and adsorption, etc, have been developed to remove metal ions from industrial wastewater [7]. However, these methods often incur high operational costs [8-10]. As an alternative low-cost absorbent material, solid wastes are generally used as adsorbent for the removal of heavy metal from wastewater. Silica fume is a by-product of silicon material or silicon alloy metal factories [2,9,10].

Methylobacterium extorquens is a major model of methylotrophic bacteria. This facultative methylotroph is able to grow on C_1- but also on multicarbon (C_2-C_4) compounds. *M. extorquens* plays an important role in metabolic pathway of plant-derived methanol [11,12]. *Methylobacterium extorquens* is not toxic and it has also been used for improving the taste of some fruits like strawberries [13]. This paper describes the use of *M. extorquens*-modified silica fume to remove cadmium from aqueous solutions. The adsorption of cadmium ions has been investigated as a function of contact time, pH, temperature and adsorbent dose. The cadmium ions have been absorbed by bacteria-modified silica fume from polluted river water and $Cd(NO_3)_2$ solution and the results have been shown on the adsorption behavior of cadmium on *M. extorquens*-modified silica fume. Equilibrium and kinetic studies have been performed to describe the adsorption process.

MATERIAL and METHODS

Adsorbent

Silica fume, also known as microsilica, is a by product of the reduction of higy-purity quartz with coal inelectric furnaces in the production of silicon and ferrosilicon alloys. It is also collected as a by product in the production of other silicon alloys such as ferrochromium, ferromanganese, ferromagnesium and calcium silicon. Silica fume has been obtained from Ferro-Chromate Factory in Antalya [14].

Adsorbent Preparation

The silica fume was thoroughly washed with distilled water until it became neutral. The suspension was wet sieved through a 200 mesh screen. The solid fraction was washed five times with distilled water following the sequence of mixing, settling, and decanting. The last suspension was filtered, and the residual solid was then dried at 105ºC, ground in a mortar, and sieved through a 200 mesh sieve. The product was used in the study. Its chemical and index properties are summarized in *Table 1*.

Table 1. *Chemical compositions and engineering properties of silica fume used in the study*

Tablo 1. *Bu çalışmada kullanılan silis dumanının kimyasal kompozisyonu ve mühendislik özellikleri*

Chemical Composition		**Engineering Properties**	
Property Compound	**Silica Fume**	**Property Density**	**Silica Fume**
SiO_2, %	85-95	Density, (mg/m^3)	2-2.5
Al_2O_3, %	1-3	**Grain size**	
Fe_2O_3	0.5-1.0	Gravel (>2000 µm), %	-
CaO, %	0.8-1.2	Sand (2000-75 µm), %	-
MgO, %	1-2	Silt, (2-75 µm), %	20
TiO_2, %	-	Clay (< 2 µm), %	80
Heat loss, %	0.5-1	*Specific surface area* Specific surface area, m^2/g	20.12

1 g of silica fume sample was shaken with 10 mL, 10^8 CFU/mL *M. extorquens* solution for approximately 1 h, and then the separated particles were stored [15].

Adsorbent Characterization

The pH values were determined with a pH meter. The scanning electron microscope (SEM) was used to examine the surface of the adsorbent. Images of native adsorbent and metal loaded adsorbent were magnified 5.000 times by SEM modeled JEOL JSM-6400 SEM. Before SEM examinations, the sample surfaces were coated with a thin layer (20 nm) of gold to obtain a conductive surface and to avoid electrostatic charging during examination. The same machine was also used for the energy dispersive X-ray (EDX) spectra analysis to know the elemental composition of the silica fume.

Adsorption Procedure

The amount of Cd was determined with dithizone at 228 nm by using spectrophotometer according to calibration curve *(Fig. 1)* [16].

The adsorption capacity of adsorbent (q_t) was calculated using Eq. 1.

$$qe = \frac{(C_o - C_t) * V}{m} \qquad (1)$$

where, q_t is the adsorption capacity of the adsorbent at time t (mg adsorbate/g adsorbent); C_o is the initial concentration of metal (mg/L); C_t is the residual concentration of metal after adsorption had taken place over a period of time t (mg/L); V is volume of metal solution in shake flask (L) and m is mass of adsorbent (g). The metal removal percentage (R %) was calculated using Eq. 2.

$$R(\%) = \frac{(C_o - C_t) * 100}{C_o} \qquad (2)$$

where (R %) is the ratio of difference in metal concentration before and after adsorption; C_o is the initial concentration

of metal (mg/L); C_t is the residual concentration of metal after adsorption had taken place over a period of time t (mg/L).

RESULTS

Linear regression is one of the most frequently used analyses in calibration. From the calibration curve in the *Fig. 1*, it was observed that there is an approximate linear relationship between absorbance and cadmium concentration in the aqueous solutions. It is observed that the regression coefficient (R^2) is quite high, and its value is 0.9881.

In order to find the optimal pH value for the sorption process, the removal of cadmium ions in the pH range 3-7 was investigated and the data were illustrated in the *Fig. 2*. The effect of temperature influencing the adsorption has been studied in the range of 10-80°C. The effect of temperature on the adsorption capacity of bacteria-modified silica fume is shown in *Fig. 3*.

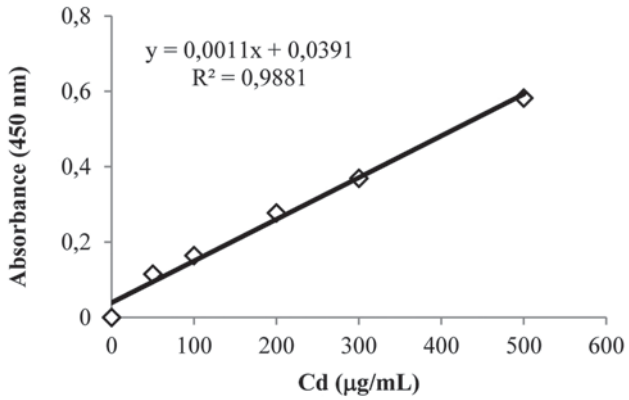

Fig 1. Calibration curve of cadmium adsorbtion

Şekil 1. Kadmiyumun adsorbsiyonu için kalibrasyon grafiği

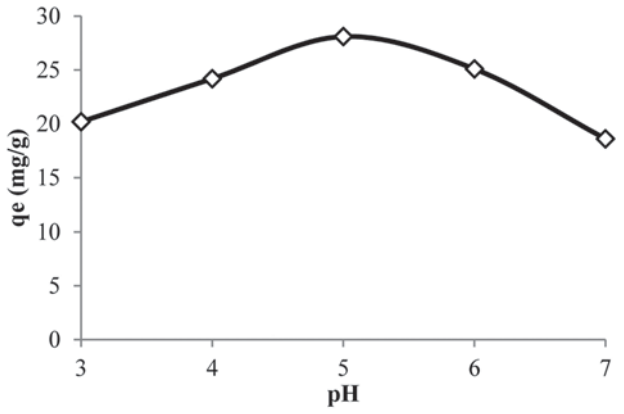

Fig 2. Effect of cadmium as a function of pH (initial cadmium concentration: 1 mg/mL, silica fume dose: 1 g/50 mL, agitation speed: 500 rpm and temperature: 25±1°C)

Şekil 2. Kadmiyum iyonu adsorpsiyonu üzerine pH değişiminin etkisi (ilk kadmiyumun konsantrasyonu 1 mg/mL, silis dumanı doz: 1 g/50 mL, çalkalama hızı: 500 rpm ve sıcaklık: 25±1°C)

Fig. 4 illustrates the effect of contact time on the adsorption process. It clearly shows that the equilibrium is attained just after only 60 min. The effect of the adsorbent dosage was studied by varying the adsorbent amounts from 0.5 to 3.0 mg/mL. The effect of bacteria-modified silica fume dosage on amount of cadmium adsorbed was shown in *Fig. 5*. The cadmium concentration of polluted river water and $Cd(NO_3)_2$ solutions treated with bacteria-modified silica fume indicate that bacteria-modified silica fume enhances adsorption capacity *(Fig. 6)*.

Images of native adsorbent and metal loaded adsorbent were magnified 20.000 times by SEM was used to examine the surface of the adsorbent. The SEM photographs showed that the progressive changes occurred in the surface of native adsorbent *(Fig. 7A)* after its surface loaded by cadmium ions *(Fig. 7B)*. The EDX measurements were recorded for qualitative analysis of the element constitution of the adsorbents in *Table 2* and the EDX spectra of native

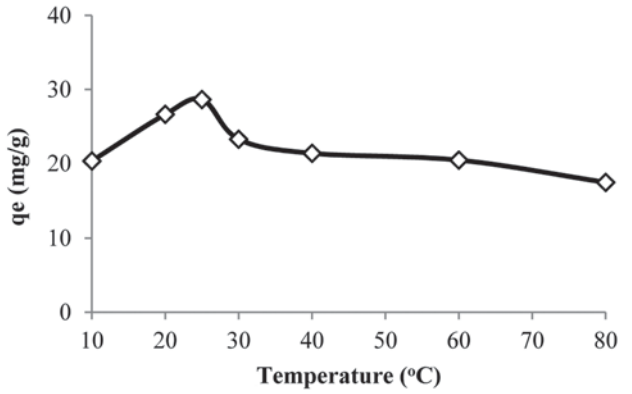

Fig 3. Effect of cadmium as a function of temperature (pH:5.0, initial cadmium concentration: 1 mg/mL, silica fume dose: 1 g/50 mL, agitation speed: 500 rpm, contact time 60 min)

Şekil 3. Kadmiyum iyonu adsorpsiyonu üzerine sıcaklık değişiminin etkisi (pH: 5.0, ilk kadmiyumun konsantrasyonu 1 mg/mL, silis dumanı doz: 1 g/50 mL, çalkalama hızı: 500 rpm ve temas süresi 60 dak)

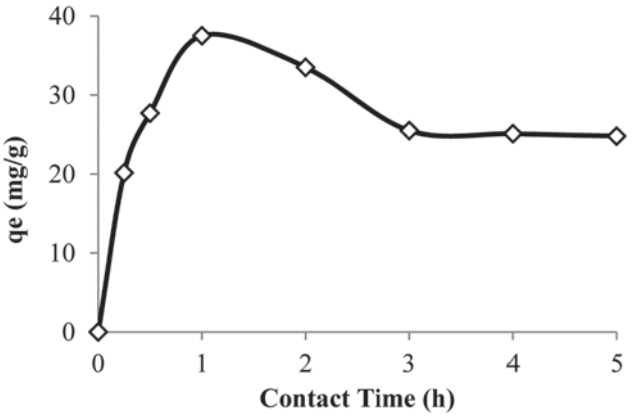

Fig 4. Removal of cadmium as a function of equilibrium time (pH:5.0, initial cadmium concentration: 1 mg/mL, silica fume dose: 1 g/50 mL, agitation speed: 500 rpm and temperature: 25±1°C)

Şekil 4. Kadmiyum'un giderilmesinde denge zamanı (pH: 5.0, ilk kadmiyumun konsantrasyonu 1 mg/mL, silis dumanı doz: 1 g/50 mL, çalkalama hızı: 500 rpm ve sıcaklık 25±1°C)

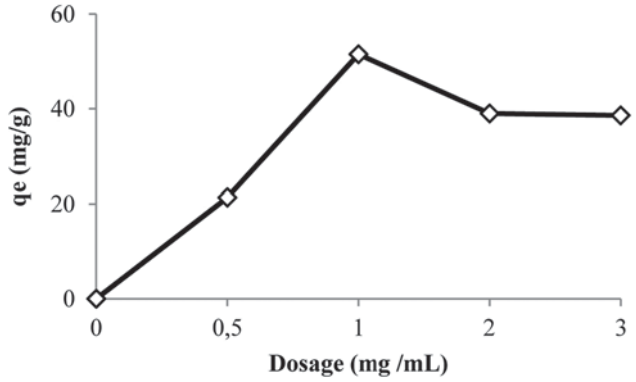

Fig 5. Effect of silica fume dosage on adsorption of cadmium (pH 5.0, initial cadmium concentration: 1 mg/mL, agitation speed: 500 rpm, contact time 60 min)

Şekil 5. Kadmiyumun adsorpsiyonu üzerine silis dumanı miktarının etkisi (pH: 5.0, ilk kadmiyumun konsantrasyonu 1 mg/mL, silis dumanı doz: 1 g/50 mL, çalkalama hızı: 500 rpm ve temas süresi 60 dak)

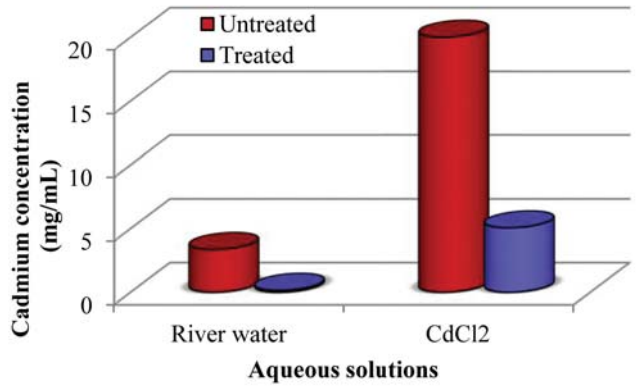

Fig 6. Variation of cadmium concentration after silica fume treating with river water and $Cu(NO_3)_2$

Şekil 6. Nehir suyu ve $Cu(NO_3)_2$ ile silis dumanının muamelesinden sonra kadmiyum konsantrasyonunun değişimi

Table 2. *Results of EDX spectrum*
Tablo 2. *EDX spektrumun sonuçları*

Elements	Native Adsorbent		Cadmium Ion Loaded Adsorbent	
	Weight (%)	Atom (%)	Weight (%)	Atom (%)
Mg	2.11	2.62	3.40	4.35
Si	77.65	83.68	74.70	82.85
K	9.21	7.13	6.21	4.95
Ca	2.19	1.66	1.09	0.85
Cr	3.11	1.81	4.27	2.56
Fe	1.17	0.64	1.47	0.82
Cu	1.88	0.89	2.53	1.24
Zn	1.82	0.84	2.56	1.22
Cd	-	-	3.64	1.01

Table 3. *Values of the Langmuir and Freundlich constant for adsorption of cadmium*
Tablo 3. *Kadmiyumun adsorbsiyonu için Langmuir and Freundlich sabitlerinin değerleri*

Adsorption Isotherm	Value
Langmuir constants	
Q_o(mg/g)	166.67
b(L/mg)	0.022
R^2	0.996
Freundlich constants	
K_F	0.181
N	0.824
R^2	0.993

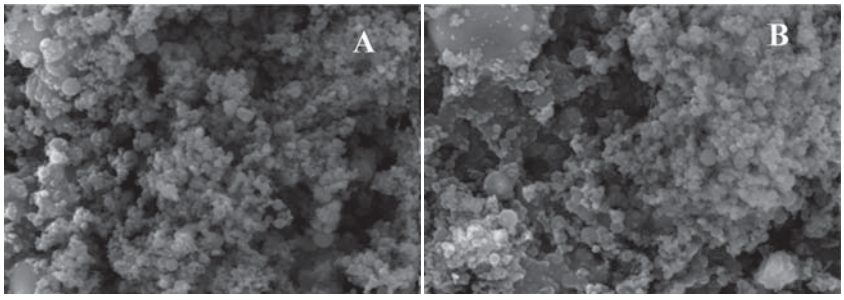

Fig 7. SEM images of native adsorbent (A) and cadmium loaded adsorbent (B)

Şekil 7. Doğal adsorbent (A) ve kadmiyum yüklenmiş adsorbent (B)'in SEM görüntüleri

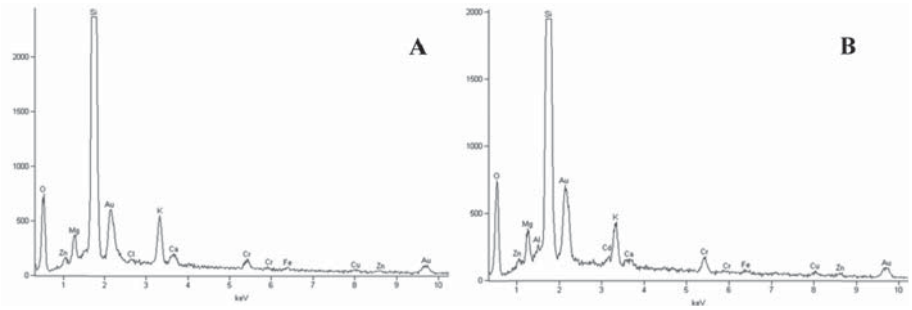

Fig 8. EDX spectra of native adsorbent (A) and cadmium loaded adsorbent (B)

Şekil 8. Doğal adsorbent (A) ve kadmiyum yüklenmiş adsorbent (B)'in EDX spektrumları

Table 4. *Thermodynamic parameters for the adsorption of cadmium onto silica fume adsorbent*

Tablo 4. *Silis dumanı üzerine kadmiyumun adsorbsiyonu için termodinamik parametreler*

Adsorbent	$\Delta H_{ads}°$ (kJmol^{-1})	$\Delta S_{ads}°$ (Jmol^{-1})	$\Delta G_{ads}°$ (kJmol^{-1})		
			298 K	303 K	313 K
Silica fume	10.634	-36.374	-205.45	-387.32	-751.06

adsorbent and cadmium ion loaded adsorbent were illustrated in the *Fig. 8A* and *8B*.

Freundlich and Langmuir adsorption isotherms model were applied to evaluate the experimental data and results are shown in *Table 3*. A comparison of the correlation coefficients results shown in *Table 3*.

Thermodynamic parameters were determined such as change in free energy *(ΔG°)* kJ/mol, enthalpy *(ΔH°)* kJ/mol, and entropy *(ΔS°)* J/Kmol from 298 to 313 K *(Table 4)*.

DISCUSSION

The uptake and percentage removal of metals from the aqueous solution are strongly affected by the pH of the solution [17,18]. The optimal pH was investigated the removal of cadmium ions in the pH range 3-7. It shows the effect of pH variation on adsorption of cadmium ions on the silica fume particle surface. It was shown that the absorption amount of cadmium increases with increasing pH and maximum adsorption of cadmium ions were obtained at pH 5.0. The uptake of cadmium increased from 24.19 to 28.1 mg/g when the pH of solution was increased from 4 to 5 *(Fig. 2)*. This is due to the surface complexation reactions, which are mostly influenced by the electrostatic force of attraction between cadmium and the surface of the silica fume [2,19,20].

Temperature affects the adsorption rate by altering the molecular interactions and the solubility of the adsorbate [21,22]. The effect of temperature on the adsorption has been determined in the range of 10-80°C *(Fig. 3)*. It is observed that the degree of adsorption increases with increasing temperature and maximum adsorption of cadmium ions are obtained at 25°C which is the temperature of the solution for the bacteria-modified silica fume [23]. The increase of the temperature can change the pore sizes which become wider, and can induce a certain activation of the surface of the solid support [24].

The contact time is inevitably a fundamental parameter in all transfer phenomena such as adsorption [24]. Therefore, it is important to study its effect on the capacity of retention of cadmium by bacteria-modified silica fume. The effect of contact time on the adsorption process was determined as 60 minutes *(Fig. 4)*. The increase in contact time has increased the cadmium uptake and this can be explained by the affinity of the support towards cadmium. As the surface adsorption sites become exhausted, the uptake rate is controlled by the rate at which the adsorbate is transported from the exterior to the interior sites of the adsorbent particles [3,25,26].

Adsorbent dosage is an important parameter because it determines the capacity of an adsorbent for a given initial concentration of the adsorbate [2]. The adsorbent dosage was studied by varying the adsorbent amounts from 0.5 to 3.0 mg/mL by using bacteria-modified silica fume as a adsorbent *(Fig. 5)*. A trend of increase in adsorption capacity with increase in adsorbent dosage was observed from 0.5 mg/mL to 1 mg/mL. Any further addition of the adsorbent beyond this did not cause any significant change in the adsorption. The amount of maximum cadmium removal was 51.5 mg/g at 1 mg/mL of adsorbent dose. Some trends were reported similar with the present study [27,28].

Without the addition of the bacteria-modified silica fume, the leachate cadmium concentration is approximately 3.345 and 20 mg/mL for polluted river water and $Cd(NO_3)_2$ solutions, respectively. The addition of bacteria-modified silica fume strongly inhibits the leaching of cadmium in the polluted river water and $Cd(NO_3)_2$ solutions *(Fig. 6)*. The increase in the adsorption capacity of aqueous solutions treated by bacteria-modified silica fume is attributed to the pH values and active components of bacteria-modified silica fume [2].

The SEM enables the direct observation of the surface microstructures of different adsorbents [27]. Images of native silica fume and metal loaded *M. extorquens*-modified silica fume were magnified 20.000 times by SEM was used to examine the surface of the adsorbent. After modified-silica fume's surface loaded by cadmium ions, the SEM photographs showed that the some changes occurred in the surface of native silica fume *(Fig. 7A* and *7B)*. From the EDX spectra, the cadmium ions were sorbed onto the adsorbent. The EDX analysis provided direct evidence for the adsorption of cadmium onto adsorbent [27]. It is shown from EDX spectra that after cadmium adsorption, cadmium concentration increased in the cadmium loaded adsorbent *(Fig. 8A* and *8B*; *Table 2)*.

Equilibrium data, commonly known as adsorption isotherms, are basic requirements for the design of an adsorption system. In an adsorption isotherm study, several equilibrium models have been developed to describe adsorption isotherm relationships [29]. The Freundlich [30] and Langmuir [31] equations are the world-widely used models because of their simplicity [32]. The Langmuir adsorption isotherm model represents one of the first theoretical treatments of non-linear sorption and suggests that the uptake occurs on a homogenous surface by monolayer sorption without interaction between the adsorbed molecules [27]. Langmuir adsorption isotherm is often used to describe the maximum adsorption capacity of an adsorbent and it is given as;

$$q_e = \frac{q_m * K_L * C_e}{1 + K_L * C_e} \quad (3)$$

where q_e (mg/g) is the adsorption amount of adsorbent at equilibrium, q_m (mg/g) is the maximum adsorption amount of metal ions, C_e (mg/L) is the equilibrium concentration of adsorbate in solution and K_L (L/mg) is the equilibrium adsorption constant which is related to the affinity of the binding sites. The Langmuir constants K_L and q_m are calculated with the following equation;

$$\frac{C_e}{q_e} = \frac{1}{K_L * q_m} + \frac{q_e}{q_m} \quad (4)$$

where C_e (mg/L) is the equilibrium concentration of adsorbate in solution, *qe* (mg/g) is the adsorption amount of adsorbent at equilibrium, q_m (mg/g) is the maximum adsorption amount of metal ions and K_L (L/mg) is the equilibrium adsorption constant which is related to the affinity of the binding sites. The Freundlich isotherm is based on the assumption that adsorption is on a heterogeneous surface and exponential distribution of sites and their energies [32], which can be expressed by the following equation;

$$q_m = K_F + C_e^{1/n} \quad (5)$$

where q_m (mg/g) is the maximum adsorption amount of metal ions, C_e (mg/L) is the equilibrium concentration of adsorbate in solution. K_F (mg/g) and n are the Freundlich constants related to the sorption capacity of the adsorbent and the energy of adsorption, respectively. They can be calculated in the following linear form;

$$\log q_e = \log K_F + \frac{1}{n} \log C_e \quad (6)$$

where q_e (mg/g) is the adsorption amount of adsorbent at equilibrium, C_e (mg/L) is the equilibrium concentration of adsorbate in solution, K_F (mg/g) and n are the Freundlich constants related to the sorption capacity of the adsorbent and the energy of adsorption. The Langmuir and Freundlich isotherms were obtained from the experiments.

The Langmuir model is based on the assumption that maximum adsorption occurs when a saturated monolayer of solute molecules is present on the adsorbent surface, and the energy of adsorption is constant and there is no migration of adsorbate molecules in the surface plane [25,33]. The Langmuir characteristics parameters and the degree of correlation of the adsorption data with respect to this equation are given in *Table 3*. The parameters K_F and *n* for cadmium calculated from experimental data and results obtained are given in *Table 3*. The magnitude of exponent *n* gives an indication of favorability and capacity adsorbent/adsorbate. The *n* value is 0.824 L/mg *(Table 3)* and it is located to range 1-10 represent good adsorption characteristic [25].

The sorption capacity of natural and bacteria-modified clay adsorbent increased with increase in the temperature of the system from 298-313 K. Thermodynamic parameters such as change in free energy (ΔG^o) kJ/mol, enthalpy (ΔH^o) kJ/ mol, and entropy (ΔS^o) J/Kmol were determined using the following equations [34]:

$$K_L = \frac{C_s}{C_e} \quad (7)$$

$$\Delta G^o = -RT \ln K_L \quad (8)$$

$$\ln K_L = \left(\frac{\Delta S^o}{R}\right) - \left(\frac{\Delta H^o}{RT}\right) \quad (9)$$

where K_L is the equilibrium constant, C_s is the solid phase concentration at equilibrium (mg/L), C_e is the liquid phase concentration at equilibrium (mg/L), *T* is the temperature in Kelvin, and *R* is the gas constant.

From the temperature variation from 298 to 313 K on the sorption, ΔH^o and ΔS^o were obtained. The ΔH_{ads}^o and ΔS_{ads}^o values obtained from the slope and intercept of Van't Hoff plots is presented in *Table 4*. Gibbs free energy (ΔG) was calculated as:

$$\Delta G^o = \Delta H^o - T\Delta S \quad (10)$$

The values of thermodynamic parameters for the sorption of cadmium on bacteria-modified silica fume adsorbent are given in *Table 4*. The Gibbs free energy change *(ΔG^o)* was calculated to be -205.45, -387.32 and -751.06 kJmol^{-1} on bacteria-modified silica fume for cadmium adsorption at 298, 303, 313 K, respectively. Negative ΔG^o values indicated the feasibility of the process and spontaneous nature of the adsorption, were obtained for cadmium at each of the temperatures studied. The $\Delta H°$ parameter was found to be 10.634 kJ/mol for cadmium adsorption on bacteria-modified silica fume. The positive values of $\Delta H°$ further confirmed the endothermic nature of adsorption process. The heat of adsorption value between 10 and 400 kJ/mol indicates the chemisorptions process [35].

The $\Delta S°$ parameter was found to be -36.374 (kJ/mol K) for cadmium adsorption on bacteria-modified silica fume. The negative value of $\Delta S°$ corresponds to a decrease in degree of freedom of the adsorbed species. During the adsorption process, the coordinated water molecules were displaced by metal cations, resulting in increased randomness in the adsorbent-adsorbate system [36,37].

In this study, *Methylobacterium extorquens*-modified silica fume was converted into an adsorbent, and the suitability of the *Methylobacterium extorquens*-modified silica fume for adsorption of cadmium from the polluted river water and $Cd(NO_3)_2$ solutions was investigated by adsorption experiments. This study shows clearly that sawdust which is a cheap and abundant material can be

used as an effective adsorbent for removal of cadmium from wastewater.

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RESEARCH ARTICLE

Kitosanla Muamelenin Dondurulmuş Karideslerin Duyusal ve Kimyasal Kalite Parametreleri Üzerine Etkisi [1]

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Özet

Bu çalışma dondurulmuş karideslerde kalitenin uzun süre korunabilmesi için kitosanla muamele edilmesinin etkisini araştırmak için yapılmıştır. Denemeler Marmara Denizinden avlanan derin su pembe karidesi (*Parapenaeus longirostris*) üzerinde gerçekleştirilmiştir. Taze karidesler sekiz gruba ayrıldıktan sonra çeşme suyu (kontrol) ile sodium metabisülfit (2500 mg/L), 4-heksilresonsinol (50 mg/L), kitosan (5 g/L), sitrik asit (50 mg/L) ve biberiye ekstraktı (50 mg/L) kombinasyonlarını içeren solüsyonlarda 10 dk. bekletilmiştir. Muamele sonrası karidesler paketlenmiş ve -18°C'de 12 ay süreyle muhafaza edilmiştir. Muhafaza sırasında periyodik olarak duyusal özellikleri, pH, TBA, TVB-N ve TMA-N değerleri ile nem oranı bakımından analiz edilmiştir. Muhafazanın sonunda bile bütün gruplara ait örnekler panelist değerlendirmesine göre yenilebilir bulunmuştur. Buna karşın kontrol grubu örnekler muamele gruplarına göre daha düşük lezzet ve koku puanları almıştır. Muhafaza sırasında kimyasal parametrelerde önemli düzeyde değişimler meydana gelmemiş, gruplar arasında farklılık gözlenmemiştir. Özellikle, başlangıçta zaten düşük seviyede olan TBA değeri muhafaza sırasında örneklerin duyusal özelliklerini değiştirecek düzeyde yükselmemiştir. Elde edilen bulgulara göre çiğ karideslerin kabuklu olarak dondurulması durumunda en az 12 ay kadar tüketilebilir özelliklerini koruduğu, muhafaza öncesi kitosanla muamele edilmesinin incelenen parametrelerde kısmi iyileşme sağlamış olsa da istatistiki anlamda önemli bir farklılık oluşturmadığı sonucuna varılmıştır.

Anahtar sözcükler: *Kitosan, Karides, Donmuş muhafaza, Bozulma*

Effects of Treatment with Chitosan on Sensory and Chemical Quality Parameters of Frozen Shrimp

Summary

This study was conducted to investigate the effects of chitosan treatment for a long time to preserve the qualities of shrimp during frozen storage. The experiments were performed on deepwater pink shrimp (*Parapenaeus longirostris*) caught from Marmara Sea. Fresh shrimps were divided into seven groups and immediately were dipped in tap water (control group) and solutions containing sodium metabisulphite (2500 mg/L), 4-hexylresorcinol (50 mg/L), chitosan (5 g/L), citric acid (50 mg/L) and rosemary extract (50 mg/L) for ten minute. After treatment, the shrimps were packaged and stored at -18°C for twelve months. Sensorial characteristics, values of pH, TBA, TVB-N and TMA-N, rate of moisture of shrimp were determined periodically during frozen storage. All samples were palatable according to the panellist evaluation at the end of storage. However control group (untreated) shrimps were received lower scores. No significant changes on chemical parameters were determined during frozen storage and no difference among groups were observed. In particular, the initial TBA value, is already low levels, not elevated level to change the sensorial characteristics of the samples during storage. The results of this study indicated that freezing of raw shelled shrimp insures the acceptability for at least 12 months, and that treatment with chitosan solutions of shrimps did not cause a statistically significant difference.

Keywords: *Chitosan, Shrimp, Frozen storage, Spoilage*

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GİRİŞ

Karidesler çok kısa sürede bozulan gıda maddeleri arasında yer almaktadır. Yakalanmalarının ardından kısa sürede canlılıklarını kaybetseler de karides dokusu hala biyokimyasal olarak aktiftir. Dolayısıyla hem bakteriyel hem de orijinal enzimlerin (otoliz) aktivitesinden dolayı son derece hızlı bir bozulma sürecine girmektedirler. Muhafaza sıcaklığı düşürüldükçe karideslerin raf ömründe dikkate değer artışlar söz konusu olmaktadır [1]. Karideslerin dondurulması durumunda ise dayanıklılık süresi önemli derecede uzatılabilmektedir. Dondurma işlemi güçlü hava akımında dondurma (şoklama), glazing, sıvı CO_2 veya N_2 kullanılarak bireysel hızlı dondurma (IQF, Individual Quick Freezing) şeklinde yapılabilmektedir [2,3]. Dondurma, gıda maddelerinin korunmasında etkili bir yöntem olmasına rağmen, muhafaza sırasında dondurulmuş gıdaların kalitesinde kayıplar meydana gelmektedir. Rengin solması, yağların oksidasyonu, proteinlerin denatürasyonu, buzun sublimasyonu ve rekristalizasyonu dondurulmuş karideslerde görülen önemli kalite değişiklikleri olup bunlar dehidrasyona, ağırlık kaybına, kötü lezzete, acılaşmaya, sululuğun azalmasına, tekstürel değişikliklere, su bağlama kapasitesinin azalmasına, uçucu azotlu bileşenlerdeki artışa neden olmaktadır [3-6].

Kitosan, adından son yıllarda sıkça bahsedilen antimikrobiyel etkili bir polimerdir. Başlıca yengeç ve karides gibi kabuklu deniz ürünlerinin dış iskeletlerinde bulunan doğal bir polisakkarit olan kitinden kısmi deasetilasyon yoluyla elde edilmektedir. Antimikrobiyel ve antioksidan etkinin yanı sıra nem tutma, film oluşturma, enzim immobilizasyonu gibi çok çeşitli fonksiyonları sayesinde çeşitli gıdaların raf ömründe önemli iyileştirmeler sağlayabilmektedir [7-13]. Kitosan biobozunurdur, toksik değildir ve FDA tarafından gıdalarda kullanımı güvenli (GRAS) olarak kabul edilmektedir [7,8].

Bu çalışma ülkemizde avcılığı yapılan derin su pembe karidesinin donmuş muhafaza sırasında kalite kaybını önlemek için doğal bir koruyucu madde olarak bilinen kitosan ile muamele edilmesinin etkisini araştırmak amacıyla gerçekleştirilmiştir. Bu kapsamda pratikte kullanılan kararma önleyici ajanlarla biberiye ekstraktı ve sitrik asidin de dahil olduğu kombinasyonlar oluşturularak denemeler gerçekleştirilmiştir.

MATERYAL ve METOT

Karides Örneklerinin Temini

Çalışmada Mayıs-Haziran 2011 tarihleri arasında Marmara Denizi'nden (Tekirdağ açıkları) yakalanan derin su pembe karidesi *(Parapenaeus longirostris)* kullanılmıştır.

Karideslerin Koruyucu Maddelerle Muamelesi

Laboratuvara ulaştırılan karidesler (her denemede yaklaşık 25 kg) geniş bir plastik kap içinde çeşme suyu ile yıkama işlemine tabi tutulmuştur ve takiben karidesler birisi kontrol olmak üzere yedi eşit gruba ayrılmıştır. Muamele grupları *Tablo 1*'de belirtilen oranlarda sodyum metabisülfit (Merck 106528), 4-heksilresorsinol (Merck, 820647), deasetile (%85) kitosan (Sigma-Aldrich 448877), asetik asit (Merck, 100056), sitrik asit (Merck 100244) ve biberiye ekstraktı (Sigma-Aldrich, W299200) içeren 6'şar litre miktarında hazırlanmış solüsyonlarda 10 dakika süreyle bekletilmiştir. Kontrol grubu olarak ayrılan karidesler ise aynı süre çeşme suyunda tutulmuştur. Kitosan içeren gruplarda, kitosan öncelikle asetik asit içinde çözündürülmüş, daha sonra diğer kimyasallar ilave edilmiştir. Muamele sonrası karidesler 10 dk. süreyle süzdürüldükten sonra strafor tabaklara dağıtılarak polietilen filmle kaplanmıştır. Bütün gruplara ait paketler derin dondurucuda (-18°C) 12 ay süreyle muhafazaya alınmıştır.

Analizler

Karidesler muamele öncesi, muamele sonrası ve donmuş muhafazanın 3, 6, 9 ve 12. aylarında analiz edilmiştir. Dondurulmuş örneklerin analizi soğutucuda 4-5 saat kadar bekletilip çözündürüldükten sonra gerçekleştirilmiştir.

***Duyusal Analizler*:** Deneme gruplarına ait karides örnekleri sekiz kişilik bir panel tarafından değerlendirilmiştir. Panelistlere her bir gruba ait karidesler çiğ ve pişmiş (kaynar suda 3 dk. bekletilmiş) olarak sunulmuş koku ve lezzet özellikleri bakımından 100 mm'lik bir çizgiden (0: kabul

Tablo 1. *Deneme grupları ve kullanılan kimyasallar*
Table 1. *Treatment groups and chemicals*

Grup	Muamele Solüsyonu					
	Asetik Asit	Kitosan	Biberiye Ekstraktı	Sitrik Asit	Sodyum Metabisülfit	4-Heksilresorsinol
A	-	-	-	-	2500 mg/L	-
B	-	-	-	-	-	50 mg/L
C	10 ml/L	5 g/L	-	-	2500 mg/L	-
D	10 ml/L	5 g/L	-	-	-	50 mg/L
E	10 ml/L	5 g/L	50 mg/L	200 mg/L	2500 mg/L	-
F	10 ml/L	5 g/L	50 mg/L	200 mg/L	-	50 mg/L
Kontrol	-	-	-	-	-	-

edilemez; 100: mükemmel) oluşan grafik derecelendirme ölçeği (görsel analog skala) üzerine işaretlemeleri istenmiştir [14]. Değerlendirmeler tamamlandıktan sonra her bir panelistin skala üzerinde işaretledikleri nokta cetvelle ölçülerek elde edilen puanlar kayıt altına alınmıştır.

***Tekstür (Shear Force) Değerinin Belirlenmesi*:** Karides örneklerinin (kabuksuz) tekstür analizleri Warner-Bratzler Share Force cinsinden ölçüm yapan tekstür ölçüm cihazı (3343 model, Instron, İngiltere) kullanılarak belirlenmiş ve sonuçlar kg/cm^2 üzerinden kaydedilmiştir [15].

***Nem Oranının Saptanması*:** Karideslerin nem oranı etüvde kurutma (115°C) yöntemi ile belirlenmiştir [16].

***Trimetilamin Azot (TMA-N) Tayini*:** Spektrofotometrik yöntem kullanılmıştır. On gram örnek 90 ml %7.5'lik triklorasetikasit içinde homojen hale getirilmiştir. Filtre edildikten sonra 4 ml ekstrakt test tüpüne aktarılmış ve üzerine 1 ml formaldehit, 10 ml toluen ve 3 ml K_2CO_3 solüsyonu ilave edilmiştir. Tüpler iyice karıştırıldıktan sonra oluşan toluen fazı pipetle alınarak üzerine 5 ml pikrik asit solüsyonu (%0.02) katılmıştır. İçerik iyice karıştırıldıktan sonra spektrofotometreye alınmıştır. Köre karşı 410 nm dalga boyunda absorbansı ölçülmüştür. Aynı zamanda hazırlanan standartların da ölçümleri yapılmıştır. TMA-N miktarı, mg/100 g örnek üzerinden hesaplanmıştır [17].

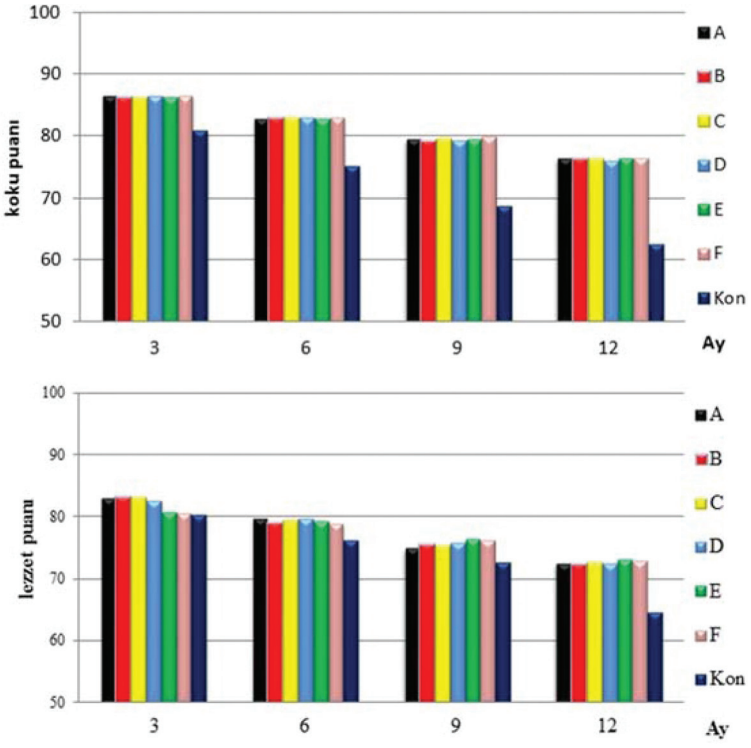
***Toplam Uçucu Bazik Azot (TVB-N) Tayini*:** Manthey ve ark.'nın [18] önerdiği su buharı destilasyon yöntemi kullanılmıştır. Kabuğu ayrılmış karideslerden hazırlanan homojen örnekten alınan 10 g, magnezyum oksit katalizorlüğünde su buharı ile ısıtılmış ve oluşan buhar soğutucudan geçirilerek destilat, içinde 10 ml 0.1 N HCl ve 40 ml su bulunan balonda toplanmıştır. Destilat 0.1N NaOH ile titre edilerek alınan sonuç formülde yerine konmuş ve matematiksel olarak mg/100 g olarak TVB-N değeri hesaplanmıştır.

***pH Değerinin Ölçülmesi*:** Karideslerin pH ölçümleri Hanna HI 9125 model pH metre ile oda sıcaklığında yapılmıştır [16].

***Tiyobarbitürik Asit (TBA) Değerinin Belirlenmesi*:** Her bir gruba ait dondurulmuş karides örnekleri iyice parçalandıktan sonra 20 g alınıp üzerine 50 ml %20'lik trikloroasetik asit (2 M fosforik asit solüsyonunda hazırlanmış) ilave edilerek ultra-turrax (ART Miccra RT) aracılığıyla homojenize edilmiştir. Deiyonize su ile 100 ml'ye tamamlanarak filtre edildikten sonra filtratın 5 ml'si, 5 ml taze hazırlanmış 0.005 M tiyobarbitürik asit solüsyonu ile karıştırılmıştır. Karışım birkaç defa çevrilmek suretiyle karıştırılmış; karanlık bir ortamda oda sıcaklığında 15 saat bekletildikten sonra oluşan rengin absorbansı UV spektrofotometrede (Chebios Optimum-One) 530 nm'de ölçülmüş ve TBA değeri reaksiyonda oluşan malondialdehid (mg MDA/kg) üzerinden hesaplanmıştır [19].

BULGULAR

Kontrol grubu ve muamele gruplarına ait donmuş muhafaza sırasındaki ortalama koku ve lezzet puanlarındaki değişimler *Şekil 1*'de gösterilmiştir. Muamele öncesi karideslerdeki ortalama duyusal koku puanı 100 üzerinden ortalama 86.97 olarak belirlenmiştir. Muamele gören bütün gruplardaki koku puanları muhafaza sırasında yaklaşık 10 puan civarında bir azalma göstermiş ve muhafazanın son günü 76.02-76.50 arasında saptanmıştır. Muamele grupları arasındaki



Şekil 1. Donmuş muhafaza sırasında kabuklu karideslerin koku ve lezzet puanlarındaki değişimler

Fig 1. Changes in odour and flavour scores of shelled shrimps during frozen storage

Tablo 2. *Donmuş muhafaza sırasında karideslerdeki fiziksel ve kimyasal parametreler*
Table 2. *Physichal and chemical parameters of shrimps during frozen storage*

Parametre	Grup	Donmuş Muhafaza (ay)			
		3	6	9	12
Tekstür (kg/cm²)	A	1.11±0.06*	1.36±0.04	1.09±0.09	0.89±0.05
	B	1.09±0.12	1.38±0.05	1.28±0.14	1.11±0.07
	C	1.18±0.09	1.19±0.10	1.34±0.07	1.12±0.08
	D	1.23±0.06	1.32±0.12	1.29±0.11	1.05±0.11
	E	1.19±0.06	1.27±0.06	1.31±0.16	1.10±0.09
	F	1.17±0.03	1.27±0.04	1.19±0.22	1.01±0.06
	Kontrol	1.25±0.07	1.29±0.06	1.06±0.26	0.88±0.08
Rutubet (%)	A	77.42±0.21*	77.05±0.21	76.47±0.15	75.82±0.22
	B	77.52±0.19	77.07±0.12	76.51±0.21	75.86±0.25
	C	77.53±0.18	77.15±0.19	76.72±0.23	76.28±0.14
	D	77.59±0.25	77.25±0.27	76.65±0.26	76.25±0.20
	E	77.65±0.17	77.33±0.13	76.81±0.24	76.16±0.16
	F	77.53±0.23	77.18±0.21	76.66±0.20	76.12±0.23
	Kontrol	77.40±0.20	77.06±0.20	76.56±0.22	75.98±0.17
pH	A	6.97±0.04*	7.09±0.06	7.24±0.07	7.31±0.80
	B	6.97±0.07	7.08±0.05	7.22±0.05	7.39±1.03
	C	6.93±0.05	7.00±0.05	7.14±0.06	7.24±1.33
	D	6.92±0.06	7.03±0.06	7.15±0.05	7.32±1.20
	E	6.90±0.08	7.03±0.05	7.18±0.06	7.33±0.89
	F	6.89±0.06	7.02±0.04	7.13±0.03	7.25±1.33
	Kontrol	6.99±0.06	7.14±0.06	7.32±0.05	7.48±1.42
TMA-N (mg/100 g)	A	0.70±0.02*	1.16±0.10	1.61±0.08	2.34±0.10
	B	0.71±0.08	1.05±0.10	1.71±0.12	2.18±0.08
	C	0.73±0.08	1.20±0.12	1.78±0.14	2.31±0.13
	D	0.66±0.10	1.14±0.13	1.58±0.12	2.10±0.10
	E	0.64±0.08	1.20±0.05	1.60±0.05	2.03±0.09
	F	0.86±0.08	1.29±0.08	1.84±0.14	2.26±0.13
	Kontrol	0.90±0.08	1.38±0.06	1.88±0.11	2.43±0.11
TVB-N (mg/100 g)	A	21.12±1.01*	25.07±0.57	30.57±0.45	34.11±0.80
	B	22.75±0.91	27.57±0.71	31.11±0.58	35.14±1.03
	C	21.57±1.27	25.95±1.18	29.75±1.26	33.55±1.33
	D	20.56±1.11	24.85±0.81	29.52±1.25	33.76±1.20
	E	21.12±1.61	24.91±1.30	30.27±0.96	33.22±0.89
	F	21.07±0.92	25.39±0.84	29.30±0.78	33.64±1.33
	Kontrol	21.64±0.39	26.94±0.61	32.49±0.81	34.85±1.42
TBA (mg MDA/kg)	A	0.19±0.02*	0.29±0.01	0.39±0.02	0.66±0.02
	B	0.14±0.02	0.24±0.02	0.34±0.02	0.58±0.02
	C	0.16±0.01	0.26±0.01	0.35±0.01	0.54±0.01
	D	0.19±0.02	0.27±0.02	0.36±0.01	0.62±0.01
	E	0.20±0.01	0.27±0.02	0.37±0.02	0.54±0.02
	F	0.18±0.02	0.25±0.01	0.33±0.02	0.60±0.02
	Kontrol	0.19±0.02	0.24±0.01	0.35±0.01	0.56±0.02

** Aynı kolonda yer alan ortalamalar arasında istatistiksel anlamda önemli bir fark bulunmamıştır (P<0.05)*

farklar istatistiki anlamda önemli bulunmamıştır. Kontrol grubu karideslerde ise koku puanlarındaki azalma 24 puan civarında gerçekleşmiş muhafazanın bütün aşamalarında muamele gruplarından önemli derecede daha düşük bulunmuştur (P<0.05). Panelistler tarafından muamele öncesi 85.30 olarak belirlenen lezzet puanı muamele gruplarında yaklaşık 13 civarında bir azalma göstererek muhafazanın sonunda 72.32-73.13 arasında saptanmıştır. Kontrol grubunda lezzet puanlarındaki azalma muamele gruplarına göre daha hızlı şekillenmiş olup donmuş muhafazanın sonunda başlangıca göre yaklaşık 21 puan daha düşük bulunmuştur. Muhafazanın üçüncü ayı E ve F grubu hariç, muhafaza sırasında kontrol grubunda saptanan ortalama lezzet puanları ile muamele gruplarına ait ortalamalar arasındaki farklar istatistiki anlamda önemli bulunmuştur (P<0.05).

Donmuş muhafaza sırasında karideslerde saptanan fiziksel ve kimyasal parametrelerdeki değişimler *Tablo 2*'de gösterilmiştir. Muamele öncesi karideslerin kesmeye karşı direnci 0.94 kg/cm^2 olarak saptanmıştır. Donmuş muhafaza sırasında tekstür değerlerinde düzensiz değişimler gözlenmiştir. Başlangıçta %77.85 olarak saptanan nem oranı kontrol ve deneme gruplarında donmuş muhafaza sırasında kısmi bir azalma göstermiştir. Muhafazanın sonunda azalma oranı başlangıca göre ancak %1.57-2.03 düzeyinde gerçekleşmiştir. Karideslerin TMA-N miktarlarında yavaş fakat düzenli bir artış görülmüştür. Muamele öncesi 0.31 mg/100 g olarak belirlenen miktar on iki aylık muhafazanın sonunda deneme gruplarında 2.03-2.34 mg/100 g arasında bulunmuştur. Sadece su ile muamele edilen kontrol grubunda ise bu değer 2.43 mg/100 g olarak saptanmıştır. TVB-N miktarı bütün gruplarda muhafaza sırasında düzenli bir artış göstermiş ve muhafazanın onikinci ayında 33.22-35.14 mg/100 g arasında saptanmış olup aralarındaki farklar istatistiki anlamda önemsiz bulunmuştur(P<0.05). Muamele öncesi 6.79 olarak saptanan pH değeri donmuş muhafaza sırasında kısmi bir artış göstermiş; muhafazanın sonunda 7.24-7.48 arasında saptanmıştır. Kontrol grubunda saptanan pH değerleri muhafazanın bütün aşamalarında deneme gruplarından yüksek saptanmış olmakla birlikte aralarındaki fark istatistiksel anlamda önemli bulunmamıştır (P<0.05). Muamele öncesi 0.117 mg MDA/kg olarak saptanan TBA değeri muhafazanın sonunda 0.54-0.66 mg MDA/kg'a ulaşmıştır.

TARTIŞMA ve SONUÇ

Duyusal değerlendirme bulgularımız karides örneklerinin donmuş muhafaza sırasında kalite kaybına uğradığı ancak kontrol grubu dahil bütün örneklerin on ikinci ayda bile tüketilebilir olduğunu göstermektedir. Tsironi ve ark.[6] da -12°C ve -15°C'de muhafaza edilen karideslerin sırasıyla sekizinci ve on birinci aylarda hala kabul edilebilir durumda olduklarını saptamışlardır. Karideslerde koku ve tat değişikliğinin başlıca sebebi mikrobiyel faaliyetlerdir. Dondurulmuş karideslerde mikrobiyel aktivite durdurulmuş olmasına rağmen duyusal analizlerde kontrol grubunun daha düşük puan alması panelistlerin muhtemelen kontrol grubuna ait örneklerin renginden (hafif kararma) etkilenmiş olabileceği şeklinde değerlendirilmiştir. Nitekim duyusal olarak belirlenen farklılık bozukluğun kimyasal olarak belirlendiği TBA-N ve TVB-N analizlerinde gözlenmemiştir *(Tablo 2)*.

Muamele edildiği kimyasallar arasında biberiye ekstraktının da bulunduğu gruplarda (E ve F) lezzet puanları fark edilebilir biberiye lezzetinden dolayı muhafazanın üçüncü ayı diğer muamele gruplarınkinden (A, B, C, D) önemli derecede daha düşük bulunmuştur **(**P<0.05). Panelistler tarafından puan düşürücü kriter olarak belirtilen biberiye lezzeti altıncı ay ve sonraki muhafaza dönemlerinde bir olumsuzluk olarak görülmemiştir. Bu durum uçucu özelliği olan biberiye ekstraktının zaman içinde üründeki seviyesinin azalması şeklinde açıklanabilir.

Genel itibariyle tekstür değerinde muhafaza sırasında ilk zamanlar sertleşmeye işaret eden bir artış, son zamanlarda ise yumuşamaya işaret eden bir azalma gözlenmiştir. Karides dokusunun sertleşmesi nem kaybıyla, yumuşaması ise bozulma sürecinin başlamasıyla ilişkilendirilmiştir. Gerek kontrol grubu gerek muamele gruplarındaki ortalama değerler birbirine yakın saptanmıştır. Bulgular karideslerin kitosan ve benzeri maddelerle muamele edilmeden de donmuş muhafaza sırasında tekstürel özelliklerini koruduğunu göstermektedir. Donmuş muhafaza sıcaklığı tekstür üzerine belirleyici etkiye sahiptir. Tsironi ve ark.[6] dondurulmuş karideslerde süre ve sıcaklığa bağlı olarak tekstürel parametrelerde değişikliklerin olduğunu gözlemlemişler, sıcaklık yükseldikçe sertlik değerlerinde daha fazla azalma olduğunu bildirmişlerdir. Yamagata ve Low [4] -10°C'de saklanan karideslerin 7 haftada yumuşadığını; -20°C'de saklananların ise altıncı aydan sonra yumuşamaya başladığını bildirmişlerdir. Çalışmamızda ise -18°C'de saklanan karides lerde entstrümental tekstür analizinde ileri düzeyde bir yumuşamaya işaret eden bir bulgu elde edilmemiştir. Karideslerde doku yumuşaması özellikle mikrobiyel kökenli enzimlerin faaliyeti sonucu protein yapısının parçalanmasıyla ilişkilidir. Dekompozisyonun en önemli kriteri olan TMA-N sayısı da muhafaza sırasında önemli bir artış saptanmamıştır. Dolayısıyla karideslerdeki TMA-N miktarları ile tekstür değerleri birbirini desteklemektedir.

Dondurulmuş karideslerle görülen önemli kalite sorunlarından birisi dehidrasyon ve buna bağlı olarak şekillenen ağırlık kaybıdır [20]. Kitosanla muamele edilmiş C, D, E ve F gruplarındaki nem oranları kontrol ve diğer deneme gruplarındakinden önemsiz de olsa muhafazanın çoğu aşamalarında daha yüksek saptanmıştır. Karideslerin kabuklu veya kabuksuz dondurulması su kaybı üzerine etkilidir. Çalışmamızda karideslerin kabuklu dondurulması su kaybının minimum düzeyde tutulmasında etkili olmuştur. Kayıp oranı zaten düşük olduğundan gruplar arası karşılaştırma yapmak, özellikle kitosanın etkisini analiz etmek mümkün olmamıştır.

Trimetilamin miktarı bozulmanın en önemli indikatör-

lerinden birisidir [21]. Varlık ve ark.[22] ise tüketime uygun su ürünlerinde TMA-N değerinin 1-8 mg/100 g TMA-N olması gerektiğini, 8 mg/100 g TMA-N değerinin bozulmuşluğu belirlediğini bildirmişlerdir. Muhafaza periyodunun bütün aşamalarında kontrol grubu dahil bütün gruplara ait TMA-N miktarı ortalamaları birbirine yakın bulunmuş olup aralarındaki farklar istatistiki anlamda önemli bulunmamıştır ($P<0.05$). Saptamış olduğumuz miktarlar muhafazanın son günü bile bozulma limiti olarak kabul edilen sınırın (8 mg/100 g) çok altında kalmıştır. Lopez-Caballero ve ark.[23] üç ay süreyle derin dondurulmuş ve çözündürülmüş karideslerde TMA-N seviyesini 0.5 mg/100 g olarak saptamışlardır. Çalışmamızda da donmuş muhafazanın üçüncü ayı saptanan TMA-N miktarı bildirilen bu değere yakın olup 0.6-0.9 mg/100 g olarak belirlenmiştir. Bulgularımızın aksine Tsironi ve ark.[6] dondurulmuş karideslerde TMA-N içeriğinin muhafaza sırasında 14 mg/100 g'a yükseldiğini saptamışlardır. Bu farklılık söz konusu çalışmada başlangıç TMA-N seviyesinin çok daha fazla (2.85 mg/100 g) ve muhafaza sıcaklığının daha yüksek olmasından (-12°C) kaynaklanmış olabilir.

Su ürünlerinde TVB-N, trimetilamin, dimetilamin, amonyak gibi dekompozisyona bağlı olarak ortaya çıkan uçucu azotlu bileşikleri kapsamaktadır [21]. Karideslerde 30 mg/100 g TVB-N düzeyi karideslerin kabul edilebilirlik limiti için yararlı bir indikatör olarak kabul edilmektedir [24,25]. Donmuş muhafaza sırasında bir mikrobiyel aktivite söz konusu olmamakla birlikte otolitik enzimler yavaş da olsa faaliyetini sürdürmekte ve uçucu bileşiklerin oluşumu devam etmektedir. Çalışmamızda da donmuş muhafaza sonunda saptamış olduğumuz TVB-N miktarları öngörülen limitlerin biraz üzerindedir. Buna karşın duyusal değerlendirmede elde edilen bulgular karideslerin hala tüketilebilir olduğunu göstermektedir. Bu durum mikroorganizmaların dahil olmadığı bir süreçte ortaya çıkan uçucu bileşiklerin karideslerin tadını ve kokusunu önemli derecede değiştirecek seviyede olmadığı şeklinde açıklanabilir. Çalışmamızda karideslerin kitosanla muamelesi TVB-N miktarındaki artışı yavaşlatmada önemli bir etki oluşturmamıştır. Donmuş muhafaza sırasında sıcaklık TVB-N miktarındaki artış üzerine etkili olmaktadır. Tsironi ve ark.[6] karideslerde başlangıçta 6.49 mg/100 g olan TVB-N miktarının -5°C'de saklananlarda 45 günde, -8°C'de saklananlarda 82 günde, -12°C'de saklananlarda 187 günde, -15°C'de saklananlarda 353 günde ve -18°C'de saklananlarda 677 günde 25 mg/100 g düzeyine ulaştığını bildirmişlerdir. Gonçalves ve Gindri Junior [20] glazing işleminin dondurulmuş (-18°C'de) karideslerinin kalitesi üzerine etkisini inceledikleri araştırmalarında başlangıçta 7.52-8.81 mg/100 g olarak belirlenen TVB-N miktarının muhafazanın ilk 90 günü önemli bir artış göstermediğini, doksanıncı günden sonra bir artış görüldüğünü fakat hiçbir grupta 180 günlük muhafazanın sonunda bu miktarın 20 mg/100 g'ı geçmediğini bildirmişlerdir. Çalışmamızda yukarıda bildirilenlerden daha fazla TVB-N miktarları saptanmıştır. Bu farklılık materyal olarak kullandığımız karideslerde başlangıçtaki TVB-N miktarının (13.46 mg/100 g) diğerlerinin yaklaşık iki katı olmasından kaynaklanmıştır.

Karideslerde muhafaza sırasında bakteriler tarafından trimetilaminoksitin trimetilamine redüksiyonu, doku proteinlerinin dekompozisyonu ve deaminasyon gibi prosesler nedeniyle meydana gelen bazik aminler pH değerinde atışa neden olmaktadır [4]. Çeşitli araştırıcılar tarafından yapılan çalışmalarda 7.5-7.7 ve üzerinde pH değerine ulaşan karidesler bozuk olarak nitelendirilmiştir [25-27]. Dondurulmuş muhafaza sırasında da biyokimyasal reaksiyonların devam ettiği bilinmektedir. Bu reaksiyonlar sonucu ortaya çıkan yan ürünlerin miktarına bağlı olarak pH değerinde artış görülmektedir. Çalışmamızda muamele öncesi normal sınırlar içinde bulunan pH değeri donmuş muhafaza sırasında yavaş bir artış göstermiştir. Ancak kontrol dahil bütün gruplar arasında önemli bir fark gözlenmemiştir. Kitosan ve diğer kimyasallarla muamele muhafaza sırasında pH değeri üzerine önemli bir etki oluşturmamıştır. Muhafazanın sonunda ulaşılan pH değerleri bozulmuş olarak değerlendirilecek seviyenin (7.5) altındadır. Muhafaza sıcaklığı ne kadar düşük olursa reaksiyonların hızı da o kadar azalmaktadır. Tsironi ve ark.[6] başlangıçta 6.95 olan pH değerinin -5°C'de saklanan karideslerde 39. gün 7.93'e, -8°C'de saklananlarda 74. gün 7.85'e yükseldiğini; buna karşın -15°C'de saklananlarda muhafazanın 300. günü bile bu değere ulaşılmadığını bildirmişlerdir.

Karideslerde yağ oranı düşük (%1 civarında) olmasına rağmen yüksek seviyede (%32.8-%47.5) çoklu doymamış yağ asitlerini içermesinden dolayı oksidasyona duyarlı olarak değerlendirilmekte, uzun süreli donmuş muhafaza sırasında karideslerin acılaşmaya maruz kalabileceğinden söz edilmektedir [5,6,28]. Acılaşma indeksi olan TBA değeri 8 mg MAD/kg değerine ulaştığı zaman ürün bozulmuş (tüketilemez) olarak kabul edilmektedir [26]. Donmuş muhafaza sırasında bütün gruplarda TBA değerinde çok düşük seviyede bir artış kaydedilmiştir. Muhafazanın sonunda belirlenen bu değerler bozulmuşluğun limiti olan seviyenin oldukça uzağında kalmıştır. Bulgularımız herhangi bir antioksidan kullanılmasa bile kabuklu karideslerin en azından 12 ay (muhtemelen daha uzun süre) donmuş muhafaza sırasında acılaşma riski taşımadığını göstermektedir. Piyasadan toplanan donmuş karides örneklerinde de düşük (1 mg MDA/kg'ın altında) TBA değerleri bildirilmiştir [29,30].

Kitosanın antioksidan etkisi et bazlı çeşitli gıda maddelerinde araştırılmış ve yağ oksidasyon oranını önemli derecede azalttığı rapor edilmiştir [31-34]. Aynı şekilde biberiye ektraktı ile muamele edilen örneklerin muhafaza sırasında kontrol grubuna göre önemli derecede daha düşük peroksit ve TBA değerlerine sahip oldukları bildirilmiştir [34-38]. Her ikisinin birlikte kullanılması durumunda antioksidan etkinin daha da arttığı saptanmıştır [39]. Çalışmamızda gerek kitosanla gerekse kitosanın biberiye ve sitrik asitle kombine edildiği solüsyonlarla muamele edilen gruplarda saptanan TBA değerleri diğerlerinden farklı bulunmamıştır. Bu durum muhtemelen kontrol dahil bütün gruplarda TBA değerinde mukayese yapabilecek düzeyde bir artış olmamasından kaynaklanmıştır.

Bu çalışmada elde edilen bulgulara göre karideslerin kabuklu olarak dondurulması sırasında en az 12 ay süreyle kabul edilebilirliği olumsuz etkileyecek değişimlere maruz kalmadığını, kitosan ile muamele edilmenin bazı parametrelerde kısmi iyileşme yapmasına rağmen ticari anlamda bir öneminin olmadığını; karideslerin herhangi bir antioksidanla muamele edilmeksizin dondurulduğunda bile yağların oksidasyonuna bağlı bir sorun yaşanmadığı sonucuna varılmıştır.

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RESEARCH ARTICLE

Simultaneous Detection of Six Different Groups of Antimicrobial Drugs in Porcine Oral Fluids Using A Biochip Array-Based Immunoassay

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Summary

The objectives of this study were 1) to determine whether a biochip array-based immunoassay could be used to detect 6 different group antimicrobials in porcine oral fluids collected under experimental conditions; 2) to determine the feasibility of antimicrobial detection in clean versus dirty oral fluid samples; and 3) to determine if the assay could be used to detect chlortetracycline in oral fluids of swine ingesting a routine diet containing chlortetracycline. Biochip array-based immunoassay is currently used for simultaneous detection and quantitation of different group six antimicrobials in milk, urine, meat, honey, and feed. The assay had not been investigated for potential use in oral fluids. Following evaluation of different extraction procedures, a centrifugation method was chosen. Results showed that of the six target drugs, norfloxacin, ceftiofur, florfenicol, streptomycin, tylosin and tetracycline could be detected in both clean and dirty oral fluid samples. However, tetracycline was not well recovered in dirty samples. Chlortetracycline from tetracycline group was detected in all oral fluid samples collected from the field at concentrations ranging from 176.0-698.6 ppb. In conclusion, this assay can detect all target antibacterials from different groups in clean and dirty oral fluid samples with the exception of tetracycline which was not well-recovered from in dirty samples.

Keywords: *Antimicrobials, Biochip array-based immunoassay, Oral fluid, Swine*

Domuz Ağız Sıvısında Altı Farklı Grup Antimikrobiyal İlacın Aynı Anda Biochip Array-Based Immunoassay ile Tayini

Özet

Çalışmanın amacı Biochip array-based immunoassay yöntemi ile 6 farklı grup antibakteriyel ilacın deneysel şartlarda toplanmış domuz ağız sıvısında tespit edilip edilemediğini; temiz ve kirli domuz ağız sularında bu antimikrobiyallerin tespit edilebilirliğini ve bu metotla, rasyonlarında sürekli olarak klortetrasiklin bulunan domuzların ağız sıvılarında klortetrasiklinin tespit edilebilirliğini belirlemektir. Bu metod, aynı anda altı farklı grup antimikrobiyalin süt, idrar, et, bal ve yem numunelerinde miktarlarının belirlenmesinde kullanılmaktadır, ancak ağız sıvılarında tespit edilebilirlikleri henüz araştırılmamıştır. Yapılan farklı ekstraksiyon çalışmalarından sonra bir santrifüj metodu seçildi. Bu metodla hedef ilaç olan norfloksasin, seftifor, florfenikol, streptomisin, taylosin ve tetrasiklin temiz ve kirli domuz ağız sıvısında tespit edildi. Ancak, kirli ağız sularında tetrasiklinin geri kazanımı oldukça düşük olarak elde edildi. Klortetrasiklin çiftlikteki domuzların ağız sıvılarında 176.0-698.6 ppb arasında tespit edildi. Sonuç olarak, bu test ile kirli ağız sıvısında tetrasiklin aranması dışında, altı farklı gruptaki hedef antibakteriyel ilaç domuzların ağız sıvısında kolaylıkla tespit edilebilmektedir.

Anahtar sözcükler: *Antimikrobiyaller, Biochip array-based immunoassay, Ağız sıvısı, Domuz*

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INTRODUCTION

Oral fluid is composed of saliva, gingival cervical fluids contained in the dentogingival sulcus, mucosal transudate, cell detritus, bacteria and food remains [1]. Human saliva is composed of 98% water. The remaining amount is made up of other compounds, such as electrolytes (Na, K, Ca, Mg, hydrogen carbonates, and phosphates), mucus (composed mainly of mucopolysaccharides and glycoproteins), antiseptic substances (hydrogen peroxide, IgA), and various enzymes (α-amylase, lysozymes, lingual lipase) [2]. Saliva also contains many other proteins such as histatin and polypeptides with antibacterial and antifungal properties [2].

Following oral or parenteral administration, antimicrobials can be transported from blood to saliva by simple diffusion and/or active transport mechanisms [3,4]. Depending upon the degree of ionization, antimicrobials that are weak bases may reach high concentrations in saliva [5].

Antimicrobial residues are of food safety concern. To protect health of consumers it is necessary to test pigs for potential antimicrobial residues on the farm before they are put on the market. Simultaneous analysis of different groups of antimicrobials is a difficult task but is highly desirable in diagnostic laboratories. A biochip array-based immunoassay test (BABIT) that can quantitatively analyze for quinolones, ceftiofur, thiamphenicol, streptomycin, tylosin and tetracyclines, simultaneously in select matrices was recently developed and has been used for analysis of honey, milk, tissue, urine, and feed matrices. The test can be used to simultaneously quantify multiple analytes from a single sample [6].

It has been demonstrated that oral fluids can be used as a matrix to detect some drugs in domestic animals [7]. Significant research has also been conducted on the use of oral fluid as a diagnostic medium for detection of some viral infections [7-9]. Recently, there is a report [10] on the detection of antibacterials such as ceftiofur and oxytetracycline in swine oral fluids. In this study [10], ceftiofur and oxytetracycline were qualitatively detected in swine oral fluid as positive or negative using a pen-side competitive ELISA. In veterinary diagnostic medicine, oral fluid specimens could potentially be used for detection of some drugs for food safety applications. At present, commonly used matrices for this purpose are milk, meat, urine and serum. There are some advantages in using oral fluids compared to traditional matrices. Collecting oral fluid specimens is animal friendly (less stressful) and non-invasive [7]. Besides, oral fluids can be collected from a single animal or from group of animals. Oral fluid is not a common diagnostic specimen for analysis of antimicrobials in domestic animals and in veterinary medicine. Therefore continued research is needed to standardize collection methods of oral fluids from swine [7]. The quality of oral fluids collected depends on sampling method, number of pigs in the pen and cleanliness of the pen.

The impact of sample quality on antimicrobial test results in domestic animals warrants continued research. There is also a need for further research on tests which can simultaneously detect multiple antimicrobials in a single oral fluid sample. The objectives of this study were: 1) to determine whether a biochip array-based immunoassay could be used to detect 6 specific antimicrobials simultaneously in porcine oral fluids collected under experimental conditions; 2) to determine the feasibility of antimicrobial detection in clean versus dirty oral fluid samples; and 3) to determine if the assay could be used to detect chlortetracycline in oral fluids of swine ingesting a routine diet containing chlortetracycline.

MATERIAL and METHODS

Animals and Animal Care, and Feeding Conditions

Clean oral fluid samples were collected from pigs housed in research facilities in the College of Veterinary Medicine at Iowa State University (Ames, IA), and all studies were approved by the Institutional Animal Care and Use Committee. The samples were collected from 8 pens of 15 pigs each. The oral fluid used in the methods development part this study was from a pooled sample containing oral fluid from each of the 8 pens. The animals were conventionally produced pigs, weighed approximately 14 kg (30 lbs), and contained both male and female pigs. The pigs were fed an antimicrobial-free diet. The field collection of oral fluid was from 20 pens of 25 animals per pen in a commercial finisher swine barn. The pigs weighed approximately 34 kg (75 lbs) and were placed in the finisher barn 3 days prior to oral fluid collection. There were a total of approximately 1200 pigs in the barn but only a pen with 20 pigs were enrolled in the study. These pigs were fed a commercial diet containing chlortetracycline (approximately 440 g/ton) and tiamulin (tiamulin hydrogen fumerate approximately 38g/ton). Animal were fed *ad libitum*, and no parenteral antimicrobial treatments were administrated to these pigs. Oral fluids were collected on January 06, 2012.

Sample Collection

Oral fluid samples were collected from pigs as described in a recent study [10] by hanging 100% cotton rope (Wep Ringing Suplly, Inc., Lake Barrington, IL, USA) in each pen for a minimum for 20 min in the morning. Oral fluids were extracted by wringing the ropes through a manual wringer. Oral fluid from each pen were pooled into a 50-ml Falcon plastic tube and frozen at -20°C until analysis. Sample quality as a colour can range from very clean to very dirty *(Fig. 1)*.

Biochip Array-Based Immunoassay Test Procedure

Oral fluid samples were tested using Antimicrobial Array II (AM II) Evidence Investigator Test Kit and the AM II Control was used as a control (EV 3524 and EV5337, Randox Laboratories Ltd., Crumlin, UK). All assays were done according to AM II manufacturer's instructions [6]. Biochips

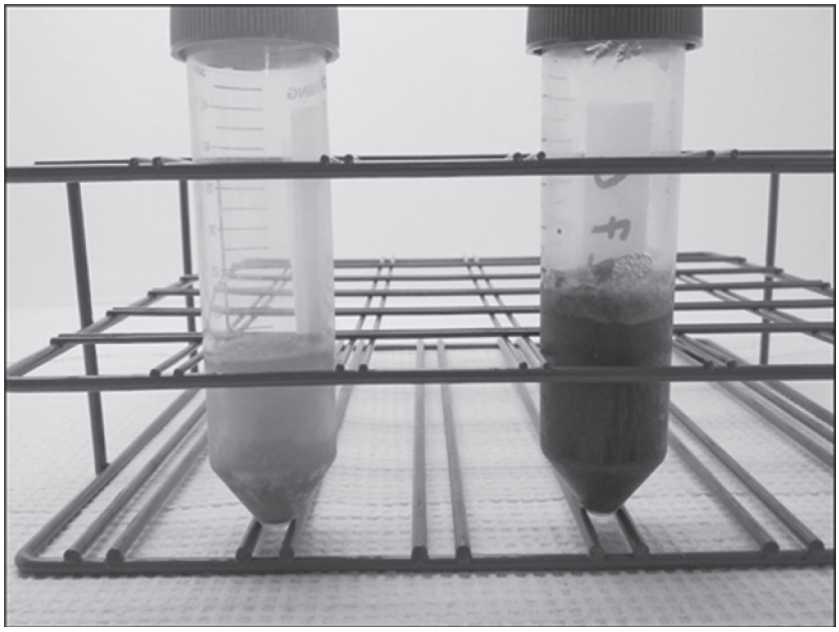

Fig 1. Clean and dirty porcine oral fluids

Şekil 1. Temiz ve kirli domuz ağız sıvısı

were equilibrated to room temperature for approximately 30 min prior to opening. After extraction, 100 µl of "assay diluent" was pipetted into the biochip wells. 100 µl of calibrator or samples was pipetted into the wells and gently taped all edges of the handling tray to mix reagents. Biochips were incubated for 30 min at 25°C on a thermo-shaker (Randox Laboratories Ltd., Crumlin, UK) 370 rpm. 100 µl of working strength conjugate was slowly mixed before use and pipetted into the wells. Biochip wells were incubated for 60 min at 25°C and 370 rpm on the thermo-shaker. Reagents were discarded to the waste container using a sharp flicking action of the handling tray. 2 quick wash cycles were immediately carried out with "diluted wash buffer" (wash buffer) with approximately 350 µl for each well. 4 additional wash cycles were used; for each cycle all edges of the handling tray were gently taped approximately 10-15 sec, then biochips were left to soak in wash buffer for 2 min. After the final wash, all the wells were filled with wash buffer and left to soak until directly prior to imaging. 250 µl "working signal reagent-EV805" was added to each well and covered to protect from light in the thermoshaker. After exactly 2 min (+/- 10 sec) the carrier was placed into the Evidence Investigator (Randox Laboratories Ltd., Crumlin, UK). Captures of images were automatically initiated as defined by the dedicated software.

Extraction Method Development for Oral Fluids of Pigs

Antibacterial standards and chemicals: In the present study, norfloxacin (FLUKA, Buchs, Switzerland), florfenicol (FLUKA, Buchs, Switzerland), tylosin tartrate (FLUKA, Buchs, Switzerland), ceftiofur (Sigma-Aldrich, Seelze, Germany), streptomycin sulfate salt (Sigma-Aldrich, Seelze, Germany), tetracycline (Sigma-Aldrich, Seelze, Germany), and chlor-tetracycline hydrochloride (Sigma-Aldrich, Seelze, Germany) were used as antimicrobial standards. Ethylenediamine tetra acetic acid (EDTA) disodium salt dehydrate (Fisher Scientific, New Jersey, USA), sodim sulfate (Fisher Scientific, New Jersey, USA) and acetic acid (glacial) (Fisher Scientific, New Jersey, USA) were used for extraction in Method 3.

Preparation of standards: Stock standard solutions were prepared as 10 mg/ml. Tylosin, tetracycline, florfenicol in methanol (Fisher Scientific, New Jersey, USA), norfloxacin in acetone (Fisher Scientific, New Jersey, USA), ceftiofur in deionized water (Water Aries High Purity Water System, West Berlin, Germany): Acetonitrile (Fisher Scientific, New Jersey, USA) (7:3), and streptomycin in deionized water were dissolved. Dilutions from stock solutions were made with the washing buffer of AM II Kit.

Extraction studies in oral fluid: Different extractions methods for antibiotics in oral fluids were investigated, including filtration, dilution, precipitation, and centrifugation. A summary of each extraction method investigated follows:

- *Method 1. Filtration and dilution:* The manufacturer's (Randox) suggested sample protocol involves filtration of 3 ml of oral fluid with 0.45 µm syringe filter (TITAN, Nylon 0.45 µm). Then, 100 µl of the filtrate is dilute with 900 µl of washing buffer.

- *Method 2. Dilution-filtration and dilution-centrifuge:* 100 µl oral fluid samples at two different spike levels were diluted with 900 µl washing buffer (n=2) or deionized water (n=2), vortexed (FISHERbrand, Vortex Genie 2™, USA), filtered through a 0.45 µm syringe filter, and the filtrate was analyzed. In *dilution-centrifuge,* 100 µl oral fluids that at two different spike levels were diluted with 900 µl washing buffer (n=2) or deionized water (n=2) and centrifuged (Eppendorf, 5417 C, USA) at 5.000 rfc for 10 min at room temperature, and the supernatant was analyzed.

- *Method 3:* 0.5 ml of 0.3 M EDTA and 4 ml acetonitrile/1%

acetic acid was added to 1 ml of oral fluid, and sample was vortexed for 1 min. Then 0.4 g sodium sulfate was added and vortexed, followed by centrifugation at 2.500 rfc for 20 min at room temperature. The supernatant was evaporated and reconstituted with 1 ml of washing buffer. Two different spike levels (2 and 4 ppb for streptomycin, and 1 and 2 ppb for the other antimicrobials) were performed (n=6).

- *Method 4. Centrifugation*: This involved a slight modification of the AM II urine method described in the manual. Briefly, 1 ml of oral fluid sample and 1.5 ml eppendorf tube was used. The 1 ml oral fluid sample was centrifuged at 5.000 rfc for 10 min at room temperature. 50 µl of centrifuged oral fluid was collected and diluted with 450 µl washing buffer, the dilution factor was 10. The experiment involved testing different centrifuge speeds (5.000, 10.000, and 15.000 rfc) (n=6). Assay ranges, recovery and precision values of clean and dirty oral fluids were performed according to Australian Pesticides and Veterinary Medicines Authority Guidelines [11]. Samples were spiked for determination of assay ranges, percent recovery and precision. Assay range studies were carried out between 0.25 and 12 ppb for norfloxacin, florfenicol, tylosin, and tetracycline; 0.25 and 20 ppb ceftiofur, and 0.5 and 100 ppb for streptomycin in clean and dirty oral fluid samples. Recovery was studied at 1.0, 1.2 and 1.5 ppb levels for tetracycline; at 1.5, 1.7 and 2 ppb levels for norfloxacin, florfenicol and tylosin; at 2.0, 2.5 and 3.0 ppb for ceftiofur, and at 10.0, 15.0 and 20.0 ppb levels for streptomycin. Precision was carried out six times at 1.0 ppb for tetracycline, at 2.0 ppb for norfloxacin, ceftiofur, florfenicol and tylosin at 20.0 ppb concentrations for streptomycin.

For field samples, chlortetracycline (representing tetracyclines as a group) concentrations in 20 porcine oral fluid samples were determined using the centrifugation method (Method 4). There was a color difference between clean and dirty oral fluid samples. The test specificity was 51% for chlortetracycline according to the kit manual [6], and recovery rates (n=3) averaged 55% for these oral fluid samples. The dilution factor was 80 (10 times are coming from method 4, and 8 times made dilution before analysis). All the results were calculated according to these factors.

Statistical Method

Descriptive statistics were performed using the Minitab Statistical Program [12].

RESULTS

The procedure recommended by the manufacturer (Method 1) did not work for both clean and dirty pig oral fluids because the oral fluids could not pass through the filter without dilution. As for method 2 (dilution and filtration), the oral fluid could be filtered, but with difficulty, and there was huge variability between results (between 13% and 500%). Results from Method 3 indicated very high recovery rates (between 790% and 5.000%) except florfenicol results (between 74% and 295%). As such, the first 3 extraction procedures did not work. Method 4, which was a modification of the urine extraction procedure, worked well. We investigated 3 centrifugation speeds, (5.000, 10.000 and 15.000 rfc) for both clean and dirty oral fluid samples, and results indicated that 5.000 rfc recovery values were slightly better than the other speeds, and this speed was adopted by the researchers.

Standard curves were prepared using AM II Kit calibrators. Assay ranges were 0-9.8 (norfloxacin), 0-20.7 (ceftiofur),

Table 1. *Assay ranges, recovery, precision and control results of norfloxacin, ceftiofur, florfenicol, streptomycin, tylosin and tetracycline in clean and dirty oral fluids of pigs (as ppb), and AM II Control and mix standard results (as ppb)*

Tablo 1. *Domuzların temiz ve kirli ağız sıvılarında norfloksasin, seftifor, florfenikol, streptomisin, taylosin ve tetrasiklinin ölçüm aralığı, geri kazanımı, kesinlik (doğruluk) ve control sonuçları (ppb olarak), AM II kontrol ve karışık standart sonuçları (ppb olarak)*

Sample	DF	Norflox	Ceftiofur	Florfen	Strep	Tylosin	Tetra
Assay ranges* AM II Kit	1	0-9.8	0-20.7	0-4.8	0-54.9	0-4.5	0-4.0
AM II controls	1	1.11 (1.21)**	2.48 (2.5)**	0.56 (0.63)**	6.22 (7.31)**	0.46 (0.54)**	0.37 (0.45)**
Mix std %Recovery	1	2.81 (140%)	4.25 (212%)	2.54 (127%)	2.65 (64%)	1.64 (82%)	0.84 (42%)
COF control	1	0.01	0.02	0	0	0.04	0.07
DOF control	1	1.62	0.61	0.14	0	0.02	0.27
COF assay ranges	1	0.5-6	0.5-9	0.25-3	2.0-75	0.25-5	0.5-1.5
DOF assay ranges	1	0.5-8	0.5-10	0.25-4	1.0-75	0.25-6	2.0-6
COF %Recovery (Mean)	1	140±7.54	130±6.24	122±8.00	71±3.46	78±9.53	171±50.26
DOF %Recovery (Mean)	1	42±7.23	117±12.74	93±12.74	69±3.51	55±1.15	5±0.57
COF Precision (Mean), %RSD	1	2.71±0.14 (5.2%)	2.74±0.19 (6.9%)	2.50±0.12 (4.9%)	13.44±0.70 (5.2%)	1.55±0.07 (4.4%)	1.21±0.07 (6.0%)
DOF Precision (Mean), %RSD	1	1.33±0.18 (13.3%)	2.74±0.25 (9.2%)	2.45±0.19 (7.7%)	14.54±0.24 (1.6%)	1.22±0.09 (7.4%)	0.09±0.05 (57.0%)

** Assay ranges that determined with AM II calibrators according to AM II Manual, ** AM II Control result: Antimicrobial II Controls were assigned with HPLC by Randox,* ***Mix std:*** *Six antibacterial standards were prepared (4 and 2 ppb for strep. and others, respectively) with diluted washing buffer from stock solution and analyzed,* ***COF:*** *Clean Oral Fluid,* ***DOF:*** *Dirty Oral Fluid,* ***DF:*** *Dilution Factor,* ***Norflox:*** *Norfloxacin,* ***Florfen:*** *Florfenicol,* ***Strep:*** *Streptomycin,* ***Tetra:*** *Tetracycline,* ***±:*** *Standard Deviation*

0-4.8 (florfenicol), 0-54.9 (streptomycin), 0-4.5 (tylosin), and 0-4.0 (tetracycline). R^2 values that determined with AM II calibrators were 0.998, 0.999, 0.999, 0.997, 0.998, and 0.993 for norfloxacin ceftiofur, florfenicol, streptomycin, tylosin, and tetracycline, respectively. Results for assay ranges, recoveries, precisions and controls of clean and dirty oral fluid's centrifuge method (Method 4), and AM II Control and mix standard results are presented in *Table 1*. Results of field assays for chlortetracycline concentrations determined in 20 oral fluid samples collected from a pig farm are between 176.0 ppb and 698.6 ppb, with a mean level of 421.3±210.6 ppb.

DISCUSSION

It turned out that developing a suitable method for simultaneous extraction of six different antimicrobials in oral fluid was challenging. The challenge with Method 1 was filtration of the oral fluids; oral fluid samples did not filter well. The oral fluid of pigs is too thick for filtration through a 0.45 µm syringe filter. In Method 2, following dilution the filtration process worked most of the time, but the recovery values were unreliable. The reasons for this are unclear, but probably antimicrobials in the oral fluid did not filter well enough thru 0.45 µm syringe filter. Method 3 did not work well either as recovery values were very high. The reasons for the extremely high recoveries are not clear. Results were acceptable for the Centrifugation method (Method 4), as recovery values were generally acceptable for both for clean and dirty oral fluid samples with the exception of the tetracycline. Recovery of tetracyclines in dirty oral fluid was low *(Table 1)*. Because of the overall performance, this extraction method was chosen for analysis of six antimicrobials with the BABIT. This extraction method is simple as it does not require adding any chemical. Simply, 1 ml of oral fluids is used for analysis. Because of its simplicity, and because it is easy to collect this sample size from pigs singly or as group, this method was adopted and is recommended.

As shown in *Table 1*, assay ranges of the AM II Kit was generally between 0 and 54.9 ppb depending on the antimicrobial. AM II control background results for this study was similar to manufacturer's background control results. This confirms that the reagent kits were working well. However, recovery values were different for individual antimicrobials. For example, for norfloxacin (140%), ceftiofur (212%), and florfenicol (127%) higher than 100%; for streptomycin (64%), tylosin (82%), and was least for tetracycline (42%). This test therefore likely has higher sensitivity for norfloxacin, ceftiofur and florfenicol, and lower sensitivity for streptomycin and tetracyclines. Dirty oral fluid samples had slightly higher, but insignificant control values for norfloxacin, ceftiofur, florfenicol and tetracycline. Reasons for this are not clear, but could likely be caused by some interference in this matrix for these antibiotics. It is interesting that both clean and dirty oral fluid samples did not contain any streptomycin *(Table 1)*. Assay ranges for dirty and clean oral fluids were generally similar except for tetracycline. For tetracyclines, the assay range of dirty oral fluid (2.0-6.0 ppb) was wider than the clean oral fluid assay range (0.5-1.5 ppb) *(Table 1)*. Tetracycline recovery was very low (5%) in dirty oral fluid *(Table 1)*. Although reasons for this are not clear, it is likely that tetracyclines easily bind to the matrix ingredients such us calcium, magnesium, aluminum, and iron [13]. It is possible that constituents in dirty oral fluid samples bind to the tetracycline group. This may be valid for norfloxacin as well because recovery for this antimicrobial in dirty oral fluid samples was low (42% versus 140%). Besides these exceptions, recovery values for streptomycin, ceftiofur and tylosin in clean and dirty oral fluid samples were close *(Table 1)*.

In the field samples, concentrations of chlortetcycline in 20 pig oral fluid samples were between 176.0 ppb and 698.6 ppb, with a mean level of 421.3±210.6 ppb. The highest level was approximately 4 times higher than the lowest level. There could be several reasons why there was so much variability in concentration of chlortetracycline in oral fluids collected from pigs fed a diet containing 440 ppm chlortetracycline, including improperly mixed feeds, time of last meal, etc. Overall, chlortetracycline was present in oral fluids at approximately 1.000 times less in oral fluid of pigs than in feeds. In this study, we did not collect blood samples from these pigs. This was not the objective of this study. However, these results indicate that tetracyclines could pass to oral fluids of pigs fed feeds containing tetracyclines. Therefore, oral fluids can be used to detected tetracyclines in pig oral fluid using this novel biochip array-based immunoassay. Oxytetracycline and ceftiofur in oral fluids of swine was also reported in a previous study [10]. However their assay was qualitative, with results reported as either positive or negative, using a pen-side competitive ELISA. In addition, if the ceftiofur pass from the blood to oral fluid as mentioned the previous study [10], ceftiofur might also be detected easily with the BABIT in field conditions. The average recovery was 55% in the oral fluids collected from pig farm. The recovery values could for tetracycline could vary between 5% and 171% *(Table 1)* depending on the samples properties, eg very clean versus dirty samples *(Fig. 1)*. More research is therefore needed to validate this assay and to determine factors affecting recovery of different antimicrobials in oral fluids.

In general, results suggest that this Biochip technology can be used to simultaneously detect and quantify the antimicrobials evaluated in this study with the exception of tetracyclines in dirty samples. Overall, the technology is can work for clean oral fluid samples like those collected under controlled research conditions, in clean facilities. However, this technology will be of value if it can be used under typical field conditions. Field oral fluid samples are reflective of the environment in which the pigs are housed. Therefore, sample cleanliness should be considered during assay development of this technology especially for tetracyclines.

In conclusion, oral fluids can be used to detect and/or monitor certain antibacterials in domestic animals. This study has demonstrated feasibility for the use of a biochip array based immunoassay for simultaneous detection and quantitation of (name the drugs) in porcine oral fluids. More field studies are recommended to characterize the technique under field conditions before it can be adopted for field applications.

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RESEARCH ARTICLE

Ergin Atlarda III, IV ve VI. Çift Kranial Sinirlerin Transversal Kesit Alanları ve Myelinli Akson Sayıları

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Özet

Ekstrinsik göz kaslarının motor uyarımını sağlayan n. oculomotorius, n. trochlearis ve n. abducens'in transversal kesit alanları ve içerdiği myelinli akson sayılarının belirlenmesi amaçlandı. Çalışmada 3 dişi, 3 erkek yetişkin at kullanıldı. Doku örnekleri sinirlerin cavum subarachnoideale'de seyreden bölümlerinden alındı. Parafin blokları hazırlanan dokular 4 µm kalınlığında transversal olarak rotary mikrotom ile kesildi, Masson trikrom ile boyandı. Sinirlerin kesit alanları Cavalieri metodu ile içerdikleri myelinli akson sayıları ise parçalama yöntemi ile araştırıldı. Sağ ve sol göze ait sinirlerin kesit alanları ve içerdikleri myelinli akson sayıları arasında istatistiki bir fark gözlenmediğinden sinirlerin akson sayıları taraf ayırt etmeksizin tek bir veri olarak (median) değerlendirildi. Sinir kesitlerinin alanları n. oculomotorius, n. trochlearis ve n. abducens için sırası ile 2.647 mm^2, 0.511 mm^2 ve 1.092 mm^2 olarak, myelinli akson sayıları ise sırası ile 13.523, 2.034 ve 4.151 adet olarak tespit edildi. Atlarda III, IV ve VI. çift kranial sinirlerin transversal kesit alanlarının ve myelinli akson sayılarının belirlendiği çalışma sonuçlarının bu alandaki bilgi birikimine katkı sağlayacağı ve gelecekte yapılacak çalışmalara ışık tutacağı sonucuna varıldı.

Anahtar sözcükler: *At, Cavalieri metodu, Kranial sinirler, Myelinli akson sayısı, Parçalama*

The Area of Cross Sections and the Number of Myelinated Axons of Cranial Nerves III, IV and VI of Adult Horse

Summary

It was aimed to determine the number of myelinated axons and the area of cross sections of oculomotor, trochlear and abducens nerves providing motor innervation of extrinsic muscles of the eye. The study included 3 male and 3 female adult horses. Tissue samples were taken from the part of nerve being in subarachnoid space. Paraffin blocks of tissues were prepared and cut with a rotary microtome transversely at a thickness of 4 µm and sections were stained with Masson's trichrome. The area of cross sections was determined with Cavalieri's method and the number of myelinated axons was calculated by fractionator technique. There were no statistically significance of cross sectional areas and the number of myelinated axons of the right and the left sides, thus the data belonging to both sides were accepted as a single data (median). The areas of cross sections of oculomotor, trochlear and abducens nerves were calculated to be 2.647 mm^2, 0.511 mm^2 and 1.092 mm^2 and the number of myelinated axons 13.523, 2.034 and 4.151 respectively. The results of the study performed to determine the area of cross sections and the number of myelinated axons of III., IV. and VI. cranial nerves of the horse will contribute to the knowledge of this area and shed light on the studies to be conducted in the future.

Keywords: *Horse, Cavalieri's method, Cranial nerves, Number of myelinated axons, Fractionator*

GİRİŞ

Göz kaslarının motor uyarımı somatik efferent sınıfında yer alan nervus oculomotorius, nervus abducens ve nervus trochlearis tarafından sağlanır. Bu sinirler genel fonksiyon, filogenetik ve köken açısından birbirlerine benzer yapıdadırlar. Nervus oculomotorius diğerlerinden farklı olarak visseral efferent (parasempatik) liflere sahiptir [1]. N. oculomotorius somatik efferent lifleri ile gözün pozisyonunda, parasempatik lifler aracılığı ile de gözün akomodasyonunda rol almaktadır [2]. Söz konusu sinirlerde hem myelinli hem de myelinsiz sinir lifleri bulunmaktadır [3].

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Göz kaslarının innervasyonunu sağlayan sinirlerde akson sayıları rat [1], kedi [4], köpek [5] ve koyunda [3] yapılan histomorfometrik araştırmalarla ortaya konmuştur. Ancak yapılan kaynak taramalarında atlarda bu sinirlerin morfometrik özellikleriyle ilgili bir bilgiye ulaşılamamıştır.

Stereolojik metotlar içerisinde yer alan parçalama yöntemi ilk defa Mayhew [6] tarafından sinir dokusunun morfometrisinin değerlendirilmesinde ve sinir liflerinin sayılmasında kullanılmıştır. Bu yöntem gelişen bilgisayar yazılımlarının desteği ile günümüzde çok daha kolay kullanılabilir hale gelmiştir. Bilgisayar destekli parçalama yöntemi sinir liflerinin sayısı, akson ve myelin kalınlıkları ve sinir liflerinin sinir kesiti içerisindeki dağılımının tespitinde etkili ve pratik bir yöntem olarak kullanılmaktadır [7].

Bu araştırma ile ergin atlarda göz kaslarının sinirsel uyarımına katılan n. oculomotorius, n. trochlearis ve n. abducens'in transversal kesit alanları ve myelinli akson sayılarının stereolojik yöntemlerle belirlenmesi amaçlandı.

MATERYAL ve METOT

Materyal

Araştırma, Selçuk Üniversitesi Veteriner Fakültesi Binicilik Tesisinden çeşitli ortopedik rahatsızlıkları gerekçesiyle ötenazisine karar verilen ve bu işlemi gerçekleştirmek ve kadavra hazırlanmak üzere Anatomi Anabilim Dalına tahsis edilen 6 attan (1 adet Pony 15 yaşlı 230 kg dişi, 2 adet arap 13 ve 5 yaşlı 300-340 kg dişi, 1 adet Belçika 15 yaşlı 480 kg erkek, 2 adet İngiliz 10 ve 12 yaşlı 420-450 kg erkek) temin edilen materyaller üzerinde gerçekleştirildi. Çalışma, Selçuk Üniversitesi Veteriner Fakültesi Hayvan Deneyleri Yerel Etik Kurulu tarafından onaylandı (SÜVFEK, 2011/100).

Metot

- Dokuların Tespiti ve Hazırlanması

Hayvanlar %10'luk kloral hidrat (80 mg/kg, IV) [8] ile genel anesteziye alındıktan sonra a. carotis communis'e yerleştirilen kanül vasıtası ile kanı boşaltılarak ötenazi gerçekleştirildi. Aynı kanül vasıtası ile fizyolojik tuzlu su verilerek damarların temizlenmesi sağlandı. Bu işlemi takiben her bir hayvan için hazırlanan %10'luk yaklaşık 40 L formalin solüsyonu usulüne uygun bir şekilde a. carotis communis' teki kanül aracılığı ile hayvanlara verilerek oda ısısında tespiti sağlandı. Tespit işlemini izleyen yedinci günde hayvanların başları atlanto-occipital eklemden ayrıldı ve 15 gün süresince %10 formalin solusyonu içeren tanklarda bekletildi. Bu süre sonunda beyinler dura mater korunarak cranium'dan uzaklaştırıldı. Bu işlemi takiben ventral yaklaşımla dura mater açıldı ve III, IV ve VI. çift kranial sinirler dura mater'e girdikleri bölgeden ensize edilerek sinirlerin cavum subarachnoideale'de seyreden bölümlerinin beyin üzerinde kalması sağlandı. Daha sonra sağ ve sol sinir çiftleri beyni terk ettikleri yerden kesilerek alındı. Söz konusu sinir çiftlerinin dura mater'e girmeden önceki 10 mm'lik bölümleri alınarak histolojik takipleri yapıldı. Bu işlemi takiben parafin blokları hazırlanan dokulardan, sinirin uzun eksenine dik olacak şekilde rotary mikrotom (Leica RM2125 RT) kullanılarak 4 µm kalınlığında kesitler alındı ve kesitler Crossman'ın modifiye üçlü boyası (triple) ve May Grunwald Giemsa ile boyandı.

- Sinir Kesitlerinin Alanı

Sinir kesitlerinin alanları Stereo Investigator (ver. 10, MicroBrightField Inc., VT, USA) yazılımının cavalieri estimator komutu kullanılarak tespit edildi. İki nokta arası 100 µm olan noktalardan oluşan noktalı alan ölçüm cetveli rastgele olarak 20x objektif altında sinir kesitleri üzerine atıldı *(Şekil 1)*. Sinirlerin transversal kesit yüzey alanları A= a/p x ∑p formülü ile hesaplandı. Bu formülde A ilgili sinirin transversal kesit alanını, a/p (10.000 µm^2) tarafsız sayım çerçevesinde yer alan bir noktanın alanını, ∑p ise kesit yüzeyine düşen toplam nokta sayısını ifade etmektedir [9].

- Parçalama İçin Sistematik Rastgele Örnekleme

Her bir kranial sinirde yer alan akson sayılarını belirlemek için parçalama sondası ve tarafsız sayım çerçevesi kullanıldı [10]. Yapılan ön çalışma ile akson sayımında kullanılacak ızgara büyüklüğü ve tarafsız sayım çerçevesinin alanı güvenilir hata katsayısı (CE) değerine uygun olarak belirlendi. Örneklenen alanlardan tarafsız olarak akson sayıları hesaplandı. Hata katsayısı değeri CE= 1/√Q x (1-sf) formülü ile hesaplandı ve CE değerinin %5'in altında olmasına dikkat edildi. Bu formülde Q sayılan taneciği, sf ise örnekleme oranını ifade etmektedir [7]. Izgara alanı uygun CE değerine ulaşmak için her bir sinir analizinde ayrı olarak belirlendi. Tarafsız sayım çerçevesinin alanı ise tüm sinirler için 20 µm x 20 µm = 400 µm^2 olarak belirlendi. Kranial sinirlerde örneklenen alanlarda 300-500 arasında akson 100 x apo immersiyon objektif (NA 1.4) altında sayıldı [8,11] *(Şekil 2)*.

İstatistiksel Analiz

Çalışma sonucunda sağ ve sol göze ait sinir kesit alanlarının karşılaştırılmasında Two Sample T test, myelinli akson sayılarının karşılaştırılmasında Mann-Whitney U test kullanıldı (SPSS inc. ver. 17). Alan sonuçları mean±SE, myelinli akson sayıları ise median şeklinde tablolarda sunuldu.

Terminolojik ifadelerin yazımında Nomina Anatomica Veterinaria kullanıldı [12].

BULGULAR

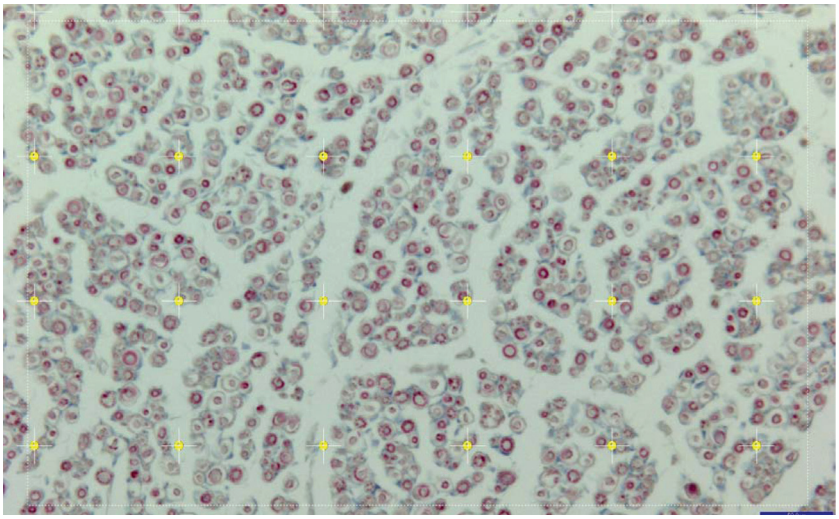
Morfometri

Araştırma sonucunda n. oculomotorius, n. trochlearis ve n. abducens'in kesit yüzey alanları, myelinli akson sayıları (median), hata katsayısı değerleri (CE) ve CV değerleri *Tablo 1*'de verildi. Sinir kesit yüzey alanları ve myelinli akson sayıları yönünden sağ ve sol tarafa ait sinirler arasında istatistiki

fark gözlenmedi (P>0.05). Sinir kesitlerinin alanları n. oculomotorius, n. trochlearis ve n. abducens için sırası ile 2.647 mm^2, 0.511 mm^2 ve 1.092 mm^2 olarak tespit edildi *(Tablo 1)*. Ergin atlarda n. oculomotorius'un 13.523, n. trochlearis' in 2.034 ve n. abducens'in 4.151 myelinli akson içerdiği belirlendi *(Tablo 1, 2)*.

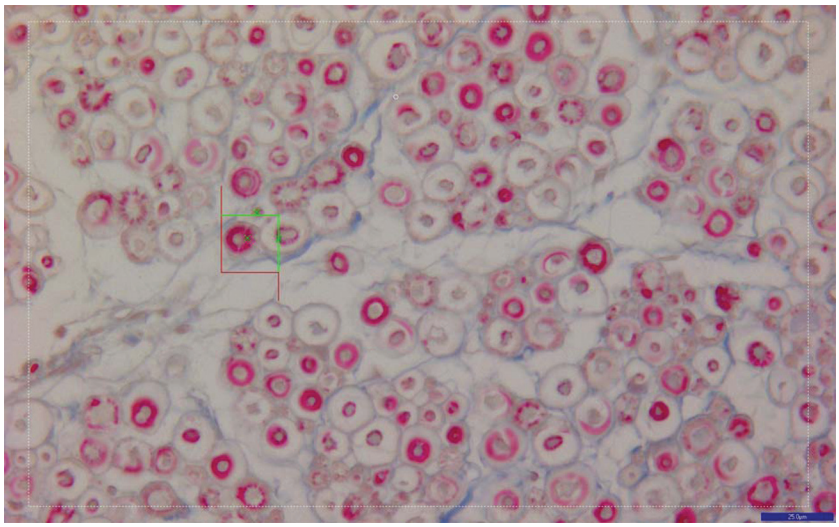
Histoloji

Enine kesitlerde her üç sinirin morfolojisinin oval olduğu gözlendi *(Şekil 3)*. N. oculomotorius'ta çok kalın olmayan epineurium'dan ayrılan bağ doku kollarının sinirin merkezine kadar devam ederek buradaki arter ve venin adventisyasına karıştığı gözlendi. Her üç sinirde de, her bir akson ve çevresindeki myelin nörokeratin rahatlıkla ayırt edilebildi. Yapılan incelemelerde üç sinir arasında sadece n. oculomotorius'un merkezinde damar bulunduğu gözlendi. N. trochlearis'te epineurium'un oldukça ince olduğu damarların ise bu sinirin daha çok çevresine yerleştiği göz-



Şekil 1. Noktalı alan ölçüm cetveli yardımı ile sinir kesit alanının hesaplanması, iki nokta arası 100 µm, Triple, bar = 50 µm

Fig 1. Calculation of cross sectional area of nerve with the help of point counting grid, distance between two points is 100 µm, Triple, bar = 50 µm



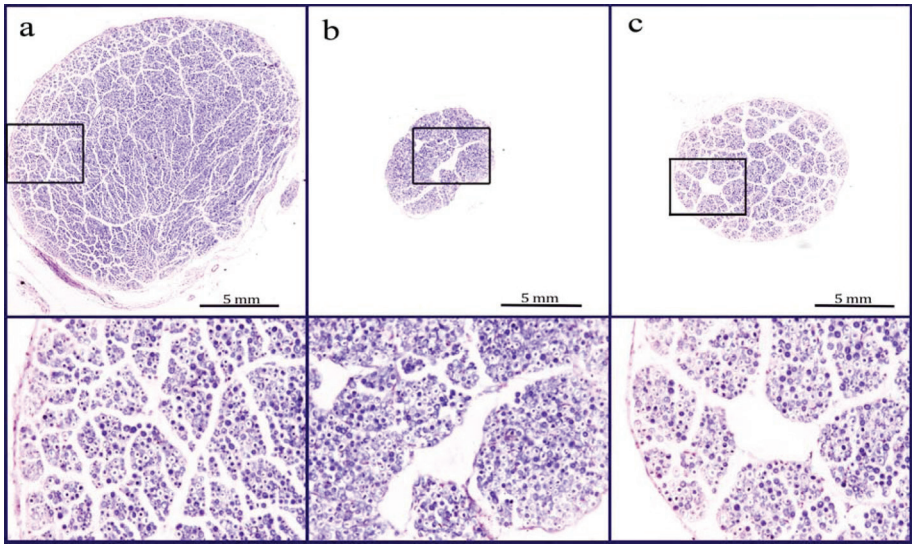
Şekil 2. Tarafsız sayım çerçevesi kullanılarak sinir kesiti üzerinde yer alan myelinli akson sayısının parçalama sondası ile hesaplanması. Triple, bar = 25 µm

Fig 2. Calculation of the number of myelinated axons with fractionator probe using unbiased counting frame on cross section of nerve. Triple, bar = 25 µm

Tablo 1. *Sağ ve sol III., IV. ve VI. çift kranial sinirlerde sinir kesit yüzey alanları (mean±SE) ve myelinli akson sayıları (median)*
Table 1. *Cross sectional areas (mean±SE) and the number of myelinated axons (median) of cranial nerves III. IV. and VI. of right and left sides*

Parametre	Yön	n	N. oculomotorius	N. trochlearis	N. abducens	CV	CE
Sinir kesit alanı (mm^2)	Sağ	6	2.633±0.27	0.467±0.04	1.127±0.13	0.25	
	Sol	6	2.662±0.12	0.555±0.05	1.058±0.12	0.21	
	Ortalama*		2.647±0.21	0.551±0.05	1.093±0.12	0.23	
Akson sayısı (median)	Sağ	6	13.593	1.994	4.091	0.15	0.04
	Sol	6	13.453	2.073	4.211	0.15	0.04
	Ortalama*		13.523	2.034	4.151	0.15	0.04

**Sağ ve sol göze ait morfometrik değerlerin ortalamasıdır.*



Şekil 3. Göz kaslarını innerve eden kranial sinirlerin virtual slice kullanılarak elde edilen genel görünümü, **a-** n. oculomotorius, **b-** n. trochlearis, **c-** n. abducens, May Grunwald Giemsa

Fig 3. General view of cranial nerves innervating eye muscles obtained using virtual slice, **a-** oculomotor nerve, **b-** trochlear nerve, **c-** abducens nerve, May Grunwald Giemsa

Tablo 2. *III, IV ve VI. çift kranial sinirlerde myelinli akson sayıları*
Table 2. *The number of myelinated axons of cranial nerves III, IV and VI*

Parametre	Tür	Özellik	N. oculomotorius (Mean±SD)	N. trochlearis (Mean±SD)	N. abducens (Mean±SD)	Kaynaklar
Akson Sayıları	Rat	Myelinli	13.88±43	282±10	329±10	[1]
	Köpek	Myelinli	8.543±1231	1.509±223	2.473±211	[5]
	Koyun	Myelinli	13.684±294	3.377±52	2.908±132	[3]
	Kedi	Myelinli	-	965	1.901	[4]
	At	Myelinli (median)	1.3523	2.034	4.151	Mevcut araştırma sonucu

Tablo 3. *Göz kaslarının innervasyonuna katılan toplam myelinli sinir akson sayısına III., IV. ve VI. çift sinirlerin katkı oranları (%)*
Table 3. *Contribution rates of cranial nerves III. IV. and VI. to the number of myelinated axons involving innervation of eye muscles (%)*

Tür	N. oculomotorius (%)	N. trochlearis (%)	N. abducens (%)	Kaynaklar
Rat	69	14	17	[1]
Köpek	68	12	20	[5]
Koyun	68	17	15	[3]
At	69	10	21	Mevcut araştırma sonucu

lemlendi. Perineurium ile çevrili sinir demetlerinin belirgin olduğu gözlenirken, bu demetlerin içindeki akson çaplarının homojen olmadığı belirlendi. N. abducens'te sinir demetlerinin çaplarının diğer iki sinire göre daha geniş olduğu ve epineurium'un burada daha kalın olduğu tespit edildi. Yapılan histolojik incelemede bu üç sinirin de bağdoku, akson ve fasikül yerleşimi yönünden farklı özellikler taşıdıkları tespit edildi.

TARTIŞMA ve SONUÇ

Biyolojik dokularda tespit sonrası meydana gelen doku hacmindeki büzüşme bilinen bir gerçektir. Parafin bloklarda dokuya bağlı olarak %40-50 oranında büzüşme görülebilmektedir [13]. Biyolojik yapılardaki tanecik sayısı histolojik işlemler sırasında şekillenen hacim değişikliklerinden etkilenmemektedir [14]. Mevcut çalışmada, 2 boyutlu kesitlerde alan örneklemesi yapılarak tanecik sayısının hesaplandığı metot olan parçalama metodu kullanıldı.

Sinir yenilenmesi ve hasarı ile ilgili çalışmalarda sinir dokusu genellikle osmium tetraoksit ile tespit edilmekte ve resin ya da araldit uygulaması yapılmaktadır [15,16]. Parafin'e gömme metodu ile resin'e gömme metodunun sonuçları karşılaştırıldığında myelinli akson sayısının tespitinde kullanılan iki metot arasında istatistiki bir farkın bulunmadığı, ancak sinirlerde yer alan akson alanı, myelin alanı,

akson ve myelin çapı gibi morfometrik değerler arasında fark görüldüğü bildirilmektedir [16]. Mevcut çalışmada ilgili sinirlerin sadece içerdikleri myelinli akson sayısı tespit edilmiş, metot kısıtlaması nedeni ile diğer histomorfometrik parametreler araştırılmamıştır.

Yapılan literatür taramaları sonucunda III, IV ve VI. çift kranial sinirlerde myelinli akson sayıları ile araştırmamızda elde edilen sonuçlar *Tablo 2*'de verildi. Araştırmada n. oculomotorius'da tespit edilen myelinli akson sayısı ile koyunda bildirilen sayı arasında yakın bir benzerlik olduğu gözlenirken diğer sinirlerde böyle bir benzerlik tespit edilmedi. Rat, köpek, koyun ve kedide adı geçen sinirler üzerinde yapılan araştırmalarda benzer metotlar kullanılarak myelinli ve myelinsiz akson sayıları ayrı ayrı tespit edilmiştir.

Rat, köpek, koyun ve atta göz kaslarının innervasyonuna katılan toplam myelinli sinir akson sayılarına adı geçen sinirlerin katkı oranları ayrı ayrı değerlendirildiğinde n. oculomotorius'un katkı oranının bütün hayvanlarda hemen hemen aynı oranda ve en yüksek olduğu belirlendi *(Tablo 3)*. Koyun dışındaki hayvanlarda n. abducens ikinci derecede katkıya sahipken n. trochlearis'in en az katkıya sahip sinir olduğu gözlendi. Rat, köpek ve atta IV. ve VI. çift sinirlerin katkı oranlarının ise birbirlerine yakın olduğu tespit edildi.

Çalışmada kullanılan yöntemin myelinli akson sayılarının tespitinde oldukça başarılı olduğu ancak myelinsiz akson sayısı ile myelin ve akson morfometrisinin belirlenmesinde yetersiz kaldığı ve konuyla ilgili yeni çalışmalara ihtiyaç duyulduğu düşünülmektedir.

Atlarda III., IV. ve VI. çift kranial sinirlerin transversal kesit alanlarının ve myelinli akson sayılarının belirlendiği çalışma sonuçlarının bu alandaki bilgi birikimine katkı sağlayacağı ve gelecekte yapılacak çalışmalara ışık tutacağı sonucuna varıldı.

Teşekkürler

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RESEARCH ARTICLE

Growth Hormone Gene Polymorphism in Four Cattle Breeds in Turkey

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Summary

This study was conducted to determine DNA-polymorphism of a *Alu*I RFLP at bovine growth hormone (bGH) gene in Zavot (n=48), East Anatolian Red (n=40), Simmental (n=94) and Brown Swiss (n=64) cattle breeds. A total of 246 cattle were genotyped for the bGH-*Alu*I polymorphism by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). In the study, two alleles (L and V) and three genotypes (LL, VV and LV) were revealed after than digestion of amplification product with *Alu*I restriction enzyme. Allelic frequencies for EAR, SIM, BS and Zavot breeds were determined as 0.775, 0.734, 0.781 and 0.760 respectively for L allele; 0.225, 0.266, 0.219 and 0.240 respectively for V allele. Otherwise, genotypic frequencies were 0.65, 0.57, 0.63 and 0.63 for LL, 0.10, 0.11, 0.06 and 0.10 for VV, and 0.25, 0.32, 0.31 and 0.27 for LV respectively. A significant deviation from Hardy-Weinberg equilibrium was not observed in the investigated breeds. As a result, this study provided information on the polymorphism of bGH in four cattle breeds. Additionally, this study reported the existence of a genetic polymorphism at bGH gene in Zavot cattle breed for the first time.

Keywords: *Growth Hormone, Cattle, Zavot, Polymorphism*

Türkiye'deki Dört Sığır Irkında Büyüme Hormonu Gen Polimorfizmi

Özet

Bu çalışma, Zavot (n=48), Doğu Anadolu Kırmızısı (n=40), Simental (n=94) ve İsviçre Esmeri (n=64) sığır ırklarında büyüme hormonu (bGH) geninin *Alu*I RFLP polimorfizminin incelenmesi amacıyla yapılmıştır. Büyüme hormonu-*Alu*I polimorfizmi için toplam 246 baş sığır polimeraz zincir raksiyonu-restriksiyon parça uzunluk polimorfizmi (PZR-RFLP) ile genotiplendirilmiştir. Bu çalışmada, *Alu*I enzim kesimi sonucu iki allel (L ve V) ve üç genotip (LL, VV ve LV) belirlenmiştir. DAK, SIM, İsviçre Esmeri ve Zavot sığır ırkları için allel frekansları; L alleli için sırasıyla 0.775, 0.734, 0.781 ve 0.760; V alleli için sırasıyla 0.225, 0.266, 0.219 ve 0.240 bulunmuştur. Diğer taraftan, genotipik frekanslar LL genotipi için sırasıyla 0.65, 0.57, 0.63 ve 0.63; VV genotipi için 0.10, 0.11, 0.06 ve 0.10; LV genotipi için ise; 0.25, 0.32, 0.31 ve 0.27 bulunmuştur. Çalışılan ırklarda Hardy-Weinberg dengesinden sapma görülmemiştir. Sonuç olarak, bu çalışmada dört sığır ırkında bGH gen polimorfizmi hakkında bilgi verilmiştir. Ayrıca, bu çalışma ile Zavot sığırında bGH gen polimorfizmi varlığı ilk defa bildirilmiştir.

Anahtar sözcükler: *Büyüme hormonu, Sığır, Zavot, Polimorfizm*

INTRODUCTION

The bovine growth hormone (bGH) gene has been intensively studied in livestock because of its effects on growth, body composition, metabolism regulation, lactation and mammary gland development [1-3]. Therefore, there is an interest in the growth hormone (GH) gene polymorphism to improve production traits in farm animals and GH gene has been suggested as a putative candidate for variability. bGH is a single copy gene spanning 1800bp on the chromosome region 19q26-qter and consists of five exons [4].

Growth hormone gene may an important candidate genetic marker for growth and milk yield traits in livestock [5]. Polymorphisms were detected in the fifth exon [6], the third intron [7,8] and the 3 'UTR region [9] of bGH gene. One of the most investigated is the *Alu*I restriction site situated in the fifth exon region. The *Alu*I restriction site polymorphisms of GH gene have been previously reported in dairy cattle [10,11], Bavarian Simmental [12], Indian native cattle breeds [13], Hereford and composite cattle breeds [14]. There are several studies on

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the relationship between bGH genotypes and milk yield traits in cattle [15,16], sheep [17] and goat [18]. The results of these studies have demonstrated that the *Alu*I polymorphism of bGH gene can be used as a potential marker for milk yield, protein and fat content in milk [19]. In addition, there are some other studies on the association between GH genotypes and growth traits in goat [20,21], in sheep [22] and in cattle [23].

Three different genotypes (LL, VV and LV) for *Alu*I restriction have been reported [24]. bGH was shown to be polymorphic in many cattle breeds, being that the distribution of GH variants (LL, LV and VV) and their frequencies differ among each breed [6,9,16,18]. Several studies have reported an association between allelic variants of this gene with high fat and high protein contents in milk [25]. In another study, the V allele was reported favorable for milk yield, fat yield and protein yield [26]. Yardibi et al.[27] reported an association between bGH-*Alu*I polymorphism and milk fat percentage in East Anatolian Red (EAR) and South Anatolian Red (SAR) cow two of the native cattle breeds of Turkey. Akyüz et al.[28] have reported that the highest frequency of the L allele has been found in the SAR breed (0.938), which has the highest milk yield in Turkish native cattle breeds, and the high L allele frequency has been found in EAR breed (0.898). Dario et al.[29] reported that daily milk yield in the LL genotype was higher than in the LV genotype.

Various cattle breeds are raised in different regions of Turkey. Nearly 50% of Turkey's cattle population is consisted of European originated cattle (Holstein, Brown Swiss, Simmental, Jersey) and their crosses [30]. Simmental has a special place among them with more meat producing capacity besides the milk yield on Anatolian highlands [30,31]. Brown Swiss have higher fattening performance [31]. East Anatolian Red (EAR) breed raised in the eastern Anatolian region. An important part of the meat requirement is provided from EAR breed in Turkey. EAR are well suited to the harsh climate and poor pasture [31]. Zavot cattle have been in Kars and Ardahan provinces in Northeast Anatolian region for more than 150 years. It is generally accepted that the Simmental and Brown Swiss genotypes have played an important role in the construction of the Zavot breed [32,33]. Zavot breed raised for milk and meat production [31].

The aim of the present study was to investigate of *Alu*I polymorphism at exon 5 of the bGH gene in Zavot, EAR, (Simmental) SIM, (Brown Swiss) BS cattle breeds by PCR-RFLP.

MATERIAL and METHODS

Samples and DNA Isolation

A total of 246 blood samples were collected from Zavot (n=48, Ardahan), SIM (n=94, Kayseri-Nevşehir), BS (n=64, Kayseri) and EAR (n=40, Erzurum and Kars) cattle breeds. The blood samples were placed into tubes containing EDTA for DNA isolation. Genomic DNA was isolated using the phenol-chloroform-isoamlyalcohol (25:24:1) method [34]. The quality of DNA was checked on 0.8% agarose gels and stained with ethidium bromide.

DNA Amplification and Genotyping

The genotyping for bGH-*Alu*I polymorphism was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) according to the method proposed by Chrenek et al.[35]. PCR products were amplified using primers (gene accession number EF592534.1): forward 5'-GCT GCT CCT GAG GGC CCT TCG-3' and reverse 5'-GCG GCG GCA CTT CAT GAC CCT-3'. PCR for the bGH gene was performed in a 25 µl reaction mixture, containing 1.5mM $MgCl_2$, 200 µM of each dNTPs, 5 pmol of each primer, 1X PCR buffer, 1U Taq polymerase and 100 ng of genomic DNA template. Thermal cycling conditions included: an initial denaturation step at 95°C for 4 min followed by 35 cycles of 94°C, 60°C, 72°C each for 40s and a final extension step at 72°C for 5 min. The PCR products were digested with 10 U of *Alu*I restriction endonuclease (Fermentas) at 37°C for at least 3.5 h. The PCR products and restriction fragments were electrophoresed on 2% and 3% agarose gels respectively and stained with ethidium bromide.

Statistical Analysis

Direct counting was used to estimate genotype and allele frequencies of bGH gene *Alu*I genetic variants. Chi-square statistic (χ^2) was used to check whether the populations were Hardy-Weinberg equilibrium. All statistical analyses were performed using PopGene32 software [36].

RESULT

By using PCR a 223 bp fragment was successfully amplified *(Fig. 1)* and this fragment was digested with *Alu*I restriction enzymes to detect the presence of L or V variants. PCR-RFLP with the *Alu*I enzyme revealed the polymorphic site. As a result of digesting the amplification product with, two alleles, L and V, were observed. Restriction digestion of 223 bp PCR products with *Alu*I enzymes revealed three genotypes of VV (223 bp), LL (171 and 52 bp) and LV (223, 171 and 52 bp). An example of gel photograph showing the polymorphisms of the amplified product cut by *Alu*I restriction enzyme were shown in *Fig. 2*. The allelic and genotypic frequencies of the bGH gene polymorphism for the EAR, SIM, BS and Zavot cattle were given in *Table 1*. The results of Chi-square statistic reflected that breeds were in Hardy-Weinberg equilibrium.

DISCUSSION

The bovine growth hormone gene can be used as potential candidate genetic marker for growth, body composition, metabolism regulation, lactation and mammary gland

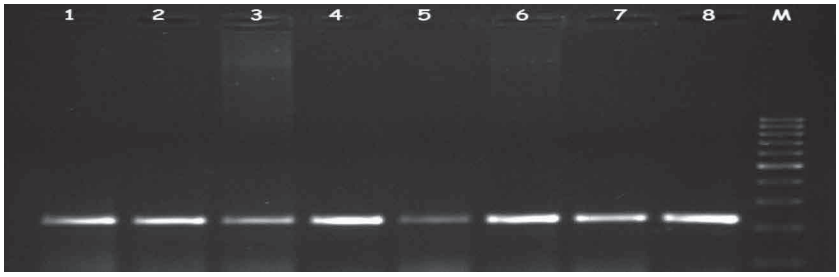

Fig 1. PCR amplifications of bGH gene (223bp, lanes 1-8). Lane M, molecular size marker (100 bp DNA ladder)

Şekil 1. bGH geninin PZR ürünleri (223bp, hat 1-2). Hat M, moleküler büyüklük belirteci (100 bç DNA ladder)

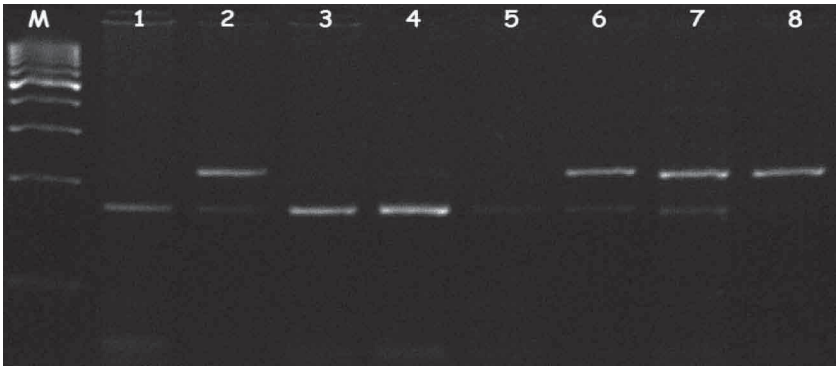

Fig 2. Photograph of *Alu*I enzyme digestion products of bovine GH gene on agarose gel. Lane 8; VV (223bp), Lane 2,6,7; LV (223bp/171bp/52bp, Lane 1,3,4,5; LL (171bp/52bp) genotypes. Lane M, molecular size marker (100 bp DNA ladder)

Şekil 2. Sığır GH geninin *Alu*I enzim kesim ürünlerinin agaroz jeldeki fotografı. Hat 8; VV (223bç), Hat 2,6,7; LV (223bç/171bç/52bç), Hat 1,3,4,5; LL (171bç/52bç) genotipleri. Hat M, moleküler büyüklük belirteci (100 bç DNA merdiveni)

Table 1.*Allele and genotype frequencies of bGH gene for AluI site in the EAR, SIM, BS and Zavot cattle breeds*
Tablo 1. *Zavot, DAK, Simental ve İsviçre Esmeri sığır ırklarında bGH geninin AluI bölgesinin allel ve genotip frekansları*

Breed	n	Genotype						Allele Frequency		χ2	χ2 P-Value	G²	G² P-Value
		LL		VV		LV		L	V				
		Obs (Exp)	F	Obs (Exp)	F	Obs (Exp)	F						
EAR	40	26 (23.937)	0.65	4 (1.937)	0.10	10 (14.126)	0.25	0.775	0.225	3.581	0.058^{NS}	3.192	0.074^{NS}
ZAV	48	30 (27.663)	0.63	5 (2.663)	0.10	13 (17.674)	0.27	0.760	0.240	3.484	0.062^{NS}	3.180	0.075^{NS}
SIM	94	54 (50.551)	0.57	10 (6.551)	0.11	30 (36.898)	0.32	0.734	0.266	3.341	0.068^{NS}	3.170	0.075^{NS}
BS	64	40 (38.977)	0.63	4 (2.976)	0.06	20 (22.047)	0.31	0.781	0.219	0.569	0.451^{NS}	0.540	0.462^{NS}

F: *Frequency;* ***NS:*** *Non significant*

development, milk yield traits in livestock. Therefore, studies were conducted genetic polymorphism of the bGH in different cattle breeds [19,28,29]. We investigated this genetic polymorphism of the bGH in EAR, SIM, BS and Zavot cattle breeds. Especially, this study showed the existence of a genetic polymorphism at bGH gene in Zavot breed for the first time. Digestion of amplification product with *Alu*I restriction enzyme for bGH gene revealed two alleles namely, L and V and three genotypes (LL, VV and LV).

The findings of the present study on allele and genotype frequencies were similar to those reported in the literature [37,38]. Jakaria et al.[39] reported that the L allele frequency of GH *Alu*I loci was higher for cattle with European origin [40]. bGH polymorphism has been investigated with 164 Jersey cows by Dario et al.[29]. They reported that the frequency of LL (0.22) genotype was found to be lower than LV (0.61) and VV (0.17) genotypes and then the genotypic frequencies of LL and VV genotypes were very close. Additionally Dario et al.[29] showed that the frequency of V (0.48) allele at bGH locus was lower compared to the frequency of L (0.52) allele. This was consistent with the result of the breeds reared in Turkey. A higher frequency of L allele (0.898 and 0.830) bGH gene was found for EAR and BS, respectively [28]. This finding on allele frequency was similar to that reported for EAR and BS (0.775 and 0.781) in this study. Ozdemir [41] has shown that L allele is predominant and its frequency is ranging from 0.893 to 0.976 in Turkish Grey Breeds and EAR, respectively. Similarly, Ozkan et al.[42] reported that L allele was predominant with frequencies 0.842 in EAR and 0.867 in Turkish Grey. However, Yardibi et al.[27] found that bGH V gene was predominant as 0.570 in EAR. Previously, polymorphism studies about bGH locus for EAR and BS breeds [28] showed higher frequencies of LL genotype in comparison with the present study. In a similar study, a higher freguency of L allele (0.976 and 0.905) bGH gene was reported for EAR and BS, respectively [41].

The revealing polymorphism of economically important genes is necessary to explain to the genetic structure of animal population and configuration of selection program. Therefore more comprehensive studies using further loci associated with economically important yields and including pedigree records are needed. Further investigation

is necessary to perform statistical analysis aiming the existence of association between bGH-*Alu*I genotypes and growth, milk traits in the Turkish breed of cattle.

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RESEARCH ARTICLE

Effects of Methionine and Lysine on Metabolic Profile in Dairy Cattle During Periparturient Period [1]

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Summary

Periparturient period is highly important in terms of health. For feeding in this stage substances providing high energy should be selected and aimed at reducing the mobilization of reserve fats. For this purpose; various feed additives such as glycogene, liquid fats, glycerol, propilene glycole, propionats, monensin, *methionine, lysine,* colin, niasin, biotin, sodium borate, conjugated linoleic acid ve xylitol can be used. Several studies showed that methionine and lysine are the two most important amino acids. In this study, effects of methionine and lysine on metabolic profile was comparatively investigated in similar age, feeding and productive traits of dairy cattle. The applications of methionine caused significant changes at the concentrations of cholesterol, triglyceride, blood urea nitrogen, high-density lipoprotein, low-density lipoprotein, very low-density lipoprotein, glucose, beta-hydroxybutyric acid and non-esterified fatty acid; applications of lysine at serum levels of total bilirubin, direct bilirubin, total protein, albumin, glucose, triglyceride, blood urea nitrogen, aspartate aminotransferase, alanine aminotransferase, low-density lipoprotein, very low-density lipoprotein and non-esterified fatty acid; and applications of methionine and lysine together at the levels of blood urea nitrogen, high-density lipoprotein, low-density lipoprotein, very low-density lipoprotein, gamma-glutamyl transferase, alanine aminotransferase, aspartate aminotransferase, glucose, total bilirubin, direct bilirubin, cholesterol, triglyceride and non-esterified fatty acid. Addition of methionine and lysine to the ration in this study had partial effects on serum values of animals however findings showed that the additive materials are not necessary for dairy cattle fed by balanced and adequate rations.

Keywords: *Negative energy balance, Transition period, Milk yield, Fatty liver, Cow*

Periparturient Dönem Sütçü Sığırlarda Metionin ve Lizinin Metabolik Profil Üzerine Etkileri

Özet

Periparturient dönem sağlık açısından son derece önemlidir. Bu dönem beslemede yüksek enerji sağlayan besin maddeleri tercih edilmeli ve rezerv yağların mobilizasyonunun azaltılması amaçlanmalıdır. Bu amaçla; glikojen, likid yağlar, gliserol, propilen glikol, propionatlar, monensin, *metionin, lizin,* kolin, niasin, biotin, sodyum borat, konjuke linoleik asit ve ksilitol gibi çeşitli yem katkı maddeleri kullanılabilir. Birçok araştırma sonucu metionin ve lizinin bu amaçla kullanılabilecek en önemli iki aminoasit olduğunu göstermiştir. Sunulan çalışmada, metionin ve lizinin metabolik profil üzerine etkileri benzer yaş, besleme ve verim özelliklerine sahip süt sığırları üzerinde karşılaştırmalı olarak araştırıldı. Metionin uygulamaların, kolesterol, trigliserit, kan üre nitrojen, yüksek dansiteli lipoprotein, düşük dansiteli lipoprotein, çok düşük dansiteli lipoprotein, glukoz, beta hidroksi bütirik asit ve esterleşmemiş yağ asidi serum konsantrasyonlarında, lizin uygulamalarının total bilirubin, direkt bilirubin, total protein, albümin, glukoz, trigliserit, kan üre nitrojen, aspartat aminotrasferaz, alanin aminotransferaz, düşük dansiteli lipoprotein, çok düşük dansiteli lipoprotein ve esterleşmemiş yağ asiti serum düzeylerinde ve metionin ve lizin birlikte uygulamasının ise kan üre nitrojen, yüksek dansiteli lipoprotein, düşük dansiteli lipoprotein, çok düşük dansiteli lipoprotein, gamma glutamil tranferaz, aspartat aminotrasferaz, alanin aminotransferaz, glukoz, total bilirubin, direkt bilirubin, kolesterol, trigliserit ve esterleşmemiş yağ asidi serum seviyelerinde önemli değişikliklere neden olduğu tespit edildi. Sunulan araştırmada, rasyona metionin ve lizin ilavesinin hayvanların serum değerleri üzerine kısmi etkisinin olduğu belirlendi. Bununla birlikte, elde edilen çalışma bulguları, dengeli ve yeterli rasyonla beslenen süt sığırlarında katkı maddelerinin gerekli olmadığını gösterdi.

Anahtar sözcükler: *Negatif enerji balansı, Geçiş periyodu, Süt verimi, Karaciğer yağlanması, Sığır*

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INTRODUCTION

Dairy cattle should be provided with main dietary needs to help them endure the periparturient period without complications [1]. For this purpose, different nutritional additives such as glycogen, liquid fats, glycerol, propylene glycol, propionates, monencine, *methionine, lysine*, choline, niacin, biotin, sodium borate, conjugated linoleic acid, or xylitol can be added to rations of the animals [2].

Among the nutritional additives, methionine is the one of the most essential amino acids of dairy cow's metabolism. Methionine is a precursor substance and required for the hepatic synthesis of apolipoproteins needed for production of very low-density lipoprotein (VLDL) [1].

Furthermore, lysine is required in all species for protein synthesis and forms L-carnitine by combining with methionine; therefore, its deficit impairs protein biosynthesis [3]. Addition of just methionine or methionine plus lysine at higher amounts to rations of the cows at transition periods is shown to protect the animals against liver lipid accumulation [4], increase milk production and milk protein rates [5]. Numerous studies point out that methionine and lysine are main confining amino acids [6].

In the present study, we aimed to comparatively study the effect of methionine and lysine on the metabolic features of dairy cattle with similar age, feedings, and production traits during periparturient period.

MATERIAL and METHODS

Animals and Treatments

In the current study, 3-5 years old, healthy, pregnant, and multiparous 24 Holstein dairy cows were used. The dairy cattle in the study groups were selected among the healthy animals with no history of enduring diseases of the periparturient period. All of the cows were clinically and systemically examined prior to their inclusion to the current study. Study groups were formed among the similar cows according to their milk production traits (liter/day), live body weight, and body condition score (3.250±0.112) [7] *(Table 1)*. The animals were obtained from a private enterprise operating in Bolvadin, Afyonkarahisar, Turkey (Korel Agriculture and Animal Husbandry Enterprises). The dairy cattle were divided into four groups of six. The groups were designated as follows: Control group (Group C), methionine group (Group M), lysine group (Group L), and lysine plus methionine group (Group LM). The groups were followed on a daily basis during the periparturient period for 30 days (15 prenatal and postnatal 15 days). The animals in Group M were orally provided with 15 g/day Mepron M85- equivalent of 12.75 g rumen-protected methionine (Degussa AG, Hanau, Germany), 50 g/day AminoShure-L-equivalent of 18 g rumen-protected lysine (Balchem, Animal Nutrition & Health Co.) was given orally to the cows in Group L. Finally, the animals in Group LM were orally provided with 15 g/day Mepron M85 and 50 g/day AminoShure-L. All of the cows included in the present study were fed with the same type of ration throughout the study. The contents of the ration are illustrated in *Table 2*.

Table 1. *Initial values of MY and BW in terms of comparison groups (mean ±SE)*

Tablo 1. *Süt verimi ve canlı ağırlık başlangıç verilerinin gruplarası karşılaştırılması*

Parameter	Grup M	Grup L	Grup LM	p
BW (kg)	697.500±17.953	697.667±17.383	697.00±9.145	NS
MY (L)	31.167±1.222	30.667±0.882	30.500±0.579	NS

BW: *live weight,* ***MY:*** *milk yield,* ***NS:*** *Not significant*

Table 2. *Ingredient and nutrient composition of prepartum and postpartum diets*

Tablo 2. *Prepartum ve postpartum rasyonun içerik ve besin kompozisyonu*

Ingredient	Prepartum	Postpartum
% DM		
Corn silage	30.1	26.0
Wet brewers grains	4.9	5.2
Alfalfa hay	14.0	18.2
Barley hay	18.5	10.3
Wheat Bran	2.9	9.2
Barley	13.4	6.8
Corn	8.4	11.0
Cottonseed meal (32%)	2.2	7.9
Cottonseed meal (48%)	4.3	2.6
Bypass fat [1]	1.0	0.6
Bypass protein [2]	0.0	0.6
Salt	0.14	0.30
Minerals and vitamins [3]	0.16	0.04
Sodium bicarbonate [4]	0.0	0.5
Yeast [5]	0.000	0.004
Marble dust ($CaCO_3$ source)	0.0	0.7
Chemical composition		
DM	60	60
CP (% DM)	12.7	17.0
Rumen Degradable Protein (% DM)	7.7	11.8
Bypass protein (% DM)	5.0	5.2
NEL (cal/g)	1.47	1.58
NDF (% DM)	45.94	39.89
ADF (% DM)	27.28	23.42
Ca (% DM)	0.46	0.72
P (% DM)	0.27	0.42

[1] Megalac (Church & Dwight Co., Inc., Rinceton. NJ); [2] Soy Pass (Borregaard LignoTech); [3] Rovimix 302-FM/20 providing by kg 15.000.000 IU vitamin A, 3.000.000 IU vitamin D_3, 20.000 mg vitamin E, 10.000 mg manganese, 10.000 mg iron, 10.000 mg zinc, 5.000 mg copper, 100 mg cobalt, 100 mg iodine; [4] $NaHCO_3$ (99%, Şişecam Chemicals Group); [5] Yeast (Beta Agriculture, Yüreğir/Adana); ***DM:*** *Dry matter;* ***CP:*** *Crude protein;* ***NEL:*** *Net energy lactation;* ***NDF:*** *Neutral detergent fiber;* ***ADF:*** *Acid detergen fiber*

Blood Collection and Analysis

Biochemical analyses were performed weekly before and after the diet application. The analyses were carried out at five separate times as described: 1st measurement was done at prepartum 2nd week prior to the diet application (0 time), 2nd measurement was obtained at prepartum 1st week prior to the diet application (1 week), 3rd measurement was taken at calving day 4th measurement was acquired at postpartum 1st week, and 5th measurement was done at postpartum 2nd week. The blood samples were collected from the jugular vein, put into dry tubes, centrifuged, and obtained serums were removed and kept at -20°C until use.

Commercially available test kits were used to measure levels of biochemical parameters (Roche Diagnostics, Germany). The kits were used according to the instructions of manufacturers using an autoanalyzer (Roche Cobas C111). The value of VLDL was calculated using the formula triglyceride/5 [8]. Moreover, beta-hydroxybutyric acid (BHBA) (Randox Laboratories Ltd. United Kingdom, Kat. No: RB 1008) and non-esterified fatty acid (NEFA) (Randox Laboratories Ltd. United Kingdom, Kat.No: FA 115) measurements were done spectrophotometrically (Shimadzu UV-1601).

Statistical Method

Statistical analyses of the data were performed using the program SPSS 10.0 (SPSS Inc, for Windows). One way ANOVA test was used to compare the groups. The control of significance within and between the groups was checked with Tukey test.

RESULTS

Methionine caused significant changes (P=0.000) at the concentrations of cholesterol, triglyceride, blood urea nitrogen, low-density lipoprotein, very low-density lipoprotein and glucose. Furthermore, serum beta-hydroxybutyric acid (P=0.004) and non-esterified fatty acid (P=0.005) levels were considerably different in Group M when compared within the groups.

We measured statistically significant at serum levels of total bilirubin (P=0.014), direct bilirubin (P=0.011), total protein (P=0.002), albümin (P=0.000), glucose (P=0.001), triglyceride (P=0.000), blood urea nitrogen (P=0.025), aspartate aminotransferase (p=0.001), alanine aminotransferase (P=0.024), low-density lipoprotein (P=0.011), very low-density lipoprotein (P=0.000) and non-esterified fatty acid (P=0.000) for applications of lysine.

Also, we measured considerable changes at the levels of blood urea nitrogen (P=0.023), high-density lipoprotein (P=0.015), low-density lipoprotein (P=0.001), very low-density lipoprotein (P=0.000), gamma-glutamyl transferase (P=0.038), alanine aminotransferase (P=0.024), aspartate aminotransferase (P=0.020), glucose (P=0.000), total bilirubin (P=0.003), direct bilirubin (P=0.005), cholesterol (P=0.012), triglyceride (P=0.000) and non-esterified fatty acid (P=0.004) in group LM.

The study results as a summary is given in the *Tables 3, 4, 5, 6,* and *7*.

Table 3. *Serum biochemical parameters (TP, ALB and BUN) in dairy cattle during periparturient period (2 weeks in prepartum and 2 weeks in postpartum) according to oral treatments (Group M: methionine (12.75 g/day); group L: lysine (18 g/day); group ML: methionine (12.75 g/day) + lysine (18 g/day); group C: negative controls) (n = 6 in each group). Results are expressed as means ± standard deviations*

Tablo 3. *Serum TP, ALB ve BUN konsantrasyonlarının gruplararası ve grupiçi değişimi*

Parameter	Group	-2 weeks	-1 week	Calving	+1 week	+2 weeks	P
TP (g/L)	C	7.883±0.101	6.917±0.150	7.467±0.199	7.917±0.305	8.102±0.551	0.096
	M	7.917±0.408	7.250±0.589	6.783±0.380	7.617±0.430	7.383±0.355	0.466
	L	6.783±0.119^{b}	7.200±0.121^{ab}	7.133±0.136^{ab}	7.567±0.279^{a}	7.800±0.089^{a}	**0.002**
	LM	6.867±0.201	7.500±0.236	7.133±0.297	7.217±0.368	7.950±0.319	0.124
	P	**0.003**	0.673	0.385	0.580	0.623	
ALB (g/dL)	C	3.450±0.763	3.550±0.342	3.617±0.703	3.400±0.816	3.317±0.167	0.223
	M	3.350±0.106	3.233±0.095	3.117±0.107	3.267±0.128	3.217±0.046	0.469
	L	3.567±0.033^{a}	3.666±0.033^{a}	3.667±0.076^{a}	3.283±0.083^{b}	3.217±0.087^{b}	**0.000**
	LM	3.350±0.067	3.483±0.031	3.550±0.095	3.133±0.108	3.366±0.051	0.073
	P	0.166	0.000	0.001	0.357	0.666	
BUN (mg/dL)	C	10.208±0.633^{b}	11.667±0.791^{ab}	14.700±1.504^{a}	13.633±0.843^{ab}	13.367±0.747^{ab}	**0.022**
	M	7.417±0.853^{c}	8.967±1.060^{bc}	13.517±2.134^{ab}	15.083±1.190^{a}	15.383±0.527^{a}	**0.000**
	L	12.600±0.865^{b}	15.100±1.114^{ab}	17.683±1.482^{a}	15.450±0.471^{ab}	13.767±1.029^{ab}	**0.025**
	LM	11.533±0.853^{a}	12.133±0.592^{ab}	10.633±1.047^{ab}	14.133±1.545^{ab}	15.783±1.347^{a}	**0.023**
	P	**0.001**	**0.001**	**0.039**	0.627	0.240	

BUN: *Blood urea nitrogen,* ***ALB:*** *Albumin,* ***TP:*** *Total protein, Different superscripts a,b in the same row indicate significant differences (P<0.05 or more) according to time during the periparturient period for a given group*

Table 4. *Serum biochemical parameters (TG, CHOL, NEFA, BHBA and GLU) in dairy cattle during periparturient period (2 weeks in prepartum and 2 weeks in postpartum) according to oral treatments (Group M: methionine (12.75 g/day); group L: lysine (18 g/day); group ML: methionine (12.75 g/day) + lysine (18 g/day); group C: negative controls) (n = 6 in each group). Results are expressed as means ± standard deviations*

Tablo 4. *Serum TG, CHOL, NEFA, BHBA ve GLU konsantrasyonlarının gruplararası ve grupiçi değişimi*

Parameter	Group	-2 weeks	-1 week	Calving	+1 week	+2 weeks	p
TG (mg/dL)	C	25.833±2.197^{a}	21.833±4.222^{a}	7.333±1.085^{b}	6.500±0.885^{b}	6.167±1.222^{b}	**0.000**
	M	23.000±3.022^{a}	24.500±0.957^{a}	10.500±2.376^{b}	10.333±1.647^{b}	10.167±1.667^{b}	**0.000**
	L	22.167±2.495^{a}	20.833±1.579^{a}	9.167±1.302^{b}	8.500±1.204^{b}	7.000±0.577^{b}	**0.000**
	LM	22.167±1.600^{a}	20.000±1.732^{a}	12.667±2.641^{b}	7.667±0.803^{b}	7.833±0.792^{b}	**0.000**
	P	0.666	0.604	0.301	0.169	**0.049**	
CHOL (mg/dL)	C	115.500±5.264^{b}	113.500±3.566^{b}	100.000±3.670^{b}	113.500±5.383^{b}	144.500±7.379^{a}	**0.000**
	M	124.667±7.154^{ab}	107.833±4.643^{bc}	85.833±3.953^{c}	114.000±9.402^{abc}	139.333±8.204^{a}	**0.000**
	L	72.333±7.112	79.333±7.356	63.000±6.061	65.833±8.268	77.167±6.710	0.439
	LM	77.500±4.072^{b}	85.000±4.823^{ab}	72.833±5.192^{b}	78.667±7.237^{b}	105.833±9.488^{a}	**0.012**
	P	0.000	0.000	0.000	0.000	0.000	
NEFA (mmol/L)	C	0.214±0.036^{a}	0.291±0.032^{a}	0.506±0.114^{a}	0.503±0.048^{a}	0.470±0.089^{a}	**0.018**
	M	0.303±0.009^{b}	0.316±0.012^{b}	0.633±0.060^{ab}	0.865±0.222^{a}	0.640±0.073^{ab}	**0.005**
	L	0.195±0.029^{b}	0.147±0.020^{b}	0.682±0.172^{a}	0.617±0.082^{a}	0.460±0.042^{ab}	**0.000**
	LM	0.163±0.028^{b}	0.145±0.026^{b}	0.518±0.101^{a}	0.368±0.053^{ab}	0.375±0.099^{ab}	**0.004**
	P	**0.014**	**0.000**	0.668	0.061	0.142	
BHBA (mmol/L)	C	0.109±0.041	0.212±0.072	0.067±0.026	0.119±0.049	0.079±0.023	0.220
	M	0.049±0.006^{b}	0.047±0.007^{b}	0.046±0.004^{b}	0.111±0.022^{a}	0.065±0.013^{ab}	**0.004**
	L	0.390±0.089	0.557±0.145	0.527±0.151	0.302±0.072	0.563±0.107	0.432
	LM	0.099±0.017	0.210±0.060	0.242±0.152	0.573±0.295	0.223±0.116	0.316
	P	**0.000**	**0.004**	**0.018**	0.151	**0.001**	
GLU (mg/dl)	C	52.000±3.864^{ab}	46.667±3.442^{ab}	58.000±7.127^{a}	34.833±8.308^{ab}	30.667±4.702^{b}	**0.013**
	M	50.500±2.487^{a}	46.167±1.939^{a}	59.333±12.068^{a}	25.000±2.757^{a}	21.167±2.725^{b}	**0.000**
	L	48.167±2.257^{ab}	55.833±2.072^{a}	46.167±5.186^{abc}	36.667±3.242^{bc}	31.000±5.228^{c}	**0.001**
	LM	53.333±3.333^{a}	53.500±2.232^{ab}	52.500±3.019^{a}	42.667±4.372^{bc}	24.833±5.890^{c}	**0.000**
	P	0.669	**0.026**	0.610	0.143	0.417	

TG: *Triglycerides,* ***NEFA:*** *Non esterified fatty acids,* ***BHBA:*** *Beta-hydroxy butyric acid,* ***GLU:*** *Glucose,* ***CHOL:*** *Cholesterol, Different superscripts a,b in the same row indicate significant differences (P<0.05 or more) according to time during the periparturient period for a given group*

DISCUSSION

The effect of adding methionine or/and lysine supplements in dairy cow diets on animals' performance and efficiency traits has been extensively studied for last 30 years [6]. Although several studies regarding the contribution of methionine or/and lysine in rations to metabolic profiles of dairy cattle during periparturient period are available [9,10], the number of the studies comparing the effects of methionine and lysine on animals' metabolic profiles are limited.

In the present study, we noticed statistically significant change in total protein (TP), only in Group L (P=0.002). Studies report no marked change in TP concentrations during prepartum and postpartum periods. Nevertheless, TP level is shown to be increased in postpartum period [11,12] but it is reduced to its lowest concentrations at calving day [13]. In the current study, although we determined no marked change in TP concentrations in control and methionine groups, we measured considerable increase in TP levels in Group L at postpartum 1 w and 2 w. At their study Chibisa et al.[14] similarly demonstrate no momentous alterations in TP concentrations during prepartum and postpartum periods. Likewise, TP concentrations in the current study were similar and within the reference range (6.2-8.2 mg/dl) [15] in all groups during prepartum and postpartum periods.

Likewise, within group comparisons albumine (ALB) concentration was noted to be considerably changed in only Group L. This observation may be associated with the initiation of lactation. Krober et al.[16] show that methionine and lysine does not affect serum albumin levels during postpartum period.

Lysine is required for protein synthesis [3]. Nevertheless, serum TP concentrations were not significantly changed in Group M and Group LM when the obtained results were

Table 5. *Serum biochemical parameters (HDL, LDL and VLDL) in dairy cattle during periparturient period (2 weeks in prepartum and 2 weeks in postpartum) according to oral treatments (Group M: methionine (12.75 g/day); group L: lysine (18 g/day); group ML: methionine (12.75 g/day) + lysine (18 g/day); group C: negative controls) (n = 6 in each group). Results are expressed as means ± standard deviations*

Tablo 5. *Serum HDL, LDL ve VLDL konsantrasyonlarının gruplararası ve grupiçi değişimi*

Parameter	Group	-2 weeks	-1 week	Calving	+1 week	+2 weeks	p
HDL (mg/dL)	C	97.333±3.750^{b}	95.283±2.985^{b}	91.283±3.287^{b}	99.183±4.899^{b}	123.066±7.725^{a}	**0.001**
	M	105.467±6.577^{ab}	90.183±2.887^{ab}	75.250±7.627^{b}	97.950±9.711^{ab}	119.850±8.410^{a}	**0.004**
	L	64.483±5.949	68.967±5.877	60.467±4.817	61.867±7.105	70.483±5.883	0.712
	LM	67.717±3.451^{ab}	70.767±3.989^{ab}	66.833±4.456^{b}	71.533±6.654^{ab}	92.150±7.200^{a}	**0.015**
	P	**0.000**	**0.000**	**0.003**	**0.003**	**0.000**	
LDL (mg/dL)	C	20.000±2.557^{a}	16.317±1.445^{ab}	11.167±0.758^{b}	13.248±1.669^{ab}	20.000±2.155^{a}	**0.006**
	M	25.083±1.724^{a}	17.033±0.697^{bc}	11.867±0.776^{c}	15.900±0.917^{c}	22.017±2.267^{ab}	**0.000**
	L	7.150±1.044^{ab}	9.617±1.590^{a}	3.450±0.742^{b}	5.533±1.114^{ab}	6.950±0.955^{ab}	**0.011**
	LM	10.150±0.907^{ab}	13.433±1.263^{a}	4.767±1.190^{b}	6.750±1.080^{b}	13.233±2.510^{a}	**0.001**
	P	NS	NS	NS	NS	NS	
VLDL (mg/dL)	C	5.167±0.439^{a}	4.367±0.844^{ab}	1.466±0.217^{c}	2.300±0.867^{bc}	1.233±0.244^{c}	**0.000**
	M	4.600±0.604^{a}	4.900±0.191^{a}	2.100±0.475^{b}	2.067±0.329^{b}	2.033±0.233^{b}	**0.000**
	L	4.433±0.499^{a}	4.167±0.316^{a}	1.833±0.260^{b}	1.700±0.241^{b}	2.701±0.276^{b}	**0.000**
	LM	4.433±0.320^{a}	4.267±0.204^{a}	2.100±0.422^{b}	1.600±0.179^{b}	2.100±0.634^{b}	**0.000**
	P	NS	NS	NS	NS	NS	

HDL: *high density lipoprotein,* ***LDL:*** *low density lipoprotein,* ***VLDL:*** *very low density lipoprotein, Different superscripts a,b in the same row indicate significant differences (P<0.05 or more) according to time during the periparturient period for a given group, NS: Not significant*

Table 6. *Serum biochemical parameters (TBIL and DBIL) in dairy cattle during periparturient period (2 weeks in prepartum and 2 weeks in postpartum) according to oral treatments (Group M: methionine (12.75 g/day); group L: lysine (18 g/day); group ML: methionine (12.75 g/day) + lysine (18 g/day); group C: negative controls) (n = 6 in each group). Results are expressed as means ± standard deviations*

Tablo 6. *Serum TBIL ve DBIL konsantrasyonlarının gruplararası ve grupiçi değişimi*

Parameter	Group	-2 weeks	-1 week	Calving	+1 week	+2 weeks	p
TBIL (mg/dL)	C	0.135±0.015	0.160±0.023	0.350±0.116	0.275±0.052	0.223±0.049	0.130
	M	0.120±0.007	0.115±0.011	0.240±0.024	0.270±0.121	0.168±0.027	0.232
	L	0.196±0.036^{b}	0.155±0.014^{b}	0.870±0.305^{a}	0.550±0.149^{ab}	0.262±0.025^{b}	**0.014**
	LM	0.173±0.019^{b}	0.143±0.016^{b}	0.597±0.142^{a}	0.360±0.092^{b}	0.233±0.051^{b}	**0.003**
	P	NS	NS	NS	NS	NS	
DBIL (mg/dL)	C	0.025±0.003	0.045±0.011	0.073±0.027	0.065±0.003	0.052±0.019	0.276
	M	0.020±0.008^{a}	0.025±0.006^{a}	0.082±0.017^{a}	0.063±0.028^{a}	0.032±0.006^{a}	**0.045**
	L	0.013±0.005^{b}	0.022±0.007^{b}	0.190±0.067^{a}	0.148±0.005^{ab}	0.083±0.008^{ab}	**0.011**
	LM	0.017±0.002^{b}	0.016±0.007^{b}	0.135±0.042^{a}	0.100±0.028^{ab}	0.055±0.016^{ab}	**0.005**
	P	NS	NS	NS	NS	NS	

TBIL: *total bilirubin,* ***DBIL:*** *direct bilirubin, Different superscripts a,b in the same row indicate significant differences (P<0.05 or more) according to time during the periparturient period for a given group,* ***NS:*** *Not significant*

compared within the groups and according to sampling times. By contrast, even though TP concentration was altered considerably in Group L, the levels of serum TP were still between the reference ranges. This situation may be accounted for the fact that all the animals used in the present study were fed with intuitionally well balanced ration and possessed similar performance and production traits.

Moreover, no marked difference in activity of creatine kinase (CK) within group evaluations may reflect no protein mobilization primarily from the muscle tissue and other body reservoirs in all present groups [17].

We noted considerable variation in BUN levels during periparturient period in all study groups. However, the levels of BUN in control, M, L, and LM groups were within the physiological reference ranges (7.8-25 mg/dl) [15]. While Sevinc et al.[11] report statistically important increase in total urea on calving day, Bauchart [18] indicate that TP and urea decrease on the day before calving. Increases during

Table 7. *Serum biochemical parameters (GGT, ALT, AST, ALP and CK) in dairy cattle during periparturient period (2 weeks in prepartum and 2 weeks in postpartum) according to oral treatments (Group M: methionine (12.75 g/day); group L: lysine (18 g/day); group ML: methionine (12.75 g/day) + lysine (18 g/day); group C: negative controls) (n = 6 in each group). Results are expressed as means ± standard deviations*

Tablo 7. *Serum GGT, ALT, AST, ALP ve CK konsantrasyonlarının gruplararası ve grupiçi değişimi*

Parameter	Group	-2 weeks	-1 week	Calving	+1 week	+2 weeks	P
GGT (U/L)	C	20.833±2.845	22.167±3.103	26.166±4.100	26.000±5.215	26.500±4.341	0.785
	M	20.833±2.242	20.833±1.922	19.333±2.788	20.833±1.249	22.667±1.977	0.863
	L	17.833±1.194	17.833±1.166	20.1667±0.872	26.167±5.717	24.000±3.454	0.244
	LM	13.167±1.222	13.500±1.384	16.000±1.460	18.333±2.060	19.333±1.763	**0.038**
	P	**0.043**	**0.033**	0.119	0.754	0.438	
ALT (U/L)	C	28.000±2.503	23.667±2.348	23.167±1.887	22.333±1.584	24.333±2.261	0.403
	M	27.333±2.917	23.666±3.138	20.000±2.294	18.167±2.762	21.000±2.280	0.172
	L	22.167±1.447^{ab}	24.833±2.151^{a}	19.833±1.922^{ab}	19.333±1.382^{ab}	17.167±.0543^{b}	**0.024**
	LM	21.500±1.544^{ab}	22.667±1.837^{a}	19.000±2.000^{ab}	16.333±0.667^{b}	17.333±0.714^{ab}	**0.024**
	P	NS	NS	NS	NS	NS	
AST (U/L)	C	70.000±4.082^{b}	68.667±5.506^{b}	81.500±3.567^{ab}	94.667±4.773^{a}	89.833±8.360^{ab}	**0.008**
	M	71.500±2.693	66.333±2.789	65.500±3.784	73.500±5.649	69.000±5.894	0.669
	L	66.667±4.997^{b}	71.500±4.588^{b}	65.500±9.172^{b}	113.167±13.197^{a}	90.333±5.643^{ab}	**0.001**
	LM	62.167±3.135^{ab}	56.000±7.928^{b}	71.833±4.728^{ab}	87.667±8.252^{a}	79.667±8.293^{ab}	**0.020**
	P	0.347	0.246	0.197	**0.030**	0.149	
ALP (U/L)	C	54.767±7.433	58.100±5.771	64.750±6.015	49.333±8.602	48.033±6.973	NS
	M	32.650±2.883	40.017±5.496	52.517±13.314	30.717±3.218	35.920±3.382	NS
	L	46.317±6.080	48.500±6.566	58.067±11.289	56.117±10.372	42.350±9.619	NS
	LM	48.400±7.024	54.800±4.972	62.450±7.976	50.367±6.033	45.650±5.659	NS
	P	NS	NS	NS	NS	NS	
CK (U/L)	C	118.000±11.778	116.383±17.781	179.916±36.875	231.467±87.509	127.250±12.709	NS
	M	90.116±14.691	89.733±18.775	311.68±203.292	107.317±6.889	166.920±45.212	NS
	L	118.217±35.546	105.983±14.127	105.150±11.640	186.133±35.992	163.117±42.247	NS
	LM	124.250±15.297	114.983±12.270	168.783±39.050	132.367±21.031	132.267±8.743	NS
	P	NS	NS	NS	NS	NS	

GGT: *gamma-glutamyl transferase,* ***ALT:*** *alanine aminotransferase,* ***AST:*** *aspartate aminotransferase,* ***ALP:*** *alkaline phosphatase,* ***CK:*** *creatine kinase, Different superscripts a,b in the same row indicate significant differences (P<0.05 or more) according to time during the periparturient period for a given group,* ***NS:*** *Not significant*

calving day are thought to be associated with calving stress and with the hemodynamic effect of stress on glomerular filtration rate [11]. The cause of reductions is considered to be due to decrease in food intake or colostrums production, and as food intake increases BUN concentration raises. Increase in dry matter taken with diet is reported to regulate blood protein and urea levels [19]. There are studies asserting that urea concentration is associated with dietary protein degradability, amount of energy and non-protein nitrogen [20], and with the number of calving [19]. Blum et al.[21] fed lactating cows with methionine for five days and observed a slight increase at the end of the treatment but the increase was not meaningful. While addition of methionine (12.4 mg/dl) to rations containing %16 proteins was shown to decrease urea levels, methionine plus lysine supplementations were not noted to change its levels [9]. Nevertheless, decrease in serum urea levels in the animals fed with rations containing lysine and methionine could be associated with increase in nitrogen cycle [22]. Krober et al.[16] demonstrated that methionine, lysine, or methionine plus lysine supplementations during early lactation period did not markedly alter serum BUN levels.

In our study, we measured statistically significant dissimilarity in serum triglyceride (TG) concentrations in all the groups among sampling day evaluations (P=0.000). TG levels in all the groups were lower at calving day, postpartum 1 w (the first week in postpartum period), and postpartum 2 w (the second weeks in postpartum period) measurements when compared to prepartum 2 w. We also accomplished considerable difference (P=0.049) at only postpartum 1w TG concentrations at evaluations among the groups, and the lowest TG level was computed in control group. TG is used for making milk fat by mammary gland during lactation period [4]; therefore, TG serum concentration is lower in lactation than dry period [23]. On the other hand,

current TG serum concentrations measured at calving day and postpartum period were between reference limits 0-14 mg/dl. Elevated prepartum TG levels measured here in all groups could be associated with reduced catabolism in dry period and/or with excessive production [24].

At their recent study, Phillips et al.[25] demonstrate that inclusion of methionine into diets of cows drops off their serum TG at postpartum 1st week. By contrast, the studies using methionine during early lactation period report no change in serum TG levels [26,27]. Xu et al.[28] discloses that the addition of rumen-protected amino acids containing methionine (13 g/day) and lysine (40 g/day) into the rations of multiparous Holstein dairy cattle reduces serum levels of TG and NEFA. In the current study, at the comparisons within the groups were calculated the lowest serum TG levels at postpartum 1 w in Group LM but at postpartum 2 w in Group M, Group L, and in control groups. At evaluations among the groups, we obtained numerically the highest TG level on calving day in Group LM, and at postpartum 1 w and 2 w in Group M.

In the present study at evaluations within the groups, serum CHOL concentrations were decreased between prepartum 2 w and calving day but increased between calving day and postpartum 2 w in all groups. CHOL levels at evaluations between the groups were the highest in control group and the lowest in Group L on calving day. When compared to other groups, the lowest serum CHOL levels were measured in Group L. We also noted numerical increase in high-density lipoprotein (HDL) levels in all study groups between calving day and postpartum 2 w. The lowest concentrations of CHOL and HDL were measured on the day of calving [29], an observation that might be associated with stress at the time of calving because cortisol, a steroid hormone and released in response to stress is derived from the cholesterol [30]. CHOL and HDL levels were measured to be lower at postpartum 2nd week in Group L than the other study groups. That 60% of HDL is made of cholesterol might be reason for this reduction [31]. Basoglu et al.[32] reported that the levels of HDL are higher in late lactation than early lactation and dry period. In another study, Yildiz et al.[13] demonstrate that serum HDL concentration is higher in the last month of pregnancy than calving day. The results of the present study are consistent with these studies mentioned here. On the other hand, methionine supplementation during early lactation in cows is shown to have no effect on serum HDL levels [26]. Likewise, methionine addition into the rations of dairy cattle during lactation period is reported not to affect CHOL levels [27]. At their study on dairy cattle with high milk production, Trinacty et al.[10] show that treatment of dairy cattle with rumen-protected methionine, lysine, or both does not influence levels of blood metabolites except for BHBA. Similarly, Ye et al.[22] report that while feeding of dairy cattle with pellet containing methionine and lysine during periparturient decrease serum NEFA and BHBA levels, it does not affect other blood parameters.

It reports that while methionine supplementation during prepartum period does not alter NEFA concentration [25], its inclusion to diets during postpartum period either reduces NEFA level [21], or does not affect its level [9]. Likewise, methionine supplementation during periparturient period in cows is reported not to affect normal concentration of NEFA [25]. In the present study, we observed statistically significant changes in serum NEFA concentrations according to all sampling days in all groups *(Table 4)*. At evaluations within the groups we determined numerically increased NEFA levels on calving day with respect to prepartum 2 w. Serum NEFA concentrations were decreased at postpartum 1 w and 2 w in Group L and Group LM when compared with calving day NEFA measurement values and the main reduction at evaluations among the groups was in Group LM. In addition, at evaluations among the groups serum NEFA concentrations was only significantly diminished at prepartum 1w measurement (P=0.000).

Furthermore, in our study serum BHBA levels were considerably different (P=0.004) in only Group M when compared within the groups. At comparisons among the groups, the changes at BHBA levels were momentous at prepartum 1 w, on calving day, and at postpartum 2 w. When compared with respect to measurement times, a reduction in BHBA levels in Group M was observed till calving day measurement when compared to prepartum 1 w.

Acute increase in NEFA levels during calving is reported to be associated with the initiation of TG infiltration [25,33]. A positive correlation between negative energy balance and NEFA concentration is demonstrated [33]. NEFA concentration is shown to reach its highest concentration one day after calving and begin to decrease till postpartum third week. The reductions in NEFA levels can be evaluated as an indication of decreased body fat mobilization or use of NEFA for synthesizing VLDL in liver [34]. It is shown that at the cases when blood glucose level is appropriate, body does not need fat deposits. In the present study, increase in NEFA concentrations can be related to statistically significant reductions in serum glucose levels at postpartum 2 w in all groups and methionine and lysine appeared to be inadequate to prevent this condition. Methionine supplementation during lactation is reported to have no impact on NEFA concentration [34]. Our present study indicates that lysine seemed to have similar effect on NEFA concentration. Leroy et al.[12] disclose that increase in BHBA in hypoglycemic animals is much higher. There is also statistical correlation between NEFA and BHBA [19]. Cheng et al.[33] determined the highest NEFA and BHBA concentrations at postpartum 1st day and postpartum 2nd week. In the present study at evaluations within the groups, the highest NEFA and BHBA concentrations in Group M were obtained at postpartum 1 w. Possible reason for this might be the use of NEFA in the synthesis of BHBA [33]. In the current study, the highest numerical postpartum BHBA concentrations were measured at postpartum 1w in control group, at postpartum 2 w in Group L, and at postpartum

1 w in Group LM. Even though BHBA concentrations are shown to be associated with negative energy balance and mobilization of stored fats in body, they are also affected by lactation itself [13]. Several studies demonstrate that various doses of methionine supplementations during prepartum and postpartum periods in cows do not change BHBA concentrations [26]. In addition, the level of BHBA in multiparous is reported to be elevated with respect to the cows at their first calving [26]. At their study where they fed the cows with low and normal energy rations, Rulquin and Delaby [5] noted no effect of methionine on serum BHBA levels in both groups. By contrast, there are also studies showing no effect of methionine and lysine supplements during lactation period on serum BHBA concentration [1,28].

Moreover, the lowest low-density lipoprotein (LDL) levels in all the study groups were calculated on calving day at the evaluations within the present study groups. At their study, Basoglu et al.[32] state that prepartum serum LDL levels are higher when compared to late lactation. In this study, the highest serum LDL concentrations were obtained at postpartum 2 w in control group, at prepartum 2 w in Group M, at prepartum 1 w in Group L and Group LM.

The study results established that serum VLDL concentrations in all groups were numerically decreased on calving day, postpartum 1 w and 2 w with regard to prepartum 1 w and 2 w. Prepartum VLDL levels are shown to be higher than those of postpartum 1st week [32]. This condition can be associated with increased VLDL catabolism in mammary glands and excessive fat accumulation in liver [2]. Davidson et al.[26] added methionine to diets of cows at their early lactation period but observed no change in their VLDL concentrations. In the current study at evaluations within the groups, we measured numerically the lowest VLDL concentration in control and methionine groups at postpartum 2 w but at postpartum 2 w in Group L and Group LM. Similarly, Ye et al.[22] fed cows with pellet containing methionine and lysine during periparturient period but noted no change in VLDL concentration when compared to the control group. In the current study at evaluations within the groups we, by contrast, determined significant differences in serum VLDL levels between the study groups (P=0.000).

Furthermore, the highest concentration of glucose (GLU) was obtained on calving day in control and methionine groups and at postpartum 1 w in Group L and Group LM. The lowest glucose level was recorded at postpartum 2w samples in all the groups. Glucocorticoids secreted on the day before calving is known to rise glucose level [35] and the increase in glucose level on calving day is thought to be associated with calving stress [30]. Reduction in postpartum serum glucose level is reported to be associated with decrease in food intake during early lactation [36], and increase in the consumption of glucose by mammary gland [37] and fetus near to calving. Studies state that decline in serum glucose level observed during the first week of lactation can be corrected in later weeks by providing animals with energetically well balanced rations [33,36]. In the present study, serum glucose concentrations at postpartum 2 w were numerically lower than those of calving day and postpartum 1 w. Moreover, postpartum serum GLU concentrations in all groups were determined to be below the reference range (42.1-74.5 mg/dl) [15]. Reductions in serum glucose levels indicate that methionine, lysine, or methionine plus lysine supplementations fail to boost the postpartum serum GLU levels up in the present study groups fed with the same type of ration. Nonetheless, measurement of lower serum NEFA levels and absence of significant differences concerning milk production among the present groups might be an indication of undeveloped postpartum NEB. In the present study, the reduction in GLU level during early lactation can be resulted from increased milk production and the use of glucose for the synthesis of lactose. While some studies show that postpartum serum GLU levels increase [35], the present study reports that postpartum glucose concentrations are reduced [12,32].

Several studies indicate that addition of methionine into diets of dairy cattle does not change serum GLU concentrations [21]. Similarly we noted increase in prepartum GLU levels when compared to postpartum period in the animals provided with methionine. Nonetheless, methionine addition to the diets of lactating cows is shown to decrease blood GLU levels [35]. Likewise, at their study Socha et al.[9] report that addition of methionine in diets of cows reduces serum glucose levels at postpartum first and third weeks and addition of lysine with methionine in rations further diminishes GLU levels, an observation consistent with our present observations.

In the current study, aspartate aminotransferase (AST) values were characterized with insignificant changes in only Group M during prepartum and postpartum periods. By contrast, AST levels were considerable increased, particularly on calving day, and postpartum first week and postpartum second week in control group, Group L, and Group LM. As a result, this can be explained, methionine was observed to prevent excessive cellular activity on calving day and postpartum days but lysine by itself or in combination with methionine was found to be inadequate for suppressing cellular activity. Nevertheless, all the obtained present values were between reference ranges (45-110 U/L) [15].

Liver cells in ruminants do not have high alanine aminotransferase activity (ALT). However, Sevinc et al.[11] report that ALT activity in the first two months of the lactation increases with respect to the calving day and last month of dry period. In our study, while ALT activity was determined to be unimportant in control and methionine groups, it was significantly reduced in Group L and Group LM. Nevertheless, all obtained values for ALT levels in all groups were within the within the reference ranges (6.9-35 U/L) [15]. This observation is consistent with literature and further

supports the concept that ALT activity is not specific in ruminants for evaluating liver functions [2,35,38].

Furthermore, we noted no statistically significant increases in the levels of serum GGT in control, methionine, and lysine groups when compared to prepartum period. The serum GGT levels in these groups were within the reference limits (4.9-26 U/L) [15]. Although there was a meaningful increase in serum GGT levels in Group LM when prepartum and postpartum periods were compared, the values were still within the reference ranges. In the present study, numerical increases in serum GGT levels could be associated with postpartum liver fat infiltration and liver metabolism condition [38].

Metabolic indicators may change depending on composition of the ration [39]. Furthermore, the results of the present study indicated that the use of dietary additives such as methionine and lysine in the rations of dairy cattle that were fed with adequate and nutritionally well balanced rations to provide animals with liver protection and increase their milk production was not essential in order to obtain optimal milk production and protect cows against the development of the periparturient period diseases even though their use affected some parameters of the serum of these animals. On the other hand, the addition of methionine and lysine to the rations of dairy cattle nourished with inadequate and nutritionally unbalanced rations and handled with management problems helps cows recover their specific amino acid needs for gaining liver protection and augmenting milk production.

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RESEARCH ARTICLE

Proliferation and LDH Leakage in Cell Cultures of Animal and Insect Origin Exposed to Insecticide Endosulfan [1]

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Summary

In the present study three different cell cultures, derived from rabbit kidney (RK13), rat liver (WBF344) and insect origin (Sf21), were used to examine the cytotoxic effect of the insecticide endosulfan. Cytotoxicity was determined on the basis of cell proliferation activity; the cellular damage was assessed by evaluation of cytopathic effect and lactate dehydrogenase (LDH) leakage. Endosulfan treatment suppressed proliferative activity in cell cultures as follows: insect Sf21 cells (10^{-1}–10^{-5} M; $P<0.01$) > WBF344 (10^{-1}–10^{-4} M) > RK13 cells (10^{-1}–10^{-3} M). LDH leakage into the medium was increased in WBF344 cells at 10^{-1}–10^{-3} M ($P<0.01$), whereas in RK13 and Sf21 cells at 10^{-1}–10^{-2} M ($P<0.05$) compared to solvent control. These results indicate cell type-dependent sensitivity to endosulfan exposure. Endosulfan caused a more pronounced decrease in insect cell proliferation in comparison with mammalian cell cultures; however, the LDH leakage and microscopical signs of cellular damage were the most intensive in liver cells.

Keywords: *Insecticide, Endosulfan, Cell cultures, Cytotoxicity, LDH, Cell proliferation*

İnsektisit Endosülfana Maruz Kalan Hayvansal ve Böcek Kökenli Hücre Kültürlerindeki Proliferasyon ve LDH Fazlalığı

Özet

Bu çalışmada, tavşan böbrek (RK13), sıçan karaciğer (WBF344) ve böcek (Sf21) kökenli üç farklı hücre kültürü, insektisit endofulfanın sitotoksik etkisini incelemek için kullanıldı. Sitotoksisite hücre proliferasyonu aktivitesine bağlı olarak belirlendi; hücre hasarı, sitopatik etkinin artması ve laktat dehidrogenaz (LDH) fazlalığı ile değerlendirildi. Endosulfan tedavisi hücre kültürlerinde proliferatif aktiviteyi baskılama değerleri; böcek kökenli Sg21 (10^{-1}–10^{-5} M; $P<0.01$) > WBF344 (10^{-1}–10^{-4} M) > RK13 hücre kültürü (10^{-1}–10^{-3} M) şeklinde belirlendi. Ortamda LDH fazlalığı, kontrol ile kıyasla WBF344 türündeki hücrelerde 10^{-1}–10^{-3} M ($P<0.01$) miktarında arttarken RK13 ve Sf21 hücre kültürlerinde 10^{-1}–10^{-2} M ($P<0.05$) miktarında belirlendi. Bu sonuçlar endosülfan maruziyetine karşı hücre tipine bağlı duyarlılığı işaret etmektedir. Endosülfan, memeli hücre kültürüne göre böcek kökenli hücre kültürlerindeki hücre sayılarında daha belirgin bir düşüşe neden olsa da LDH fazlalığı ve hücredeki mikroskobik hasar belirteçleri en belirgin olarak karaciğer hücrelerinde görülmektedir.

Anahtar sözcükler: *: İnsektisit, Endosülfan, Hücre kültürleri, Sitotoksisite, LDH, Hücre proliferasyonu*

INTRODUCTION

Organochlorine pesticides continue to be used in several developing countries despite concerns regarding their toxicity profile. One such organochlorine compound is an insecticide and acaricide endosulfan. This colourless

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solid has emerged as a highly controversial agrichemical due to its acute toxicity [1], persistence in the environment, potential for bioaccumulation, and role as an endocrine disruptor [2]. Banned in more than 63 countries, it is still used extensively to control insect pests including whiteflys, aphids, leafhoppers, Colorado potato beetles and cabbage worms in vegetables, cotton, and fruits in some other countries including India and Brasil. Because of its unique mode of action, it is useful in resistance management [3]. On the other hand, endosulfan is one of the most toxic pesticides on the market today, responsible for many fatal pesticide poisoning incidents in humans and animals around the world. It is absorbed through the intestinal tract, the lungs, and the skin. Toxicokinetics of ^{14}C-endosulfan in rats was described by Chan et al.[4]. Monitoring of the residue levels of insecticide revealed its accumulation in various tissues and fluids [5,6]. The commonest manifestations of endosulfan intoxication are neurological although other organ dysfunction also occurs [1]. Hepatotoxicity and nephrotoxicity in humans and animals exposed to endosulfan have been documented in many studies [7-9].

The aim of our study was to compare the direct effect of different endosulfan concentrations on proliferative activity and cellular damage of mammalian and insect cell cultures.

MATERIAL and METHODS

The Insecticide Tested

Chemical data on endosulfan are depicted in *Fig. 1* [3]. Endosulfan was dissolved in dimethylsulfoxide (DMSO, Lachema, Brno, Czech Republic), of which the final concentration in the maintenance medium was 1%. The basic molar concentrations of insecticide, freshly prepared before each experiment, were 10^{-1}–10^{-5} M and added to cell cultures at the rate of 1% of total cell volume; i.e. the actual doses were 100x lower than the basic ones. After endosulfan exposure, cell proliferation, lactate dehydrogenase release and cell desintegration were evaluated in cell cultures of mammalian and insect origin.

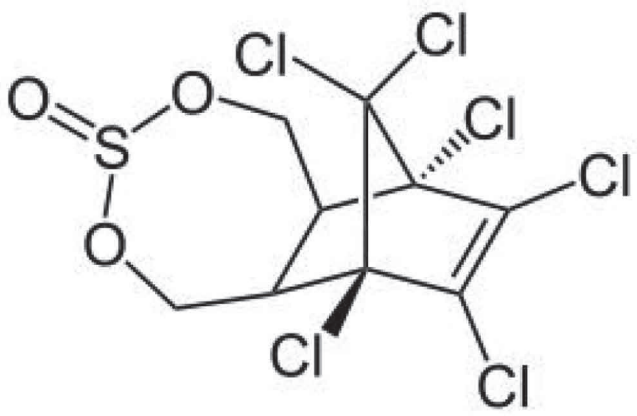

Fig 1. Chemical data on endosulfan [3]

CAS No. 115-29-7; Molecular formula: $C_9H_6Cl_6O_3S$; Mol. wt.: 406.9; Chemical family: organochlorines; Use: insecticide, acaricide; Producer, Purity: Supelco, Bellefonte, USA; 99%

Şekil 1. Endosulfan'ın kimyasal bilgileri [3]

CAS No. 115-29-7; Moleküler formül: $C_9H_6Cl_6O_3S$; Mol. ağırlık: 406.9; Kimyasal aile: organochlorines; Kullanım: insektisit, akarisit; Üretici, Saflık: Supelco, Bellefonte, ABD; 99%

Cell Cultures

Cell lines (kindly provided from Virological Institute, Bratislava, Slovakia) RK13 (rabbit kidney), WBF344 (rat liver) and the IPLBSF-21 (the pupal ovarian cells of the fall army worm, *Spodoptera frugiperda* – Sf21) were used in the study. RK13 cells were cultured in minimal essential medium (MEM) supplemented with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) antibiotic solution of Penicillin G Sodium Salt (PNC) and Streptomycin Sulphate (STM) (Gibco, Invitrogen, Corp., USA) at 37°C. WBF344 cells were cultured in Dulbecco´s modified Eagle´s medium (D-MEM) with 10% (v/v) FCS, 1% (v/v) PNC and STM in humidified 5% CO_2 at 37°C. Sf-900 II SFM medium was used for insect cell line (Gibco, Invitrogen, Corp., USA) supplemented with 1% (v/v) PNC and STM. Sf21 cells were cultured at 27°C.

Cell Density and Cytopathic Effect

Cell density and cytopathic effect determined on the basis of microscopical signs of cellular damage (granulation and vacuolisation of cytoplasm, rounding off and detachment of cells from the bottom of cultivation vessel, rupture of cells) were evaluated by standard counting technique using an inverted microscope (Carl Zeiss, Germany) at magnifications of 400 x after 24 h exposure to endosulfan.

Proliferation Test (PT)

Cells were seeded in 100 ml of cell culture medium in 96-multiwell culture plate (Corning, Inc., USA) at a density of 2x10^5/ml. After 24 h incubation different endosulfan concentrations were then added and treated cells were incubated for 48 h. There were five replicates of each treatment. After the exposure period a colorimetric immunoassay was used to quantify cell proliferation (Cell Proliferation ELISA Kit, BrdU-colorimetric, Roche Diagnostics, GmbH, Germany). This was based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis. Twenty four hours before the end of the cultivation, 10 µM BrdU was added and the cells were reincubated. After removing the culture medium, denaturation of DNA and fixation of the cells on the bottom of wells, 100 ml of anti-BrdU-peroxidase labelled conjugate was added and allowed to react for 90 min at 25°C. The immune complexes were detected by the subsequent substrate reaction (100 ml substrate solution) for 30 min at room temperature. The reaction was stopped by 25 ml 1 M H_2SO_4 and the optical density (OD) was measured in an ELISA-multiwell reader (BIO-RAD Laboratories, Inc., USA) at 450 nm (OD_{450}). The mean optical densities were converted into percentage of

residual cell viability expressed as percentage of proliferative activity (% PA): %PA = [$OD^{endosulfan}$ / OD^{DMSO}] x 100; where $OD^{endosulfan}$ is the mean value of OD of cells treated with insecticide and OD^{DMSO} is the mean value of OD of cells treated with the solvent control, measured at 450 nm.

Cytotoxicity Assay

Cells were seeded in 100 ml of complete medium in 96-multiwell culture plate (Corning, Inc., USA) at a density of $2x10^5$/ml and incubated for 24 h. Growth medium was changed to maintenance medium with 1% (v/v) FCS and different endosulfan concentrations were then added and cells were incubated for additional 24 h. There were five replicates of each treatment. After the exposure period a colorimetric assay was used to quantify cytotoxicity/cytolysis by measuring LDH activity released from damaged cells (Cytotoxicity Detection Kit^{PLUS}, Roche Diagnostics, GmbH, Germany). To each well on the 96-well plate 100 µl reaction mixture was added and plate was incubated for 30 min at room temperature. After incubation 50 µl of stop solution was added to each well. Optical density (OD) was measured in an ELISA-multiwell reader (BIO-RAD Laboratories, Inc., USA) at 450 nm (OD_{450}). To calculate percent cytotoxicity in each plate low control (LC) and high control (HC) were set up and the percentage of cytotoxicity was calculated according to the formula: Cytotoxicity (%) = [$OD^{endosulfan}$ – OD^{LC} / OD^{HC} – OD^{LC}] x 100; where $OD^{endosulfan}$ is the mean value of OD of cells treated with insecticide, OD^{LC} (low control) is the mean value of OD cells treated with the solvent control (DMSO) and OD^{HC} (high control) is the mean value of OD cells treated with the lysis buffer (the maximum releasable LDH activity in the cells) measured at 450 nm.

Statistical Analysis

Data were analysed by ANOVA, followed by Dunnett´s test. Results are presented as mean ± SD, $P<0.05$ was considered to be statistically significant.

RESULTS

Cytotoxic effect of endosulfan on cell cultures determined by proliferation activity is shown in *Table 1*. Proliferative activity of mammalian cell cultures was significantly suppressed in liver WBF344 (10^{-1}–10^{-4} M) and kidney cells RK13 (10^{-1}–10^{-3} M). Insect Sf21 cells were the most sensitive to this insecticide with significant suppression of their proliferative activity ranging from 10^{-1}–10^{-5} M ($P<0.01$), with PA = 1.6–65.6% *(Table 1)*.

Cytopathic effect, determined on the basis of microscopical signs of cellular damage and the LDH leakage into the medium, was observed in WBF344 in the concentrations

Table 1. *Cytotoxic effect of insecticide endosulfan on cell cultures tested determined by proliferation test*
Table 1. *İnsektisit endosulfan'ın hücre kültürleri üzerinde proliferasyon testi ile belirlenen sitotoksik etkileri*

Cell Culture	Test Parameters	Concentration of Endosulfan in M (Mean Values)					Solvent Control (DMSO)
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
RK13	OD_{450}±SD %PA±SD	0.04±0.02 12.1±6**	0.02±03 6.1±9**	0.25±0.16 75.8±48.5*	0.37±0.07 115.2±21.2	0.35±0.04 75.8±6.0	0.33±0.05 -
WBF344	OD_{450}±SD %PA±SD	0.02±0.005 7.4±1.9**	0.03±0.01 11.1±3.7**	0.11±0.07 40.7±25.9**	0.19±0.03 70.4±11.1*	0.32±0.07 118.5±25.9	0.27±0.09 -
Sf21	OD_{450}±SD %PA±SD	0.005±0.003 1.6±0.96**	0.005±0.004 1.6±1.3**	0.006±0.004 1.9±9.5**	0.13±0.03 40.6±9.4**	0.21±0.007 65.6±2.2**	0.32±0.04 -

RK13 *- Rabbit kidney cell line;* ***WBF344*** *- epithelial rat liver cell line;* ***Sf21*** *- Spodoptera frugiperda pupal ovarian tissue;* ***DMSO*** *- dimethylsulfoxide;* ***OD_{450}*** *- optical density;* ***SD*** *- standard deviation;* ***%PA*** *- percentage of proliferative activity;* ***M*** *- molar concentration; ** P<0.01; * P<0.05*

Table 2. *Cytotoxic effect of insecticide endosulfan determined by evaluation of LDH activity released from cells*
Table 2. *İnsektisit endosulfan'ın hücrelerden serbest kalan LDH aktivitsinin değerlendirilmesi ile baelirlenen sitotoksik etkileri*

Cell Culture	Test Parametres	Concentration of Endosulfan in M (Mean Values)				Solvent Control (DMSO)
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	
RK13	OD_{450}±SD % Cytotoxicity±SD CPE	1.81±0.18 60.7±6.0* +	1.9±0.1 66.8±3.5* +	1.23±0.05 21.6±0.9 -	1.2±0.06 6.08±10.1 -	1.24±0.46 22.27±8.3 -
WBF344	OD_{450}±SD % Cytotoxicity±SD CPE	1.86±0.04 83.6±1.8** +	1.76±0.03 76.6±1.3** +	1.51±0.13 59.4±5.1** +	0.94±0.14 20.0±3.0 -	0.94±0.13 23.43±4.0 -
Sf21	OD_{450}±SD % Cytotoxicity±SD CPE	0.91±0.03 24.6±0.8** +	0.84±0.07 20.1±1.7* +	0.78±0.08 16.2±1.7 -	0.61±0.05 6.0±0.5 -	0.62±0.31 6.01±3.0 -

RK13 *- Rabbit Kidney cell line;* ***WBF344*** *- epithelial rat liver cell line;* ***Sf21*** *- Spodoptera frugiperda pupal ovarian tissue;* ***DMSO*** *- dimethylsulfoxide;* ***OD_{450}*** *- optical density;* ***SD*** *- standard deviation;* ***CPE*** *- cytopathic effect;* ***M*** *- molar concentration; ** P<0.01; * P<0.05*

of 10^{-1}–10^{-3} M, in RK 13 cells and the insect cell line Sf21 in the concentrations of 10^{-1}–10^{-2} M compared to solvent control *(Table 2)*. Cytopathic effect and LDH leakage into the medium were strongly limited in the insect cell line Sf21 and RK 13 cells in comparison to the inhibition of proliferative activity.

DISCUSSION

The indiscriminate and injudicious use of pesticides particularly endosulfan in agriculture and animal husbandry practices has considerably increased the risk of health hazard. Traditionally, animal models have been used to examine the toxic potential of pesticides. For screening purposes, there is increasing interest in the development of *in vitro* methods to replace conventional animal toxicity tests. Established mammalian and insect cell lines may contribute to rapid screening of pesticides and discover their modes of action or may be useful for toxicity prediction of target organs on chemicals or drug exposure [10].

The liver is known to be the main site of xenobiotic biotransformation, therefore, cell lines of liver origin are widely used in biomedical research involving xenobiotic metabolism and hepatotoxicity [11]. For our study we used WBF344 established from a single cloned non-parenchymal epithelial cell isolated from a normal male adult rat liver.

Kidney is the main organ for elimination of xenobiotics from the body and the use of the cultured cells of kidney origin to assess specific cytotoxic effects of nephrotoxins described Li et al.[12]. Rabbit kidney (RK13) cell line was used in the present study.

Continuous cell line Sf21 developed from ovarian tissue of the *Spodoptera frugiperda*, a moth species that is an agricultural pest on corn and other grass species, was chosen for evaluation of direct effect of endosulfan on insect cells. Cell lines used in this study were also used as model systems for testing of various xenobiotics, e.g. WBF344 for food and perfume additives [13], RK13 for mycotoxins [14], and the cell line Sf21 for testing of insect cell extracts [15] or novel insecticides [16]. Manji and Friesen [17] and Tseng et al.[18] studied induction of apoptosis and Takahashi et al.[19] cell cycle arrest induced by radiation in SF21 cell line. The present work was undertaken to evaluate the direct toxic effect of endosulfan on model cell cultures mentioned above.

Cytotoxic effects of endosulfan were previously studied in various cell cultures. Holovská et al.[20] evaluated proliferative activity of cell lines Madin-Darby Bovine Kidney (MDBK), Rabbit Kidney (RK13), Porcine Kidney (PK15), and semi-continual line of Bovine Embryonic Pulmonary Cells (BEPC) after exposure to endosulfan. From these cell cultures cell proliferative activity was suppressed most intensively in PK15 culture at the concentrations of 10^{-1}–10^{-6} M of endosulfan. Krovel et al.[21] observed moderate cytotoxicity and steatosis in a dose-dependent manner in the primary cultures of hepatocytes isolated from Atlantic salmon exposed to concentrations of 0.001–100 microM endosulfan for 48 h. Skandrani et al.[22] investigated endosulfan toxicity in neuronal SH-SY5Y and pulmonary A549 cell lines exposed to increasing pesticide concentrations for 3 days. This experiment showed that neuronal cells were more sensitive than pulmonary cells. Insecticide was found to cause concentration-dependent (50-400 microM) apoptotic cell death in SH-SY5Y human neuroblastoma cells [23]. Kannan et al.[24] also recorded the ability of endosulfan to induce apoptosis in a human T-cell leukemic line. In our previous studies we determined the cytotoxic and immunotoxic effects of endosulfan on sheep peripheral blood phagocytes and lymphocytes on the basis of functional immunological assays under *in vitro* conditions [25,26]. Results of our present *in vitro* study showed that endosulfan significantly suppressed mainly proliferation of insect Sf21 cells, whereas from mammalian cell cultures WBF344 liver cells were the most sensitive to insecticide exposure.

The quantitative relationship between lactate dehydrogenase (LDH) release and the loss of animal cell viability or proliferation was described by several authors [27-30]. LDH is an important glycolytic enzyme, present in the cells of almost all body tissues, and changes in the enzyme activity may provide direct and indirect evidence of the cellular damage and can indicate the toxic mechanism [31]. The LDH release is a parameter, which reflects the membrane integrity. In our study the cytopathic effect of endosulfan and LDH leakage were the most intensive in WBF344 cell culture, whereas RK 13 and insect Sf21 cells showed higher resistance to endosulfan. Study of El-Shenawy [29] also showed that incubation of rat hepatocytes with 10 or 100 microM of endosulfan for 2 h induced cell membrane damage as indicated by increasing the leakage percentages of LDH. Correlation of LDH activity with loss of cell viability in Sf-9 insect cell cultures was observed by Wu et al.[32]. However, our study showed a highly significant proliferative suppression of insect Sf21 cells (10^{-1}–10^{-5} M), but without correlation with LDH leakage into the maintenance medium and cytopathic effect (10^{-1}–10^{-2} M).

The molecular mechanisms of endosulfan cytotoxicity have not yet been elucidated and require further studies. One of causes of cytotoxicity might be the result of the intrinsic toxicity of pesticides that could lead to the generation of reactive oxygen species (ROS) during the metabolisation of certain substances or interactions with secondary targets. Some reports showed that many organochlorine and carbamate insecticides are capable of inducing oxidative stress by overproduction of ROS in experimental systems. Cells possess enzymatic and nonenzymatic antioxidant system, which detoxify reactive oxygen species (ROS) that are generated during oxidative stress [33,34].

It can be concluded that endosulfan caused a more pronounced decrease in insect cell proliferation in comparison

with mammalian cell cultures; however the LDH leakage and microscopical signs of cellular damage were limited on the first two highest concentrations of insecticide. The most intensive decrease of cell proliferation and the LDH leakage of mammalian cells was observed in liver cells. Our results revealed different sensitivity of mammalian and insect cells to endosulfan exposure under *in vitro* conditions, probably caused by different metabolisation pathways of the insecticide in the studied cells.

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RESEARCH ARTICLE

Allelic Frequency of Kappa-Casein, Growth Hormone and Prolactin Gene in Holstein, Brown Swiss and Simmental Cattle Breeds in Turkey [1]

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Summary

The purpose of this study was to examine the kappa-casein (κ-CN), growth hormone (bGH) and prolactin hormone (PRL) gene polymorphisms in the Holstein (n=150), Simmental (n=50) and Brown Swiss (n=50) cattle breeds in Turkey. In order to determine the κ-CN-*Hind*III, PRL-*Rsa*I and bGH-*Alu*I polymorphisms, polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) were performed. A 443 bp fragment of κ-CN, a 223 bp fragment of bGH and a 156 bp fragment of PRL were amplified. In this study, two types of alleles, A and B for κ-CN, V and L for bGH, and A and B for PRL, were observed. The cattle breeds in which the highest frequencies of the alleles were estimated, were the HL breed (0.82) for κ-CN-A, the BS breed (0.55) for κ-CN-B, the HL breed (0.85) for bGH-L, the S breed (0.34) for bGH-V, the HL breed (0.87) for PRL-A, and the BS breed (0.24) for PRL-B. According to the results of the chi-square test, a significant deviation from the Hardy-Weinberg equilibrium was determined only for the bGH locus in the investigated breeds. The present study is the first report that examines three loci (κ-CN, bGH and PRL) in three cattle breeds of European origin (Holstein, Simmental and Brown Swiss) raised in Turkey.

Keywords: *Cattle, Genetic polymorphism, Growth hormone, Kappa-casein, Prolactin*

Türkiye'deki Holştayn, İsviçre Esmeri ve Simmental Sığır Irklarında Kapa-Kazein, Büyüme Hormonu ve Prolaktin Genlerinin Allel Frekansları

Özet

Bu çalışmada Türkiye'de yetiştirilen Holştayn (n=150), Simental (n=50) ve İsviçre Esmeri (n=50) sığır ırklarında kapa-kazein (κ-CN), büyüme hormonu (bGH) ve prolaktin hormonu (PRL) gen polimorfizimlerinin belirlenmesi amaçlanmıştır. Kapa-kazein-*Hind*III, PRL-*Rsa*I ve bGH-*Alu*I polimorfizimleri polimeraz zincir reaksiyonu ve restriksiyon parçacık büyüklük polimorfizimi (PCR-RFLP) ile belirlenmiştir. Kapa-kazein geni için 443 bç'lik bir bant, bGH geni için 223 bç'lik bir bant ve PRL geni için 156 bç'lik bir bant PZR ile yükseltgenmiştir. Bu çalışmada κ-CN için A ve B; bGH için L ve V; PRL için A ve B olarak olarak adlandırılan iki allel belirlenmiştir. En yüksek κ-CN-A allel frekansı HL ırkında (0.82), en yüksek κ-CN-B allel frekansı ise BS ırkında (0.55) belirlenmiştir. En yüksek bGH-L allel frekansı HL ırkında (0.85), en yüksek bGH-V allel frekansı ise S ırkında (0.34) belirlenmiştir. En yüksek PRL-A allel frekansı HL ırkında (0.87), en yüksek PRL-B allel frekansı ise BS ırkında (0.24) belirlenmiştir. Ki-kare sonuçlarına göre, incelenen ırklarda Harrdy-Weinberd dengesinden istatiksel olarak sapma sadece bGH lokusunda gözlenmiştir. Bu çalışma Türkiye'de yetiştirilen Holştayn, Simmental ve İsviçre Esmeri sığır ırklarında κ-CN, bGH ve PRL genotiplendirilmesinin yapıldığı ilk çalışmadır.

Anahtar sözcükler: *Büyüme hormonu, Genetik polimorfizim, Kapa-kazein, Prolaktin, Sığır*

INTRODUCTION

The accurate prediction of the future yields of breeder animals is one of the most significant, yet, complicated issues of farm animal breeding. The lengthiness of the generation interval prevents the achievement of a rapid

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genetic improvement through the use of available selection methods in farm animal breeding. Therefore, researchers are striving to develop efficient methods for livestock, including cattle, sheep, goats and horses, which would aid in the selection of potential breeders within a shorter time period and with greater accuracy. In recent years, research on molecular markers and the correlation between gene polymorphisms and different yield traits have gained increasing importance in farm animal breeding [1].

On the global scene, selection related research conducted in dairy cattle, generally focuses on milk yields, milk components and dairy technology [2]. Milk yield is a polygenic trait, which is affected by environmental factors. In recent years, the presence of several genes, which determine the correlation between milk protein polymorphism and the physiological and biochemical traits affecting milk yield, has been reported. It has been indicated that the allelic structure of the genes coding several hormones, such as the growth hormone (GH) and prolactin (PRL), as well as milk proteins, such as kappa-casein (κ-CN), are correlated with milk yield, milk components and milk processing products. For this reason, it is considered that the allelic structure of the κ-CN, GH and PRL genes could be used as a candidate gene in predicting the lactation performance of potential bovine breeders [2].

In cattle, it has been ascertained that the κ-CN gene has 12 alleles, named as A, B, B2, C, E, F, F1, G, H, I, A(1) and J [3]. While the majority of these alleles have been determined to exist in only a few cattle breeds at a low frequency, it has been ascertained that the A and B alleles are very common and are found in almost all cattle breeds [3]. It has been reported that, of the casein proteins, κ-CN has affect on milk yield, milk protein composition and milk fat content [4].

In addition to milk protein polymorphisms, another subject studied extensively in farm animals is the growth hormone gene [5]. The growth hormone is involved in multiple physiological processes, including the regulation of growth, development of the mammary glands, onset of lactation, glucogenesis, activation of lypolysis, and regulation of muscle development [6]. In view of the above-mentioned effects of the growth hormone, both GH concentration and the allelic variations of the gene coding this hormone have drawn the attention of researchers. It has been reported that, in cattle, the bGH gene is correlated with certain yield traits, in particular with milk yield and quality, growth [6], carcass composition and quality [7]. In this context, it is considered that it could be of use as a candidate gene in animal improvement programmes targeted at increasing milk and meat yields [8]. Previous molecular genetic studies have demonstrated that yield traits such as milk yield and body weight gain are correlated with certain polymorphisms of the bGH gene [6].

In mammals, prolactin is responsible for the onset and maintenance of lactation, the growth of the mammary glands, and lactogenesis [8]. In view of the effects described above, it is considered that this gene could be used as a potential genetic marker of milk yield in cattle [8]. However, information available on polymorphisms of the PRL gene in cattle remains limited. A few polymorphisms have been reported for the bovine PRL gene [9]. Literature reports are available, which report correlation to both exist [8] and not exist [10] between PRL-*Rsa*I polymorphisms and several traits, including milk yield, milk fat rate and milk protein content in the particular animal species and breeds investigated. For, it is considered that the variants of this gene could be used in the development of an appropriate test method for genetic improvement programmes and animal breeding [10].

The present study was aimed at the determination of the allelic structure of the κ-CN, bGH and PRL genes for the first time in Turkey, using the restriction fragment length polymorphism (RFLP) method in the Holstein, Simmental and Brown Swiss breeds.

MATERIAL and METHODS

Cattle of the Holstein (HL, n=150, from Kayseri, Kahramanmaraş, Ankara), Simmental (S, n=50, from Kayseri) and Brown Swiss (BS, n=50, from Kayseri, Çorum) breeds, including those obtained by the Cattle Breeders Association of Turkey from several different farms in 2010, constituted the material of the study. The DNA used in the study was isolated using the phenol-chloroform extraction method. The determination of the κ-CN-*Hind*III, bGH-*Alu*I and PRL-*Rsa*I polymorphisms in the cattle breeds investigated was performed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as described by Chrenek *et al.*[11].

The genotypic structure and allele frequency of the individual animals used in the trial were determined by means of gene counts. The Hardy-Weinberg equilibrium of the breeds included in the trial for the loci investigated was analysed using the Chi-square test. This statistical analysis was performed using the Pop Gene software package program version 1.32 [12].

RESULTS

The PCR performed for the κ-CN gene produced a single band of 443 bp length for the samples assayed. Following enzymatic digestion with *Hind*III endonuclease for the kappa-casein gene, in animals of the homozygote BB genotype two bands of a length of 348 bp and 95 bp; in animals of the AB genotype three bands of 443, 348 and 95 bp length, and in animals of a homozygote AA genotype a single band of 443 bp length were observed *(Fig. 1)*.

Following enzymatic digestion with *Hind*III for the kappa-casein gene, the highest rates of the genotypes AA and

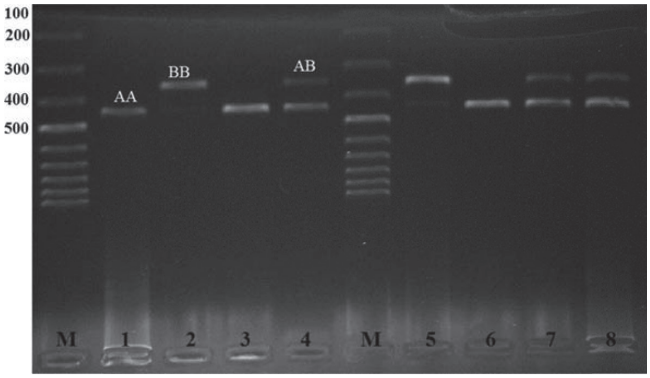

Fig 1. *Hind*III enzyme digestion products of different κ-CN genotypes. M 100 bp DNA ladder; 1, 3 and 6 AA (443 bp) individuals genotyped: 2 and 5 BB (348, 95 bp) individuals genotyped: 4, 7 and 8 AB (443, 348, 95 bp) individuals genotyped

Şekil 1. *Hind*III enzimi ile kesim sonucu elde edilen farklı κ-CN genotipleri. M 100 bç'lik DNA cetveli; 1, 3 ve 6 AA (443 bç) genotipli bireyler: 2 ve 5 BB (348, 95 bç) genotipli bireyler: 4, 7 ve 8 AB (443, 348, 95 bç) genotipli bireyler

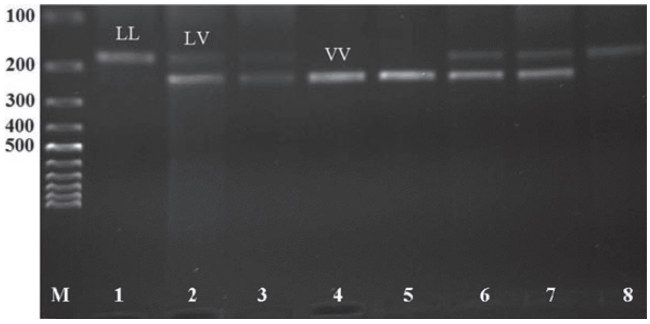

Fig 2. *Alu*I enzyme digestion products of different bGH genotypes. M; 100 bp DNA ladder; 1 and 8; individuals of the LL (171, 52 bp) genotype: 4 and 5; individuals of the VV (223 bp) genotype: 2, 3, 6 and 7; individuals of the LV (223, 171, 52 bp) genotype

Şekil 2. *Alu*I enzimi kesim sonucu elde edilen farklı κ-CN genotipleri. M 100 bç'lik DNA merdiveni; 1 ve 8; LL (171, 52 bç) genotipli bireyler: 4 and 5; VV (223 bç) genotipli bireyler: 2, 3, 6 and 7; LV (223, 171, 52 bç) genotipli bireyler

BB were determined in the HL and BS breeds, respectively, whilst the highest rate of the genotype AB was ascertained in the S and BS breeds. The analysis of the samples pertaining to the breeds investigated revealed that the frequency of the A allele was greater than that of the B allele in the S and HL breeds, whilst in the BS breed the frequency of the B allele was greater than that of the A allele. The genotype and allele frequencies of the cattle breeds investigated are shown in *Table 1*.

PCR performed for the growth hormone gene produced a single band of 223 bp length in the samples. Digestion of the PCR products obtained for the growth hormone gene with the enzyme *Alu*I endonuclease resulted in a single band of 223 bp in animals of the VV genotype, three bands of 223, 171 and 52 bp length in animals of the LV genotype, and two bands of 171 and 52 bp length in animals of the LL genotype *(Fig. 2)*.

According to the bands produced by the digestion of the PCR products pertaining to the samples analysed for the growth hormone gene by the enzyme *Alu*I endonuclease, the highest frequency of the genotype LL was determined in the BS breed, whilst the highest frequency of the genotypes VV and LV was determined in the S breed. In all of the cattle breeds investigated, the frequency of the L allele was higher than that of the V allele. However, the frequency of the V allele was highest in the S breed, compared to the other two cattle breeds investigated. The genotypes and alleles of the cattle breeds investigated in the present study are given in *Table 2*.

PCR analysis for the prolactin gene produced a single band of 156 bp length in the samples assayed. Digestion for the prolactin gene by *Rsa*I endonuclease resulted in a

Table 1. *Allele and genotype frequencies of the κ-CN locus in the S, BS and HL cattle breeds*
Tablo 1. *S, BS ve HL sığır ırklarında κ-CN lokusunun allel ve genotip frekansları*

Breed	n	Genotype						Allele Frequency		χ2	χ2 p-value
		AA		BB		AB		A	B		
		Obs (Exp)	F.	Obs (Exp)	F.	Obs (Exp)	F.				
S	50	27 (25.8182)	0.54	5 (3.8182)	0.10	18 (20.3636)	0.36	0.7200	0.2800	0.694249	0.404723^{NS}
BS	50	9 (9.5556)	0.18	15 (15.5556)	0.30	26 (24.8889)	0.52	0.4400	0.5600	0.101744	0.749746^{NS}
HL	150	105 (101.6087)	0.70	8 (4.6087)	0.05	37 (43.7826)	0.25	0.8233	0.1767	3.659409	0.055753^{NS}

n: *number of animals;* ***S:*** *Simmental;* ***BS:*** *Brown Swiss;* ***HL:*** *Holstein;* ***Obs:*** *Observed;* ***Exp:*** *Expected;* ***χ2:*** *Chi-square;* ***NS:*** *Non Significant;* ***F:*** *Frequency*

Table 2. *Allele and genotype frequencies of the bGH locus in the S, BS and HL cattle breeds*
Tablo 2. *S, BS ve HL sığır ırklarında bGH lokusunun allel ve genotip frekansları*

Breed	n	Genotype						Allele Frequency		χ2	χ2 P-value
		LL		VV		LV		L	V		
		Obs (Exp)	F.	Obs (Exp)	F.	Obs (Exp)	F.				
S	50	25 (21.6667)	0.50	9 (5.6667)	0.18	16 (22.6667)	0.32	0.6600	0.3400	4.434	0.035*
BS	50	38 (35.2121)	0.76	4 (1.2121)	0.08	8 (13.5758)	0.16	0.8400	0.1600	8.922	0.002**
HL	150	115 (109.1639)	0.77	9 (3.1639)	0.06	26 (37.6722)	0.17	0.8533	0.1467	14.693	0.000***

n: *number of animals;* ***S:*** *Simmental;* ***BS:*** *Brown Swiss;* ***HL:*** *Holstein;* ***Obs:*** *Observed;* ***Exp:*** *Expected;* ***χ2:*** *Chi-square;* ****,**,***** *significant (P<0.05, P<0.01, P<0.001);* ***F:*** *Frequency*

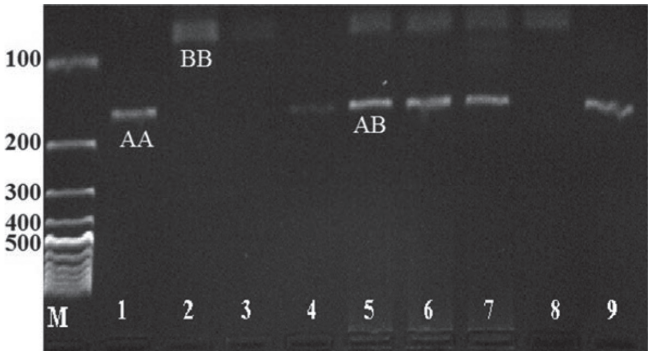

Fig 3. *Rsa*I endonuclease enzyme digestion products of different PRL genotypes. M; 100 bp DNA ladder: 1, 4 and 9; individuals of the AA (156 bp) genotype: 2, 3 and 8 individuals of the BB; (82, 74 bp) genotype: 5, 6 and 7; individuals of the AB (156, 82, 74 bp) genotype

Şekil 3. *Rsa*I enzimi ile kesim sonucu elde edilen farklı PRL genotipleri. M 100 bç'lik DNA cetveli; 1, 4 ve 9; AA (156 bç) genotipli bireyler: 2, 3 ve 8 BB; (82, 74 bç genotipli bireyler: 5, 6 ve 7; AB (156, 82, 74 bç) genotipli bireyler

breeds raised in Turkey, namely, the HL, S and BS breeds, for the κ-CN, PRL and bGH genes.

Kappa-casein (κ-CN)

It has been reported that the alleles of the kappa-casein gene, excluding the A and B alleles, are found in only certain cattle breeds and at a rather low frequency [3]. The most frequently observed alleles in cattle breeds, namely, the A and B alleles, have emerged consequential to a base change in the κ-CN gene [14]. In domestic cattle breeds, the frequency of the κ-CN-A allele has been found to be highest in the HL, compared to the dairy breeds Jersey and Guernsey [10]. Similarly, reports on HL cattle raised in the United Kingdom [15], Argentina [10] and Italy [16] point out to the frequency of the κ-CN-A allele being greater than that of the B allele. In agreement with these reports, it was ascertained in the present study that the frequency of the κ-CN-A allele

Table 3. *Allele and genotype frequencies of the PRL locus in the S, BS and HL cattle breeds*
Tablo 3. *S, BS ve HL sığır ırklarında PRL lokusunun allel ve genotip frekansları*

Breed	n	Genotype						Allele Frequency		χ2	χ2 P-value
		AA		BB		AB		A	B		
		Obs (Exp)	F.	Obs (Exp)	F.	Obs (Exp)	F.				
S	50	32 (32.7273)	0.64	1 (1.7273)	0.02	17 (15.5455)	0.34	0.8100	0.1900	0.458480	0.498335^{NS}
BS	50	27 (28.7879)	0.54	1 (2.7879)	0.02	22 (18.4242)	0.44	0.7600	0.2400	1.951590	0.162416^{NS}
HL	150	115 (114.3512)	0.77	3 (2.3512)	0.02	32 (33.2977)	0.21	0.8733	0.1267	0.233304	0.629085^{NS}

n: *number of animals;* ***S:*** *Simmental;* ***BS:*** *Brown Swiss;* ***HL:*** *Holstein;* ***Obs:*** *Observed;* ***Exp:*** *Expected;* ***χ2:*** *Chi-square;* ***NS:*** *Non Significant;* ***F:*** *Frequency*

single band of 156 bp length in animals of the genotype AA, three bands of 156, 82 and 74 bp length in animals of the genotype AB, and two bands of 82 and 74 bp length in animals of the genotype BB *(Fig. 3)*.

In the samples analysed for the prolactin gene, the highest frequency of the genotype AA was determined in the HL breed. In all three cattle breeds investigated in the present study, the frequency of the genotype BB was found to be equal. The highest frequency of the genotype AB was determined in the BS breed. While the frequency of the A allele was greater than that of the B allele in the HL breed, the frequency of the B allele was highest in the BS breed. The genotypes and the allele frequencies of the cattle breeds investigated in the present study are shown in *Table 3*.

DISCUSION

Today, in farm animal breeding, it is aimed to predict the genetic value of potential breeder animals with greater accuracy and to achieve rapid genetic improvement through selection. In this context, the hypothesis suggesting that variations in certain genes, which affect physiological processes, are correlated with variations in quantitative traits has made a breakthrough in animal improvement efforts [13]. The present study was aimed at the investigation of the genetic structure of the three major imported cattle

(0.82) was greater than that of the B allele in the HL breed. In a study conducted on milk protein polymorphism, Oner and Elmaci [17] reported that, in HL cattle raised in Bursa province, the frequency of the genotype AA was greater than that of the other genotypes. Similarly, in the present study, the frequency of the genotype AA was higher than that of the other genotypes. However, Oner and Elmaci [17] reported that the animals they investigated displayed deviation from the Hardy-Weinberg (HW) equilibrium for the κ-CN locus. The underlying reason may be the material of the study comprising only HL cattle raised in Bursa province. In the present study, in which 150 cattle raised in Kayseri province and its vicinity was investigated, it was ascertained that the HW equilibrium was maintained for the κ-CN locus. However, no information was available on the phylogenetic relation between the individuals. In the present study, both female animals raised in the Kayseri province and female animals supplied by the Cattle Breeders' Association from different provinces were used. Particular attention was paid to avoid the animals being dam and daughter and progeny of the same sire. Thereby, it is considered that an appropriate representation of the HL breed raised in Turkey was achieved in the present study. Compared to studies conducted in the HL breed, there are very few literature reports available on the κ-CN gene in the S and BS breeds. In very few studies conducted in the BS breed, it has been reported that, differently from

the HL, the frequency of the κ-CN-B allele was higher than that of the A allele [15]. In compliance with these reports, in the present study, in which 50 BS cattle were investigated, the frequency of the B allele (0.55) was higher than that of the A allele. Previous research has shown that, in the S, which is a dual-purpose breed, the frequency of the κ-CN-A allele is higher than that of the B allele. However, it has been reported that, differently from the HL, in the S, the difference between the frequencies of the two alleles is not big [18]. In the present study, the frequency of the κ-CN-A allele (0.62) was greater than that of the B allele. However, this frequency was found to be lower than that of the HL (0.82). The data obtained for the alleles of the κ-CN gene in all three cattle breeds investigated in the present study were in compliance with data previously reported from different parts of the world.

In research aimed at the genetic characterization of cattle breeds, it was determined that the frequency of the κ-CN-B allele was higher in breeds originating from the *Bos taurus*, compared to breeds originating from the *Bos indicus* [19]. These results show that data related to κ-CN gene polymorphism could be used in the genetic identification of cattle breeds as well as in the determination of the genetic origin of breeds and the genetic relations between different breeds. However, the frequency of neither the κ-CN allele nor genotypes should be assessed alone when determining differences or phylogenetic relations between breeds. For such targets, other loci should also be assessed. The present study is the first research, in which the κ-CN alleles have been determined in the HL, S and BS breeds raised in Turkey.

Growth Hormone (bGH)

In cattle breeding, it has been reported that polymorphisms of the bGH gene are correlated with milk yield traits such as milk yield and milk composition [20] as well as with meat yield traits such as carcass composition and quality [21]. In the bGH gene, enzymatic digestion with *Alu*I following PCR amplification has revealed the presence of two alleles, namely, the V and L alleles [1], whilst enzymatic digestion with *Msp*I has revealed the presence of two alleles, namely, the +/- alleles [7]. In the present study, which was aimed at the investigation of the allelic structure of the bGH gene in HL, S and BS cattle raised in Turkey, digestion was performed using the enzyme *Alu*I. In the HL cattle raised in Poland [20], Hungary [2], Russia [10] and Australia [22], it has been determined that the frequency of the bGH-L allele is higher than that of the V allele. Similarly, in the present study, in which 150 female HL were investigated, the frequency of the bGH-L allele (0.85) was found to be higher than that of the V allele. In previously conducted studies, the frequency of the genotype LL has been reported to be higher than that of the other genotypes [2,20]. Similarly, in the present study, it was ascertained that the frequency of the genotype LL (0.77) was higher than that of the other genotypes. On the other hand, previous studies have shown that in the S and BS breeds, the frequency of the bGH-L allele is higher than the frequency of the V allele [23]. Similarly, in the present study, the frequency of the L allele was higher than that of the V allele in both the S (0.66) and the BS (0.84) breeds. Of the three cattle breeds investigated, the S displayed the highest frequency for the genotype LV. In another study, in which of the milk-type cattle breeds, the HL, and of the meat-type cattle breeds, the Limousine, Charolaise, Piemontese, Angus and Hereford were investigated for the bGH locus, it was determined that the frequency of the L allele (0.86) was higher in the HL, whilst in the meat-type breeds the frequency of the V allele was higher (0.62) [10]. These results show that, in selection programmes, the bGH locus could be used to select breeder animals with a genotype appropriate for the type of breeding. It has been reported that, the S, which is of the LV genotype, is characterized by greater body weight gain and richer carcass composition, and has a V allele frequency of 0.32 [24]. Similarly, in the present study, in the S, the frequency of the V allele (0.34) was higher than that of the other two cattle breeds investigated.

Furthermore, in a study conducted in seven cattle breeds raised in Brazil and originating from the *Bos taurus* and *Bos indicus*, which were investigated for the bGH gene, in the cattle breeds originating from the *Bos taurus*, two alleles, namely, the L and V alleles were determined to exist; whilst the cattle breeds originating from the *Bos indicus* were monomorphic and possessed only the L allele [10]. In another study, it was demonstrated that, in cattle breeds originating from the *Bos indicus*, the L allele was either monomorphic or had a higher frequency than that of the V allele [25]. Therefore, it is considered that the bGH locus could be used in research on the origin of breeds.

Prolactin (PRL)

The bovine PRL locus is reported to have two alleles, namely, the A and B alleles [8]. Previous research has shown that in HL cattle raised in Russia, South Korea [15] and Lithuania [21] the frequency of the PRL-A allele is higher than that of the B allele. In the HL cattle investigated in the present study, the frequency of the PRL-A allele (0.87) was higher than that of the B allele. In BS cattle raised in Slovakia, it was found out that the frequency of the PRL-A allele was higher than the frequency of the B allele, yet, the frequency of the A allele was not as high as that determined in cattle of the HL breed [4]. In the present study, in the 50 BS cattle investigated, the frequency of the PRL-A allele (0.76) was found to be higher than that of the B allele, yet of the three cattle breeds investigated, the BS displayed the highest frequency for the PRL-B allele (0.24). Again, in the present study, in samples pertaining to the 50 S cattle investigated, the frequency of the PRL-A allele (0.81) was higher than the frequency of the B allele. The number of studies on the PRL gene in the S is rather limited. Therefore, it is considered that the results of the present study will contribute to literature by providing

data on the allelic structure and allele frequencies of the PRL gene in the S breed.

The association between bGH genotypes and milk yield traits has been reported in cattle [5]. It is considered that the bGH-AluI polymorphism can be used as a potential marker for the milk yield traits [10]. The polymorphism of the κ-CN gene has been studied extensively in cattle breeds [26, 27]. The relationship between the κ-CN alleles and some milk yield traits such as the total protein content of milk, fat percentage of milk and milk production, has been reported in previous studies [28]. The κ-CN-B allele has been indicated to have a significant effect on milk yield traits [16]. Also, it has been reported that the κ-CN-B allele has a favourable effect on technological milk properties [26]. Several authors have examined the effect of the PRL-*Rsa*I AA, AB and BB genotypes on some milk yield traits [10]. The highest milk and milk fat yields were obtained in cows of the BB genotype [19].

Currently available data is not sufficient to confirm the use of the κ-CN, PRL and bGH gene polymorphisms as markers for selection in cattle breeding. Further studies are required to determine the correlation between the variants of the genes coding the κ-CN protein and bGH and PRL hormones, which affect milk yield, and milk yield parameters. Furthermore, it is considered that these variants could be used for the development of appropriate test systems for use in genetic research and animal breeding programmes. The present study has demonstrated, for the first time, polymorphisms of the κ-CN, PRL and bGH genes in HL, S and BS cattle raised in Turkey.

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RESEARCH ARTICLE

Chewing Lice (Phthiraptera) of Magpie (*Pica pica* L.) (Aves: Passeriformes: Corvidae) in Turkey

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Summary

This study was performed to detect chewing lice on Magpie, between the dates of August 2011-February 2012 in Konya, Central Anatolian Region of Turkey. For this aim, 64 Magpie individuals were examined for the louse. In order to sample chewing lice, the feathers of the birds were carefully examined macroscopically. All lice were collected and placed in tubes with 70% alcohol, cleared in 10% KOH mounted in Canada balsam on slides and identified under a light microscope. Of birds examined, 20 out of 64 (31.25%) were infested with at least one chewing louse species. Three lice species which belongs to the three genera, *Menacanthus eurysternus* (Burmeister, 1838), *Myrsidea picae* (Linnaeus, 1758) and *Brueelia biocellata* (Piaget, 1880) were found. *Brueelia biocellata* is the first ever recorded in Turkey.

Keywords: *Menacanthus eurysternus, Myrsidea picae, Brueelia biocellata, Magpie, Turkey*

Türkiye'de Saksağanlarda (*Pica pica* L.) (Aves: Passeriformes: Corvidae) Görülen Bit (Phthiraptera) Türleri

Özet

Bu çalışma saksağanlardaki (Pica pica) bit türlerini belirlemek amacıyla Ağustos 2011-Şubat 2012 tarihleri arasında Konya'da yapılmıştır. Bu amaçla 64 saksağan, bit yönünden incelenmiştir. Bitleri toplamak için kuşların tüyleri makroskopik olarak dikkatli bir şekilde incelenmiştir. Toplanan bitler, %70 alkol bulunan tüplere konulup %10 KOH'de saydamlaştırıldıktan sonra Kanada balsam ile lamlar üzerine yapıştırılarak ışık mikroskobunda teşhis edilmişlerdir. İncelenen 64 kuşun 20 (%31.25)'si en azından bir bit türü ile enfeste bulunmuştur. Enfeste kuşlarda üç cinse ait üç bit türü; *Menacanthus eurysternus* (Burmeister, 1838), *Myrsidea picae* (Linnaeus, 1758) ve *Brueelia biocellata* (Piaget, 1880) tespit edilmiştir. Bu türlerden *Brueelia biocellata* Türkiye'den ilk kez bildirilmektedir.

Anahtar sözcükler: *Menacanthus eurysternus, Myrsidea picae, Brueelia biocellata, Saksağan, Türkiye*

INTRODUCTION

Magpie (*Pica pica*) is a bird which belongs to the family Corvidae, in the order Passeriformes. Its length is about 46 cm, the feathers on the ventral side of abdomen and shoulders are white, the other sides are black and metallic green on the tail, and their apical parts are metallic blue. Magpies live on the trees in the parks, gardens and shrubbery and feed by insects, roundworms. This bird is found very common in Turkey, Chine and Palearctic Region [1]. Magpies were shot by hunters and some farmers, because they usually destroyed planted lands.

Approximately 4000 species of chewing lice (Phthiraptera) have been recorded on birds worldwide [2]. Price et al.[2] reported that, *Menacanthus eurysternus* (Burmeister, 1838), *Myrsidea picae* (Linnaeus, 1758), *Brueelia biocellata* (Piaget, 1880) and *Philopterus picae* (Denny, 1842) were found on magpie over the world. However, the chewing lice fauna of magpie is little known in Turkey. Although, three magpies were examined for louse up today, two of them were found to be infested by the lice, and two lice species; *Menacanthus eurysternus* and *Myrsidea picae* were detected on the magpies in the study [3].

This study was performed to detect the lice species found on Magpie in Turkey.

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MATERIAL and METHODS

This study was done between the months August 2011-February 2012 in Konya. No Magpie was examined in September, in this period. In this study, 64 Magpies shot by the hunters in this season were examined for chewing lice. In order to collect the lice, the feathers of each bird were carefully examined. All lice were collected and placed in tubes with 70% alcohol. The lice specimens were cleared about 24 h in 10% KOH, and then kept for one day in distilled water. Following dehydration in a graded alcohol series (70%, 80%, 90% and 99%, in consecutive days), the specimens were mounted in Canada balsam on the slides. After dried in incubator, the lice specimens were identified under a light microscope (Leica DM750). The identification of the lice was carried out according to relevant literatures [4-7].

RESULTS

Of the birds examined, 20 out of 64 (31.25%) were infested with at least one chewing louse species and three lice species; *Brueelia biocellata* (Piaget, 1880), *Myrsidea picae* (Linnaeus, 1758) and *Philopterus picae* (Denny, 1842) were identified. *Menacanthus eurysternus (Fig.1)* was the most common species and it was found on 15 magpies. *Brueelia biocellata* (*Fig. 2* and *Fig. 3*) and *M. picae (Fig. 4)* were detected on three and two birds, respectively. In all cases, only one louse species was detected on the birds.

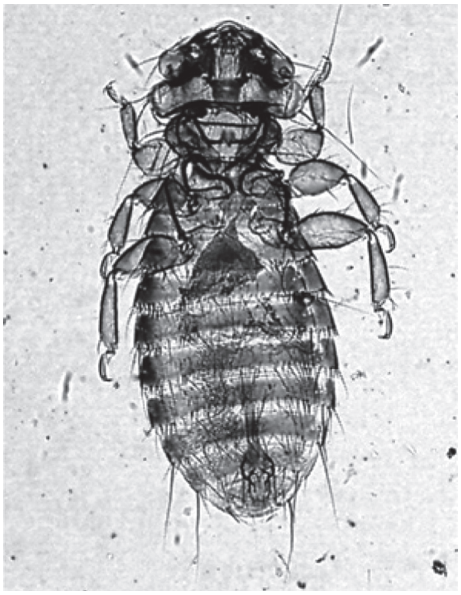

Fig 1. *Menacanthus eurysternus*, male, original
Şekil 1. *Menacanthus eurysternus*, erkek, orijinal

Fig 2. *Brueelia biocellata*, male, original
Şekil 2. *Brueelia biocellata*, erkek, orijinal

Fig 3. *Brueelia biocellata*, female, original
Şekil 3. *Brueelia biocellata*, dişi, orijinal

DISCUSSION

Approximately, 4000 valid lice species were found on the birds throughout the world [2]. There are 468 bird species recorded in Turkey so far and the actual total is likely to exceed 500 species [8]. However, almost all of the louse fauna of these birds are still unknown.

Price et al.[2] stated that four lice species; *M. eurysternus, M. picae, B. biocellata* and *P. picae* were found on magpies. Dik et al.[3] reported that the louse infestation rates on songbirds' (Passeriformes) are very low rates, and only two lice species were detected on the magpies in a study in Kars, Turkey. In this study, three lice species; *M. eurysternus, M. picae* and *B. biocellata* were detected on the magpies,

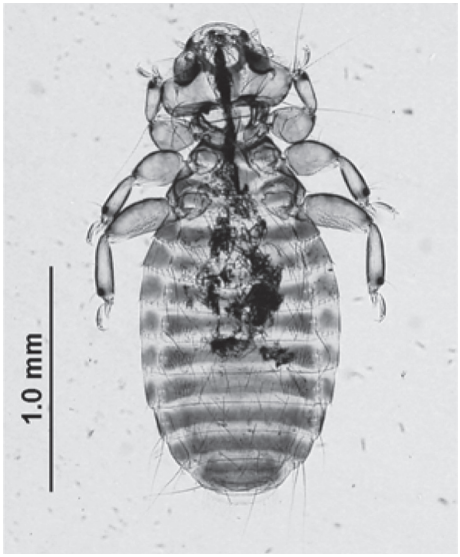

Fig 4. *Myrsidea picae*, female, original
Şekil 4. *Myrsidea picae*, dişi, orijinal

while *P. picae* was not found on the birds. *Menacanthus eurysternus* was the very common species and it was found on 15 magpies. This species was collected in huge numbers on the birds. *Brueelia biocellata* and *M. picae* were found on a few birds and they were collected lower numbers.

Menacanthus eurysternus is a cosmopolitan species and it has been occurred on many bird species in the order Passeriformes [2,6]. Kettle [9] stated that the menoponids louse species on starlings were found the most prevalent in August and September, while philopterids were found in June and July. So; *M. eurysternus* had been collected on the starlings the most numbers in August and September [9]. Chandra et al.[10] reported that *M. eurysternus* has detected the lowest rate in January on myna *(Acridotheres tristis)* and infestation rate of this species has decreased in autumn and winter seasons, increased in spring and summer seasons. Although, no literatures found about the seasonal variations of this louse species on magpies. Recently, this species was found on European robin *(Erithacus rubecula)*, sparrow *(Passer domesticus)* and starlings *(Sturnus vulgaris)* in Turkey in autumn and winter seasons [11]. In this study, 13 out of 20 magpies examined in August and two of 23 in October were found to be infested by the louse specimens. No louse individual was detected on 11 and 10 magpies examined in December and January, respectively. *M. eurysternus* and *M. picae* was found in August and October, *B. biocellata* was found on the magpie in August. Hunting many of the bird species including magpie is forbidden between the middle of August until the end of February in Turkey [12]. 64 magpies were examined in autumn and winter seasons, while no magpie samples were examined due to hunting was forbidden in spring and summer seasons in Turkey. In spite of this, *B. biocellata* on three, *M. picae* on one of magpies examined in October were found in this study. Both species could not be detected on the magpies in the other months.

It was reported that *P. picae* was occurring on magpie [13]. This species also recorded from a magpie in Iran [14]. But, *P. picae* was not detected on the magpie in this study.

As a result, it was detected that 20 out of 64 (31.25%) were infested by the louse and three lice species; *M. eurysternus, M. picae* and *B. biocellata* were detected on magpies and *B. biocellata* was recorded for the first time in Turkey.

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RESEARCH ARTICLE

Tocolytic Effects of Diclofenac Potassium and Diclofenac Sodium on Cattle Myometrium Pre-Incubated with PCB-153 [1]

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[1] *This study was presented as a poster presentation at the 12th International Congress of the European Association for Veterinary Pharmacology and Toxicology (EAVPT) from 08 to 12 July, 2012 in Noordwijkerhout, the Netherlands*

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Summary

In this study, the tocolytic effects of the potassium and sodium salts of diclofenac, a non-steroidal anti-inflammatory drug (NSAID), on cattle myometria were investigated in the presence of an estrogenic polychlorinated biphenyl congener 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153). The uterine samples were obtained from newly slaughtered, non-pregnant cattle more than two years old and in the anoestrus stage. Eight groups were constituted for testing (each group contained 8 strips from two animals, with 4 from each); there were four control groups (diclofenac potassium 24 h and 48 h; diclofenac sodium 24 h and 48 h) and four groups for PCB-153 (diclofenac potassium 24 h and 48 h; diclofenac sodium 24 h and 48 h). The control groups were incubated in physiological salt solution (PSS) for 24 or 48 hours and the PCB-153 groups were incubated in PSS which contained 100 ng/mL of PCB-153, for either 24 h or 48 h. After the incubation period, the strips were hung and incubated in the tissue bath system until spontaneous contractions occurred. Oxytocin (OT) was administered at 0.5 nM to the strips in all groups for the stimulation of spontaneous contractions. Diclofenac potassium and diclofenac sodium were then administered cumulatively in the range of $1x10^{-7}$ to $7x10^{-4}$ M to the strips of all groups to the maximum inhibitory effect was observed. After each application, isometric uterine contractions were recorded for 20 min. Mean peak amplitude (P_{MAX}), frequency (beats per minutes, BPM) and area under the curve (AUC) values of the myometrial contractions, which are criteria for determining drug effects, were calculated from the curve and evaluated statistically. The inhibitory concentration 50 (IC_{50}) values were calculated for the BPM, P_{MAX} and AUC values of the myometrial contractions. Finally, the tocolytic effects of diclofenac potassium and diclofenac sodium on the contractions of cattle uterine strips pre-incubated with PCB-153, were determined.

Keywords: *Diclofenac potassium, Diclofenac sodium, PCB-153, Cattle, Tocolysis*

PCB-153 İle İnkübe Edilen Sığır Miyometriumu Üzerine Diklofenak Potasyum ve Diklofenak Sodyumun Tokolitik Etkisi

Özet

Bu çalışmada, steroid yapıda olmayan ağrı kesici ve yangı giderici ilaçlardan (non steroidal anti-inflammatory drugs, NSAIDs) diklofenakın potasyum ve sodyum tuzlarının poliklorlu bifenil (PCB)'lerden östrojenik etkili 2,2',4,4',5,5'-hekzaklorobifenil (PCB-153) varlığında sığır uterus düz kaslarını gevşetici etkisi araştırılmıştır. Uterus örnekleri mezbahada iki yaşın üzerinde, yeni kesilen, anöstrüs evresinde, gebe olmayan ineklerden sağlanmıştır. İlaçların etkisini ortaya koymak için, dört kontrol (diklofenak potasyum 24 ve 48 saat, diklofenak sodyum 24 ve 48 saat) ve dört PCB-153 (diklofenak potasyum 24 ve 48 saat, diklofenak sodyum 24 ve 48 saat) olmak üzere sekiz ayrı grup oluşturulmuştur. Her bir grup iki farklı hayvandan dörder olmak üzere toplam sekiz miyometrium şeridi içermektedir. Kontrol grupları ayrı ayrı 24 ve 48 saat süre ile fizyolojik tuz solüsyonu (physiological salt saline, PSS) içinde, PCB-153 grupları ise aynı sürelerle 100 ng/mL PCB-153 içeren PSS içinde bekletilmiştir. Bu süreler sonrasında miyometrium şeritleri izole doku banyosu sistemine asılarak spontan kasılmalar oluşuncaya kadar beklenmiştir. Tüm gruplara spontan kasılmaları teşvik etmek amacı ile 0.5 nM yoğunluğunda oksitosin (OT) uygulanmıştır. Daha sonra diklofenak potasyum ve diklofenak sodyum kendileri için oluşturulan kontrol ve PCB-153 gruplarına $1x10^{-7}$ ve $7x10^{-4}$ M arası yoğunluklarda en yüksek inhibitör etki görülünceye kadar kümülatif olarak uygulanmıştır. Her uygulama sonrası uterus kasılmaları 20 dk süre ile kaydedilmiştir. Elde edilen grafiklerden ilaç etkisinin ölçütleri olan ortalama pik yükseklikleri (P_{MAX}), frekansları (dakikadaki pik sayısı, beats per minute-BPM) ve eğrinin altında kalan alan (EAA) değerleri hesaplanmış ve istatistiksel olarak değerlendirilmiştir. Ayrıca miyometrium kasılmalarının BPM, P_{MAX} ve EAA değerleri için inhibitör yoğunluk 50 (IC_{50}) değerleri hesaplanmıştır. Çalışma sonunda diklofenak potasyum ve diklofenak sodyumun önceden PCB-153 ile inkübe edilen sığır miyometrium şeritleri üzerine tokolitik etkisi ortaya konmuştur.

Anahtar sözcükler: *Diklofenak potasyum, Diklofenak sodyum, PCB-153, Sığır, Tokoliz*

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INTRODUCTION

Polychlorinated biphenyls (PCBs) are a class of chlorinated organic compounds which was widely used, particularly in the manufacturing of electrical equipment, because of their favourable dielectric properties and chemical stability [1]. Although many countries have banned or restricted the use of PCBs, their distribution in nature is still ubiquitous. PCBs are bioaccumulated in lipid tissue and biomagnified along food chains. A variety of the 209 PCB congeners are, to different extents, detectable in environmental sampling and in human and animal tissues [2]. These compounds may be responsible for shortening the duration of pregnancy in women. Moreover, a mixture of PCBs (Aroclor 1254) was found to induce abortion in monkeys [3]. PCBs are also suspected of affecting cell function via the estradiol receptor (ER), as agonists or antagonists, and via the aryl-hydrocarbon receptor (AhR). It is well-known that PCB 153, and to some extend PCB 77, have estrogen - like properties. Although some PCBs have a lower affinity for the ER than estradiol, they can stimulate OT secretion from granulosa cells at a higher level than estradiol [4].

OT, a non-peptide hormone, produces uterine and mammary gland contractions. Both tissues contain OT receptors (OTRs). The number of these receptors is increased by estrogen and decreased by progesterone [5]. OT is secreted from granulosa cells in cattle is estradiol-dependent and is involved in growth, maturation and ovulation of follicles [6]. Several studies in ewes have shown that estradiol administration *in vivo* can stimulate endometrial OTR expression [7]. Thus, the concomitant rise in estrogen and fall in progesterone levels that occur before parturition can cause increased sensitivity to OT and result in labour. The drug is used clinically for the induction of labour and to stimulate lactation [5]. In the first days of the estrus cycle, OT is involved in the establishment of the corpus luteum and then OT of luteal origin amplifies luteolysis at the end of the cycle. Hence, disruptions to the synthesis or secretion of OT can impair the estrus cycle and reduce fertility of cattle [6].

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is released from the uterus in large amounts during parturition, and this is commonly assumed to initiate labour contractions in pregnant cows [8]. PGs are well-known uterine contraction inducer by enhancing myometrial gap junction and increasing intracellular calcium concentration [9]. The increase in PG during labour causes increased uterine contractility and inhibitors of PG synthesis delay the onset of labour [10]. OT is a much more potent myometrial stimulant than $PGF_{2\alpha}$ and causes contractions in picomolar concentrations rather than at nanomolar levels. Moreover, the release of PGs from intact tissues *in vivo* is rarely spontaneous and usually occurs in response to some kind of stimulus [8]. The force of myometrial contractions is increased by OT and $PGF_{2\alpha}$. The ability of OT to stimulate contractions of the myometrium rises around ovulation because of an estradiol-dependent increase in the number of OTRs [11].

In obstetrics and gynecology, NSAID's have long been used to control acute and chronic postoperative pain, menstrual pain, pain related to medical abortions, menorrhagia, intrauterine device, assist in fertility treatment, and administered as tocolytics in preterm labor [10]. But their adverse effects, including premature closure of the ductus arteriosus and reduction in renal perfusion that result in decreased amniotic fluid volume, have limited their use in preterm labour [12]. Diclofenac, one of the NSAIDs, is used for pain control in major surgery. The exact mechanism of action is not entirely understood, but it is thought that the primary mechanism responsible for its anti-inflammatory, antipyretic and analgesic activity is inhibition of PG synthesis by inhibition of cyclooxygenase (COX) activity [13] like the other NSAIDs. COX is divided into three subgroups: COX-1, COX-2, and COX-3. COX-1 is constitutively expressed in many mammalian tissues. On the other hand, COX-2 is induced in response to various stimuli, such as cytokines, bacterial lipopolysaccharide (LPS) and growth factors. COX-3 is a splicing variant derived from the COX-1 gene. This protein is expressed in the central nervous system and is involved in the sensitive neuronal pathway [14]. Diclofenac has a low to moderate ability to block COX-2 and is therefore reported to cause a somewhat lower incidence of gastrointestinal complaints than noted with indomethacin and aspirin [13]. Assessment of inhibitory concentration 50 (IC_{50}) values for COX-1 and COX-2 and the calculation of COX-1/COX-2 IC_{50} ratios for different COX inhibitors show that their biochemical selectivity is a continuous variable. Thus, it is quite difficult to establish COX-1 and COX-2 IC_{50} values that separate non-selective from selective NSAIDs. However, for a cluster of compounds including the profens (ibuprofen, ketoprofen, flurbiprofen) and naproxen, it is apt to classify them as non-selective NSAIDs for their inhibitory effects on both COX-1 and COX-2 activity. The cluster of COX-2 inhibitors should also include the traditional NSAIDs, meloxicam, nimesulide and diclofenac (which are from 18 - to 29 - fold more potent towards COX-2 *in vitro*), and coxibs [15].

When estrogenic activity increases, the risk of preterm labour increases in pregnant animals. PCB-153 is an estrogenic compound. Therefore, the aim of this study was the determination of the tocolytic effect of diclofenac potassium and diclofenac sodium on cattle myometrium pre-incubated with PCB-153 in the isolated tissue bath system.

MATERIAL and METHODS

In this study, the methods described by Çelik et al.[16] and Wrobel et al.[3] were employed. Myometrial strips were obtained from newly slaughtered, non-pregnant cattle more than two years old and in the anoestrus stage at the time of sampling. Extra vascularisation, thickening and caruncles were not observed in the uterine endometriums. Also there was no asymmetry between two uterine horns,

macroscopically. The uterine samples were immediately brought to the laboratory in physiological salt solution (PSS) at 4°C. The PSS for transportation and incubation of the strips in the tissue bath was constituted as follows: 116 mM NaCl, 4.6 mM KCl, 1.16 mM NaH_2PO_4 x $2H_2O$, 1.16 mM $MgSO_4$ x $7H_2O$, 21.9 mM $NaHCO_3$, 1.8 mM $CaCl_2$ x $2H_2O$ and 11.6 mM dextrose (Merck, Germany). Longitudinal myometrial strips (1 cm x 0.2 cm x 0.2 cm) were prepared from the curvature major of the uterine horns. The eight test groups, each containing 8 strips as 4 strips from each of two animals (a Brown Swiss and a Holstein), were constituted as four control groups (diclofenac potassium 24 h and 48 h; diclofenac sodium 24 h and 48 h) and four groups for PCB-153 (diclofenac potassium 24 h and 48 h; diclofenac sodium 24 h and 48 h). The control groups were incubated in PSS and the PCB-153 groups were incubated in PSS which contained 100 ng/mL of PCB-153 [3] (Dr.Ehrenstorfer, Germany) at 4°C for either 24 h or 48 h. After the incubation period, the strips were hung on force transducers (Commat, Turkey) and 2 g of basal tension was applied to the strips in 10 mL tissue baths (95% O_2 + 5% CO_2, pH 7.4, 38°C). They were incubated in tissue baths until spontaneous contractions occurred. OT (Sigma, USA) was administered at 0.5 nM to the strips in all groups to stimulate spontaneous contractions. After that, diclofenac potassium and diclofenac sodium (Novartis, Turkey) were administered cumulatively at $1x10^{-7}$, $1x10^{-6}$, $1x10^{-5}$, $1x10^{-4}$, $2x10^{-4}$, $3x10^{-4}$, $4x10^{-4}$, $5x10^{-4}$, $6x10^{-4}$ and $7x10^{-4}$ M to the strips of both the control and PCB-153 groups until the maximum inhibitory effect was observed. After each application, isometric uterine contractions were recorded with a data acquisition system (Biopac, USA) for 20 min.

P_{MAX}, BPM and AUC values of the myometrial contractions, which are criteria for determining drug effects, were calculated from the curve. Factorial Analysis of Variances (ANOVA), followed by the Tukey's Post Hoc Test, were used for statistical evaluation. The effects of PCB-153 on AUC, P_{MAX} and BPM values for spontaneous cattle uterine contractions before and after OT application were determined with the Independent Samples T Test. Differences were considered significant when P values were less than 0.05. In addition, the IC_{50} values of diclofenac potassium and diclofenac sodium were calculated for the BPM, P_{MAX} and AUC values of the myometrial contractions with the GraphPad Prism (5.0) program.

RESULTS

The effects of diclofenac potassium and diclofenac sodium on the isometric contractions of cattle myometrium pre-incubated with PCB-153 for a 24 h period and stimulated with OT are shown in *Fig. 1* and *2* as examples.

The statistical analyses of AUC, P_{MAX} and BPM showed that the time x drug relationship for both diclofenac potassium and diclofenac sodium was significant ($P<0.05$), while the dose x time, dose x group (control and PCB-153), time x group and dose x time x group relationships were not found significant ($P>0.05$).

The effects of diclofenac potassium and diclofenac sodium on the AUC values of the isometric cattle myometrial contractions are shown in *Fig. 3*. The mean AUC value of the PCB-153 48 h group was found significantly

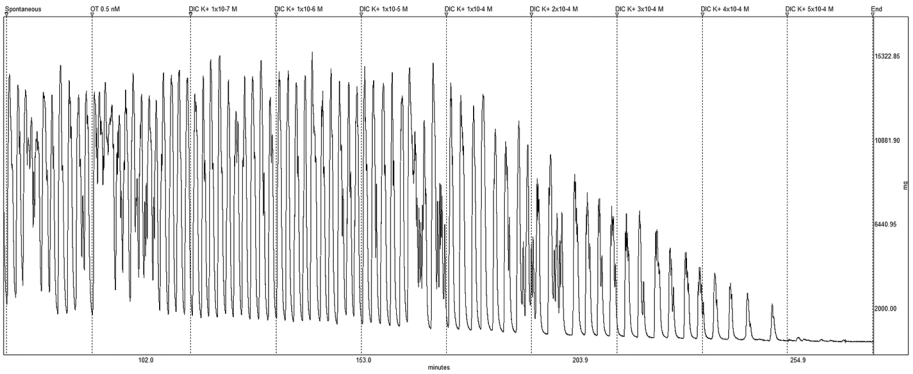


Fig 1. The effects of diclofenac potassium on the isometric contractions of cattle myometrium pre-incubated with PCB-153 for a 24 h period and stimulated with OT

Şekil 1. 24 saat süresince PCB-153 ile inkübe edilen ve OT ile uyarılan izometrik sığır miyometrium kasılmaları üzerine diklofenak potasyumun etkisi

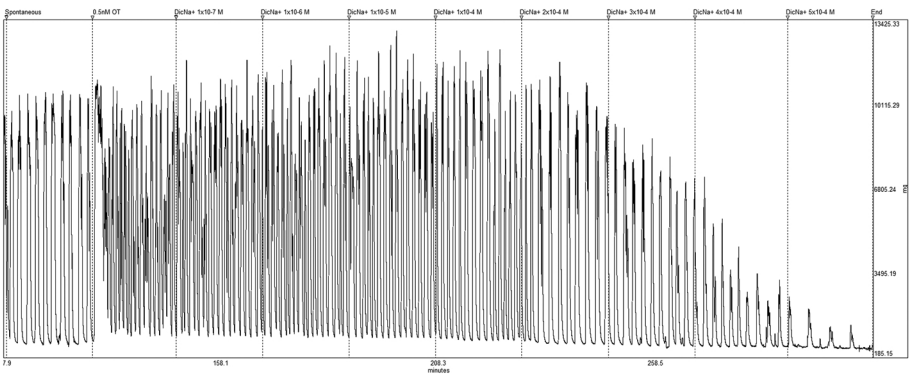


Fig 2. The effects of diclofenac sodium on the isometric contractions of cattle myometrium pre-incubated with PCB-153 for a 24 h period and stimulated with OT

Şekil 2. 24 saat süresince PCB-153 ile inkübe edilen ve OT ile uyarılan izometrik sığır miyometrium kasılmaları üzerine diklofenak sodyumun etkisi

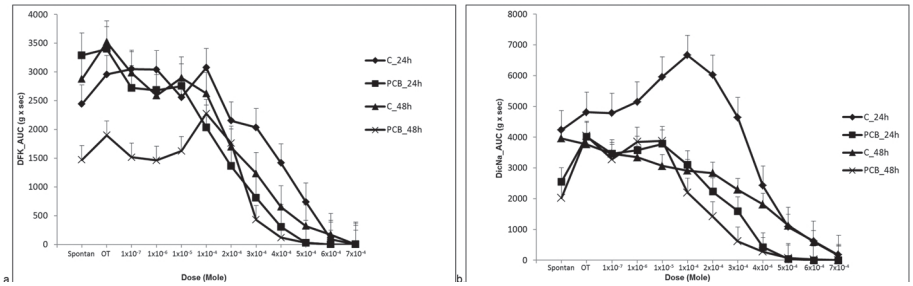
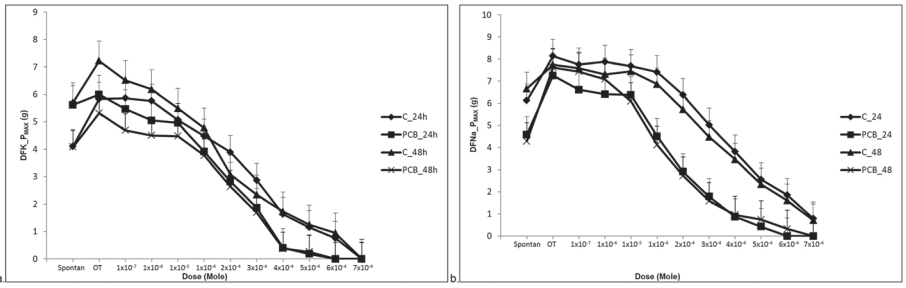


Fig 3. The effects of (a) diclofenac potassium and (b) diclofenac sodium on the AUC values of isometric cattle myometrial contractions

Şekil 3. Diklofenak potasyum (a) ve diklofenak sodyumun (b) izometrik sığır miyometrium kasılmalarının EAA değerine etkisi

Fig 4. The effects of (a) diclofenac potassium and (b) diclofenac sodium on the P_{MAX} values of isometric cattle myometrial contractions

Şekil 4. Diklofenak potasyum (a) ve diklofenak sodyumun (b) izometrik sığır miyometrium kasılmalarının P_{MAX} değerine etkisi



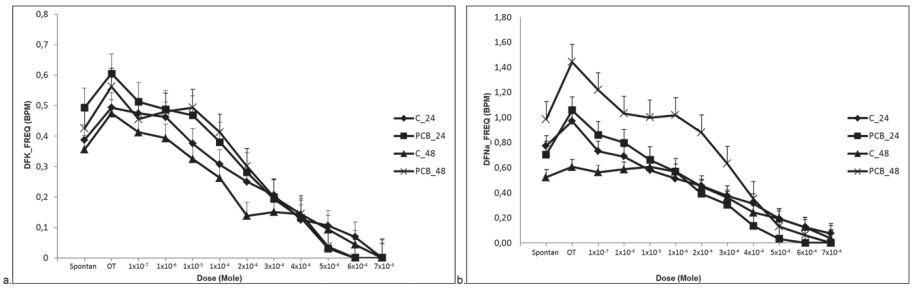


Fig 5. The effects of (a) diclofenac potassium and (b) diclofenac sodium on the BPM values of isometric cattle myometrial contractions

Şekil 5. Diklofenak potasyum (a) ve diklofenak sodyumun (b) izometrik sığır miyometrium kasılmalarının BPM değerine etkisi

Table 1. *The IC_{50} values with 95% confidence limit for mean (CLM) of diclofenac potassium and diclofenac sodium for BPM, P_{MAX} and AUC for cattle uterine contractions*

Tablo 1. *Diklofenak potasyum ve diklofenak sodyumun sığır uterus kasılmalarının BPM, P_{MAX} ve EAA'ları üzerine % 95 güven aralığı ile birlikte IC_{50} değerleri*

Values	Diclofenac Potassium				Diclofenac Sodium			
	Control 24 h	PCB-153 24 h	Control 48 h	PCB-153 48 h	Control 24 h	PCB-153 24 h	Control 48 h	PCB-153 48 h
P_{MAX}	$1.75x10^{-3}$	$5.32x10^{-4}$	$5.19x10^{-4}$	$2.51x10^{-4}$	$5.38x10^{-4}$	$2.25x10^{-4}$	$6.31x10^{-4}$	$2.02x10^{-4}$
95% CLM	$0.46\text{-}6.68x10^{-3}$	$2.28\text{-}12.41x10^{-4}$	$2.50\text{-}10.77x10^{-4}$	$1.95\text{-}3.21x10^{-4}$	$4.00\text{-}7.25x10^{-4}$	$1.81\text{-}2.80x10^{-4}$	$4.00\text{-}9.94x10^{-4}$	$1.32\text{-}3.08x10^{-4}$
BPM	$6.62x10^{-4}$	$7.68x10^{-4}$	$3.05x10^{-4}$	$1.26x10^{-3}$	$1.78x10^{-3}$	$5.25x10^{-4}$	$4.76x10^{-4}$	$3.50x10^{-4}$
95% CLM	$2.11\text{-}20.75x10^{-4}$	$4.10\text{-}14.38x10^{-4}$	$0.10\text{-}9.32x10^{-4}$	$0.42\text{-}3.73x10^{-4}$	$0.23\text{-}13.97x10^{-3}$	$1.99\text{-}13.88x10^{-4}$	$2.34\text{-}9.67x10^{-4}$	$2.61\text{-}4.70x10^{-4}$
AUC	$5.32x10^{-4}$	$2.29x10^{-4}$	$2.86x10^{-4}$	$2.80x10^{-4}$	$3.77x10^{-4}$	$2.75x10^{-4}$	$1.19x10^{-3}$	$1.57x10^{-4}$
95% CLM	$1.46\text{-}19.36x10^{-4}$	$1.76\text{-}2.98x10^{-4}$	$2.04x3.99x10^{-4}$	$0.72\text{-}10.88x10^{-4}$	$3.11\text{-}4.48x10^{-4}$	$2.04\text{-}3.72x10^{-4}$	$0.51\text{-}2.77x10^{-3}$	$0.98\text{-}2.51x10^{-4}$

Table 2. *The effect of PCB-153 on AUC, P_{MAX} and BPM values for spontaneous cattle uterine contractions before and after OT application (%)*

Tablo 2. *PCB-153'ün spontan sığır uterus kasılmalarının AUC, P_{MAX} and BPM değerleri üzerine OT uygulama öncesi ve sonrası etkisi (%)*

Values	Control 24 h	PCB-153 24 h	Control 48 h	PCB-153 48 h
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
AUC	68.54±36.20	37.66±13.08	37.82±19.93 [a]	156.63±44.7 [b]
P_{MAX}	39.24±6.35	32.17±7.68	31.64±8.98 [a]	80.33±21.18 [b]
BPM	34.83±10.87	40.77±8.82	26.83±6.81	43.92±10.57

[a,b] Means within the same row with different letters are significantly different (P<0.05)

different from the other 3 groups (P<0.05) and there was no difference between the other groups for diclofenac potassium (P>0.05). In the 24 h control group, diclofenac sodium was found significantly different from the other groups (P<0.05). For the various doses, there was no difference between the AUC value for spontaneous

contraction and the dose until $1x10^{-4}$ M (P>0.05). However, a significant tocolytic effect was determined after the $2x10^{-4}$ M dose in both diclofenac sodium and diclofenac potassium applications (P<0.05).

The effects of diclofenac potassium and diclofenac sodium on the P_{MAX} values of isometric cattle myometrial contractions are shown in *Fig. 4*. There was no significant difference between the respective control groups and diclofenac potassium and diclofenac sodium applications (P>0.05). However, the PCB-153 24 h and the PCB-153 48 h groups were significantly different from the control groups (P<0.05) for both diclofenac potassium and diclofenac sodium. A significant difference was also observed between the P_{MAX} values for spontaneous contraction and after OT application (P<0.05). This difference disappeared in the $2x10^{-4}$ and $3x10^{-4}$ M concentrations (P>0.05), and a significant tocolytic effect occurred at the doses higher than $4x10^{-4}$ M concentration (P<0.05).

The effects of diclofenac potassium and diclofenac sodium on the BPM values of isometric cattle myometrial contractions are shown in *Fig. 5*. There was no difference between the control and PCB-153 groups for diclofenac potassium (P>0.05) and between the control groups (P>0.05). However, the PCB-153 24 h and the PCB-153 48 h groups were significantly different from their respective control groups (P<0.05) for diclofenac sodium. In addition, the PCB-153 24 h group was significantly different from the PCB-153 48 h group (P<0.05). For doses, the significant difference between BPM values of spontaneous contraction and after OT application (P<0.05) disappeared between the doses from $1x10^{-7}$ to $1x10^{-4}$ M (P>0.05), and the tocolytic effect was significant at higher molarities than $2x10^{-4}$ M for both diclofenac potassium and sodium (P<0.05). The tocolytic effect was significant from $4x10^{-4}$ to $7x10^{-4}$ M (P<0.05), and it peaked in $6x10^{-4}$ and $7x10^{-4}$ M.

The IC_{50} values of diclofenac potassium and diclofenac sodium for the BPM, P_{MAX} and AUC values of cattle myometrial contractions are shown in *Table 1*. The lowest IC_{50} values for P_{MAX} and AUC were determined in the PCB-153 48 h group of diclofenac sodium and the lowest IC_{50} value of BPM was determined in the 48 h control group of diclofenac potassium. The highest IC_{50} value for AUC was in the 48 h control group of diclofenac sodium. The highest IC_{50} value for P_{MAX} was in the 24 h control group of diclofenac potassium and the highest IC_{50} value of BPM was in the 24 h control group of diclofenac sodium.

The effects of PCB-153 on AUC, P_{MAX} and BPM values for spontaneous cattle uterine contractions before and after OT application were shown in *Table 2*. There was no significant difference between the control 24 h group and the PCB-153 24 h group (P>0.05). However, a significant increase was observed in the AUC and P_{MAX} values of the PCB-153 48 h group when compared with the control 48 h group (P<0.05). There was also an increase in the BPM value of the PCB-153 48 h group when compared with the control 48 h group, but that change was not significant (P>0.05).

DISCUSSION

Wrobel et al.[3] investigated the *in vitro* effects of PCBs on the contractility of bovine myometrium from the periovulatory stage of the estrus cycle. Uterine strips were incubated with Aroclor 1248, a mixture of PCBs, or with one of three PCBs (PCB-77, -126 or -153), all at doses of 10 or 100 ng/mL. Aroclor 1248 increased the force of spontaneous contractions after 24 h but it decreased after 48 h. Pretreatment with the estrogen-like PCB-153 increased the frequency of both spontaneous and OT-induced myometrial contractions. The authors concluded that PCBs may impair both fertilization and blastocyst implantation in cows.

Wrobel et al.[17] reported that PCB-153 did not alter myometrial contractility in an acute manner (90 min) in rats but it increased the force of contractions in cattle after 24, 48 and 72 h. They also reported that an increase of $PGF_{2\alpha}$ secretion is involved in causing the adverse effects of PCBs on myometrial contractions.

Tsai et al.[18] reported that 4-Hydroxy-2',4',6'-Trichlorobiphenyl (4-OH - TCB or 4-OH - PCB-30) increased the contractile responses of mid-gestation uteri to OT by an ER-mediated mechanism. A 20 h exposure to either 4-OH - PCB-30 (0.1, 1 or 10 μM) or estradiol-17β (10 μM) failed to alter the contractile response to cumulative additions 10^{-10} to 10^{-7} M. However 42 h exposure to 1 μM 4-OH - PCB-30 or 10 μM estradiol-17β significantly elevated the contractile response to OT.

Kotwica et al.[19] investigated the influence of PCBs and phytoestrogens *in vitro* on the functioning of the reproductive tract in cows. They reported that PCBs increased OT secretion from granulosa cells but paradoxically each congener decreased FSH-stimulated OT secretion. Likewise, congeners and Aroclor 1248 stimulated OT secretion from luteal cells, although that effect was dependent on the stage of the cycle and type of congener, while the effect of PCBs on LH-stimulated OT secretion was equivocal during the course of the estrus cycle.

Thaina et al.[5] investigated the uterine relaxant effects of *Curcuma aeruginosa* Roxb. rhizome extracts on both non-stimulated, agonist- and KCl-stimulated rat uteri. In the non-stimulated uteri, the two extracts (10 and 400 mg/mL) had no significant effect. In contrast, in the stimulated uteri, the chloroform and methanol extracts exerted concentration-dependent inhibition of the contractions induced by OT, $PGF_{2\alpha}$, acetylcholine and KCl with IC_{50}s of 31.4, 58.59, 56.21 and 29.28 μg/mL; and 57.79, 69.3, 223.8 and 69.19 μg/mL, respectively. They also reported that the IC_{50} of diclofenac for $PGF_{2\alpha}$-induced contractions was 31.36 μg/mL.

Lee et al.[20] studied the therapeutic effects of COX in-

hibitors with different isoform selectivity on LPS-induced preterm birth in mice. Preterm birth occurred in 90% of mice after intra-peritoneal LPS injection and in 20% of mice after phosphate-buffered saline solution injection. Indomethacin and meloxicam, but not diclofenac, significantly decreased the incidence of preterm birth induced by LPS to 33.3% and 33.3%, respectively. They concluded that meloxicam appeared to have no advantage over indomethacin with regard to tocolysis and maternal side effects.

In the current study, the tocolytic effects of diclofenac potassium and diclofenac sodium on the contractions of cattle uterine tissues pre-incubated with PCB-153 were similar to those reported by Thaina et al.[5]. However, the results of the *in vivo* study performed by Lee et al.[20] contradict the results of the present study. The estrogenic effects of PCB-153 on AUC and P_{MAX} values were statistically significant after a 48 h incubation period. The BPM value was also affected but not significantly *(Table 2)*. These results are supported by those of Wrobel et al.[3] and Tsai et al.[18]. Our results are also similar to the results of the study performed by Wrobel et al.[17] in terms of 48 h incubation period, but they contradict to us for 24 h period.

Diclofenac potassium is claimed to dissolve faster and hence absorbed faster, than the sodium salt and is recommended for the treatments that need short onset of action, mainly for its analgesic properties [21]. Although there were differences between IC_{50} values of sodium and potassium salts of diclofenac, a statistical evaluation was not performed *(Table 1)*. Because, some of IC_{50} values were low for potassium salt, while some of IC_{50} values were low for sodium salt and there was no correlation between those data. This result may be due to realisation of the study *in vitro* condition.

Although diclofenac is a member of traditional NSAIDs, its 29 fold COX-2 selectivity is satisfactory. Diclofenac may therefore be a useful tocolytic drug for reducing the risk of preterm labour in cases of exposure to PCB-153 or other estrogenic agents. However, *in vivo* studies should be performed on cattle for confirmation of the present results.

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RESEARCH ARTICLE

Molecular Analysis of Cattle Isolates of *Echinococcus granulosus* in Manisa Province of Turkey [1]

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Summary

Echinococcus granulosus is the causative agent of cystic echinococcosis (CE) in humans and many domestic animals, and still one of the most important global health problem in the world and in Turkey. Infection with metacestode causes severe illness and high economic losses. Several strains of *Echinococcus* have been identified based on the epidemiological and biological characteristics of strains. In this study, a total of 18 individual hydatid cyst samples from cattle were examined. They were obtained from central slaughterhouse in the province of Manisa/Turkey between 2010-2012. The total genomic DNA (gDNA) was extracted using RTA-DNA Isolation Kit (Gebze/Kocaeli, Turkey) according to manufacturer instructions from protoscoleces and cystic germinal membranes. The aim of this study was to provide molecular characterization of *E. granulosus* isolates which were obtained from cattles by using polymerase chain reaction (PCR) in Manisa province of Turkey. After PCR, to investigate the genetic characteristics of isolates, deoxyribonucleic acid sequencing of the mitochondrial cytochrome c oxidase subunit 1 (CO1) and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (NAD1) genes were performed with ABI Prism Genetic Analyzer 3100 instrument. As a result of our study, all (18) cattle isolates were detected as *E. granulosus* sensu stricto (G1-G3 complex). This is the first molecular study report genotyping of *Echinococcus* isolates from cattle in Manisa province.

Keywords: *Echinococcus granulosus, Cattle, Genotyping, PCR, DNA Sequence, Turkey*

Manisa İlinde *Echinococcus granulosus*'un Sığır İzolatlarının Moleküler Analizi

Özet

Echinococcus granulosus insanda ve birçok evcil hayvanda kistik ekinokokkozise (KE) neden olan etkendir ve hala dünyada ve Türkiye'de en önemli sağlık problemlerinden biridir. Metasestodlarla infeksiyon şiddetli hastalıklara ve yüksek ekonomik kayıplara neden olur. Bazı *Echinococcus* suşları, suşların epidemiyolojik ve biyolojik karakteristiklerine dayanarak tanımlanmaktadır. Çalışmamızda sığırlardan elde edilen toplam 18 örnek incelenmiştir. Örnekler 2010-2012 yılları arasında Manisa merkez mezbahasından elde edilmiştir. Total genomik DNA (gDNA) üretici firmanın talimatları doğrultusunda protoskoleks ve kistik germinal membranlardan RTA-DNA İzolasyon Kiti kullanılarak (Gebze/Kocaeli, Türkiye) izole edilmiştir. Bu çalışmanın amacı, Türkiye'de Manisa ilindeki sığırlardan elde edilen *E. granulosus* izolatlarının Polimeraz Zincir Reaksiyonu (PZR) ile moleküler karakterizasyonunun elde edilmesidir. PZR'dan sonra, izolatların genetik karakteristiklerini araştırmak için mitokondrial sitokrom c oksidaz alt ünite 1 (CO1) ve nikotinamid adenin dinükleotit dehidrogenaz alt ünite 1 (NAD1) genleri deoksiribonükleik asit dizileme ile ABI Prism Genetik Analizör 3100 cihazıyla çalışıldı. Çalışmamızın sonucu olarak, tüm (18) sığır izolatları *E. granulosus* sensu stricto (G1-G3 kompleksi) olarak teşhis edildi. Bu çalışma Manisa ilindeki sığırlardan elde edilen *Echinococcus* izolatlarının ilk moleküler genotiplendirme çalışmasıdır.

Anahtar sözcükler: *Echinococcus granulosus, Sığır, Genotiplendirme, PZR, DNA Dizileme, Türkiye*

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INTRODUCTION

Cystic echinococcosis (CE) is quite widespread in the world. It is one of the most important cestode infections causing significant morbidity and mortality in humans as well as significant economic losses in livestock animals.

The extensive intraspecific variation in *E. granulosus* is associated with change in life cycle pattern, host specificity, geographical distribution, transmission dynamics, infectivity to human, antigenicity and sensitivity to chemotherapeutic agents [1,2]. This may have important implications for the design and development of diagnostic reagents, vaccines and control of echinococcosis. At least ten genotypically defined strains (G1–G10) were described within the *E. granulosus* complex, some of which exhibit marked biological and morphological differences. Such genotypes were recently proposed to merit species status, namely *E. granulosus* sensu stricto (G1–G3), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6–G10*). E. granulosus* sensu stricto is composed of three closely related genotypes, G1–G3. *E. granulosus sensu stricto* is known to be highly infective for humans [3].

To determine the perpetuation of echinococcosis, investigation must be done its spread in the definitive and intermediate hosts [4,5]. Being largely confined with life cycles involving sheep and dogs, exposure of humans to *E. granulosus* is common in Turkey. The majority of people lives in rural areas and is engaged in animal husbandry. High prevalences of CE have been reported in animals in Turkey: 24% (at autopsy) and 62% (by ELISA) recorded in dogs, 66.4% (by Western blotting, EITB) and 51.9% (at autopsy) in sheep, 63.3% (by ELISA), 54.7% (by IFAT) and 39.7% (at autopsy) in cattle, and 22.1% (at autopsy) in goats [4,6].

Turkey is one of the countries where CE is of public health and economic importance. Despite its public health impact, relatively little informations avaliable on the presence of the different genotypes (strains, species) of *E. granulosus*. In Turkey, many studies have been performed regarding the prevalence of the disease in sheep and cattle but only few studies have been performed about genetic characterization of *Echinococcus* variants [3-9]. Therefore the aim of the present study was to provide molecular characterization of *E. granulosus* isolates from cattle in Manisa province of Turkey.

MATERIAL and METHODS

Collection of Cyst Materials

In this study, a total of 18 individual hydatid cyst samples from cattle were examined. They were obtained from central slaughterhouse in the province of Manisa/Turkey. All livestock isolates of *E. granulosus* were obtained from liver (16) and lung (2) hydatid cysts. Protoscoleces were detected under light microscope and all cysts were examined for their fertility (1 fertile, 17 sterile kist). Protoscoleces and cyst walls (germinal and laminar layer) were washed three times with phosphate buffered saline solution. The sediment was preserved in 70% ethanol and stored at -20ºC until used. Cyst walls were rinsed in sterile distilled water and then fixed in 70% ethanol and were stored at same conditions such as protoscoleces.

Molecular Analysis

Before the DNA isolation, protoscoleces and cut cyst walls were rinsed several times with sterile distilled water to remove ethanol. In order to determine the average number of protoscoleces in milliliters of a sample, the bottle was thoroughly shaked, 10 µl fluid was placed between the microscope slide and coverslip and then protoscoleces were counted under the light microscope. gDNA was extracted from samples which had 200 protoscoleces or upon [10]. The total genomic DNA (gDNA) was extracted using RTA-DNA Isolation Kit (Gebze/Kocaeli, Turkey) according to manufacturer instructions from protoscoleces and cystic germinal membranes. Then the gDNA was examined with spectrophotometer (NanoDrop- ND1000) for qualitative and quantitative analyses (between 50-70 ng/µl). The gDNA was stored -20ºC until use.

The isolates were analyzed using amplification of two mitochondrial DNA regions which were cytochrome c oxidase subunit 1 (CO1) and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (NAD1) genes separately. Amplicons of the CO1 mitochondrial gene were amplified using the JB3 (forward) (5′ -TTTTTTGGGCATCCT GAGGTTTAT- 3′)/JB4.5 (reverse) (5′-TAAAGAAAGAACATAA TGAAAATG- 3′) primers [6] and NAD1 mitochondrial gene's amplicons were amplified using the MS1 (5′-CGTAGGTA TGTTGGTTTGTTTGGT-3′)/MS2 (5′-CCATAATCAAATGGCGTA CGAT- 3′) primers [11].

PCR amplification for CO1 carried out in a final volume of 25 µl including 3 µl gDNA, 2 µl of each primers (20 pmol), 12.5 µl of Amplitaq Gold Master Mix (Roche, Branchburg, New Jersey/USA), 4 µl GC enhancer (GML, Wollerau/ Switzerland) 2.5 µl molecular grade water and 1 µl Hotstart Taq DNA polymerase (MBI, Fermantas, Lithuania). The PCR conditions were: 10 min at 95ºC (initial denaturation), 35 cycles of 50 s at 95ºC, 50 s at 47ºC and 50 s at 72ºC and finally 10 min at 72ºC (final extension). NAD1 carried out in a final volume of 25 µl including between 2.5 - 4 µl DNA, 2 µl each primers (20 pmol), 12.5 µl of Amplitaq Gold Master Mix (Roche, Branchburg, New Jersey/USA), 3 µl GC Enhancer (GML, Wollerau/Switzerland) and between 3.5-5 µl molecular grade water. The PCR conditions were: 10 min at 95ºC (initial denaturation), 35 cycles of 30 s at 95ºC, 30 s at 51ºC and 40 s at 72ºC and finally 10 min at 72ºC (final extension).

After the PCR, amplicons were fractionated in 1.5%

agarose gel which was including 5 µl ethidium bromide and then visualized under the UV light with gel imaging system (SYNGENE). For purification step, all PCR amplicons of both CO1 and NAD1 genes were purified with ExoSap-IT (GML, Wollerau/Switzerland) in a final volume of 7 µl including 5 µl each PCR product and 2 µl ExoSap-IT. The prufication step conditions were: 30 min at 37ºC and 15 min at 80ºC.

Forward and reverse primers which employed in the PCR were used in the Cycle Sequencing step. Cycle Sequencing carried out in a final volume of 10 µl including 2 µl BigDye Terminator v3.1 (Applied Biosystems, USA), 2 µl 5x sequencing buffer (Applied Biosystems, USA), 2 µl forward and reverse primers, 2 µl PCR product (prufied with ExoSap-IT) and 2 µl molecular grade water. The Cycle Sequencing conditions were: 10 min at 96ºC (initial denaturation), 25 cycle of 10 s at 96ºC, 5s at 47ºC and 4 min at 60ºC. In PCR applications, DNA which previously identified as sheep strain by DNA sequence analysis was used as a positive control and distilled water was used as a negative control.

RESULTS

Eighteen hydatid cyst samples belonged lung and liver, CO1 and NAD1 genes were successfully amplified for all isolates. The CO1 and NAD1 amplicons produced ~450 [6] and 400 [11] bp bands by PCR, respectively *(Fig.1)*. Individual amplicons represented single bands on agarose gels, indicating the specificity of the PCR and the conditions used. There was no detectable size variation on agarose gels among amplicons derived from cysts from the same host. The reference sequences determined in this study from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers: EU178103 (CO1) and AB677822 (NAD1). The consensus sequence lengths determined were 444 and 378 bp for CO1 and NAD1. The electropherogram of each sequence was verified by eye, and the sequences were aligned using the program SeqScape v2.6. All 18 examined CO1 and NAD1 sequences were identified as corresponding to the *E.granulosus sensu stricto* (G1-G3 cluster).

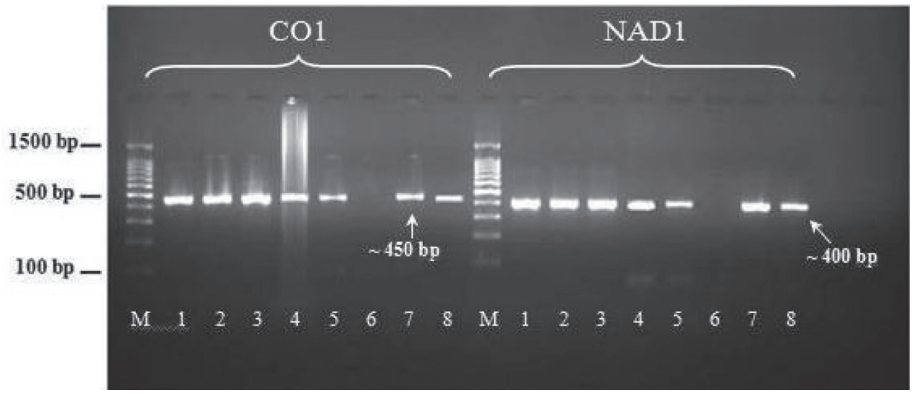


Fig 1. PCR amplification of CO1 (~450bp) and NAD1 (~400bp) genes and run on 1.5% agarose gel **M:** marker, **1:** Positive control (reference strain), **6:** Negative control (distilled water), **2-5:** Cattle isolates, **7-8:** Cattle isolates

Şekil 1. CO1 (~450bp) and NAD1 (~400bp) genlerinin 1.5% agarose jelde PZR ürünleri **M:** marker, **1:** Positif kontrol (referans suş), **6:** Negatif kontrol (distile su), **2-5:** Sığır izolatları, **7-8:** Sığır izolatları

Fig 2. The figure shows sequencing results of the silent mutation which position is 78 C → T

Şekil 2. Sekans sonuçlarında 78 C → T pozisyonundaki sessiz mutasyonu göstermektedir

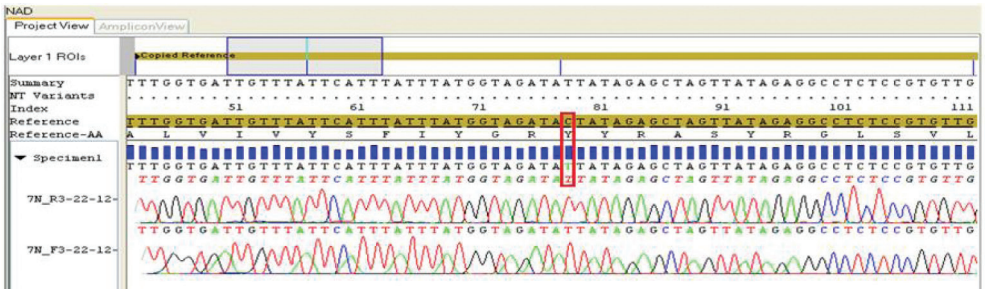
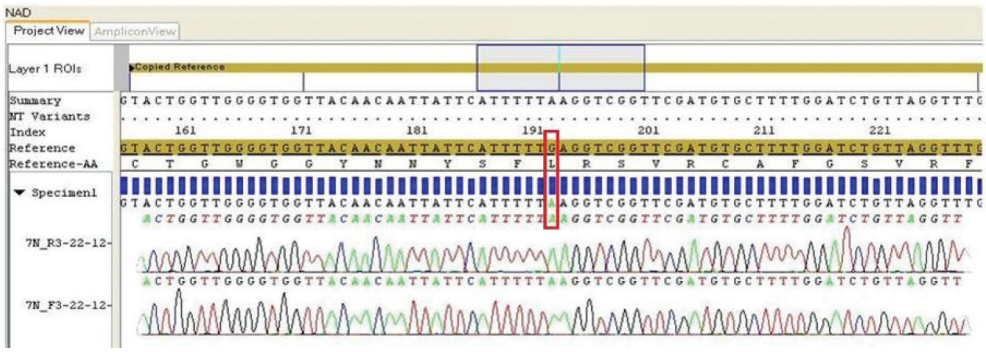


Fig 3. The figure shows sequencing results of the silent mutation which position is 192 G → A

Şekil 3. Sekans sonuçlarında 192 G → A pozisyonundaki sessiz mutasyonu göstermektedir



The results of the sequence that we have obtained from 18 cattle isolates were compared with the Genbank reference sequences (EU178103 for CO1 and AB677822 for NAD1) by SeqScape V2.6 program. Two silent mutations [78 C → T *(Fig. 2)* and 192 G → A *(Fig. 3)* silent mutations] were obtained from this study by Sequencing images from second cattle isolate which is lane 2 in *Fig. 1*.

DISCUSSION

Since several years extensive literature on the application of molecular biological methods has been published in order to discriminate *Echinococcus* strains/species. PCR is one of the method used for molecular characterization of *Echinococcus* isolates. The advantage of PCR method which has been used the diagnosis of *E. granulosus* is to provide operability with the large amount of samples and easy application [12].

DNA sequencing has become the reference method. Because nowadays, there is no another technique that allows measurement of the difference in genotype. Stability of the intra- and inter-laboratory reproducibility of the technique is good and it also has high power separation and excellent typing [10].

Until now limited reports have been published on the strain characteristics of *E. granulosus* in Turkey. For this purpose, different molecular techniques such as PCR, PCR-RFLP (restriction fragment length polymorphism), RAPD-PCR (random amplified polymorphic DNA), PZR-SSCP (single stranded conformation polymorphism) and DNA sequencing have been used to detect different strains of *E. granulosus* [10].

Nucleotide sequences of fragments of the mitochondrial CO1 gene provides valuable information about identification and variability of *E. granulosus* [1]. The occurrence and host preference of the sheep strain (G1 genotype) of *E. granulosus* in different countries have been shown by previous molecular epidemiologic studies based on mitochondrial gene sequences [13]. From many countries in the Mediterranean area including our country, G1 genotype has been reported as the most prevalent genotype in both of human and animals [14-16].

In Sardinia, a total of 91 pieces of sheep, cattle and pig isolates examined with PCR-RFLP and DNA sequencing techniques and 89 isolates determined as G1 strain, 2 pig isolates determined as G7 strain [17]. In Iran, 50 human, 166 cattle, 153 sheep and 3 camel isolates examined with PCR-RFLP technique. All of human, cattle and sheep isolates determined with CO1 gene sequencing (37 isolates) which were G1 strain but 3 camel isolates were G6 strain [18]. In Bulgaria, as a result of examination nuclear and mitochondrial gene sequences of *E. granulosus* of cattle, sheep, pigs, jackals and wolves isolates, G1 strain was found the predominant strain in intermediate and final hosts [19]. Bagcı et al.[20], investigated *E. granulosus* strains in 100 sheep isolates which were brought to Istanbul from various regions of Turkey. CO1 gene sequence analysis performed from all of collected samples and they found G1 strain in 98 of 100 sheep isolates, G3 (buffalo) strain in 2 of them.

Simsek et al.[21] examined 220 hydatid cysts from cattle by PCR of 12S rRNA gene and sequencing of partial mt-CO1 gene. They detected *E. granulosus* s.s. (G1-G3) in 147 of 220 isolates with 12S rRNA-PCR and confirmed G1 strain by sequencing of mt-CO1 genes of 28 cattle isolates. And also they detected buffalo (G3) strain in seven cattle isolate. Simsek and Eroksuz [22] detected sheep strain (G1) infection in a Turkish mouflon (*Ovis gmelinii anatolica*) in Malatya province by partial sequencing of mt-CO1 gene.

The molecular analysis shows that all isolates are G1 and G1variants until now. Previous studies which have been done from different isolates of intermediate hosts (sheep, goat, cattle, camel, and human isolates) showed the presence of G1 strain [6]. Vural et al.[23] showed the presence of G1 and G3 strains in the isolates from sheep and cattle. Snabel et al.[3] found *E. canadensis* and *E. granulosus* s.s. groups and it is the first report of the pig strain in humans in Turkey.

In our study, the described PCR and sequencing using well characterized primers provides a simple, rapid, sensitive and specific method for detection of the strains of *E. granulosus*. Eighteen hydatid cyst samples obtained lung and liver and CO1 and NAD1 genes were successfully amplified for all isolates. And the results showed paralelism with the other studies had been done [10,21,24]. Individual amplicons represented single bands on agarose gels (CO1 (~450 bp) and NAD1 (~400 bp)) indicating the specificity of the PCR and the conditions used. There was no detectable size variation on agarose gels among amplicons derived from cysts from the same host.

In this study, generally the CO1 and NAD1 sequencing results were highly homologous with few differences, corresponding to punctual base substitution. Therefore our study is the first sequencing report indicating that the *E. granulosus* s.s. is present in Manisa province of Turkey.

As a conclusion; the present study is the first report about the genetic characterization of *Echinococcus* isolates from cattle in Manisa province of Turkey. But further molecular studies are necessary to performed for defining the strains of *E. granulosus* in different intermediate hosts and get more data on epidemiology to establish the control programmes in Turkey.

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RESEARCH ARTICLE

Isolation, Virulence Genes and Antimicrobial Susceptibilities of Shiga Toxin-Producing *Escherichia coli* O157 from Slaughtered Cattle in Abattoirs and Ground Beef Sold in Elazığ

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Summary

Shiga Toxin-Producing *Escherichia coli* O157 (STEC O157) is a foodborne pathogen. Contaminated meat and meat products have an important role in human STEC O157 outbreaks. The aims of this study were to investigate the presence of STEC O157 in slaughtered cattle in abattoirs and ground beef sold in Elazığ, and to determine virulence genes and antimicrobial resistance patterns of STEC O157 isolates. A total of 540 rectal swab samples were collected immediately after slaughter. In addition, 100 ground beef samples were obtained from the butcher shops. Selective enrichment, immunomagnetic separation and plating on Sorbitol MacConkey Agar with cefixime and tellurite (CT-SMAC Agar) were used for the culture. Presence of genes encoding shiga toxin 1 and 2 (*stx1* and *stx2*), H7 flagella (*fliCh7*), enterohemolysin (*hlyA*), intimin (*eae*) and O157 (*rfbE*) in the isolates was detected by Polymerase Chain Reaction (PCR). In the PCR analysis of rectal swab samples., 34 of 82 sorbitol negative isolates were positive for *E. coli* O157. 22 (64.7%) of *E. coli* O157 isolates belonged to *E. coli* O157:H7. STEC O157 was detected in 18 (3.3%) of rectal swab samples. STEC O157:H7 was isolated from 2 (2%) of ground beef samples. All STEC O157 isolates contained *hlyA* and *eae* genes. All STEC O157 isolates obtained from both rectal swab and ground beef samples were resistant to four or more antimicrobials. All STEC O157 isolates were resistant to penicillin, clindamycin, tiamulin and tilmicosin. Two STEC isolates were resistance to ampicillin. Six STEC O157 isolates were resistance to chlortetracycline and sulphadimethoxine. One STEC O157 isolate was resistant to enrofloxacin, florfenicol and oxytetracycline.

Keywords: *E. coli O157, Cattle, Ground beef, Virulence genes, Antibiotic resistance*

Elazığ'da Mezbahalarda Kesilen Sığırlardan ve Piyasada Satılan Kıymalardan Shiga Toksin Üreten *Escherichia coli* O157'nin İzolasyonu, Virulens Genleri ve Antibiyotiklere Duyarlılıkları

Özet

Shiga toxin üreten *Escherichia coli* O157 (STEC O157) gıda kaynaklı enfeksiyonlara yol açan bir patojendir. Kontamine et ve et ürünleri insanlarda salgınların görülmesinde önemli bir rol oynamaktadır. Bu çalışma Elazığ'da mezbahalarda kesilen sığırlardan ve piyasada satılan kıymalardan STEC O157'nin izolasyonu, izolatların virulens genlerinin ve antibiyotiklere duyarlılıklarının belirlenmesi amacıyla yapılmıştır. Kesimden sonra 540 rektal swap örneği ve kasaplardan 100 kıyma örneği toplanmıştır. Etken izolasyonu için selektif zenginleştirme ve immunomagnetik separasyondan sonra sefiksim ve tellürit suplementi içeren Sorbitol MacConkey Agar kullanıldı. Polimeraz zincir reaksiyonu(PCR) ile izolatlarda shiga toxin 1 ve 2 (*stx1* and *stx2*), H7 flagella (*fliCh7*), enterohemolysin (*hlyA*), intimin (*eae*) ve O157 (*rfbE*) genlerinin varlığı araştırıldı. Rektal sıvap örneklerinin PCR testinde, 82 sorbitol negatif izolatın 34'ü *Escherichia coli* O157 yönünden pozitif bulundu. *Escherichia coli* O157 izolatlarının 22'si (%64.7) *Escherichia coli* O157:H7 olarak tiplendirildi. Rektal swap örneklerinin 18'inde (%3.3) STEC O157 saptandı. Kıyma örneklerinin %2'sinden STEC O157 izole edildi. STEC O157 izolatlarının tümünde *hlyA* and *eaeA* genleri tesbit edildi. Rektal sıvap ve kıyma örneklerinden elde edilen tüm STEC O157 izolatları penisilin, klindamisin, tiamulin ve tilmikosin'e dirençli bulundu. Altı STEC O157 izolatı klortetrasiklin ve sülfadimetoksin'e, iki STEC O157 izolatı ampisilin'e ve bir STEC O157 izolatı enrofloksasin, florfenikol ve oksitetrasiklin'e dirençli bulundu.

Anahtar sözcükler: *E. coli O157, Sığır, Kıyma, Virulens genler, Antibiyotik direnci*

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INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) strains are the most important emerged group of foodborne pathogens worldwide. *E. coli* O157:H7 is considered a highly pathogenic serotype responsible for severe human diseases [1]. Cattle are the main reservoir of STEC and shed the bacteria through their feces spreading these pathogens among cattle herds and the environment [2]. Most infections caused by *E. coli* O157:H7 result from the consumption of food and water contaminated with animal feces [3]. Elderly and pediatric patients are at an increased risk of developing *E. coli* O157:H7 associated conditions such as hemorrhagic colitis (HC), haemolytic-uremic syndrome (HUS), thrombotic thrombocytopenic purpura and death [4,5].

The main virulence factor of STEC is the production of shiga toxins encoded by *stx*1 and *stx*2 genes. Additional virulence factors have also been described including intimin (encoded by the *eaeA* gene) and EHEC hemolysin (encoded by EHEC *hlyA* gene) [1,6]. The widespread use of antibiotics in food animals has resulted in an increase in resistant strains of bacteria. Development of resistance in zoonotic bacteria constitutes a public health risk. Antibiotic resistance strains of STEC have been reported in many countries [7-9]. The presence of *E. coli* O157 in cattle [10-13], ground beef [14-17] and red meat samples [18] has previously been reported in Turkey.

This study was carried out to determine the occurrence of virulence genes and antimicrobial resistance patterns of STEC O157 isolates from slaughtered cattle and ground beef samples in Elazığ.

MATERIAL and METHODS

Sampling

Rectal swab samples were collected immediately at slaughter during the period of December 2011 to June 2012 from 540 cattle at two abattoirs (named A and B) located in Elazığ. The abattoirs were visited once weekly. At each visit, 20 rectal swab samples were taken. The animals sampled were randomly selected. The swabs were placed in a modified tryptone soya broth (mTSB) (LAB165; Lab M) supplemented with novobiocin in 10 ml tubes and then transported immediately to the laboratory. A total of 100 ground beef samples were obtained from the butcher shops. The ground beef samples were brought into the laboratory within sterile containers preserved in ice cold packs.

Isolation of STEC O157

Aproximately 25 g of ground beef samples taken under aseptic conditions were homogenized within 225 ml of mTSB. The mTSB medium containing rectal swab and ground beef samples was incubated at 41.5°C for 24 h. Then immunomagnetic separation (IMS) was performed according to the manufacturer's instructions (Captive O157, Lancashire, UK). The IMS samples were plated onto Sorbitol MacConkey Agar supplemented with cefixime and tellurite (CT-SMAC Agar) (LAB161; Lab M). The agar plates were incubated at 37°C for 24 h. Sorbitol negative colonies on CT-SMAC Agar were considered presumptive *E. coli* O157. Presumptive *E. coli* O 157 colonies were confirmed by amplification of the gene encoding O157 somatic antigen (*rfbE*) by PCR [19].

Detection of Virulence Genes, O157 and Flagellar H7 Gene by PCR

Cultures were grown overnight at 37°C on nutrient agar. A small amount of the culture was resuspended in 200 µl of distilled water, heated to 99°C for 15 min, and centrifuged for 2 min at 12.000 x g. The resulting supernatant was used as a template for PCR. Shiga toxin genes *stx*1 and *stx*2 were detected by multiplex PCR. Single gene PCR was used to determine the presence of genes encoding H7 flagella *(fliCh7)*, enterohemolysin *(hlyA)*, intimin *(eae)* and O157 (rfbE). The primers used in this study are listed in *Table 1*. Reaction contents for each PCR (11-µl total reaction volume) consisted of 3 µl of template DNA, 0.5 µM of primers, 0.18 mM concentration of each deoxyribo-

Table 1. *The primers used in the study*
Tablo 1. *Çalışmada kullanılan primerler*

Target Gene	Sequence of Primers (5'-3')	Size (bp) of PCR Product	Reference
*stx*1	F: ACA CTG GAT GAT CTC AGT GG R: CTG AAT CCC CCT CCA TTA TG	582	Paton and Paton [21]
*stx*2	F: GGC ACT GTC TGA AAC TGC TCC R: TCG CCA GTT ATC TGA CAT TCT G	255	Paton and Paton [21]
eae	F: GTG GCG AAT ACT GGC GAG ACT R: CCC CAT TCT TTT TCA CCG TCG	890	Gannon et al.[22]
$rfbE_{O157}$	F: AAC GGT TGC TCT TCA TTT AG R: GAG ACC ATC CAA TAA GTG TG	678	Nagano et al.[23]
fliCh7	F: TAC CAC CAA ATC TAC TGC TG R: TAC CAC CTT TAT CAT CCA CA	560	Nagano et al.[23]
hlyA	F: AGC CGG AAC AGT TCT CTC AG R: CCA GCA TAA CAG CCG ATG T	525	Fratamico et al.[24]

nucleotide, 4 mM $MgCl_2$, 0.4 U of *Taq* DNA polymerase, 50 mM Tris (pH 8.3), 250 µg/ml Bovine Serum Albumin (BSA), 2% sucrose, and 0.1 mM cresol red. The PCR was performed using rapid-cycle DNA amplification method. The reactions consisted of 30 cycles of template denaturation 94°C, primer anneling at 54°C, and primer extension at 74°C for 30 s. Amplified products were electrophoresed in 1% agarose gels at 200 V for 30 min. The gels were stained with ethidium bromide and were visualized under ultraviolet light. Positive samples were identified based on the presence of bands of the expected sizes compared with results with a positive control strain *(E. coli* ATCC 43895) [7,20].

Antimicrobial Susceptibility Testing

A total of 20 STEC O157 isolates were examined for antimicrobial susceptibility. Minimum inhibitory concentrations (MIC) were measured using the Sensititre Susceptibility System. The following antimicrobial agents were used: ampicillin, ceftiofur, chlortetracycline, clindamycin, danofloxacin, enrofloxacin, florfenicol, gentamicin, neomycin, oxytetracycline, penicillin, spectinomycin, sulphadimethoxine, tiamulin, tilmicosin, trimethoprim + sulphamethoxazole, tulathromycin and tylosin. All plates were inoculated following the guidelines recommended by the Clinical and Laboratory Standarts Institute (CLSI), and CLSI breakpoints for interpretation of MIC results [25].

RESULTS

A total of 82 sorbitol-negative isolates were obtained from rectal swab samples from 540 slaughtered cattle. Of these isolates, 34 were positive for *E. coli* O157. 22 (64.7%) of *E. coli* O157 isolates belonged to *E. coli* O157:H7. STEC O157 was detected in 18 (3.3%) of rectal swab samples. *Stx*2 gene was only detected alone in 14 STEC O157 isolates. Four STEC O157 isolates were positive for both *stx*1 and *stx*2 genes. PCR products of *stx*1 (582 bp) and *stx*2 (255 bp) were shown in *Fig. 1*.

All STEC O157 isolates from rectal swab samples were positive for both *eae* and *hlyA* genes. STEC O157 was detected in 2.59% (7/270) and 4.07% (11/270) of rectal swab samples collected from the abattoir A and the abattoir B, respectively. All of the STEC O157 isolates from rectal swab samples were susceptible to ceftiofur, danofloxacin, gentamicin, neomycin, spectinomycin, trimethoprim-sulphamethoxazole, tulathromycin and tylosin. But all of them were resitant to penicillin, clindamycin, tiamulin and tilmicosin. Two STEC O157 isolates were resistance to ampicillin. Six STEC O157 isolates were resistant to chlortetracycline and sulphadimethoxine. One STEC O157 isolate was resistant to enrofloxacin, florfenicol and oxytetracycline *(Table 2)*.

Two sorbitol-negative isolates from ground beef samples were positive for STEC O157:H7. These isolates were positive for *stx*2, *eae* and *hlyA* genes, but none were positive for *stx*1. All of the two STEC O157 isolates from ground beef samples were resistance to penicillin, clindamycin, tiamulin and tilmicosin *(Table 3)*.

DISCUSSION

STEC O157 has been recognized as a growing public health all around the world. Although it is isolated from many animal species, it was reported that STEC O157 is largely hosted in cattle intestines without showing any symptoms [6]. It was reported that cattle beef, milk and the products obtained from them play an important role in the development of human STEC infections [1].

In the studies conducted in several countries for determining STEC O157 prevalence in cattle faecal samples,

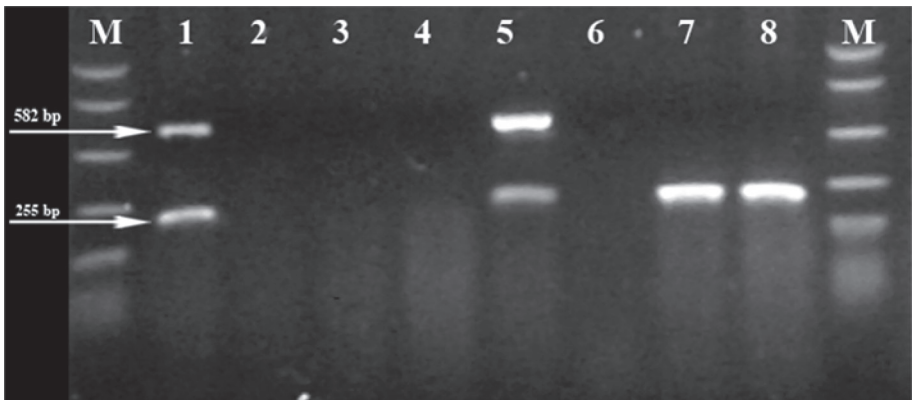

Fig 1. Agarose gel electrophoresis of PCR products of *E. coli* O157 isolates by multiplex PCR

M: Marker (50, 150, 300, 500, 750, 1.000 bp) Lane 1: Positive control, Lane 2: Negative control, Lane 3, 4, 6: Negative samples, Lane 5: Positive sample for *stx*1 and *stx2*, Lane 7, 8: Positive samples for *stx*2

Şekil 1. Multiplex PCR ile *E. coli* O157 izolatlarından elde edilen ürünlerinin agaroz jel elektroforezi

M: Marker (50, 150, 300, 500, 750, 1.000 bp) 1: Pozitif kontrol, 2: Negatif kontrol, 3, 4, 6: Negatif örnekler 5: *Stx*1 ve *stx*2 pozitif örnek, 7, 8: *Stx*2 pozitif örnekler

Table 2. *Virulence genes and antimicrobial resistance of E. coli O157 isolates from cattle*
Tablo 2. *Sığırlardan izole edilen E. coli O157 izolatlarının virulens genleri ve antibiyotik direnci*

Abattoir	Isolate No.	*O157 rfbE*	*fliCh7*	*Stx1*	*Stx2*	*eaeA*	*hlyA*	Resistance[a] Pattern
A	1	+	-	-	-	ND	ND	ND
A	2	+	-	-	-	ND	ND	ND
B	3	+	-	-	-	ND	ND	ND
A	4	+	-	-	-	ND	ND	ND
B	5	+	+	+	+	+	+	CLI, PEN, SUL,TIL
A	6	+	-	-	-	ND	ND	ND
A	7	+	-	-	-	ND	ND	ND
A	8	+	-	-	-	ND	ND	ND
A	9	+	-	-	-	ND	ND	ND
B	10	+	+	+	+	+	+	CHL,CLI, PEN, SUL, TIA,TIL
B	11	+	+	+	+	+	+	CHL,CLI, PEN, SUL,TIA,TIL
B	12	+	+	+	+	+	+	CHL,CLI, PEN, SUL, TIA,TIL
B	13	+	-	-	-	ND	ND	ND
B	14	+	-	-	-	ND	ND	ND
A	15	+	-	-	-	ND	ND	ND
B	16	+	+	-	+	+	+	AMP, CLI, ENF, PEN, SUL,TIA, TIL, TRM
B	17	+	+	-	-	ND	ND	ND
B	18	+	+	-	-	ND	ND	ND
B	19	+	+	-	+	+	+	CHL, CLI, PEN, TIA, TIL
B	20	+	+	-	+	+	+	CHL, CLI, PEN, SUL,TIA,TIL
B	21	+	+	-	+	+	+	AMP, CHL, CLI, FLO, OXY, PEN, SUL, TIA, TIL
A	22	+	-	-	-	ND	ND	ND
B	23	+	+	-	+	+	+	CLI, PEN, TIA, TIL
B	24	+	+	-	-	ND	ND	ND
B	25	+	+	-	-	ND	ND	ND
A	26	+	+	-	+	+	+	CLI, PEN, TIA, TIL
A	27	+	+	-	+	+	+	CLI, PEN, TIA, TIL
A	28	+	+	-	+	+	+	CLI, PEN, TIA, TIL
A	29	+	+	-	+	+	+	CLI, PEN, TIA, TIL
A	30	+	+	-	+	+	+	CLI, PEN, TIA, TIL
A	31	+	+	-	+	+	+	CLI, PEN, TIA, TIL
B	32	+	+	-	+	+	+	CLI, PEN, TIA, TIL
A	33	+	+	-	+	+	+	CLI, PEN, TIA, TIL
B	34	+	+	-	+	+	+	CLI, PEN, TIA, TIL

[a] ***AMP,*** *ampicillin;* ***CHL,*** *chlortetracycline;* ***CLI,*** *clindamycin;* ***ENF,*** *enrofloxacin;* ***FLO,*** *florfenicol;* ***OXY,*** *oxytetracycline;* ***PEN,*** *penicillin;* ***SUL,*** *sulphadimethoxine;* ***TIA,*** *tiamulin;* ***TIL,*** *tilmicosin;* ***TRM,*** *trimethoprim+sulphamethoxazole,* ***ND,*** *not done*

Table 3. *Virulence genes and antimicrobial resistance of E. coli O157 isolates from ground beef*
Tablo 3. *Kıymalardan izole edilen E. coli O157 izolatlarının virulens genleri ve antibiyotik direnci*

Isolate No.	*O157 rfbE*	*fliCh7*	*Stx1*	*Stx2*	*eaeA*	*hlyA*	Resistance[a] Pattern
1	+	+	-	+	+	+	CLI, PEN, TIA, TIL
2	+	+	-	+	+	+	CLI, PEN, TIA, TIL

[a] ***CLI,*** *clindamycin;* ***PEN,*** *penicillin;* ***TIA,*** *tiamulin;* ***TIL,*** *tilmicosin*

prevalence rates varying by the countries were obtained. It was reported that the worldwide prevalence is between 0.3 and 27.3% for beef cattle and between 0.2 and 48% for dairy cattle [6,26]. Hancock *et al.*[27] detected STEC O157 in 1.8% of cattle faeces in the USA, while Islam *et al.*[28] reported detection of the bacterium in 7.2% of the cattle slaughtered in slaughter houses in Bangladesh, Manna *et al.*[29] in 2% of cattle slaughtered in slaughter houses in India, Sasaki *et al.*[30] in 8.9% of beef cattle in Japan, and Zhou *et al.*[31] in 1.7% of cattle faeces in China. In studies conducted in several regions of Turkey, it is reported that *E. coli* O157 was detected in cattle faeces with rates varying between 0.6% and 25% [10-13]. However, in some of these studies the presence of shiga toxin producing *E. coli* O157 was examined. In the study by Inat and Siriken [13] conducted in Samsun city, the authors reported they had isolated STEC O157 from 18% of the rectal swab samples taken from 100 slaughtered cattle. In the study conducted by Aslantas *et al.*[11] in Hatay city, it was reported that in 11% of 565 cattle faecal samples, STEC O157 was detected. In another study carried out in Turkey, STEC O157 was isolated from 1.2% of cattle faecal samples of 251 cattle [12]. In this presented study, STEC O157 was detected in 3.3% of 540 cattle rectal swab samples. In comparison with the studies conducted in Turkey and in other countries, this rate is higher than the findings of some researchers [12,27,29,31] and lower than the findings of others [11,13,28,30]. This may result from the differences in seasons, ages of animals, method of breeding and geographic differences. It was reported that also sampling method and isolation technique affect prevalence [28,32]. It is reported that IMS method is one of the most sensitive methods for STEC O157 isolation from faeces and food samples [12,13]. Also in the present study the IMS method was employed for isolation. However, samples were collected in winter and spring months. It was reported that in ruminants the prevalence of *Escherichia coli* O157:H7 is the highest in summer months and decreases in winter months [6,26].

Pathogenic STEC strains produce toxins that cause human illnesses and can produce other virulence factors that may increase the severity of illnesses. These factors include intimin and enterohemolysin, which are encoded by the *eae* and *hlyA* genes, respectively [6]. In the present study, *eae* and *hlyA* genes were detected as well as shiga toxin genes (4 *stx*1 and *stx*2, 14 only *stx*2) in 18 of the total 34 *E. coli* O157 isolates obtained from rectal swab samples. *Stx*2-producing strains are often more related to HUS than *stx*1-producing strains [4,6,33]. In this study, the predominant stx type found was *stx*2, in aggrement with previous studies [2,28,32]. The presence of a combination *eae, stx* and *hlyA* genes is generally regarded as a highly virulent genetic mix [34]. Results of the presented study show that seemingly healthy cattle contain *E. coli* O157 strains that are highly pathogenic for humans. *E. coli* O157 was isolated from diarrheal human faeces in Turkey and the suspected source of contamination was reported to be foodstuff [35,36]. However, there is a need for a comprehensive study that examines the relation of human *E. coli* O157 infections in Turkey with foodstuffs. In the studies conducted in Turkey on faecal samples of cattle, Aslantas *et al.*[11] reported that 74 of a total of 77 *E. coli* O157 isolates contained *hly*A, while 72 of them contained *eae,* 62 contained *stx*2 and 3 contained both *stx*1 and *stx*2 genes. In the study by Kuyucuoglu *et al.*[37], it was reported that all of 5 *E. coli* O157:H7 isolates were positive for *hly*A, while 2 of them were positive for *eae* gene. In another study conducted by Ongor *et al.*[12], it was determined that 2 of a total of 4 *E. coli* O157 isolates contained *eae, stx*1 and *stx*2, while one contained *eae* and another contained *eae* and *stx*2 genes.

In studies conducted in several countries with the purpose of determining *E. coli* O157 in ground beef samples it was reported that in Italy Conedera *et al.*[38] isolated STEC O157 from 0.43% of 931 samples, in the Netherlands Heuvelink *et al.*[39] from 1.1% of 571 samples and in Peru Mora *et al.*[40] isolated *E. coli* O157 from 23% of 102 samples. In a study conducted in Argentina, *E. coli* O157 was isolated from 3.8% of a total of 160 samples [41]. In England, *E. coli* O157 was detected in 0.35% of 1979 samples [42]. In the studies carried out in Turkey, Alisarli and Akman [14] reported that they isolated *E. coli* O157 from 4.6% of 150 ground beef samples, Sarimehmetoglu *et al.*[15] reported 7.6% isolation from 255 ground beef samples in Ankara city, Cadirci *et al.*[17] reported 1% of isolation from 100 ground beef samples in Samsun city and Aksu *et al.*[16] reported that they isolated *E. coli* O157 from 6% of 50 ground beef samples in Istanbul city. In the presented study, STEC O157 was isolated from 2% of 100 ground samples. In many countries, low rates similar to the results of the present study were obtained. When compared with the other studies conducted in Turkey, the rate determined with the present study is lower than the rates found in some studies [14-16]. However, Cadirci *et al.*[17] reported a rate (1%) close to the rate determined in this study. The differences in isolation rates from ground beef samples may occur due to the differences in sampling method, isolation method, season and geography. Also inadequate hygienic implementations at butcher shops and slaughter houses can affect isolation rate. Sarimehmetoglu *et al.*[15] reported that one of the total 19 *E. coli* O157 strains isolated from ground beef contained *stx*1, *stx*2, *eae*, *hlyA* and *fliCh7* genes, while the genes of *stx*1, *eae, hlyA* and *fliCh7* genes were found in all other strains. In the present study, *stx*1, *stx*2, *eae, hlyA* and *fliCh7* genes were detected in all ground beef isolates.

Antimicrobial resistance in animal STEC isolates may be spread to humans through the food chain. Strains of STEC are commonly found in the ruminant gastrointestinal tract and can serve as indicator organisms for the development of antibiotic resistance [7,8]. In the present study, all STEC O157 isolates were found to be resistant to penicillin, clindamycin, tiamulin and tilmicosin. Howewer, all the isolates were susceptible to ceftiofur, danofloxacin, gentamicin, neomycin,

spectinomycin, trimethoprim-sulphamethoxazole, tulatromycin and tylosin. Six (33.3%) of the isolates were resistant to chlortetracycline and sulphadimethoxine. Cephalosporins and fluoroquinolones often are the drugs of choice for treatment of infections in humans. Although no resistance against ceftiofur was found in the isolates in this study, one isolate was found to be resistant against enrofloxacin. In Japan, resistance to dihydrostreptomycin in 241 STEC O157 isolates from beef cattle was detected most frequently (9.5%), followed by resistance to oxytetracycline (7.9%) and ampicillin (5.4%) [9]. In aggrement with our results, In the USA, all *E. coli* O157:H7 isolates from cattle were found to be susceptible to ceftiofur, gentamicin and trimethoprim-sulphamethoxazole [7]. It was reported that 100% of the 6 STEC O157 strains isolated from ground beef in the Czech Republic were resistant against ampicilline, cephazolin and tetracycline, while 83% were resistant against chloramphenicol and colistin, and 50% were resistant against cefuroxime and cefoxitine [43]. There are very limited numbers of studies in Turkey concerning the determination of the resistances of the STEC O157 strains isolated from cattle faeces and ground beef samples. In a study conducted by Aksoy *et al.*[44], it was reported that all of the 4 STEC O157 strains isolated from cattle were resistant against all antibiotics. Sasaki *et al.*[9] determined that the antibiotics resistance of STEC O157 isolates that contain both *stx*1 and *stx*2 was higher than the resistance of the isolates that contain only *stx*2. However, no significant relation could be found in this study between antibiotics resistance and type of stx.

In conclusion, this study showed cattle are an important reservoir of STEC O157 in Turkey. Cross contamination of carcasses may occur during the slaughter of cattle. This constitutes a serious hazard to human health as it may lead to outbreaks of human STEC O157 infections. Appropriate hygienic measures in food industries including abattoirs may be implemented to reduce the risk of STEC O157 infection. Consumers should take proper care for prevention of the organism such as cold temperature and cooking before consumption. More studies should be carried out to understand a genetic relationship between food, animal and human isolates.

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RESEARCH ARTICLE

İsviçre Esmeri, Holştayn, Simental ve Doğu Anadolu Kırmızısı Irkı İneklerde Prob İlaç Olarak Debrizokin Kullanılarak *in vivo* CYP2D6 Enzim Aktivitesinin Fenotipik Belirlenmesi [1]

Fatih SAKİN * Ersoy BAYDAR ** Kadir SERVİ ***
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Özet

Mevcut araştırma; İsviçre Esmeri (İE), Holştayn (HOL), Simental (Sİ) ve Doğu Anadolu Kırmızısı (DAK) ırkı ineklerde prob ilaç olarak debrizokin (DEB) kullanılarak sitokrom P450 2D6 (CYP2D6) enzim aktivitesinin fenotipik belirlenmesi amacıyla yapıldı. Çalışmada her ırktan 15 adet olacak şekilde toplam 60 adet inek kullanıldı. İneklere DEB, 0.5 mg/kg dozunda uygulandı. Uygulamayı takiben 12 ve 24. saatlere kadar çıkarılan idrar örnekleri toplandı. İdrar örneklerinde DEB metabolik oranları (DMO) ve DEB rekoveri oranları (DRO) hesaplandı. 12. saat DMO değerleri DAK ırkı ineklerde diğer ırklara göre anlamlı şekilde yüksek olarak bulunurken ($P<0.01$), DRO değerleri DAK ırkı ineklerde diğer ırklara göre anlamlı şekilde düşük tespit edildi ($P<0.01$). DAK ırkı ineklerde CYP2D6 enzim aktivitesinin fenotipi zayıf metabolizer (ZM) olarak değerlendirilirken; İE, HOL ve Sİ ırkı ineklerde ise yaygın metabolizer (YM) olarak değerlendirildi. Sonuç olarak; ineklerde *in vivo* CYP2D6 enzim aktivitesi fenotipinin belirlenmesinde prob ilaç olarak DEB'in 0.5 mg/kg dozunda uygulandıktan sonra 12. saatte kadar alınan idrar örneklerinin kullanılabileceği ifade edilebilir. Ayrıca, DAK ırkı ineklerde CYP2D6 sübstratı olan ilaçlarla tedavi uygulanmasında bu ilaçların daha yavaş metabolize olacağı, vücutta kalış ve etki sürelerinde artış olacağı sonucuna varılabilir.

Anahtar sözcükler: *CYP2D6, İnek, Debrizokin, Fenotiplendirme*

Phenotyping Determination of *in vivo* CYP2D6 Enzyme Activity Used as A Probe Debrisoquine in Swiss Black, Holstein, Simmental and Eastern Anatolian Red Cow Breeds

Summary

In the current study was carried out to determine phenotyping of *in vivo* CYP2D6 enzyme activity used as a probe debrisoquine (DEB) in Swiss Black (SB), Holstein (HOL), Simmental (SI) and Eastern Anatolian Red (EAR) cows. In the study, totally 60 cows, fifteen cows from each breed, were used. DEB was application at 0.5 mg/kg. Urine samples were collected throughout 12. and 24th h after DEB application. The metabolic (DMR) and recovery (DRR) rates of DEB in urine samples were calculated to evaluate the *in vivo* activity of CYP2D6 enzyme activity. The DMR value of EAR at 12th h were significantly higher than those others ($P<0.01$) while this value significantly lower in EAR compared to that of others ($P<0.01$). The phenotyping CYP2D6 enzyme activity of EAR at 12th h was considered as poor metaboliser (PM) while this phenotype was extensive metabolizer (EM) in SB, HOL and SI cows. In conclusion, it can be considered that urine samples taken at 12th h after DEB administration at dose of 0.5 mg/kg as probe can be used to determine *in vivo* phenotyping of CYP2D6 enzyme activity in cows. Besides, in implementation of treatment with drugs that are substrates of CYP2D6 in cows, it can be concluded that these drugs will be metabolized more slowly, and the duration time of body and effect durations of action will be increased in EAR cows.

Keywords: *CYP2D6, Cows, Debrisoquine, Phenotyping*

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GİRİŞ

Ksenobiyotik-metabolize eden enzimler, steroidler ve safra asitleri gibi belli endojen bileşiklerin olduğu kadar hayvan vücuduna giren kimyasalların da detoksifikasyonu ve biyoaktivasyonunda önemli bir rol oynarlar [1]. Sitokrom P450 enzim grubu ince bağırsak, karaciğer ve diğer dokularda bulunan, bir sübstrat içine moleküler oksijenden bir oksijen atomunun birleştirilmesini katalize eden, çok sayıda ilaç ve ksenobiyotik sübstansın biyoaktivasyonu ve detoksifikasyonunda kilit rol oynayan monooksijenazların bir süperfamilyasını temsil eder [2-4]. Biyotransformasyon enzimlerinin aktiviteleri esas olarak ilaç ve diğer ksenobiyotiklerin ya farmakolojik ya da toksikolojik tesirlerini etkiler [5]. Memelilerde, sitokrom enzim süperfamilyasının çoğu üyeleri temel olarak expresedir; ve diyet, cinsiyet, tür, fizyolojik durum ile ksenobiyotiklere maruziyet gibi bir çok faktör onların ekspresiyonunu ve/veya metabolik aktivasyonu değiştirilebilir [1]. İnsan ve rodentlerde CYP450 enzim aktiviteleri için kullanılabilir geniş veritabanın aksine veteriner hekimliğindeki hayvan türleri (sığır, koyun, keçi, köpek vs) ile ilgili veriler çok yetersizdir [3].

İlaç-ilaç etkileşimleri açısından önemli bir sitokrom izoformu olan CYP2D6, bütün ilaçların yaklaşık %30'unun metabolizmasından sorumludur [4] ve genetik varyasyonları ilaç metabolizması, farmakokinetikleri ve farmakodinamiklerinde önemli bireylerarası değişkenliklere sebep olabilir [6]. CYP2D6 son derece polimorfik olduğu için farmakogenetiklerin model özelliklerinden biri haline gelmiştir ve antidepresanlar (mianserin, nortriptilin ve venlafaksin), nöroleptikler (levomepromazin, perfenazin, risperidon ve tioridazin), antiaritmikler (enkainid, flekainid, propafenon ve spartein), beta-blokörler (metoprolol, propranolol ve timolol), antihipertansifler (debrizokin, indoramin), dekstrometorfan ve kodein dahil olmak üzere terapötik olarak kullanılan ilaçların geniş bir kısmının metabolizmasından sorumludur [7,8]. Günümüzde, insanlarda CYP2D6 ilaç-metabolizma aktivitesinde bireylerarası ve ırklararası değişkenliğe yol açan CYP2D6 geni için 90'dan fazla allelik varyantları identifiye edilmiştir [9]. Bireylerde CYP2D6 aktivitesi bozulduğunda veya eksildiğinde CYP2D6 tarafından metabolize edilen ilaçların standart dozları advers ilaç reaksiyonları veya terapötik başarısızlık riskini artırabilmektedir [9]. *İn vitro* ve *in vivo* CYP2D6 aktivitesini belirlemek için uygun fenotip problarının kullanımı, ilaç bulma ve geliştirilmesinin daha iyi olması ve bireysel tedavi başarısı için önemli olabilir [6,10,11]. 1970'lerde debrizokin/spartein oksidasyon polimorfizminin keşfinden beri çeşitli etnik gruplardaki farmakogenetik etkilerini araştıran çok sayıda çalışmalar yapılmıştır [12].

CYP2D6 tarafından seçici olarak metabolize edilen spesifik sübstratlar, metabolik aktivitenin değerlendirilmesi için sıklıkla kullanılmaktadır. Spesifik sübstrat ve onun metabolitinin vücut sıvılarındaki konsantrasyonu (metabolik oran) bireysel sitokrom aktivitesinin ölçülmesi için hizmet eder [13]. CYP2D6 için uygun sübstratlar DEB, bufuralol, tramadol, dekstrometorfan, metoprolol ve sparteini içermektedir [11,14]. DEB metabolizma tarafından yaygın olarak elimine edilir. CYP2D6 tarafından katalize edilen 4 pozisyonundaki hidroksilasyon tamamen idrarla atılan 4-OH DEB oluşumu ile sonuçlanır [15]. Bu kapsamlı hidroksilasyon genetik olarak kontrol edilir ve popülasyonlarda YM ve ZM gibi mevcut iki fenotip olarak görülmektedir [16]. CYP2D6'nın *in vivo* aktivitesi tek bir per oral doz debrizokin uygulamasından sonra 6-8. saatlerde toplanan idrardaki DRO (DRO=4-OHD/D+4-OHD) veya DMO (DMO=D/4-OHD) hesaplanarak değerlendirilebilir. DMO parametresi, sıklıkla klinik çalışmalarda kullanılmasına rağmen *in vivo* CYP2D6 aktivitesinin linear bir ölçümünü temsil etmez. DRO, CYP2D6 aktivitesi ile orantılıdır ve 4-OH DEB oluşumu ile daha ilişkilidir. 0.12'den daha düşük DRO veya 12.6'dan daha büyük DMO'lı bireyler ZM olarak dikkate alınır [17,18].

Yapılan literatür incelemelerinde bugüne kadar sığır ırkları arasında *in vivo* CYP2D6 enzim aktivitesinin fenotipik belirlenmesi ile ilgili bir çalışmaya rastlanılmamıştır. Bu çalışmanın amacı prob olarak DEB kullanılarak İE, HOL, Sİ ve DAK ırkı ineklerde CYP2D6 enzim aktivitesinin fenotipik belirlenmesi ve ırklar arasında bu enzim aktivitesi bakımından polimorfizmin olup olmadığını ortaya koymaktır.

MATERYAL VE METOT

Kimyasallar

DEB sülfat, 4-OH DEB ve sodyum hidroksit Sigma (St Louis, MO, USA) şirketinden temin edildi. HPLC-gradeli asetonitril Merck'ten (Darmstadt, Germany) satın alındı. Analitik gradeli diklorometan, izopropanol ve sodyum asetat Merck'ten (Darmstadt, Germany) temin edildi. Mevcut çalışmada kullanılan su ELGA'dan (Bucks, UK) bir Milli-Q sistemi kullanılarak elde edildi.

Hayvanlar, Deneysel Dizayn ve Örnek Toplama

Çalışmada klinik olarak sağlıklı oldukları tespit edilen ve fenotipik özellikleri belirlenen HOL (350-400 kg), İE (350-400 kg), Sİ (360-420 kg) ve DAK (200-300 kg) ırklarından 18-24 aylık her ırktan 15'er inek olacak şekilde toplam 60 adet inek kullanıldı. Hayvanlar aynı şartlarda 1 ay bakım ve beslenmeye alındı. Hayvanlar bireysel bölmelerde barındırıldı ve kuru ot, saman, sığır süt yemi ve arpa tanesi ile beslendi. İçme suyu *ad libitum* olarak verildi. Bu süre boyunca hayvanlara herhangi bir ilaç uygulaması yapılmadı. Çalışma, Fırat Üniversitesi Hayvan Deneyleri ve Etik Kurulunun resmi onayı (Protokol no: 2007/17) alınarak gerçekleştirildi.

Hayvanlara 0.5 mg/kg dozunda DEB sülfat 500 ml serum fizyolojik içerisinde çözdürüldükten sonra bir sonda aracılığıyla direkt olarak rumene verildi. Uygulamayı takiben 12 ve 24. saatlere kadar çıkarılan idrarlar toplanarak 10-15 ml örnek alındı. Alınan idrar örnekleri analizler yapılıncaya kadar -20°C'de saklandı.

Cihazlar ve Sıvı Kromatografisi Şartları

DEB ve 4-OH DEB'in kromatografik analizleri HPLC sistemi (Shimadzu Corporation International Marketing Division, Tokyo, Japan) kullanılarak gerçekleştirildi. HPLC sistemi; sıvı kromatografi LC-20AT solvent dağıtıcı modülü, SIL-20A HT oto örnekleyici, DGU-20A5 degazer, RF-10AXL spektroflumetrik dedektör ve CTO-20A kolon fırınından oluşturuldu. Ayrıca analiz sonuçlarını değerlendirmek için Shimadzu LC Workstation LC-Solution kromatografik data sistemi yazılımı kullanıldı.

Analitlerin kromatografik ayrımları Inertsil ODS-3 kolon (5.0 µm particle size, 4.6 mm × 250 mm, GL Sciences Inc., Tokyo, Japan) kullanılarak gerçekleştirildi. Mobil faz 0.25 M asetat tamponu (pH 5.0) ve asetonitril (9:1, h/h, son pH = 5.4) kullanılarak oluşturuldu. Akış hızı 0.7 ml/dakika olarak kullanıldı. Kolon akışı eksitasyon dalga boyu 210 nm ($\lambda_{eksitasyon}$) ve emisyon dalga boyu 290 nm ($\lambda_{emisyon}$) olarak izlendi. Her örnek için toplam analiz süresi 40 dakika olarak gerçekleşti.

Örnek Hazırlama

İdrarda DEB ve 4-OH DEB konsantrasyonları Pereria ve ark.[19] tarafından bildirilen metoda göre gerçekleştirildi. Özetle; 400 µL idrar numunesi 80 mg sodyum klorür ilavesiyle purifiye edilerek 0.4 M sodyum hidroksit (20 µmol) ilavesiyle pH 9.0 alkanize edildi ve diklorometan: izopropanol (6:4, v/v) karışımı bir vorteks tüp içinde 1 dak. karıştırılarak ekstraksiyon gerçekleştirildi. Ekstraksiyondan sonra 10 dakika için 3000 rpm'de santrifüj edildi ve sulu faz organik fazdan aspirasyonla ayrılarak atıldı. Geri kalan organik faz 10 saniye için sıvı azota daldırılarak organik ekstrakt temiz ve kuru bir konik tüpe aktarıldı. Ekstraktlar azot akışı altında 37°C'de su banyosu içinde kurutularak yoğunlaştırıldı. Kurutulmuş ekstrakt daha sonra 100 µL mobil fazda çözdürüldü ve bunun 50 µL'si kolon içine injekte edildi.

İstatistiksel Analizler

İstatistiksel değerlendirmeler SPSS for Windows 10.0 paket programı kullanılarak yapıldı. Varyans analizi için en uygun kareler toplamı yöntemi belirlendi [20]. Grupların karşılaştırılması tek yönlü varyans analizini takiben grup içi farklılıklar *Tukey* testi ile değerlendirildi. Sonuçlar ortalama ± standart hata (SH) olarak ifade edildi ve P<0.05 değerleri istatistiksel olarak anlamlı kabul edildi.

BULGULAR

Hayvanlarda prob ilaç olarak kullanılan 0.5 mg/kg dozunda DEB sülfat ile ilgili herhangi bir yan etki gözlenmedi.

CYP2D6'nın *in vivo* aktivitesini değerlendirmek için 0.5 mg/kg tek per oral doz DEB uygulamasından sonra 12 ve 24. saatlerde toplanan idrardaki DMO ve DRO hesaplandı. Hesaplanan DMO ve DRO değerlerine ait istatistiksel değerlendirmeler *Tablo 1*'de sunuldu. 24. saatte hesap edilen DMO ve DRO değerleri bakımından inek ırkları arasında istatistiksel olarak anlamlı bir fark tespit edilmedi (P>0.05). 12. saat DMO değerleri DAK ırkı ineklerde diğer ırklara göre anlamlı şekilde yüksek bulundu (P<0.01). 12. saat DMO değerleriyle uyumlu şekilde 12. saat DRO değerleri DAK ırkında diğer ırklara göre anlamlı şekilde düşük tespit edildi (P<0.01). DAK ırkı ineklerde DMO değeri 12.6'dan büyük ve DRO değeri de 0.12'den daha küçük olduğundan bu ırklar ZM olarak belirlendi. İE, HOL ve Sİ ırkı ineklerde DMO değerleri 12.6'dan küçük ve DRO değerleri de 0.12 den büyük olduğundan bu ırklarda YM olarak değerlendirildi.

TARTIŞMA ve SONUÇ

CYP2D6 enzim aktivitesinin değerlendirilmesinde bugüne kadar çeşitli prob ilaçlar kullanılmıştır. Bunlar içerisinde DEB, CYP2D6 ultra hızlı metabolizerlerinin belirlenmesinde kullanılan en iyi prob ilaçtır [21]. Antihipertansif bir ilaç olarak kullanılan DEB farmakogenetik tarihinde bir dönüm noktası olarak görülmektedir [22]. CYP2D6 insanlarda DEB hidroksilasyonunu katalize eden major enzim olarak tanımlanmakta [23] ve CYP2D6'nın genetik değişkenliğinde önemli bir rol oynamaktadır [24]. CYP2D6 genotiplenmesi, CYP2D6 enzim aktivitesini değerlendirmek için yaygın olarak kullanılmasına rağmen CYP2D6 metabolik kapasitesi dekstrometorfan, DEB, metoprolol, tramadol, spartein gibi spesifik

Tablo 1. *İsviçre Esmeri, Holştayn, Simental ve Doğu Anadolu Kırmızısı ineklerde (n=15) 0.5 mg/kg dozunda debrizokin sülfat uygulandıktan sonra 12 ve 24. saatlere ait idrar debrizokin metabolik oranları (DMO) ve debrizokin rekoveri oranları (DRO) ortalama±SH değerleri*

Table 1. *The debrisoquine metabolic ratio (DMR) and debrisoquine recovery ratio (DRR) values of urine samples taken at 12th and 24th hours after debrisoquine sulfate administration at dose of 0.5 mg/kg in Swiss Black, Holstein, Simmental, and Eastern Anatolian Red cows (n=15) (Mean±SE)*

İnek Irkları	DMO		DRO	
	12. saat	24. saat	12. saat	24. saat
İsviçre Esmeri	4.38±0.41^{b}	6.46±0.80	0.20±0.02^{ab}	0.16±0.02
Holştayn	4.56±1.18^{b}	6.25±0.93	0.27±0.03^{a}	0.17±0.02
Simental	6.01±0.94^{b}	6.21±0.68	0.17±0.02^{b}	0.15±0.01
Doğu Anadolu Kırmızısı	14.41±1.69^{a}	6.42±0.34	0.08±0.01^{c}	0.14±0.01
P Değeri	0.000	0.993	0.000	0.557

^{a,b,c} *Aynı sütundaki farklı satırlar arasındaki fark istatistiksel olarak önemlidir (P<0.05)*

bir ilacı içeren bir fenotipik test sonucunun değerlendirilmesi aracılığıyla belirlenebilir [25]. DEB, metoprolol ve dekstrometorfan *in vivo* CYP2D6 aktivitesinin değerlendirilmesi için yaygın olarak kullanılan prob sübstratlardır [26]. Ancak, bu sübstratlar varolan pKa değerleriyle (sırasıyla; 11.9, 9.7 ve 8.3) farklı iyonizasyon özelliklerine sahiplerdir [27]. Buna ilave olarak, idrar pH'sı 4.5-8.5 değerleri arasında değişiklik gösterdiğinden bu ilaçların metabolik oranları kullanılarak enzim aktivitesinin tahmin edilmesinde idrar pH'sına bağımlı böbrek klerensi değişkenliklere yol açmaktadır. Daha önce yapılan çalışmada [28] idrar pH'sındaki değişkenliğin dekstrometorfan ve metoprolol'un metabolik oranlarında %20 ile %80 arasında bireylerarası farklılığa yol açtığı bildirilmiştir. Özdemir ve ark.[29] tarafından yapılan bir diğer çalışmada ise, dekstrometorfan ve metoprolol'un metabolik oranlarının idrar pH'sına bağlı olarak önemli düzeyde değişiklik gösterdiği ancak DEB'in metabolik oranlarının idrar pH değişkenliğinden etkilenmediği rapor edilmiştir. Mevcut çalışmada da CYP2D6 enzim aktivitesinin fenotipik değerlendirilmesi için prob ilaç olarak DEB tercih edilmiştir.

Günümüzde, CYP2D6 polimorfizmi, aktivitesinin 4 seviyesinden birine göre; iki fonksiyonel alleli ile yaygın metabolizerler (YM), fonksiyonel alleli olmayan ve enzim aktivitesinin tam bir eksikliği ile zayıf metabolizerler (ZM), bir aktif heterozigot taşıyıcısında ve bir eksik allel ile orta metabolizerler (OM), son derece yüksek aktivite ile ultra hızlı metabolizerler (UM) sınıflandırılırlar. Buna ilave olarak; YM fenotipi popülasyonun çoğunluğu tarafından ifade edilir ve bu nedenle normal olarak kabul edilir. ZM, iki CYP2D6 allelinden yoksun olduğundan onlar ilaçları daha düşük bir oranda metabolize ederler. Dolayısıyla metabolize olmamış ilaçların seviyelerinde yükselmeye neden olurlar. UM fenotipi aktif CYP2D6'nın duplikasyonuna veya amplikasyonuna neden olurlar. UM genotipi olan bireyler ultra hızlı bir oranda ilaçları metabolize edeceğinden ilaçların standart dozlardaki terapötik etkilerinde bir kayba yol açarlar. Defektif bir CYP2D6 alleline sahip olan bireyler genellikle OM fenotipinde görülmektedir. Bu fenotip, ZM fenotipinden daha iyi ve YM fenotipine yakın bir metabolik aktiviteye sahip olmasıyla geniş bir spektruma sahiptir [30-32]. Farklı etnik gruplarda YM, OM, ZM ve UM'lerin değişken yüzdelerinden dolayı CYP2D6 sübstratları ile ilaç tedavisinin klinik sonuçları değişkenlik göstermektedir [33]. Mevcut çalışmada; hesap edilen DMO ve DRO değerlerine göre, DAK ırkı ineklerde DMO değeri 12.6'dan büyük ve DRO değeri de 0.12'den daha küçük tespit edildiği (Table 1) için bu ırklarda CYP2D6 enzim aktivitesinin fenotipi ZM olarak belirlenmiştir. Buna göre DAK ırkı ineklerde CYP2D6 sübstratı olan ilaçlarla tedavi uygulandığında, enzim aktivitesi zayıf olduğundan ilaçların daha yavaş metabolize olacağı, ilacın vücutta kalış sürelerinin ve etkilerinin uzayacağı sonucuna varılabilir. Çalışmada İE, HOL ve Sİ ırkı ineklerde ise DMO değerleri 12.6'dan küçük ve DRO değerleri de 0.12 den büyük olduğundan bu ırklarda CYP2D6 enzim aktivitesinin fenotipi YM olarak değerlendirilmiştir *(Table 1)*. YM popülasyonun çoğunda görülmekte ve normal olarak kabul edilmektedir.

İnsanlarda ilaç metabolizmasında rol alan enzimlerin aktivitelerinde etnik farklılıkların varlığı birçok çalışmada gösterilmiş olmasına rağmen özellikle besin değeri olan çiftlik hayvanları başta olmak üzere hayvanlarda biyotransformasyon enzimlerinin ırklar arası karşılaştırılması ile ilgili çok az sayıda çalışma mevcuttur. Bu çalışmanın prob ilaç olarak DEB kullanılarak farklı inek ırklarında *in vivo* CYP2D6 enzim aktivitesinin fenotipik belirlenmesi ile ilgili bu dizaynda hazırlanmış ilk çalışma olduğu söylenebilir. Bu çalışmada, İE, HOL, Sİ ve DAK ırkı ineklerde prob ilaç olarak kullanılan DEB'in metabolizmasından sorumlu olduğu kabul edilen CYP2D6 enziminin aktivitesi fenotipik olarak belirlenmiş ve enzim aktivitesi bakımından ırklar arasında farklılık olup olmadığı ortaya konulmuştur.

Sonuç olarak; ineklerde *in vivo* CYP2D6 enzim aktivitesinin fenotipinin belirlenmesinde prob ilaç olarak DEB'in 0.5 mg/kg dozunda uygulandıktan sonra 12. Saatte kadarki çıkarılan idrar örnekleri kullanılabilir. CYP2D6 enzim aktivitesinin fenotipi DAK ırkı ineklerde ZM; buna karşın İE, HOL ve SI ırkı inekler de ise YM olarak tanımlanmıştır. Buna bağlı olarak; ineklerde CYP2D6 sübstratı olan ilaçlarla tedavi uygulanmasında DAK ırkı ineklerde bu ilaçların daha yavaş metabolize olacağı, vücutta kalış ve etki sürelerinde artış olacağı sonucuna varılmıştır. Hayvan sağlığında kullanılan ilaçların klinik etkinlikleri ve halk sağlığını etkileyecek ilaç kalıntısı problemlerini aydınlatabilmek için özellikle çiftlik hayvanları başta olmak üzere tüm hayvan ırklarında CYP450 enzimlerinin metabolizma yolaklarının *in vitro* ve *in vivo* çalışmalarla ortaya konulması, yeni tedavi seçeneklerinin geliştirilmesi, ilaç etkinliğinin arttırılması ve ilaç etkileşimlerinin tespiti gibi optimum tedavi uygulayabilmek için çok önem arz etmektedir.

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RESEARCH ARTICLE

Yerli Ördek *(Anas Platyrhynchase)* ve Kaz'ın *(Anser anser)* Alt Solunum Yolları ve Akciğerlerinde Bulunan Mast Hücrelerinin Dağılımı ve Heterojenitesi Üzerine Morfolojik ve Histometrik Araştırmalar [1]

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Özet

Bu çalışma ördek ve kazlarda alt solunum yolları ve akciğerde bulunan mast hücrelerinin dağılımı ve heterojenitesini belirlemek amacıyla yapıldı. Çalışmada 6 adet kaz ve 6 adet ördek kullanıldı. Kanatlı hayvanların solunum sisteminde bulunan trakeya, syrinks, bronş ve akciğerden uygun büyüklükte parçalar alındı. BLA (Basic Kurşun Asetat - Mota), Carnoy, IFAA (Izotonik Formaldehit Asetik Asit)'da tespit edildikten sonra rutin doku takibi yapılarak paraplast ile bloklandı. 6-7 µm kalınlığında alınan kesitler %0.5'lik toluidine blue ve alcian blue-safranin O (AB/SO) kombine boyalarında boyandılar. Toluidine blue ile boyanan kesitlerde 40'lık objektifte 1mm^2'lik alandaki mast hücre sayıları belirlendi. Her iki hayvan türünde de tespitlere göre değişmekle beraber incelenen organlardan akciğerde en fazla sayıda mast hücresine rastlandı. Sayısal veriler için en uygun tespitin IFAA olduğu, granül yapısını belirlemek için de en uygun tespitin BLA olduğu saptandı. Alcian blue- safranin O boyamasında ise kazlarda trakeya, syrinks, bronş ve akciğerde SO (+), AB (+) ve miks granüllü mast hücrelerine rastlanırken, ördeklerde trakeyada AB (+) hücreler, syrinks, bronş ve akciğerde de AB (+), SO (+), miks özellikte mast hücreleri görüldü.

Anahtar sözcükler: *Kaz, Ördek, Işık mikroskobu, Mast hücresi, Alt solunum yolları, Akciğer, Heterojenite*

Morfological and Histometric Studies on Mast Cell Distribution and Heterogeneity, Present in the Lower Respiratory Tract and in the Lung of Local Duck *(Anas platyrhnchase)* and Goose *(Anser anser)*

Summary

This study was performed to determine mast cell distribution and heterogeneity present in the lower respiratory tract and in the lung of duck and goose. In this study, 6 ducks and 6 goose were used. Sufficient amount of tissue pieces from trachea, syrinks, bronches and lungs were taken from the choosen poultries. The samples were fixed in BLA (Basic Lead Acetate - Mota), Carnoy, IFAA (Isotonic Formaldehyde Acetic Acid), then, after routin tissue follow up, the samples were blocked with paraplast. Obtained sections (6-7 µm) were stained with 0.5% toluidine blue and alcian blue - safranin O (AB/SO). Sections stained with toloudine blue were examined under microscope (40 objective) and the number of mast cell in 1 mm^2 were determined. In both animal species; number of mast cells were changable according to fixation technique, with the highest number in lung samples. For numerical results; the most appropriate fixation technique was IFAA and BLA respectively. In AB/SO staining in trachea, syrinks bronches and lungs of geese, mast cells with SO (+), AB (+) and mix granules were obserwed. On the other hand; with the same staining in ducks; AB (+) cells in trachea, AB (+), SO (+) and mix charecteristic mast cells in syrinks, bronch and lungs, were obserwed.

Keywords: *Goose, Duck, Light microskobe, Mast cell, Lower respiratory tract, Lung, Heterogeneity*

GİRİŞ

Mast hücreleri bağdokunun en iri hücreleridir [1]. Bulundukları organlar özellikle akciğer, deri, bağırsak, nazal mukoza, uterus, tuba uterina, meme derisi, aksillar lenf düğümleri ve midedir [2]. Mast hücreleri ördeklerde ovalimsi,

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yuvarlak, mekik şeklinde, çekirdeği yuvarlak, oval ve segmentsiz [3], çekirdeği bazı türlerde segmentli, çentikli ve ekzantirik yerleşimli görülebilmektedir [4].

Mast hücreleri bulundukları yere ve granüllerindeki kimyasal içeriklerine göre Mukozal Mast Hücreleri (MMC) ve Konnektif Doku Mast Hücreleri (CTMC) olmak üzere ikiye ayrılmaktadır [5].

Mast hücrelerinin heterojenitesini belirlemek amacıyla Carnoy, BLA gibi tespitlere ihtiyaç vardır. Formol içeren tespit solusyonları ise MMC'nin belirlenmesine olumsuz etki etmektedirler. Fakat CTMC formol içeren tespit solusyonlarıyla da tespit edilebilmektedirler. MMC ve CTMC'ler, Carnoy gibi her iki tür hücreyi tespit edebilen solüsyonlarla tespit edildikten sonra Alcian blue/Safranin O (AB/SO) boyamasıyla Alcian blue ile hem MMC, hemde CTMC' ler Alcian blue (+) boyanmakta fakat Safranin O ile sadece CTMC'ler Safranin O (+) boya almaktadırlar [6]. Heterojenite yalnızca boyanma türü ve tespit türü ile değil, hücrenin bulunduğu yer ile de belirlenebilmektedir. Heterojeniteyi belirlemede insanlarda mast hücrelerinde bulunan kimaz ve triptazın varlığı da etkili olmaktadır [7].

Mast hücrelerinin önemi; granüllerinden salgılanan, heparin, histamin, prostaglandin, nötral proteaz, β-glukorunidaz, aryl sülfataz, triptaz, anaflaksinin euzonofil kemotaktik faktörü (ECF-A), anaflaksinin yavaş reaksiyon maddesi olan (SRS-A) gibi hayati öneme sahip faktörleri içermeleridir [1,2,6,8,9].

Bu çalışma ile ördek ve kazlarda alt solunum yollarında mast hücrelerinin dağılım ve heterojenitesini belirlemek, literatüre ve bilime katkı sağlamak amaçlanmıştır.

MATERYAL ve METOT

Çalışmada 6 adet ergin kaz, 6 adet ergin ördek kullanıldı. Hayvanlar Yüzüncü Yıl Üniversitesi Deney Hayvanları ve Etik Kurulu'ndan görevlendirilen denetleyiciler gözetiminde kesildikten sonra alt solunum sistemi organlarından alınan doku örnekleri histolojik yöntemlerle işlendi. Çalışma, Yüzüncü Yıl Üniversitesi Deney Hayvanları ve Etik Kurulu' nun 26.05.20011 tarih ve 2011-05-15 sayılı kurul kararı onay alınarak yürütülmüştür.

Işık Mikroskopik İnceleme

Trakeyanın proksimal, medial ve distal bölgelerinden, sağ ve sol primer bronştan, syrinksten ve akciğerlerin kranial, medial ve kaudal bölgelerinden alınan doku örnekleri daha önceden hazırlanmış olan Carnoy tespiti (60 ml absolut alkol, 30 ml kloroform, 10 ml glasial asetik asit) [3,10], IFAA tespiti (40 ml formaldehit, 100 ml distile su, 0.5 ml glasial asetik asit) (IFAA, pH 2.9) [11] ve BLA tespiti (Basic Lead Asetat) (1 g basic lead asetat, 50 ml etanol, 50 ml distile su, 0.5 ml glasial asetik asit) [12] içerisine konuldu. Alınan bu doku parçaları IFAA' da 24 saat, Carnoy'da 12 saat, BLA'da 24 saat süreyle tespit edildi. Carnoyda yıkama yapmadan dokular direkt %70 alkolde 12 saat bekletilerek, diğer iki tespitte ise yıkama işleminden sonra rutin doku takibinin ardından paraplast ile bloklandılar [11]. Hazırlanan bloklardan 6 µm kalınlığında seri kesitler alındı. %0.5'lik konsantrasyonda olan toluidine-blue ile 10 dk. (pH 0.5) [6,13,14], alcian blue 8GX- safranin O (pH 1.42) kombine boyaları ile 30 dk boyandılar [15,16]. Boyanan preparatlar araştırma mikroskobunda (Nikon Optiphot-2, Japan) heterojenite ve dağılım açısından incelendi. Gerekli yerlerin fotoğrafları çekildi.

Hücre Sayımları ve İstatistiksel Analizler

Toluidine blue ile boyanan preparatlarda mast hücrelerinin dağılımını belirlemek için yapılan hücre sayımında 100 kare oküler mikrometre (eyepice graticule) kullanıldı. Kırklık (40) objektif büyütmesinde oküler mikrometrenin 100 kare birim alanındaki mast hücreleri sayıldı. Seri kesitlerdeki mast hücrelerinin sayılması sonucu bu rakamların aritmetik ortalaması alındı. Böylece 100 kare oküler mikrometrenin kapsadığı alandaki ortalama mast hücresi saptandı. Kırklık (40) objektif büyütmesi için mikrometrik lam yardımıyla, 100 kare oküler mikrometrenin alanı hesaplandı. Daha sonra bu veriler 1 mm^2'lik birim alandaki mast hücre sayısına dönüştürüldü [17]. Kaz ve ördeklerde mast hücre sayımları SAS v. 12.0 paket programı kullanarak varyans analizleri yapıldı. Farklılıklar Duncan Testi ile belirlendi [18].

BULGULAR

Kazlarda Bulgular

BLA, Carnoy ve IFAA tespiti uygulanan trakeya, syrinks, bronşlar ve akciğer dokularındaki mast hücreleri metakromatik boyanmaları ile dikkati çekti. Mast hücreleri solunum sisteminin incelenen organlarında çoğunlukla küçük kan damarları etrafında, kıkırdaklara yakın bağ doku alanlarında ve kas demetlerinin aralarında, yoğunlaşmış olarak yerleşim gösterdikleri tespit edildi.

Trakeyanın bütününde seyrek olarak mast hücreleri görüldü. Lamina propriya ve submukoza bir bütün kabul edilerek istatistiki olarak değerlendirildi. Lamina propriya bölgesinde mast hücrelerinin daha çok epitele yakın yerlerde, kıkırdağa yakın ve kan damarlarına yakın olarak yerleştiği belirlendi. Tunika adventisyada da kan damarları ve kaslara yakın yerleşimli oldukları saptandı. Yerleşim yerlerine göre yuvarlak, oval, mekik şeklinde ve seyrek oldukları görüldü. Trakeyanın proksimalinden distaline doğru gidildikçe mast hücre sayısının arttığı izlendi. Mast hücrelerinde heterojeniteyi belirlemek amacıyla AB/SO boyaması yapıldı. Trakeyada AB (+) hücrelere, SO (+) hücrelere ve miks mast hücrelerine rastlandı.

Syrinkste trakeya ile benzer yerleşimli ve şekilli metakromatik hücrelere rastlandı. Sayısal olarak en fazla mast hücresi uygulanan tespitlerden IFAA'da tespit edilmiş kesitlerde belirlendi. AB/SO boyamasında AB (+) mast hücreleri,

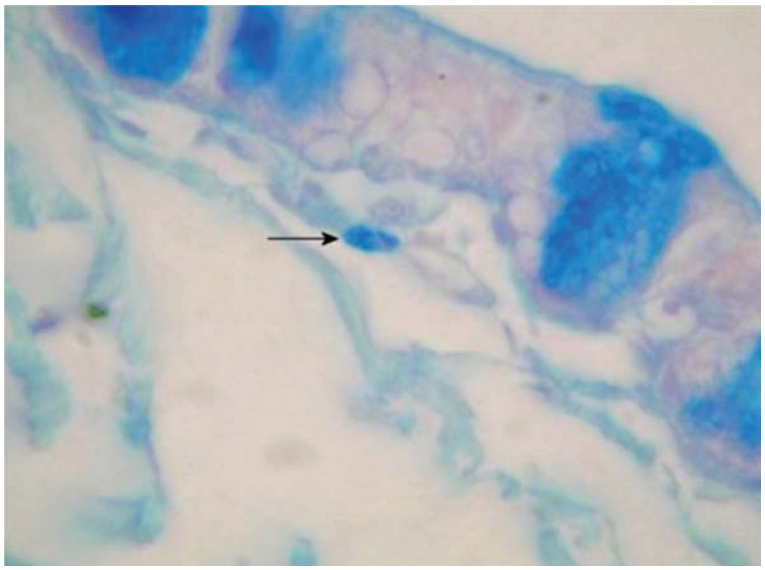
SO (+) mast hücreleri ve miks özellikte granüle sahip mast hücreleri görüldü *(Şekil 1)*.

Primer bronşlardan sağ ve sol bronştan ayrı ayrı alınan doku örnekleri tek bir organ olarak değerlendirildi. Mast hücreleri çoğunlukla lamina propriyada subepiteliyal alanda, kıkırdağa yakın kısımlarda yerleşmişti. Mast hücreleri oldukça küçük ve seyrekti *(Şekil 2)*. İstatistiksel değerlendirme tek bir organ olarak yapılmasına rağmen, sağ ve sol bronş arasında istatistiksel bir fark belirlenmedi. Sayısal olarak ve özelliklerini belirleyebilmek için en uygun tespitin IFAA olduğu saptandı. Mast hücre heterojenitesini belirlemek için yapılan AB/SO boyamasında AB (+) hücreler, çok seyrek olarak da SO (+) hücreler ve miks granüllere sahip hücreler belirlendi.

İntrapulmoner primer bronşlar, sekonder bronşlar ve parabronşlara ait peribronşial bağdoku başta olmak üzere akciğerde çok sayıda mast hücresine rastlandı. Mast hücreleri tüm akciğer dokusu içinde dağınık olarak yayılmış fakat sıklıkla kan damarları çevresinde ve BALT dokusuna yakın olarak yerleştikleri görüldü. Solunum sistemi organlarından en fazla sayıda mast hücresi akciğerlerde belirlendi. Sayısal veriler incelendiğinde en fazla mast hücresi IFAA ile tespit edilen doku örneklerinde görüldü. Akciğerde AB/SO boyamasında AB (+) hücreler yanında SO (+) mast hücreleri ve miks granüllü mast hücrelerine rastlandı *(Şekil 3)*.

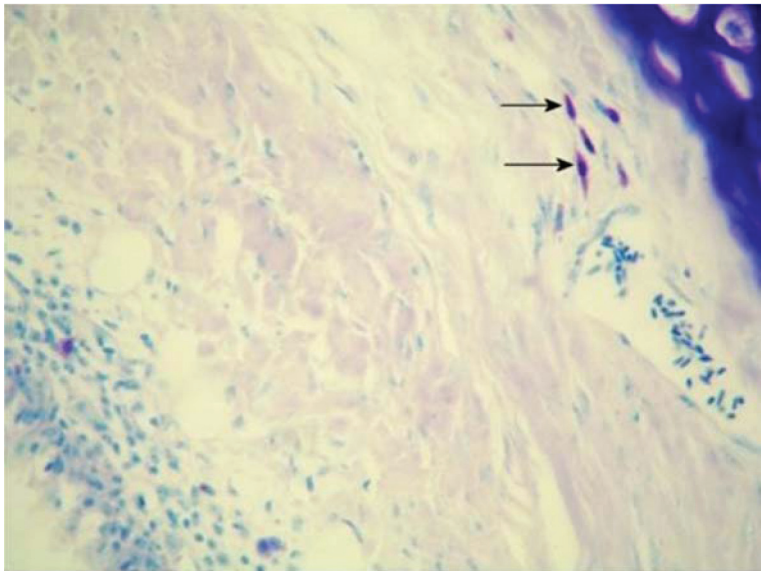
Ördeklerde Bulgular

Ördek mast hücrelerinin toluidine blue ile belirlenmesi ve demonstrasyonu için kullanılan solüsyonlardan BLA ve IFAA tespitlerinin daha uygun olduğu görüldü. Burada da mast hücrelerinin incelenen solunum sistemi organlarında özellikle kan damarları çevresinde, subepitelyal alanda, kıkırdak yakınında ve kas demetleri arasında yerleştiği belirlendi. Heterojeniteyi belirlemede ördekler için en uygun tespitin IFAA ve BLA olduğu saptandı.



Şekil 1. Kaz syrinks AB (+) mast hücreleri *(ok)*. BLA, Alcian blue safranin O. X 1350

Fig 1. Goose syrinks AB (+) mast cells *(arrow)*. BLA, Alcian blue safranin O. X 1350



Şekil 2. Kaz bronş mast hücreleri *(ok)*. BLA, Toluidine blue. X 540

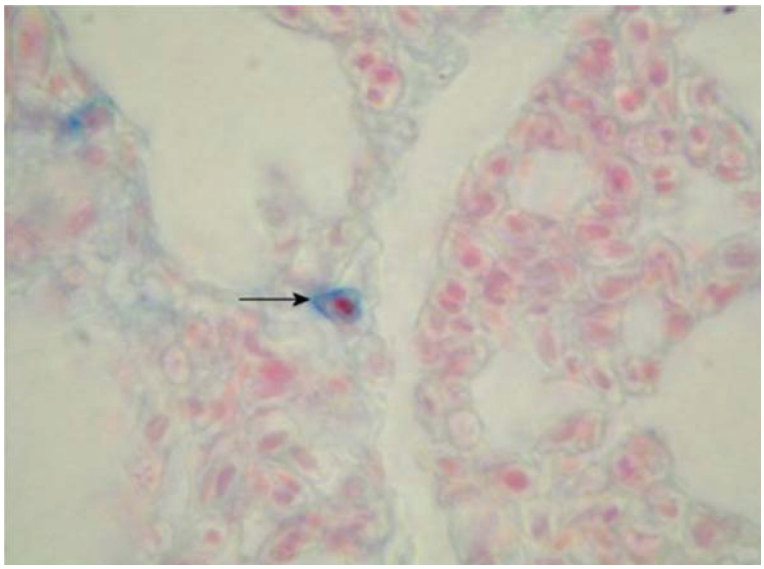
Fig 2. Goose bronchial mast cells *(arrow)*. BLA, Toluidine blue. X 540

Trakeya kıkırdağının iç tarafında lamina propriya ve submukoza, kıkırdağın dış tarafında ise tunika adventisya katmanı incelendi. Kıkırdağın iç yüzünde mast hücrelerinin epitele ve kıkırdağa yakın yerleşimde olduğu, tunika adventisyada ise kan damarlarına komşu olarak yerleştiği görüldü. Yerleşim yerine göre mekik şeklinde veya yuvarlak şekilli olarak görüldüler. Mast hücre çekirdekleri ekzantirik yerleşimli olduğu ve genellikle stoplazmik granüller tarafından örtülü olduğu dikkati çekti *(Şekil 4)*. Mast hücre sayısının en fazla BLA tespitinde olduğu belirlendi AB/SO boyamasında AB (+) mast hücreleri tespit edilirken, SO (+) mast hücresine rastlanılamadı.

Syrinkste daha çok yuvarlak şekilli olarak gözlenen mast hücreleri kan damarlarına, subepitelyal alana ve kıkırdağa yakın bölgelerde gözlendi. Sayısal olarak en fazla mast hücresi IFAA ile tespit edilen doku örneklerinde saptandı. Kullanılan tespit solüsyonlarından BLA tespit sıvısının mast hücrelerinin özelliklerinin ortaya konmasında daha ideal olduğu gözlemlendi. Heterojeniteyi belirlemek için ise IFAA tespitinin daha uygun olduğu sonucuna varıldı. AB/SO boyamasında AB (+) mast hücreleri, SO (+) mast hücreleri, miks mast hücreleri belirlendi *(Şekil 5)*.

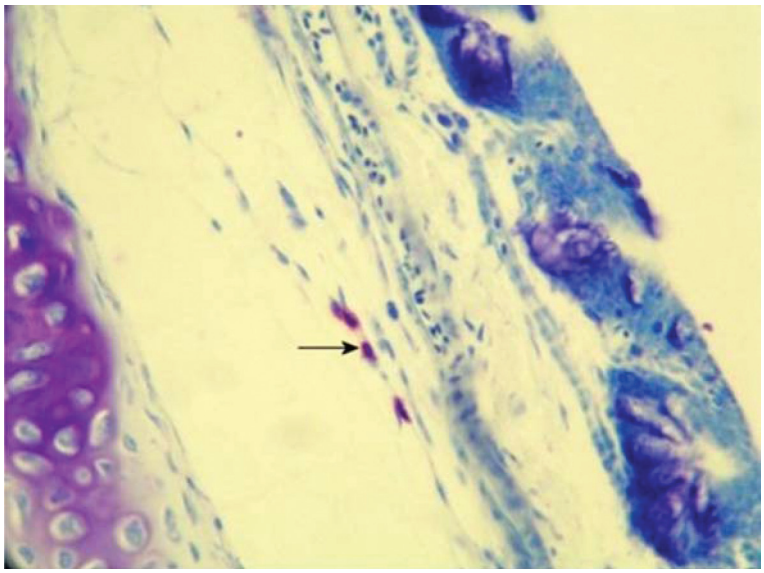
Bronşlarda ekstrapulmoner kısımlarda mast hücreleri daha çok kıkırdağa yakın olarak bulunurken, intrapulmoner alanlarda ise kıkırdak ve bezlerin çevresi ile peribronşial bağ dokuda yaygın oldukları gözlendi. Sayısal olarak en fazla mast hücresi IFAA ile tespit edilen dokularda belirlendi. AB/SO boyamasında AB (+) hücreler, SO (+) hücreler ve miks granüllere sahip mast hücreleri görüldü.

Akciğerdeki mast hücrelerinin yuvarlak ve mekik şekilli, çekirdeklerinin belirgin ve kenara yakın yerleşimde olduğu görüldü. Ördeklerde incelenen organlar arasından en fazla mast hücresi akciğer dokusunda belirlendi *(Şekil 6)*. IFAA'da tespit edilen dokularda mast hücre yoğunluğu daha fazlaydı ($P<0.05$). BLA ile tespit edilen dokularda da



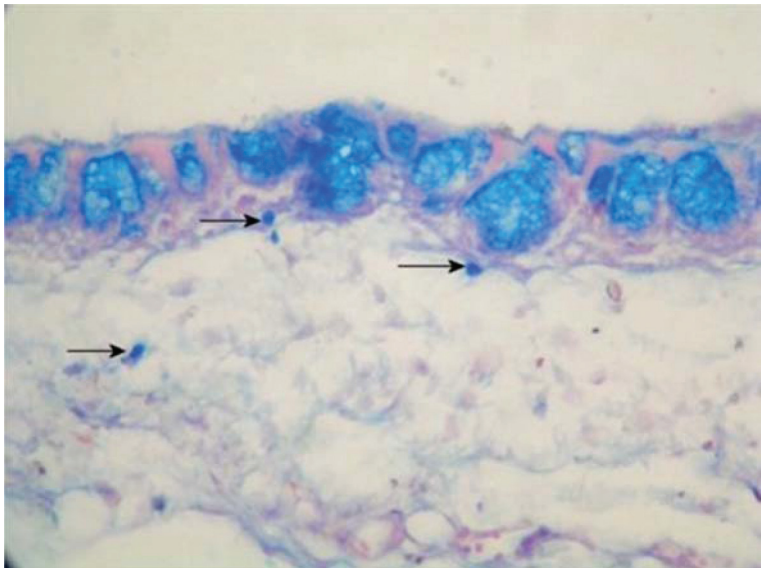
Şekil 3. Kaz akciğer miks mast hücreleri *(ok)*. BLA, Alcian blue safranin O. X 1350

Fig 3. Goose lung mix mast cells *(arrow)*. BLA, Alcian blue safranin O. X 1350



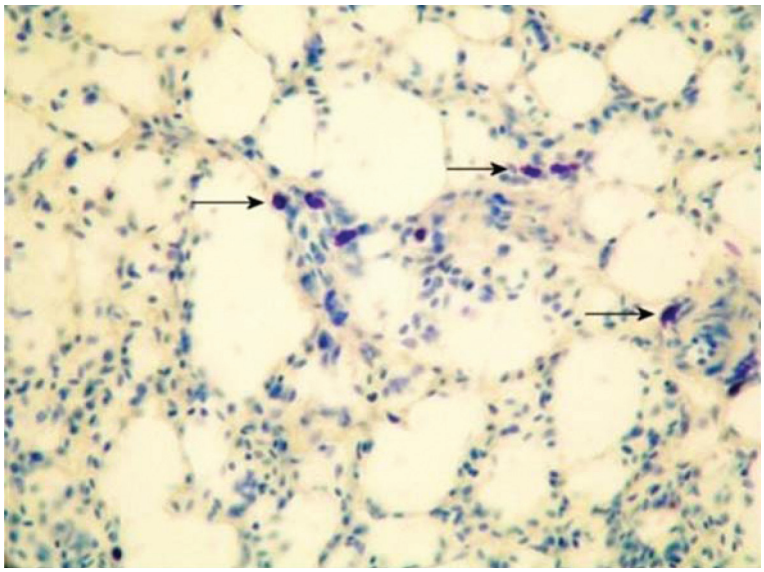
Şekil 4. Ördek trakeya mast hücreleri *(ok)*. Carnoy, Toluidine Blue. X 540

Fig 4. Duck tracheal mast cells *(arrow)*. Carnoy, Toluidine Blue. X 540



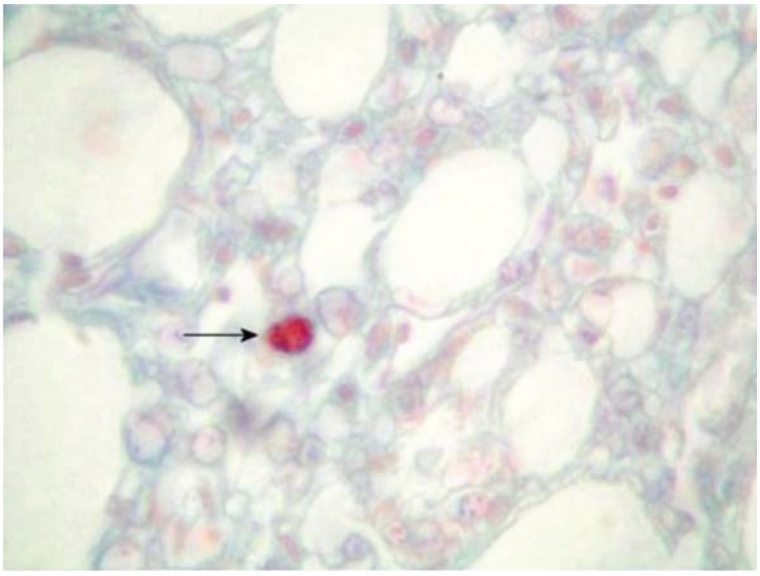
Şekil 5. Ördek syrinks AB (+) mast hücreleri *(ok)*. BLA, Alcian blue safranin O. X 540

Fig 5. Duck syrinks AB (+) mast cells *(arrow)*. BLA, Alcian blue safranin O. X 540



Şekil 6. Ördek akciğer mast hücreleri *(ok)*. BLA, Toluidine blue. X 540

Fig 6. Duck lung mast cells *(arrow)*. BLA, Toluidine blue. X 540



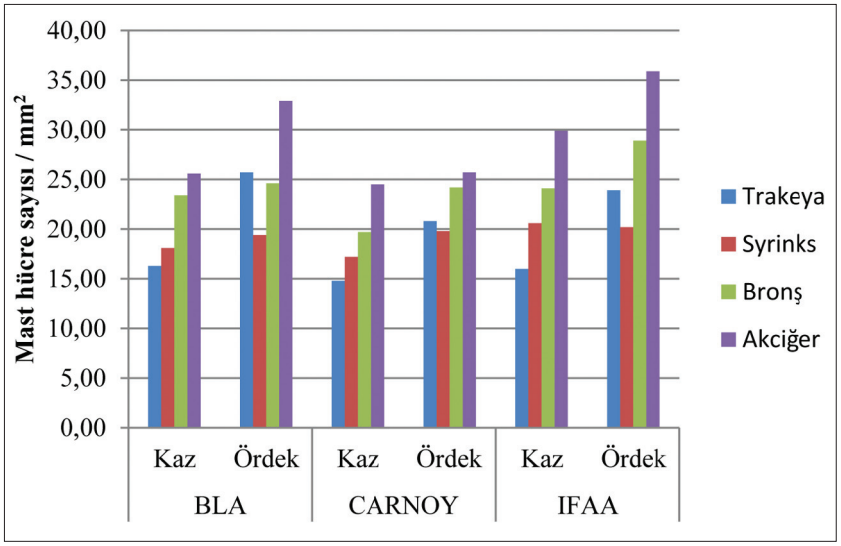
Şekil 7. Ördek akciğer SO (+) mast hücresi *(ok)*. BLA, Alcian blue safranin O. X 1350

Fig 7. Duck lung SO (+) mast cell *(arrow)*. BLA, Alcian blue safranin O. X 1350

Tablo 1. *Ördeklerde ve kazlarda BLA, Carnoy ve IFAA tespitlerinde mast hücrelerinin dağılımı (X±XS) (n=6) hücre/mm²*
Table 1. *Ducks and geese BLA, IFAA, Carnoy's of findings and distribution of mast cells (X ± XS) (n = 6) cells/mm²*

Tespitler	BLA		CARNOY		IFAA	
Organlar	**Kaz**	**Ördek**	**Kaz**	**Ördek**	**Kaz**	**Ördek**
Trakeya	$16.3\pm1.94^{b,A}$	$25.7\pm2.93^{ab,B}$	14.8 ± 1.03^{bA}	20.8 ± 2.23^{B}	$16.0\pm1.64^{b,A}$	$23.9\pm2.16^{bc,B}$
Syrinks	18.1 ± 3.19^{b}	19.4 ± 3.63^{b}	17.2 ± 2.11^{ab}	19.8 ± 2.34	20.6 ± 3.84^{ab}	20.2 ± 3.56^{c}
Bronş	23.4 ± 1.34^{ab}	24.6 ± 2.28^{ab}	19.7 ± 2.75^{ab}	24.2 ± 1.69	24.1 ± 2.63^{ab}	28.9 ± 2.70^{ab}
Akciğer	$25.6\pm1.85^{a,A}$	$32.9\pm5.54^{a,B}$	24.5 ± 3.14^{a}	25.7 ± 1.86^{A}	29.9 ± 3.68^{a}	$35.9\pm1.84^{a,B}$

a, b, c, : *Aynı sütunda farklı harfleri taşıyan grup ortalamaları arası fark önemlidir (P<0.05),* ***A, B:*** *Aynı satırda farklı harfleri taşıyan grup ortalamaları arası fark önemlidir (P<0.05)*



Şekil 8. Ördeklerde ve kazlarda BLA, Carnoy ve IFAA tespitlerinde mast hücrelerinin dağılımı

Fig 8. Ducks and geese BLA, IFAA, Carnoy's of findings and distribution of mast cells

mast hücre granüllerinin IFAA ve Carnoy ile tespit edilen dokulara göre oldukça belirgin olduğu görüldü. AB/SO boyamasında AB (+) hücrelere, SO (+) hücrelere ve miks granüllü hücrelere rastlandı *(Şekil 7)*.

İstatistiksel Bulgular

Ördek ve kazlarda alt solunum yolu organlarında ve akciğerlerinde bulunan mast hücrelerinin tespit organ ve katmanlardaki dağılımlarını belirlemek amacı ile yapılan hücre sayımlarında ulaşılan ortalama değerler *Tablo 1*'de ve *Şekil 8*'de verilmiştir. Kaz ve ördekler alt solunum yollarındaki mast hücreleri sayısal olarak karşılaştırıldığında, ördeklerde kazlara göre daha fazla sayıda mast hücresi olduğu belirlendi *(Tablo 1, Şekil 8)*.

Tablo 1'de gösterilen kaz ve ördeklerde BLA, Carnoy, IFAA tespitlerinde mast hücrelerinin dağılımı *Şekil 8*'de grafik halinde gösterilmiştir.

TARTIŞMA ve SONUÇ

Bu çalışmada, kaz ve ördeklerde alt solunum sistemi organlarında mast hücrelerinin dağılımı, yerleşimi, morfolojileri ve boyanma özellikleri belirlendi. Organların histolojik yapısına göre hücre sayımları ve istatistiksel analizler yapıldı. AB/SO boyamasında da kaz ve ördeklerde mast hücrelerinin heterojenitesi belirlenmeye çalışıldı.

Toluidine blue, thionin, azura A, mast hücrelerini metakromatik olarak boyanmasında ve demonstrasyonunda kullanılan bazik boyalardandır [11,19].

Trakeya, syrinks ve bronşlarda mast hücrelerinin genellikle lamina propriyada, kıkırdaklara yakın bölgeler, tunika adventisya ve kasların arasında kan damarlarına yakın olarak yerleşim yaptıkları belirlendi. Valsala ve ark.[3], ördeklerin çeşitli organlarında yapmış oldukları çalışma, sunulan bu çalışma ile paralellik göstermekteydi.

Kaz ve ördeklerde akciğer incelendiğinde mast hücrelerinin bronşlar etrafındaki peribronşial bağ dokuda, alveoller etrafında küçük kılcallara yakın olarak yerleştiği belirlendi. Çeşitli hayvan türlerindeki çalışmalarda da benzer bulguların olduğu bildirilmiştir [3,20-21].

Kurtdede ve ark.[22], Ankara keçilerinin alt solunum yolları mast hücreleri üzerine yaptıkları çalışmada, trakeyada mast hücrelerinin lamina propriya ve subepitelyal alanda, akciğerde ise interalveoler septumda, bronşiollerin ve kan damarlarının çevresinde yerleştiklerini bildirmişlerdir. Kaz ve ördeklerde yaptığımız çalışmada, alt solunum yollarında bulunan mast hücre şekillerinin bulunduğu bölgeye ve

organa göre değişebildiği belirlenmiştir. Her iki hayvan türünde de trakeya, bronşlar, syrinks ve akciğerde bulunan mast hücrelerinin daha çok yuvarlak, ovalimsi veya mekik şeklinde olduğu görülmüştür. Kurtdede ve ark.[22], Ankara keçilerinde yaptıkları çalışmalarında bizim çalışmamızda görüldüğü gibi yerleşim yerine bağlı olarak mast hücrelerinin yuvarlak, oval, mekik şeklinde ve farklı büyüklüklerde olabildiğini belirlemişlerdir.

Mast hücrelerini belirlemek için çeşitli çalışmalarda farklı tespit solüsyonları kullanılmış ve birçok farklı sonuca ulaşılmıştır [3,21,23]. Yapılan bu çalışmada üç farklı tespit, BLA, IFAA ve Carnoy solüsyonları kullanılmış, organlara göre en uygun tespit türü belirlenmiştir. Çalışmamızda mast hücre özelliklerinin en iyi belirlenebildiği ve granül yapısının daha iyi görüldüğü tespit solüsyonu BLA ve Carnoy'dur. Fakat IFAA'da tespit edilen dokularda sayısal olarak daha fazla mast hücresine rastlanılmıştır.

Valsala ve ark.[3], ördeklerde ve tavuklarda mast hücrelerinde metakromaziyi belirlemede en uygun tespitin Carnoy olduğunu belirtmişler; aynı çalışmada %10 luk formolle tespit edilen ördeklere ait çeşitli dokuların toluidine blue ile metakromatik boyanmadığını bildirmişlerdir. Karaca [13], tavuk ve bıldırcınlarda mast hücreleri için BLA ve Carnoy tespitinin en uygun olduğunu bildirmiştir. Karaca'nın [13] çalışmasında her iki tespit solüsyonunda metakromazi iyi derecede görülürken, BLA tespitinin granül yapısının korunması ve belirlenmesinde daha uygun olduğu bildirilmiştir.

Solunum sisteminde yer alan mast hücreleri, sindirim ve genital sistemlerde bulunanlarla sayısal olarak karşılaştırıldığında daha az sayıda olduğu belirlenmiştir [3]. Yine yapılan literatür taramalarında rat, koyun, sığır ve at [21,24,25] gibi hayvanlarla karşılaştırılınca kaz ve ördeklerde solunum sisteminde daha az sayıda mast hücresinin olduğu tespit edilmiştir. Ördekler ve kazlar kendi arasında karşılaştırılınca ise ördeklerde solunum sisteminde daha fazla sayıda mast hücresi belirlenmiştir.

Wight [10], tavukların çeşitli organlarında yaptığı çalışmada akciğer ve trakeyada mast hücre dağılımlarını incelemiştir. Buna göre trakeyada mast hücresine rastlanmamış ama akciğer dokusunda çok az sayıda mast hücresi görmüştür. Wight'ın [10] bu çalışmasında akciğerde mast hücresine rastlaması bizim bulgularımız ile paralelken, trakeyada mast hücresine rastlamaması bulgularımıza paralel değildir. Yapılan bu çalışmada her iki hayvan türünde de trakeyada ve akciğerde mast hücresine rastlanmıştır.

Karaca ve ark.[26], tavuklarda trakeya ve akciğerlerde bulunan mast hücreleri üzerine yaptıkları bir araştırmada az sayıda mast hücresi olduğu sonucuna varmışlardır. Bizim çalışmamızda da kaz ve ördekte tespitlere göre değişmekle beraber, trakeyada akciğerden daha az sayıda mast hücresi belirlenmiştir. Sunulan bu çalışmada mast hücrelerinin yerleşim yerleri de Karaca ve ark.[26], bulguları ile paraleldir. Bununla beraber Valsala ve ark.[3], ördeklerde yaptıkları mast hücre çalışmalarında akciğer dokusunda çok az sayıda mast hücresi olduğunu belirtmişlerdir.

Kurtdede ve ark.[22], Ankara keçilerinin solunum yollarında yaptıkları çalışmalarında trakeya bölümleri, bronşlar ve akciğerlerdeki mast hücrelerini toluidine blue boyaması ile belirleyip sayısal karşılaştırma yapmışlar ve bizim çalışmamızla paralel sonuçlara ulaşmışlardır. Trakeyanın üç bölgesindeki mast hücre sayısının istatistiksel farkı anlamsızken, bronş ile akciğer arasındaki farkın istatistiksel olarak anlamlı olduğu saptanmıştır.

Mair ve ark.[25], tekparmaklılarda solunum yollarında mast hücrelerinin dağılımı ve yapısını belirlemek için yaptıkları çalışmalarında, Carnoy tespitini kullanmış ve %1'lik toluidine blue boyaması yaparak sayısal verileri değerlendirmişlerdir. Bu sonuçlara göre, trakeyanın üç bölgesi arasında en fazla mast hücresi proksimal kısmında, akciğer dokusunun ise incelenen bölgelerinden en yüksek sayıda mast hücresi sekonder bronşların yakınlarında ve kan damarlarına yakın olarak bulunmuştur. Bu çalışmaya göre en az sayıda mast hücresi akciğerin periferal bölümlerinden alınan doku örneklerinde belirlenmiştir.

Mast hücrelerine granül içeriklerindeki farklılığı belirlemek amacıyla çeşitli boyamalar uygulanabilir. Sunulan bu çalışmada, mast hücre heterojenitesini belirlemek için AB/SO boyaması yapıldı. Kazlarda trakeya, syrinks, bronş ve akciğerlerde AB (+) hücreler ve SO (+) hücreler ve miks granüllü mast hücrelerine rastlanıldı. Ördeklerde ise trakeyada AB (+) hücreler görülürken syrinks, bronşlar ve akciğerde AB (+), SO (+) ve miks hücreler görüldü. Ayrıca, SO (+) hücrelerin syrinks, bronşlar ve akciğerlerde az sayıda olduğu belirlendi. SO (+) hücreler ve miks özellikteki mast hücrelerinin IFAA, BLA ve Carnoy ile tespit edilen dokularda görülebildiği saptandı. Yapılmış olan çalışmalarda da solunum sisteminde bizim çalışmamızla uyumlu veya uyumlu olmayan birçok sonuca ulaşılmıştır. Buna göre; Bachelet ve ark.[20], ratlarda ve kobaylarda alt solunum yollarındaki mast hücrelerinin heterojenitesini incelemişlerdir. Yapılan araştırmada ratların trakeya, bronş ve akciğerlerinde AB (+), SO (+) mast hücrelere ve miks yapıdaki granüle sahip mast hücrelerine rastlanırken, kobaylarda da yalnızca AB (+) mast hücreleri saptanmıştır.

Chen ve ark.[21] koyunlarda, Chen ve ark.[27] sığırlarda alt solunum yollarında mast hücrelerinin heterojenitelerinin üzerine yaptıkları çalışmalarında, AB/SO boyamasında yalnızca AB (+) hücreler gözlemişler fakat aynı çalışmanın kontrolü amacıyla ratlarda aynı sisteme ait organ boyamalarında AB (+) ve SO (+) hücreler saptamışlardır.

Koçak-Harem [28], tavukların alt solunum yollarında yaptığı çalışmasında, tavuklarda alt solunum yollarında AB/SO boyamasında AB (+), SO (+) ve miks özellikteki mast hücrelerini belirlemiştir.

Sonuç olarak, kaz ve ördeklerin alt solunum yollarına

ait organlar incelenmiş; mast hücre morfolojileri, yerleşim yerleri, sayısal dağılımları ve heterojeniteleri ortaya konmaya çalışılmıştır. Yapılan bu çalışmada kaz ve ördeklerde elde ettiğimiz sonuçlar, farklı hayvan türlerinde solunum sisteminde veya bu sisteme ait organlarda yapılan çalışmalardaki literatür bilgisi ile uyum içindedir. Yapılan literatür araştırmalarında kaz ve ördeklerde alt solunum yollarında mast hücreleri ile ilgili çalışmaya rastlanamadığından elde edilen bulguların bu konudaki literatüre katkıda bulunacağı düşünülmektedir.

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RESEARCH ARTICLE

Merinos Irkı Sağlıklı Gebe Koyunların Perifer Kan Lenfositlerinde Alfa Naftil Asetat Esteraz ve Asit Fosfataz Aktivitelerinin Belirlenmesi

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Özet

Bu çalışma, Merinos ırkı koyunlarda gebeliğin perifer kan lenfositlerinin alfa naftil asetat esteraz (ANAE) ve asit fosfataz (ACP-az) aktiviteleri ile perifer kan lenfosit oranları üzerindeki etkilerinin belirlenmesi amacıyla yapıldı. Hayvanlardan her grupta 20'şer adet olacak şekilde gebe olmayan-kontrol, bir aylık, iki aylık, üç aylık, dört aylık ve beş aylık gebeler olmak üzere toplam 6 dönemde perifer kanlar alındı. Histolojik incelemeler sonucunda en düşük ANAE pozitif-lenfosit oranı (%63.5) bir aylık gebe koyunlarda tespit edilirken aynı dönemde null lenfosit sayısı da en yüksek seviyede (%12.75) belirlendi. Gebeliğin son dönemindeki koyunların perifer kan lenfosit (%42.9) ve ACP-az pozitif lenfosit (%43.35) oranlarında da istatistiksel olarak önemli düşüşler gözlendi. Tüm gebelik dönemlerinde perifer kan lenfosit oranlarında hormonal değişimlerin neden olduğu düşünülen dalgalanmalar olsa da en belirgin değişimler gebeliğin ilk ve son dönemlerinde gözlendi. Bu çalışmadan elde edilen bulguların maternal toleransın olası mekanizmalarının anlaşılmasına katkı sağlayabileceği sonucuna varıldı.

Anahtar sözcükler: *ANAE, ACP-az, Null lenfosit, Gebelik, Koyun*

Determination of the Activity of Alpha Naphthyl Acetate Esterase and Acid Phosphatase of Peripheral Blood Lymphocytes in Healty Pregnant Merino Sheep

Summary

This study was performed to determine the effects of pregnancy on the activities of alpha naphthyl acetate esterase (ANAE) and acid phosphatase (ACP-ase) of the peripheral blood lymphocytes in pregnant Merino sheep. Peripheral blood lymphocyte percentages were also estimated. Periferal blood samples were taken from animals in six different gestational stages as non-pregnant control, in the first, the second, the third, the fourth and the fifth month of pregnancy. Each group was contained 20 animals. The lowest ANAE (+) lymphocytes percentage (63.5%) was determined in the first month of pregnancy whereas the highest null lymphocytes proportion (12.75%) was detected in the same gestational period. There were statistically decreases in the proportions of peripheral blood lymphocyte (42.9%) and the ACP-ase (+) lymphocytes (43.35%) in the last gestational stages. Although the posibble hormonal changes may cause the fluctuation of peripheral blood lymphocyte proportions in all gestational periods, the most distinctive changes were observed at the begining and at the end of the pregnancy. It was concluded that the data was obtained from this study was useful for understanding of the possible mechanisms of maternal tolerance.

Keywords: *ANAE, ACP-ase, Null lymphocyte, Pregnancy, Sheep*

GİRİŞ

Memelilerde türün devamlılığı sağlıklı bir gebelikle mümkündür. Embriyonun uterusa tutunması -implante olması- ve gelişmesi sürecinde meydana gelen en kritik olaylardan biri de "immün tolerans"tır. Zira plasentayı oluşturan

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trofoblast hücreleri yarı yarıya babaya ait antijenleri taşımaktır. Allojen antijenleri taşıyan trofoblast hücrelerine karşı annenin bağışıklık sistemi tepkisinin belirli sınırlar içerisinde tutulmasını sağlayan tüm olaylar dizisi "maternal tolerans" olarak bilinir [1,2].

Sağlıklı bir gebelikte annenin bağışıklık sistemi hücrelerinin embriyoya karşı olumsuz tepki göstermemesi için perifer kanda, endometriyumda ve plasenta dokusunda bir takım değişiklikler meydana gelir. Özellikle T-lenfosit alt tipleri ve doğal katil hücrelerinin (natural killer-NK) kan, endometriyum ve plasenta dokusundaki sayıları ve aktivitelerinde gözlenen farklılıklar söz konusu bu değişimin temelini oluşturmaktadır [2,3]. Fizyolojik ve patolojik herhangi bir neden olmaksızın karşılaşılan infertilite olaylarında, yavrunun anne tarafından kabul edilmemesi yani maternal tolerans yetersizliği sorumlu tutulabilir [4,5].

Mahmoud ve ark.[6], sağlıklı hamile ve hamile olmayan bayanlarda yaptıkları bir çalışmada, gebelik süresince perifer kan toplam lenfosit sayılarının yanı sıra B-lenfosit sayısı ile doğal katil hücrelerin (Natural killer-NK) sayısında belirgin düşüşler gözlediklerini bildirirlerken; Medina ve ark.'nın [7] farelerde yaptığı bir çalışmada da gebelikle birlikte B-lenfosit yapımının düştüğü bildirilmiştir. Agricola ve ark.'nın [8] gebe ve postpartum kısraklarda yaptıkları bir çalışmada da perifer kan toplam lökosit sayılarının yanı sıra, toplam T-lenfosit, yardımcı T-lenfosit ve sitotoksik T-lenfosit sayılarında düşüşlerin meydana geldiği gözlenmiştir.

Alfa naftil asetat enzimi (ANAE), lizozomal bir enzim olup; insanlarda, sığırlarda, farelerde ve tavuklarda T- ve B-lenfositler ile monositlerin birbirlerinden ayırt edilmesinde kullanılır [9–12]. Asit fosfataz enzimi (ACP-az) de lizozomal bir enzim olup; memelilerde çoğunluğunu T-lenfositlerinin oluşturduğu hücre populasyonları için spesifiktir [13].

Bu çalışmada Merinos ırkı koyunların perifer kan lenfosit oranları ile ANAE- ve ACP-az-pozitif lenfosit oranlarında gebeliğin farklı dönemlerinde meydana gelen değişimler tespit edilerek, insanlarda ve deney hayvanlarında üzerinde sıkça çalışılan maternal tolerans konusuna çiftlik hayvanları açısından yaklaşılmaya çalışılmış ve bu tür çalışmalarda model hayvan olarak önerilen koyunlardan elde edilecek verilerin ileride yapılacak olan daha detaylı çalışmalara ışık tutması amaçlanmıştır.

MATERYAL VE METOT

Hayvan Materyali

Çalışma, Selçuk Üniversitesi Veteriner Fakültesi Etik Kurulu'nun 23.12.2009 tarih ve 2009/77 sayılı kararı ile alınan Etik Kurul Onayı ile gerçekleştirildi. Çalışmanın hayvan materyalini Selçuk Üniversitesi Veteriner Fakültesi Araştırma ve Uygulama Çiftliğinde bulunan 20 adet Merinos ırkı koyun oluşturdu. Kontrol grubunu oluşturmak amacıyla gebe olmayan hayvanlardan kan örnekleri alındı. Daha sonra arama koçları ile östrusları belirlenen koyunlar aynı ırka ait koçlarla elde aşım yöntemiyle çiftleştirildi. Hayvanların gebelik dönemleri, çiftleşme tarihi ve ultrasonografik (Falco 100, Pie medical, Maastrich, The Netherlands) muayenelerle belirlenerek gebeliklerinin birinci, ikinci, üçüncü, dördüncü ve beşinci ayında olan hayvanlardan kan örnekleri alındı.

Kan Materyali

Hayvanların jugular venasından klasik yöntemle heparinli tüplere yaklaşık 2'şer ml kan alındı. Alınan kanlardan 6'şar adet froti hazırlandı ve bu frotilerden ikisi klasik May Grünwald-Giemsa boyama yöntemi ile boyanırken, ikisi ANAE, ikisi de ACP-az enzimi demonstrasyonları için kullanıldı. Havada kurutulan frotiler -10°C'deki glutaraldehid-aseton tespit solüsyonunda (pH=4.8) 3 dak. süreyle tespit edildiler. Bu sürenin sonunda distile su ile 3 kez yıkanan frotiler oda sıcaklığında kurutuldu [14].

Kurumayı takiben frotiler ANAE ve ACP-az enzimi için hazırlanan inkübasyon solüsyonlarında gerekli sürelerde inkübe edildiler. İnkübasyon süresinin sonunda birkaç kez distile suyla yıkanan preparatlara, asetat tamponunda (pH=4.8) hazırlanmış olan %1'lik methyl-green (Merck) ile çekirdek boyası uygulandı [14].

Enzim demonstrasyonu yapılan kan preparatlarının her birinde toplam 200 adet lenfosit sayılarak pozitif lenfosit oranları belirlenirken, May-Grünwald-Giemsa yöntemi ile boyanan diğer kan preparatlarında lenfosit oranları (%) tespit edildi. Hazırlanan preparatlar DFC-320 model kamera ataçmanı olan Leica DM-2500 model ışık mikroskobu ile incelendikten sonra gerekli bölgelerin dijital görüntüleri kaydedildi.

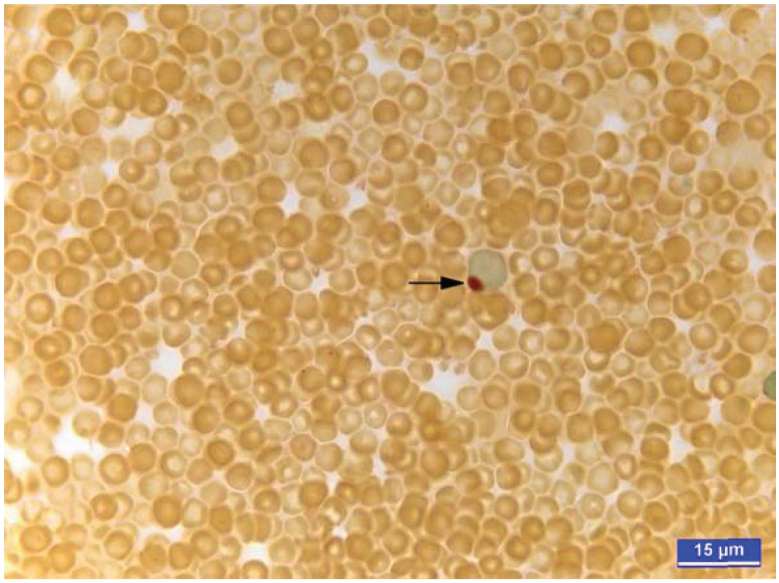
İstatistik

Perifer kan enzim pozitivite oranları ile lenfosit oranları Açı (Arc Sinus) dönüşüm metodu kullanılarak analiz edildiler. Bu metoda göre transforme edilen parametrelerinin birbirleriyle karşılaştırmalarında SPSS 10.0 [15] istatistik programı yardımıyla DUNCAN testi kullanıldı. Verilerin tablolaştırılmasında dönüşüm öncesi gerçek değerler kullanıldı.

BULGULAR

Frotiler üzerinde yapılan ışık mikroskobik incelemeler sonucunda, perifer kan lenfositlerinde 2 farklı ANAE enzimi aktivitesi belirlendi. Sayıları 1-4 arasında değişen kırmızı-kahverengi granüller içeren lenfositler ANAE pozitif lenfositler olarak değerlendirilirken *(Şekil 1)*; 5 ve daha fazla sayıda granül içeren lenfositler "null lenfositler" olarak değerlendirildi *(Şekil 2)*. ACP-az enzimi aktivitesinin ise 1-3 adet pembe-kırmızı granül şeklinde olduğu dikkati çekti *(Şekil 3)*.

Çalışmada elde edilen perifer kan lenfosit oranları ile

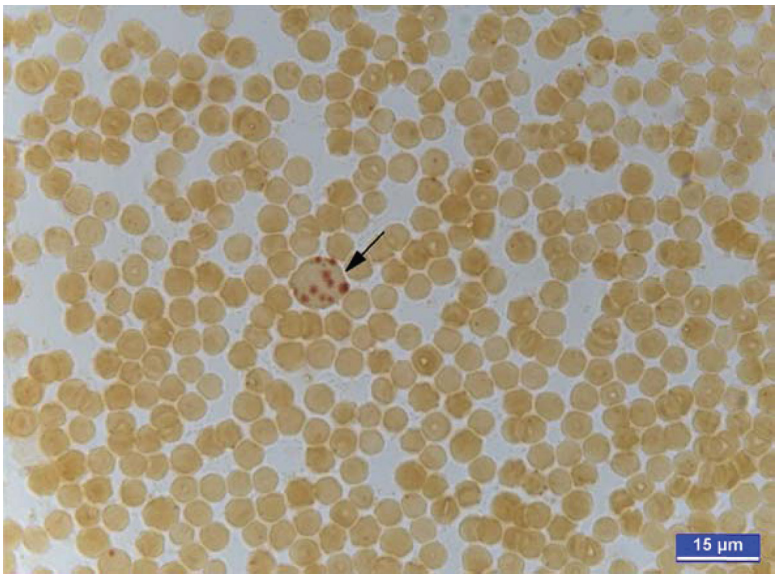


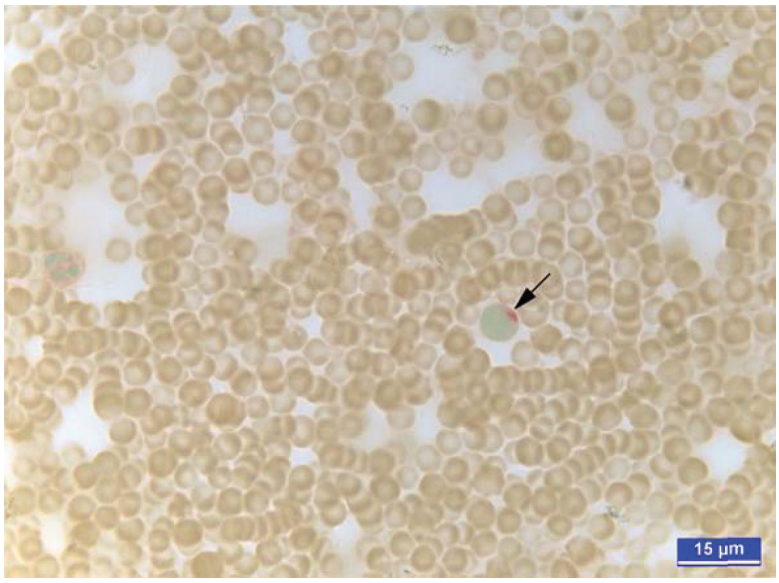
Şekil 1. Kontrol grubundan bir koyuna ait perifer kan frotisinde ANAE demonstrasyonu. *Ok:* ANAE-pozitif lenfosit. Büyütme çizgisi: 15 µm

Fig 1. An ANAE-positive peripheral blood lymphocyte in animal from control group ANAE demonstration. *Arrow:* ANAE-positive peripheral blood lymphocyte. Bar: 15 µm

Şekil 2. Gebeliğin birinci ayındaki bir koyuna ait perifer kan frotisinde ANAE demonstrasyonu. *Ok:* Null lenfosit. Büyütme çizgisi: 15 µm

Fig 2. A null lymphocyte in animal at the first month of pregnancy. ANAE demonstration. *Arrow:* Null lymphocyte. Bar: 15 µm





Şekil 3. Kontrol grubundan bir koyunun perifer kan frotisinde ACP-az enzimi demonstrasyonu. *Ok:* ACP-az-pozitif lenfosit. Büyütme çizgisi: 15 µm

Fig 3. An ACP-ase-positive peripheral blood lymphocyte in animal from control group ACP-ase demonstration. *Arrow:* ACP-ase-positive peripheral blood lymphocyte. Bar: 15 µm

Tablo 1. *Gebeliğin farklı dönemlerinde perifer kan lenfosit, null lenfosit ve ANAE- ve ACP-az lenfosit oranları*
Table 1. *Proportion of peripheral blood lymphocyte, null lymphocyte, and ANAE and ACP-ase positive lymphocyte in different gestational stages*

Gruplar (n=120)	Lenfosit (%) X±SE	ANAE-Pozitif Lenfosit (%) X±SE	Null Lenfosit (%) X±SE	ACP-az Pozitif Lenfosit (%) X±SE
Grup–1 Kontrol (Gebe olmayan hayvanlar)	49.25±8.74^{ab}	73.00±5.41^{a}	4.50±1.70^{b}	63.05±6.88^{a}
Grup–2 (1 aylık gebe hayvanlar)	45.00±4.69^{bc}	63.50±6.23^{c}	12.75±3.14^{a}	62.70±6.38^{a}
Grup–3 (2 aylık gebe hayvanlar)	51.25±7.64^{a}	68.75±6.95^{ab}	5.85±3.51^{b}	60.45±9.00^{a}
Grup–4 (3 aylık gebe hayvanlar)	49.25±7.78^{ab}	72.00±6.96^{a}	4.25±2.07^{b}	59.35±7.15^{a}
Grup–5 (4 aylık gebe hayvanlar)	47.90±7.53^{abc}	67.15±7.24^{bc}	4.40±2.26^{b}	58.95±8.08^{a}
Grup–6 (5 aylık gebe hayvanlar)	42.90±12.78^{c}	68.75±8.22^{ab}	6.20±3.90^{b}	43.35±10.75^{b}

a-c: *Aynı sütunda farklı harfler taşıyan gruplar arasındaki farklılıklar istatistiksel açıdan önemlidir (P<0.05)*

ANAE pozitif lenfosit, null lenfosit ve ACP-az-pozitif lenfosit oranları *Tablo 1*'de verilmiştir. Tabloda da görüldüğü gibi gebeliğin ilk dönemindeki koyunlarda en belirgin düşüşler ANAE pozitif lenfosit oranlarında tespit edilmiştir. Buna karşın aynı dönemde null lenfosit oranı önemli ölçüde yüksek bulunmuştur. Gebeliğin son döneminde ise perifer kan lenfosit oranındaki belirgin düşüşe paralel olarak ANAE ve ACP-az pozitif lenfosit oranlarında da düşüşler tespit edilmiştir. Gebeliğin 2., 3. ve 4. aylarında elde edilen veriler incelendiğinde hormonal değişimlerle ilgili olduğu düşünülen farklılıklar dikkati çekmiştir.

TARTIŞMA ve SONUÇ

Memelilerde gebelikle birlikte yeni bir östrus siklusunun başlamasını önleyip embriyoya karşı annenin bağışıklık sistemi hücrelerinin saldırısını engelleyen ve embriyonun gelişmesine olanak tanıyan bir dizi olaylar sonucunda "maternal immün tolerans" sağlanmış olur [1-2,16]. İmplantasyon olarak bilinen embriyonun uterus endometriyumuna tutunması olayı, temelde yangısal sitokinlerin salınmasıyla karakterizedir [17]. Diğer sistemlere ait mukozalarda olduğu gibi uterus mukozası da normalde T- ve B-lenfositlerinin yanı sıra makrofajlar, dendritik hücreler ve doğal katil hücreleri (natural killer cell-NK) içerir. Martinez ve ark.'nın [18] gebe ve gebe olmayan keçi uterusları üzerinde yaptıkları çalışmada gebe olmayan uterusların içerdiği lenfositlerin pek çoğunun T-lenfosit olduğu; buna karşın gebe uterusların karunkular bölgesinde tüm lenfosit alt tiplerinin hemen hemen tamamının gözden kaybolduğu bildirilmiştir.

ANAE enzimi, içerisinde insanın da yer aldığı pek çok hayvan türünde T-lenfositleri için spesifik bir enzimdir [10-12]. Koyunlarda söz konusu enzimin T-lenfositleri için spesifik bir enzim olmadığı bildirilmekle birlikte [19] diğer türlerde yapılan çalışmalarda lenfositlerde iki farklı ANAE enzimi pozitivitesinden bahsedilmektedir. Bunlardan birisi T-lenfositleri için spesifik olan 1-4 adet lokalize granülden oluşan boyanma şekli, bir diğeri ise çok sayıda dağınık yerleşimli boyanma şeklidir. Bu son pozitivitenin "null" lenfositleri için özel olduğu bildirilmektedir [12,20]. Null lenfositlerinin, NK hücrelerinin öncüllerinin yanı sıra farklı gelişim aşamalarındaki T- ve B-lenfosit serilerine ait hücreleri de içeren bir lenfosit alt tipi olduğu bildirilmektedir [21,22]. Perifer kandaki bu hücrelerin dokulara geçerek tümör hücreleri, virusla enfekte hücreler ve allojen antijenlere karşı organizmayı savunduklarını bildirilirken, uterus dokusuna gelen NK hücrelerinin de uterusta olgunlaşarak büyük ve granüllü hücre formuna dönüştükleri bu andan itibaren de uterus doğal katil hücreleri (uNK) olarak adlandırıldıkları bildirilmektedir [23]. NK hücrelerinin bir bölümünün gebeliğin ilk dönemlerinde sitotoksik T-lenfositlerle birlikte fötusa saldırdığı, ancak düzenleyici NK hücreleri olarak adlandırılan bir diğer bölümünün ise düzenleyici T-lenfositlerle birlikte fötusu bu saldırılardan koruduğu bildirilmektedir [24].

Bazı araştırıcılar null lenfositlerinin NK hücreleri olarak değerlendirilmesi gerektiğini ileri sürmektedirler [25]. Null lenfositlerinin NK hücreleri olarak değerlendirilmesinin ve ANAE histokimyası ile bunların belirlenebilmesinin klinik-laboratuar teşhiste önemli olabileceği; zira gebelikte NK hücrelerinin gerek perifer kan ve gerekse uterus dokusundaki sayılarında önemli değişimlerin olabileceği ileri sürülmektedir. Koç ve Kanter'in [26] gebe sıçanlarda yaptıkları bir çalışmada ANAE pozitivitesi gösteren uterus doğal katil hücrelerinin (uNK) desidual alan içerisinde implantasyonun ilk üç gününde giderek arttığı bildirilmektedir.

Sur ve ark.'nın [14] gebeliğin farklı dönemlerindeki Holstein ırkı süt sığırlarında yapmış oldukları bir çalışmada, 5-8 adet dağınık ANAE (+) granüller içeren ve null lenfosit olarak değerlendirilen hücrelerin oranı gebe olmayan kontrol grubu hayvanlarda %1.45 olarak tespit edilirken, gebeliğin I. trimesterindeki hayvanlarda bu hücrelerin oranının %8.1'e yükseldiği bildirilmektedir. Yine Sur ve ark.'nın [27] fareler üzerinde yaptıkları bir başka çalışmada ise perifer kan null lenfosit oranının kontrol grubu hayvanlarda %5.66, gebeliğin 3. gününe karşılık gelen erken dönemde ise %11.5 olarak tespit edildiği ileri sürülmektedir. Akbulut [28] ise sağlıklı hamile ve hamile olmayan bayanlarda yapmış olduğu bir çalışmada söz konusu hücrelerin oranını kontrol grubunu oluşturan bayanlarda %2 olarak tespit ederken, I. trimesterdeki bayanlarda bu oranın %11'e yükseldiğini ifade etmektedir. Perifer kan null lenfositlerinin gebeliğin farklı

dönemlerindeki oransal değişimlerinin klinik açıdan önemli olduğu bildirilmektedir. Andalip ve ark.'nın [29] sağlıklı bir gebelik süreci geçiren kadınlar ile tekrarlayan spontan düşük (Recurrent Spontan Abortus-RSA) geçmişi olan kadınlarda yaptıkları bir çalışmada sağlıklı gebelerin perifer kan NK oranı %9.21 olarak bulunurken RSA geçmişi olanlarda bu oranın %13.48 olduğu tespit edilmiştir. Bu çalışmada da koç katımı öncesinde kanları alınan ve kontrol grubunu oluşturan koyunlarda perifer kan null lenfositlerinin oranı %4.5 olarak bulunurken, gebeliğin birinci ayındaki hayvanlarda bu oranın %12.75'lere kadar yükseldiği tespit edilmiştir *(Tablo 1)*. Null lenfosit ve NK hücreleri arasındaki ilişki, bu hücrelerin perifer kan ve uterus dokusundaki oranlarının gebeliğe bağlı değişimleri ve klinik bilgiler dikkate alındığında, gebeliğin ilk dönemindeki perifer kan null lenfositlerindeki artışın embriyoya karşı şekillenen doğal tepkinin bir sonucu olabileceği sonucuna varılabilir. İlerleyen dönemlerde bu oranın kontrol grubuna yakın seviyelere gerilemesi de gebeliğin maternal kabulünün bir sonucu olarak kabul edilebilir.

Bu çalışmada perifer kan ANAE-pozitif lenfosit oranları dikkate alındığında gruplar arasında istatistiksel açıdan önemli farklar tespit edilmiştir (P<0.05; *Tablo 1*). En yüksek perifer kan ANAE-pozitif lenfosit oranı gebe olmayan kontrol grubu hayvanlarda tespit edilirken (%73); en düşük ANAE-pozitif lenfosit oranına gebeliğin birinci ayındaki koyunlarda rastlanmıştır (%63.5). Sur ve ark.'nın [14] Holstein ırkı süt sığırlarında yapmış oldukları çalışmada ise perifer kan ANAE-pozitif lenfosit oranları açısından gruplar arasında istatistiksel açıdan önemli bir fark olmamasına karşın en düşük oran I. trimesterdeki hayvanlarda tespit edilmiştir. Sur ve ark.'nın [27] fareler üzerinde yaptıkları çalışmada ise kontrol grubu farelerde perifer kan T-lenfosit oranı en yüksek bulunurken (%64.67), en düşük T-lenfosit oranı gebeliğin 3. gününe karşılık gelen erken dönemde tespit edilmiştir (%43.83). Akbulut'un [28] insanlarda yaptığı bir çalışmada hamile olmayan kontrol grubu bayanlarda %70 olan perifer kan T-lenfosit oranının hamileliğin başlaması ile birlikte hızla düştüğünü bildirmektedir.

Sur ve ark.'nın [14] Holstein ırkı süt sığırlarında, Akbulut'un [28] ise insanlarda yapmış oldukları çalışmalarda perifer kan ACP-az (+)-lenfositlerde I. ve III. trimesterlerde belirgin düşüşler bildirilmiştir. Bu çalışmada ise perifer kan ACP-az (+)-lenfosit oranındaki en belirgin düşüşe gebeliğin son dönemindeki koyunlarda rastlanmıştır (P<0.05; *Tablo 1*).

Pisek ve ark.'nın [30] koyunlarda yapmış oldukları bir çalışmada gebelik süresince perifer kan toplam akyuvar sayısında önemli düşüşlerin meydana geldiği ve düşüşün asıl kaynağının nötrofil ve lenfosit sayılarındaki düşüşler olduğu kanıtlanmıştır. Sur ve ark.'nın Holstein ırkı süt sığırlarında [14] ve farelerde [27] yapmış oldukları çalışmalarda en düşük perifer kan lenfosit oranına gebeliğin erken dönemlerinde rastlandığı bildirilmiştir. Akbulut [28] ise insanlarda yaptığı çalışmada en düşük perifer kan lenfosit oranını III. trimesterde tespit ettiğini bildirmektedir. Bu çalışmada da en düşük perifer kan lenfosit oranına gebeliğin son dönemindeki koyunlarda rastlanmıştır (P<0.05; *Tablo 1*).

Çalışmada gebeliğin ilk döneminde perifer kan ANAE pozitif lenfosit, lenfosit ve ACP-az pozitif lenfosit oranlarında dikkati çeken düşüşlerin gebelik hormonu olarak da bilinen progesteron hormonundaki artışla ilişkili olduğu düşünülmektedir. Söz konusu hormonun, immünosupresif etkisi nedeniyle gebeliğin erken dönemlerinde maternal toleransın gelişiminde kritik bir role sahip olduğu ileri sürülmektedir. Progesteron hormonunun baskın olduğu luteal fazda ve eğer şekillenmişse tüm gebelik süresince lenfositlerde yer alan progesteron reseptörlerinin arttığı bildirilmektedir [31]. Progesteron hormonunun lenfositlerin proliferatif aktivitelerini baskılayıcı etkisi dikkate alındığında söz konusu düşüşlerin progesteron hormonundan ileri gelebileceği düşünülmektedir. Gebeliğin son dönemlerinde meydana gelen ve en belirgin olarak perifer kan lenfosit ve ACP-az pozitif lenfosit oranlarında dikkati çeken düşüşlerin ise doğuma yakın dönemlerde artan fötal kortizol seviyesi ile ilişkili olabileceği düşünülmektedir. Bu süreçte fötal hipotalamus-hipofiz-adrenal korteks etkileşimi sonucu salgılanan kortizol hormonu bir seri hormonal değişimleri tetikleyerek doğumu başlatmaktadır [32]. Yapılan deneysel çalışmalar, koyunlarda oluşturulan stresin fötal ve maternal plazma kortizol seviyesini yükselterek erken doğuma neden olduğunu ortaya koymaktadır [33]. Kortizol hormonunun lenfositler üzerindeki baskılayıcı etkisi göz önüne alındığında [34,35], çalışmada gebeliğin son dönemindeki koyunlarda tespit edilen perifer kan lenfosit oranlarındaki düşüşler ile buna bağlı olduğu düşünülen ANAE ve ACP-az pozitif lenfosit oranlarındaki düşüşlerinin mekanizması bir ölçüde açıklanabilir.

Koyunlar, canlı ağırlıkları, embriyonik gelişim süreçleri, hormonal değişimler, doğumun aşamaları ve mekanizması ile doğum ağırlıkları dikkate alındığında insanlarla benzer sonuçlar veren uygun bir model hayvan olarak değerlendirilmektedir [36]. Fötus ve annenin hayatını tehlikeye atmaksızın uzun süre monitörize edilebilen ve post-operatif süreci sorunsuz yaşayan bu hayvanlar üzerinde uygun anatomileri nedeniyle kolaylıkla kalıcı kateter ve benzeri işlemler de yapılabilmektedir. Aynı zamanda uysal bir karaktere sahip olan koyunlar, periyodik aralıklarla kan alma işlemi gerektiren gebelik, fötal gelişim ve endokrinolojik çalışmalar gibi uzun süreli deneysel çalışmalarda eskiden beri tercih edilen bir tür olarak değerlendirilmektedirler [37]. Sayılan bu avantajlardan dolayı koyunlardan elde edilen bulguların, insanlarda prenatal gelişim ile normal ve premature doğum olgularının anlaşılmasına önemli katkılar sağladığı bildirilmektedir [36]. Bu bilgiler doğrultusunda, maternal toleransın olası mekanizmalarının anlaşılabilmesi için yapılacak daha detaylı deneysel çalışmalarda da koyunların model hayvan olarak kullanılabileceği ve elde edilen verilerin bundan sonra yapılacak olan çalışmalara katkı sağlayacağı düşünülmektedir.

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RESEARCH ARTICLE

Plasma Malondialdehyde, Thyroid Hormones and Some Blood Profiles in Ovine Babesiosis

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Summary

Thyroid hormone and plasma malondialdehyde (MDA) levels as well as other blood parameters were performed in ovine babesiosis. The *Babesia* genus comprises pathogen parasites that cause economic problems in livestock management. Many published studies have suggested its complications, but few studies have assessed blood biochemistry. Hence, a survey of the changes in blood parameters in animals with babesiosis may be useful. Sheep with acute babesiosis were identified based on clinical signs and the observation of *Piroplasms* in red blood cells with Giemsa staining of blood smears. Blood samples were obtained from the jugular veins of 46 babesiosis-infected sheep and 46 healthy sheep without babesiosis. Malondialdehyde (MDA), Paraoxonase (PON), low density lipoprotein (LDL), high density lipoprotein (HDL), Cholestrol (Chol), Total plasma protein (TPP) and glucose (GL) in plasma and triiodothyronine (T3), thyroxine (T4) measured in serum. Levels of erythrocyte superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) and total antioxidant capacity (TAC) were also measured. The results indicated significant increases ($P<0.01$) in MDA and GL and significant decreases ($P<0.01$) in the levels of antioxidant enzymes (PON, GSH-Px, CAT, TAC [except SOD], TPP, T3, andT4, LDL, HDL, and Chol levels when compared with healthy group. The results suggested that hypothyroidism with concurrent oxidative stress are significant signs of ovine babesiosis.

Keywords: *Ovine Babesiosis, Malondialdehyde, Thyroid hormones , Blood values*

Babesiosisli Koyunlarda Plazma Malondialdehid, Tiroid Hormonları ve Bazı Kan Profilleri

Özet

Babeziyozlu koyunlarda bazı kan parametreleri ile birlikte tiroid hormonu ve plazma malondialdehit (MDA) düzeyleri incelendi. Babesia cinsindeki parazitler çiftlik hayvancılığında ekonomik sorunlara neden olurlar. *Babesia* ile ilgili yayınların çoğu hastalığın komplikasyonlarını ile ilgili olup, kan biyokimyası üzerine yapılan çalışma sayısı azdır. Bu nedenle, Babeziyozlu hayvanlarda kan parametrelerindeki değişikliklerin araştırılması yararlı olabilir. Bu çalışmadaki akut babeziyozlu koyunlar, hastalığa ait klinik belirtileri ve Giemsa ile boyanmış preparatlarda enfekte eritrositleri görerek teşhis edilmiştir. Kan örnekleri, 46 enfekte ve 46 sağlıklı koyunun juguler veninden alınmıştır. Plazmada malondialdehit (MDA), paraoksonaz (PON), LDL, HDL, kolesterol (Chol), toplam plazma protein (TPP), glikoz (GL) ve serumda triiyodotironin(T3) ve tiroksin(T4), eritrosit süperoksit dismutaz (SOD), glutatyon peroksidaz (GSH-Px), katalaz (CAT) ve total antioksidan kapasite (TAK) düzeyleri ölçülmüştür. Sağlıklı grup ile karşılaştırıldığında, enfekte hayvanlarda MDA ve GL düzeylerinde belirgin bir artış ve anti oksidant enzimleri (SOD hariç), TPP, T3, T4, LDL, HDL ve Chol düzeylerinde belirgin bir azalma görülmüştür. Bu sonuçlar hipotiroidizm ile beraber görülen oksidatif stresin, koyun babeziyozunda önemli bir gösterge olduğunu göstermektedir.

Anahtar sözcükler: *Koyun babesiosu, Malondialdehid, Tiroid hormonları, Kan profilleri*

INTRODUCTION

Babesiosis is a tick-borne hemoprotozoan disease with host-specific features carried by members of the *Babesia* genus. *Babesia ovis* induces the expression of severe pathogenic traits in sheep and causes sheep babesiosis,

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which is characterized by fever, anemia, icterus and hemoglobinuria [1,2]. *B. ovis* has been observed in most regions of Iran [1]. A high prevalence among sheep and goats in north-eastern of Iran was reported by Razmi et al.[3]. Studies of blood profiles changes in sheep babesiosis are scarce. Hematological and biochemical alterations in splenectomized sheep with experimentally induced *B. motasi* infection were investigated by Alani and Herbert [4]. In 2010 Apaydin et al.[5] investigated serum protein fractions in sheep naturally infected with babesiosis.

Anemia is a common consequence of babesiosis, which causes parasitemia and erythrocyte destruction [6]. Anemia results from the physical effects on erythrocytes, activated macrophage-mediated erythrocyte phagocytosis, antibody-affected erythrocytes and enhanced cell membrane permeability [7]. Moreover, severe cases are not always accompanied by parasitemia [6], while non-parasitic factors may also destroy erythrocytes in animals with parasite infections. Free radicals are thought to play a role in erythrocyte demolition; the assessment of free radicals in the pathogenesis of parasitic infections garnered increasing attention in recent years [7-10].

Free radicals and Reactive Oxygen Species (ROS) are continually produced during metabolic processes; levels may increase under pathological conditions and cause tissue damage [6,11]. Oxidative stress as a result of the excessive generation of free radicals, which overwhelms the antioxidants available (redox imbalance), has been reported in some animal diseases, such as pneumonia, pig's sepsis and airway disorders in horses [11-13]. Oxidative stress causes macromolecular damage, erythrocyte oxidation (cell destruction) and poly unsaturated fatty acid oxidation, which results in lipid peroxidation. Malondialdehyde (MDA) is a by-products of lipid peroxidation. The measurement of MDA levels can be used to indirectly measure the degree of lipid peroxidation and the levels of free radicals [6,9,10]. Literature regarding MDA is scarce but includes reports on elevated MDA levels in sheep naturally infected with *Babesia ovis* and cattle infected with *B. bovis* [9,14]. The thyroid gland plays a vital role in several functions, such as, oxygen consumption by cells, energy utilization, biologic processes (metabolism, growth and nervous improvement) and the productive performance of domestic animals (hair, milk, fiber). Hence, thyroid hormone levels can be investigated as markers of certain animal conditions. The thyroid hormones include of tri-iodothyronine (T3) and tetra-iodothyronine (T4). The chief secretory hormone in adult sheep is T4. Several factors influence thyroid function: e.g seasonal changes, reproductive status, age and mercury-induced hypothyroidism [15-17]. Although related research is abundant, investigations of *Babesiosis*-induced blood profile alterations are scarce.

There are no published literatures on thyroid hormones alterations in sheep babesiosis. Thus, the recent study aimed to evaluate thyroid hormones (T3, T4), MDA as a lipid peroxidation index, some blood profiles and probable interrelationship of these factors each other in ovine babesiosis.

MATERIAL and METHODS

This study was conducted in western Azerbaijan province (North west of Iran) in 2011. The animals were examined for the presence of ticks and clinical signs including hyperthermia (39.9-40.8), anorexia, petechial bleeding, icterus, and hemoglobinuria. Anemia, which is associated with pallor of the mucosal membrane, was observed in 46 sheep. Blood samples were collected via the jugular vein and blood smear staining was performed with Giemsa solution 5%. Microscopic examination in the immersion objective (X100) revealed *Piroplasms* in the same 46 sheep. The other 46 sheep without any clinical or paraclinical signs of babesiosis were selected as the healthy group. Blood samples with and without EDTA were centrifuged at 3000 RPM for 10 minutes at 4°C. MDA and PON activity were detected using the Satoh [18] and Furlong [19] method (spectrophotometer, model Cecil,Italy), respectively. T3 and T4 were measured in serum using a Roche Co. Elecsys 2010 in accordance with the electrochemiluminescence method; GL, TPP, cholesterol, HDL, and LDL (Pars azmoon Co. kits, Tehran, Iran) levels were evaluated in plasma using a Hitachi-917 Auto analyzer (Japan). SOD, GSH-Px, and TAC were measured in isolated and lysed red blood cells (by auto-analyzer, Alcyon-300, USA), (Ransod® and Ransel kits, Randox laboratories Ltd. G.B) and CAT was measured manually according to a previously described method, Abei [20]. Statistical analysis was performed for all of the data completed during the study. The Mean ± SD and the determination of variation between the data points were carried out with Student's *t*-test with SAS v9.1 (SAS Institute Inc., Cary, NC, USA). The significance level was specified at $P<0.01$.

RESULTS

All of the altered parameters are presented in *Table 1*. A significant elevation of MDA ($P<0.01$) was observed in the infected sheep compared to the healthy ones. In contrast, the level of antioxidant enzymes activity (involving CAT, GSH-Px, TAC or PON) was reduced in sheep with babesiosis compared to the healthy group ($P<0.01$). No changes in SOD activity were observed. In the case of thyroid hormones, decreases ($P<0.01$) in T3, T4, lipid profiles, and TPP were observed in infected sheep. Finally, glucose levels were increased in sheep with babesiosis as compared with the healthy group.

DISCUSSION

Lipid peroxidation plays an essential role in erythrocyte oxidation and anemia [9]. Several studies have indicated

Table 1. *Some blood parameters of sheep with babesiosis*
Tablo 1. *Babeziyozlu koyunların bazı kan parametreleri*

Parameters	Control Group	Patient Group
MDA (nmol/ml)	3.13±0.78	8.30±0.86†
T_3 (ng/ml)	1.50±0.2	0.69±0.17†
T_4 (µg/dl)	4.71±0.5	1.63±0.71†
PON (U/L)	68±12.51	26.44±4.82†
SOD (U/gHb)	714.55±74.60	678.33±90.49
CAT (k/gHb)	87.80±14.46	49.96±10.69†
GSH-Px (U/mgHb)	91.39±11.09	49.37±8.04 †
TAC (mmol/L)	0.74± 0.12	0.44±0.9†
GL (mg/dl)	57.77±7.57	93.33±10.50†
TPP (g/dl)	7.06±0.7	5.23±0.36†
Chol (mg/dl)	122.77±17.93	63.22±9.47†
HDL (mg/dl)	26.66±6.34	7.77±1.92†
LDL (mg/dl)	93.55±13.70	57.44±8†

Data are expressed as mean ± standard deviation, † Significantly different from the control group (P<0.01)

the importance of lipid peroxidation in blood parasitic diseases [6,8,10]. Various others have reported babesiosis-induced MDA elevation in domestic animals such as, sheep naturally infected with *Babesia ovis* [14]. The same authors demonstrated the important role of *B. ovis* in the induction of oxidative damage to RBCs and anemia. Moreover, Saleh [9] asserted that various causes can provoke radical species and cause lipid peroxidation in crossbred cattle naturally infected with *Babesia bigemina*: Examples include the potential role of RBCs in free- radical generation and iron`s role in sensitizing the RBC`s membrane. In this study, plasma MDA levels were found to be high in sheep with babesiosis. The likely cause of this elevation is the over-production of free radicals (ROS) and their induced lipid peroxidation. All of the above- mentioned findings are in accordance with the findings presented here. One of the other probable causes of MDA enhancement might be associated with hypothyroidism and increasing in lipid peroxidation, as documented in rats and humans. However, no association between MDA elevation and hypothyroidism has been observed in sheep. Erdamar et al. [21] demonstrated MDA elevation and lipid peroxidation in overt hypothyroidism and hyperthyroidism and concluded that thyroid hormones play an important role in oxidative stress. Moreover, Cano-Europa [22] reported a correlation between thyroid dysfunction and the induction of selective oxidative stress in the rat amygdala and hippocampus. Other authors have reported increases in oxidative stress in the context of hypothyroidism [23,24]. In this report, we observed decreases in T3 and T4. The following factors may influence hypothyroidism-induced lipid peroxidation in sheep: 1) Increased production of hydrogen peroxide (H_2O_2) via low levels of thyroid hormones through the over-activity of superoxide dismutase (manganese dependent) and a decrease in catalase activity in hepatocyte mitochondria [22]. 2) A decline in Na^+/K^+ ATPase activity in the cell membrane. Pachcco-Rosado et al.[25] and Carageorgiou et al.[26] described a decrease in hippocampus Na^+/K^+ ATPase activity in hypothyroid rats and Yur et al.[2] reported Na^+/K^+ ATPase activity decreases in sheep naturally infected with babesiosis. It is likely that decreased levels of Na^+/K^+ ATPase activity would have been observed in the animals studied here, eventually resulting in lipid peroxidation via unknown mechanism(s).

Several studies demonstrated diminishing of antioxidant enzymes (SOD, CAT, GSH-Px, TAC) in hemoprotozoan diseases in domestic animals [14,30] which is in accordance with our study except for SOD activity. Chaudhuri et al.[6] reported a significant elevation of CAT and SOD in canine babesiosis compared with non-babesiosis dogs, which is also in contrast to the results presented here.

PON is a hydrolase that plays a major role in xenobiotic metabolism such as involving in the detoxification of organophosphorus compounds and inhibiting lipid peroxidation [31,32]. There are many reports about PON activity in humans, but reports in the context of veterinary medicine are scarce [31,33]. PON levels were found to be low in this study. The following represent potential reasons for the decrease: 1) A protective role of PON in the peroxidation of lipids and inhibition of the harmful effects of MDA. 2) Decreased HDL levels. Various studies have reported an association between the N-terminal of paraoxonase and apolipoprotein A1 (Apo A1) of HDL [31]. Hence, a decrease in PON might be simultaneously correlated with HDL decreases.

Hypothyroidism is the most prevalent thyroid disturbances in small ruminants and causes disorders that reduce the animal's ability to defend against infections and render it vulnerable to ketosis [34]. Diverse factors such as, iodine deficiency and chronic disease can induce hypothyroidism [15]. Certain situations may cause hypothyroidism without clinical signs, as is typical of secondary euthyroid conditions: for example, Pituitary-Thyroid(HPT) axis disorders and the possible effects on hormone- binding proteins in serum, disturbances in hormone metabolism, disorders in the peripheral distribution of hormones, the inhibition of hormone synthesis and an increase in the release of cytokines (e.g., interleukin-1, interleukin-2, interferon gamma and tumor necrosis factor α) that may be caused anorexia as well as the inhibition of secretion by the glandular portion of the thyroid [35]. Issi et al.[36] reported low levels of free T3, free T4, total T3 and total T4 in the pre-treatment of cattle naturally infected with *Theileria annulata*. A similar study performed by Badiei et al.[37] in crossbred Holstein cattle naturally infected with *Theileria annulata* revealed low levels of T3, T4, free T3 and free T4. The authors postulated a paramount effect of anorexia.

Kumar et al.[35] reported decreases in T3 and T4 in dogs with concurrent *Erlichia canis* and *Babesia gibsoni* infections and hypothesized that the body adapts by decreasing cellular metabolism during the period of disease. In recent

studies the levels of T3 and T4 were low. It is likely that certain factors have induced diminished levels in sheep with bebesiosis. These factors include low iodine levels in soil and food, cytokines elevations due to disease that may cause anorexia and thyroid activity suppression, deficiencies of trace elements (e.g selenium [37], and bodily adaptations such as decreased cellular metabolism during periods of disease as well as seasonally induced alterations [35,38].

Thyroid hormones influence lipid and lipoprotein metabolism in diverse species such as sheep, goat, horse and camel. Moreover, thyroid hormones increase lipolysis in adipose tissue and stimulate lipogenesis and the associated enzyme activity. The relationship between thyroid hormones and cholesterol remains to be elucidated. Notably, triglyceride levels do not correlate with cholesterol or thyroid hormone levels in camels and goats [30,38,39]. Bartley [40] reported cholesterol decreases resulting from the catabolic effect of liver mediated by thyroid hormones. Ibrahim et al.[41] demonstrated the inverse correlation of triglycerides, cholesterol and phospholipid concentrations with hyperthyroidism in stricken Nubian goats. Santi [42] reported an inverse association between hypothyroidism and total cholesterol, triglyceride and LDL -cholesterol. Conflicting data exist regarding the relationship between thyroid function and cholesterol and triglyceride levels. We observed decreases in all aspects of the lipid profile, as reported by Bartley [40]. Various factors may have affected this decrease, such as hypothyroidism, malnutrition and /or seasonal effects.

Thyroid hormones are considerable mediators of glucose metabolism and their roles in provoking glucose utilization have been established [43,44]. Forhead et al.[45] described the effects of thyroid hormones in decreasing liver glycogen storage during late pregnancy in the ovine fetus and suggested a possible influence of hypothyroidism and the related difficulties with euglycemia regulation. Keller et al.[46] reported hypoglycemia in many dogs that suffered from babesiosis and indicated the importance of prompt hypoglycemia detection in canine babesiosis. The significant increase in glucose levels in these animals compared with the non-babesiosis group observed in this study may have been due to disease - the stress induced and/or intravascular hemolysis [47].

Infected animals exhibited a decrease in TPP when compared to healthy ones. Several studies have demonstrated TPP decreases in dog and sheep with babesiosis [5,10,48,49]. Moreover, Barrera et al.[50] suggested that TPP decreases may be due to anorexia in horse babesiosis. Our results generally support this assertion.

In conclusion, on the basis of similar published studies in various species of domestic animals, the results presented here suggest that oxidative stress is a common complication of ovine babesiosis. Moreover, low levels of T3 and T4, which denote hypothyroidism may exacerbate oxidative stress. Thus, it can be concluded that the concurrent occurrence of oxidative stress and hypothyroidism may negatively impact farm animals and thereby meat or dairy production.

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RESEARCH ARTICLE

Comparative Pharmacokinetics of Gentamicin in Laying Hens [1]

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Summary

The aim of this study was to compare pharmacokinetics of gentamicin sulphate (5 mg/kg body weight) after single intravenous, intramuscular and subcutaneous administration in laying hens. Blood samples were collected at time 0 (pretreatment), and at 0.083, 0.166, 0.25, 0.5, 0.75, 1, 2, 4, 8, 12, 24, 36 and 48 h after drug administration in 24 laying hens. Gentamicin concentrations were determined using the HPLC method recommended by the European Union by some modifications. The total concentration of the gentamicin (C1, C2 and C1a) was calculated. The lowest detection limit was 0.01 µg/ml. Noncompartmental pharmacokinetic analyses were performed using Excel add-in program, PK solver. Following IV administration the area under the plasma time-concentration curve from time zero to infinity (AUC0-∞), first-order elimination rate constant (λz), terminal half-life (t1/2λz) and mean residence time (MRT) were 224.46 µg/mL h, 0.06 h-1, 11.52 h and 9.50 h, respectively. After i.m. and s.c. dosing, the mean maximum plasma concentrations (C_{max}) were 26.64 and 36.92 µg/mL, achieved at a same post-injection times (T_{max}) of 0.75 h, respectively. The t1/2λz was 8.35 and 8.24 h, the MRT was 11.05 and 9.79 h, respectively, after IM and SC administration. There are no significant between IM and SC administration excluding the Cmax values and between i.v. and other administration excluding the t1/2λz values.

Keywords: *Gentamicin, Pharmacokinetics, Laying hens*

Yumurta Tavuklarında Gentamisinin Karşılaştırmalı Farmakokinetiği

Özet

Çalışmada, yumurtlayan tavuklara damar içi, kas içi ve deri altı yolla verilen gentamisin sülfatın (5 mg/kg canlı ağırlık) karşılaştırmalı farmakokinetiğinin belirlenmesi amaçlandı. 24 tavuğa ilaç uygulandıktan sonra kan örnekleri 0 (uygulama öncesi), 0.083, 0.166, 0.25, 0.5, 0.75, 1, 2, 4, 8, 12, 24, 36 ve 48. saatlerde toplandı. Gentamisin konsantrasyonları Avrupa Birliği tarafından önerilen yöntemde modifikasyon yapılarak belirlendi. Gentamisinin toplam konsantrasyonu (C1, C2 ve C1a) ölçüldü. En düşük belirlenme limiti 0.01 µg/ml olarak hesaplandı. Non-kompartmental farmakokinetik analizleri Excel ile çalışan program olan PK solver ile belirlendi. Damar içi uygulamayı takiben plazma konsantrasyonu zaman eğrisinin altında kalan alan (AUC0-∞), ilk hız atılma sabitesi (λz), yarı-ömür (t1/2λz) ve ortalama tutulma zamanı (MRT) sırasıyla 224.46 µg/mL saat, 0.06 saat-1, 11.52 saat ve 9.50 saat olarak ölçüldü. Kas içi ve deri altı uygulamadan sonra ortalama maksimum plazma konsantrasyonu (C_{max}) sırasıyla26.64 ve 36.92 µg/mL, maksimum konsantrasyona ulaşmak için geçen zaman (T_{max}) ise aynı (0.75 saat) olarak belirlendi. Yine kas içi ve deri altı uygulamadan sonra sırasıyla t1/2λz 8.35 ve 8.24 saat, MRT ise 11.05 ve 9.79 saat olarak ölçüldü. Kas içi ve deri altı uygulama arasında Cmax değerleri hariç ve damar içi ile diğer uygulamalar arasında t1/2λz değerleri hariç tutulursa önemli farklılıklar olmadığı sonucuna varılmıştır.

Anahtar sözcükler: *Gentamisin, Farmakokinetik, Yumurta tavuğu*

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INTRODUCTION

Gentamicin is an aminoglycoside antibiotic for treating a variety of bacterial infections in pigs, cattle, poultry and horses. In veterinary medicine it is normally used as the sulphate salt [1]. It is effective against gram-negative and some gram-positive bacteria, but not anaerobic bacteria [2,3]. In view of their polar nature and high aqueous solubility, aminoglycosides are poorly absorbed after oral administration. However, the absorption after intramuscular (IM) or subcutaneous (SC) administration in most species is good with peak blood concentrations occurring within 30 to 90 min. Aminoglycosides are not metabolized and are eliminated unchanged in the urine by glomerular filtration. Within 24 h 80 to 90% of the administered dose is eliminated [4]. In mammals and birds, systemic administration of aminoglycosides is complicated by their nephrotoxicity [5,6]. There are no avian-specific data on the pharmacokinetics of systemic aminoglycosides, but as avian and mammals both exhibit aminoglycoside-induced nephrotoxicity, it is likely that elimination occurs via the renal pathway in avian as it does in mammals [2,7].

Therapeutic use of antibiotics in laying hens poses a particular problem because it may result in drug residues in the eggs that are laid during and after treatment. The elimination of gentamicin residues in eggs was reported by Filazi et al.[8]. When administered to laying hens via IM or SC routes, gentamicin was deposited in egg yolk and albumen, with residues persisting for longer periods in the yolk [8,9].

Although the aminoglycosides have been extensively reviewed, few studies on the pharmacokinetics of gentamicin are available in chickens, but none in laying hens. Therefore, the aim of this study was to compare pharmacokinetics of gentamicin sulphate (5 mg/kg body weight) after single intravenous (IV), IM and SC administration.

MATERIAL and METHODS

Chickens

Twenty-four ISA Brown laying hens, 30 weeks of age, were kept individually in fibre cages (30 cm x 35 cm x 45 cm), within a ventilated, heated room (20°C) and given 14 h of light a day. The animals were monitored for 3 weeks for any apparent clinical signs and to ensure that they were free from antibiotics before drug administration. They received a standard commercial layer mash (120 g/d) and water *ad libitum*. The study was authorized by the official ethical committee of Faculty of Veterinary Medicine in Ankara University (2004/17-45)

Drug

A veterinary drug containing 50 mg gentamicin in 1 mL was used (Gentavet, Vetaş Company, Istanbul, Turkey).

Experimental Design

Chickens were individually weighed before drug administration (1.6-2.0 kg body weight) and doses were calculated accordingly. They were divided into three equal groups (8 birds/group). Chickens of groups 1, 2 and 3 were given a single dose of gentamicin (5 mg/kg bw) by IV, IM and SC administration, respectively. Gentamicin was given in the left brachial vein, pectorals muscle and neck for IV, IM and SC administration, respectively. Blood samples (1-1.5 ml) were collected from brachial and cutaneous ulnar veins into heparinized tubes at time 0 (pretreatment) and at 0.083, 0.166, 0.25, 0.5, 0.75, 1, 2, 4, 8, 12, 24, 36 and 48 h after drug administration in 24 laying hens. They were directly centrifuged at 3000 rpm for 5 min to obtain clear plasma and were stored at -21°C until assayed. Gentamicin concentrations were determined using the HPLC method recommended by the European Union by some modifications. The total concentration of the gentamicin (C1, C2 and C1a) was calculated [8,10]. The lowest detection limit was 0.01 µg/ml.

Statistics and Data Analysis

Variance analysis was applied to all data and a multiple range test was used to determine whether or not there were differences among the groups (SPSS Release 17). Non-compartmental pharmacokinetic analyses were performed using Excel add-in program, PK solver [11].

RESULTS

All chickens were clinically healthy throughout the experimental period. The mean plasma concentration-time profiles of gentamicin (5 mg/kg bw) after IV, IM and SC administration are shown in *Fig. 1*.

The pharmacokinetics parameters of gentamicin after single IV, IM and SC administration are shown *Table 1*.

DISCUSSION

Parenterally administered gentamicin is much more bioavailable than when given orally; therefore, it is generally administered by IV, IM and SC routes [12]. Thus, all of the AUC values were high.

After all routes of administration of gentamicin, the terminal half-life was higher than values reported in turkeys [12,13], eagles [14], roosters [15] and broiler chicks [16]. This may due to differences in drug formulation and the gentamicin assay.

As shown *Table 1*, gentamicin is rapidly absorbed after IM and SC administration with C_{max} of 26.64 and 36.92 µg/mL at 0.75 h. These results are higher than values reported in turkeys [12], roosters [13] and broiler chicks [14]. This may due to individual differences that interfere with the drug

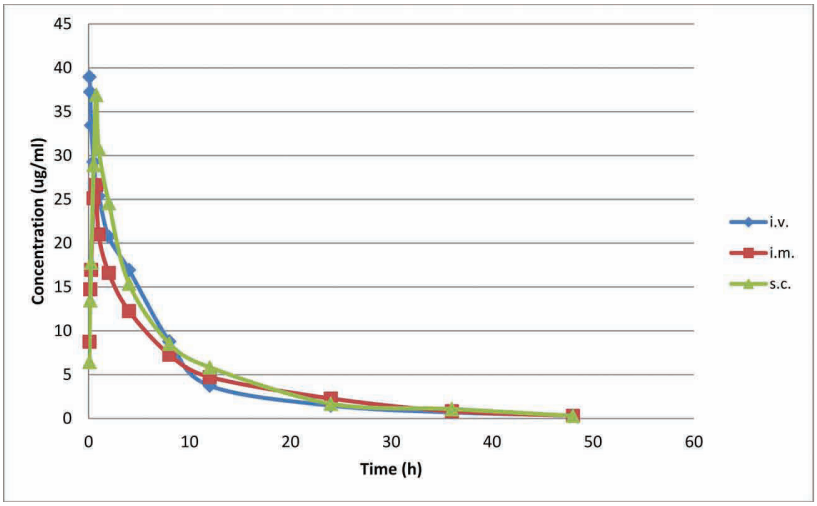

Fig 1. Plasma concentration-time profile of gentamicin after IV, IM and SC administration of 5 mg/kg bw as determined by HPLC, Values are mean ±SE (n=8)

Şekil 1. Damar içi, kas içi ve deri altı yolla 5 mg/kg canlı ağırlık dozunda uygulanan gentamisinin HPLC ile ölçülen plazma konsantrasyonu-zaman grafiği, Değerler ortalama±SE olarak verilmiştir (n=8)

Table 1. *Pharmacokinetic parameters of gentamicin (5 mg/kg bw) in laying hens after a single parenteral administration*
Tablo 1. *Tek bir parenteral uygulamayla yumurta tavuklarına 5 mg/kg dozunda verilen gentamisinin farmakokinetik parametreleri*

Parameter	Units	Administration Route		
		Intravenous	Intramuscular	Subcutaneous
λz	h^{-1}	0.06	0.08	0.08
$t_{1/2\lambda z}$	h	11.52*	8.35	8.24
MRT	h	9.50	11.05	9.79
C_{max}	µg/mL	-	26.64	36.92**
T_{max}	h	-	0.75	0.75
$AUC_{0-\infty}$	µg/mL.h	224.46	202.56	242.86

λz: *First-order elimination rate constant,* $t_{1/2\lambda z}$***:*** *Terminal half-life, MRT: Mean residence time,* C_{max}***:*** *Mean maximum plasma concentration,* T_{max}***:*** *time of peak plasma concentration,* $AUC_{0-\infty}$***:*** *The area under the plasma time-concentration curve from time zero to infinity, * Values differ significantly compared with IM and SC (P<0.05), ** Values differ significantly compared with IM (P<0.05)*

distribution, which will result in delay or acceleration of the drug elimination.

Since the effect of gentamicin is concentration dependent, such that the antimicrobial drug kills bacteria to a greater extent at increasing exposure concentration, the efficacy of gentamicin is achieved when C_{max} reaches 8-10 times above the MIC of it against the susceptible microorganisms [15]. The reported MICs of gentamicin against susceptible microorganisms isolated from different species of animals were 2, 1.2, 8, 4 and 0.8 µg/ml for *Escherichia coli*, *Proteus* spp., *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Staphylococcus aureus*, respectively [18] and 1 µg/ml for both *E. coli* and *Salmonella* species isolated from diseased chickens [12]. The results of this study showed that (C_{max}/MIC>10) for most susceptible bacteria after parenteral administration. Therefore, a dose of 5 mg/kg bw seems to be suitable therapeutic dose of gentamicin in laying hens.

It should be noted that therapeutic use of gentamicin in laying hens poses a particular problem because it may result in drug residues in the eggs that are laid during and after treatment [7]. In contravention of the regulations in European Union, Turkey and some other countries, there is a tendency for withdrawal times for drugs used for laying hens to be ignored, because of the producer's financial loss in large poultry flocks. In cases where it is necessary to use gentamicin, an appropriate period must be taken into consideration by producers and the eggs containing gentamicin residues should not enter the human food chain during this withdrawal period. Obeying the legislation regarding drug residue withdrawal periods is essential to protect consumer health.

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RESEARCH ARTICLE

Impacts of Climate Parameters on Physiological Characteristics of Karayaka Sheep

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Summary

Objectives of this research were to investigate the variations in adaptation parameters of Karayaka rams and ewes to cold winter conditions, to determine mutual interactions among them and to put forward their physiological responses against climate parameters. Average body temperatures of rams and ewes were respectively determined as 39.51°C and 39.31°C in the morning, 39.64°C and 39.50°C in the evening; pulse rates were determined as 88.86 and 83.04 pulse/min in the morning, 91.68 and 86.96 pulse/min in the evening; respiration rates were determined as 39.71 and 43.06 times/min in the morning, 41.36 and 44.35 times/min in the evening. Effects of sex and measuring time on body temperature, pulse and respiration rates were found to be significant (P<0.01), interaction between sex and measuring time was found to be insignificant (P>0.05). A negative correlation was observed between relative humidity and physiological parameters. Correlations between air velocities and physiological parameters were found to be insignificant (P>0.05).

Keywords: *Environmental conditions, Physiological response, Body temperature, Respiration rate, Pulse rate, Ewe, Ram*

İklim Parametrelerinin Karayaka Koyunlarının Fizyolojik Özellikleri Üzerine Etkisi

Özet

Bu çalışma soğuk kış koşullarında iklim parametreleri ile Karayaka koç ve koyunlarının adaptasyon parametreleri değişimlerinin incelenmesi, birbirlerine olan etkilerinin belirlenmesi ve fizyolojik tepkilerinin ortaya konması amacıyla yapılmıştır. Erkek ve dişi bireylerde ortalama vücut sıcaklığı sırasıyla sabah 39.51°C, 39.31°C; akşam 39.64°C, 39.50°C; nabız sayısı sabah 88.86 adet/dak, 83.04 adet/dak; akşam 91.68 adet/dak, 86.96 adet/dak, solunum sayısı sabah 39.71 adet/dak, 43.06 adet/dak; akşam 41.36 adet/dak, 44.35 adet/dak olarak belirlenmiştir. Vücut sıcaklığı, nabız sayısı ve solunum sayısı ortalaması üzerine cinsiyet ve ölçüm zamanının etkisi önemli (P<0.01), cinsiyet ve ölçüm zamanı arasındaki interaksiyon istatistiki olarak önemsiz bulunmuştur (P>0.05). Bağıl nem ve fizyolojik parametreler arasında negatif bir korelasyon tespit edilmiş, rüzgar hızı ile fizyolojik parametreler arasında belirlenen korelasyonlar istatistiki olarak önemsiz bulunmuştur (P>0.05).

Anahtar sözcükler: *Çevre koşulları, Fizyolojik tepki, Vücut sıcaklığı, Solunum sayısı, Nabız sayısı, Koç, Koyun*

INTRODUCTION

Climate is one of the most significant factors effective in animal adaptation to surrounding environment. It has several parameters like ambient temperature, relative humidity and air movement all with considerable impacts either alone or in combination on both biological and yield activities of livestock. Therefore, breeding studies mostly deal with the adaptation capacities of livestock to various environmental conditions.

Impacts of climate on animal performance vary based on age, sex and breed of animals. Changes in environmental and climate conditions create some problems in adaptation of animals already adapted to previous conditions. Such changes even result in fatal losses in young animals. Therefore, environmental conditions have critical importance in livestock production activities. Climatic biological capacities of sheep are especially more flexible than the other livestock [1].

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Basic objectives of environmental studies are to comprehend the effects of environmental changes on animal performance, to improve the adaptation capacities of animals to variable conditions through breeding activities and to provide support to knowledge in environmental physiology. All these objectives should be taken into consideration in relation with each other rather than considering them one by one. Primary aim of livestock production is to get sufficient yield with sufficient quality at low-cost. Beside the hereditary characteristics, environmental conditions have also significant impacts on the amount and quality of products in sheep breeding. Varied sheep breeds are raised in different regions of Turkey for milk, meat and mutton production. Karayaka sheep is one of the major indigenous sheep breeds of Black Sea Region in Turkey. This breed is commonly raised in Tokat, Samsun and Amasya Provinces of the region. It is highly adapted to harsh environment of the region. The total population of the breed in the region is about 1.300.000. Being a non-fat tailed sheep in a harsh environment makes the Karayaka sheep more interesting. This breed is mainly kept for its high meat quality. Characteristics of products vary considerably with climatic environmental conditions.

Adaptation capacities of animals to climatic environment may be understood by determining some physiological characteristics such as body temperature, pulse and respiration rates. There are no reports on effects of climate parameters on physiological characteristics of Karayaka ewes and rams. This study was conducted to investigate the impacts of yield-related climate parameters on physiological characteristics of Karayaka ewes and rams and to evaluate the relationships among these variables.

MATERIAL and METHODS

The study was carried out at the experimental farm of the Gaziosmanpasa University, Tokat, Turkey (40°31 'N, 36° 53' E and 650 m above the sea level). Data were collected from an experimental nucleus flock of Karayaka sheep at college farm of Gaziosmanpasa University in the year 2011. Experiments were carried out during the month January with the lowest temperatures of the year. A total of 30 Karayaka sheep were used in experiments. Of these, 10 were rams with an average weight of 50.9 kg and 20 were ewes with an average weight of 35.54 kg.

Experiments were carried out in an open-sided shelter type barn with open North side. Rams and ewes were housed at separate pens with 2 m^2 space per sheep. Sheep were exposed to cold stress during the experiments. Hourly air temperature and relative humidity in sheltered yard were continuously measured with data loggers (HOBO Type: U12-012- temperature measuring range between -20°C and 70°C with ±0.35°C accuracy; relative humidity range of 5-95% with ±2.5% accuracy) placed at a level equal to animal height. Air velocities were measured with a digital anemometer (Testo 425 hand-held hotwire anemometer - measuring range of 0-15 m/s with ±0.05 m/s accuracy).

Physiological adaptation mechanisms (body temperature, pulse and respiration rates) were determined to evaluate the impacts of climate. These adaptation mechanisms were measured every day at 8 a.m. and 6 p.m. Sheep were kept at resting position for a while before to take such measurements and were made ready for measurements. Pulse rate was determined by pressing over *arteria femoralis* with hand and counting the pulse for 60 sec. Respiration rate was measured by counting nose expressions for 60 sec. Body temperatures were measured with a digital thermometer by inserting it 4 cm into rectum. The thermometer was kept inside the rectum until a steady temperature was observed [2].

Sheep were fed with 200 g concentrated feed (93.1% dry matter, 15.2% crude protein, 29.41% ADF, 30.22% NDF, 2.23% crude oil, 8.6% crude ash, 2.605 cal/kg metabolic energy) daily during the experimental period. As a fodder, 1.1 kg medium quality dry clover forage (94% dry matter, 15% crude protein, 59.75% ADF, 58.22% NDF, 0.74% crude oil, 10.30% crude ash, 1878 cal/kg metabolic energy) was provided to each sheep daily.

Criteria specified by NIH (National Institute of Health Guide for the Care and Use of Laboratory Animals) were obeyed during the experiments carried on animals.

Experiments were conducted in 2x2 factorial randomized block design and SPSS 17.0 [3] was used for statistical analysis.

RESULTS

Daily average temperatures, relative humidity and air velocity values are presented in *Fig. 1*. Air temperatures, relative humidity, dew points, air velocity and enthalpy values measured in this study are given in *Table 1*.

During the research period, average temperature was determined as 2.3°C, the lowest as -7.8°C and the highest as 13.9°C. Temperature was below 4°C at 71% of the measuring period, below 0°C at 27.9% and below -5°C at 2.6%. Dew point temperature varied between -10.4 and 4.5°C and was below 0°C at 79.4% of the measuring period. The lowest average temperature during the hours in which adaptation parameters of animals are measured was determined as -6.2°C in morning measurements and -0.9°C in evening measurements.

Another significant environmental parameter, average relative humidity was determined as 73.7% and the value was above 80% at 40.1% of measuring period, below 60% at 17.7% of the time, below 80% at 59.8% and below 50% at 4.0% of the time.

Average air velocity was 1.25 m/s and morning values (1.3 m/s) were higher than evening values (0.8 m/s). The

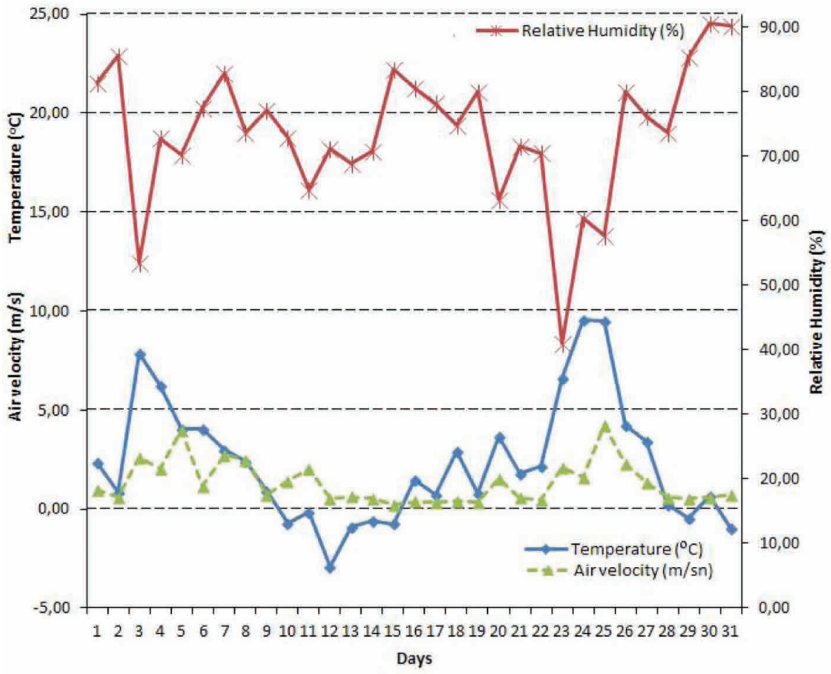

Fig 1. Daily average temperature, relative humidity and air velocity

Şekil 1. Günlük ortalama sıcaklık, bağıl nem ve hava akım hızı

air was still-windless at 26.1% of the measuring period. Air velocity was below 0.4 m/s at 49.8% of the time, above 5 m/s at 4.51% and above 3 m/s at 13.6% of the time.

Average body temperatures, pulse and respiration rates of rams and ewes in morning and evening hours are given in *Table 2*. The relationships between climate parameters and physiological characteristics are provided in *Table 3*.

Average body temperatures of rams and ewes were respectively measured as 39.5°C and 39.3°C in the mornings, 39.6°C and 39.5°C in the evenings. Body temperatures of ewes were found to be lower than rams ($P<0.01$). Measuring time and sheep sex had significant effects on body temperature ($P<0.01$). The interaction between measuring time and sheep sex was not found to be significant ($P>0.05$).

A correlation coefficient of -0.480 was observed between relative humidity and body temperature of ewes ($P<0.01$). Changes in air velocity did not have significant impacts on body temperatures ($P>0.05$).

Average pulse rates of rams and ewes of Karayaka sheep were respectively counted as 88.86 and 83.04 pulse/min in the morning, as 91.68 and 86.96 pulse/min in the evening. The lowest pulse rate was observed in ewes during morning measurements. Measuring time and sex had significant effects on pulse rates ($P<0.01$). Interaction between measuring time and sex was found to be insignificant ($P>0.05$).

While the effects of morning temperatures on pulse and respiration rates of rams and ewes were significant ($P<0.01$), effects on body temperature was not significant ($P>0.05$). Effects of evening temperatures on pulse rate and body temperatures were significant ($P<0.01$), effects on respiration rate were not significant ($P>0.05$).

A negative correlation was observed between relative humidity and physiological parameters. However, negative correlations between relative humidity and morning respiration rates of rams and ewes ($P<0.05$), between relative humidity and evening pulse rates of rams and ewes ($P<0.01$), between relative humidity and evening body temperature of ewes ($P<0.01$) were found to be significant.

Correlations between air velocity and physiological parameters of rams and ewes were found to be insignificant ($P>0.05$).

DISCUSSION

Air temperature, relative humidity and air velocity have direct and indirect impacts on animals. Such climate parameters significantly affect both heat exchange of animals with surrounding ambient atmosphere and physiological characteristics of animals. Therefore, climate parameters of the region should definitely be analyzed before the initiation of any sheep breeding facility. Optimum temperature range for sheep is specified as 10-13°C [4,5]. Convenient temperature ranges were implied as 4-24°C by [6,7], as 8-17°C by [8]. Lower limit of optimum temperature was indicated as 7°C by [4,5], as 5°C by [8]. It was also observed that sheep grown at temperatures between -27°C and 6°C had higher feed consumptions but lower weight gains than sheep raised at 15°C [7].

Another significant environmental factor to be considered for sheep health is relative humidity. Average relative humidity was determined as 73.7% in current study. Optimum relative humidity ranges were specified as 60-80% by Mutaf and Sonmez [8]. Ekmekyapar [7] indicated that 80% relative humidity at severely cold regions did not have significant

Table 1. *Climate parameters and enthalpy values*
Table 1. *İklim verileri ve entalpi değerleri*

Day	Temperature (°C)			Dew Point (°C)			Humidity (%)			Air Velocity (m/s)			Enthalpy (kcal/kg dry air)		
	a.m.	p.m.	Mean	a.m.	p.m.	Mean	a.m.	p.m.	Mean	a.m.	p.m.	Mean	a.m.	p.m.	Mean
1	1.6	3.0	2.4	0.1	-1.0	2.3	90	75	81	0.4	0.4	0.9	11.1	11.8	11.5
2	-3.4	1.9	0.8	-5.0	-3.4	0.4	89	68	86	0.0	0.9	0.6	3.1	9.3	9.4
3	7.6	10.3	7.8	-2.4	0.1	-1.1	49	49	54	4.0	2.7	2.6	15.5	19.9	16.7
4	7.2	5.3	6.2	0.4	3.5	1.6	62	88	73	2.2	0.0	2.0	17.0	17.5	17.0
5	4.3	3.4	4.0	-1.5	-1.2	-0.9	66	72	70	5.8	0.9	4.0	12.8	12.1	12.9
6	1.7	4.9	4.0	-0.4	1.2	0.4	86	77	78	0.0	1.8	1.2	10.9	15.2	13.8
7	3.2	2.7	3.0	1.5	-1.1	0.4	89	76	83	0.9	3.6	2.7	13.8	11.4	12.7
8	2.6	1.9	2.4	-2.1	-2.1	-1.9	71	75	74	5.4	0.4	2.5	10.7	10.0	10.6
9	1.2	-0.9	0.9	-1.2	-4.3	-2.7	84	78	77	0.0	0.0	0.7	9.8	6.0	8.7
10	-2.3	-0.5	-0.7	-6.3	-4.6	-5.0	74	74	73	4.0	0.4	1.4	3.6	6.2	5.8
11	-1.3	-0.9	-0.1	-6.5	-6.9	-6.1	68	64	65	4.9	0.4	2.1	4.5	4.7	5.9
12	-6.2	-1.7	-2.9	-8.9	-5.9	-7.5	81	73	71	0.4	0.4	0.5	-1.5	4.3	2.5
13	-5.3	1.2	-0.9	-7.3	-4.7	-6.3	86	65	69	0.0	0.4	0.6	0.1	7.9	5.2
14	-5.3	0.9	-0.6	-7.6	-4.0	-5.5	84	70	71	0.0	0.0	0.5	- 0.0	7.9	5.8
15	-1.9	0.6	-0.8	-4.4	-1.5	-3.2	83	86	84	0.0	0.0	0.2	4.9	9.1	6.7
16	0.7	1.1	1.4	-0.7	-2.8	-1.7	90	75	81	0.4	0.4	0.4	9.6	8.7	9.9
17	-1.1	2.9	0.7	-3.3	-2.0	-2.7	85	70	78	0.4	0.0	0.3	6.3	11.0	8.5
18	0.6	5.2	2.9	-1.1	-1.1	-1.4	88	64	75	0.4	0.4	0.4	9.3	14.0	11.6
19	-0.8	2.6	0.8	-3.0	-1.3	-2.3	85	76	80	0.0	0.0	0.4	6.7	11.2	8.8
20	1.1	3.9	3.6	-3.8	-2.3	-2.9	70	64	63	3.6	0.0	1.5	8.2	11.9	11.4
21	-1.4	2.6	1.8	-3.9	-2.3	-3.0	83	70	72	0.0	0.0	0.6	5.6	10.6	9.5
22	-2.3	3.9	2.2	-4.5	-1.2	-2.9	85	69	71	0.0	0.0	0.5	4.4	12.5	9.9
23	0.7	10	6.6	-2.7	-0.8	-1.5	78	47	41	0.4	2.2	2.1	8.4	19.0	12.9
24	9.5	10.6	9.5	1.9	2.2	2.1	59	56	60	0.0	3.1	1.6	20.4	21.8	20.7
25	10.1	10.2	9.5	0.4	1.8	1.3	51	56	58	4.0	1.8	4.3	19.9	21.1	20.2
26	3.2	5.2	4.2	1.7	0.4	1.0	90	71	80	0.0	0.4	2.3	13.9	14.9	14.5
27	2.7	3.6	3.4	0.7	-2.4	-0.5	87	65	76	0.0	0.9	1.3	12.7	11.5	12.6
28	-2.0	0.7	0.2	-4.3	-4.1	-4.0	84	70	74	0.0	0.0	0.6	4.8	7.6	7.3
29	-0.6	-0.2	-0.5	-3.6	-1.7	-2.7	80	90	85	1.3	0.9	0.5	6.6	8.1	7.3
30	0.3	0.7	0.6	-0.9	-0.6	-0.7	92	91	91	0.0	0.4	0.6	9.2	9.7	9.6
31	-1.7	-0.6	-1.0	-2.9	-2.0	-2.4	91	90	90	0.9	0.9	0.7	5.8	7.5	6.9
Average	0.7	3.0	2.3	2.6	-1.8	-1.9	79	71	74	1.3	0.8	1.3	11.1	11.8	11.5

negative impacts on physiological characteristics of sheep when the optimum temperatures were provided.

Temperature and relative humidity values of current study were out of the ranges specified by previous researches. These values indicate that sheep were exposed to high relative humidity and low temperature stress during most of the research period.

The air velocities measured in present study were generally above the recommended values. Although the rates between 0.15-0.40 m/s are found to be convenient for sheep, values above 2.5 m/s may have negative impacts on animals [7].

Body temperature findings of current study are in compliance with the findings of Williamson and Payne [11], AbiSaab and Sleiman [9], DaSilva and Minomo [10] and Ceyhan et al.[2]. A 1°C increase or decrease in body temperature is sufficient to drop the performance of sheep. Body temperature is a physiological response of animal to heat stress [12].

Srikandakurmar et al.[13] observed the body temperatures of cold-sheltered sheep as 39.5°C for Merino sheep and 39°C for Omani sheep. Increasing air temperatures raised the body temperatures of Merino sheep to 39.8°C and body temperatures of Omani sheep to 39.7°C.

Results indicated that sheep were under ambient temperature stress. Since the air temperatures were relatively lower in second and third weeks of the study,

Table 2. *Body temperatures, pulse and respiration rates*
Tablo 2. *Vücut sıcaklıkları, nabız ve solunum değerleri*

Parameter	Time	Ram	Ewe	Mean	P
Body Temperature	Morning	39.51±0.050	39.31±0.027	39.38±0.024	**
	Evening	39.64±0.047	39.50±0.031	39.54±0.026	
	Mean	39.57±0.034	39.40±0.021	39.46±0.018	
	P	**			
Pulse Rate	Morning	88.86±0.821	83.04±0.580	84.98±0.482	**
	Evening	91.68±0.898	86.96±0.640	88.54±0.526	
	Mean	90.27±0.611	85.00±0.435	86.76±0.359	
	P	**			
Respiration Rate	Morning	39.71±0.324	43.06±0.231	41.94±0.195	**
	Evening	41.36±0.330	44.35±0.227	43.35±0.193	
	Mean	40.53±0.234	43.70±0.163	42.64±0.138	
	P	**			

***P<0.01*

Table 3. *Correlation coefficients between physiological characteristics of Karayaka sheep*
Tablo 3. *Karayaka koyunlarının bazı fizyolojik parametreleri arasındaki korelasyon katsayıları*

Time	Parameter	Ram			Ewe		
		Body Temp.	Pulse	Respiration	Body Temp.	Pulse	Respiration
Morning	Air Temperature	0.350	0.591**	0.627**	0.271	0.520**	0.623**
	Relative Humidity	-0.261	-0.304	-0.357*	-0.455	-0.351	-0.371*
	Air Velocity	0.068	0.025	0.058	0.141	0.180	0.162
Evening	Air Temperature	0.560**	0.503**	0.397	0.596**	0.568**	0.276
	Relative Humidity	-0.336	-0.526**	-0.309	-0.480**	-0.581**	-0.280
	Air Velocity	0.189	0.047	0.191	0.327	0.028	0.194

** P<0.05; ** P<0.01*

body temperatures were also found to be lower in second and third weeks. Increase in total heat capacity of the air also increased the body temperatures of sheep.

Sheep are homo-thermic animals and they need stable body temperature to sustain their physiological functions. They can keep steady body temperature in certain ranges of ambient temperature. Sheep can arrange heat transfer between the ambient atmosphere and their bodies without hyperthermia or hypothermia and they do not need much energy to conserve body temperature within thermo-neutral (comfort) zone. Sheep do not sweat due to insufficient sweat glands and it is hard for them to adapt hot conditions. Therefore, they are better adapted to cold conditions than hot conditions [7,14].

Pulse rates observed in present study are in compliance with the values observed by Altan and Sendil [15], for sheep (60-90), by AbiSaab and Sleiman [9] for breeding and Ivesi sheep (99.0 and 93.1 pulse/min).

Eyal [16], reported the pulse rate as 80 pulse/min at 5 a.m., as 100 pulse/min at 7 p.m. and decreased rates later on. Naqvi et al.[17], investigated the physiological impacts of ambient temperatures on Bharat Merino sheep and observed the pulse rates of sheep as 73.7 pulse/min at 8 a.m. and as 93 pulse/min at 2 p.m.

Changes in metabolisms of small ruminant animals cause changes in pulse rates. Results indicated slowed metabolism and consequently lower pulse rates with decreasing ambient temperatures. Such rates indicate that Karayaka sheep could adapt to local conditions of Tokat Province. The point here is to have increasing pulse rates with increasing temperatures as expected and to have the highest values in evening hours.

Respiration rate was proven to be a significant factor in adaptation capacities of an animal to variable climate conditions [18-20]. The very first response of animals is to reduce their pulse rates when they are exposed to ambient temperatures below the comfort zone.

Respiration rates of rams and ewes of Karayaka sheep were respectively observed as 39.71 and 43.06 times/min in the morning, as 41.36 and 44.35 times/min in the evening. Measuring time and sex had positive impacts on respiration rates (P<0.01). The interaction between measuring time

and sex was found to be insignificant (P>0.05). The highest respiration rate was observed in ewes during evening measurements. The highest rates were seen in the days with the highest temperatures and the lowest rates were observed in the days with low temperature and high relative humidity. Results indicated that Karayaka sheep decreased their respiration rates as temperature decreases to sustain their body temperatures *(Table 3)*.

Silanikove [21], reported respiration rates of sheep in cold conditions as 40-60 times/min. Respiration rates of current study were similar to findings of AbiSaab and Sleiman [9], for breeding and Ivesi sheep (52.4 and 49.0 times/min) and lower than the findings of Ceyhan et al.[2] for Kıvırcık, SBA, (SBA X Kıvırcık) F1 and (SBA X F1) G1 sheep (50.379, 54.281, 55.186 and 56.673 times/min, respectively).

Heat loss of livestock decreases with decreasing temperature due to decreasing respiration rate. Reduced respiration is the first response of animal against decreasing temperatures. Temperature and humidity of air inhaled into lungs increase and the heat removed from the body decrease with decreasing volume of inhaled air [22].

Sheep are homo-thermic animals and need to perform some behavioral and physiological adaptations based on the trend and level of change in ambient temperature (either hot or cold) to maintain a steady body temperature against fluctuating environmental conditions. Decreasing ambient temperature decreases respiration and pulse rates of a homo-thermic animal through active heat release mechanisms.

There are several factors effective on production activities in sheep breeding. Temperature, relative humidity and air velocities are among the significant ones of these factors. In this study, relationships between such environmental factors and body temperature, pulse and respirations rates of sheep were investigated and environmental conditions were evaluated with regard to animal demands.

Results indicated decreasing body temperatures and consequent decrease in pulse and respiration rates with increasing ambient temperatures during the cold winter months. Such increases in temperature therefore cause some physiological changes in animals.

Negative effects of variable climate conditions may be decreased by improved adaptation capacity of animals through some physiological characteristics. Sheep try to minimize such negative impacts with instant physiological response against changes in environmental conditions. Karayaka sheep of the current study were able to have physiological response against changes in ambient temperature, relative humidity and air velocities by changing their body temperature, pulse and respiration rates. They were able to develop proper adaptation mechanisms for environmental conditions of Tokat Province.

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RESEARCH ARTICLE

Effects of Aflatoxin on *AgNOR* Activity of Cells in Different Regions of Kidney, and Protective Effectiveness of Esterified Glucomannan in Ram [1] [2]

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Summary

In this study, the effects of total aflatoxin (AF) given orally on silver-staining nucleolus organizer regions (*AgNORs*) activity were studied in glomerulus and tubular epithelial cells of kidney in Merino rams. In addition, this study was conducted in order to evaluate the efficacy of an esterified glucomannan (EG) for protection against to aflatoxicosis. As materials, 1 year-old 32 Merino rams were used. Rams were fed through the 92 days. Control group (C) fed with the commercial feed. AF group fed with commercial feed added 250 µg/day of total AF. EG group fed with commercial feed added 2 g/day of EG daily. AF+EG group fed with commercial feed added 250 µg/day of total AF and 2 g/day of EG. At the end of the 92nd day the animals were sacrificed, and tissue samples were taken from the kidneys. Whereas ratio of nuclear area of the *AgNOR* area of cells in examinated regions of kidney was found decreased significantly ($P<0.05$) in the AF group compared to the control group, AF+EG group was found similar to control group ($P>0.05$). In conclusion, the adverse effects causing by aflatoxicosis on the kidney *AgNOR* activity could be ameliorated by adding EG to the ration.

Keywords: *Aflatoxin, AgNOR, Glucomannan, Kidney, Ram*

Koçlarda Aflatoksinin Böbreğin Farklı Bölgelerindeki Hücrelerin *AgNOR* Aktivileri Üzerine Etkileri ve Glukomannanın Koruyucu Etkinliği

Özet

Bu çalışmada Merinos ırkı koçlara oral yolla verilen total aflatoksinin (AF) glomerulus ve böbrek tubuler epitel hücrelerindeki gümüşleme metoduyla çekirdekçik organizatör bölgelerinin (*AgNORs*) aktiviteleri üzerine olan etkileri ve AF ile birlikte verilen glukomannanın (EG) aflatoksikozun neden olduğu etkileri azaltıcı/engelleyici etkinliğinin belirlenmesi amaçlandı. Hayvan materyali olarak, 32 adet 1 yaşlı Merinos ırkı koç kullanıldı. Beslemeye 92 gün süreyle devam edildi. Kontrol (K) grubuna ticari yem, AF grubuna ticari yem ile günlük 250 µg AF, EG grubuna ticari yem ile günlük 2 g EG, AF+EG grubuna ise ticari yemle birlikte günlük 250 µg AF ve 2 g EG verildi. 92 günlük besleme periyodunu takiben hayvanlar kesildi. Böbreğin incelenen bölgelerindeki hücrelerin *AgNOR* alanının çekirdek alanına oranları AF grubunda kontrol grubuna göre önemli oranda düşük ($P<0.05$); AF+EG grubunda ise kontrol grubuna benzer ($P>0.05$) bulunmuştur. Sonuç olarak; elde edilen veriler, yemle birlikte alınan AF'ın böbrek *AgNOR* aktiviteleri üzerinde olumsuz etki oluşturabileceğini, bu etkilerin yeme EG ilave edilerek önemli oranda önlenebileceğini ortaya koymuştur.

Anahtar sözcükler: *Aflatoksin, AgNOR, Böbrek, Glukomannan, Koç*

INTRODUCTION

One of the most important problems in human and animal nutrition is contamination of human foods and animal feeds with molds and mycotoxins [1]. Aflatoxins are a group of mycotoxins produced by the strains of *Aspergillus*

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flavus and *A. parasiticus* [2]. They are produced on cereal grains during growth, harvest, storage, or transportation [3]. It is well known AFs are teratogenic [4], immunosuppression [5], mutagenic, and carcinogenic [6]. There is evidence that it has harmful effects on the kidneys, though not as much as on the liver [7].

Prevention of feed and feedstuffs from possible mold growth and AF contamination is very important. Practical and cost-effective methods for detoxification of AF containing feed and feedstuff are in great demand [8]. Since the early 1990s, the adsorbent-based several studies have been performed to detoxify AF in contaminated food and foodstuffs and to minimize the deleterious effects of AF [9]. An approach to the problem has been the usage of non-nutritive and inert adsorbents in the diet to bind AF and reduce the absorption of AF from the gastrointestinal tract [10]. The non-nutritive clays such as aluminosilicates, zeolites, bentonites, and clinoptilolite were preferred by the researchers [5,11]. Recent years, researchers suggested that the best approach for decontamination would be biological degradation [12]. Live yeast (*Saccharomyces cerevisiae)* initially used as a performance promoter in the early 1990s, was found to have beneficial effects on aflatoxicosis [13]. Esterified glucomannan (EG) showed considerably high binding ability (80-97%) with AF [14], and it has been preferred for detoxification of AF in poultry animals [6].

Nucleolar-organizer regions (NORs) are loops of DNA containing ribosomal RNA genes [15]. These regions can be easily stained with silver methods to appear as black dots (*AgNORs*) in the cell nucleus since they are argyrophilic. NORs are used by cytogeneticists for studying chromosomal disorders. This staining technique is very simple and does not require any special instruments or costly reagents [16]. Additionally, the size, number and dispersion of the silver deposits on the NOR reflect the degree of transcriptional, nucleolar and proliferative activity of the cells [17,18].

The aim of the study was to determine the effects of total AF given orally on *AgNOR* activity of cells in different regions of kidney in Merino rams. In addition, this study was conducted in order to evaluate the efficacy of EG for protection against to aflatoxicosis.

MATERIAL and METHODS

Animals and Diet

Approval for the present study was obtained from the Animal Ethics Committee of the Faculty of Veterinary Medicine of the Selcuk University (2008/061). Thirty-two Merino rams were approximately purchased 1-year-old (12-14 months old). Animals were examined for general health. Antiparasitic ivermectin injection (Avromec-F, 1 ml/50 kg) and oksifendazol (oxa-F, 1 tablet/50 kg) were performed. In addition, enterotoxemia (Pluritoxiven-8, 1 ml) and smallpox vaccines were performed. For adaptation to the environment and the implementation of a new 15-day training program was applied to feeding. Individually weighted rams were divided into four equal groups. Experimental feeding was continued throughout ninety-two days. The rams were fed a commercial food *(Table 1)*. Water and alfalfa were given *ad libitum*.

Experimental Design

The experimental design consisted of four dietary treatments. Control group (C) fed with the commercial feed. AF group fed with commercial feed added 250 µg/day of total AF. EG group fed with commercial feed added 2 g/day of EG (Alltech, Turkey). AF+EG group fed with commercial feed added 250 µg/day of total AF and 2 g/day of EG. AF dose was determined by the views of the researches [19-21] who were articles on the effects of AF and the pharmacologists who were studies on AF in our faculty. EG dose was also determined according to the dose in the prospectus (Alltech, Turkey). To make sure feed consumed, AF and EG that were mixed of 250 g commercial feed were given to animals before morning feeding and then morning feeding was continued.

Aflatoxin

The AF was produced from *Aspergillus parasiticus* NRLL 2999 culture (USA, Agricultural Research Service, Peoria, IL) via fermentation of rice by the method of Shotwell et al.[22] with minor modifications by Demet et al.[23]. Fermented rice was sterilized in autoclave, dried at 70°C, and ground to a fine powder. According to the method reported by Vicam [24] extraction and cleaning of AF in fermented rice was used immunoaffinity column (Down Test®; Vicam). The amount of AF carried out by high performance liquid chromatography (HPLC) according to the method reported by Stroka et al.[25]. The amount of total AF in the fermented rice was found 73.96 ppm. The AF within the rice consisted of 84.15% AFB_1, 6.29% AFB_2, 9.13% AFG_1 and 4.25% AFG_2 (rate of return method 97.4%; sensitivity 0.4 ppb).

Collection and Processing of Tissue Samples

At the end of the 92nd day, after sacrificed of the all animals

Table 1. *Composition of the commercial feed*
Tablo 1. *Ticari yemin içeriği*

Chemical Composition	Percentage Rate	Chemical Composition	Percentage Rate
Dry matter	%88	Na	%0.1-0.4
Crude protein	%12	NaCl	%1.0
Crude Celluluos	%12	Metabolic energy	2750 kcal/kg
Crude ash	%9	Vit A	7000 IU-kg
İnsoluble ash in HCL	%1.0	Vit D3	700 IU-kg
Ca	%0.6-1.6	Vit E	25 mg/kg
P	%0.4		

tissue samples were taken from the kidney in 10% neutral-buffered formalin solution. The tissues were processed and paraffin sections (6 µm) were stained with a solution containing one volume of 2% gelatin in 1% aqueous formic acid and two volumes of 50% silver nitrate (Merck). The staining was performed at 37ºC in the dark for 20-30 min [26]. The histological preparations were examined with a light microscope (Leica DM-2500 attached to a DFC-320 digital camera). In different regions of the kidney (glomerulus, proximal tubules, distal tubules and collecting tubules) of each animal, 25 cells having nuclei were evaluated. The nuclear area and the *AgNOR* area (*Fig. 1* and *2*) were analyzed with an image analysis program (IM-50). Also, the percentage of the *AgNOR* area relative to the nuclear area was calculated.

Statistical Analysis

The obtained results were statistically analyzed using Duncan test in SPSS software [version 17; SPSS Inc., Chicago, IL, USA]. The level of significance was $P<0.05$.

RESULTS

We obtained the nuclear area *(Table 2)*, the *AgNOR* area *(Table 3)*, and the ratio of the nuclear of the *AgNOR* area *(Table 4)* of cells in different regions of the kidney. Nuclear areas and ratio of nuclear area of the *AgNOR* area of glomerulus and tubular epithelial cells of kidney decreased significantly ($P<0.05$) in the AF group compared to the other groups. Furthermore, AF group was lowest ($P<0.05$) *AgNOR* area.

DISCUSSION

The liver and kidney are considered the main target organ for aflatoxicosis [7,27-29]. Lakkawar et al.[30] reported that liver and kidney were the most affected organs in rabbits which fed an AFB_1 contaminated diet. The effects of AFs on histopathological changes are directly correlated with the concentration of AF and the duration of the exposure [31].

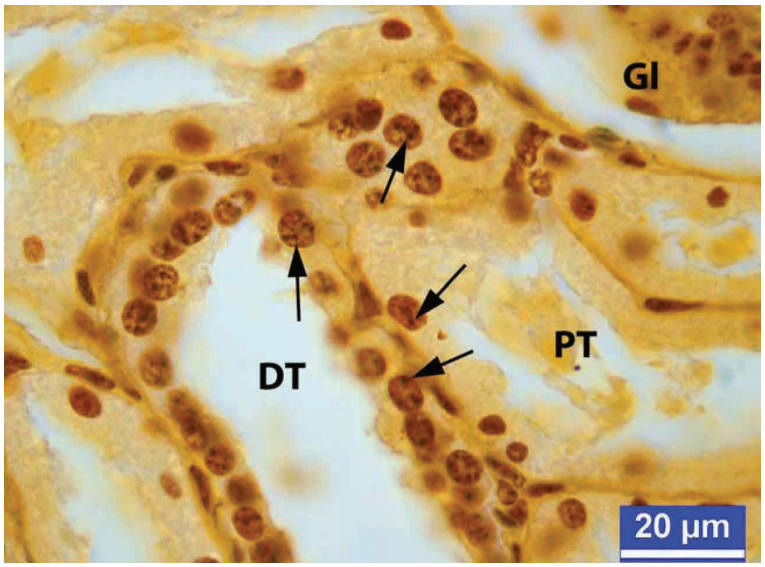

Fig 1. A section from cortex of kidney. **DT:** Distal tubule, **GL:** Glomerulus, **PT:** Proximal tubule, *Arrows:* AgNOR areas, AgNOR staining

Şekil 1. Böbreğin korteks bölgesinden bir kesit. **DT:** Distal tubul, **GL:** Glomerulus, **PT:** Proksimal tubul, *Oklar:* AgNOR alanları, AgNOR boyama

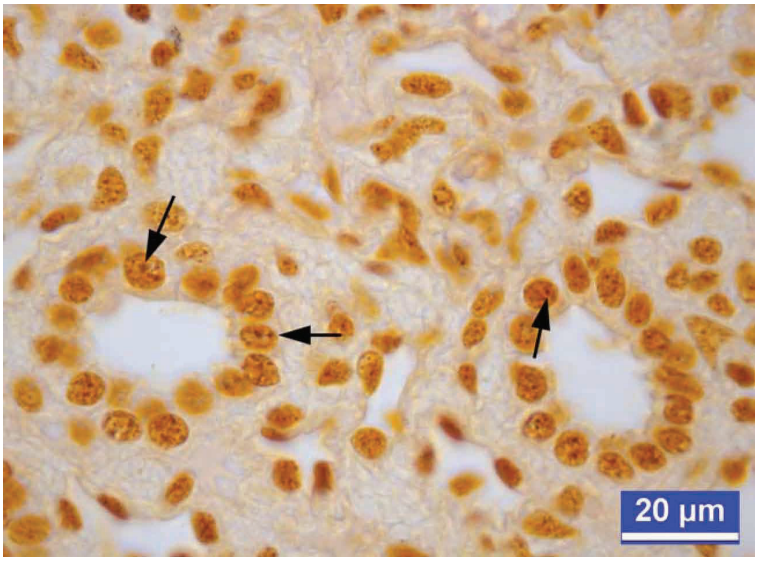

Fig 2. Collecting tubules in medulla of kidney. *Arrows:* AgNOR areas, AgNOR staining

Şekil 2. Böbreğin medula bölgesindeki toplayıcı borucuklar. *Oklar:* AgNOR alanları, AgNOR boyama

Table 2. *Nuclear area of cells in different regions of kidney*
Tablo 2. *Böbreğin farklı bölgelerindeki hücrelerin çekirdek alanı*

Groups (n=8)	Glomerulus (µm²)±SE	Proximal Tubules (µm²)±SE	Distal Tubules (µm²)±SE	Collecting Tubules (µm²)±SE
C	15.75±0.34^{a}	26.02±0.46^{a}	23.11±0.32^{a}	28.95±0.48^{a}
AF	13.13±0.28^{c}	22.20±0.29^{b}	20.32±0.32^{c}	25.33±0.30^{c}
EG	15.17±0.32^{ab}	25.30±0.39^{ab}	22.29±0.33^{ab}	28.24±0.35^{abc}
AF+EG	14.33±0.31^{b}	22.33±0.36^{b}	21.65±0.30^{b}	27.00±0.29^{b}

C: *Control,* ***AF:*** *Aflatoxin,* ***EG:*** *Glucomannan,* ***AF+EG:*** *Aflatoxin+glucomannan;* ***a-c*** *Values within a column with no common superscripts are significantly (P<0.05) different*

Table 3. *AgNOR area of cells in different regions of kidney*
Tablo 3. *Böbreğin farklı bölgelerindeki hücrelerin AgNOR alanı*

Groups (n=8)	Glomerulus (µm²)±SE	Proximal Tubules (µm²)±SE	Distal Tubules (µm²)±SE	Collecting Tubules (µm²)±SE
C	0.23±0.06^{a}	0.43±0.01^{a}	0.41±0.01^{a}	0.51±0.01^{a}
AF	0.17±0.00^{c}	0.27±0.01^{c}	0.26±0.01^{c}	0.38±0.01^{b}
EG	0.22±0.01^{a}	0.41±0.01^{a}	0.38±0.01^{a}	0.49±0.01^{a}
AF+EG	0.19±0.01^{b}	0.33±0.01^{b}	0.29±0.01^{b}	0.39±0.01^{b}

C: *Control,* ***AF:*** *Aflatoxin,* ***EG:*** *Glucomannan,* ***AF+EG:*** *Aflatoxin+glucomannan;* ***a-c*** *Values within a column with no common superscripts are significantly (P<0.05) different*

Table 4. *The ratio of the nuclear of the AgNOR area of cells in different regions of kidney*
Tablo 4. *Böbreğin farklı bölgelerindeki hücrelerin AgNOR alanının çekirdek alanına oranı*

Groups (n=8)	Glomerulus (%) ±SE	Proximal Tubules (%) ±SE	Distal Tubules (%) ±SE	Collecting Tubules (%) ±SE
C	1.80±0.09^{a}	1.53±0.07^{a}	1.50±0.06^{b}	1.76±0.07^{a}
AF	1.23±0.05^{c}	1.18±0.05^{b}	1.27±0.05^{c}	1.44±0.06^{b}
EG	1.60±0.09^{a}	1.79±0.09^{a}	2.08±0.09^{a}	1.82±0.06^{a}
AF+EG	1.59±0.07^{a}	1.49±0.07^{a}	1.49±0.06^{b}	1.68±0.08^{a}

C: *Control,* ***AF:*** *Aflatoxin,* ***EG:*** *Glucomannan,* ***AF+EG:*** *Aflatoxin+glucomannan;* ***a-c*** *Values within a column with no common superscripts are significantly (P<0.05) different*

Nuclear areas of glomerulus and tubular epithelial cells were significantly (P<0.05) decreased in all regions of AF group compared to the C group. Measurements in glomerulus, distal and collecting tubule regions of AF+EG group were similar compared with the C group. But, it was observed that measurements in proximal tubule regions in AF+EG group were significantly (P<0.05) different compared with the C group. It was known that AFs have toxic effect on p53 gene which is a protective effect against DNA damage in cells [32,33]. The findings of the study were showed that AF was significantly decreased nuclear areas of cells and formed negative impact on metabolic activity of cells.

AgNOR areas of glomerulus and tubular epithelial cells of kidney were similar with C and EG groups. We were observed AF group was lowest (P<0.05) *AgNOR* area. *AgNOR* area of AF+EG group was found statistically higher (P<0.05) than that of AF group. These data were in agreement with findings of the researches [34,35] who reported that AFs caused genetic disorders in cells. In this study, we found the lowest *AgNOR* area of the AF group since AF caused to modification [36] and reduce number of ribosome. Ultimately, this situation causes to reduce synthesis of proteins [37,38]. On the other hand, *AgNOR* area of AF+EG group to be higher than those of AF group, it shows aflatoxin is relatively eliminated pressure on protein synthesis by EG.

The ratio of nuclear area of the *AgNOR* area of glomerulus and tubular epithelial cells of kidney decreased significantly (P<0.05) in the AF group compared to the other groups. This result reveals that AFs which are caused DNA damage [28,33,39] reduce activity of cell's protein synthesis [37,38]. Zaczek et al.[17] have reported that *AgNOR* parameters associated with the proliferation activity of the epithelium.

As a conclusion, reason of decline in protein synthesis activity of the cells, we can be said AF is partly eliminated the negative impact on the cells by used EG. We were concluded EG is an agent which can be used successfully to prevent aflatoxicosis. Obtained data are showed that there are important changes in the *AgNOR* parameters. Therefore, we think that *AgNOR* parameters will also utilize taking into account besides other histopathological assessments for future similar studies.

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RESEARCH ARTICLE

In vitro Efficacy of *Quercus infectoria* Oliv. and *Achillea millefolium* L. Extracts Against *Blastocystis spp*. Isolates [1]

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Summary

Blastocystis is a common intestinal parasite that can inhabit the intestinal tract of humans and many animals. Despite it was firstly described almost 100 years ago; many subjects are still under debate about *Blastocystis*, including its life-cycle, pathogenic potential and treatment of infected individuals. Historically, local plant species have been used for therapeutic purposes by the local people of Anatolia. Here, hexane and methanol extracts of two local plants, *Quercus infectoria (Fagaceae)* and *Achillea millefolium*, which have been used against diarrhea in Anatolia, were examined for their *in vitro* efficacies against *Blastocystis*. LC_{50} and EC_{50} values of the plant extracts were determined by Brine Shrimp and Graphpad Prism 5® methods, respectively. The results showed that LC_{50} (500 µg/ml) and EC_{50} (198.8 µg/ml) concentrations of the methanol extract of *A. millefolium* were lowest compared to other extracts, its *anti-Blastocystis* activity was found to be comparable to metronidazole and it showed no cytotoxic activity. These initial results suggest that the methanol extract of *A. millefolium* may be a novel option for the treatment of *Blastocystis* infections in humans in future, if confirmed by further, larger-scale studies.

Keywords: *Blastocystis, treatment, Medicinal plants, Quercus infectoria, Achillea millefolium*

Quercus infectoria Oliv. ve *Achillea millefolium* L. Ekstrelerinin *Blastocystis spp*. İzolatlarına *in vitro* Etkileri

Özet

Blastocystis spp, insanların ve birçok hayvanın gastrointestinal sistemine yerleşen yaygın bir bağırsak parazitidir. Yaklaşık 100 yıl önce tanımlanmış olmasına rağmen, yaşam döngüsü, patojenitesi ve tedavisini içeren birçok konu halen gizemini korumaktadır. Geçmişten bugüne Anadolu'da çok sayıda bitki halk tarafından tedavi amacıyla kullanılmıştır. Bu projede ishale karşı kullanılan bitkilerden ülkemizde yetişen *Quercus infectoria (Fagaceae)* ve *Achillea millefolium*'un hekzan ve metanol ile hazırlanan ekstrelerinin *in vitro* ortamda Blastocystis spp.'lerin üremesi üzerine etkileri incelenmiştir. Bitki ekstrelerinin LC_{50} değeri "Brine Shrimp" yöntemi, EC_{50} değeri Graphpad Prism 5® istatistik yöntemi kullanılarak saptanmıştır. Sonuç olarak, *A. millefolium*'un metanol ekstresinin LC_{50} (500 µg/ml) ve EC_{50} (198.8 µg/ml) konsantrasyonları diğer ekstrelerle kıyaslandığında en düşük bulunmuş, *anti-Blastocystis* aktivitesinin ise metronidazol grubunun değerlerine en yakın olduğu ve sitotoksik aktivite göstermediği saptanmıştır. Bu sonuçlar *A. millefolium*'un metanol ekstresinin, ileride yapılacak geniş kapsamlı çalışmalarla doğrulandığında, *Blastocystis spp*. enfeksiyonlarının tedavisinde yeni bir seçenek olabileceğini göstermektedir.

Anahtar sözcükler: *Blastocystis, Tedavi, Tıbbi bitkiler, Quercus infectoria, Achillea millefolium*

INTRODUCTION

Blastocystis spp. was firstly described as yeast in 1911 by Alexieff; however, issues concerning its taxonomy, life cycle and pathogenic potential have long been debated. Its prevalence is 30-50% in developing countries, and it is

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probably the most common intestinal parasite in humans. However, there are contradictory reports about its clinical significance as it is isolated from both symptomatic and non-symptomatic patients. *Blastocystis spp.* has been reported as the only pathogenic agent in patients with gastrointestinal complaints that resolved after effective treatment [1]. Recent molecular studies have shown extensive genetic diversity among *Blastocystis* spp. isolates, which may explain why some patients showed symptoms, while others not [2]. Due to its varying clinical manifestations, many laboratories report *Blastocystis* spp. only when more than 5 parasites were identified under x400 magnification on the microscope using saline - Lugol direct examination method of the stool [3]. Direct Fluorescent Antibody Method, which is fast and practical, was also considered as a diagnostic method [4].

Treatment is suggested for symptomatic patients, if other gastrointestinal pathological agents can be excluded, infected with *Blastocystis* spp. and metronidazole is the drug of choice in the treatment. However, metronidazole may cause common side effects, which may refrain the patient from complying with treatment effectively [5-7].

In traditional medicine in Anatolia, leaves, bodies, fruits and seeds of many plant species have long been used as anti-diarrheic agents. As medical agents are relativey expensive and may cause significant side effects in patients, herbal compounds are used commonly by local people. Some of these herbal compounds may have significant potential therapeutic effects against different parasites, and may turn out to be registered drugs for these infections in the future.

Achillea millefolium is traditionally used against skin inflammations, hepatobiliary disorders and gastrointestinal complaints. It is mainly preferred for its spasmolytic, digestive, carminative, antiphlogistic and cholagoge effects. Its efficacy against dyspeptic complaints has been attributed to the presence of compounds in *A. millefolium* which could stimulate the digestive fluids in stomach, pancreas, and liver by increasing the tone of the vagal system [8]. The antioxidant and antimicrobial properties and the chemical profile of the essential oil obtained from *A. millefolium* have also been reported. Flavonoids, phenolic acids and sesquiterpene lactones are considered to be the most important groups of pharmacologically active compounds present in *Achillea* species [9-12]. The chemical composition of *Achillea* species has been analyzed in detail and extracts of this plant have been demonstrated to contain a number of pharmacological active ingredients, including alkaloids, such as choline, and flavonoids such as rutin and apigenin. Among these, choline was reported to be the active compound for the pharmacological effects of *A. millefolium* [9].

Quercus infectoria is a small tree widely distributed in Greece, Asia Minor and Iran. It has been evaluated in terms of its pharmacological effects and it was found that it had antiparkinsonian, antitremorine, antiinflammatory, antidiabetic and antioxidant effects. The constituents of the galls of *Q. infectoria* comprise a large amount of tannins, gallic acid, syringic acid, ellagic acid, beta sitosterol, amentoflavone hexamethyl ether, isocryptomerin, methyl betulate, methyl olenate, and hexagalloyl glucose [13]. Larvacidal activity of the gall extracts of *Quercus infectoria* was initially reported against *Anopheles stephensi* [14].

The aim of the present study was to assess the *in vitro* efficacies of the extracts of two local plants *Quercus infectoria* Oliv. belonging to *Fagaceae* family and *Achillea millefolium* L.(Yarrow) from *Asteraceae* family that have been used traditionally against diarrhea, on *Blastocystis* spp. isolates diagnosed by three different methods. In addition, genotyping was employed to identify any relationship between *Blastocystis* spp. subtypes and sensitivity to *A. millefolium* and *Q. infectoria*.

MATERIAL and METHODS

Blastocystis spp. Isolates

Cryopreserved stool samples of six patients found to be infected with *Blastocystis* spp. in Parasitology Laboratory of Celal Bayar University Medical School's Hospital were used in the study. These positive samples were read and cultured on the same day, and the remaining samples were kept for two weeks at +4°C before genotyping.

These stool samples were inoculated into Jones medium [15], which was commonly preferred for *Blastocystis* spp. culture. The cultures were kept at 37°C for 48-72 h, and one drop of culture fluid was then examined microscopically to detect whether *Blastocystis* spp. were reproduced.

Amplification and Genotyping of Blastocystis spp. Isolates

Both stool and culture samples were used for the molecular assessments. DNA isolation was conducted with QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany), according to the instructions of the manufacturer. Two µls of DNA were taken for PCR analysis, using the primers F1 and BHCRseq that targeted the small subunits of ribosomal RNA, and standard conditions [16]. The amplicons were separated on 1.5% of agarose gel, and PCR products of 550-590 bp were considered positive for *Blastocystis* spp. PCR products were gel-purified using the UltraClean™ Gel Spin DNA Purification Sample kit (SANBIO, Uden, The Netherlands) and dideoxy sequenced in one direction using the BHCRseq3 primer as the sequencing primer. Sequence chromatograms were analyzed and aligned using the software program Bio Edit Sequence Alignment Editor [17]. Distance-based analysis was conducted with MEGA 3.1 [18], and trees were constructed using the UPGMA algorithm with the Kimura 2-parameter model;

Proteromonas lacertae (U37108) was used as the out-group. *Blastocystis* spp. subtype terminology as described [19]. Sequences were blasted against those in the National Centre for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Plant Materials

The samples of two plant species used in the present study were collected in Manisa province in western Turkey. The aerial parts of *Achillea millefolium* were collected from Spil Mountain (almost 1150 meters above sea level), and nut galls of *Quercus infectoria* were collected from Yagcilar village (almost 250 m asl), which was located 20 km away from the city centre. Collected plant materials were separated and identified technically [20], and voucher specimens for plant materials have been deposited in the Herbarium of Celal Bayar University, School of Science and Letters, Department of Biology.

Preparation of Plant Extracts and Determination of Cytotoxic Activity

The air dried and ground aerial parts of *Achillea millefolium* and nut galls of *Quercus infectoria* were extracted using n-hexane and methanol under stirring. The organic phases were filtered through 0.45 µm and distilled *in vacuo* to yield n-hexane and methanol extracts. Brine Shrimp Method was used to assess the biological activities of the plant extracts [21-23].

In vitro Sensitivity Tests and Determination of the Effective Concentration

Plant extracts were prepared at different concentrations ranging between 62.5 and 4000 µg/ml, while the control drug, metronidazole was between 0.6 and 40 µg/ml. Saline solution was used as control and 10^5 *Blastocystis* spp./ml were added to the tubes containing extract and saline solution. All tubes were cultivated for 48 h at 37°C and tube samples were suspended in 0.1% of eosine solution to count the living cells. Reproduction of *Blastocystis* spp. isolates as well as the presence of living cells were checked in all concentrations, and 1 ml of culture fluid was drawn from the tube just before the concentration presenting with no living cells or reproduction, and inoculated in a new culture tube for testing. Thus, lethal concentrations (LC) of each plant extracts *on Blastocystis* spp. isolates, if present, were determined. Effective concentrations (EC_{50}) were also assessed using Graphpad Prism 5® statistical method.

RESULTS

Blastocystis spp. isolates were thawed in water bath at 37°C after cryopreservation, and immediately inoculated into Jones medium. All six isolates reached 10^3 parasites/ml concentration within 48 h *(Fig. 1)*.

Genotypic assessments of the isolates revealed three subtypes; two Subtype 1, one Subtype 2 and three Subtype 3. No differences were noted in terms of the subtypes between stool and culture samples. No significant differences were identified between the isolates for reproduction efficacies of parasites (One-way variance analysis, P>0.05); each subtype showed similar reaction to each extract at the same concentrations. LC_{50} levels of the methanol extracts of *Q. infectoria* and *A. millefolium* were found to be 1000 µg/ml and 500 µg/ml, respectively. The methanol extract of *A. millefolium* was found to have the lowest EC_{50} value (198.8), compared to others *(Table 1, Fig. 2)*.

Cytotoxic activity assessments with Brine Shrimp Method revealed that the LC_{50} value of the methanol extract of *Q. infectoria* was 190.8605; no cytotoxicity was defined

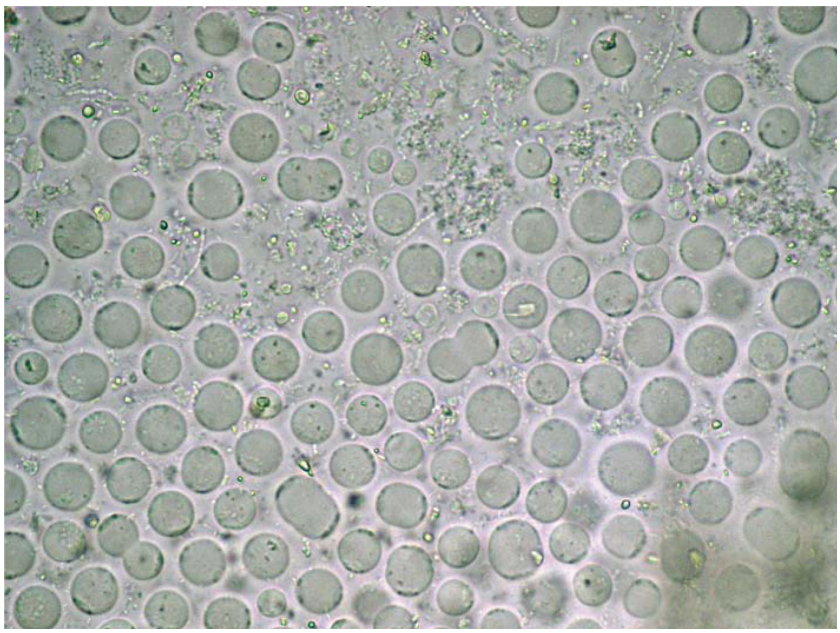

Fig 1. *Blastocystis* spp. isolates grown in Jones medium after cryopreservation

Şekil 1. Kriyoprezervasyondan sonra Jones besiyerinde üreyen *Blastocystis* spp. izolatları

Table 1. *Average numbers of Blastocystis spp. isolates 48 h after addition of plant extracts to study groups at different concentrations (Blastocystis spp. number x 10^7/ml)*

Table 1. *Çalışma gruplarına farklı konsantrasyonlarda bitki ekstresi eklendikten sonraki 48. saatte saptanan canlı Blastocystis sayısının ortalama değerleri (Blastocystis sayısı x 10^7/ml)*

Extract (µg/ml)	*Quercus infectoria* n-hexane	*Quercus infectoria* Methanol	*Achillea millefolium* n-hexane	*Achillea millefolium* Methanol	Extract (µg/ml)	Metronidazole	Control (Saline solution)
4000	0.98±0.08	0.00±0.00	0.00±0.00	0.00±0.00	**40.00**	0.00±0.00	5.02±0.08
2000	2.48±0.08	0.00±0.00	0.50±0.06	0.00±0.00	**20.00**	0.00±0.00	5.02±0.08
1000	2.98±0.08	0.00±0.00	1.03±0.05	0.00±0.00	**10.00**	0.00±0.00	5.05±0.14
500	4.03±0.10	1.00±0.06	2.05±0.12	0.00±0.00	**5.00**	0.00±0.00	5.07±0.14
250	4.98±0.12	2.02±0.08	2.48±0.08	1.03±0.05	**2.50**	0.48±0.08	5.00±0.11
125	5.05±0.08	2.50±0.06	4.05±0.05	2.52±0.08	**1.30**	1.05±0.05	4.98±0.08
62.5	5.08±0.08	3.03±0.08	5.03±0.10	3.07±0.08	**0.60**	2.48±0.12	5.00±0.11
EC_{50}*	3.458e+6	336.8	~ 546.5	198.8		0.1100	

EC_{50}: *Effective Concentration*

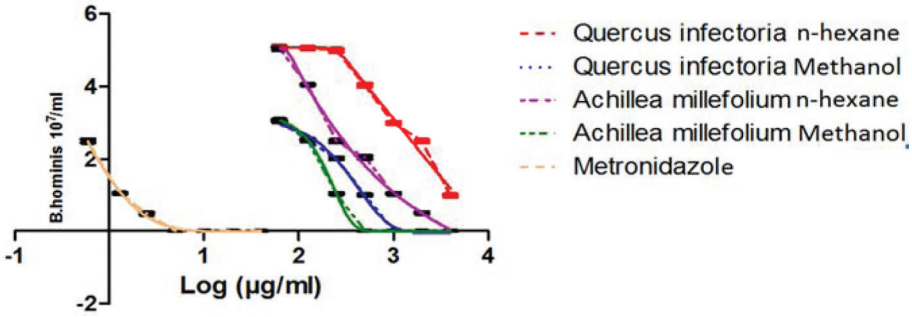

Fig 2. LC_{50} values of plant extracts

Şekil 2. Bitki ekstrelerinin LC_{50} değerleri

for n-hexane or methanol extracts of *A. millefolium* or n-hexane extract of *Q. infectoria*.

DISCUSSION

Metronidazole is a first line drug against intestinal protozoal infections, including blastocystosis. However, it has some drawbacks which are more severe in HIV-infected patients, such as nasty side effects, metallic taste, and headache [5-7,24,25]. Despite some other agents such as co-trimaxazole [26] was shown to be effective against *Blastocystis* spp., they are not commonly used and thus there is a need for new anti-protozoal agents which are safe and effective.

Medicinal plants have been used commonly in developing countries due to their availability, inexpensiveness and traditional use for centuries. In a study from Tailand, extracts of anti-diarrheic *Acacia catechu resin*, *Amaranthus spinosus* wholeplant, *Brucea javanica* seed (Bjs), *Piper longum* fruit (Plf) and *Quercus infectoria* nut gall (Qin) were assessed against *Blastocystis* spp. *in vitro* and dichloromethane and methanol extracts of Bjs were found to be effective [21]. Efficacy of the water extract of Bjs against an axenic strain of *Blastocystis* spp. was also reported [27]. In addition, anti-amebic [28] and anti-*Plasmodial* [29] activities were reported for Bjs.

Isolates of *Blastocystis* spp. have varying responses to plant extracts; this is probably due to different karyotypic features or isoenzyme patterns of the isolates [30-32]. *Quercus infectoria* nut gall (Qin) has been used as anti-diarrheic in traditional Taylandese medicine but there is no enough scientific data to support it. Its methanol extract showed anti-amebic activity in mice [33].

Sawangjaroen et al.[21] assessed *in vitro* anti-amebic activities of some plants. They reported that anti-amoebic activities of plants were dose-dependent and 1.000 mg/kg of Plf extract had the highest activity which was also achieved by 125 mg/kg of metronidazole. Lower doses of Plf could not kill the amoebas but limited their pathogenic effects in gut. Methanol extract of Qin showed efficacy against ceacum involvement of amebiasis in mice, but lower compared to Plf.

In another study, essential oils obtained from *Lavandula angustifolia* and *Lavandula intermedia* showed anti-parasitic activities against *Giardia lamblia*, *Trichomonas vaginalis* and a fish parasite, *Hexamita inflata* under 1% of concentrations [34]. Water, dichloromethane and methanol extracts of *Brucea javanica* and the methanol extracts of *Q. infectoria* had inhibitory effects against *Blastocystis* spp., which required further studies [21,35].

A. millefolium L. has been used traditionally in the treatment of inflammatory and spasmodic intestinal diseases, and hepatobiliary complaints [36]. It is used as a

deworming agent in animals; its anti-helminthic activity was shown in a study on sheep against gastrointestinal nematodes [35].

In vitro screening tests are essential for new drug assessments. In the present study, successful 24-month cryopreservation of *Blastocystis* spp. isolates followed by application of microscopy and culture for *in vitro* screening tests were demonstrated. Despite only six isolates were assessed in the study, it is noteworthy to report that no subtype differences were identified after the genotyping of stool samples and culture material.

No cytotoxic activity was demonstrated for n-hexane and methanol extracts of *A. millefolium* or n-hexane extract of *Q. infectoria*, which was significant for their reliabilities in biological investigations. Since the methanol extract of *Q. infectoria* had cytotoxic activity, and LC_{50} and EC_{50} concentrations of the methanol extract of *A. millefolium* had higher values, it was considered that the methanol extract of *A. millefolium* had higher anti-*Blastocystis* spp. activity, which warranted further assessments. In addition, the compounds that are responsible for the cytotoxic activity against *Blastocystis* spp. in the methanol extract of *Q. infectoria* should be identified.

This is the first study that involves the assessment of the efficacies of some plant extracts grown in Turkey and used as anti-diarrheic agents by local people, against cultured *Blastocystis* spp. isolates. Initial results, if confirmed by further assessments, demonstrate that the methanol extract of *A. millefolium* gave promising results and, could be used as an anti-protozoal agent in future.

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RESEARCH ARTICLE

Phenotypic and Genotypic Determination of Antibiotic Resistant and Biofilm Forming *Staphylococcus aureus* Isolated in Erzincan Tulum Cheese [1]

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Summary

In this study, biofilm formation and antibiotic resistance of *Staphylococcus aureus* strains isolated from Erzincan tulum cheeses were phenotypically and genotypically investigated. Samples collected from 100 different Erzincan tulum cheese were inoculated into Baird-Parker agar to isolate *S. aureus*. Identification of *S. aureus* was performed with Gram staining, hemolysis or coagulase tests. Determination of the *nuc, mecA, vanA,* and *vanB* genes in isolates was performed by Polymerase Chain Reaction (PCR). Congo red agar was used for the biofilm formation of *S. aureus*. Antibiotic resistance was determined by antibiotic discs of oxacillin (1 µg), cefoxitin (30 µg), vancomycin (30 µg), amoxicillin-clavulanic acid (20 µg), and penicillin (10 units). A total of 72 of 100 (72%) samples were positive for *Staphylococcus* spp. Of 72 samples, 61 (84.7%) were phenotypically and genotypically identified as *S. aureus*. Of 61 isolates, 37 (60.6%) formed a biofilm. Of 61 isolates, 49 were determined to resistant to antibiotics of oxacillin (methicillin) (9), cefoxitin (8), amoxicillin-clavulanic acid (4), and, penicillin (28). Vancomycin-resistance was not detected. Only the *nuc* and *mecA* genes were detected in 10 of 61 (16.3%) strains of *S. aureus*. In this study, the rate of *S. aureus* determined in Erzincan tulum cheeses was high. Considering the high rate of contamination and antibiotic resistance due to poor hygienic conditions, it was concluded that Erzincan tulum cheese, now a PDO cheese, should be considered to be great risk for public health.

Keywords: *Antibiotic resistance, Biofilm, Staphylococcus aureus, Polymerase Chain Reaction, Tulum cheese*

Erzincan Tulum Peynirinden İzole Edilen *Staphylococcus aureus* İzolatlarında Antibiyotik Direncinin ve Biyofilm Oluşturma Özelliğinin Fenotipik ve Genotipik Olarak Belirlenmesi

Özet

Bu çalışmada Erzincan tulum peynirinden izole edilen *Staphylococcus aureus*'ların biyofilm oluşturabilme yetenekleri ve antibiyotik dirençlilikleri fenotipik ve genotipik yöntemlerle araştırıldı. Araştırmada 100 adet Erzincan tulum peyniri numunesi toplandı. Peynir örneklerinden *Staphylococcus aureus* izolasyonu için Baird-Parker agar'a ekim yapıldı. İzolatlar Gram boyama, hemoliz ve koagülaz testleriyle *S. aureus* olarak identifiye edildi. Polimeraz Zincir Reaksiyonu ile izolatlar *nuc, mecA, vanA* ve *vanB* genleri yönünden incelendi. *S. aureus*'ların biyofilm oluşturma yeteneği için Kongo Red agar ve oksasilin (1 µg), sefoksitin (30 µg), vankomisin (30 µg), amoksisilin-klavulonik asit (20 µg) ve penisilin (10 unit) diskleri antibiyotik dirençliliğin saptanması amacıyla kullanıldı. Test edilen örneklerin 72 (%72)'si *Staphylococcus* spp. pozitif bulundu. Bunlardan 61 (%84.7) örnek fenotipik ve genotipik olarak *S. aureus* olarak identifiye edildi. İzolatların 37 (%60.6)'sinin biyofilm oluşturduğu saptandı. Disk difüzyon yönteminde 61 izolattan 9 (%14.7)'unda oksasilin (metisilin), 8 (%13.1)'inde sefoksitin, 4 (%6.5)'ünde amoksisilin-klavulonik asit, 28 (%45.9)'inde ise penisilin dirençliliği tespit edilirken vankomisin dirençli izolata rastlanmadı. Polimeraz Zincir Reaksiyonu sonucunda 61 örnekten 10 (%16.3)'unda *nuc* ve *mecA* genleri saptanırken *vanA* ve *vanB* genlerine rastlanmadı. Bu araştırmada Erzincan tulum peynirinde *S. aureus*'un yüksek bir oranda bulunduğu görülmüştür. Kontaminasyon oranının yüksek olması ve izolatların antibiyotik dirençliliği göz önünde bulundurulduğunda hijyenik şartlarda üretilmeyen bu peynirlerin halk sağlığı açısından büyük risk oluşturabileceği kanaatine varılmıştır.

Anahtar sözcükler: *Antibiyotik direnci, Biyofilm, Staphylococcus aureus, Polimeraz Zincir Reaksiyonu, Tulum peyniri*

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INTRODUCTION

Tulum cheese is produced commonly in Turkey. However, there is no standard production methods including ripening time. Therefore, quality of tulum cheese varies. Tulum cheese is traditionally made of sheep's milk. Following milking, raw milk is filtered without any heat treatment or pasteurization, and then fermentation process is started. Fermentation temperature of milk varies from 28°C to 30°C. Fermentation process is completed between 1.5 and 4 hours depending on the strength and amount of yeast [1].

Staphylococcus aureus, a health-threatening pathogen associated with food can cause many diseases including mild skin infections, pneumonia or septicemia in people and animals. The most common carriers of these pathogenic bacteria are human being. Approximately 50% of people are commensal carriers of *S. aureus*. Antibiotic-resistant pathogens can be contaminated by milk and milk products, and meat and meat products. *Staphylococcus aureus* strains may have an adhesive protein known as biofilm made of polysaccharide. It was indicated that strains with biofilm were more virulent compared to those without biofilm; therefore, strains with biofilm become resistant to antibiotics [2]. Food-borne contamination can play an important role in transporting methicillin-resistant *S. aureus* (MRSA) which has been frequently encountered in recent years [3]. Methicillin-resistant strains may become multiresistant; therefore, treatment of these strains can be very difficult. Vancomycin has been used successfully in the treatment of methicillin-resistant strains of *S. aureus*; however, in recent years, only medium-level resistance was determined. Therefore, determination of vancomycin-resistant strains is of critical importance [4].

The purpose of this study was to investigate phenotypically and genotypically biofilm formation and antibiotic resistance of *Staphylococcus aureus* isolated from Erzincan tulum cheese with high consumption rate in Turkey.

MATERIAL and METHODS

Study materials consisted of 100 samples of Erzincan tulum cheese in Erzincan province. Samples as 200 g in weight were collected from markets via aseptic conditions in the sterile bags and then were brought to the laboratory in the cold chain.

Ten grams of each sample was placed in sterile stomacher bags and then 90 ml 0.1% sterile peptone water was added. Stomacher bags were mixed for homogenization at least 3 minutes. One ml of homogenates was transferred in 9 ml peptone water tubes. Decimal dilutions were performed to determine the exact structure of the colonies due to unknown bacterial density in the sample. Dilution in 0.1 ml was incubated for aerobic culture on Baird-Parker agar at 37°C for 24-48 h. After incubation growth typical and atypical colonies were isolated for analyzing [5].

Phenotypic Methods

Gram staining was performed for growth colonies on Baird-Parker [6]. Hemolysis and coagulase tests were performed on isolates identified as Gram-positive [5].

Investigation of Resistance to Antibiotics by the Disc Diffusion Method

Bacterial suspension was prepared in accordance with the recommendations of the Clinical Laboratory Standards Institute (CLSI) [7]. Briefly, suspension from the 24-h culture of bacteria in 0.9% NaCl solution which is equal to the 0.5 McFarland turbidity standards (1×10^8 CFU/ml) was prepared by using the direct colony suspension method. Suspension was spread onto the surface of Mueller-Hinton agar (Oxoid CM337) plates using sterile swabs. After the medium surface dried oxacillin, cefoxitin, vancomycin, amoxicillin-clavulanic acid and penicillin discs were placed on the plates. The zone diameters of the discs were measured after incubation at 35°C for 24 h. The results were evaluated on the basis of standards set, in accordance with recommendations of the Clinical Laboratory Standards Institute [7]. Because oxacillin (1 µg) resistance also determines methicillin resistance, an oxacillin antibiotic disc (1 µg) was used in the resistance investigation of methicillin.

Genotypic Methods

DNA extraction was performed with the phenol/chloroform extraction method [8].

The nuc Gene Detection by Polymerase Chain Reaction

According to the protocol of Maes et al.[9] primer pairs of the *nuc* gene sequence were used to follow 279 bp DNA fragments. Preparation of the reaction mixture was made using $MgCl_2$ (Sigma) 2 mM dNTPs (Sigma) 250 µM, primers 20 pmol/µl (Sigma), 0.4 µM of each of the primers, *Taq* polymerase (Sigma) 2 U, and DNA 5 µl. The amplification process was performed with 30 cycles in a thermalcycler (Techne, UK). The amplification cycle was performed as initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, connecting at 51°C for 1 min, elongation at 72°C for 2 min, and final elongation at 72°C for 2 min. Products were stained with 6 µM ethidium bromide and electrophoresis (Thermo, USA) 120 V for 40 min was performed. The products were examined under an UV-transilluminator (Vilber Lourmat, France). *Escherichia coli* ATCC 11230 was used as the *nuc* (-) control strain [9].

Detection of Methicillin Resistance by Polymerase Chain Reaction

The same procedures of detection of the *nuc* gene were applied to follow the 533 bp DNA fragment. In the test, *S. aureus* ATCC 46300 was used as the *mecA* (+) control and

the *S. aureus* ATCC 1065 strain was used as the *mecA* (-) control [9].

Detection of Vancomycin Resistance by Polymerase Chain Reaction

The protocol indicated by Clark et al.[10] was used to perform PCR by using primer pairs (Sigma) forming the *vanA* and *vanB* gene sequence to follow 1030 bp DNA fragments for *vanA*, and 433 bp DNA fragments for *vanB*. The reaction mixture was consisted of 2.5 U *Taq* polymerase (Sigma), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM dNTPs mix (Sigma), 0.5 µM of each primer (Sigma primers), 1.5 mM $MgCl_2$, and 5 µl DNA. The amplification process was performed with 30 cycles in thermalcycler (Techne, UK). The amplification cycle was performed as initial denaturation at 94°C for 10 min, denaturation at 94°C for 30 sec, connecting at 58°C for 30 sec, elongation at 72°C for 30 sec, and final elongation at 72°C for 10 min. The PCR products were stained in 1.8% agarose gel with 10 ml of ethidium bromide and electrophoresis (Thermo, USA) 110 V for 1 hour was performed. The products were examined under a UV-transilluminator (Vilber Lourmat, France). 1030 bp for *vanA* and 433 bp for *vanB* were determined as positive [10]. *E. faecium* ATCC 51559 (*vanA*) and *E. faecalis* ATCC 700802 (*vanB*) strains were used for positive control [11].

Determination of Biofilm Creation Feature

Congo Red agar (CRA) method developed by Freeman et al was used to determine the slime-posivity [12]. Colonies isolated as *S. aureus* were incubated on CRA at 37°C for 24 h. After incubation black-colored colonies were determined as biofilm positive.

Statistics

The SPSS (Statistical Package for Social Sciences for Windows, 16.0) program was used for the statistical analysis. Frequency distributions and the chi-square statistical methods were applied. Results were evaluated at 95% confidence interval at a level of $P<0.05$.

RESULTS

Staphylococcus spp. was determined in 72 of 100 Tulum cheese samples on the market after incubation on Baird-Parker's agar. Moreover, all 72 isolates were determined as Gram positive and cocci by Gram-staining. Of 72 isolates, 61 (84.7%) were identified as *S. aureus* by hemolysis and coagulase tests.

When 61 isolates of *S. aureus* were tested phenotypically, 9 (14.75%) were oxacillin resistant, 8 (13.11%) were cefoxitin resistant, 4 (6.55%) were amoxicillin-clavulanic acid resistant, and 28 (45.90%) were penicillin resistant.

Although the *nuc* gene was detected in all 61 *S. aureus* isolates the *mecA* gene was only detected in 10 (16.39%) isolates *(Fig. 1)*. On the other hand, neither the *vanA* nor *vanB* genes were not detected.

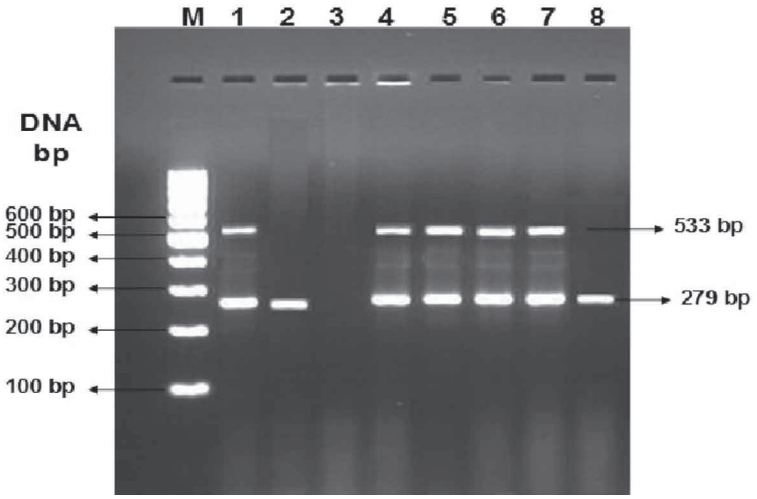

Fig 1. Electrophoresis View of the *nuc* and *mecA* Genes. M: Marker (Gene Ruler Set-Promega, Madison, USA) DNA Ladder Plus (G210A) 100 bp-3000 bp Blue/Orange Loading Dye (G190A), 1: *Staphylococcus aureus* ATCC 46300 (533 bp *mecA* positive and 279 bp *nuc* positive control strains), 2: *Staphylococcus aureus* ATCC 1065 (*mecA* negative and 279 bp *nuc* positive control strains), 3: *E. coli* ATCC 11230 (negative control strain) 4: 533 bp *mecA* positive, 279 bp *nuc* positive study isolates, 5: 533 bp *mecA* positive, 279 bp *nuc* positive study isolates, 6: 533 bp *mecA* positive, 279 bp *nuc* positive study isolates, 7: 533 bp *mecA* positive, 279 bp *nuc* positive study isolates, 8: *mecA* negative, 279 bp *nuc* positive study isolates

Şekil 1. *nuc* ve *mecA* Genlerinin Elektroforez Görünümü. M: Marker (Gene Ruler Seti-Promega, Madison, USA) DNA Ladder Plus (G210A) 100 bp-3000 bp Blue/Orange Loading Dye (G190A), 1: *Staphylococcus aureus* ATCC 46300 (533 bp'da *mecA* pozitif ve 279 bp'da *nuc* pozitif kontrol suşları), 2: *Staphylococcus aureus* ATCC 1065 (*mecA* negatif ve 279 bp'da *nuc* pozitif kontrol suşlar), 3: *E. coli* ATCC 11230 (negatif kontrol suşu) 4: 533 bp'da *mecA* pozitif, 279 bp'da *nuc* pozitif çalışma izolatları, 5: 533 bp'da *mecA* pozitif, 279 bp'da *nuc* pozitif çalışma izolatları, 6: 533 bp'da *mecA* pozitif, 279 bp'da *nuc* pozitif çalışma izolatları, 7: 533 bp'da *mecA* pozitif, 279 bp'da *nuc* pozitif çalışma izolatları, 8: *mecA* negatif, 279 bp'da *nuc* pozitif çalışma izolatları

Table 1. *The results of analysis of antibiotic resistance of biofilm positive and negative S. aureus*
Tablo 1. *Biyofilm pozitif ve negatif S. aureus'larda antibiyotik direnç analiz sonuçları*

Antibiotic	Biofilm Positive 37 (60.65%)		Biofilm Negative 24 (39.25%)		Total 61 (100%)		P*<0.05 Significant
	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	
Oxacillin	9 (24.32%)	28 (75.68%)	0 (0%)	24 (100%)	9 (14.75%)	52 (82.25%)	P*=0.05
Cefoxitin	8 (21.62%)	29 (78.38%)	0 (0%)	24 (100%)	8 (13.11%)	53 (86.89%)	P=0.06
Vancomycin	0 (0%)	37 (100%)	0 (0%)	24 (100%)	0 (0%)	61 (100%)	P=0.19
Amoxicillin-Clavulanic acid	4 (10.81%)	33 (89.19%)	0 (0%)	24 (100%)	4 (6.55%)	57 (93.45%)	P=0.11
Penicillin	25 (67.56%)	12 (32.44%)	3 (12.50%)	21 (87.50%)	28 (45.90%)	33 (54.10%)	P*=0.01

Of 61 *S. aureus* isolates, 37 (60.65%) were positive for biofilm formation. Of 37 biofilm positive isolates, 9 (24.32%) were oxacillin resistant, 8 (21.62%) were cefoxitin resistant, 4 (10.81%) were amoxicillin-clavulanic acid resistant, and 25 (67.56%) were penicillin resistant. On the other hand, vancomycin resistance was not detected *(Table 1)*.

DISCUSSION

Staphylococcus aureus threats public health by causing serious hospital infections. Most of the nosocomial infections caused by methicillin-resistant *S. aureus* resulted in substantial losses in the world. Identification of m*ecA* gene in determination of methicillin-resistant is a gold standard. Methicillin-resistant strains are multi-resistant. It is known that genes resistant to vancomycin were transferred from *Enterococcus* to *Staphylococcus*. Wide spread usage of vancomycin resulted in decreased sensitivity of *S. aureus* strains to vancomycin. Therefore, treatment of this infection is difficult. Antibiotic-resistant strains can infect humans via the ingestion of foods contaminated with these resistant strains. Therefore, contaminated foods have great risk potential to human health [13].

There are many studies that presence of *Staphylococcus* spp. and *S. aureus* in cheese has been investigated. Yasar [14] reported that *Staphylococcus* spp. was determined in 47 of 99 cheese samples on the market and in 45 of 72 fresh cheese samples. Of 45 strains, 12 were *S. aureus*. Önganer et al.[15] indicated that *S. aureus* was determined in 30 of 100 fresh curd cheese in Diyarbakır. Öksüztepe et al.[16] indicated that *S. aureus* was determined in 37 of 40 curd cheese on market in Elazığ province. Another study indicated that *S. aureus* was determined in 14 of 181 different cheese samples [17].

In the present study, high contamination rates of *Staphylococcus* spp. and *S. aureus* in cheese are probably associated with raw milk usage, improper use of starter cultures, and lack of sanitary conditions during production and storage, and insufficient ripening period. In addition, different methods and the medium used in the isolation and identification of the organisms may have effects on the results.

Staphylococcus aureus caused many lethal infections before discovery of antibiotics. In addition, staphylococci strains which have gained resistance to penicillin and many other antibiotics became an infection agent causing increased rate of hospital infections in the world. Rosengren et al.[18] indicated that *S. aureus* was determined in 6 of 96 pasteurized milk, and in 38 of 55 non-pasteurized cheese samples; moreover, 39% of these isolates were penicillin resistant. In another study, *S. aureus* was identified in 17 of 24 cheese samples. While these isolates were resistant to penicillin (60%) and oxacillin (5%) none of them were resistant to vancomycin [19]. Spanu et al.[20] reported that *S. aureus* were isolated in 36 cheese samples made of raw sheep's milk. While one third of those strains were penicillin resistant none of them were resistant to neither oxacillin nor vancomycin. In another study, *S. aureus* strains were isolated in 40 of 81 samples consisted of raw milk, cheese and whey. While 50% of them were penicillin resistant only 15% of them were oxacillin resistant; however, none of them were vancomycin resistant [21]. Nohutçu et al.[22] indicated that 79 *S. aureus* isolated in cheese samples were examined for antibiotic resistance; 13.9% were resistant to penicillin and 6.3% were resistant to methicillin. In the present study, high rates of penicillin resistance can be associated with usage of penicillin for the treatment of mastitis and other infections. In addition, antibiotic resistance could be related to usage of milk of animals treated with antibiotics without waiting for the recommended ripening period in humans and calves. Bacteria found in many live and dead surfaces can form a biofilm which is a problem for the pharmaceutical and dairy industries. Biofilm bacteria exhibit resistance to the effect of antibiotics in a variety of ways. Limited diffusion of the antibiotic into the biofilm, different growth rates of bacteria in the biofilm, and the negative effect of micro-environmental changes to the antibiotics are a few example of bacterial resistant. To the best of our knowledge, no information is available in the literature regarding biofilm forming ability of *S. aureus* strains isolated from different varieties of cheese. In this study, of 60.65% *S. aureus* strains formed biofilm; 24.32% were resistant to oxacillin, 21.62% were resistant to cefoxitin, 10.81% were resistant to amoxicillin-clavulanic acid, and 67.56% were resistant to penicillin. Vancomycin resistance was not detected in any of these strains. This

is the first study about biofilm-positive *S. aureus* strains isolated from tulum cheese.

According to these results and statistical data, it was observed that the production of biofilm may be an effective factor for the resistance gaining phenomenon of bacteria against oxacillin and penicillin.

The high rate of *S. aureus* in Erzincan tulum cheese in the present study was concluded as a result of improper hygienic conditions and shortening ripening time. The high rates of antibiotic resistance and the biofilm formation in isolated *S. aureus* indicates that these strains can cause diseases that are difficult to treat. It is thought that Erzincan tulum cheese manufactured with improper hygienic conditions is a threat for public health.

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RESEARCH ARTICLE

Cloning and Expression of β-1,3-Glucanase Gene from *Cellulosimicrobium cellulans* in *Escherichia coli* DH5α [1] [2]

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Summary

In this study, β-1,3-glucanase gene of *Cellulosimicrobium cellulans* was amplified by PCR and cloned in pUC18 cloning vector to construct the recombinant plasmids pTEG5 and pTEG11. The recombinant plasmids pTEG5 and pTEG11 were transformed into competent *Escherichia coli* cells. Digestion of recombinant plasmids with *Sac*I produced 1.9 kbp β-1,3-glucanase gene band on agarose gel which indicated the gene integration. β-1,3-Glucanase gene amplification on the recombinant vectors also indicated 1.9 kbp gene insert. Recombinant enzyme was produced by *E. coli* intracellularly. Intracellular components of recombinant *E. coli* strains with pTEG5 or pTEG11 dropped on LB-laminarin-agar plate, showed clear positive zones by Congo-red staining revealing the activity of secreted protein. Based on the zymogram analysis, the intracellular produced recombinant β-1,3-glucanase enzymes exhibited the same activity bands with *C. cellulans* enzyme with respect to molecular weight.

Keywords: *β-1,3-Glucanase, Cellulosimicrobium cellulans, Cloning, Escherichia coli*

Cellulosimicrobium cellulans'dan β-1,3-Glukanaz Geninin *Escherichia coli* DH5α'da Klonlanması ve Ekspresyonu

Özet

Bu çalışmada *Cellulosimicrobium cellulans* bakterisine ait β-1,3-glukanaz geni PCR ile amplifiye edilerek pUC18 klonlama vektörüne klonlanmış, böylece pTEG5 ve pTEG11 rekombinant plazmit DNA'lar elde edilmiştir. Rekombinant plazmit DNA'lar pTEG5 ve pTEG11 kompetent *Escherichia coli* hücrelerine transfer edilmişlerdir. *Sac*I enzimi ile kesilmiş rekombinant plazmitlerin agaroz jelde elektroforezi sonucu 1.9 kbç büyüklüğündeki β-1,3-glukanaz gen bandının görünmesi, gen entegrasyonunun tamam olduğunu göstermiştir. β-1,3-glukanaz geninin rekombinant vektörlerden amplifikasyonu da 1.9 kbç büyüklüğündeki geni göstermiştir. Rekombinant enzim *E. coli* tarafından hücre içi olarak üretilmiştir. LB-laminarin-agar plağına damlatılan rekombinant *E. coli* suşlarının intraselüler içerikleri Congo-red boyaması ile pozitif zonlar üretmiş, böylece rekombinant bakterilerce üretilen proteinin aktif olduğu anlaşılmıştır. Zimogram analizinde, hücre içi üretilen rekombinant β-1,3-glukanaz enzimleri *C. cellulans* enzimi ile aynı moleküler ağırlığa ait aktivite bantları sergilemişlerdir.

Anahtar sözcükler: *β-1,3-Glukanaz, Cellulosimicrobium cellulans, Klonlama, Escherichia coli*

INTRODUCTION

The yeast cell wall consists mainly of glucan, mannoprotein, and chitin [1-3]. Among these compounds, the glucans are essential structural components, responsible for mechanical strength, shape, and elasticity of the yeast cell wall [3].

Endo-1,3-β-glucanases (EC 3.2.1.6 and EC 3.2.1.39) are widely distributed among bacteria and higher plants [4]. These enzymes catalyse the hydrolysis of β-1,3-glucan component found in the yeast cell wall and other β-1,3-

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glucans such as laminarin, curdlan and pachyman [3,5]. The bacterium *Cellulosimicrobium cellulans* (also known with the synonyms *Cellulomonas cellulans*, *Oerskovia xanthineolytica*, and *Arthrobacter luteus)* has been regarded as a major source of yeast-lytic enzymes, particularly endo-β-1,3-glucanases, proteases and mannanases [6]. Commercially available yeast-lytic glucanases preparations derived from this organism, namely Lyticase, Zymolyase, and Quantazyme, have been produced and widely used for yeast protoplast preparations and yeast DNA isolation [6-8]. Of these preparations, only Quantazyme (Quantum Biotechnology, Canada) is produced recombinantly and protease-free [6,7]. The β-1,3-glucanase gene from *C. cellulans* (ATCC 21606) was cloned and then sequenced previously [2].

In the present study, we amplified the β-1,3-glucanase gene from *C. cellulans* genome via PCR. The DNA insert carrying the gene of interest was subcloned and expressed in *Escherichia coli* strain DH5α to lead for our further studies.

MATERIAL and METHODS

Strains of Bacteria and Growth Conditions

Cellulosimicrobium cellulans (*Oerskovia xanthineolytica*, ATCC 21606) was cultured in GYM Streptomyces medium (glucose (0.4% wt/v), yeast extract (0.4% wt/v), malt extract (1% wt/v), pH 7.2) at 28°C. Agar (1.2% wt/v) and $CaCO_3$ (0.2% wt/v) were added into GYM Streptomyces medium for preparation of GYM Streptomyces agar. *Escherichia coli* strain DH5α was cultured in LB-broth (10 g bacto tryptone (Merck), 5 g yeast extract (Merck) and 10 g NaCl (Merck) per L, pH 7.5) at 37°C. Agar (1.5% wt/v) was added into LB medium for LB-agar. Both LB-broth and LB-agar were supplemented with ampicillin (50 μg mL^{-1}) for culturing of recombinant *E. coli* strains. For activity testing on LB-laminarin-agar plates, intracellular proteins of cultured recombinant *E. coli* strains obtained by sonication (50-60 kHz, Bandelin Electronic Sonopuls UWV 2070, Germany) and dropped onto the plate and air-dried for 15-20 min. After drying, the plate was incubated at 30°C for 4-5 h. The plate was stained with Congo-red solution (0.1% wt/v Congo-red) for 15 min and then destained with 1 M NaCl solution for 15 min. Clear bands at the dropped area indicated the presence of β-1,3-glucanase activity [9].

Plasmids

pUC18 (ampicillin resistant; amp®) was used to create recombinant vectors pTEG5 and pTEG11, harbouring β-1,3-glucanase gene. The recombinant plasmids pTEG5 and pTEG11 were used for *E. coli* transformation. Recombinant *E. coli* strains, carrying pTEG5 or pTEG11, were grown on LB-agar at 37°C supplemented with ampicillin (50 μg mL^{-1}) as a selective agent. Recombinant plasmids were isolated from *E. coli* cells containing pTEG5 or pTEG11 as described previously [10].

DNA Modification

The following modifying enzymes were purchased and used for DNA modifications; *Sac*I, *Sma*I, *Eco*RI and bacterial alkaline phosphatase, bacteriophage T4 DNA ligase as well as Pfu DNA polymerase (Fermentas, Vivantis and Promega Corporation). Restriction enzyme reactions were monitored by examining digestion by agarose gel electrophoresis using standard methods [11]. Linearized plasmid DNA and PCR product were excised from gels and purified using Genomic DNA Purification Kit (Fermentas).

PCR and Cloning Procedures

The sequences of the primers used for amplifying of β-1,3-glucanase gene were 5'-AGAGCTCGTGGCACTGCAC TCGTTCGAGTCT-3' (forward) and 5'-AGAGCTCGACGGGC GCGGTCAGAGCGTCCAG-3' (reverse) based on the gene sequence [2]. The PCR mixture consisted of 5 μL of reaction buffer, 1 μL of 40 mM dNTP mix (200 μM each final), 1 μL each of forward and reverse primers (20 pmol each primer), 0.5 μL of Pfu DNA polymerase (2.5 U/μL), 1 μL of 50% wt/v DMSO (1% wt/v final), and 650 ng of template in a total volume of 50 μL. The following amplification program was used: Initial denaturation step at 94°C for 2 min, then 30 cycles of denaturation at 98°C for 10 s, annealing and elongation at 68°C for 5 min. A final extension step was performed as 72°C for 5 min. The blunt ended PCR product was ligated into *Sma*I digested pUC18 plasmid DNA to create pTEG5 and pTEG11 using standard methods [11]. The ligation mixes contained approximately 360 ng of PCR product and 630 ng of linearized plasmid.

Recombinant plasmids pTEG5 and pTEG11 were transformed into *E. coli* DH5α strain using the method as described previously [12].

Electrophoretic Analysis of Extracellular and Intracellular Proteins

To obtain the extracellular proteins of *C. cellulans*, *E. coli* DH5α and recombinant *E. coli* strains from growth medias, the cells were pelleted by centrifuge. The extracellular extracts (supernatants) were mixed with 1:1 volume of 20% wt/v TCA for precipitation. After the incubation at room temperature for overnight, protein pellets were obtained by centrifuge. Air-dried protein pellets were dissolved in 0.1 M Tris-HCL buffer (pH 8.0). To obtain the intracellular proteins of recombinant *E. coli* strain from LB-broth, the cells were pelleted by centrifuge and then the pellets were dissolved in equal volume of water. After the sonication process (50-60 kHz) the samples were centrifuged. The protein pellets were obtained from the supernatant using 20% wt/v TCA as described above.

SDS-PAGE and SDS-Laminarin-PAGE (0.2% laminarin) were done as described previously [13] with slab gels (12% wt/v acrylamide). After the electrophoresis, the gel was stained for 1 h with Coomassie blue R 250 dye in methanol–

acetic acit–water solution (4:1:5 by volume) and destained in the same solution without dye [14,15]. For activity staining (zymogram analysis), SDS was removed by washing the gel at room temperature in solutions containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol 20% v/v for 1 h and 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2) for 1 h, respectively. Renaturation of enzyme proteins was carried out by keeping the gel overnight in a solution containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), 5 mM β-mercaptoethanol and 1 mM EDTA at 4°C. Gel was then transferred onto a glass plate, sealed with a film, and incubated at 30°C for 4 h. Gel was stained in a solution of Congo-red (0.1% wt/v Congo-red, 0.2 M NaOH), for 1 h, and destained in 1 M NaCl for 30 min. Clear bands indicated the presence of β-1,3-glucanase activity [16-18].

RESULTS

Transformation of Escherichia coli

The β-1,3-glucanase gene of *C. cellulans* was cloned into pUC18 and thus recombinant plasmids pTEG5 and pTEG11 were created *(Fig. 1)*.

The pTEG5 and pTEG11 recombinant plasmids were then transformed into *E. coli* DH5α strain to express the β-1,3-glucanase gene. Intracellular supernatant of recombinant *E. coli* strains showed β-1,3-glucanase activity on LB-agar plate supplemented with ampicillin (50 μg mL^{-1}) and laminarin (0.1% wt/v) by producing clear zones *(Fig. 2)*.

Recombinant pTEG5 and pTEG11 plasmids were isolated from *E. coli* cells. They were, then subjected to restriction fragment length analysis together with PCR amplified DNA fragment on agarose gel electrophoresis (0.8% wt/v) encoding the gene. β-1,3-Glucanase gene fragment (~1.9 kbp) amplified by PCR and restriction endonuclease digested recombinant plasmids confirmed the success of the cloning experiments *(Fig. 3)*. Recombinant plasmids digested with *Eco*RI were both yielded the same DNA fragments consisting of pUC18 and β-1,3-glucanase gene (both are the same plasmid).

Culture supernatants of *C. cellulans*, recombinant *E. coli*/pTEG5, and non-recombinant *E. coli* DH5α strains were applied to SDS-PAGE and SDS-Laminarin-PAGE to visualize total proteins and zymogram analysis, respectively. For

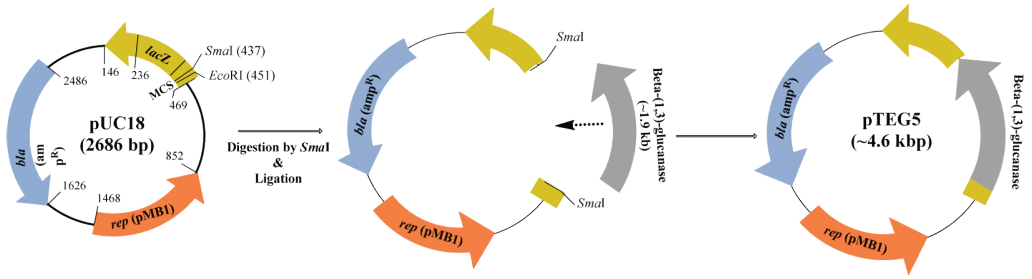

Fig 1. Construction of pTEG5 plasmid (~4.6 kbp) from β-1,3-glucanase gene (~1.9 kbp) and pUC18 plasmid (~2.7 kbp)
Şekil 1. β-1,3-glukanaz geni (~1.9 kbç) ve pUC18 plazmidinden (~2.7 kbç) pTEG5 plazmidinin (~4.6 kbç) oluşturulması

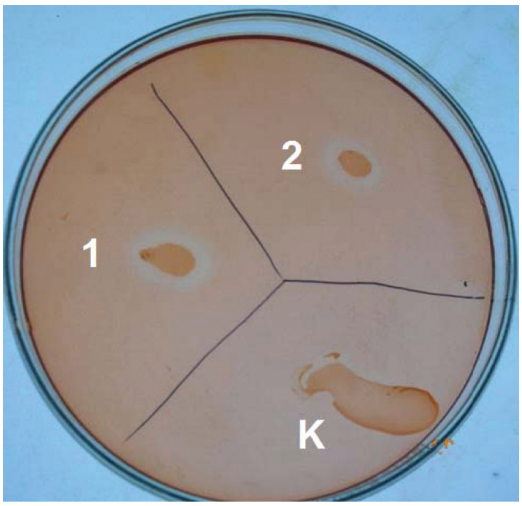

Fig 2. Intracellular supernatant of recombinant *E. coli* strains showing clear β-1,3-glucanase enzyme activity with Congo red staining (1: *E. coli*/pTEG5, 2: *E. coli*/pTEG11, K: *E. coli* as control)

Şekil 2. Rekombinant *E. coli* suşlarına ait hücre içi süpernatantların Congo-red boyaması ile β-1,3-glukanaz aktivitesi göstermesi (1: *E. coli*/pTEG5, 2: *E. coli*/pTEG11, K: *E. coli* kontrol)

zymogram analysis denaturated proteins were renaturated on SDS-Laminarin-PAGE after removing denaturating agents from the gel and then allowing to the enzyme to digest substrate, thereby producing clear zones on the gel. On zymogram analysis, only β-1,3-glucanase protein band of *C. cellulans* with 54.5 kDa in size was showed a clear zone together with intracellular protein counterparts of all other recombinant *E. coli* strains *(Fig. 4)*.

DISCUSSION

With this study, β-1,3-glucanase gene of *C. cellulans* was cloned and expressed in *E. coli*. The enzyme secreted from recombinant *E. coli* strains were found to be active, showing intracellularly clear zones on LB-agar plate containing laminarin. On the other hand, zymogram analysis clearly indicated that activity bands surrounded with clear zones confirming renaturation of denatured enzyme.

β-1,3-Glucanase gene of *C. cellulans* (ATCC 21606) was well studied and enzymatic properties were revealed [19].

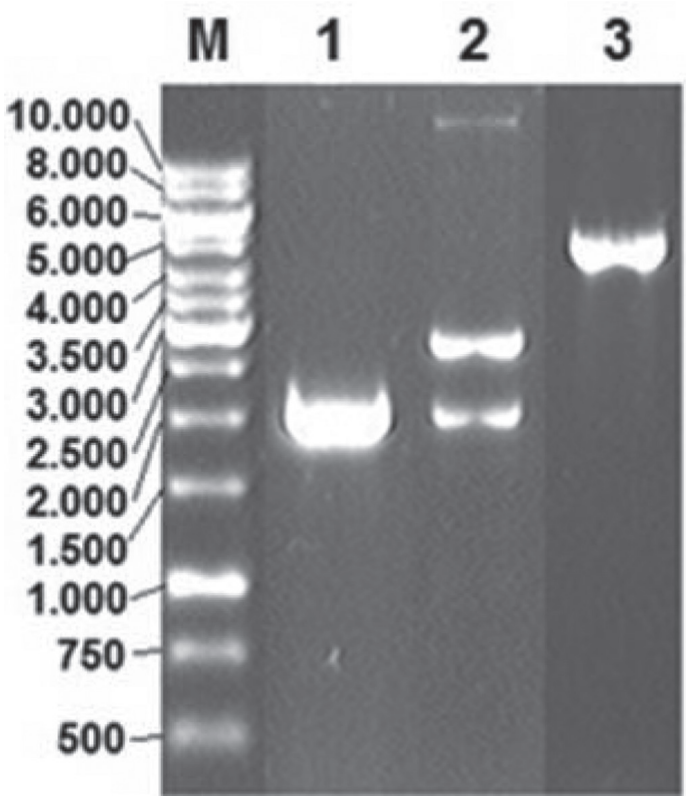

Fig 3. Insert and PCR analysis of *E. coli*/pTEG5 (M: 1 kbp DNA markers, 1- PCR amplified fragment of β-1,3-glucanase gene from pTEG5, 2- pTEG5/*Sac*I, 3- pTEG5/*Eco*RI)

Şekil 3. *E. coli*/pTEG5'e ait insört ve PCR analizleri (M: 1 kbç DNA markır, 1- pTEG5'den PCR ile amplifiye edilmiş β-1,3-glukanaz genine ait fragment, 2- pTEG5/*Sac*I, 3- pTEG5/*Eco*RI)

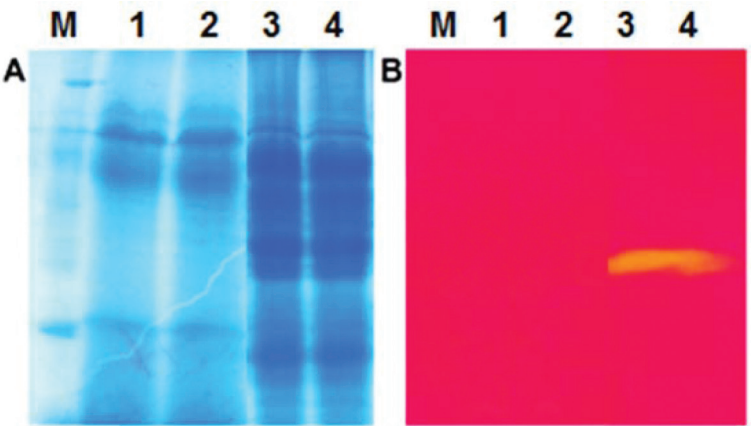

Fig 4. SDS-PAGE (A) and SDS-Laminarin-PAGE (B) analysis of recombinant and non-recombinant bacterial proteins (M: Marker, 1- *E. coli*, 2- *E. coli*/pTEG5 (extracellular proteins), 3- *E. coli*/pTEG5 (intracellular proteins), 4- *C. cellulans* culture supernatant)

Şekil 4. Rekombinant ve rekombinant olmayan bakterilere ait proteinlerin SDS-PAGE (A) ve SDS-Laminarin-PAGE (B) analizleri (M: Markır, 1- *E. coli*, 2- *E. coli*/pTEG5 (hücre dışı proteinler), 3- *E. coli*/pTEG5 (hücre içi proteinler), 4- *C. cellulans* kültür süpernatantı)

Herbal β-1,3-glucanase genes from soybean [20], jujube fruit [21] and rice [22] were cloned in different organisms. The *C. cellulans* β-1,3-glucanase gene was also cloned and expressed in *Bacillus subtilis* and *E. coli* [2-5,23]. In one of these studies, 75% of the recombinant protein was released to the extracellular space [3]. On the other hand, in another study, it has been reported that *E. coli* cells secreted the recombinant β-1,3-glucanase into the periplasm as a mature enzyme [2].

Although restriction digestion and PCR analysis showed the β-1,3-glucanase gene was cloned into *E. coli* DH5α, no lytic activity was observed on LB-laminarin-agar plates with Congo-red staining. But revealing of β-1,3-glucanase activity on LB-laminarin-agar plates, after dropping of extracellular compounds of disrupted bacteria and Congo-red staining, showed that the enzyme was produced by recombinant bacteria intracellularly. It was noted that, compared to other hosts, *E. coli* does not naturally secrete high amounts of proteins [24,25]. Nevertheless, protein secretion in *E. coli* is a complex process [26-28] and so secretion of recombinant proteins can face several problems [28]. Among these problems, incomplete translocation across the inner membrane [28,29], insufficient capacity of the export machinery [30,31], and proteolytic degradation [32] are the most frequently problems encountered [28]. Additionally, protein size may influence secretion efficiency [28,33] and large cytoplasmic proteins may be physically impossible to translocate [28,29]. The amino acid composition of the leader peptide also could be important for segregation of proteins [28,34]. We observed intracellular activity of β-1,3-glucanase on the agar plates containing laminarin. On the other hand, the recombinant enzyme is 54.5

kDa in size and there are several studies demonstrated extracellularly secretion of recombinant proteins with similar size in *E. coli* [35-37]. In one of these studies, endo-1,4-β-glucanase gene of *Bacillus licheniformis* subcloned and 52.2 kDa corresponding enzyme was extracellularly secreted by *E. coli* strain [35]. Therefore, we do not consider the proteolytic degradation and protein size about the enzyme secretion. Generally, premature proteins found in the outer membrane or periplasmic space contain a short specific amino acid sequence (signal sequence) that allows proteins to be exported outside the cytoplasm and during transportation, the signal sequence is cleaved by signal peptidase to yield a mature protein product [38]. Proteins without a signal peptide are made within the cell cytoplasm. Our results most likely indicate that signal peptide of the enzyme can not be recognized by the *E. coli* secretion machinery. It was reported that, the *rsda* gene of a *Cytophaga* sp. was cloned and expressed in *E. coli* DH5α and the enzyme was produced intracellularly by the transformant [39]. Similarly, the *Bacillus subtilis* β-1,4-glucanase gene (a similar enzyme hydrolyzing β-1,4-linkages present in noncrystalline cellulosic substrates such as carboxymethyl cellulose and trinitrophenyl carboxymethyl cellulose) was cloned in *E. coli* expressing the intracellularly [40]. These reported results are supporting our thesis.

As a conclusion, the β-1,3-glucanase gene was isolated from *C. cellulans* genome and cloned in *E. coli*. Our results indicate that, recombinant *E. coli* strains secreted the enzyme intracellularly.

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RESEARCH ARTICLE

Effects of Early and Late Lactation Period on Plasma Oxidant/Antioxidant Balance of Goats

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Summary

The purpose of the study was to investigate effects on oxidant and antioxidant balance of early and late lactation period in goats. Blood samples used in study were obtained from Halep goats in early and late lactation period. Oxidative stress index (OSI) and lipid hydroperoxide (LOOH) and total oxidative status (TOS) and total antioxidant status (TAS) were analyzed to determined of oxidant and antioxidant balance. Plasma TOS, OSI and LOOH levels in early lactation period were significantly higher (P<0.001) than late lactation period of goats, while lower (P<0.001) level of TAS. It was concluded that lipid peroxidation decreased and antioxidant defence system mechanism in late lactation period in goats served as more positive than early lactation period.

Keywords: *Goat, Lactation, Total antioxidant status, Total oxidant status, Oxidative stress*

Erken ve Geç Laktasyon Periyodunun Keçilerde Plazma Oksidan/Antioksidan Dengesi Üzerindeki Etkileri

Özet

Bu çalışmada keçilerin oksidan ve antioksidan dengesi üzerinde erken ve geç laktasyon periyodunun etkilerinin araştırılması amaçlandı. Çalışmada kullanılan kan örnekleri erken ve geç laktasyon periyodunda bulunan Halep keçilerinden elde edildi. Oksidan ve antioksidan dengenin değerlendirilmesi için oksidatif stres indeksi (OSİ), lipid hidroperoksid (LOOH), total oksidan (TOS) ve total antioksidan düzeyleri (TAS) analiz edildi. Erken laktasyon periyodunda plazma TOS, OSİ ve LOOH düzeylerinin geç laktasyon periyodundaki keçilere göre daha yüksek (P<0.001) olduğu belirlenirken, TAS düzeyinin daha düşük olduğu belirlendi (P<0.001). Sonuç olarak, geç laktasyon periyodundaki keçilerde erken laktasyon periyoduna göre lipid peroksidasyonunun azaldığı ve antioksidan savunma sistemi mekanizmasının daha olumlu yönde işlediği kanısına varıldı.

Anahtar sözcükler: *Keçi, Laktasyon, Total antioksidan seviye, Total oksidan seviye, Oksidatif stres*

INTRODUCTION

Lactation period is a process that makes metabolic and physiological regulations necessary for maintaining the homeostasis during postpartum [1]. Lactation stages including early and late lactation period since the catabolic reactions increases at cellular level may be effected on some metabolic functions related to the level of the free radicals [2,3]. It has been reported that differences can be observed in the level of oxidative stress with respect to the stages of lactation period [2,4]. The changes occurring at the composition of the milk in lactation period are resulted from the various factors such as epithelial cell proliferations, secretion activity, supplying of the nutrients and removing of the metabolic wastes by means of the blood [5,6].

General knowledge about oxidative stress related-metabolic disorders increases, and the pathologic effects of these disorders generally are associated with the free radical molecules [7-9]. While free radicals are continuously produced in metabolic processes, their levels increase in a remarkable rate as a result of the various pathological and non-pathological conditions [7]. When the balance between oxidant and antioxidant status is disturbed, health problems

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may appear due to the oxidation of the biological substances [8-10]. Oxidative stress can be detected by some biological markers, but new methods have been developed that measure each antioxidant parameter to evaluate the TAS [11,12]. The ratio of total peroxide to total antioxidant capacity is accepted as a measure to define the OSI [13]. It is reported that only measurement of TAS can be used to identify the dynamic balance between the plasma pro-oxidant and antioxidant status [9,14]. When polyunsatured fatty acids are oxidized with free radicals, lipid peroxyl radicals are formed via chain reactions which also produces LOOH [15].

Changes may occur in oxidant and antioxidant status in the blood during transitional period from early lactation to late lactation period that could affect both the animal and the health of others which benefit from the milk and meat [16,17]. In addition, it has not reached to sufficient number of studies relation to lactation period and comprehensive oxidative status on goats. Therefore, in this study, TAS, TOS, OSI and LOOH levels was examined to understand the balance between oxidants and antioxidants in the blood.

MATERIAL and METHODS

Two-three years old 25 Halep goats lived in early and late lactation period were used in this study. Goats were allowed to graze on natural pasture from 07:30 to 17:30 and kept in pens from 17:30 to 07:30 during the trial. During the study, they were fed with 75% tender whole crop barley and 25% mustard straw (23% dry matter, 9.2% organic matter, 14% crude protein, 0.5% crude lipid, 24% crude cellulose, 7.2% crude ash, and 54.3% nitrogen free extract). Fresh water was available *ad libitum*. Kids were not weaned and goats were not milked throughout lactation period. The management of the goat did not change during the experimental period.

The blood samples were taken from *V. jugularis* at the end of March, the first week of April and first week of August. The samples taken into the tube with EDTA were centrifuged for 10 minutes at 3000 rpm. Plasma samples were kept at -20ºC until the analysis. TAS, TOS, OSI and LOOH levels were evaluated by the methods stated below.

Measurement of Total Antioxidant Status

TAS levels were measured by using commercially available diagnostic kits (Gaziantep, Turkey) via auto analyzer (Aeroset®, Abbott®, Illinois, USA) [18]. In this method, hydroxyl radicals are produced, which are very strong biological radical. In the analysis, ferrous ion solution which is found in Reagent 1 is mixed with H_2O_2 which is found in Reagent 2. Then consecutively, by hydroxyl radicals strong radicals are produced like the brown coloured dianisidyl radical cation. With this method, antioxidant status of the sample is measured against the strong free radical reactions, which is initiated by producing hydroxyl radical. Results are expressed as mmol Trolox Eq/L.

Measurement of Total Oxidant Status

TOS level was measured by using commercial diagnostic kit (Gaziantep, Turkey) at auto analyzer (Aeroset®, Abbott®, Illinois, USA) [19]. The oxidants found in the sample, convert ferrous ion-o-dianisidine complex into ferric ion. Oxidation reaction is increased by glycerol molecules during the reaction. Ferrous ion forms a colour complex with xylenol orange in acidic media. Density of the colour is related to total quantity of oxidant molecules which are found in the sample. Measurement was calibrated by hydrogen peroxide (H_2O_2) and the results are given as micro moles in a liter H_2O_2 equivalent (µmol H_2O_2 Eq/L)

Measurement of Lipid Hydroperoxide

Lipid hydroperoxide level is evaluated in "xylenol orange" containing media by using ferrous ion oxidation method [20].

Oxidative Stress Index

The rate of TOS and TAS were used as oxidative stress index (OSI).

Statistical Analysis

Results of all parameters were expressed as mean (X) ± standard deviation (SD) for each group. The results were evaluated statistically using paired samples t-test on SPSS packet programme (SPSS 16.0 for Windows). A p-value less than 0.001 was considered to be statistically significant.

RESULTS

Levels of TOS, OSI and LOOH in early and late lactation period of goats were shown in *Fig. 1* and also levels of TAS in *Fig. 2*. In early lactation period according to late lactation period was determined that TAS levels were significantly decreased while TOS, OSI and LOOH levels were significantly increased.

In the blood samples taken from early and late lactation period, OSI and LOOH levels were 2.53±0.32 and 1.46±0.25 arbitrary unite, 10.92±1.41 and 8.31±0.63 µmol H_2O_2/L ($P<0.001$), respectively. TOS levels was determined to be 20.01±1.69 and 14.14±2.36 µmol H_2O_2/L while TAS levels was determined to be 0.83±0.04 and 0.99±0.07 mmol trolox Eq/L ($P<0.001$).

DISCUSSION

Free radicals including reactive oxygen and nitrogen species (ROS and RNS) are produced continuously during aerobic metabolism and their levels may increase dramatically during increased production requirments or as a result of some pathological events [21,22]. In the free radical level may be changes during the postpartum and lactation stages including early and late lactation periods

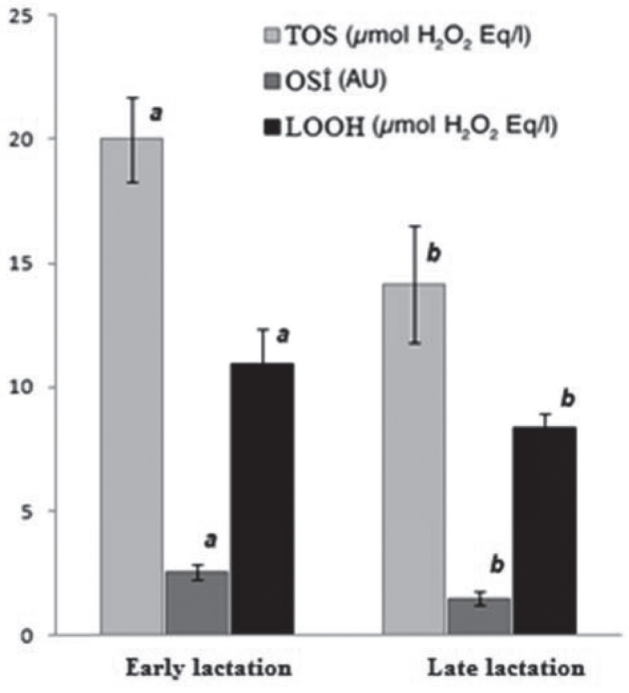

Fig 1. TOS, OSI and LOOH levels in early and late lactation period of goats, **a,b:** Values with different letter indicates significant differences (P<0.001)

Şekil 1. Keçilerde erken ve geç laktasyon periyodunda TOS, OSİ ve LOOH düzeyleri, **a,b:** Farklı harf ile gösterilen değerler istatistiksel olarak farklıdır (P<0.001)

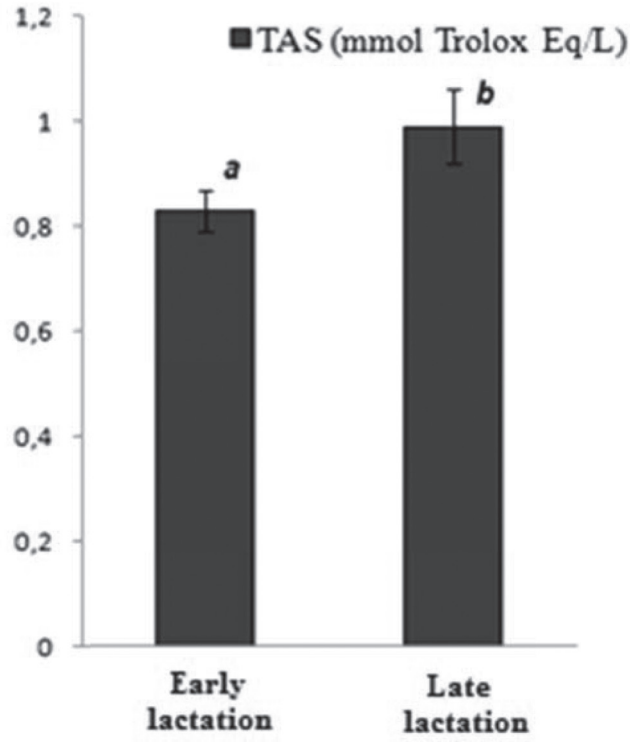

Fig 2. TAS levels in early and late lactation period of goats. **a,b:** Values with different letter indicates significant differences (P<0.001)

Şekil 2. Keçilerde erken ve geç laktasyon periyodunda TAS düzeyleri. **a,b:** Farklı harf ile gösterilen değerler istatistiksel olarak farklıdır (P<0.001)

since the catabolic reactions increases at cellular level with metabolic and physiological regulations necessary for maintaining the homeostasis [1-3]. While molecular oxygen is need to continue of normal cellular functions in mammals, excess level of ROS can cause cell and tissue damages and lead to a case referred to as oxidative stres [3,7,22]. It is the antioxidant capability including several antioxidative matters that will ensure to protect cells from the disruptive effects of oxidative stress. Given the multiplicity of antioxidant matters and pathways, their centrality in the prevention of oxidant stress, and the influences of diet on overall antioxidant capacity types, it is a serious point to be able to quantitatively measure TAS or antioxidant power inside of biological specimens [21,22]. Evaluation of the oxidative and antioxidative status can also be performed by the measurement of TAS, TOS, OSI and LOOH levels [18,20]. In this study, it was used assay of TAS levels to determine combined action of all antioxidants present in the sample was based upon to measured against the strong free radical reactions, which is initiated by producing hydroxyl radical. The level of TAS was lower during early lactation, in agreement with other reports related with antioxidants, and is presumably due, in part, to the utilization of antioxidants in colostrums production period [23]. Changes in free radical and antioxidant concentration appear to represent homeorhetic processes that normally occur in early lactation. Given the lack of reference values for oxidative stress indicators and TAS levels for lactation periods in goats, and the fact that few studies have been carried out in this topic the causes of oxidative stres are difficult to determine.

It was also claimed that animals in the early lactation period were shown more effort to meet its energy needs and so, there is a decrease in total protein and lipid content of the adipose tissue [24,25]. Transition phase from early lactation period to late lactation period is regulated by a homeostatic mechanism comprising metabolic interactions and body fuel distribution, and this mechanism is primarily based on glucose [26]. Turk et al.[28] reported that in lactating cow serum glucose concentrations were in low levels in early lactation period and these levels increased towards to the middle of the lactation period. In early lactation period, non-esterified fat acids (NEFA), which form as the result of lipid mobilization, are used to meet energy need [27,28]. It has been stated that free radical level will also be higher because of NEFA oxidation that increases in hepatocyte mitochondrium [29]. A decrease in the antioxidant capacity may be possible depending on the function of secretion and synthesis in the liver of ruminant in which hepatomegaly is seen due to elevated intracellular lipid rate resulting from increased NEFA synthesis [30,31]. The relationship between blood NEFA and triglycerides in the peripartum period by performed studies has been well documented [32,33] and reported that increase in the oxidative metabolism has implied peroxidation of fatty acids leading to formation of lipid peroxides [34]. One way to determine

if injury originated from ROS is occurring within tissues is to measure end products of free radical oxidation patways. Lipid peroxidation consists when ROS react with poly-unsaturated fatty acids (PUFA) [22,26]. The evaluation of LOOH levels in plasma would be an sign of early stages of this lipid peroxidation injury. Castillo et al.[9] reported that there is no statistically change in mean serum TAS levels between late lactation period and the first weeks of the early lactation periods, and they reported that TAS levels could show remarkable fluctuations during the first weeks of early lactation period. It has been stated that glutathion peroxidase (GSH-Px) activity of plasma connected with TAS as an indirect indicator of oxidative stress has showed higher values 4 weeks after calving compared with those measured 2 weeks before calving [35]. It was found an increase in malondialdehyde (MDA) levels related to TOS known as an oxidative stres indicator one week before and one week after calving compared to earlier and later time points, however with wide individual variations [34,36]. Measurement of LOOH levels in this study indicated significant differences between goats early and late lactation periods ($P<0.001$). This finding are consistent with study reported in late lactation animals where MDA levels, another biomarker of lipid peroxidation [34]. Results for the present study suggest that antioxidant potential reduced and oxidative stress increased in early lactation according to goats at late lactation period. The depletion of critical antioxidant defense components in tissues may predispose to metabolic changes originated from oxidative stress as reported in fish [37]. Our results are coherent with similar studies related to lactation and oxidative parameters [34,35]. Concentrations of the oxidant and antioxidant parameters are measured separately by using different methods and it can be seen that with the methods used in this study oxidant and antioxidant balance can be evaluated in a shorter time period, with a lower cost and with safer results [18,38]. In our study it was identified that TAS levels are higher in late lactation period goats compared to early lactation period goats, and also TOS, OSI and LOOH levels are significantly lower. This situation may be related with decrease in lipid mobilization occuring in adipose tissue due to the transition from negative energy balance (NEB) to positive energy balance (PEB) in late lactation period.

In studies were focused on energy balance and milk yield and feeding system categories to avoid from this uncertainty [30,39]. Both early lactation and after parturition in dairy animals is a period of severe NEB characterized by variables such as reduced blood glucose and insulin concentrations and elevated blood growth factors concentrations [2,4,40]. In addition, NEB has been related to altered hormonal levels in the ability of the hypothalamus-hypophyseal axis that do not support a functional reproductive system during early lactation [40]. The energy requirements of a lactating animals are met through a combination of dietary intake and mobilization of body reserves [40,41]. In a study performed by Soryal et al.[16] in goats, it was found that lipid and content of additive substances reach the highest level in late lactation period [16]. It has reported that oxidative stress increases due to NEB in early lactation period and the reason of this increase may effected from decreasing elements of antioxidant defence system [30]. The time taken to re-establish PEB after parturition is affected by the extent of fatty tissue reserves and the efficiency with which these reserves can be mobilized [42]. It has been reported that differences in the level of oxidative stress can be observed with respect to the stages of lactation period as a result of NEB that take places after the parturition [2,4]. According to a study performed for the energy balance category, the results indicated that an increased energy reserve mobilization affected the level of oxidative stress but only in cows fed to achieve restricted milk production. Therefore, it has been defined that oxidative stress levels were related to lower biological antioxidant potential in animals fed to achieve restricted milk production, a finding that might suggest that only animals with very high body reserve mobilization experience oxidative stress [30]. It was reported that animals in good body condition at calving and in high NEB in early lactation had higher oxidative stress [2]. In the study evaluated the role of diet during pregnancy and lactation in rats in the induction of metabolic abnormalities, especially an oxidant/anti-oxidant imbalance was found that level of total antioxidant capacity and levels of antioxidant component increased following days in lactation period. In addition, it strongly has suggested that maternal fat and energy intake condition during lactation can play an important role in the development of metabolic disorders observed in their offspring, and that maternal oxidative stress can be singled out as the factor involved [43].

In conclusion, elevated TOS, OSI and LOOH levels in early lactation period and increased TAS levels in late lactation period suggest that lipid peroxidation and oxidative stres decrease in late lactation period.

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SHORT COMMUNICATION

Changes of Volatile Compounds of Herby Cheese During the Storage Period

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Summary

Changes in the composition of volatile compounds of the control and four different types of herby cheeses (Control, cheese with no added herbs; CH, cheese containing Helis (*Ferule* sp.); CK, cheese containing Kekik (*Thymus* sp.); CS, cheese containing Sirmo (*Allium* sp.) and CM, cheese containing Mendo (*Anhriscus* sp.)) were investigated during 28 days of storage at 4°C. A total of 60 compounds were tentatively identified during the storage period, including aldehydes, ketones, alcohols, acids, esters, terpenes, aliphatic hydrocarbons, aromatic hydrocarbons, furans and other compounds.

Keywords: *Herby cheese, Volatile compounds, SPME-GC/MS*

Otlu Peynirlere Ait Uçucu Bileşenler Profilinin Depolama Süresince Değişimi

Özet

Kontrol ve 4 farklı otlu peynirin (Kontrol, otsuz üretilen peynir; CH, Helis içeren peynir (*Ferule* sp.); CK, Kekik içeren peynir (*Thymus* sp.); CS, Sirmo içeren peynir (*Allium* sp.) ve CM, Mendo içeren peynir (*Anhriscus* sp.)) uçucu bileşenler profili 4°C'de 28 günlük depolama periyodu boyunca belirlenmiştir. Çalışmanın sonucunda aldehit, keton, alkol, asit, ester, terpen, alifatik hidrokarbon, aromatik hidrokarbon, furan ve diğer bileşenleri içeren toplam 60 bileşik tespit edilmiştir.

Anahtar sözcükler: *Otlu peynir, Uçucu bileşikler, SPME-GC/MS*

INTRODUCTION

Herby cheese, which is called "Otlu peynir" in Turkish, is mainly produced in eastern and south-eastern regions of Turkey [1]. The cheese is produced by adding specific aromatic herbs, which have been commonly used for many years [2]. Generally, more than 20 kinds of herbs have been used in the production of herby cheeses, with the most commonly used being *Allium* sp. (Sirmo), *Thymus* sp (Kekik), *Ferule* sp. (Helis), *Anhriscus* sp. (Mendo), *Rannunculus* sp. (Cünk), *Anethum* sp. (Dereotu) and *Mentha* sp. (Nane) [3]. Flavour is one of the most important characteristics that determine the quality of cheese. The flavour compounds in cheese are primarily derived from carbohydrate, citrate, protein and milk fat as a result of glycolysis, citrate fermentation, proteolysis and lipolysis by microorganisms [4].

Analysis of the volatile components of herby cheese samples were conducted by a purge and trap connected to a gas chromatography with a mass spectrometer and disclosed a total of 60 components belonging to the following chemical families aldehydes, ketones, alcohols, acids, esters, terpenes, aliphatic hydrocarbons, aromatic hydrocarbons, furans and other compounds. Therefore, the aim of this research was to determine the changes of volatile compounds during the storage period in five different batches of herby cheeses.

MATERIAL and METHODS

Materials

The milk that is used in the production of cheese

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was obtained from the pilot milk-processing plant of the Agricultural Collage of Atatürk University. Jars for the packaging of samples were bought from the local markets of Erzurum, Turkey. Herbs used for the production of herby cheeses were purchased from Van, Turkey.

Manufacture of Experimental Herby Cheese

For the production of cheese, a total of 75 kg of whole-fat milk was used. For the production of cheese, milk was left for 24 h to turn sour. Afterwards, the sour milk was heat-treated at 80-85°C for 15 min and the formed curd was cooled to 25-30°C. Afterwards, the cheese was divided into five equal parts and each herb was added to the curds at a ratio of 2%, with the exception of the control, and salted. For analyses, samples were packed into the sterile jars and stored at 4±1°C for 28 days and analysed for volatile compounds.

Analysis of Volatile Compounds

The extraction of headspace volatile compounds was carried out using a SPME device (Supelco, Bellefonte, PA), using fibres of 75 ml, carboxen/polydimethylsiloxane (CAR/PDMS). Before the analysis, the fibres were pre-conditioned in the injection port of the GC as indicated by the manufacturer's guidelines. For each analysis, 5 g of herby cheese was minced and weighed into a 40 ml headspace vial and sealed with a PTFE-faced silicone septum (Supelco, Bellefonte, PA, USA). The vial was left at 30°C in a thermo block (Supelco, Bellefonte PA, USA) for 1 hour to equilibrate its headspace. Next, the fibre was exposed to the head-space while maintaining the sample at 30°C for 1 h. Analyses were performed on a gas chromatograph (Agilent 6890 N) coupled to a mass selective detector (Agilent 5973) [5].

Statistical Analysis

The data were analysed statistically using the SPSS statistical software programme version 13 (SPSS Inc., Chicago, IL, USA). Analysis of Variance (ANOVA) [6] and Duncan's multiple range test was used to determine significant differences among results.

RESULTS

The mean values of volatile compounds of herby cheeses during the storage period are presented in *Table 1*. The most of volatile compounds showed significant differences at the levels of P<0.01, P<0.05 during storage at ±4°C.

DISCUSSION

In herby cheeses, acetaldehyde, pentanal, nonanal, hexanal, heptanal, benzaldehyde and octanal were detected during storage. However, the most important of these were acetaldehyde and hexanal in the cheese samples. The highest mean acetaldehyde percentage was detected in CM cheese, while the lowest mean percentage value was determined in CS. There were no significant (P>0.05) differences between the experimental cheeses in terms of acetaldehyde values. As can be seen *Table 1*, relative percentage of acetaldehyde decreased after 14 days of storage and this difference was found to be statistically significant (P<0.05). This result could be explained by the presence of antimicrobial effects of sirmo on bacteria because aldehydes are produced by microorganisms in cheeses [7,8]. The hexanal percentage of the CH had a higher value than those of other samples and this was found to be statistically significant (P<0.01). Generally, the lowest hexanal percentage was determined in CS. This situation can be explained as a result of the lower β-oxidation regarding the reduced microbial activity in CS [9,10]. As can be seen *Table 1*, a relative percentage of hexanal showed a decrease during the storage period and this was statistically significant (P <0.01) *(Table 1)*.

Herby cheeses were found to contain a range of ketones. All of these ketones were formed at different levels in all herby cheeses during storage at 4°C and showed significant differences (P<0.01, P<0.05) both among samples and during the storage periods statistically *(Table 1)*. The highest mean percentage value of acetoin was found in CC and the lowest mean percentage value was in CS. These results could be due to the antimicrobial effect of herbs. Conversely, the highest mean percentage amount of aceton was detected in CS and the lowest value was found in CC. The acetoin and aceton levels of the samples were completely different (P<0.01) from each other and the throughout ripening period *(Table 1)*. The fluctuations in the concentrations of the ketones in herby cheeses during ripening were conceivably the result of the inter-conversions of the methyl ketones and their corresponding secondary alcohols [11].

Ethanol was the principal and most abundant volatile aromatic compound produced during the storage of the experimental cheeses. Similar results were found by Kaminarides et al.[12], Bintsis and Robinson [13]. The highest mean percentage value of ethanol was found in CC, while the lowest mean percentage value was determined in CK *(Table 1)*, although the differences between the cheese samples were insignificant (P>0.05). This result could be due to the antimicrobial effects of kekik on lactic acid bacteria and yeasts. As can be seen *Table 1*, a relative percentage of ethanol showed irregular changes during storage and this was statistically significant (P<0.01).

Acetic and butyric acid were detected in all samples during the storage period. Acetic acid showed irregular changes among samples, which was statistically significant (P<0.01). The highest mean acetic acid percentage was determined in CM, whereas the lowest mean percentages were in CC and CH. It was observed that the acetic acid value increased (P<0.05) at the end of storage. These

Table 1. *The mean peak area ratio of volatile compounds in the control and herby cheese samples during storage*
Tablo 1. *Kontrol ve Otlu peynir örneklerine ait uçucu bileşenlerin ortalama pik alanlarının depolama süresince değişimi*

Volatile Compounds	Herby Cheese Samples					Days of Storage				
	CC	CH	CK	CS	CM	1.	7.	14.	21.	28.
Aldehydes										
Acetaldehyde	8.67±3.93a	8.42±4.07a	7.70±5.07a	6.43±3.15a	8.90±3.18a	7.84±4.61b*	8.69±3.48a*	10.51±3.91a*	6.39±2.76b*	6.69±3.71b*
Pentanal	0.95±1.06a**	0.93±0.91a**	nd**	0.49±0.64b**	0.87±1.01a**	1.75±0.69a**	1.50±0.55b**	0.40±0.46c**	0.08±0.12d**	0.00±0.01d**
Nonanal	0.30±0.46a	0.37±0.33a	0.23±0.32a	0.49±0.72a	0.52±0.48a	0.70±0.78a*	0.34±0.29b*	0.24±0.32b*	0.24±0.38b*	0.39ab*
Hexanal	5.09±5.62a**	6.26±4.89a**	1.40±1.82c**	1.39±1.63c**	3.15±3.75b**	7.91±4.56a**	6.19±4.32b**	2.24±2.88c**	0.61±0.65d**	0.35±0.21d**
Heptanal	0.33±0.39a*	0.28±0.15ab*	0.15±0.24bc*	0.09±0.14c*	0.26±0.41ab*	0.47±0.28a**	0.44±0.38a**	0.14±0.14b**	0.06±0.13b**	nd**
Benzaldehyde	0.42±0.46a**	0.01±0.04c**	1.37±0.36bc**	0.30±0.12ab**	0.30±0.33ab**	0.47±0.51a**	0.14±0.15b**	0.15±0.12b**	0.16±0.30b**	0.23±0.31b**
Octanal	0.12±0.28ab**	0.17±0.17a**	0.06±0.13c**	0.01±0.05c**	0.13±0.26ab**	0.32±0.28a**	0.13±0.20b**	nd**	0.03±0.06c**	nd**
Ketones										
2-propanone (Aceton)	0.48±0.31b**	0.88±0.87b**	1.33±2.65b**	3.56±613a**	0.99±1.69b**	0.48±0.25b**	0.55±0.75b**	0.71±1.43b**	3.98±5.97a**	1.52±2.67b**
2-butanone	0.78±1.28b**	0.76±1.58b**	0.85±1.40b**	0.16±0.14b**	2.42±3.67a**	0.06±0.07c**	0.05±0.10c**	2.23±3.91a**	1.83±1.59a**	0.81±0.69b**
2-pentanone	15.71±18.60bc**	9.41±11.35c**	35.06±34.15a**	25.36±24.20ab**	17.89±21.15bc**	2.14±0.67c**	2.64±1.69c**	23.47±19.10b**	45.30±28.67a**	29.89±20.83b**
2-butanone, 3-hydroxy (Acetoin)	0.40±0.36a**	0.30±0.28b**	0.23±0.26b**	0.20±0.28b**	0.22±0.29b**	0.62±0.22a**	0.42±0.25b**	0.25±0.26c**	0.06±0.09d**	nd**
2-hexanone	0.77±1.05bc*	1.00±1.30abc*	1.57±1.79a*	1.32±1.50ab*	0.59±0.82c*	0.05±0.08c**	0.07±0.13c**	0.84±0.68b**	2.25±1.59a**	2.05±1.35a**
2-heptanone	32.76±40.00b**	25.53±36.40b**	67.67±60.79a**	55.58±57.16a**	22.86±28.42a**	2.03±0.48c**	2.64±0.69c**	32.67±28.68b**	81.03±51.22a**	86.03±41.87a**
2-octanone	0.42±0.61b**	0.63±0.86b**	nd**	1.63±2.51a**	0.25±0.35b**	0.02±0.05b**	0.01±0.05b**	0.15±0.16b**	1.29±1.95a**	1.46±1.77a**
2-nonanone	10.78±14.81b*	14.94±22.70b*	22.36±28.40ab*	34.80±61.34a*	5.69±8.91b*	0.11±0.14c**	0.27±0.13c**	4.50±3.49c**	32.56±39.79b**	51.12±45.40a**
2-undecanone	0.41±0.62b**	0.78±1.24b**	0.73±1.22b**	3.16±5.29a**	0.24±0.37b**	nd**	nd**	0.06±0.18b**	1.97±3.31a**	3.31±4.10a**
Alcohols										
Ethanol	30.30±39.86a	17.70±9,94b	17.41±9.49b	17.54±12.13b	20.59±28.98ab	31.78±11.95b**	44.59±35.33a**	14.66±9.21c**	4.94±5.66c**	7.57±12.44c**
Butyl alchol	0.36±0.67a*	0.07±0.10b*	0.16±0.26ab*	0.08±0.14b*	0.29±0.40a*	0.30±0.19b**	0.52±0.71a**	0.11±0.14bc**	0.03±0.11c**	nd**
2-pentanol	0.26±0.57bc*	0.09±0.21c*	0.74±1.21a*	0.65±1.07ab*	0.44±0.68abc*	nd**	nd**	0.04±0.11c**	0.75±1.08b**	1.39±0.95a**
1-pentanol	0.13±0.39b*	0.46±0.54a*	0.21±0.30b*	0.21±0.33b*	0.16±0.40b*	0.33±0.41ab**	0.32±0.48ab**	0.39±0.57a**	0.11±0.17bc**	0.03±0.07c**
2-butanol	0.39±0.61a**	0.39±0.34a**	0.23±0.44b**	0.20±0.34b**	0.05±0.11c**	0.48±0.44a**	0.48±0.62a**	0.31±0.26b**	nd**	nd**
Nonanol	0.04±0.08b**	nd**	0.32±0.54a**	0.05±0.17b**	0.01±0.05b**	nd*	nd*	0.05±0.10b*	0.25±0.42a*	0.13±0.42a*
1-hexanol	0.10±0.17b**	0.51±0.49a**	0.74±1.16a**	0.07±0.12b**	0.12±0.18b**	0.06±0.11b*	0.03±0.08b*	0.35±0.59ab*	0.48±0.68a*	0.62±0.96a*
2-heptanol	0.45±0.81b**	0.57±1.22b**	3.11±4.51a**	4.45±6.41a**	0.41±0.58b**	nd**	nd**	0.16±0.28c**	3.23±4.36b**	5.61±5.68a**
Acids										
Acetic acid	0.23±0.40b**	0.23±0.17b**	0.42±0.89b**	0.91±1.04a**	1.13±1.49a**	0.60±0.61b*	0.42±0.77b*	0.49±0.70b*	0.30±0.38b*	1.12±1.72a*
Butyric acid	0.64±0.49b**	0.66±0.38b**	0.53±0.34b**	1.65±2.23a**	0.56±0.24b**	0.84±0.34a	0.71±0.28a	0.58±0.13a	1.14±2.22a	0.76±1.08a
Esters										
Ethyl acetate	6.83±6.26a	7.39±5.31a	5.08±4.08a	6.66±5.97a	4.92±5.15a	11.49±4.02a**	10.28±4.02a**	6.41±3.51b**	1.41±1.71c**	1.30±2.37c**
Methyl butyrate	0.56±0.47abc**	0.33±0.29bc**	0.62±0.46ab**	0.78±0.72a**	0.28±0.28c**	0.56±0.28a	0.52±0.27a	0.34±0.14a	0.56±0.80a	0.58±0.67a
Isobutylacetate	1.41±0.87ab	2.17±0.74a	1.81±2.45ab	0.55±1.52b	1.82±1.93ab	1.06±1.39a	1.64±1.97a	1.55±1.94a	1.54±1.94a	1.97±1.08a
Ethyl butyrate	0.44±0.40b**	0.44±0.14b**	1.62±0.74a**	1.58±1.70a**	0.36±0.32b**	0.84±0.50ab	1.05±0.84ab	1.16±0.91a	0.46±0.54b	0.91±1.77ab

Table 1. *The mean peak area ratio of volatile compounds in the control and herby cheese samples during storage (continue)*
Tablo 1. *Kontrol ve Otlu peynir örneklerine ait uçucu bileşenlerin ortalama pik alanlarının depolama süresince değişimi (devam)*

Volatile Compounds	Herby Cheese Samples					Days of Storage				
	CC	CH	CK	CS	CM	1.	7.	14.	21.	28.
Hexanoic acid, ethyl ester	0.07±0.09b**	0.15±0.12b**	0.72±0.39a**	0.69±0.73a**	0.06±0.10b**	0.26±0.38a	0.27±0.31a	0.46±0.45a	0.34±0.36a	0.37±0.78a
Ethyl octanoate	nd**	0.04±0.09bc**	0.23±0.18a**	0.09±0.20b**	nd**	0.01±0.05c**	0.05±0.11bc**	0.10±0.21ab**	0.07±0.12bc**	0.13±0.20a**
Terpenes										
1R-.alpha.-Pinene	1.59±2.46bc**	9.34±6.49a**	4.32±7.91b**	0.52±0.36c**	1.23±1.32bc**	3.88±5.85a	4.89±6.23a	2.76±3.69a	3.66±8.40a	1.82±2.40a
beta-Pinene	0.27±0.36ab	0.29±0.26ab	0.39±0.76a	0.08±0.13b	0.28±0.22ab	0.23±0.26ab	0.41±0.34a	0.33±0.22a	0.31±0.76ab	0.05±0.10b
.beta.-Myrcene	0.06±1.21b**	0.38±0.19b**	13.07±8.57a**	nd**	0.12±0.21b**	1.00±1.79b*	3.30±6.54ab*	4.69±9.51a*	2.39±6.27ab*	2.24±5.77b*
.DELTA. 3-Carene	1.07±2.48b**	3.09±3.59a**	1.18±0.54b**	0.22±0.29b**	0.19±0.37b**	0.26±0.45b**	0.54±0.71b**	1.98±3.33a**	1.19±2.11a**	1.77±2.62a**
.alpha.-Phellandrene	0.05±0.14c**	0.31±0.32b**	1.49±0.81a**	0.01±0.02c**	0.16±0.47bc**	0.08±0.21b**	0.52±0.73a**	0.49±0.76a**	0.46±0.70a**	0.45±0.94a**
.beta.-Phellandrene	0.23±0.62c**	2.08±0.54a**	0.91±0.51b**	0.16±0.35c**	0.28±0.38c**	0.34±0.60c**	0.69±0.90b**	0.76±0.95b**	0.71±0.96b**	1.15±0.82a**
D-limonene	0.58±0.73c**	3.15±0.67a**	2.11±0.93b**	0.41±0.27c**	3.60±1.15a**	1.49±1.16b**	2.44±1.85a**	1.94±1.38ab**	1.96±1.42ab**	2.01±1.75ab**
Benzene, 1-methyl-2-(1-methylethyl)	2.82±3.70b**	5.20±1.94b**	48.44±16.95a**	2.86±3.76b**	1.81±0.83b**	6.77±10.52b**	12.52±17.58a**	15.60±24.46a**	13.73±21.75a**	12.52±23.34a**
gamma-Terpinene	0.11±0.20a	0.06±0.07ab	nd	nd	0.06±0.16ab	nd	0.08±0.22a	0.00±0.01a	0.07±0.12a	0.07±0.10a
Eucalyptol	nd**	nd**	0.48±0.39a**	nd**	nd**	0.17±0.36a**	0.15±0.34a**	0.11±0.24ab**	0.05±0.19bc**	nd**
Linalool	nd**	nd**	0.38±0.31a**	nd**	nd**	0.07±0.16a	0.09±0.19a	0.14±0.33a	0.04±0.15a	0.04±0.16a
Aliphatic Hydrocarbons										
Hexane	27.87±35.40a**	11.47±13.99bc**	7.50±9.29c**	7.85±9.76c**	18.26±25.00b**	7.16±6.67b**	6.82±4.23b**	45.97±32.28a**	8.98±11.36b**	4.07±2.62b**
n-Octane	0.31±0.22b**	0.58±0.39a**	0.16±0.20c**	0.06±0.12d**	0.21±0.19c**	0.42±0.22ab**	0.44±0.35a**	0.33±0.33b**	0.05±0.14c**	0.09±0.14c**
Heptane	10.26±10.95a	6.99±8.29b	7.35±9.22b	8.83±10.84ab	7.94±9.10ab	18.41±5.73a**	19.29±6.28a**	2.68±2.21b**	0.65±0.69b**	0.33±0.39b**
Undecane	0.34±0.66a	0.22±0.39ab	0.13±0.19ab	0.22±0.47ab	0.07±0.11b	0.41±0.75a**	0.22±0.23ab**	0.26±0.40ab**	0.03±0.10b**	0.06±0.12b**
Dodecane	0.32±0.49a	0.44±0.53a	0.37±0.31a	0.24±0.31a	0.26±0.37a	0.47±0.52a**	0.43±0.43a**	0.36±0.45a**	0.30±0.26a**	0.06±0.18b**
Decane	0.08±0.15a	0.10±0.24a	0.13±0.13a	0.12±0.19a	0.09±0.07a	0.19±0.21a**	0.16±0.19ab**	0.07±0.17bc**	0.07±0.11bc**	0.04±0.07c**
n-Dodecane	0.07±0.13ab	0.08±0.23ab	nd	0.21±0.46a	0.13±0.33ab	0.31±0.48a	0.14±0.33b	0.04±0.10b	nd	0.01±0.02b
Tridecane (Eicosane)	0.73±0.72ab	0.86±0.61ab	1.07±0.44a	0.97±0.74ab	0.60±0.36b	1.13±0.68a**	0.81±0.46ab**	0.80±0.64ab**	0.89±0.72ab**	0.58±0.39b**
2-Decanone	0.06±0.12a	0.09±0.16a	nd	11.70±42.97a	0.03±0.08a	nd	nd	nd	0.39±0.70a	11.49±43.03a
Pentadecane	0.33±0.58cd**	0.69±0.70bc**	1.19±0.55a**	1.03±1.32ab**	0.25±0.37d**	0.32±0.60b**	0.35±0.53b**	0.45±0.71b**	1.25±0.98a**	1.12±0.88a**
Aromatic Hydrocarbons										
Toluene	6.64±5.42a	4.82±1.85a	6.50±4.51a	4.19±1.52a	5.10±1.93a	5.82±4.33a	6.17±5.27a	6.60±1.95a	4.68±2.56a	3.97±1.45a
Ethylbenzene	0.28±0.56a	0.14±0.16a	0.38±0.45a	0.11±0.13a	0.24±0.51a	0.38±0.42a**	0.51±0.66a**	0.10±0.14b**	0.12±0.22b**	0.04±0.11b**
Benzene, 1,2-dimethyl o-Xylene)	0.04±0.08b**	0.05±0.13b**	0.03±0.06b**	0.05±0.09b**	0.14±0.11a**	0.02±0.09bc**	nd**	0.08±0.14b**	0.08±0.10b**	0.14±0.08a**
p-Xylene	0.95±1.60a	0.60±0.44a	1.09±1.32a	0.46±0.23a	0.93±1.66a	1.07±1.30ab*	1.62±2.07a*	0.62±0.39b*	0.43±0.41b*	0.27±0.18b*
Styrene	0.77±1.46a	0.69±0.46a	1.14±1.21a	0.55±0.18a	1.15±1.84a	0.81±1.23b*	1.64±2.15a*	0.71±0.54b*	0.67±0.49b*	0.47±0.15b*
Furans										
Benzofuran	0.04±0.10b**	3.37±1.22a**	0.20±0.29b**	0.02±0.04b**	0.09±0.11b**	0.52±1.01b**	1.15±2.01a**	0.70±1.33b**	0.69±1.45b**	0.65±1.32b**

Mean values ± standard deviations of Conrol and Herby cheeses manufacturing with duplicate samples. The letters a, b, c and d indicate means that significantly differ at P<0.01 and P<0.05. nd, not detected

results could be due to the microbial loads of the herbs added to the cheeses [11]. The highest mean percentage value of butyric acid was found in CS and the lowest mean value was in CK. Butyric acid concentrations of samples shown to be statistically different (P<0.01) to each other, although the storage period affected the butyric acid percentages and were found to be statistically insignificant (P>0.05) *(Table 1)*. This could be explained by the presence of diversified flora in the herbs used. Similar results were reported by Foda et al.[14].

Ethyl acetate was the principal and most abundant volatile aromatic compound produced during the storage of herby cheeses. The highest mean percentage value of ethyl acetate was found in CH and the lowest mean percentage value was in CM. Differences among the samples were found to be insignificant (P>0.05) statistically *(Table 1)*. However, ethylacetates percentage values showed a regular decrease during storage and the storage period's effect on this compound was found to be statistically significant. Isobutylacetate was the second highest ester found in experimental herby samples. It had the highest percentage in CH as with the ethylacetate percentage, although the lowest mean percentage value was detected in CS. Both the herb and storage period effect on the isobutylacetate percentage were found to be statistically insignificant (P>0.05) *(Table 1)*.

11 terpenes were identified in the control and herby cheeses during the storage period. Among the terpenes, 1R-.alpha.-pinene and benzene, 1-methyl-2-(1-methylethyl) were the most abundant compounds in the experimental cheeses. The highest mean percentage value of 1R-.alpha.-Pinene was found in CH and the lowest mean percentage value was in CS. Differences among the samples were significant (P<0.01) statistically, although the effect of the storage period was found to be insignificant (P>0.05) *(Table 1)*. This might be explained by the properties of the herbs used. The highest mean value of benzene, 1-methyl-2-(1-methylethyl) was found in CK and the lowest mean value was in CM. Differences between the samples and the storage period were found to be statistically significant (P<0.01) *(Table 1)*. The differences of terpenes in experimental cheeses can be probably derived from the pasture and herbs used in cheese production [15].

Hexane was the most abundant aliphatic hydrocarbon among the experimental cheeses during the storage period. The highest mean percentage value was found in CC, while the lowest mean value was found in CK. Differences among the samples and the storage period were found to be significant (P<0.01) statistically *(Table 1)*.

The main aromatic hydrocarbon was the toluene in herby cheese samples. Similarly, toluene had already been identified at high levels in Feta-type [13] cheese. The highest mean percentage value of toluene was determined in CC, while the lowest mean percentage was detected in CS. Differences between the control, herby samples and the storage period were found to be statistically insignificant (P>0.05). Conversely, toluene values generally showed an irregular change during the storage period *(Table 1)*. These results could be explained by the sample differences.

In this research study, 1 furan compound was only determined in cheeses during storage time. Benzofuran was found in all samples. This compound reached the highest mean percentage in CH, while the lowest mean percentage was determined in CS cheese. Differences among the samples and the storage period were found to be significant (P<0.01) statistically *(Table 1)*. Differences of furans in experimental cheeses can be derived from the herbs used in cheese production.

This study clearly indicates that the different herbs used in cheese production affected the volatile composition of the samples. The SPME GC/MS technique was used to analyse the aroma compounds in the experimental cheeses. During storage more than 60 volatile components were detected in all cheeses. At the same time, it was observed that the changes of ratios of compounds during storage were different. According to the results obtained, acetaldehyde, hexanal, ethanol, ethylacetate, 2-pentanone, 2-heptanone, 2-nonanone, hexane, heptane and toluene were considered to be the major compounds of experimental cheeses and they were detected in all samples during storage. These compounds showed differences among the samples due to the herbs used.

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SHORT COMMUNICATION

Seroprevalence of Brucellosis in Horses in Kars and Ardahan Provinces of Turkey Where Ruminant Brucellosis is Endemic and Prevalent

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Summary

The prevalence of brucellosis was investigated in horses in Kars and Ardahan Districts of Turkey between 2008-2010. In order to achieve this, a total of 361 horse serum samples from 23 different villages were examined for Brucella antibodies by Rose Bengal Plate Test and Serum Agglutination Test. Of the 361 sera obtained from horses, 48 (13.29%) were determined positive by RBPT and 52 (14.40%) by SAT. The positive titers varied between 1/40 and 1/320 for brucellosis by SAT. Among the positive samples, the titers were 1/40 in 24, 1/80 in 6, 1/160 in 18 and 1/320 in 4 samples.

Keywords: *Brucellosis, Horse, Seroprevalence, RBPT, SAT*

Ruminant Brusellozisinin Endemik ve Yaygın Olduğu Kars ve Ardahan Yörelerinde Atlarda Brusellozisin Seroprevalansı

Özet

Kars ve Ardahan yörelerinde 2008 ile 2010 yılları arasında atlarda brusellozisin seroprevalansının araştırılmasının amaçlandığı bu çalışmada, 23 farklı yerleşkeden elde edilen toplam 361 at serum örneği Rose Bengal Plate Test (RBPT) ve Serum Aglütinasyon Testi (SAT) ile incelendi. İncelenen 361 at serum örneğinin 48 (%13.29)' i RBPT ve 52 (%14.40)' si SAT ile pozitif saptandı. SAT ile pozitif saptanan serum örneklerinde antikor titrelerinin 1/40 ile 1/320 arasında değişkenlik gösterdiği (24 örnek 1/40, 6 örnek 1/80, 18 örnek 1/160 ve 4 örnek 1/320 olarak) belirlendi.

Anahtar sözcükler: *Brusellozis, At, Seroprevalans, RBPT, SAT*

INTRODUCTION

Brucellosis is an important zoonotic disease worldwide and endemic in many regions of Turkey. Although several control and eradication measures have been established, the disease continues to produce large economic losses especially in cattle and small ruminants and cause a serious public health problem in Turkey [1]. Naturally acquired Brucella infection in horses associated with infected cattle *(B. abortus)* and swine *(B. suis)* horizontal transfer has been demonstrated [2]. Brucellosis is uncommon and generally asymptomatic in horses. But in some cases it is associated with diseases, inflammation of the supraspinal bursa (fistulous withers) and atlantal bursa (poll evil), and also tenosynovitis, osteomyelitis or osteoarthritis and, rarely, abortion and infertility [3-5]. The horses infected with *B. abortus,* even they do not show signs of illness, they excrete the organism in purulent discharges, milk and urine [4]. So that horse brucellosis may be a potential source of infection in human beings and other animals.

National Control and Eradication Programme for Brucellosis in domestic animals run by the Ministry of Food, Agriculture and Animal Livestock has been implemented in Turkey since 1984. However, the prevalence of brucellosis still ranges from region to region throughout Turkey. Kars

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and Ardahan regions have about 650.000 cattle, 230.000 sheep, 17.000 goats and 12.000 horses population mainly raised on family-operated farms and human population in these regions is 406.000 and 75% of them deal with animal husbandry. It can be expressed that almost every farmer has also a horse kept together with the farm animals in these regions used especially for purposes of harvesting and transporting of crops in July and August. In addition, they are commonly used in racing games and for other recreational purposes for the rest of the year during which no agricultural activities are carried out [6]. The prevalence of brucellosis in cattle, Turkey was investigated previously and the most prevalent figures with 20.8% and 7.9% [7] were reported from Kars and Ardahan Provinces, respectively that located in the Northeast Anatolia Region of Turkey. However, no research has been conducted in horses from these two regions.

In this study, we aimed to determine seroprevalence of brucellosis in horses in the Kars and Ardahan district by RBPT and SAT, where ruminant brusellosis is endemic and prevalent.

MATERIAL and METHODS

Horse Serum Samples

This study was carried out between January 2008 and January 2010. A total of 361 horse serum samples from 23 different villages were evaluated for brucellosis in Kars and Ardahan Provinces in Northeast Anatolia Region of Turkey *(Fig. 1)*. The animals were treated according to the Animal Care and Use Regulation (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purpose). Horses are traditionally raised together with cattle, sheep and other animals in the same stable in these provinces. Native breed horses investigated in this study aged between 3-8 years and approximately 10 ml of blood samples from each horses were taken and transported to the Laboratory of Microbiology Department, Faculty of Veterinary Medicine, Kafkas University. The serum samples were kept at -20°C until tested.

Serological Detections of Brucella Antibodies

Rose Bengal Plate Test (RBPT) and Serum Agglutination Test (SAT) were used to detect Brucella antibodies for all serum samples. *Brucella abortus* antigen used in the study for both RBPT and SAT was obtained from Pendik Veterinary Control and Research Institute, Istanbul. The RBPT and SAT were carried out according to the method described by Alton et al.[8]. In RBPT, 25 µl of antigen was mixed with an equal volume of horse serum on a clean glass slide and examined for agglutination after 4 min. In SAT, 0.5 ml of Brucella SAT antigen was added to 0.5 ml of each serum sample serially diluted in 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320. The mixture was incubated at 37ºC overnight. The agglutination ++ and stronger, observed in sera at dilution 1:40 and higher, was considered to be positive [3].

RESULTS

Of the 361 sera obtained from horses, 48 (13.29%) were determined positive by RBPT and 52 (14.40%) by SAT. The positive titers varied between 1/40 and 1/320 for brucellosis by SAT. Among the positive samples, the titers were 1/40 in 24, 1/80 in 6, 1/160 in 18 and 1/320 in 4 samples. Number and results of the distribution of the samples according to regions are given in *Table 1*.

DISCUSSION

Brucellosis is prevalent in some middle-eastern countries such as Iran, Iraq, Saudi Arabia, Egypt, Syria, Pakistan and

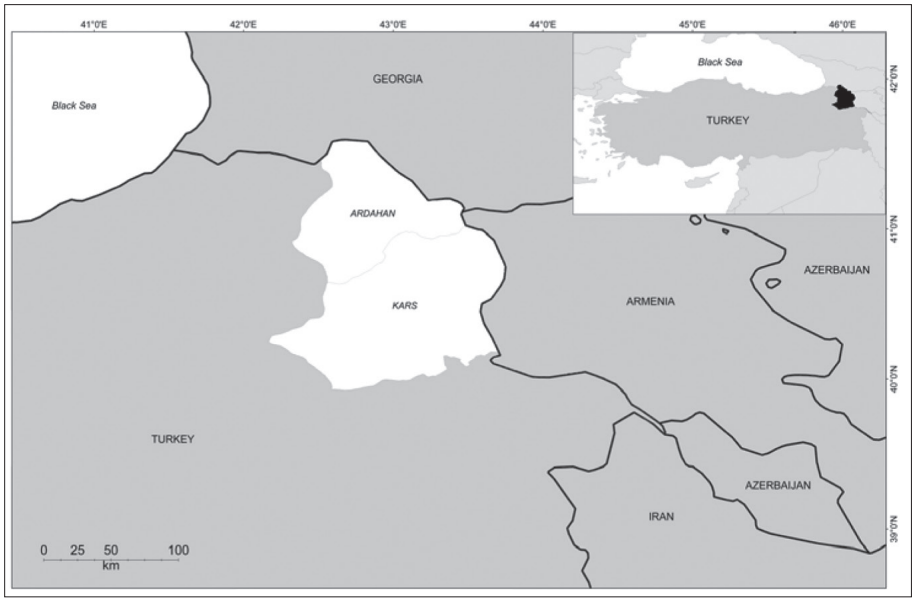

Fig 1. Map of Turkey and geographical location of Kars and Ardahan Districts

Şekil 1. Türkiye haritası ve Kars ile Ardahan illerinin coğrafi konumu

Table 1. *Provinces, number and results of the horse serum samples analyzed by RBPT and SAT*
Tablo 1. *At serum örneklerinin RBPT ve SAT sonuçları*

Province	Number of Samples	Assay and Numbers of Positive and Negative Serum Samples							
		RBPT				SAT			
		Positive		Negative		Positive		Negative	
		n	%	n	%	n	%	n	%
Kars	225	31	13.77	194	86.23	33	14.66	92	85.33
Ardahan	136	17	12.50	119	87.50	19	13.97	117	86.02
Total	361	48	13.29	313	86.71	52	14.40	309	85.60

some south-eastern European countries. Turkey borders with several of these countries and is situated within this geography. Therefore, Turkey lies within the risky area between Middle East and Europe.

Turkey has a relatively large area and is divided into seven geographical regions. Various prevalence rates of brucellosis have been reported for human, cattle and sheep population from different parts of Turkey. However, there are few researches conducted for the determination of the prevalence of brucellosis in horses. In a study from Hakkari region, Eastern part of Turkey, 9.5% Brucella seropositivity was reported for horses with RBPT and presence of anti *B. abortus* antibodies was confirmed by SAT [9]. Solmaz et al.[10] reported a seroprevalance of 60.59% (123/203) in horses raised in Van province of East Turkey by RBPT and out of these 123 positive serum samples 98 (79.67%) showed 1/40 or higher titers by SAT. The result of another study on horse brucellosis in Southeast Turkey was 13.68 and 0.51% by RBPT and SAT, respectively [11]. If we take a look at the situation in neighbouring countries; in a study conducted in North-East of Iran, the seroprevalence of brucellosis was determined as 2.5% in horses by RBPT and SAT [12]. In another study seroprevalence of brucellosis on 393 sera of horses in Iraq has been found to be 16.28% by RBPT but nine of them were found positive by SAT [13]. Wadood et al.[14] determined the disease's seroprevalence in horses in and around Fisalabad- Pakistan, as 20.7 and 17.7% by RBPT and SAT, respectively. In another study, Abo-Shehada [15] determined the corresponding figures for horses and donkeys in Jordan as 1% and 8.5%, respectively.

In the current study, the seroprevalence of brucellosis was determined to be 48 (13.29%) by RBPT and 52 (14.40%) by SAT in 361 horse serum samples. The results of our study and the studies mentioned above show that horse brucellosis is very common in both the some regions of Turkey and the Middle East which is also a common case for other animal species like cattle and sheep. In previous studies performed in our laboratory in the same region, the seroprevalence of brucellosis was determined to be 32.92% by RBPT and 34.64% by SAT in 407 serum samples of cattle collected from the herds with a history of abortions and 32 (13%), 35 (14.22%) and 44 (17.88%) of the farmers' sera were found positive for brucellosis by RBPT, SAT and ELISA, respectively [1]. In addition, the culture isolation of the organism supported these serological findings. As the previous results in the region from our laboratory, Sahin et al.[16] isolated *B. abortus* from 48 (32.21%) of 149 lung samples and stomach contents of the aborted cattle fetuses. Therefore, these findings in horses were not surprising. Since no clinical or microbiological examination was performed on the horses, the ratio of active infection on seropositive animals cannot be determined. However there are several reports about isolation of *B. abortus* from infected horses [17,18]. The results of this study show that there is a need for further clinical and microbiological studies on horse brucellosis and a possible clonal relationship among different *B. abortus* isolates from these two regions should be verified by molecular typing methods. Nonetheless, when considering the possibility of risk for Brucella transmission from horses to humans (especially horse riders, caretakers, farmers and veterinarians) as well as to other animals, horses should be avoided to raise together with other farm species. These issues are suggested to be taken into account for determining strategies for Brucella control and eradication programs.

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CASE REPORT

T Cell Lymphoma in An Akkaraman Sheep

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Summary

Lympocytic T cell lymphoma was detected in liver and kidneys of a 3 year old Akkaraman sheep which was submitted to the pathology department for diagnosis. Macroscopically, nodular formations with white colour and about 1 cm size were observed in the cortex of both kidneys. The irregular nodules were observed in the liver, similar to the lesions in the kidneys. Microscopically, the nodular lesions in the liver and kidneys were consisted of uniform lymphocytic accumulations. These lymphocytes were similar to the mature lymphocytes although they were bigger and had less chromatin. Immunohistochemically, tumor cells had positivity with CD3, but negativity with CD20 and CD79a. Thus, the tumor was described as type of lymphocytic T cell lymphoma.

Keywords: *Akkaraman sheep, Immunohistochemistry, Lymphoma*

Bir Akkaraman Koyunda T Hücreli Lenfoma

Özet

Teşhis amacıyla Patoloji Anabilim dalına karaciğer ve böbrekleri getirilen 3 yaşında Akkaraman ırkı bir koyunda lenfositik tipte T hücreli lenfoma olgusu tanımlandı. Makroskobik olarak, her iki böbreğin korteksinde, beyaz renkli, yaklaşık 1 cm çapında, nodüler formasyonlar saptandı. Karaciğerde de böbreklerdeki lezyonlara benzeyen düzensiz nodüller mevcuttu. Mikroskobik olarak; karaciğer ve böbrekteki nodüler lezyonların, üniform yapıda lenfosit birikimlerinden oluştuğu gözlendi. Olgun lenfositlere benzeyen ancak boyut olarak daha büyük ve daha az kromatin içeren bu hücreler lenfositik tipte lenfoma hücreleri olarak değerlendirildi. İmmunohistokimyasal olarak tümör hücreleri, CD3 ile pozitif, CD20 ve CD79a ile negatifti. Bundan dolayı, tümör T hücreli lenfositik tipte lenfoma olarak tanımlandı.

Anahtar sözcükler: *Akkaraman koyun, Immunohistokimya, Lenfoma*

INTRODUCTION

Lymphoma is a rare neoplastic condition in sheep [1-3]. Although many cases have been reported in older sheeps, lymphoma was also identified in lambs [4]. No predisposition has been found in sheep in terms of breed and gender. However, the number of affected female cases has been reported to be higher among adult animals [1]. Both enzootic and sporadic forms of lymphoma occur in sheep [5]. Retrovirus infection has been suggested to be a cause of enzootic lymphoma in sheep, and bovine leukemia virus (BLV) can be experimentally transmitted to sheep and ovine lymphoma has been reported to develop within 3 years following the inoculation of BLV [6]. Retroviral etiology has also been documented epidemiologically and virologically in spontaneous lymphoma cases in sheep [7].

The distribution of lesions in sheep is usually similar to cattle. Ovine lymphoma has several anatomic forms including primarily the multicentric type, the alimentary form and, rarely thymic and skin forms [8]. Multicentric form results in skin form in rare cases [5]. Although the most constant feature of ovine lymphoma is the enlargement of affected lymph nodes and progressive spread of neoplastic tissue to other lymphoid and non-lymphoid organs, enlargement of peripheral lymph nodes may not be observed in some cases. Organs next to the lymph nodes such as spleen, liver, kidney, digestive tract, skeletal muscles and heart are commonly affected in sheep [1]. While the liver and the spleen are the most commonly affected organs in spontaneous lymphoma cases in sheep [9], common involvement of the

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uterus, heart and abomasum has been reported in experimental infections [10]. Sometimes the only macroscopical lesions are detected in kidney associated with ovine lymphoma [7].

Focal or generalized diffuse accumulation of neoplastic lymphocytic cells are generally observed microscopic changes in affected organs [1,6,7]. The most common observed lesion of lymphoma is the existence of undifferentiated neoplastic cells in other animal species, but well differentiated lymphoid cells are reported to be dominant in sheep [1].

This is the first report about the diagnosis of T cell lymphoma with the aid of immunophenotypic methods in an Akkaraman sheep in Turkey.

CASE HISTORY

The study material was composed of the liver and the kidneys of a 3 year old Akkaraman sheep which was submitted to the pathology department for diagnosis. Following examination of the organs, tissue samples obtained from the liver and the kidneys were fixed in 10% neutral buffered formalin, embedded in paraffin, and cross sections were stained with hematoxylin and eosin (HE). Liver and kidney sections were immunohistochemically stained with the avidin-biotin-peroxidase complex (ABC-P) for CD3, CD20 and CD79a monoclonal antibodies. Rabbit anti-human CD3 (Early T cell marker) (Thermo Scientific, Cat No: RM-9107-S0), mouse anti-human CD20 (Dako Corp, Carpinteria, CA, Cat No: M7254, IR604) and mouse anti-human CD79a/mb1 B cell marker (Thermo Scientific, Cat No: MS-1171-P0) kits were used. A mediastinal lymh node from a sheep with ovine pulmonary adenomatosis served as a positive control for all antibodies, and nonimmune serum was used as a negative control. The primary antibodies were diluted to 1:50. The immune complexes were stained with diaminobenzidine tetrahydrochloride (DAB) and counterstained with Mayer's hematoxylin (M-H).

Macroscopically, multiple nodular formations with yellow-white colour and about 1 cm size were observed in the cortex of both kidneys *(Fig. 1A)*. Evaluation of the cross section surface in the kidneys revealed thinning of the medulla and the enlargement of the pelvis *(Fig. 1B)*. The liver was observed to be enlarged, pale and fatty; capsular fibrosis and nodules with irregular size were detected on both surfaces *(Fig. 2A)*. In addition, prominent enlargement was observed in the hilar lymph node of the liver *(Fig. 2B)*.

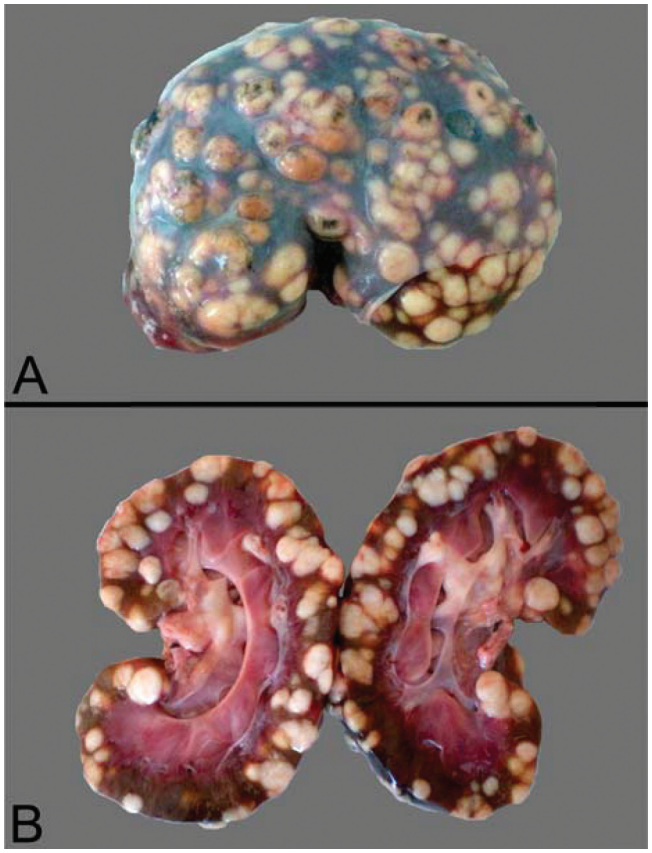

Fig 1. A- Nodular formations in kidney kortex, **B-** Macroscopical appearance sagittal section of kidney

Şekil 1. A- Böbrek korteksinde nodül formasyonları, **B-** Böbreğin kesit yüzünün görünümü

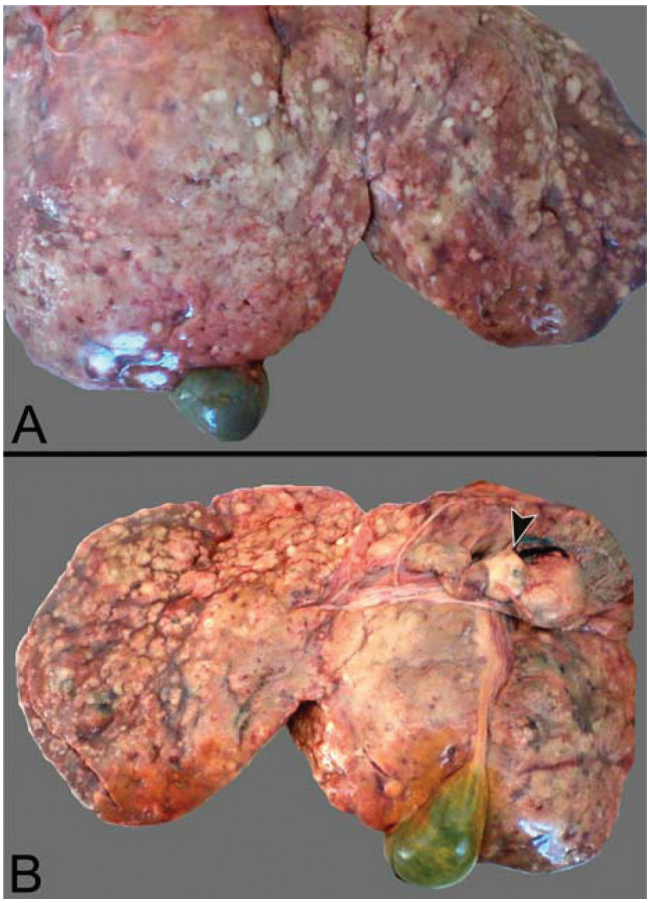

Fig 2. A- Nodular formations and capsular fibrosis on diaphragmatic surface of liver, **B-** Pale and fatty appearance on visseral surface of liver with enlargement hilar lymph node *(arrow head)*

Şekil 2. A- Karaciğerin diafragmatik yüzünde kapsülar fibrozis ve nodül formasyonları, **B-** Karaciğerin visseral yüzünün solgun ve yağlı görünümü ile birlikte hilar lenf düğümünde büyüme *(ok başı)*

Microscopically, the nodules in the liver and the kidneys were observed to be composed of neoplastic lymphocyte accumulations, similar to uniform and mature lymphocytes though they were bigger and had less chromatin. Lymphocyte accumulations were seen in the overall liver parenchyma, while the infiltration was especially more severe in the portal area *(Fig. 3A)*. Due to the neoplastic cell infiltrations, the normal liver structure was completely destroyed in the midzonal and periacinar regions and small islands of hepatocytes were seen between the affected areas. Although intrasinusoidal accumulation was observed in the liver, no intravascular neoplastic cells were

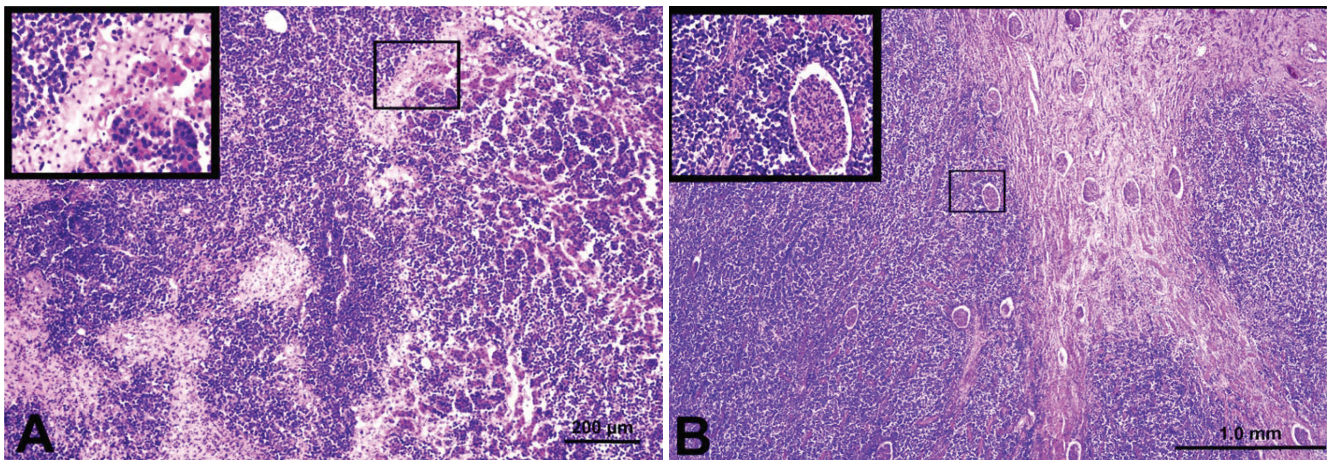

Fig 3. A- Diffuse lymphocytic infiltration in liver parenchyma, H-Ex10 **B-** Diffuse infiltration in intersititium of kidney and remains of destroyed tubules, H-Ex4

Şekil 3. A- Karaciğer parankiminde diffüz lenfositik infiltrasyon, H-Ex10, **B-** Böbrek intersitisyumunda diffüz infiltrasyon ve yıkımlanmış tubulus kalıntıları, H-Ex4

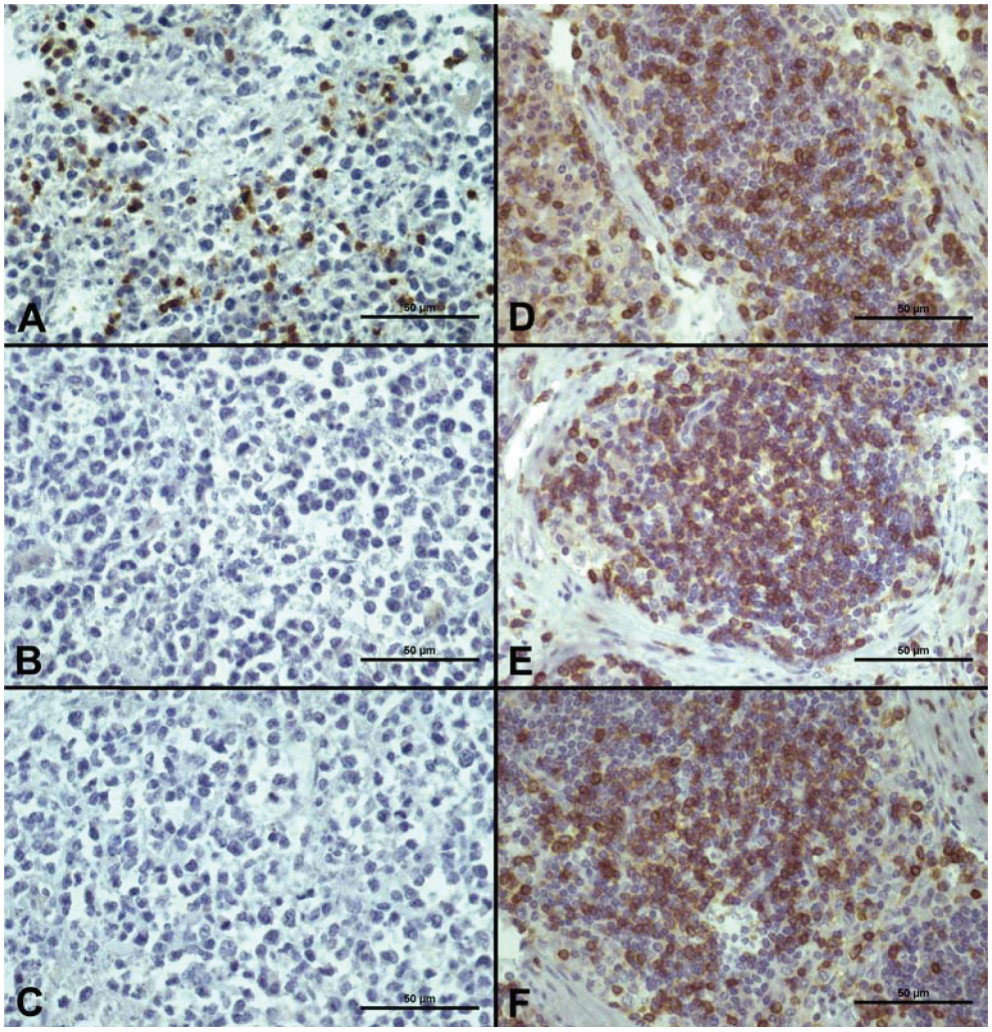

Fig 4. A- CD3 positivity in neoplastic lymphocytes, M-H, liver, ABC-Px40, **B-** CD20 negative reaction in lymphoma cells, M-H, liver, ABC-Px40, **C-** CD79a negative reaction in neoplastic cells, M-H, liver, ABC-Px40, **D-** CD3 positivity in T cell lymphocytes, M-H, lymph node, ABC-Px40, **E-** CD20 positivity in B cell lymphocytes and follicular dentritic cells, M-H, lymph node, ABC-Px40, **F-** CD79a positivity in B cell lymphocytes, M-H, lymph node, ABC-Px40

Şekil 4. A- Neoplastik lenfositlerde CD3 pozitifliği, M-H, karaciğer, ABC Px40, **B-** Neoplastik lenfositlerde CD20 negatifliği, M-H, karaciğer, ABC Px40, **C-** Neoplastik hücrelerde CD79a negatifliği, M-H, karaciğer, ABC-Px40, **D-** T lenfositlerde CD3 pozitifliği, M-H, lenf düğümü, ABC-Px40, **E-** B lenfositleri ve foliküler dentritik hücrelerde CD20 pozitifliği, M-H, lenf düğümü, ABC-Px40, **F-** B lenfositlerinde CD79a pozitifliği, M-H, lenf düğümü, ABC-Px40

detected. In the kidney, progressive lymphocytic infiltration of cortical interstitial tissues led to the eventual destruction of the affected region *(Fig. 3B)*.

Immunohistochemical staining showed that the tumor cells had positive results with CD3, a specific marker for T lymphocytes *(Fig. 4A)*, and had negative results with CD20 *(Fig. 4B)* and CD79a *(Fig. 4C)*, a specific marker for B lymphocytes. The antibodies employed accurately identified B, T, follicular dentritic cells in the control sheep lymph node *(Fig. 4 D,E,F)*.

DISCUSSION

Although the anatomical classification differs in sporadic or enzootic cases in sheep, macroscopic changes are mainly observed in the liver and kidneys [1,6,7]. In the multicentric form, varying numbers of enlarged profound and superficial lymph nodes are observed, accompanied by alterations in the spleen, liver, kidneys, lungs, heart, digestive tract and bone marrow [6]. In the alimentary form, together with the intestines, Peyer's patches, mesenteric lymph nodes and the liver are mainly affected [1,6]. Only the thymus is affected in some cases of thymic form, but in most cases, enlarged mediastinal lymph nodes, spreading to the internal organs are observed [6]. Because the material comprised only two organs in the presented case no comments could be made about the form of lymphoma.

Intravascular neoplastic cells, characteristic finding of leukemia [5], were determined in the liver and spleen in sheep with lymphoma in sporadic cases [11]. It has been reported that although leukemia and bone marrow were not involved in ovine lymphoma in a study [8], but chronic lymphocytic leukemia and bone marrow involvement were described in sheep in another study [10]. Development of persistent lymphocytosis was reported in sheep infected by BLV [4]. Furthermore, leukemia was reported to develop following the late stages of solid lymphoma [5]. In the present case, macroscopic and microscopic findings in the liver and the kidneys show similarity to previous studies; however, intravascular neoplastic cell groups associated with leukemia were not found.

Lymphomas are histologically classified as lymphoblastoid, prolymphocytic, lymphocytic, histiocytic or reticular types [5,6]. The most common type in sheep has been reported as the lymphoblastoid type [7]. Based on atypical mononuclear cells found in the liver and the kidneys in our case exhibiting similar characteristics to mature lymphocytes, the case was regarded as lymphocytic lymphoma.

Lymphoma originates from T and B lymphocytes and histiocytes, which comprise the immune system. Differentiation of these cells is made by revealing the phenotypic and molecular characteristics of the tumor cells [8]. Immunophenotypic and haematological studies indicate that enzootic bovine lymphoma are mostly B cell origin [12,13]. Immunophenotypic studies in sheep show that lymphoma is the B and T cell origin; however, B cell lineage was identified in most cases [5]. In addition, alimentary form lymphomas are usually originated from B cell [14]. A lymphoma case in a sheep was examined by immunohistochemical staining with CD79a, CD20, CD3, CD68 kits and, the only positivity was determined with CD79a, a diagnostic marker of B cell leukemia [11]. In the present case, the case was regarded as T cell lymphoma due to the neoplastic cells showed positivity with CD3, but negativity with CD20 and CD 79a.

In conclusion, lymphocytic T cell lymphoma was described by histological and immunophenotypical examination for the first time in the Akkaraman sheep in Turkey. It is therefore believed that this finding may provide significant contribution to the literature in this area.

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ERRATUM

The Effects of Hen Age, Genotype, Period and Temperature of Storage on Egg Quality (Tavuk Yaşı, Genotip, Depolama Süresi ve Sıcaklığının Yumurta Kalitesine Etkisi)

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Makale Kodu (Article Code): 2009/041-A

Formula present in the paragraph 5 in the Material and Methods section page 518 in Volume 15, Number 4, Year 2009 were written erroneously. Corrected formula was given below.

Dergimizin 2009 Yılı, 15. Cilt, 4. sayısının, 517-524. sayfalarında basılan makalenin 518. sayfasındaki MATERIAL and METHODS bölümünün 5. paragrafındaki formüller hatalı basılmıştır. Düzeltilmiş formüller aşağıda yeniden verilmiştir.

Wrong Formula/Hatalı Formül

Haugh units = [HU = 100 log (H-1.7$W^{0.37}$ + 7.57)] [18]

Albumen index = yolk height/(long diameter of albumen + short diameter of albumen/2) x100 [16]

Yolk index = yolk height/yolk width x 100 [32]

Shell density = shell weight/3.9782 x egg weigh$W^{0.662}$ x shell thickness [33]

Shell weight per unit surface = shell weight/4.835 W3.9782 x egg $weight^{0.7056}$ [34]

Corrected Formula/Düzeltilmiş Formüller

Haugh units = [100 log (H-1.7$W^{0.37}$ + 7.57)] [18]

Albumen index = Albumen height/albumen width [16]

Yolk index = Yolk height/yolk width [32]

Shell density = Shell weight/(3.9782 x $egg\ weight^{0.7056}$) x shell thickness [33]

Shell weight per unit surface = Shell weight/(3.9782 x $egg\ weight^{0.7056}$) [34]

In addition, there are some mistakes in *Table 1, Table 2* and *Table 3* on pages 519, 520 and 521 of the same article. Corrected tables were also given below.

We sincerely apologize for the mistake.

Ayrıca, aynı makalenin 519, 520 ve 521. sayfalarında yer alan *Tablo 1, Tablo 2* ve *Tablo 3*'teki verilerin bir kısmı hata içermektedir. Düzeltilen tablolar aşağıda yeniden verilmiştir.

Yanlışlıklardan dolayı özür dileriz.

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Table 1. *Main and interactive effects on egg weight and weight loss*
Tablo1. *Yumurta ağırlığı ve ağırlık kaybına ana ve interaktif faktörlerin etkisi*

Treatments				n	Egg Weight (g)		Weight Loss (g)
Genotype	**Hen Age (Weeks)**	**Storage Temperature**	**Storage Period (Weeks)**		**Fresh Egg**	**Stored Egg**	
μ					64.22	62.91	1.31
Lohman White				1687	64.00 [b]	62.73 [b]	1.28
Isa Brown				1715	64.45 [a]	63.09 [a]	1.35
	33			1762	62.37 [b]	61.11 [b]	1.26 [b]
	64			1640	66.07 [a]	64.70 [a]	1.37 [a]
		4ºC		2364	64.57	63.82 [a]	0.75 [b]
		24ºC		1038	63.88	61.99 [b]	1.89 [a]
			0	878	64.03	64.38 [a]	-
			1	987	64.60	63.83 [ab]	0.78 [c]
			3	891	64.57	62.92 [ab]	1.66 [b]
			5	646	63.69	60.84 [b]	2.84 [a]
ANOVA					**Probability**		
Genotype (G)					*	*	-
Hen age(HA)					**	*	*
Storage temperature (ST)					-	*	**
Storage period (SP)					-	**	**
GxHA					*	*	*
GxST					-	-	-
G x SP					-	*	-
HA x ST					-	-	-
HA x SP					-	-	*
ST x SP					-	*	**
SEM					0.30	0.26	0.08
R^2					0.16	0.16	0.21

[a-b] Means in a column and treatment variable with no common superscript differ significantly (P<0.05)

Table 2. *Main and interactive effects on internal egg quality traits*
Tablo 2. *Yumurta iç kalite özelliklerine ana ve interaktif faktörlerin etkisi*

Treatments				n	Albumen			Yolk			Haugh Unit
Genotype	Hen Age (Weeks)	Storage Temperature	Storage Period (Weeks)		Width (mm)	Height (mm)	İndex	Width (mm)	Height (mm)	Index	
µ					87.97	3.89	0.05	36.00	17.55	0.49	50.00
Lohman White				1687	82.69^{b}	4.08^{a}	0.05^{a}	36.53^{a}	17.59	0.48^{b}	51.96^{a}
Isa Brown				1715	93.25^{a}	3.70^{b}	0.04^{b}	35.18^{b}	17.53	0.50^{a}	47.05^{b}
	33			1762	87.26^{b}	3.95^{a}	0.05^{a}	35.15^{b}	17.35^{b}	0.50^{a}	51.52^{a}
	64			1640	88.69^{a}	3.83^{b}	0.05^{b}	36.57^{a}	17.76^{a}	0.49^{b}	47.48^{b}
		4°C		2364	81.76^{b}	4.52^{a}	0.05^{a}	35.29^{b}	19.09^{a}	0.55^{a}	58.11^{a}
		24°C		1038	94.14^{a}	3.26^{b}	0.04^{b}	36.43^{a}	16.02^{b}	0.45^{b}	40.90^{b}
			0	878	77.21^{c}	4.70^{a}	0.06^{a}	36.77^{a}	18.82^{a}	0.52^{a}	60.23^{a}
			1	987	87.24^{b}	4.13^{a}	0.05^{b}	36.56^{a}	17.67^{b}	0.49^{b}	53.02^{a}
			3	891	92.58^{ab}	3.32^{b}	0.04^{c}	35.75^{b}	17.40^{b}	0.49^{c}	41.52^{b}
			5	646	94.86^{a}	3.41^{b}	0.04^{c}	34.34^{b}	16.35^{c}	0.48^{d}	43.25^{b}
ANOVA					Probability						
Genotype (G)					**	**	**	**	-	**	**
Hen age(HA)					**	*	*	**	**	**	**
Storage temperature (ST)					**	*	**	**	**	**	**
Storage period (SP)					**	**	**	**	**	**	**
GxHA					**	**	**	-	-	*	**
GxST					-	**	-	-	**	**	-
G x SP					**	**	**	*	*	**	**
HA x ST					-	*	**	-	**	**	**
HA x SP					-	**	**	*	**	**	**
ST x SP					**	**	*	**	**	**	**
SEM					0.70	0.09	0.06	0.19	0.10	0.00	1.17
R^2					0.40	0.17	0.25	0.25	0.32	0.36	0.18

$^{a-d}$ Means in a column and treatment variable with no common superscript differ significantly (P<0.05)

Table 3. *Main effects on external egg quality traits*
Tablo 3. *Yumurta dış kalite özelliklerine ana faktörlerin etkisi*

Treatments				n	Egg Shell			Shell Weight Per Unit Surface (mg/cm²)
Genotype	Hen Age (Weeks)	Storage Temperature	Storage Period (Weeks)		Weight (g)	Thickness (µm)	Density (g/cm³)	
µ					6.02	400.92	2.03	81.15
Lohman White				463	5.92^{b}	399.30^{b}	2.01^{b}	80.25^{b}
Isa Brown				486	6.11^{a}	402.55^{a}	2.04^{a}	82.05^{a}
	33			473	5.95^{b}	403.10^{a}	2.03	81.62^{a}
	64			476	6.07^{a}	398.73^{b}	2.03	80.68^{b}
		4°C		443	5.99	399.97	2.03	80.94
		24°C		506	6.04	401.88	2.03	81.36
			0	263	5.97	402.40	2.01	80.82
			1	234	6.07	400.21	2.04	81.67
			3	221	6.05	398.95	2.05	81.71
			5	231	5.97	402.14	2.00	80.41
ANOVA					Probability			
Genotype (G)					*	**	*	**
Hen age(HA)					**	**	-	*
Storage temperature (ST)					-	-	-	-
Storage period (SP)					-	-	-	-
SEM					0.02	0.23	0.01	0.28
R^2					0.05	0.02	0.03	0.03

$^{a-b}$ Means in a column and treatment variable with no common superscript differ significantly (P<0.05)

YAZIM KURALLARI

1- Yılda 6 (Altı) sayı olarak yayımlanan Kafkas Üniversitesi Veteriner Fakültesi Dergisi'nde (Kısaltılmış adı: Kafkas Univ Vet Fak Derg) Veteriner Hekimlik ve Hayvancılıkla ilgili (klinik ve paraklinik bilimler, hayvancılıkla ilgili biyolojik ve temel bilimler, zoonozlar ve halk sağlığı, hayvan besleme ve beslenme hastalıkları, hayvan yetiştiriciliği ve genetik, hayvansal orijinli gıda hijyeni ve teknolojisi, egzotik hayvan bilimi) orijinal araştırma, kısa bildiri, ön rapor, gözlem, editöre mektup, derleme ve çeviri türünde yazılar yayımlanır. Dergide yayımlanmak üzere gönderilen makaleler Türkçe, İngilizce veya Almanca dillerinden biri ile yazılmış olmalıdır.

2- Dergide yayımlanması istenen yazılar Times New Roman yazı tipi ve 12 punto ile A4 formatında, 1.5 satır aralıklı ve sayfa kenar boşlukları 2.5 cm olacak şekilde hazırlanmalı ve resim, tablo, grafik gibi şekillerin metin içindeki yerlerine Türkçe ve yabancı dilde adları ve gerekli açıklamaları mutlaka yazılmalıdır.

Dergiye gönderilecek makale ve ekleri (şekil vs) *http://vetdergi.kafkas.edu.tr* adresindeki online makale gönderme sistemi kullanılarak yapılmalıdır.

Başvuru sırasında yazarlar yazıda yer alacak şekilleri (13 X 18 cm boyutlarından büyük olmamalı) online makale gönderme sistemine yüklemelidirler. Yazının kabul edilmesi durumunda tüm yazarlarca imzalanmış *Telif Hakkı Devir Sözleşmesi* editörlüğe gönderilmelidir.

3- Yazarlar yayınlamak istedikleri makale ile ilgili olarak gerekli olan etik kurulu onayı aldıkları kurumu ve onay numarasını Materyal ve Metot bölümünde belirtmelidirler. Yayın kurulu gerekli gördüğünde etik kurul onay belgesini ayrıca isteyebilir.

4- ***Makale Türleri***

Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır.

Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, giriş, materyal ve metot, bulgular, tartışma ve sonuç ile kaynaklar bölümlerinden oluşmalı ve metin, tablo, şekil vs dahil) 10 sayfayı aşmamalıdır. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler ile birlikte Türkçe makale yazım kurallarında belirtilen diğer bölümlerden oluşur. Türkçe ve yabancı dilde özetlerin her biri yaklaşık 200±20 sözcükten oluşmalıdır.

Kısa Bildiri, konu ile ilgili yeni bilgi ve bulguların bildirildiği fakat orijinal araştırma olarak sunulamayacak kadar kısa olan yazılardır. Kısa bildiriler, orijinal araştırma makalesi formatında olmalı, fakat özetlerin her biri 100 sözcüğü aşmamalı, referans sayısı 15'in altında olmalı ve 4 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.

Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 6 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.

Gözlem, uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri giriş, olgunun tanımı, tartışma ve sonuç ile kaynaklardan oluşmalı ve 4 sayfayı geçmemelidir.

Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 1 sayfayı geçmemelidir.

Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri giriş, metin ve kaynaklardan oluşmalı ve 10 sayfayı geçmemelidir.

Çeviri, makalenin orijinal formatı dikkate alınarak hazırlanmalıdır.

Yazarla ilgili kişisel ve kuruma ait bilgiler ana metin dosyasına değil, on-line başvuru sırasında sistemdeki ilgili yerlere unvan belirtilmeksizin eklenmelidir.

5- Makale ile ilgili gerek görülen açıklayıcı bilgiler (tez, proje, destekleyen kuruluş vs) makale başlığının sonuna üst simge olarak işaret konularak makale başlığı altında italik yazıyla belirtilmelidir.

6- Kaynaklar, metin içinde ilk verilenden başlanarak numara almalı ve metin içindeki kaynağın atıf yapıldığı yerde parantez içinde yazılmalıdır.

Kaynak dergi ise, yazarların soyadları ve ilk adlarının başharfleri, makale adı, dergi adı (orijinal kısa ad), cilt ve sayı numarası, sayfa numarası ve yıl sıralamasına göre olmalı ve aşağıdaki örnekte belirtilen karakterler dikkate alınarak yazılmalıdır.

Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.

Editörlü ve çok yazarlı olarak yayınlanan kitaptan bir bölüm kaynak olarak kullanılmışsa, bölüm yazarları, bölüm adı, editör(ler), kitap adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı sırası dikkate alınarak aşağıdaki örneğe göre yazılmalıdır.

Örnek: **Mcllwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. 339-447, Lea and Febiger, Philadelphia, 1988.

Online olarak ulaşılan kaynaklarda web adresi ve erişim tarihi kaynak bilgilerinin sonuna eklenmelidir.

Diğer kaynakların yazımında bilimsel yayın ilkelerine uyulmalıdır.

Kaynak listesinde "et al." ve "ve ark." gibi kısaltmalar yapılmaz.

7- Bakteri, virus, parazit ve mantar tür isimleri ve anatomik terimler gibi latince ifadeler orijinal şekliyle ve italik karakterle yazılmalıdır.

8- Editörlük, dergiye gönderilen yazılar üzerinde gerekli görülen kısaltma ve düzeltmeleri yapabileceği gibi önerilerini yazarlara iletebilir. Yazarlar, düzeltilmek üzere yollanan yazıları online sistemde belirtilen sürede gerekli düzeltmeleri yaparak editörlüğe iade etmelidirler. Editörlükçe ön incelemesi yapılan ve değerlendirmeye alınması uygun görülen makaleler ilgili bilim dalından bir yayın danışmanı ve iki raportörün olumlu görüşü alındığı takdirde yayımlanır.

9- Yayınlanan yazılardan dolayı doğabilecek her türlü sorumluluk yazarlara aittir.

10- Yazarlara telif ücreti ödenmez.

11- Resim ve baskı masrafları için yazarlardan ücret alınır. Ücret bilgileri *http://vetdergi.kafkas.edu.tr/* adresinden öğrenilebilir.

12- Yazarlara 50 adet ayrı baskı ücretsiz olarak yollanır.

INSTRUCTIONS FOR AUTHORS

1- Kafkas Üniversitesi Veteriner Fakültesi Dergisi (Journal of the Faculty of Veterinary Medicine, Kafkas University) (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly, covers original papers, short communication and preliminary scientific reports, case reports, observation, letter to the editor, review and translation on all aspects of veterinary medicine and animal science (clinical and paraclinical sciences, biological and basic sciences, zoonoses and public health, animal feeding and nutritional diseases, animal breeding and genetics, hygiene and technology of food from animal sources, exotic animal science). Manuscripts submitted for publication should be written in Turkish, English or German.

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure, table and graphic must clearly be written in both Turkish and foreign language and their appropriate position should be indicated in the text.

The manuscript and its supplementary (figure etc.) should be submitted by using online manuscript submission system at the address of *http://vetdergi.kafkas.edu.tr/*

During the submission, the authors should upload the figures of the manuscript (the dimensions must not to exceed 13 X 18 cm) to the online manuscript submission system. If the manuscript is accepted for publication, the copyright transfer agreement form signed by all the authors should be send to the editorial office.

3- Authors should indicate the name of institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, editorial board may also request the official document of the ethical commission report.

4- Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts written in Turkish consist of the title in Turkish, summary and keywords in Turkish, introduction, material and methods, results, discussion and references and it should not exceed 10 pages including text, tables and illustrations. Manuscripts written in a foreign language should follow the title in foreign language, summary and keywords in foreign language, title in Turkish, summary and keywords in Turkish and the remaining sections described above for the manuscripts written in Turkish.

Summaries written in Turkish or foreign language should contain 200±20 words.

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but each of the summaries should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are short description (maximum 6 pages) of partially completed original research findings at interpretable level. These should be prepared in the format of full-length original articles.

Case reports describe rare significant findings encountered in the application, clinic and laboratory of related fields. The title and summary of these articles should be written in the format of full-length original articles and the remaining sections should follow introduction, case history, discussion and references without exceeding the total of 4 pages.

Letters to Editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases without exceeding 1 page.

Reviews are original manuscripts gather the literature on current and significant subject along with the commentary and findings of the author on the particular subject. The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should follow introduction, text and references without exceeding 10 page.

Translations should be prepared based on the format of original document being translated.

The information about author/s and institution/s should be added during the online submissoin and the main documant should be free of these information.

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6- References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: **McIlwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. 339-447, Lea and Febiger, Philadelphia, 1988.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must be complied with the other references.

Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

7- The Latin expression such as species names of bacterium, virus, parasite and fungus and anatomical terms must be written in italic character keeping their original forms.

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