ISSN 1300 - 6045
(e-ISSN: 1309-2251)

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

The Journal of the Faculty of Veterinary Medicine, University of Kafkas

(Yılda altı sayı yayımlanır)

(Published Bi-monthly)

http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Cilt / Volume : 21

Sayı / Number : 2

MART - NİSAN
MARCH - APRIL

Yıl / Year : 2015

ISSN: 1300-6045
e-ISSN: 1309-2251

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

JOURNAL OF THE FACULTY OF VETERINARY MEDICINE, KAFKAS UNIVERSITY

(MART - NİSAN)
(MARCH - APRIL)

Cilt/Volume: 21 Sayı/Number: 2 Yıl/Year: 2015

This journal is indexed and abstracted by Thomson Reuters Services beginning with Volume 13 (1) 2007 in the followings:

- **Science Citation Index Expanded (also known as SciSearch®)**
- **Journal Citation Reports/Science Edition**

This journal is also indexed and abstracted in:

- **THOMSON REUTERS - ZOOLOGICAL RECORD**
- **ELSEVIER - SCOPUS**
- **CAB Abstracts**
- **TÜRKİYE ATIF DİZİNİ**
- **TÜBİTAK - ULAKBİM Yaşam Bilimleri Veri Tabanı**
- **EBSCO**

YAZIŞMA ADRESİ (ADDRESS FOR CORRESPONDENCE)

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

E-ISSN: 1309-2251

ELEKTRONİK BASKI (ELECTRONIC EDITION)
http://vetdergi.kafkas.edu.tr

ONLINE MAKALE GÖNDERME (ONLINE SUBMISSION)
http://vetdergikafkas.org

Bu dergi Kafkas Üniversitesi Veteriner Fakültesi tarafından iki ayda bir yayımlanır
This journal is published bi-monthly, by the Faculty of Veterinary Medicine, University of Kafkas

BASKI (Print)
ESER OFSET MATBAACILIK Tel: +90 442 2334667 ERZURUM

DANIŞMA KURULU
(Advisory Board)

Bu Sayının Hakem Listesi *(alfabetik sıra)*
The Referees List of This Issue *(in alphabetical order)*

KORKMAZ AĞAOĞLU Özgecan	Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi
AKÇAY Aytaç	Erciyes Üniversitesi Veteriner Fakültesi
AKDOĞAN KAYMAZ Alev	İstanbul Üniversitesi Veteriner Fakültesi
ALGAN CAVULDAK Özge	Bülent Ecevit Üniversitesi Mühendislik Fakültesi
ALKAN Fahrettin	Selçuk Üniversitesi Veteriner Fakültesi
ARSLAN Cavit	Kafkas Üniversitesi Veteriner Fakültesi
ATABAY H. İbrahim	İzmir Yüksek Teknoloji Enstitüsü
ATASEVEN Veysel Soydal	Mustafa Kemal Üniversitesi Veteriner Fakültesi
AVCI Mehmet	Harran Üniversitesi Veteriner Fakültesi
AYAŞAN Tugay	Doğu Akdeniz Tarımsal Araştırma Enstitüsü
AYDIN Rahim	Balıkesir Üniversitesi Veteriner Fakültesi
AYHAN Veysel	Süleyman Demirel Üniversitesi Ziraat Fakültesi
AZKUR Ahmet Kürşat	Kırıkkale Üniversitesi Veteriner Fakültesi
BAĞCIGİL Arzu Funda	İstanbul Üniversitesi Veteriner Fakültesi
BARAN Murat Sedat	Dicle Üniversitesi Veteriner Fakültesi
BAŞAĞAÇ GÜL R. Tamay	Ankara Üniversitesi Veteriner Fakültesi
BELGE Ali	Adnan Menderes Üniversitesi Veteriner Fakültesi
BEŞOLUK Kamil	Selçuk Üniversitesi Veteriner Fakültesi
BOSTAN Kamil	İstanbul Aydın Üniversitesi Mühendislik Fakültesi
BOYDAK Murat	Selçuk Üniversitesi Veteriner Fakültesi
BÜYÜK Fatih	Kafkas Üniversitesi Veteriner Fakültesi
COŞKUN Behiç	Selçuk Üniversitesi Veteriner Fakültesi
ÇAKIR Ahmet	Ankara Üniversitesi Veteriner Fakültesi
ÇARLI Tayfun	Uludağ Üniversitesi Veteriner Fakültesi
ÇELEBİ Özgür	Kafkas Üniversitesi Veteriner Fakültesi
ÇİFTCİ Alper	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
ÇOLAK Armağan	Atatürk Üniversitesi Veteriner Fakültesi
DAĞ Serpil	Kafkas Üniversitesi Veteriner Fakültesi
ELMAS Muammer	Selçuk Üniversitesi Veteriner Fakültesi
ERENER Güray	Ahi Evran Üniversitesi Ziraat Fakültesi
ERTUĞRUL Okan	Ankara Üniversitesi Veteriner Fakültesi
EYDURAN Ecevit	Iğdır Üniversitesi Ziraat Fakültesi
GENÇ Oktay	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
GÜL Mehmet	Atatürk Üniversitesi Veteriner Fakültesi
GÜLER Leyla	Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi
GÜMÜŞSOY Kadir Semih	Erciyes Üniversitesi Veteriner Fakültesi
GÜRBULAK Kutlay	Erciyes Üniversitesi Veteriner Fakültesi
İKİZ Serkan	İstanbul Üniversitesi Veteriner Fakültesi
İMİK Halit	Atatürk Üniversitesi Veteriner Fakültesi
KAÇAR Cihan	Kafkas Üniversitesi Veteriner Fakültesi
KARADAĞ SARI Ebru	Kafkas Üniversitesi Veteriner Fakültesi
KARAHAN Murat	Cumhuriyet Üniversitesi Veteriner Fakültesi
KARAMAN Musa	Kafkas Üniversitesi Veteriner Fakültesi
KENAR Beytullah	Afyon Kocatepe Üniversitesi Veteriner Fakültesi

Bu Sayının Hakem Listesi *(alfabetik sıra)*
The Referees List of This Issue *(in alphabetical order)*

KÖROĞLU Ergün	Fırat Üniversitesi Veteriner Fakültesi
KÜPLÜLÜ Özlem	Ankara Üniversitesi Veteriner Fakültesi
MACRI Adrian	The University of Agricultural Science and Veterinary Medicine/Romania
MUZ Adile	Fırat Üniversitesi Veteriner Fakültesi
ÖNER Zübeyde	Süleyman Demirel Üniversitesi Mühendislik Fakültesi
ÖZEN Hasan	Kafkas Üniversitesi Veteriner Fakültesi
ÖZMEN TOGAY Sine	İstanbul Medipol Üniversitesi Sağlık Bilimleri Fakültesi
ÖZSOY Bülent	Mustafa Kemal Üniversitesi Veteriner Fakültesi
SAÇAKLI Pınar	Ankara Üniversitesi Veteriner Fakültesi
SANAL Şule	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
SAREYYÜPOĞLU Barış	Ankara Üniversitesi Veteriner Fakültesi
SARI Barış	Kafkas Üniversitesi Veteriner Fakültesi
SEKKİN Selim	Adnan Menderes Üniversitesi Veteriner Fakültesi
SERBESTER Uğur	Niğde Üniversitesi, Bor Meslek Yüksekokulu
SUR Emrah	Selçuk Üniversitesi Veteriner Fakültesi
ŞENLİK Bayram	Uludağ Üniversitesi Veteriner Fakültesi
ŞİMŞEK Sami	Fırat Üniversitesi Veteriner Fakültesi
TUNCA Recai	Adnan Menderes Üniversitesi Veteriner Fakültesi
UZLU Erdoğan	Kafkas Üniversitesi Veteriner Fakültesi
ÜNVER Ahmet	Çanakkale Onsekiz Mart Üniversitesi Tıp Fakültesi
YILDIZ Ahmet	Atatürk Üniversitesi Veteriner Fakültesi
YÖRÜK Mecit	Yüzüncü Yıl Üniversitesi Veteriner Fakültesi

İÇİNDEKİLER (Contents)

Kafkas Univ Vet Fak Derg
21 (2): 147-153, 2015
DOI: 10.9775/kvfd.2014.11767

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Osteometric Examination of Metapodial Bones in Sheep *(Ovis aries* L.) and Goat *(Capra hircus* L.) Unearthed from the Yenikapı Metro and Marmaray Excavations in Istanbul [1]

Gülsün PAZVANT [1] Vedat ONAR [1] Hasan ALPAK [1] Nazan GEZER İNCE [1]
K. Oya KAHVECİOĞLU [1] Altan ARMUTAK [2] Zeynep KIZILTAN [3]

[1] *This work was supported by the TUBİTAK (Project No: 107 O 518)*

[1] Osteoarchaeology Laboratory of the Department of Anatomy, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Avcılar, Istanbul - TURKEY
[2] Department of Veterinary History and Deontology, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Avcılar, Istanbul - TURKEY
[3] Istanbul Archaeological Museums, TR-34122 Gülhane, Istanbul - TURKEY

Article Code: KVFD-2014-11767 Received: 10.06.2014 Accepted: 16.09.2014 Published Online: 22.09.2014

Abstract

In this study, the metapodial bones of sheep and goats which were found out at Yenikapı Metro and Marmaray archaeological excavations in İstanbul were used. The variability of characteristic features of mentioned metapodia (CV values) were examined and calculations of shoulder heights were made with using metapodial evaluations. The results which were reached were compared with the data of modern and archaeological sheep and goats which belong to previous studies about this topic. Hereat, it is observed that the shoulder heights of Byzantine sheep are in same data range with Iron Age sheep and are in same size with one of the modern breeds Tuj sheep. It is confirmed that the shoulder heights of the Byzantine goats are higher than Iron Age goats and are in same size with one of the modern breeds Anatolian Black Goats.

Keywords: *Metapodial bones, Yenikapı Metro and Marmaray, Sheep, Goat*

Istanbul Yenikapı Metro ve Marmaray Kazılarında Ortaya Çıkan Koyun ve Keçi Metapodial Kemiklerinin Osteometrik İncelenmesi

Özet

Çalışmada, İstanbul Yenikapı Metro ve Marmaray bölgesi arkeolojik kazılarında ortaya çıkarılan koyun ve keçi metapodium'ları kullanıldı. Söz konusu metapodium'ların ana özelliklerinin değişkenlikleri (CV değerleri) incelendi ve metapodial ölçümler kullanılarak omuz yüksekliği hesaplamaları yapıldı. Elde edilen sonuçların bu konu üzerinde yapılmış çalışmalardaki modern ve arkeolojik koyun-keçi verileri ile karşılaştırılması yapıldı. Bunun sonucunda; Bizans dönemi koyunların omuz yüksekliğinin, Demirçağ koyunları ile aynı veri aralığında, modern ırklardan ise Tuj ırkı koyunlarının ebatında olduğu gözlendi. Bizans dönemi keçilerin omuz yüksekliği, Demirçağ keçilerinden daha yüksek, modern ırklardan ise Anadolu Kıl keçisi ebatlarında olduğu tespit edildi.

Anahtar sözcükler: *Metapodial kemikler, Yenikapı Metro ve Marmaray, Koyun, Keçi*

INTRODUCTION

Yenikapı Metro and Marmaray excavations has started with Marmaray sub-sea tunnel project in 2004 and is still going on. Excavation area is 58.000 m^2 wide and with many archaeological material, animal bones were also found [1]. The ongoing excavation confirmed that the region is ancient harbour of Theodosius [1-3]. Together with dating the archaeological material in the excavation area, animal bones were also radiocarbon dated and the mentioned skeletons belong to different ages between early (4-7th century) and late (15th century) Byzantine [4].

The excavation bones are not only giving information about only animal-human relations, but also the parameters such as visual morphological features, diseases of

İletişim (Correspondence)
+90 212 4737070/17193
nazan@istanbul.edu.tr

animal population and animal husbandry.

By examining the obtained ruins determination of many morphological parameters were provided (weight, shoulder height, age, sex, physical structure of animal) [5-10]. Information about visual morphology was obtained by using evaluation of metapodia specially [10,11]. For the enlightenment of history of domestication of sheep [10-12] and goats [10,13], metapodia were often used to determining morphological changes in this duration. In the studies, estimated shoulder heights were calculated by using evaluations of metapodia which obtained in archaeological excavations and classification of species was tried to be identified by comparing with archaeological data and actual breeds.

In this study, the metapodial slenderness index which classificates the individuals as ''slender" or ''thick" and playing a role for determining of visual features was used (SD/GL*100) [5,14,15]. Distribution of CV was examined for the osteometric evaluation of aforementioned metapodiums and the data which were used in identification of breed were obtained by using these values [11,16,17].

We believe that the metapodia data obtained will enlighten the definition of sheep and goat population of Byzantine age, socio-economic condition of region and ancient animal husbandry of Istanbul where is the heart of Byzatine.

MATERIAL and METHODS

In this study, the metapodial bones of adult sheep and goat, which were found in Yenikapı Metro and Marmaray excavations were used. For this purpose, sheep with maximum numbers of 148 metacarpus and 219 metatarsus bones were examined while maximum number of metapodial bones for goats were 65 and 92 respectively which were examined.

Measurements of metapodial bones were taken by digital calliper with precision of 0.01 mm. The measurements were made as described in Onar et al.[10], Berteaux and Guintard [14], Davis [16], Guintard and Lallemand [17], Daugnora [18], Driesch [19], Haak [20], Rowley-Conwy's [21 studies and taken from measuring points which stated below.

Morphometric measurements (Fig. 1)

GL: Greatest length
Bp: Width of proximal end
Dp: Depth of proximal end
SD: Smallest width of diaphysis in the medio-lateral axis
d: Mid-shaft width of diaphysis
e: Mid-shaft depth of diaphysis in the dorso-palmar axis
DD: Smallest depth of diaphysis in the dorso-palmar axis
Bd: Width of distal end
Be: Greatest width of metaphysis in the medio-lateral axis
De: Greatest depth of metaphysis in the dorso-palmar axis
Dd: Depth of distal end
DIM: Antero-posterior diameter of the internal trochlea of the medial condyle
DEM: Antero-posterior diameter of the external trochlea of the medial condyle
DIL: Antero-posterior diameter of the internal trochlea of the lateral condyle
DEL: Antero-posterior diameter of the external trochlea of the lateral condyle
WCM: Medio-lateral width of the medial condyle
WCL: Medio-lateral width of the lateral condyle

Calculation of mean values, SD values and CV values of above mentioned measurements were made for the two breeds. SPSS 8.0 and Excel programs were used for the calculation of statistical data. (SD/GL*100) metapodial slenderness index which was used for determining the

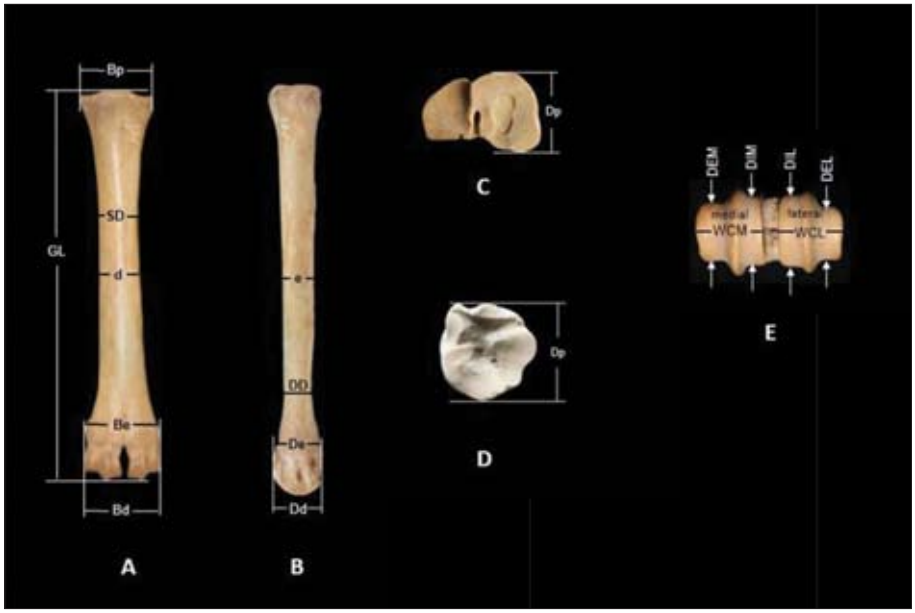


Fig 1. Measurements taken from the metapodial bones (sheep-goat), **A-** Dorsal view of metacarpus; **B-** Lateral view of metacarpus; **C-** Proximal view of metacarpus; **D-** Proximal view of metatarsus; **E-** Distal view of metatarsus

Şekil 1. Metapodium kemiklerinden alınan ölçümler (koyun-keçi), **A-** Metacarpus'un dorsal görünüşü; **B-** Metacarpus'un lateral görünüşü; **C-** Metacarpus'un proksimal görünüşü; **D-** Metatarsus'un proksimal görünüşü; **E-** Metatarsus'un distal görünüşü

thinness of skeleton were calculated by using metapodial calculation of bones of both species [5,14,15].

Shoulder height estimation which provides to estimate the visual morphology, has been calculated by using metapodial values taken from sheep and goats [20,22]. Visual morphology was tried to be estimated by comparing the data of shoulder heights of the two species with current data of goat and sheep [23,24].

RESULTS

Mean values, SD values and mean of CV values of osteometric values of metapodial bones of sheep and goats which were obtained from excavation for this study was given in *Table 1* and *Table 2*. Due to some of the metapodial bones have only distal or proximal fragments, material numbers which were used for each evaluation was subject to vary.

According to osteometrical evaluation results of sheep metacarpi, it was determined that the minimum variability was in Dp measurement (CV: 8.40%) while the maximum variability was observed in DD measurements (CV: 12.03%). When metatarsi were examined, it was determined that maximum variability was in DD (CV: 8.49%) and minimum variability was in Bd (CV: 5.91%) *(Table 1)*.

In the measurements of metapodium of goats for metacarpus, it was found that maximum variability was in Bd measurement (12.32%) and minimum variability was in WCM (7.24%) measurement. The maximum variability of goat metatarsi was in De measurements (%9.78) and minimum variability was observed in DIL measurements (6.79%) *(Table 2)*.

Metapodial slenderness index was calculated for metacarpi and metatarsi of both species seperately. While metapodial slenderness index for metacarpi of sheep was observed 11.70±1.12, index of metatarsi was determined 9.45±0.73 *(Table 3)*. Aforementioned index values for metacarpi and metatarsi were calculated 14.89±1.24 and 11.11±0.79 for goats respectively.

In this study, estimated shoulder heights of sheep and goats were calculated by using metacarpi and metatarsi which were obtained. Thus shoulder heights data were acquired for both species *(Table 3, Table 4)*. Mean value for the shoulder height of sheep was estimated as 60.79 cm while it was determined as 65.55 cm for goats. It was found that the value of CV (7.89%) lower in shoulder height results which were calculated with the values of metatarsi of sheep was lower. For goats, unlike the sheep CV value (8.18%) of estimated shoulder height was the lowest which were determined by using results of metacarpus measurements.

DISCUSSION

In this study, osteometric measurements were made on metapodial bones of sheep and goats which were found in İstanbul Yenikapı Metro and Marmaray excavations. The variabilities of main features of metapodia were researched and these features were compared with the

Table 1. *Morphometrical data of metapodial bones of sheep and calculation of CV %*
Tablo 1. *Koyunların metapodium kemiklerinin morfometrik verileri ve % CV hesaplanması*

Bone	Statistical Values	Measurements																
		GL	Bp	Dp	SD	d	e	DD	Bd	Be	De	Dd	DIM	DEM	DIL	DEL	WCM	WCL
Metacarpus	N	136	147	147	148	148	148	148	136	145	145	135	135	137	135	136	135	134
	Mean	124.91	24.51	17.51	14.56	14.71	10.98	9.98	26.74	27.00	14.10	16.42	14.24	11.98	14.32	11.06	12.66	12.14
	SD	11.04	2.45	1.47	1.74	1.52	1.13	1.20	2.49	2.41	1.41	1.43	1.29	1.26	1.35	1.18	1.21	1.20
	Min	105.85	20.36	14.74	10.79	10.82	8.70	7.83	22.63	22.38	11.61	13.10	11.73	9.26	11.68	8.70	10.72	10.25
	Max	180.31	47.31	28.03	25.73	25.64	19.09	18.49	48.85	45.12	24.04	25.15	23.86	21.82	24.99	20.40	23.27	22.52
	%CV	8.84	10.00	8.40	11.92	10.32	10.25	12.03	9.31	8.93	10.03	8.69	9.06	10.52	9.40	10.70	9.55	9.87
Metatarsus	N	203	219	217	219	219	219	217	208	216	216	207	209	210	207	208	207	207
	Mean	134.05	21.33	21.15	12.61	12.90	12.15	10.51	25.31	25.38	14.46	16.28	14.06	11.25	14.07	10.36	12.11	11.11
	SD	10.57	1.45	1.48	1.02	1.08	0.95	0.89	1.50	1.69	1.18	1.16	0.92	0.84	0.92	0.76	0.77	0.67
	Min	104.09	18.20	13.69	10.00	10.21	10.14	8.66	21.36	21.10	11.68	12.98	11.39	8.94	11.56	8.52	10.19	9.35
	Max	161.29	28.87	25.28	15.47	15.94	15.36	14.45	29.51	30.84	18.55	20.36	17.31	14.53	17.55	12.84	14.37	13.14
	%CV	7.89	6.78	6.98	8.05	8.33	7.86	8.49	5.91	6.64	8.20	7.10	6.57	7.47	6.57	7.38	6.33	6.05

GL: *Greatest length,* ***Bp:*** *Width of proximal end,* ***Dp:*** *Depth of proximal end,* ***SD:*** *Smallest width of diaphysis in the medio-lateral axis,* ***d:*** *Mid-shaft width of diaphysis,* ***e:*** *Mid-shaft depth of diaphysis in the dorso-palmar axis,* ***DD:*** *Smallest depth of diaphysis in the dorso-palmar axis,* ***Bd:*** *Width of distal end,* ***Be:*** *Greatest width of metaphysis in the medio-lateral axis,* ***De:*** *Greatest depth of metaphysis in the dorso-palmar axis,* ***Dd:*** *Depth of distal end,* ***DIM:*** *Antero-posterior diameter of the internal trochlea of the medial condyle,* ***DEM:*** *Antero-posterior diameter of the external trochlea of the medial condyle,* ***DIL:*** *Antero-posterior diameter of the internal trochlea of the lateral condyle,* ***DEL:*** *Antero-posterior diameter of the external trochlea of the lateral condyle,* ***WCM:*** *Medio-lateral width of the medial condyle,* ***WCL:*** *Medio-lateral width of the lateral condyle*

Table 2. *Morphometrical data of metapodial bones of goats and calculation of CV %*
Tablo 2. *Keçilerin metapodium kemiklerinin morfometrik verileri ve % CV hesaplanması*

Bone	Statistical Values	Measurements																
		GL	Bp	Dp	SD	d	e	DD	Bd	Be	De	Dd	DIM	DEM	DIL	DEL	WCM	WCL
Metacarpus	N	57	64	64	65	54	53	63	57	53	51	54	57	57	55	55	57	55
	Mean	115.52	26.04	17.81	17.30	17.95	11.42	11.31	28.66	28.77	14.54	17.19	14.75	11.07	14.85	10.51	13.44	13.01
	SD	9.44	2.15	1.47	1.80	1.85	1.09	1.19	3.53	3.08	1.40	1.33	1.40	0.89	1.40	0.82	0.97	1.08
	Min	99.57	21.77	14.82	13.34	14.05	9.56	9.36	15.86	18.64	11.69	14.12	12.00	9.66	12.10	8.76	11.59	10.94
	Max	142.92	30.51	21.15	22.10	22.92	15.88	15.03	35.50	34.15	17.14	19.70	19.79	13.33	19.49	12.55	15.48	15.71
	%CV	8.18	8.25	8.24	10.41	10.33	9.52	10.52	12.32	10.72	9.65	7.72	9.49	8.05	9.45	7.82	7.24	8.27
Metatarsus	N	78	90	88	92	76	76	87	75	69	69	76	77	77	77	78	76	75
	Mean	121.13	20.83	19.67	13.53	13.88	11.62	10.36	25.03	25.42	13.87	16.04	13.77	10.44	13.87	9.97	11.83	11.24
	SD	11.40	1.53	1.70	1.23	1.27	1.13	0.93	1.88	2.12	1.36	1.29	0.95	0.83	0.94	0.71	0.91	0.92
	Min	97.78	17.73	15.92	9.06	9.26	8.63	8.10	21.90	20.32	11.66	13.28	12.01	9.07	11.73	8.53	10.44	9.85
	Max	165.19	24.24	24.48	16.39	16.87	14.61	12.55	28.94	29.71	16.63	19.17	16.59	13.15	16.21	11.50	14.10	14.84
	%CV	9.41	7.36	8.64	9.12	9.12	9.70	8.97	7.49	8.32	9.78	8.04	6.90	7.91	6.79	7.11	7.70	8.19

GL: *Greatest length,* ***Bp:*** *Width of proximal end,* ***Dp:*** *Depth of proximal end,* ***SD:*** *Smallest width of diaphysis in the medio-lateral axis,* ***d:*** *Mid-shaft width of diaphysis,* ***e:*** *Mid-shaft depth of diaphysis in the dorso-palmar axis,* ***DD:*** *Smallest depth of diaphysis in the dorso-palmar axis,* ***Bd:*** *Width of distal end,* ***Be:*** *Greatest width of metaphysis in the medio-lateral axis,* ***De:*** *Greatest depth of metaphysis in the dorso-palmar axis,* ***Dd:*** *Depth of distal end,* ***DIM:*** *Antero-posterior diameter of the internal trochlea of the medial condyle,* ***DEM:*** *Antero-posterior diameter of the external trochlea of the medial condyle,* ***DIL:*** *Antero-posterior diameter of the internal trochlea of the lateral condyle,* ***DEL:*** *Antero-posterior diameter of the external trochlea of the lateral condyle,* ***WCM:*** *Medio-lateral width of the medial condyle,* ***WCL:*** *Medio-lateral width of the lateral condyle*

Table 3. *Metapodial slenderness index of sheep and calculation of estimated shoulder height*
Tablo 3. *Koyunların metapodial incelik indeksi ve tahmini omuz yüksekliğinin hesaplanması*

Animal	Statistical Values	Metacarpus		Metatarsus	
		MSI	SH (cm)	MSI	SH (cm)
Sheep	N	136	136	203	203
	Mean	11.70	60.58	9.45	60.99
	SD	1.12	5.36	0.73	4.81
	Min	9.80	51.34	7.65	47.36
	Max	19.66	87.45	11.80	73.39
	%CV	9.56	8.84	7.77	7.89

MSI: *Metapodial Slenderness Index,* ***SH:*** *Estimated Shoulder Height*

Table 4. *Metapodial slenderness index of goats and calculation of estimated shoulder height*
Tablo 4. *Keçilerin metapodial incelik indeksi ve tahmini omuz yüksekliğinin hesaplanması*

Animal	Statistical Values	Metacarpus		Metatarsus	
		MSI	SH (cm)	MSI	SH cm)
Goat	N	57	57	78	78
	Mean	14.89	66.42	11.11	64.68
	SD	1.24	5.43	0.79	6.09
	Min	11.73	57.25	8.79	52.21
	Max	17.06	82.18	12.75	88.21
	%CV	8.34	8.18	7.11	9.41

MSI: *Metapodial Slenderness Index,* ***SH:*** *Estimated Shoulder Height*

previous studies about modern and archaeological sheep-goat population.

CV values which were obtained from the measuring points of metapodia of sheep and goats were calculated *(Table 5)*. While CV values of metacarpus measurements of sheep varies between 12.03% (DD) and 8.40% (Dp), these values were between 8.49% (DD) and 5.91% (Bd) for metatarsus. The aforementioned values varies between 12.32% (Bd) and 7.24% (WCM) for metacarpi relating to goats, where as 9.78% (De) and 6.79% (DIL) for metatarsi. Obtained CV values were found as higher than the maximum values which Haak [20] and Davis [16] and lower than the maximum values that Guintard and Lallemand [17] defined. Because there is not any sampling method for data in archaeological excavations, homogeneous distribution may not be expected. In this study, variation in CV values suggests the bone samples probably belong to different breeds and genders. The CV values are not high as much as in modern breed studies [11,17] so that it supports the obtained sampling may be close to homogeneous.

Guintard and Lallemand [17] noted in their studies that GL is the main measurement for the assesment of slenderness of bone. They used this parameter for categorizing breeds as "tall" for higher and "short" for lower than 135 mm. Considering the GL values of metacarpus and metatarsus of sheep and goats which we used in our study, these values vary between 115.52 mm and 134.05 mm and therefore individuals belong to both species must be categorized as "short".

Table 5. *Comparison of the coefficient of variation (CV%) of osteometric measurements of sheep metapodial bones with the modern and archeological values*

Tablo 5. *Koyun metapodium kemiklerinin osteometrik ölçümlerinin varyasyon katsayısının modern ve arkeolojik veriler ile karşılaştırılması*

Bone	Sheep															Goat			
	Yenikapı Metro and Marmaray		Upper Anzaf Castle		Lallemand 2002				Guintard and Lallemand 2003				Davis 1996 (Cvall) Female		Davis 1996 (Cvav+good) Female		Yenikapı Metro and Marmaray		Upper Anzaf Castle	
					Female		Male		Female		Male									
Metacarpus	DD	12.03	De	7.84	d	15.04	d	19.61	d	15.04	d	19.61	DEM	5.8	DEM	5.8	Bd	12.32	GL	9.61
	SD	11.92	DD	7.34	SD	14.52	SD	18.85	SD	14.52	SD	18.85	DEL	5.4	DEL	5.4	Be	10.72	De	9.23
	DEL	10.70	SD	6.92	Bp	10.79	e	15.24	Bp	10.79	e	15.24	SD	5	SD	5	DD	10.52	e	8.09
	DEM	10.52	e	6.74	GL	9.56	DD	14.32	GL	9.56	DD	14.32	DIL	4.9	DIL	4.9	SD	10.41	Dp	7.59
	d	10.32	Dd	6.38	e	9.55	Bp	13.76	e	9.55	Bp	13.76	DIM	4.7	DIM	4.7	d	10.33	Bp	7.46
	e	10.25	GL	6.36	DD	9.37	Dp	13.32	DD	9.37	Dp	13.32	Bp	3.9	Bp	3.9	De	9.65	DD	7.04
	De	10.03	Be	5.64	Be	9.07	WCL	13.09	Be	9.07	WCL	13.09	GL	3.8	GL	3.8	e	9.52	SD	6.17
	Bp	10.00	Bp	5.61	Dp	8.56	Be	12.98	Dp	8.56	Be	12.98	WCM	3.6	WCM	3.6	DIM	9.49	Dd	5.94
	WCL	9.87	Dp	5.21	WCM	8.42	DEL	12.75	WCM	8.42	DEL	12.72	WCL	3.6	WCL	3.6	DIL	9.45	Be	5.91
	WCM	9.55	Bd	4.13	DEL	8.32	WCM	12.69	DEL	8.32	WCM	12.69	Bd	3	Bd	3	WCL	8.27	Bd	4.83
	DIL	9.40			De	8.21	DEM	12.32	De	8.21	DEM	12.32					Bp	8.25		
	Bd	9.31			DEM	8.14	Bd	11.96	DEM	8.14	Bd	11.96					Dp	8.24		
	DIM	9.06			WCL	8.04	DIM	11.75	WCL	8.04	DIM	11.75					GL	8.18		
	Be	8.93			Bd	7.79	DIL	11.74	Bd	7.97	DIL	11.74					DEM	8.05		
	GL	8.84			Dd	7.73	GL	11.58	Dd	7.73	GL	11.58					DEL	7.82		
	Dd	8.69			DIL	7.36	De	11.24	DIL	7.36	De	11.24					Dd	7.72		
	Dp	8.40			DIM	7.18	Dd	9.97	DIM	7.18	Dd	9.97					WCM	7.24		
Metatarsus	DD	8.49	DD	9.36	d	13.73	d	18.54	d	13.73	d	18.54	SD	5.1	SD	4.5	De	9.78	e	11.14
	d	8.33	SD	8.32	SD	12.66	SD	17.68	SD	12.66	SD	17.68	DIL	4.9	DIL	4.1	e	9.70	DD	10.01
	De	8.20	e	8.29	e	11.49	DEM	16.83	e	11.49	DEM	16.83	DIM	4.4	GL	3.7	GL	9.41	SD	9.71
	SD	8.05	Dp	8.04	GL	10.41	e	16.64	GL	10.41	e	16.64	GL	4.1	DIM	3.5	SD	9.12	Dp	8.67
	GL	7.89	De	7.86	DEM	10.2	DEL	16.3	DEL	10.20	DEL	16.30	Bd	3.2	Bd	2.9	d	9.12	De	8.55
	e	7.86	GL	7.71	DEL	10.2	DD	16.14	DEM	10.20	DD	16.14					DD	8.97	Bp	8.38
	DEM	7.47	Be	7.26	DD	10.18	Bp	15.3	DD	10.18	Bp	15.3					Dp	8.64	GL	7.87
	DEL	7.38	Bp	5.95	Dp	10.04	Dp	15.16	Dp	10.04	Dp	15.16					Be	8.32	Dd	7.14
	Dd	7.10	Dd	5.81	WCM	9.99	DIL	14.82	WCM	9.90	DIL	14.82					WCL	8.19	Be	6.68
	Dp	6.98	Bd	5.45	WCL	9.96	GL	14.55	WCL	9.96	GL	14.55					Dd	8.04	Bd	5.86
	Bp	6.78			Bp	9.95	WCL	14.47	Bp	9.95	WCL	14.47					DEM	7.91		
	Be	6.64			Be	9.52	DIM	14.45	Be	9.52	DIM	14.45					WCM	7.70		
	DIL	6.57			Dd	9.44	De	14.39	Dd	9.44	De	14.39					Bd	7.49		
	DIM	6.57			De	9.17	Be	14.23	De	9.17	Be	14.23					Bp	7.36		
	WCM	6.33			Bd	8.95	WCM	14.23	Bd	8.95	WCM	14.23					DEL	7.11		
	WCL	6.05			DIL	8.5	Bd	13.71	DIL	8.50	Bd	13.71					DIM	6.90		
	Bd	5.91			DIM	8.25	Dd	12.91	DIM	8.25	Dd	12.91					DIL	6.79		

GL: *Greatest length,* ***Bp:*** *Width of proximal end,* ***Dp:*** *Depth of proximal end,* ***SD:*** *Smallest width of diaphysis in the medio-lateral axis,* ***d:*** *Mid-shaft width of diaphysis,* ***e:*** *Mid-shaft depth of diaphysis in the dorso-palmar axis,* ***DD:*** *Smallest depth of diaphysis in the dorso-palmar axis,* ***Bd:*** *Width of distal end,* ***Be:*** *Greatest width of metaphysis in the medio-lateral axis,* ***De:*** *Greatest depth of metaphysis in the dorso-palmar axis,* ***Dd:*** *Depth of distal end,* ***DIM:*** *Antero-posterior diameter of the internal trochlea of the medial condyle,* ***DEM:*** *Antero-posterior diameter of the external trochlea of the medial condyle,* ***DIL:*** *Antero-posterior diameter of the internal trochlea of the lateral condyle,* ***DEL:*** *Antero-posterior diameter of the external trochlea of the lateral condyle,* ***WCM:*** *Medio-lateral width of the medial condyle,* ***WCL:*** *Medio-lateral width of the lateral condyle*

Table 6. *Comparison of the estimated shoulder heights of sheep and goats unearthed at the Yenikapı Metro and Marmaray excavations with the current breeds and archeological values*

Tablo 6. *Yenikapı Metro ve Marmaray kazılarından elde edilen koyun ve keçilerin tahmini omuz yüksekliğinin günümüz ırklar ve arkeolojik verilerle karşılaştırılması*

Breeds	Morphological Appearance and Pattern			
	Shoulder Height (cm)	Distribution	Phenotypic Description	Size
Yenikapı Metro ve Marmaray Sheep	**60.79**			
Upper Anzaf Castle Sheep	59.93			
White Karaman[1]	Male: 62-67; Female: 63-66	Eastern/Central Anatolia	Fat-tailed	Medium-size
Red Karaman[1]	Male: 65-71; Female: 66-68	Eastern/Central Anatolia	Fat-tailed	Medium-size
Awassi[1]	Male: 70-80; Female: 60-70	Eastern/Central Anatolia	Fat-tailed	Medium-size
Karakul[2]	65	Eastern/Central Anatolia	Fat-tailed	Medium-size
Tuj[2]	60	Eastern Anatolia	Fat-tailed	Medium-size
Dağlıç[2]	58	Central-west Anatolia	Fat-tailed	Small
Yenikapı Metro ve Marmaray Goat	**65.55**			
Upper Anzaf Castle Goat	56.97			
Angora Goat[1]	Male: 55-60; Female: 50-55	Central Anatolia		
Anatolian Black Goat[1]	Male: 65-70; Female: 65-73	All region in Turkey		
Kilis Goat[1]	Male: 65-75; Female: 60-70	South-Eastern Anatolia		

[1] Soysal et al.[23], [2] Yalcin [24]

Comparing with the literature that categorize the individuals as ''heavy" and ''light" according to metacarpus Bp values higher and lower than 27 mm, the values of individuals which belong to our study were evaluated lower than 27 mm thus small ruminants from Byzantine age defined as ''light". The results of GL and Bp of sheep and goats in the zooarchaeological study of Onar et al.[10] are same with our results and show that same breeds of that age are also ''short" and ''light". The metapodial bones were also used in osteological researches on development of sheep in South England during post medieval period [12]. According to the results of metapodial data, authors indicated that the sheep of that region were ''light" and ''short".

The morphological image of the sheep breeds in that study have parallelism with the ones in our study. Metapodial slender index was used in this study (SD/GL*100) which classified the individuals as ''slender" and ''thick"and effective for the evaluation of visual morphology [5,14,15]. While slenderness index varies between 9.45 and 11.70 for sheep, it was determined between 11.11 and 14.89 for goats. It is determined that results showed similarities with the results of animal bones which Onar et al.[10] stated as ''short" and ''slender" breeds and obtained from Upper Anzaf Castle excavations.

The comparison of modern and archaeological sheep goat data is given particularly in this study which shoulder heights of both species are evaluated *(Table 6)* [10,23,24]. The size of the sheep of Byzantine age appear to be similar according to modern Tuj sheep breeded in Eastern Anatolia and archaeological Iron Age sheep. However the goats are 10 cm higher than archaeological Iron Age goats and in the same data range with modern breed "Anatolian Black Goat". It is known that gender and breeding regions are importantly effective on the size of goats [13].

Considering the examined metapodium numbers, breeding and consumption of sheep are higher compared to goats, same as Upper Anzaf Castle excavation studies. Metapodial values obtained which enables to determine the position of the population of sheep and goat and the visual morphological structure of this population, are of nature to enlighten the history of livestock in Istanbul which is the heart of Byzantine.

Acknowledgment

We would like to thank Prof. Dr. Bülent EKİZ for statistical analysis.

REFERENCES

1. Onar V, Alpak H, Pazvant G, Armutak A, Gezer İnce N, Kızıltan Z: A bridge from Byzantium to modern day Istanbul: An overview of animal skeleton remains found during Metro and Marmaray excavations. *Istanbul Univ Vet Fak Derg,* 39, 1-8, 2013.

2. Başaran S: "Iron ways" and an ancient harbour on the Marmara coast. **In,** Kocabaş U (Ed): The "Old Ships" of the "New Gate" 1. Ege Publishing, İstanbul, 1-22, 2008.

3. Müller-Wiener W: Bizans'tan Osmanlı'ya İstanbul Limanı (Die Hafen von Byzantion 4 Konstantinupolis). History Foundation Yurt Publications, Istanbul, 1998.

4. Onar V, Pazvant G, Armutak A: Radiocarbon dating results of the animal remains uncovered at Yenikapı Excavations. **In,** *Istanbul Archaeological Museums, Proceedings of the 1st Symposium on Marmaray-Metro Salvage Excavations,* 5th-6th May, Istanbul, 249-256, 2008.

5. Guintard C: Osteometrie des metapodes de bovins. *Rev Med Vet,* 149, 751-770, 1998.

6. Harcourt RA: The dog in prehistoric and early historic Britain. *J Archaeol Sci,* 1, 151-175, 1974. DOI: 10.1016/0305-4403(74)90040-5

7. Onar V, Armutak A, Belli O, Konyar E: Skeletal remains of dogs unearthed from the Van-Yoncatepe necropolises. *Int J Osteoarchaeol,* 12, 317-334, 2002. DOI: 10.1002/oa.627

8. Onar V, Belli O: Estimation of shoulder height from long bone measurements on Dogs unearthed from the Van-Yoncatepe early Iron Age necropolis in Eastern Anatolia. *Rev Med Vet,* 156, 53-60, 2005.

9. Onar V, Janeczek M, Pazvant G, Gezer Ince N, Alpak H, Armutak A, Chrószcz A, Kızıltan Z: Estimating the body weight of Byzantine dogs from the Theodosius Harbour at Yenikapı, Istanbul. *Kafkas Univ Vet Fak Derg,* 21, 55-59, 2015. DOI: 10.9775/kvfd.2014.11693

10. Onar V, Pazvant G, Belli O: Osteometric examination of metapodial bones in sheep *(Ovis aries L.*) and goat *(Capra hircus L.*) unearthed from the Upper Anzaf Castle in Eastern Anatolia. *Rev Med Vet,* 159, 150-158, 2008.

11. Lallemand M: Etude ostéométrique de métapodes de mouton *(Ovis aries, L*). *PhD Thesis,* Ecole Nationale Veterinaire de Nantes: Nantes, 2002.

12. Vann S, Grimm J: Post-medieval sheep *(Ovis aries)* metapodia from southern Britain. *J Archaeol Sci,* 37, 1532-1542, 2010. DOI:10.1016/j.jas.2010.01.014

13. Zeder MA: A metrical analysis of a collection of modern goats *(Capra hircus aegargus* and *C. H. Hircus)* from Iran and Iraq: Implications for the study of caprine domestication. *J Archaeol Sci,* 28, 61-79, 2001. DOI: 10.1006/jasc.1999.0555

14. Berteaux D, Guintard C: Osteometric study of the metapodials of Amsterdam Island feral cattle. *Acta Theriol,* 40, 97-110, 1995.

15. Davis SJM: The effect of castration and age on the development of the Shetland sheep skeleton and a metric comparison between bones of males, females and castrates. *J Archaeol Sci,* 27, 373-390, 2000. DOI:10.1006/jasc.1999.0452

16. Davis SJM: Measurements of a group of adult female Shetland sheep Skeletons from a single flock: A baseline for Zooarchaeologists. *J Archaeol Sci,* 23, 593-612, 1996. DOI: 10.1006/jasc.1996.0056

17. Guintard C, Lallemand M: Osteometric study of metapodial bones in sheep. *Ann Anat,* 185, 573-583, 2003. DOi: 10.1016/S0940-9602(03)80131-0

18. Daugnora L: Analysis of Cattle metacarpal bones dated from the XIII to XVIIth centuries. *Vet Zootec,* 18, 22-28, 2002.

19. Driesch A Von den: A guide to the measurements of animal bones from Archaeological Sites. *Peabody Museum Bulletins 1*, Harvard University, Massachusetts, 1976.

20. Haak D: Metrische Untersuchungen an Röhrenknochen bei deutschen Merinoslandschafen und Heidschnucken. *Th Med Vet,* Munchen, 1965.

21. Rowley-Conwy P: Improved separation of Neolithic metapodials of sheep *(ovis)* and goats *(capra)* from Arene Candide cave, Liguria, Italy. *J Archaeol Sci,* 25, 251-258, 1998. DOI: 10.1006/jasc.1997.0204

22. Schramm Z: Long bones and height in withers of goat (Poln., Engl., u. Russ., Ausz). *Roczniki Wyzszej Szkoly Rolniczej w Poznaniu, Posen*, 36, 89-105, 1967.

23. Soysal MI, Özkan E, Gürcan EK: The status of native farm animal genetic diversity in Turkey and in the world. *Trakia J Sci,* 1, 1-12, 2003.

24. Yalçın BC: Sheep and goats in Turkey. *FAO Animal Production and Health Pape*, 60, Rome, 1986.

Kafkas Univ Vet Fak Derg
21 (2): 155-162, 2015
DOI: 10.9775/kvfd.2014.11889

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Effects of Supplementary Mineral Amino Acid Chelate (ZnAA - MnAA) on the Laying Performance, Egg Quality and Some Blood Parameters of Late Laying Period Layer Hens

Bilgehan YILMAZ DİKMEN [1] Arda SÖZCÜ [1] Aydın İPEK [1] Ümran ŞAHAN [1]

[1] Department of Animal Science, Faculty of Agriculture, Uludağ University, TR-16059 Bursa - TURKEY

KVFD-2014-11889 Received: 07.07.2014 Accepted: 10.11.2014 Published Online: 10.12.2014

Abstract

This study was carried out to determine the effect of supplementary mineral amino acid chelate (ZnAA - MnAA) on the performance, egg quality and some blood parameters of late laying period layer hens. A total of 320, 64 wk of age Lohmann LSL laying hens, were divided into 4 groups with four replicates with 20 hen within each group. The control group was fed with basal diet and treatment groups were fed basal diet containing 1, 2 and 4 g/kg mineral amino acid chelate (ZnAA - MnAA) for 12 weeks. At the end of the experiment, there were no statistically difference among the groups in live weight, feed intake, feed conversion ratio, damaged egg ratio, mortality (P>0.05). Egg weight (P<0.01), albumen weight (P<0.01), shell weight (P<0.05), yolk index (P<0.01), yolk ratio (P<0.01), albumen ratio (P<0.01), and shell ratio (P<0.05) were found significant. The dietary supplementation of amino acid chelate (ZnAA - MnAA) were not significantly affected serum total protein, glucose, total cholesterol, Calcium (Ca), Phosphorus (P), Zinc (Zn) (P>0.05). The only statistical difference was found on the plumage score of the back region of hens (P<0.05). Also plumage coloring difference was statistically significant among groups (P<0.01). In conclusion, the supplementation of 1 g/kg mineral amino acid chelate (ZnAA-Mn AA) increased egg weight and had favourable effects on interior and exterior egg quality of laying hens in the late laying period.

Keywords: *Chelate, Egg production, Egg quality, Mn-Aminoacid, Zn-Aminoacid*

Geç Yumurtlama Dönemindeki Yumurtacı Tavuk Yemlerine Mineral Aminoasit Şelat (ZnAA - MnAA) Katkısının Performans, Yumurta Kalitesi ve Bazı Kan Parametreleri Üzerine Etkileri

Özet

Bu çalışma geç yumurtalama dönemindeki yumurtacı tavukların yemlerine mineral aminoasit şelat (ZnAA - MnAA) katkısının performans, yumurta kalitesi ve bazı kan parametreleri üzerine etkisini belirlemek amacıyla yapılmıştır. Çalışmada 320 adet 64 haftalık yaşta lohmann LSL beyaz yumurtacı tavuk 1 kontrol ve 3 uygulama grubu olacak şekilde 4 gruba ve her grup 20 tavuk içeren 4 tekerrür içermektedir. Kontrol grubu bazal rasyon ile, uygulama grupları ise 1, 2 ve 4 g/kg mineral amino asit şelat (ZnAA - MnAA) içeren bazal rasyonla 12 hafta beslenmiştir. Araştırma sonunda, canlı ağırlık, yem tüketimi, yemden yararlanma oranı, hasarlı yumurta oranı, ölüm oranı bakımından gruplar arasında istatistik açıdan bir farklılık görülmemiştir (P>0.05). Yumurta ağırlığı (P<0.01), ak ağırlığı (P<0.01), kabuk ağırlığı (P<0.05), sarı indeksi (P<0.01), sarı oranı (P<0.01), ak oranı (P<0.01), kabuk oranı (P<0.05) değerleri istatiski açıdan önemli bulunmuştur. Rasyona mineral aminoasit şelat (ZnAA - MnAA) ilavesi grupların kan serumu toplam protein, glukoz, toplam kolesterol, Kalsiyum (Ca), Fosfor (P), Çinko (Zn) değerlerini istatistiki açıdan etkilememiştir (P>0.05). Tüy skorlaması bakımından gruplar arasında farklılık sadece sırt bölgesi tüylerinde istatistiki açıdan önemli bulunmuştur (P<0.05). Ayrıca gruplar arasında tüy renklenmesi de istatistiki açıdan önemli bulunmuştur (P<0.01). Sonuç olarak, geç yumurtlama dönemindeki tavuk rasyonuna 1 g/kg mineral amino asit şelat katkısı (ZnAA-Mn AA) yumurta ağırlığını artırmış ve yumurta iç ve dış kalite özellikleri üzerine olumlu etkileri bulunmuştur.

Anahtar sözcükler: *Mn-Aminoasit, Şelat, Yumurta kalitesi, Yumurta verimi, Zn-Aminoasit*

INTRODUCTION

The drastic changes in egg quality is observed with the increasing flock age. As the hen ages, the thickness of the shell usually declines. Older flocks lay larger eggs, which break easily [1]. Maintaining eggshell quality is a complex activity, it is impossible, even with current knowledge, to correct all eggshell quality problems. Most of the scientific

İletişim (Correspondence)
+90 224 2941569
bilgehan@uludag.edu.tr

papers main topic is the improving the production and quality of eggs specially late laying period [2,3]. It has been suggested that part of losses in egg production are directly connected with low eggshell quality [4] and also the internal quality of eggs is very important for the consumers [5].

Traditionally trace minerals have been supplemented to animal diets as inorganic salts. However, because of their preintestinal absorption and after absorption in cellular metabolism due to higher bioavailability when compared with the same minerals in inorganic form, in recent years organic minerals have began to gain importance [6,7]. The term "organic mineral" is used to denote minerals forms chelated to an organic molecule, with the intention of increasing mineral bioavailability in animal diets [8].

Zn and Manganese are the main trace minerals involved in the metabolic process of eggshell formation. *Zn* plays an important role in poultry, particularly for layers, as a component of a number of metalloenzymes such as carbonic anhydrase which is essential for eggshell formation in the hens shell gland [9]. The Zn deficiency symptoms are suppressed immune system, poor feathering and dermatitis, infertility and poor shell quality in poultry. Deficiency of manganese could perosis, bone shortening [9] and increases incidence of thin-shelled and shell-less eggs in laying hens [10]. Therefore, supplementing the diet with highly bioavailable minerals like trace elements mineral-amino acid chelated or complexes were expecteted by increases the eggshell quality [2]. Researchers are reported that amino acids in the organic-mineral chelate are also used as a source of amino acid [11,12].

The trace minerals have been supplemented to animal diets as inorganic salts. In recent years, there has been studies on use of chelated or organic form of trace minerals in animal diets. This is because use of organic trace minerals in animal diets is improved growth, reproduction and health in ruminants, broilers and laying hens [13]. Lima *et al.*[14] reported that supplementation of Zn-Met chelate in single or in combination with Mn-Met chelate increased specific gravity of egg, eggshell strength and eggshell thickness. Park *et al.*[15] and Hudson *et al.*[16], in excess, Zn has detrimental effect on egg production, affecting the absorption of nutrients, and causing lesions in the pancreas and gizzard of laying hens. Abnormal accumulation of Zn in the liver, result with decrease in plasma Zn concentration [17].

Feather cover is important for its insulation value and protection from scratches and injury to the hen's skin. Severe feather pecking has been demonstrated in hens that were fed with low mineral, protein or amino acid level in the diet [18]. Increasing the dietary protein and amino acid contents resulted in improved plumage score [19].

Although many studies have demonstrated benefits of metal amino chelate on animal metabolism, but the research on their effects on late laying hen performence is limited. This study was conducted to determine the effect of supplementary mineral amino acid chelate (ZnAA - MnAA) on the performance, egg quality and some blood parameters of laying hens in the late laying period.

MATERIAL and METHODS

The experiment was carried out on 320 hens of Lohmann LSL white laying strain at the age of 64 weeks and experiment was conducted during 12 week. Before the experiment hens were placed cages for adaptation and controlled their egg production values during two week.

The hens were weighed on a digital scale and randomly assigned into 4 dietary treatment groups which are all reared at first floor of the battery cages. One control (0 g/kg) and three levels of L1 (1 g/kg), L2 (2 g/kg) and L3 (4 g/kg) mineral amino acid chelate containing diet were used to feed the hens in each group (n=80). The size of each cage was 50x45x45 cm. Each treatment group consisted of 4 replicates in 4 different cages (5 birds per cage).

All experimental procedures were in accordance with established standards for the care and use of animals for research purposes. The experiment was conducted under the protocol which was approved by Uludağ University Animal Use Local Ethical Committee (No: 2014-07-01).

Management

In the study a standard commercial layer diet was used for a basal diet (16% CP and 2.700 ME kcal/kg). The diet was formulated to meet or exceed National Research Council [20] specifications. The chemical composition of the basal diet fed to the hens were given in *Table 1*. The control group (Control) was feed with a basal diet and other groups were fed with basal diet supplemented with 1 (L1) g/kg, 2 (L2) g/kg and 4 (L3) g/kg mineral amino acid chelate (5.000 mg/kg Zn Amino acid - ZnAA, 3.500 mg/kg Mn Amino acid-MnAA and 85% $CaCO_3$ as carriers). During to trial, feed and water were offered adlibitum to all hens. The hens were placed into the cages and kept under 16L:8D light. The environmental conditions were the same for all groups.

Data

The laying hens were weighed individually with a digital scale (±1 g) at the beginning and at the end of the experiment. Eggs from each cage were collected 1 times a day. Egg production as hen day (EP), number of damaged eggs (cracked and soft-shelled eggs), mortality were monitored daily. Feed intake (FI) and egg weight (EW) were recorded on weekly basis. Based on the collected data, the basic production parameters (egg production, feed conversion ratio, and feed intake) were calculated. Egg mass (EM) was calculated with formula as EM=(EP*EW)/100. Feed conversion ratio (FCR) was calculated with

Table 1. *Chemical composition of diet used in the experiment*
Tablo 1. *Denemede kullanılan rasyonun kimyasal komposizyonu*

Ingredient,%	**Layer Diet**
Ground corn	64.74
Soybean meal (46% CP)	25.03
Dicalcium phosphate	1.42
Calcium carbonate	8.12
DL-methionine	0.02
NaCl	0.37
Vitamin and Trace mineral premix*	0.30
Chemical Composition	
Crude protein,%	16.0
ME, kcal/ kg	2700
Ca,%	3.00
Phosphorus,%	0.70
Metionin,%	0.33
Lysine,%	0.70
Cystine,%	0.31

** Ingredients in 1 kg of premix: Vitamin A 8.000 IU; Vitamin D_3 2.000 IU; Vitamin B_2 4 mg; Vitamin B_{12} 10 mg; Vitamin E 15 mg; Vitamin K_3 2 mg; Vitamin B_1 3 mg; Niacin 30 mg; Cal-D-pantothenic acid 10 mg; Vitamin B_6 5 mg; Folic acid 1 mg; D-biotin 0.05 mg; Vitamin C 50 mg; Choline Chloride 300 mg; Mn 60 mg; Zn 50 mg; Fe 60 mg; Cu 5 mg; Co 0.5 mg; Iodine 2 mg; Se 0.15 mg*

formula as FCR= FI/EM. The FI and FCR were determined for each replicate weekly.

Egg quality was assessed at 2, 4, 8, and 12th week of the experiment, eighty eggs from each treatment group were randomly collected to determine the egg interior and exterior quality parameters. Subsequently the eggs were weighed then broken, and the yolks were separated from the albumen. The chalazae were carefully removed from the yolk, using forceps, prior to weighing the yolk. The shells were carefully washed and dried 24 h in a drying oven at 105°C then weighed. Albumen weight was calculated by subtracting yolk and shell weights from the original egg weight. Eggshell thickness was measured at three different egg points (air cell, sharp end, and any side of the equator) using a caliper. An average of three different thickness measurements from each egg was used to estimate the eggshell thickness. The weights of egg components and shell thickness were measured to the precision of 0.01 g or 0.01 mm, respectively. The data of egg weight, yolk weight, shell weight (g) were recorded using digital scale.

The egg shape index (%) was determined by equipment developed by Rauch and egg shell strength (kg/cm^2) was measured by special equipment. Egg yolk diameter, albumen length, albumen width (mm) were measured with digital caliper.

The albumen and yolk height (mm) were measure using tripod micrometer. The proportion of eggshell, albumen and yolk were calculated as (shell or albumen or yolk weight/egg weight) x 100). Egg yolk index was calculated as (yolk height/yolk diameter) x100. Albumen index was calculated as (albumen height/(albumen length + albumen width)/2)) x 100. The Haugh unit (HU) was calculated by the formula HU = 100 Log (H + 7.57-1.7W $^{0.37}$), where W refers to measurements of egg weight (g) and H refers to albumen height (mm). The egg yolk color was measure using color fans [21].

At the end of the treatment blood samples were taken from nine hen from each treatment group for determination of serum total protein, glucose, total cholesterol, Ca, P and Zn level. Blood samples were taken from the wing vein and immediately centrifuged at 3.000 rpm for 15 min and the serum was removed in vacutainer tubes [2]. Serum levels of total protein, glucose, total cholesterol, Ca and P were determined by use of an Roche autoanalyser (Cobas 6000 series C501 module, Roche Diagnostic, Indianapolis, IN, USA) and Roche kits used. The serum Zn level was determined by use of atomic absorption spectrophotometer (Atomic Absorption Spectrophotometer AAnalyst 300, PerkinElmer, Shelton, CT, USA).

At the end of the study randomly selected 40 hens from each treatment group was observed for plumage condition and scored. Plumage condition was scored using a 4 point scoring system [22] for 6 different areas of the body (neck, breast, back, wings, tail, and vent). A score of 4 indicated very good feathering with few worn or otherwise deformed feathers. Score 3 was used when feathers showed deterioration but when complete feather coverage was observed. Score 2 indicated areas of the body that showed marked deterioration with some part being denuded. Score 1 indicated areas with little or no feather coverage and when feathers were present they were severely damaged.

The average plumage condition for each bird was calculated by adding over all 6 areas, to yield a total score ranging from 6 to 24 points. Also same person did a macroscopical subjective scoring of the melanin colorotion of feathers each individual hen at the end of the trial, giving a score between 3 - point scoring system for colured feathers of the body (back). A score 3 was used when feathers intensive coloured with light grey-dirty white colour. Score 2 was used when feathers few colured with light grey-dirty white colour. Score 1 indicated no any coloured feather on hen body.

Statistical Analysis

The parametric data (live weight, feed intake, egg production, egg weight, egg mass, damaged egg ratio, FCR, egg quality traits, blood plasma values) were analysed by using PROC GLM procedure of statistical analysis software (SAS v9.4) [23]. The non-parametric (Plumage score) data were analyzed with Wilcoxcon scores (Rank Sums) test

with using PROC NPAR1WAY procedure of SAS 9.4 and Kruskal Wallis test was used to determine the differences among groups [24]. Differences were considered significant at P≤0.05. Mortality data were analyzed with using PROC FREQ procedure and Chi-Square test was used to determine the differences among groups. All statistical analyzes were done with using SAS v9.4 [23].

RESULTS

The mean live weight, feed intake, hen day egg production, egg mass, feed conversion, damaged egg ratio and mortality ratio of treatment groups were shown in *Table 2*. There were no statistically differences among the groups in live weight, feed intake, FCR, damaged egg ratio, and mortality (P>0.05). The difference in hen day egg production, egg weight and egg mass were statistically significant (P<0.01).

The mean egg interior and exterior quality of treatment groups were given in *Table 3*. The highest yolk index was found in control group (P<0.01). The egg weight (P<0.01), albumen weight (P<0.01), shell weight (P<0.05), yolk ratio (P<0.01), albumen ratio (P<0.01), and shell ratio (P<0.05) were found significant in the treatment groups.

The mean blood plasma total protein, glucose, total

Table 2.*Mean live weight, egg production, feed intake, egg mass, FCR and mortality values of groups (n=80 hen)*
Tablo 2. *Grupların ortalama canlı ağırlık, yumurta üretimi, yem tüketimi, yumurta kütlesi, YYO ve ölüm oranı değerleri (n=80 tavuk)*

Traits	Diets[1]				P
	Control	L1	L2	L3	
Initial live weight, kg	1.74±0.04	1.81±0.05	1.77±0.03	1.83±0.04	n.s.
Final live weight, kg	1.88±0.04	1.86±0.04	1.82±0.03	1.87±0.05	n.s.
FI, g/d/hen	131.81±5.14	126.99±3.52	126.18±3.69	126.47±3.47	n.s.
EP, %	70.71±1.13^{a}	71.42±0.63^{a}	66.47±1.01^{b}	66.64±0.98^{b}	**
EM, g/d/hen	48.41±0.92^{ab}	51.00±0.46^{a}	45.35±0.73^{c}	46.49±0.72^{bc}	**
FCR	2.75±0.13	2.50±0.08	2.80±0.10	2.73±0.09	n.s.
Damaged Egg Ratio, %	3.02±0.67	2.66±0.12	3.20±0.74	2.32±0.31	n.s.
Mortality, %	5	5	0	2.5	n.s.

*$^{a-c}$within column, values with different superscript letters differ significantly (P<0.01); ** P<0.01; **n.s.:** not significant; [1] Control: Fed with basal diet, L1, L2, and L3: Fed with basal diet containing 1, 2 and 4 g/kg mineral amino acid chelate (Zn Amino acid - Mn Amino acid; ZnAA - MnAA), respectively; **EP:** Henday egg production, **FI:** Feed intake, **EM:** Egg mass, **FCR:** Feed conversion ratio, FCR= FI/EM*

Table 3.*Mean interior and exterior egg quality trait values of groups (n=80 egg)*
Tablo 3. *Grupların ortalama yumurta iç ve dış kalite özellik değerleri (n=80 yumurta)*

Traits	Diets[1]				P
	Control	L1	L2	L3	
Egg weight, g	68.71±0.43^{b}	70.96±0.61^{a}	68.61±0.56^{b}	69.38±0.59^{ab}	**
Yolk weight, g	19.16±0.16	18.92±0.16	18.69±0.14	19.07±0.18	n.s.
Albumen weight, g	43.45±0.38^{b}	45.78±0.48^{a}	43.82±0.46^{b}	44.35±0.47^{ab}	**
Shell weight, g	6.09±0.08^{ab}	6.26±0.07^{a}	6.10±0.06^{ab}	5.97±0.07^{b}	*
Shell Thickness, mm	0.3774±0.00	0.3776±0.00	0.3763±0.00	0.3706±0.01	n.s.
Shell breaking strength, kg/cm^{2}	0.85±0.09	0.78±0.07	0.88±0.07	0.71±0.08	n.s.
Shape index, %	75.38±0.25	75.08±0.28	74.44±0.27	74.81±0.28	n.s.
Albumen Index, %	7.61±0.15	7.58±0.19	7.66±0.17	7.54±0.16	n.s.
Yolk Index, %	45.07±0.29^{a}	43.82±0.36^{b}	43.48±0.34^{b}	43.56±0.31^{b}	**
Haugh Unit	75.80±0.78	75.55±1.04	76.74±0.89	76.27±0.88	n.s.
Yolk Color	12.08±0.17	11.65±0.15	11.91±0.13	11.66±0.16	n.s.
Yolk Ratio, %	27.94±0.25^{a}	26.72±0.21^{b}	27.30±0.18^{ab}	27.53±0.21^{a}	**
Albumen Ratio, %	63.19±0.26^{b}	64.45±0.22^{a}	63.80±0.20^{ab}	63.85±0.23^{ab}	**
Shell Ratio,%	8.87±0.10^{ab}	8.83±0.06^{ab}	8.91±0.07^{a}	8.61±0.08^{b}	*

*$^{a-c}$within column, values with different superscript letters differ significantly (P<0.05, P<0.01); * P<0.05; ** P<0.01; n.s.: not significant; [1] Control: Fed with basal diet, L1, L2, and L3: Fed with basal diet containing 1, 2 and 4 g/kg mineral amino acid chelate (Zn Amino acid – Mn Amino acid; ZnAA - MnAA), respectively*

cholesterol, Serum Ca, P and Zn values of groups were given in *Table 4*. The dietary supplementation of amino acid chelate (ZnAA - MnAA) were not significantly affected plasma total protein, glucose, total cholesterol, Ca, P, Zn (P>0.05).

The mean plumage score values of six regions of hen body and plumage coloring were given in *Table 5*. The only back region plumage score of hens was found significant (P<0.05). Plumage coloring was greater in all treatment groups and this difference was statictically significant (P<0.01). But numerically highest plumage coloring was found in 4 gr/kg mineral amino acid chelate (ZnAA-Mn AA) supplemented group (L3) and there was not any plumage coloring in control group.

DISCUSSION

Recently, organic trace minerals and especially mineral amino acid complex or chelate have become the focus of attention for the role in the high quality egg production of layers and breeders [11,25,26]. The organic trace minerals have a greater bioavability compared with inorganic trace minerals. This availability caused increased solubility and decreased interaction with other nutrients during absorption in gastrointestinal tract [27]. However mineral amino acid chelate should be supplemented to diet of layer hens for the optimal performance, in the present study suplementation of ZnAA - Mn-AA chelate on diet did not significally affect of groups live weight, feed intake, FCR, damaged egg ratio, and mortality (P>0.05). Rossi *et al.*[28] reported that increased level of organic Zn (0, 15, 30, 45 and 60 ppm) supplementation had no effect on live weight, feed intake, FCR and mortality in broilers which was similar to the results of our study. According to those authors, lack of consistent effects of dietary Zn on performance of birds may be due to the amount of Zn present in the basal diet or to the amount and sources such as phytate, which forms insoluble complexes with Zn and prevents its absorption, added. On the other hand, Ferket *et al.*[29] reported that 20 and 40 ppm Zn and Manganese-methionine supplementation to turkey diets improved FCR and also reduced mortality when compared to 80 ppm zinc and 120 ppm manganese as sulfates. The percentage of cracked egg was reduced by supplementation of Zn and manganese chelate to the layer diets depend on the significant effects of Zn and Manganese on carbonic anhydrase activity levels which is essential for eggshell formation, in the shell gland of laying hens [11].

In the present study, supplementation of ZnAA - MnAA on diet significantly affect hen day egg production and egg mass of treatment groups (P<0.01). It has been reported that the egg production [30] and egg weight [31]

Table 4. *Blood plasma values of groups (n=9 hen)*
Tablo 4. *Grupların kan plazma değerleri (n=9 tavuk)*

Traits	Diets[1]				P
	Control	L1	L2	L3	
Total Protein, g/dL	5.12±0.16	5.53±0.15	5.03±0.07	5.50±0.27	n.s.
Glucose, mg/dL	208.70±7.97	230.27±8.55	237.67±13.30	233.87±3.53	n.s.
Total Cholesterol, mg/dL	131.03±13.05	173.23±21.92	102.10±16.86	181.73±31.30	n.s.
Ca, mg/dL	25.83±0.62	28.73±0.77	23.80±1.73	28.30±1.74	n.s.
P, mg/dL	4.03±0.17	5.77±0.03	5.27±0.82	5.60±0.76	n.s.
Zn, µg/dL	421.67±41.47	423.33±13.64	496.67±22.31	398.33±14.53	n.s.

n.s.: *not significant;* [1] *Control: Fed with basal diet, L1, L2, and L3: Fed with basal diet containing 1, 2 and 4 g/kg mineral amino acid chelate (Zn Amino acid – Mn Amino acid; ZnAA - MnAA), respectively*

Table 5. *Mean plumage score and plumage coloring values of groups (n=40 hen)*
Tablo 5. *Grupların ortalama tüy scoru ve tüy renklenme değerleri (n=40 tavuk)*

Diets[1]	Body Region						Total	Plumage Coloring
	Neck	Breast	Vent	Back	Wings	Tail		
Control	2.30±0.15	2.40±0.18	2.25±0.16	2.30±0.31[b]	2.80±0.17	2.10±0.12	14.15±0.71	1.00±0.00[b]
L1	2.40±0.15	2.50±0.18	2.30±0.15	2.90±0.18[ab]	2.60±0.11	1.90±0.07	14.60±0.60	2.50±0.11[a]
L2	2.40±0.15	2.50±0.15	2.40±0.18	3.50±0.19[a]	2.40±0.11	1.90±0.07	15.10±0.54	2.50±0.15[a]
L3	2.60±0.21	2.80±0.17	2.60±0.18	3.20±0.23[ab]	2.70±0.15	2.20±0.14	16.10±0.75	2.70±0.12[a]
P	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	**

*[a,b] within row, values with different superscript letters differ significantly (P<0.05, P<0.01); * P<0.05; ** P<0.01;* ***n.s.:*** *not significant; Plumage scoring: 1 no feather - 4 feathered; Plumage coloring: 1 no color- 3 colored;* [1] *Control: Fed with basal diet, L1, L2, and L3: Fed with basal diet containing 1, 2 and 4 g/kg mineral amino acid chelate (Zn Amino acid – Mn Amino acid; ZnAA - MnAA), respectively*

were increased with supplementation of layers diets with organic form of Mn and Zn replacing inorganic forms of minerals, depend on higher bioavability of organic form of minerals. And also, Xavier *et al*.[32] verified beneficial effects on the performance and egg quality of brown layers during the second laying cycle with the use of organic selenium, Zn, and manganese combinations than inorganic forms. In contrast to our findings some researchers have found that supplementation of eggshell-49 (contained organic Mn and Zn chelate) did not affect performance of layers [14,33]. Mineral depended results were also reported by Lim and Paik [2] who have been found that egg production was increased by Cu-Met chelate supplementation but decreased by Zn-Met chelate supplementation. According to authors it might be due to negative interactions between minerals, such as; Zn has a strong antagonism with Cu. Also Bülbül *et al*.[34] found that both organic and inorganic sources of Zn and Mn decreased the oxidative stress in the laying hens, whereas organic Cu source increased it.

Park *et al*.[15] observed that at high dietary concentrations, Zn can reduce the use of calcium by the hen, because this element would be the first limiting factor for ovulation. In the present study better results in 1 mg/kg ZnAA - MnAA chelate group (L1) might be a result of sufficient requirement need may be met to the hens at this level. On the other hand, the similar results at higher levels of Zn in mineral amino acid chelate might be a result of disruption of feed ingredients in the digestive organs. Thus, Hudson *et al*.[16] observed that high level Zn supplementation to diets had detrimental effects on egg production affecting absorption of nutrients and causing lesions in the pancreas and gizzard of laying hens.

Deficiency of manganese increases incidence of thin shelled eggs [10]. The use of organic complexes of Zn and Mn could alleviate the negative effect of hen age on eggshell breaking strength [35]. However improvement of eggshell quality was expected by supplementation of Zn or Mn in diets. In the present study, the difference in egg weight ($P<0.01$), albumen weight ($P<0.01$), shell weight ($P<0.05$), yolk ratio ($P<0.01$), albumen ratio ($P<0.01$), and shell ratio ($P<0.05$) were found significant in the treatment groups. But egg shell thickness and shell breaking strength were similar to the control. A similar result have been reported by Nys *et al*.[36] and Mabe *et al*.[37] who have been observed that organic or inorganic Mn, Cu and Zn combinations supplemented with diets, did not affect egg shell quality. Zhao *et al*.[25] found that basal diet supplemeted with methionine trace mineral chelates improved egg quality and physiological function of laying hens, also yolk weight and proportion increased significantly. Ceylan and Scheideler [11] found that Mn and Zn chelate supplementation to layer diets increased egg shell quality after 40 wks of age but, egg production and FCR did not affected by supplementation of Mn and Zn chelate. Lima *et al*.[14] reported that supplementation of Zn-Met chelate in single or in combination with Mn-Met chelate increased eggshell strength and eggshell thickness. However Lim and Paik [2] found that supplementation of Zn-Met or its combination with other mineral chelates (Cu-Met, Mn-Met) had no beneficial effects on laying performance, but Zn-Mn-Met treatment showed significantly better eggshell strength than the control. Also Hudson *et al*.[38] found that dietary Zn in high concentration reduced shell thickness of eggs of hens at 66 weeks of age.

The abnormal accumulation of Zn in the liver causes decrease in plasma Zn concentration [17]. The plasma total protein was increased with Zn-Gly dietary supplementation in broilers [39] and Zn proteinat in laying hens [40]. Uyanik *et al*.[41] indicated that Zn supplementation decreased serum cholesterol concentration of broilers and Abd-El-Samee [42] of quails. However in the present study dietary supplementation of ZnAA - MnAA chelate were not significantly affected plasma total protein, glucose, total cholesterol, Ca, P, Zn level ($P>0.05$). Our results are in agreement with the findings of Güçlü and İşcan [43] who have been reported that organic Mn and Zn chelate supplementation did not affect live weight, feed intake, FCR, haugh unit and plasma Ca and P level. But in contrast to our findings organic Zn supplementation to broiler diets consistenly increased plasma Ca levels, that show that interactive effects between Zn and Ca metabolism [39,44]. Thus Dobrzanski *et al*.[45] found that organic form of Fe and Mn supplementation to diet did not cause any significant changes in the content of Fe, Cu, Mn and Zn in blood. Also Aksu Sarıpınar *et al*.[46] found that using at much lower level organic forms of minerals (Cu, Zn and Mn) in broiler diets instead of inorganic forms of those minerals did not created a negative impact on blood parameters.

Since feathers are 89-97% protein, dietary amino acids play a critical role in feather development. Severe feather pecking has been demonstrated in birds that were fed with low mineral, protein or amino acid levels in diet [18]. In the present study only back region plumage score of hens was found significant ($P<0.05$). The better results in treatment groups might be an effect of Zn and aminoacid on feather development. However, increasing the dietary protein and amino acid contents resulted in improved plumage condition [19]. Also supplementation of Zn had an effect on improved the feather score from poor to good [47]. In constrast to these results, it has been reported that dietary amino acid or trace mineral treatment did not show any significant effects on feather scores [12].

Studies have shown that there is a high level of correlation between the level of metal concentration in bird's diet and the level found in its feathers (melanic pigmentation) [48,49]. Dobrzanski *et al*.[45] found that organic form of Fe and Mn supplementation to diet resulted with significantly higher level of Mn concentration in feathers of hen than control group. Birds can eliminate heavy metals in their feathers. The darker portions of the

feather were enriched with Zn and Fe, which supports the suggestion that feather melanins are efficient ligands of some metals found in the environment, sequestering potentially harmful particles away from the body [50]. In consistent with Chatelain *et al*.[51] showed that darker individuals had higher Zn in their feathers and effectiveness of detoxification via melanic feathers may depend on level of metal intake. During to present study we have been observed that the hens white feather color was changed to grey-dirty white color. In the present study plumage coloring was greater in all treatment groups and this difference was statictically significant (P<0.01). But numerically highest plumage coloring was found in L3 group while there was not any plumage coloring in control group. Indeed, high levels of mineral amino acid chelate (ZnAA - MnAA) supplementation may have been resulted toxic effects and performance loss and reduction in egg quality could be considered due to this situation.

Animals absorb, digest and use mineral chelate better than inorganic form of minerals. This means that lower concentrations of organic trace minerals can be used in animal feeds. In addition, animals fed chelated sources of essential trace minerals excrete lower amounts in their feces and so there is less environmental contamination. The results of the current study showed that, the supplementation of 1 g/kg mineral amino acid chelate (ZnAA - MnAA) to diet increased egg weight, egg production and had favourable effects on interior and exterior egg quality of laying hens in the late laying period.

REFERENCES

1. Washburn KW: Incidence, cause, and prevention of eggshell breakage in commercial production. *Poult Sci*, 61, 2005-2012,1982. DOI: 10.3382/ps.0612005

2. Lim HS, Paik IK: Effects of supplementary mineral methionine chelates (Zn, Cu, Mn) on the performance and eggshell quality of laying hens. *Aust J Anim Sci,* 16, 1804-1808, 2003. DOI: 10.5713/ajas.2003.1804

3. Fernandes JIM, Murakami AE, Sakamoto MI, Souza LMG, Malaguido A, Martins EN: Effects of organic mineral dietary supplementation on production performance and egg quality of white layers. *Rev Bras Cienc Avic*, 10, 59-65, 2008. DOI: 10.1590/S1516-635X2008000100009

4. Klecker D, Zeman L, Jelinek P, Bunesova A: Effect of manganese and zinc chelates on the quality of eggs. *Acta Univ Agric Silvic Mendelianae Brun,* 50, 59-68, 2002.

5. Tumova E, Gous RM: Interaction between oviposition time, age, and environmental temperature and egg quality traits in laying hens and broiler breeders. *Czech J Anim Sci,* 57, 541-549, 2012.

6. Trindade Neto MA, Pacheco BHC, Albuquerque R, Schammass EA: Lysine and zinc chelate in diets for brown laying hens: Effects on egg production and composition. *R Bras Zootec*, 40, 377-384, 2011. DOI: 10.1590/S1516-35982011000200020

7. Aksu T, Özsoy B, Sarıpınar Aksu D, Yörük MA, Gül M: The effects of lower levels of organically complexed zinc, copper and manganese in broiler diets on performance, mineral concentration of tibia and mineral excretion. *Kafkas Univ Vet Fak Derg*, 17, 141-146, 2011.

8. Torres CA: Influence of maternal flock age, maternal trace mineral nutrition and incubation temperature on bone development of embryos and chicks. *PhD Thesis* of University of Alberta, Edmenton, Alberta, Canada, 2013.

9. Scheideler SE: Trace mineral balance in poultry. *Midwest Poultry Federation Convention, St. Paul, Minnesota,18-20 March, 2008.*

10. Scott ML, Nesheim MC, Young RJ: Nutrition of the Chicken. 3rd ed., 321, Ithaca, New York, USA, 1982.

11. Ceylan N, Scheideler SE: Effects of eggshell 49, dietary calcium level and hen age on performance and egg shell quality, biotechnology in the feed industry. *Alltech's 15th Annual Symposium in Biotechnology in the Feed Industry,* Lexington, USA, 20-22 April, 1999.

12. Scheideler SE, Weber S, Shields S, Stilborn H: Effects of trace mineral supplementation on egg production and feathering in laying hens. *Poult Sci,* 87, (Abstr.), 176-177, 2008.

13. Ayaşan T: Hayvan beslemede organik iz mineraller. *Çukurova Üniv Zir Fak Derg*, 22, 21-28, 2007.

14. Lima FR, Mabe I, Albuquerque R: Zinc and manganese additional supplementations may benefit egg production and quality. *Poult Sci,* 79, 46, 2000.

15. Park SY, Birkhold SG, Kubena LF, Nisbet DJ, Ricke SC: Review on the role of dietary zinc in poultry nutrition, immunity, and reproduction. *Biol Trace Elem Res,* 102, 147-163, 2004.

16. Hudson BP, Dozier III WA, Wilson JL: Broiler live performance response to dietary zinc source and the influence of zinc supplementation in broiler breeder diets. *Anim Feed Sci Technol,* 118, 329-335, 2005. DOI: 10.1016/j.anifeedsci.2004.10.018

17. Beisel WR: Single nutrients and immunity. *Am J Clin Nutr,* 35, 442-451, 1982.

18. Krimpen MM van: Impact of nutritional factors on eating behavior and feather damage of laying hens. *PhD Thesis,* Wageningen University, The Netherlands, 2008.

19. Al Bustany Z, Elwinger K: Comparison between barley/fish meal and maize/soybean meal based diets with various lysine and protein levels fed to different strains of laying hens. *Acta Agricult Scand*, 37, 41-49, 1987. DOI:10.1080/00015128709436542

20. NRC: Nutrient Requirements of Poultry. 9th rev. ed., National Academy Press, Washington, DC, 1994.

21. Sarıca M, Erensayın C: Tavukçuluk ürünleri. **In,** Türkoğlu M, Sarıca M (Eds): Tavukçuluk Bilimi Yetiştirme, Besleme, Hastalıklar. 3rd ed., 103-109, Bey Ofset Matbacılık, Ankara, 2009.

22. Tauson R: Plumage conditions in SCWL laying hens kept in conventional cages of different designs. *Acta Agric Scand*, 34, 221-230, 1984. DOI:10.1080/00015128409435391

23. SAS: SAS® v9.4 User's Guide. SAS Inst., Inc., Cary, NC, 2013.

24. Reijrink IAM, Van Duijvendijk LAG, Meijerhof R, Kemp B, van den Brand H: Influence of air composition during egg storage on egg characteristics, embryonic development, hatchability, and chick quality. *Poult Sci,* 89, 1992-2000, 2010. DOI: 10.3382/ps.2009-00610

25. Zhao B, Ding XM, Zhang KY, Chen DW: Effects of amino acid trace mineral chelates on egg quality and physiological function of laying hens. *J Sichuan Agric Univty*, 2, 238-243, 2005.

26. Favero A, Vieira SL, Angel CR, Bos-Mikich A, Lothhammer N, Taschetto D, Cruz RFA, Ward TL: Development of bone in chick embryos from Cobb 500 breeder hens fed diets supplemented with zinc, manganese, and copper from inorganic and amino acid-complexed sources. *Poult Sci*, 92, 402-411, 2013. DOI: 10.3382/ps.2012-02670

27. Cao J, Henry PR, Davis SR, Cousins RJ, Miles RD, Little RC, Ammerman CB: Relative bioavailabity of organic zinc sources based on tissue zinc and metallothionein in chicks fed conventional dietary zinc concentrations. *Anim Feed Sci Technol,*101, 161-170, 2002. DOI: 10.1016/S0377-8401(02)00051-2

28. Rossi P, Rutz F, Anciuti MA, Rech JL, Zauk NHF: Influence of graded levels of organic zinc on growth performance and carcass traits of broilers. *J Appl Poult Res,* 16, 219-225, 2007. DOI: 10.1093/japr/16.2.219

29. Ferket PR, Nicholson L, Robertson KD, Yoong CK: Effect of level of inorganic and organic zinc and magnesium on the performance and leg abnormalities of turkey toms. *Poult Sci,* 71 (Suppl. 1): 18 (Abstr.): 1992.

30. Klecker D, Zeman L, Siske V, Basauri GJ: Influence of trace mineral proteinate supplementation on egg shell quality. *Poult Sci*, 76 (Suppl. 1): 116 -131, 1997.

31. Zeman L, Klecker M, Lichovnikova M, Tvrznik P: Effect of proteinated trace mineral elements (eggshell-49) on reproductive traits of hens. *Alltech's 15th Annual Symposium in Biotechnology in the Feed Industry,* 20-22April, Lexington, USA, 1999.

32. Xavier GB, Rutz F, Dionello NJL, Duarte AD, Goncalves FM, Trouk NHF, Ribiero CLG: Performance of layers fed diets containing organic selenium, zinc and manganese during a second cycle of production. *20th Nutritional Biotechnology in the Feed and Food Industry Symposium,* 22-26 May, Lexington, USA, 2004.

33. Ceylan N, Scheideler SE, Sefton T: Effects of calcium level and chelate manganese and zinc on performance and egg quality in layers at different periods of lay. *XXI World's Poultry Congress,* 20-24 August, Montreal, Canada, 2000.

34. Bülbül A, Bülbül T, Küçükersan S, Şireli M, Eryavuz A: Effects of dietary supplementation of organic and inorganic Zn, Cu and Mn on oxidant/antioxidant balance in laying hens. *Kafkas Univ Vet Fak Derg*, 14, 1, 19-24, 2008.

35. Swiatkiewicz S, Koreleski J: The effect of zinc and manganese source in the diet for laying hens on eggshell and bones quality. *Vet Med Czech*, 53, 555-563, 2008.

36. Nys Y, Mabe I, Rapp C: Supplementation of a corn-soybean meal diet with manganese, copper and zinc from organic or inorganic sources improves eggshell mechanical properties in aged hens. *IX European Symposium on the Quality of Eggs and Egg Products,* 9-12 September, Kuşadası, Turkey, 2001.

37. Mabe I, Rapp C, Bain MM, Nys Y: Supplementation of a corn-soybean meal diet with manganese, copper, and zinc from organic or inorganic sources improves eggshell quality in aged laying hens. *Poult Sci*, 82, 1903-1913, 2003.

38. Hudson BP, Dozıer WA, Wilson JL, Sander JE, Wardt L: Reproductive performance and immune status of caged broiler breeder hens provided diets supplemented with either inorganic or organic sources of zinc from hatching to 65 wk af age. *J Appl Poult Res,* 13, 349-359, 2004.

39. Feng J, Ma WQ, Niu HH, Wu XM, Wang Y, Feng J: Effects of zinc glycine chelate on growth, hematological and immunological characteristics in broilers. *Biol Trace Elem Res,* 133, 203-211, 2010. DOI: 10.1007/s12011-009-8431-9

40. Bülbül T, Küçükersan S: Yumurta tavuğu rasyonlarına organik ve inorganik çinko katılmasının yumurta verimi ve kalitesi ile bazı kan parametreleri üzerine etkisi. *Vet Bil Derg,* 20, 53-60, 2004.

41. Uyanik F, Eren M, Atasever A, Tunçoku G, Kolsuz AH: Changes in some biochemical parameters and organs of broilers exposed to cadmium and effect of zinc on cadmium induced alterations. *Israel J Vet Med,* 56, 128-134, 2001.

42. Abd-El-Samee DL, El-Wardany I, Nematallah GA, Abo-El-Azab OM: Effect of dietary organic zinc and prebiotic on productive performance and immune response of growing quails. *Iranian J Appl Anim Sci,* 3, 761-767, 2013.

43. Güçlü KB, İşcan MK: Farklı düzeylerde kalsiyum içeren yumurta tavuğu rasyonuna eggshell-49 ilavesinin performans, yumurta kalitesi ve bazı kan parametrelerine etkisi. *Ankara Üniv Vet Fak Derg,* 51, 219-224, 2004.

44. Supplee WC, Combs GF, Blamberg DL: Zinc and potassium effects on bone formation, feathering and growth of poults. *Poult Sci,* 37, 63-67, 1958. DOI: 10.3382/ps.0370063

45. Dobrzanski Z, Korczynski M, Chojnacka K, Gorecki H, Opalinski S: Influence of organic forms of copper, manganese and iron on bioaccumulation of these metals and zinc in laying hens. *J Elementol*, 13, 309-319, 2008.

46. Aksu Sarıpınar D, Aksu T, Özsoy B: The effects of lower supplementation levels of organically complexed minerals (zinc, copper and manganese) versus inorganic forms on hematological and biochemical parameters in broilers. *Kafkas Univ Vet Fak Derg*, 16 (4): 553-559, 2010.

47. Salim HM, Lee HR, Jo C, Lee SK, Lee BD: Effect of sex and dietary organic zinc on growth performance, carcass traits, tissue mineral content, and blood parameters of broiler chickens. *Biol Trace Elem Res,* 147, 120-129, 2012. DOI: 10.1007/s12011-011-9282-8

48. Niecke M, Rothlaender S, Roulin A: Why do melanin ornaments signal individual quality? Insights from metal element analysis of barn owl feathers. *Oecologia*, 137, 153-158, 2003.

49. Burger J, Gochfeld M, Jeituer C, Snigaroff D, Snigaroff R, Stamm T, Volz C: Assessment of metals in down feathers of female common eiders and their eggs from the Aleutians: arsenic, cadmium, chromium, lead, manganese, mercury and selenium. *Environ Monit Assess,* 143, 247-256, 2008. DOI 10.1007/s10661-007-9973-y

50. Martin RR, Naftel SJ, Doucet SM, Hanleyd D, Weisner CG: Synchrotron radiation analysis in the study of pollution in the ring-billed gull (*larus delawarensis*): A novel application of the technique. *X-ray Spectrum,* 42,437-441, 2013. DOI: 10.1002/xrs.2500

51. Chatelain M, Gasparini J, Jacquin L, Frantz A: The adaptive function of melanin-based plumage coloration to trace metals. *Biol Lett,* 10, 3, 20140164, 2014. DOI: 10.1098/rsbl.2014.0164

Kafkas Univ Vet Fak Derg
21 (2): 163-167, 2015
DOI: 10.9775/kvfd.2014.11967

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Excretion of *Coxiella burnetii* in Cows with Secretion Disorder

Miodrag RADINOVIĆ [1] Stanko BOBOŠ [1] Marija PAJIĆ [1]
Annamaria GALFI [1] Branka VIDIĆ [2] Živoslav GRGIĆ [2]

[1] University of Novi Sad, Faculty of Agriculture, Department of Veterinary Medicine, SERBIA
[2] Scientific Veterinary Institute, Novi Sad, SERBIA

KVFD-2014-11967 Received: 12.07.2014 Accepted: 23.12.2014 Published Online: 24.12.2014

Abstract

Coxiella burnetii is the causative agent of a very important disease with zoonotic potential. Domestic ruminants are very important factor in transmission of this pathogen to humans. This pathogen can be found in feces, urine, lochia, placenta and milk of infected animals. Of all these routes, the excretion through milk lasts the longest, and can last over several lactations. In this paper, we examined the excretion dynamics of *Coxiella burnetii* through milk of seropositive cows and the levels of immunoglobulin G in milk and blood serum. Correlation between these parameters is also examined. Milk samples from infected cows were used for somatic cell count determination. The PCR method was used for determination of *Coxiella burnetii* genome. RID plates were used for determination of concentration of immunoglobulin. The excretion of the pathogen through milk differed between different stages of lactation: it was the lowest in the first stage (16.6%) and the highest in the second stage (80.0%). In the second stage the lowest concentration of immunoglobulin G was detected in milk serum (4.0 g). Somatic cell count was increased, some samples had very high values.

Keywords: *Coxiella burnetii, Cow, Immunoglobulin, Somatic cells*

Salgı Bozukluğu Olan İneklerde *Coxiella burnetii*'nin Atılımı

Özet

Coxiella burnetii zoonotik potansiyele sahip çok önemli bir hastalığa neden olan bir ajandır. Etkeni insanlara aktarmada evcil ruminantlar oldukça önemlidir. Etken enfekte hayvanların dışkı, idrar, vajinal akıntılar, plasenta ve sütlerinde bulunabilir. Tüm bu yollar arasında süt ile salınım en uzun süren salınım yolu olup birkaç laktasyon periyodu süresince devam edebilir. Bu makalede, seropozitif ineklerde süt yoluyla *Coxiella burnetii* atılımı ile süt ve kan serumunda immunglobulin G seviyelerinin dinamikleri incelendi. Ayrıca bu parametreler arasındaki ilişki de değerlendirildi. Enfekte ineklerden elde edilen süt örneklerinde somatik hücre sayımı yapıldı. *Coxiella burnetii* genomunu belirlemek amacıyla PCR metodu uygulandı. Immünoglobulin konsantrasyonunu belirlemek için RID plaklar kullanıldı. Patojenin süt ile salınımı laktasyon devreleri arasında farklılık gösterdi; birinci evrede en düşük olup (%80.0) ikinci evrede en yüksek seviyedeydi (%16.6). Süt serumunda immunglobulin G konsantrasyonu en düşük ikinci evrede belirlendi (4.0 g). Somatik hücre sayısı artmış olup bazı örneklerde çok yüksekti.

Anahtar sözcükler: *Coxiella burnetii, İnek, İmmunglobulin, Somatik hücre*

INTRODUCTION

Q fever is a zoonosis caused by an obligate intracellular microorganism, *Coxiella burnetii*. The disease is present throughout the world. The most important source for human infection is marked to be domestic animals. Dogs and cats are responsible for spread of disease primarily in urban areas. Ruminants are known to be the most important source of infection in humans [1,2]. In domestic animals, Q fever mostly passes as a latent disease and the commonest clinical symptoms are abortions and reduced fertility. In addition to these symptoms related to the reproductive tract, occurrence of pneumonia, mastitis and polyarthritis have also been observed [2]. The causative agent of Q fever - *Coxiella burnetii* is an immobile Gram negative bacterium, its life cycle is completed in phagosomes of infected cells [3]. It has a cell membrane similar to that of other Gram-negative bacteria. It is normally stained with Giemsa since they stain poorly with the Gram stain [4]. *Coxiella burnetii* is very virulent, so the infectious dose could be only one microorganism [5].

The pathogenesis of this disease is characterized with primary replication in the lymph nodes, followed

İletişim (Correspondence)
+38 160/597-3600
radinovic@polj.uns.ac.rs

by stage of bacteremia and after that, localization of agent in predilection organs: primarily in mammary gland and uterus in pregnant animals [6]. Localization of pathogens in the mammary gland is critical for long-term secretion through milk, so the cows can excrete the agents through milk more than a year and even during successive lactations [7], while secretion through feces and vaginal discharge lasts for a few weeks. Reservoirs of the agent are only partially known, but certainly include mammals, birds and arthropods, especially ticks. Although 40 species of ticks can be naturally infected with *Coxiella burnetii*, they obviously do not have a great importance for the infection of animals and humans [8]. However, the pathogen replicates in cells of the tick's gut and it is excreted in large number through the feces. Leather and wool contaminated with feces of ticks can be a source of infection either through direct contact or after inhalation of dry feces. The farm animals - cattle, sheep and goats - are the most common source for human infections. Pets like dogs, cats or rabbits can also be source of infection with *Coxiella burnetii*. There is a report on the occurrence of disease in humans as a result of direct or indirect contact with cats during parturition [9]. In the case of dairy cows, occupational exposure of humans to infection should be highlight. Most exposed are veterinarians, farmers, milkmen and workers in slaughterhouses and dairies. In the general population, categories at risk are smokers and immunocompromised persons [10]. Some authors [11] have also reported a significant association between seropositivity in humans and intake of non-pasteurized milk and milk products, whether people were in contact with animals or not.

While *Coxiella burnetii* is shed for extended periods in milk of dairy cattle, and has been shown to be immunogenic in dairy cattle, potential associations with clinical or subclinical mastitis only rarely have been examined [12]. A more recent report suggested that the prevalence of *Coxiella burnetii* infections was higher among dairy cattle with reproductive problems including mastitis [13]. For detection of *Coxiella burnetii* PCR method is highly sensitive and specific detection method that has been used previously to trace *Coxiella burnetii* in clinical samples. A PCR performed with primers based on a repetitive, transposonlike element (Trans-PCR) proved to be highly specific and sensitive, but extraction of DNA from milk samples took considerable effort and there was a high risk of contamination due to the numerous preparation steps [14]. The objective of this work was to explore the potential association between *Coxiella burnetii* detection in milk by PCR and elevated SCC in milk in successive lactation stages and also to recognize relation between immunoglobulin G in milk serum and PCR detection in milk. Findings of agent in milk does not always coincides with serological finding, so seronegative animals can also excrete *Coxiella burnetii* in milk [15].

MATERIAL and METHODS

Serological screening of blood serum samples for antibodies to *Coxiella burnetii* was performed on a farm with 200 holstein-friesian dairy cows by ELISA. Serum samples were tested for Q fever antibodies using the indirect ELISA kit (Idexx Switzerland, Switzerland), according to the protocol recommended by the manufacturer. Sera were prepared at 1:400 dilution, and specific antibodies were detected using a peroxidase-labeled anti-ruminant immunoglobulin G (IgG) conjugate. Results were expressed as a percentage of the optical density reading of the test sample (value), calculated as value = 100 × (S–N)/(P–N), where S, N, and P are the OD of the test sample, the negative control, and the positive control, respectively. Sera were considered to be ELISA positive if they had a value of 40% or more, suspect if the value was between 30% and 40%, and negative if the value was <30%.

Based on the results of the ELISA tests, an experimental group of cows serologically positive for *Coxiella burnetii* was formed. In total, the experiment included nine dairy cows. The cows were in good body condition and showed no clinical signs of disease. In beginning of experiment all cows were in first lactation, and three of them newer got pregnant again. From another six, who got pregnant, one had abortion and five had normal calving.

From the experimental animals, milk samples during lactation, pregnancy and the postpartum period were collected during regular milking. With use of true-testers, from each cow two milk samples were taken. One sample was used for performing PCR and for determining of immunoglobulin G concentration in milk serum. Another sample was used for determination of somatic cell count.

In total 65 milk samples were taken during different stages of lactation. Along with milk samples, blood samples were also taken for determination of presence of *Coxiella burnetii* in serum.

After arriving in the laboratory, milk samples were placed in an incubator for 24-48 h. Incubation is carried out at a temperature of 38°C to form coagulum and milk serum. Blood samples were stored in room temperature for 48 h to segregate the serum. The PCR method was used to determine the presence of *Coxiella burnetii* genome in milk and blood serum samples. For serum samples, a 200-µl sample volume was used. Cells were lysed with proteinase K (final concentration, 200 µg/ml) at 56°C overnight. DNA was prepared with a Prep-A-Gene purification kit (Bio-Rad, Munich, Germany) by using 10 µl of silica matrix. DNA was eluted from the silica matrix by adding 100 µl of Prep-A-Gene elution buffer. To increase the yield, DNA was eluted at 56°C for 5 min and centrifuged again. One microliter of supernatant containing DNA was used for amplification. Followed

primers were used: Trans1: 5'-TGGTATTCTTGCCGATGAC-3'; Trans 2: 5'-GATCGTAACTGCTTAAT AAACCG-3'.

To determine the concentration of immunoglobulin G in milk and blood serum immunodiffusion method with RID plates was used. The RID plates with monospecific antiserum to bovine immunoglobulin G were provided by INEP (Belgrade, Serbia). Sample of milk serum was poured in wells of RID plate, and after incubation for 48 h in room temperature reading of results was done. Reading was done by measuring the diameter of the precipitation ring. Diameter was measured by RID meter with an accuracy of 0.1 mm. The value obtained using the following formula calculates the concentration of immunoglobulin in the tested serum.

The formula for the calculation is: $C = ((R^2-b)/a) \times 30$, where R is the radius of precipitation ring, b is a constant whose value is 8.69, a is a constant with a value of 47.48. The resulting value is the concentration of immunoglobulin in the serum.

The correlation coefficient between IgG concentration and presence of *Coxiella burnetii* in milk serum was calculated using Statistica v. 7.5 software.

Research is approved by Ethics Commission to safeguard the welfare of experimental animals of the University of Novi Sad, number 01-153/7-3.

RESULTS

Processing of blood serum samples from 200 cows on tested farm by ELISA test has shown antibodies for *Coxiella burnetii* in 9 cows. These animals accounted for 4.5% of total herd.

From seropositive cows, 65 samples of milk serum were collected by successive lactation stages. The results of the analysis of these samples using the PCR method are shown in *Table 1*. During lactation, the excretion of bacteria was greatest in the second stage when 80% of milk serum samples were positive for *Coxiella burnetii*. In the colostrums stage, there was a high percentage of *Coxiella burnetii* excretion through milk (50% of positive milk serum samples). The lowest percentage of excretion through milk was in the first stage of lactation *(Table 1)*.

In *Table 2* presence of *Coxiella burnetii* in blood serum of infected cows is shown. From *Table 2* it can be seen that during all lactation stages there was a small oscillation in presence of agent in blood serum.

Concentration of immunoglobulin G in milk serum of infected cows is shown in *Table 3*. Highest concentration was in colostrums stage and significantly lower concentration was measured in successive lactation stages.

Somatic cell count in cumulative milk samples was measured during lactation stages and results are shown in *Table 4*. It is evident that SCC in milk samples from infected cows was increased during all lactation stages and some samples had very high values.

The correlation coefficient between presence of *Coxiella burnetii* genome in blood serum and excretion in colostrums and milk during all lactation stages was 0.072.

The correlation coefficient between excretion of *Coxiella*

Table 1. *Excretion of Coxiella burnetii through milk in different lactation stages*
Tablo 1. *Farklı laktasyon evrelerinde süt yoluyla Coxiella burnetii atılımı*

Stage of Lactation	Colostral Stage First 10 Days	First Stage 10-60 Days	Second Stage 60-180 Days	Third Stage Over 180 Days
Number of samples	4	8	20	33
C. burnetii excretion	50%	16.6%	80%	40.6%

Table 2. *Presence of Coxiella burnetii in blood serum in different lactation stages*
Tablo 2. *Farklı laktasyon evrelerinde kan serumunda Coxiella burnetii varlığı*

Stage of Lactation	Colostral Stage First 10 Days	First Stage 10-60 Days	Second Stage 60-180 Days	Third Stage Over 180 Days
Number of samples	8	21	30	6
C. burnetii in blood serum	50 %	66.6%	66.6%	48.6%

Table 3. *Concentration of immunoglobulin G in milk serum in different lactation stages*
Tablo 3. *Farklı laktasyon evrelerinde süt serum immünoglobülin G konsantrasyonu*

Stage of Lactation	Colostral Stage First 10 Days	First Stage 10-60 Days	Second Stage 60-180 Days	Third Stage Over 180 Days
Number of samples	4	8	20	33
IgG concentration g/L	153.1±31.2	5.0±0.9	4.0±2.2	8.4±5.4

Table 4. *Somatic cell count in cumulative milk samples from infected cows*
Tablo 4. *Enfekte ineklerin kümülatif süt örneklerinde somatik hücre sayısı*

Lactation Stage	First Stage 10-60 Days	Second Stage 60-180 Days	Third Stage Over 180 Days
SCC x 10^3/mL	147	526	1024
	778	1795	103
	2277	900	2000
	1004	588	1193
	419	169	836
	1092	685	236
	113	743	1236
	361	1485	1048
	-	1240	981
Average	773±711	903±512	961±558

burnetii through milk and of immunoglobulin G concentration in milk serum at each stage of lactation was 0.072.

DISCUSSION

Q fever disease caused by *Coxiella burnetii*, is an important zoonosis found worldwide. In humans, it causes a variety of diseases such as acute flu-like illness, pneumonia, hepatitis, and chronic endocarditis. In animals, *Coxiella burnetii* is found in the reproductive system, both uterus and mammary glands, and may cause abortion or infertility [16].

The high prevalence of *Coxiella burnetii* infection in dairy cattle with reproductive problems showed that these infected cattle play an important role in maintaining the infection and in disseminating the pathogenic agent to environment. Thus, such excretions (milk, colostrums, urine, and birth fluid) are considered to be potential sources of infection in animals and humans via inhalation of infectious aerosols or airborne dust [13].

According to our results *(Table 1)*, the lowest percentage of excretion of *Coxiella burnetii* was in the first stage of lactation, amounting to 16.6%. With the transition to the next stage of lactation, a striking increase in the percentage of excretion was noticed. In the second stage it was 80%. In the third stage there was decrease to 40.6%. In colostrums stage percentage of excretion through milk was 50%. Similar results are published by Rodolakis et al.[17] who claim that excretion of *Coxiella burnetii* through milk starts after eight to twelve weeks of lactation in most cows. This period coincides with second stage of lactation as we divide it.

In blood serum of infected cows presence of *Coxiella burnetii* was similar during all four lactation stages ranging from 48.6% to 66.6%. Presence of *Coxiella burnetii* genome in blood serum is consequence of lysis of infected cells by antibody-dependent system. So bacteria became free in blood serum [18]. Reflecting these percents to whole herd it can be calculated that 2.4% to 3.3% of all animals have positive blood serum to *Coxiella burnetii*. This is similar to findings of Kirkan et al.[19]. Concentration of immunoglobulin G in milk serum was highest in colostrums stage *(Table 3)*, this is in accordance with Leyton et al.[20]. Through the next two stages there was significantly lower concentration of immunoglobulin so the lowest was in second lactation stage (4.0 g/L). This is also the stage with highest excretion of *Coxiella burnetii* through milk. In third lactation stage there was increased immunoglobulin concentration. These values are in accordance with allegations of Bobos [21].

Analyzing values of somatic cell count in milk samples from infected cows it can be concluded that during whole lactation infected cows had increased number of somatic cells, with some samples having very high values. Barlow et al.[12] and Radinović et al.[22] had similar finding examining the milk from cows with *Coxiella burnetii* infection.

Infected cows shed *Coxiella burnetii* through milk during whole lactation with highest intensity in the second stage, while presence of pathogen in blood serum is similar in all lactation stages. Concentration of immunoglobulin G in milk serum corresponds to values in uninfected cows. Somatic cell count is increased in infected cows during all lactation stages.

Acknowledgement

Funding for this research is provided through project "Control of animal health in the production chain in terms of food safety" of Provincial Secretariat for Science and Technological Development, Autonomous Province of Vojvodina, Republic of Serbia, No. 114-451-3419.

REFERENCES

1. Arricau-Bouvery N, Rodolakis A: Is Q fever an emerging or re-emerging zoonosis. *Vet Res*, 36 (3): 327-349, 2005. DOI: 10.1051/vetres: 2005010

2. Vidić B, Boboš S, Savić S, Prica N: Findings of *Coxiella burnetii* in milk and her significance for human infection. *Contemp Agri*, 57 (3-4): 208-215, 2008.

3. Woldehiwet Z: Q fever (coxiellosis): epidemiology and pathogenesis. *Res Vet Sci*, 77 (2): 93-100, 2004. DOI: 10.1016/j.rvsc.2003.09.001

4. Gimenez DF: Staining rickettsiae in yolk-sack cultures. *Stain Technol,* 39 (3): 135-140, 1964.

5. McQuiston JH, Childs JE, Thompson HA: Q fever. *J Am Vet Med Assoc,* 221 (6): 796-799, 2002.

6. Babudieri B: Q fever: A zoonosis. *Adv Vet Sci*, 5, 82-182, 1959.

7. Muskens J, van Engelen E, van Maanen C, Bartels C, Lam TJ: Prevalence of *Coxiella burnetii* infection in Dutch dairy herds based on testing bulk tank milk and individual samples by PCR and ELISA. *Vet Rec*, 168 (3): 79, 2011. DOI: 10.1136/vr.c6106

8. Maurin M, Raoult D: Q fever. *Clin Microbiol Rev*, 12 (4): 518-553, 1999.

9. Marrie TJ, Raoult D: Update on Q fever, including Q fever endocarditis. *Curr Clin Top Infect Dis,* 22, 97-124, 2002.

10. Van Der Hoek W, Dijkstra F, Schimmer B, Schneeberger PM, Vellena P, Wijkmans C, ter Schegget R, Hackert V, van Duynhoven Y: Q fever in the Netherlands: An update on the epidemiology and control masures. *Euro Surveill*, 15 (12), pii: 19520, 2010.

11. Cerf O, Condron R: *Coxiella burnetii* and milk pasteurization: An early application of the precautionary principle? *Epidemiol Infect*, 134 (5): 946-951, 2006. DOI: 10.1017/S0950268806005978

12. Barlow J, Rauch B, Welcome F, Kim SG, Dubovi E, Schukken Y: Association between *Coxiella burnetii* shedding in milk and subclinical mastitis in dairy cattle. *Vet Res,* 39, 23, 2008. DOI: 10.1051/vetres:2007060

13. To H, Htwe KK, Kako N, Kim HJ, Yamaguchi T, Fukushi H, Hirai K: Prevalence of *Coxiella burnetii* infection in dairy cattle with reproductive disorders. *J Vet Med Sci,* 60 (7): 859-861, 1998. DOI: 10.1292/jvms.60.859

14. Lorenz H, Jager C, Willems H, Baljer G: PCR detection of *Coxiella burnetii* from different clinical specimens, especially bovine milk, on the basis of DNA preparation with a silica matrix. *Appl Environ Microbiol,* 64, 4234-4237, 1998.

15. Rousset E, Berri M, Durand B, Dufour P, Prigent M, Delcroix T, Touratier A, Rodolakis A: *Coxiella burnetii* shedding routes and antibody response after outbreaks of Q fever-induced abortion in dairy goat herds. *Appl Environ Microbiol,* 75 (2): 428-433, 2009. DOI: 10.1128/AEM.00690-08

16. Porter S, R, Czaplicki G, Mainil J, Guatteo R, Saegerman C: Q fever: Curent state of knowledge and perspectives of research of a neglected zoonosis. *Int J Microbiol*, 2011. DOI: 10.1155/2011/248418

17. Rodolakis A, Berri M, Héchard C, Caudron C, Souriau A, Bodier CC, Blanchard B, Camuset P, Devillechaise P, Natorp JC, Vadet JP, Arricau-Bouvery N: Comparison of *Coxiella burnetii* shedding in milk of dairy bovine, caprine, and ovine herds. *J Dairy Sci*, 90 (12): 5352-5360, 2007. DOI: 10.3168/jds.2006-815

18. Jerrells TR: Mechanisms of immunity to Rickettsia species and *Coxiella burnetii*. **In,** Walker DH (Ed): The Biology of the Rickettsiae. Vol. II. 79-100, CRC Press, Boca Raton, FL, 1988.

19. Kirkan S, Kaya O, Tekbiyik S, Parin U: Detection of *Coxiella burnetii* in cattle by PCR. *Turk J Vet Anim Sci*, 32 (3): 215-220, 2008.

20. Leyton W, Gapper, David EJ, Copestake, Don E, Otter, Indyk Harvey E: Analysis of bovine immunoglobulin G in milk, colostrums and dietary supplements: A review. *Anal Bioanal Chem*, 389 (1): 93-109, 2007. DOI: 10.1007/s00216-007-1391-z

21. Boboš S, Vidić B: Mlečna žlezda preživara- morfologija- patologija-terapija. Novi Sad: Poljoprivredni fakultet- Departman za veterinarsku medicinu, Naučni istitut za veterinarstvo „Novi Sad"; 2005.

22. Radinović M, Boboš S, Pajić M, Vidić B, Rašić Z, Davidov I, Špegar R: Blood count and number of somatic cells in milk of cows infected with *Coxiella burnetii*. *Vet Glasnik*, 65 (5-6): 367-374, 2011. DOI: 10.2298/VETGL1106367R

Kafkas Univ Vet Fak Derg
21 (2): 169-172, 2015
DOI: 10.9775/kvfd.2014.11977

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

A Novel Polymerase Chain Reaction to Detect *Brucella canis* in Dogs

Zeki ARAS [1] Mehmet TAŞPINAR [2] İbrahim AYDIN [3]

[1] Department of Microbiology, Faculty of Veterinary Medicine, Aksaray University, TR-68100, Campus, Aksaray - TURKEY
[2] Department of Medical Biology, Faculty of Medicine, Yuzuncu Yil University, TR-65080 Campus, Van - TURKEY
[3] Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Selçuk University, TR-42075 Campus Konya - TURKEY

KVFD-2014-11977 Received: 14.07.2014 Accepted: 01.10.2014 Published Online: 09.10.2014

Abstract

In this study, the specific polymerase chain reaction has been standardized and evaluated for the direct diagnosis of *Brucella canis* in vaginal swab samples from dogs. The specific primer sets are directed to the 16S-23S rRNA inter-space region of *Brucella* spp. and the deletion of 351 bp in BMEI1426-BMEI1427 in *B. canis*. A total of 21 references and field strains and 35 vaginal swab samples were used for the evaluation of the polymerase chain reaction. It found that polymerase chain reaction is positive for *B. canis* DNA indicated by only amplification of 214 bp product. It detected at least 2.7 x 10^1 CFU/g of bacteria diluted in vaginal swab samples indicates that the polymerase chain reaction can be used as a practical alternative for bacterial isolation. The novel polymerase chain reaction provides a simple and rapid for the detection of *B. canis* in clinical and field samples in one step and in short time about 24 h.

Keywords: *Specific PCR, Brucella canis, Vaginal swab samples*

Köpeklerde *Brucella canis*'in Teşhisi Amacıyla Yeni Polimeraz Zincir Reaksiyonu Metodunun Geliştirilmesi

Özet

Bu çalışmada, köpeklerin vajinal sıvap örneklerinden *Brucella canis (B. canis)*'in direk teşhisi amacıyla spesifik polimeraz zincir reaksiyonu metodu standardize edildi ve değerlendirildi. *Brucella* spp.'in 16S-23S inter-space bölgesine ve *B. canis*'in BMEI1426-BMEI1427 gen bölgesinde ki 351 bp'lik delesyon alanına özgü primer çiftleri seçildi. polimeraz zincir reaksiyonu yönteminin değerlendirilmesinde toplam 21 referans ve saha suşu ile 35 vajinal sıvap örneği kullanıldı. Sadece 214 bp'lik amplifiye polimeraz zincir reaksiyonu ürününün görüntülenmesi *B. canis* için pozitif olarak kabul edildi. Vajinal sıvap örneklerinden en az DNA tespit limiti yeni metot için 2.7 x 10^1 CFU/g bakteri olarak bulundu. Bu yöntem bakteriyel izolasyon metoduna pratik bir alternatif olabilir. Yeni polimeraz zincir reaksiyonu testi *B. canis*'in klinik veya saha örneklerinden 24 saat sürede ve tek adımda hızlı ve basit tespitini sağlamaktadır.

Anahtar sözcükler: *Spesifik PZR, Brucella canis, Vajinal sıvap örneği*

INTRODUCTION

Brucella (B.) canis is main etiologic agent of canine brucellosis and induces various reproductive failures in dogs and in human. It was first isolated from dogs in 1966 by Leland Carmichael. Canine brucellosis is an important disease because it causes the great economic losses in commercial breeding kennels and threats public health. The other smooth *Brucella* species *(B. abortus* and *B. melitensis)* have occasionally been isolated from dogs in various regions of the world [1]. Canine brucellosis is found most of the world but Australia and New Zealand appear to be free [1] and the global prevalence of the disease is unknown [2]. Normally, *B. canis* infections in humans have been seen through either laboratory accidents or contact with positive dogs [1,3,4].

The main clinical findings of canine brucellosis are reproductive failure such as late abortion, birth of weak offspring, epididymitis, orchitis, and testicular atrophy. Lymphadenitis is a common finding in both sexes, affecting most lymph nodes in the body [1,5]. The only definitive method for diagnosis of canine brucellosis is based on the isolation of *B. canis* from various clinical samples [3,6,7].

İletişim (Correspondence)
+90 382 2882941
zekiaras@hotmail.com

However, microbiological culture method has some disadvantages such as it is time consuming, includes complex tests, requires skilled personnel and is hazardous for laboratory workers [1,6,7]. Generally, serology can be used for diagnosis of infection but cross-reactions between *B. canis* and other bacteria can occur in some serological tests. Nonspecific agglutination reactions also cause false-positive results in some dogs [4,8,9].

In fact, the polymerase chain reaction (PCR) is a rapid, very specific, highly sensitive, and inexpensive technique for *Brucella* DNA detection. Hence, it is an alternative to bacteriological isolation for direct diagnosis of canine brucellosis [10,11]. Several studies were carried out in recent years in order to standardize PCR assays for the detection of *B. canis* DNA in various clinical samples including canine blood, semen, vaginal swab, blood serum, lymphoid tissue [11-16]. However, the PCR assays were used *Brucella* genus-specific primer pairs that were directed to the 16S-23S rRNA interspace and/or the *virB2* gene regions of *Brucella* spp. The Bruce-ladder multiplex PCR assay has been developed as a rapid and one-step molecular test for identification, typing of *Brucella* species and enhancing to distinguish between *B. suis* and *B. canis* [17,18]. However, this method is rarely used for direct detection of *Brucella* species DNA in clinical samples, because it was designed for bacterial isolates [19]. It is recently reported that, Bruce-ladder multiplex assay does not yield ideal results when DNA is extracted directly from clinical samples because of uneven amplification pattern [19].

With the above consideration in mind, the aim of this study is to use the novel PCR assay to detect *B. canis* in pure bacterial culture and vaginal swab samples, for the first time. The primers are directed to the 16S-23S rRNA interspace region of *Brucella* spp. and the deletion of 351 bp in BMEI1426-BMEI1427 in *B. canis* [15,20]. It proved that the PCR assay discriminates *B. canis* from other *Brucella* species and also detects the other *Brucella* species as *Brucella* spp.

MATERIAL and METHODS

Bacterial Strains

Strains examined in this study are lisited in *Table 1*. *Brucella* isolates were classified according to standard microbiological procedures, as described by Alton et al.[3].

Dogs

Vaginal swab samples were collected from 35 bitches from the city's pound of Konya Province of west-central Turkey in the years 2012. Breeds included Boxer (2), Golden Retriever (1), Kangal (3), and Mongrel (29). The ages of the dogs were in the range of 1 to 10 years. Clinical signs that suggested canine brucellosis were investigated in these dogs by questionnaire forms that were obtained from the Veterinarian of the city pound. These data stated that generalized lymphadenitis, metritis, vaginal discharge, still-birth, osteomyelitis, uveitis, lethargy, decreased appetite, weight loss, and hyperthermia were seen and some of the dogs were reported to have received antibiotics.

Table 1. *Strains examined in this study and PCR pattern*
Tablo 1. *Bu çalışmada kullanılan suşlar ve elde edilen PZR bantları*

Strain	Source	Biovar	PCR Result (bp)
Brucella canis	NCTC 10854	-	214
Brucella canis	field isolate 1	-	214
Brucella canis	Field isolate 2	-	214
Brucella canis	field isolate 3	-	214
Brucella canis	field isolate 4	-	214
Brucella abortus	S99	biovar 1	214. 774
Brucella abortus	field isolate	biovar 1	214. 774
Brucella abortus	field isolate	biovar 3	214. 774
Brucella abortus	field isolate	atypical	214. 774
Brucella melitensis	16M ATCC 10094	biovar 1	214. 774
Brucella melitensis	field isolate	biovar 1	214. 774
Brucella melitensis	field isolate	biovar 3	214. 774
Brucella melitensis	field isolate	atypical	214. 774
Brucella suis	Unknown	biovar 1	214. 774
Brucella ovis	ATCC 25840	-	214. 774
Brucella neotomae	ATCC 23459	-	214. 774
Rhizobium tropici	CIAT 899	-	-
Escherichia coli	field isolates	-	-
Pasteurella haemolytica	field isolates	-	-
Staphylococcus aureus	field isolates	-	-
Listeria monocytogenes	field isolated	-	-

Vaginal swab samples were collected in duplicate from the 35 bitches by sterile swabs. One of the swab samples was placed in a tube containing 2 mL of Brucella Broth (Sigma, B3051, MO, USA) for bacterial isolation. The other sample was put into a tube containing 2 mL of TE buffer (10mM Tris-HCl pH 8.0, 1mM disodium EDTA pH 8.0) and was kept at -20°C until used for the PCR assay.

Vaginal swab samples from three dogs were contaminated with *B. canis* NCTC 10854 (supplied by Refik Saydam Hifzissihha Institute, Ankara, Turkey) to determine the detection limit of the PCR assay. The dogs were previously diagnosed as negative to canine brucellosis by clinical, serological and culture examinations. Tested samples were transported to the laboratory under cool condition. The study protocol was approved by Selcuk University Veterinary Faculty Ethical Committee (2007/24).

Bacteriological Examinations

Samples were processed using the method described by Alton et al.[3]. Vaginal swab sample was immediately cultured onto Blood Agar Base (Oxoid, CM0271, Hampshire,

UK) plates containing 5% defibrinated sheep blood with an antibiotic mixture. The cultures were incubated at 37°C under aerobic conditions for 7 days. *B. canis* were identified by morphological and biochemical characteristics.

DNA Extraction

First, reference and field strains were grown in Brain Heart Infusion Broth (Oxoid, CM225, Hampshire, UK). Bacteria were killed by addition of 0.5% formaldehyde. After that DNA was extracted using the protocol provided in Promega Wizard Genomic DNA purification Kit (Promega, A1120, WI, USA). DNA concentration was determined spectrophotometrically (Eppendorf, Model 6131, Germany) by absorbance readings in the range of 260 to 280 nm.

B. canis DNA from vaginal swab samples was extracted using the protocol reported by Leal-Klevezas et al.[21]. Two mL of Tris EDTA (TE) buffer containing swab samples was used for this purpose. The DNA samples were kept at -20°C until used as templates for amplification.

PCR Assay

The two primer sets of the 16S-23S rRNA inter-space region of *Brucella* spp.[15] and the deletion of 351 bp in BMEI1426-BMEI1427 in *B. canis* [20] used in this study are recorded in *Table 2*.

The amplification reaction mixture was prepared in a total volume of 50 µL containing 5 µL of 10 x PCR buffer, 250 µM each of the four dNTPs (Fermentas, Vilnius, Lithuania), 1.5 mM $MgCl_2$, 1.5 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 0.5 µM of each primer (IDT, USA) and 5 µL of template DNA. The amplifications were performed in a thermal cycler (Ependorf, Mastercycler *gradient,* Germany) with the following steps: 1 × 7 min at 95°C, 30 × 45 s at 95°C, 45 s at 63°C, 120 s at 72°C, and a final extension at 72°C for 5 min. DNA extracted from *B. canis* NCTC 10854 and nuclease free water was served as positive and negative controls, respectively. The PCR product (10 µL) was further analyzed by electrophoresis on 2% agarose gel, and the gel was stained with ethidium bromide (1.5 µg/mL) and photographed. Reactions were considered positive for *B. canis* when they yielded unique PCR product of 214 bp but products of 214 and 774 bp were accepted positive for the other *Brucella* species.

Table 2. *Primers used in this study*
Tablo 2. *Bu çalışmada kullanılan primerler*

Primers	DNA Sequence (5'-3')	Length (bp)	Source
ITS66	ACATAGATCGCAGGCCAGTCA	214	Keid et al.[15]
ITS279	AGATACCGACGCAAACGCTAC		
BMEI1426	TCGTCGGTGGACTGGATGAC	774	Zygmunt et al.[20]
BMEI1427	ATGGTCCGCAAGGTGCTTTT		

Determination of Detection Limit of the PCR

Detection limit of the PCR assay was evaluated using *B. canis* NCTC 10854 reference strain. The suspension of the 72 h culture of *B. canis* was prepared in sterile saline and 10-fold dilutions (10^{-1} to 10^{-10}) for determine of colony-forming unit (CFU) and concentration of undiluted *B. canis* culture calculated as 2.1 x 10^7 CFU/mL by microbiological culture method. To determine the sensitivity of the assay, decreasing numbers of *B. canis* culture (methanol killed) were added to the 1 mL of the vaginal swab samples (obtained by three number of non-infected bitches). The final concentrations of *B. canis* in mixture were 2.1 x 10^7, 2.1 x 10^6, 2.1 x 10^5, 2.1 x 10^4, 2.1 x 10^3, 2.1 x 10^2 and 2.1 x 10^1 CFU/g. DNA extraction was performed with all dilutions of mixture, as described previously [21]. Then DNA samples processed by PCR assay as described above.

RESULTS

The PCR assay was evaluated with 21 reference and field isolates *(Table 1)*. All *B. canis* strains exhibited unique PCR band of 214 bp. However, other *Brucella* species such as *B. abortus, B. melitensis, B.ovis, B. suis* and *B. neotomae* showed 2 bands of 214 and 774 bp. No PCR products were amplified with DNAs from bacteria genetically related to *Brucella* such as *Rhizobium tropici* and other non-Brucella organism commonly associated with animals *(Table 1)*.

To determine the analytical sensitivity of the assay, decreasing numbers of *B. canis* were added to the vaginal swab samples. A positive PCR product for *B. canis* always achieved with different amounts containing at least 2.7 x 10^1 CFU/g of vaginal swab samples. The limit of PCR detection of *B. canis* was determined to be 2.7 x 10^1 CFU/g at least.

A total of 35 vaginal swab DNA samples were tested by the novel PCR assay. Namely, *B. canis* DNA was not detected from any vaginal swab samples in addition to that the samples were bacteriological negative.

DISCUSSION

Microbiological culture method and serological tests are widely used for diagnosis of canine brucellosis. The isolation of agent from different tissues of dogs is still considered as the gold standard for the definitive diagnosis of infection [3,7,22,23]. These methods have some disadvantages, while the PCR assay is fast, simple, highly sensitive and specific for detection of *B. canis* [11,15]. A very specific, highly sensitive and reliable diagnostic PCR assay for *B. canis* is very important for controlling the spread of infection in animal population and public health. In the present study, a species-specific PCR assay was designed and evaluated for detection and differentiation of *B. canis* in vaginal swab samples.

The primers that used in the novel PCR assay were directed to the 16S-23S rRNA inter-space region of *Brucella* spp. and the deletion of 351 bp in BMEI1426-BMEI1427 in *B. canis* [15,20]. This PCR assay discriminated *B. canis* from other *Brucella* species in a single reaction. The specificity of the species-specific PCR assay was explored with bacteria related to *Brucella* including *Rhizobium tropici, Escherichia coli, Pasteurella haemolytica, Staphylococcus aureus* and *Listeria monocytogenes (Table 1)* and it demonstrated a remarkable good specificity. However, *B. canis* is main etiological agent of canine brucellosis but the other smooth *Brucella* species *(B. abortus* and *B. melitensis)* have occasionally been isolated from dogs [1]. A major advantage of the present assay is that it can directly identify *B. canis* at species level and also detected all other *Brucella* species as *Brucella* spp. in clinical samples.

In our study, decreasing numbers of *B. canis* were added to the vaginal swab samples to determine the analytical sensitivity of the assay. The novel PCR assay detected at least 2.7 x 10^1 CFU/g of bacteria diluted in vaginal swab sample. This good analytical sensitivity agreed with the analytical sensitivity of *Brucella* genus-specific PCR assay from vaginal swab samples as previously described [14].

The degree of sensitivity of PCR assay is a key issue for its effective use in detection of brucellosis [24]. In our study, to detect the sensitivity and specificity of PCR assay, a total of 35 vaginal swab samples were tested with the PCR and culture methods. All samples were found negative for *B. canis* by two methods and in a full consistence with data between the PCR assay and culture method, the gold standard method for direct diagnosis of *B. canis*.

Finally, we concluded that, the novel species-specific PCR assay has been developed for the direct diagnosis of *B. canis* in vaginal swab samples from dogs. It was proved that the PCR is highly specific and sensitive for detection of *B. canis*. This technique open a new gate to detect alternative for bacterial isolation. We strongly believe that the PCR assay provides a simple and rapid tool for detection of *B. canis* in one step and one day. It is suitable for routine diagnosis of this disease and can be used for confirmation of *B. canis* cultures.

REFERENCES

1. Wanke MM: Canine brucellosis. *Anim Reprod Sci,* 82-83, 195-207, 2004.

2. Corbel M: Brucellosis: An overview. *Emerg Infect Dis*, 3, 213-221, 1997.

3. Alton GG, Jones LM, Angus RD, Verger JM: Techniques for the brucellosis laboratory. 149-153, INRA, Paris, 1988.

4. Köylü Ö, Aras Z, Uçan US: Seroprevalance of *Brucella canis* infection in a risky human population in province Konya. *Turkish J Infect,* 23, 73-77, 2009.

5. Carmichael LE, Kenney RM: Canine abortion caused by *Brucella canis*. *J Am Vet Med Assoc*, 152, 605-616, 1968.

6. Aras Z, Uçan US, Ateş M: Identification and biotyping of *Brucella* strains. *Eurasian J Vet Sci*, 25, 51-59, 2009.

7. Aras Z, Uçan US: Diagnosis of *Brucella canis* by Polymerase Chain Reaction. **In,** DeGiovine VM (Ed): Dogs: Biology, Behavior and Health Disorders. 201-218, Nova Science Publishers, New York, 2011.

8. Lucero NE, Escobar GI, Ayala SM, Lopez G: Sensitivity and specificity of an indirect enzyme-linked immunoassay for the diagnosis of *Brucella canis* infection in dogs. *J Med Microbiol,* 51, 656-660, 2002.

9. Uçan US, Aras Z, Zorlutuna M: Detection of canine brucellosis by a rapid agglutination test using *Rhizobium tropici* as antigen. *Revue Med Vet,* 161, 51-56, 2010.

10. Bricker BJ: PCR as a diagnostic tool for brucellosis. *Vet Microbiol,* 90, 435-446, 2002. DOI: 10.1016/S0378-1135(02)00228-6

11. Aras Z, Uçan US: Detection of *Brucella canis* from inguinal lymph nodes of naturally infected dogs by PCR. *Theriogenology,* 74, 658-662, 2010. DOI: 10.1016/j.theriogenology.2010.03.023

12. Kim S, Lee DS, Suzuki H, Watari M: Detection of *Brucella canis* and *Leptospira interrogans* in canine semen by multiplex nested PCR. *J Vet Med Sci*, 68, 615-618, 2006.

13. Keid LB, Soares RM, Vasconcellos SA, Chiebao DP, Megid J, Salgado VR, Richtzenhain LJ: A polymerase chain reaction for the detection of *Brucella canis* in semen of naturally infected dogs. *Theriogenology*, 67, 1203-1210, 2007. DOI: 10.1016/j.theriogenology.2007.01.003

14. Keid LB, Soares RM, Vasconcellos SA, Chiebao DP, Salgado VR, Megid J, Richtzenhain LJ: A polymerase chain reaction for detection of *Brucella canis* in vaginal swabs of naturally infected bitches. *Theriogenology*, 68, 1260-1270, 2007. DOI: 10.1016/j.theriogenology.2007.08.021

15. Keid LB, Soares RM, Vieira NR, Megid J, Salgado VR, Vasconcellos SA, da Costa M, Gregori F, Richtzenhain LJ: Diagnosis of canine brucellosis: Comparison between serological and microbiological tests and a PCR based on primers to 16S-23S rDNA interspacer. *Vet Res Commun*, 31, 951-965, 2007.

16. Keid LB, Soares RM, Vasconcellos SA, Salgado VR, Megid J, Richtzenhain LJ: Comparison of a PCR assay in whole blood and serum specimens for canine brucellosis diagnosis. *Vet Rec,* 167, 96-99, 2010. DOI: 10.1136/vr.c3811

17. Garcia-Yoldi D, Marin CM, de Miguel MJ, Munoz PM, Vizmanos JL, Lopez-Goni I: Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev.1. *Clin Chem*, 52, 779-781, 2006. DOI: 10.1373/clinchem.2005.062596

18. Lopez-Goni I, Garcia-Yoldi D, Marin MC, De Miguel MJ, Barquero-Calvo E, Guzman-Verri C, Albert D, Garin-Bastuji B: New Bruce-ladder multiplex PCR assay for the biovar typing of *Brucella suis* and the discrimination of *Brucella suis* and *Brucella canis. Vet Microbiol*, 154, 152-155, 2011. DOI: 10.1016/j.vetmic.2011.06.035

19. Sánchez-Jiménez MM, Ortiz-Román LF, Castrillón-Salazar LL, Giraldo-Echeverri CA, Olivera-Angel M: Application of a polymerase chain reaction test for the detection of *Brucella* canis from clinical samples of canines and humans. *Rev Colom Cienc Pecua*, 27, 3-11, 2014.

20. Zygmunt MS, Blasco JM, Letesson JJ, Cloeckaert A, Moriyon I: DNA polymorphism analysis of *Brucella* lipopolysaccharide genes reveals marked differences in O-polysaccharide biosynthetic genes between smooth and rough *Brucella* species and novel species-specific markers. *BMC Microbiol,* 13, 92-95, 2009. DOI: 10.1186/1471-2180-9-92

21. Leal-Klevezas DS, Martinez-Vazquez IO, Lopez-Merino A, Martinez-Soriano JP: Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. *J Clin Microbiol*, 33, 3087-3090, 1995.

22. Büyük F, Şahin M: Investigation of *Brucella* species from various samples of aborted cattle in Kars province (Turkey) by cultural and molecular methods and epidemiological analysis of cases. *Kafkas Univ Vet Fak Derg*, 17, 809-816, 2011.

23. Erdenliğ Gürbilek S, Baklan EA, Aksoy HY, Stack J: Comparative production of Rapid Slide Agglutination Test (RSAT) antigen used in serological diagnosis of *Brucella canis* in different culture media in the fermenter. *Kafkas Univ Vet Fak Derg*, 20, 273-278, 2014. DOI: 10.9775/kvfd.2013.10019

24. Mukherjee F, Jain J, Patel V, Nair M: Multiple genus-specific markers in PCR assays improve the specificity and sensitivity of diagnosis of brucellosis in field animals. *J Med Microbiol,* 56, 1309-1316, 2007. DOI: 10.1099/jmm.0.47160-0

Kafkas Univ Vet Fak Derg
21 (2): 173-178, 2015
DOI: 10.9775/kvfd.2014.11983

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Bazı Yabani Kuşların Beyin Dokularında *Toxoplasma gondii* ve *Neospora caninum*'un Moleküler Tanısı

Mustafa Necati MUZ [1] Özlem Orunç KILINÇ [2] Cafer Tayer İŞLER [3]
Enes ALTUĞ [3] Muhammet KARAKAVUK [4]

[1] Namık Kemal Üniversitesi, Veteriner Fakültesi, Parazitoloji Anabilim Dalı, TR-59030 Tekirdağ - TÜRKİYE
[2] Yüzüncü Yıl Üniversitesi, Özalp Meslek Yüksekokulu, TR-65800 Van - TÜRKİYE
[3] Mustafa Kemal Üniversitesi, Veteriner Fakültesi, Cerrahi Anabilim Dalı, TR-31001 Hatay - TÜRKİYE
[4] Ege Üniversitesi, Tıp Fakültesi, Parazitoloji Anabilim Dalı, TR-35040 İzmir - TÜRKİYE

Article Code: KVFD-2014-11983 Received: 15.07.2014 Accepted: 01.10.2014 Published Online: 09.10.2014

Özet

Çiftlik hayvanlarının ekonomik öneme sahip protozoonlarından *Toxoplasma gondii* ve *Neospora caninum*'un ara konak yabani kuşlardaki moleküler tanısı hakkında az sayıda araştırma bulunmaktadır. Türkiye'de bu parazitlerin yabani kuşların beyin dokularındaki moleküler prevalansı bilinmemektedir. Bu çalışmada, Türkiye'nin iki farklı ekocoğrafyasında bulunan 20 yabani kuş türüne ait 101 adet beyin dokusunda *T. gondii* ve *N. caninum*'un varlığı polimeraz zincir reaksiyonu (PZR) metodu ile araştırılmıştır. Araştırma sonucunda yabani kuşların beyin dokularında *T. gondii* %9, *N. caninum* %14, her iki türe aynı anda rastlanma oranı ise %4 olarak tespit edilmiştir. Elde edilen verilere Ki kare testi uygulanmıştır. Sonuç olarak bu çalışmada *T. gondii*'nin *Corvus corone (Gri leş kargası), Melanitta fusca (Kadife ördek), Aquila heliaca (Şah kartal), Aquila pomarina (Küçük orman kartalı), Buteo rufinus (Kızıl şahin), Accipiter nisus (Bayağı atmaca), Strix aluco (Alaca baykuş)*'un beyin dokularında ve *N. caninum*'un *Larus genei (İnce gagalı martı), Corvus corone, Melanitta fusca, Anas clypeata (Bayağı kaşık gaga), Perdix perdix (Çil keklik), Aquila heliaca* ve *Buteo rufinus*'un beyin dokularında PZR metoduyla dünya'da ilk tespiti yapılmıştır. Araştırma Türkiye'deki yaban kuşlarında *T. gondii* ve *N. caninum*'un moleküler tanısı amacıyla yapılan ilk çalışmadır.

Anahtar sözcükler: *Toxoplasma gondii, Neospora caninum, Yabani Kuş, Beyin, PZR*

Molecular Diagnosis of *Toxoplasma gondii* and *Neospora caninum* in Brain Tissues of Some Wild Birds

Abstract

There are limited molecular studies about *Toxoplasma gondii* and *Neospora caninum* which are economically important livestock protozoons in wild birds investigated by polymerase chain reaction (PCR) method. Molecular prevalance of both parasites in brain tissues of wild birds in Turkey is unknown. Prevalance of *T. gondii* was 7%, *N. caninum* was 14% and mix infection was found 4% in brain tissues of 101 wild birds under 20 species from two different regions of Turkey. The chi-square test has been applied to the acquired data. This is the first molecular biologic investigation for the aim of PCR diagnosis of *T. gondii* in brain tissues of *Corvus corone, Melanitta fusca, Aquila heliaca, Aquila pomarina, Buteo rufinus, Accipiter nisus, Strix aluco* and *N. caninum* in brain tissues of *Larus genei, Corvus corone, Melanitta fusca, Anas clypeata, Perdix perdix, Aquila heliaca, Buteo rufinus* in the world. This also is the first molecular diagnostic investigation of *T. gondii* and *N. caninum* in brain tissues of wild birds in Turkey.

Keywords: *Toxoplasma gondii, Neospora caninum, Wild bird, Brain, PCR*

GİRİŞ

Toxoplasma gondii ara konak insan dahil tüm sıcak kanlı canlılarda ve kuşlarda görülen, gebelerde abortlara yol açabilen, kedigillerin son konak olduğu sistemik protozoal bir enfeksiyon etkenidir. *Neospora caninum* ise son konak köpeklerde sinirsel bozukluk ve felç semptomlarına, ara konak sığır ve atlarda değişen oranlarda aborta neden olabilen protozoal bir enfeksiyon etkenidir [1,2]. Her iki parazit türünün dünyadaki prevalansı yüksektir. Özellikle evcil çiftlik hayvanlarında neden oldukları verim kayıpları ciddi ekonomik zarara yol açmaktadır [3].

Yabani kuş türleri *N. caninum* ve *T. gondii*'nin enfektif formlarını, avladıkları küçük hayvanlar, yedikleri leş, bit-

İletişim (Correspondence)
+90 282 2504714; Mobile: +90 532 5481532
mustafamuz@nku.edu.tr

kisel besin veya su gibi kontamine gıdalarla alırlar. Yırtıcı memelilerin besin zincirinde bulunan yabani kuş türleri *T. gondii* ile *N. caninum*'a konaklık yaparlar. Son yıllarda yapılan araştırmalar, kuş türlerinin *N. caninum* ve *T. gondii*'nin epidemiyolojisinde aldığı role dikkat çekmektedir [3,4]. Bıldırcın, keklik ve tavuklarda *N. caninum* ile yapılan deneysel enfeksiyon çalışmalarında kekliklerde mortalite oranı %100 olarak tespit edilirken bıldırcın ve tavuklarda sinirsel semptomlar ve doza bağlı değişen mortalite oranları bildirilmiştir [5-7]. Yabani kuşların beslenme ve üreme amacıyla farklı ekocoğrafyalarda bulunmaları, avlanan kuşların kısmen ya da tamamen doğaya atılması gibi faktörler *T. gondii* ve *N. caninum*'un epidemiyolojisinde önem kazanmaktadır [8-12].

Dünya'da *T. gondii* ve *N. caninum'un* yabani kuşlardaki moleküler tanısı ve prevalansı hakkında az sayıda araştırmaya rastlanırken Türkiye'ye ait moleküler tanı kaydına rastlanmamıştır. İspanya'da yapılan bir araştırmada 14 farklı kuş türüne ait 200 adet örnek incelenmesi sonucunda *T. gondii* %6, *N. caninum* %1.5 oranında PZR pozitif olarak bildirilmiştir [4]. İtalya'da üç farklı ördekgil türüne ait 103 örnekten üçünde *T. gondii* PZR pozitifliği saptanmıştır [13]. *Toxoplasma gondii* ve *N. caninum'un* kuşlardaki teşhisi genellikle parazitin konvansiyonel teknikler ile izole edilmesi veya serolojik metotlar ile yapılmaktadır. Araştırma konusu hakkında az sayıda moleküler biyolojik tanı çalışması bulunmaktadır. Bu araştırmada 20 farklı kuş türünün beyin dokularında *T. gondii* ve *N. caninum*'un moleküler tanısı PZR metodu ile araştırılarak bazı yabani kuş türlerine ait dünyadaki ilk veriler elde edilmiştir.

MATERYAL ve METOT

Çalışma Alanlarının Özellikleri

Ilıman Akdeniz iklimine sahip olan Hatay ili, 36°14 Kuzey ve 36°10 Doğu koordinatlarında yaklaşık 6.000 km^2 yüzölçümüne sahiptir. Cebelitarık boğazından sonra dünyada ikinci önemli göçmen kuş güzergahı üzerinde yer alan Hatay ilinde bulunan Amik Gölü ve Asi Nehri çevresinde çok sayıda ve dağınık sulak alanlar bulunmaktadır. Van ili karasal iklime sahip Doğu Anadolu bölgesinde, 38°29 Kuzey ve 43°40 Doğu koordinatlarında yaklaşık 19.000 km^2 yüzölçümüne sahiptir. Van ilinde yer alan dünyanın en büyük sodalı gölü olan Van Gölü çevresinde çok sayıda sulak alanlar bulunmaktadır. Her iki il sahip oldukları ekocoğrafya ile çok sayıda ve türde, yerli ve göçmen kuşun yaşamasına uygun habitat sağlamaktadır.

Çalışma Materyalini Oluşturan Kuşların Toplanması ve Türlerinin Tanımlanması

Bu araştırmada incelenen örnekler Hatay ve Van illerini temsil etmektedir. Hatay ilinde 2007-2014 yılları arasında ateşli silah ya da travma sonucu yaralanan 79 adet yabani kuş Mustafa Kemal Üniversitesi, Veteriner Fakültesi Hayvan Sağlığı Araştırma ve Uygulama Merkezinin cerrahi kliniğine getirilmiştir. Bu kuşlardan acil olarak müdahale edilen ancak tedavi ve operasyonlara rağmen kurtarılamayan 24 adeti araştırmaya dahil edilmiştir. Yüzüncü Yıl Üniversitesi, Yaban Hayatı Koruma ve *Rehabilitasyon* Merkezine acil tıbbi yardım amacıyla getirilen yabani kuşlardan tedavi ve operasyonlara rağmen kurtarılamayanlar ile ölü olarak bulunan *Anas clypeata*, *Melanitta fusca*, *Anser anser* türleri ve 2012-2013 yılı av sezonunda yakalanan yabani kuşlara ait toplam 77 adet kuşun baş kısımları araştırmaya dahil edilmiştir. Kuş türlerinin teşhisinde ilgili literatürlerden faydalanılmıştır [14-18]. Araştırmada yer alan türlerin sayısal dağılımı şöyledir. Van iline ait yabani kuş türü sekiz, örnek sayısı 77 olup, *Perdix perdi (n=14), Anser anser (n=7), Columba oenas (n=5), Pica pica (n=1), Anas clypeata (n=9), Melanitta fusca (n=3), Corvus corone (n=21), Larus genei (n=17) dir.*

Hatay iline ait yabani kuş türü 12, örnek sayısı 24 olup, *Aquila heliaca (n=3), Hieraaetus pennatus (n=2), Aquila pomarina (n=1), Buteo rufinus (n=5), Buteo buteo (n=4), Accipiter nisus (n=1), Phoenicopterus roseus (n=1), Ciconia ciconia (n=3), Caprimulgus europaeus (n=1), Apus apus (n=1), Strix aluco (n=1) ve Merops apiaster (n=1)*'dir.

Genomik DNA Ekstraksiyonu ve PZR

Araştırmada kullanılan örnekler -24°C'de ya da %70'lik etil alkol içerisinde muhafaza edilmiştir. Kuş kafalarının makroskopik temizliğinden sonra kafatasına yapılan ensizyon ile beyin dokusuna ulaşılmıştır. Her kuştan alınan 20 mg beyin dokusu örneği genomik DNA (gDNA) eldesinde kullanılmıştır. gDNA eldesi, bir ticari kitin (Thermo, GeneJET Genomic DNA Purification Kit) kendi kullanma kılavuzu araştırmaya uyarlanarak gerçekleştirilmiştir. Steril 1.5 ml hacimli şeffaf tüp (1.5 ml) içerisine alınan beyin dokusu örneğine, kit içeriğinde bulunan *digestion solution* 'dan 180 µl eklenerek steril çubuk ile ezilmiştir. Tüp içerisine 0.5 mm çapında steril cam boncuklardan 30 mg eklenerek, beyin dokusu parçacıklarının tamamen homojen hale gelmesi amacıyla tüpler 2 dk süre ile kuvvetli şekilde vortekslenmiştir. Her tüp oda ısısında bir dakika süre bekletilerek, üstteki sıvı steril tüp içerisine aktarılmış, kit içerisinde bulunan ve -24°C'de muhafaza edilen *Proteinaz K* enziminden 20 µl eklenen tüpler vortekslenerek, 56°C'de 6 saat süre ile ısı bloğunda bekletilmiş, saat başı vortekslenmiştir. Kit protokolü takip edilerek sırasıyla 20 µl *RNase A* solüsyonundan eklenmiş oda ısısında 10 dk bekletilmiştir. Kit içeriğinde bulunan *lysis buffer*'dan 200 µl eklenip 15 sn vortekslendikten sonra %50'lik etil alkolden 400 µl eklenerek vortekslenmiştir. Karışımın tamamı kit içeriğinde bulunan *GeneJet Genomic DNA purification column*'a aktarılarak mikrosantrifüjde 1 dk süre ile 6.000 g/dk. devirde santrifüj edilmiştir. Santrifüj sonucunda dolan sıvı toplama haznesi yenisi ile değiştirilmiştir. Kit içeriğinde bulunan *wash buffer I*'den tüplere 500 µl eklenerek 8.000 g/dk devirde tekrar santrifüj edilmiştir. Sıvı toplama haznesi yenisi ile değiştirilerek tüplere 500 µl *wash buffer'*II eklenerek 12.500 g/dk'da 3 dk süre ile santrifüj edilmiştir. Tüpler yeni 1.5 ml hacimli steril tüplere yerleştirildikten sonra 200 µl *elution buffer* eklenerek oda ısısında 5 dk süre bekletilmiş ve 10.000

g/dk'da 1 dk süre santrifüj edilerek genomik DNA örnekleri elde edilmiştir. Örnekler –24°C'de muhafaza edilmiştir.

Toxoplasma gondii'nin moleküler tanısı amacıyla nested PZR metodu kullanılmıştır. Hedef gen bölgesinin iki aşamalı eldesini sağlayan bu metot konvansiyonel PZR'dan daha özgün olduğu için tercih edilmiştir. Araştırmada *T. gondii* DNA sının 18S ssrRNA bölgesindeki B1 genini hedefleyen ve daha önce bildirilen primer çiftleri kullanılmıştır [19]. *Neospora caninum*'un moleküler tanısı tek aşamalı Nc5 genine spesifik primerlerin kullanıldığı konvansiyonel PZR metodu ile gerçekleştirilmiştir [20]. PZR koşulları ilgili literatürlere göre bazı değişiklikler yapılarak uyarlanmıştır [19,20]. Buna göre *T. gondii* spesifik nested PZR nin birinci aşamasında 5 µl 10xTaq bufer, 2.5mM $MgCl_2$, 0.2mM dNTP (Fermentas, Litvanya), 1.5U Taq DNA polimeraz (Fermentas, Litvanya), 0.3 µM forward ve reverse primerleri, 3 µl gDNA olarak toplam reaksiyon hacmi 50 µl olacak şekilde moleküler biyolojik kalitede su ile tamamlanarak hazırlanan karışım kullanılmıştır. PZR koşulları; 95°C de 5 dk, 94°C'de 1 dk, 50°C de 1 dk, 72°C de 1 dk kullanılarak toplam 35 döngü gerçekleştirilmiş ve son olarak 72°C de 5 dk tutularak birinci aşama tamamlanmıştır. Birinci aşama sonucunda elde edilen PZR ürünü ikinci aşama PZR'de kalıp DNA kaynağı olarak kullanılmıştır. İkinci aşamada PZR koşulları ve konsantrasyonları birinci basamak ile aynı, farklı olarak P3-P4 primerleri kullanılarak çalışılmıştır. *Neospora caninum* spesifik Np6 ve Np21 oligonükleotid primerlerin kullanıldığı reaksiyonda ise yukarıdaki hacimlerin aynıları kullanılarak bağlanma ısısı 59°C'de 1 dk olarak uygulanmıştır.

Amplifikasyon ürünleri elektroforezde, 0.5 µg/ml ethidium bromide içeren %1'lik agaroz jelde yürütülüp, UV transillimünatörde görüntülenerek, amplikon büyüklüklerine göre değerlendirilmiştir. *Toxoplasma gondii*'nin moleküler tanısı amacıyla yapılan nested PZR'ın birinci turunda kullanılan P1-P2 olügonükleotid primerleri ile 194 bp'lik bölgenin, ikinci turunda kullanılan P3-P4 primerleri ile 97 bp'lik kısmı ampliye edilmiştir [19]. *Neospora caninum* 337 bp bölgesi önceden bildirilen [20] Np6 ve Np21 oligonükleotid primerler kullanılarak amplifiye edilmiştir *(Tablo 1)*.

İstatistik Analiz

Bu araştırmada Van ilinden sekiz kuş türüne ait 77 adet örnek, Hatay ilinden ise 12 kuş türüne ait 24 adet örnek kullanılmıştır. Toplam kuş türü 20, toplam örnek sayısı ise 101 dir. Bu kuşların beyin dokularındaki varlığı tek ve miks olarak araştırılan *T. gondii* ve *N. caninum*'a rastlanma oranları arasındaki farklılıklar ki kare test istatistiği ile değerlendirilerek, tartışma bölümünde gerekli açıklamalara yer verilmiştir.

Yasal İzin

Doğa Koruma ve Milli Parklar Genel Müdürlüğünden 72784983-488.04-171653 sayılı yasal izni alınmıştır.

BULGULAR

Hatay ilinden örneklenen 12 tür yaban kuşunun iki türünde *N. caninum (Şekil 1)*, beş türünde *T. gondii (Şekil 2)* bir türde ise her iki parazit birlikte tespit edilmiş, yedi kuş türünde bu parazitler tespit edilememiştir.

Van ilinden örneklenen sekiz tür yaban kuşunun beş türünde *N. caninum*, iki türünde *T. gondii* ayrı ayrı, iki türde ise her iki parazit birlikte tespit edilmiş, üç kuş türünde bu parazitler tespit edilememiştir.

Bu araştırmada *Neospora caninum*'un *Larus genei, Corvus corone, Melanitta fusca, Anas clypeata, Perdix perdix, Aquila heliaca*, *Buteo rufinus* türü yabani kuşlara ait beyin örneklerinde dünyadaki ilk moleküler tanısı yapılmıştır.

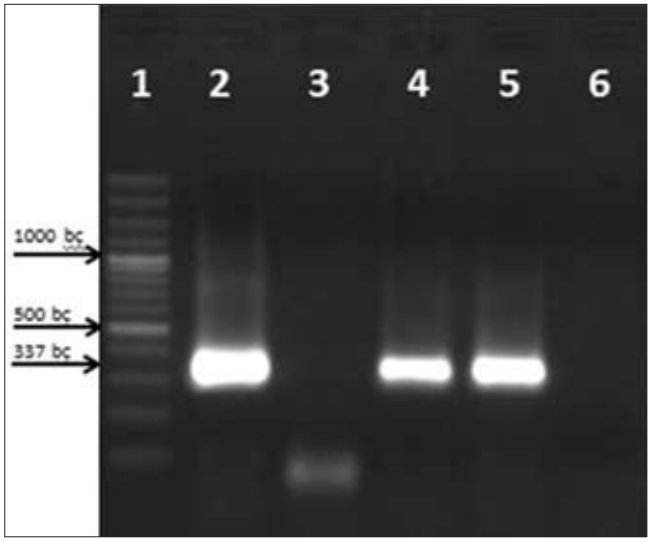
Toxoplasma gondii'nin ise *Corvus corone, Melanitta fusca, Aquila heliaca, Aquila pomarina, Buteo rufinus*, *Accipiter nisus, Strix aluco* türü yabani kuşlara ait beyin örneklerinde dünyadaki ilk moleküler tanısı gerçekleştirilmiştir.

Her iki parazite *Corvus corone, Melanitta fusca ve Buteo rufinus* türü yabani kuşlara ait beyin dokularında birlikte dünyada ilk defa rastlanmıştır.

Buna göre 20 farklı kuş türünün yedisinde *N. caninum*, yedisinde *T. gondii* tespit edilmiş, üçünde her iki parazit birlikte tespit edilmiş, 10 türde ise hiç birisi tespit edilememiştir *(Tablo 2)*. Van ve Hatay illerinde örneklenen 20 türe ait 101 adet yabani kuş türünde tek ya da miks olarak *T. gondii*, *N. caninum*'a rastlanma oranları arasında her üç grup için de istatiksel olarak anlamlı farklılıklar bulunmuştur (her üç grup $P<0.05$).

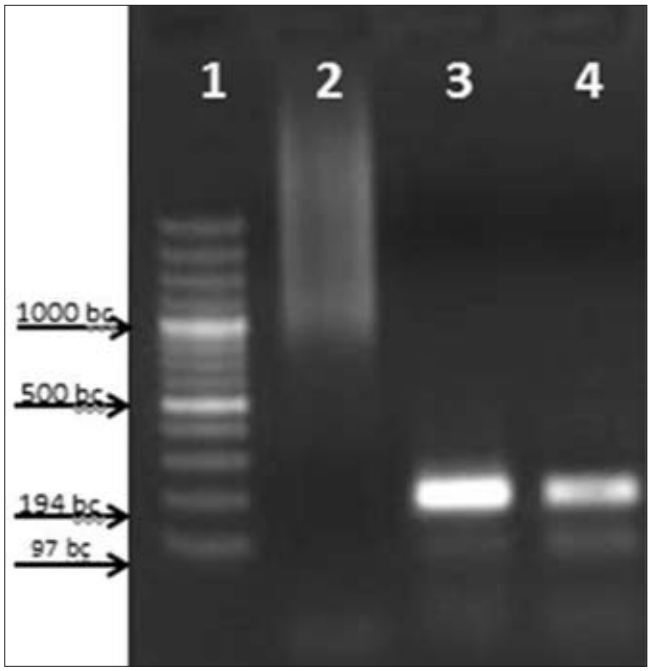
Tablo 1. *Primerlerin özellikleri*
Table 1. *Characteristics of the Primers*

Oligonükleotid Primerler	Dizilişi (5'-3')	Dizinin Yeri
P1 Outer primer (sense strand)	GGAACTGCATCCGTTCATGAG	694-714
P2 Outer primer (antisense strand)	TCTTTAAAGCGTTCGTGGTC	887-868
P3 Inner primer (sense strand)	TGCATAGGTTGCAGTCACTG	757-776
P4 Inner primer (antisense strand)	GGCGACCAATCTGCGAATACACC	853-831
Np6 Forward primer (sense strand)	CTCGCCAGTCAACCTACGTCTTCT	806-824
Np21 Reverse primer (antisense)	CCCAGTGCGTCCAATCCTGTAAC	550-568



Şekil 1. *Neospora caninum* jel elektroforezi sonucu elde edilen bantlar
1- DNA merdiveni, 2, 4- *N. caninum* pozitif bulunan örnekler, 3- *N. caninum* negatif bulunan örnek, 5- *N. caninum* pozitif kontrol, 6- Negatif kontrol

Fig 1. *N. caninum* bands on gel electrophoresis
1- Gene ruler DNA ladder, 2, 4- *N. caninum* positive samples, 3- *N. caninum* negative saples, 5- *N. caninum* pozitive control, 6- Negative control



Şekil 2. *Toxoplasma gondii* için yapılan elektroforez sonucu elde edilen bantlar
1- DNA merdiveni , 2- *T. gondii* negatif bulunan örnek, 3- *T. gondii* pozitif bulunan örnek, 4- *T. gondii* pozitif kontrol örnek

Fig 2. *T. gondii* bands on gel electrophoresis
1- Gene ruler DNA ladder, 2- *T. gondii* negative samples, 3- *T. gondii* positive samples, 4- *T. gondii* positive control

TARTIŞMA ve SONUÇ

Tıp ve veteriner hekimliğindeki önemi nedeniyle çok sayıda araştırmaya konu olan toksoplazmozis, Sağlık Bakanlığı'nın bildirime esas bulaşıcı hastalıklar listesinde "Grup C hastalıklar" bölümünde yer almaktadır. Hayvanlarda *T. gondii*'nin neden olduğu abortlar özellikle koyun ve keçilerde ciddi ekonomik kayıplara yol açmaktadır. Süt sığırlarının en önemli paraziter abort etkeni *N. caninum*'un A.B.D'inde yol açtığı ekonomik kayıp miktarı yılda 2.38 milyar dolardır [3]. İspanya'da *T. gondii* ye bağlı koyun abortları 4/74, *N. caninum*'a bağlı olanlar ise 5/74 oranında tespit edilmiştir. Keçilerde görülen abortlarda *N. caninum*'a 3/26, *T. gondii*'ye ise 1/26 oranında rastlanmıştır [24]. İnsanlar ve evcil hayvanlar açısından önemi oldukça fazla olan her iki parazitin de doğal yaşam döngüsünde yer alan yabani kanatlılar hakkında yapılan moleküler tanı ve prevalans çalışması çok azdır.

Bu araştırmada *T. gondii* ve *N. caninum* PZR ile dünyada ilk defa yedişer farklı kuş türünün beyin dokularında tek olarak, üç kuş türünde ise ikisi birlikte tespit edilmiştir. *Neospora caninum*'a en fazla oranda *Buteo rufinus*'ta, *T. gondii*'ye *Aquila pomarina, Accipiter nisus* ve *Strix aluco*'da rastlanmıştır. *Neospora caninum* ve *T. gondii*'ye en az oranda *Corvus corone*'da rastlanmıştır.

Benzer bir araştırma 2012 yılında İspanya'da yapılmış, on beş farklı türe ait toplam 201 adet yabani kuşun beyin dokusunun %6'sında *T. gondii* DNA'sı, %1.5'inde *N. caninum* DNA'sı tespit edilmiştir [4]. Mevcut araştırmada ise 20 farklı türe ait 101 adet yabani kuşun beyin dokusunun %7'sinde *T. gondii*, %14'ünde ise *N. caninum* DNA'sı tespit edilmiştir. İspanya'da *N. caninum* DNA'sının tespit edildiği iki kuş türü *Buteo buteo* ve *Pica pica* olup Türkiye'de her iki parazite ait DNA'ya bu kuşlarda rastlanmamıştır. İtalya'da yapılan bir araştırmada *Anas crecca*, *Anas platyrhynchos* ve *Anas clypeata* türü yaban kuşlarının beyinlerinde nested PZR metodu ile *T. gondii* pozitifliği tespit edilmiştir [13]. Mevcut araştırmada ise *Anas clypeata* türünde sadece *N. caninum* DNA'sı tespit edilmiştir.

Yırtıcı kuşların besin zincirinde yer alan fare gibi kemirgenler her iki parazit türü için konaklık yapabilen canlılardır. İngiltere'de PZR metodu ile beyin dokuları incelen ara konak farelerde *N. caninum* %3, *T. gondii* %53, ratlarda ise *N. caninum* %4.4, *T. gondii* %42.2 oranında tespit edilmiştir [22]. İtalya'da *N. caninum* DNA'sına ratlarda %13.6, farelerde ise %3.6 oranında rastlanmıştır [23]. Her iki parazitin yaşam döngüsünde yer alan bu kemirgenler de tıpkı kuşlar gibi kedilerin besin zincirinde yer almaktadır.

Sulak alanlarda yaşayan kuşlar da beslenmeleri esnasında bu parazitlere ara konak olabilecek böcek ve diğer küçük canlıları bol miktarda tüketir. Bu kuşları ve kemirgenleri avlayan yırtıcılardan olan kediler sulak alanlara yakın yerlere dışkılayarak *T. gondii* nin doğal döngüsüne katkı sağlar. Böylece dışkı parçalarının yağmur suları ya da diğer doğa etkenlerine bağlı olarak su ve yaşam kaynaklarını kontamine etmesi mümkün olur. Karadeniz Bölgesindeki dere ve içme suyu kaynaklarının *T. gondii* DNA'sı yönünden PZR metodu ile analiz edildiği bir araştırmada dere sularında *T. gondii* DNA'sına rastlanma oranı

Tablo 2. *Yabani kuşlarda T. gondii ve N. caninum'un moleküler prevalansı*
Table 2. *Molecular prevalance of T. gondii and N. caninum in wild birds*

Yörelere Göre Kuş Türleri			PZR Pozitif Örnek Sayısı		
Van İli Yaban Kuşları	**Türkçe Karşılığı**	**Örnek Sayısı**	***T. gondii***	***N. caninum***	**Miks**
Larus genei	İnce Gagalı Martı	17	-	3	
Corvus corone	Gri Leş Kargası	21	1	1	1
Melanitta fusca	Kadife Ördek	3	1	2	1
Pica pica	Saksağan	1	-	-	-
Anas clypeata	Bayağı Kaşık Gaga	9	-	3	-
Columba oenas	Gökçe Güvercin	5	-	-	-
Anser anser	Boz Kaz	7	-	-	-
Perdix perdix	Çil Keklik	14	-	1	-
Hatay İli Yaban Kuşları					
Aquila heliaca	Şah Kartal	3	1	1	-
Hieraaetus pennatus	Küçük Kartal	2	-	-	-
Aquila pomarina	Küçük Orman Kartalı	1	1	-	-
Buteo rufinus	Kızıl Şahin	5	1	3	1
Buteo buteo	Bayağı Şahin	4	-	-	-
Accipiter nisus	Bayağı Atmaca	1	1	-	-
Strix aluco	Alaca Baykuş	1	1	-	-
Caprimulgus europaeus	Çoban Aldatan	1	-	-	-
Ciconia ciconia	Leylek	3	-	-	-
Merops apiaster	Arı Kuşu	3	-	-	-
Apus apus	Ebabil	1	-	-	-
Phoenicopterus roseus	Flamingo	1	-	-	-

%35.7 olarak tespit edilmiştir [21]. Yabani kuşlar *T. gondii* ve *N. caninum*'a ait sporlanmış ookistleri ya da doku bradizoitlerini beslenme yolu ile alarak enfekte olabilmekte ve bu parazitlere ait DNA'ların, beyin dokusu gibi vücudun fizyolojik olarak korunaklı bölgelerinde PZR metodu ile tespit edilmesi tanıda değerli kabul edilmektedir.

Türkiye'de Niğde yöresinde evcil *(Columba livia domestica)* ve yabani (Columba livia livia) güvercinlerde *T. gondii*'nin seroprevalansının Sabin Feldman boya testi ile yoklanması amacıyla yapılan bir araştırmada sırasıyla %0.95 ve %0.90 oranlarında seropozitiflik tespit edilmiştir [25]. Kayseri yöresinde ise Sabin Feldman boya testi ile serolojik yoklaması yapılan yırtıcı kuşlardan 10 şahinin 1'inde (%10.00), 9 doğanın 1'inde (%11.11), 8 baykuşun 1 (%12.50) ve 9 atmacının 1'inde (%11.11) olmak üzere toplam 44 yırtıcı kuşun 4'ünde (%9.09) *T. gondii* spesifik antikorları tespit edilmiştir. Buna karşılık 7 kartal ve 1 balıkçılda seropozitiflik saptanamamıştır [26].

Yabani kuşlar, evcil kanatlılara göre birçok patojene karşı daha dayanıklıdırlar. Özellikle yabani göçmen kuşlar, taşıdıkları hastalık etkenlerini gittikleri yerlerdeki kanatlılara bulaştırarak, ekonomik kayıplara neden olabilmektedirler [27]. Sulak alanların yoğun olarak bulunduğu göl ve benzeri coğrafik yerler, bulaşıcı hastalıklar ve kontaminasyon tehlikesi açısından yüksek riskli noktalar arasında gösterilmektedir. Yabani kuşların önemli göç yolları üzerinde bulunan, sulak alan ve kuş türü zengini olan Türkiye'nin de bu açıdan değerlendirildiğinde önemli risk noktalarına sahip olduğu görülmektedir [28-31].

Yırtıcı kuşlar ya da nesli tükenmekte olan kuşların da yer aldığı araştırmalarda, kullanılacak örneklere ait kesin rakamların önceden belirlenememesi yaban hayatı dengesi ve yasal araştırma izinleri açısından önemlidir. Çünkü yaban hayatından elde edilen tür ve örnek sayıları arasında kesin bir denge kurulması kolay değildir. Bu nedenle benzer araştırma sonuçlarının istatistiki olarak değerlendirilmesi de bilimsel açıdan kolay olmamaktadır. Bu araştırmada enfeksiyonlara rastlanma oranları anlamlı çıkmış olsa da, yabani kuş türleri ve örnek sayılarının dengeli olmaması, bu sonuçların farklılıklara göre değerlendirilmesine engel olmaktadır. Örneğin bu araştırmada sadece bir adet rastlanan *A. pomarina* da *T. gondii* ye rastlanma oranı 1/1 olarak belirlenirken 17 adet örneklenen *L. genei* de *T. gondii*'ye rastlanma oranı 17/0 olarak bulunmuştur. Bu durum enfeksiyona rastlama yüzdesini %100 ya da %0 olarak ifade edilmesine neden olacaktır. *Tablo 2* incelendiğinde benzer örnekler olduğu görülmektedir. Ayrıca bu araştırmada yer alan Hatay ve Van illerine ait örnekler arasında tür benzerliği de bulunmamaktadır. Bu tip verilerin tür ve örnek

sayıları arasında tam bir denge kurulmadan istatistiksel değerlendirmeye tabi tutulması farklı deneysel hatalara ve bilimsel yanlışlıklara yol açacaktır. Bu nedenle verilerin istatistiki değerlendirilmesi araştırmayı inceleyecek bilim insanlarının değerlendirmesine bırakılmıştır.

Bu araştırma göçmen kuşların kuzey-doğu ve güney göç yollarında yer alan en önemli dinlenme, beslenme ve üreme alanlarından Van ve Hatay illerinde gerçekleştirilmiştir. Yabani kuşların tercih ettiği doğal sulak alanlar belli mevsimlerde göçmen kuşlar tarafından da tercih edilebilmektedir. Bu durum *T. gondii* ve *N. caninum*'un göçmen kuşlar, kanatlı kuş türleri ve diğer canlılar arasındaki bulaşma olasılığını yükseltmektedir.

Sonuç olarak yabani kuşların *T. gondii* ve *N. caninum*'un doğal yaşam döngüsü içerisinde önemli yeri olduğu ancak konu hakkında ileri araştırmalar gerektiği görülmektedir.

Teşekkür

Bu araştırmada Namık Kemal Üniv., Veteriner Fak., Bioistatistik Anabilim Dalı Başkanı Yrd.Doç.Dr. Doğan NARINÇ'e istatistiki analiz katkıları, Yüzüncü Yıl Üniv., Veteriner Fak., Patoloji Anabilim Dalı Öğretim Üyesi Yrd Doç.Dr. Serkan YILDIRIM'a nekropsi çalışması, 19 Mayıs Üniv., Veteriner Fak., Patoloji Anabilim Dalı Öğretim Üyesi Yrd Doç.Dr. Yonca BETIL KABAK'a *Toxoplasma gondii* örneği, Kırıkkale Üniv., Veteriner Fak., Patoloji Anabilim Dalı Başkanı Prof.Dr. Oğuz KUL'a *Neospora caninum* örneği gönderdikleri için teşekkür ederiz.

KAYNAKLAR

1. Altay K, Dumanlı N: Neosporosis, Toxoplasmosis. *Türkiye Klinikleri J Vet Sci*, 3 (2): 21-26, 2012.

2. Leon A, Richard E, Fortier C, Laugier C, Fortier G, Pronost S: Molecular detection of *C. burnetii* and *N. caninum* in equine aborted foetuses and neonates. *Prev Vet Med*, 1 (1-2): 179-183, 2012. DOI: 10.1016/j.prevetmed.2011.11.001

3. Reichel MP, Alejandra Ayanegui-Alcérreca M, Gondim LF, Ellis JT: What is the global economic impact of *N. caninum* in cattle - The billion dollar question. *Int J Parasitol*, 43 (2): 133-142, 2013. DOI: 10.1016/j.ijpara.2012.10.022

4. Darwich L, Cabezón O, Echeverria I, Pabón M, Marco I, Molina López R, Alarcia-Alejos O, López-Gatius F, Lavín S, Almería S: Presence of *T. gondii* and *N. caninum* DNA in the brain of wild birds. *Vet Parasitol*, 10 (3-4): 377-381, 2012. DOI: 10.1016/j.vetpar.2011.07.024

5. Mansuorian M, Namavari M, Khodakaram-Tafti A, Rahimian A: Experimental *N. caninum* infection in domestic bird's embryonated eggs. *J Parasitic Dis*, 37, 1-4. 2013. DOI: 10.1007/s12639-013-0327-0

6. Mansourian M, Khodakaram-Tafti A, Namavari M: Histopathological and clinical investigations in *N. caninum* experimentally infected broiler chicken embryonated eggs. *Vet Parasitol*, 23 (3-4): 185-190, 2009. DOI: 10.1016/j.vetpar.2009.09.041

7. Furuta PI, Mineo TW, Carrasco AO, Godoy GS, Pinto AA, Machado RZ: *N. caninum* infection in birds: Experimental infections in chicken and embryonated eggs. *Parasitology*, 134 (14): 1931-1939, 2007.

8. Bartels CJ, Wouda W, Schukken YH: Risk factors for *N. caninum* associated abortion storms in dairy herds in The Netherlands (1995 to 1997). *Theriogenology*, 15 (2): 247-257, 1999. DOI: 10.1016/S0093-691X(99)00126-0

9. Otranto D, Llazari A, Testini G, Traversa D, Frangipane di Regalbono A, Badan M, Capelli G: Seroprevalence and associated risk factors of neosporosis in beef and dairy cattle in Italy. *Vet Parasitol*, 1 (1-2): 7-18, 2003. DOI: 10.1016/j.vetpar.2003.10.008

10. McGuire AM, McAllister M, Wills RA, Tranas JD: Experimental inoculation of domestic pigeons *(C. livia)* and zebra finches *(P. guttata)* with *N. caninum* tachyzoites. *Int J Parasitol*, 29 (10): 1525-1529, 1999. DOI: 10.1016 S0020-7519(99)00103-4

11. Mineo TW, Carrasco AO, Raso TF, Werther K, Pinto AA, Machado RZ: Survey for natural *N. caninum* infection in wild and captive birds. *Vet Parasitol*, 15 (2-4): 352-355, 2011. DOI: 10.1016/j.vetpar.2011.05.022

12. Elmore SA, Jones JL, Conrad PA, Patton S, Lindsay DS, Dubey JP: *Toxoplasma gondii*: Epidemiology, feline clinical aspects, and prevention. *Trends Parasitol*, 26 (4): 190-196, 2010. DOI: 10.1016/j.pt.2010.01.009

13. Mancianti F, Nardoni S, Mugnaini L, Poli A: *T. gondii* in Waterfowl: The First Detection of this parasite in *A. crecca* and *A. clypeata* from Italy. *J Parasitol*, 99 (3): 561-563, 2013.

14. Heinzel H, Fitter R: Türkiye ve Avrupa'nın Kuşları. Doğal Hayatı Koruma Derneği Yayınları, İstanbul, 1995.

15. Kiziroğlu İ: Türkiye Kuşları Cep Kitabı. GeoTurka, İstanbul, 2013.

16. Kiziroğlu İ: Türkiye Kuşları. Orman Genel Müdürlüğü Yayınları. Ankara, 1989.

17. Porter RF, Christensen S, Schiermacker-Hansen P: Türkiye ve Orta-doğu'nun Kuşları. Uzerler Matbaası, Ankara, 2009.

18. Svensson L, Mullarney K, Zetterström D, Grant PJ: Collins Bird Guide, 2nd ed., Harper Collins Publishers Ltd, Printer, Trento, Italy. 2010.

19. Jones CD, Okhravi N, Adamson P, Tasker S, Lightman S. Comparison of PCR detection methods for B1, P30, and 18S rDNA genes of *T. gondii* in aqueous humor. *Invest Ophthalmol Vis Sci*, 41 (3): 634-644, 2000.

20. Müller N, Zimmermann V, Hentrich B, Gottstein B: Diagnosis of *N. caninum* and *T. gondii* infection by PCR and DNA hybridization immunoassay. *J Clin Microbiol*, 34 (11): 2850-2862, 1996.

21. Koloren Z, Demirel E: Detection of *T. gondii* in Turkish river and drinking water samples by different PCR and LAMP methods. *Clean - Soil Air Water*, 41 (10): 963-968, 2013. DOI: 10.1002/clen.201200468

22. Hughes JM, Williams RH, Morley EK, Cook DA, Terry RS, Murphy RG, Smith JE, Hide G: The prevalence of *N. caninum* and co-infection with *T. gondii* by PCR analysis in naturally occurring mammal populations. *Parasitology*, 1328 (1): 29-36, 2006. DOI: 10.1017/S0031182005008784

23. Ferroglio E, Pasino M, Romano A, Grande D, Pregel P, Trisciuoglio A: Evidence of *Neospora caninum* DNA in wild rodents. *Vet Parasitol*, 30 (148): 346-349, 2007. DOI: 10.1016/j.vetpar.2007.06.031

24. Moreno B, Collantes-Fernández E, Villa A, Navarro A, Regidor-Cerrillo J, Ortega-Mora LM: Occurrence of *Neospora caninum* and *Toxoplasma gondii* infections in ovine and caprine abortions. *Vet Parasitol*, 8 (1-2): 312-318, 2012. DOI: 10.1016/j.vetpar.2011.12.034

25. Karatepe M, Kılıç S, Karatepe B, Babür C: Niğde yöresinde evcil *(Columba livia domestica)* ve yabani güvercinlerde *(Columba livia livia) Toxoplasma gondii* antikorlarının prevalansı. *Türkiye Parazitol Derg,* 35, 23-26, 2011. DOI: 10.5152/tpd.2011.06

26. İnci A, Babür C, Çam Y, İça A: Kayseri yöresinde bazı yırtıcı kuşlarda Sabin-Feldman boya testi ile *Toxoplasma gondii* (Nicolle ve Manceaux, 1908) seropozitifliğinin araştırılması. *F Ü Sağlık Bil Derg*, 2, 177-179, 2002.

27. Sentürk B, Güler H: Financial effects of HPAI H5N1 cases on backyard poultry in the Kızılırmak Delta. *Kafkas Univ Vet Fak Derg*, 20 (1): 73-78, 2014. DOI: 10.9775/kvfd.2013.9454

28. Boynukara B, Gulhan T, Adizel O, Ilhan Z, Aksakal A, Durmus A, Ekin IH, Ogun E, Coven F, Solmaz H: Determination of Avian Influenza A Viruses in some avian species in Van Lake basin by Real Time-PCR, isolation and subtyping. *J Anim Vet Adv,* 10 (4): 502-510, 2011. DOI: 10.3923/javaa.2011.502.510

29. Hénaux V, Samuel MD: Avian influenza shedding patterns in waterfowl: Implications for surveillance, environmental transmission, and disease spread. *J Wild L Dis.* 47(3): 566-578, 2011.

30. Mehrabanpour MJ, Rahimian A, Shirazinezhad A, Moein H, Shayanfar MA: Avian influenza virus in migratory and resident birds during migratory season in Boushehr, Iran. *TurkJ Vet Anim Sci,* 36 (4): 446-450, 2012. DOI: 10.3906/vet-1105-25

31. Hénaux V, Samuel MD, Dusek RJ, Fleskes JP, Ip HS: Presence of avian influenza viruses in waterfowl and wetlands during summer 2010 in California: Are resident birds a potential reservoir? *PLoS One,* 7 (2): 2012. DOI: 10.1371/journal.pone.0031471

Kafkas Univ Vet Fak Derg
21 (2): 179-186, 2015
DOI: 10.9775/kvfd.2014.11999

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Epidemiology and Evolutionary Characteristics of Avian Infectious Bronchitis Virus in China

Zhao-xiong WANG [1,2] Shi-min GAO [3] Qiao-wei ZHENG [2]
Min-hua SUN [2] Wei-xin JIA [2] Tao REN [2]

[1] College of Animal Science, Yangtze University, 88 Jing Mi Road, Jingzhou, Hubei 434023, P. R. CHINA
[2] Key Laboratory of Animal Diseases Control and Prevention of the Ministry of Agriculture; College of Veterinary Medicine, South China Agricultural University, 483 Wu Shan Road, Tian He District, Guangzhou 510642, P. R. CHINA
[3] College of Animal Science and Veterinary Medicine, Shanxi Agricultural University, Taigu, Shanxi 030801, P.R. CHINA

KVFD-2014-11999 Received: 17.07.2014 Accepted: 08.01.2015 Published Online: 09.01.2015

Abstract

To examine the epidemiology and evolutionary characteristics of avian infectious bronchitis virus (IBV) in mainland China, the S1 gene of 63 IBVs isolated after 1993 was amplified by reverse transcription (RT) –polymerase chain reaction (PCR) and sequenced. Our results showed that the sequence length of 63 IBV strains ranged from 1.608 to 1.635 nucleotide (nt). Compared to the published representative strains, the homology of S1 nt sequences and their deduced amino acid (aa) residues between these isolates and reference strains ranged from 74.6% to 99.9% and 71.7% to 100%, respectively. Analysis of S proteins demonstrated that 63 isolates had eight kinds of cleavage sites (RRFRR, RRIRR, RRSKR, RRTGR, RRHRR, HRRRR, RRSRR and RRLRR), and the amount of RRFRR and HRRRR reached 46. Furthermore, the study showed that there was no direct relation between cleavage sites and genotypes, and cleavage sites were unable to decide the pathology types. Compared to vaccine strain H120, S1 nt sequences of 63 isolates had four sites deleted or inserted frequently, and the S1 proteins had three hypervariable regions. A comprehensive study was also carried out to study the S1 nt sequences of 63 isolates, 210 published reference strains and 10 vaccine strains; the investigation showed that all these stains could be divided into two types including 11 kinds of genotypes by the phylogenetic analysis. However, 63 isolates belonged to Mass, American, LX4, LHLJ/95 I, LDT3/03, J, BJ and LDL/97 I genotypes, and the LX4 genotype was co-circulated dominantly in chicken flocks over an 18-year period. It indicated that there were several IBV genotypes with new changes circulating in China, emphasizing the importance of continued IBV surveillance.

Keywords: *IBV, S1 sequence, Genetic variation, Phylogenetic analysis*

Epidemiology and Evolutionary Characteristics of Avian Infectious Bronchitis Virus in China

Özet

Avian Enfeksiyöz Bronşitis Virüsünün (IBV) epidemiyolojisini ve evrimsel ezelliklerini araştırmak amacıyla Çin'de 1993 yılından sonra izole edilen 63 IBV S1 geni ters transkriptaz (RT)-polimeraz zincir reaksiyonu (PCR) ile amlifiye edilerek sekansı yapıldı. Çalışma sonuçları 63 IBV suşunun sekans boyutunun 1.608 ile 1.635 nükleotide (nt) arasında değiştiğini gösterdi. Yayınlanmış benzer suşlar ile karşılaştırıldığında bu izolatlar ile referans suşları arasında S1 nt sekanslarının homolojisi ve açığa çıkan amino asit (aa) rezidüleri sırasıyla %74.6 ile %99.9 ve %71.7 ile %100 arasında değişti. S proteinlerinin analizi 63 izolatın 8 çeşit bölünme bölgesine (RRFRR, RRIRR, RRSKR, RRTGR, RRHRR, HRRRR, RRSRR ve RRLRR) sahip olduğunu ve RRFRR ve HRRRR miktarının 46'ya ulaştığını ortaya koydu. Ayrıca, bölünme bölgesi ile genotipler arasında doğrudan bir ilişki olmadığı ve bölünme bölgelerinin patoloji tiplerini belirlemediği tespit edildi. Aşı suşu H120 ile karşılaştırıldığında 63 izolatın S1 nt sekanslarının sıklıkla çıkarılmış veya eklenmiş 4 bölgeye sahip olduğu belirlendi. S1 proteinleri üç adet oldukça değişken bölgeye sahip olduğu gözlemlendi. 63 izolatın S1 nt sekansları, 210 yayınlanmış referans suşu ve 10 aşı suşunu araştırmak amacıyla ayrıca geniş çaplı bir çalışma yürütüldü. Tüm bu suşlar filogenetik analiz ile 11 çeşit genotipi de içeren iki tipe ayrılabilir. Ancak, 63 izolat Mass, American, LX4, LHLJ/95 I, LDT3/03, J, BJ ve LDL/97 I genotiplerine aitti ve LX4 genotipinin 18 yıldan daha uzun süredir tavuk sürülerinde baskın olarak bulunduğu belirlendi. Bu çalışma ile Çin'de yeni değişimlerle birlikte birkaç IBV genotipinin bulunduğu ve sürekli takibin önemi ortaya konuldu.

Anahtar sözcükler: IBV, S1 sekansı, Genetik varyasyon, Filogenetik analiz

İletişim (Correspondence)
+86 20 85283054; Fax: +86 20 85280242
rentao6868@126.com

INTRODUCTION

Avian infectious bronchitis (IB) is caused by IBV, which is a member of the *Coronaviridae* family, is recognized as one of the significant diseases of poultry, and leads to great economic losses to the poultry industry all over the world [1,2]. As a highly contagious pathogen, IBV not only brings chicken high pathogenicity rate with upper respiratory disease, nephritis or enteritis, but also causes fertility problems accompanied by low egg production and poor quality. Furthermore, the disease is a risk for increasing susceptibility to infections with other pathogens, resulting in an even higher morbidity and mortality rate [3,4].

IBV is an enveloped, non-segmented and single-stranded positive RNA virus with a genome around 27.6-kb in length [6,7]. The genome encodes four structural proteins, including spike glycoprotein (S), integral membrane glycoprotein (M), small membrane protein (E) and phosphorylated nucleocapsid (N) protein. Additionally, the genome also encodes the replicase complex, which carries out the unique discontinuous transcription process, leading to a nested set of 3' coterminal subgenomic mRNAs [8-10]. The S glycoprotein is proteolytically processed into two non-covalently bound peptide chains, S1 and S2. The S1 subunit located on the outside of virion is responsible for the fusion between the virus envelope and the cell membrane of the host, and, it contains epitopes and determinants for virus neutralizing antibodies, protective immunity, cell attachment and serotype specificity [11-13]. Classification of genotype has been done on the base of the antigenic site especially the hypervariable regions (HVR) of S1 nt sequence. The difference of several aa residues in S1 sequence can produce a new serotype strain and result in poor cross-protection between the strains. So, analysis of the S1 nt sequences has been usually used for differentiating IBV genotypes and serotypes [14,15].

More than 20 serotypes of IBV have been found around the world, and many IBV variant strains have been identified in various regions, leading to clinical symptom of birds infected by IBV varies greatly. The complex epidemiology characteristics of IB raised the difficulty in controlling this disease. These IBVs have no or low degree cross-protection for their serotype's diversity and virus genome varied frequently [16,17]. There are at least two reasons for explaining IBV continuous evolution. Firstly, it is thought that nucleotide deletions, insertions or point mutations within IBV genome result in errors made by viral RNA-dependent RNA polymerase [18,19]. Secondly, it can be explained by the IBV genome recombination in field strains or even between field strains and vaccine strains by the extensive and continuing use of live vaccine on chicken flock. Therefore, it is important to choose an appropriate vaccine with a serotype/genotype that is consistent with the epidemic virus to prevent and control the disease in each geographical region or country [5,11].

In China, after the first IBV has been identified by virus isolation since the early 1980s, more and more strains have been isolated in different regions. It becomes more difficult to prevent and control IB when novel IBV variants are circulating in chicken flocks. A vaccine programme with live-attenuated and inactivated vaccines of Massachusetts (Mass) serotype has been carried out widely and is partially successful in China. But in recent years, the phenomenon of IBV outbreaks occurred frequently in different vaccinated and non-vaccinated chicken flocks showed that the vaccines had poor or no protection against field virus [20-24]. Bing et al.[25] found that the proportion of nephro-pathogenic type IBV is higher than any other types by analysis of the strains isolated from China in these years. How to avoid enormous economic loss caused by nephro-pathogenic IB through selecting appropriate vaccine comes to be very important.

In this study, to reveal the epidemiology and evolutionary characteristics of recent IBV field isolates and to demonstrates which IBV genotypes are circulating in China, we investigated 63 IBVs isolated from 1993 to 2010 in mainland China, analyzed the sequences of whole S1 nt and took sequence alignment and phylogenetic analysis compared with other reference strains available in the GenBank database.

MATERIAL and METHODS

Virus

IBV was isolated from kidney, proventriculus and trachea from chicken flocks suspected to be infected with IBV during 1993-2010, covering 16 provinces, which occupied most of the chicken-raising regions of China *(Table 1)*.

Primer Design

S1 oligonucleotide primers were designed using eight IBV reference strains (Beaudette, Mass41, Cal99, BJ, KQ6, GD/S14/2003, SAIBK, LX4)logged in GenBank, for amplifying the entire S1 region (including leading sequence) and S1/S2 cleavage sites (1750 bp). The sense primer was S1-F (5'-TTGAAAACTGAA CAAAAGACCG-3'), and the sequence S1-R (5'-TACAAAACCTGCCATAACTAACAT-3') was used as antisense primer. The PCR conditions included 5 min incubation at 95°C followed by 30 cycles at 94°C for 1 min, annealing at 52°C for 1min, and extension at 72°C for 2 min. A final extension step was performed at 72°C for 10 min.

RT-PCR Amplification and Sequencing

Sixty-three IBVs were propagated in 9-day-old specific pathogen-free chicken embryos. Then the allantoic fluids of the infected embryos were harvested and total RNA was extracted with TRIzol reagent (Invitrogen, Grand Island, USA). RT-PCR products were analyzed on 1% agarose gel and sequenced after cloning into the pMD18-T (Takara,

Table 1. *63 IBV Strains isolated during 1993-2010 in China*
Tablo 1. *Çin'de 1993-2010 yılları arasında izole edilen 63 IBV suşu*

IBV Isolates	Year of Isolation	Province	Organs Used for Virus Isolation	Cleavage Recognition Motifs	Genotypes	IBV Isolates	Year of Isolation	Province	Organs Used for Virus Isolation	Cleavage Recognition Motifs	Genotypes
CK/CH/HIBV97	1997	Shandong	kidney	RRFRR	LDT3/03-type (VI)	CK/CH/GX-98 I	1998	Guangxi	trachea	RRSRR	American group (II)
CK/CH/SWIBV97	1997	Shandong	kidney	RRFRR	LDT3/03-type (VI)	CK/CH/HeN-93 II	1993	Henan	kidney	HRRRR	LX4-type (IV)
CK/CH/JNIBV98	1998	Shandong	kidney	RRIRR	BJ-type (VIII)	CK/CH/SC-93 II	1993	Sichuan	kidney	RRFRR	Mass-type (I)
CK/CH/QXIBV2	1998	Shandong	proventriculus	RRFRR	Mass-type (I)	CK/CH/GD-98 VI	1998	Guangdong	kidney	RRHRR	J-type (VII)
CK/CH/GNIBV98	1998	Shandong	kidney	RRFRR	J-type (VII)	CK/CH/GX-09 I	2009	Guangxi	kidney	HRRRR	LHLJ/95 I-type (V)
CK/CH/HeBIBV98	1998	Hebei	kidney	RRIRR	BJ-type (VIII)	CK/CH/GX-08 I	2008	Guangxi	kidney	HRRRR	LHLJ/95 I-type (V)
CK/CH/HeNIBV98	1998	Henan	kidney	RRFRR	LDT3/03-type (VI)	CK/CH/GX-08 II	2008	Guangxi	kidney	HRRRR	J-type (VII)
CK/CH/JSNJ97	1997	Jiangsu	kidney	RRSKR	LHLJ/95 I-type (V)	CK/CH/HaN-09 I	2009	Hainan	kidney	RRFRR	J-type (VII)
CK/CH/SDPL-01 I	2001	Shandong	kidney	RRTGR	LDL/97 I-type (IX)	CK/CH/GX-08 III	2008	Guangxi	kidney	HRRRR	J-type (VII)
CK/CH/SDZC-01 I	2001	Shandong	proventriculus	RRFRR	Mass-type (I)	CK/CH/GD-09 I	2009	Guangdong	kidney	HRRRR	LX4-type (IV)
CK/CH/SDTA-01 I	2001	Shandong	kidney	RRTGR	LDL/97 I-type (IX)	CK/CH/GX-07 I	2007	Guangxi	trachea	RRFRR	Mass-type (I)
CK/CH/SDTA-01 II	2001	Shandong	kidney	RRFRR	Mass-type (I)	CK/CH/GX-08 IV	2008	Guangxi	kidney	RRTGR	LDL/97 I-type (IX)
CK/CH/JX-99I	1999	Jiangxi	kidney	RRHRR	LDT3/03-type (VI)	CK/CH/HaN-09 III	2009	Hainan	kidney	RRFRR	J-type (VII)
CK/CH/JL-97 I	1997	Jilin	trachea	RRFRR	Mass-type (I)	CK/CH/GX-09 IV	2009	Guangxi	kidney	HRRRR	LX4-type (IV)
CK/CH/JS-97 I	1997	Jiangsu	kidney	RRSKR	LHLJ/95 I-type (V)	CK/CH/HaN-09 IV	2009	Hainan	kidney	HRRRR	LHLJ/95 I-type (V)
CK/CH/JS-95 III	1995	Jiangsu	kidney	RRFRR	Mass-type (I)	CK/CH/GX-09 V	2009	Guangxi	kidney	HRRRR	LX4-type (IV)
CK/CH/TJ-96 II	1996	Tianjin	kidney	RRFRR	LDT3/03-type (VI)	CK/CH/CQ-09 I	2009	Chongqing	kidney	RRTGR	LDL/97 I-type (IX)
CK/CH/SD-97 I	1997	Shandong	trachea	RRFRR	Mass-type (I)	CK/CH/GX-08 VII	2008	Guangxi	kidney	RRLRR	LDT3/03-type (VI)
CK/CH/SD-97 II	1997	Shandong	kidney	HRRRR	LX4-type (IV)	CK/CH/GX-08 VIII	2008	Guangxi	kidney	HRRRR	J-type (VII)
CK/CH/HeN-96 IV	1996	Henan	kidney	RRFRR	Mass-type (I)	CK/CH/HaN-09 VI	2009	Hainan	kidney	HRRRR	LHLJ/95 I-type (V)
CK/CH/GDGZ-97 I	1997	Guangdong	trachea	RRFRR	J-type (VII)	CK/CH/GX-08 IX	2008	Guangxi	proventriculus	RRFRR	Mass-type (I)
CK/CH/SD-97 IV	1997	Shandong	kidney	RRFRR	LDT3/03-type (VI)	CK/CH/GX-08 X	2009	Guangxi	proventriculus	RRFRR	Mass-type (I)
CK/CH/HLJ-98 I	1998	Heilongjiang	kidney	HRRRR	LHLJ/95 I-type (V)	CK/CH/GD-09 II	2009	Guangdong	trachea	RRFRR	Mass-type (I)
CK/CH/SD-96 III	1996	Shandong	kidney	HRRRR	LX4-type (IV)	CK/CH/GD-09 III	2009	Guangdong	kidney	RRFRR	J-type (VII)
CK/CH/SX-96 I	1996	Shanxi	trachea	RRFRR	Mass-type (I)	CK/CH/GX-09 VI	2009	Guangxi	proventriculus	RRFRR	Mass-type (I)
CK/CH/HeB-96 I	1996	Hebei	trachea	RRFRR	Mass-type (I)	CK/CH/HaN-09 VIII	2009	Hainan	kidney	RRIRR	BJ-type (VIII)
CK/CH/SD-96 VI	1996	Shandong	trachea+muscle	RRSRR	American group (II)	CK/CH/GD-09 IV	2009	Guangdong	kidney	RRFRR	J-type (VII)
CK/CH/AH-97 I	1997	Anhui	kidney	RRSKR	LHLJ/95 I-type (V)	CK/CH/GD-10 I	2010	Guangdong	kidney	HRRRR	LX4-type (IV)
CK/CH/HeN-99 III	1999	Henan	kidney	RRFRR	LDT3/03-type (VI)	CK/CH/GD-10 II	2010	Guangdong	kidney	HRRRR	LX4-type (IV)
CK/CH/XJ-99 II	1999	Xinjiang	trachea+muscle	RRSRR	American group (II)	CK/CH/GD-10 III	2010	Guangdong	kidney	HRRRR	LX4-type (IV)
CK/CH/HaN-95 I	1995	Hainan	trachea	RRSRR	American group (II)	CK/CH/GD-10 IV	2010	Guangdong	kidney	RRFRR	LDT3/03-type (VI)
CK/CH/HaN-95 II	1995	Hainan	trachea	RRFRR	Mass-type (I)						

RRFR: *Arg-Arg-Phe-Arg-Arg,* ***RRIRR:*** *Arg-Arg-Ile-Arg-Arg,* ***RRSKR:*** *Arg-Arg-Ser-Lys-Arg,* ***RRTGR:*** *Arg-Arg-Thr-Gly-Arg,* ***RRHRR:*** *Arg-Arg-His-Arg-Arg,* ***HRRRR:*** *His-Arg-Arg-Arg-Arg,* ***RRSRR:*** *Arg-Arg-Ser-Arg-Arg,* ***RRLRR:*** *Arg-Arg-Leu-Arg-Arg*

Dalian, China). Each region of the S1 nt sequence in each IBV isolate was sequenced in the forward and reverse directions at least five times and the consensus sequence was determined.

Genetic Variability Analysis

Length and homology of S1 nt sequences and their deduced aa residues were analyzed by using MEGALIGN program in DNAStar. Furthermore, the homology, variation and cleavage sites of S1 nt and their aa sequences were determined between 36 reference strains (including 10 vaccine strains and 26 mainly genotypes derived from China) and 63 isolates in this study. Simultaneously, sequence variation characteristics of glycoprotein of 63 isolates were analyzed by comparing to vaccine strain H120.

Phylogenetic Analysis of IBV S1 nt

Comparative analysis of S1 nt sequences between 63 isolates and 233 references strains (including 210 main epidemic strains in mainland China, 10 vaccine strains and 13 IBV representative strains from other countries) was performed, and the phylogenetic tree was obtained by using the neighbour-joining method with 1000 boot-strapping replicates integrated in the MEGA software version 4.1.

RESULTS

Length and Homology Analysis

Length and homology of S1 nt and aa sequence were analyzed in this study. The results showed that the length of S1 nt sequence (from initiation codon ATG to S gene precursor protein cleavage sites) included 1608, 1611, 1617, 1620, 1623, 1626, 1629, 1632 and 1635 nt, and the length of their deduced amino acid residues accordingly were 536, 537, 539, 540, 541, 542, 543, 544 and 545 aa.

Homology of the S1 nt sequences and their deduced aa residues between 63 isolates ranged from 75.5% to 99.9% and 72.5% to 100%, respectively, the similarity of S1 nt sequences and their deduced aa residues between 99 strains (including 63 isolates in this study plus the 36 reference strains) were from 74.6% to 99.9% and 71.7% to 100%, respectively. It indicated low homology and high sequence variation due to nt point mutations, insertions and deletions on S1 sequences.

Cleavage Sites and Mutation Analysis

Analysis of S1 nt and their deduced aa sequences between 63 isolates and 36 reference strains indicated that the variant regions focused mainly on S1 nt 5' tail end, which were identical to the published data. Ten kinds of S1/S2 cleavage sites could be found in these 99 IBV strains, and the cleavage site sequences were RRFRR (29/44), RRIRR (3/4), RRSKR (3/3), RRTGR (4/6), RRHRR (2/2), HRRRR (17/22), RRSRR (4/14), RRLRR (1/1), HRFRR (0/1) and HRSRR (0/2), respectively (numerator, number of cleavage sites in IBV isolates; denominator, total number of cleavage sites in IBV isolates and reference strains). Most of the isolates had S1/S2 cleavage sites, RRFRR, HRRRR (46/63). The cleavage site RRFRR was common for Mass genotype (24/25), RRIRR for BJ genotype strains (4/4), RRTGR for LDL/97 I genotype strains (6/6), and HRRRR for LX4 genotype strains (13/13). Six cleavage sites RRIRR, RRSKR, RRTGR, RRHRR, HRRRR and RRLRR were found only in mainland China *(Table 1)*. Some strains with high gene similarity had the same cleavage sites while some strains lied in same genotype had different cleavage sites, indicating that cleavage sites played a limited role in the IBV S1 nt sequence typing.

Compared to vaccine strain H120, S1 nt sequences of 63 isolates had four regions, which were inserted or deleted frequently and located between nt 64-75, 210-214, 355-358 and 417-418 (numbered according to the S1 sequence of H120 strain), respectively. S1 protein of 63 isolates had three hypervariable regions and had 7 aa deletion or insertion mainly among residues 3-25, 52-154 and 266-294. Behind the residue 24, aspartic acid (N) was inserted in 12 isolates and serine (S) was deleted in four isolates. Amino acids (NYTNGNSD) were inserted between residues 71 and 72 in 18 isolates and 5-6 aa were inserted in the site of residue 141 in four isolates. Amino acid (KKSVVGPSD) were inserted behind the residue 138 and SD was inserted behind the residue 141 in IBV isolate GX-98 I, which was consistent with international reference strain Holte with classical amino acid sequence FKKKSVVGPSD after residue 138.

Phylogenetic Analysis of S1 nt between 63 IBV Isolates and 233 Reference Strains

Sixty-three IBV isolates and 233 reference strains were divided into two groups by analyzing S1 nt sequences. One group had 49 strains including 20 IBV isolates, 10 vaccine strains, seven representative strains isolated from other countries and 12 reference strains in China. The second group had 247 strains including 43 isolates, six international represent strains isolated from other countries and 198 reference strains in China *(Fig. 1)*.

In this research, all IBV strains could be divided into 11 genotypes by homology analysis of S1 nt sequences. Sixty-three isolates were clustered into eight genotypes, including genotype I (Mass-type), genotype II (American type), genotype IV (LX4 type), genotype V (LHLJ/95 I type), genotype VI (LDT3/03 type), genotype VII (J type), genotype VIII (BJ type) and genotype IX (LDL/97 I type) respectively while they did not appeared in genotype III (4/91 type), genotype X (LGD04/III type) and genotype XI (Taiwan group). Sixteen isolates belonged to genotype I, 10 isolates were genotype VII, 9 isolates lied in genotypes VI, respectively, and eight isolates existed in genotype V? The proportion of the five kinds of genotypes to all isolates was 82.5%, which was a little close to mainland China

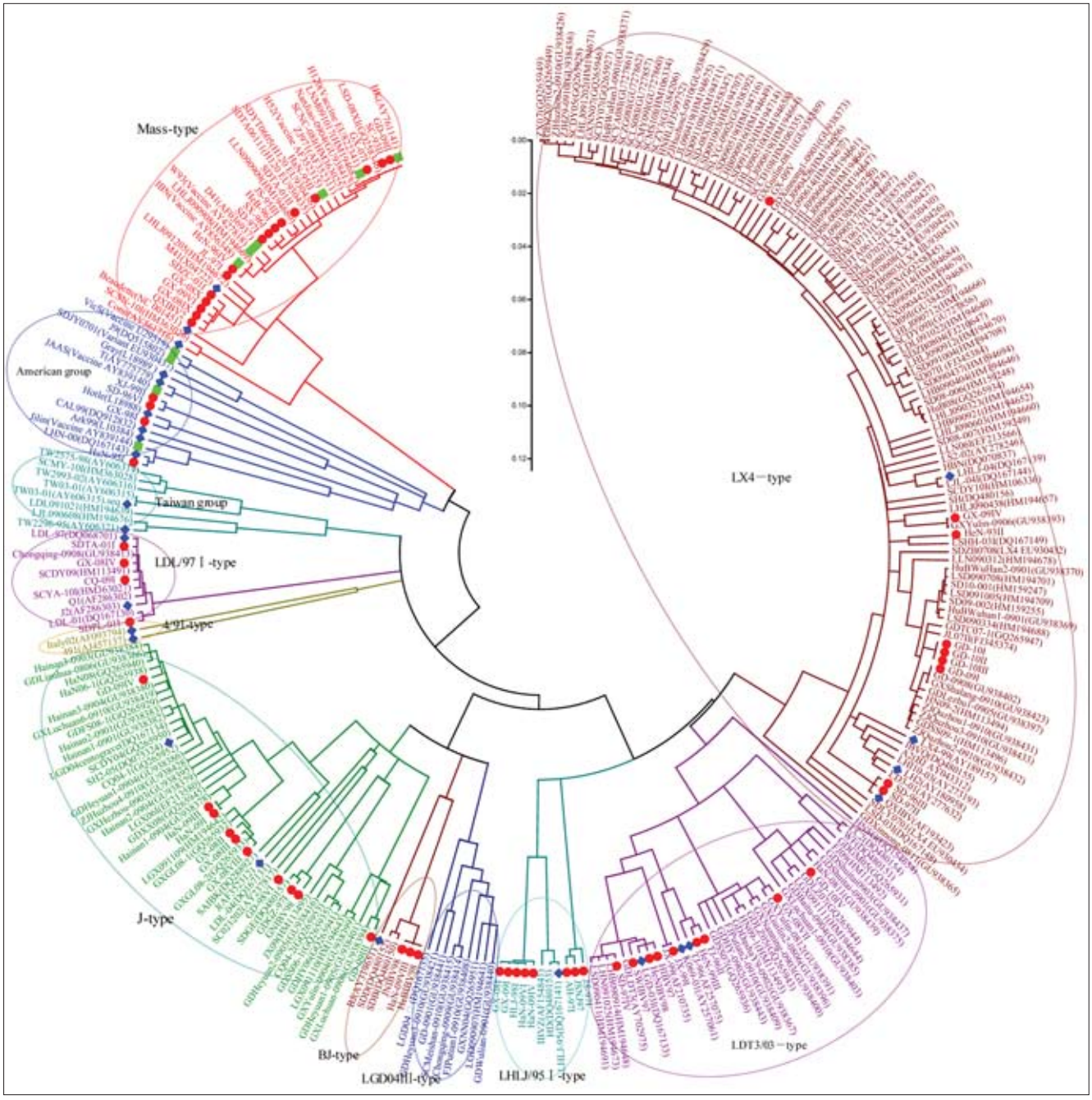


Fig 1. The phylogenetic tree constructed based on IBV S1 nucleotide sequences by N-J method
"●" IBV isolate strains, "■" vaccine strains "◆" international represent strains

Sekil 1. N-J metodu kullanılarak IBV S1 nükleotid sekanslarına dayanarak oluşturulan filogenetik ağaç
"●" IBV izolat suşlar, "■" aşı suşlar "◆" uluslararası tanımlı suşlar

reference strains (87.6%) *(Table 1, Fig. 2)*. It was noteworthy that the proportion of genotype V (12.7%) was much higher than that of the reference strains (1.4%) in mainland China. It all suggested that the genotype of the virus had new changes.

DISCUSSION

Infectious bronchitis (IB) has become one of the most common diseases to outbreaks frequently and persistently in commercial chicken farms of China. The disease is difficult to be controlled by IBV vaccines, which have been used widely in different chicken farms, because of the rapid circulation and complicated evolution of IBV strains. The chicken flocks have potential danger of IB outbreak when the serotype/genotype of vaccine strains are inconsistent to infective strains [20-24]. Therefore, it is important to choose a suitable IBV candidate vaccine strain to prevent the disease based on profound understanding of IBV epidemiology in China. In this work, we examined the epidemiology and

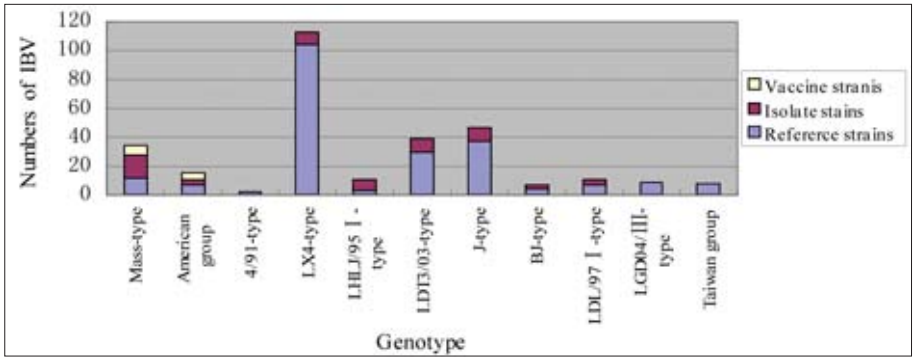


Fig 2. The gene quantitative statistics of isolates, vaccine and reference strains genotypes based on IBV S1 nucleotides

Şekil 2. IBV S1 nükleotidlerine dayanarak oluşturulan izolatlar, aşı ve referans genotiplerinin gen kuantitatif istatistikleri

evolutionary characteristics of IBV by analyzing S1 nt of 63 IBVs isolated from 16 geographic provinces in China over an 18-year period from 1993 to 2010. Our results showed that the S1 nt and their aa had significant characteristics with low homology and high variation between the isolates and reference strains, indicating that new changes have taken place in IBV genotypes circulating in China.

The previous studies suggested that site mutation, insertion and deletion in S1 nt sequence were very important factors resulting in poor immune effect of conventional IBV vaccine [11,26]. In order to learn S1 nt of IBV isolated from China, we analyzed the differences in S1 nt sequences of 63 isolates and vaccine strain H120, which belonged to Mass serotype. Our study found that, compared to vaccine strain H120, S1 nt of 63 isolates had four regions, which were inserted or deleted frequently between nt 64-75, 210-214, 355-358 and 417-418, respectively. In addition, the S1 protein of 63 isolates had three hypervariable regions between aa 3-25, 52-154 and 266-294, which was consistent with the published data [13,26-28]. Differences on S1 nt sequences suggest that vaccine contained H120 strain has limited protection on chickens.

IBV S protein has a proteinase cleavage site; it can be cleaved into S1 and S2 subunits when the virus particles are replicating [11,13]. Our investigations showed that S protein of 63 isolates and 36 reference strains had 10 kinds of different cleavage sites, among which RRFRR and HRRRR were prominent, and the cleavage sites HRRRR, RRIRR, RRHRR, RRLRR, RRSKR and RRTGR only existed in strains coming from China. This result revealed that the IBV lied in continuing change status in China, but the role of these cleavage sites to virus pathogenicity and vaccine immunity was still uncertain. Highly similar IBV S1 proteins often had the same cleavage sites [11,13], but we found that some IBVs possessed different cleavage sites though they were laid in the same genotype, implying that genotyping of IBV by S1 cleavage sites has limited application. Just as Jackwood et al.[29] reported, IBV genotype can not only be decided by cleavage sites and pathogenicity and tissue tropism.

The 63 Chinese IBV isolates and 233 reference strains had been divided into two groups by analysis of the homology of S1 nt. We found that one group had 49 strains including 20 IBV isolates, 10 vaccine strains, seven representative strains isolated from other countries and 12 Chinese reference strains, and the other group had 247 strains including 43 IBV isolates, six international representative strains isolated from other countries and 198 Chinese reference strains. All the strains were divided into 11 genotypes, and the 63 isolates came from eight different genotypes. Han et al.[20] reported that they found 13 IBV variant strains, making the IBV epidemiology more intricate. Moreover, we could not find IBV variant strains by analyzing isolates sequence. The reason most probably was that we used different reference strains.

At the present time, only the Mass type vaccine has been permitted to be used in the market in China, which had controlled the disease effectively [11], but IB still broke out in some chicken flocks though they had been immunized with Mass type IBV vaccine, causing chicken death or deterioration in the quality of eggs. The investigations in IBV isolated from immunized chickens discovered that immunity effect declined partly owing to the genetic variation in the process of IBV evolution [7,22,23,25,30]. We found that more than 40% IBVs belonged to LX4 type, which was circulating prevalently in chicken flocks in China, the result corresponded with the reported literature [20,22,30]. The study also indicated that IBV S1 gene of LX4 type had point mutation, insertion and deletion, probably because virus evolved in different regions. Compared to Mass type vaccine strains, the homology of nt and aa sequences of the field strain of LX4 type derived from China were less than 78.8% and 78.3%, respectively, which helps to explain the failure of the vaccine. As the second and third major types of IBV circulating in chicken flocks in China, the homology of aa sequences of J and LDT3/03 types were less than 82% compared to Mass type vaccine strains.

It should be noted that Mass type IBV can be isolated frequently and be considered as the fourth type to appear in chicken flock even though the Mass type IBV vaccine has been used for several years in China. A similar situation also appeared in other countries [31]. The vaccine virus was isolated frequently from chicken flocks, The reason maybe the wide use of attenuated vaccine in farms. This study discovered that the homology of nt and aa

sequences of isolates and vaccine strains were more than 94.6%, and S1 nt of most isolates had point mutations and deletions compared to vaccine strain. It was reported by Cavanagh et al that a small change of IBV S1 glycoprotein could result in a change in virus serotype [11]. Amino acid substitutions might be caused by immune pressure due to comprehensive use of vaccines. Therefore, it can partially explain why IBV still broke out and circulated in chicken flocks immunized or not with Mass type vaccine. Other IBV genotypes such as BJ, LDL/97 I? and LGD04/III? spread widely around the world except for four genotypes LX4, J, LDT3/03 and Mass described above. It indicated that new variant strains might be generated by gene recombination from different IBV genotypes, bringing enormous pressures for IBV control and prevention.

As serological tests (virus neutralization test and immune poisoning test) serve as effective tools for determining the exact protective effect of the vaccine strains to field virus, deep sequence analysis of IBV S1 becomes necessary to predict the efficiency of vaccine. However, due to the diversity of IBV serotypes and genotypes, it is particularly important to re-screen immune effects of Mass type vaccine strains and re-assess and analyze IBV isolates on the basis of laboratory and field tests.

Acknowledgments

This work was supported by a grant from the National Natural Science Foundation of China (31072319, 31372412) and the Specialized Research Fund for the Doctoral Program of Higher Education of China (20124404110016).

REFERENCES

1. Cavanagh D: Severe acute respiratory syndrome vaccine development: experiment of vaccination against avian infectious bronchitis coronavirus. *Avian Pathol,* 32, 567-582, 2003. DOI: 10.1080/0307945031000162119 8

2. Ignjatovic J, Gould G, Sapats S: Isolation of a variant infectious bronchitis virus in Australia that further illustrates diversity among emerging strains. *Arch Virol,* 151, 1567-1585, 2006. DOI: 10.1007/s00705-006-0726-y

3. Ambali AG, Jones RC: Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. *Avian Dis,* 34 (4): 809-817, 1990.

4. Ziegler AF, Ladman BS, Dunn PA, Schneider A, Davison S, Miller PG, Lu H, Weinstock D, Salem M, Eckroade RJ, Gelb Jr J: Nephropathogenic infectious bronchitis in Pennsylvania chickens 1997-2000. *Avian Dis,* 46 (4): 847-858, 2002.

5. Cavanagh D, Davis PJ, Cook JK: Infectious bronchitis virus: Evidence for recombination with in the Massachusetts serotype. *Avian Pathol,* 21 (3): 401-408, 1992. DOI: 10.1080/03079459208418858

6. Boursnell ME, Brown TD, Foulds IJ, Green PF, Tomley FM, Binns MM: Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. *J Gen Virol,* 68 (1): 57-77, 1987.

7. Liu XL, Sun JL, Zhao JX, Zhang GZ: Complete genome sequence analysis of a predominant infectious bronchitis virus (IBV) strain in China. *Virus Genes,* 38 (1): 65-65, 2009. DOI: 10.1007/s11262-008-0282-5

8. Stern DF, Kennedy SI: Coronavirus multiplication strategy. II. Mapping the avian infectious bronchitis virus intracellular RNA species to the genome. *J Virol,* 36 (2): 440-449, 1980.

9. Stern DF, Burgess L, Sefton BM: Structural analysis of virion proteins of the avian coronavirus infectious bronchitis virus. *J Virol,* 42 (1): 208-219, 1982.

10. Lai MM, Cavanagh D: The molecular biology of coronaviruses. *Adv Virus Res,* 48, 1-100, 1997. DOI: 10.1016/S0065-3527(08)60286-9

11. Cavanagh D, Davis PJ, Cook JK, Li D, Kant A, Koch G: Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. *Avian Pathol,* 21 (1): 33-43, 1992. DOI: 10.1080/03079459208418816

12. Cavanagh D, Davis PJ: Coronavirus IBV: Removal of spike glycopolypeptide S1 by urea abolishes infectivity and haemagglutination but not attachment to cells. *J Gen Virol,* 67 (1): 1443-1448, 1986.

13. Cavanagh D: Coronavirus IBV: Structural characterization of the spike protein. *J Gen Virol,* 64 (12): 2577-2583, 1983. DOI: 10.1099/0022-1317-64-12-2577

14. Gelb J Jr, Keeler CL Jr, Nix WA, Rosenberger JK, Cloud SS: Antigenic and S1 genomic characterization of the Delaware variant serotype of infectious bronchitis virus. *Avian Dis,* 41 (3): 661-669, 1997. DOI: 10.2307/1592158

15. Bourogaa H, Miled K, Gribaa L, El Behi I, Ghram A: Characterization of new variants of avian in factious bronchitis virus in Tunisia. *Avian Dis,* 53 (3): 426-433, 2009. DOI: 10.1637/8988.1

16. Ladman BS, Loupus AB, Gelb J Jr: Infectious bronchitis Virus S1 nucleotides sequence comparison is a better predictor of challenge of immunity in chickens than serotyping by virus neutralization. *Avian Pathol,* 35 (2): 127-133, 2006. DOI: 10.1080/03079450600597865

17. Wang CH, Huang YC: Relationship between serotypes and genotypes based on the hypervariable region of the S1 nucleotides of infectious bronchitis virus. *Arch Virol,* 145 (2): 291-300, 2000. DOI: 10.1007/s007050050024

18. Jenkins GM, Rambaut A, Pybus OG, Holmes EC: Rates of molecular evolution in RNA viruses: aquantitative phylogenetic analysis. *J Mol Evol,* 54 (2): 156-165, 2002. DOI: 10.1007/s00239-001-0064-3

19. Duffy S, Shackelton LA, Holmes EC: Rates of evolutionary change in virus's patterns and determinants. *Nat Rev Genet,* 9 (4): 267-276, 2008. DOI: 10.1038/nrg2323

20. Han Z, Sun C, Yan B, Zhang X, Wang Y, Li C, Zhang Q, Ma Y, Shao Y, Liu Q, Kong X, Liu S: A 15-year analysis of molecular epidemiology of avian infectious bronchitis coronavirus in China. *Infect Genet Evol,* 11 (1): 190-200, 2011. DOI: 10.1016/j.meegid.2010.09.002

21. Ji J, Xie JW, Chen F, Shu DM, Zuo KJ, Xue CY, Qin J, Li H, Bi Y, Ma J, Xie Q: Phylogenetic distribution and predominant genotype of the avian infectious bronchitis virus in China during 2008-2009. *J Virol*, 8, 184, 2011. DOI: 10.1186/1743-422X-8-184

22. Liu SW, Zhang QX, Chen JD, Han ZX, Liu X, Feng L, Shao YH, Rong JG, Kong XG, Tong GZ: Genetic diversity of avian infectious bronchitis coronavirus strains isolated in China between 1995 and 2004. *Arch Virol,* 151 (6): 1133-1148, 2006. DOI: 10.1007/s00705-005-0695-6

23. Liu S, Zhang X, Wang Y, Li C, Han Z, Shao Y, Li H, Kong X: Molecular characterization and pathogenicity of infectious bronchitis coronaviruses: complicated evolution and epidemiology in China caused by cocirculation of multiple types of infectious bronchitis coronaviruses. *Intervirology,* 52 (4): 223-234, 2009. DOI: 10.1159/000227134

24. Li LL, Xue CY, Chen F, Qin JP, Xie QM, Bi YZ, Cao Y: Isolation and genetic analysis revealed no predominant new strains of avian infectious bronchitis virus circulating in South China during 2004-2008. *Vet Microbiol,* 143 (2-4): 145-154, 2010. DOI: 10.1016/j.vetmic.2009.11.022

25. Bing GX, Liu X, Pu J, Liu QF, Wu QM, Liu JH: Different genotypes of nephropathogenic infectious bronchitis viruses co-circulating in chicken population in China. *Virus Genes,* 35 (2): 333-337, 2007. DOI: 10.1007/s11262-007-0100-5

26. Moore KM, Bennett JD, Seal BS, Jackwood MW: Sequence comparison of avian infectious bronchitis virus S1 glycoproteins of the Florida serotype and five variant isolates from Georgia and California.

Virus Genes, 17 (1): 63-83, 1998. DOI: 10.1023/A:1008057118625

27. Li H, Yang HC: Sequence analysis of nephropathogenic infectious bronchitis virus strains of the Massachusetts genotype in Beijing. *Avian Pathol,* 30 (5): 535-541, 2001. DOI: 10.1080/03079450120078734

28. Schikora BM, Shih LM, Hietala SK: Genetic diversity of avian infectious bronchitis virus California variants isolated between 1988 and 2001 based on the S1 subunit of the spike glycoprotein. *Arch Virol,* 148 (1): 115-136, 2003. DOI: 10.1007/s00705-002-0904-5

29. Jackwood MW, Hilt DA, Callison SA, Lee CW, Plaza H, Wade E: Spike glycoproteion cleavage recognition site analysis of infectious bronchitis virus. *Avian Dis,* 45 (2): 366-372, 2001.

30. Liu SW, Kong XG: A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non-vaccinated flocks in China. *Avian pathol,* 33 (3): 321-327, 2004.

31. Dolz R, Pujols J, Ordonez G, Porta R, Majo N: Antigenic and molecular characterization of isolates of the Italy 02 infectious bronchitis virus genotype. *Avian Pathol,* 35 (2): 77-85, 2006. DOI: 10.1080/03079450600597295

Kafkas Univ Vet Fak Derg
21 (2): 187-194, 2015
DOI: 10.9775/kvfd.2014.12004

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

The Effects of Propetamphos, Cypermethrin and Propetamphos-Cypermethrin Combination on Some Biochemical and Histopathological Parameters in Mice [1]

Murat KANBUR [1] Gökhan ERASLAN [1] Sinan İNCE [2]
Levent ALTINTAŞ [3] Bilal Cem LİMAN [1] Latife ÇAKIR BAYRAM [4]

[1] *A part of this study was presented to 22th Turkish Pharmacology Congress in 19-22 October 2011, Eskişehir - Turkey*

[1] Erciyes Üniversitesi, Veteriner Fakültesi, Farmakoloji ve Toksikoloji Anabilim Dalı, TR-38039 Kayseri - TÜRKİYE
[2] Afyon Kacatepe Üniversitesi, Veteriner Fakültesi, Farmakoloji ve Toksikoloji Anabilim Dalı, Ahmet Necdet Sezer Kampüsü, TR-03200 Afyon - TÜRKİYE
[3] Ankara Üniversitesi, Veteriner Fakültesi, Farmakoloji ve Toksikoloji Anabilim Dalı, TR-06110 Dışkapı, Ankara - TÜRKİYE
[4] Erciyes Üniversitesi Veteriner Fakültesi, Patoloji Anabilim Dalı, TR-38039 Kayseri - TÜRKİYE

Article Code: KVFD-2014-12004 Received: 08.07.2014 Accepted: 30.10.2014 Published Online: 03.12.2014

Abstract

This study was aimed at investigating the toxic effects of subchronic and chronic exposure to propetamphos (PRO) and cypermethrin (CYP) combination in mice. Seventy male Swiss albino mice were used for this study. Group 1 was maintained for control and given insecticide-free feed for 60 days. Group 2 was administered with 5.0 mg/kg/bw/day of PRO (LD50/20); Group 3 with 10.0 mg/kg/BW/day of CYP (LD50/20), Group 4 with PRO and CYP combination at the same doses in feed for 60 days. Blood samples, liver and kidney were taken on days 45 and 60 from 10 animals. Serum samples were analysed for biochemical parameters, and liver and kidney tissues were examined histopathologically. When compared to the control group with insecticide-treated groups, it was determined that cholesterol, triglyceride, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) levels increased and albumin levels decreased ($P<0.05$), bilirubin levels increased ($P<0.05$) in Group 4. It was seen that triglyceride and bilirubine levels and ALP and GGT activities were more high ($P<0.05$) in Group 4 than other groups; also ALT activity and bilirubine level were high ($P<0.05$) in Group 4 at 60 day. In all treatment groups, necrosis of hepatocytes, cytoplasmic vacuolation, bile duct hyperplasia and mononuclear cellular infiltration were presented in liver. In Group 4 at 60 day, tigroid basophilic cytoplasm and hepatocellular hypertrophy of liver; necrosis of the tubular epithelial cells, cytoplasmic vacuolation, cellular infiltration and glomerular atrophy of kidney were seen. As a result, it was concluded that the exposure of mice to PRO and CYP for periods of 45 and 60 days cause to adverse effects on both liver and kidney functions and protein and lipid metabolism. These effects were observed to be more severe in the case of PRO-CYP exposure for 60 days.

Keywords: *Cypermethrin, Propetamphos, Toxicity, Mice, Serum biochemical parameter, Histopathology*

Sipermetrin, Propetamfos ve Sipermetrin + Propetamfos Karışımının Farelerde Bazı Biyokimyasal ve Histopatolojik Parametreler Üzerine Etkileri

Özet

Bu çalışmada propetamfos (PRO), sipermetrin (CYP) ve bunların karışımlarının farelerde subkronik ve kronik toksik etkilerinin incelenmesi amaçlandı. Çalışmada 70 adet Swiss albino ırkı erkek fare kullanıldı. Grup 1 kontrol olarak tutuldu ve gruptaki hayvanlara 60 gün boyunca insektisit içermeyen yem verildi. Grup 2'ye 5 mg/kg/ca/gün PRO (LD50/20), Grup 3'e 10 mg/kg/ca/gün CYP (LD50/20), Grup 4'e aynı dozda PRO-CYP karışımı 60 gün boyunca yem içinde verildi. Çalışmanın 45 ve 60. günlerinde 10'ar farenin kan örnekleri ile karaciğer ve böbrekleri alındı. Serum örneklerinde biyokimyasal analizler, karaciğer ve böbrekte histopatolojik incelemeler yapıldı. İnsektisit uygulanan gruplar kontrol grubu ile karşılaştırıldığında, serum kolesterol ve trigliserit düzeyleri ile aspartat aminotransferaz (AST), alanin aminotransferaz (ALT), alkalin fosfataz (ALP) ve gama glutamil transferaz (GGT) aktivitelerinde yükselme ($P<0.05$), albumin düzeyinde azalma ($P<0.05$), grup 4'te bilirubin düzeyinde yükselme ($P<0.05$) olduğu belirlendi. Grup 4'de trigliserit ve bilirubin düzeyleri ile ALP ve GGT aktivitelerinin diğer gruplara göre daha yüksek olduğu ($P<0.05$); ayrıca 4. grupta 60. gün ALT aktivitesi ve bilirubin düzeylerinin yüksek seyrettiği ($P<0.05$) gözlendi. İnsektisit uygulanan tüm gruplarda karaciğerde hepatositlerde nekroz, sitoplazmik vakuolleşme, safra kanallarında hiperplazi ve hücre infiltrasyonu olduğu gözlendi. Bunlara ilaveten grup 4'te 60 gün insektisit uygulaması sonrasında karaciğerde tigroid bazofilik sitoplazma ve hepatoseluler hipertrofi; böbrekte tubuler epitelyal hücrelerde nekroz, sitoplazmik vakuolleşme, hücre infiltrasyonu ve glomerular atrofi gözlendi. Sonuç olarak farelerde 45 ve 60 gün boyunca PRO, CYP ve PRO-CYP karışımı verilmesinin karaciğer ve böbrek fonksiyonları ile protein ve lipid metabolizmasını olumsuz yönde etkilediği; bu etkilerin 60 gün PRO-CYP verilen hayvanlarda daha şiddetli olduğu gözlendi.

Anahtar sözcükler: *Sipermetrin, Propetamfos, Zehirlenme, Fare, Serum biyokimya, Histopatoloji*

İletişim (Correspondence)
+90 352 2076666/29972
kanburm@hotmail.com

INTRODUCTION

PRO is an organic phosphorus insecticide of a phosphoroimidate group, which has a strong insecticidal effect and weak anticholinesterase activity [1]. Similar to other organophosphate insecticides, PRO irreversibly blocks the activity of acetyl cholinesterase (AChE), which breaks down acetylcholine (ACh), and thereby, leads to the accumulation of ACh at the neuromuscular junctions, postganglionic nerve terminals in smooth muscle, the myocardium, etc., and in all autonomic ganglia and the cholinergic synapses of the central nervous system, causing intoxication [2].

Owing to its strong insecticidal activity, broad spectrum, and low toxicity in mammals, CYP has commonly used in treatment of parasitic infestations [3]. The LD_{50} of CYP in mice is 82-779 mg/kg [4]. The major target area of CYP and other pyrethroids in the body is the voltage-dependent sodium channels located in the membrane of nerve cells. Pyrethroids affect the opening and closure of these channels, resulting in their remaining open for an extended time period, the acceleration of Na^+ transport, and eventually nervous discharge and blockage. Apart from the Na^+ channels, pyrethroids also affect voltage-sensitive Ca^{+2} channels, by inducing the secretion of neurotransmitters and inhibiting Ca^{+2}-ATPases and Mg^{+2}-ATPases [2,3].

Insecticides are widely used in agriculture, environmental health, human and animal health and many insecticide formulations contain two or more active ingredient. Because of this reason, humans and animals are constantly exposed to multi-insecticide residues. Chronic exposure to insecticides has been associated with many hazardous effect on nervous, endocrine, reproductive and immune system [5-7]. It has been showed that chronic exposure of PRO, CYP and PRO-CYP combination cause lipid peroxidation in mice [5]. There are several insecticide formulations containing CYP and PRO for veterinary medicine in Turkey. PRO and CYP poisonings have been reported for human by Turkey Public Health Corporation [8]. There is not any study about effects of chronic administration to PRO, CYP and PRO-CYP combination on serum biochemical parameters, and liver and kidney in mice. With this study, subchronic and chronic effects of PRO, CYP and their mixtures on serum biochemical parameters and, liver and kidney histopathology in mice to understand the possible health effects to animals and human was investigated.

MATERIAL and METHODS

The trial was performed in compliance with the instructions of Local Ethical Board of Erciyes University Veterinary Faculty (040/051). Forty-day old male Swiss albino mice, each weighing 35-40 g and the progeny of parent mice, constituted the material of the study. The mice were raised under a daily 12-h light and 12-h dark regime, in a heat- (20-22°C) and ventilation-regulated room, in polyethylene cages, each containing a maximum of 10 animals. Feed and water were provided as *ad libitum* [5]. For this research, technical standards of cypermethrin (70%) and propetamphos (90%) obtained by Topkim Drug Corporation, Istanbul, Turkey.

Four groups were established. While the control group comprised 10 animals, each treatment group was composed of 20 animals. The control group (Group 1) was provided *ad libitum* with insecticide-free feed for 60 days. The mice in Groups 2, 3 and 4 were administered with 5.0 mg/kg/BW/day of PRO, 10.0 mg/kg/BW/day of CYP, and 5.0 mg/kg/BW/day of PRO in combination with 10.0 mg kg/BW/ day of CYP, respectively, in feed for a period of 60 days [5].

Blood samples were collected by cardiac puncture on day 60 from the control group and on days 45 and 60 from 10 mice included in each of the treatment groups. The blood samples were centrifuged at 3.000 rpm for 10 min by using Sigma 3K-30 and serum was transferred in eppendorph tubes. Serum samples were maintained at - 80°C in a deep freezer until analysed. The serum samples were analysed for cholesterol, triglyceride, total protein, albumin, and bilirubin levels, and AST, ALT, ALP and GGT activities. The analyses were performed using Johnson & Johnson kits in a Vitros 750 model auto-analyser. AST, ALT, ALP and GGT were given in U/L, while the results of the other analyses were given in mg/dL.

The mice, which were sampled for blood, were euthanized by cervical dislocation. After extracted, the liver and kidneys were examined for macroscopic lesions. Subsequently, samples taken from the hepatic and renal tissues were fixed in neutral formalin for 24 h, subjected to routine processing, and embedded in paraffin. The paraffin blocks were cut into sections of 5-6 µm thickness and stained with haematoxylene and eosin (H&E) for microscopic examination.

Statistical analysis of biochemical parameters were made with SPSS 16.0 software. Data were given as arithmetic means and standard deviation. Significance of among groups were employed with one-way variance analysis and Tukey test.

RESULTS

When compared to the control group, in the treatment groups it was observed that, the cholesterol and triglyceride levels, and AST, ALT, ALP and GGT activities had increased ($P<0.05$) and the albumin levels had decreased ($P<0.05$); also the bilirubin level had increased ($P<0.05$) in PRO-CYP treated group (Group 4) *(Table 1)*. It was seen that triglyceride and bilirubine levels and ALP and GGT activities were more high ($P<0.05$) in PRO-CYP treated group (Group 4) than other groups; also ALT activity and bilirubine level were high ($P<0.05$) in PRO-CYP treated group (Group 4)

at 60 day *(Table 1)*. When compared to the PRO treated (Group 2) and CYP treated (Group 3) groups, triglyceride level and AST activity were high (P<0.05) in CYP treated group (Group 3) *(Table 1)*. When compared to the PRO-CYP treated group (Group 4), cholesterol, triglyceride and bilirubin levels and AST, ALT and GGT activities decreased (P<0.05) in PRO treated group (Group 2); also triglyceride and bilirubin levels and ALP and GGT activities decreased (P<0.05) in CYP treated group (Group 3); also triglyceride and bilirubin levels and GGT activities were low (P<0.05) both PRO treated group (Group 2) and CYP treated group (Group 3) *(Table 1)*.

Degenerative changes were present in all of the treatment groups and were classified as severe (+++), moderate (++), and mild (+), according to their severity *(Table 2)*. Microscopically, severe lesions were observed in the kidney and liver samples of Group 4 on days 45 and 60 *(Table 2)*.

The presence of many focal inflammatory cells and degeneration were observed in the hepatic tissues. Degenerative changes were characterized by parenchymatous degeneration and the disintegration of hepatocytic nuclei along with the hyperplasia of the bile

Table 1. *Biochemical parameters of groups (mean±sd)*
Tablo 1. *Gruplardaki biyokimyasal parametreler (ort±ss)*

Parameter	Group I (Control)	Group II (PRO)		Group III (CYP)		Group IV (PRO-CYP)	
		45 DAY	60 DAY	45 DAY	60 DAY	45 DAY	60 DAY
Cholesterol (mg/dL)	88.2±8,1^{d}	116.3±12.2^{c}	118.7±13.1^{c}	127.3±11.9^{bc}	129.3±11.9^{bc}	142.2±4.0^{ab}	147.7±5.7^{a}
Triglyceride (mg/dL)	107.7±8.2^{d}	160.0±19.5^{c}	161.0±14.8^{c}	207.8±27.1^{b}	210.8±26.6^{b}	260.5±38.6^{a}	265.0±26.9^{a}
Total protein (mg/dL)	7.6±0.6	7.3±0.5	7.2±0.2	7.1±0.5	7.2±0.4	7.4±0.3	7.5±0.2
Albumin (mg/dL)	4.6±0.2^{a}	4.0±0.2^{b}	3.7±0.2^{bcd}	4.0±0.4^{bc}	3.5±0.2^{bcd}	3.5±0.2^{bcd}	3.4±0.1^{bcd}
AST (U/L)	106.7±16.0^{e}	225.3±42.6^{d}	242.2±30.8^{d}	300.3±8.5^{c}	311.5±10.2^{bc}	343.8±21.0^{ab}	362.3±19.5^{a}
ALT (U/L)	21.3±2.9^{d}	36.2±6.7^{c}	36.7±2.9^{c}	40.7±8.2^{bc}	47.8±5.4^{b}	50.2±6.2^{b}	69.3±6.7^{a}
ALP (U/L)	49.7±1.0^{d}	77.2±8.5^{c}	80.0±7.3^{bc}	76.0±5.4^{c}	78.8±8.8^{c}	94.0±7.7^{ab}	97.3±5.6^{a}
GGT (U/L)	1.7±0.8^{c}	14.7±2.1^{b}	15.3±2.0^{b}	15.3±3.2^{b}	16.0±3.3^{b}	25.2±4.8^{a}	27.2±1.0^{a}
Bilirubin (mg/dL)	2.4±0.2^{c}	2.6±0.2^{c}	2.8±0.1^{c}	2.8±0.3^{c}	2.9±0.1^{c}	3.4±0.5^{b}	4.0±0.3^{a}

PRO: *Propetamphos,* ***CYP:*** *Cypermethrin,* ***PRO-CYP:*** *Propetamphos and cypermethrin combination,* ***AST:*** *Aspartate aminotransferase,* ***ALT:*** *Alanine aminotransferase,* ***ALP:*** *Alkaline phosphatase,* ***GGT:*** *Gamma-glutamyl transferase;* ***a,b,c,d,e:*** *Different characters indicate statistically significant differences in the same line (P<0.05)*

Table 2. *Histopathological findings of groups*
Tablo 2. *Gruplardaki histopatolojik bulgular*

Organ /Lesion	Group II		Group III		Group IV	
	PRO-45	PRO-60	CYP-45	CYP-60	CYP-PRO-45	CYP-PRO-60
Liver						
Cytoplasmic vacuolation					+++	++++
Condensed/pyknotic nuclei				+++		
Necrosis		+++		+++	+++	++++
Cellular infiltration		+++	++	+++	++	++++
Hyperplasia of bile duct						
Passive congestion		++	++		++	++++
Kidney						
Pyknotic nuclei						
Necrosis						++++
Deposition of casts						++++
Epithelium cytoplasmic vacuolation				+++		++++
Glomerulus atrophy						++++
Cellular infiltration	+++					++++

CYP: *Cypermethrin,* ***PRO:*** *propetamfos,* ***CYP-PRO:*** *Cypermethrin and propetamfos combination*

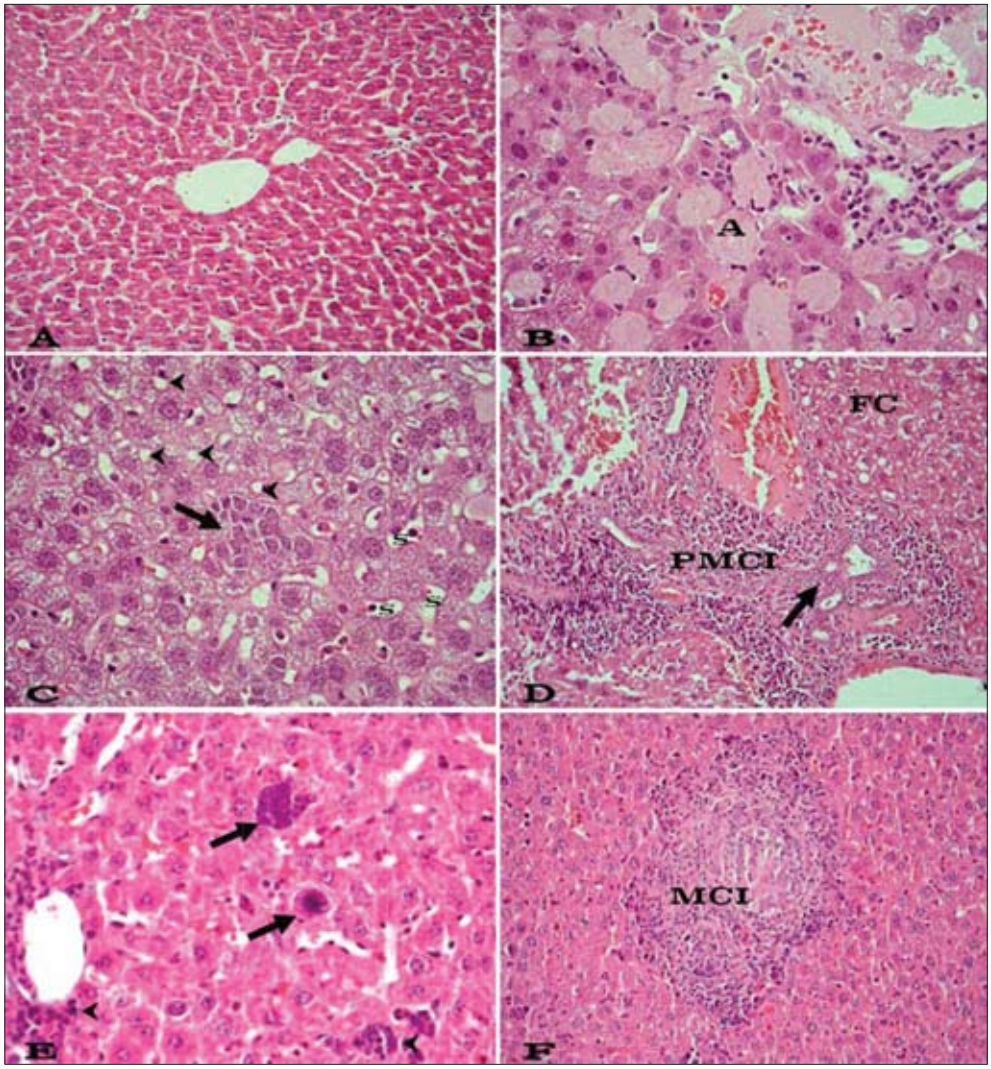


Fig 1. Histopathological findings of liver in Group 4. **A:** Histology of control group liver, **B-F:** Pronounced histopathological abnormalities observed in Group 4, **B:** Perisinusoidal amyloidosis (A), **C:** Hepatocellular hypertrophy with cytoplasmic basophilia, Fatty changes *(arrowheads)* and dilated sinusoids (S), **D:** Perivascular cell infiltration and fatty changes, **E:** Parenchymatous degeneration of hepatocytes with mild necrosis. Mononuclear cell infiltration and multinucleation of hepatocytes *(arrow),* **F:** Focal cellular granulomatous lesions. PMCI: Perivasküler round cell infiltration; FC: Fatty change; MCI: Mononuclear cell infiltration. Magnification: A&D&F, x200; B-C-E, x400

Şekil 1. Gruplarda karaciğere ait histopatolojik bulgular. **A:** Kontrol grubuna ait karaciğerlerin histolojisi, **B-F:** Grup 4'te gözlenen belirgin histopatolojik bulgular, **B:** Perisinuzoidal amiloidler (A), **C:** Sitoplazmik bazofiler içeren hepatoselüler hipertorifi. Yağlı değişimler *(ok uçları)* ve genişlemiş sinüzoidler (S), **D:** Perivasküler hücre infiltrasyonu ve yağlı değişimler, **E:** Hepatositlerde orta şiddetli nekrozla karakterize paranşimatöz dejenerasyon. Mononuklear hücre infiltrasyonu ve hepatositlerde multinükleasyon *(oklar)*, **F:** Fokal hücresel granülomatöz lezyonlar. PMCI: Perivasculer hücre infiltrasyonu; FC: Yağlı değişimler; MCI: Mononüklear hücre infiltrasyonu. Büyütme: A&D&F, x200; B-C-E, x400

ducts. The cytoplasm of the hepatocytes was of a light colour and foamy appearance and was filled with vacuoles. The cell size had increased, the nuclear chromatin was more compact, and the slightly smaller nucleoli were not conspicuous. The necrotic hepatocytic nuclei were contracted, pyknotic, filled with condensed chromatin, and the nuclear cytoplasm was strongly acidophilic. The accumulation of mononuclear cells was observed in the vicinity of sinusoids. Histopathological alterations included perivascular round cell infiltration, marked degeneration of the hepatic cords, and increased incidence of vacuolar degeneration *(Fig. 1, Table 2)*. Hepatocellular hypertrophy, multinucleated hepatocytes and the deposition of pale, homogeneous and amorphous eosinophilic material in the periportal and perisinusoidal areas and the blood vessel wall were observed on day 60 in only Group 4. Microscopically, the kidneys showed toxic tubular necrosis characterized by pyknotic cells, sloughing of the tubular epithelium and epithelial casts in the tubular lumen associated with the atrophy of the glomeruli in Group

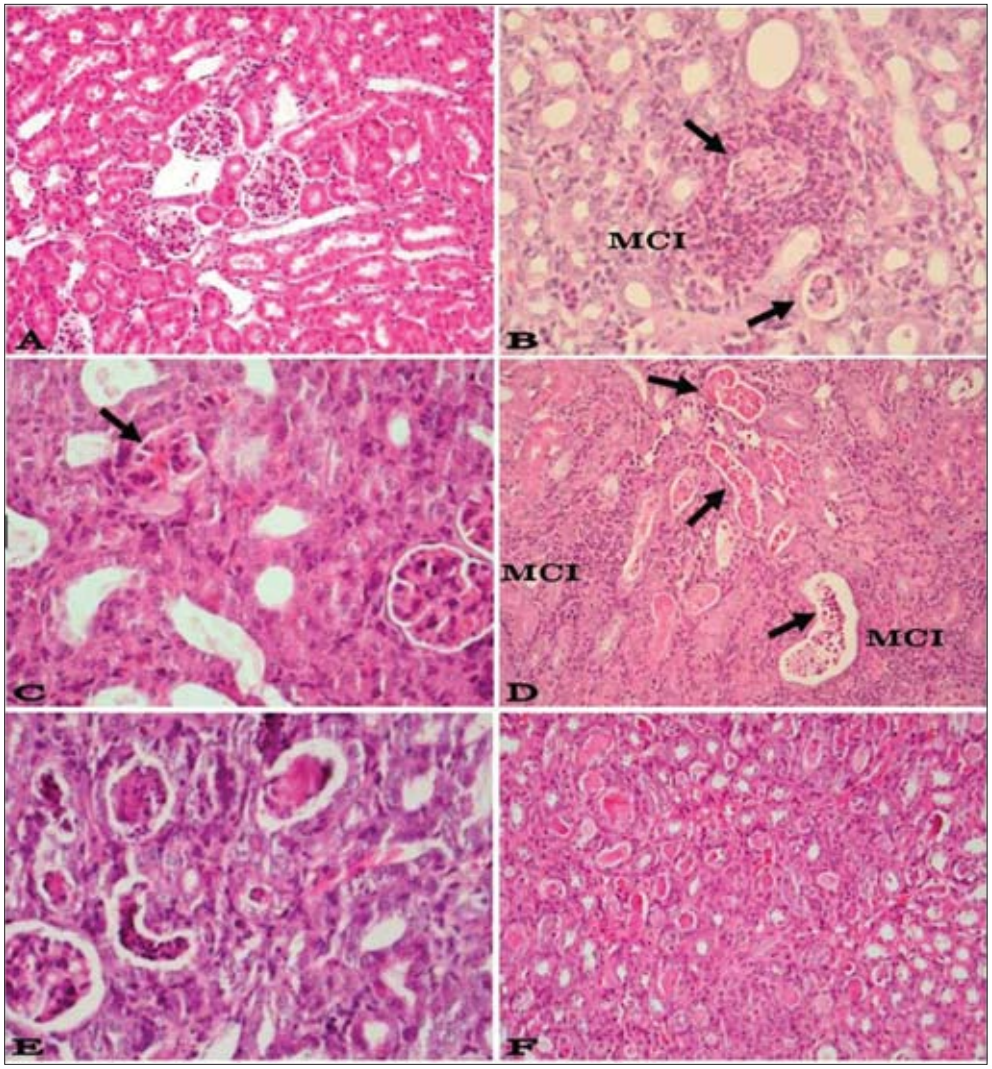


Fig 2. Histopathological findings of kidney on Group 4. **A:** Histology of control group kidney, **B-F:** Pronounced histopathological abnormalities in Group 4 on day 60, **B:** Tubular necrosis *(arrows)* and focal mononuclear cell infiltration, **C:** Glomerular necrosis *(arrow)* and tubular dilatation, **D:** Tubules containing necrotic cells *(arrows)* and mononuclear cell infiltration, **E:** Tubular necrosis, **F:** Hyaline casts in tubule lumens. MCI: Mononuclear cell infiltration. Magnification: A&D&F, x200; B-C-E, x400

Şekil 2. Gruplarda böbreklere ait histopatolojik bulgular. **A:** Kontrol grubuna ait böbreklerin histolojisi, **B-F:** Grup 4'de 60. günde gözlenen belirgin histopatolojik değişimler, **B:** Tubuler nekroz *(oklar)* ve fokal mononuclear hücre infiltrasyonu, **C:** Glomeruler nekroz *(oklar)* ve tubuler genişleme, **D:** Tubuler nekrotik hücreler ve mononuclear hücre infiltrasyonu *(oklar),* **E:** Tubuler nekroz, **F:** Tubul lümenlerinde hiyalinize bölgeler. MCI: Mononüklear hücre infiltrasyonu. Büyütme: A&D&F, x200; B-C-E, x400

4. Karyorrhexis, and in some cells karyolysis and cellular infiltration, were also observed in Group 4 *(Fig. 2, Table 2)*.

DISCUSSION

In the present study, when compared to the control group, it was observed that in the PRO treated group (Group 2) for 45 and 60 days, the serum cholesterol and triglyceride levels and AST, ALT, ALP and GGT activities had increased, while the albumin levels had decreased. In particular, the increases observed in the activity of ALT, an enzyme specific to hepatic damage [9], as well as in the activities of AST, ALP and GGT demonstrated that PRO caused hepatic damage in mice. It has been reported that PRO undergoes desulphuration by the liver P-450 enzymes and is converted into oxygen-derived metabolites, while by hydrolytic reactions it is converted into acetoacetate, acetone and CO_2 in rats and mice [10]. In the present study, increasing of serum cholesterol and triglyceride levels in PRO treated group (Group 2) demonstrate that PRO have adverse effects on the lipid metabolism. It is reported that serum ALP activity, and cholesterol and triglyceride levels also increase in cases of cholestasis [10]. In addition to increase of ALP activity and cholesterol and triglyceride levels in the PRO treated group (Group 2) suggest that subchronic exposure to PRO causes cholestasis in mice.

Albumin is an enzyme synthesized in the liver and found at a high level in the serum [10]. This study showed that the administration of PRO decreased serum albumin levels, and this decrease may be associated with hepatic damage.

Cetin et al.[11] reported that, following the administration of 15 mg/kg of PRO for 28 days to rats, serum triglyceride levels, and AST, ALP, and ALT activities increased, while total protein levels decreased. Upon administering propetamphos at doses of 7.5 and 15 mg/kg to rats for 28 days, Kanbur et al.[12] reported that plasma MDA levels had increased while erythrocyte SOD, CAT and GSH-Px activities had decreased. It is suggested that the adverse effects of PRO on the liver could be related to the active metabolites of the drug, which are generated in the liver, and to the lipid peroxidation induced by these metabolites.

The present study demonstrated that, when compared to the control group, the administration of CYP at a dose of 10 mg/kg to mice for periods of 45 and 60 days led to increased serum cholesterol and triglyceride levels, and AST, ALT, ALP and GGT activities and decreased albumin levels. Bhushan et al.[13] reported that the acute (300 mg/kg) and subchronic (10.7 mg/kg) exposure of rats to CYP caused increases in serum AST, ALT, ALP, and LDH activities, and total lipid, triglyceride, total protein, cholesterol, and bilirubin levels particularly on day 28. Gomaa et al.[14] indicated that the administration of rats with 14.5 mg/kg of CYP for 30 days elevated serum liver enzymes, cholesterol and MDA levels, and reduced total protein, albumin, triglyceride and LDL levels as well as antioxidant enzyme (SOD, CAT, GSH-Px) activities. It has been reported that, in rats, exposure to CYP causes haemolytic anaemia and hyperbilirubinaemia due to erythrocytic membrane disease [13]. In a study conducted by Khan [15], in which mice were given 20 mg/kg of CYP by gavage for 15 days, serum ALP, AST and ALT activities were determined to have increased, while liver SOD, CAT, GSH-Px and GSH-S-transferase activities were ascertained to have decreased. Manna et al.[16] determined that, when rats were administered with alpha-CYP at a dose of 14.5 mg/kg for 30 days, serum AST, ALT, and ALP activities, and blood glucose and liver MDA levels increased, and CAT and SOD activities decreased. ALT activity is considered as an indicator of general hepatocellular damage, whilst AST activity is used as an indicator of mitochondrial damage. On the other hand, the increase of blood ALP activity indicates the presence of cholestasis in the liver. Furthermore, increased serum LDH levels are considered to be an indicator of hepatic necrosis [17]. Both the results of the present study and those reported in previous research suggest that CYP leads to hepatic damage and induces cholestasis. The results of this study also show that the damaging effect of CYP on the liver is more severe than that of PRO. Xenobiotics activate the sympathetic nervous system, inducing the release of adrenaline from the adrenal medulla, thus increasing both the mobilisation of lipids from tissues and serum triglyceride levels [13]. The triglyceride-increasing effects of CYP may also be related to this mechanism.

Manna et al.[18] reported that CYP caused moderate histopathological alterations in the liver and kidneys. Gomaa et al.[14] observed congestion of the central and portal veins and hydropic degeneration of the hepatocytes in the liver, and suggested hepatic damage to be associated with free radicals generated as a result of the oxidation of CYP by the CYP-450 enzyme system. In various research, rats administered with CYP were observed to present with the enlargement of the hepatic sinusoids, degeneration of the hepatic cords and hepatocytes in the centrilobular areas, dilatation and desquamation, cytoplasmic accumulation of eosinophils, and erythrocyte accumulation in the cerebellar and meningeal blood vessels of the brain, and these effects were considered to be associated with the generation of free radicals [19-21]. Apart from causing free radical generation in the liver, CYP also inhibits the activity of hepatic ATPase, resulting in the necrosis, inflammation and cytoplasmic hypertrophy of hepatocytes. It has been reported that synthetic pyrethroids lead to the formation of unstable cyanides and aldehydes in the liver [22]. Thus, these metabolites may be responsible for the cellular damage caused by CYP in the liver. The hepatic damage caused by cypermethrin in the present study may be related to these metabolites as well as to free radical-induced lipid peroxidation and reduced ATPase activity.

Compared to the PRO and CYP treated group (Group 2 and Group 3), cholesterol, triglyceride, and bilirubin levels, and AST, ALT and GGT activities increased ($P<0.05$) in PRO-CYP treated group particularly in 60 days (Group 4). The results of the present study demonstrated that the combined administration of CYP and PRO caused synergistic interaction in mice and aggravated hepatic damage. The results of the present study also showed that while the administration of PRO and CYP alone did not cause any statistically significant difference in serum bilirubin levels, the combined use of PRO and CYP led to a statistically significant increase in serum bilirubin levels. Increased bilirubin levels are considered an indicator of hepatobiliary disorders [9]. This data confirms PRO-CYP treatment aggravate the liver damage.

In a study in which mice were administered with CYP (10 mg/kg), PRO (5 mg/kg) and a combination of CYP and PRO [5] it has been reported that MDA and NO significantly increase, SOD, CAT and GSH-Px activities significantly decrease in all groups; CYP, PRO and CYP+PRO combination induce lipid peroxidation. In our study, triglyceride and bilirubine levels and ALP and GGT activities were more high in PRO-CYP treated group (Group 4) than other groups; also ALT activity and bilirubine level were high in PRO-CYP treated group (Group 4) at 60 day. Results of

this study show that PRO-CYP treatment exacerbates the liver degeneration. PRO-CYP induced hepatic damage could be related to lipid peroxidation as mentioned in previous studies.

In the present study, the combined administration of PRO and CYP, particularly for a period of 60 days, was observed to be associated with passive congestion, necrosis, mononuclear cell infiltration and hyperplasia of the bile ducts in the liver, and with cellular infiltration and degenerative alterations in the kidneys. Bile duct hyperplasia may result from hepatic injury and repair, and is often associated with evidence of these phenomena. Mononuclear cells with areas of hepatocyte necrosis can be considered as an indication of the chronic inflammation of the liver. The infiltration of inflammatory cells is a typical response to parenchymal cell death following exposure to toxicants and their toxic metabolites [23]. Similar but more severe lesions were observed in the Group 4.

In the Group 4, degenerative and aggressive lesions including necrosis, multinucleated and disarranged hepatocytes and amyloid degeneration, were characteristic pathological changes. Hepatocellular hypertrophy following enzyme induction was considered to be an adaptive response to chemical stress. Tubular necrosis and focal mononuclear cell infiltration, glomerular necrosis and hyaline casts in tubule lumens with tubular dilatation, and perivascular round cell infiltration were observed in the Group 4. The histopathological changes show consistency with our biochemical results *(Table 1)* and previous studies [24,25].

Enzymes responsible for metabolic interactions (biotransformation), which are one of the major mechanisms of drug interactions, are in general enzymes belonging to the cytochrome P-450 group. When drugs, which are the substrate of the same enzyme group, are administered in combination, there is a possibility of metabolic interactions occurring [26,27]. It has been reported that pyrethroids were rapidly metabolized by esterases in the body and that their metabolic rate could be reduced by esterase inhibitors, such as organic phosphorous insecticides, increasing the toxicity of pyrethroids [5,27]. Similarly, the present study demonstrated that the combined administration of PRO and CYP to mice for 60 days caused to synergistic effect on biochemical parameters and at histopathological findings. The aggravation of adverse effects with combined use may not only be related to metabolic interactions, but also to the two pesticides prolonging the elimination of each other from the body.

In the present study, it was concluded that the exposure of mice to PRO, CYP and PRO-CYP combinations for subchronic and chronic periods caused adverse effects on liver and kidney functions as well as on protein and lipid metabolism; PRO-CYP combination aggravated these adverse effects; chronic exposure of PRO, CYP and their mixture are more toxic than subchronic exposure. The results show that usage of insecticide combinations can increase insecticide induced toxic effects on mammals and duration time affects toxicity of insecticides. So, *in vivo* and *in vitro* researches are required to understand toxicities of insecticides and insecticide mixtures on mammals.

REFERENCES

1. Kasagami T, Miyamoto T, Yamamoto I: Activated transformations of organophosphorus insecticides in the case of non-AChE inhibitory oxons. *Pest Manag Sci*, 58, 1107-1117, 2002.

2. Kaya S: İnsektisidler. **In,** Kaya S, I Pirincci I, Bilgili A (Eds): Veteriner Hekimliğinde Toksikoloji. 2. Baskı. 401-453. Medisan Yayınları No: 53. Ankara, 2002.

3. Giray B, Gürbay A, Hincal F: Cypermethrin-induced oxidative stress in rat brain and liver is prevented by vitamin E or allopurinol. *Toxicol Lett*, 118, 139-146, 2001. DOI: 10.1016/S0378-4274(00)00277-0

4. Extoxnet: Cypermethrin. http://extoxnet.orst.edu/pips/cypermet.htm, 1996. *Accessed:* 17.07.2014.

5. Kanbur M, Liman BC, Eraslan G, Altınordulu S: Effects of cypermethrin, propetamphos, and combination involving cypermethrin and propetamphos on lipid peroxidation in mice. *Environ Toxicol*, 23, 473-479, 2008. DOI: 10.1002/tox.20360

6. Raizada RB, Srivastava MK, Kaushal RA, Singh RP, Gupta KP: Subchronic oral toxicity of a combination of insecticide (HCH) and herbicide (ISP) in male rats. *J Appl Toxicol*, 21, 75-79, 2001. DOI: 10.1002/jat.733

7. Wade MG, Parent S, Finnson KW, Foster W, Younglai E, McMahon A, Cyr DG, Hughes C: Thyroid toxicity due to subchronic exposure to a complex mixture of 16 organochlorines, lead, and cadmium. *Toxicol Sci,* 67, 207-218, 2002. DOI: 10.1093/toxsci/67.2.207

8. Cesaretli Y, Kaya S, Essiz D, ve Otogeçim N: Türkiye Ulusal Zehir Danışma Merkezi kayıtlarına göre 1995-2004 yıllarında veteriner ilaçları ile meydana gelen zehirlenme vakalarının değerlendirilmesi. *I. Ulusal Veteriner Farmakoloji ve Toksikoloji Kongresi*, 22-24 Eylül, Ankara, s.272-273, 2005.

9. Turgut K: Klinik enzimoloji. **In,** Turgut K (Ed): Veteriner Klinik Laboratuar Teşhis. 2. Baskı. 179-201. Bahçıvanlar Basımevi, Konya, 2000.

10. Ecobichon DJ: Toxic effects of pesticides. **In,** Klaassen CD (Ed): Casarett and Doull's Toxicology-The Basic Science of Poisons. 643-690. McGraw-Hill, New York, 1996.

11. Çetin E, Kanbur M, Silici S, Eraslan G: Propetamphos-induced changes in haematological and biochemical parameters of female rats: Protective role of propolis. *Food Chem Toxicol*, 48, 1806-1810, 2010. DOI: 10.1016/j.fct.2010.04.010

12. Kanbur M, Eraslan G, Silici S: Antioxidant effect of propolis against exposure to propetamphos in rats. *Ecotoxicol Environ Saf*, 72, 909-915, 2009. DOI: 10.1016/j.ecoenv.2007.12.018

13. Bhushan B, Pande S, Saxena N, Saxena PN: Serum biochemical responses under stress of cypermethrin in albino rat. *Environ Exp Biol,* 11, 81-89, 2013.

14. Gomaa MS, Alla MAA, Sameer MM: The possible protective effect of propolis (bbe glue) on cypermethrin-induced hepatotoxicity in adult albino rats. *Forensic Med Clin Toxicol*, 19, 17-32, 2011.

15. Khan SM: Protective effect of black tea extract on the levels of lipid peroxidation and antioxidant enzymes in liver of mice with pesticide-induced liver injury. *Cell Biochem Funct*, 24 (4): 327-332, 2006.

16. Manna S, Bhattacharyya D, Mandal TK, Das S: Repeated dose toxicity of alfa-cypermethrin in rats. *J Vet Sci*, 5, 241-245, 2004.

17. Kanbur M, Eraslan G, Çakir L, Silici S, Liman BC, Altınordulu S, Atasever A: The effects of royal jelly on liver damage induced by paracetamol in mice. *Exp Toxicol Pathol*, 61 (2): 123-132, 2009. DOI: 10.1016/j.etp.2008.06.003

18. Manna S, Bhattacharyya D, Basak DK, Mandal TK: Single oral dose toxicity study of α-cypermethrin in rats. *Indian J Pharmacol*, 36, 24-28, 2004.

19. Sankar P, Telang AG, Manimaran A: Protective effect of curcumin on cypermethrin-induced oxidative stress in wistar rats. *Exp Toxicol Pathol*, 64 (5): 487-493, 2012. DOI: 10.1016/j.etp.2010.11.003

20. Yavasoğlu A, Sayım F, Uyanikgil Y, Turgut M, Karabay-Yavasoglu NÜ: The pyrethroid cypermethrin-induced biochemical and histopathological alterations in rat liver, *J Health Sci*, 52 (6): 774-780, 2006.

21. Muthuviveganandavel V, Muthuraman P, Muthu S, Srikumar K: A study on low dose cypermethrin induced histopathology, lipid peroxidation and marker enzyme changes in male rat. *Pest Biochem Physiol*, 91 (1): 12-16. 2008. DOI: 10.1016/j.pestbp.2007.11.010

22. WHO: Cypermethrin, Environmental Health Criteria. International Programme on Chemical Safety, No 82. Geneva, 1989.

23. Renne R, Harkema J, Herbert R, Kittel B, Lewis D, March T, Nagano K, Pino M, Rittinghausen S, Rosenbruch M, Tellier P, Wohrmann T: Proliferative and nonproliferative lesions of the rat and mouse respiratory tract. Proliferative and nonproliferative lesions of the rat and mouse respiratory tract. *Toxicol Pathol*, 37, 5-73, 2009. DOI: 10.1177/0192623309353423

24. Khana, A, Faridia HAM, Alia M, Khan MZ, Siddique M, Hussain I, Ahmad M: Effects of cypermethrin on some clinico-hemato-biochemical and pathological parameters in male dwarf goats *(Capra hircus)*. *Exp Toxicol Pathol*, 61, 151-160, 2009.

25. Aslam F, Khan A, Khan MZ, Sharaf S, Gul ST, Saleemi MK: Toxico-pathological changes induced by cypermethrin in broiler chicks: Their attenuation with Vitamin E and selenium. *Exp Toxicol Pathol*, 62, 441-450, 2010. DOI: 10.1016/j.etp.2009.06.004

26. Dulger G: Farmakokinetik etkileşmeler. **In,** O Kayaalp (Ed): Tıbbi Farmakoloji. 121-128, Hacettepe-Taş Kitabevi, Ankara, 2002.

27. Audegond L, Catez D, Foulhoux P, Fournex R, Le Rumeur CL, Hotellier M, Stepniewski JP: Potentiation of the toxicity of deltamethrin by organophosphorus insecticides. *J Toxicol Clin Exp*, 9, 163-176, 1989.

Kafkas Univ Vet Fak Derg
21 (2): 195-201, 2015
DOI: 10.9775/kvfd.2014.12015

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Effects of Kefir, Koumiss, Milk and Yoghurt Administration on Distribution of Plasma Cells and Mast Cells in Mice Spleen [1]

Sevda ELİŞ YILDIZ [1] Funda YİĞİT [2] Berna DUMAN AYDIN [3]
Ebru KARADAĞ SARI [4] Turgay DEPREM [4] Serap KORAL TAŞÇI [4]

[1] *This research was presented at XII National Histology and Embriyology Congress with International Contribution, P:030, 27-30 May, 2014, Ankara, Türkiye*

[1] Kafkas Üniversitesi, Kars Sağlık Yüksekokulu, TR-36100 Kars - TÜRKİYE
[2] İstanbul Üniversitesi, Veteriner Fakültesi, Histoloji ve Embriyoloji Anabilim Dalı, TR-34320 İstanbul - TÜRKİYE
[3] Gıda Tarım ve Hayvancılık İl Müdürlüğü, TR-21100 Diyarbakır - TÜRKİYE
[4] Kafkas Üniversitesi, Veteriner Fakültesi, Histoloji ve Embriyoloji Anabilim Dalı, TR-36300 Kars - TÜRKİYE

KVFD-2014-12015 Received: 20.07.2014 Accepted: 04.12.2014 Published Online: 10.12.2014

Abstract

The present study was conducted to investigate the effect of kefir, koumiss, milk and yoghurt administration on plasma cells, which synthesize specific antibodies against antigens, and mast cells, which have a role in the regulation of humoral and cellular events, in the mice spleen tissues. Mice (n=30) were divided into 5 groups. As a result of statistical evaluation, live weights of mice in kefir, koumiss, milk and yoghurt groups were found to be increased compared to the control group. When numbers of plasma cells in spleens of mice treated with kefir, koumiss, milk and yoghurt were checked with stereological methods, significant difference was found to be increased in number of plasma cells of kefir-treated mice compared to other groups at the end of 15 days. At the end of day 15, a significant difference was found to be increased in the number of mast cells in the spleen of milk-treated mice compared to control group. As the number of plasma cells in the spleen is at higher level with kefir, than with koumiss, milk and yoghurt; also there being a significant difference between the numbers of mast cells in the spleens of mice treated with milk compared to control group, this is remarkable for healthy eating/functional food.

Keywords: *Kefir, Koumiss, Milk, Yoghurt, Plasma cell, Mast cell*

Kefir, Kımız, Süt ve Yoğurt Uygulamasının Fare Dalağında Plazma Hücresi ve Mast Hücrelerinin Dağılımı Üzerine Etkileri

Özet

Bu çalışma, kefir, kımız, süt ve yoğurt uygulamasının fare dalak dokusunda, karşılaştıkları antijene spesifik antikor sentezleyen plazma hücreleri ile humoral ve hücresel olayların düzenlemesinde rolü olan mast hücrelerinin sayısal dağılımını belirlenmeyi amaçlanmıştır. Fareler (n=30) her grupta 6 adet olacak şekilde 5 gruba ayrıldı. İstatiksel değerlendirmeler sonucunda kefir, kımız, süt ve yoğurt grubunda bulunan farelerin canlı ağırlıklarının kontrol grubuna göre arttığı tespit edildi. Kefir, kımız, süt ve yoğurt uygulanan farelerin sterelojik yöntemle dalaktaki plazma hücreleri sayısına bakıldığında 15. günün sonunda kefir uygulanan farelerin dalağında plazma hücrelerinin sayısında diğer gruplara göre anlamlı bir artışın olduğu tespit edildi. 15. günün sonunda süt uygulanan farelerin dalağında mast hücrelerinin sayısında kontrol grubuna göre anlamlı bir artışın olduğu belirlendi. Yararlı mikroorganizmalardan oluşan kefirin, dalaktaki plazma hücre sayısına bakıldığında kımız, süt ve yoğurttan daha yüksek düzeyde bulunması; ayrıca süt uygulanan farelerin dalağında mast hücrelerinin sayısında kontrol grubuna göre anlamlı bir farkın olması sağlıklı beslenme/fonksiyonel gıda kapsamında dikkat çekicidir.

Anahtar sözcükler: *Kefir, Kımız, Süt, Yoğurt, Plazma hücresi, Mast hücresi*

INTRODUCTION

Kefir, obtained from kefir granules or main culture of granules, is a refreshing, slightly acidic fermented dairy product with combination of ethyl alcohol and lactic acid fermentation [1]. Kefir has been reported to have antitumoral effect, effect on immune system and digestive system, effect against lactose intolerance and cholesterol, as well

İletişim (Correspondence)
+90 474 2251567
sevdaelis36@hotmail.com

as relaxing effect [2-6]. Koumiss is a dairy product produced from fermented mare's milk in Central Asia, especially in Turkestan and Mongolia [7]. Koumiss is an old Turkish drink derived from mare's milk. Koumiss ferment also includes yeasts such as Torulasp in addition to lactic acid bacteria such as *Lactobacillus bulgaricus* and *Saccharomycetales* [8]. Milk is a fluid with peculiar smell and flavor which is secreted from milk glands according to animal species at different times in order to feed new breeds and it contains essential amino acids, fat, lactose, essential minerals and vitamins that baby animals have to get until they can feed themselves [9]. Yoghurt is a fermented dairy product obtained by lactic acid fermentation with addition of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* to milk [10]. It has carbohydrates, lipids, quality protein, important minerals and B vitamins and is known to have an important place in human nutrition [11]. Plasma cells typically represent less than 1% of the cells in lymphoid organs, yet they are responsible for all antibody in circulation [12]. Because it has more phagocytic cells, spleen is an important place for defense against antigens reaching blood circulation. Spleen is an important filter for blood and antibody-producing organ as it catches blood borne antigens and reacts quickly [13,14]. Mast cells are found in the skin and in all mucosal tissues at homeostasis, and numbers are elevated in asthmatics lungs and gastrointestinal tract of inflammatory bowel disease [15]. These cells are particularly found in body surfaces such as gastrointestinal tract mucosa, skin and peritoneum in proximity to blood vessels and nerves [16] and mast cells are necessary for the development of allergic reactions, through crosslinking of their surface receptors for IgE leading to degranulation and the release of vasoactive, pro-inflammatory and nociceptive and proteolytic enzymes [17,18].

In this study, it was aimed to determine the effects of kefir, koumiss, milk and yoghurt on number of plasma cells that synthesize specific antibodies against antigens and mast cells that play important role in regulation of immune system and defense of organism in mice spleen tissue.

MATERIAL and METHODS

Experimental Animals

This study, approved by the Animal Testing Local Ethics Committee of Mehmet Akif Ersoy Universty use Committee Protocol (12/03/2014, 11/69). In the study, a total of 30 male mice *(Swiss albino)*, weighing approximately 22-35 g, which were never mated and never used in any studies, were used. Mice were randomly divided into 5 groups each containing 6 mice and fed ad libitum for fifteen days with normal rat chow and tap water, at 22±2°C ambient temperature, 12 h of light/dark cycle in standard cages.

Establishment of Experimental Groups

Group I (Kefir), kefir drink was prepared by adding kefir granules into 3% sterile milk and fermentation for 24 h at 30°C. The obtained kefir was diluted at a 1/3 rate and prepared fresh for every administration. Group II (Koumiss), koumiss obtained from Alaş Koumiss Farm (Kemalpaşa, İzmir) was used in its original form. Group III (Milk), sterile milk was given to rats in their original form, as sold in the market as UHT milk. Sterile milk used during the study contained 3% fat, 3% protein and 4.5% carbohydrates. Group IV (Yoghurt), lyophilized yoghurt culture was added into sterile milk and fermentation was achieved at 43°C for 3-4 h and yoghurt was provided to complete 24 h at +4°C. At the end, yoghurt was applied following 1/3 dilution rate. Group V (Control), this group of mice was fed with mice chow and tap water.

Live Weight Measurements

Live weight of all animals in all groups was weighed before starting 15 days administration and at the end of study. Mice were euthanized by cervical dislocation under anesthesia with diethyl ether and spleen tissues were collected. Statistical Package for Social Sciences 15.0 (SPSS 2006) program was used for statistical comparison of live weight measurements between groups. Using the Wilcoxon Signed Ranks Test, possible differences were detected.

Histological Studies

A portion of the spleen tissue fragments was fixed in alcohol-formalin fixing solution in order to determine plasma cells and pyroninophilic cells, and fixed in 10% formaldehyde solution for 48 h in order to determine mast cells. Then they were passed from graded alcohols, methyl benzoate and benzene series and blocked in paraplast embed. 5 µm sections from these blocks were stained by methyl green-pyronin staining [19] to demonstrate plasma cells and Toluidine Blue (pH 0.5) staining method to demonstrate mast cells [20].

Cell Counts and Statistical Analysis

For stereological analysis of plasma cells in spleen tissue preparations; digital camera (MBF/Bioscience, Qimaging), automatically controlled sample stepper, light microscope (Leica, DM400B) and the software program (MBF Bio-science, Stereo investigator, version 9) were used. Each region was determined to be 200.000 μm^2 in 5 different regions of spleen preparations obtained from each animal. In this field, number of plasma cells was determined in a 1 mm^2 area on the entire surface with a random systematic with 70 µm x70 µm step interval and 900 μm^2 unbiased counting frame [21]. 100 square ocular micrometer (eye piece graticule) was used in order to determine the numerical distribution of mast cells in spleen preparations stained with Toluidine Blue. The area of 100 square ocular micrometer was calculated with the help of micrometer slide at hundred (100) lens zoom of Olympus CX22-type

light microscope. Mast cell count was done with 100x lens magnification in 25 randomly selected different regions of each section. Then all the obtained data was converted to number of mast cells per 1 mm^2 unit area [19]. SPSS 15.0 was used for statistical analysis of plasma and mast cell counts in the spleen and One-Way ANOVA and Duncan's multiple comparison tests were performed.

RESULTS

Live Weight Results

As a result of the statistical data, live weights of kefir, koumiss, milk and yoghurt groups were increased compared to the control group *(Table 1)*. When groups were inter-compared, a significant difference was detected regarding live weight gain in kefir-control, koumiss-control, milk-control and koumiss-yoghurt groups *(Table 2)*.

Table 1. *A comparison of the live weight between groups*
Tablo 1. *Gruplar arasında canlı ağırlıklarının karşılaştırılması*

Groups	Number (n)	Mean ± Standard Deviation
Kefir	6	37.71±3.34
Koumiss	6	38.80±2.85
Milk	6	39.08±3.84
Yoghurt	6	36.63±2.26
Control	6	33.58±3.76

Histological Findings

In spleen tissue of all groups, specific plasma cells were detected as a result of methyl green-pyronin staining *(Fig. 1, 2, 3)*. Plasma cells were determined to be found in greater amounts in the red pulp compared to white pulp. Considering the number of plasma cells, distribution was observed to differ between the groups. When number of plasma cells in 1 mm^2 of spleen was examined by stereological methods at the 15th day a significant difference was found in number of plasma cells in the kefir group compared to koumiss, milk, yoghurt and the control group ($P<0.05$). Also increased number of plasma cells was found in koumiss, milk and yoghurt groups compared to control group ($P<0.05$) *(Table 3)*.

Specific mast cells were detected in spleen tissues of all groups *(Fig. 4, 5)*. Mast cells were determined in the red pulp in greater amount than in white pulp of spleen. It was observed that they especially concentrated around blood vessels. Considering the number of mast cells, significant differences between groups were observed regarding distribution. When number of mast cells in 1 mm^2 of spleen was examined by stereological methods at the 15th day groups, a significant difference was found in number of mast cells in the milk group compared to other groups ($P<0.05$). The largest increase in the number of mast cells of groups was determined to be in the milk group ($P<0.05$) *(Table 4)*.

Table 2. *Comparison of inter-group live weights with Wilcoxon signed-range test*
Tablo 2. *Gruplar arası canlı ağırlıkların Wilcoxon signed rang testi ile karşılaştırılması*

Test	Kefir Koumiss (g)	Kefir Milk (g)	Kefir Yoghurt (g)	Kefir Control (g)	Koumiss Milk (g)	Koumiss Yoghurt (g)	Koumiss Control (g)	Milk Yoghurt (g)	Milk Control (g)	Yoghurt Control (g)
Z value	-0.94	-0.52	-0.73	-2.20	-0.11	-1.99	-1.99	-0.73	-1.57	-1.57
P value	0.35	0.60	0.46	0.03*	0.92	0.046*	0.046*	0.46	0.03*	0.12

** P<0.05*

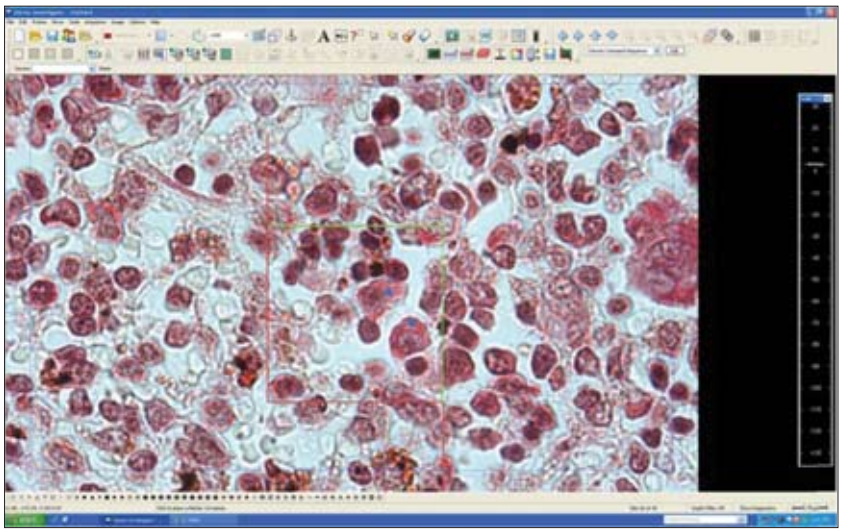


Fig 1. Specific plasma cells in mice spleen tissue as a result of methyl green-pyronin staining, 1000x

Şekil 1. Fare dalak dokusunda metil green-pironin boyaması sonucu spesifik plazma hücreleri, 1000x

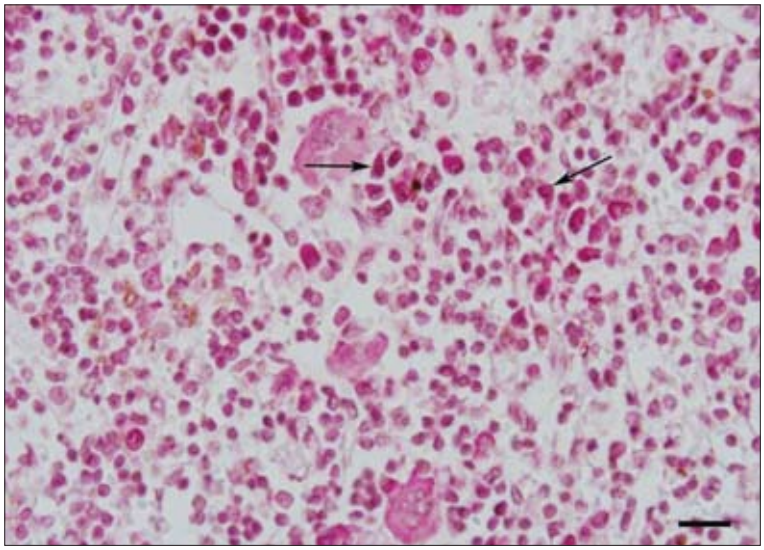


Fig 2. Specific plasma cells in mice spleen tissue as a result of methyl green-pyronin staining, Bar: 50 µm

Şekil 2. Fare dalak dokusunda metil green-pironin boyaması sonucu spesifik plazma hücreleri, Bar: 50 µm

Fig 3. Specific plasma cells in mice spleen tissue as a result of methyl green-pyronin staining, Bar: 10 µm

Şekil 3. Fare dalak dokusunda metil green-pironin boyaması sonucu spesifik plazma hücreleri, Bar: 10 µm

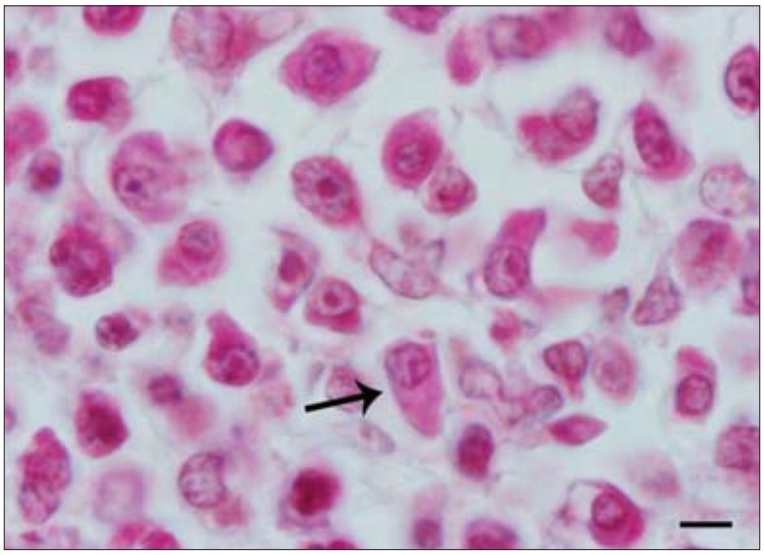


Table 3. *The number of plasma cells in the spleen*
Tablo 3. *Dalakta plazma hücre sayısı*

Cell	Control X±S	Koumiss X±S	Yoghurt X±S	Milk X±S	Kefir X±S	Significant
Plazma cells	417.5±39.0^{c}	543.5±31.4^{b}	583.5 ±33.1^{b}	551.1±49.0^{b}	732.3±35.3^{a}	P<0.001

^{a,b,c} *Differences between average values, represented by different letters in the same row, is important (P<0.05)*

DISCUSSION

Teruya et al.[22] found that kefir both increase the growth and live weight, in parallel to study of Carnevell et al.[23], in a study on the protective effects of kefir and fermented milk in case of X-ray irradiation-induced intestinal damage. Also in this study, similar to studies [23,24] reporting that beneficial microbial food increase live weight, it was detected that kefir, koumiss, milk and yoghurt administration increases live weight and that this increase is statistically significant (P<0.05). Live weight gain in kefir, koumiss, milk and yoghurt group being more compared to the control group suggests that it arise from kefir, koumiss, milk and yoghurt containing different beneficial organisms [25]. Beyond the inherent high nutritional value as a source of protein and calcium, kefir is believed to be beneficial to health in the countries where kefir is an essential part of the diet culture for a long time [26,27]. Effects of kefir on antioxidant and growth factors have been shown in several studies [22,28-33] found that the protective effect of kefir is more than vitamin E in oxidative damage induced by CCl_4 in mice, by reducing lipid peroxidation and increasing reduced glutathione and glutathione peroxidase levels. In recent years in the mentality of a healthy diet, nutrition support with antioxidant-rich foods to prevent damages causing

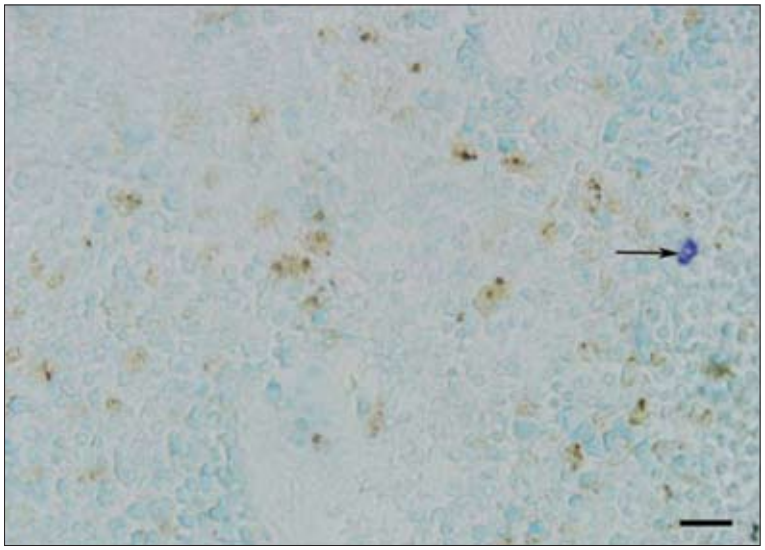
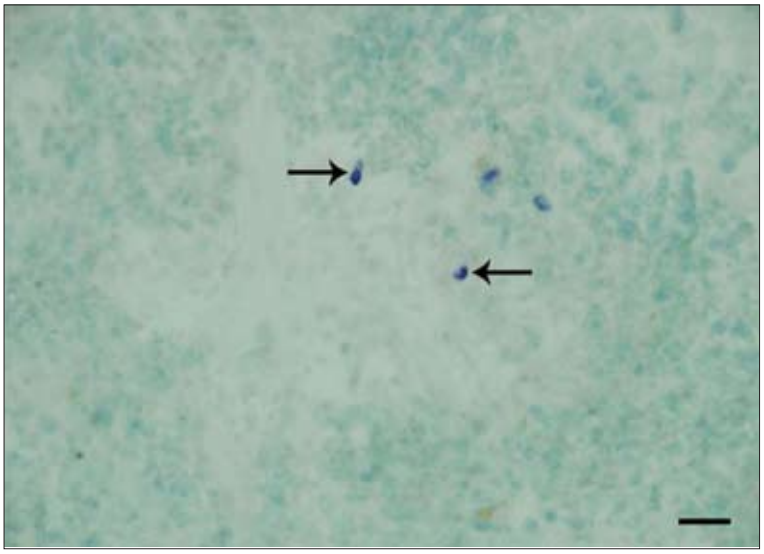


Fig 4. Specific mast cells in mice spleen tissue as a result of Toluidine Blue, Bar: 50 µm

Şekil 4. Fare dalak dokusunda Toluidine Blue boyaması sonucu spesifik mast hücreleri, Bar: 50 µm

Fig 5. Specific mast cells in mice spleen tissue as a result of Toluidine Blue, Bar: 50 µm

Şekil 5. Fare dalak dokusunda Toluidine Blue boyaması sonucu spesifik mast hücreleri, Bar: 50 µm



Tablo 4. *Dalakta mast hücre sayısı*
Table 4. *The number of mast cells in the spleen*

Cell	Control X±Sx	Koumiss X±Sx	Yoghurt X±Sx	Milk X±Sx	Kefir X±Sx	Significant
Mast cell	4.56±0.32^{c}	6.40±0.71^{b}	6.11 ±0.42^{bc}	8.12±0.46^{a}	5.95±0.63^{bc}	P<0.001

$^{a, b, c}$ Differences between average values, represented by different letters in the same row, is important (P<0.05)

oxidative stress in the body has risen to the forefront. In this direction, antioxidant substances may avoid oxidation damages by preventing the formation of free radicals or stopping or decreasing the activity of formed free radicals [34]. In our study, the number of plasma cells in the spleen of mice treated with kefir is significantly higher than mice in koumiss, milk, yoghurt, and control group. Likewise plasma cell number of mice treated with koumiss, milk and yoghurt is significantly greater than control group. When the groups were compared, plasma cell counts in spleen, which has a very important role in organism's defense by containing antigen presenting cells to T and B lymphocytes and macrophages, being significantly more in kefir group than koumiss, milk and yoghurt group is suggesting that kefir has an effect on natural immune system, modulates the immune system and also it has ability to stimulate immune cells.

Koumiss is rich in fatty acids as linoleic acid and arachidonic acid. These fatty acids being high enhance the importance in nutritional terms. Healing effect of koumiss is brought about intermediates as a result of fermentation of vitamins C, carbondioxide, lactic acid and alcohol contained in mare milk [35]. In our study, koumiss, milk and yoghurt were found to significantly increase plasma cell count in spleen, which response by starting

immune response that then activates T and B cells against blood antigens, compared to control group. Mare milk has essential features for human nutrition due to high amounts of polyunsaturated fatty acids, low cholesterol content and different protein structure [36]. Kefir, koumiss, milk and yoghurt, comprising useful microorganisms, are thought to potentiate the mucosal defenses, induce mucosal antibody response and cellular immune response and play immune modulatory role by stimulating cell activity.

It wasn't come across so many literature about mast cells population and functions in mammalian lymphoid organs. In our study, the number of mast cells in the spleen of mice treated with milk was significantly higher than the control group. Although number of mast cells of mice treated with kefir, koumiss and yoghurt were more than the control group, no statistically significant difference was detected. In this study, mast cells in the spleen of mice treated with milk showed numerical differences. Mast cells are the effector cells of the immune system found in all of connective tissue. They play a very critical role in allergic reactions [37,38]. Despite the entire literature search, no study investigating the effect of milk administration on mast cells in the spleen in a mice model and anti-allergic effects of milk was observed. The role of mast cells in allergic tissue inflammation is well known. IL-4 plays a role in mast cell proliferation and release of IgE-dependent mast cell mediators. Activated mast cells secrete Th2-type cytokines such as IL-3, IL-5, and IL-13 and leads to the accumulation of eosinophils and other inflammatory cells [39]. Milk, necessary in all stages of human life, is thought to cause contraction of smooth muscles and neurogenic vasodilatation by increasing vascular permeability of histamine, the most important and best-known mast cell mediator, and have profound effects on natural and acquired immune system by their effects on T lymphocytes.

In conclusion; as the number of plasma cells in the spleen is at higher level with Kefir, consisting of beneficial bacteria, than with koumiss, milk and yoghurt; also there being a significant difference between the numbers of mast cells in the spleens of mice treated with milk compared to control group, this is remarkable for healthy eating/ functional food. As there are limited researches on this subject, new researches will undoubtedly be important.

REFERENCES

1. Karatepe P, Yalçın H, Patır B, Aydın I: Kefir ve kefirin mikrobiyolojisi. *Elekt Mikrobiyol Derg*, 10 (1): 1-10, 2012.

2. Guzel-Seydim ZB, Greene AK, Taş T: Determination of antimutagenic properties of some fermented milks including changes in the total fatty acid profiles including CLA. *Int J Dairy Tech,* 59 (3): 209-215, 2006. DOI: 10.1111/j.1471-0307.2006.00265.x

3. Hertzler SR, Clancy SM: Kefir improves lactose digestion and tolerance in adults with lactosemal digestion. *J Am Dietetic Assoc*, 103 (5): 582-587, 2003. DOI: 10.1053/jada.2003.50111

4. Liu JR, Chen MJ, Lin CW: Antimutagenic and antioxidant properties of milk-kefir and soymilk-kefir. *J Agric Food Chem*, 53 (7): 2467-2474, 2005.

5. Otles S, Cagindi O: Kefir: A probiotic dairy composition, nutritional and therapeutic aspects. *Pakistan J Nutrition*, 2 (2): 54-59, 2003. DOI: 10.3923/pjn.2003.54.59

6. Sarkar S: Potential of kefir as a dietetic beverage: A review. *Br Food J*, 109 (4): 280-90, 2007. DOI: 10.1108/00070700710736534

7. Kurdal E: Kımız. *Atatürk Üniv Ziraat Fak Derg,* 24 (1): 233-225, 1993.

8. Dilanyan SH: Utilisation of mares', ewes', camels and yaks' milk in the USSR. Report Int. Comm. Dairying in warmcountries. Brussels, Belgium: *Int Dairy Federation*, 1959.

9. Demirci M: Süt Teknolojisine Giriş: Yayın No: 105, Ders Notu: 68, Trakya Üniv. Tekirdag Ziraat Fakültesi, Tekirdağ, 1992.

10. Perdigon G, De Moreno de Le Blanc A, Valdez J, Rachid M: Role of yoghurt in the prevention of colon cancer. *Eur J Clin Nutr*, 56 (3): 65-68, 2002. DOI: 10.1038/sj.ejcn.1601490

11. Plessas S, Bosnea L, Alexopoulos A, Bezirtzoglou E: Potential effects of probiotics in cheese and yoghurt production. *Eng Life Sci*, 12 (4): 433-440, 2012. DOI: 10.1002/elsc.201100122

12. Kallies A, Hasbold J, Tarlinton DM, DietrichW, Corcoran LM, Hodgkin PD: Plasma cell ontogeny defined by quantitative changes in Blimp-1 expression. *J Exp Med*, 200, 967-977, 2004. DOI: 10.1084/jem.20040973

13. Junqueira LC, Carneiro J: Temel Histoloji. Solakoğlu S, Aytekin Y (Çeviri Editörleri), 99-274, Nobel Tıp Kitap Evleri, İstanbul, 2009.

14. Berne RM, Levy MN, Koeppen BM, Stanton BA: Fizyoloji. Türk Fizyolojik Bilimler Derneği. 5th ed., 520-521, Güneş Tıp Kitapevleri, Ankara, 2008.

15. Amin K: The role of mast cells in allergic inflammation. *Respir Med,* 106 (1): 9-14, 2012. DOI: 10.1016/j.rmed.2011.09.007

16. Mierke CT, Ballmaier M, Werner U, Manns MP, Welte J, Bischoff SC: Human endothelial cells regulate survival and proliferation of human mast cells. *J Exp Med,* 192 (6): 801-812, 2000. DOI: 10.1084/jem.192.6.801

17. Kobayashi H, Ishizuka T, Okayana Y: Human mast cells and basophils as sources of cytokines. *Clin Exp Allergy,* 30 (9): 1205-1212, 2000. DOI: 10.1046/j.1365-2222.2000.00808.x

18. Galli SJ, Wedemeyer J, Tsai M: Analyzing the roles of mast cells and basophils in host defense and other biological responses. *Int J Hematol*, 75: 363-369, 2002.

19. Böck P: Romeis Mikroskopische Technik. 17th ed., 561, Urban and Schwarzenhferg Verlag, München, 1989.

20. Enerback L: Mast cells in rat gastrointestinal mucosa. I. effect of fixation. *Acta Pathol Microbiol Scand,* 66 (3): 289-302, 1966.

21. Yigit F, Aktas A, Daglioglu S: Effects of bisphenol A and diethyl-stilbestrol on the involution of bursa of fabricius in the hens. *Istanbul Univ Vet Fak Derg,* 39 (2): 168-174, 2013.

22. Teruya K, Myojin-Maekawa Y, Shimamoto F, Watanabe H, Nakamichi N, Tokumaru K, Tokumaru S, Shirahataa S: Protective effects of the fermented milk kefir on X-Ray irradiation- induced intestinal damage in B6C3F1 mice. *Biol Pharm Bull,* 36 (3): 352-359, 2003.

23. Carnevali, O, Vivo L, Sulpizio R, Gioacchini GI, Olivotto I, Silvi S, Cresci A: Growth improvement by probiotic in European seabassjuveniles (*Dicentrarchuslabrax*, L.) with particular attention to IGF-1, myostatin and cortisol gene expression. *Aquacult,* 258 (1-4): 430-438, 2006. DOI: 10.1016/j.aquaculture.2006.04.025

24. Kabir SML, Rahman MM, Rahman MB, Rahman MM, Ahmed SU: The dynamics of probiotics on growth performance and immune responce in broiler. *Int J Poult Sci,* 3 (5): 361-364, 2004.

25. Farnworth ER: Kefir a complex probiotic. *Food Sci Technol Bull: Funct Foods,* 2 (1): 1-17, 2005. DOI: 10.1616/1476-2137.13938

26. Vinderola G, Perdigon G, Duarte J, Thangavel D, Farnworth E, Matar C: Effects of kefir fractions on innate immunity. *Immunobiology*, 211 (3): 149-156, 2006. DOI: 10.1016/j.imbio.2005.08.005

27. Yaman H, Elmalı M, Kamber U: Observation of lactic acid bacteria and yeast populations during fermentation and cold storage in cow's,

ewe's and goat's milk kefirs. *Kafkas Univ Vet Fak Derg,* 16 (Suppl-A): S113-S118, 2010.

28. Lin MY, Change FJ: Antioxidativeeffect of intestinal bacteria *bifido bacterium longum* ATCC 15708 and *lacto bacillus acidophilus* ATCC 4356. *Dig Dis Sci*, 45 (8): 1617-1622, 2000. DOI: 10.1023/A:1005577330695

29. Güven A, Güven A, Gülmez M: The Effect of kefir on the activities of GSH-Px, GST, CAT, GSH and LPO levels in Carbon Tetrachloride-induced mice tissues. *J Vet Med*, 50 (8): 412-416, 2003. DOI: 10.1046/j.1439-0450.2003.00693.x

30. Sozmen M, Erginsoy SD, Cenesiz S, Devrim AK: The protective effect of kefir and vitamin C on Azoxymethane induced toxicity and induction of metallothionein in mice. *Scand J Lab Anim Sci,* 32 (4): 211-220, 2005.

31. Liu JR,Wang SY, Lin YY, Lin CW: Antitumoractivity of milk kefir and soy milk kefir in tumor-bearing mice. *Nutr Cancer*, 44 (2): 183-187, 2002.

32. Sari EK, Bakir B, Aydin BD, Sozmen M: The effects of kefir, koumiss, yoğurt and commercial probiotic formulations on PPARα and PPAR-β/δ expressions in mouse kidney. *Biotech Histochem,* 84 (4): 287-295, 2014. DOI: 10.3109/10520295.2013.844274

33. Güven A, Güven A, Gülmez M, Beytut E, Erişir M: Aterosklerotik farelerde kefir ve yoğurdun lipidperoksidasyonuna ve antioksidan enzimlere etkileri. *Kafkas Univ Vet Fak Derg,* 9 (1): 79-83, 2003.

34. Singh S, Singh RP: *In vitro* methods of assay of antioxidants: An overview. *Food Rev Int*, 24 (4): 393-415, 2008.

35. Yaygın H: Kımızın nitelikleri ve sağlık ile ilgili özellikleri. *Gıda*, 16 (2): 111-115, 1991.

36. Kucukcetin A, Yaygin H, Hinrichs J, Kulozik U: Adaptation of bovine milk towards mares' milk composition by means of membrane technology for koumiss manufacture. *Int Dairy J,* 13 (12): 945-951, 2003. DOI: 10.1016/S0958-6946(03)00143-2

37. Frungieri MB, Weidinger S, Meineke V, Köhn FM, Mayerhofer A: Proliferative action of mast-celltryptase is mediatedby PAR2, COX2, prostaglandins, and PPAR gamma: Possible relevance to human fibrotic disorders. *Proc Natl Acad Sci USA,* 12, 99 (23): 15072-15077, 2002.

38. Kurum A, Özen A, Karahan S, Özcan S: Investigation of mast cell distribution in the ovine oviduct during oestral and luteal phases of the oestrous cycles. *Kafkas Univ Vet Fak Derg*, 20 (6): 915-920, 2014. DOI: 10.9775/kvfd.2014.11398

39. Untersmayr E, Jarolim JJ: Mechanism of type I food allergy. *Pharmacol Ther*, 112 (3): 787-789, 2006.

Kafkas Univ Vet Fak Derg
21 (2): 203-209, 2015
DOI: 10.9775/kvfd.2014.12020

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Hypolipidemic Effects of *Satureja khuzistanica* Essential Oil in Broiler Chicken are Realized Through Alteration in Steroid Hormones

Heshmatollah KHOSRAVINIA [1]

[1] Department of Animal Science, Faculty of Agricultural, Lorestan University, Khorramabad- 68137-17133, Lorestan - IRAN

Article Code: KVFD-2014-12020 Received: 21.07.2014 Accepted: 17.09.2014 Published Online: 22.09.2014

Abstract

Two experiments were conducted to evaluate the effects of high and low doses of *Satureja Khuzistanica* essential oil (SEO) on blood lipid constituents and sex hormones in broiler chicks. In trail 1, 420 one-day-old Cobb 500 chicks of both sexes were randomly placed in 21 floor pens to examine the effect of supplementation of drinking water with 0, 0.5, 1.0, 1.5, 2.0, and 2.5 g/L SEO or 3.0 g/L Polysorbate-80 (control+) in 3 replicates of 20 birds each. In Experiment 2, 720 newly hatched male Arian chicks were randomly assigned to 36 floor pens to examine the effect of 0, 0.2, 0.3, 0.4 and 0.5, g/L SEO or 0.5 g/L Polysorbate-80 (control+) in 6 replicates of 20 birds each. As a result of this study, SEO at 0.5 and 0.3 g/L in Experiments 1 and 2, respectively, modulated serum cholesterol and high density lipoproteins (HDL) levels, albeit the differences were not significant when compared to appropriate control groups ($P>0.05$). The SEO at 0.5 g/L caused significant decrease (16%) in abdominal fat percentage of the birds at 28 d (Trial 1; $P<0.05$). In trail 2, inclusion of SEO into drinking water at 0.2, 0.3, 0.4 and 0.5 g/L significantly reduced the serum estradiol to 50, 50 and 45 percent, respectively, and increased serum testosterone level by about 2, 4, 4 and 5 folds, respectively, in comparison with the control- birds ($P>0.05$). The results propose the possibility of testosterone-coupled hypolipidemic properties for SEO in broiler chicken.

Keywords: *Broiler chicken, Carvacrol, Estradiol, Hypolipidemic effect, Testosterone*

Broyler Tavuklarda *Satureja khuzistanica* Esansiyel Yağın Hipolipidemik Etkileri Steroid Hormonlardaki Değişim Yoluyla Oluşturulur

Özet

Yüksek ve düşük doz *Satureja Khuzistanica* esansiyel yağ (SEO) uygulamasının broyler civcivlerde kan lipid bileşenleri ve seks hormonları üzerine etkilerini değerlendirmek üzere iki çalışma yürütüldü. Birinci çalışmada, 420 adet her iki cinsiyetten bir günlük Cobb 500 civciv rastgele olarak her birinde 20 adet civciv bulunan 21 adet yer kümeslerine yerleştirildi ve 3 tekrar olmak üzere içme suyu içerisinde 0, 0.5, 1.0, 1.5, 2.0 ve 2.5 g/L SEO veya 3.0 g/L Polysorbate-80 (kontrol+) uygulamasının etkileri incelendi. İkinci çalışmada, 720 adet yumurtadan yeni çıkmış erkek Arian civciv rastgele olarak her birinde 20 adet civciv bulunan 36 adet yer kümeslerine yerleştirildi ve 6 tekrar olmak üzere 0, 0.2, 0.3, 0.4 ve 0.5, g/L SEO veya 0.5 g/L Polysorbate-80 (kontrol+) uygulamasının etkileri incelendi. Çalışma sonucunda 1 çalışmada 0.5 ve 2. Çalışmada 0.3 g/L SEO uygulamasının serum kolesterol ve yüksek yoğunluklu lipoprotein (HDL) seviyelerini azalttığı ancak farkların kontrol grupları ile karşılaştırıldığında anlamlı olmadığı ($P>0.05$) tespit edilmiştir. 0.5 g/L SEO 28. günde civcivlerin abdominal yağ yüzdesinde (%16) anlamlı bir düşmeye neden oldu (Deney 1; $P<0.05$). İkinci deneyde, kontrol grubuyla karşılaştırıldığında 0.2, 0.3, 0.4 ve 0.5 g/L SEO azalmaları serum östradiyol seviyelerini yüzde 50, 50 ve 45 oranlarında anlamlı derecelerde azaltırken serum testosteron seviyelerini sırasıyla 2, 4, 4 ve 5 katlarında arttırdı ($P>0.05$). Elde edilen sonuçlar broyler tavuklarda SEO için testosteron ilişkili hipolipidemik etkilerin olabileceğini göstermektedir.

Anahtar sözcükler: *Broyler tavuk, Carvacrol, Östradiyol, Hipolipidemik etki, Testosteron*

INTRODUCTION

The essential oil derived from *Satureja Khuzistanica* contains above 90 percent carvacrol. Carvacrol is described as a phenolic, caustic and bitter tasting compound [1] which demonstrates significant antioxidant [2] and antimicrobial [3] properties. Accordingly, it has been reported that the carvacrol-reached essential oils from Lamiaceae

İletişim (Correspondence)
+98 0661 2252111 (Res.), +98 0661 4231917/4200191 (Off.), +98 0916 6673705 (Mob.)
khosravi_fafa@yahoo.com

family plants such as savory has antioxidant [4,5], antiviral [6], antibacterial [7] and antifungal [8] effects.

Recently, particular attention has been focused on hypolipidemic effects of phytogenic remedies in poultry meat and egg. Among many herbal spices or extracts examined, essential oils of onion and garlic [9,10], thyme [11-13], turmeric [14,15] and oregano [16] exhibited superior hypocholesterolemic effects in chicken. It has been suggested that such effects are mainly induced through the inhibition of the key enzymes in cholesterol and lipid synthesis [17-19]. On the other hand, many clinical investigations showed that herbal extracts are able to alter the reproductive functions in mice [20] and white plymouth rock-mini cocks [21] through affecting sex hormones secretion and their physiological balance. Considering the anabolic effects of androgens, the hypolipidemic effects of herbal extracts may be the consequence of abovementioned alterations in the sex hormones.

In spite of a significant decrease in serum triglyceride levels observed with a carvacrol reached plant extract from savory in diabetic and hyperlipidemic rats and no change in cholesterol level in hyperlipidemic rats [4], the hypolipidemic properties of carvacrol and carvacrol-reached plant extracts remain largely uninvestigated. In view of the scarce experimental results on hypolipidemic properties of carvacrol in connection with sex hormones in avian species, two studies were undertaken to examine the effect of high and low doses of *Satureja khuzistanica* essential oil containing 94.16 percent carvacrol, on blood fat constituents and sex hormones, while it was administrated through drinking water into broiler chicks.

MATERIAL and METHODS

Preparation of Essential Oil

The essential oils used for this experiment freshly provided from a particular species of savory herbs known as *Satureja khuzistanica* Jamzad, an endemic plant distributed in southern part of Iran [22,23]. The aerial parts of the plant collectively contain up to 3 percent of essential oils which is spectacularly rich in carvacrol (up to 95 percent) [24]. The aerial parts of *Satureja khuzistanica* were manually harvested during the flowering stage of plant. The collected materials were air dried at ambient temperature in the shade and hydrodistilled using a Clevenger type apparatus for 5 h, giving yellow oil in 3 percent yield. The oils were dried over anhydrous sodium sulfate and stored at 4ºC. A random sample of the stored oil was analyzed for the composition of essential oils using the methods described by Hadian et al.[22]. The resulting composition verified that it is highly-reached in carvacrol by >94 percent. The major constituents in the remaining impurity were determined as *p*-Cymene (0.96%) and γ-Terpenene (0.51%) *(Table 1)*.

Experimental Flocks

In Experiment 1, 420 day-old Cobb 500, broiler chicks (43.65±1.2 g) were provided from a local commercial hatchery. The birds were housed in a concrete floor, cross-ventilated windowless shed where they were randomly placed in 21 pens (90×180 cm; at density of 0.08 m^2/bird). Each pen was equipped with an infra-red brooder. The treatments were arranged into 3 blocks to account for variations in the ventilation system. Seven experimental treatments including 0 (control-), 0.5, 1.0, 1.5, 2.0, and 2.5 g/L SEO or 3.0 g/L Polysorbate-80 as emulsifier agent (control+) were administrated *ad libitum* via drinking water to 3 replicate pens of 20 birds each, up to the day 28 of age. The solution was prepared for each treatment in a daily basis and the remaining was discarded. The chicks were maintained on a 24-h light schedule. Feed and water supplied to the birds through a tube feeder and a manual waterer in each pen, respectively. Corn and soybean meal based starter and grower diets were formulated using UFFDA software according to the NRC [25] recommendations *(Table 2)*. The Diets and water were provided for *ad libitum* consumption throughout the 28-d experimental period.

In the second experiment, 720 one-day-old Arian broiler chicks were obtained from a commercial hatchery and housed in the same shed with similar flocking density as Experiment 1 up to 42 days of age. The chicks were randomly assigned to 36 pens arranged in 6 rows (blocks/replicates). Corn and soybean meal based pre starter, starter, grower and finisher diets *(Table 2)* and water was provided for *ad libitum* consumption throughout the 42-d experimental period. Diets were pelleted and the pellet sizes adjusted to the age of the birds. The six experimental treatments consisting 0 (control-), 0.2, 0.3, 0.4 and 0.5 g/L SEO or 0.5 g/L Polysorbate-80 (at 1:1 ratio *v/v*; control+) were continuously provided (through drinking water) for 6 replicate pens of 20 birds each, up to 42 days of age.

Table 1. *Composition of Satureja khuzistanica essential oil*
Tablo 1. *Satureja khuzistanica esansiyel yağın bileşeni*

Compound	RI[1]	Composition (%)	Identification[2]
Carvacrol	1282	94.16±0.46	RI, MS, CoI
p-Cymene	1017	0.96±0.86	RI, MS, CoI
γ-Terpenene	1053	0.51±0.23	RI, MS, CoI
(Z)- β-Oeimene	1036	0.42±0.08	RI, MS
α -terpinole	1175	0.32±0.45	RI, MS
Myreene	981	0.21±0.19	RI, MS
α-Terpinene	1013	0.18±0.12	RI, MS, CoI
α-Thujene	925	0.14±0.14	RI, MS
α- Pinene	933	0.12±0.05	RI, MS, CoI

[1] RI; Retention indices determined relative to n-alkanes (C_6-C_{24}) on a DB-5GC column
[2] RI; Retention indices, MS; mass spectra, CoI; co-injection

Table 2. *The ingredients and the nutrient composition of the experimental diets*
Tablo 2. *Deneysel diyetin içerik ve besin bileşenleri*

Item	Experiment 1		Experiment 2			
	Starter (1-14d)	Grower (15-28d)	Prestarter (1-7d)	Starter (8-21d)	Grower (22-35d)	Finisher (36-42d)
Ingredient (%)						
Corn	55.00	63.00	45.3	47.9	46.7	47.8
Soybean meal	36.00	28.10	34.8	33.9	26.9	23.6
Fish meal	3.17	3.20	-	-	-	-
Wheat	-	-	7	12	20	22
Soybean oil	3.40	3.20	1.3	1.2	1.3	1.4
Corn gluten	-	-	6	-	-	-
Calcium carbonate	-	-	1.20	1.10	1.11	1.15
Dicalcium phosphate	1.20	1.00	2.16	1.94	2.01	2.07
DL-methionine	0.10	0.15	0.34	0.31	0.32	0.33
L-lysine	0.15	0.15	0.15	0.13	0.14	0.14
Vitamin permix[1]	0.25	0.30	0.28	0.25	0.26	0.27
Mineral permix[2]	0.30	0.30	0.28	0.25	0.26	0.27
Salt	0.25	0.25	0.39	0.35	0.36	0.38
Coline cloride	-	-	0.14	0.13	0.13	0.14
Calculated value						
ME (kcal/kg)	3100	3220	2962	2880	2952	2993
Crude protein (%)	23.00	19.12	24.28	21.15	18.82	17.63
Crude fat (%)	3.90	3.70	4.32	3.13	3.50	3.74
Crude fiber (%)	3.01	2.87	3.74	3.75	3.48	3.33
Calcium (%)	0.85	1.00	1.10	1.00	1.00	1.00
Available P (%)	0.42	0.50	0.55	0.50	0.50	0.50
Methionine (%)	0.51	0.40	0.59	0.51	0.45	0.43
Lysine (%)	1.44	1.03	1.29	1.09	0.95	0.88

[1] Supplied per kg of diet: Mn, 55 mg; Zn, 50 mg; Fe, 80 mg; Cu, 5 mg; Se, 0.1 mg; I, 0.18 mg
[2] Supplied per kg of diet: vitamin A, 18000 IU; vitamin D_3, 4000 IU; vitamin E, 36 mg; vitamin K_3, 4 mg; vitamin B_{12}, 0.03 mg; thiamine, 1.8 mg; riboflavin, 13.2 mg; pyridoxine, 6 mg; niacin, 60 mg; calcium pantothenate, 20 mg; folic acid, 2 mg; biotin, 0.2 mg; choline chloride, 500 mg

Data Collection

At the end of Experiment 1 (28 d) two male and two female birds per pen, ±50 g of the mean pen weight for each sex, and at close of Experiment 2 (42 d), one male bird with the closest mean to the mean pen weight for males were killed for blood and abdominal fat collection. Abdominal fat (in Experiment 1) was manually collected and recorded as the summation of fat deposited around proventriculus and gizzard plus fat pad for each bird. Serum low-density lipoprotein (LDL), High-density lipoprotein (HDL), total cholesterol (TC), and triglyceride (TG) concentrations were estimated in both experiments using SEPPIM Diagnostic Kits (SEPPIM S.A.S., Zone Industrielle, 61500, SEES, France) in two replicates/sex per pen, at 25ºC. The concentration of estradiol and testosterone in serum were measured by a solid-phase RIA in Experiment 2 using reagents provided by IMMUNOTECH kits (IMMUNOTECH SAS, 130 av. De Lattre de Tassugny – B.P. 177 – 13276 Marseille Cedex 9 France) in 6 male birds per treatment.

Statistical Analysis

The collected data were analyzed using PROC MIXED of SAS 9.3 [26]. The LSD test was used for multiple treatment comparisons using the LSMEANS statement of SAS 9.1 [26] with letter grouping obtained using the SAS pdmix800 macro [27]. For all variables in Experiment 2, the effect of birds' live weight before slaughter, as a continuous random variable, was also included in the statistical model. For the different statistical tests, significance was declared at $P \leq 0.05$. Linear and quadratic contrasts were used for the effects of SEO levels on the studied variables.

RESULTS

Experiment 1

Data for Experiment 1 are presented in *Tables 3* and *4*, and *Fig. 1* and *Fig. 2*. Administration of SEO through

drinking water had no significant effect on serum TG, LDL, HDL and total cholesterol (TC) levels of the birds at day 28 of age (P>0.05). However, the concentrations of TC and HDL (pooled data over sexes) were reduced by 1.41 and 8.50%, respectively, in the birds received 0.5 g/L SEO compared to control- birds *(Table 3)*. In sexwise analysis of data, male and female serum LDL, HDL and TC levels, but not triglycerides, were affected by SEO-treated water in dissimilar ways *(Table 4)*. As shown in *Fig. 1*, accumulation of fat in abdominal cavity of the birds was reduced by SEO-added water (P=0.0262). Supplementation of 0.5 g/L SEO caused approximately 16% decrease in abdominal fat-to-body weight ratio (AFP) at 28 d. Addition of SEO in drinking water at doses >0.5 g/L exhibited adverse effect of AFP *(Fig. 1)*. The serum TC and HDL level was significantly influenced by the sex of the birds. The male broilers were found to have 8.55 and 9.01 percent lower TC and HDL, respectively, in serum. No parameter of consideration was affected by sex × SEO interaction in the Experiment 1 *(Table 3)*.

Table 3. *Effect of high doses of Satureja khuzistanica essential oil (SEO) in drinking water on serum concentration of triglycerides (TG), total cholesterol (TC), low density lipoproteins (LDL) and high density lipoproteins (HDL) in broiler chicks at day 28 of age (Experiment 1)*

Tablo 3. *İçme suyu içerisinde yüksek doz Satureja khuzistanica esansiyel yağ (SEO) uygulamasının 28. günde Broyler tavukların trigliserid (TG), total kolesterol (TC), düşük yoğunluklu lipoproteinler (LDL) ve yüksek yoğunluklu lipoproteinler (HDL) serum konsantrasyonlarına olan etkileri (Deney 1)*

Factor\Level	TG	TC	LDL	HDL
	mg/ 100 ml			
SEO (g/ L)				
Control+[1]	35.50	150.16[a]	62.25	75.58[a]
0.0	36.83	157.26[a]	66.83	74.91[a]
0.5	40.17	122.75[b]	52.08	60.92[b]
1.0	37.08	125.51[b]	56.50	65.25[ab]
1.5	38.16	132.59[ab]	55.83	71.50[ab]
2.0	40.50	128.83[ab]	56.67	66.25[ab]
2.5	37.33	129.41[ab]	57.58	65.25[ab]
Sex				
Male	38.77	135.46	57.39	68.97
Female	37.11	136.35	52.32	68.10
SEM[2]	1.087	2.546	1.300	1.321
	P > F			
ANOVA results				
SEO	0.8859	0.0094	0.1463	0.0270
Sex	0.4765	0.8464	0.1135	0.7257
SEO × Sex	0.2480	0.0511	0.0115	0.0344
Trends[3]				
Linear	0.8721	0.0923	0.1291	0.1007
Quadratic	0.5710	0.0946	0.0874	0.0426

[1] Control+; The birds received drinking water supplemented with 3.0 g/L polysorbate-80 throughout the experiment, and Control-; The birds received drinking water with no additive
[2] Standard error for overall mean
[3]Trend analysis was done for SEO levels
[a-b] Means within a column for each factor without a common superscript differ significantly (P<0.05)

Table 4. *Effect of high doses of Satureja khuzistanica essential oil (SEO; g/L) in drinking water on serum concentration of triglycerides (TG), total cholesterol (TC), low density lipoproteins (LDL) and high density lipoproteins (HDL) in male and female broiler chicks at day 28 of age (Experiment 1)*

Tablo 4. *İçme suyu içerisinde yüksek doz Satureja khuzistanica esansiyel yağ (SEO; g/L) uygulamasının 28. günde dişi ve erkek Broyler tavukların trigliserid (TG), total kolesterol (TC), düşük yoğunluklu lipoproteinler (LDL) ve yüksek yoğunluklu lipoproteinler (HDL) serum konsantrasyonlarına olan etkileri (Deney 1)*

Factor\Level	TG	TC	LDL	HDL
	mg/ 100 ml			
Males				
Control+[1]	37.37	141.25	55.62	71.62
0.0	41.00	131.33	56.00	68.67
0.5	42.50	130.33	53.17	62.83
1.0	38.67	140.50	60.17	72.67
1.5	40.33	156.83	70.50	78.50
2.0	41.25	126.62	55.62	65.25
2.5	50.33	123.33	51.17	62.83
SEM[2]	2.606	3.944	1.940	1.881
P>F	0.8893	0.3009	0.1597	0.2223
Trends				
Linear	0.0985	0.3491	0.3251	0.2008
Quadratic	0.1529	0.1528	0.1280	0.1134
Females				
Control+[1]	31.75	125.50	53.00	61.00
0.0	35.44	122.22	45.22	63.67
0.5	37.83	115.17	51.00	59.00
1.0	35.50	118.83	52.83	57.83
1.5	36.00	125.00	51.17	64.50
2.0	39.00	138.25	58.75	68.25
2.5	44.17	137.17	62.33	67.67
SEM[2]	1.492	2.637	1.823	1.422
P>F	0.5365	0.1874	0.1598	0.3944
Trends				
Linear	0.5201	0.4612	0.2381	0.3011
Quadratic	0.0970	0.1036	0.0872	0.1905

[1] Control+; The birds received drinking water supplemented with 3.0 g/L polysorbate-80 throughout the experiment
[2] Standard error for overall mean
[a-b] Means within a column for each factor without a common superscript differ significantly (P<0.05)

Experiment 2

Incorporation of low doses of SEO (ranging from 0.2 to 0.5 g/L) in drinking water did not affect plasma TG, LDL, HDL and TC levels of the birds at the day 42 of age

(P>0.05; *Table 5*). The concentrations of TC, LDL, and HDL, nevertheless, were modulated by approximately 8, 9 and 5%, respectively, by SEO-treated water at 0.3 g/L compared to the control- birds *(Table 5)*. In contrast to the Experiment 1, live body weight of the birds before slaughter showed significant effects on serum LDL and TC levels.

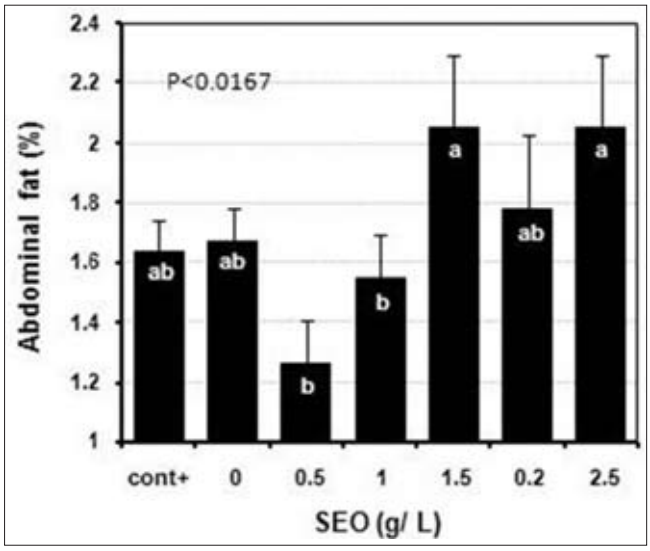


Fig 1. Effect of high doses of *Satureja khuzistanica* essential oil (SEO) in drinking water on abdominal fat (%) in broiler chicks at 28 days of age (Experiment 1). Means without a common superscript ($^{a-b}$) differ significantly (P<0.017)

Şekil 1. İçme suyu içerisinde yüksek doz *Satureja khuzistanica* esansiyel yağ (SEO) uygulamasının 28. günde Broyler tavukların abdominal yağı (%) üzerine etkileri (Deney 1). Aynı üstsimgeye ($^{a-b}$) sahip olmayan gruplar anlamlı oranda birbirlerinden farklıdır (P<0.017)

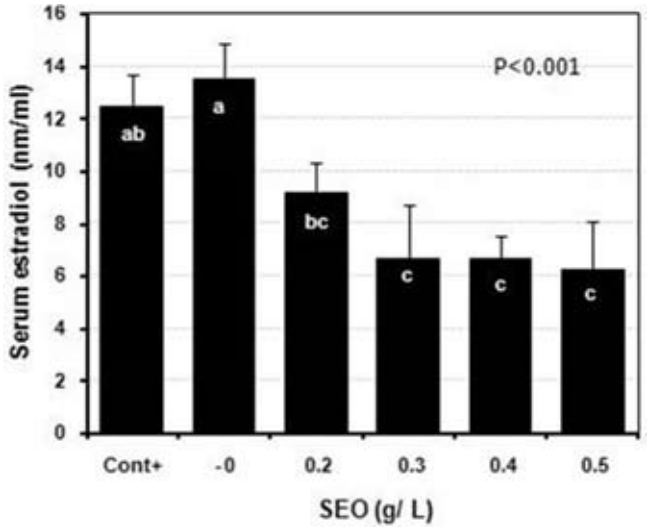


Fig 2. Effect of low doses of *Satureja khuzistanica* essential oil (SEO) in drinking water on serum estradiol level in male broiler chicken at day 42 of age (Experiment 2). Means without a common superscript ($^{a-c}$) differ significantly (P<0.001)

Şekil 2. İçme suyu içerisinde düşük doz *Satureja khuzistanica* esansiyel yağ (SEO) uygulamasının 42. günde erkek Broyler tavukların serum östradiyol seviyeleri üzerine etkileri (Deney 2). Aynı üstsimgeye ($^{a-c}$) sahip olmayan gruplar anlamlı oranda birbirlerinden farklıdır (P<0.001)

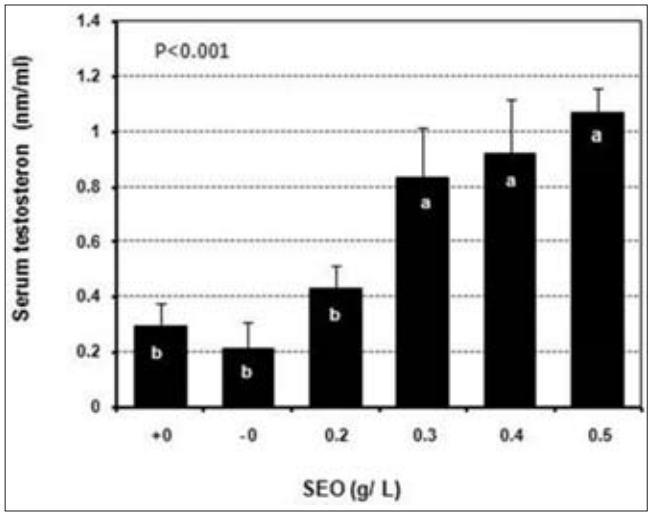


Fig 3. Effect of low doses of *Satureja khuzistanica* essential oil (SEO) in drinking water on serum testosterone level in male broiler chicken at 42 days of age (Experiment 2). Means without a common superscript ($^{a-b}$) differ significantly (P<0.001)

Şekil 3. İçme suyu içerisinde düşük doz *Satureja khuzistanica* esansiyel yağ (SEO) uygulamasının 42. günde erkek broyler tavukların serum testosteron seviyeleri üzerine etkileri (Deney 2). Aynı üstsimgeye ($^{a-c}$) sahip olmayan gruplar anlamlı oranda birbirlerinden farklıdır (P<0.001)

Table 5. *Effect of low doses of Satureja khuzistanica essential oil (SEO) in drinking water on serum concentration of triglycerides (TG), total cholesterol (TC), low density lipoproteins (LDL) and high density lipoproteins (HDL) in male broiler chicks at day 42 of age (Experiment 2)*

Tablo 5. *İçme suyu içerisinde düşük doz Satureja khuzistanica esansiyel yağ (SEO) uygulamasının 42. günde erkek broyler tavukların trigliserid (TG), total kolesterol (TC), düşük yoğunluklu lipoproteinler (LDL) ve yüksek yoğunluklu lipoproteinler (HDL) serum konsantrasyonlarına olan etkileri (Deney 2)*

Factor\Level	TG	TC	LDL	HDL
	µg/ 100 ml			
SEO (g/ L)				
Control+[1]	80.66	147.33^{ab}	70.17^{ab}	74.00^{ab}
0.0	84.50	155.01^{a}	74.50^{a}	78.33^{a}
0.2	83.50	134.33^{bc}	63.83^{abc}	71.17^{abc}
0.3	76.83	132.33^{bc}	56.50^{c}	66.83^{bc}
0.4	81.83	152.83^{c}	58.67^{bc}	66.17^{bc}
0.5	80.50	122.67^{c}	53.83^{c}	61.17^{c}
SEM[2]	0.412	2.833	1.692	1.974
	P > F			
ANOVA Result				
SEO	0.9886	0.0004	0.0064	0.0445
Trends				
Linear	0.3461	0.3471	0.0016	0.0876
Quadratic	0.1023	0.0120	0.2468	0.0432

[1] Control+; The birds received drinking water supplemented with 0.5 g/L polysorbate-80 throughout the experiment
[2] Standard error for overall mean
[a-b] Means within a column for each factor without a common superscript differ significantly (P<0.05)

Significant differences were found among treatments in mean serum estradiol and testosterone levels. Administration of SEO into drinking water at 0.3, 0.4 and 0.5 g/L significantly reduced the serum estradiol to 52, 50 and 48 percent, respectively, in comparison with the control- birds *(Fig. 2)*. The mean serum testosterone significantly elevated in the birds received 0.2, 0.3, 0.4 and 0.5 g/L SEO by about 2, 4, 4 and 5 folds, respectively, compared to the control- birds *(Fig. 3)*.

DISCUSSION

The results of analysis of variance in the Experiments 1 and 2 *(Table 3, 4* and *5)* indicated that the administrated doses of SEO had no effect on plasma lipid constituents. However, the serum cholesterol and HDL levels were the lowest for the birds receiving 0.5 g/L SEO *(Table 3)*. These observations were coincided with the significantly reduced abdominal fat at 0.5 g/L SEO in *Fig. 1*, indicating the potential of SEO as a hypolipidemic water additive. Thus, from the results in *Table 3* and *Fig. 3*, it appears that the "optimum inclusion level" for SEO in water for broilers is between 0.3 to 0.5 g/L water.

Although there was no alteration in plasma lipids, the pronounced decrease in abdominal fat of the birds received 0.5 g/L SEO in Experiment 1 *(Fig. 1)*, indicates that SEO may affect lipid metabolism in broiler chicken. In broiler, lipids and especially triglycerides are mainly stored in adipocytes of the abdominal fat. It has been shown that *de novo* lipogenesis, i.e., synthesis of fatty acids, is very limited in abdominal fat [28]. Thus, triglyceride storage in abdominal fat compartments depends on the availability of a plasma lipid substrates originating from either the diet or lipogenesis in the liver [29,30]. We suggest the significant decrease (15%) in the abdominal fat of the 0.5 g/L SEO- treated birds was a response to decreased plasma LDL and HDL. Carvacrol as the main component of SEO, seems to affect LDL and/or HDL metabolism in extra hepatic metabolic routes [31]. These results are compliant with other reports which shown that dietary carvacrol significantly affect fat metabolism in chicken [11,32]. It has been revealed that oregano extract, which it is also rich in carvacrol, exhibit significant hypocholesterolemic effects in chicken [16]. From the results of the second experiment, remarkable decrease *(Table 5)* in plasma cholesterol, LDL, and HDL by 8, 9 and 5%, respectively, with 0.3 g/L SEO were concur with opposite alteration in plasma estradiol and testosterone levels *(Fig. 2* and *Fig. 3)*. These results are in consistent with the finding of Haeri et al.[33] who reported that oral administration of 150 and 225 mg/kg per day *Satureja khuzistanica* essential oils through drinking water significantly increased plasma testosterone concentration in male rats. In the study of caponization and testosterone effects on blood lipid in male chicken it has been demonstrated that testosterone decreases lipid storage capacity and inhibit lipid accumulation in male chicks [34]. These results are interesting since the current knowledge proposed that the inhibitory action of essential oils on lipid metabolism regulatory enzymes is independent of the diurnal cycle of many hormones such as insulin, glucocortocoids, T3 and glucagons [35].

The modulated serum LDL and cholesterol in the first experiment could be attributed to the elevated serum testosterone level in the birds received 0.3 g/L SEO-added water. The results from sex-disconnect analysis of data in Experiment 1 also supported the above conclusion where an apparent dose-dependent response in serum LDL, HDL and TC levels were exhibited in male chicks, but not in females *(Table 4)*. Chen et al.[34] in conformity with the idea confirmed by Whitehead et al.[36] reported that testosterone implantation in capons decreased the serum LDL and cholesterol level while triglycerides remained unaffected. Considering all variables in Experiments 1 and 2, it is barely credible to attribute the differences between the treated and control- birds as regards blood fat constituents to random variability. Therefore, two reasons could be pointed out to propose the possibility of hypolipidemic properties for SEO in broiler chicken under the circumstances which the current experiments were conducted. 1) The decreased abdominal fat in 0.5 g/L SEO-treated birds could be caused by modulated serum cholesterol, LDL, and HDL in trial 1. 2) The decreased levels of the same blood lipid constituents in 0.3 g/L SEO-treated birds could be associated with elevated serum testosterone, as an anabolic hormone, in trial 2.

Results propose the possibility of testosterone-linked hypolipidemic properties for carvacrol as well as carvacrol-reached plant extracts in broiler chicken under the circumstances which the current experiments were conducted.

Aknowledgment

This study was supported by a grant from Lorestan Medical Plant Lab., Khorraman, Khorramabad-68135-579, Iran

REFERENCES

1. Agricultural Research Service (ARS): Dr. Duke's phytochemical and ethnobotanical databases. Accessed at June, 2002. http://www.ars-grin.gov/duke, 2002.

2. Cuppett SL and Hall CA: Antioxidant activity of Labiatae. *Adv Food Nutr Res*, 42, 245-271, 1998. DOI: 10.1016/S1043-4526(08)60097-2

3. Burt S: Essential oils: Their antibacterial properties and potential applications in food-A review. *Int J Food Microbiol*, 94, 223-253, 2004. DOI: 10.1016/j.ijfoodmicro.2004.03.022

4. Abdollahi M, Salehnia A, Mortazavi SH, Ebrahimi M, Shafiee A, Fouladian F: Antioxidant, antidiabetic, antihyperlipidemic, reproduction stimulatory properties and safety of essential oil of *Satureja khuzestanica* in rat *in vivo*: A oxicopharmacological study. *Med Sci Monit*, 9, 331-335, 2003.

5. Radonic A, Milos M: Chemical composition and in vitro evaluation of antioxidant effect of free volatile compounds from *Satureja montana* L.

Free Radic Res, 37, 673-679, 2003.

6. Yamasaki K, Nakano M, Kawahata T, Mori H, Otake T, Ueba N, Oishi I, Inami R, Yamane M, Nakamura M, Murata H, Nakanishi T: Anti-HIV-1 activity of herbs in Labiatae. *Biol Pharm Bull*, 21, 829-833, 1998.

7. Azaz D, Demirci F, Satil F, Kurkcuoglu M, Baser KH: Antimicrobial activity of some Satureja essential oils. *Z Naturforsch,* 57, 817-821, 2002.

8. Skocibusic M, Bezic N: Phytochemical analysis and *in vitro* antimicrobial activity of two Satureja species essential oils. *Phytother Res*, 18, 967-970. 2004.

9. Sklan D, Berner YN, Rabinowitch HD: The effect of dietary onion and garlic on hepatic lipid concentration and activity of antioxidative enzymes in chicks. *J Nutr Biochem*, 3, 322-325, 1992. DOI: 10.1016/0955-2863(92)90022-B

10. Konjufca VH, Pesti GM, Bakalli RI: Modulation of cholesterol levels in broiler meat by dietary garlic and copper. *Poult Sci*, 76, 1264-1271, 1997. DOI: 10.1093/ps/76.9.1264

11. Case GL, He L, Mo H, Elson CE: Induction of geranyle pyrophosphatase activity by cholesterol-suppressive isoprenoids. *Lipids*, 30, 357-359, 1995.

12. Lee KW, Everts H, Beynen AC: Essential oils in broiler nutrition. *Int J Poult Sci*, 3, 738-752, 2004.

13. Lee KW, Everts H, Kappert HJ, Frehner M, Wouterse H, Beynen AC: Cinnamanaldehyde, but not thymol, counteracts the carboxymethyl cellulose-induced growth depression in female broiler chickens. *Int J Poult Sci*, 3, 608-612, 2004.

14. Honda SF, Aoki F, Tanaka H, Kishida H, Nishiyama T, Okada S, Matsumoto I, Abe K, Mae T: Effects of ingested turmeric oleoresin on glucose and lipid metabolisems in obese diabetic mice: A DNA microarrey study. *J Agric Food Chem,* 54, 9055-9062, 2006. DOI: 10.1021/jf061788t

15. Sugiharto I, Widiastuti E, Prabowo NS: Effects of turmeric extract on blood parameters, feed efficiency and abdominal fat content in broilers. *J Indonesian Trop Anim Agric,* 36, 21-26, 2011.

16. Brenes A, Roura E: Essential oils in poultry nutrition: Main effects and modes of action. *Anim Feed Sci Tech*, 158, 1-14, 2010. DOI: 10.1016/j.anifeedsci.2010.03.007

17. Qureshi AA, Din ZZ, Abuirmeileh N, Burger WC, Ahmed Y, Elson CE: Suppression of avian hepatic lipid metabolisem by solvent extracts of garlic: Impact on serum lipids. *J Nutr*, 113, 1746-1755, 1983.

18. Elson CE, Qureshi AA: Coupling the cholesterol- and tumor-suppressive actions of palm oil to the impact of its minor constituents on 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. *Prostaglandins Leukot Essent Fatty Acids*, 52, 205-208, 1995. DOI: 10.1016/0952-3278(95)90024-1

19. Crowell PL: Prevention and therapy of cancer by dietary monoterpenes. *J Nutr*, 129, 775S-778S, 1999.

20. Dehghani F, Azizi M, Panjehshahin MR: Toxic effects of hydro-alcoholic extract of *Citrullus colocynthis* on pregnant mice. *Iranian J Vet Res*, 9, 42-45, 2008.

21. Grigorova G, Kashamov B, Sredkova V, Surdjiiska S, Zlatev H: Effect of tribulus terrestris extract on semen quality and serum total cholesterol content in white plymouth rock-mini cocks. *Biotech Anim Husband*, 24, 139-146, 2008.

22. Hadian J, Mirjalili MH, Kanani MR, Salehnia A, Ganjipoor P: Phytochemical and morphological characterization of *Satureja khuzistanica* Jamzad populations from Iran. *Chem Biodiver*, 8, 902- 915, 2011. DOI: 10.1002/cbdv.201000249.

23. Zargari A: Medicinal Plants. 4th ed., 42-45, Tehran University Publications, Tehran, 1990.

24. Khosravinia H, Salehnia A, Rafiei Alavi E: Toxicity and safety assessment of *Satureja khuzistanica* essential oils in broiler chicks up to 28 days of age. Pp.178-179 in Proc. *The 1st National Medicinal Plants Congress,* 27-28 March, Iran, Sari. Univ. Mazandaran. 2010.

25. National Research Council (NRC): Nutrient Requirements of Poultry. 9th ed., p.155, National Academy Press, Washington, DC., USA., ISBN: 9780309048927, 1994.

26. SAS Institute: SAS/STAT® Guide for personal computers. Version 9.1 Edition. SAS Institute, Inc., Cary, NC, 2002.

27. Saxton AM: A macro for converting mean separation output to letter grouping in Proc Mixed. pp.1243-1264 in Proc. 23rd SAS User Group Intl. SAS Institute, Cary, NC, 1998.

28. Saadoun A, Leclercq B: *In vivo* lipogenesis of genetically lean and fat chicken: Effects of nutritional state and dietary fat. *J Nutr*, 117, 428-435, 1987.

29. Griffin HD, Guo K, Windsor D, Butterwith SC: Adipose tissue lipogenesis and fat deposition in leaner broiler chickens. *J Nutr*, 122, 363-368, 1992.

30. Hermier D: Lipoprotein metabolism and fattening in poultry. *J Nutr*, 127, 805S-808S, 1997.

31. Hotta M, Nakata R, Katsukawa M, Hori K, Takahashi S, Inoue H: Carvacrol, a component of thyme oil, activates PPAR and suppresses COX-2 expression. *J Lipid Res*, 51, 132-139, 2010. DOI: 10.1194/jlr.M900255-JLR200

32. Can Baser KH: Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. *Curr Pharm Des*, 14, 3106-3119, 2008. DOI: 10.2174/138161208786404227

33. Haeri S, Minaie B, Amin G, Nikfar S, Khorasani R, Esmaily H, Salehnia A, Abdollahi M: Effect of *Satureja khuzistanica* essential oil on male rat fertility. *Fitoterapia*, 77, 495-499, 2006.

34. Chen KL, Chi WT, Chiout PWS: Caponization and testosterone implantation effects on blood lipid and lipoproteins profile in male chicken. *Poult Sci*, 84, 547-552, 2005.

35. Middleton B, Hui KP: Inhibition of hepatic S-3-hydroxy-3-methyl-glutaryl-CoA reductase and *in vivo* rates of lipogenesis by a mixture of pure cyclic monoterpenes. *Biochem Pharm,* 31, 2897-2901, 1982.

36. Whitehead CC, Hood RL, Heard GC, Pym RAE: Comparison of plasma very low density lipoproteins and lipogenic enzymes as predictors of fat content and food conversion efficiency in selected lines of broiler chickens. *Br Poult Sci*, 72, 1479-1491, 1984.

Kafkas Univ Vet Fak Derg
21 (2): 211-218, 2015
DOI: 10.9775/kvfd.2014.12035

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Effects of Dietary Sodium Bentonite and Mannan Oligosaccharide Supplementation on Performance, Egg Quality, Blood and Digestion Characteristics of Laying Hens Fed Aflatoxin Contaminated Diet

Engin YENİCE [1] Cengizhan MIZRAK [2] Necmettin CEYLAN [3]
Turgay YILDIZ [4] Meltem GÜLTEKİN [1] Zafer ATİK [1]

[1] Poultry Research Station, TR-06042 Yenimahalle, Ankara - TURKEY
[2] General Directorate of Agricultural Research and Policy, TR-06042 Yenimahalle, Ankara - TURKEY
[3] Ankara University, Faculty of Agriculture, Department of Animal Science, TR-06110 Diskapi, Ankara - TURKEY
[4] North Anatolian Development Agency, TR-37100 Kastamonu - TURKEY

Article Code: KVFD-2014-12035 Received: 23.07.2014 Accepted: 16.09.2014 Published Online: 22.09.2014

Abstract

In this experiment, sodium bentonite (SB) (0.5% and 1%) and mannan oligosaccharide (MOS, 0.1%) were fed to laying hens each receiving approximately 120 ppb total aflatoxin (AF), and were compared to AF contaminated negative control (NC) and control without AF (C) groups. A total of 180 hens at 26 weeks of age from Barred Rock were tested for 12 weeks. No significant differences in liveability, feed intake, feed conversion ratio, egg quality characteristics except for egg yolk redness (a*) and blood parameters were observed among the groups. When compared NC with C, egg weight, egg mass and body weight gain were decreased. The addition of 0.5% SB (SB-1) increased egg production and egg mass compared to NC and MOS. The addition of 1% SB (SB-2) increased egg mass compared to NC. The AF contaminated diet (NC) caused a significant decrease in a* compared to C. Aflatoxin was not detected in eggs obtained from any of the treatments. Faeces pH was higher in NC than in C, SB-1 and SB-2 and similar to that of MOS. The proportion of dry matter of the faeces in C was higher than that of NC. As a result, SB appears to be more effective than MOS as a toxin-binding agent in counteracting the adverse effects of AF in laying hens.

Keywords: *Aflatoxin, Bentonite, Laying hen, Mannan oligosaccharide, Performance and egg quality, Blood and digestion parameters*

Aflatoksin İçeren Yumurta Tavuğu Yemlerine Sodyum Bentonit ve Mannan Oligosakkarit İlavesinin Performans, Yumurta Kalitesi, Kan ve Sindirim Özelliklerine Etkileri

Özet

Bu araştırmada, yaklaşık 120 ppb toplam aflatoksin (AF) içeren yumurta tavuğu yemlerine sodyum bentonit (SB) (%0.5 ve %1) ve mannan oligosakkarit (MOS, %0.1) ilavesi yapılmış, bu gruplar AF bulaşık negatif kontrol (NC) ve AF içermeyen kontrol (C) grupları ile karşılaştırılmıştır. Barred Rock hattından 26 haftalık yaşlı toplam 180 adet tavuk 12 hafta süresince denenmiştir. Gruplar arasında yaşama gücü, yem tüketimi, yem değerlendirme oranı, yumurta sarısı kırmızılık değeri (a*) haricindeki yumurta kalite kriterleri ve kan parametreleri bakımından önemli farklılıklar gözlenmemiştir. Yumurta ağırlığı, yumurta kütlesi ve canlı ağırlık kazancı NC de, C ile karşılaştırıldığında azalmıştır. İlave edilen %0.5 SB (SB-1) yumurta verimi ve kütlesini NC ve MOS gruplarına göre artırmıştır. İlave edilen %1 SB (SB-2) yumurta kütlesini NC grubuna göre artırmıştır. Aflatoksin bulaşık yem (NC) C ile karşılaştırıldığında a* değerinde önemli bir azalmaya sebep olmuştur. Hiçbir grubun yumurtasında aflatoksin tespit edilmemiştir. Dışkı pH'ı NC grubunda, C, SB-1 ve SB-2 gruplarından daha yüksek ve MOS grubu ile benzerdir. Dışkı kuru madde oranı C grubunda, NC grubundan daha yüksektir. Sonuç olarak, bir toksin bağlayıcı ajan olarak SB yumurta tavuklarında AF'nin zararlı etkilerini önlemede MOS'dan daha etkili görünmektedir.

Anahtar sözcükler: *Aflatoksin, Bentonit, Yumurta tavuğu, Mannan oligosakkarit, Performans ve yumurta kalitesi, Kan ve sindirim parametreleri*

İletişim (Correspondence)
+90 312 6515171
enginyenice@hotmail.com

INTRODUCTION

The occurrence of mycotoxins in foods and feeds is a problem of major concern all over the world. It has been estimated that 25% of the world's crop production is contaminated with mycotoxins. The process of pre or post-harvest, storage and the steps of feed production are all potential sources of microbial contamination [1-3]. Acute mycotoxicosis outbreaks are rare events in modern poultry production; however, low mycotoxin doses which very often are not detected are responsible for reduced efficiency of production and increased susceptibility to infectious disease [2]. Aflatoxins (AF), a group of closely related and biologically active mycotoxins, are produced by strains of *Aspergillus flavus* and *Aspergillus parasiticus* [3]. Aflatoxicosis in poultry also cause listlessness, hepato-toxicosis, haemorrhage, anorexia with lowered growth rate, poor feed utilization and decreased egg production and fertility [4-8].

Practical and cost-effective methods for detoxifying AF contaminated feed and feedstuffs are in high demand. In recent years, in particular, effective results were seen with the adding of organic and inorganic additives to feeds containing toxins. Detoxifying agents bind aflatoxins from the digestive-tract and thus reduce their absorption into the organism [9]. One of the inorganic materials used for such purpose is the natural bentonite. Bentonite is an aluminosilicate compound, used as adsorbent, and can be added to poultry feed without harmful effects to the animals [10]. *In vitro* and *in vivo* studies have indicated that natural sodium bentonite (SB) has a strong ability to absorb AF [11,12]. Previous works have shown that the adverse effects of AF on broiler performance can be minimized by supplementing their feed rations with the various levels of SB (0.3 to 1.5%) [6,13,14]. Incorporation of SB (0.25 to 0.5%) reduces the incidence and severity of the hepatic histopathology changes associated with aflatoxicosis in broilers [14,15]. Mannan oligosaccharides (MOS) used as organic additives, which are derived from the cell wall of yeast (*Saccharomyces cerevisiae*), also have considerably high AF binding capability [11,16]. Studies using MOS (0.1%) in broiler [17,18] and laying hens [5,19] indicate that MOS partially or completely reversed the effects of AF on performance and blood biochemistry.

Reports on the effects of MOS and SB as AF binder on the performance, egg quality, blood and digestion characteristics in laying hens are lacking. The studies so far have focused on broilers. Therefore the present study was designed to observe the possible adverse effects of AF on laying hens, and to evaluate the possible beneficial effects of dietary SB and MOS as a toxin-binder.

MATERIAL and METHODS

Experimental Design and Diets

Sodium bentonite (SB) (0.5% and 1%) and mannan oligosaccharide (MOS, 0.1%) were supplemented to the feed of laying hens receiving approximately 120 ppb total aflatoxin (AF), and were compared to control (C) and negative control (NC) groups *(Table 1)*. Prior to the experiment, hens were given commercial layers' diet for one week. During this period, egg production and egg weight was monitored and hens of similar body weight and egg production were selected. A total of 180 Barred Rock laying hens were randomly distributed to the individual cages (25 × 47 × 55 cm) in the form of 5 groups having 6 replicates each (6 hens in each replicate). The experiment began at 26 weeks of hen age and continued for 12 weeks. Feed and water was supplied to the hens *ad libitum*. The environment of the hen house was fully controlled with a 14 h light period. Yellow corn contaminated with AF was obtained from a private company (Adana, Turkey) and mixed to the trial diets in a proportion of 60% in order to increase the amount of AF. Diets were analysed [20] for AFB_1 and total AF (B_1+B_2+G_1+G_2) composition *(Table 1)*. Sodium bentonite with Turkey origin was obtained from Çanbensan A.Ş. (Ankara, Turkey). The composition of the fine-grained powder was nearly 61.3% SiO_2, 17.8% Al_2O_3, 3.0% Fe_2O_3, 2.7% Na_2O, 2.1% MgO, 1.3% K_2O and 4.5% CaO. The loss of ignition at 1050°C was 8%. Specific gravity was 2.5 g/cm^3, humidity was 8%, and the swelling index was 18-25. Mannan oligosaccharide (Bio-Mos; Alltech Inc, Nicholasville, Kentucky) is a product derived from

Table 1. *Experimental design and aflatoxin composition of diets*
Tablo 1. *Deneme deseni ve karma yemlerin aflatoksin içerikleri*

Experimental Groups	Sodium Bentonite (%)	Mannan Oligosaccharide (%)	Aflatoxin B_1 (ppb)	Total Aflatoxin (B_1+B_2+G_1+G_2) (ppb)
C	0	0	2.0	2.0
NC	0	0	107.3	121.9
SB-1	0.5	0	106.2	118.9
SB-2	1	0	106.1	120.6
MOS	0	0.1	103.3	122.3

C: *Control, without aflatoxin;* ***NC:*** *Negative control, aflatoxin contaminated diet;* ***SB-1:*** *Contaminated diet plus 0.5% sodium bentonite;* ***SB-2:*** *Contaminated diet plus 1% sodium bentonite;* ***MOS:*** *Contaminated diet plus 0.1% mannan oligosaccharide*

the outer cell wall of a selected strain of *Saccharomyces cerevisiae* yeast.

The experimental diets were formulated according to NRC requirements [21] *(Table 2)*. Feeds were prepared as mash using a cracker-mixer machine with 300 kg/h capacity. Crude nutrient, starch, and sugar analyses of feed ingredients were performed according to AOAC [22] procedures and their metabolisable energy were calculated using the equations of Janssen [23]. The resulting values were used in the calculation of diet composition and chemical components.

Production and Egg Quality Parameters

Hen body weight was measured individually at the beginning and at the end of the experiment, and was used in the calculation of body weight gain. Egg production, broken-cracked eggs and hen deaths were recorded daily. Feed intake and egg weight were determined every 2 weeks. Egg mass was calculated from laying rate and egg weight [egg production (hen-day, %) × egg weight (g)/100], and feed conversion ratio was determined from feed intake and egg mass [feed intake (g/hen/day)/egg mass (g/hen/day)]. Twenty four randomly selected eggs from each group were collected every 4 weeks, and egg quality characteristics were determined 24 h after collection of the eggs. Eggshell thickness was measured after peeling off the membrane under the shell with Mitutoyo digital micrometer (digital 395 series with 0.001 mm sensitivity, Kawasaki, Japan) on three points at the equatorial region

Table 2. *Compositions of the experimental diets*
Tablo 2. *Deneme yemlerinin kompozisyonu*

Feed Ingredients	**Experimental Diets (%)**				
	C	**NC**	**SB-1**	**SB-2**	**MOS**
Yellow corn	60	60	60	60	60
Wheat	2.64	2.64	1.34	0.84	2.34
Soybean meal (42% CP)	19.8	19.8	19.3	18.4	19.7
Full fat soya (32% CP)	5.7	5.7	7.0	7.9	6.0
Vegetable oil	0.5	0.5	0.5	0.5	0.5
Ground limestone	8.6	8.6	8.6	8.6	8.6
Di calcium phosphate	1.7	1.7	1.7	1.7	1.7
Salt (NaCl)	0.35	0.35	0.35	0.35	0.35
DL-Methionine	0.26	0.26	0.26	0.26	0.26
Vitamin-mineral premix[1]	0.2	0.2	0.2	0.2	0.2
Antioxidant	0.05	0.05	0.05	0.05	0.05
Salmonella inhibitor	0.2	0.2	0.2	0.2	0.2
Sodium bentonite	0	0	0.5	1.0	0
Mannan oligosaccharide	0	0	0	0	0.1
Nutrient Content					
Crude protein (%)	16.56	16.52	16.49	16.45	16.53
Metabolizable energy (kcal/kg)	2780	2773	2770	2770	2774
Dry matter (%)	88.41	88.40	88.45	88.50	88.42
Crude ash (%)	12.46	12.46	12.49	12.51	12.46
Ether extract (%)	3.58	3.58	3.79	3.96	3.62
Calcium (%)[2]	3.70	3.70	3.70	3.70	3.70
Available phosphorus (%)[2]	0.40	0.40	0.40	0.40	0.40
Methionine (%)[2]	0.50	0.50	0.50	0.50	0.50
Methionine+cystine (%)[2]	0.78	0.78	0.78	0.78	0.78
Lysine (%)[2]	0.85	0.83	0.83	0.83	0.83

[1] Vitamin-mineral premix provided per kilogram of diet; vitamin A, 15.000 IU; vitamin D_3, 5.000 IU; vitamin E, 50 mg; vitamin K_3, 10 mg; thiamine, 4 mg; riboflavin, 8 mg; pyridoxine, 5 mg; vitamin B_{12}, 0.025 mg; niacin, 50 mg; Ca-pantothenate, 20 mg; folic acid, 2 mg; biotin, 0.25 mg; ascorbic acid, 75 mg; choline, 175 mg; Mg, 35 mg; Mn, 56 mg; Zn, 140 mg; Fe, 56 mg; Cu, 10.5 mg; I, 1 mg; Co, 0.28 mg; Se, 0.28 mg; Mo, 0.7 mg
[2] Calculated values from NRC [21] tables
C: *Control, without aflatoxin;* ***NC:*** *Negative control, aflatoxin contaminated diet;* ***SB-1:*** *Contaminated diet plus 0.5% sodium bentonite;* ***SB-2:*** *Contaminated diet plus 1% sodium bentonite;* ***MOS:*** *Contaminated diet plus 0.1% mannan oligosaccharide;* ***CP:*** *Crude protein*

of the egg, and expressed as an average value. Eggshell breaking strength and haugh unit were measured by using Futura 3/A egg quality measuring system (Futura, Lohne, Germany). Redness (a*) and yellowness (b*) of egg yolk was determined by CR-10 Konica Minolta Color Reader (Osaka, Japan).

Collection and Analysis of Samples

Blood samples of 10 hens from each group were taken individually from brachial vein using injector at the end of the experiment. Serum was isolated by centrifuging the blood at 4500 × g for 10 min. Serum samples were analyzed for total protein, albumin, bilirubin, total cholesterol, calcium, phosphorus, aspartate amino transferase (AST) and alanine amino transferase (ALT) using Roche Cobas Integra original kits by a Roche Cobas Integra 800 automatic analyzer (Roche, Switzerland) [24]. The methodology and reagents were those recommended by the manufacturer of the system [25]. In addition, 6 randomly selected eggs from each group were analyzed for AFB_1 and total AF (B_1+B_2+G_1+G_2) by Agilent 1100 HPLC system [20]. At the end of the experiment, faeces pH and dry matter content of 6 hens from each group was measured. Faeces were collected in plates, which were placed under the cages for 24 h. Feed and feathers were carefully removed from fecal samples. Dry matter was determined according to AOAC [22], pH was measured by a digital pH meter (Hanna pH 211, Italy) calibrated at 22°C. Absorption rate of AFB_1 and total AF were determined, by mixing chromium oxide in 0.3% ratio to the feed of six hens from each treatment, and for 3 days. Faeces from the last 2 days were collected and analyzed for chromium oxide and AF content [20]. Digestibility of AF was calculated on the basis of the equation by Maynard and Loosli [26]: Nutrient digestibility (%) = (Indicator in feed/Indicator in faeces) × (Nutrient in faeces/Nutrient in feed). The animal care protocol used in this study was reviewed and approved by the Ethics Committee of the Poultry Research Station, Ankara, Turkey (22.01.09-2009/06).

Statistical Analysis

The results of all experiments were analysed using the analysis of variance procedures of the statistical program MINITAB Release 14 and the means were compared for differences using Duncan's multiple range test [27] at P<0.05.

RESULTS

The effects of SB and MOS supplementation on the performance of laying hens fed an AF contaminated diet are shown in *Table 3*. No difference in liveability, feed intake and feed conversion ratio were observed among the experimental groups (P>0.05). The AF contaminated diet (NC) caused significant decreases in egg weight, egg mass and body weight gain compared to the control (C) (P<0.05). The addition of 0.5% bentonite (SB-1) resulted in increased egg production (7.6% and 6.3%) and egg mass (10.9% and 7.7%) compared to NC and MOS (P<0.05). However, the addition of 1.0% bentonite (SB-2) increased egg mass (7.3%) compared to NC (P<0.05).

Egg quality characteristics are presented in *Table 4*. There were no significant differences in broken-cracked egg rate, eggshell breaking strength, eggshell thickness, haugh unit and egg yolk yellowness (b*) observed among groups (P>0.05). NC caused a significant decrease in egg yolk redness (a*) compared to C (P<0.05). The eggs collected on the last day of the experiment, when subjected to HPLC analysis, indicated no detectable levels of AFB_1 or total AF residues in any of the treatments.

No significant differences were observed in serum total protein, albumin, bilirubin, total cholesterol, calcium, phosphorus, AST and ALT levels (P>0.05) *(Table 5)*. The measured digestion parameters are given in *Table 6*. Faeces pH of NC was higher than those of C, SB-1 and SB-2 (P<0.05) and similar to that of MOS (P>0.05). The proportion of dry matter in the faeces of C was higher than that of NC (P<0.05).

Table 3. *Effects of dietary sodium bentonite and mannan oligosaccharide supplementation on performance traits of laying hens fed aflatoxin contaminated diet*
Tablo 3. *Aflatoksin içeren yumurta tavuğu yemlerine sodyum bentonit ve mannan oligosakkarit ilavesinin performans ölçütleri üzerine etkileri*

Treatment	Liveability (%)	Egg Production (%/hen/day)	Egg Weight (g/egg)	Egg Mass (g/hen/day)	Feed Intake (g/hen/day)	Feed Conversion Ratio (g feed/g egg)	Body Weight Gain (g/hen)
C	94.4	85.2 ^{ab}	61.9 ^{a}	52.7 ^{a}	119.6	2.27	134.4 ^{a}
NC	94.4	80.2 ^{b}	59.4 ^{b}	47.7 ^{c}	113.8	2.39	46.7 ^{b}
SB-1	94.4	86.3 ^{a}	61.2 ^{ab}	52.9 ^{a}	118.6	2.24	115.6 ^{ab}
SB-2	100	83.7 ^{ab}	61.2 ^{ab}	51.2 ^{ab}	116.1	2.27	116.3 ^{ab}
MOS	94.4	81.2 ^{b}	60.5 ^{ab}	49.1 ^{bc}	113.8	2.32	53.3 ^{b}
SEM	5.56	1.22	0.48	0.72	2.4	0.05	19.4
P	0.903	0.039	0.006	0.003	0.433	0.417	0.006

^{a,b,c} Means within columns with no common superscripts are significantly different (P<0.05); ***C:*** *Control, without aflatoxin;* ***NC:*** *Negative control, aflatoxin contaminated diet;* ***SB-1:*** *Contaminated diet plus 0.5% sodium bentonite;* ***SB-2:*** *Contaminated diet plus 1% sodium bentonite;* ***MOS:*** *Contaminated diet plus 0.1% mannan oligosaccharide;* ***SEM:*** *Standard error of means*

Table 4. *Effects of dietary sodium bentonite and mannan oligosaccharide supplementation on egg quality characteristics of laying hens fed aflatoxin contaminated diet*

Tablo 4. *Aflatoksin içeren yumurta tavuğu yemlerine sodyum bentonit ve mannan oligosakkarit ilavesinin yumurta kalite özelliklerine etkileri*

Treatment	Broken-Cracked Egg (%)	Eggshell Breaking Strength (Newton)	Eggshell Thickness (10^{-2} mm)	Haugh Unit	Egg Yolk Redness (a*)	Egg Yolk Yellowness (b*)
C	0.91	38.0	32.2	75.0	5.91 ^{a}	13.96
NC	1.38	37.5	31.7	74.6	5.54 ^{b}	13.94
SB-1	0.85	38.0	31.9	76.8	5.71 ^{ab}	13.67
SB-2	1.14	37.8	32.2	76.8	5.68 ^{ab}	13.70
MOS	1.35	37.6	31.7	75.1	5.70 ^{ab}	13.28
SEM	0.39	0.95	0.31	1.13	0.07	0.18
P	0.862	0.996	0.634	0.518	0.006	0.069

^{a,b} Means within columns with no common superscripts are significantly different (P<0.05); ***C:*** *Control, without aflatoxin;* ***NC:*** *Negative control, aflatoxin contaminated diet;* ***SB-1:*** *Contaminated diet plus 0.5% sodium bentonite;* ***SB-2:*** *Contaminated diet plus 1% sodium bentonite;* ***MOS:*** *Contaminated diet plus 0.1% mannan oligosaccharide;* ***SEM:*** *Standard error of means*

Table 5. *Effects of dietary sodium bentonite and mannan oligosaccharide supplementation on some blood parameters of laying hens fed aflatoxin contaminated diet*

Tablo 5. *Aflatoksin içeren yumurta tavuğu yemlerine sodyum bentonit ve mannan oligosakkarit ilavesinin bazı kan parametrelerine etkileri*

Treatment	Total Protein (g/dL)	Albumin (g/dL)	Bilirubin (mg/dL)	AST (U/L)	ALT (U/L)	Total Cholesterol (mg/dL)	Calcium (mg/dL)	Phosphorus (mg/dL)
C	5.50	2.25	0.02	163	1.67	194	36.1	6.60
NC	5.70	2.23	0.03	164	1.50	164	33.0	6.60
SB-1	5.80	2.29	0.02	159	1.67	162	34.8	6.60
SB-2	5.80	2.25	0.02	161	1.70	167	35.4	7.30
MOS	5.70	2.22	0.03	160	1.63	166	33.9	6.50
SEM	0.18	0.05	0.006	6.98	0.19	18.12	1.84	0.38
P	0.674	0.889	0.511	0.978	0.952	0.696	0.792	0.572

C: *Control, without aflatoxin;* ***NC:*** *Negative control, aflatoxin contaminated diet;* ***SB-1:*** *Contaminated diet plus 0.5% sodium bentonite;* ***SB-2:*** *Contaminated diet plus 1% sodium bentonite;* ***MOS:*** *Contaminated diet plus 0.1% mannan oligosaccharide;* ***SEM:*** *Standard error of means;* ***AST:*** *Aspartate amino transferase;* ***ALT:*** *Alanine amino transferase*

Table 6. *Effects of dietary sodium bentonite and mannan oligosaccharide supplementation on some digestion parameters of laying hens fed aflatoxin contaminated diet*

Tablo 6. *Aflatoksin içeren yumurta tavuğu yemlerine sodyum bentonit ve mannan oligosakkarit ilavesinin bazı sindirim parametrelerine etkileri*

Treatment	Faeces pH	Faeces Dry Matter (%)	Digestibility of Aflatoxin B_1* (%)	Digestibility of Total Aflatoxin * (B_1+B_2+G_1+G_2) (%)
C	7.80 ^{bc}	26.0^{a}	95.4	95.3
NC	8.56 ^{a}	22.2^{b}	71.8	78.5
SB-1	7.30 ^{c}	23.9^{ab}	66.0	64.2
SB-2	7.87 ^{b}	23.2^{ab}	58.5	55.1
MOS	8.05 ^{ab}	22.7^{ab}	65.0	66.3
SEM	0.12	1.25		
P	0.0001	0.047		

** In the determination of the digestive rate of total AF and AFB_1, samples taken from six hens were mixed for analysis without repetition, and statistical evaluation was not carried out;* *^{a,b,c} Means within columns with no common superscripts are significantly different (P<0.05)* ***C:*** *Control, without aflatoxin;* ***NC:*** *Negative control, aflatoxin contaminated diet;* ***SB-1:*** *Contaminated diet plus 0.5% sodium bentonite;* ***SB-2:*** *Contaminated diet plus 1% sodium bentonite;* ***MOS:*** *Contaminated diet plus 0.1% mannan oligosaccharide;* ***SEM:*** *Standard error of means;*

DISCUSSION

The results of present study indicate that feed contaminated with AF (120 ppb total AF; 106 ppb AFB_1) caused adverse effects on egg weight, egg mass and body weight gain of laying hens. However, liveability, egg production, feed intake and feed conversion ratio were not affected by AF contamination. Similarly, Pandey and Chauhan [8] reported that body weight gain of laying hens were significantly lower in the contaminated groups with AF compared to control group. In another study, the inclusion of dietary AFB_1 from 0 to 2.0 ppm resulted in lowered egg weight and nitrogen retention [28]. However, in 2-week a short feeding study, AF contamination did not have any detrimental effect on body weight gain [29]. Aflatoxin appears to exert its negative effect on animal performance chiefly by depressing the DNA and RNA synthesis and eventually protein synthesis [28,30]. Ali et al.[13] reported that the toxicity of aflatoxin was characterized by reduction in body weight gain as aflatoxins interfere with normal metabolic pathway through the inhibition of protein synthesis and enzyme system that is involved in carbohydrate metabolism and energy release. The results of present study agree with data showing that AF contamination did not affect the liveability, egg production and feed intake of laying hens [5,19,28,29,31]. However, some researchers did report that dietary AF decreased egg production and feed intake [8,32,33]. The different results in the studies may be due to causes as the AF concentration, its form, the length of trial period and poultry genotype and age.

In the present study, dietary SB supplementation ameliorated the effects of AF on performance of laying hens. Previous studies reported that SB supplementation to AF contaminated broiler diets significantly improved their performance [6,13]. However, there are currently no studies on the effect of dietary SB supplementation on laying hen performance with which to compare our results. Our results suggest that MOS supplementation to the AF contaminated diet had no a positive effect on performance of laying hens, which agrees with the findings of previous reports [5,33]. However, in other studies, the addition of MOS allowed significant recovery from the adverse effects of AF on the performance of broilers [17,18]. Detoxifying agents as bentonite and MOS form a complex with the toxin thus preventing the absorption of aflatoxin across the intestinal epithelium [1]. Therefore, these agents may ameliorate the negative effects of AF on performance of poultry.

The present study showed that an AF contaminated diet did not have adverse effects on egg quality characteristics except for egg yolk redness (a*). Similarly, previous studies reported that different AF doses did not affect egg quality [8,28,33], but had a negative effect on egg yolk colour parameters [5,8]. The decrease in the value of colour parameters may be connected to AF interference with lipid metabolism [34], carotenoid absorption, or deposition in yolk [35]. The supplementation of SB and MOS numerically increased a* according to NC in the present study. Similarly, Zaghini et al.[5] reported that MOS supplementation to diets contaminated with AF did not affect eggshell rate and eggshell thickness, but improved a*.

No AFB_1 or total AF residues were detected in the eggs of any of the treatments in the present study. Similarly, in other trials, no measurable residual AF or metabolites were found in eggs despite the consumption of different doses of AF (100 ppb to 2.5 ppm) [1,5,19,33]. Conversely, the residues of AFB_1 and total AF were detected in the eggs of hens given 500 ppb AFB_1 [31] and the inclusion of dietary total AF from 190 to 900 ppb [32]. These contrasting results may be ascribed to the administration of naturally contaminated feeds or diets containing different AF with different levels of toxicity [31].

The blood parameters of laying hens were not effect by the experimental treatments in the present study. Similarly, AF contaminated diet and the addition of MOS to this diet did not affect the serum total protein, albumin, bilirubin, total cholesterol, calcium, phosphorus, AST and ALT levels of laying hens [33]. SB supplementation (0.5-1%) to a contaminated broiler diet in another study did not affect serum albumin and ALT levels [6]. However, some studies demonstrated a decrease in serum total protein, albumin, total cholesterol, triglyceride, calcium and phosphorus levels, and an increase in bilirubin, ALT and AST levels of broilers given AF contaminated diets [14,15,17,36-38]. In these studies, total protein, albumin, cholesterol, enzymes as ALT and AST are consistent indicators of the hepatocellular damage [15,37]. AF may cause alteration of calcium and inorganic phosphorus metabolism. It may directly alter the renal, intestine and parathyroid regulation of calcium and inorganic phosphorus [36]. In many studies, dietary SB and MOS supplementations resulted in significant improvements in blood biochemical parameters adversely affected by AF ingestion [14,15,17,36,37]. However, Ghahri et al.[38] reported that the biochemical parameters for broilers fed diets containing SB+AF and MOS+AF did not completely return to normal values. But, MOS supplementation counteracted the observed increase in liver enzymes.

Our results indicate that the AF contaminated diet negatively affected the pH and dry matter of laying hen faeces. Dietary SB and MOS supplementations were partially effective in counteracting the adverse effects of AF on these digestive parameters. Some studies reported that the differences in the digestive parameters of hens receiving AF may be due to the alteration in intestinal morphology [29] or organic matter digestibility [8].

The digestibility of AFB_1 and total AF in C, NC, MOS, SB-1 and SB-2 were 95.4, 71.8, 65.0, 66.0, 58.5 and 95.3, 78.5, 66.3, 64.2, 55.1%, respectively. A portion of the received AF became bound by MOS or SB in the digestive tract and was

discarded in the faeces. *In vitro* studies have indicated that SB and MOS have a strong ability to absorb AF (95-98 and 80-97%, respectively) [11,16]. However, the measurements taken under laboratory conditions had been difficult to reproduce in experiments with animals *(in vivo)* [39].

Results of this study demonstrated that approximately 120 ppb dietary AF contamination resulted in adverse effects on some performance, egg yolk colour and digestion parameters of laying hens. SB and MOS were inert and non-toxic and SB appears to be more effective than MOS in counteracting the adverse effects of AF for layers. These findings suggest that dietary supplementation with detoxifying agents, such as SB, may be a solution to the problem of dietary AF contamination and toxicity in laying hens.

REFERENCES

1. Manafi M: Evaluation of different mycotoxin binders on aflatoxin B_1 *(Aspergillus parasiticus)* produced on rice *(Oriza sativa)* on fertility, hatchability, embryonic mortality, residues in egg and semen quality. *Adv Environ Biol,* 5, 3818-3825, 2011.

2. Surai PF, Mezes M, Dvorska JE: Mycotoxin-Antioxidant interactions: Theoretical considerations and practical applications. *Proceedings of 1st Mediterranean Summit of WPSA, Advances and Challenges in Poultry Science,* 7-10 May, Chalkidiki, Greece, pp.134-143, 2008.

3. Colakoglu F, Donmez HH: Effects of aflatoxin on *AgNOR* activity of cells in different regions of kidney, and protective effectiveness of esterified glucomannan in ram. *Kafkas Univ Vet Fak Derg,* 19, 505-509, 2013. DOI: 10.9775/kvfd.2012.8176

4. Çelik K, Uluocak AN, Ayaşan T, Doran F, Öztürkcan O: Farklı dozlardaki mycotoxinin Japon bıldırcınlarının *(Coturnix Coturnix Japonica)* besi performansı ile histopatolojik özelliklerine etkileri. *VIV Yutav'99 Uluslararası Tavukçuluk Fuarı ve Konferansı,* 3-6 Haziran, İstanbul, Turkey, Bildiriler Kitabı, s.222-230, 1999.

5. Zaghini A, Martelli G, Roncada P, Simioli M, Rizzi L: Mannan-oligosaccharides and aflatoxin B_1 in feed for laying hens: Effects on egg quality, aflatoxins B_1 and M_1 residues in eggs, and aflatoxin B_1 levels in liver. *Poult Sci,* 84, 825-832, 2005.

6. Manafi M, Umakantha B, Narayanswamy HD: Investigation on aflatoxicosis using high grade sodium bentonite in broilers. *Proceedings of XXIII World's Poultry Congress,* 30 June-4 July, Brisbane, Australia, Abstract's Book, pp.222, 2008.

7. Ortatatli M, Oguz H: Ameliorative effects of dietary clinoptilolite on pathological changes in broiler chickens during aflatoxicosis. *Res Vet Sci,* 71, 59-66, 2001. DOI: 10.1053/rvsc.2001.0487

8. Pandey I, Chauhan SS: Studies on production performance and toxin residues in tissues and eggs of layer chickens fed on diets with various concentrations of aflatoxin AFB_1. *Br Poult Sci,* 48, 713-723, 2007.

9. Huwig A, Freimund S, Kappeli O, Dutler H: Mycotoxin detoxication of animal feed by different adsorbents. *Toxicol Lett,* 122, 179-188, 2001. DOI: 10.1016/S0378-4274(01)00360-5

10. Kurnick AA, Reid BK: Poultry nutrition studies with bentonite. *Feedstuffs,* 32, 34, 1989.

11. Diaz DE, Jr Hagler WM, Hopkins BA, Whitlow LW: Aflatoxin binders I: *In vitro* binding assay for aflatoxin B_1 by several potential sequestering agents. *Mycopathologia,* 156, 223-226, 2002.

12. Rosa CA, Miazzo R, Magnoli C, Salvano M, Chiac SM, Ferrero S, Saenz M, Carvalho EC, Dalcero A: Evaluation of the efficacy of bentonite from the south of Argentina to ameliorate the toxic effects of aflatoxin in broilers. *Poult Sci,* 80, 139-144, 2001.

13. Ali H, Hag A, Rasool S, Shah TH, Anjum I: Effect of sodium bentonite as aflatoxin binder in broiler feeds containing fungal infected grains. *Pak J Agri Sci,* 37, 3-4, 2000.

14. Miazzo R, Peralta MF, Magnoli C, Salvano M, Ferrero S, Chiacchiera SM, Carvalho EC, Rosa CA, Dalcero A: Efficacy of sodium bentonite as a detoxifier of broiler feed contaminated with aflatoxin and fumonisin. *Poult Sci,* 84, 1-8, 2005.

15. Eraslan G, Essiz D, Akdogan M, Karagoz E, Oncu M, Ozyıldız Z: Efficacy of dietary sodium bentonite against subchronic exposure to dietary aflatoxin in broilers. *Bull Vet Inst Pulawy*, 50, 107-112, 2006.

16. Mahesh BK, Devegowda G: Ability of aflatoxin binders to bind aflatoxin in contaminated poultry feed-an *in vitro* study. *Proceedings of 20th Worlds Poultry Congress,* 2-5 September, New Delhi, pp.296, 1996.

17. Khatke PA, Singh R, Mandal AB, Tyagi PK: Ability of biological adsorbents to ameliorate aflatoxicosis in broiler chicken: Effect on blood biochemicals and organ weights. *Indian J Poult Sci,* 48, 117-121, 2013.

18. Raju MVLN, Devegowda G: Influence of esterified-glucomannan on performance and organ morphology, serum biochemistry and haematology in broilers exposed to individual and combined mycotoxicosis (aflatoxin, ochratoxin and T-2 toxin). *Br Poult Sci,* 41, 640-650, 2000.

19. Rizzi L, Zaghini A, Roncada P: Aflatoxin B_1 oral administration to laying hens: Efficacy of modified mannanoligosaccharide (MTB-100) to prevent mycotoxicosis. *Poster Presented at the 14th Annual Symposium on Biotechnology in the Feed Industry,* 20-22 April, Lexington, KY, 1998. http://www.vetaque.com/per/news/Mycotoxins%20in%20layers.pdf, *Accessed:* 26.08.2009.

20. AOAC: Official Methods of Analysis. Association of Analytical Communities. 17th ed., 2nd Revision, Gaithersburg, MD, USA, 2003.

21. NRC: Nutrient Requirements of Poultry. 9th revised ed., 19-34, National Academy Press, Washington, DC, USA, 1994.

22. AOAC: Official Methods of Analysis. Association of Official Analytical Chemists. 15th ed., Arlington, Virginia, 1990.

23. Janssen WMMA: European Table of Energy Values for Poultry Feedstuffs. 3rd ed., Beekbergen, Netherlands: Spelderholt Center for Poultry Research and Information Services, 1989.

24. Sribhen C, Choothesa A, Songserm T, Sribhen K: Age-Related differences in levels of blood chemistry parameters and cardiac marker proteins in commercial broilers. *Kasetsart J (Nat Sci),* 37, 321-326, 2003.

25. Roche Diagnostics: Cobas Integra 800 Kullanıcı Eğitimi El Kitabı, SW versiyon 9763.A, Roche Diagnostics GmbH, İsviçre. 213 s., 2001.

26. Maynard LA, Loosli JK: Animal Nutrition, 6th ed., McGraw-Hill Company, New York, 1969.

27. Duncan DB: Multiple range and multiple F tests. *Biometrics*, 11, 1-42, 1955.

28. Galkate UV, Rokde SN: Effect of dietary aflatoxin on certain egg production and quality parameters in white leghorn layers. *Anim Nutr Feed Techn,* 10, 121-126, 2010.

29. Applegate TJ, Schatzmayr G, Pricket K, Troche C, Jiang Z: Effect of aflatoxin culture on intestinal function and nutrient loss in laying hens. *Poult Sci,* 88, 1235-1241, 2009. DOI: 10.3382/ps.2008-00494

30. Venkata RK, Gopalakrishan RD, Rama RP: Effect of aflatoxin feeding and its withdrawal effect on the growth rate of broilers and layers under long term feeding trials. *Indian Vet J,* 65, 113, 1988.

31. Olivera CA, Kobashigawa E, Reis TA, Mestieri L, Albuquerque RC, Correa B: Aflatoxin B_1 residues in eggs of laying hens fed a diet containing different levels of the mycotoxin. *Food Addit Contam,* 17, 459-462, 2000. DOI:10.1080/02652030050034037

32. Herzallah SM: Aflatoxin B_1 residues in eggs and flesh of laying hens fed aflatoxin B_1 contaminated diet. *Am J Agric Biol Sci,* 8, 156-161, 2013. DOI : 10.3844/ajabssp.2013.156.161

33. Mızrak C, Yenice E, Kahraman Z, Tunca M, Yıldırım U, Ceylan N: Effects of dietary sepiolite and mannanoligosaccharide supplementation on the performance, egg quality, blood and digestion characteristics of laying hens receiving aflatoxin in their feed. *Ankara Üniv Vet Fak Derg,* 61, 65-71, 2014.

34. Tung HT, Donaldson WE, Hamilton PB: Altered lipid transport during aflatoxicosis. *Toxicol Appl Pharmacol,* 22, 97-104, 1972. DOI: 10.1016/0041-008X(72)90229-3

35. Genedy SGK, El-Naggar NM, Isshak NS, Qota EMA: Effectiveness of available commercial products to alleviate the toxic severity of aflatoxin diets on egg production and its quality of two local hen strains. *Egyptian Poult Sci J,* 19, 569-589, 1999.

36. Eraslan G, Essiz D, Akdogan M, Sahindokuyucu F, Altıntas L: The effects of aflatoxin and sodium bentonite combined and alone on some blood electrolyte levels in broiler chickens. *Turk J Vet Anim Sci*, 29, 601-605, 2005.

37. Kececi T, Oguz H, Kurtoglu V: Effects of polyvinylpolypyrrolidone, synthetic zeolite and bentonite on serum biochemical and hematological characters of broiler chickens during aflatoxicosis. *Br Poult Sci,* 39, 452-458, 1998.

38. Ghahri H, Habibian R, Fam MA: Effect of sodium bentonite, mannan oligosaccharide and humate on performance and serum biochemical parameters during aflatoxicosis in broiler chickens. *Global Veterinaria*, 5, 129-134, 2010.

39. Taylor D: Mycotoxin binders: What they are and how they work. *Proceedings of Oil-Dri Animal Health Technical Symposium,* 25 August, Montreal, Canada, pp.1-17, 2000.

Kafkas Univ Vet Fak Derg
21 (2): 219-224, 2015
DOI: 10.9775/kvfd.2014.12054

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Siyah Alaca Sığırlarda 305 Günlük Süt Verimini Etkileyen Faktörlerin Path (İz) Analizi İle Belirlenmesi

Öznur İŞÇİ GÜNERİ [1] Çiğdem TAKMA [2] Yavuz AKBAŞ [2]

[1] Muğla Üniversitesi, Fen Edebiyat Fakültesi, İstatistik Bölümü, TR-48000 Kötekli, Muğla - TÜRKİYE
[2] Ege Üniversitesi, Ziraat Fakültesi, Zootekni Bölümü, Biyometri ve Genetik Anabilim Dalı, TR-35100 Bornova, İzmir - TÜRKİYE

Article Code: KVFD-2014-12054 Received: 30.07.2014 Accepted: 26.11.2014 Published Online: 15.12.2014

Abstract

Kantitatif bir özellik üzerinde çeşitli faktörlerin doğrudan etkileri yanı sıra, faktörler arasındaki ilişkiler sonucu dolaylı etkiler de bulunabilmektedir. Doğrudan ve dolaylı etkilenme şekillerinin birbirinden ayrılması için söz konusu ilişkilerin ayrıntılı bir şekilde ortaya konması gerekmektedir. Bu çalışmada 5047 Siyah Alaca sığırın 7 laktasyonuna ait toplam 11647 adet 305 günlük süt verim kaydı kullanılarak süt verimi (Y) üzerine laktasyon sırası (X1), buzağılama yılı (X2) ve laktasyon süresinin (X3) doğrudan ve dolaylı etkileri iz analizi ile incelenmiştir. 305 günlük süt verimi ile laktasyon sırası, buzağılama yılı ve laktasyon süresi arasındaki korelasyonlar sırasıyla 0.17, 0.43, 0.54 ve istatistiksel olarak önemli bulunmuştur (P<0.01). Laktasyon sırası, buzağılama yılı ve laktasyon süresi değişkenlerinin 305 günlük süt verimi üzerine doğrudan etkileri de sırasıyla PY1=0.12, P21=0.10, P31=0.46 ve istatistiksel olarak önemli saptanmıştır (P<0.01). Laktasyon sırasının 305 günlük süt verimine buzağılama yılı üzerinden U ve laktasyon süresi üzerinden S etkileri sırasıyla 0.01 ve 0.04 düzeyindedir. Buzağılama yılının 305 günlük süt verimine laktasyon süresi üzerinden U ve laktasyon sırası üzerinden S etkileri ise sırasıyla 0.31 ve 0.02 düzeyindedir. Ayrıca, laktasyon süresinin süt verimine buzağılama yılı üzerinden U ve laktasyon sırası üzerinden S etkilerinin sırasıyla 0.07 ve 0.01 düzeylerinde ve düşük olduğu tespit edilmiştir.

Keywords: *305 günlük süt verimi, Regresyon analizi, İz analizi, İz katsayısı*

Determination of Factors Affecting 305-Day Milk Production via Path Analysis on Holstein Friesians

Özet

A quantitative trait was affected directly or indirectly by several factors due to relationships among them. It is necessary to identified direct and indirect effects of a factor to reveal all relationships in a detailed way. In this study, total of 11647 305-day milk yield records from the 7 parities of 5047 Holstein Friesian cows were statistically evaluated for determining direct and indirect effects of parity (X1), year of calving (X2) and lactation length (X3) on 305-day milk production (Y) via path analysis. Correlations among 305 day milk yield, parity, year of calving and lactation length were calculated 0.17, 0.43, 0.54 respectively and found statistically significant ((P<0.01). The direct effects of the parity, year of calving and lactation length on 305-day milk production were found PY1=0.12, P21=0.10, P31=0.46 respectively and statistically significant (P<0.01). The indirect U effect of parity via year of calving and S effect via lactation length on 305 day milk yield were found 0.01 and 0.04, respectively. The indirect U effect of year of calving via lactation length and S effect via parity on 305 day milk yield were found 0.31 and 0.02, respectively. Moreover, the indirect U effect of lactation length via year of calving and S effect via parity were found 0.07 and 0.01, respectively and lower.

Anahtar sözcükler: *305-day milk production, Regression analysis, Path analysis, Path coefficient*

GİRİŞ

Islah çalışmalarının amacı, ekonomik özellikler bakımından popülasyonun ortalama düzeyini iyileştirmektir. Bu nedenle, ekonomik özellikleri belirlemek ve bu özellikleri etkileyen değişkenleri etki şekilleriyle birlikte tanımlamak gerekir. İncelenen verim ile ilişkili olan bir değişken, verim üzerine doğrudan etki yaptığı gibi, diğer değişkenler üzerinden dolaylı bir etkiye de sahip olabilir. Bu durumda söz konusu verim ile verimi etkileyen değişkenler arasındaki ilişkilerin korelasyon katsayıları ile tam olarak açıklanabilmesi olanaksızdır [1]. Bu ilişkileri daha iyi açıklayabilmek

İletişim (Correspondence)
+90 232 3112714
cigdem.takma@ege.edu.tr

için iz (path) analizine ihtiyaç duyulur [2]. İz analizinde amaç, değişkenlerin doğrudan veya dolaylı etkilerinin iz katsayıları ile belirlenmesi ve söz konusu katsayıların önemliliğinin ve büyüklüğünün tahmin edilmesidir [3].

İz analizi ilk kez Dr. Sewall Wright tarafından ortaya konulup, Li [4] tarafından geliştirilmiş ve birçok konuya uygulanabilir hale getirilmiştir. İz analizinin temeli, çoklu regresyon analizine dayanmaktadır [5-7]. Çoklu regresyon analizinde, bir bağımlı değişken tüm bağımsız değişkenler üzerinden analiz edilirken, iz analizinde bağımlı değişken her bir bağımsız değişken üzerinden analiz edilmekte; başka bir ifadeyle birden fazla regresyon analizi yapılmaktadır. Nitekim tahminlenen standardize edilmiş regresyon katsayıları iz katsayılarına eşittir [8]. İz analizi, bağımlı ve bağımsız değişkenler arasında tek yönlü bir sebep-sonuç ilişkisini ele almakta, ölçümlerin kantitatif yapıda ve hatasız elde edilmiş olduğunu varsaymaktadır [9]. Ayrıca, iz analizinde değişkenler arasındaki ilişkiler, doğrudan, dolaylı, U ve S olmak üzere dört farklı türde etkileşim şeklinde incelenebilmektedir. Bu analizlerde, değişkenler arasındaki etkileşimler iz katsayıları olarak hesaplanmakta ve bir diyagramla gösterilmektedir [10].

Hayvan ıslahında süt verimine etki eden faktörlerin genellikle doğrudan etkileri üzerinde durulmaktadır. Bu amaçla varyans analizi ve korelasyon katsayılarından yararlanılmaktadır. Bununla birlikte fizyolojik ve çevresel faktörlerin süt verimi üzerine doğrudan etkilerinin yanında dolaylı etkilerinin de olduğu bilinmektedir [11,12]. Sığırlarda süt verimi üzerine söz konusu etkilerin düzeyini ortaya çıkarmak için iz analizinden yararlanan çalışmalar (İşçi ve ark.[13], Tahtalı ve ark.[14] ve Orhan ve Kaşıkçı [15]) az sayıdadır.

Bu çalışma ile Siyah alaca süt sığırlarının 305 günlük süt verimi üzerine laktasyon sırası, buzağılama yılı ve laktasyon süresinin doğrudan ve dolaylı etkilerinin iz analizi kullanılarak incelenmesi amaçlanmıştır.

MATERYAL ve METOT

Araştırmada 2010-2011 yılları arasında yetiştirilen 5047 Siyah Alaca sığırın 7 laktasyonuna ait toplam 11647 adet 305 günlük süt verim kaydı kullanılmıştır. 305 günlük süt verimi üzerine laktasyon sırası, buzağılama yılı ve laktasyon süresi etkilerinin iz analizi, SPSS [16-18] istatistik paket programı ile yapılmıştır. Bu araştırmada 305 günlük süt verimi bağımlı; laktasyon sırası, buzağılama yılı ve laktasyon süresi ise bağımsız etkiler olarak incelemeye alınmıştır. Bağımlı ve bağımsız değişkenler arasındaki doğrudan, dolaylı, U ve S etkilerine ait iz diyagramları aşağıdaki tanımlamalara göre oluşturulmuştur [19].

Doğrudan Etki

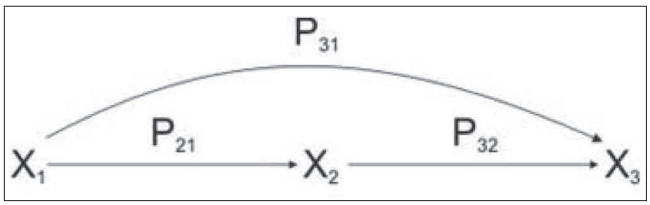
Doğrudan etki (D), bir değişkenin diğer bir değişkene yapmış olduğu doğrudan etkidir. Doğrudan etkiye ait katsayı yani P_{21}, iki değişken arasındaki korelasyon katsayısına eşittir ($r_{12} = P_{21}$).

Dolaylı Etki

Şekil 1'de X_1 değişkeninin X_3 değişkeni üzerinde yapmış olduğu doğrudan etki (P_{31}) ve dolaylı etkiler (P_{21} ve P_{32}) görülmektedir. X_1 ve X_3 değişkenleri arasındaki korelasyon katsayısı, doğrudan (D) ve dolaylı etkilerin (I) toplamına eşittir. X_1 değişkeninin X_3 değişkeni üzerine yapmış olduğu dolaylı etki (I) ise X_1 değişkeninin X_2 değişkeni üzerine yapmış olduğu doğrudan etkiyi gösteren iz katsayısı (P_{21}) ile X_2 değişkeninin X_3 üzerine yapmış olduğu doğrudan etkiyi gösteren iz katsayısının (P_{32}) çarpımına eşittir ($r_{13} = D + I = P_{31} + P_{21} \times P_{32}$).

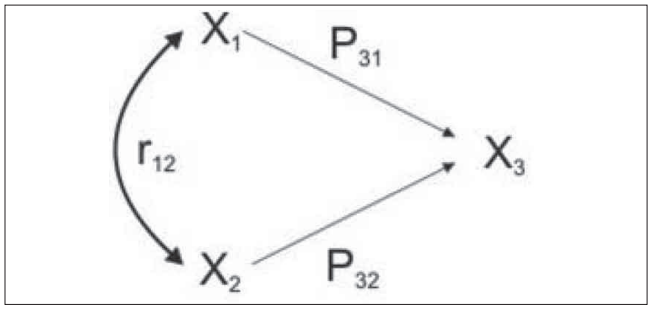
U Etkisi

Sebep değişkenleri arasında karşılıklı bir etkileşim söz konusu olduğunda ortaya çıkan etki U etkisidir. *Şekil 2*'de X_1 ve X_3 değişkenleri arasındaki U etkisi görülmektedir. Bu etkiye göre, X_1 değişkeni X_3 değişkeni üzerinde doğrudan etkiye sahip olup, X_2 değişkeni ile arasında karşılıklı etkileşime sahiptir. Buna göre, X_1 değişkeninin X_3 değişkeni üzerinden yapmış olduğu doğrudan etki, bu iki değişken arasındaki iz katsayısına eşittir ($D = P_{31}$). X_1 değişkeninin X_2 değişkeni üzerinden X_3 değişkenine yapmış olduğu U etkisi ise X_1 ve X_2 değişkenleri arasındaki korelasyon katsayısı ve X_2 değişkeninin X_3 değişkeni üzerinde yapmış olduğu doğrudan etkiyi gösteren iz katsayısının çarpımına eşittir ($U = r_{12} \times P_{32}$). Bu durumda, X_1 ile X_3 değişkenleri arasındaki korelasyon (r_{13}), doğrudan (D) ve U etkisinin toplamına eşittir ($r_{13} = D + U = P_{31} + r_{12} \times P_{32}$). Aynı durum *Şekil 2*'deki



Şekil 1. Değişkenler arasındaki doğrudan ve dolaylı etkileri gösteren iz diyagramı

Fig 1. The path diagram displays direct (D) and indirect (I) effects among variables



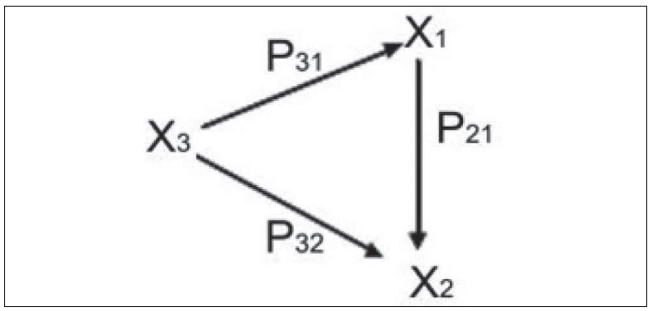
Şekil 2. Değişkenler arasındaki U etkisini gösteren iz diyagramı

Fig 2. The path diagram displays U effect among variables

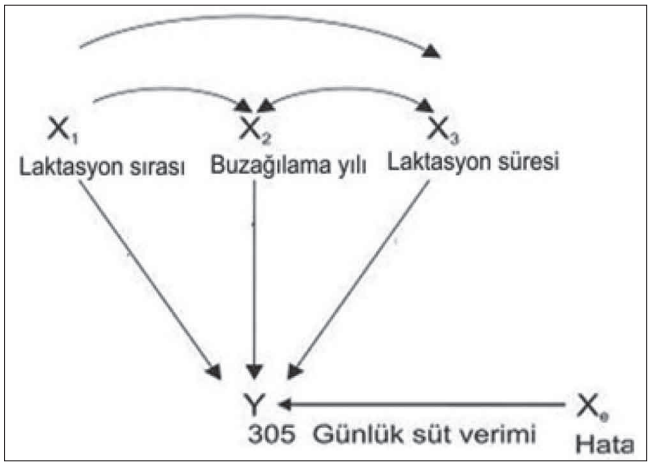
X_2 değişkeni ile X_3 değişkeni arasındaki etkileşimde de söz konusudur ($r_{23} = D + U = P_{32} + r_{12} \times P_{31}$).

S Etkisi

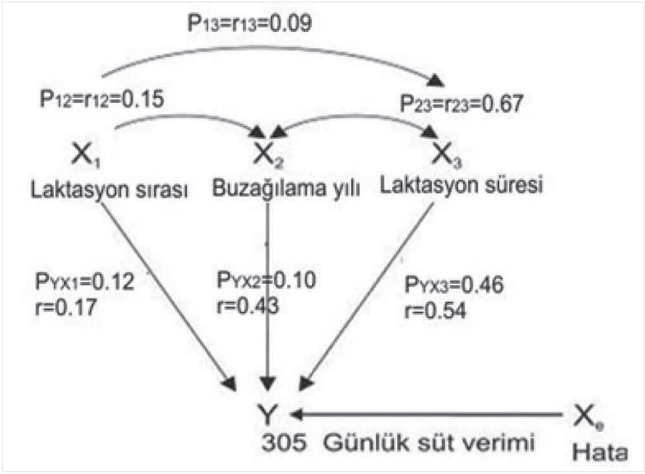
İki değişkeni etkileyen ortak bir sebep değişkeni olduğunda görülen etkiye S etkisi (S) denir [19]. *Şekil 3*'te yer alan X_2 değişkeni, X_3 değişkenini iki şekilde etkilemektedir. Birincisi, X_2 değişkeninin X_3 değişkenine yapmış olduğu doğrudan etki (P_{32}), ikincisi ise X_1 değişkeninin, X_2 ve X_3 değişkenlerinin her ikisini de etkileyen ortak bir sebep değişkeni olmasından kaynaklanan S etkisidir. *Şekil 3*'teki X_1 değişkeninin X_3 değişkeni üzerindeki doğrudan etkisi, bu değişkenler arasındaki iz katsayısına eşittir ($D = P_{31}$). X_2 değişkeninin X_3 değişkeni üzerindeki S etkisi ise; X_1 değişkeninin X_3 değişkeni üzerinde yapmış olduğu doğrudan etkiyi gösteren iz katsayısı (P_{31}) ile X_1 değişkeninin X_2 değişkeni üzerindeki doğrudan etkisini gösteren iz katsayısının (P_{21}) çarpımına eşittir ($S = P_{21} \times P_{31}$). Bu etkilerin toplamı X_2 ile X_3 arasındaki korelasyon katsayısına eşittir ($r_{23} = D + S = P_{32} + P_{21} \times P_{31}$).



Şekil 3. Değişkenler arasındaki S etkisini gösteren iz diyagramı
Fig 3. The path diagram displays S effect among variables



Şekil 4. Değişkenler arasındaki ilişkileri gösteren iz diyagramı
Fig 4. The path diagram displays relationships among variables



Şekil 5. Laktasyon sırası, buzağılama yılı ve laktasyon süresinin 305 günlük süt verimi etkisine ait iz diyagramı
Fig 5. Path diagram of parity, calving year, lactation lenght effects on 305 day milk yields

Araştırmada incelenen laktasyon sırası (X_1), buzağılama yılı (X_2), laktasyon süresi (X_3) ile 305 günlük süt verimi (Y) arasındaki ilişkileri gösteren iz diyagramı *Şekil 4*'te verilmiştir.

Şekil 5'teki iz diyagramında gösterilen iz katsayıları aşağıdaki eşitliklerle hesaplanmıştır [4,19]. Burada, P_{YX}, X bağımsız değişkeninin Y bağımlı değişken üzerinde yapmış olduğu doğrudan etkiyi gösteren iz katsayısı; b, kısmi regresyon katsayısı; S_X, X değişkenine ait standart sapma, S_Y, Y değişkenine ait standart sapmadır.

$$P_{YX} = b\frac{S_X}{S_Y} \qquad S_X = \sqrt{\frac{1}{n-1}\left(\sum\left(X-\overline{X}\right)^2\right)} \qquad S_Y = \sqrt{\frac{1}{n-1}\left(\sum\left(Y-\overline{Y}\right)^2\right)}$$

BULGULAR

Bu çalışmada incelenen laktasyon sırası, buzağılama yılı, laktasyon süresi ve 305 günlük süt verimi değişkenlerinin birbirleri arasındaki doğrudan ve dolaylı etkilerin belirlen-

Tablo 1. *Bağımlı ve Bağımsız değişkenler arasında tahminlenen korelasyonlar*
Table 1. *Estimated correlations between dependent and independent variables*

Değişkenler	Laktasyon Sırası (X_1)	Buzağılama Yılı (X_2)	Laktasyon Süresi (X_3)	305.Günlük Süt Verimi (Y)
Laktasyon sırası (X_1)	1	0.15**	0.09**	0.17**
Buzağılama yılı (X_2)	0.15**	1	0.67**	0.43**
Laktasyon süresi (X_3)	0.09**	0.67**	1	0.54**
305 günlük süt verimi (Y)	0.17**	0.43**	0.54**	1

*** Korelasyonlar 0.01 seviyesinde önemli*

Tablo 2. *Laktasyon sırası, buzağılama yılı ve laktasyon süresinin 305 günlük süt verimi üzerine regresyon analizi*
Table 2. *Regression analysis of parity, calving year and lactation length effects on 305 day milk yields*

Model	Standardize Edilmemiş Katsayılar	Standardize Edilmiş Katsayılar	t	p
	b (Standart hata)	B		
Sabit	-11.45 (95.19)		-0.12	0.90
Laktasyon sırası	105.87 (7.1)	0.12	14.84	0.00
Buzağılama yılı	0.62 (0.1)	0.10	9.56	0.00
Laktasyon süresi	14.13 (0.3)	0.46	43.69	0.00

Tablo 3. *Laktasyon sırasının buzağılama yılı ve laktasyon süresi üzerine regresyon analizi*
Table 3. *Regression analysis of parity effect on calving year and lactation length*

Model	Laktasyon Sırası (X)-Buzağılama Yılı (Y)		t	p
	Standardize Edilmemiş Katsayılar	Standardize Edilmiş Katsayılar		
	b (Standart Hata)	b		
Sabit	1942.73 (3.29)		591.29	0.00
Laktasyon sırası	22.83 (1.36)	0.15	16.85	0.00
Laktasyon sırası (X) - Laktasyon süresi (Y)				
Sabit	262.75 (0.66)		397.51	0.00
Laktasyon sırası	2.58 (0.28)	0.09	9.45	0.00

Tablo 4. *Değişkenler arası etki değerleri*
Table 4. *The values of effects among variables*

Etki Şekilleri	Etki Değerleri $P_{X/Y}$	Toplamdaki Payı (%)
Laktasyon sırasının 305 günlük süt verimine etki şekilleri		
Laktasyon sırasının doğrudan etkisi (DE) $DE = P_{Y1}$	0.12	70.59
Laktasyon sırasının buzağılama yılı üzerinden U etkisi (UE) $UE = r_{12} \times P_{2Y} = P_{21} \times P_{Y2} = 0.15 \times 0.10$	0.01	5.88
Laktasyon sırasının laktasyon süresi üzerinden S etkisi (SE) $SE = r_{13} \times P_{3Y} = P_{31} \times P_{Y3} = 0.09 \times 0.46$	0.04 +	23.53
Laktasyon sırası ile 305 günlük süt verimi arasındaki toplam korelasyon	0.17	100
Buzağılama yılının 305 günlük süt verimine etki şekilleri		
Buzağılama yılının doğrudan etkisi (DE) $DE = P_{Y2}$	0.10	23.26
Buzağılama yılının laktasyon süresi üzerinden U etkisi (UE) $UE = r_{23} \times P_{Y3} = P_{32} \times P_{Y3} = 0.67 \times 0.46$	0.31	72.09
Buzağılama yılının laktasyon sırası üzerinden S etkisi (SE) $SE = r_{12} \times P_{Y1} = P_{21} \times P_{Y1} = 0.15 \times 0.12$	0.02 +	4.65
Buzağılama yılı ile 305 günlük süt verimi arasındaki toplam korelasyon	0.43	100
Laktasyon süresinin 305 günlük süt verimine etki şekilleri		
Laktasyon süresinin doğrudan etkisi (DE) $DE = P_{Y3}$	0.46	85.19
Laktasyon süresinin buzağılama yılı üzerinden U etkisi (UE) $UE = r_{23} \times P_{Y2} = P_{32} \times P_{Y2} = 0.67 \times 0.10$	0.07	12.96
Laktasyon süresinin laktasyon sırası üzerinden S etkisi (SE) $SE = r_{13} \times P_{Y1} = P_{31} \times P_{Y1} = 0.09 \times 0.12$	0.01 +	1.85
Laktasyon süresi ile 305 günlük süt verimi arasındaki toplam korelasyon	0.54	100

mesinde kullanılacak olan korelasyonlar *Tablo 1*'de verilmiştir. Görüldüğü gibi hem 305 günlük süt verimi ile laktasyon sırası, buzağılama yılı ve laktasyon süresi arasındaki ilişkiler hem de laktasyon sırası, buzağılama yılı ve laktasyon süresinin birbirleri arasındaki ilişkiler istatistiksel olarak önemli ($P<0.01$) bulunmuştur.

305 günlük süt verimi üzerine laktasyon sırası, buzağılama yılı ve laktasyon süresinin doğrudan etkilerine ait standardize edilmiş regresyon katsayıları ise *Tablo 2*'de verilmiştir. Laktasyon sırası, buzağılama yılı ve laktasyon süresinin 305 günlük süt verimi üzerine doğrudan etkileri (sırasıyla $P_{Y1} = 0.12$, $P_{21} = 0.10$ ve $P_{31} = 0.46$) istatistiksel olarak önemli bulunmuştur ($P<0.01$). Bu bilgiler *Şekil 5* üzerinde de verilmiştir.

Laktasyon sırasının buzağılama yılı ve laktasyon süresine regresyonundan elde edilen sonuçlar *Tablo 3*'te verilmiştir. Laktasyon sırasının buzağılama yılı ve laktasyon süresi üzerindeki doğrudan etkileri (sırasıyla $P_{21} = 0.15$, $P_{31} = 0.09$) istatistiksel olarak önemli ($P<0.01$) bulunmuştur.

Diğer yandan, laktasyon sırası, buzağılama yılı ve laktasyon süresi değişkenlerinin 305 günlük süt verimine etkilerini açıklayan iz diyagramı, iz ve korelasyon katsayıları ile birlikte *Şekil 5*'te verilmiştir. *Şekil 5*'te verilen diyagramda 305 günlük süt verimi ile bağımsız değişkenler (X_1, X_2, X_3) arasındaki doğrudan, dolaylı, U ve S etkilerine ilişkin hesaplamalar ise *Tablo 4*'te toplu halde sunulmuştur.

Bu çalışmadaki laktasyon sırası değişkeninin buzağılama yılı ve laktasyon süresi üzerindeki doğrudan etkisi ($P_{21} = 0.15$, $P_{31} = 0.09$) önemlidir *(Şekil 5)*. Bunun yanı sıra, değişkenler arasında tahminlenen korelasyonlara göre en yüksek ilişkinin buzağılama yılı ile laktasyon süresi arasında olduğu (0.67) görülmektedir. Bu ilişkiyi laktasyon süresi ile 305 günlük süt verimi arasındaki ilişki (0.54) izlemektedir *(Tablo 1, Şekil 5)*.

Değişkenler arasındaki korelasyon katsayısının düzeyi, bu iki değişken arasındaki doğrudan ilişki yanında, bunlarla ilişkili olduğu başka değişkenler üzerinden dolaylı, U ve S olarak ifade edilen diğer ilişkiler toplamı ile şekillenmektedir. Nitekim bu çalışmada incelenen değişkenlerle süt verimi arasındaki doğrudan ve dolaylı ilişki düzeylerinin hesabı toplu olarak *Tablo 4*'te de görülmektedir.

Laktasyon sırası ile 305 günlük süt verimi arasındaki 0.17 düzeyindeki toplam ilişki, onu oluşturan kısımlara ayrıldığında 0.12'lik düzeyle en büyük payın laktasyon sırasının süt verimi üzerine doğrudan etkisine ait olduğu görülmektedir. Söz konusu doğrudan etkinin toplam korelasyondaki payı %70.59'dur. Laktasyon sırasının buzağılama yılı ve laktasyon süresi üzerinden 305 günlük süt verimine yapmış olduğu dolaylı etkiler ise sırasıyla 0.01 ve 0.04 olarak hesaplanmıştır. Laktasyon sırasının 305 günlük süt verimine buzağılama yılı üzerinden dolaylı etkisinin toplamdaki payı %5.88 iken, laktasyon süresi üzerinden dolaylı etkisinin payı ise %23.53 olarak belirlenmiştir *(Tablo 4)*. Benzer şekilde buzağılama yılı ile 305 günlük süt verimi arasındaki 0.43'lük ilişki, onu oluşturan bileşenlere parçalandığında buzağılama yılının 305 günlük süt verimine doğrudan etkisinin 0.10 olduğu belirlenmiştir. Bu etkinin toplamdaki payı ise %23.26 bulunmuştur. Toplam korelasyon içinde dolaylı etkiler parçalandığında, buzağılama yılının laktasyon süresi üzerinden U etkisi 0.31 (%72.09), buzağılama yılının laktasyon sırası üzerinden S etkisi ise 0.02 (%4.65) düzeyinde düşük etkili bulunmuştur *(Tablo 4)*.

Laktasyon süresi ile 305 günlük süt verimi arasındaki korelasyon 0.54 büyüklüğündedir. Bu ilişki doğrudan ve dolaylı ilişkilere ayrıldığında laktasyon süresinin 305 günlük süt verimine doğrudan etkisi 0.46 düzeyinde bulunurken, laktasyon süresinin buzağılama yılı üzerinden U ve laktasyon sırası üzerinden S etkileri ise sırasıyla 0.07 ve 0.01 düzeylerinde saptanmıştır. Laktasyon süresinin süt verimi üzerine olan bu üç etki şeklinin toplam içindeki payları sırasıyla %85.19, %12.96 ve %1.85'tir.

TARTIŞMA ve SONUÇ

Korelasyon analizi ile değişkenler arasındaki doğrudan etkilere ait katsayılara ulaşılmaktadır. Fakat değişkenler arasındaki doğrudan etkilerin yanında dolaylı etkilerin de ortaya konması önemlidir. Korelasyon katsayılarının ayrıntılarını görebilmek, değişkenlerin birbirleriyle hangi nedensel ilişkilere sahip olduğunu değerlendirebilmek ve söz konusu ilişkileri bir diyagramla açıklayabilmek için iz analizi kullanılmaktadır. Bu çalışmada iz analizi ile 305 günlük süt verimi üzerine laktasyon sırası, buzağılama yılı ve laktasyon süresi değişkenlerinin etkili olduğu belirlenmiştir. Bu sonuç İşçi ve ark.[13] tarafından sürü, buzağılama yılı ve buzağılama yaşı değişkenlerinin 305 günlük süt verimi üzerine etkilerini iz analizi kullanarak inceleyen araştırma ile uyumlu bulunmuştur. Tahtalı ve ark.[13] da laktasyon süresi, 305 günlük süt verimi, buzağılama yaşı ve buzağılama aralığının gerçek süt verimi üzerine yapmış olduğu etkileri iz analizi ile belirleyerek gerçek süt verimi ile 305 günlük süt verimi ve laktasyon süresi arasındaki korelasyon katsayılarını bu çalışmadaki gibi pozitif ve önemli bulmuştur. Orhan ve Kaşıkçı [14] ise laktasyon süt verimi üzerine laktasyon süresi, 305 günlük süt verimi, yaş, servis periyodu ve günlük ortalama süt miktarının doğrudan ve dolaylı etkilerini araştırmış, yaş ve servis periyodu dışındaki değişkenlerin laktasyon süt verimi üzerine pozitif ve önemli etkilerini saptamışlardır.

Path katsayıları verilen sonuçlar incelendiğinde, uygulanan iz analizinin değişkenler arasındaki ilişkilerin tamamını dikkate aldığı görülmektedir. Nitekim değişkenler arasında gözlenen doğrudan, dolaylı, U ve S etkilerinin toplamının değişkenler arasındaki korelasyona eşit olduğu saptanmıştır. Yine doğrudan etki değerleri incelendiğinde Siyah Alacalarda 305 günlük süt veriminin en çok (%85.19)

laktasyon süresinden etkilendiği görülmektedir. Orhan ve Kaşıkçı [14] ve Tahtalı ve ark.[13] tarafından laktasyon süresi sırasıyla laktasyon süt verimi ve gerçek süt verimine de en çok etkili bulunmuştur. Bunun yanı sıra, 305 günlük süt verimine laktasyon sırasının etkisi ikinci sırada büyük bulunmuştur (%70.59). 305 günlük süt verimine buzağılama yılının etkisi ise düşüktür (%23.26).

Sonuç olarak, çok sayıda değişkenlerle çalışıldığında incelenen değişkenler arası ilişkilerin sadece korelasyon katsayıları üzerinden değerlendirilmesi yeterli olmayabilir. Söz konusu ilişkilerin iz analizi gibi teknikler kullanılarak doğrudan ve dolaylı etkiler biçiminde detaylı ele alınması daha yararlı sonuçlar verebilecektir. Bu tip ayrıntı içeren sonuçlar uygulamada pratik kullanımlar sağlayabilir. Örneğin hayvan ıslahında ele alınan özellikler arasındaki doğrudan ve dolaylı ilişkilerin biliniyor olması, dolaylı seleksiyon gibi yöntemlerin uygulanmasına zemin oluşturacaktır. Nitekim bu çalışmada ele alınan Siyah Alaca ırkı ineklerin 305 günlük süt verimlerini artırmaya yönelik yapılacak bir seleksiyon çalışmasında laktasyon süresi ve buzağılama yılının seleksiyon kriterleri olarak ele alınmaları fayda sağlayacaktır.

KAYNAKLAR

1. Bal C, Doğan N, Doğan İ: Path analizi ve bir uygulama. *5. Ulusal Biyoistatistik Kongresi*, 13-15 Eylül, Eskişehir, Osmangazi Üniversitesi Basımevi, s.376, 2000.

2. Brannick MT: Path analysis. http://luna.cas.usf.edu/~mbrannic/files/regression/Pathan.html, *Accessed:* 21.10.2013.

3. Ender P: Path analysis. http://www.gseis.ucla.edu/courses/ed230bc1/notes2/path1.html, *Attached:* 11.02.2007.

4. İkiz F, Şengonca H: Path analizi. *Ege Üniv Elektronik Hesap Bil Enst Derg*, 1 (1): 1-17, 1978.

5. Li CC: Path Analysis: A Primer. The Boxwood Press, 346, Pacific Grove, California, 1975.

6. Düzgüneş O, Eliçin A, Akman N: Hayvan Islahı. III. Baskı, *Ankara Üniv. Ziraat Fakültesi Yayınları*, No: 1437, Ders Kitabı: 419, s.197, Ankara, 1996.

7. Dewey DR, Lu KH: A correlation and path analysis of crested wheat grassseed production. *Agronomy J,* 51, 515-518, 1959. DOI: 10.2134/agronj1959.00021962005100090002x

8. Efe E, Bek Y: Path katsayıları analizi ve çözüm için bir makro. *Tarımda Bilgisayar Uygulamaları Sempozyumu,* 3-7 Ekim, İzmir, *Bildiriler Kitabı*, s.35-43, 1994.

9. Kaygısız Z, Saraçlı S, Dokuzlar KU: İllerin gelişmişlik düzeyini etkileyen faktörlerin path analizi ve kümeleme analizi ile incelenmesi. *VII. Ulusal Ekonometri ve İstatistik Sempozyumu*, İstanbul Üniv. İktisat Fakültesi, 26-27 Mayıs, İstanbul, 2005.

10. Pedhazur EJ: Multipleregression in Behavioral Research: Explanation and Prediction. 3rd ed., Forth Worth, Harcourt Brace College Publishers, New York, 1997.

11. Perochon L, Coulon JB, Lescourret F: Modelling lactation curves of dairy cows with emphasis on individual variability. *J Anim Sci,* 63, 189-200, 1996. DOI: 10.1017/S1357729800014740

12. Aktürk D, Bayramoğlu Z, Savran F, Tatlıdil FF: The factors affecting milk production and milk production cost: Çanakkale case-Biga. *Kafkas Univ Vet Fak Derg,* 16 (2): 329-335, 2010.

13. İşçi Ö, Takma Ç, Akbaş Y: Siyah alaca sığırlarda 305 günlük süt verimi üzerine etkili faktörlerin path analizi ile incelenmesi. *4. Ulusal Zootekni Bilim Kongresi*, Süleyman Demirel Üniversitesi, Ziraat Fakültesi, 1-4 Eylül, Isparta, 2004.

14. Tahtalı Y, Şahin A, Ulutaş Z, Şirin E, Abacı SH: Esmer ırkı sığırlarda süt verimi üzerine etkili faktörlerin path analizi ile belirlenmesi. *Kafkas Univ Vet Fak Derg,* 17 (5): 859-864, 2011.

15. Orhan H, Kaşıkçı D: Path, korelasyon ve kısmi regresyon katsayılarının karşılaştırılmalı olarak incelenmesi. *Hayvansal Üretim,* 43 (2): 68-78, 2002.

16. SPSS: Inc. Released, SPSS for Windows, Version 16.0. Chicago, SPSS Inc., 2007.

17. Stephen L: PSY6003 advanced statistics: Multivariate analysis II:Manifest variables analyses topic 3: Path analysis. http://people.exeter.ac.uk/SEGLea/multvar2/pathanal.html, *Accessed:* 12.08.2014.

18. Şahinler S, Görgülü Ö: Path analizi ve bir uygulama. *Mustafa Kemal Üniv Ziraat Fak Derg*, 5 (1-2): 87-102, 2000.

19. Turan ZM: Regresyon Analizleri. *Lisansüstü Eğitimi Ders Notları* (Yayınlanmamış), Bursa, 2000.

Kafkas Univ Vet Fak Derg
21 (2): 225-228, 2015
DOI: 10.9775/kvfd.2014.12083

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Effect of Omega-3 Resource on Glucose and Total Protein in Ostriches

Mahdi KHODAEI MOTLAGH [1]

[1] Department of Animal Science, Faculty of Agriculture and Natural Resources, Arak University, Arak 38156-8-8349, IRAN

Article Code: KVFD-2014-12083 Received: 04.06.2014 Accepted: 22.09.2014 Published Online: 30.09.2014

Abstract

The purpose of the study was to evaluate the effectiveness of the canola oil on the some metabolites ostriches. In order to study the metabolic profile of ostriches in relation to diet, Six blue-neck male ostriches (Struthio camelus) were fed omega-3 resource (canola oil =3%) throughout a 60-day experiment. Blood samples were collected from ostriches on days 0 and 60 of the experiment to measure levels of total serum protein, albumin, total immunoglobulin, cholesterol, the activity of ASP, ALT, insulin and glucose. The results showed that from days 0 to 60 of the experiment, glucose and total protein levels increased significantly (P<0.05). whereas total immunoglobulins insulin, albumin, ALT and AST did not change.

Keywords: *Ostrich, Omega-3 resource, Glucose, Total protein*

Devekuşlarında Omega 3 Kaynağının Glukoz ve Total Protein Üzerine Etkisi

Özet

Bu çalışmanın amacı devekuşlarında kanola yağının bazı metabolic değerler üzerine etkisini araştırmaktır. Diyetle ilişkili olarak devekuşlarında metabolik değerleri araştırmak amacıyla altı adet Mavi-boyunlu devekuşu (Struthio camelus) 60 günlük çalışma periyodu süresince omega-3 kaynağı (%3'lük kanola yağı) ile beslendi. Devekuşlarından çalışmanın 0 ve 60. günlerinde total serum protein, albümin, total immunglobulin, kolesterol, ASP, ALT, insülin ve glukoz seviyelerini tespit etmek amacıyla kan örnekleri alındı. Çalışmanın sonuçları 0. ile 60. günler arasında glukoz ve total protein seviyelerinin arttığını (P<0.05) buna karşın total immunglobulin, insülin, albümin, ALT ve AST seviyelerinin ise değişmediğini ortaya koymuştur.

Anahtar sözcükler: *Devekuşu, Omega-3 kaynağı, Glukoz, Total protein*

INTRODUCTION

The ostrich industry in South Africa dates back to the 1860's when ostriches were domesticated in the Klein Karoo, Western Cape [1]. The industry was initially focused on the feathers and hides for the fashion industry. Over the last decade however focus has shifted and is now aimed at further developing and sustaining the meat production sector of the South African ostrich industry [2].

It is well recognized that a direct relationship exists between a high intake of fat in the diet, particularly saturated and the so-called 'diseases of the western world, with the latter predominantly being characterized by an increased incidence of heart disease [3]. A higher PUFA content of meat the higher meat's nutritive quality, which has a direct effect on human health, for example, the long-chain n-3 PUFA docosahexaenoic acid (DHA) is reported to contribute to brain and liver development in human infants and may also play a role in the prevention and treatment of various diseases [4].

Ostrich meat is gaining in popularity worldwide due to its high nutritive value [5,6]. In recent years ostriches *(Struthio camelus)* have been increasingly farmed in Europe with more than 40 000 head in Italy alone [7]. Ostrich industries have recently been developing in many countries around the world, especially for the production of meat due to its favorable fatty acid profile and low fat content as compared with other kinds of red meat [8]. Commercial breeding of farmed ostriches in Iran was started from 1999 [9] and at present ostrich farms are distributed in all

İletişim (Correspondence)
+98 863 2761007
m-motlagh@araku.ac.ir

regions and climates of the country, especially in the central regions. Blood profiling, initially used to detect subclinical disorders due to incorrect feeding, has recently been used more widely to evaluate the effects of different treatments on metabolic, nutritional and welfare conditions of animals [10]. Use of fats in animal feed has many benefits. Some of these benefits are increase in energy level and palatability of diet, improvement of growth rate, feed efficiency, absorption of fat soluble vitamin and decrease of metabolic heat production during heat stress. Dietary fats are also source of essential fatty acids [11]. Various studies have been conducted on the inclusion of fish oil or fish meal in the diet of broilers [12], pigs [13] and beef [14] in order to try and manipulate the lipid composition of the meat. It is known that the fatty acid composition of animal products is the result not only of biosynthesis in tissue, but also the fatty acid composition of ingested lipids [15], being stronger in monogastrics, where stored lipids in adipose tissue not only reflect ingested lipids [16]. Canola oil has a high content of α-linolenic acid, which is susceptible to oxidation, and has very rich source of monounsaturated oleic acid, it contains considerable amounts of linoleic (LA) and alpha-linoleic (ALA) acids, the precursors of omega-6 and omega-3 fatty acids and is poor of saturated fatty acid [15]. There is no report to show an academic research to evaluate the effects of canola oil on ostriches. To our knowledge, this is the first study of effects of canola oil on plasma biochemical parameters of ostriches. The purpose of the study was to evaluate the effectiveness of the canola oil on the health of ostriches.

Table 1. *Ingredients composition of the diet*
Tablo 1. *Diyet içeriğinin kompozisyonu*

Ingredients	(%)
Corn, Grain	37.4003
Alfalfa Meal-20	28.0502
Soybean Meal-44	27.5827
Dical. Phos	2.3004
Canola Oil	3
Sunflower Oil	0
Limestone	0.6924
Common Salt	0.3101
Vitamin Premix	0.2338
Mineral Premix	0.2338
DL-Methionine	0.1314
L-Lysine HCl	0.0636

Table 2. *The chemical composition of d ration*
Tablo 2. *Rasyonun kimyasal kompozisyonu*

Chemical Composition	Content
E	2600 Kcal/kg
CP (%)	22
EE (%)	5/65
CF (%)	10/69
Lysin(%)	1/19
Met + Cys(%)	0/79
Ca (%)	1/30
P (%)	0/52
Na(%)	0/18

MATERIAL and METHODS

Animals and Diet

Field work was conducted from November to January 2013 at the farming and animal husbandry station near Arak, Markazi province, Iran. Six birds of ostrichs, approximately 4 months of age were used in this investigation. The experiment was approved by the animal group committee of the Agriculture faculty of Arak University (Approval No: 2013-07/02 -366). The ostriches received increasing amounts of supplementary feed *(Table 1*, *Table 2)* that contained 3% unrefined canola oil and for period of around 60 days. This supplementary diet had a calculated energy value of 2.600 kcal/kg and a protein content of 22%. All the supplementary feed was consumed by the birds. The feed was mixed on a weekly basis and stored in bags in a cold feed storage shed. No anti-oxidants were added to diets. Water was provided *ad libitum*. They were kept in an enclosure with 1.000 m^2 of open space and 100 m^2 of covered space.

Plasma Biochemical Analyses

Blood samples for the determination of some parameters were obtained between 8 and 9 am, to avoid diurnal influences, following about a 12-h fasting by wing vein into vacutainers with heparin. The plasma was prepared by centrifugation at 2.500×g for 15 min. plasma were stored at −21°C until analysis. The biochemical parameters were measured using a standard autoanalyser (Hitachi 717, Boehringer. Mannheim, Germany). The level of total protein by Biuret reaction, albumin by Bromocresol green dye binding method, and the activity of AST and ALT was measured by the colorimetric method of Reitman and Frankel [17]. All results of enzyme activities were expressed in international units per liter written as IU/l [18].

Statistical Analysis

All results are expressed as means ±standard error of mean (SEM). Raw data were checked for normal distribution using Kolmogorov-Smirnov method. All analyses utilized parametric statistical methods. A value was considered to be statistically significant if the associated P value was less than 0.05. Paired t tests were performed using SAS software (2001).

RESULT

The effects of Omega-3 resource feeding on weight change indicated on *Table 3*. All ostrich chicks exhibited proper growth and active ingestion during the feeding period. Initial weight and final weight indicated the weight on 0 and 60 days respectively. At two month gain for six ostrich chicks were 93.900 kg. Total feed intake at this period was 743 kg. Average daily gain (ADG) was 260 g/day and feed conservation ratio (FCR) was 7.913 kg in this study.

The effects of Omega-3 resource ingestion on plasma biochemical variables *(Table 4)* are reported. There was no significant difference in total immunoglobulins, insulin, albumin, ALT and AST at two times. But from days 0 to 60 total protein levels and glucose increased significantly ($P \leq 0.05$).

DISCUSSION

Results indicated that ADG and FCR in this study was agreement with others. Trebušak et al.[19] indicated that body weight gain was not influence while feed intake was decreased and feed efficiency was improved when the rabbits fed on diets content linseed oil. Body weight gain was increased in this study Studies in the US [20] on ostriches reported that grew at only 180 g/day with a FCR of 8.7 compared with this study was low. Final body weight of sunflower oil groups (poultry) were increased [21].

Average feed intake per day, water consumption per day, feed conversion ratio (FCR) and weight gained were different from that estimated by Mushi et al.[22]. Also, Kreibich and Sommer [23] explained that feed conversion ranged from 1.4:1 to 1.6:1 for younger birds 4 to 6 months old, while for older birds ranging from 4:1 to 6:1. Studies [24,25] showed that increasing dietary energy or fat supplementing decreased feed intake and improved Feed Conversion Ratio (FCR) of broiler chicks [25].

Findings of this study showed that feeding of Canola oil in growing ostriches can significantly increase plasma levels of glucose and total protein in the 60th day, compared to that of day 0 *(Table 4)*. Total proteins play an important role in transport of vitamins, hormones, enzymes and electrolytes. In our study, total protein values increased on day 60. This accords with earlier observations, which showed that total protein values increased with age in male ostriches until 24-36 months [26]. Total protein values also tend to increase with age in emus, masai ostriches, and broilers [27]. The protein content of the feed may raise total proteins in ostriches. In our study, total immunoglobulin values did not change in day 60.

Enzyme activities in birds are variable and originate from different organs. There were no significant differences between albumin, AST, and ALT. These findings may indicate that canola oil supplementation to feed had effect on the health of ostriches during the experiment. The major finding of current study was a significant increased on glucose along with a significant rise in total protein concentration. This finding may reflect the beneficial effects of canola oil on health. This research will serve as a base for future studies on the effects of types of oils on health. A limitation of this study is that the numbers of ostriches were relatively small. More broadly, research also needed to determine where the effects of canola oil on other metabolit profile content of ostrich muscle, liver and plasma.

It was concluded, dietary supplementation with 3% canola oil can increase body weight and FCR in ostrich.

Table 3. *Effects of omega-3 resource feeding on weight gain at two months period*
Tablo 3. *İki aylık süreçte omega-3 kaynağı kullanımının kilo kazanımı üzerine etkisi*

Animal	Initial Weight (kg)	Final Weigh (kg)	Weight Gain (kg)	ADG (g)	FCR (kg)
Ostrich	258.1	352	93.900	260	7.913

Table 4. *Comparison between two blood sampling (day 0–60) for some plasma parameters, results are expressed as mean ± SEM (n=6)*
Tablo 4. *Bazı kan parametrelerinin iki kan örneklemesi(0 -60 gün)arasındaki karşılaştırması Sonuçlar ortalama±SEM (n=6)*

Variable	Unit of Measurement	Day 0	Day 60	P-Value	
Insulin	Mg/dl	11.98±2.39	12.48±1.44	0.28	NS
Glucose	Mg/dl	108.80±18.90	138.10±17.60	0.05	*
ALT	IU/l	38.20±2.81	37.89±2.46	0.24	NS
AST	IU/l	30.32±6.20	30.32±4.81	0.99	NS
Total protein	Mg/dl	4.31±0.25	4.95±0.40	0.05	*
Albumin	Mg/dl	4.22±0.92	4.80±0.70	0.17	NS
Total immunoglobulin	Mg/dl	0.84±0.49	0.83±0.35	0.95	NS

*AST (aspartate amino transferase), ALT (alanine amino transferase), NS not significant (P>0.05) and *P≤0.05*

In other hand omega-3resource increased total protein and glucose level on plasma.

Acknowledgment

The authors gratefully acknowledge the financial support for this study that was provided by the Arak University. We are also grateful to Mr. Ali Hasani and Mr. Sepehr nematpour for all their helps during the field work.

REFERENCES

1. Smit DJ: Ostrich farming in the Little Karoo. *Bull Dep Agric Tech,* 358, 1963.

2. Cooper RG, Horbańczuk JO: Anatomical and physiological characteristics of ostrich (*Struthio camelus* var. *domesticus*) meat determine its nutritional importance for man. *Anim Sci J,* 73, 167-173, 2002. DOI: 10.1046/j.1344-3941.2002.00024.x

3. Scollan ND, Choi NJ, Kurt E, Fisher AV, Enser M, Wood JD: Manipulating the fatty acid composition of muscle and adipose tissue in beef cattle. *Br J Nut,* 85, 115-124, 2001. DOI: 10.1079/BJN2000223

4. Zhang W, Xiao S, Samaraweera H, Lee EJ, Ahn DU: Improving functional value of meat products. *Meat Sci,* 86, 15-31, 2010. DOI: 10.1016/j.meatsci.2010.04.018

5. Sales J, Horbanczuk J: Ratite meat. *World's Poult Sci* J, 54, 59-67, 1998. DOI: 10.1079/WPS19980005

6. Sales J, Horbańczuk JO, Dingle J, Coleman R, Sensik S: Carcass characteristics of emus *(Dromaius novaehollandiae)*. *Br Poult Sci,* 40, 145-147, 1999. DOI: 10.1016/j.meatsci.2014.05.027

7. ISTAT: La consistenza degli allevamenti di struzzi in Italia. Statistiche in breve, www.istat.it. 2000.

8. Zotte DA, Brand TS, Hoffman LC, Schoon K, Cullere M, Swart R: Effect of cottonseed oilcake inclusion on ostrich growth performance and meat chemical composition. *Meat Sci,* 93, 194-200, 2012. DOI: 10.1016/j.meatsci.2012.08.027

9. Hajibabaei A, Mosavi SM, Vahedi H: Ostrich breeding in Iran. *Proceedings of the 3rd International Ratite Science Symposium of the World's Poultry Science Association (WPSA),* Madrid, Spain, 14th-16th October, 359-361, 2005.

10. Bertoni G, Piccioli Cappelli F, Bald, A, Borghese A, Duranti E, Falasachini A, Formigoni A, Grasso F, Lacetera N, Lupi P, Meluzzi A, Pinna W, Rosi F, Stefanon B, Zicarelli L, Bernabucci U, Campanile G, Moniello G, Trombetta MF: Interpretation of metabolic profiles in farming animals. *Progr Nutr*, 2, 51-76, 2000.

11. Baião NC, Lara LJC: Oil and fat in broiler nutrition. *Rev Bras Cienc Avic,* 7 (3): 129-141, 2005. DOI: 10.1590/S1516-635X2005000300001

12. Herstad O, Øverland M, Haug A, Skrede A, Thomassen MS, Egaas E: Reproductive performance of broiler breeder hens fed n-3 fatty acid-rich fish oil. *Acta Agric Scand,* 50, 121-128, 2000. DOI:10.1080/09064700412331312331

13. Leskanich CO, Matthews KR, Warkup CC: The effect of dietary oil containing (n_3) fatty acids on the fatty acid, physicochemical, and organoleptic characteristics of pig meat and fat. *J Anim Sci,* 75, 673-683, 1996.

14. Choi NJ, Enser M, Wood JD, Scollan ND: Effect of breed on the deposition in beef muscle and adipose tissue of dietary n 3 poly-unsaturated fatty acids. *Anim Sci,* 71, 509-519, 2000.

15. Mourot J, Hermier D: Lipids in monogastric animal meat. *Reprod Nutr Dev,* 42, 109-118, 2001.

16. Chilliard Y, Bauch D, Lessire M, Schmidely P, Mourot J: Qualité des produits: modulation par l'alimentation des animaux la composition en acides gras du lait et de la viande. *INRA Prod Anim,* 21, 95-106, 2008.

17. Mansour MM, Farid Z, Bassily S, Salah LH, Watten RH: Serum enzyme tests in hepatosplenic schistosomiasis. *Trans Royal Soci Tropic Med Hyg,* 76 (1): 109-111, 1982.

18. Burtis CA, Ashwood ER: Textbook of Clinical Chemistry. 2nd ed., 735-888, Saunders, Philadelphia, 1994.

19. Trebušak T, Levart A, Voljč M, Tomažin U, Pirman T: The effect of linseed oil supplementation on performance, fatty acid composition and oxidative stat us of rabbits. *Acta Argic Slovenica,* 98, 119-125, 2011. DOI: 10.2478/v10014-011-0028-2

20. Baltmanis MS, Blue-McLendon AB, Angel R: The effect of feeding a lay supplement diet versus a complete-pelleted diet on the performance of growing ostriches. *Am Ostrich Res,* 20-24, 1997.

21. Karakaş Oğuz F, Numan Oğuz M, Büyükoğlu T: The effects of using chicken drippings oil instead of the sunflower oil on performance, blood parameters, cholesterol and fatty acid composition of egg yolk in laying Japanese quail *(Coturnix coturnix japonica)*. *Kafkas Univ Vet Fak Derg,* 18 (6): 945-950, 2012.

22. Mushi EZ, Binta MG, Chaba RG, Lsa JF, Modisa L: Serum biochemical values of farmed ostrich *(Struthio camelus)* in Botswana, onderstepoort. *J Vet Res,* 65 (3): 189-193, 1998.

23. Kreibich A, Sommer M: Ostrich Farm Management. pp.450, Landwirtschaftsver GmbH, Munster-Hilt up, 1995.

24. Harms RH, Russell GB, Sloan DR: Performance of four strains of commercial layers with major changes in dietary energy. *J Appl Poult Res,* 9, 535-541, 2000. DOI: 10.1093/japr/9.4.535

25. Bryant M, Wu G, Roland DA: Optimizing dietary energy for profits and performance of two strains of white leghorns. *International Poultry Scientific Forum Abstracts,* Atlanta, GA, USA, pp.23, 2005.

26. Quintavalla F, Bigliardi E, Bertoni P: Blood biochemical baseline values in the ostrich *(Struthio camelus). Annali Facoltà Med Vet,* 21, 61-71, 2001.

27. Samour J, Naldo J, Libanan N, Rahman H, Sakkir M: Age-related hematology and plasma chemistry changes in captive masai ostriches *(Struthio camelus massaicus)*. *Comp Clin Pathol,* 20 (6): 659-667, 2010.

Kafkas Univ Vet Fak Derg
21 (2): 229-232, 2015
DOI: 10.9775/kvfd.2014.12116

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Isolation and Identification of an Exopolysaccharide Producer *Streptococcus thermophilus* Strain from Turkish Yogurt

Enes DERTLİ [1]

[1] Department of Food Engineering, Faculty of Engineering, Bayburt University, TR-69000 Bayburt - TURKEY

Article Code: KVFD-2014-12116 Received: 05.08.2014 Accepted: 30.10.2014 Published Online: 07.11.2014

Abstract

Production of exopolysaccharides (EPS) by starter Lactic acid bacteria (LAB) gained special interest due to technological role of these natural polymers. Yogurt samples were collected from different households in Turkey and bacterial strains were isolated and evaluated for EPS production. Strains potentially producing EPS were then identified to species level with 16S rRNA sequencing. An EPS producer *Streptecoccus thermophilus* strain was identified from yogurt samples. EPS was extracted from culture supernatants of *S. thermophilus* and partially purified and structural analysis of the crude EPS by FTIR spectroscopy revealed the presence of typical functional groups related to exopolysaccharides. This study explains the identification of a potential starter culture for yogurt production with EPS production.

Keywords: *Exopolysaccharides, Streptococcus thermophilus, yogurt, 16S rRNA, FTIR*

Yoğurttan Ekzopolisakkarit Üreten *Streptococcus thermophilus* Suşunun İzolasyonu ve Tanımlanması

Özet

Starter Laktik Asit Bakterileri (LAB) tarafından ekzopolisakkarit (EPS) üretimi bu doğal polimerlerin teknolojik rollerinden dolayı son yıllarda önem kazanmıştır. Bu çalışmada yoğurt örnekleri yerel kaynaklardan toplanmış, bakteriler izole edilmiş ve bu türlerin EPS üretimi test edilmiştir. EPS üretim potansiyeli gösteren suşlar 16S rRNA sekanslama tekniği ile suş seviyesine kadar tanımlanmıştır. Bu çalışmalar neticesinde EPS üretimi gösteren bir *Streptecoccus thermophilus* suşu tanımlanmıştır. EPS *S. thermophilus*'un kültür süpernatantından ekstrakte edilmiş, kısmi olarak saflaştırılmış ve FTIR spektreskopi ile gerçekleştirilen yapısal analiz ekstraktın ekzopolisakkaritler ile alakalı tipik fonksiyonel grupları içerdiğini göstermiştir. Bu çalışma yoğurt üretimi için EPS üretme kabiliyetinde olan potansiyel bir starter kültürün tanımlanmasını açıklamaktadır.

Anahtar sözcükler: *Ekzopolisakkaritler, Streptococcus thermophilus, Yoğurt, 16S rRNA, FTIR*

INTRODUCTION

Several lactic acid bacteria (LAB) strains, generally used in fermentation processes as starter cultures, are capable of producing exopolysaccharides (EPS) that can either form a capsule as an outer layer or directly being secreted to the environment [1]. The structure of EPS produced by LAB strains has a wide diversity depending on the sugar monomers that EPS are composed of and those containing only one type of sugar monomer are described as homo-polysaccharides and those containing two or more sugar molecules are described as heteropolysaccharides [2]. EPS of LAB have several ecological functions such as playing important roles on colonization, adhesion, stress resistance, host-bacteria interactions and immunomulation [2]. But EPS are also of special interest in food industry due to their technological roles and their GRAS (generally recognised as safe) status [3].

Yogurt is one of the most consumed fermented milk products worldwide and production of healthier yogurt with less food additives sustaining its technological properties is an important issue matching with consumer's demands. For this reason the use of EPS producing ropy starter cultures in yogurt production is a common practice in order to replace food additives with an improved yogurt viscosity and *in situ* EPS production during yogurt fermentation may also result in a smooth and creamy texture in the final product [4]. Importantly it was shown that these positive effects in technological properties of yogurt is more pronounced when EPS was formed *in situ* rather than when added as an additive [5]. Thus, finding

İletişim (Correspondence)
+90 458 2111153/3225
edertli@bayburt.edu.tr

new EPS producer yogurt starter cultures is crucial in order to develop more desired products as EPS structure, production level of different LAB strains are different and directly affects the functions of EPS [1].

The aim of this study was to isolate and identify an EPS producer yogurt starter culture from Turkish yogurt collected from different households and investigate the EPS structure produced by this potential starter culture.

MATERIAL and METHODS

Isolation of LAB from Yogurt Samples

Yogurt samples (n=8) were randomly collected from four different households in Düzce province of Turkey and samples were stored at 4°C and analysed within 48 h as previously described [6]. Basically, 10 g of yogurt samples were taken aseptically and transferred to separate sterile bags. In order to homogenise yogurt samples, 90 ml of sterile saline solution (0.85%, pH: 7.0) were added to each sample. Serial dilutions were prepared and aliquots of these dilutions were plated to MRS (de Man, Rogosa and Sharpe) and BHI (Brain-heart infusion) agars and incubated at 37 and 42°C for 48 h. Colonies with typical slimy characteristics were randomly selected from MRS and BHI agar plates and tested for Gram stain, cell morphology and catalase reaction as described elsewhere [6]. Potential LAB were selected from these colonies and further investigated for their genotypic identification.

Bacterial Identification by 16S RNA Sequencing

Seven slimy colonies were selected as potential EPS producer LAB cultures using the previously described method [7] and grown in MRS broth and further inoculated to MRS agar to have single pure fresh colonies. Single bacterial colonies were resuspended in 10 ml sterile H_2O and 1 ml aliquots were taken from these suspensions as a DNA template in a PCR reaction that contained 1 µl DNA template, 10 µl 5× PCR buffer for Taq polymerase (Go Taq, Promega), 0.4 µl dNTPs (Bioline), 1 µl of 20 mM primers AMP_F (5'- GAGAGT TTGATYCTGGCTCAG - 3') and AMP_R (5'-AAGGAGGTGATCCARCCGCA - 3') [8], 0.25 µl 5U Taq polymerase and up to 50 µl of sterile H_2O in order to amplify complete 16S rDNA with a final product of c. 1.5 kb. PCR was performed using a thermocycler (Biometra) with the following programme: 95°C for 2 min, 20 cycles of 95°C for 30 s, 55°C for 20 s, and 72°C for 30 s and 72°C for 5 min final extension. PCR products were run on a gel to check the amplication and amplicons were further purified using SureClean kit (Bioline). Sequencing reactions were prepared using primers AMP_F/AMP_R at 1.6 µM concentrations and the ABI Prism BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems) according to the manufacturer's protocol. Sequences obtained were interrogated by using Ribosomal Database Project II [9] and the identities of the isolates were determined on the basis of more than 0.98 matching score.

Isolation of Exopolysaccharides

Exopolysaccharides (EPS) were isolated from bacterial strain using the method described previously [10]. Briefly identified *Streptecoccus thermophilus* strain was grown in 500 ml MRS culture, inoculated at 1% (v/v) with an overnight culture then incubated at 37°C for 2 d. The bacterial supernatant was collected after centrifugation at 6.000 × g for 30 min at 4°C and an equal volume of chilled ethanol was added to the supernatant to precipitate bacterial EPS and stored at 4°C overnight. Sample was centrifuged at 10.000 × g for 30 min at 4°C and the pellet of the precipitates was retained. The sample was resuspended in H_2O with gentle heating (50°C) and EPS was recovered by precipitation upon the addition of 2 volumes of chilled ethanol. After centrifugation at 10.000× g for 30 min at 4°C the resulting EPS was resuspended in distilled H_2O with gentle heating (less than 50°C) followed by dialysis for 72 h (12.000-14.000-Da dialysis membrane) at 4°C, with two changes of H_2O per day. The contents of the dialysis tubing were freeze-dried to provide EPS. This was further purified by dissolving in 10% TCA and stirring overnight. The precipitated protein was removed by centrifugation at 10.000 × g for 15 min at 4°C. The pH of the supernatant was adjusted to 7 with 1 M NaOH and EPS was precipitated again with 2 volumes of chilled ethanol. The pellet was dissolved in distilled water and then lyophilized by freeze drying. The EPS samples were stored at 4°C for further analysis.

FTIR Spectroscopy Analysis of EPS

Fourier transform infrared (FTIR) spectra of the pure capsular EPS isolated from wild type and mutants cell pellets were measured with a FTS 175C Digilab FT-IR spectrometer (Bio-Rad, US) equipped with a MCT detector and a single-reflection diamond ATR sampling accessory (GoldenGate, Specac). The spectra were recorded in the region of 4000-800 cm^{-1} with 128 scans at 4 cm^{-1} resolution and processed by the spectrometer software. The finger-print region of 800-1800 cm^{-1} spectra of EPS samples were analysed in detail.

RESULTS

Identification of EPS Producer LAB Strain Isolated from Traditional Yogurt

Yogurt samples that were collected for this study were produced with traditional methods and it should be noted that bacterial populations from four yogurt samples were low and ranged between 10^5 - 10^6 CFU/ml suggesting the traditional yogurt production conditions were not that appropriate. A total of seven bacterial isolates from thirty isolates were selected as potential EPS producing

LAB strains after morphological and chemical tests and subjected to 16S rRNA sequencing and as can be seen in *Fig. 1*, the 1.5 kb region of 16S gene from each isolate were successfully amplified. From this seven isolates three strains were identified as *Streptecoccus thermophilus* as can be expected and three strains were identified as *Staphylococcus hominis* and one strain were matched with an uncultured bacterium (data not shown). Isolation of *S. hominis* as a slimy colony from yogurt was not that surprising as this strain was isolated from traditionally fermented milk in South Africa and its EPS production for the first time was reported recently [11]. *S. hominis* was not our target strain in this study but it should be noted that this strain could preserve its ropiness for a period of cell transfers [11] which is not always the case for the ropy LAB as genetic biosynthesis mechanism of EPS production is not that stable. After isolation of EPS from the new *S. thermophilus* strain structural analysis has been conducted in order to confirm the polymer as EPS.

Structural Analysis of EPS by FTIR Analysis

In this study structural and functional groups of the crude EPS were determined by FTIR analysis *(Fig. 2)*. The EPS sample showed a wide absorption peak around 3200-3400 cm^{-1}, indicating typical hydroxyl groups (O-H) of polysaccharides suggesting that the analysed sample is a polysaccharide [12]. The peak from 2800 to 2950 cm^{-1} showed a weak C-H stretching frequency for exopolysaccharide of *S. thermophiles* ED1 [13]. The region around 1500-1600 cm^{-1} did not show the intense peak which is assigned to N-H bending and C-N stretching in proteins [14] *(Fig. 2)*. The amide C=O stretching and carboxyl groups were detected from the corresponding peak at 1600-1700 cm^{-1}. The EPS sample showed an intense peak around 1000 cm^{-1} which

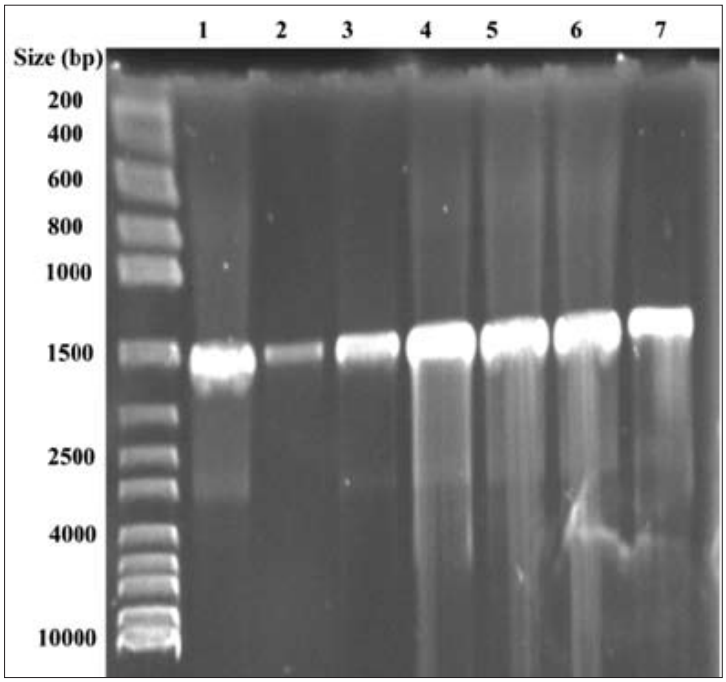
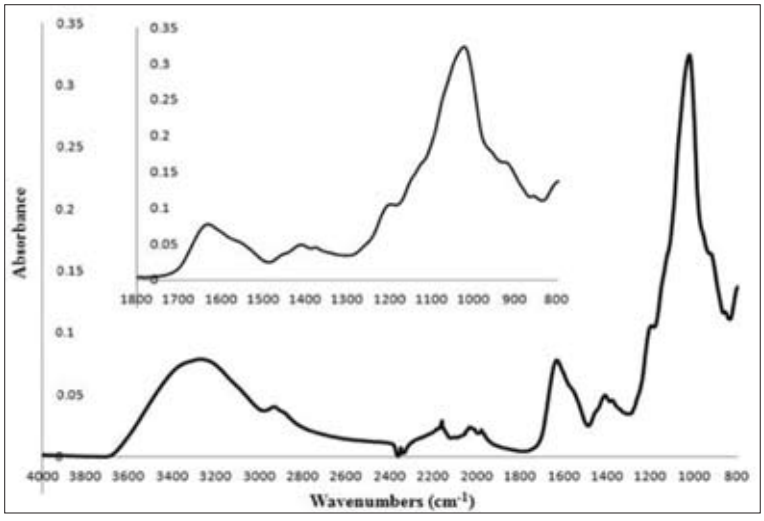


Fig 1. Agarose gel (0.8%) showing the amplication of 16S rRNA genes from seven yogurt isolates

Şekil 1. 16S rRNA geninin 7 yogurt izolatında çoğaltılarak elde edilmesini gösteren agaroz (%0.8) jeli

Fig 2. FTIR spectra of EPS isolated from yogurt isolate *S. thermophilus* strain. The inner figure represents the spectra of the fingerprint region

Şekil 2. Yoğurt izolatı *S. thermophilus* suşundan izole edilen EPS'in FTIR analiz görüntüsü. İçteki şekil parmak izi bölgesinin analiz görüntüsünü vermektedir



indicated the characteristic C-O bond of polysaccharides [13]. Overall FTIR spectrum confirmed the EPS production of yogurt isolate *S. thermophilus* strain.

DISCUSSION

In this study a new *S. thermophilus* strain is identified with EPS production ability as a potential starter culture in yogurt production. It was possible to isolate and identify several other strains from yogurt but our target was finding an EPS producer LAB strain as EPS production in yogurt is critical for yogurt rheology, texture and its microbiological characteristics [1]. Previously several studies also showed the EPS production of *S. thermophilus* strains [15,16] and importantly *S. thermophilus* was the first food related organism in which the *eps* gene cluster was identified [17]. Structural analysis in different studies revealed that *S. thermophilus* strains were able to produce heteropolymeric type EPS in which glucose, galactose, rhamnose, N-acetylgalactosamine and fucose were the sugar monomers comprising the EPS repeating units [15,18-20]. Following the isolation and identification of an EPS producer *S. thermophilus* strain, EPS from culture supernatants of *S. thermophilus* were extracted and subjected to FTIR structural analysis and the polymer was confirmed as an EPS.

Recent interest in food industry especially in dairy industry increased the attention to EPS producer LAB strains due to the technological role of *in situ* EPS production. We should note that more studies are required in order to find new EPS structures and new EPS producer strains as technological performance of EPS is dependent on EPS production levels and EPS structures. Research is ongoing with the identified *S. thermophilus* strain in this study in order to determine its EPS production levels under different conditions and its potential as a new starter culture for yogurt production under industrial scale.

In conclusion, an EPS producer *S. thermophilus* strain was isolated from Turkish yogurt as a potential ropy starter culture and the structural analysis of the crude EPS was analysed by FTIR Spectroscopy. Studies on EPS production levels of this strain under different conditions such as incubation temperature, carbon source and pH are definitely required in order to optimise the EPS production. Additionally identification of the sugar monomers that EPS is composed of as well as the potential *eps* genes is also in our future plans. Moreover technological properties of the yogurt produced with this stain will be studied.

REFERENCES

1. De Vuyst L, Degeest B: Heteropolysaccharides from lactic acid bacteria. *FEMS Microbiol Rev*, 23 (2): 153-177, 1999.

2. Delcour J, Ferain T, Deghorain M, Palumbo E, Hols P: The biosynthesis and functionality of the cell-wall of lactic acid bacteria. *Antonie Van Leeuwenhoek*, 76 (1-4): 159-184,1999. DOI: 10.1007/978-94-017-2027-4_7

3. Badel S, Bernardi T, Michaud P: New perspectives for Lactobacilli exopolysaccharides. *Biotechnol Adv*, 29 (1): 54-66,2011. DOI: 10.1016/j.biotechadv.2010.08.011

4. Guzel-Seydim ZB, Sezgin E, Seydim AC: Influences of exopolysaccharide producing cultures on the quality of plain set type yogurt. *Food Control*, 16 (3): 205-209, 2005. DOI: 10.1016/j.foodcont.2004.02.002

5. Doleyres Y, Schaub L, Lacroix C: Comparison of the functionality of exopolysaccharides produced in situ or added as bioingredients on yogurt properties. *J Dairy Sci*, 88 (12): 4146-4156, 2005. DOI: 10.3168/jds.S0022-0302(05)73100-3

6. Aslim B, Onbasili D, Yuksekdag ZN: Determination of lactic acid production and antagonistic activity against *Helicobacter pylori* of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains. *Kafkas Univ Vet Fak Derg*, 17 (4): 609-614, 2011.

7. Ruas-Madiedo PCG, de los Reyes-Gavilan: Invited review: Methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. *J Dairy Sci*, 88 (3): 843-856, 2005. DOI: 10.3168/jds.S0022-0302(05)72750-8

8. Baker GC, Smith JJ, Cowan DA: Review and re-analysis of domain-specific 16S primers. *J Microbiol Methods*, 55 (3): 541-555, 2003. DOI: 10.1016/j.mimet.2003.08.009

9. Cole JR, Chai1 B, Farris RJ, Wang Q, Kulam SA, McGarrell DM, Garrity GM J. M. Tiedjeet JM: The ribosomal database project (RDP-II): Sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res*, 33 (Suppl. 1): D294-D296, 2005. DOI: 10.1093/nar/gki038

10. Horn N, Wegmann U, Dertli E, Mulholland F, Collins SR, Waldron KW, Bongaerts RJ, Mayer MJ, Narbad A: Spontaneous mutation reveals influence of exopolysaccharide on *Lactobacillus johnsonii* surface characteristics. *PLoS One*, 8 (3): e59957, 2013. DOI: 10.1371/journal.pone.0059957

11. Bauer R, Bekker JP, Wyk N, du Toit C, Dicks LMT, Kossmann J: Exopolysaccharide production by lactose-hydrolyzing bacteria isolated from traditionally fermented milk. *Int J Food Microbiol*, 131 (2-3): 260-264, 2009. DOI: 10.1016/j.ijfoodmicro.2009.02.020

12. Kanmani P, Satish Kumar R, Yuvaraj N, Paari KA, Pattukumar V, Arul V: Production and purification of a novel exopolysaccharide from lactic acid bacterium Streptococcus phocae PI80 and its functional characteristics activity *in vitro. Bioresour Technol*, 102 (7): 4827-4833, 2011. DOI: 10.1016/j.biortech.2010.12.118

13. Wang Y, Ahmed Z, Feng W, Li C, Song S: Physicochemical properties of exopolysaccharide produced by *Lactobacillus kefiranofaciens* ZW3 isolated from Tibet kefir. *Int J Biol Macromol*, 43 (3): 283-288, 2008. DOI: 10.1016/j.ijbiomac.2008.06.011

14. Yuan SJ, Sun M, Sheng GP, Li Y, Li WW, Yao RS, Yu HQ: Identification of key constituents and structure of the extracellular polymeric substances excreted by *Bacillus megaterium* TF10 for their flocculation capacity. *Environ Sci Technol*, 45 (3): 1152-1157, 2011. DOI: 10.1021/es1030905

15. Lemoine J, Chirat F, Wieruszeski JM, Strecker G, Favre N, Neeser JR: Structural characterization of the exocellular polysaccharides produced by *Streptococcus thermophilus* SFi39 and SFi12. *Appl Environ Microbiol*, 63 (9): 3512-3518, 1997.

16. Petersen BL, Dave RI, McMahon DJ, Oberg CJ, Broadbent JR: Influence of capsular and ropy exopolysaccharide-producing *Streptococcus thermophilus* on mozzarella cheese and cheese whey. *J Dairy Sci*, 83 (9): 1952-1956, 2000. DOI: 10.3168/jds.S0022-0302(00)75071-5

17. Stingele F, Neeser JR, Mollet B: Identification and characterization of the eps (Exopolysaccharide) gene cluster from Streptococcus thermophilus Sfi6. *J Bacteriol*, 178 (6): 1680-1690, 1996.

18. Bubb WA, Urashima T, Fujiwara R, Shinnai T, Ariga H: Structural characterisation of the exocellular polysaccharide produced by *Streptococcus thermophilus* OR 901. *Carbohydrate Res*, 301 (1-2): 41-50, 1997. DOI: 10.1016/S0008-6215(97)00083-9

19. Doco T, Wieruszeski JM, Fournet B, Carcano D, Ramos P, Loones A: Structure of an exocellular polysaccharide produced by S*treptococcus thermophilus. Carbohydr Res*, 198 (2): 313-321, 1990. DOI: 10.1016/0008-6215(90)84301-A

20. Low D, Ahlgren JA, Horne D, McMahon DJ, Oberg CJ, Broadbent JR: Role of *Streptococcus thermophilus* MR-1C capsular exopolysaccharide in cheese moisture retention. *Appl Environ Microbiol*, 64 (6): 2147-2151, 1998.

Kafkas Univ Vet Fak Derg
21 (2): 233-239, 2015
DOI: 10.9775/kvfd.2014.12197

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Determination of Volatile Compounds of Sucuk with Different Orange Fiber and Fat Levels [1]

Barış YALINKILIÇ [1] Güzin KABAN [1] Özlem ERTEKİN [2] Mükerrem KAYA [1]

[1] *This study has been supported by the Research Council of Atatürk University (BAP 2008/66)*

[1] Atatürk University, Faculty of Agriculture, Department of Food Engineering, TR-25240 Erzurum - TURKEY
[2] Tunceli University, Tunceli Vocational School, Department of Food Processing, TR-62000 Tunceli - TURKEY

Article Code: KVFD-2014-12197 Received: 22.09.2014 Accepted: 02.01.2015 Published Online: 07.01.2015

Abstract

In this study, the effects of fat (10, 15 and 20%) and orange fiber (0, 2 and 4%) levels on volatile compounds of sucuk (Turkish dry-fermented sausage) were investigated. The volatile compound profile of sucuk samples was analyzed by gas chromatography/mass spectrometry (GC-MS) by using a solid phase microextraction (SPME). 75 volatile compounds were identified in the sucuk samples. The volatile compounds identified were 11 aldehydes, 7 aliphatic hydrocarbons, 2 acids, 10 esters, 1 furan, 5 alcohols, 8 aromatic hydrocarbons, 6 ketones, 6 sulphur compounds, and 19 terpenes. It was determined that the use of orange fiber had significant ($P<0.05$) effects on a few volatile compounds (allyl methyl sulfide, copaene and caryophyllene). Alpha-thujene was significantly affected by fat level ($P<0.05$). Fat level also showed very significant effect on p-xylene and allyl methyl sulfide ($P<0.01$). As result, orange fiber and fat level had no significant effects on volatile profile of sucuk samples.

Keywords: *Sucuk, SPME, Volatile compounds, Orange fiber, Fat*

Farklı Oranlarda Portakal Lifi ve Yağ İçeren Sucukların Uçucu Bileşiklerinin Belirlenmesi

Özet

Bu çalışmada, sucuğun (Türk tipi kuru-fermente sosis) uçucu bileşikleri üzerine yağ (%10, 15 ve 20) ve portakal lifi (% 0, 2 ve 4) oranlarının etkileri araştırılmıştır. Sucuk örneklerinin uçucu bileşik profili katı faz mikroekstraksiyon (SPME) tekniği kullanılarak gaz kromatografisi/kütle spektrometresi (GC/MS) ile analiz edilmiştir. Sucuk örneklerinde 75 uçucu bileşik tanımlanmıştır. Uçucu bileşik olarak 11 aldehit, 7 alifatik hidrokarbon, 2 asit, 10 ester, 1 furan, 5 alkol, 8 aromatik hidrokarbon, 6 keton, 6 sülfürlü bileşik ve 19 terpen belirlenmiştir. Portakal lifi kullanımının az sayıda uçucu bileşik (allil metil sülfit, kopaen ve karyofillen) üzerinde önemli ($P<0.05$) etkiye sahip olduğu belirlenmiştir. Alfa-thujen, yağ oranından önemli şekilde etkilenmiştir ($P<0.05$). Ayrıca yağ oranı p-ksilen ve allil metil sülfit üzerine çok önemli ($P<0.01$) etki göstermiştir. Sonuç olarak, portakal lifi ve yağ oranının sucuk örneklerinin uçucu bileşikleri üzerinde önemli bir etkiye sahip olmadığı belirlenmiştir.

Anahtar sözcükler: *Sucuk, SPME, Uçucu bileşikler, Portakal lifi, Yağ*

INTRODUCTION

Sucuk is a dry fermented sausage, one of the most important of traditional Turkish meat products. It is produced with beef meat and/or water buffalo meat/sheep meat, sheep tail fat and/or beef fat. Curing ingredients (nitrite and/or nitrate), salt, sucrose, and various spices including cumin, garlic, pimento, red and black peppers are also used in sucuk production. After stuffing the mixture into natural sausage casings (air-dried bovine small intestines) or small casings of similar characteristics, fermentation and drying steps are carried out under controlled or natural climatic conditions [1,2]. According to Communique of Meat and Meat Products of Turkish Food Codex (No: 2012/74) [3], sucuk must have 40% maximum moisture content and 5.4 maximum pH value.

Volatile compounds generated during processing of dry fermented sausage have great importance on aromatic character of the product. Raw meat contains a large number of aroma precursors which are converted

İletişim (Correspondence)
+90 442 2312794; Fax: +90 442 2360958
mkaya@atauni.edu.tr

to volatile compounds during ripening [4]. These compounds have the ability to influence the quality of products due to their effects on sensorial properties. Volatile compounds generated during production and storage can occur as a result of a) ingredients used in formulation, b) conditions used in production, c) microbial activities of starter and endogenous microorganisms such as amino acid catabolism, carbohydrate fermentation and d) lipid oxidation [5,6].

Sucuk has a large number of volatile compounds. This product is especially rich in terms of terpens from spices which could play an important role in the overall aroma. On the other hand, sulfur compounds (diallyl disulfide, 1, propene 3-thiobis, disulfide methyl 2-phenyl), acids (especially acetic acid), and aldehydes (p-cumic aldehyde) are also found at significant levels. The profile of these compounds could display some differences due to differences in raw materials, spices and other ingredients, and production conditions [7].

Turkey is an important orange producer [8]. Citrus fruits such as orange are mainly processed to fruit juice and millions of tones waste are released every year around the world which could cause an environmental problem. In order to eliminate this problem, citrus wastes are processed to valuable by products like dietary fibers [9]. As well as their physiological properties, dietary fibers could improve the product's texture and decrease the cooking loss with their technological properties [10].

In order to understand the effects of dietary fiber in dry-fermented sausages, many researches have been carried out in the last decade [11-13]. However, the effects of fiber addition and decrease of fat level on volatile compound profile of sucuk have not been examined, yet. In this study, the effects of different levels of fat and orange fiber on volatile compound profile of sucuk were investigated by using gas chromatography/mass spectrometry (GC-MS) with solid phase microextraction (SPME).

MATERIAL and METHODS

Production of Orange Fiber

Cooked and dried orange fiber was obtained according to a method offered by Fernandez-Gines et al.[14].

Sausage Formulation and Processing

Two replicates were designed for the study (Experiment I and Experiment II). Nine sucuk batters were prepared for each experiment based on fat level (90% lean meat + 10% sheep tail fat, 85% lean meat + 15% sheep tail fat, and 80% lean meat + 20% sheep tail fat) and orange fiber (0%, 2% and 4%). As a parallel research project to that of Yalınkılıç et al.[12], the following ingredients (g/kg) and ripening conditions were used; 25 g/kg NaCl, 10 g/kg garlic, 7 g/kg red pepper, 4 g/kg sucrose, 5 g/kg black pepper, 2.5 g/kg pimento, 9 g/kg cumin, and 0.15 g/kg $NaNO_2$ [1]. The amount of orange fiber was calculated over the total mixture and added to batters at different levels (0, 2 and 4%). *Lactobacillus plantarum* GM77 and *Staphylococcus xylosus* GM92 strains [15] were used as starter culture approximately in the level of 10^6 cfu/g, as explained by Kaban and Kaya [1]. Sucuk batters were prepared in a laboratory-type cutter (MADO Typ MTK 662, Dornhan, Schwarzwald). Prepared batters were stuffed into collagen casings (38 mm, NaturinDarm, Germany) using a laboratory-type filling machine (MADO Typ MTK 591, Dornhan/ Schwarzwald). Sucuk samples were fermented and dried in an automatic climate unit (Reich, Klima-Rauchertechnik, Stuttgart). The ripening process was carried out under the following conditions: first day 22°C, second and third days 20°C and 18°C for the following days. In the first three days relative humidity (RH) was 90±2% and on the other days the RH was decreased gradually to 82±2%.

Sampling Procedure

Volatile compound profile examination was carried out using ripened sucuk samples from nine different groups at day 9.

Analysis of Volatile Compounds

Analysis of volatile compounds was done according to a method specified by Kaban [7]. The extraction of headspace volatile compounds was performed using a SPME device (Supelco, Bellefonte, PA), using fibers of 75 µm, carboxen/polydimethylsiloxane (CAR/PDMS). For each sample, 5 g minced sample was 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) during 1 h to equilibrate its headspace. Then, a SPME fiber was exposed to the headspace while maintaining the sample at 30°C during 2 h.

The volatile compounds adsorbed by the fiber were desorbed from the injection port of the gas chromatography (GC, Agilent Technologies 6890N) for 6 min at 250°C with the purge valve off (splitless mode). DB-624 (J&W Scientific, 30 m, 0.25 mm i.d., 1.4 µm film) capillary column was used in separation of volatile compounds. The GC was equipped with a mass selective detector (MS, Agilent Technologies 5973). Helium was used as carrier gas. The GC oven temperature was started to increase as programmed when the fiber was inserted and held at 40°C for 5 min and subsequently programmed from 40 to 110°C at 3°C/min and at a rate of 4°C/min from 150°C, then at a rate of 10°C/min from 210°C where it was held for another 15 min. GC-MS interface was maintained at 240°C. Mass spectra were obtained by electron impact at 70 eV, and data were acquired across the range 30-400 amu. The compounds were determined by comparing the

results with mass spectra from a database developed by NIST and WILEY or standards molecules (for calculating Kovats Index, Supelco 44585-U, Bellefonte PA, USA) and by matching their retention indices with those in the literature. Quantification was based on a total ion chromatogram on an arbitrary scale.

Statistical Analysis

All data from Experiment I and Experiment II were subjected to variance analysis (complete randomized design, two replications) and differences between means were evaluated by Duncan's multiple range test using the SPSS 13.0.0.246 for Windows (SPSS, Inc., Chicago, III., USA). The results of statistical analysis are shown as mean values ± standard deviation in tables.

RESULTS

Seventy five volatile compounds including eleven aldehydes, two acids, five alcohols *(Table 1)*, seven aliphatic hydrocarbons, eight aromatic hydrocarbons, one furan *(Table 2)*, ten esters, six ketones, six sulphur compounds *(Table 3)*, and nineteen terpenes *(Table 4)* were identified in sucuk samples.

Eleven different aldehyde compounds were identified and quantified (Table 1). Both fat and fiber level had no effect on aldehyde profile (P>0,05). Within aldehyde compounds, 2-methyl-3-phenyl propanal had the highest peak area and it was followed by hexanal. An increase in fat level from 10 to 20% increased acetaldehyde amount which was not statistically significant (P>0.05). On the other hand, an increase in fiber level caused a slight rise in levels of pentanal, hexanal, heptanal, 2-methyl-3-phenyl propanal, 2-hexanal and 2-heptenal. In contrast to aldehydes, only two acids; acetic acid and butanoic acid were identified *(Table 1)*. Both fiber and fat levels had no effects on acid profile (P>0.05). Five different alcohols were detected by solid phase microextraction (SPME) method *(Table 1)*. Each identified alcohol was not significantly affected by both fiber and fat levels (P>0.05). The highest peak was obtained for 4-(1-methylethyl) benzene methanol. Second highest peak was obtained for ethanol.

Seven aliphatic hydrocarbons were determined in samples, but these compounds were not affected by fat

Table 1. *Aldehyde, acid and alcohol compounds of sucuk produced by using different fat and orange fiber levels*
Tablo 1. *Farklı yağ ve portakal lif oranları kullanılarak üretilen sucukların aldehit, asit ve alkol bileşikleri*

Compound	KI	S	Fat Level (%)			S	Fiber Level (%)			Fat Level x Fiber Level S
			10	15	20		0	2	4	
Aldehydes										
Acetaldehyde	623	NS	0.18±0.09a	0.24±0.15a	0.45±0.54a	NS	0.26±0.14a	0.19±0.17a	0.41±0.54a	NS
Pentanal	742	NS	0.40±0.33a	0.38±0.33a	0.42±0.28a	NS	0.29±0.22a	0.33±0.32a	0.57±0.31a	NS
Hexanal	849	NS	0.76±0.56a	0.80±0.84a	0.79±0.43a	NS	0.53±0.28a	0.63±0.42a	1.20±0.81a	NS
2-Furancarboxaldehyde	902	NS	0.01±0.01a	0.00±0.00a	0.01±0.01a	NS	0.01±0.01a	0.01±0.01a	0.01±0.01a	NS
2-Hexanal	922	NS	0.01±0.01a	0.03±0.02a	0.03±0.03a	NS	0.01±0.02a	0.02±0.02a	0.03±0.03a	NS
Heptanal	955	NS	0.38±0.30a	0.47±0.61a	0.42±0.34a	NS	0.27±0.25a	0.31±0.24a	0.69±0.57a	NS
2-Heptenal	1022	NS	0.04±0.03a	0.04±0.02a	0.05±0.04a	NS	0.03±0.02a	0.04±0.03a	0.06±0.04a	NS
Octanal	1054	NS	0.16±0.14a	0.44±0.48a	0.17±0.16a	NS	0.23±0.21a	0.32±0.51a	0.24±0.19a	NS
Nonanal	1163	NS	0.54±0.15a	0.54±0.13a	0.53±0.12a	NS	0.50±0.16a	0.50±0.06a	0.61±0.12a	NS
Propanal, 2-methyl-3-phenyl-	1334	NS	2.65±1.03a	2.30±0.80a	2.94±1.34a	NS	2.19±0.59a	2.45±0.10a	3.25±1.29a	NS
Benzaldehyde, 4-methoxy	1369	NS	0.10±0.04a	0.08±0.04a	0.10±0.09a	NS	0.12±0.08a	0.09±0.04a	0.07±0.05a	NS
Acids										
Acetic acid	717	NS	2.07±0.91a	1.54±0.26a	1.52±1.03a	NS	1.76±0.41a	1.74±0.59a	1.64±1.29a	NS
Butanoic acid	890	NS	0.05±0.02a	0.05±0.02a	0.04±0.02a	NS	0.04±0.01a	0.05±0.01a	0.05±0.03a	NS
Alcohols										
Ethanol	539	NS	0.35±0.44a	0.12±0.10a	0.11±0.10a	NS	0.26±0.45a	0.17±0.18a	0.14±0.08a	NS
1-Hexanol	931	NS	0.03±0.03a	0.05±0.02a	0.05±0.03a	NS	0.04±0.03a	0.05±0.03a	0.04±0.03a	NS
Benzyl Alcohol	1136	NS	0.15±0.06a	0.12±0.04a	0.08±0.05a	NS	0.12±0.03a	0.12±0.05a	0.12±0.08a	NS
Phenylethyl Alcohol	1211	NS	0.22±0.25a	0.06±0.02a	0.09±0.04a	NS	0.15±0.25a	0.13±0.12a	0.09±0.04a	NS
Benzenemethanol, 4-(1-methylethyl)	1382	NS	2.06±0.70a	1.23±0.85a	1.95±0.91a	NS	2.00±0.81a	1.63±0.76a	1.60±1.09a	NS

S: *significance,* ***NS:*** *Not Significant; Results are expressed in Arbitrary Area Units (×10⁻⁶) as means of 3 replicates of each sausage;* ***KI:*** *Kovats index calculated for DB-624 capillary column (J & W scientific: 30 m, 0.25 mm id, 1.4 lm film tickness) installed on a gas chromatograph equipped with a mass selective detector*

Table 2. *Aliphatic hydrocarbon, aromatic hydrocarbon and furan compounds of sucuk produced by using different fat and orange fiber levels*
Tablo 2. *Farklı yağ ve portakal lif oranları kullanılarak üretilen sucukların alifatik hidrokarbon, aromatik hidrokarbon ve furan bileşikleri*

Compound	RI	S	Fat Level (%)			S	Fiber Level (%)			Fat Level x Fiber Level S
			10	15	20		0	2	4	
Aliphatic hydrocarbons										
Hexane	600	NS	0.19±0.17a	0.29±0.43a	0.09±0.11a	NS	0.23±0.18a	0.24±0.42a	0.10±0.18a	NS
Heptane	700	NS	0.03±0.03a	0.04±0.04a	0.04±0.03a	NS	0.03±0.03a	0.04±0.04a	0.04±0.04a	NS
Undecane	1100	NS	0.10±0.10a	0.07±0.04a	0.13±0.18a	NS	0.18±0.17a	0.06±0.04a	0.05±0.03a	NS
Dodecane	1200	NS	0.10±0.08a	0.07±0.05a	0.11±0.04a	NS	0.13±0.07a	0.09±0.04ab	0.06±0.05b	NS
Tridecane	1300	NS	0.12±0.06a	0.10±0.04a	0.17±0.12a	NS	0.15±0.06a	0.11±0.06a	0.14±0.13a	NS
Tetradecane	1400	NS	0.11±0.04a	0.11±0.20a	0.12±0.05a	NS	0.14±0.03a	0.11±0.04a	0.10±0.03a	NS
Pentadecane	1500	NS	0.17±0.06a	0.19±0.02a	0.19±0.04a	NS	0.19±0.03a	0.18±0.05a	0.18±0.04a	NS
Aromatic hydrocarbons										
Toluene	789	NS	0.48±0.49a	0.29±0.06a	0.40±0.15a	NS	0.36±0.15a	0.35±0.10a	0.46±0.49a	NS
p-Xylene	892	**	0.05±0.03b	0.06±0.01b	0.11±0.04a	NS	0.08±0.06a	0.08±0.02a	0.06±0.01a	**
Styrene	933	NS	0.13±0.05a	0.10±0.07a	0.16±0.09a	NS	0.14±0.10a	0.16±0.04a	0.09±0.06a	NS
1-methyl-2-(1-methylethyl)-benzene	1062	NS	6.71±1.50a	10.18±3.39a	10.63±4.43a	NS	8.45±2.54a	8.58±3.51a	10.49±4.75a	NS
Eugenol	1460	NS	0.12±0.12a	0.04±0.03a	0.06±0.09a	NS	0.10±0.09a	0.07±0.10a	0.06±0.09a	NS
1,2-dimethoxy-4-(2-propenyl)-benzene	1482	NS	1.34±0.60a	0.87±0.60a	0.97±0.42a	NS	1.22±0.58a	0.93±0.43a	1.03±0.69a	NS
1-methoxy-4-(1-propenyl)-benzene	1342	NS	0.21±0.07a	0.13±0.10a	0.17±0.07a	NS	0.20±0.10a	0.13±0.05a	0.17±0.09a	NS
2-Methoxy-1,3-bis(trimethylsilyl)benzene	1491	NS	0.06±0.02a	0.03±0.02a	0.04±0.02a	NS	0.05±0.03a	0.04±0.02a	0.04±0.02a	NS
Furan										
Furan, 2-pentyl-	1021	NS	0.05±0.09a	0.03±0.02a	0.02±0.02a	NS	0.02±0.2a	0.02±0.02a	0.06±0.09a	NS

S: *significance,* ***NS:*** *Not Significant, ** P<0.01; Results are expressed in Arbitrary Area Units (×10^{-6}) as means of 3 replicates of each sausage;* ***KI:*** *Kovats index calculated for DB-624 capillary column (J & W scientific: 30 m, 0.25 mm id, 1.4 lm film tickness) installed on a gas chromatograph equipped with a mass selective detector*

and orange fiber levels (P>0.05) *(Table 2)*. On the other hand, eight aromatic hydrocarbons were detected in all groups *(Table 2)*. Among these compounds, 1-methyl-2-(1-methylethyl)-benzene had the highest peak. Only p-xylene was very significantly (P<0.01) affected by both fat level and fat x fiber interaction. Only one furan compound was identified in the samples *(Table 2)*.

Ten ester compounds were identified in samples *(Table 3)*. None of them were affected by fiber and fat levels (P>0.05). On the other hand, six ketone compounds were detected in samples *(Table 3)* and only 3,5-octadien-2-one was significantly (P<0.05) affected by interaction of fat level and fiber level.

A total of six sulphur compounds were identified and quantified in experimental sucuk samples *(Table 3)*. Only allyl methyl sulfide was affected very significantly by fat level (P<0.01) and significantly by orange fiber level (P<0.05). In contrast, interaction of fat level and fiber level had no statistically significance (P>0.05).

Terpens were the largest volatile group in the present study with their nineteen identified compounds *(Table 4)*. β-myrcene had the highest peaks among all terpen compounds identified in sucuk samples. D-limonene, 3-carene, linalool and caryophyllene were also standing out with their high peak areas.

DISCUSSIONS

Biochemical reactions including glycolysis, proteolysis, lipolysis, autoxidation etc. play an important role in transformation of meat to fermented meat products. Volatile compounds resulted from such reactions have significant impact on characteristic flavor and consumer acceptance [16]. As a result of these reactions and components of sucuk formulation, seventy five volatile compounds were identified in experimental samples. In parallel to our findings, Kaban [7] identified ninety-two volatile compounds in sucuk belonging to different brands. In addition, forty volatile compounds were also identified in a different study on sucuk produced with same starter culture as in our study [1]. Besides, Olivares et al.[5] identified ninety five volatile compounds by using SPME in dry-fermented sausages. These differences in number and amount of volatile compounds in various

Table 3. *Ester, ketone and sulphur compounds of sucuk produced by using fat and orange fiber levels*
Tablo 3. *Farklı yağ ve portakal lif oranları kullanılarak üretilen sucukların ester, keton ve sülfürlü bileşikleri*

Compound	KI	S	Fat Level (%)			S	Fiber Level (%)			Fat Level x Fiber Level S
			10	15	20		0	2	4	
Esters										
Acetic acid ethenyl ester	639	NS	0.06±0.08a	0.06±0.06a	0.02±0.02a	NS	0.07±0.09a	0.05±0.06a	0.02±0.02a	NS
Ethyl acetate	640	NS	0.28±0.10a	0.23±0.09a	0.15±0.14a	NS	0.24±0.11a	0.22±0.07a	0.20±0.17a	NS
Butanoic acid, ethyl ester	843	NS	0.07±0.09a	0.05±0.05a	0.02±0.02a	NS	0.03±0.03a	0.03±0.02a	0.07±0.09a	NS
Ethyl lactate	867	NS	0.04±0.05a	0.06±0.05a	0.04±0.03a	NS	0.04±0.05a	0.05±0.03a	0.05±0.05a	NS
Hexanoic acid, ethyl ester	1024	NS	0.06±0.10a	0.15±0.19a	0.14±0.22a	NS	0.09±0.20a	0.08±0.15a	0.17±0.17a	NS
2,4-Hexadienoic acid, methyl ester	1077	NS	0.54±0.31a	0.52±0.32a	0.47±0.35a	NS	0.60±0.33a	0.46±0.25a	0.47±0.37a	NS
Hexanoic acid, propyl ester	1126	NS	0.43±0.13a	0.34±0.22a	0.42±0.16a	NS	0.39±0.14a	0.45±0.23a	0.34±0.13a	NS
2,4-Hexadienoic acid, ethyl ester	1154	NS	0.17±0.18a	0.18±0.16a	0.13±0.12a	NS	0.13±0.13a	0.13±0.12a	0.21±0.20a	NS
Butanoic acid, hexyl ester	1224	NS	0.25±0.11a	0.26±0.20a	0.22±0.16a	NS	0.26±0.14a	0.27±0.18a	0.20±0.16a	NS
Octanoic acid, ethyl ester	1239	NS	0.06±0.06a	0.05±0.04a	0.07±0.03a	NS	0.06±0.05a	0.05±0.04a	0.07±0.06a	NS
Ketones										
2-Butanone, 3-methyl-	637	NS	0.06±0.05a	0.03±0.03a	0.04±0.03a	NS	0.04±0.03a	0.03±0.04a	0.06±0.04a	NS
2-Butanone, 3-hydroxy-	779	NS	0.32±0.08a	0.24±0.16a	0.22±0.21a	NS	0.26±0.17a	0.26±0.17a	0.25±0.17a	NS
2-Heptanone	948	NS	0.34±0.58a	0.03±0.01a	0.03±0.02a	NS	0.06±0.05a	0.07±0.09a	0.28±0.60a	NS
6-Methyl-5-hepten-2-one	1042	NS	0.07±0.11a	0.04±0.01a	0.04±0.02a	NS	0.03±0.02a	0.03±0.02a	0.07±0.11a	NS
3,5-Octadien-2-one	1141	NS	0.12±0.11a	0.13±0.06a	0.09±0.10a	NS	0.11±0.09a	0.14±0.08a	0.09±0,11a	*
2-Nonanone	1147	NS	0.04±0.07a	0.09±0.08a	0.05±0.03a	NS	0.05±0.09a	0.08±0.05a	0.04±0.03a	NS
Sulphur compounds										
Thiirane, methyl	574	NS	0.17±0.24a	0.04±0.05a	0.04±0.07a	NS	0.11±0.14a	0.03±0.03a	0.10±0.23a	NS
Sulfide, allyl methyl	731	**	0.39±0.29a	0.10±0.08b	0.12±0.13b	*	0.15±0.15b	0.12±0.13b	0.34±0.32a	NS
1-Propene, 1-(methylthio)	762	NS	0.05±0.06a	0.06±0.06a	0.06±0.06a	NS	0.07±0.07a	0.05±0.06a	0.06±0.05a	NS
1-Propene, 3,3'-thiobis-	888	NS	0.14±0.07a	0.12±0.10a	0.09±0.05a	NS	0.14±0.10a	0.09±0.05a	0.11±0.07a	NS
Disulfide, methyl 2-propenyl	958	NS	0.12±0.09a	0.09±0.06a	0.06±0.03a	NS	0.10±0.03a	0.07±0.03a	0.10±0.10a	NS
Diallyl disulphide	1139	NS	1.27±0.40a	0.96±0.25ab	0.89±0.24b	NS	1.20±0.32a	1.01±0.43a	0.90±0.21a	NS

S: *significance,* ***NS:*** *Not Significant, * P<0.05, ** P<0.01; Results are expressed in Arbitrary Area Units (×10⁻⁶) as means of 3 replicates of each sausage;* ***KI:*** *Kovats index calculated for DB-624 capillary column (J & W scientific: 30 m, 0.25 mm id, 1.4 lm film tickness) installed on a gas chromatograph equipped with a mass selective detector*

dry-fermented sausages can be explained by the differences in formulation, processing conditions, starter culture and biochemical reactions taking place during production [1,17,18].

Aldehydes are final products of lipid oxidation and have importance in volatile profile of food products in which they are present. Because of their low perception thresholds, aldehydes can affect the aroma even in trace amounts [19]. Hexanal was one of the outstanding aldehyde compound in samples. This compound has fatty, grassy and fruity odor which is also found in nearly 300 natural sources including meat, cheese and fruits [20]. Short chain aldehydes such as acetaldehyde are mostly originated from carbohydrate metabolism. Besides, some of aldehydes shown in *Table 1* are probably formed by the autoxidation of oleic and linoleic acids [19].

Acetic acid, had the highest peak area in samples, is a colorless volatile liquid with its strong vinegar odor [20]. This volatile acid is generally produced by microbial metabolism [6]. Likewise, 4-(1-methylethyl) benzene methanol and ethanol had the highest peaks, respectively. Within these alcohols, 4-(1-methylethyl) benzene methanol has an aromatic, burning taste and intense, caraway-like odor [20]. On the other hand, ethanol is mainly produced by carbonhydrate fermentation and catabolism of amino acids, and lipids are also responsible for its production [19].

Aromatic hydrocarbons are important volatile components for dry-fermented meat products and 1-methyl-2-(1-methylethyl)- benzene had the highest peak in current study. In contrast, 1,2-dimethoxy-4-(2-propenyl)-

benzene was found by Kaban and Kaya [1] in sucuk as the most abundant aromatic hydrocarbon in terms of peak area. p-xylene and toluene which were also detected in samples could be originated from animal feed and from catabolism of phenylalanine, respectively [19].

2,4-Hexadienoic acid methyl ester and hexanoic acid propyl ester were detected in high levels in samples. 2,4-Hexadienoic acid methyl ester has a fruity, sweet, anise odor. Hexanoic acid propyl ester has an ether-like odor with a pineapple-blackberry undertone. Moreover, hexanoic acid propyl ester is naturally found in some cheeses and some fruits [20].

Ketones are formed by β-oxidation and autoxidation of free fatty acids found in fermented meat products [19]. 3,5-octadien-2-one was the only ketone compound which was affected by interaction of main variation sources (fiber and fat). This compound has a pungent, herbaceous odor and soluble in fats. It's aroma and taste threshold values are 0.15 ppm (in water) and 1.0 ppm, respectively [20]. 3-hydroxy,2-butanone was another significant ketone compound found in samples. This compound known as acetoin is a yellowish liquid with a bland, woody, yogurt odor and a fatty creamy "tub" butter taste [20]. 3-hydroxy,2-butanone is also known to be produced by fermentation of sugars by lactic acid bacteria [19]. Similar ketones were also found in commercial sucuk samples but in low proportions [7].

Diallyl disulphide was the significant sulphur compound in sucuk samples. This compound is the characteristic garlic odor [20] and sulphur compounds are probably originated from garlic. Garlic is one of the main ingredient used in sucuk production [7].

Terpenes are important share in volatile profile of sucuk. As indicated by Kaban and Kaya [1] and Kaban [7], sucuk contains higher level of terpens originated from spices. β-myrcene which is known for its pleasant, sweet, balsamic, plastic odor [20] was one of the terpenes with its higher peak. D-limonene, 3-carene and caryophyllene were also outstanding among these nineteen terpenes detected in the study. D-limonene, 3-carene and caryophyllene are some of the major compounds found in essential oil of black pepper [19]. D-limonene is known as the most important and widespread terpene and has a pleasant, lemon-like odor. 3-carene is colorless or very pale-yellow liquid oil. Linalool has a typical nice floral odor. Caryophyllene's three different isomers are found in

Table 4. *Terpene compounds of sucuk produced by using different fat and orange fiber levels*
Tablo 4. *Farklı yağ ve portakal lif oranları kullanılarak üretilen sucukların terpen bileşikleri*

Compound	KI	S	Fat Level (%)			S	Fiber Level (%)			Fat Level x Fiber Level S
			10	15	20		0	2	4	
Terpenes										
alpha-thujene	944	*	0.23±0.05b	0.34±0.04a	0.26±0.06b	NS	0.26±0.08a	0.27±0.06a	0.29±0.07a	NS
α-pinene	957	NS	0.60±0.07a	0.63±0.10a	0.61±0.13a	NS	0.64±0.09a	0.62±0.09a	0.59±0.11a	*
Camphene	970	NS	0.04±0.02a	0.05±0.01a	0.04±0.02a	NS	0.06±0.03a	0.04±0.01a	0.03±0.01a	NS
β-pinene	988	NS	0.86±0.83a	0.75±0.81a	0.77±0.79a	NS	0.83±0.88a	0.71±0.78a	0.83±0.76a	NS
β-myrcene	1005	NS	5.92±2.34a	7.05±3.07a	6.97±4.95a	NS	7.35±3.81a	6.16±4.48a	6.43±2.20a	NS
α-phellandrene	1019	NS	0.72±0.22a	0.86±0.29a	0.61±0.55a	NS	0.68±0.29a	0.92±0.45a	0.58±0.32a	NS
3-carene	1026	NS	1.82±0.18a	1.98±0.45a	2.04±0.72a	NS	2.15±0.57a	1.89±0.61a	1.79±0.13a	NS
D-limonene	1054	NS	2.35±0.82a	3.36±1.03a	3.33±1.99a	NS	2.98±1.34a	3.11±1.93a	2.94±0.98a	NS
β-phellandrene	1065	NS	0.58±0.11a	0.66±0.14a	0.58±0.37a	NS	0.59±0.25a	0.67±0.25a	0.57±0.19a	NS
o-cymene	1070	NS	0.44±0.20a	0.43±0.17a	0.43±0.32a	NS	0.52±0.23a	0.42±0.24a	0.37±0.20a	NS
α-carene	1114	NS	0.05±0.05a	0.08±0.02a	0.06±0.05a	NS	0.07±0.05a	0.06±0.04a	0.07±0.05a	NS
Linalol	1161	NS	1.10±0.19a	1.13±0.35a	0.91±0.50a	NS	1.20±0.27a	1.05±0.25a	0.88±0.49a	NS
p-cymene	1138	NS	0.21±0.04a	0.23±0.06a	0.24±0.05a	NS	0.23±0.06a	0.02±0.05a	0.23±0.05a	NS
4-terpineol	1240	NS	0.18±0.12a	0.12±0.09a	0.05±0.08a	NS	0.10±0.11a	0.11±0.09a	0.14±0.13a	NS
α-terpineol	1267	NS	0.19±0.10a	0.08±0.07a	0.13±0.13a	NS	0.13±0.12a	0.11±0.08a	0.15±0.14a	NS
Carvacrol	1428	NS	0.06±0.03a	0.06±0.03a	0.04±0.01a	NS	0.06±0.02a	0.06±0.04a	0.04±0.02a	NS
Copaene	1433	NS	0.36±0.05a	0.26±0.09b	0.35±0.10a	*	0.40±0.07a	0.28±0.07b	0.29±0.08b	NS
β-elemene	1453	NS	0.11±0.08a	0.09±0.07a	0.12±0.08a	NS	0.15±0.10a	0.10±0.06a	0.07±0.05a	NS
Caryophyllene	1495	NS	2.89±1.11a	2.39±0.90a	2.98±0.85a	*	3.56±0.77a	2.61±0.55ab	2.09±0.90b	NS

S: *significance,* ***NS:*** *Not Significant, * P<0.05; Results are expressed in Arbitrary Area Units (×10⁻⁶) as means of 3 replicates of each sausage;* ***KI:*** *Kovats index calculated for DB-624 capillary column (J & W scientific: 30 m, 0.25 mm id, 1.4 lm film tickness) installed on a gas chromatograph equipped with a mass selective detector*

nature. Furthermore, 1R-alpha-pinene, another terpene found in samples, has a characteristic odor of pine [20].

Fat is a major component in dry-fermented sausages and has an important role both in development of textural parameters and sensory properties of dry-fermented sausages. It is known to be effective in volatile compound formation in such products. But taking into consideration of sucuk samples produced in current study, effects of different fat levels did not appear well, maybe due to the short ripening time of nine days. Thus, extending the ripening time with different fat levels may reveal more information about the exact role of fat in aroma formation in sucuk. On the other hand, orange fiber had little impact on volatile compound profile of sucuk. This result may be explained by processing steps applied in orange fiber production. Because of several washing steps and pasteurization process, many important volatile compounds could be moved away from the fiber. It could be suggested that sucuk samples with orange fiber has similar volatile profile with traditional one.

Acknowledgments

This study has been supported by the Research Council of Atatürk University (BAP 2008/66). The financial support of Atatürk University is gratefully acknowledged.

REFERENCES

1. Kaban G, Kaya M: Effects of *Lactobacillus plantarum* and *Staphylococcus xylosus* on the quality characteristics of dry fermented sausage "Sucuk". *J Food Sci,* 74 (1): S58-S63, 2009. DOI: 10.1111/j.1750-3841.2008.01014.x

2. Yalçın H, Can ÖP: Geleneksel yöntemle üretilen sucuklarda *Listeria monocytogenes*, *Staphylococcus aureus* ve koliform varlığının araştırılması. *Kafkas Univ Vet Fak Derg,* 19 (4): 705-708, 2013. DOI: 10.9775/kvfd.2013.8610

3. FAOLEX - legislative database of FAO Legal Office: Notification of Meat and Meat Products of Turkish Food Codex (No: 2012/74) *Resmi Gazete,* Number: 28.488, 2012.

4. Demeyer D, Stahnke L: Quality control of fermented meat products in meat processing improving quality. **In,** Kerry J, Kerry J, Ledward D (Eds): Meat Processing. 359-382, CRC Press, Cambridge, 2002.

5. Olivares A, Navarro JL, Flores M: Effect of fat content on aroma generation during processing of dry fermented sausages. *Meat Sci*, 87, 264-273. 2011. DOI: 10.1016/j.meatsci.2010.10.021

6. Summo C, Caponio F, Pasqualone A, Gomes T: Vacuum-packed ripened sausages: evolution of volatile compounds during storage. *J Sci Food Agric*, 9, 950-955, 2011. DOI: 10.1002/jsfa.4272

7. Kaban G: Volatile compounds of traditional Turkish dry fermented sausage (sucuk). *Int J Food Proper,* 13, 525-534, 2010. DOI: 10.1080/10942910802688184

8. Subaşı SK: Yaş Meyve ve Sebze. T. C. Başbakanlık Dış Ticaret Müsteşarlığı İhracatı Geliştirme Etüd Merkezi. Ankara, 2008.

9. Marín FR, Soler-Rivas C, Benavente-García O, Castillo J, Pérez-Alvarez JA: By-products from different citrus processes as a source of customized functional fibres. *Food Chem*, 100 (2): 736-741, 2007. DOI: 10.1016/j.foodchem.2005.04.040

10. Ekici L, Ercoşkun H: Et ürünlerinde diyet lif kullanımı. *Gıda Tekn Elek Derg*, 1, 83-90, 2007.

11. Coksever E, Saricoban C: Effects of bitter orange albedo addition on the quality characteristics of naturally fermented Turkish style sausages (sucuks). *J Food Agric Environ,* 8 (1): 34-37, 2010.

12. Yalınkılıç B, Kaban G, Kaya M: The effects of different levels of orange fiber and fat on microbiological, physical, chemical and sensorial properties of sucuk. *Food Microbiol*, 29 (2): 255-259, 2012. DOI: 10.1016/j.fm.2011.07.013

13. Eim VS, Garcia-Perez JV, Rossello C, Femenia A, Simal S: Influence of the addition of dietary fiber on the drying curves and microstructure of a dry fermented sausage (Sobrassada). *Drying Techn*, 30, 146-153, 2012. DOI: 10.1080/07373937.2011.628428

14. Fernandez-Gines JM, Fernandez-Lopez J, Sayas-Barbera E, Sendra E, Perez-Alvarez JA: Effect of storage conditions on quality characteristics of Bologna sausages made with citrus fiber. *J Food Sci,* 68 (2): 710-715, 2003. DOI: 10.1111/j.1365-2621.2003.tb05737.x

15. Kaban G, Kaya M: Identification of lactic acid bacteria and Gram-positive catalase-positive cocci isolated from naturally fermented sausage (Sucuk). *J Food Sci,* 73 (8): M385-M388, 2008. DOI: 10.1111/j.1750-3841.2008.00906.x

16. Ercoşkun H, Ertaş AH: Fermente et ürünlerinin lezzet bileşenleri ve oluşumları. *Gıda Müh Derg,* 7 (16): 38-45, 2003.

17. Lücke FK: Fermented sausages. **In**, Wood BJB (Ed): Microbiology of Fermented Foods. 441-483, Blackie Academic& Professional, London, 1998.

18. Toldra F, Sanz Y, Flores M: Meat Fermentation Technology. **In,** Hui YH, Nip WK, Rogers RW, Young OA (Eds): Meat Science and Applications. 538-558, Marcel Dekker Inc, New York, 2001.

19. Ordóñez JA, Hierro EM, Bruna JM, Hoz LDL: Changes in the components of dry-fermented sausages during ripening. *Crit Rev Food Sci Nutr*, 39 (4): 329-367, 1999. DOI: 10.1080/10408699991279204

20. Burdock GA: Fenaroli's Handbook of Flavor Ingredients. 6th ed., CRC press, New York, 2010.

Kafkas Univ Vet Fak Derg
21 (2): 241-246, 2015
DOI: 10.9775/kvfd.2014.12210

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Genotoxic and Cytotoxic Effects of the Aglepristone, A Progesteron Antagonist, in Mid-gestation Pregnancy Termination in Rabbits

Özgür VATAN [1] Deniz BAĞDAŞ [2] Nilüfer ÇİNKILIÇ [1]
Axel WEHREND [3] Gözde Rabia ÖZALP [4]

[1] Department of Biology, Faculty of Science and Arts, Uludag University, TR-16059 Görükle, Bursa - TURKEY
[2] Experimental Animals Breeding and Research Center, Faculty of Medicine, Uludag University, TR-16059 Görükle, Bursa - TURKEY
[3] Clinic for Obstetrics, Gynecology and Andrology of Large and Small Animals, Justus-Liebig-University, 35392, Giessen - GERMANY
[4] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Uludag University, TR-16059 Görükle, Bursa - TURKEY

KVFD-2014-12210 Received: 01.09.2014 Accepted: 16.12.2014 Published Online: 22.12.2014

Abstract

Aglepristone is an antiprogestin using for pregnancy termination in veterinary medicine. The information about side effects of aglepristone is limited. The aim of the study was to investigate cytotoxicity and genotoxicity of aglepristone in mid-gestation pregnancy termination in rabbits. Fifteen New Zealand White rabbits were used and pregnant does were randomly divided into three groups. Group I (n=5) was treated with saline as the control. The does in group II (n=5) and group III (n=5) were treated with aglepristone (10 mg/kg) on 15th day and 15th-16th days of pregnancy, respectively. The rabbits were sacrificed by guillotine 24 h after last treatment. Bone marrow and blood samples were immediately collected. Cytotoxic and genotoxic potential were tested by micronucleus and Comet assays. No genotoxicity and cytotoxicity were found in micronucleus test with single aglepristone administration. In contrast, two consecutive treatments of aglepristone showed high genotoxic and cytotoxic effects on bone marrow in animals. While comet assay of blood samples did not show any significant difference between groups; the results from comet assay of bone marrow cells showed the single injection of aglepristone did not induce any DNA damage but two injections group increased the DNA damage.

Keywords: *Aglepristone, Cytotoxic, Genotoxic, Micronucleus test, Comet assay*

Tavşanlarda Orta Dönem Gebelikleri Sonlandırılmasında Kullanılan Bir Progesteron Antagonisti Olan Aglepriston'un Genotoksik ve Sitotoksik Etkileri

Özet

Aglepriston, veteriner hekimlikte gebeliklerin sonlandırılmasında kullanılan bir antiprogestindir. Aglepriston'un yan etkileri ile ilgili bilgiler sınırlıdır. Çalışmamızın amacı; orta gebelikte gebeliği sonlandırılan tavşanlarda aglepriston'un potansiyel sitotoksik ve genotoksik etkilerinin araştırılmasıdır. Çalışmada 15 Yeni Zelanda beyaz tavşanı kullanılmıştır ve gebe tavşanlar rastgele üç gruba ayrıldı. I. Grup (n=5) kontrol grubu olarak tuz çözeltisi ile muamele edildi. II. Grup (n=5) gebeliğin 15. gününde aglepriston (10 mg/kg) enjekte edilen III. Grup (n=5) ise gebeliğin 15. ve 16. günlerinde aglepristone enjekte edilen gruptu. Tavşanlar son enjeksiyonlardan 24 saat sonra giyotin ile sakrifiye edilerek hızlı bir şekilde kemik iliği ve kan örnekleri alındı. Sitotoksik ve genotoksik potansiyel mikroçekirdek ve komet yöntemleri ile araştırıldı. Tek aglepriston uygulaması ile gebeliği sonlandırılan tavşanlarda mikroçekirdek testi ile herhangi bir sitotoksik ve genotoksik etki belirlenemedi. Bunun tersine iki aglepriston uygulanan tavşanların kemik iliği hücrelerinde yüksek sitotoksik ve genotoksik etki belirlendi. Komet yönteminde kullanılan kan örneklerinde gruplar arasında bir fark belirlenemedi. Kemik iliği hücrelerinin kullanıldığı komet yönteminde tek enjeksiyon grubunda herhangi bir DNA hasarı belirlenmemesine rağmen çift enjeksiyon grubunda DNA hasarının arttığı belirlendi.

Anahtar sözcükler: *Aglepriston, Sitotoksik, Genotoksik, Mikroçekirdek testi, Komet yöntemi*

İletişim (Correspondence)
+90 224 2940825, Fax: + 90 224 2940873
rgozalp@uludag.edu.tr , rgozalp@gmail.com

INTRODUCTION

Aglepristone, developed as antiprogestin, has high potential activity in blocking action of progesterone in veterinary therapy. Anti-progesterone activity of the synthetic steroid has been clinically tested in small animal veterinary practices. Based on the clinical experiences, aglepristone can be safely used in specific situations such as termination of unwanted pregnancies, induction of parturition, treatment of pyometra and fibroadenomatosis in dogs and cats [1-6]. The successful clinical features in induction abortion during mid-gestation and implantation have been thoroughly investigated in rabbits [7-9]. Progesterone antagonists have similar structure and show high binding affinity to the receptor with hydrophobic side chain at C17. The administration of aglepristone leads conformational changes leading to a suppression of transcription at additional aromatic ring at C11 with a dimethyl-amino group, resulting incomplete signaling cascade and non-biologic effect of progesterone [1,10,11].

In general, *in vivo* genotoxicity test systems contain micronucleus (MN) and comet tests in experimental rodent models [12]. Micronuclei arise from chromosome breakage (a result of clastogenic activity) or lagging chromosomes (due to aneugenic activity) in the anaphase stage of cell division. Generally, mammals may produce spontaneously micronucleated cells at background level and their presence might increase when organisms are exposed to genotoxic compounds [13,14]. MN test can be conducted from peripheral blood samples, liver and bone marrow [15-18]. Especially, MN tests are carried out on bone marrow samples in adult rabbits, because the spleen quickly removes damaged micronucleated erythrocytes from the peripheral blood [19]. DNA damage can be detected by embedding single cells in agarose gel and staining with a fluorescent-DNA binding dye after electrophoresis, named single-cell gel electrophoresis (comet assay) method [20].

Mifepristone, lilopristone and onapristone are the counterparts of aglepristone. There is no information about the genotoxicity of mifepristone but lilopristone and onapristone were studied with negative results [21]. On the other hand, many exogenous hormonal steroids may cause genotoxic or cytotoxic effects and chromosome breakage [21]. Some natural estrogens, estradiol and estrone and medroxyprogesterone group of synthetic progestins with cyproterone acetate are prominent examples that are gene mutation or genotoxic activity in different tests [22].

To our knowledge, there is no information available about the toxicity of aglepristone. So the objective of this study was to investigate possible genotoxic or cytotoxic effects of aglepristone by MN test and comet assay in mid-gestation termination in does.

MATERIAL and METHODS

Study Design and Animals

Fifteen healthy, 12 months old, New Zealand White rabbits weighing 2.800-3.400 g were housed individually under day-light conditions. All does were fed a standard commercial dry food once (5 g/100 g BW) daily and given water *ad libitum*. Approval of the Ethical Committee of the Uludag University for using the animals was obtained (2013-01/06). The does were brought to a cage of a fertile buck and the first mating was observed. Next day after mating was recorded as first day of pregnancy. Pregnancy confirmation was carried out by ultrasonographic examination (5-7.5 MHz linear array transducer; Siemens Sonoline Prima, Siemens Medical System, USA) on day 14 after mating. The does were examined in dorsal recumbency in order to detect gestational sacs and fetal heart beats. The pregnant does were randomly divided into three groups. Pregnant does in group I (n=5) were injected the same volume of 0.9% sodium chloride solution (Eczacibasi, Baxter, Turkey) whereas the animals in group II (n=5) and group III (n=5) were treated with aglepristone at a dose of 10 mg/kg (Alizin®,Virbac, Germany) once on day 15 post-mating and twice on days 15 and 16 post-mating, respectively.

Micronucleus Test

Micronucleus test was used for the detection of genetic damage that probably induced by compounds [23]. The rabbits were sacrificed by guillotine. Femur was immediately removed from animals with forceps and scissors and bones were cleaned off adhering tissues. The epiphysis of femur was dissected; bone marrow was taken from medullar canal and placed in 2 ml fetal bovine serum. Then bone marrow was thoroughly mixed to obtain a fine suspension and centrifuged at 1000 rpm for 5 min. The pellet was then re-suspended in fresh bovine albumin and smears were prepared on clean glass slides. The smear slides were kept dark for overnight, stained with May-Gruenwald and Giemsa at pH 6.8. For the determination of the frequency of micronucleated polychromatic erythrocytes (MNPCEs), 1000 polychromatic erythrocytes (PCEs) per animal were analyzed by light microscopy. Cytotoxicity was assessed by scoring the relative proportion of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE). This ratio was determined by counting a total of 2000 erythrocytes for each animal.

The Single-cell Gel Electrophoresis (Comet) Assay

Rabbit bone marrow was taken from medullar canal from both femur and suspended in chilled PBS; blood sample was taken from ear vein and suspended in PBS. The cell suspensions were then mixed with an equal amount of 1% low melting agarose and the slides were prepared [24]. The slides were kept overnight at 4°C in lysis solution (2.5

M NaCl, 100 mM Na_2EDTA, 10 mMTris (pH 10), with 1% Triton X-100 (added just before use). Slides were subjected to DNA unwinding for 20 min and then electrophoresis was performed at 0.7 V/cm and 300 mA at 4°C for 25 min in freshly prepared electrophoresis buffer (1 mM EDTA sodium salt and 300 mM NaOH). After electrophoresis the slides were neutralized with Tris buffer and were stained with 20 µg/ml ethidium bromide. Slides were scored at a final magnification of 400x using an image analysis system (Microsystem®, Istanbul, Turkey) attached to a microscope (Nikon Eclipse 80i) equipped with a fluorescence attachment of a CCD camera. The comet parameters were used to measure DNA damage in the cells was Olive Tail Moment (OTM), tail DNA (%) and tail length (µm). Images from 100 random cells (50 from each replicate slides) were analyzed for each experiment.

Statistical Analysis

Data are expressed as mean ± SEM (standard error of the mean) in all cases. Five rabbits were tested in each group. Data were analyzed using one-way ANOVA followed by Tukey test. A P value of less than 0.05 was considered significant.

RESULTS

The means, standard errors, and significance of the MNPCE and PCE/NCE frequencies were given in *Table 1* and photographic presentation of micronucleated polychromatic erythrocyte was shown in *Fig. 1*. When single aglepristone injected rabbits (Group II) was compared with the control (Group I), no significant differences (P>0.05) were observed in the number of MNPCE and PCE/NCE ratio. Two consecutive injections of aglepristone (Group III) resulted significantly higher MNPCE number (P<0.0001) and lower PCE/NCE ratio (P<0.0001) compared with control animals. When animals in Group II were compared with the animals in Group III, significant differences were observed in the number of MNPCE (P<0.0001) and ratio of PCE/NCE (P<0.001).

The comet assay results were presented in *Table 2* and photographic presentation of slides were shown in *Fig. 2*. There was no significant difference in single aglepristone injected group (Group II) compared with control group (Group I) for OTM, tail % DNA and tail length results (P>0.05). Two injections group (Group III) were shown statistically significant elevation of OTM (P<0.0001), tail % DNA (P<0.0001) and tail length frequencies (P<0.0001).

DISCUSSION

In the present investigation, no genotoxicity and cytotoxicity were detected in MN test with single aglepristone

Table 1.*Genotoxic and cytotoxic effect of aglepristone in micronucleus test*
Tablo 1. *Aglepristone'nin mikroçekirdek testindeki genotoksik ve sitotoksik etkileri*

Groups	n	MNPCE	NCE/PCE
Group I	5	5.80±2.79	0.98±0.40
Group II	5	7.40±3.06	0.92±0.38
Group III	5	15.80±6.55 [a,b]	0.75±0.32 [a,c]

One pregnant rabbit per treatment; data are expressed as mean ± standard error; ***n:*** *number of rabbits;* ***MNPCE:*** *micronucleated polychromatic erythrocytes;* ***PCE:*** *polychromatic erythrocytes;* ***NCE:*** *normochromatic erythrocytes;* [a] *P<0.0001 vs. Group I;* [b] *P<0.0001 vs. Group II;* [c] *P<0.01 vs. Group II*

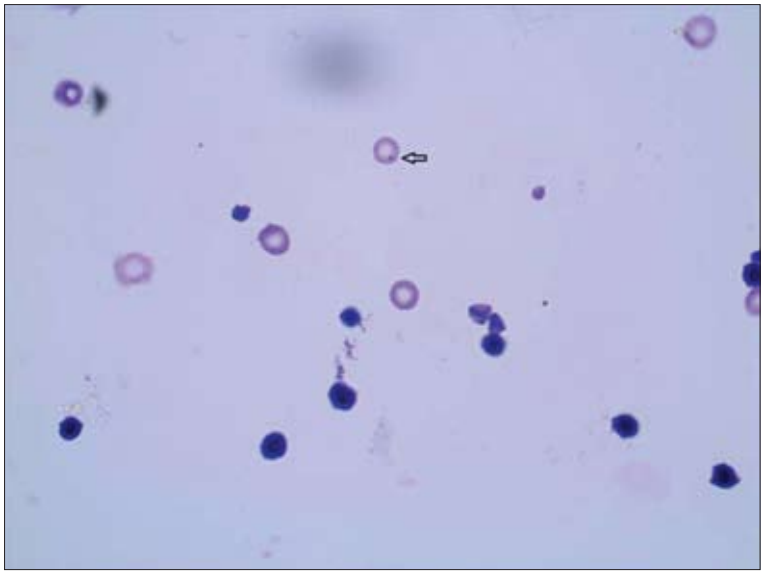


Fig 1. Photographic image of example of MN test under light microscopy (x100 magnification). *Arrow* indicated a MN in PCE in Group II (single injection group)

Şekil 1. Işık mikroskobualtında (X100) MN testi örneğinin fotoğrafik görseli. *Ok* Grup II (tek enjeksiyon grubu)'de PCE içerisindeki MN'yi işaret etmektedir

Table 2. *DNA damaging effect of aglepristone in comet assay*
Tablo 2. *Aglepristone'nin komet yöntemindeki DNA hasar etkileri*

Statistical Data	Group I			Group II			Group III		
	Tail Length (µm)	Tail % DNA	OTM	Tail Length (µm)	Tail % DNA	OTM	Tail Length (µm)	Tail % DNA	OTM
Mean^{BM}	7.81	6.90	0.39	7.82^{a}	7.39^{a}	0.45^{a}	26.20^{b}	17.4^{b}	3.84^{b}
SE	0.17	0.31	0.02	0.17	0.35	0.02	0.56	0.49	0.16
Mean^{BL}	10.07	5.13	0.175	10.2^{a}	5.38^{a}	0.21^{a}	10.43^{a}	5.39^{a}	0.21^{a}
SE	2.95	1.74	0.143	2.97	3.01	0.26	2.68	1.78	0.09

One pregnant rabbit per treatment; data are expressed as mean ± standard error; ***OTM:*** *Olive Tail Moment;* ***SE:*** *Standard Error;* ***BM:*** *Bone Marrow;* ***BL:*** *Blood;* ^{a} *P>0.05 vs. Group I;* ^{b} *P<0.0001 vs. Group I*

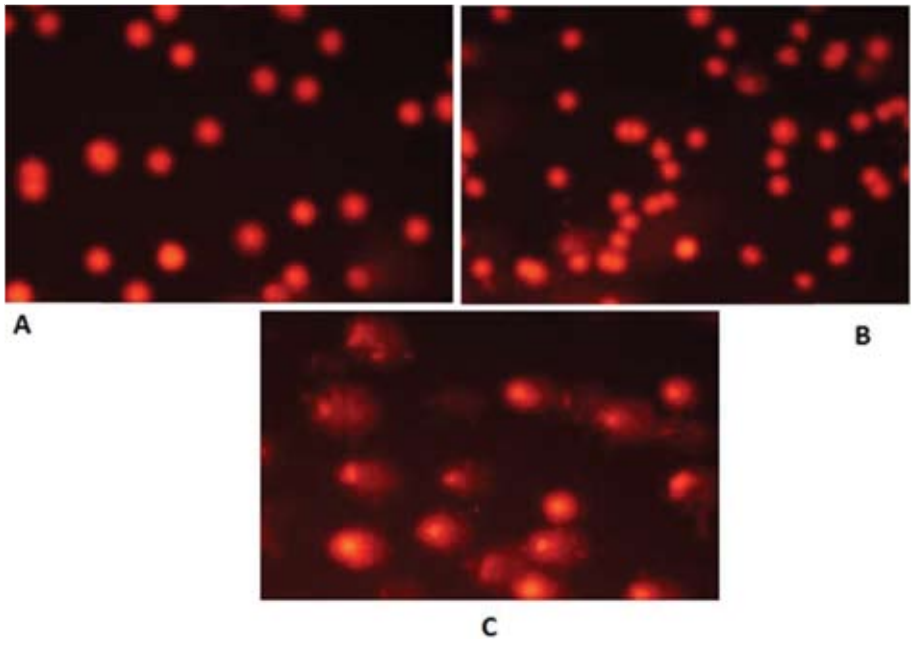


Fig 2. Photographic image of example of comet assay under fluorescence microscopy (x20 magnification). **a-** Group I (control), **b-** Group II (single injection group), **c-** Group III (double injection group)

Şekil 2. Floresans mikroskop altında (x20) komet yöntemi örneğinin fotoğrafik görseli. **a-** Grup I (Kontrol), **b-** Grup II (Tek enjeksiyon grubu), **c-** Grup III (Çift enjeksiyon grubu)

administration (Group II) whereas two consecutive injections of aglepristone (Group III) showed high genotoxic and cytotoxic effects on bone marrow in mid-gestation pregnancy termination in rabbits. No such differences were found between rabbits in control rabbits (Group I) and those treated once with aglepristone (Group II). Additionally, the comet assay results showed the single injection of aglepristone did not induce any DNA damage in bone marrow cells but two injections group increased the DNA damage according to OTM, tail % DNA and tail length frequencies significantly. Finally, comet assay results from blood samples did not show any significant difference between all groups.

The efficiency of aglepristone had been investigated in many species but no serious side effects or transient adverse effects after applications were reported [1-9]. The prominent side effects were noted as a short non-receptive period and irregular mating behavior after abortion, short time decrease in food consumption and the tendency of increase of some hematologic parameters during abortion duration in rabbits [7-9]. Such side effects had also been reported in dogs and cats but the reason of these unwanted effects remains unclear [4,5,25]. No information is available about the genotoxicity of aglepristone *in vitro* or *in vivo*. Other important antiprogestins are mifepristone, onapristone and lilopristone which exhibit a high chemical similarity with aglepristone. No clastogenicity and no genotoxic effects of these anti-progesterone compounds had been found [21,22]. There is no information on mifepristone; on the other hand lilopristone and onapristone have been shown to be inactive in gene mutation tests [26]. In this sense, the current study provides an important contribution to the literature with investigating potential genotoxic and cytotoxic profile of aglepristone.

Micronucleated erythrocytes could be spontaneously produced in peripheral blood in mammals but the frequency of erythrocytes might increase when they meet any genotoxic compounds [13]. Bone marrow samples are used for MN tests in rabbits, as micronucleated erythrocytes are removed by spleen from peripheral blood [19]. In our study two consecutive injection of aglepristone showed increased frequency of micronucleus in bone marrow of

rabbits. The elevation in the incidence of MNPCE in bone marrow results from chromosome damage in a short period of time and this allow us to evaluate short time exposure to chemicals [27]. It was also reported that binding affinity of the compounds could vary between species so it was suggested that the effects of antiprogestins must vary depending on receptor expression, the affinity to the receptor and the dose applied [10]. This could be connected with our results; high genotoxic, cytotoxic effects and DNA damage on bone marrow of two aglepristone applications. But the only one application of aglepristone cannot be effectively used in treatment of any pathologic case or pregnancy termination. General usage for termination the pregnancy in rabbits is double administration of aglepristone (10 mg/kg each dose) for a final concentration of 20 mg/kg. One of the limitations of our study is being not to investigate a dose of 20 mg/kg of aglepristone as a single dose. We choose a two consecutive administration in accordance with clinical use.

Aglepristone is recommended to use twice subcutaneous injection with a 24 h interval precisely. In the therapy of some pathologic cases such as pyometra, fibroadenomatous hyperplasia, more than two applications are used to cure effectively [2,3,19]. So we cannot recommend one application of aglepristone in order to minimize the genotoxic, cytotoxic effects and DNA damage. Uncontrolled reactive oxygen species (ROS) which is generated as a by-product of normal mitochondrial activity causes severe DNA damage response in aerobic cells. Cellular responses are described as the alterations in the cellular redox state during hypoxia or oxidative stress [28]. Superoxide dismutase (SOD), reduced glutathione (GSH) and malondialdehyde (MDA) are important intracellular antioxidants and they are used as markers for detecting severity of oxidative stress [29,30]. These important markers were measured by our working group in mid-pregnancy termination in rabbits previously [31]. Marked increase of MDA after two injections of aglepristone were found and this result was statistically significant from one injection of aglepristone and control group. On the other hand contrary decrease had been detected in SOD and GSH in both aglepristone groups which are statistically significant compared to control group. The detected decrease in GSH after two injections of aglepristone was also significant from other two groups [31]. According to these results we speculated that the application of aglepristone on two consecutive days in abort induction could both trigger oxidative stress and change antioxidant enzyme activities in rabbits. Therefore, as an extension to our previous study, in the current study, aglepristone induced DNA damage on bone marrow of two injection group of rabbits and this was not seen in peripheral blood cells [31]. High level of ROS in a cell could increase the level of DNA damage [32]. We could also explain aglepristone induced DNA damage via the increased ROS level. The contradictory results between blood and bone marrow comet assays are interesting. We already know that the genotoxicity in blood cells reflects long term exposure to clastogenic chemicals unlike the bone marrow genotoxicity situation [13]. It has also been suggested in the report of Vasquez [33] that the *in vivo* comet assay from different tissues may show varied results. Thus aglepristone induced DNA damage in bone marrow cells may reflect short term effects with double dose treatment.

In conclusion the present study show the feasibility of bone marrow MN and comet assays in rabbits to determine the potential genotoxicity of aglepristone administered for abortion. The use of the aglepristone for abortion in small animals proved promising at single lower doses, however, long-term studies would be required to understand the carcinogenic effects for this compound.

REFERENCES

1. Galac S, Kooistra HS, Butinar J, Bevers MM, Dieleman SJ, Voorhout G, Okkens AC: Termination of mid-gestation pregnancy in bitches with aglepristone, a progesterone receptor antagonist. *Theriogenology,* 53, 941-950, 2000. DOI: 10.1016/S0093-691X(00)00241-7

2. Wehrend A, Hospes R, Gruber AD: Treatment of feline mammary fibroadenomatous hyperplasia with a progesterone-antagonist. *Vet Rec,* 148, 346-347, 2001. DOI: 10.1136/vr.148.11.346

3. Jurka P, Max A: Treatment of fibroadenomatosis in 14 cats with aglepristone-changes in blood parameters and follow-up. *Vet Rec,* 165, 657-660, 2009.

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

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

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

7. Ozalp GR, Seyrek-Intaş K, Calişkan C,Wehrend A: Mid-gestation pregnancy termination in rabbits by the progesterone antagonist aglepristone. *Theriogenology,* 69, 1056-1060, 2008. DOI: 10.1016/j.theriogenology.2008.01.016

8. Ozalp GR, Calişkan C, Seyrek-Intaş K, Wehrend A: Effects of the progesterone receptor antagonist aglepristone on implantation administered on days 6 and 7 after mating in rabbits. *Reprod Domest Anim,* 45, 505-508, 2010. DOI: 10.1111/j.1439-0531.2008.01282.x

9. Ozalp GR, Temizel EM, Özocak-Batmaz E: Clinical, ultrasonography and haematology of aglepristone-induced mid-gestation pregnancy terminations in rabbits. *J S Afr Vet Assoc,* 84, 1-4, 2013. DOI: 10.4102/jsava.v84i1.998

10. Hoffmann B, Schuler G: Receptor blockers - General aspects with respect to their use in domestic animal reproduction. *Anim Reprod Sci,* 60-61, 295-312, 2000. DOI: 10.1016/S0378-4320(00)00129-9

11. Hoffmann B, Goericke-Pesch S, Schuler G: Antiprogestins - High potential compounds for use in veterinary research and therapy: A review. *Eurasian J Vet Sci,* 27, 77-86, 2011.

12. Kirkland D, Pfuhler S, Tweats D, Aardema M, Corvi R, Darroudi F, Elhajouji A, Glatt H, Hastwell P, Hayashi M, Kasper P, Kirchner S, Lynch A, Marzin D, Maurici D, Meunier JR, Müller L, Nohynek G, Parry J, Parry E, Thybaud V, Tice R, van Benthem J, Vanparys P, White P: How to reduce false positive results when undertaking *in vitro* genotoxicity testing and thus avoid unnecessary follow-up animal tests: Report of an ECVAM Workshop. *Mutat Res,* 628, 31-55, 2007. DOI: 10.1016/j.mrgentox.2006.11.008

13. Gómez-Meda BC, Zúñiga-González GM, Zamora-Perez A, Ramos-Ibarra ML, Batista-González CM, Torres-Mendoza BM: Folate supplementation of cyclophosphamide-treated mothers diminishes micronucleated erythrocytes in peripheral blood of newborn rats. *Environ Mol Mutagen,* 44, 174-178, 2004. DOI: 10.1002/em.20037

14. Zúñiga-González G, Torres-Bugarín O, Ramos-Ibarra ML, Zamora-Perez A, Gómez-Meda BC, Ventura-Aguilar AJ, Ramos-Mora A, Ortíz GG, Alvarez-Moya C, González-Rodríguez A, Luna-Aguirre J, Gallegos-Arreola MP: Variation of micro nucleated erythrocytes in peripheral blood of Sciurusaureogaster in relationtoage: An increment of micronucleated polychromatic erythrocytes after the administration of colchicine. *Environ Mol Mutagen,* 37, 173-177, 2001. DOI: 10.1002/em.1025

15. Hayashi M, MacGregor JT, Gatehouse DG, Blakey DH, Dertinger SD, Abramsson-Zetterberg L, Krishna G, Morita T, Russo A, Asano N, Suzuki H, Ohyama W, Gibson D; In Vivo Micronucleus Assay Working Group, IWGT: *In vivo* erythrocyte micronucleus assay III. Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes, with discussion of non-hematopoietic target cells and a single dose-level limit test. *Mutat Res,* 627, 10-30, 2007. DOI: 10.1016/j.mrgentox.2006.08.010

16. Özkan O, Üstüner O: Investigations about genotoxicity of deltamethrin. *Kafkas Univ Vet Fak Derg,* 18 (1): 69-74, 2012.

17. Aksu P, Doğan A, Gül S, Kanıcı A: Farelerde 3-Metilkolantren ile indüklenen fibrosarkoma üzerine sisteminin etkileri: Genotoksisitenin araştırılması. *Kafkas Univ Vet Fak Derg,* 19 (6): 955-961, 2013. DOI: 10.9775/kvfd.2013.9172

18. Polat F, Bingöl G, Turaçlar N: An investigation of micronucleus induction by butylated hydroxytoluene in wistar rat bone marrow cells. *Kafkas Univ Vet Fak Derg,* 20 (4): 527-531, 2014. DOI: 10.9775/kvfd.2013.10521

19. Zúñiga-González G, Torres-Bugarín O, Zamora-Perez A, Gómez-Meda BC, Ramos Ibarra ML, Martínez-González S, González-Rodríguez A, Luna-Aguirre J, Ramos-MoraA, Ontiveros-Lira D, Gallegos-Arreola MP: Differences in the number of micronucleated erythrocytes among young and adult animals including humans. Spontaneous micronuclei in 43 species. *Mutat Res,* 494, 161-167, 2001. DOI: 10.1016/S1383-5718(01)00180-2

20. Speit G, Hartmann A: The comet assay: A sensitive genotoxicity test for the detection of DNA damage and repair. *Methods Mol Biol,* 920, 79-90, 2012. DOI: 10.1007/978-1-61779-998-3_6

21. Reimann R, Kalweit S, Lang R: Studies for a genotoxic potential of some endogenous and exogenous sex steroid. II. Communication: examination for the induction of cytogenetic damage using the chromosomal aberration assay on human lymphocytes *in vitro* and the mouse bone marrow micronucleus test *in vivo*. *Environ Mol Mutagen,* 28, 133-144,1996.

22. Joosten HF, van den Dobbelsteen DJ, Horbach GJ, Krajnc EI: Genotoxicity of hormonal steroids. *Toxicol Lett,* 151, 113-134, 2004. DOI: 10.1016/j.toxlet.2004.01.018

23. MacGregor JT, Heddle JA, Hite M, Margolin BH, Ramel C, Salamone MF, Tice RR, Wild D: Guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes. *Mutat Res,* 189, 103-112, 1987. DOI: 10.1016/0165-1218(87)90016-4

24. Singh NP, McCoy MT, Tice RR, Schneider EL: A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res,* 175, 184-191, 1988. DOI: 10.1016/0014-4827(88)90265-0

25. Pettersson CH, Tidholm A: Safety and efficacy of mid-term pregnancy termination using aglepristone in dogs. *J Small Anim Pract,* 50, 120-123, 2009. DOI: 10.1111/j.1748-5827.2008.00692.x

26. Lang R, Reimann R: Studies for a genotoxic potential of some endogenous and exogenous steroids. I. Communication: examination for the induction of gene mutations using the Ames Salmonella/Microsome Test and the HGPRT Test in V79 cells. *Environ Mol Mutagen,* 21, 272-304, 1993.

27. Heddle JA, Hite M, Kirkhart B, Mavournin K, MacGregor JT, Newell GW, Salamone MF: The induction of micronuclei as a measure of genotoxicity. A report of the U.S. environmental protection agency Gene-Tox program. *Mutat Res,* 123, 61-118, 1983. DOI: 10.1016/0165-1110(83)90047-7

28. Jackson AL, Chen R, Loeb LA: Induction of microsatellite instability by oxidative DNA damage. *Proc Natl Acad Sci,* 95, 12468-12473, 1998. DOI: 10.1073/pnas.95.21.12468

29. Barzilai A, Yamamoto K: DNA damage responses to oxidative stress. *DNA Repair (Amst),* 3, 1109-1115, 2004. DOI: 10.1016/j.dnarep.2004.03.002

30. Ozden M, Maral H, Akaydın D, Cetinalp P, Kalender B: Erythrocyte glutathione peroxidase activity, plasma malondialdehyde and erythrocyte glutathione levels in hemodialysis and CAPD patients. *Clin Biochem,* 35, 269-273, 2002. DOI: 10.1016/S0009-9120(02)00307-7

31. Sonat FA, Bağdaş D, Gül Z, Özalp GR: Tavşanlarda orta dönem gebeliklerin sonlandırılmasında kullanılan aglepriston'un bazı oksidatif stres parametreleri üzerine etkisi. *Uludag Univ J Fac Vet Med,* 32, 1-6, 2013.

32. Cooke MS, Evans MD, Dizdaroglu M, Lunec J: Oxidative DNA damage: mechanisms, mutation, anddisease. *FASEB J,* 17, 1195-1214, 2003. DOI: 10.1096/fj.02-0752rev

33. Vasquez MZ: Combining the *in vivo* comet and micronucleus assays: A practical approach to genotoxicity testing and data interpretation. *Mutagenesis*, 25, 187-199, 2010. DOI: 10.1093/mutage/gep060

Kafkas Univ Vet Fak Derg
21 (2): 247-252, 2015
DOI: 10.9775/kvfd.2014.12212

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Investigation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in Fecal and Bulk Milk Samples from Dairy Farms in Thrace Region of Turkey

Haydar ÖZPINAR [1] İsmail Hakkı TEKİNER [1] Olcay KARAMAN [1] Yunus KURT [1]

[1] Food Engineering Department, İstanbul Aydın University, Florya Campus, TR-34295 Sefaköy/Küçükçekmece, İstanbul - TURKEY

KVFD-2014-12212 Received: 28.08.2014 Accepted: 07.10.2014 Published Online: 04.11.2014

Abstract

The Thrace, which is Turkey's European part as well as the adjoining parts of Southern Bulgaria and north-east Greece has a strategic importance for being a vaccination buffer zone for Europe as declared by FAO in 1999. The objective of the study was to better understand the occurence of MAP in this animal disease free area of Turkey by applying *F57* Real time PCR assay and *IS900* nested-PCR. In this study, 270 feces samples from the dairy cattles over 2 years old in 30 randomly selected dairy farms, 45 raw milk samples from each of the bulk tanks belonging to these dairy farms and the villages located in Thrace were collected. Nine fecal samples were used to create the pooled fecal sample for a dairy farm before performing DNA extraction. All the samples were initially subjected to a *F57*-Real time PCR analysis, and subsequently an insertion sequence *IS900* nested-PCR was performed to verify the results. However, the results revealed that MAP genom could not be detected in any pooled fecal and milk samples. In conclusion, the occurrence of MAP in this part of Turkey may likely be very lower than the capability of the detection limit of the used Real time PCR assay. Furthermore, the results once more confirmed the difficulty of MAP detection in asymptomatic animals and milk samples by performing PCR technique only.

Keywords: *Mycobacterium avium subsp. paratuberculosis, Real time PCR, Nested-PCR, Feces, Milk*

Türkiye Trakya Bölgesindeki Süt İşletmelerinden Toplanan Fekal ve Çiğ Süt Örneklerinde *Mycobacterium avium* subsp. *paratuberculosis* (MAP) İncelemesi

Özet

Trakya Türkiye'nin Avrupa topraklarında olup, Güney Bulgaristan ve Kuzeydoğu Yunanistan sınırlarının keşiştiği bir bölgedir. FAO 1999 yılında bu bölgeyi zoonozları erken önlemek bakımından stratejik önemi olan Avrupa aşılama tampon bölgesi olarak duyurmuştur. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) varlığı Trakya'da net şekilde anlaşılamamıştır. Bu çalışmada zoonozlardan ari olarak kabul edilen Trakya'da MAP varlığını *F57* Real time PCR assay ve *IS900* nested-PCR yöntemlerini uygulayarak incelemek amaçlandı. Araştırmada, rastgele seçilen 30 adet süt işletmesinde 2 yaş ve üzeri sığırlardan 270 adet fekal örnek ile bu işletmelerin ve bulundukları köylerin süt toplama tanklarından 45 adet çiğ süt örnekleri toplandı. Bir süt işletmesini temsilen 9 adet fekal örnek tek havuz örnek olacak şekilde karıştırıldı. Örneklerden DNA izolatları elde edildi. İzolatlara *F57*-Real time PCR assay uygulandı. Elde edilen sonuçların *IS900* nested-PCR yöntemi kullanılarak doğrulaması yapıldı. Sonuç olarak, havuz fekal örnekleri ve çiğ süt örneklerinde MAP genomu tespit edilmedi. Trakya bölgesinde MAP varlığının *F57*-Real time PCR yönteminin tespit sınırının altında kaldığı sonucuna varıldı ve asemptomatik hayvanlar ve süt örneklerinde yalnızca PCR yöntemi ile MAP tespitinin zorluğu doğrulanmış oldu.

Anahtar sözcükler: *Mycobacterium avium subsp. paratuberculosis, Real time PCR, Nested-PCR, Fekal, Süt*

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis called Johne´s Disease (JD), which is an infectious, chronic, and granulomatous enteropathy of the ruminants providing milk and/or meat as food source for human consumption [1]. JD is characterized by severe symptoms such as diarrhoea, reducing milk production and weight loss [2,3]. During a long pre-clinical period more than 2 years it persists and multiplies in subepithelial macrophages to lead to a chronic transmural inflammatory reaction. This pathogen is periodically shed in feces, milk and semen of MAP infected dairy cattles [4,5].

İletişim (Correspondence)
+90 212 4441428
haydarozpinar@aydin.edu.tr

Although a causal link between MAP and Crohn's disease (CD) has not been proved, MAP may be involved in CD in humans, and unfortunately no cure for CD is currently known [6,7]. The incidence rates for CD in some European Countries (EU) were given as $5.2/10^5$ in Germany, $6.4/10^5$ in France, $2.3/10^5$ in Italy, and $5.9\text{-}11.7/10^5$ in England, respectively [8]. In an epidemiological study conducted in Turkey the incidence of CD was found to be $1.4/10^5$ while its prevalence was estimated as 7.7%, which was higher than in Asia but lower than in Europe [9].

The importance of food as a source of exposure to MAP has been assessed by many research groups. Milk and dairy products might be a likely food source for human exposure to MAP as well as a direct contact to the MAP infected animal [10,11]. The MAP occurrence in milk worldwide was estimated to be 1-3% [11,12]. Thus, exposure of humans to MAP should be minimized as a precautionary measure [13]. Due to this fact, the EU has compulsorily developed effective control programs for monitoring it [14].

The epidemiological situation of MAP in Turkey has not been well-understood. A study in 1968 indicated that sheeps in İzmit-Turkey were infected with MAP. This result was followed by another study performed for goats [15]. Towards 2000, MAP was initially found in goats [16]. Subsequently, mycobacterial DNA in milk samples of 500 dairy cows in Elazığ was detected by a polymerase chain reaction (PCR) based on IS900 [17]. A recent study for the dairy cattles in Uşak-Turkey showed that MAP prevalence was ranged between 9.5-17.0% in feces whereas it was between 5.5-17.5% in milk samples [18]. In Kars-Turkey the sero-prevalence of subclinical paratuberculosis in the cattles was found to be 3.5% while it was 41.6% in farms [19].

Culture-based methods are time-consuming as well as having insufficient effectiveness of decontaminating methods. On the other hand, it still holds the advantage of specificity for MAP detection [20]. Recently, molecular-based methods such as PCR has become important for the evaluation of MAP-infected animals and the products of animal origin instead of using culture-based methods as well as immuno-based diagnosis [21].

The objective of the study was to investigate the occurence of MAP in pooled fecal and bulk milk samples collected from the Thrace, which is considered to be the animal disease free area of Turkey by using *F57* Real time PCR and *IS900* nested-PCR assays.

MATERIAL and METHODS

Sample Collection

In this study, 2 dairy farms per village, a total of 30 dairy herds from 15 villages in Thrace were randomly selected. From October 2011 to December 2011, 270 fecal samples from 9 cattles over 2 years per a farm and 45 bulk milk samples from the dairy farms and the villages were collected *(Table 1)*. All samples were placed into sampling bags, and immediately transported to the laboratory in a container at 4°C for sample preparation.

Table1. *Sampling data*
Tablo 1. *Numune bilgileri*

Name of Village	No of Herds	Herd's Cattle Population	No of Animals > 2 years in herd	Distribution of Collected Samples		
				No of Fecal Samples	No of Bulk Milk Samples	No of Bulk Milk Samples from Bulk Milk
Bahçeköy	2	35	20 (57.1%)	18	2	1
Çamlıca	2	44	22 (50%)	18	2	1
Çobançeşme	2	70	34 (48.6%)	18	2	1
İzzetiye	2	34	18 (52.9%)	18	2	1
Karahisar	2	54	32 (59.3%)	18	2	1
Karasatı	3	102	53 (51.9%)	27	3	1
Karlıköy	2	51	28 (54.9%)	18	2	1
Kılıçköy	1	200	60 (30%)	9	1	1
Küçükdoğanca	1	24	11 (45.8%)	9	1	1
Lalacık	2	50	25 (50%)	18	2	1
Orhaniye	3	92	53 (57.6%)	27	3	1
Paşayiğit	2	38	22 (57.9%)	18	2	1
Pırnar	3	94	44 (46.8%)	27	3	1
Siğilli	1	22	10 (45.4%)	9	1	1
Türkmen	2	130	85 (65.4%)	18	2	1
Total	30	1040	517 (49.7%)	270	30	15

Number of Dairy Farms

Minimum number of the herds to be sampled was calculated as 30 [22]. The criteria for decision were selected to be an expected herd level-prevalence of 0.80 (EU prevalance of MAP in herds), a maximum acceptable error rate of 0.10, a probability of Type I error of 0.05 and Z statistic for a level of confidence of 1.96 were choosen, respectively.

Number of Animals

Minimum number of the animals to be samples was taken as 270 [22,23]. The criteria was decided to be an expected animal level prevalence proportion of 0.05, Z statistic for a level of confidence of 1.96, precision of 0.025 [23]. The average number of animals per dairy farm was directly obtained as 9.

Extraction Procedure of DNA from Pooled Fecal and Bulk Milk Samples

Approximately 2 g from each of 9 fecal samples per herd was put into a clean tube for pooling, i.e. a total of 18 g pooled mixture (9 randomly selected fecal samples/pool; 1 pooled sample/herd). It was homogenized for 5 min by vortexing (Daihan Scientific, South Korea). Then, 1.5 g of pooled feces was mixed with 5 ml of Roche S.T.A.R Buffer Solution in a 15 ml falcon tube followed by vortexing for 30 s (Daihan Scientific, South Korea). The tubes were allowed to stand at room conditions till a clear supernatant is observed. Following that 200 µL of this supernatant was transferred to a 2 ml Eppendorf tube, in which 200 µl of Roche Lysozyme was. Finally, it was incubated at 37°C for 1 h in an incubator (Binder, Germany). In a similar way, 10 ml of bulk milk sample were pippetted into a 15-ml falcon tube. It was centrifuged at 2.500 ×g for 30 min (Hettich, Germany). The pellets were resuspended in 200 µl of Roche Lysozyme, well-mixed by vortexing, following that incubated at 37°C for 1 h. DNA isolation was made according to GENESpin DNA Isolation Kit protocol (Eurofins GeneScan GmbH, Germany). Then, eluted DNA was kept at 4°C for direct use or at −20°C for further processing.

F57 Real-time PCR Application

The kit procedure of MAPsureEasy® (TransMIT GmbH, Germany) was followed. *F57* Real time PCR analysis was performed in a 96-well plate format on Agilent Stratagene Mx3000P Real-time PCR (Stratagene, USA). A 5 µl aliquot of DNA was mixed with 20 µl of Master Mix (12.5 µl of qPCR Master Mix, 1 µl of MAP Oligonucleotid Mix, 1 µl of IAC Solution and 5.5 µl of A. dest). HEX fluorescence was selected because its emission peaks do not extend over each other due existence of inhibitory impurities in feces matrix. FAM was used for IAC. Thermal processing parameters were adjusted as 1 cycle at 95°C for 15 s and 45 cycles at 60°C for 1 min. Reference strain ATCC 19698 as positive control, DNA of a non-MAP mycobacteria as negative control as well as a master mix blank control were included. Each measurement was performed in duplicate with *IS900* nested-PCR method by using primers and PCR conditions [24]. Threshold cycle (C_T) of the assay $C_t \leq 40$ was accepted to be positive in MAP [25].

Determination of Detection Probability

The detection limit of Real-time PCR assay was initially determined by analyzing serial dilutions including MAP reference strain ATCC 19698. Subsequently, the inoculum for spiking was prepared by inoculating 10 ml Middlebrook 7H9 broth (Difco Laboratories, Germany) containing 10% OADC supplement (Becton-Dickinson, Germany), 2 µg/mL mycobactin J (Allied Monitor, USA), 0.05% Tween 80 (Sigma-Aldrich, Germany) and 2.5% glycerol with a colony of the reference strains of MAP from a slant of Herrold's Egg Yolk Medium (BD HEYM, Germany). MAP reference strains were grown in a shaker incubator for 6-8 weeks at 37°C. Broth cultures were centrifuged at 2500 x *g* for 15 min and the pellets were re-suspended in phosphate buffered saline (PBS) by vortexing with a few sterile glass beads (VWR International, Germany) to yield a suspension containing approximately 10^7 MAP cfu/mL. An initial MAP concentration of about 10^7 cfu/mL was set using a photometer and a counting chamber. Serial dilutions prepared from the MAP stock solution ranged from 10^7 to 10^1 cfu/ml using PBS and 1 ml of each of the dilutions were serially added to 10 ml of raw milk and 10 g of feces homogenate. Positive controls used were "raw milk" and "feces" spiked with 10^7 cfu/ml MAP whereas negative controls as raw milk and feces were spiked with equivalent volume of sterile PBS, respectively. Serial dilutions for spiking were also confirmed by DNA extraction, and subsequently by *F57* Real time PCR as described above. All experiments were repeated in triplicate format.

RESULTS

This study assessed the occurrence of MAP in pooled fecal samples from the cattles over 2 years and the bulk milk samples from each of the collection tanks installed in the randomly selected dairy farms and the villages located in the Thrace Region of Turkey by using a *F57* Real time PCR and *IS900* nested-PCR assays. The results revealed that MAP genom could not be detected in any pooled fecal and milk samples

DISCUSSION

The Thrace, i.e. Turkey's European part and the adjoining parts of Southern Bulgaria and north-east Greece has a strategic importance as a vaccination buffer zone for Europe [26]. In literature, the studies related to understanding MAP prevalance in this region are significantly limited. MAP could not be detected in fecal samples of 2 years-old and/or older cattle from Thrace by PCR based on *IS900* [27].

In 2007, Turkey firstly reported Food and Mouth Disease in Thrace whereas there was no information of MAP in this reporting [28]. A low prevalance of MAP in animals from Thrace part of Greece was determined by *F57* Real time PCR and *IS900* n-PCR. But, in this study, no MAP could be detected in the collected fecal and bulk milk samples in a defined area of Thrace [29].

In MAP prevalence studies, sampling size is arranged by randomly selecting herds and animals from these herds as wells as considering sensitivity and specificity of the diagnostic test [30]. In this study, sampling size calculations were performed based on prevalences of both MAP-infected herd and MAP-infected cattle as 80% and 5% according to EU Data due to the lack of official data indicating MAP prevalance in Turkey. In this study, minimum numbers of the herds and the animals to be sampled were found as 30 and 270, respectively [22,23].

Estimation of the apparent prevalence of MAP in the dairy herds varies significantly among studies, depending on number of the herd, number of the animals to be tested and the method to be performed [31]. Sensitivity of detection for MAP was greater with a smaller pool size, i.e. 5 versus 10 samples per pool whereas 10 cows per pool was recommended by another study [32,33]. In this study, we performed 9 fecal samples per pool as well as the bulk milk samples from the holding tanks were already pooled naturally. Thus, our MAP negative results might likely be due to the dilutions in the pooled feces and the bulk milks. In this way, probability density for low prevalence herds and infected animals would not be distributed within the reference prevalence of MAP in EU as 80%.

The effective diagnosis of MAP is a challenge due to lack of the clinical signs from sub-clinically infected animals, the difficulties in primary isolation of this hardy bacillus, and possible unknown kinds of the MAP strains [34]. Real time PCR has significant advantages over other methods [20,35]. On the other hand, success of a Real time PCR is dependent on a well-performed DNA extraction from test matrix [25,36]. Thus, commercial kits for DNA extraction would detect MAP from feces with high sensitivity without cultivating bacteria [37]. Bead beating in a lysis solution for cell distruption as well as use of spin column technology can perform more effective DNA extraction especially from fecal samples [38]. In this study, DNA extraction was also conducted by commercial kit supported with lysis solution and spin column technology for the most accurate diagnosis of MAP.

There are the genomes highly homologous to other environmental *Mycobacterium* species. It highly affects the reliability of PCR application [39]. *F57* and *IS900* are MAP-specific genetic elements [40]. Insertion sequence *IS900* is a reference marker for confirmation of MAP whereas it may lead to cross-reactions and possible false positive results [24]. Due to this fact, another alternative genetic element *F57* is used [41]. In contrast to *IS900*, *F57* is not similar to genes on other related organisms [42]. But, *F57* does not provide for as high a sensitivity as *IS900* element with less false positive results [43]. DNA extraction followed by Real-time PCR is a sensitive method making possible a detection limit of MAP like such as log 2.0 cfu/mL or g of raw milk and feces within one day only [44]. In this study, *F57* Real time PCR assay was very reliable because it was tested for specificity by including an internal amplification control (IAC) to exclude false-negative PCRs for smaller *MAP* DNA amounts. In addition to that, *IS900* n-PCR was applied despite of being time-consuming with high risks of cross-contamination [45]. Thus, any other available PCR system was not able to detect any MAP DNA [46].

Diagnostic strategies to detect MAP super-shedder cows in dairy herds have been minimally studied [47]. Similar to our study based on Real time PCR, MAP was determined at a level of 10^4 cfu/g of spiked feces, 1-10 cfu/ml of milk, 2.42×10^1 MAP cells in 1 ml of artificially contaminated raw milk, and 10^2 cfu/g of feces and 10^2 cfu/10 ml of bulk milk, respectively [42,48-50]. Hence, in our study we concluded that number of MAP cells in samples might be lower than detection levels of the assays used.

In this study, MAP negative results might be arised from some limitations. These limitations would be lack of information for MAP prevalance in Turkey, no clinical signs on sampled animals over 2 years, MAP cells in smaller amounts, lower sensitivity of *F57*-Real time PCR assay, dilution effect of pooling, and lack of culture-based method.

In conclusion, screening of MAP should be extended to cover the whole region by increasing sampling size of herds, animals, fecals and bulk milk samples in parallel to including the culture based microbiological method together with molecular-based methods for an effective investigation of MAP throughout this animal disease free area of Turkey in Europe.

REFERENCES

1. Berger ST, Griffin FT: A comparison of ovine monocyte-derived macrophage function following infection with *Mycobacterium avium* ssp. *avium* and *Mycobacterium avium* ssp. *Paratuberculosis*. *Immunol Cell Biol*, 84, 349-356, 2006. DOI: 10.1111/j.1440-1711.2006.01431.x

2. Klee W: Paratuberkulose (Johne'sche Krankheit). **In,** Dirksen G, Grunder HD, Stober M (Eds): Innere Medizin und Chirurgie des Rindes. 586-591, Parey Verlag 5, 2012.

3. Çetinkaya B, Erdoğan HM, Morgan KL: Relationships between the presence of Johne's Disease and farm and management factors in dairy cattle in England. *Prev Vet Med*, 32, 253-266, 1997. DOI: 10.1016/S0167-5877(97)00028-7

4. Clarke CJ: The pathology and pathogenesis of paratuberculosis in ruminants and other species. *J Comp Pathol,* 116, 217-261, 1997. DOI: 10.1016/S0021-9975(97)80001-1

5. Manning EJ, Collins MT: *Mycobacterium avium* subsp. *paratuberculosis*: pathogen, pathogenesis and diagnosis. *Rev Sci Tech,* 20, 133-150, 2001.

6. Nacy C, Buckley M: *Mycobacterium avium paratuberculosis*: Infrequent Human Pathogen or Public Health Threat? A Report from American

Academy of Microbiology. 1752 N Street, NW Washington DC, 2008.

7. Eltholth MM, Marsh VR, Van Winden S, Guitian FJ: Contamination of food products with *Mycobacterium avium paratuberculosis*: A systematic review. *J Appl Microbiol,* 107 (4): 1061-1071, 2009. DOI: 10.1111/j.1365-2672.2009.04286.x

8. Economou M, Pappas G: New global map of Crohn's Disease: Genetic, environmental, and socioeconomic correlations. *Inflamm Bowel Dis,* 14 (5): 709-720, 2008. DOI: 10.1002/ibd.20352

9. Dağlı Ü: Turkish Inflammatory Bowel Disease Society recommendations on selected topics of Crohn's Disease. *Turk J Gastroenterol,* 21 (1): 78-79, 2010. DOI: 10.4318/tjg.2010.0059

10. Behr MA: Paratuberculosis: Organism, Disease, Control. **In,** Collins DM (Ed): Paratuberculosis: Organism, Disease, Control. AgResearch, New Zealand, ISBN: 978 1 84593 613 6, 2010.

11. NAC: Assessment of food as a source of exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP). *J Food Prot,* 73 (7): 1357-1397, 2010.

12. Mihajlovic B, Klassen M, Springthorpe S, Couture H, Farber J: Assessment of sources of exposure for *Mycobacterium avium* subsp. *paratuberculosis* in food and water. *Int Food Risk Anal J,* 1 (2): 1-22, 2011.

13. Gill CO, Saucier L, Meadus WJ: *Mycobacterium avium* subsp. *paratuberculosis* in dairy products, meat, and drinking water. *J Food Prot,* 74 (3): 480-499, 2011. DOI: 10.4315/0362-028X.JFP-10-301

14. Khol JL, Baumgartner W: Examples and suggestions for the control of paratuberculosis in European cattle. *Jpn J Vet Res,* 60 (Suppl.): S1-S7, 2012.

15. Alibaşoğlu M, Ertürk E, Yücel N: Türkiye'de rastlanan ilk keçi paratüberküloz olayları üzerinde patolojik incelemeler. *Ankara Üniv Vet Fak Derg*, 20 (1): 43-63, 1973.

16. Karadaş E, Metün, N, Yaman Ü: Elazığda bir keçide gözlenen ilk paratüberküloz olgusunda patolojik bulgular. *Turk J Vet Anim Sci*, 24, 317-323, 2000.

17. Çetinkaya B, Muz A, Ertaş HB, Öngör H, Sezen İY, Gülcü HB: Süt ineklerinde Paratüberküloz prevalansının polimeraz zincir reaksiyonu (PZR) ile saptanması. *Turk J Vet Anim Sci*, 24, 371-379, 2000.

18. Yıldırım D, Civelek T: Prevalence of subclinical Paratuberculosis in dairy cattle in Uşak region. *Kafkas Univ Vet Fak Derg,* 19 (1): 121-126, 2012.

19. Makav M, Gökçe E: Seroprevalence of subclinical Paratuberculosisin cattle in Kars region. *Kafkas Univ Vet Fak Derg,* 19 (5): 913-916, 2013. DOI: 10.9775/kvfd.2013.8529

20. Ayele WY, Svastova P, Roubal P, Bartos M, Pavlik I: *Mycobacterium avium* subsp. *paratuberculosis* cultured from locally and commercially pasteurized cow's milk in the Czech Republic. *Appl Environm Microbiol*, 71, 1210-1214, 2005. DOI: 10.1128/AEM.71.3.1210-1214.2005

21. Fang Y, Wu WH, Pepper JL, Larsen JL, Marras, SAE, Nelson EA, Epperson WB, Christopher-Hennings J: Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples. *J Clin Microbiol,* 40 (1): 287-291, 2002. DOI: 10.1128/JCM.40.1.287-291.2002

22. Johnson-Ifearulundu YJ, Kaneene JB: Management-related risk factors for *M. paratuberculosis* infection in Michigan, USA, dairy herds. *Prev Vet Med,* 37: (1-4), 41-54, 1998. DOI: 10.1016/S0167-5877(98)00110-X

23. Naing L, Winn T, Rusli BN: Practical issues in calculating the sample size for prevalaence studies. *Arch Orofac Sci,* 1, 9-14, 2006.

24. Bull TJ, McMinn EJ, Sidi-Boumedine K, Skull A, Durkin D, Neild P, Rhodes G, Pickup R, Hermon-Taylor J: Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *J Clin Microbiol,* 41, 2915-2923, 2003. DOI: 10.1128/JCM.41.7.2915-2923.2003

25. Donaghy JA, Johnston J, Rowe MT: Detection of *Mycobacterium avium* ssp. *paratuberculosis* in cheese, milk powder and milk using IS900 and f57-based qPCR assays. *J Appl Microbio,* 110 (2): 479-489, 2011. DOI: 10.1111/j.1365-2672.2010.04905.x

26. FAO: Serosurveillance for Foot-and-Mouth Disease in Bulgaria. Session of the Research Group of the Standing Technical Committee of EuFMD. Appendix 10, 1999.

27. Ikiz S, Bagcigil AF, Seyyal AK, Ozgur NY, Lgaz A: Paratuberculosis in cattle in Turkey detected by PCR. *Med Weter,* 61, 881-883, 2005.

28. OIE - World Organisation for Animal Health: Word Animal Health in 2006. pp.7, Paris, France, 2006.

29. Mpatziou R, Ntafis V, Liandris E, Ikonomopoulos J, Xylouri E: Serological ınvestigation of IBR and MAP in dairy cows in Greece. *The 61st Annual Meeting of The European Association for Animal Production*, 23-27 August, Heraklion-Greece, pp.235, 2010.

30. Humphry RW, Cameron A, Gunn GJ: A practical approach to calculate sample size for herd prevalence surveys. *Prev Vet Med,* 65, 173-188, 2004. DOI: 10.1016/j.prevetmed.2004.07.003

31. Çetinkaya B, Erdoğan HM, Morgan KL: Risk factors for bovine Paratuberculosis. II. The multiple analysis of risk ractors for bovine Paratuberculosis. *Turk J Vet Anim Sci*, 21, 303-306, 1997.

32. Raizman EA, Wells SJ, Muñoz-Zanzi CA, Tavornpanich S: Estimated within-herd prevalence (WHP) of *Mycobacterium avium* subsp. *paratuberculosis* in a sample of Minnesota dairy herds using bacterial culture of pooled fecal samples. *Can J Vet Res,* 75 (2): 112-116, 2011.

33. van Schaik G, Pradenas FM, Mella NA, Kruze VJ: Diagnostic validity and costs of pooled fecal samples and individual blood or fecal samples to determine the cow- and herd-status for *Mycobacterium avium* subsp. *paratuberculosis*. *Prev Vet Med,* 82, 159-165, 2007. DOI: 10.1016/j.prevetmed.2007.05.018

34. Sohal JS, Sing SV, Subhodh S, Singh AV, Singh PK, Sheoran N, Sandhu K, Narayansamy K, Maitra A: *Mycobacterium avium* subspecies *paratuberculosis* diagnosis and strain typing - Present status and future developments. *Indian J Exp Biol,* 45, 843-852, 2007.

35. Özpınar H, Tezmen G, Gökçe İ, Tekiner İH: Detection of animal species in some meat and meat products by comparatively using DNA microarray and real time PCR methods. *Kafkas Univ Vet Fak Derg,* 19 (2): 245-252, 2013. DOI: 10.9775/kvfd.2012.7616

36. Özpınar H, Turan B, Tekiner İH, Tezmen G, Gökçe İ, Akıneden Ö: Evaluation of pathogenic *Escherichia coli* occurrence in vegetable s amples from district bazaars of İstanbul using Real Time PCR. *Lett Appl Microbiol*, 57 (4): 362-367, 2013. DOI: 10.1111/lam.12122

37. Christopher-Hennings J, Dammen MA, Weeks SR, Epperson WB, Singh SN, Steinlicht GL, Fang Y, Skaare JL, Larsen JL, Payeur JB, Nelson EA: Comparison of two DNA extractions and nested PCR, real-time PCR, a new commercial PCR assay, and bacterial culture for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces. *J Vet Diagn Invest,* 15 (2): 87-93, 2003.

38. Leite FL, Stokes KD, Robbe-Austerman S, Stabel JR: Comparison of fecal DNA extraction kits for the detection of *Mycobacterium avium* subsp. *paratuberculosis* by polymerase chain reaction. *J Vet Diagn Invest,* 25 (1): 27-34, 2013. DOI: 10.1177/1040638712466395

39. Englund S, Bolske G, Johansson KE: An IS900-like sequence found in a *Mycobacterium* sp. other than *Mycobacterium avium* subsp. *paratuberculosis. FEMS Microbiol Lett,* 209, 267-271, 2002. DOI: 10.1111/j.1574-6968.2002.tb11142.x

40. Akineden Ö, Fernández-Silva JA, Weirich S, Abdulmawjood A, Bülte M: Comparison of two decontamination procedures, three culture media, and real time-PCR assay for the detection of *Mycobacteriumavium* subsp. *paratuberculosis* (MAP) from artificially contaminated raw sausage. *Archiv fur Lebensmittelhygiene,* 62,150-156, 2011.

41. Tasara T, Stephan R: Development of an F57 sequence-based Real-Time PCR assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. *Appl Environ Microbio,* 71 (10): 5957-5968, 2005. DOI: 10.1128/AEM.71.10.5957-5968.2005

42. Herthnek D, Bölske G: New PCR systems to confirm real-time PCR detection of *Mycobacterium avium* subsp. *paratuberculosis. BMC Microbiol,* 6, 87, 2006. DOI: 10.1186/1471-2180-6-87

43. Slana I, Paolicchi F, Janstova B, Navratilova P, Pavlik I: Detection methods for *Mycobacterium avium* subsp. *paratuberculosis* in milk and

milk products: A review. *Vet Med Czech,* 53 (6): 283-306, 2008.

44. Schönenbrücher H, Abdulmawjoo A, Failing K, Bülte M: New triplex Real-Time PCR assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces. *Appl Environ Microbiol,* 74 (9): 2751-2758, 2008. DOI: 10.1128/AEM.02534-07

45. Ricchi M, Manini F, Cammi G, Donaghy J, Arrigoni N: Comparison of four different PCR methods for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. *Proceedings of the 10th International Colloquium on Paratuberculosis*, August 9-14, Minneapolis, Minnesota-USA. pp.56-59, 2009.

46. Diéguez FJ, González AM, Menéndez S, Vilar MJ, Sanjuán ML, Yus E, Arnaiz I: Evaluation of four commercial serum ELISAs for detection of *Mycobacterium avium* subspecies *paratuberculosis* infection in dairy cows. *Vet J,* 180, 231-235, 2009. DOI: 10.1016/j.tvjl.2007.11.004

47. Aly SS, Anderson RJ, Whitlock RH, Fyock TL, McAdams SC, Byrem TM, Jiang J, Adaska JM, Gardner IA: Cost-effectiveness of diagnostic strategies to identify *Mycobacterium avium* subspecies *paratuberculosis* super-shedder cows in a large dairy herd using antibody enzyme-linked immunosorbent assays, quantitative real-time polymerase chain reaction, and bacterial culture. *J Vet Diagn Invest*, 24 (5): 821-832, 2012. DOI: 10.1177/1040638712452107

48. Herthnek D, Nielsen SS, Lindberg A, Bölske G: A robust method for bacterial lysis and DNA purification to be used with real-time PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. *J Microbiol Methods,* 75 (2): 335-340, 2008. DOI: 10.1016/j.mimet.2008.07.009

49. Dzieciol M, Volgger P, Khol J, Baumgartner W, Wagner M, Hein I: A novel real-time PCR assay for specific detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis* in milk with the inherent possibility of differentiation between viable and dead cells. *BMC Res Notes,* 3, 251, 2010. DOI: 10.1186/1756-0500-3-251

50. Kralik P, Slana I, Kralova A, Babak V, Whitlock RH, Pavlik I: Development of a predictive model for detection of *Mycobacterium avium* subsp. *paratuberculosis* in faeces by quantitative real time PCR. *Vet Microbiol,* 149 (1-2): 133-138, 2011. DOI: 10.1016/j.vetmic.2010.10.009

Kafkas Univ Vet Fak Derg
21 (2): 253-258, 2015
DOI: 10.9775/kvfd.2014.12216

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

The Effect of Field Pea *(Pisum sativum)* Replacement in Starch- and Fiber-Based Post Weaning Transition Diets for 7.5 Month Old Beef Calves and Subsequent Effect on Feedlot Finishing Performance, Carcass Quality and Net Return [1]

Songül ŞENTÜRKLÜ [1,2] Douglas G. LANDBLOM [1]

[1] *Infrastructure for this study was provided by the Dickinson Research Extension Center (ND06253) and for the USDA/CSREES Cool Season Food Legume Special Program (BAK792-01)*

[1] Dickinson Research Extension Center, North Dakota State University, Dickinson, North Dakota, 58601 USA
[2] Çanakkale Onsekiz Mart Üniversitesi, BMYO, TR-17200 Çanakkale - TURKEY

KVFD-2014-12216 Received: 30.08.2014 Accepted: 30.12.2014 Published Online: 08.01.2015

Abstract

The research objective, during a 3-yr study, was to compare starch- and fiber-based 38 d weaning transition diets (WTD) to identify the effect on calf performance, feed intake and feed efficiency (FE). Subsequently, the effect of WTD on feedlot finishing performance and cow and calf net return was determined. Crossbred steer and female calves (Angus x Hereford x Gelbvieh; n=405; Age=7.5 month; BW=278±4.3 kg) were randomly assigned to six treatments (4 pen replicates/treatment) based on age and weaning weight. Starch-based WTD were formulated with soybean meal (SBM) and field pea (FP), and fiber-based diets were formulated with increasing levels of FP (0-30%). Pelleted WTD treatments were: 1-(CSBM) starch-base+SBM, 2-(CPEA) starch-base+FP, 3-(0PEA) fiber-base+0% FP, 4-(10PEA) fiber-base+10% FP, 5-(20PEA) fiber-base+20% FP, and 6-(30PEA) fiber-base+30% FP. The CSBM, 0PEA, 10PEA, and 20PEA treatments had greater gain and average daily gain (ADG) compared to the CPEA and 30PEA (P<0.01), and the CPEA treatment had the lowest gain and ADG (P<0.001). Dry matter intake (DMI) was greatest for the fiber-based 0PEA and 20PEA WTD treatments and lowest for the starch-based CSBM and CPEA (P<0.001), and WTD did not affect FE (P=0.39). In addition, feedlot finishing performance and carcass measurements were not affected by WTD (P>0.10). Therefore, we conclude that FP can replace up to 20% of fiber-based ingredients in WTD without affecting DMI and ADG. However, the highest cow-calf net return was from the CSBM, 20PEA and 30PEA WTD treatments (P<0.001).

Keywords: *Beef calf post weaning transition diet, Fiber-based diet, Field pea, Feedlot performance, Soybean meal, Starch-based diet*

Nişasta ve Lif Esaslı Sütten Kesim Sonrası Geçiş Rasyonlarına İkame Edilen Yemlik Bezelyenin 7.5 Aylık Yaştaki Besi Danalarının Daha Sonraki Besi Bitirme Performansı, Karkas Kalitesi ve Net Getirisi Üzerine Etkisi

Özet

Araştırmanın amacı, 3 yıllık çalışma süresince 38 gün nişasta ve lif esaslı sütten geçiş dönemi rasyonlarını (WTD) karşılaştırmak ve rasyonların buzağı performansı, yem tüketimi ve yemden yararlanma (FE) üzerine etkisini belirlemektir. Bunu takiben, WTD'lerinin besi bitirme performansı, inek ve buzağı net getirisi üzerine etkisi belirlenmiştir. Melez erkek ve dişi danalar (Angus x Hereford x Gelbvieh; n=405; Yaş=7.5 ay; BW=278±4.3 kg) yaş ve sütten kesim ağırlığı homojen olacak şekilde rastgele altı gruba (4 tekrar/grup) ayrılmıştır. Nişasta esaslı WTD'leri soya küspesi (SBM) ve yemlik bezelye (FP) ile lif esaslı WTD'leri de artan seviyelerde (%0-30) yemlik bezelye (FP) ile formüle edilmiştir. Peletlenmiş WTD grupları sırasıyla 1-(CSBM) nişasta-esaslı+SBM, 2-(CPEA) nişasta-esaslı+FP, 3-(0PEA) lif-esaslı+%0 FP, 4-(10PEA) lif-esaslı+%10 FP, 5-(20PEA) lif-esaslı+%20 FP, and 6-(30PEA) lif-esaslı+%30 FB şeklindedir. Canlı ağırlık ve ortalama günlük canlı ağırlık artışı (ADG) CSBM, 0PEA, 10PEA ve 20PEA gruplarında CPEA ve 30PEA gruplarına oranla daha yüksek (P<0.01) ve CPEA grubunda ise ağırlık ve ADG daha düşük bulunmuştur (P<0.001). Kuru madde tüketimi (DMI) lif esaslı 0PEA ve 20PEA WTD gruplarında en yüksek, nişasta esaslı CSBM ve CPEA gruplarında ise en düşüktür (P<0.001) ve WTD FE'i etkilememiştir (P=0.39). Ayrıca, besi sonu performansı ve karkas kriterleri WTD'den etkilenmemiştir (P>0.10). Bu nedenle, DMI ve ADG'ni etkilemeden, lif esaslı WTD'lerinde hammaddelerinin %20'lik kısmının yerini yemlik bezelyenin alabileceği sonucuna varılmıştır. Bununla birlikte, en yüksek inek-buzağı net getirisi CSBM, 20PEA ve 30PEA gruplarında bulunmuştur (P<0.001).

Anahtar sözcükler: *Etçi melez danaların sütten kesim sonrası geçiş diyeti, Lif-esaslı diyet, Yemlik bezelye, Açık besi, Soya küspesi, Nişasta-esaslı diyet*

İletişim (Correspondence)
+90 286 3162878
ssenturklu@comu.edu.tr; songuls2011@hotmail.com

INTRODUCTION

Beef cattle calves in the USA routinely nurse milk from their mothers for 6-7 months before weaning. Weaning is stressful. Therefore, management procedures that minimize stress during the critical change over, or transition period utilize weaning feeds that are part of a sound weaning management program. Calf weaning feed formulations are prepared with a variety of highly digestible fiber-based co-product ingredients (soybean hulls [1,2], wheat middlings [2] and barley malt sprouts [2]. Although North Dakota research with field pea (FP) has shown FP to be an excellent feedstuff in creep feeds for grazing calves [3,4], and as a source of protein and energy in growing and finishing diets [5,6], FP have received less attention as an ingredient in weaning transition diets (WTD).

The FP contained more than 50% starch and are a protein and energy dense feed ingredient containing 20-27% crude protein (CP), 88-90% total digestible nutrients (TDN), 7-8% acid detergent fiber, and 1.40 Mcal ME/kg for cattle on a dry matter (DM) basis [7]. FP protein is 78-94% rumen degradable [8]. The starch content of FP grain is of concern when FP are to be used as an ingredient in WTD, since starch, when introduced in forage-based diets, has been shown to decrease forage intake and (or) digestibility resulting in reduced performance [9,10] due to changes in TDN and rumen pH changes associated with starch-based grains like corn and FP. Canadian research with dairy cattle suggests that the degradability rate of pea starch is slower than that of conventional cereal grains such as barley, wheat and oats, and is similar to corn [11]. Due to the slower degradability of corn and FP, these ingredients may be nutritionally compatible with fiber-based ingredients.

The purpose of this calf weaning management research was to compare corn- and fiber-based 38 d WTD diets formulated with either soybean meal (SBM) or increasing levels of FP (0-30%) that replaced wheat middlings (midds) and barley malt sprouts. We hypothesized that dry matter intake (DMI) would increase with increasing FP level during the WTD period after weaning, but that WTD would not affect post weaning calf performance, feedlot finishing performance, carcass quality, or net return to retained ownership.

MATERIAL and METHODS

This research project was conducted at the Dickinson Research Extension Center, Ranch Headquarters, Manning, North Dakota, USA (47º11'34" N 102º50'17" W) in accordance with guidelines approved by The North Dakota State University Institutional Animal Care and use Committee (Protocol Approval Number A0209).

Over a 3-year period, six annually replicated treatment groups (n=405) of 7.5 month old crossbred steer and female calves (Angus x Hereford x Gelbvieh) with an average weight of 278±4.3 kg were weaned and randomly assigned to one of six pelleted WTD treatments based on age and weaning weight. The experimental treatment diets, which are described in detail in *Table 1*, consisted of two starch-based diets formulated with either corn or a combination of corn and FP. The starch-based diets were compared to fiber-based diets containing the highly digestible fiber ingredients soybean hulls, wheat middlings, and barley malt sprouts that were blended with FP. The WTD treatments evaluated were: 1- (CSBM) starch-base + SBM, 2- (CPEA) starch-base + FP, 3- (0PEA) fiber-base + 0% FP, 4- (10PEA) fiber-base + 10% FP, 5- (20PEA) fiber-base + 20% FP, and 6- (30PEA) fiber-base + 30% FP.

To test the WTD, four pen replicates of six to eight calves per pen were randomly assigned to treatment based on a 2-day starting weight and fed an average 38 d each year over the three year period. The pelleted weaning feeds *(Table 1)* were prepared as complete feeds and were medicated with decoquinate for coccidiosis control at the rate of 22.5 mg/45.4 kg body weight (BW). The calves were fed chopped alfalfa-bromegrass hay *(Medicago sativa* and *Bromus inermis*, 10.0% CP). The pelleted supplements shown in *Table 1* were formulated to be isonitrogenous, but not isocaloric, and were top dressed over the chopped alfalfa-bromegrass hay [12]. Using challenge feeding, chopped hay was replaced with the experimental weaning supplements until calves in the grain-based treatments (CSBM and CPEA) were consuming from 4.99 to 6.35 kg/head/day and up to 9.53 kg/head/day among the calves receiving the fiber-based FP supplements (0PEA, 10PEA, 20PEA, and 30PEA). Once on full supplement intake, the CSBM and CPEA treatments were consuming 67% of their intake as supplement and for the fiber-based treatments, 88% of their DMI was from supplement. The amount of supplement offered to calves receiving the SBM/Corn and FP/Corn treatments were limited due to the high level of starch present in these two supplements.

Five weeks before weaning all calves were vaccinated with Zoetis Bovi-Shield Gold-5 for bacterial and viral diseases and booster vaccinated at weaning with Zoetis One Shot Ultra for clostridial myonecrosis diseases and pneumatic pasturellosis.

At the end of the 38-d WTD evaluation, an average 2-day ending weight was recorded and the only the steer calves were shipped to a commercial feedlot (Decatur County Feed Yard, Inc., Oberlin, Kansas, USA) where they were grown and finished. At the end of the finishing period, the steers were slaughtered at the federally inspected Cargill Packing Plant, Ft. Morgan, Colorado, USA. The Decatur County Feedlot uses the ACCU-TRAC electronic cattle management system to determine slaughter time, which has been previously described by Senturklu and Landblom [13].

Data was analyzed using procedures of the Statistical

Table 1. *WTD ingredient composition and nutrient analysis (Dry matter)*
Tablo 1. *WTD ham madde kompozisyonları ve besin analizleri (Kuru madde)*

Ingredient Composition	**CSBM***	**CPEA***	**0PEA***	**10PEA***	**20PEA***	**30PEA***
Corn, %	77.801	31.365	10.0	10.0	10.0	10.0
FP*, %	0.0	62.046	0.0	10.0	20.0	30.0
SBM*, %	15.601	0.0	0.0	0.0	0.0	0.0
Soybean Hulls, %	0.0	0.0	39.421	38.226	37.086	35.628
Wheat Middlings, %	0.0	0.0	24.56	20.748	16.888	13.346
Barley Malt Sprouts, %	0.0	0.0	20.0	15.0	10.0	5.0
Limestone, %	0.85	0.85	0.3	0.3	.03	.03
Decoquinate (6.0%), %	0.0489	0.0386	0.0269	0.0269	0.0269	0.0269
Other**, %	5.7	5.7	5.7	5.7	5.7	5.7
Total, %	100.0	100.0	100.0	100.0	100.0	100.0
Nutrient Analysis						
CP*, %	16.0	16.1	16.5	16.2	16.0	15.7
TDN*, %	85.2	79.2	69.3	69.9	70.7	77.5
Crude Fiber, %	2.8	5.0	18.0	17.9	17.9	17.7
Fat, %	3.6	2.2	2.4	2.4	2.3	2.2
NEm, Mcal/kg	2.12	1.92	1.61	1.63	1.65	1.68
NEg, Mcal/kg	1.43	1.30	1.02	1.04	1.06	1.08

** **CSBM:** Starch-base+SBM, **CPEA:** Starch-base+FP, **0PEA:** Fiber-base+0% FP, **10PEA:** Fiber-base+10% FP, **20PEA:** Fiber-base+20% FP, and **30PEA:** Fiber-base+30% FP, **SBM:** Soybean meal, **FP:** Field pea, **CP:** Crude protein, **TDN:** Total digestible nutrients; ** Other; Beet Molasses, 5.0%; Salt, 0.50%, Dicalcium Phosphate (21%), 0.10%, Feedlot Trace Mineral Premix, 0.075%, Feedlot Vitamin Premix, 0.025%*

Analysis System [14]. Transition, finishing, carcass, income and expense data were analyzed as a randomized complete block design using the PROC GLM procedure of SAS and USDA quality grade was analyzed using Chi-Square procedures in PROC GENMOD. Orthogonal contrasts were made to compare Starch and Fiber, SBM and FP, and for linear (L), quadratic (Q), and cubic (C) effects. Effects for L, Q, and C were only discussed when a significant *F*-test was detected. Pen served as the experimental unit. Differences between the experimental treatment groups were considered significant at P≤0.05.

RESULTS

Weaning Transition Period

The effect of WTD over a period of three years on steer and female calves' performance during the 38-d post weaning period is shown in *Table 2*. The mean starting weight of the steer and female calves in the study was 278 kg (P=0.73). Ending weight comparison between fiber-based treatments and starch-based (corn) treatments did not differ (P=0.28). Comparing starch- versus fiber-based diets, calves that were fed the CSBM, 0PEA, 10PEA, and 20 PEA had greater gain and average daily gain (ADG) compared to the CPEA and 30PEA (P=0.0001) feed treatments. The calves fed the CPEA feed treatment had the slowest ADG. It is critically important that newly weaned calves begin eating as soon as possible after weaning, if a post-weaning growth depression is to be avoided. Daily DMI was greatest for calves receiving the fiber-based 0PEA and 20PEA WTD and lowest for those calves that were fed the starch-based CSBM and CPEA WTD, and intermediary for the 10PEA and 30PEA WTD (P=0.0001).When feed treatments formulated with either SBM or FP were compared, there was no difference for gain or ADG; however, there was a significant DMI difference for FP (P=0.05). Orthogonal L, Q, and C fiber analysis of the data was unremarkable for Q and C; however, a L fiber feed treatment relationship for gain (P=0.0001), ADG (P=0.0002), and DMI (P=0.0001) were identified. Among treatments, greater DMI corresponded to greater ADG and smaller DMI also corresponded to smaller ADG. Therefore, for feed efficiency (FE), there was no statistical difference identified between treatments (P=0.39). When pea replaced 30% of the fiber ingredients (30PEA), gain and ADG were reduced (P=0.0001), DMI was intermediary, but FE did not differ (P=0.39). Feed cost per unit of gain was lowest for the CSBM and 0PEA WTD; however, the feed cost per unit of gain for the starch-based CSBM diet was 11.6% less than the fiber-based 0PEA WTD.

Feedlot Finishing Period

For finishing, only the steer calves from each treatment were fed to finish and slaughtered for performance and carcass data evaluation *(Table 3)*. Steers that received a 20% FP replacement diet during the 38 d transitioning required numerically fewer days to slaughter (129 vs. 134 d). Feedlot starting weight did not differ (P=0.79) and averaged 333.0

kg. Ending weight also did not differ (P=0.99) and averaged 531.0 kg. ADG was uniform across treatments and there was no statistical difference between WTD treatments at the end of finishing. Due to the uniform performance of steers during the growing-finishing period, WTD fed immediately after weaning did not affect subsequent feedlot FE, which was similar across treatments (P=0.55).

Carcass Measurements

Carcass measurements have also been summarized in *Table 3*. Carcass measurements for hot carcass weight (P=0.97), rib eye area (P=0.33), marbling score (P=0.14), USDA yield grade (P=0.72) and USDA quality grade (P=0.77) were not affected by treatment. Across treatments 54.5% of steers graded choice compared to 68% among steers receiving a 20% FP replacement diet during the 38 d WTD period. The 13.5% increase in the number of carcasses grading USDA Choice or better was not significantly greater (P=0.77).

Enterprise Analysis

Enterprise analysis with respect to revenue that is returned to the cow-calf business, when ownership of the cattle remains in the cow-calf operator's possession, has been summarized in *Table 4*. Income from finished carcasses and direct production expenses to including WTD, weaning transition yardage charge, animal health care (vaccine and antibiotic), ACCU-TRAC electronic cattle management fee, transportation charge, and annual cow cost of production were used to estimate net return to the cow and calf enterprise. Highest return to the cow/calf enterprise of $247 was obtained with the CSBM WTD

Table 2. *Effect of 38 d WTD on calf growth performance and economics (3-year)*
Tablo 2. *38 günlük WTD'nin buzağı büyüme performansı ve ekonomisi üzerine etkisi (3-yıl)*

Calf Growth Performance	CSBM*	CPEA*	0PEA*	10PEA*	20PEA*	30PEA*		P-Value [e]			
							SEM*	TRT*	Starch vs Fiber	SBM* vs FP*	Fiber (L)*
Number of Calves	68	68	68	68	67	66					
Starting Weight, kg	275	279	277	276	286	274	7.25	0.73			
Ending Weight, kg	319	313	324	322	330	312	7.40	0.28			
Gain, kg	44[a]	34[c]	47[a]	46[a]	44[a]	38[b]	1.81	0.0001	0.0004	0.23	0.0001
ADG*, kg	1.16[a]	0.90[c]	1.24[a]	1.21[a]	1.16[a]	1.0[b]	0.05	0.0001	0.0003	0.25	0.0002
DMI*, kg	7.59[c]	6.88[d]	8.78[a]	8.24[b]	8.46[a]	7.80[bc]	0.21	0.0001	0.0001	0.05	0.0001
Feed:Gain, kg	6.54	7.64	7.08	6.81	7.29	7.80	0.50	0.39	0.18	0.35	0.12
Feed Cost/Head,$	37.53	35.03	46.14	44.21	44.30	41.10	0.96				
Feed Cost/kg Gain, $	0.85	1.03	0.98	0.96	1.00	1.08	0.03				

[a-d] Means with different superscripts within a line are significantly different, (P<0.05); [e] P-Values for treatment and orthogonal contrasts. Only linear (L) P-Value is shown; Quadratic and Cubic were NS; ****CSBM:*** *Starch-base+SBM,* ***CPEA:*** *Starch-base+FP,* ***0PEA:*** *Fiber-base+0% FP,* ***10PEA:*** *Fiber-base+10% FP,* ***20PEA:*** *Fiber- base+20% FP, and* ***30PEA:*** *Fiber-base+30% FP,* ***SEM:*** *Pooled standard error of the mean,* ***TRT:*** *treatments,* ***SBM:*** *Soybean meal,* ***FP:*** *Field pea,* ***L:*** *Linear,* ***ADG:*** *Average daily gain,* ***DMI:*** *Dry matter intake*

Table 3. *Feedlot finishing performance and carcass measurements (3-year)*
Tablo 3. *Besi sonu performansı ve karkas parametreleri (3-yıl)*

Feedlot Finishing Performance	CSBM*	CPEA*	0PEA*	10PEA*	20PEA*	30PEA*	SEM*	P-Value
Days at Feedlot	135	141	131	135	129	136	4.0	0.40
Starting Weight, kg	331	328	332	333	339	329	12.7	0.79
Ending Weight, kg	531	530	529	532	528	531	13.9	0.99
ADG*, kg	1.48	1.43	1.50	1.47	1.47	1.49	0.07	0.75
Feed:Gain	6.0	6.0	5.85	6.0	6.0	5.81	0.10	0.55
Carcass Measurements								
Hot Carcass Weight, kg	335	331	335	334	331	332	10.0	0.97
Ribeye Area, sq.cm.	77	75	75	78	76	75	0.16	0.33
Marbling Score	49.0	54.4	52.8	52.7	54.3	48.0	51.9	0.14
Yield Grade	3.15	3.21	3.27	3.11	3.21	3.14	0.07	0.72
Quality Grade	2.46	2.44	2.53	2.46	2.36	2.48	0.08	0.77
Percent Choice, %	56.0	55.3	53.2	55.8	68.0	52.0	56.8	0.15

** **CSBM:** Starch-base+SBM, **CPEA:** Starch-base+FP, **0PEA:** Fiber-base+0%FP, **10PEA:** Fiber-base+10%FP, **20PEA:** Fiber-base+20%FP, and **30PEA:** Fiber-base+30%FP, **SEM:** Pooled standard error of the mean, **ADG:** Average daily gain*

Table 4. *Feedlot income, expenses and net return to the cow and calf enterprise (3-year)*
Tablo 4. *İnek ve buzağı işletmesinin besi gelir, giderleri ve net getirisi (3-yıl)*

Parameter	CSBM*	CPEA*	0PEA*	10PEA*	20PEA*	30PEA*	SEM*	P-Value
Feedlot Economics								
Income								
Carcass Value, $	886	849	871	863	871	880	16.6	0.71
Expenses								
Calf Cost/Head, $	633	624	632	634	644	630	8.1	0.70
Feedlot Cost/Head, $	236	242	231	233	226	233	7.7	0.81
Finishing Net Return/Head, $	17	17	8	4	1	17		
Cow-Calf Enterprise Analysis								
Income								
Carcass Value, $	886	849	871	863	871	880		
Expenses								
Transition Feed Cost/Head, $	38	35	46	44	44	41		
Feedlot Feed Cost/Head, $	236	242	231	233	226	233		
Other**, $	365	365	365	365	365	365		
Total Expense, $	639	642	642	642	635	639		
Net Return to Cow and Calf Enterprise	247	207	229	221	236	241		

** **CSBM:** Starch-base+SBM, **CPEA:** Starch-base+FP, **0PEA:** Fiber-base+0% FP, **10PEA:** fiber-base+10% FP, **20PEA:** Fiber-base+20% FP, and **30PEA:** Fiber-base+30% FP, **SEM:** Pooled standard error of the mean; ** Other; Transition period yardage cost, $8.00/steer; annual cow maintenance cost, $341.00/cow; cattle transportation to feedlot cost, $16.00/steer*

supplement followed by $241 and $236 for the 30PEA and 20PEA WTD supplements, respectively. The CPEA WTD yielded the smallest return to the cow/calf enterprise of $207. The WTD tested that were formulated with 0PEA and 10PEA resulted in net returns per cow of $229 and $221, respectively.

DISCUSSION

Weaning protocols that promote DMI and ADG minimize weaning growth depression and help calves transition from grazing to a feedlot environment. In this evaluation of weaning transition protocols, the ratio of starch to fiber in the concentrate supplements ranged from high starch (CPEA) to high fiber (0PEA). Soybean hulls, wheat midds, and barley malt sprouts were the sources of highly digestible fiber and corn was the primary starch source. Dietary energy from grain is primarily from starch or nonstructural carbohydrate. Energy from forage is primarily from fiber or structural carbohydrate. When small amounts of starch-based ingredients (0.25-0.40% of BW or less) are fed in fiber-based diets, forage intake and digestibility are not adversely affected or may improve slightly. At higher starch intake, forage intake and digestibility decline affecting performance [9,10]. Changes in TDN that result from the partial replacement of fiber-based ingredients with starch-based ingredients is referred to as a negative associative effect and is dependent on forage quality and amount of grain (starch) fed. The onset of a negative relationship is variable, but when the production goal is to achieve maximum forage intake and digestibility, adding a starch-based grain to forage-based diets in which starch intake will be 0.4-0.5 percent of BW may lead to reduced forage digestibility [9,10,15].

In *Table 2*, FP replaced up to 30% of the fiber-based ingredients (soybean hulls, wheat middlings, and barley malt sprouts). With increasing level of FP, ADG and DMI increased up to 20% FP, but declined at the 30% FP level. Total weight gain was affected, but not FE. For CSBM, DMI declined, but ADG was not affected (P=0.0001), and for CPEA both DMI and ADG declined (P=0.0001).

Paralleling this current field investigation a companion intake and digestibility study was conducted to evaluate the effect of replacing fiber-based ingredients with increasing level of FP from 0-45% [16] in medium concentrate diets. The findings of Soto-Navarro [16] serve to explain the response observed in this study. In the companion study, DMI declined linearly with increasing FP level (P<0.07), which is in agreement with the current study and others [17] who supplemented gestating cows with increasing levels of FP grain, but does not agree with others who reported an increase in DMI when barley and SBM [18] or barley and canola meal [19] were replaced with FP. Explanation for the decline in DMI in our study may be explained by reported results for fluid dilution rate [16], in which DMI declined linearly with increasing FP level. Compared to the current study, the highest fluid dilution rate was associated with the 0% FP level, which was also associated with the greatest DMI. Decreased DMI has also been associated

with decreased passage rate [20]. Similarly, for the CPEA and CSBM, DMI was reduced and could be explained by fluid dilution rate in which substrate passage was probably slowed compared to the WTD supplemented with increasing levels of FP. In the paralleling digestibility study, organic matter (OM) intake and total-tract OM digestibility were not affected by increasing FP level, which was not expected, because the TDN level of FP is higher than the combined TDN value of the fiber ingredients that FP replaced.

In a 39-d weaned calf receiving diet study, intake, digestibility, and feedlot performance were evaluated [21]. Compared to our experiment in which DMI and ADG declined when corn-pea (CPEA) was fed, they [21] measured an increase in DMI (P≤0.07) and BW gain (P≤0.04) when corn- FP, corn-chickpea, and corn-lentil receiving diets were fed. Considering the results of others that studied receiving (42-d) [5,22], growing [23,24] and finishing [6,25] cattle diets supplemented with FP, many have reported no change or increases in DMI, gain, and gain efficiency.

In our study, the carryover effect of WTD on ending carcass measurements was unremarkable and consistent with others [6,25,26] that found no difference between treatments that evaluated the effect of FP on carcass measurements. However, FP has been shown to improve meat tenderness [25,26] when fed in high grain diets without altering performance.

In conclusion, based on the results of the current study and the results of the paralleling digestibility study and others, up to 20% FP can replace fiber-based ingredients in 38 d WTD without negatively affecting DMI or ADG. Moreover, our data suggests that when ownership of calves continues through finishing and slaughter, the highest net return to the cow and calf enterprise will be from WTD feed treatments where DMI and ADG were less and feed cost per kilogram of gain was lowest. The decision to consider using FP in WTD should be based on cost per unit of protein compared to other protein-energy sources.

REFERENCES

1. Ferreira EM, Pires AV, Susin I, Mendes CQ, Gentil RS, Araujo RC, Amaral RC, Loerch SC: Growth, feed intake, carcass characteristics, and eating behavior of feedlot lambs fed high-concentrate diets containing soybean hulls. *J Anim Sci*, 89, 4120-4126, 2011. DOI: 10.2527/jas.2010-3417

2. Lardy GP, Anderson VL: Alternative feeds for ruminants. North Dakota State University Extension Bulletin, AS-1182, 2007.

3. Anderson VL: Field peas in creep feed for beef calves. North Dakota State University Carrington Research Extension Center Beef and Bison Field Day Proc. 22:1, 1999.

4. Landblom DG, Poland WW, Lardy GP: Application of salt-limited pea/wheat midds creep diets in Southwestern North Dakota. North Dakota Cow/Calf Conference and Beef Cattle Range Research Report, Bismarck, North Dakota, 2000.

5. Anderson VL, Stoltenow CS: Field peas in preconditioning diets for beef calves. *J Anim Sci,* 82 (Suppl.1): 65 (Abstr), 2004.

6. Lardy GP, Loken BA, Anderson VL, Larson DM, Maddock-Carlin KR, Ilse BR, Maddock R, Leupp JL, Clark R, Paterson JA, Bauer ML: Effects of increasing field pea *(Pisum sativum)* level in high-concentrate diets on growth performance and carcass traits in finishing steers and heifers. *J Anim Sci,* 87, 3335-3341, 2009. DOI: 10.2527/jas.2009-1785

7. Anderson VL, Lardy GP, Ilse B: Field pea grain for beef cattle. http://www.ag.ndsu.edu/ pubs/ansci/beef/as1301.pdf. *Accessed*: 08.01.2014.

8. Aufrere J, Graviou D, Michalet-Doreau B: Degradation in the rumen of proteins of 2 legumes: Soybean meal and field pea. *Reprod Nutr Dev*, 34, 483, 1994. DOI: 10.1051/rnd:19940509

9. Chase CC Jr, Hibberd CA: Utilization of low-quality native grass hay by beef cow fed increasing quantities of corn grain. *J Anim Sci,* 65, 557-566, 1987.

10. Sanson DW, Clanton DC: Intake and digestibility of low-quality meadow hay by cattle receiving various levels of whole shelled corn. *J Anim Sci,* 67, 2854-2862, 1989.

11. Robinson PH, McQueen RE: Non-structural carbohydrates in rations for dairy cattle. *Proc. 10th Western Nutr. Conf. Univ. Saskatchewan, Saskatoon, Saskatchewan,* Canada. p.153, 1989.

12. NRC: Nutrient Requirements of Beef Cattle. 7th ed., Natl. Acad. Press, Washington, DC, 1996.

13. Senturklu S, Landblom DG: Effect of post-weaning steer diets supplemented with field pea, flaxseed and a field pea-flaxseed combination on feedlot finishing performance, carcass quality and immune response. *Kafkas Univ Vet Fak Derg*, 20 (2): 295-300, 2014. DOI: 10.9775/kvfd.2013.10078

14. SAS: SAS/STAT Institute Inc. Cary, NC 27513, 2008.

15. Pordomingo AJ, Wallace JD, Freeman AS, Galyean ML: Supplemental corn grain for steers grazing native rangeland during summer. *J Anim Sci,* 69, 1678-1687, 1991.

16. Soto-Navarro SA, Williams GJ, Bauer ML, Lardy GP, Landblom DG, Caton JS: Effect of field pea replacement level on intake and digestion in beef teers fed byproduct-based medium concentrate diets. *J Anim Sci,* 82, 1855-1862, 2004.

17. Encinias AM, Scheaffer AN, Radunz AE, Bauer ML, Lardy GP, Caton JS: Influence of field pea supplementation on intake and performance of gestating beef cows fed grass hay diets. *Can J Anim Sci,* 80, 766 (Abstr.), 2000.

18. Poland WW, Landblom DG: Feeding value of field pea and hull-less oat in growing calf diets. *J Anim Sci*, 74 (Suppl. 1): 279 (Abstr), 1996.

19. Okine E: Feeding peas to backgrounding cattle. Western Forage/Beef Group. http://www1.agric.gov.ab.ca/$department/newslett.nsf/all/wfbg33?Open, *Accessed:* 08.01.2014, 2013.

20. Guthrie MJ, Wagner DG: Influence of protein or grain supplementation and increasing levels of soybean meal on intake, utilization and passage rate of prairie hay in beef steers and heifers. *J Anim Sci,* 66, 1529-1537, 1988.

21. Gilbery TC, Lardy GP, Soto-Navarro SA, Bauer ML, Anderson VL: Effect of field peas. Chickpeas, and lentils on rumen fermentation, digestion, microbial protein synthesis, and feedlot performance in receiving diets for beef cattle. *J Anim Sci,* 85, 3045-3053, 2007.

22. Anderson VL, Schoonmaker JP: Effect of pulse grains on performance of newly weaned steer calves. North Dakota State University Carrington Research Extension Center Beef Production Field Day Proceedings, 27:6, 2004.

23. Reed JJ, Lardy GP, Bauer ML, Gilbery, TC Caton JS: Effect of field pea level on intake, digestion, microbial efficiency, ruminal fermentation, and in situ disappearance in beef steers fed growing diets. *J Anim Sci,* 82, 2123-2130, 2004.

24. Chen JQ, Okine EK, Price MA, Khorasani GR: Feeding value of peas for backgrounding heifers. *Can J Anim Sci,* 83, 779-786, 2003. DOI: 10.4141/A03-010

25. Jenkins KH, Vasconcelos JT, Hinkle JB, Furman SA, De Mello AS Jr, Senaratne LS, Calkins CR: Evaluation of performance, carcass characteristics, and sensory attributes of beef from finishing steers fed field peas. *J Anim Sci,* 89, 1167-1172, 2011. DOI: 10.2527/jas.2009-2552

26. Carlin KM, Lardy GP, Maddock RJ, Ilse B, Anderson VL: Field pea inclusion in high grain diets for beef heifers improves beef tenderness without altering performance. *J Anim Sci,* 84 (Suppl. 1): 36 (Abstr.), 2006.

Kafkas Univ Vet Fak Derg
21 (2): 259-263, 2015
DOI: 10.9775/kvfd.2014.12233

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Determining Gene Expression Profile of GPX 1 in the Liver of Diabetic Rats Treated with Capsaicin by Real-Time PCR [1]

Aytül KÜRÜM [1] Hakan KOCAMIŞ [1] Turgay DEPREM [2] Mehmet Ulaş ÇINAR [3]

[1] *This study was supported by Kirikkale University Scientific Research Coordination Unit (Project no. 2012/51)*

[1] Kirikkale University, Faculty of Veterinary Medicine, Department of Histology and Embryology, TR-7145 Kirikkale - TURKEY
[2] Kafkas University, Faculty of Veterinary Medicine, Department of Histology and Embryology, TR-36100 Kars - TURKEY
[3] Erciyes University, Faculty of Agriculture Department of Animal Science, TR-38039 Kayseri - TURKEY

Article Code: KVFD-2014-12233 Received: 03.09.2014 Accepted: 05.12.2014 Published Online: 11.12.2014

Abstract

The purpose of the present study was to determine the Glutathione Peroxidase 1 (GPX 1) gene expression by Real Time PCR in the liver of healthy and diabetic rats treated with capsaicin. Twenty Sprague-Dawley rats were used in our study. Rats were divided into four groups: Group I: diabetic rats (n=5), Group II: capsaicin injected rats (n=5), Group III: Capsaicin injected diabetic rats (n=5), and Group IV: control rats (n=5). Capsaicin injection began 72 h after streptozotocin (STZ) injection. Capsaicin (1 mg/kg) was prepared in 10% ethanol, 1% Tween 20, and 80% sterile water and subcutaneously injected daily in both Groups II and III for two weeks. The results of RT-PCR conducted to determine GPX 1 gene expression indicated that capsaicin treated diabetic rats had higher GPX 1 expression compared to all groups including control ($P<0.05$). As a result, capsaicin causes an increase in GPX 1 gene expression at transcription level in the diabetic rat liver. Further investigation is needed to determine if such an increase occurs at protein level using various methods such as western-blot analysis.

Keywords: *GPX 1, RT-PCR, Capsaicin, Diabetes, Liver, Rat*

Kapsaisin Uygulanan Diabetik Sıçanların Karaciğer Dokusunda GPX 1'in Real Time-PCR ile Expresyon Profilinin Çıkarılması

Özet

Bu çalışmada, Kapsaisin (CAP) uygulanan, sağlıklı ve diabetik sıçanların karaciğer dokusunda Glutatyon Peroksidaz 1'in (GPX 1) RT-PCR ile belirlenmesi amaçlanmıştır. Araştırmada 20 adet Sprague-Dawley ırkı sıçan, Diyabet (Grup I), Sadece Kapsaisin Uygulanan (Grup II), Kapsaisin uygulanmış Diabetli (Grup III) ve Kontrol (Grup IV) olmak üzere dört gruba ayrıldı. Kapsaisin uygulamasına, streptozotosin (STZ) enjeksiyonundan 72 saat sonra başlanıp, 2 hafta boyunca hem diabetik gruba hem de sadece kapsaisin uygulanan gruba her gün 1 mg/kg kapsaisin, %10 ethanol, %1 Tween 20 ve %80 distile su ile çözdürüldükten sonra subkutan olarak uygulandı. Karaciğerdeki GPX 1 geninin ekspresyonu için yapılan RT-PCR analizi sonucunda; kapsaisin uygulanan diabet grubunda GPX 1'in gen ekspresyonunun kontrol grubu da dahil diğer gruplara göre daha yüksek olduğunu belirlendi ($P<0.05$). Sonuç olarak diabette kullanılan kapsaisinin GPX 1 transkripsiyon artışına neden olduğu ancak protein seviyesinde bir artışa yansıyıp yansımadığının, western-blot gibi metodlarla da belirlenmesi gerektiği sonucuna varıldı.

Anahtar sözcükler: *GPX 1, RT-PCR, Kapsaisin, Diabet, Karaciğer, Sıçan*

INTRODUCTION

Diabetes mellitus is a metabolic disorder with three metabolic types that occurs due to insulin resistance or, rarely, deficient insulin secretion and is characterized by hyperglycemia [1,2]. Type I or the juvenile type develops as a result of autoimmune inflammation against pancreatic β cells while Type II occurs later in life, secondary to insulin resistance. Type III, also called the gestational diabetes, only occurs during pregnancy and is due to insulin resistance, or rarely to β cell failure. Oxidative stress has been implicated in both the pathogenesis and complications of three types of diabetes [1]. Under normal circumstances, the rates of formation and clearance of free radicals are in a fine

İletişim (Correspondence)
+90 532 7280729
aytululum@hotmail.com

equilibrium, which is referred to as oxidative equilibrium. An increase in the rate of formation or a decrease in the rate of clearance of free radicals is called oxidative stress [3,4]. In parallel to increasing oxidative stress, the amount of free radicals also increases [5]. The latter event causes lipid peroxidation, DNA damage, and inactivation of multiple enzymes [4]. While low levels of reactive oxygen species play a role in intracellular signaling pathways involving cell differentiation, cell progression, or halt of cell growth and apoptosis, their increasing doses such as those in oxidative stress causes metabolic disorders and damage to biological macromolecules [6]. Former studies have shown that diabetes leads to oxidative stress by lowering the antioxidant potential and increasing free radical formation rate [7]. It has been reported that hyperglycemia particularly induces oxidative stress in the cell [8,9]. Oxidative stress emerges not only in diabetes, but also in certain other pathological conditions such as cardiovascular diseases, aging, cancer, and neurological disorders [7]. In human body, there are plenty of endogenous and exogenous defensive mechanisms collectively called antioxidants to prevent formation of free radicals and neutralize their detrimental effects. Such mechanisms can be grouped as agents that prevent free radical formation or neutralize already formed free radicals; they may also be classified as enzymes and non-enzymes [10]. Glutathione peroxidase (GPX), which has been reported to exist in mitochondria and cytosol [11], is an endogenous enzyme [10], which is responsible from degradation of hydroperoxides [10,11]. It has been formerly reported that, depending on the tissue types, it has at least 5 isoforms in mammalian cells and GPX 1 is especially abundant in erythrocytes, kidneys, and liver [12]. As a chronic metabolic disorder, diabetes is characterized by increased oxidative stress. As a result, increased free radicals engage an interaction with nucleic acids, proteins, and lipids, ultimately causing loss of membrane integrity, functional and structural alterations in proteins, and genetic mutations [13]. Diabetes-induced oxidative stress renders liver and other tissues more susceptible to various complications [14]. Increased lipid peroxidation and reduced glutathione levels are characteristic in diabetes [15]. Moreover, oxidative stress plays an important role in eliciting diabetic complications and underlies its pathogenesis [16]. Majority of the data suggesting a role of oxidative stress in initiating diabetes comes from animal studies that have employed alloxan and streptozotocin (STZ) to induce diabetes [10]. In this study STZ an agent that destroy β cells in pancreas, by inhibiting N-Acetyl-β-D-Glucosaminidase enzyme, was used in order to induce diabetes [17].

Capsaicin, which was used in the study, is the active ingredient of hot pepper [18,19]. It has been suggested that capsaicin has certain effects on gastrointestinal, cardiovascular, respiratory, limbic, and thermoregulatory systems [20]. Particularly used for arthritis management, capsaicin inhibits superoxide anion formation and changes the redox state of the cell [18]. Capsaicin metabolism is similar in human, dog, and rat and it is rapidly metabolized by hepatic enzymes. In addition to the main metabolites in these species, namely 16-hydroxycapsaicin, 17- hydroxycapsaicin, and 16,17- dehydrocapsaicin; microsomes and S9 fractions in rats also produce vanillylamine and vanillic acid. It has been reported that capsaicin is activated in liver by mix-function oxidase systems and turned into an electrophilic intermediary substance, which is able to covalently bind to hepatic proteins [21]. It has been observed that capsaicin has *in vitro* regulatory functions on cellular growth and collagenase and prostaglandin synthesis in rheumatoid arthritis. It also regulates lymphocyte proliferation, antibody production, and neutrophil chemotaxis [18]. Capsaicin has been shown to be effective in diabetic neuropathies [22]. In a study performed in rats, it has been observed that capsaicin induced lipid mobilization in fatty tissue and lowered triglyceride levels in serum [23] and liver [18]. As a regulatory molecule having certain effects on fat and energy metabolism, capsaicin has also been reported to possess some anti-obesity properties by lowering the blood fat content and inhibiting proliferation of the white fat cells [24]. Furthermore, it has been suggested that capsaicin has oxidative stress lowering effects by increasing the levels of antioxidant molecules and enzymes especially in liver and erythrocytes [25].

In the present study, it was aimed to determine the mRNA expression of Glutathion Peroxidase 1 (GPX 1), an antioxidant enzyme, in liver tissues of capsaicin administered healthy rats and rats with STZ-induced experimental diabetes.

MATERIAL and METHODS

Experimental Animals

This study was conducted after obtaining the approval from Kirikkale University Animal Experiments Local Ethics Committee (No:23.02.2012/12). It enrolled 20 female Sprague-Dawley rats with an average age of 8-12 weeks. The rats were housed in standard cages with alternating 12-h light-dark cycles at a temperature of 22±2°C and an average humidity of 50±5%. They were fed ad libitum with standard rat feed and water. The rats were grouped into 4 groups each containing 5 rats. Group I: STZ Diabetes Group (n=5); Group II: Capsaicin only Group (n=5); Group III: Capsaicin administered STZ Diabetes Group (n=5); Group IV: Control Group (n=5). Group I and Group III were administered STZ (Sigma, St Louis, MO, USA) dissolved in fresh citrate tampon (pH 4.5; 0.1 M) via intraperitoneal (IP) route in a single dose of 45 mg/kg [26]. Then, a blood sample was obtained from the tail veins of the animals following an 8-h fasting period 72 h after STZ injection and rats having a blood glucose level of 200 mg/dl or higher measured by a hand glucometer (Accu-Chek-Go, Roche, Switzerland) were considered diabetic [27] and included in the study.

After the third day, when diabetes was confirmed, Group II and Group III were subcutaneously injected with capsaicin 1 mg/kg (Sigma, St Louis, MO, USA) (dissolved in 10% ethanol, 1% Tween 20, and 80% distilled water) every day for 2 weeks [28].

Tissue Sampling

Liver tissue samples were taken at 14th day after sacrifice of rats with cervical dislocation under ether anesthesia. Liver tissues harvested for molecular analysis were homogenized in Tri-Reagent (Sigma, St Louis, MO, USA) and stored at +4°C until the day of analysis.

RNA Isolation and c-DNA Synthesis

Total RNA isolation was performed using the Tri-Reagent (Sigma, St. Louis, MO, USA) obtained by modification of the guanidine isothiocyanate/Phenol-chloroform method described by Chomczynski and Sacci [29]. RNA concentration per microliter was measured at a wavelength of 260 nm. From each total RNA, a 4 µg sample was taken and cDNAs were obtained using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). The cDNAs were then stored at -20°C to be later used in real time PCR.

RT-PCR Analysis

Gene expression was carried out using Real-time PCR. Each PCR reaction contained 3 µl water, 1 µl forward primer (5'CAGTTCGGACATCAGGAGAAT3'), 1 µl reverse primer (5'AGAGCGGGTGAGCCTTCT 3') [30] and 10 µl SYBR Green Supermix (Roche) and 5 µl cDNA to a total volume of 20 ml. mRNA quantification was performed in LightCycler 480 instrument by using SYBR Green I reagents (Roche diagnostics, USA) PCR reaction was induced as 5 min in 95°C, 45 cycles in 10 sec at 95°C. Each analysis was conducted with 5 biological repeats and 3 technical repeats. Glyceraldehyde 3 Phosphate Dehydrogenase (GAPDH) was used for normalization of GPX I gene expression. Forward 5'ACCACAGTCCATGCCATCAC3' and reverse 5'TCCACCACC CTGTTGCTGTA 3' [31] primers were used for GAPDH gene expression. Normalization of gene expression was performed as described by Kayan et al.[32]. Normalization was done with the Delta Ct method ($\Delta Ct = Ct_{target\ gene} - Ct_{housekeeping\ gene}$).

Statistical Analysis

The arithmetical means of the technical repeats was compared with the SPSS software package using the t-test.

RESULTS

Examination of the gene expression in the samples revealed that only the capsaicin-administered diabetes group demonstrated a significant difference *(Table 1, Fig. 1)*. Compared with the control group, the capsaicin only group and the diabetic group had no significant differences in GPX I expression. In the capsaicin-administered diabetes group, on the other hand, GPX I's gene expression was found significantly higher compared to the other groups ($P<0.05$)

DISCUSSION

Under normal circumstances, the rate of production of reactive oxygen species (ROS) and the antioxidant defense system are in a fine equilibrium; an imbalance in favor of ROS results in oxidative stress [4]. There are enzymatic and non-enzymatic cellular antioxidant defense mechanisms to reduce the detrimental effects of ROS [33]. The main enzymes of the antioxidant defense mechanism, namely

Table 1. *Ct levels of the groups*
Tablo 1. *Grupların Ct değerleri*

Groups	Control	Capsaicin	Diabetes	Diabetes + Capsaicin
Ct Levels	9.07	8.58	9.79	4.56*

** P<0.05, n=5*

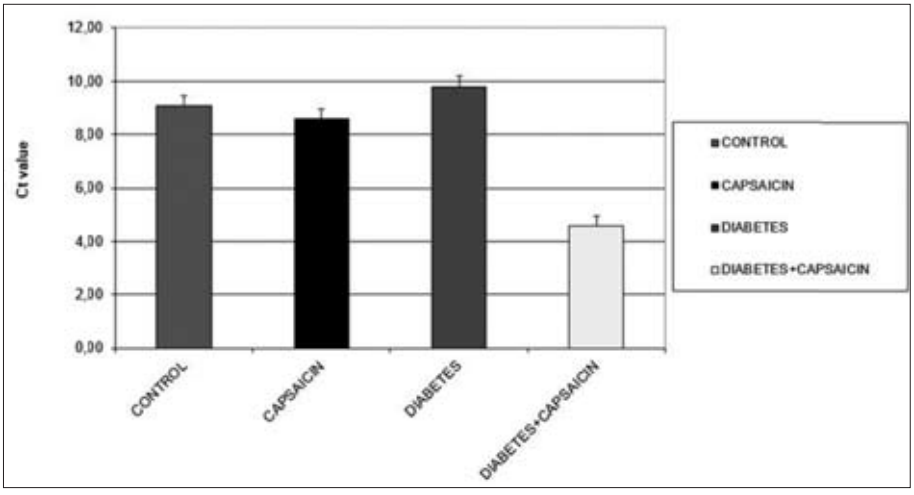


Fig 1. Ct levels of the groups (diabetes + capsaicin),* P<0.05, n=5

Şekil 1. Gruplara ait Ct değerleri (diabet + kapsaisin), * P<0.05, n=5

superoxide dismutase (SOD), glutathione peroxidase, and catalase are overwhelmed by excessive expression of ROS or chronic hyperglycemia. As a result, a vicious cycle is created in which ROS and RNS (Reactive nitrogen species) are incrementally produced and the oxidative stress pathways are activated [34].

Glutathione peroxidase (GPX), an enzymatic antioxidant [33], reportedly possess 5 isoforms in mammals and its levels vary by tissue type [6]. It has in vivo protective action against reactive oxygen species and is the first selenoprotein described in mammals [1]. It has cytosolic and mitochondrial forms and it reduces hydroperoxides of fatty acids [6]. Having been reported to possess regulatory functions on apoptotic signal pathways in various cells and tissues, GPX I has been implicated in the pathogenesis of many disease states including diabetes [1]. Studies on mice have demonstrated that GPX I overexpression strengthens cells against oxidative stress, while its absence promotes susceptibility to oxidative stress [11].

Liver is the main organ for free radical reactions, oxidation and detoxification processes. Therefore, biomarkers of oxidative stress are found elevated at early stage of many disorders [35]. It has been reported that the activity of many antioxidants are reduced [14,34], leading to increased oxidative stress in diabetes [34]. On the other hand, there are also some studies suggesting increased activity of antioxidant system [4,15]. Moreover, conflicting data have been reported by different studies on GPX expression during oxidative stress in rats with experimentally formed diabetes. For instance, both decreased [16,36] and increased [37,38] GPX enzyme expression compared to controls have been reported by separate studies in rats with STZ-induced diabetes. On the other hand, it has also been reported that, when compared with the controls, no significant difference was observed for GPX at hepatic mRNA level [39]. This study also observed that there was no significant difference between diabetic rats and the controls with respect to GPX expression. Such different results obtained in GPX expression of diabetic rats was attributed to various experimental conditions such as age and race of the rats and the duration of the experiment [16].

In the present study, no difference was found in the capsaicin only group compared to the control group with regard to GPX expression. Additionally, GPX I gene expression was found quite elevated in the capsaicin administered diabetes group compared to the controls. This was attributed both to capsaicin's inhibitory effects on oxidative stress [25] and, partially, free radical formation [40] by augmenting antioxidant molecules and enzymes, and also to its antioxidant actions originating from its phenolic OH groups [41] and its stimulant effect on antioxidant defense system [40].

In conclusion, this study suggests that capsaicin has a potential protective effect against hepatic oxidative stress in diabetes via triggering GPX I gene expression. It can be suggested that it needs to be verified by certain methods such as Western-Blot that that increase in transcription is reflected as an augmentation in protein level.

REFERENCES

1. Lei XG, Cheng WH, McClung JM: Metabolic regulation and function of glutathione peroxidase-1. *Annu Rev Nutr*, 27, 41-61, 2007. DOI: 10.1146/annurev.nutr.27.061406.093716

2. Maritim AC, Sanders RA, Watkins III JB: Diabetes, oxidative stress, and antioxidants: A review. *J Biochem Mol Toxicol*, 17 (1): 24-38, 2003. DOI: 10.1002/jbt.10058

3. Serafini M, Del Rio D: Understanding the association between dietary antioxidants, redox status and disease: Is the total antioxidant capacity the right tool? *Redox Rept*, 9 (3): 145-152, 2004. DOI: http://dx.doi.org/10.1179/135100004225004814

4. Sanders RA, Rauscher FM, Watkins JB III: Effects of quercetin on antioxidant defense in streptozotocininduced diabetic rats. *J Biochem Mol Toxicol*, 15, 143-149, 2001.

5. Şekeroğlu MR, Şahin H, Dülger H, Algün E: The effect of dietary treatment on erythrocyte lipid peroxidation, superoxide dismutase, glutathione peroxidase, and serum lipid peroxidation in patients with type 2 diabetes mellitus. *Clin Biochem,* 33 (8): 69-74, 2000. DOI: 10.1016/S0009-9120(00)00190-9

6. Mates JM, Perez-Gomez C, Castro IN: Antioxidant enzymes and human diseases. *Clin Biochem*, 32, 595-603, 1999. DOI: 10.1016/S0009-9120(99)00075-2

7. Modak MA, Ghaskadbi SS: Tissue specific oxidative stres profile in relation to glycaemic regulation in mice. *Diabetes Metab Res Rev,* 30, 31-41, 2014. DOI: 10.1002/dmrr.2460

8. Ceriello A: Oxidative stress and glycemic regulation. *Metabolism,* 49, 27-29, 2000. DOI: 10.1016/S0026-0495(00)80082-7

9. King GL, Loeken MR: Hyperglicemia-induced oxidative stress in diabetic complications. *Histochem Cell Biol,* 122, 333-338, 2004. DOI: 10.1007/s00418-004-0678-9

10. Akkuş I: Serbest Radikaller ve Fizyopatolojik Etkileri. Mimoza Yayınları, Konya, 1995.

11. McClung JP, Roneker CA, Mu W, Lisk DJ, Langlais P, Liu F, Lei XG: Development of insulin resistance and obesity in mice overexpressing cellular glutathione peroxidase. *PNAS*, 101 (24): 8852-8857, 2004. DOI: 10.1073/pnas.0308096101

12. Mates JM: Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicology,* 153, 83-104, 2000. DOI: 10.1016/S0300-483X(00)00306-1

13. Vincent AM, Russell JW, Low P, Feldman EL: Oxidative stress in the pathogenesis of diabetic neuropathy. *Endocr Rev,* 25, 612-628, 2004. DOI: 10.1210/er.2003-0019

14. Lim J, Ali ZM, Sanders RA, Snyder AC, Eells JT, Henshel DS, Watkins JB III: Effect of low-level light therapy on hepatic antioxidant defense in acute and chronic diabetic rats. *J Biochem Mol Toxicol,* 23, 1-8, 2009. DOI: 10.1002/jbt.20257

15. Rauscher FM, Sanders RA, Watkins JB III: Effect of coenzyme Q10 treatment on antioxidant pathways in normal and streptozotocin-induced diabetic rats. *J Biochem Mol Toxicol,* 15, 41-46, 2001. DOI: 10.1002/1099-0461(2001)15:1<41::AID-JBT5>3.0.CO;2-Z

16. Guerra JFC, Magalhaes CLB, Costa DC, Silva ME, Pedrosa ML: Dietary açai modulates ROS production by neutrophils and gene expression of liver antioxidant enzymes in rats. *J Clin Biochem Nutr,* 49 (3): 188-194, 2011. DOI: 10.3164/jcbn.11-02

17. Bingöl SA, Kocamış H: Sağlıklı ve diabet oluşturulmuş farelerin böbrek dokusunda katalaz enziminin RT-PCR ile gen ve immunohistokimyasal olarak protein ekspresyonu. *Kafkas Univ Vet Fak Derg,* 16 (5): 825-834, 2010.

18. Ahmad N, Mukhtar H, Aggarwal BB: Spices as potent antioxidant with therapeutic potential. **In,** Cadenas E, Packer L (Eds): Handbook of Antioxidants. Revised and Expanded 2nd ed. 437-453, CRC Press, Marcel

Dekker, New York-Basel, 2002.

19. Surh YJ, Lee SS: Capsaicin, a double-edged sword: Toxicity, metabolism and chemopreventive potential. *Life Sci,* 56, 1845-1855, 1995. DOI: 10.1016/0024-3205(95)00159-4

20. Şener E, Şahin S: Kapsaisin: Farmakokinetik, toksikolojik ve farmakolojik özellikleri. *Hacettepe Üniv Eczacılık Fak Derg,* 29, 149-163, 2010.

21. Chanda S, Bashir M, Babbar S, Koganti A, Bley K: *In vitro* hepatic and skin metabolismof capsaicin. *Drug Metabol Dispos,* 36, 670-675, 2008. DOI: 10.1124/dmd.107.019240

22. Forst T, Pohlmann T, Kunt T, Goitom K, Schulz G, Löbig M, Engelbach M, Beyer J, Pfützner A: The influence of local capsaicin treatment on small nerve fibre function and neurovascular control in symptomatic diabetic neuropathy. *Acta Diabetol,* 39, 1-6, 2002. DOI: 10.1007/s005920200005

23. Deprem T, Gülmez YN: Immunohistochemical localization of glutathione peroxidase 1 enzyme and its gene expression by RT-PCR in the liver tissue of healthy and diabetic mice. *Turk J Vet Anim Sci,* 38, 363-369, 2014. DOI: 10.3906/vet-1401-44

24. Baek J, Lee J, Kim K, Kim K, Kim D, Kim C, Tusutomu K, Ochir S, Lee K, Park CH, Lee YJ, Choe M: Inhibitory effects of capsicum annum l. water extracts on lipoprotein lipase activity in 3T3-L1 cells. *Nutr Res Pract,* 7 (2): 96-102, 2013. DOI: 10.4162/nrp.2013.7.2.96

25. Kempaiah RK, Srinivasan K: Influence of dietary curcumin, capsaicin and garlic on the antioxidant status of red blood cells and the liver in high-fat-fed rats. *Ann Nutr Metab,* 48, 314-320, 2004. DOI: 10.1159/000081198

26. Zafar M, Naqvi SNH: Effects of STZ-induced diabetes on the relative weights of kidney, liver and pancreas in albino rats: A comparative study. *Int J Morphol*, 28 (1): 135-142, 2010.

27. Kanitkar M, Bhonde R: Existence of islet regenerating factors within the pancreas. *Rev Diabet Stud,* 1, 185-192, 2004. DOI: 10.1900/RDS.2004.1.185

28. Moran C, Morales L, Roza RS, Apolonio J, Quiroz U, Chavira R, Dominguez R: Effects of sensorial denervation induced by capsaicin injection at birth or on day three of life, on puberty, induced ovulation and pregnancy. *Life Sci,* 73, 2113-2125, 2003. DOI: 10.1016/S0024-3205(03)00598-8

29. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem,* 162, 156-159, 1987. DOI: 10.1016/0003-2697(87)90021-2

30. Xiong Q, Xie P, Li H, Hao L, Li G, Qiu T, Liu Y: Acute effect of microcystins exposure on the transcription of antioxidant enzyme genes in three organs (liver, kidney, and testis) of male wistar rats. *J Biochem Mol Toxicol,* 24 (6): 361-367, 2010. DOI: 10.1002/jbt.20347

31. Nishikawa Y, Doi Y, Watanabe H, Tokairin T, Omori Y, Su M, Yoshioka T, Enomoto K: Transdifferentiation of mature rat hepatocytes into bile duct-like cells *in vitro*. *Am J Pathol*, 166 (4): 1077-1088, 2005. DOI: 10.1016/S0002-9440(10)62328-0

32. Kayan A, Çınar MU, Uddin MJ, Phatsara C, Wimmers K, Ponsuksili S, Tesfaye D, Looft C, Juengst H, Tholen E, Schellander K: Polymorphism and expression of the porcine tenascin C gene associated with meat and carcass quality. *Meat Sci*, 89, 76-83, 2011. DOI: 10.1016/j.meatsci.2011.04.001

33. Scandalios JG: Oxidative stress: Molecular perception and transduction of signals triggering antioxidant gene defenses. *Braz J Med Biol Res*, 38, 995-1014, 2005. DOI: 10.1590/S0100-879X2005000700003

34. Monroy MLdV, Fernández-Mejía C: Oxidative stress in diabetes mellitus and the role of vitamins with antioxidant actions. **In,** Morales-Gonzalez JA (Ed): Oxidative Stress and Chronic Degenerative Diseases - A Role for Antioxidants InTech. ISBN 978-953-51-1123-8 Available from: http://www.intechopen.com/books/oxidative-stress-andchronic-degenerative-diseases-a-role-for-antioxidants/oxidative-stress-in-diabetes-mellitus-and-the-role-of-vitamins-with-antioxidant-actions. DOI: 10.5772/51788

35. Stadler K, Jenev V, Bolcsh GV, Somogyi A, Jakus J: Increased nitric oxide levels as an early sign of premature aging in diabetes. *Free Radic Biol Med,* 35 (10): 1240-1251, 2003. DOI: 10.1016/S0891-5849(03)00499-4

36. Sindhu RK, Koo JR, Roberts CK, Vaziri ND: Dysregulation of hepatic superoxide dismutase, catalase and glutathione peroxidase in diabetes: response to insulin and antioxidant therapies. *Clin Exp Hypertens,* 26 (1): 43-53, 2004.

37. El-Bahr SM, El-Sabagh IM: Hepatic gene expresion of insulin like growth factor and selected antioxidants in diabetic rats treated with turmeric or black cumin seed. *British Biotechnol J,* 4 (7): 778-793, 2014. DOI: 10.9734/BBJ/2014/11121

38. Matsunami T, Sato Y, Sato T, Ariga S, Shimomura T, Yukawa M: Oxidative stress and gene expression of antioxidant enzymes in the streptozotocin-induced diabetic rats under hyperbaric oxygen exposure. *Int J Clin Exp Pathol*, 3 (2): 177-188, 2010.

39. Sadi G, Güray T: Gene expressions of mn-sod and gpx-1 in streptozotocin induced diabetes: Effect of antioxidants. *Mol Cell Biochem,* 327, 127-134, 2009. DOI: 10.1007/s11010-009-0050-4

40. Hassan MH, Edfawy M, Mansour A, Hamed AA: Antioxidant and antiapoptotic effects of capsaicin against carbon tetrachloride-induced hepatotoxicity in rats. *Toxicol Ind Health*, 28 (5): 428-438, 2011. DOI: 10.1177/0748233711413801

41. Okada Y, Tanaka K, Sato E, Okajima H: Kinetic and antioxidative site of capsaicin in homogeneous solution. *J Am Oil Chem Soc,* 87, 1397-1405, 2010. DOI: 10.1007/s11746-010-1628-4

Kafkas Univ Vet Fak Derg
21 (2): 265-269, 2015
DOI: 10.9775/kvfd.2014.12239

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Molecular Detection and Prevalence of *Chlamydophila psittaci* in the Blood, Liver and Muscle Tissue of Urban Pigeons *(Columba livia domestica)* in Iran

Meysam KHODADADI [1] Behsan HEMMATINEZHAD [1] Abbas DOOSTI [2]
Faham KHAMESIPOUR [3] Babafela AWOSILE [4]

[1] Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, IRAN
[2] Biotechnology Research Center, Shahrekord Branch, Islamic Azad University, Shahrekord, IRAN
[3] Young Researchers and Elite Club, Shahrekord Branch, Islamic Azad University, Shahrekord, IRAN
[4] Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, CANADA

KVFD-2014-12239 Received: 03.09.2014 Accepted: 09.01.2015 Published Online: 11.01.2015

Abstract

Chlamydophila psittaci (C. psittaci) is a widespread pathogenic bacterium in pigeons. These animals are mostly infected without any clinical signs. Pigeons are probably the most commonly reported chlamydia-infected avian species. Shedding of *Chlamydia* from infected birds has been widely reported. This study was conducted to detect and to determine the prevalence of *C. psittaci* in the blood, liver and muscle tissue of urban pigeons in Iran using conventional polymerase chain reaction. In this study, authors used 90 pigeons from different retail shops across Iran. The study was including 26 female and 64 male pigeons with suspected Chlamydiosis based on clinical signs. During examination of the corpses we took 270 samples in total, including blood, liver and muscle tissue from each animal. *C. psittaci* was detected in 16 (17.78%) blood samples, 14 (15.56%) liver samples and 5 (5.56%) samples of muscle tissue. This study supports the fact that pigeons serve as carriers of *C. psittaci*. Therefore, continuous surveillance of this bacterium will go along way in understanding the distribution and risks associated with *Chlamydia* infected pigeons. This will be beneficial in prevention and control risks of infection in humans.

Keywords: *Chlamydophila psittaci, Molecular detection, Prevalence, Urban pigeons, Iran*

İran'da Şehir Güvercinlerinde *(Columba livia domestica)* Kan, Karaciğer ve Kas Dokularında *Chlamydophila psittaci*'nin Moleküler Metotlarla Belirlenmesi ve Prevalansı

Özet

Chlamydophila psittaci (C. psittaci) güvercinlerde yaygın olarak bulunan patojen bir bakteridir. Bu hayvanlar çoğunlukla klinik belirti göstermeksizin enfektedirler. Güvercinler muhtemelen en çok Klamidya ile enfekte olan kanatlı türleridir. Enfekte kuşlardan Klamidyaların yayılımı sıklıkla rapor edilmiştir. Bu çalışma ile konvensiyonel polimeraz zincir reaksiyonu kullanılarak İran'da şehir güvercinlerinin kan, karaciğer ve kas dokularında *C. psittaci*'nin belirlenerek prevalansının ortaya konulması amaçlanmaktadır. Çalışmada İran'da değişik satış yerlerinden elde edilen 90 güvercin kullanıldı. Klinik belirtilere dayanarak klamidiyozis şüpheli olan bu güvercinlerin 26'sı dişi ve 64'ü erkekti. Her hayvanın kan, karaciğer ve kas dokularını içeren toplam 270 örnek hayvanlardan elde edildi. *C. psittaci* 16 (%17.78) kan örneğinde, 14 (%15.56) karaciğer örneğinde ve 5 (%5.56) kas dokusunda tespit edildi. Bu çalışma güvercinlerin *C. psittaci* için taşıyıcı olarak görev yaptığı bilgisini desteklemektedir. Bu nedenle sürekli takip ve kontrolün yapılması Klamidya ile enfekte güvercinlerin yaygınlığı ve buna ilişkin risklerin anlaşılması için uzun süreli devam ettirilmelidir. Böyle bir uygulama aynı zamanda insanlara enfeksiyonun yayılmasını kontrol altına almada da yararlı olacaktır.

Anahtar sözcükler: *Chlamydophila psittaci, Moleküler tespit, Prevalans, Şehir güvercini, İran*

INTRODUCTION

Chlamydophila psittaci (*C. psittaci*) is a Gram-negative and obligate intracellular bacterium, with nine (A to F, E/B, M56, and WC) known genotypes [1,2]. *C. psittaci* has been identified in 465 different bird species [3], but the highest rate of infection was found in parrots *(Psittacidae)* and pigeons *(Columbiformes)* [4,5]. The family *Chlamydiaceae*

İletişim (Correspondence)
+98 0913 4132858
dr_faham@yahoo.com; F.Khamesipour@iaushk.ac.ir

is divided into two genera: Genus *Chlamydia* with the species *C. muridarum*, *C. suis* and *C. trachomatis*, and genus *Chlamydophila* with the species *C. abortus*, *C. caviae*, *C. felis*, *C. pecorum*, *C. pneumoniae* and *C. psittaci* [6]. Genotypes are distinguished by sequencing of the outer membrane protein A (*ompA*) gene [7]. These bacteria are obligate intracellular organisms that are transmitted by biologically inactive particles called elementary bodies (EBs) [8]. *C. psittaci* is a bacterium that can be transmitted from pet birds to humans [9] and pigeons, but other bird species can be infected by same bacteria as well [10].

Urban or street pigeons are known to be reservoirs of *Chlamydia* and their zoonotic potentials have already been reported for decades [11]. Pigeons *(Columba livia domestica)* which are mostly located in towns and cities, especially of tourist attractions, are commonly infected with this bacterium. In human medicine this is the causative agent of psittacosis (also known as ornithosis) [12].

Many people especially children derived much pleasure in feeding pigeons during their leisure in city parks. Sometimes, these birds are kept as pets and are also housed within the living rooms, childcare facilities, garden centers and rest homes, which brings about a close interaction with humans [12,13].

Today, the increased pigeon population in major cities of the world is not only a major concern on environmental hygiene due to fecal droppings and fouling odor of buildings and monuments, but also associated risk of transmission infection from animals to humans. The most important pathogenic organism transmissible from feral pigeons to humans is *C. psittaci*, with 101 cases of disease reported in the literatures [10,14].

Exposure to *C. psittaci*-contaminated dust, pigeon feeding, and direct contact with pigeons to a lesser extent have been identified as risk of exposures in many of the human cases [15]. The principal route of human infection with *C. psittaci* is via the respiratory system, by inhaling infected aerosols of dried feces or respiratory secretions from infected birds. Other possible route of infection have been identified including direct contact with the feathers, tissue or secretions of infected birds, mouth-to-beak contact, or by bite wounds and the other open skin wounds, as well [16,17]. Person-to-person transmission is also possible [18] but it is thought to be rare.

Most infected pigeons are asymptomatic and they shed the organism occurs in feces as well as in respiratoric and conjunctival secretions. The clinical signs are often viewed after triggers like a stress, so this asymptomatic flow makes it difficult to assess the risk of bacteria transmission to other animals and humans [19,20].

Up until the 1990s, most epidemiological *C. psittaci* studies were based on serology. However, the significance in terms of worldwide dissemination of the agent is unclear [14]. The use of molecular techniques has enabled researchers in understanding the epidemiology of this pathogen in the past years [14]. There are several studies describing the *C. psittaci* carrier status of urban pigeon populations, especially from fecal droppings have been reported recently [10,21,22]. In Iran, there are available reports on molecular detection of *C. psittaci* in feces of pigeons [23-25]. However, in this study, we examined urban pigeons for detection of *C. psittaci* from the blood, liver and muscle tissue using molecular techniques in Iran.

MATERIAL and METHODS

All experiments were carried out under the ethical guidelines of the Islamic Azad University of Shahrekord Branch (92/910, in 2013).

Sample Collection

The pigeons were bought from different pigeon retail shops across Iran, where they were sold for food. Experiment criteria include pigeons those show clinical signs such as lethargy, anorexia, ruffled feathers, nasal discharge, diarrhea, and excretion of green to yellow-green feces. A total of 90 birds comprising of 26 female and 64 male pigeons were sampled between December 2013 and February 2014. The total number of samples were 270, including 90 blood, 90 muscle tissue and 90 liver samples and these were aseptically collected into well labeled sample bottles for detection of *ompA* gene of *C. psittaci* using PCR.

DNA Extraction

Genomic DNA was extracted from each sample with DNA extraction kit (CinnaGen, Iran), according to the manufacturer's instructions. The quality and quantity of extracted DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell [26]. The extracted DNA of each sample was kept frozen at -20°C until used. *C. psittaci* strain ATCC VR-125 (Genekam Biotechnology AG, Germany) was used as positive control and a negative-DNA control was performed by adding 1 µl of sterile ultrapure deionized water.

Gene Amplification

The *ompA* region was amplified by PCR using primers CPsitt-F (5′-GCTACGGGTTCCGCTCT-3′) and CPsitt-R (5′-TTTG TTGATYTGAATCGAAGC-3′) as described by Heddema for *ompA* region (accession number AB512087.1) [10]. Primers were analyzed at the NCBI using the experimental GENINFO BLAST Network Service to assess degree of homology between these primers and other reported sequences. The samples were placed in a thermal cycler (Mastercycler gradient, Eppendrof, Germany) with an initial denaturation step for 5 min at 95°C, then amplified for 30 cycles of denaturation for 1 min at 94°C, alignment for 1 min at 57°C,

extension for 1 min at 72°C and, final extension step for 7 min at 72°C. PCR products were separated by 2% agarose gel electrophoresis stained with solution of Ethidium Bromide and examined under Ultra Violet illumination (Uvitec, UK). The DNA molecular weight marker was used as a size marker.

Analysis

The prevalence analysis was computed in percentage and presented using simple frequency.

RESULTS

From a total of 90 urban pigeons *C. psittaci* was detected in 16 (17.78%) blood samples, 14 (15.56%) liver samples and 5 (5.56%) samples of muscle tissue *(Table 1)*. Higher prevalence was observed in the blood while lowest detection was recorded in the muscle tissue. The rate of detection was higher in the male compared to the female pigeons for all samples *(Fig. 1)*. The expected size of amplicons for *C. psittaci* is 1041 bp *(Fig. 2)*.

DISCUSSION

C. psittaci is a lethal intracellular bacterial species that causes avian Chlamydiosis, epizootic outbreaks in mammals and respiratory psittacosis in humans. The surveillance and its detection is essential in understanding the epidemiology of this bacteria and associated risks to humans. The detection of *C. psittaci* from pigeons in Iran as observed in this study further reinforce the fact that pigeon served as reservoir of infection and sometimes without clinical signs. To the best of our knowledge, this is the first study in Iran that detected *C. psittaci* from sample sources other than fecal droppings in pigeons. Available reports such as Doosti et al.[27], Doosti and Arshi [23] and Madani et al.[24], have all worked on the detection from cloacal swabs and fecal droppings. The prevalence of *C. psittaci* observed in this study (5.56-17.78%) were closely similar to that reported by Hedemma et al.[14], Doosti et al.[27] and Doosti and Arshi [23] but, lower than 23.5% reported by Madani et al.[24] in Iran. The reason for higher prevalence of *C. psittaci* in male pigeons from all the samples more than female pigeons is not clear. However, this may suggest that infection with *C. psittaci* in pigeons is sex dependent and this may incriminates sex as a risk factor of infection among pigeons. This may also suggest the increase risk of exposure to *C. psittaci* in humans who keep male pigeons as pet or come in contact with male pigeons frequently.

Aerosol transmission has been considered as the primary way of bacteria entry [28] causing respiratory disease in both mammals and birds [29]. Exposure to infected birds' feces, nasal discharges, and aerosol droplets are important transmission way as well. The detection of *C. psittaci* from muscle tissue and liver, as observed in this study, may suggest ingestion or food borne route as another means of exposure especially among animals who preyed on pigeons or human who eat pigeon meat (squab) as delicacies. The possibilities of occupational

Table 1. *Prevalence of C.psittaci in samples determined by PCR*
Tablo 1. *PCR ile doku örneklerinde belirlenen C. psittaci prevalansı*

Sex	Number of Samples	Prevalence N (%)		
		Muscle Tissue	Liver	Blood
Female	26	1 (3.85)	4 (15.39)	3 (11.54)
Male	64	4 (6.25)	10 (15.63)	13 (20.31)
Total	90	5 (5.56)	14(15.56)	16 (17.78)

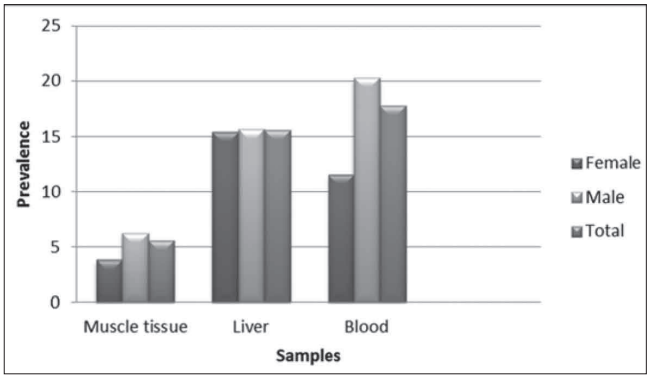


Fig 1. Distribution of *C. psittaci* in pigeons in the different samples and different sexes

Şekil 1. Güvercinlerin değişik örneklerinde ve cinsiyete göre *C. psittaci*'nin yaygınlığı

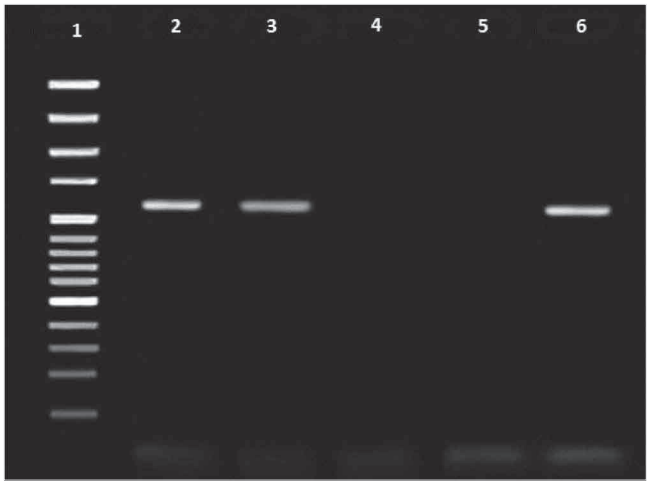


Fig 2. Ethidium bromide-stained agarose gel electrophoresis of PCR products (1041 bp) for detection of *ompA* gene in *Chlamydophila psittaci* in pigeon samples. Lane 1: DNA ladder (100 bp Ladders, Fermentas, Germany); lanes 2 and 3 (1041 bp): positive samples; lanes 4 and 5: negative samples and negative control. And lanes 6: positive control (1041 bp)

Şekil 2. Güvercin doku örneklerinde etidyum bromür ile boyanarak agaroz jel elektroforez ile belirlenen *Chlamydophila psittaci* ompA genini gösteren PCR ürünleri (1041 bp). 1. sütun: DNA merdiveni (100 bp merdiven, Fermentas, Almanya); 2. ve 3. sütunlar (1041 bp): pozitif örnekler; 4. ve 5. sütunlar sırasıyla negatif örnek ve negatif kontrol. 6. sütun: pozitif kontrol (1041 bp)

exposure during processing of pigeons for human consumption need to be considered as highest prevalence of *C. psittaci* spread. Dickx et al.[21] has reported detection of *C. psittaci* among the employees, chicken and turkey in a slaughterhouse in Belgium, and this further reinforce *C. psittaci* as an occupational hazard.

The detection of *C. psittaci* in the blood, liver and muscle tissue of pigeons may be very important in the pathogenesis of *C. psittaci* in pigeons. Page [30], in his work on experimental infection of turkey with *C. psittaci*, reported that *Chlamydia* were present in the blood, liver, spleen and kidney 48 h post inoculation and 72 h post inoculation in muscles, testes and ovaries. Later, *Chlamydia* was found in large number in cloaca and nasal turbinate. Furthermore, Vanrompay et al.[31], from their experiment on pathogenesis of *C. psittaci* in turkey, reported that Chlamydaemia was observed in these turkey before chlamydial replication could be detected in the digestive tracts, 3-5 days post infection. The higher detection of *Chlamydia* in blood in this study supported the possibility of early detection of *Chlamydia* in birds before detection from feces or cloacal swabs.

The prevalence of chlamydial infections in pigeons has been reported worldwide and is consistently high. The actual risk to humans of the infection from these birds is difficult to quantify. From this study, we concluded that pigeons serve as a reservoir of *C. psittaci* for other animals and humans. Also, male pigeons had higher prevalence of *C. psittaci* and possibly higher risk of infection to humans than female pigeons. Continuous surveillance of this bacterium will go along way in understanding the distribution and risks associated with *Chlamydia* infected pigeons. This will be beneficial in prevention and control of the infection in humans.

Acknowledgement

The authors would like to express their deep sense of gratitude and sincere thanks to the staff of the Biotechnology Research Center of Islamic Azad University of Shahrekord Branch in Iran. Also the authors would like to acknowledge the valuable contribution of Dr. Ismar Lutvikadic.

REFERENCES

1. Geens T, Desplanques A, Van Loock M, Bonner BM, Kaleta EF, Magnino S, Andersen AA, Everett KD, Vanrompay D: Sequencing of the *Chlamydophila psittaci ompA* gene reveals a new genotype, E/B, and the need for a rapid discriminatory genotyping method. *J Clin Microbiol,* 43, 2456-2461, 2005. DOI: 10.1128/JCM.43.5.2456-2461.2005

2. Van Lent S, Piet JR, Beeckman D, Van Der Ende A, Van Nieuwerburgh F, Bavoil P, Myers G, Vanrompay D, Pannekoek Y: Full genome sequences of all nine *Chlamydophila psittaci* genotype reference strains. *J Bacteriol*, 194, 6930-6931, 2012. DOI: 10.1128/JB.01828-12

3. Kaleta EF, Taday EM: Avian host range of *Chlamydophila* spp. based on isolation, antigen detection and serology. *Avian Pathol*, 32, 435-462, 2003. DOI: 10.1080/0307945031000159361 3

4. de Freitas RT, Júnior AB, Pinto AA: Evidence of *Chlamydophila psittaci* infection in captive amazon parrots in Brazil. *J Zoo Wildl Med*, 33, 118-121, 2002. DOI: 10.1638/1042-7260(2002)033[0118:EOCPII]2.0.CO;

5. Vanrompay D, Harkinezhad T, van de Walle M, Beeckman, D, van Droogenbroeck C, Verminnen K, Leten R, Martel A, Cauwerts K: *Chlamydophila psittaci* transmission from pet birds to humans. *Emerg Infect Dis*, 13, 1108-1110, 2007.

6. Everett KDE, Bush RM, Andersen AA: Emmended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol*, 49, 415-440, 1999.

7. Vanrompay D, Butaye P, Sayada C, Ducatelle R, Haesebrouck F: Characterization of avian *Chlamydia psittaci* strains using *omp1* restriction mapping and serovar-specific monoclonal antibodies. *Res Microbiol*, 148, 327-333, 1997. DOI: 10.1016/S0923-2508(97)81588-4

8. Binet R, Maurelli AT: Frequency of development and associated physiological cost of azithromycin resistance in *Chlamydia psittaci* 6BC and C. trachomatis L2. *Antimicrob Agents Chemother*, 51, 4267-4275, 2007.

9. Johnston WB, Eidson M, Smith KA, Stobierski MG: Compendium of measures to control *Chlamydia psittaci* infection among humans (psittacosis) and pet birds (avian chlamydiosis). *M M W R Weekly*, 49, 1-17, 2000.

10. Heddema ER, Ter Sluis S, Buys JA, Vandenbroucke-Grauls CM, van Wijnen JH, Visser CE: Prevalence of *Chlamydophila psittaci* in fecal droppings from feral pigeons in Amsterdam, The Netherlands. *Appl Environ Microbiol*, 72, 4423-4425, 2006. DOI: 10.1128/AEM.02662-05

11. Meyer KF: Pigeons and barn yard fowls as possible sources of human psittacosis or ornithosis. *Schweiz Med Wochenschr*, 44, 1377-1379, 1941. DOI 10.1099/jmm.0.034025-0

12. Magnino S, Haag-Wackernagel D, Geigenfeind I, Helmecke S, Dovc A, Prukner-Radovcic E, Residbegovic E, Ilieski V, Laroucau K, Donati M, Martinov S, Kaleta EF: Chlamydial infections in feral pigeons in Europe: review of data and focus on public health implications. *Vet Microbiol*, 135, 54-67, 2009. DOI: 10.1016/j.vetmic.2008.09.045

13. Haag D: Ein Beitrag zur Ökologie der Stadttaube. Dissertation, Phil. Nat. Fakultät der Universität Basel, Verlag Medizinische Biologie, 260 S, 1984.

14. Sachse K, Kuehlewind S, Ruettger A, Schubert E, Rohde G: More than classical *Chlamydophila psittaci* in urban pigeons. *Vet Microbiol*, 157, 476-480*, 2012. DOI: 10.1016/j.vetmic.2012.01.002*

15. Magnino S1, Haag-Wackernagel D, Geigenfeind I, Helmecke S, Dovc A, Prukner-Radovcić E, Residbegović E, Ilieski V, Laroucau K, Donati M, Martinov S, Kaleta EF: Chlamydial infections in feral pigeons in Europe: Chlamydial infections in feral pigeons in Europe: Review of data and focus on public health implications. *Vet Microbiol*, 135, 54-67, 2009. DOI: 10.1016/j.vetmic.2008.09.045

16. Crosse BA: Psittacosis: A clinical review. *J Infect,* 21, 251-259, 1990. DOI: 10.1016/0163-4453(90)93909-C

17. Salisch H, von Malottki K, Ryll M, Hinz KH: Chlamydial infections of poultry and human health. *World Poult Sci J*, 52, 279-308, 1996. DOI: 10.1079/WPS19960021

18. Ito I, Ishida T, Mishima M, Osawa M, Arita M, Hashimoto T, Kishimoto T: Familial cases of psittacosis: Possible person-to-person transmission. *Internal Med*, 41, 580-583, 2002.

19. Andersen AA, Vanrompay D: Avian chlamydiosis (psittacosis, ornithosis). **In,** Saif YM (Ed): Diseases of Poultry. 11th ed., 863-879, Iowa State University Press, Iowa, USA, 2003.

20. National Association of State Public Health Veterinarians (NASPHV): Compendium of measures to control *Chlamydophila psittaci* infection among humans (psittacosis) and pet birds (avian chlamydiosis). 2006.

21. Dickx V, Beeckman DS, Dossche L, Tavernier P, Vanrompay D: *Chlamydophila psittaci* in homing and feral pigeons and zoonotic transmission. *J Med Microbiol*, 59, 1348-1353, 2010. DOI: 10.1099/jmm.0.023499-0

22. Geigenfeind I, Haag-Wackernagel D: Detection of *Chlamydophila psittaci* from feral pigeons in environmental samples: Problems with currently available techniques. *Integr Zool*, 5, 63-69, 2010. DOI: 10.1111/j.1749-4877.2010.00187.x

23. Doosti A, Arshi A: Determination of prevalence of Chlamydophila psittaci by PCR in Iranian pigeons. *Int J Biol,* 3 (4): 79-82, 2011. DOI: 10.5539/ijb.v3n4p79

24. Madani SA, Peighambari SM, Barin A: Isolation of *Chlamydophila psittaci* from pet birds in Iran. *Iranian J Vet Med*, 5 (2): 95-98, 2011.

25. Mahzonieh M, Heidari khoei H, Ghasemi Shamsabadi M, Heidari F: Prevalence of *Chlamydophila psittaci* in pigeons in Chaharmahal va Bakhtiari and Yazd provinces of Iran, by nested-PCR, 2012. *Iran J Microbiol*, 7 (1): 1-6, 2013.

26. Sambrook J, Russell DW: Molecular Cloning: A Laboratory Manual. 3rd ed., 137-450, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2011.

27. Doosti A, Arshi A, Ghasemi Dehkordi P: Molecular study for detection of *Chlamydophila psittaci* in feces of pigeons in Chaharmahal Va Bakhtiari province. *J Microbial World*, 2 (4): 249-255, 2010.

28. Harkinezhad T, Geens T, Vanrompay D: *Chlamydophila psittaci* infections in birds: A review with emphasis on zoonotic consequences. *Vet Microbiol*, 135, 68-77, 2008. DOI: 10.1016/j.vetmic.2008.09.046

29. Smith KA, Bradley KK, Stobierski MG, Tengelsen LA: Compendium of measures to control *Chlamydophila psittaci* (formerly *Chlamydia psittaci*) infection among humans (psittacosis) and pet birds, 2005. *J Am Vet Med Assoc*, 226 (4): 532-539, 2005.

30. Page LA: Experimental omithosis in turkeys. *Avian Dis*, 3, 51-66, 1959.

31. Vanrompay D, Mast J, Ducatelle R, Haesebrouck F, Goddeeris BM: *Chlamydia psittaci* infections in turkeys: Pathogenesis of infections in avian serovar A, B and D. *Vet Microbiol*, 47, 245-256, 1995. DOI: 10.1016/0378-1135(95)00125-5

Kafkas Univ Vet Fak Derg
21 (2): 271-276, 2015
DOI: 10.9775/kvfd.2014.12251

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Comparison of Manual and Automated Nucleic Acid Extraction Methods for Detection of Peste Des Petits Ruminants Virus RNA [1]

Murat ŞEVİK [1] Oğuzhan AVCI [2] Ömer Barış İNCE [3]

[1] *This study has been presented at 5 th European Congress of Virology, 11-14 September 2013, Lyon, France*

[1] Molecular Microbiology, Veterinary Control Institute, TR-42080 Meram, Konya - TURKEY
[2] Department of Virology, Faculty of Veterinary Medicine, Selçuk University, TR-42000 Konya - TURKEY
[3] Agriculture and Rural Development Support Institution, TR-03000 Afyonkarahisar - TURKEY

KVFD-2014-12251 Received: 06.09.2014 Accepted: 29.10.2014 Published Online: 06.11.2014

Abstract

Peste des petits ruminants (PPR) is an economically important contagious disease of small ruminants. PCR-based techniques have been successfully used for rapid diagnosis of PPR. The method used for isolation of RNA from tissue samples is an important concern when using reverse transcription-PCR (RT-PCR) methods for the detection of PPR virus (PPRV). In this study, a commercial kit for manual preparation and an automated processing technique for RNA extraction were compared in terms of performance. Thirty-two small ruminants, each from different flocks, with PPR suspect submitted to laboratory were chosen to compare manual and automated extraction methods for the detection of PPRV. Vero cells were used for PPRV isolation. One-step RT-PCR was used for the detection of PPRV RNA. From the 32 submitted samples, CPE was observed in 11 samples. PPRV nucleic acid was detected in 11 of 32 samples that were manually extracted, while viral RNA was detected in 9 of 32 extracts prepared by the robot. Two samples that were negative with automated extraction were weakly positive in manual extraction. RNA quality and quantity were assessed using a spectrophotometer. According to the results, difference in quantity among two methods was statistically significant ($P<0.0001$, two-tailed paired t-test), and manual extraction method is suitable for detection of low amounts of PPRV RNA in clinical samples.

Keywords: *RNA purification, Manual, Automated, Quality, Quantity, RT-PCR*

Peste Des Petits Ruminants Virus RNA'sının Tespitinde Manuel ve Otomatik Nükleik Asit Ekstraksiyon Yöntemlerinin Karşılaştırılması

Özet

Koyun ve keçi vebası (PPR), küçük ruminantların ekonomik açıdan önemli, bulaşıcı bir hastalığıdır. Günümüzde PCR tabanlı teknikler, PPR'ın hızlı tanısı için başarıyla kullanılmaktadır. PPR virusunun (PPRV) tespitinde reverse transkripsiyon PCR (RT-PCR) metotları kullanılır iken, doku örneklerinden RNA izolasyonu için kullanılan yöntem önem arz etmektedir. Bu çalışmada, RNA ekstraksiyonu için ticari manuel bir kit ve otomatik bir işleme tekniği performans açısından karşılaştırılmıştır. Laboratuvara PPR şüphesi ile gönderilen herbiri farklı sürüden otuz iki küçükbaş hayvan, PPRV tespitinde manuel ve otomatik ekstraksiyon yöntemlerinin karşılaştırılması için seçilmiştir. Vero hücreleri PPRV izolasyonu için kullanılmıştır. PPRV RNA'sının tespiti için one step RT-PCR metodu kullanılmıştır. Otuz iki örnekten, 11 âdetinde sitopatojenik efekt (CPE) gözlenmiştir. Manuel ekstraksiyon metodu ile 32 örneğin 11'inde PPRV nükleik asidi tespit edilirken, robot kullanılarak yapılan otomatik ekstraksiyon metodu ile 9 örnekte viral RNA tespit edilmiştir. Otomatik ekstraksiyon metodu ile negatif tespit edilen 2 örnek, manuel ekstraksiyon metodu sonucu zayıf pozitif olarak tespit edilmiştir. Manuel ve otomatik ekstraksiyon sonucu elde edilen RNA miktarı ve kalitesi spektrofotometre cihazı kullanılarak karşılaştırılmıştır. Elde edilen sonuçlara göre, iki metot arasında elde edilen RNA miktar farkı istatiksel olarak önemli bulunmuş ($P<0.0001$, iki - kuyruklu t - testi) olup, klinik örneklerdeki düşük miktardaki PPRV RNA'sını tespit etmek için manuel ekstraksiyon metodu daha uygundur.

Anahtar sözcükler: *RNA purifikasyonu, Manuel, Otomatik, Kalite, Miktar, RT-PCR*

İletişim (Correspondence)
+90 332 3224741
dr_muratank@hotmail.com

INTRODUCTION

Peste des petits ruminants (PPR) is an acute and highly contagious viral disease of domestic and wild small ruminants that is characterized by fever, purulent ocular and nasal discharge, diarrhoea and enteritis [1]. Cattle, buffaloes and camels can become infected, although they are not susceptible to clinical disease [2].

The causative agent, peste des petits ruminants virus (PPRV), belongs to the genus *Morbillivirus* within family *Paramyxoviridae* along with rinderpest virus (RPV), measles virus (MeV), canine distemper virus (CDV), and morbilliviruses of marine mammals [3,4]. Based on the basis of partial sequence analysis of the fusion protein (F) and nucleoprotein (N) genes, PPRV can be grouped into four lineages [5-7]. PPR viruses belonging lineages I and II have been found exclusively in west and central Africa [7,8]. Lineage III has been isolated from eastern Africa and Arabian Peninsula whereas lineage IV has been isolated in Asia, Middle East and northern Africa [6,9-13].

Serological assays can be used to detect the presence or absences of antiviral antibodies [14]. Neutralization and isolation of virus in cell culture are time-consuming, and require special laboratory requirements, so they aren't suitable for routine diagnosis [6]. Recent advances in molecular biology have led to the development of reliable and faster diagnostic tests for diagnosis of PPR. Reverse transcription-PCR (RT-PCR) provides rapid, sensitive and reliable diagnosis of the disease [15,16]. Samples of nasal and ocular discharge and anticoagulant-treated blood from live animals or lymph nodes, especially the mesenteric and bronchial nodes, lungs, spleen and intestinal mucosa from necropsied animals are used for diagnosis PPR [14].

The method used for isolation of RNA from tissue samples is an important concern when using reverse transcription-PCR (RT-PCR) methods for the detection of PPRV. The aim of this study was to compare the performance of a manual extraction method, QIAamp (Qiagen), and an automated extraction instrument, MagNA Pure LC (Roche Applied Sciences), with each other for the detection of low amounts of PPRV RNA in clinical samples. The time, effort, and reagent costs for both methods were analysed. Furthermore, various routine RT-PCR methods were tested and compared using RNA extracted with both methods.

MATERIAL and METHODS

Samples and Positive Control

During January-December 2012, 32 animals (20 sheep and 12 goats), each from different flocks with no vaccination history, suspected to have PPR were submitted to the Veterinary Control Institute, Konya, Turkey. Tissue samples (lung, liver, spleen and mesenterial lymph node) were collected from 32 animals, aged between 1 and 24 months, were tested for PPR virus by RT-PCR. All tissue samples were kept at -85°C prior to sample preparation and the RT-PCR assays. Lyophilized freeze-dried live PPR vaccine (Nigeria75/1 vaccine strain) obtained from the Division of Virology, Etlik Central Veterinary Control and Research Institute, Ankara, Turkey, was used as the positive control.

Virus Isolation

Tissue samples of each animal were combined and homogenised in PBS containing antibiotics using tissue rupture (Qiagen, Valencia, CA) to give a 10% suspension. The suspensions were then centrifuged at 3.000 g for 15 min at 4°C. Supernatants were filtered (0.2-µm pore size) and then inoculated on to Vero cells, and maintained in Dulbecco's minimum essential medium supplemented with 5% foetal bovine serum. The cultures were incubated at 37°C in 5% CO_2 atmosphere and daily examined for appearance of cytopathic effect (CPE). All materials were passaged in Vero cell cultures for three times. If CPE was not observed even after 3 blind passages, the sample was considered negative. Supernatants of CPE-positive cultures were examined for nucleic acid of PPRV using RT-PCR.

Analytical Sensitivity and Specificity Experiments

Supernatants of CPE-positive cultures were collected and virus titres (PFU/ml) were determined on Vero cells in a standard plaque assay. Panels of PPRV were created by serial dilution (10^0 to 10^{-4} PFU/ml) in nuclease-free water (Qiagen, Valencia, CA). Virus dilutions were extracted by each method and PPRV RNA was detected by RT-PCR. Nuclease-free water (negative control) was used per extraction method.

RNA Extraction Methods

Viral RNA was extracted from supernatants of CPE-positive cultures using two different methods. Manual RNA extraction of the supernatants was performed with the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (140 µl sample input, 50 µl output). The MagNA Pure LC 2.0 system (Roche Applied Science, Indianapolis, IN, USA) was applied for RNA extraction from supernatants using with the Magna Pure LC total nucleic acid isolation kit (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's instructions (200 µl sample input, 100 µl sample output).

RNA Quantity and Purity

A spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Wilmington, U.S.A.) was used for the RNA concentration of each sample after RNA extraction. The ratio of the absorbance at 260nm and 280 nm was used to assess purity of RNA. A ratio of ~ 2.0 was used as a standard for pure RNA. Additionally, the ratio of the absorbance at

260 nm and 230 nm was calculated to assess purity of RNA, and considered to represent pure RNA within the range of about 1.8-2.0 [17].

RT-PCR Methods

The quality of manually and robotically extracted RNA was tested in two different RT-PCR methods. One-step RT-PCR was performed with primers (PPRVF1b/PPRVF2d) which amplify a 448 bp fragment on fusion (F) protein gene sequence [5], and primers (N1/N2) which amplify a 463 bp fragment on nucleocapsid (N) protein gene sequence [6] using One-step RT-PCR kit (Qiagen, Hilden, Germany). The assay was carried out in a 20 µl reaction mixture containing, 20 pmol of each primer, 4 µl of the 5Xone step RT-PCR buffer (Qiagen, Germany), 10 mmol dNTPs, 0.8 µl of One Step RT-PCR enzyme mix (Qiagen, Germany) and 3 microliters of the extracted RNA using a PTC 100 Thermal cycler (MJ Research Inc., USA). The amplification conditions used were reverse transcription step of 30 min at 50ºC and 15 min at 95ºC, followed by 40 cycles at 94ºC for 1 min, 50ºC for 1 min and 72ºC for 2 minutes and final extension step in 72ºC for 10 min. The PCR products were analysed on 1.5% agarose gel after electrophoresis at 90 V for 45 min.

Time and Cost Analysis

The cost analysis per sample for both manual and automated techniques was performed described by elsewhere [17]. The cost analysis per sample were included the commercial kit, reagents, consumables, and the total time requirements for processing.

Statistical Analysis

Pairwise comparison of manual and automated techniques was performed by using two-tailed paired t-test. $P<0.05$ was considered to be statistically significant. All statistical analysis was performed with GraphPad InStat version 3.10 (GraphPad Software, San Diego, CA, USA).

RESULTS

Virus Isolation

The PPRV isolates were successfully isolated in Vero cells at passage level one after 3-4 days of infection. From the 32 submitted animals, CPE was observed in tissue samples of 11 animals.

RNA Quantity and Purity

Supernatants of CPE-positive cultures (n=11) were used for the comparative analysis of RNA extraction and purification of the RNeasy Mini kit (Qiagen, Hilden, Germany) and the MagNA Pure LC 2.0 system (Roche Applied Science, Indianapolis, IN, USA). The quantity of RNA obtained with manual extraction was significantly ($P<0.0001$) higher than automated extraction. Samples that were manually extracted displayed a mean concentration of 303.3±38.7

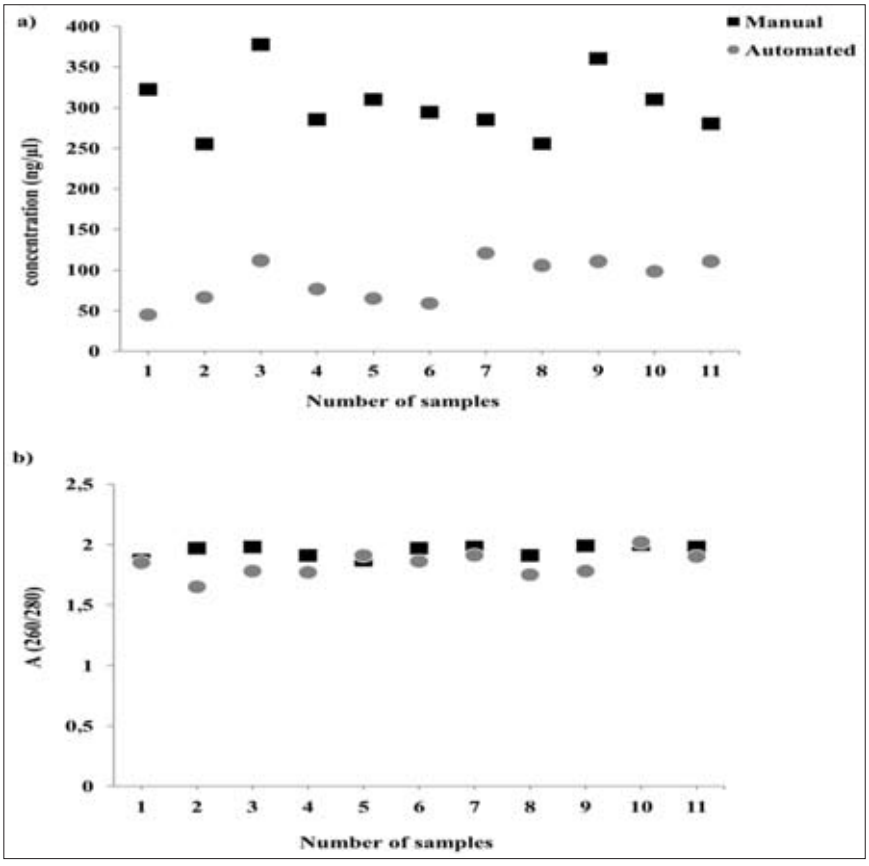


Fig 1. a- RNA concentration of eluate measured by spectrophotometer, **b-** RNA purity measured by spectrophotometer

Şekil 1. a- Spektrofotometre ile ölçülen eluatın RNA konsantrasyonu, **b-** Spektrofotometre ile ölçülen RNA saflığı

Table 1. *Analytical sensitivity of manual and automated methods*
Tablo 1. *Manuel ve otomatik metotların analitik sensitivitesi*

Extraction Method	No. Positive/Total no. Tested at a Viral Input (PFU/ml) of:				
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}
RNeasy Mini kit	11/11	11/11	10/11	5/11	0/11
MagNA Pure	11/11	10/11	9/11	4/11	0/11

Table 2. *Time and cost comparison for manual and automated RNA extraction*
Tablo 2. *Manuel ve otomatik RNA ekstraksiyonlarının zaman ve maliyet karşılaştırması*

Extraction Method	No. of Extracted Specimens	Total Time (min)/run[a]	Hands-on Time (min)/run	Cost (U.S. Dollars)/Sample
RNeasy Mini kit	32	100	85	5.31
MagNA Pure	32	95	20[b]	7.89[c]

[a] Total time includes hands-on time, [b] Hands-on time for the MagNA Pure does not include time necessary to homogenize stored tissue samples, [c] List price for kits with reagents for 96 extractions (the cost of plastics is not included)

ng/µl (range = 250-377.6 ng/µl) whereas extracts prepared by the automated extraction showed a mean concentration of 88±26.1 ng/µl (range 45-120.3 ng/µl; *Fig. 1a*).

Furthermore, the quality of extracts was assessed by comparing the 260/280 ratios of RNAs. The mean value of the manual extracts was 1.94±0.04 (range=1.87-2.0), and the automated extracts displayed a mean value of 1.78±0.23 (range=1.12–2.02; *Fig. 1b*).

The ratio of the absorbance at 260 and 280 nm (A260/280) was significantly different between manual and automated techniques (P=0.0327). Next, the ratio of the absorbance at 260 and 230 nm (260/230), used as a secondary measure of nucleic acid purity, ratio was determined. RNA extracted with both methods showed ratios below the optimum (manual: 1.16±0.09; automated: 1.7±0.4; P=0.0003).

Analytical Sensitivity of PPRV RT-PCR after Extraction of RNA by Manual and Automated Methods

The sensitivity of PPRV detection by RT-PCR after extraction by manual and automated methods was compared *(Table 1)*.

All CPE-positive samples (n=11) at 10^0 PFU/ml were detected by RT-PCR after manual and automated extraction, but no replicates of PPRV at 10^{-4} PFU/ml were detected by RT-PCR after extraction by all two methods. None of the negative controls was positive by RT-PCR after manual and automated extraction.

PPRV Detection in Clinical Specimens after Extraction by Manual and Automated Methods

In order to compare the efficacies of PPRV RNA extraction from different clinical specimens, tissue samples (including lung, liver, spleen and mesenterial lymph node) were extracted by each method and PPRV RT-PCR was performed with using F and N gene specific primers. The results of RT-PCR (based upon the PPRV F and N genes) were concordant in 30 of 32 samples that were extracted by all two methods (9 of 32 positive for PPRV RNA; 21 of 32 PPRV RNA were not detected). PPRV RNA was weakly detected in the two samples that were extracted manually whereas not detected by automated method.

Time and Cost Analysis

The amount of time required for sample processing and costs were compared for both manual and automated methods *(Table 2)*.

The times required for extraction of a comparable number of samples by the manual RNeasy and MagNA Pure methods were equivalent. However, the actual hands-on time was less for the automated compared to the manual extraction method. MagNA Pure reagents for RNA extraction were more expensive than RNeasy Mini kit.

DISCUSSION

Peste des petits ruminants is one of the important viral disease of small ruminants, and has been detected in all regions of Turkey since it was first officially reported in 1999 [18,19]. Different types of tests are available for diagnosis of PPR such as virus isolation, ELISA and RT-PCR. Virus isolation is not routinely available in diagnostic laboratories because of time consuming. RT-PCR methods are recommended by OIE for confirmation of clinical cases. Therefore, RT-PCR methods have been commonly used to diagnose PPR in many diagnostic laboratories in Turkey.

Extraction is the first step in RT-PCR methods. Extracted nucleic acid concentration and purity are important to obtain reliable results. In recent years, different manual and automated extraction methods are used for diagnosis of diseases. In this study, we assessed RNA extracts generated

by the manual (Qiagen, Hilden, Germany) and automated extraction (Roche Applied Science, Indianapolis, IN, USA) methods.

Our data demonstrate that manual and automated methods specificities were identical at 100%, and manual extraction's analytical sensitivity of PPRV detection by RT-PCR was better than the automated extraction *(Table 1)*. These results are consistent with those of Riemann et al.[17] who reported that quantity and quality of the generated DNAs were slightly higher using the manual extraction method. However, Knepp et al.[20] reported that analytical sensitivity of enterovirus detection by RT-PCR is similar after RNA extraction by manual and automated methods. A possible explanation for this result may be the differences between kits which were used for manual extraction. In this study, we used RNeasy Mini kit (Qiagen, Hilden, Germany), but they used QIAamp Viral RNA kit (Qiagen, Hilden, Germany) for manual extraction. For both extraction methods no false positive results were obtained when negative controls were tested. These findings in agreement with previous reports [20-22], but seems contrary to previous study that reported false positive results were obtained by using MagNA Pure system [23]. Possible explanation for this result may be contamination happened during the pipetting steps of the extraction protocol.

Furthermore, in the present study the yield of manually prepared RNAs was 344% higher than the yield of automated extracts when the procedures were performed according to the supplier's manual. Similarly, the purity of manually extracted RNAs was closer to the optimum value compared to RNAs produced by the MagNA Pure. However, the 260/230 ratios determined for both methods are far below the optimum. Remaining salts in the eluate usually account for these low values. Ionic strength is known to influence the absorbance of nucleic acids, especially the absorbance at 260 nm [24]. It may be explain why low 260/230 ratios obtained by MagNA Pure. Since Magna Pure LC total nucleic acid isolation kit contains high ionic strength buffers.

Discordant results of PPRV RT-PCR were observed from two samples that were previously demonstrated to contain PPRV by virus isolation. Two samples extracted with the MagNA Pure failed to generate fragments. The lack of concordance appears to correlate with the effectiveness of the extraction technique (as defined by sensitivity studies above). Studies with serially diluted PPRV demonstrated a trend of higher sensitivity after extraction by manual method.

In this study, PPRV RNA was detected by F and N gene based RT-PCR methods. It has been reported that N gene based primers are more sensitive than F gene based primers [6]. However, we obtained consentient results between F and N gene based RT-PCR methods. All CPE-positive samples were found positive with both methods. Also, no false positive results were obtained when negative controls were tested.

In our experience, when we used manual and automated methods for direct extraction from tissue samples, we obtained much and pure nucleic acid (especially RNA) concentrations by manual method than automated method. We suggest that magnetic beads in automated methods influence the concentration of nucleic acids. In automated methods, magnetic beads coated with nucleic acids (DNA or RNA), and these stuck nucleic acids can't fully separated from magnetic beads. Therefore, obtained concentrations of nucleic acids are low.

A major concern in the implementation of manual methods to extract nucleic acid for use in amplification assays is the potential for contamination. We did not observe any contamination after extraction by RNeasy Mini kit. Likewise, MagNA Pure reduce the chances of contamination of samples. MagNA Pure provides an integrated tip guard to prevent dripping by the tips and UV sterilization between runs.

From a cost perspective, the MagNA Pure and RNeasy Mini kit extraction differed minimally at approximately $2.5/sample each. Less hands-on time and the fact that approximately two to three times the number of specimens can be processed at once make the MagNA Pure a real alternative for larger sample preparations, even though the cost per sample is higher than that for RNeasy extractions.

Nowadays, manual and automated extraction methods can be used for detection of PPRV RNA from field samples. Automated extraction methods minimize the potential sample contamination compared to the manual methods. Also, they demanded much less hands-on time than the manual methods. The first and most important step in molecular diagnosis of PPRV infection is the nucleic acid isolation. From our data, it was concluded that both RNA extraction methods (manual; 11/32, automated; 9/32) demonstrated similar performance, with no significant difference (P=0.7879). However, manual extraction performed slightly better analytical sensitivity, by 5%, than the automated extraction *(Table 1)*. Accordingly, our results suggest that manual extraction is suitable for RNA extraction when small numbers of tissue samples needed to be examined.

REFERENCES

1. Lefevre PC, Diallo A: Peste des petits ruminants virus. *Rev Sci Tech Off Int Epiz*, 9, 951-965, 1990.

2. Anderson J, McKay JA: The detection of antibodies against peste des petits ruminants virus in cattle, sheep and goats and the possible implication to rinderpest control programme. *Epidemiol Infect*, 112, 225-231, 1994.

3. Gibbs EPJ, Taylor WP, Lawman MPJ, Bryant J: Classification of peste des petits ruminants virus as a fourth member of the genus Morbillivirus. *Intervirology*, 11, 268-274, 1979. DOI: 10.1159/000149044

4. Barrett T, Visser IKG, Mamaev L, Goatley L, Bressem MF, Van Osterhaus ADM: Dolphin and porpoise morbilliviruses are genetically distinct from phocine distemper virus. *Virology*, 193, 1010-1012, 1993.

5. Ozkul A, Akca Y, Alkan F, Barrett T, Karaoglu T, Dagalp SB, Anderson J, Yesilbag C, Cokcaliskan C, Gencay A, Burgu I: Prevalence, distribution, and host range of peste des petits ruminants virus, Turkey. *Emerg Infect Dis*, 8, 708-712, 2002.

6. Kerur N, Jhala MK, Joshi CG: Genetic characterization of Indian peste des petits ruminants virus (PPRV) by sequencing and phylogenetic analysis of fusion protein and nucleoprotein gene segments. *Res Vet Sci*, 85, 176-183, 2008. DOI: 10.1016/j.rvsc.2007.07.007

7. Munir M, Zohari S, Saeed A, Khan QM, Abubakar M, LeBlanc N, Kanu S, Sankoh FA, Berg M, Barrie ML, Ståhl K: Genetic characterization of peste des petits ruminants virus, Sierra Leone. *Emerg Infect Dis*, 18, 193-195, 2012. DOI: 10.3201/eid1801.111304

8. Luka PD, Erume J, Mwiine FN, Ayebazibwe C, Shamaki D: Molecular characterization and phylogenetic study of peste des petits ruminants viruses from North central states of Nigeria. *BMC Vet Res*, 7, 32, 2011. DOI: 10.1186/1746-6148-7-32

9. Shaila MS, Shamaki D, Forsyth MA, Diallo A, Goatley L, Kitching RP, Barrett T: Geographic distribution and epidemiology of peste des petits ruminants viruses. *Virus Res*, 43, 149-153, 1996. DOI: 10.1016/0168-1702(96)01312-3

10. Wang Z, Bao J, Wu X, Liu Y, Li L, Liu C, Suo L, Xie Z, Zhao W, Zhang W, Yang N, Li J, Wang S, Wang J: Peste des petits ruminants virus in Tibet, China. *Emerg Infect Dis*, 15, 299-301, 2009. DOI: 10.3201/eid1502.080817

11. Kwiatek O, Ali YH, Saeed IK, Khalafalla AI, Mohamed OI, Obeida AA, Abdelrahman MB, Osman HM, Taha KM, Abbas Z, Harrak M, Lhor Y, Diallo A, Lancelot R, Albina E, Libeau G: Asian Lineage of Peste des Petits Ruminants Virus, Africa. *Emerg Infect Dis*, 17, 1223-1231, 2011. DOI: 10.3201/eid1707.101216

12. Munir M, Zohari S, Saeed A, Khan QM, Abubakar M, LeBlanc N, Berg M: Detection and phylogenetic analysis of peste des petits ruminants virus isolated from outbreaks in Punjab, Pakistan. *Transbound Emerg Dis*, 59, 85-93, 2012. DOI: 10.1111/j.1865-1682.2011.01245.x

13. De Nardi M, Lamin Saleh SM, Batten C, Oura C, Di Nardo A, Rossi D: First evidence of peste des petits ruminants (PPR) virus circulation in Algeria (Sahrawi Territories): Outbreak investigation and virus lineage identification. *Transbound Emerg Dis*, 59, 214-222, 2012. DOI: 10.1111/j.1865-1682.2011.01260.x

14. OIE: Peste des petits ruminants. **In,** OIE (Ed): Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.7.11. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.07.11_PPR.pdf, Accessed: 06/09/2014.

15. Forsyth MA, Barrett T: Evaluation of polymerase chain reaction for the detection and characterisation of rinderpest and peste des petits ruminants viruses for epidemiological studies. *Virus Res*, 39, 151-163, 1995. DOI: 10.1016/0168-1702(95)00076-3

16. Couacy-Hymann E, Roger F, Hurard C, Guillou JP, Libeau G, Diallo A: Rapid and sensitive detection of peste des petits ruminants virus by a polymerase chain reaction assay. *J Virol Methods*, 100, 17-25, 2002. DOI: 10.1016/S0166-0934(01)00386-X

17. Riemann K, Adamzik M, Frauenrath S, Egensperger R, Schmid KW, Brockmeyer NH, Siffert W: Comparison of manual and automated nucleic acid extraction from whole-blood samples. *J Clin Lab Anal,* 21, 244-248, 2007. DOI: 10.1002/jcla.20174

18. OIE: OIE Disease Information. 12, 137, 1999.

19. Sevik M: Molecular Detection of Peste des petits ruminants virus from different organs/tissues of naturally infected animals. *Kafkas Univ Vet Fak Derg,* 20, 165-168, 2014. DOI: 10.9775/kvfd.2013.9706

20. Knepp JH, Geahr MA, Forman MS, Valsamakis A: Comparison of automated and manual nucleic acid extraction methods for detection of enterovirus RNA. *J Clin Microbiol*, 41, 3532-3536, 2003. DOI: 10.1128/JCM.41.8.3532-3536.2003

21. Marshall JA, Bruggink LD: Laboratory diagnosis of norovirus. *Clin Lab*, 52, 571-581, 2006.

22. Kurar E, Atlı MO, Guzeloglu A, Ozsensoy Y, Semacan A: Comparison of five different RNA isolation methods from equine endometrium for gene transcription analysis. *Kafkas Univ Vet Fak Derg,* 16, 851-855, 2010.

23. Chranioti A, Aga E, Margari N, Kottaridi C, Pappas A, Panayiotides I, Karakitsos P: Performance evaluation of manual and automated (MagNA pure) nucleic acid isolation in HPV detection and genotyping using Roche Linear Array HPV Test. *Infect Dis Obstet Gynecol*, 2011: 931281, 2011. DOI: 10.1155/2011/931281

24. Wilfinger WW, Mackey K: Chomczynski P: Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques*, 22, 474-476, 478-481, 1997.

Kafkas Univ Vet Fak Derg
21 (2): 277-282, 2015
DOI: 10.9775/kvfd.2014.12309

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Comparison of PCR and Culture Methods for Diagnosis of Subclinical Mastitis in Dairy Cattle [1]

Zafer CANTEKİN [1] Yaşar ERGÜN [2] Gökhan DOĞRUER [2]
Mustafa Kemal SARIBAY [2] Hasan SOLMAZ [3]

[1] *This study was supported by Mustafa Kemal University Research Fund with project number 1101 M 0103*

[1] Department of Microbiology, Veterinary Faculty, Mustafa Kemal University, TR-31040 Hatay - TURKEY
[2] Department of Gynaecology and Obstetrics, Veterinary Faculty, Mustafa Kemal University, TR-31040 Hatay - TURKEY
[3] Department of Pharmaceutical Microbiology, Faculty of Pharmacy, YuzuncuYıl University, TR-65100 Campus, Van - TURKEY

KVFD-2014-12309 Received: 16.09.2014 Accepted: 14.11.2014 Published Online: 11.12.2014

Abstract

Bovine mastitis is the greatest source of economic loss in the dairy industry. Rapid and definitive detection of causative agent is very important for treatment and control of the disease. The aim of this study was to compare culture and polymerase chain reaction techniques for diagnosis of agents in subclinical bovine mastitis. For this purpose, after conducting the California Mastitis Test on 540 cows, 79 milk samples were analysed by the classical culture method and simplex polymerase chain reaction. Forty-three of samples were found positive by both methods, differences were found only seven samples. While coagulase negative staphylococci these seven samples were determined by culture method, coagulase negative staphylococci and *S. dysgalactiae* were determined together by polymerase chain reaction. The results of this study indicate that the polymerase chain reaction is more sensitive than culture method and could detect pathogens at the species level within a few hours from directly milk samples. Rapid and relieable molecular techniques can be useful method in farm level detection for fast decision about the culling or treatment.

Keywords: *Bovine mastitis, Culture, Polymerase Chain Reaction*

Süt Sığırlarında Sub-klinik Mastitisin Tanısında Kültür ve PCR Yöntemlerinin Karşılaştırılması

Özet

Sığır mastitisleri süt sığırcılığı endüstrisinde ekonomik kayıpların en önemli nedenidir. Mastitiste etkeninin hızlı ve kesin tanısı hastalığın tedavisi ve kontrolü açısından çok önemlidir. Bu çalışmanın amacı subklinik sığır mastitislerinde etkenin tanısı için kültür ve polimeraz zincir reaksiyonu tekniklerinin karşılaştırılmasıdır. Bu amaçla, 540 ineğin California Mastitis Test ile muayenesinden sonra, pozitif bulunan 79 adet süt örneği, klasik kültür metodu ve direkt sütten yapılan polimeraz zincir reaksiyonu ile analiz edildi. Kırk üç örnekte her iki yöntemde de mikroorganizma belirlenirken, sadece 7 örnekte iki teknik arasından fark görüldü. Bu 7 örnekte kültür yöntemiyle sadece Koagülaz Negatif Stafilok belirlenirken, polimeraz zincir reaksiyonu ile Koagülaz Negatif Stafilok dışında *S. dysgalactiae* yönünden de pozitif bulundu. Bu çalışmanın sonuçları polimeraz zincir reaksiyonunun kültür yönteminden daha duyarlı olduğunu ve direkt olarak süt numunesinden hedef etkenin bir kaç saat içinde saptanabileceğini gösterdi. Hızlı ve güvenilir moleküler tekniklerin kullanımı mastitiste sürüden çıkarma veya tedavi etme konusunda hızlı karar verilebilmesinde yardımcı olabilir.

Anahtar sözcükler: *Sığır mastitisi, Kültür, Polimeraz Zincir Reaksiyonu*

INTRODUCTION

Mastitis is one of the most common causes of economic loss in dairy cattle farming. It has been reported that the economic loss in a mastitis case can range from 107 to 344 (U.S. Dolars) per cow in some countries [1,2].

More than 130 different microorganisms have been identified in cases of bovine mastitis [3]. According to primary sources, the bacteria responsible for bovine mastitis can be divided into environmental *(Escherichia coli, Streptococcus dysgalactiae*, *Streptococcus parauberis* and *Streptococcus uberis)* and contagious *(Staphylococcus*

İletişim (Correspondence)
+90 326 2455845/1552; Fax +90 326 2455704
zcantekin@hotmail.com

aureus, *Streptococcus agalactiae, Trueperella pyogenes* (formerly *Arcanobacterium pyogenes*, *Actinomyces pyogenes*, *Corynebacterium pyogenes)* and *Mycoplasma* spp.) categories [4]. In recent years, the increasing importance of Coagulase-Negative *Staphylococci* (CNS) has been emphasised [5,6]. In addition; yeast such as *Candida* spp. has been reported to be more isolated in areas with higher moisture than in other regions [7].

Mastitis is classified as clinical or subclinical according to clinical appearance. The diagnosis of subclinical mastitis cannot be made from symptoms; diagnosis requires special tests. Due to latent infection in subclinical mastitis, it may spread throughout the farm and causes a high level of economic loss [4,8]. Early diagnosis of subclinical mastitis is important in order to treat and prevent the spread of the disease by applying the appropriate security measures [9,10].

Although the milk culture is considered the gold standard test for diagnosing mastitis, there are several disadvantages associated with this technique. Growing microorganisms *in vitro* can be suppressed by factors such as antibiotic residue, inflammatory cells and mediators in milk, and infectious agents can be very low in number in subclinical mastitis [11]. The prevalence of false negative results using bacterial culture methods has increased the importance of Polymerase Chain Reaction (PCR) analysis; its usage in diagnosing mastitis has been proposed for the rapid and reliable detection of agents with high sensitivity. And a few of microorganisms in the samples can be detected by PCR [11-15]. In addition, the use of molecular techniques has been suggested for detecting fastidious microorganisms such as *Mycoplasma* spp.[16-18] and for discriminating of strictly related microorganisms such as *S. parauberis* and *S. uberis* [19]. For the prevention and control of the disease, routine and periodical control and detection of mastitis agens is very important point [20].

The aim of this study was to compare the reliability of classical culture and PCR as means of detecting bacterial or yeast agents in bovine subclinical mastitis.

MATERIAL and METHODS

Sampling

Prior to sampling, ten well-managed farms with an average fifty cattle per farm were selected from around Hatay, Turkey. Somatic Cell Counts in the bulk milk sampled farm were in the range between 125.000 and 380.000/ml. The average clinical mastitis in the sampled herd rate was below 2%, the rate of subclinical mastitis was 14.6%. Totally 2160 milk samples from 540 cows were tested with California Mastitis Test (CMT). CMT was performed according to Schalm et al.[21]. According to visible reactions the results were classified into 5 scores: (0) = negative, (±) = trace, (+1) = weak positive, (+2) = distinct positive, and (+3) = strong positive. In the study, the milk samples were only taken from subclinical infected udder halves, but clinical infected milk (positive with strip-cup test) was not taken into consideration for this study. Totally CMT positive 79 subclinical milk samples were collected aseptically according to a standard procedure [22] and transferred to the laboratory within 1-3 h in a 4-8°C cooler for the microbiological analyses (The clinical samples were taken with permission with MKÜ Local Ethics Commitee. Meeting Date 17.06.2010: Meeting No: 2010/02: Decision No: 30).

Bacteriological Culture

The milk samples were mixed and 100 µl of milk were streaked onto Blood Agar and (supplemented with 7% defibrinated sheep blood) and Mac Conkey's Lactose Agar and Sabouraud Dextrose Agar. Bacteriological and mycological isolation and identification were performed by the classical culture method and standard biochemical tests according to accepted standards [23-25]. For the isolation of yeast, after incubation 5 days at 30°C, colonies growth in the Sabouraud Dextrose Agar were stained Gram method and they were identified according to their macroscopic and microscopic properties. For the bacterial identification, after incubation for 24 and 48 h at 37°C, colonies in Blood Agar Plates were examined for colony characteristics, morphology and haemolysis properties. Mixed colonies in the plates were sub-cultured by transferring into new agar plates for the obtain pure culture. Sub-cultured pure colonies were evaluated macroscopically and stained with Gram method. Then, catalase and oxidase test put in the implement for all of isolates.

Catalase positive and oxidase negative, coccus-shaped isolates were included member of *Staphylococcus* spp. After tube coagulase test with rabbit plasma, *Staphylococcal* isolates were classified as coagulase positive and coagulase negative. Further characterisation of coagulase positive isolates were made with thermostable nuclease test and mannitol fermentation.

Gram positive, coccus-shaped, catalase negative and oxidase negative isolates were included member of *Streptococcus* spp. For the further characterization of these isolates, the Christie-Atkins-Munch-Petersen (CAMP) reaction, esculin hydrolysis on Edwards Medium (Oxoid, Basel, Switzerland), sodium hippurate hydrolysis.

Gram positive, small curved rod-shaped, catalase negative and oxidase negative isolates were confirmed as *T. pyogenes* (formerly *Arcanobacterium pyogenes)*. And other routine biochemical tests, nitrate reduction, gelatin hydrolyzation, urease production, Oxidation-Fermentation, were carried out to identify the isolates.

For the *Mycoplasma* spp. isolation, 1 ml of milk sample was transferred to 9 ml of PPLO broth medium (supplemented with horse sera, thallium acetate, and penicillin) and incubated at 37°C for two weeks under

microaerophilic conditions. After the incubation, 100 µl aliquots were transferred from the PPLO broth medium to PPLO agar (supplemented with horse sera, thallium acetate, and penicillin) and incubated at 37°C for two weeks under microaerophilic conditions according to Carter [24] and Quinn et al.[25].

Molecular Diagnosis

For the PCR analyses, *S. aureus* (ATCC 25923) and *S. epidermidis* (ATCC 12228) from department collection, and *Mycoplasma bovis* ATCC 25025 DNA (Dr. Jessie Trujillo, IOWA State University of Science and Technology, College of Veterinary Medicine, Department of Veterinary Microbiology and Preventive Medicine) were used as positive control DNA. One ml of milk sample was transferred into a sterile plastic tube and centrifuged at 5.000 x g for 5 min, after which the pellets were resuspended with 1 ml of sterile PBS (Phosphate-Buffered Saline, pH 7.4). This washing was performed three times to remove calcium ions and other inhibitors [15]. The pellets were then resuspended in 300 µl of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and a nucleic acid extraction was implemented according to the method of Sambrook and Russell [26]. Extracted DNA pellet was dissolved in 100 µl of TE buffer and stored –20°C until used in the PCR analyses. The properties of the primers are shown in *Table 1*. The simplex PCR protocols and procedures were carried out according to their references. After amplification, ten microliters of each amplification reaction mixture was analysed by electrophoresis performed with a 1.5% (wt/vol) agarose gel stained with ethidium bromide (0.7 µg/ml). After migration with 160 volts for 30 min, amplification products were visualized under ultraviolet light.

Cost Analyses

The total costs for culture and PCR analyses were determined for only reagents and plastic consumables used in the analyses, not labor costs and laboratory equipment. PCR reagents were calculated for 79 samples and ten simplex PCR analyses because of the all samples were used PCR analyses for each studied primer sets. In the culture, cost for the first isolation were calculated for 79 samles, but costs for biochemical test were calculated for 43 isolates after first isolation. Price quotations were obtained two commercial companies, and in the calculation the most lower prieces were used.

Statistical Analysis

Kappa analysis was used the determination of agreement in the results in the both methods used in this study. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) for Windows Statistical Package Version 14.0.

Table 1. *Properties of primers used in the study*
Tablo 1. *Çalışmada kullanilan primerlerin özellikleri*

Agent	Target Gene	Primer Name	Primer Sequences	Annealing Temp (°C)	Reference
Staphylococcus spp.	*16s rRNA*	Staph294-318	5′-GCCGGTGGAGTAACCTTTTAGGAGC-3′	55	[27]
		Staph 1522-1540	5′-AGGAGGTGATCCAACCGCA-3′		
E. coli	23S r RNA	Eco 2083	5′-GCTTGACACTGAACATTGAG-3′	64	[19]
		Eco 2745	5′-GCACTTATCTCTTCCGCATT-3′		
S. aureus	23S rRNA	Sau 327	5′-GGACGACATTAGACGAATCA-3′	64	[19]
		Sau 1645	5′-CGGGCACCTATTTTCTATCT-3′		
Strep. agalactiae	16S rRNA gene	Sag 40	5′-CGCTGAGGTTTGGTGTTTACA-3′	60	[19]
		Sag 445	5′-CACTCCTACCAACGTTCTTC-3′		
Strep. dysgalactiae	16S rRNA gene	Sdy 105	5′-AAAGGTGCAACTGCATCACTA-3′	57	[19]
		Sdy 386	5′-GTCACATGGTGGATTTTCCA-3′		
Strep. parauberis	23S rRNA gene	Spa 301	5′-GCGACGTGGGATCAAATACT-3′	57	[19]
		Spa 1219	5′-TACCATTACCTCTAAAGGTA-3′		
Strep. uberis	23S rRNA gene	Sub 302	5′-CGAAGTGGGACATAAAGTTA-3′	56	[19]
		Sub 396	5′-CTGCTAGGGCTAAAGTCAAT-3′		
M. bovis	membrane lipoprotein P81gene	Mb 1113–1133	5′-TATTGGATCAACTGCTGGAT-3′	55	[28]
		Mb 1542–1560	5′-AGATGCTCCACTTATCTTAG-3′		
T. pyogenes	Plo gene	Plo1	5′-GGCCCGAATGTCACCGC-3′	55	[29]
		Plo2	5′-AACTCCGCCTCTAGCGC-3′		
Candida spp.	rRNA gene	Cab1	5′-TATTAAAGTTGTTGCAG-3′	52	[30]
		Cab2	5′-CCTGCTTTGAACACTCTAATTT-3′		

RESULTS

In the result of the analyses, 43 (54.43%) of 79 CMT positive samples were found positive by both culture method and simplex PCR. Of 36 (45.57%) samples were not any isolation by culture method and they were also found negative by PCR. Totally eight different results profiles were determined both PCR and culture methods. The types of result profiles were shown in *Table 2*. Forty-three of samples were found positive by both methods, differences were found only seven samples. While coagulase negative staphylococci these seven samples were determined by culture method, coagulase negative staphylococci and *S. dysgalactiae* were determined together by polymerase chain reaction. Comparison of results for each samples were shown in the *Table 3*.

In the statistical analysis of eight results profiles, results for CNS and *S. dysgalactiae* in PCR (26 samples positive) and culture (19 samples positive) were found as substantial agreement (κ 0.785) and other results were found as almost perfect agreement (κ 1.000).

In the result of cost analyses, total cost for culture method were calculated to be 465.28 ₺ (Turkish Liras aproximately 206.42 U.S. Dollars), and for PCR analyses were determined 1076.52 ₺ (1076.52 Turkish Liras aproximately 477.60 U.S. Dolars). Of the costs in the PCR were consisted of 210.62 ₺ (93.44 U.S. Dollars) and 865.90 ₺ (384.16 U.S. Dollars) PCR tests.

Table 2. *Comparison results of culture method and PCR from milk samples*
Tablo 2. *Süt örneklerinden kültür ve PCR sonuçlarının karşılaştırılması*

Results	PCR Method	Culture Method
1 *CNS + S. dysgalactiae*	26 samples	19 samples
2 CNS	4 samples	4 samples
3 *S. dysgalactiae*	2 samples	2 samples
4 *S. aureus + S. dysgalactiae*	2 samples	2 samples
5 *CNS + S. uberis*	5 samples	5 samples
6 *CNS + S. agalactiae*	1 samples	1 samples
7 *CNS + S. dysgalactiae + T. pyogenes*	1 samples	1 samples
8 *Candida* spp.	2 samples	2 samples

DISCUSSION

The early and accurate diagnosis is important for treatment and control of mastitis that can effects animal and human health. The PCR method is widely used for the diagnosis of mastitis [11-19]. In this study, it was compared the results of diagnosis of subclinical mastitis using the bacterial culture and PCR analysis methods. Although seven samples positive for only CNS by culture method, these seven samples in the PCR analyses were found positive CNS and *S. dysgalactiae*. Other results for each samples in the culture method were the same with results of PCR analyses. In the result of this study, PCR was found more sensitive than culture method on the analysing of milk samples from subclinical bovine mastitis. Similarly our study, Phuektes et al.[11] found that multiplex PCR was more sensitive than culture for *S. aureus* and *S. uberis*, but not significantly different for *S. agalactiae* and *S. dysgalactiae* in bovine milk samples. Amin et al.[31] reported that simplex and multiplex PCR were more sensitive than culture in detecting *S. aureus*, *E. coli*, and *S. agalactiae* in milk. And, they suggested that PCR could be used as a rapid and sensitive method for detecting those microorganisms. Karahan et al.[32] compared the culture and multiplex PCR methods for diagnosing bovine mastitis and reported that multiplex PCR was more successful than culture for detecting *S. aureus* and *S. agalactiae*. And, Gillespie and Oliver [33] reported that, the real-time PCR technique correctly identified 91.7% of *S. aureus*, 98.2% of *S. agalactiae*, and 100% of *S. uberis*. They noted that multiplex real-time PCR has the potential for simultaneous identification of these agents with 95.5% sensitivity and 99.6% specificity. Above all Koskinen et al.[34] reported that PCR was more sensitive than culture methods especially multiple species in the milk samples. And their results supported that in this study,altough seven samples were positive for CNS in culture method, but in the PCR analyses these seven samples were positive for CNS and *S. dysgalactiae*. All of these studies about comparing PCR and culture in milk samples from bovine mastitis suggested that PCR had more useful than conventional culture in for speed, interpretation of results, and sensitivity.

Nelson et al.[35] compared that phenol-chloroform extraction method with two different commercial DNA extraction kits in the bacterial DNA extraction from human fecal specimens for analyses by Real Time PCR. They reported that phenol-chloroform extraction method was te cheapest (0.25 Australian Dollars per samples) extraction methods. Turenne et al.[36] compared costs of culture method and bacterial 16S rRNA gene targeted a fluorescence-based PCR–single-strand conformation polymorphism (SSCP) protocol for the identification of bacteria from blood samles. They calculated all costs for reagents and labor costs in their study, and time for each analyses. Researchers reported that the average cost for conventional identification per blood culture isolate was ranged from $39 to $45 (U.S. currency) and in the molecular analyses wiht SSCP was to be $21 (U.S. currency). Also, they reported the identifaciton time was 24 h SSCP, but in the culture was changed 1 to 8 days. In this study, PCR costs (477.60 U.S. Dollars) found nearly 2,3 times higher than culture costs (206.42 U.S. Dollars). These differences may be caused from high prices of PCR reagents in our country or didn't calculation of labor cost in this study.

In conclusion, PCR might has the potential for the rapid and reliable diagnosis of a large number of milk samples. And also, it would be beneficial for use as an

Table 3. *Comparison of culture method and PCR results for the each sample*
Tablo 3. *Her bir örnek için kültür ve PCR sonuçlarının karşılaştırılması*

No	SC	Staph		Sau		Tp		Sag		Sdy		Sub		Can	
		CCM	PCR	CCM	PCR	CCM	PCR	CCM	PCR	CCM	PCR	CCM	PCR	CCM	PCR
1	2	+	+	-	-	-	-	-	-	-	-	+	+	-	-
2	9	+	+	-	-	-	-	-	-	-	-	+	+	-	-
3	10	+	+	-	-	-	-	-	-	-	-	+	+	-	-
4	11	+	+	-	-	-	-	-	-	-	-	-	-	-	-
5	12	+	+	-	-	-	-	-	-	-	-	+	+	-	-
6	14	+	+	-	-	-	-	-	-	-	-	+	+	-	-
7	16	+	+	-	-	-	-	-	-	-	+	-	-	-	-
8	20	+	+	-	-	-	-	-	-	+	+	-	-	-	-
9	21	+	+	-	-	-	-	-	-	+	+	-	-	-	-
10	22	+	+	-	-	-	-	-	-	+	+	-	-	-	-
11	24	+	+	-	-	-	-	-	-	-	-	-	-	-	-
12	25	+	+	-	-	-	-	-	-	+	+	-	-	-	-
13	28	+	+	-	-	-	-	-	-	+	+	-	-	-	-
14	29	+	+	-	-	-	-	-	-	+	+	-	-	-	-
15	32	+	+	-	-	-	-	-	-	+	+	-	-	-	-
16	35	+	+	-	-	-	-	-	-	+	+	-	-	-	-
17	36	+	+	-	-	-	-	-	-	-	-	-	-	-	-
18	38	+	+	-	-	-	-	-	-	+	+	-	-	-	-
19	42	+	+	-	-	-	-	-	-	-	-	-	-	-	-
20	43	+	+	+	+	-	-	-	-	+	+	-	-	-	-
21	46	+	+	+	+	-	-	-	-	+	+	-	-	-	-
22	47	+	+	-	-	-	-	-	-	+	+	-	-	-	-
23	48	+	+	-	-	-	-	-	-	+	+	-	-	-	-
24	51	+	+	-	-	-	-	-	-	+	+	-	-	-	-
25	52	+	+	-	-	-	-	-	-	-	+	-	-	-	-
26	53	+	+	-	-	-	-	-	-	-	+	-	-	-	-
27	54	+	+	-	-	-	-	-	-	-	+	-	-	-	-
28	55	+	+	-	-	-	-	-	-	-	+	-	-	-	-
29	56	-	-	-	-	-	-	-	-	+	+	-	-	-	-
30	57	+	+	-	-	-	-	-	-	-	+	-	-	-	-
31	58	+	+	-	-	-	-	+	+	-	-	-	-	-	-
32	60	+	+	-	-	-	-	-	-	-	+	-	-	-	-
33	62	-	-	-	-	-	-	-	-	+	+	-	-	-	-
34	63	+	+	-	-	-	-	-	-	+	+	-	-	-	-
35	64	+	+	-	-	-	-	-	-	+	+	-	-	-	-
36	66	+	+	-	-	-	-	-	-	+	+	-	-	-	-
37	67	+	+	-	-	+	+	-	-	+	+	-	-	-	-
38	68	+	+	-	-	-	-	-	-	+	+	-	-	-	-
39	71	+	+	-	-	-	-	-	-	+	+	-	-	-	-
40	72	-	-	-	-	-	-	-	-	-	-	-	-	+	+
41	73	-	-	-	-	-	-	-	-	-	-	-	-	+	+
42	77	+	+	-	-	-	-	-	-	+	+	-	-	-	-
43	79	+	+	-	-	-	-	-	-	+	+	-	-	-	-
Total	PR	39	39	2	2	1	1	1	1	24	31	5	5	2	2
	NR	4	4	41	41	42	42	42	42	19	12	38	38	41	41

SC: *Sample Code,* ***Staph:*** *Staphylococcus spp.,* ***Sau:*** *Staphylococcus aureus,* ***Tp:*** *Trueperella pyogenes,* ***Sag:*** *Streptococcus agalactiae,* ***Sdy:*** *Streptococcus dysgalactiae,* ***Sub:*** *Streptococcus uberis,* ***Can:*** *Candida spp.* ***CCM:*** *Conventional Culture Method,* ***PCR:*** *Polymerase Chain Reaction,* ***+*** *positive,* ***-*** *negative,* ***PR:*** *Positive results,* ***NR:*** *Negative results*

auxiliary diagnosis technique to culture. Further studies on developing advanced molecular tecniques based PCR analyses for contagious or major mastitis agents can be useful tool for carrying out the checks at farm level.

ACKNOWLEDGEMENT

The authors would like to thank to Dr. Radhwane Saidi (Department of Agronomy, Telidji Amar University, Laghouat, ALGERIA) for critical reading of the manuscript.

REFERENCES

1. Wellenberg GJ, Van Der Poel WHM, Van Oirschot GJ: Viral infections and bovine mastitis: A review. *Vet Microbiol,* 88, 27-45, 2002. DOI: 10.1016/S0378-1135(02)00098-6

2. Friedman S, Shoshanı E, Ezra E: Economical loses from clinical mastitis in 4 dairy herds in Israel. *Israel J Vet Med,* 59, 1-2, 2004.

3. Watts JL: Etiological agents of bovine mastitis. *Vet Microbiol,* 16, 41-66, 1988. DOI: 10.1016/0378-1135(88)90126-5

4. Bramley AJ: Current concepts of bovine mastitis. National Mastitis Council, Madison, Wisconsin, 1996.

5. Huxley JN, Greent MJ, Green LE, Bradley AJ: Evaluation of the efficacy of an internal teat sealer during the dry period. *J Dairy Sci,* 85, 551-561, 2002. DOI: 10.3168/jds.S0022-0302(02)74108-8

6. Taponen S, Simojoki H, Haveri M, Larsen HD, Pyorala S: Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. *Vet Microbiol,* 115, 199-207, 2006.

7. NMC: Laboratory and Field Handbook on Bovine Mastitis. National Mastitis Council, Madison, WI, 139, 1999.

8. Rajala-Schultz PJ, Smith KL, Hogan JS, Love BC: Antimicrobial susceptibility of mastitis pathogens from first lactation and older cows. *Vet Microbiol*, 102, 33-42, 2004. doi:10.1016/j.vetmic.2004.04.010

9. Başt an A: İneklerde Meme Hastalıkları. Hatiboğlu Basımevi, Ankara, 2013.

10. Pyörälä S: New strategies to prevent mastitis. *Reprod Domest Anim,* 37, 211-216, 2002. DOI: 10.1046/j.1439-0531.2002.00378.x

11. Phuektes P, Mansell PD, Browning GF: Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and Streptococcal causes of bovine mastitis. *J Dairy Sci,* 84, 1140-1148, 2001. DOI: 10.3168/jds.S0022-0302(01)74574-2

12. Türkyılmaz S, Yıldız Ö, Oryaşın E, Kaynarca S, Bozdoğan B: Molecular identification of bacteria isolated from dairy herds with mastitis. *Kafkas Univ Vet Fak Derg*, 16 (6): 1025-1032, 2010.

13. Tel Y, Bozkaya F: Identifying the bacteria causing ovine gangrenous mastitis and detection of *Staphylococcus aureus* in gangrenous milk by PCR. *Kafkas Univ Vet Fak Derg*, 18 (3): 401-406, 2012.

14. Forsman P, Tilsalatimisjarvi A, Alatossava T: Identification of Staphylococcal and Streptococcal causes of bovine mastitis using 16S-23S rRNA spacer regions. *Microbiology,* 143, 3491-3500, 1997. DOI: 10.1099/00221287-143-11-3491

15. Khan MA, Kim CH, Kakoma I, Morin E, Hansen RD, Hurley WL, Tripathy DN, Baek BK: Detection of *Staphylococcus aureus* in milk by use of polymerase chain reaction analysis. *Am J Vet Res,* 59, 807-813, 1998.

16. Ghadersohi A, Coelen RJ, Hirst RG: Development of a specific DNA probe and PCR for the detection of *Mycoplasma bovis. Vet Microbiol,* 56, 87-98, 1997. DOI: 10.1016/S0378-1135(96)01343-0

17. Büyük F, Şahin M: Kars yöresinde atık yapan ineklerin çeşitli örneklerinden *Brucella* etkenlerinin kültürel ve moleküler yöntemlerle araştırılması ve olguların epidemiyolojik analizi. *Kafkas Univ Vet Fak Derg,* 17 (5): 809-816, 2011.

18. Akan M, Babacan, O, Torun E, Müştak HK, Öncel T: Diagnosis of *Mycoplasma bovis* infection in cattle by ELISA and PCR. *Kafkas Univ Vet Fak Derg*, 20 (2): 249-452, 2014. DOI: 10.9775/kvfd.2013.9959

19. Riffon R, Sayasith K, Khalil H, Dubreuil P, Drolet M, Lagace A: Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. *J Clin Microbiol,* 39 (7): 2584-2589, 2001. DOI: 10.1128/JCM.39.7.2584-2589.2001

20. Ergün Y, Aslantaş O, Kirecci E, Ozturk F, Ceylan A, Boyar Y: Antimicrobial susceptibility, presence of resistance genes and biofilm formation in coagulase negative Staphlococci isolated from subclinical sheep mastitis. *Kafkas Univ Vet Fak Derg*, 18 (3): 449-456, 2012.

21. Schalm OW, Carrol EJ, Jain NC: Bovine Mastitis. Lea and Febiger, Philadelphia, USA. 360, 1971.

22. International Dairy Federation, Milk and Milk Products: Guidance on Sampling. International Dairy Federation, Standard 50B, Brussels, Belgium, 1985.

23. Hogan JS, Gonzalez RN, Harmon RJ, Nickerson SC, Oliver SP, Pankey JW, Smith KL: Laboratory Handbook on Bovine Mastitis. Rev. ed., NMC Inc., Madison, WI, 1999.

24. Carter GR: Isolation and identification of bacteria from clinical specimens. **In,** Carter GR, Cole JR (Eds): Diagnostic Procedures in Veterinary Bacteriology and Mycology. 5th ed., Academic Press Inc. San Diego, California, 1990.

25. Quinn PJ, Carter ME, Markey BK, Carter GR: Clinical Veterinary Microbiology. 209-236, Mosby-Year Book Europe Limited, Lynton House, London WC1H9LB, England. 1994.

26. Sambrook J, Russell W: Molecular Cloning: A Laboratory Manual. 3rd ed., Cold Spring Harbor Press, New York, NY, 2002.

27. Schmitz FJ, MacKenzie CR, Hofmann B, Verhoef J, Finken-Eigen, M, Heinz HP, Kohrer K: Specific information concerning taxonomy, pathogenicity and methicillin resistance of staphylococci obtained by a multiplex PCR. *J Med Microbiol,* 46, 773-778, 1997. DOI: 10.1099/00222615-46-9-773

28. Foddai A, Idini G, Fusco M, Rosa, N, de la Fe C, Zinellu S, Corona L, Tola S: Rapid differential diagnosis of *Mycoplasma agalactiae* and *Mycoplasma bovis* based on a multiplex-PCR and a PCR-RFLP. *Mol Cell Probes,* 19 (3): 207-212, 2005. DOI: 10.1016/j.mcp.2004.11.007

29. Billington SJ, Post KW, Jost BH: Isolation of *Arcanobacterium (Actinomyces) pyogenes* from cases of feline otitis externa and canine cystitis. *J Vet Diagn Invest,* 14, 159-62, 2002. DOI: 10.1177/104063870201400212

30. Niesters HGM, Goessens WHF, Meis JFMG, Quint WGV: Rapid, polymerase chain reaction-based identification assays for *Candida* species. *J Clin Microbiol,* 31, 904-910, 1993.

31. Amin AS, Hamouda RH, Abdel-all AAA: PCR assays for detecting major pathogens of mastitis in milk samples. *WJDFS,* 6 (2): 199-206, 2011.

32. Karahan M, Açık MN, Kalın R, Çetinkaya B, Taşdemir B, Koç O: Development of a novel multiplex polymerase chain reaction assay for direct detection of major pathogens in milk samples of cows with mastitis. Abstract Book, *X. National Congress of Veterinary Microbiology (with International Guestspeakers),* 24-27 September, Aydın, Turkey, 2012.

33. Gillespie BE, Oliver SP: Simultaneous detection of mastitis pathogens, *Staphylococcus aureus, Streptococcus uberis,* and *Streptococcus agalactiae* by multiplex real time polymerase chain reaction. *J Dairy Sci,* 88 (10): 3510-3518, 2005. DOI: 10.3168/jds.S0022-0302(05)73036-8

34. Koskinen MT, Wellenberg GJ, Sampimon OC, Holopainen J, Rothkamp A, Salmikivi L, van Haeringen WA, Lam TJ, Pyörälä S: Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. *J Dairy Sci,* 93 (12): 5707-5715, 2010. DOI: 10.3168/jds.2010-3167

35. Nelson EA, Palombo EA, Knowles SR: Comparison of methods for the extraction of bacterial DNA from human faecal samples for analysis by real-time PCR. **In,** Mendez V (Ed): Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. Vol. 2, 1479-1485, Formatex Research Center, Hawthorn, Australia, 2010.

36. Turenne CY, Witwicki E, Hoban DJ, Karlowsky JA, Kabani AM: Rapid identification of bacteria from positive blood cultures by fluorescence-based PCR-single-strand conformation polymorphism analysis of the 16S rRNA gene. *J Clin Microbiol*, 38 (2): 513-520, 2000.

Kafkas Univ Vet Fak Derg
21 (2): 283-285, 2015
DOI: 10.9775/kvfd.2014.11957

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Short Communication

PCR Amplification of *Helicobacter pullorum* 16S rRNA Gene in Cecal Content of Pet Birds

Majid GHOLAMI-AHANGARAN [1] Reza AZARGOON [2]
Morteza Mohammad Hosseini ANARI [2]

[1] Department of Poultry Diseases, Veterinary Medicine Faculty, Shahrekord Branch, Islamic Azad University, Shahrekord, IRAN
[2] Graduated of Veterinary Medicine Faculty, Shahrekord Branch, Islamic Azad University, Shahrekord, IRAN

Article Code: KVFD-2014-11957 Received: 12.07.2014 Accepted: 25.11.2014 Published Online: 10.12.2014

Abstract

For evaluation of pet birds as a resource of *Helicobacter pullorum*, 100 cecal content samples were collected from 50 finches and 50 Australian parrots, with no clinical signs suggestive of systemic and/or enteric disease, in Iran. DNA was extracted from cecal samples and PCR was carry out for identification of *H. pullorum* 16S rRNA gene. Results showed, *H. pullorum* DNA was detected in 3 of the 50 (6%) cecal samples of finch and 6 out of 50 (12%) cecal samples of Australian parrot. This study represents that finch and Australian parrot can be a source of *H. pullorum* infection and serve as reservoirs for the *H. pullorum*.

Keywords: *Australian Parrot, Finch, Helicobacter pullorum*

Evcil Kuşların Sekum İçeriklerinden *Helicobacter pullorum* 16S rRNA Geninin Amplifikasyonu

Özet

Helicobacter pullorum kaynağı olarak evcil kuşların değerlendirilmesi amacıyla İran'da sistemik veya enterit klinik belirtileri göstermeyen 50 ispinoz ve 50 Avustralya papağanından toplam 100 sekum içeriği örneği toplandı. Sekal örneklerden DNA ekstraksiyonu yapıldı ve *H. pullorum* 16S rRNA geninin identifikasyonu amacıyla PCR gerçekleştirildi. *H. pullorum* geni 50 ispinozun 3'ünde (%6) ve 50 Avustralya papağanının 6'sında (%12) belirlendi. Çalışmanın sonuçları ispinoz ve Avustralya papağanının *H. pullorum* enfeksiyonu kaynağı ve bu etken için reservuar olabileceğini göstermiştir.

Anahtar sözcükler: *Avustralya papağanı, İspinoz, Helicobacter pullorum*

INTRODUCTION

Helicobacter pullorum is a gram-negative species, slightly curved rod with monopolar, nonsheathed flagella, classified in ureas-negative enterohepatic group of Helicobacters [1] which naturally colonize the gastro-intestinal tract surface [2]. *H. pullorum* has been linked with enteritis and hepatitis in broiler chickens and laying hens, and diarrhea, gastroenteritis, and liver disease in humans [1]. This bacterium can be considered a foodborne human pathogen [1]. Although there are some studies on the prevalence of *H. pullorum* in poultry [1,3,4] limited information is present in other birds [5]. Until now, there are no report on the presence of H. pullorum in pet birds. To reduce contamination and illness caused by *H. pullorum*, the finding of *Helicobacter* resources is very important. Hence, the aim of this study was to investigate the frequency of *Helicobacter* in cecal content of finch and Australian parrot in Chaharmahal-va-Bakhtiyari and Mazandaran Provinces, West and North of Iran. The aim of this study was to determine the occurrence of *H. pullorum* in pet birds by using polymerase chain reaction (PCR) to clarify the role of pet birds as reservoir of this bacterium in Iran.

MATERIAL and METHODS

Fifty cecal content samples from 50 finches and Australian parrots (each of them) was collected from 20 pet markets in Chahrmahal-va-Bakhtiyari and Mazandaran province, west and north of Iran. The sampled birds had

İletişim (Correspondence)
+98 381 3361000
mgholamia@yahoo.com

no clinical signs suggestive of systemic and/or enteric disease. DNA was extracted from cecal content samples using a commercial DNA extraction kit (AccuPrep**,** Bioneer co., South Korea).

PCR was carried out to amplify a fragment of 447 bp of the 16S rRNA gene of *H. pullorum*. The sequence of the primers was as follows: forward primer: 5′-ATGAA TGCTAGTTGTTGTCAG; reverse primer: 5′-GATTGGCTCC ACTTCACA [6]. PCR amplification was performed in PCR buffer containing 1.5 m*M* $MgCl_2$, 200 µ*M* each dNTPs, 10 p*M* each primer, and 1.0 unit of *Taq* polymerase (Fermentas, Germany) in a 25 µL total reaction volume. The gene ruler 100-bp DNA ladder plus (Fermentas, Germany) was used. The amplification was carried out in a thermal cycler (Mastercycler Gradient, Eppendorf, Germany) under the following conditions: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation, annealing, and extension at 94°C for 1 min, 60°C for 90 sec, and 72°C for 90 sec, respectively, and a final extension at 72°C for 5 min. The PCR product was then analyzed by electrophoresis in 1.5% agarose gel and visualized under UV light after staining with ethidium bromide.

RESULTS

In the inspection of sampled birds no clinical signs were observed. In PCR, a 447 bp fragment of *H. Pullorum* 16S rRNA gene was amplified in 3 out of 50 finches (6%) and 6 out of 50 Australian parrot (12%) *(Fig. 1)*. The positive samples were collected from 5 different pet markets in both Chahrmahal-va-Bakhtiyari and Mazandaran provinces in Iran.

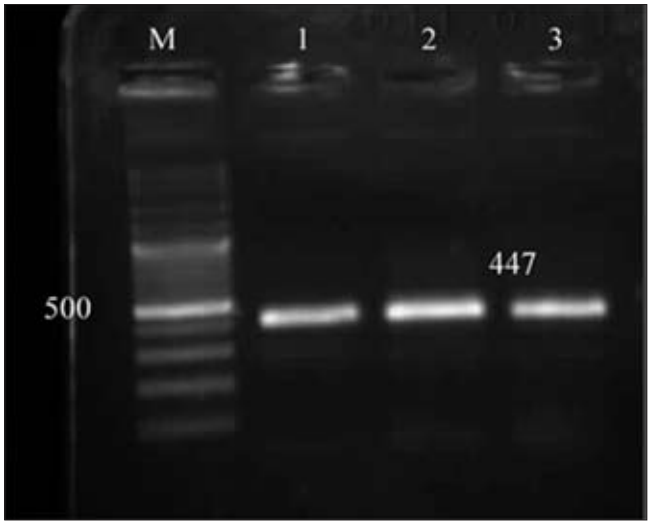


Fig 1. PCR amplification of the 16S rRNA gene of *Helicobacter pullorum* in cecal content of finch and Australian parrot (M: DNA ladder marker; lanes 1, 2 and 3: positive samples)

Şekil 1. İspinoz ve Avustralya papağanının sekum içeriklerinden *Helicobacter pullorum* 16S rRNA geninin PCR amplifikasyonu (M: DNA merdiven markırı; şerit 1, 2 ve 3: pozitif örnekler)

DISCUSSION

This study showed the infection of digestive contents of Australian parrot and finch by *H. pullorum* and demonstrated that 6% cecal content samples of finch and 12% cecal content samples of Australian parrot were positive for the 16SrRNA gene of *H. pullorum*. Presence of *H. pullorum* in the cecal contents of these bird species indicates its ability to colonize in digestive system of these birds. Previous reports showed that *H. Pullorum* could infect the digestive system of human and avian species. This bacterium was isolated from digestive contents of broiler chicken, laying hens [1,7], guinea fowl [8] and in a psittacin bird [5]. Moreover, *H. pullorum* has been isolated in fecal samples from humans with gastroenteritis in the UK, Canada, Germany and Switzerland [9] and its DNA was detected from gallbladder of a woman suffering from chronic cholecystitis and from livers of patients suffering from cirrhosis and/or hepatocarcinoma [10,11]. However, lack of knowledge about pathogenic mechanisms of the bacterium has made it difficult to relation of bacteria with disease signs. In infected persons, no history of human contact with birds was reported so far. Nevertheless, recent studies demonstrated that *H. pullorum* could be an initiator of the intestinal inflammation, because of the releasing cytolethal distending toxin, leading to activation of kB-necrozing factor pathway in the mucous intestine cells [12]. However, Infection of apparently healthy finch and Australian parrots with *H. pullorum* indicated that these birds might be referred as a source enterohepatic pathogenic bacteria in humans and causing digestive problems to humans.

Several studies have investigated the infection status of the birds with *H. pullorum* [9,13], which their comparisons are more complex because of using different samples and techniques, like culture or PCR. Several epidemiologic studies demonstrated prevalence of this micro-organism in cecal content of birds, especially layer and broiler chicken, ranging from 4% [9] to 100% [13] that depending on the bird species, diet type, keeping and growing system, different samples and techniques for the bacteria detection. This is the first report refer to infection rate of *H. pullorum* from cecal contents of the pet birds (finch and Australian parrot) in Iran. It is necessary to conduct more studies on carriage of the *H. pullorum* in different bird species and its pathogenicity in humans.

REFERENCES

1. Ceelen LM, Decostere A, Van den Bulck K, On SLW, Baele M, Ducatelle R, Haesebrouck F: *Helicobacter pullorum* in chickens, Belgium. *Emerg Infect Dis,* 12 (2): 263-267, 2006. DOI: 10.3201/eid1202.050847

2. Manfreda G, Parisi A, Lucchi A, Zanoni RG, De Cesare A: Prevalence of Helicobacter *pullorum* in conventional, organic, and free-range broilers and typing of isolates. *Appl Environ Microbiol,* 77 (2): 479-484, 2011. DOI: 10.1128/AEM.01712-10

3. Burnens AP, Stanley J, Nicolet J: Possible association of *Helicobacter pullorum* with lesions of vibrionic hepatitis in poultry. **In,** Newell DG,

Ketley JM, Feldman RA (Eds): *Campylobacter*, *Helicobacter* and Related Organisms. 291-293, Plenum Press, New York, 1996.

4. Kahraman BB, Ak S: Investigation of *Helicobacter pullorum* occurence in chicken in the Marmara region of Turkey. *Istanbul Univ Vet Fak Derg,* 39 (1): 63-66, 2013.

5. Ceelen L, Decostere A, Martel A, Pasmans A, Haesebrouck F: First report of *Helicobacter pullorum* in the faeces of diarrhoeic psittacine bird *(Psephotus haematogaster). Vet Rec,* 159, 389-390, 2006. DOI: 10.1136/vr.159.12.389

6. Stanley J, Linton D, Burnens AP, Dewhirst FE, On SLW, Porter A, Owen RJ, Costas M: *Helicobacter pullorum* sp. nov.-genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. *Microbiol,* 140, 3441-3449, 1994. DOI: 10.1099/13500872-140-12-3441

7. Zanoni RG, Rossi M, Giacomucci D, Sanguinetti V, Manfreda G: Occurrence and antibiotic susceptibility of *Helicobacter pullorum* from boiler chickens and commercial laying hens in Italy. *J Food Microbiol,* 116, 168-173, 2007. DOI:10.1016/j.ijfoodmicro.2006.12.007

8. Nebbia P, Tramuta C, Ortoffi M, Bert E, Cerruti Sola S, Robino P: Identification of enteric helicobacter in avian species. *Schweis Arch Tierheilkd,* 149 (9): 403-407, 2007. DOI: 10.1024/0036-7281.149.9.403

9. Burnens AP, Stanley J, Morgenstern R, Nicolet J: Gastroenteritis associated with *Helicobacter pullorum*. *Lancet,* 344, 1569-1570, 1994.

10. Fox JG, Dewhirst FE, Shen Z, Feng Y, Taylor NS, Paster BJ: Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. *Gastroenterology,* 114, 755-763, 1998. DOI: 10.1016/S0016-5085(98)70589-X

11. Pellicano R, Mazzaferro V, Grigioni WF, Cutufia MA, Fagoonee S, Silengo L, Rizzetto M, Ponzetto A: *Helicobacter* species sequences in liver samples from patients with and without hepatocellular carcinoma. *World J Gastroenterol,* 10, 598-601, 2004.

12. Young VB, Knox KA, Schauer DB: Cytolethal distending toxin in avian and human isolates of *Helicobacter pullorum*. *J Infect Dis,* 182, 620-623, 2000. DOI: 10.1086/315705

13. Pilon C, Prouzel-mauleon V, Menard A, Megraud F: Development of a real-time quantitative PCR specific to *Helicobacter pullorum*. *Abstract of Scientific Presentations in 13th International Workshop on Campylobacter, Helicobacter and Related Organisms.* Queensland, 2005.

Kafkas Univ Vet Fak Derg
21 (2): 287-289, 2015
DOI: 10.9775/kvfd.2014.12166

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Case Report

Anorectal Malformation with Colovesical and Colourethral Fistula in Two Calves

Ahmet ÖZAK [1] H. Özlem NİSBET [1] Cenk YARDIMCI [1] Yonca B. KABAK [2]

[1] Department of Surgery, Faculty of Veterinary Medicine, Ondokuz Mayıs University, TR-55139 Samsun - TURKEY
[2] Department of Pathology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, TR-55139 Samsun - TURKEY

KVFD-2014-12166 Received: 17.08.2014 Accepted: 24.12.2014 Published Online: 07.01.2015

Abstract

This paper reports the evaluation of anorectal malformations with two different fistula in two calves. During clinical examination atresia ani and meconium coming from preputium was observed in both calves. Distention of intestines was viewed in radiological examination. Surgical treatment had been preferred in both calves. During surgery it was observed that colon was connected to the bladder with tube-like formation in calf 1. In calf 2 colo-urethral fistula was detected during necropsy. The clinical, surgical and necropsy findings of anorectal malformations with colovesical and colourethral fistula in two calves has been reported for the first time. As a conclusion, the localization of the fistula and the condition of the animal could affect results of surgical treatment.

Keywords: *Anorectal malformation, Colovesical, Colourethral fistula, Calf*

İki Buzağıda Karşılaşın Anorektal Malformasyon İle Birlikte Gözlenen Kolovesikal ve Kolouretral Fistül Olgusu

Özet

Bu makalede iki ayrı buzağıda iki farklı fistülü bulunan anorektal malformasyonun değerlendirilmesi sunulmuştur. Her iki buzağının klinik muayenesi sırasında atresia ani ve mekonyumun prepisyumdan geldiği belirlendi. Radyografik muayenede ise bağırsakların genişlediği gözlendi. Her iki buzağının opeartif tedavisine karar verildi. Birinci buzağının operasyonunda kolonun tüp şeklinde bir yapı ile idrar kesesine bağlandığı belirlendiği. İkinci buzağıda ise kolonun urertraya fistülleştiği nekropsi sırasında anlaşıldı. Kolovesikal ve koloüretral fistüle sahip iki buzağının klinik, cerrahi ve nekropsi bulguları ilk defa sunulmaktadır. Sonuç olarak, fistülün lokalizasyonu ve hayvanın genel durumu cerrahi tedavinin sonucunu etkileyebilmektedir.

Anahtar sözcükler: *Anorektal malformasyon, kolovesikal, kolouretral fistül, buzağı*

INTRODUCTION

Congenital defects, abnormalities of structure or function present at birth, may be caused by genetic or environmental factors, or a combination of both, however, in many cases, the causes are unknown. Developmental defects may be lethal, semi-lethal, or compatible with life, causing esthetic defects or may have no effect on the animal [1,2]. Congenital intestinal malformations occur frequently in cattle. There are several types, each with its own prognosis. Atresia ani and atresia recti are hereditary malformations, which occur often in calves and sometimes in sheep [3]. Urogenital anomalies are most commonly induced as a consequence of cloacal membrane defects during embryogenesis [4]. Atresia ani or recti with recto-vaginal (recto-vestibular) fistula are reported in calves as necropsy findings [1,4-6], however, there are several studies reporting the surgical treatment of atresia ani with recto-vaginal fistula in alpaca and calves [3,7,8].

The aim of this study is to report the surgical treatment results and prognosis of anorectal malformation with colo-vesical and colourethral fistulas in two calves. According to authors' knowledge, these type anomalies have not ever been reported.

CASE HISTORY

Calf 1

The calf was referred to our clinics with a complaint of defecation. In clinical evaluation of two-day-old male Simental breed calf, lack of anal opening and meconium

İletişim (Correspondence)
+90 532 7203366
aozak@hotmail.com

coming from the prepuce were observed *(Fig. 1)*. In radiographic examination only gas and feces were detected. Complete blood cell count and blood serum biochemical analysis were normal. However, urinalysis released feces particles, leucocyte and protein. General anesthesia was induced using isoflurane (2-3%) in oxygen delivered through a mask held over the nose and mouth, and maintained with isoflurane in oxygen through an endotracheal tube. A 16 gauge catheter was placed to jugular vein for intraoperative fluid therapy and postoperative medication. Trimethoprim Sülfadimetilprimidin 40 mg/kg (Triprim, Interhas, Ankara, Turkey) and flunixin meglumine 1.1 mg/kg (Fundamin, Bavet, Istanbul, Turkey) were administered before surgery. Epidural anesthesia was performed with bupivacaine 0.2 ml/kg (Marcaine, Astra Zeneca, Istanbul, Turkey) at the level of the sacrococcygeal space. Surgery was performed by median laparotomy. The colon was connected to the bladder in tube-like formation *(Fig. 2)*. Two sutures were placed and after transection of the connection, the end of openings was sutured separately in routine manner. Right flank colostomy was performed, because distance between anus and colon was not appropriate to connect colon to the perineal region.

During the first 24 h following surgery, except defecation incontinence, the calf was seen to defecate normally. Three and six months following surgery, the calf was healthy and well growing, however, the esthetic appearance of colostomy was not seemed well.

Calf 2

In clinical evaluation of one-day-old male Simental breed calf, lack of anal opening and meconium coming from the prepuce and tenesmus were observed. In radiography of the abdomen megacolon and enlargement of secum with gas were detected. Complete blood cell count and blood serum biochemical analysis were normal. Same anesthesia procedure of calf one was used in calf two. Trimethoprim Sülfadimetilprimidin 40 mg/kg (Triprim, Interhas, Ankara, Turkey) and flunixin meglumine 1.1 mg/kg (Fundamin, Bavet, Istanbul, Turkey) were administered before surgery. Epidural anesthesia was performed with bupivacaine 0.2 ml/kg (Marcaine, Astra Zeneca, Istanbul, Turkey) at the level of the sacrococcygeal space. Surgery was performed by median laparotomy. During exploration of abdomen, secum and large intestines were full of gas, especially ascending colon was enlarged and full of

Fig 1. In case one, the meconium was seen in and around the prepuce

Şekil 1. Birinci olguda prepüsyumun içinde ve etrafında mekonyum görülmekte

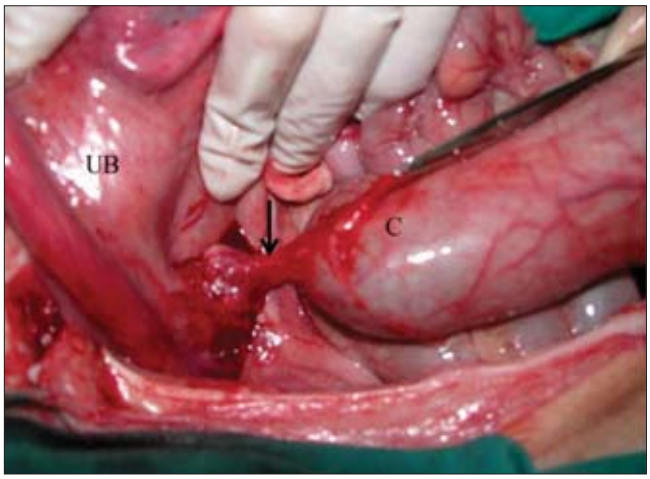


Fig 2. Connection of colon to urinary bladder with tube-like formation. **C-** Colon, **UB-** Urinary bladder, *arrow:* Tube-like formation connecting colon and urinary baldder

Şekil 2. Kolonun idrar kesesine tüp şeklinde bir bağlantıyla bağlanması. **C-** Kolon, **UB-** İdrar kesesi, *Ok:* Kolon ve irdrar kesesini bağlatınsını sağlayan tüp

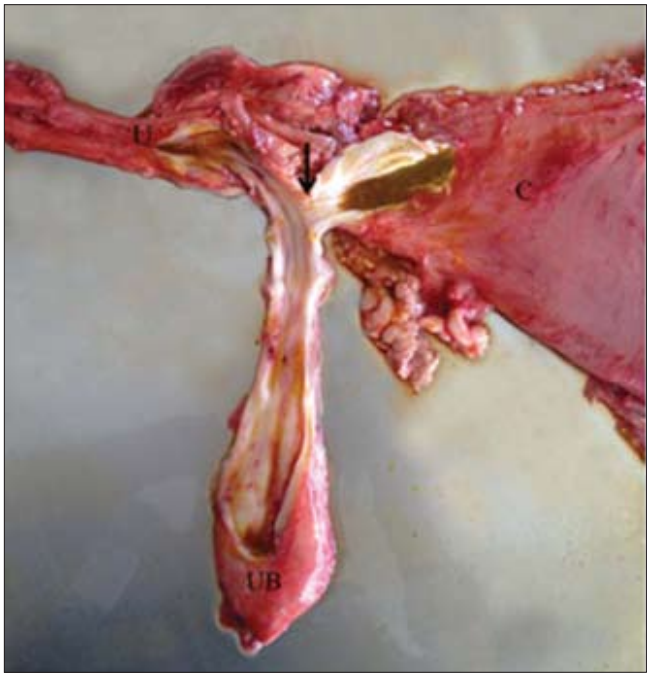


Fig 3. The appearance of connection colon to the urethra. **C-** Colon, **UB-** Urinary Bladder, **U-** Urethra, *Arrow:* connection site

Şekil 3. Kolonun üretraya bağlantısının görünümü. **C-** Kolon, **UB-** İdrar Kesesi, **U-** Üretra, *Ok:* birleşim yeri

meconium. The urinary bladder was incised and urinary catheter was applied from urinary bladder through urethra. Although, urinary bladder was empty catheter was filled with meconium when the catheter passed through the urethra. During surgery cardiac arrest was occurred, despite cardiopulmonary resuscitation calf was died. According to necropsy finding colon was opened to urethra with a small fistula *(Fig. 3)*.

DISCUSSION

Intestinal atresia is considered to be associated with interruption of the blood supply to a localized segment of embryonic intestine resulting in atrophy, disappearance of the affected segment, or failure of the affected to undergo further development [9,10]. Stenosis and atresia of the lumen are the most common congenital malformations of the gastrointestinal tract in most species. These malformations can occur at any level and are usually single rather than multiple abnormalities [11]. Failure of the urorectal fold to properly divide into the embryonic cloaca, can lead to the persistence of cloacal remnant. This malformation is usually associated with atresia ani and in these cases the terminal rectum joins the dorsal aspect of either the caudal vagina or the vestibule and thus, rectovagina or rectovestibular fistula is formed [8]. In case one, atresia ani-recti and opening of the colon to the urinary bladder, which has not been reported before, were diagnosed.

Several literatures, which usually necropsy findings are presented, have been reported the anorectal malformations with multiple disorders [1,4-6,12-14]. Successful surgical treatment of anorectal malformation with recto-vaginal (recto-vestibular) fistula is reported only in two case reports [7,8]. According to the authors' knowledge, successful surgical treatment results of anorectal malformation with multiple disorders in calves have not been presented before. Survival is largely dependent on the calf's condition at referral and on the intestinal segment affected [12]. The main causes of survival after surgical treatments were; early surgical intervention and normal general condition at referral. It is believed that general condition was not deteriorated due to evacuation of feces by the fistula. Correction of atresia ani may not be warranted in animals with severe multiple deformities [12]. Fistula that accompanies anorectal malformations was not severe deformities; moreover these positively affected the condition of the calves.

The surgical treatment of atresia ani implies the achievement of a patent opening the intestinal tract in the perineal region. Several surgical methods have been used to correct atresia coli or jejuna. Steenhaut recommended right flank colostomy if the entire colon and rectum are involved. Postoperative complications such as prolapsed of the colon and unacceptable esthetic results limited the feasible application of the procedure [15]. Flank colostomy was preferred, because it was impossible to attach the colon to the perineal region. The only major complication in flank colostomy was the esthetic appearance as mentioned in the literature [2,15].

If economic value is considered, surgical treatment choice can be preferred in anorectal malformation with fistulas. Early surgical repair, general condition and localization of the fistula are the effective points of the prognosis. However, further retrospective studies should be performed to confirm this statement.

REFERENCES

1. Ghanem M, Yoshida C, Isobe N, Nakao T, Yamashiro H, Kubota H, Miyake Y, Nakada K: Atresia ani with diphallus and separate scrota in a calf: A case report. *Theriogenology*, 61, 1205-1513, 2004. DOI: 10.1016/j.theriogenology.2003.04.002

2. Gokçe AP, Besaltı Ö, Özak A, Tong S, Yagcı B: Buzağılarda atresia kolinin operatif sağaltımı: 11 Olgu (1996-1998). *Vet Cerrahi Derg,* 3-4, 9-13, 1999.

3. Özaydın İ, Kılıç E, Aksoy Ö, Cihan M, Güngör E: Bir buzağıda üçlü malformasyon; atresia ani, rektouretral fistül ve pygomelia. *Kafkas Univ Vet Fak Derg*, 12, 189-191, 2006.

4. Jeong WI, Lee CS, Ryu SY, Willliams BH, Jee YH, Jung CY, Park SJ, Jeong DH, Lee SK, Jeong YS: Renal and ureteral fusion in a calf with atresia ani. *J Vet Med Sci,* 65, 413-414, 2003. DOI: 10.1292/jvms.65.413

5. Kilic N, Sarierler M: Congenital intestinal atresia in calves: 61 cases (1999-2003). *Revue Med Vet*, 155, 381-384, 2004.

6. Kılıç E, Özaydın İ, Aksoy Ö, Yayla S: Üç buzağıda karşılaşılan çoklu ürogenital sistem anomalisi. *Kafkas Univ Vet Fak Derg*, 12, 193-197, 2006.

7. Carraro DB, Dart AJ, Hudson NP, Dart CM, Hodgson DR: Surgical correction of anorectal atresia and rectovaginal fistula in an alpaca cria. *Aust Vet J,* 74, 352-354, 1996. DOI: 10.1111/j.1751-0813.1996.tb15443.x

8. Del Campo CH, Vits L, Del Campo MR, Ferguson JG: A case of atresia ani with rectovestibular fistulae in an alpaca *(L. pacos)*. *Dtsch Tierärz Wochenschr,* 100, 495-497, 1993.

9. Jubb TF: Intestinal atresia in Friesian calves. *Aust Vet J*, 67, 382, 1990. DOI: 10.1111/j.1751-0813.1990.tb07419.x

10. Smith DF, Ducharme NG, Fubini SL, Donawick WJ, Erb HN: Clinical management and surgical repair of atresia coli in calves: 66 cases (1977-1988). *J Am Vet Med Assoc,* 199, 1185-1190, 1991.

11. Noden DM, De Lahunta A: The Embryology of the Domestic Animal: Developmental Mechanism and Malformations. Baltimore, USA, Williams and Wilkins, 1985.

12. Dreyfuss DJ, Tulleners EP: Intestinal atresia in calves: 22 cases (1978-1988). *J Am Vet Med Assoc*, 195, 508-513, 1989.

13. Ghanem ME, Yoshida C, Nishibori M, Nakao T, Yamashiro H: A case of freemartin with atresia recti and ani in Japanese Black calf. *Anim Reprod Sci*, 85, 193-199, 2005. DOI: 10.1016/j.anireprosci.2004.04.044

14. Forzán MJ, McClure JT: Congenital aganglionosis in a 3-day-old Holstein calf. *Can Vet J*, 46, 342-344, 2005.

15. Steenhaut M, De Moor A, Verschooten F, Desmet P: Intestinal malformations in calves and their surgical correction. *Vet Rec*, 98, 131-133, 1976. DOI: 10.1136/vr.98.7.131

Kafkas Univ Vet Fak Derg
21 (2): 291-294, 2015
DOI: 10.9775/kvfd.2014.12238

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Case Report

Visceral Gout (Uricosis) and Urolithiasis Caused by Dehydration in Laying Hen Farm, Necropsy and Histopathology Findings

Agim REXHEPI [1] Corrie BROWN [2] Kurtesh SHERIFI [1] Behlul BEHLULI [1]

[1] University of Prishtina "Hasan Prishtina", Faculty of Agricultural and Veterinary, Department of Veterinary Medicine, Bill Clinton Boul, 10000 Prishtina - KOSOVA
[2] University of Georgia Athens, The College of Veterinary Medicine, Department of Pathology, GA 30602-7388 - USA

KVFD-2014-12238 Received: 03.09.2014 Accepted: 11.10.2014 Published Online: 13.10.2014

Abstract

This case report describes visceral gout in laying hens caused from deprivation of water supply as a consequence of limiting access for drinking water. In two different layer farms, nineteen week old pullets had a sudden and increased mortality rate. Gross pathology revealed typical uricosis lesions including: deposition of uric acid and its salts in kidney, pericardium, liver, muscle atrophy and dehydration. The changes seen microscopically were consistent with end stage renal failure due to chronic renal disease and other organs had lesions consistent with visceral gout. Subsequent to the diagnosis, owners instituted improved water access, and mortalities decreased. This case report emphasizes the importance of early diagnosis of chicken gout, need of training for poultry farmers and field veterinarians for early and proper prevention of visceral gout in laying hens.

Keywords: *Visceral gout, Urolithiasis, Dehydration, Pullet*

Yumurtacı Tavuk Çiftliğinde Dehidrasona Bağlı Visseral Gut (Ürikozis) ve Ürolitiazis, Nekropsi ve Histopatolojik Bulgular

Özet

Bu vaka sunumunda yumurtacı tavuklarda içme suyu eksikliğine bağlı olarak şekillenen visseral gut olgusu tanımlanmıştır. İki ayrı işletmede ani ve artmış mortalite oranı ile toplam 19 adet piliç bildirildi. Makroskobik bakıda böbreklerde, perkardiyumda ve karaciğerde ürik asit ve tuzlarının yığıntıları, kas atrofisi ve dehidrasyon ile karakterize tipik ürikozis lezyonları gözlemlendi. Mikoskobik bulgular kronik böbrek hastalığına bağlı son safha böbrek yetmezliği bulguları ile uyumlu olup diğer organlardaki lezyonlar visseral gut işaret etmekteydi. Teşhisi takiben çiftlik sahiplerinin hayvanların su alımlarını iyileştirmeleri sonucunda ölümler azaldı. Bu vaka takdimi visseral gutun yumurtacı tavuklarda erken teşhis ile çiftlik yöneticilerinin ve veteriner hekimlerin konu hakkında eğitilmelerinin hastalığın erken ve uygun bir şekilde önlenebilmesi açılarından önemini vurgulamaktadır.

Anahtar sözcükler: *Visseral gut, Ürolitiazis, Dehidrasyon, Piliç*

INTRODUCTION

The commercial laying hen industry is one of best developed branches of agriculture in Kosova, comprising about 1 million laying hens [1]. In former time egg production was mostly centralized in state cooperatives, and recently this industry is growing as private business. However, many farmers entering this field lack relevant experience, and so there are numerous health problems surfacing.

Visceral gout occurs secondary to kidney damage which can have numerous etiologic reasons, including: nutritional and metabolic factors, infectious causes, toxicity and other factors. One prominent cause is water deprivation which leads to concentration of uric acid and other minerals in the blood and later in the kidney. The infectious causes, such as nephrotropic strains of infectious bronchitis virus and renal cryptosporidiosis; and noninfectious factors, such as vitamin A deficiency [2,3], treatment with sodium bicarbonate, mycotoxins, such as oosporein [4]. Another cause of visceral gout is also if the feeding growing birds layer rations that are high in calcium and protein [2,4,5]. Whenever there is kidney damage, excretion of uric acid gets affected and uric acid starts accumulating in the blood and later in tissues.

İletişim (Correspondence)
+377 044 206269
behlul.behluli@uni-pr.edu

Visceral gout and urolithiasis are reported as an important cause of renal failure in pullets and caged laying hens [6]. Lack of access to water is a primary contributing cause and this can happen during transport, blockage of nipples, inadequate number of nipples, overcrowding etc. Severe or persistent dehydration increases resorption of water causing a subsequent\reduction in urine flow. As uric acid secretion decreases, urates may precipitate in renal tubules and ureters leading to impaction and potentially renal failure [6,7].

The cause of gout is often difficult to determine. The original kidney damage may occur long before the onset of gout mortality. Visceral gout is rarely diagnosed ante mortem and usually diagnosed only at necropsy and findings are generally sufficient to diagnose gout in poultry [2,7].

The purpose of this report is to describe importance of proper diagnosis of visceral gout caused by dehydration in order to reduce mortality outbreaks, and lessons from this study can be applied to further improvements of disease prevention and control.

CASE HISTORY

Clinical Signs

In 2012 and again in 2014, we were contacted by farmers having increased mortality in their large laying farms. The first farm had 17.000 pullets and the second had 20.000 pullets. The clinical signs were characterized by sudden onset of depression, low feed intake, dehydration, and rise in mortality. In both farms the onset of the mortality in flock of 19 to 28 weeks old is reported. In week 19 mortality rate was about 20 pullets per day with peak of mortality on weeks 20-26 with about 90 pullets per day. From week 26 until the mortality has reduced in week 28 compared to normal mortality rate. In the first farm the total number of dead pullets was 3.000 pullets (out of 17.000 initially), or 17.9%. At the second farm, mortality was 3.500 pullets (from 20.000 pullets initially), or 17.5%. Mortality has been reduced and stopped within short period after proper access to the water has improved for 2 to 4 weeks. In both farms pullets were vaccinated with complete program, including protection against infectious bronchitis virus.

Pathological Findings

The necropsy is done in dead layers (45 layers respectively 22 in first farm and 23 pullets in second farm) aged between 19 to 24 weeks-old.

Macroscopical Findings

The most prominent change was observed on the serosal surface of organs of multiple organs, where there is a diffuse deposition of white chalky material *(Fig. 1)*. Kidneys were irregularly shaped and often markedly enlarged, but still with atrophy of selected lobules *(Fig. 2)*. Dead birds were dehydrated and in poor body condition, the breast muscles were dry and atrophic *(Fig. 3)*. The ureters were markedly enlarged with irregular white uroliths *(Fig. 4)*. The large uroliths often completely filled and greatly expanded the affected ureter.

Fig 1. Advanced visceral gout with extensive deposits of urates on the pericardium, surface of the liver and peritoneum

Şekil 1. Perikardiyum, karaciğer ve periton yüzeylerinde yoğun ürat depozitleri ile karakterize ileri seviyede visseral gut

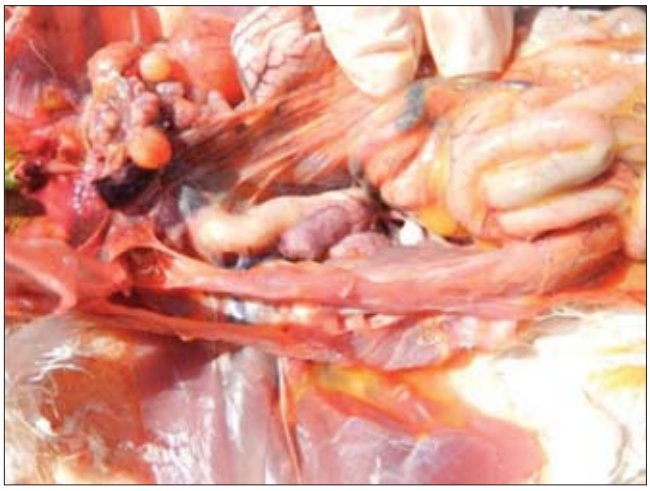


Fig 2. Urolithiasis involving right ureter and kidney atrophy with compensatory hypertrophy of the left side of the kidney

Şekil 2. Sağ üreterde ürolitiazis ve atrofi ile sol böbrekte kompanse hipertofi

Histopathology findings: The changes seen in the kidneys were consistent with end stage renal failure due to chronic renal disease. In kidney the numerous tubules have degenerate heterophils in the lumen while other tubules have proteinous casts in the lumen. Some tubules were ruptured due to crystalline array formation with multi-nucleated giant cells (tophi formation). A few tubules were acutely necrotic with intact heterophilic infiltrates. Many

Fig 3. Pullet dehydration and musculature atrophy
Şekil 3. Piliçde dehidrasyon ve kas atrofisi

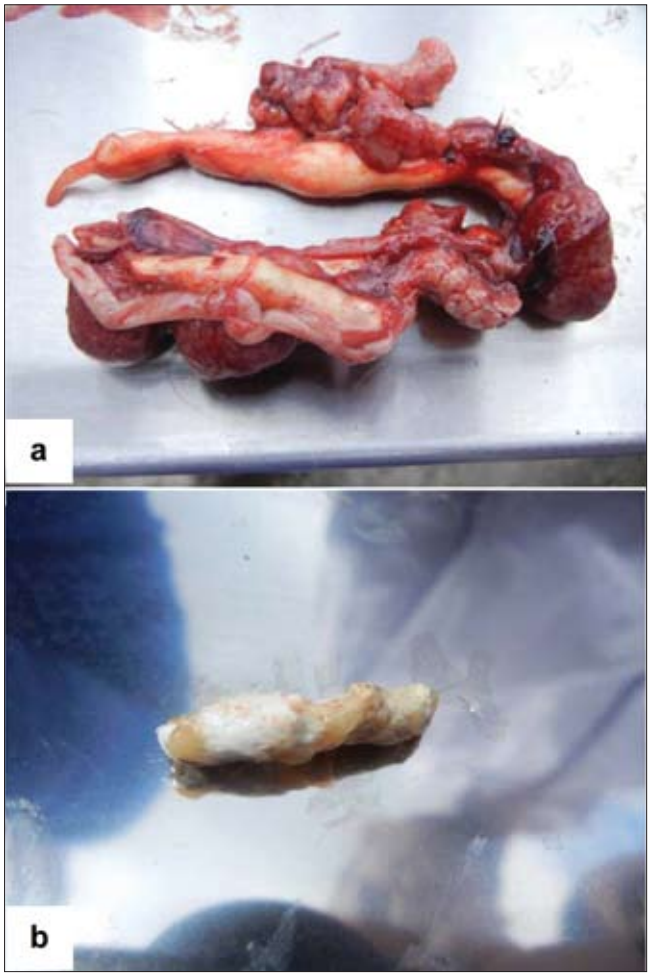


Fig 4. Picture showing, **a-** Bilateral urolithiasis and **b-** Urolith
Şekil 4. a- Bilateral ürolitiazis, **b-** Ürolit

of the organs showed lesions consistent with visceral gout. In ureters the lumen was dilated and lined by flattened epithelium that was often sloughing, with abundant eosinophilic material in the lumens, occasionally mineralizing. There were multiple areas of fibrosis in the tubules near the ureters and surrounding the ureters. The proventriculus had a few submucosal glands with focal areas of necrosis and hemorrhage. In sections of spleen, there were multiple tophi throughout the parenchyma. The liver had sections with individual hepatocyte dropout/necrosis. Fibrin thrombi were evident in sinusoids multifocally. The heart had crystals on the epicardial surface.

DISCUSSION

Dehydration due to water deprivation is a common cause of visceral urate deposition in domestic poultry [6]. Dehydration in cage farming is generally caused by inability to reach the water or failure to provide adequate amount of water. In the cases presented here, mortality decreased after it was ensured that pullets had sufficient water.

In presented case report the onset of mortality in both farms was on week 19, and continued for 5 to 6 weeks with total mortality of 17.9% of pullets (3.050 from 17.000) in first farm and 17.5% from total flock (3.500 from 20.000) in second farm.

The mortality rate was slightly higher comparing with figures with case of uricosis described by Blaxland et al.[8], where mortality was 10 to 15% of the birds with highest mortality on 19 to 24 weeks old. The mortality rate it is also reported from total flock ranging between 2% and 50% in severely affected flocks [9].

The pathogenesis of visceral gout is not completely understood but generally is considered to be the acute form of disease causing huge mortality characterized by the urate deposits on serosal surfaces, most often in the liver, kidney, pericardium, heart and air sacs reported in different authors [2,4]. Similar necropsy and histopathology findings are presents in this case report. In many cases of second most evident finding were urolithiasis characterized by blockage of one or both ureters by urate concretions with attendant atrophy of one or more lobes of the kidney drained by the obstructed ureter. The presence of uroliths in the kidney leads to compensatory hypertrophy of remaining renal tissue. Affected birds often appear normal until ureteral flow from the contralateral kidney is blocked, leading to lethargy, straining, and death [6]. Urolithiasis is condition seen particularly in caged laying hens [9].

Visceral gout and urolithiasis as a cause of pullet and layer mortality continues to be a diagnostic challenge. For the field veterinarians still seem to be difficult to react until etiologic factors are better defined it is difficult to make specific recommendations. In order to prevent visceral gout Charlton *et al.*[9] recommended to observe reasonable limits of calcium and available phosphorus in rations during grow-out and to avoid electrolyte imbalance, mycotoxins and water deprivation.

REFERENCES

1. MAFRD (Ministry of Agriculture, Forestry and Rural Development): Agriculture and Rural Development Plan 2010-13, 103 pp., Prishtina, 2010.

2. McGavin MD, Zachary JF: Pathologic Basis of Veterinary. 6th ed., p.1488, Mosby, Inc. Elsevier Inc, Missuori, 2007.

3. Shane SM: Handbook on Poultry Diseases. 2nd ed., p.210, American Soybean Association, Singapore, 2005.

4. Saif YM: Diseases of Poultry. 12th ed., pp.1409, Blackwell Publishing Ltd. Iowa, 2008.

5. Guo X, Huang K, Tang J: Clinicopathology of gout in growing layers induced by high calcium and high protein diets. *Br Poult Sci,* 46, 641-646, 2005.

6. Pollock CH: Diagnosis and treatment of avian renal disease. *Vet Clin Exot Anim,* 9, 107-128, 2006. DOI: 10.1016/j.cvex.2005.10.007

7. Lierz M: Avian renal disease: Pathogenesis, diagnosis, and therapy. *Vet Clin Exot Anim,* 6, 29-55, 2003.

8. Blaxland JD, Edith DB, Siller WG, Martindale L: An investigation of urolithiasis in tow flocks of laying fowls. *Avian Pathology,* 9, 5-19, 1980. DOI: 10.1080/03079458008418381

9. Charlton BR, Bermudez AJ, Boulianne M, Halvorson DA, Schrader JS, Newman LJ, Sander JE, Wakenell PS: Avian disease Manual. 6th ed., p.42, American Association of Avian Pathologists, Georgia, 2006.

Kafkas Univ Vet Fak Derg
21 (2): 295-297, 2015
DOI: 10.9775/kvfd.2014.12260

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Case Report

First Report on Heavy *Uncinaria (Dochmoides)* sp. (Nematoda: Ancylostomatidae) Infection in Brown Bear *(Ursus arctos)* Cub, in Van Province, Eastern Anatolian Region of Turkey

Özlem ORUNÇ KILINÇ [1] Yaşar GÖZ [2] Ali Bilgin YILMAZ [2] Loğman ASLAN [3]

[1] Yüzüncü Yıl University, Özalp Vocational School, TR-65090 Van - TURKEY
[2] Yüzüncü Yıl University, Van School of Health, TR-65090 Van - TURKEY
[3] Yüzüncü Yıl University, Wild Animal Protection Center, TR-65090 Van - TURKEY

Article Code: KVFD-2014-12260 Received: 09.09.2014 Accepted: 06.11.2014 Published Online: 11.11.2014

Abstract

On April 2014, 6 months old male Brown bear cub that found in the Saray district of Van province was brought into Directorship of Wild Animal Protection of University of Yüzüncü Yıl. Despite all the intervation, bear cub, very poor condition in clinically, dead after two days. In postmortem patological and parasitological examination were detecded severe hemorrhagic enteritis and many hookworms with white colour and approximately 1 cm diameter in surface of the small intestine. In stereo microscopic examinations, it was concluded that this worms are *Uncinaria* spp. This case report represents the first time *Uncinaria* spp. have been reported in Brown bear in Turkey.

Keywords: *Uncinaria, Brown bear cub, Turkey*

Türkiye'nin Doğu Anadolu Bölgesi Van İlinde Bir Boz Ayı Yavrusunda Şiddetli *Uncinaria (Dochmoides)* sp. (Nematoda: Ancylostomatidae) Enfeksiyonu İle İlgili İlk Bildiri

Özet

Nisan 2014'te 6 aylık erkek boz ayı yavrusu Van'ın Saray ilçesinde bulunarak Yüzüncü Yıl Üniversitesi Yaban Hayvanlarını Koruma Müdürlüğüne getirildi. Klinik olarak çok kötü durumdaki ayı yavrusu tüm müdahalelere rağmen 2 gün sonra öldü. Postmortem patolojik ve parazitolojik incelemelerde yavru ayının ince bağırsakları yüzeyine tutunmuş olarak çok sayıda beyaz renkli ve yaklaşık 1 cm uzunluğunda kancalı kurtlar ve şiddetli hemorajik enterit saptandı. Mikroskopla yapılan incelemeler sonucunda nematodların *Uncinaria* spp. olduğu sonucuna varıldı. Türkiye'de boz ayılarda *Uncinaria* spp. enfeksiyonu ilk defa bu olgu sunumuyla bildirilmiştir.

Anahtar sözcükler: *Uncinaria, Boz ayı yavrusu, Türkiye*

INTRODUCTION

The Brown bear (*Ursus arctos*) is the national animal for a number of states in North America, Europe and Asia. The Brown bear is the largest carnivore living in Turkey. Its present distrubution is mainly confined to the intact natural habitats of the Black Sea and Eastern Anatolian regions [1]. Brown bears are omnivorous and oppurtunistic, feeding on fruit, roots, insects, mammals and carcasses [2].

Many intestinal parasites including protozoa, tapeworms, cestodes and nematodes are found in bears. *Ascaris* and *Baylisascaris* are common ascaridoid nematodes in bears. Members of *Baylisascaris* are common in bear and have been reported all species except *Tremarctos ornatus*. The hookworms detected in bears are *Ancylostoma* and *Uncinaria* species. Four species of hookworm in *Ancylostoma* (*Ancylostoma brasiliens*, *A. ceylanicum*, *A. malayanum* and *A. caninum*) were reported from captive sloth bears in India [3,4]. Four species of *Uncinaria* (*U. yukonenensis*, *U. rauschi*, *U. stenocephala* and *U. ursi*) have been reported from bears [4-6].

Hookworm infections in bears like in any other gastro-intestinal parasite it is likely that the most severe effects may be seen in newborn cubs and impaired absorbtions from the intestines and can cause severe clinical sings. In

İletişim (Correspondence)
+90 530 3271928
yasargoz@hotmail.com

infected animals, there are bloody faeces, anorexia and weight loss. In juveniles, severe infection may result in poor body condition and can be fatal [2,7].

Uncinaria spp. is a common parasite of carnivores. Adult form of *Uncinaria* spp. live in the intestine and feed on blood, which can lead mucosal damage, serious clinical signs that may occur with *Uncinaria* spp. including haemorragic diarrhoea, anemia and weight loss due to malabsorption [8].

CASE HISTORY

On April 2014, 6 months old male bear cub that found in the Saray district of Van province in wooded area by shepherds was brought into Directorship of Wild Animal Protection of University of Yüzüncü Yıl *(Fig. 1)*. In bear cub, despite all the interventions that can be kept alive for 2 days, hypothermia, bloody diarrhea, apathy and anorexia were clinically present.

In pathological examination severe ulcerative hemorrhagic enteritis was detected. In postmortem macroscopic parasitological examination numerous worms with white color about 1 cm in length in surface of small intestine was detected *(Fig. 2)*. A large number of hookworm eggs were seen in microscopic faecal examination *(Fig. 3)*.

Fig 1. Brown bear cub in poor condition clinically
Şekil 1. Klinik olarak kötü durumdaki boz ayı yavrusu

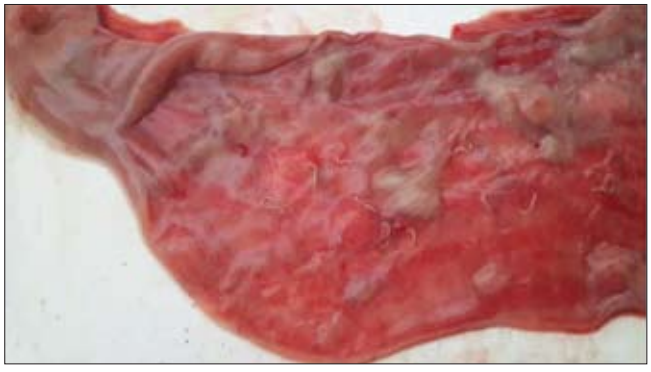


Fig 2. Many hookworms and severe hemorrhagic enteritis in small intestine of bear cub
Şekil 2. Yavru ayının ince barsaklarında çok sayıda kancalıkurt ve şiddetli hemorajik enterit

Measurements of some morphofological properties of worms that was found in small intestine of bear cub: The length of males was 7.4-8.2 mm and the diameter was 0.340-0.387 mm. Oesophagus was club-shaped, 0.784-0.820 mm long and 0.178-0.196 mm thick *(Fig. 4)*. Both spicules were slender, long, equal in lenght as 0.813-0.889 mm. The bursa comprised two big lateral lobes and a single small dorsal lobe *(Fig. 5)*. Buccal capsule was keyhole shaped, has cutting plate *(Fig. 4)*. Buccal capsule was 0.179-0.191 mm long and 0.128-0134 mm wide.

The length of females was 8.4-9.6 mm and the diameter was 0.348-0.439 mm. The distance of anus from the back-end was 0.205-0.212 mm. Female's tail was short and tapered and has a pointed terminal end *(Fig. 6)*.

DISCUSSION

Uncinaria species are small nematodes, classifed in the order of Strongylata, family *Ancylostomatidae*, and infect

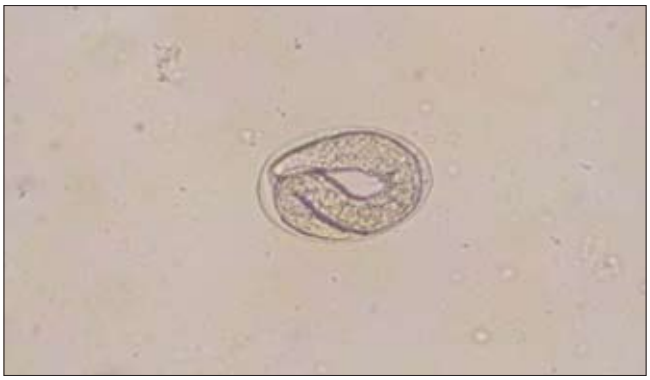


Fig 3. *Uncinaria* spp. egg containing larvae
Şekil 3. Embriyonlu *Uncinaria* spp. yumurtası

Fig 4. Buccal cavity and oesophagus
Şekil 4. Ağız boşluğu ve yemek borusu

Fig 5. Bursa copulatrix and spicules
Şekil 5. Bursa copulatrix ve spiküller

Fig 6. Posterior end of the female
Şekil 6. Dişinin arka ucu

many animal species including bear in various parts of the world [9]. The northern carnivore hookworm *(Uncinaria stenocephala)* was found in Brown bears from the vicinity of the Caspian Sea [4]. In North America, a new species of hookworm, *Uncinaria* (=Dochmoides) *yukonensis*, was described from specimens collected from wild black bear in Yukon Territory, Canada [6]. A new species of hookworm, *U. rauschi*, was described by Olsen in both black and brown bears in Alaska [10]. *Uncinaria* species, originally described as *Dochmius ursi* were collected from polar bear [4]. *Dochmius* was considired a synonyms of *Uncinaria* in later years [11].

Bursa copulatrix and spicules of male *Uncinaria* and posterior end of female *Uncinaria* are similar to *Ancylostoma*, but *Uncinaria* species in the bear cub that was detected in this study are smaller than *Ancylostoma* species in lenght.

Uncinaria species that was found in bear cub were very similar to *Uncinaria rauchi* in terms of size and many morphological characteristics. The length of the males of *Uncinaria rauchi* is 7.4-9.1 mm and the diameter is 0.243-0.297 mm. Oesophagus was club-shaped 0.763-0.856 mm long and 0.167-0.191 mm thick through bulb. Spicules were slender and 0.819-0.954 mm long. The length of the females was 7.2-10.5 mm and the width was 0.275-0.381 mm at the level of vulva [10]. But because of not having the possibility for molecular diagnosis, we also approved to name as *Uncinaria* spp. the worm that we detected in bear cub.

The importance of *Uncinaria* species in terms of public health is not known exactly, but human infections with *Uncinaria* species were reported [12]. Thus, people who handle bears should use of disposable gloves during clean up of bear faecal material.

REFERENCES

1. Can ÖE, Togan I: Status and management of Brown bears in Turkey. *Ursus*, 15, 48-53, 2004. DOI: 10.2192/1537-6176(2004)015<0048:SAMOBB>2.0.CO;2

2. Partridge J: Management Guidelines for Bears and Raccons. Assosiation of British Wild Animal Keepers, Bristol, UK, 1992.

3. Baylis HA, Daubney R: Report on the parasitic nematodes in the collection of the zoological survey of India. *Mem Indian Mus*, 7, 263-347, 1922.

4. Rogers LL Rogers SM: Parasites of bears: A review. *Third International Conference on Bears*. Binghamton, New York, USA, Paper 42, p.414, 1974.

5. Kutz S: An oppurtunistic parasitological examination of 16 nuisance black bears shot in the Dehcho in 2003. Prepared for: department of Resources, Wildlife and Economic Development, Goverment of the Northwest Territories, 8 pp, 2004.

6. Wolfgang RW: *Dochmoides yukonensis* sp. nov. from the brown bear *(Ursus americanus)* in the Yukon. *Can J Zool*, 34, 21-27, 1956.

7. Fowler ME: Zoo and Wild Animal Medicine Current Therapy 3. W.B. Saunders and Co, Philadelphia, USA, 1993.

8. Deborah J, Nicholas CL, Brett E, Danny GA: An Opportunistic Parasitological and Serological Examination of Nuisance Black Bears in the Dehcho Region of Northwest territories. Department of Environment and Natural Resources Government of the Northwest Territories, Manuscript report, no: 227, pp.30-31, 2013.

9. Schmidt GD, Roberts LA: Foundations of parasitology, 4th ed., Time Mirror/Mosby College, St. Louis, 1989.

10. Olsen OW: *Uncinaria rauschi* (Strongyloidea: Nematoda), a new species of hookwors from Alaskan bears. *Can J Zool*, 46, 1113-1117, 1968.

11. Levine ND: Nematode parasites of domestic animals and man. Minneapolis: Burgess Publishing Co. 600 pp, 1968.

12. Gadirian E: Human infection with Uncinaria in North of Iran. *Iranian J Parasitol*, 2 (3): 38-41, 2007.

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 şekil ve tablo gibi görsel öğelerin 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 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 oluşur ve toplam (metin, tablo, şekil vs dahil) 12 sayfayı geçemez. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler dışında 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 6 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.
Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 4 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.
Gözlem (Olgu Sunumu), 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 Kaynaklar bölümlerinden oluşmalı ve 4 sayfayı geçmemelidir.
Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 2 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, Sonuç ve Kaynaklar bölümlerinden oluşmalı ve 12 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: **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.
DOI numarası bulunan kaynaklarda bu bilgi ilgili kaynak künyesinin sonuna eklenmelidir.
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.

ABONE KOŞULLARI

Yıllık: 60 TL
Banka Hesap No: T.C. Ziraat Bankası Kars Şubesi, 26146825-5001 Nolu Hesap, Kars - TÜRKİYE
IBAN: TR830001000147261468255001

INSTRUCTIONS FOR AUTHORS

1- The Journal of the Faculty of Veterinary Medicine, University of Kafkas (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 and table 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 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, abstract and keywords in Turkish, Introduction, Material and Methods, Results, Discussion, and References and it should not exceed 12 pages including text, tables and illustrations. Manuscripts written in a foreign language should follow the title in foreign language, abstract and keywords in foreign language, title in Turkish, abstract and keywords in Turkish and the remaining sections described above for the manuscripts written in Turkish.

Abstracts 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 abstracts should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages. Additionally, they should not contain more than 4 figures or tables.

Preliminary Scientific Reports are short description (maximum 4 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 the Editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases without exceeding 2 pages.

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, Conclusion, and References without exceeding 12 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.

5- The necessary descriptive information (thesis, projects, financial supports etc) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of title.

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: **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.

DOI number should be added to the end of the reference.

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.

8- The editorial board has the right to perform necessary modifications and reduction on the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by editorial board, the article can only be published after the approval of the publication advisor and two referees specialized in the particular field.

9- All responsibilities from published articles merely belong to the authors.

10- There is no copyright fee for the authors.

11- A fee is charged from the authors to cover printing cost and other expenses. This payment information can be found at *http://vetdergi.kafkas.edu.tr/*

12- Reprints (in multiples of 50) of the article are sent to the authors for free.

SUBSCRIPTION

Yearly: $40

Account: T.C. Ziraat Bank, Kars Branch, Account No: 26146825-500

IBAN: TR830001000147261468255004