ISSN 1300 - 6045
e-ISSN: 1309-2251

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Journal of the Faculty of Veterinary Medicine, Kafkas University

(Yılda altı sayı yayımlanır)

(Published Bi-monthly)

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

Cilt / Volume : 19
Sayı / Number : 2 MART - NİSAN / MARCH - APRIL
Yıl / Year : 2013

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

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

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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

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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)
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Kafkas Univ Vet Fak Derg
19 (2): 183-190, 2013
DOI: 10.9775/kvfd.2012.6792

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Cytologic-Enzymologic Diagnosis of Experimental Pneumonia Induced by *Klebsiella pneumoniae* Serotype II in Rats and Its Treatment with Free and Liposomal Enrofloxacin [1]

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Makale Kodu (Article Code): KVFD-2012-6792

Summary

Enrofloxacin (ENR) rapidly localizes in eukaryotic cells *in vitro* but does not remain for prolonged periods, thereby reducing the ENR efficacy of defense against intracellular pathogens. Delivery of ENR in a liposome-encapsulated form may enhance its intracellular residence time. In this study, experimental pneumonia was induced in healthy and dexamethasone-treated rats using *Klebsiella pneumoniae* serotype II. Free and liposome-encapsulated ENR were injected intravenously into the infected animals at a dose of 7.5 mg/kg/day for 5 days. Samples of tissue, plasma and bronchoalveolar lavage (BAL) fluid were obtained at 1, 2, 3 and 4 days and 1, 2, 3 and 4 weeks after the first antibiotic treatment. All of the samples were evaluated cytologically, enzymologically, microbiologically and pathologically. It was determined that cytologic and enzymologic diagnoses of BAL fluid are not meaningful for evaluating the treatment of the experimental pneumonia in rats. However, it was established that the use of ENR in liposomal form at a dose of 7.5 mg/kg for 5 days is more effective than the free form both in the treatment of *K. pneumoniae* infections and in the prevention of recurrent infections. Liposome-encapsulated antimicrobial agents should provide another choice for antimicrobial therapy in the future, but further investigation must be completed before clinical use.

Keywords: *Enrofloxacin, Experimental pneumonia, Klebsiella pneumoniae, Liposome, Rat, Treatment*

Ratlarda *Klebsiella pneumoniae* Serotip II İle Oluşturulan Deneysel Pnömonilerin Sitolojik-Enzimolojik Teşhisi ve Serbest ve Lipozomlanmış Enrofloksasin İle Tedavisi

Özet

Enrofloksasin (ENR) *in vitro* ortamlarda ökaryotik hücrelerde hızla lokalize olur, ancak uzun süre kalamadığı için hücre içi patojenlere karşı onun etkinliği azalır. Lipozomla kapsüle edilmiş formda ENR uygulanması hücre içi ortamlarda kalış zamanını artırabilir. Bu çalışmada, sağlıklı ve deksametazon uygulanmış ratlarda *Klebsiella pneumoniae* serotip II kullanılarak deneysel pnömoni oluşturuldu. Serbest ve lipozomla kapsüle edilmiş ENR enfekte hayvanlara intravenöz olarak beş gün boyunca 7.5 mg/kg/gün dozda enjekte edildi. İlk antibiyotik uygulamasından sonraki 1, 2, 3, 4. gün ve 1, 2, 3, 4. haftalarda plazma, doku ve bronko-alveolar lavaj (BAL) sıvısı örnekleri alındı. Tüm örnekler sitolojik, enzimolojik, mikrobiyolojik ve patolojik olarak değerlendirildi. Ratlarda deneysel pnömoninin tedavisinin değerlendirilmesinde BAL sıvısının sitolojik ve enzimolojik teşhisinin anlamsız olduğu belirlendi. Ancak, enrofloksasinin beş gün boyunca 7.5 mg/kg dozda lipozomal formda kullanımının hem *K. pneumoniae* enfeksiyonlarının tedavisinde hem de tekrarlayan enfeksiyonlarının önlenmesinde serbest formdan daha etkili olduğu tespit edildi. Lipozomla kapsüle edilmiş antimikrobiyal ajanlar gelecekte antimikrobiyal tedavide diğer bir seçenek sağlayacaklardır, ancak klinik kullanımdan önce çok sayıda araştırma yapılmalıdır.

Anahtar sözcükler: *Enrofloksasin, Deneysel pnömoni, Klebsiella pneumoniae, Lipozom, Rat, Tedavi*

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INTRODUCTION

The greatest problem of fluoroquinolone antibiotics in the treatment of infections caused by intracellular bacteria is that the antibiotics cannot become effectively concentrated in the cells [1], and they cannot sustain a sufficient intracellular level long enough to display the required antibacterial effect. The cellular/extracellular (C/E) concentration rate of enrofloxacin (ENR) was defined as 9 in polymorphic mononuclear leucocytes (PMNLs) and as 5 in alveolar macrophages (AMs). When the cells were exposed to the environment without ENR, 80% of the intracellular AMs and 90% of the PMNLs moved out of the cell within the first ten minutes. In addition, *Staphylococcus aureus* isolated from the alveolar macrophages was found to be less sensitive to the bactericidal effect of ENR compared to the bacteria outside of the cell [2]. Various studies have shown that liposomal forms of fluoroquinolones can significantly increase the effectiveness of the antibacterial action compared to free forms [3,4].

Klebsiella pneumoniae is an opportunistic, pathogenic microorganism that causes nosocomial infections in people with immune system deficiency. *K. pneumoniae* infections occur in a wide range of presentations, from mild urinary tract infections to severe bacteremia as well as pneumonia associated with high mortality and morbidity [5]. Pneumonia caused by *K. pneumoniae* is generally complicated and, depending on the presence of pulmonary abscesses and multilobular dispersion, should be treated quickly with antibiotics that have fast clinical results and efficiency [6].

Cytologic examination of the bronchoalveolar lavage (BAL) fluid is commonly used for the diagnosis of respiratory diseases. It is used to determine the cellular and humoral elements of the lower respiratory airways [7,8]. The BAL provides important information for the identification of cellular structures and the definition of the scope of the inflammation [9]. Direct examination and cultures of BAL can also be used to define the etiologic agent and aid in the selection of the appropriate antimicrobial drug [10]. In pulmonary diseases, the concentration of some cytoplasmic enzymes, such as lactate dehydrogenase (LDH), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP), which are found in the cell membrane, and some lysosomal enzymes, including beta-glucuronidase and beta-N-acetyl glucosaminidase, are increased. Microprotein (MP) and urea (UREA) concentrations in the BAL are regarded as indicators of changes in the respiratory membrane and in vascular permeability [11,12].

The purpose of this study was to evaluate the clinical effectiveness of free (F-ENR) and liposomal ENR (L-ENR) microbiologically, cytologically, enzymologically and pathologically in experimental respiratory tract infections induced by *K. pneumoniae* serotype II in rats.

MATERIAL and METHODS

Preparation and Purification of Liposomes: ENR loaded multilamellar vesicles (MLVs) and large unilamellar vesicles (LUVs) were prepared using a modified dry film method [13,14]. The F- and L-ENR were separated by gel filtration on a Sephadex G-50 column at 25°C (1.0 cm in diameter, a gel bed height of 20 cm, Sigma Chemical Co., USA), and PBS (pH 7.4) was used as the medium [15].

Visualization and Transmission Electron Microscopy (TEM) Study: The final liposomes were visualized under an electron microscope using a negative staining technique (JEOL, Tokyo, Japan) at 10.000x magnification [13].

Determination of Liposome Size and Zeta Potential: Size and zeta potential measurements were performed at 25°C using a Malvern Zetasizer (Nano ZS, Malvern Instruments, Worcestershire, UK) and the results are expressed as the mean of three measurements [14,16].

Encapsulation Capacity: Triton X-100 solution (0.1 ml, 1% v/v) was added to 1 ml freshly prepared liposomal suspension and vortexed. Then, a 0.1 ml sample was adjusted to 10 ml with PBS (pH 7.4). The amount of drug present in the resulting solution was measured spectrophotometrically at 270 nm (BioSpec-1601, Shimadzu, Japan). Each measurement was performed in triplicate [17].

In Vitro Release Study: The *in vitro* drug release from the liposomal systems was determined using dialysis sacks (Sigma, 250-7U USA) and 5 ml liposomal suspension was poured into the sack. The dialysis sacks containing the liposomal preparations were suspended vertically in a beaker containing 200 ml PBS (pH 7.4), and the contents of the beaker were stirred using a magnetic stirrer at 37±1°C. The samples were withdrawn periodically and determined spectrophotometrically at 270 nm [17].

Animals: In this study, 280 Sprague-Dawley female rats (4-5 months of age, 250-300 g) were used (Selcuk University, Medicine Faculty, Kombassan Experimental Animals Unit). The experimental animals were maintained in polysulfone cages including five rats in each cage, and heat, light and humidity (24±2°C, 12/12 h light/darkness and 55±5% relative humidity) were controlled. Food and water were provided *ad libitum*. Before the experiment, approval of the Ethical Committee (No: 2005/011) was obtained from Selcuk University, Veterinary Faculty Ethical Committee.

Bacteria and Infection Model: *K. pneumoniae* serotype II (Dr. Sylvain Brisse, Unité Biodiversité des Bactéries Pathogènes Emergentes et Plate-forme Santé Publique Institut Pasteur, France) was used to infect the rats. We defined a $1x10^6$ bacteria/ml concentration as sufficient to cause experimental pulmonary infection. After the general

health of the rats was examined, experimental pulmonary infection was induced in healthy rats and in rats that were given 0.5 mg/kg dexamethasone (0.4% dexamethasone with drinking water for 1 week; Richter Pharma AG) [18], using *K. pneumoniae* serotype II according to the method stated by Bakker-Woudenberg [19].

In Vivo Experimental Design and Sampling Procedure: Bolus doses of F-ENR and L-ENR were given intravenously into the tail vein at a dosage of 7.5 mg/kg b.w. in rats infected with *K. pneumoniae* serotype II. Antibiotic applications started 24 h after bacterial inoculation into the trachea and continued at 24 h intervals for five days. The rats were divided into two groups as control and experimental groups. The control groups were divided into three groups with 8 rats in each group as follows: group I, positive control group with experimental infection but no antibiotic treatment (PC1); group II, positive control group with experimental infection after dexamethasone application but no antibiotic treatment (PC2); and group III, negative control group with healthy rats that did not receive any drug treatment (NC). The experimental groups were divided into the four following groups with 64 rats in each group: group I, F-ENR-treated group with experimental infection (FE); group II, F-ENR-treated group with experimental infection after receiving dexamethasone (FED); group III, L-ENR-treated group with experimental infection (LE); and group IV, L-ENR-treated group with experimental infection after receiving dexamethasone (LED). The PC1 and PC2 groups were euthanized by CO_2 inhalation on the fifth day following bacteria inoculation into the trachea. The experimental groups were euthanized by CO_2 inhalation following drug application on day 1, 2, 3 and 4 and week 1, 2, 3, and 4. The chest cavities were opened under sterile conditions, and the BAL fluid, lungs, liver, kidney and spleen were removed. Microbiologic, histopathologic and cytologic-enzymologic analyses of the samples were performed.

Microbiological Analysis: The quantitative bacteria numbers were defined for the BAL and lung samples by a microdilution method [20]. In addition, the susceptible colonies were identified using classic microbiology methods, and we determined whether they were *K. pneumoniae*. The samples of the other tissues (livers, kidney and spleen) were cultured on blood agar and MacConkey agar to isolate *K. pneumoniae* and other bacteria.

Analysis of BAL Fluid: The BAL fluid samples were evaluated cytologically and enzymologically [21,22]. The cytological differences and differential cell numbers were defined using two different staining techniques (Wright-Giemsa and Papanicolaou). LDH, AST, GGT, MP and urea concentrations were measured. These analyses were performed using a commercial kit with an autonalyzer (BioMerieux ILAB 300 Plus).

Pathological Analysis: After the BAL fluid was taken, the macroscopic findings were recorded. Tissue samples were collected and stored in 10% buffered formaldehyde. The tissues were prepared in paraffin blocks using an automatic tissue processor (Leica TP 1020, Nussloch, Germany) according to routine laboratory procedures, stained with hematoxylin and eosin (HE) [23] and examined using a light microscope (Olympus, Model BX51TF, Olympus, Tokyo, Japan).

Statistical Analysis: The values for bacteria numbers as defined micro-biologically were calculated. The results of the microbiology and the BAL fluid analysis were evaluated using one-way ANOVA. The differences between the groups were defined using *Duncan's* test. The results obtained in the microscopic examinations of the lung and liver tissues were evaluated using a *Mann-Whitney U* test. $P<0.05$ was accepted as statistically significant.

RESULTS

Liposome Characterization: The ENR–loaded liposomes were obtained using the dry lipid film method [19]. The characterization of the liposome shape and surface was evaluated by TEM, and spherical liposomes were obtained, as observed in *Fig. 1*. The particle size of the liposomes, with surface charges of +50.3±2.6 mV and +55.3±1.7 mV, were 2.58±0.29 mm and 4.65±0.37 mm. The encapsulation capacities of the liposomes were 83.7±0.6% and 95.3±0.1% *(Table 1)*. The slowest ENR release and highest encapsulation drug amounts can be obtained by using the LUVs rather than the MLVs.

Microbiological Findings: When the number of bacteria isolated from the BAL samples of infected rats was compared between the groups, we observed significant differences between the results obtained on different days. No bacterial isolation was performed on the BAL samples in the 4th week *(Table 2)*. We determined that the

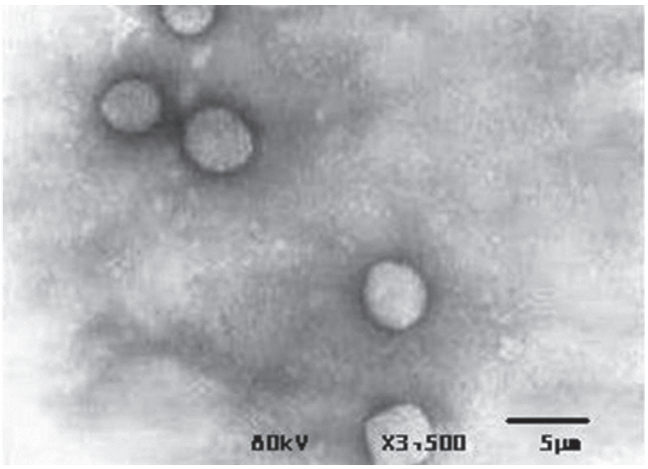


Fig 1. TEM photograph of LUV formulation (The molar ratio of DPPC: cholesterol:ENR:SA is 4.7:17.3:1.0:2.0)

Şekil 1. LUV formülasyonunun TEM fotoğrafı (DPPC:cholesterol:ENR:SA molar oranları 4.7:17.3:1.0:2.0'dir)

number of re-isolated bacteria was higher in the lung samples of the infected rats than in the BAL samples *(Table 2)*. When the days and groups were considered, the highest morbidity was found on the 3rd day in the LED group and on the 2nd day in the FED group *(Table 3)*.

Findings of BAL Cytology and Enzymology: Results of cytologic analysis between the 6 groups of rats with experimental respiratory tract infection are given in *Table 4*. The comparisons of the measured enzyme activities in the BAL fluid (LDH, GGT, and AST) with the urea and MP concentrations are presented in *Table 5* and *Table 6*.

Pathological Findings: Although the pathological findings depended on infection development were found in the control and experiment groups after histopathological examination of the tissue samples, there were no statistically significant differences among the groups. Some of the macroscopic findings are shown in *Fig. 2*, and the histopathological findings are shown in *Fig. 3*.

DISCUSSION

In this study, ENR-loaded MLVs and LUVs, which were prepared using the optimum formulations of our previous studies [13,14], were given to rats with experimental pneumonia, and we evaluated the efficiency of the liposomal treatment. The characterization of the liposomes' shape and surface was evaluated using a transmission electron microscope (TEM). The liposomes were spherical in shape and were found to be multilamellar and large unilamellar forms. The MLVs were smaller and had lower encapsulation capacity than the LUVs ($P<0.05$) *(Table 1)*. The ENR release from the LUVs was much slower than that observed from the MLVs (data not given). This might be due to the particle size and encapsulation capacity of the liposomes. When we

Table 1. *Codes and formulations of ENR-loaded MLVs and LUVs*
Tablo 1. *ENR yüklenen MLV ve LUV'ların kodları ve formülasyonları*

Codes	Molar Ratios of DPPC: Cholesterol:ENR:SA	α-tocopherol (mg)	Internal Phase [Chloroform (ml)]	External Phase [PBS, pH 7.4 (ml)]	Mean Particle Size (mm ± SD)	mV (± SD)	Encapsulation Capacity (% ± SD)
MLV	7:26.0:1.5:3.0	15	5	5	2.58±0.29	55.3±1.7	83.7±0.6
LUV	4.7:17.3:1.0:2.0	15	5	5	4.65±0.37	50.3±2.6	95.3±0.1

Table 2. *The comparisons of re-isolation of K. pneumoniae in the BAL and lung samples of the experimental groups*
Tablo 2. *Deney gruplarının BAL ve akciğer örneklerinde K. pneumoniae'nın yeniden izolasyonlarının karşılaştırılması*

Sample	Group	Day 1	Day 2	Day 3	Day 4	Day 5	Week 2	Week 3	Week 4
BAL	FE	$2.69\pm1.10^{a,y}$	$1.84\pm0.96^{a,xy}$	$0.66\pm0.44^{a,x}$	$0.00\pm0.00^{a,x}$	$0.00\pm0.00^{a,x}$	$0.87\pm0.87^{a,xy}$	$0.30\pm0.30^{a,x}$	$0.00\pm0.00^{a,x}$
	FED	$3.03\pm0.74^{a,y}$	$0.64\pm0.45^{a,x}$	$2.53\pm0.80^{a,y}$	$2.56\pm0.48^{c,y}$	$0.00\pm0.00^{a,x}$	$0.23\pm0.23^{a,x}$	$0.00\pm0.00^{a,x}$	$0.00\pm0.00^{a,x}$
	LE	$2.68\pm0.81^{a,y}$	$0.97\pm0.68^{a,x}$	$1.30\pm0.70^{a,xy}$	$0.60\pm0.41^{ab,x}$	$0.32\pm0.32^{a,x}$	$0.00\pm0.00^{a,x}$	$0.00\pm0.00^{a,x}$	$0.00\pm0.00^{a,x}$
	LED	$7.48\pm0.47^{b,z}$	$2.27\pm0.88^{a,y}$	$6.50\pm0.73^{b,z}$	$1.29\pm0.53^{b,xy}$	$0.59\pm0.39^{a,x}$	$0.00\pm0.00^{a,x}$	$0.00\pm0.00^{a,x}$	$0.00\pm0.00^{a,x}$
Lung	FE	$4.65\pm0.85^{a,x}$	$3.05\pm0.93^{a,wx}$	$2.47\pm1.08^{a,vwx}$	$2.08\pm0.95^{a,vw}$	$0.78\pm0.52^{a,vw}$	$0.37\pm0.37^{a,v}$	$0.36\pm0.36^{a,v}$	$1.66\pm0.87^{a,vw}$
	FED	$7.73\pm0.60^{b,x}$	$6.70\pm0.29^{b,x}$	$7.33\pm0.51^{c,x}$	$5.76\pm0.83^{b,wx}$	$0.37\pm0.37^{a,v}$	$1.66\pm0.71^{a,v}$	$2.19\pm0.75^{b,v}$	$4.71\pm0.91^{b,w}$
	LE	$4.62\pm0.35^{a,x}$	$1.75\pm0.73^{a,w}$	$1.24\pm0.63^{a,vw}$	$0.73\pm0.48^{a,vw}$	$0.00\pm0.00^{a,v}$	$1.62\pm0.81^{a,w}$	$0.00\pm0.00^{a,v}$	$0.00\pm0.00^{a,v}$
	LED	$6.60\pm0.45^{b,z}$	$3.37\pm0.82^{a,xy}$	$5.13\pm0.79^{b,yz}$	$2.21\pm0.95^{a,wx}$	$0.96\pm0.64^{a,vw}$	$0.50\pm0.50^{a,vw}$	$0.00\pm0.00^{a,v}$	$0.00\pm0.00^{a,v}$

Different letters in the same column (a, b, c) and line (v, w, x, y, z) are significantly different ($P<0.05$), Data is presented as mean±SD ***FE,*** *free enrofloxacin;* ***FED,*** *free enrofloxacin+dexamethasone;* ***LE,*** *liposomal enrofloxacin;* ***LED,*** *liposomal enrofloxacin + dexamethasone, Isolated bacteria numbers were calculated by transforming ln*

Table 3. *Morbidity[a] and mortality[b] levels in the experimental groups*
Tablo 3. *Deney gruplarında morbidite[a] ve mortalite[b] düzeyleri*

Group	Parameter	Day 1	Day 2	Day 3	Day 4	Day 5	Week 2	Week 3	Week 4
FE	Morbidity	8/8	5/8	2/8	2/8	1/8	0/8	0/8	3/8[c]
	Mortality	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
FED	Morbidity	8/8	6/8	7/8	6/8	1/8	2/8	2/8	4/8
	Mortality	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
LE	Morbidity	8/8	3/8	2/8	2/8	0/8	0/8	0/8	0/8
	Mortality	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
LED	Morbidity	8/8	5/8	8/8	4/8	2/8	1/8	1/8	1/8
	Mortality	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8

[a] The number of infected animals after bacteria was introduced into the trachea and the treatment was given; [b] the number of animals that died after bacteria was introduced into the trachea and the treatment was given; and [c] the number of dead and infected animals, ***FE,*** *free enrofloxacin;* ***FED,*** *free enrofloxacin+dexamethasone;* ***LE,*** *liposomal enrofloxacin;* ***LED,*** *liposomal enrofloxacin+dexamethasone*

Table 4. *Cytological evaluation of the BAL fluids of the control and experimental groups (n=8)*
Tablo 4. *Kontrol ve deney gruplarının BAL sıvılarının sitolojik değerlendirilmesi (n=8)*

Parameter	NC	PC	FE	LE	FED	LED
Macrophage %	76.97±1.57^{a}	38.16±2.45^{c}	40.02±2.30^{c}	34.43±3.02^{c}	56.49±3.49^{b}	61.40±3.02^{b}
Lymphocyte %	3.70±1.67^{bc}	9.39±2.80^{a}	6.22±0.98^{abc}	6.65±0.88^{abc}	7.09±1.31^{ab}	3.34±0.55^{a}
Neutrophil %	9.37±1.88^{c}	46.31±2.12^{a}	46.12±2.32^{a}	50.92±3.32^{a}	28.11±3.51^{b}	25.68±3.22^{b}
Eosinophil %	0.00±0.00	0.00±0.00	0.00±0.00	0.49±0.34	0.66±0.36	0.00±0.00

Different letters (a, b, c) in the same line are significantly different (P<0.05), Data is presented as mean±SD ***NC,*** *negative control;* ***PC:*** *positive control;* ***FE,*** *free enrofloxacin;* ***FED,*** *free enrofloxacin+dexamethasone;* ***LE,*** *liposomal enrofloxacin;* ***LED,*** *liposomal enrofloxacin+dexamethasone*

Table 5. *Enzyme levels in the bronchoalveolar lavage fluids of the control and experimental groups (n=8)*
Tablo 5*. Kontrol ve deney gruplarının bronko-alveolar lavaj sıvılarında enzim düzeyleri (n=8)*

Parameter	Group	Day 1	Day 2	Day 3	Day 4	Day 5	Week 2	Week 3	Week 4
LDH (U/l)	NC	10.44±2.51^{c}	10.44±2.51^{c}	10.44±2.51^{cd}	10.44±2.51^{de}	10.44±2.51^{e}	10.44±2.51^{b}	10.44±2.51^{cd}	10.44±2.51^{c}
	PC1	12.40±3.03^{c}	12.40±3.03^{c}	12.40±3.03^{cd}	12.40±3.03^{de}	12.40±3.03^{de}	12.40±3.03^{b}	12.40±3.03^{cd}	12.40±3.03^{c}
	PC2	4.90±0.77^{c}	4.90±0.77^{c}	4.90±0.77^{d}	4.90±0.77^{e}	4.90±0.77^{e}	4.90±0.77^{b}	4.90±0.77^{d}	4.90±0.77^{c}
	FE	5.33±0.94^{c}	8.00±2.74^{c}	19.40±2.25^{c}	14.50±1.12^{cd}	22.70±2.07^{bc}	19.40±1.49^{b}	17.60±1.61^{bc}	34.10±21.02^{bc}
	FED	52.90±9.64^{b}	48.50±7.68^{a}	42.40±4.90^{a}	43.40±5.56^{a}	35.64±4.96^{a}	53.80±15.97^{a}	37.50±7.00^{a}	83.80±27.25^{ab}
	LE	68.70±7.79^{a}	23.50±2.25^{b}	28.80±2.90^{b}	22.90±2.43^{bc}	21.22±2.05b^{cd}	25.36±2.36^{b}	28.00±4.71^{ab}	25.20±2.15^{bc}
	LED	45.67±2.82^{b}	39.20±1.93^{a}	30.10±3.28^{b}	29.10±3.28^{b}	29.40±3.06^{ab}	67.45±10.65^{a}	32.27±3.61^{a}	115.91±41.9^{a}
GGT (U/l)	NC	3.33±0.44^{c}	3.33±0.44^{d}	3.33±0.44^{b}	3.33±0.44^{c}	3.33±0.44^{d}	3.33±0.44^{c}	3.33±0.44^{bc}	3.33±0.44^{c}
	PC1	4.40±0.58^{c}	4.40±0.58^{bcd}	4.40±0.58^{d}	4.40±0.58^{bc}	4.40±0.58^{d}	4.40±0.58^{c}	4.40±0.58^{abc}	4.40±0.58^{bc}
	PC2	5.30±0.86^{c}	5.30±0.86^{bcd}	5.30±0.86^{bcd}	5.30±0.86^{abc}	5.30±0.86c^{d}	5.30±0.86^{abc}	5.30±0.86^{ab}	5.30±0.86^{abc}
	FE	5.11±0.77^{c}	7.10±1.44^{b}	5.60±0.64^{bcd}	6.90±0.81^{abc}	7.50±1.27^{abc}	6.00±0.94^{abc}	4.70±0.76^{abc}	4.60±0.54^{bc}
	FED	8.70±1.94^{ab}	11.20±0.99^{a}	14.10±3.17^{a}	9.00±2.77^{a}	9.82±0.92^{ab}	7.80±1.29^{a}	6.40±1.54^{a}	6.70±1.15^{a}
	LE	10.10±1.30^{a}	7.00±2.11^{bc}	9.10±1.04^{b}	7.70±1.36^{ab}	10.22±1.22^{a}	7.45±0.99^{ab}	4.40±0.79^{abc}	5.40±0.56^{ab}
	LED	5.56±1.04^{bc}	5.20±0.81^{bcd}	9.10±1.04^{bc}	7.20±1.33^{abc}	5.00±0.92^{cd}	5.18±1.09^{abc}	2.60±0.45^{c}	3.27±0.38^{c}
AST (U/l)	NC	13.11±1.01^{c}	13.11±1.01^{bcd}	13.11±1.01^{c}	13.11±1.01^{bc}	13.11±1.01^{bc}	13.11±1.01^{b}	13.11±1.01^{bc}	13.11±1.01^{cd}
	PC1	22.80±1.97^{a}	22.80±1.97^{a}	22.80±1.97^{a}	22.80±1.97^{a}	22.80±1.97^{a}	22.80±1.97^{a}	22.80±1.97^{a}	22.80±1.97^{a}
	PC2	18.20±1.37^{ab}	18.20±1.37^{ab}	18.20±1.37^{ab}	18.20±1.37^{ab}	18.20±1.37^{ab}	18.20±1.37^{a}	18.20±1.37^{ab}	18.20±1.37^{abc}
	FE	14.33±1.39^{bc}	13.00±1.62^{bcde}	14.00±1.45^{bc}	13.20±0.61^{bc}	17.70±1.33^{abc}	14.20±0.36^{b}	11.90±2.23^{c}	4.20±2.20^{e}
	FED	4.30±1.22^{d}	7.60±2.18^{e}	6.20±1.10^{d}	13.60±2.29^{bc}	5.73±1.34^{c}	16.50±3.27^{a}	14.30±3.46^{bc}	21.30±4.69^{ab}
	LE	13.20±1.69^{c}	14.60±2.65^{bc}	11.70±2.45^{c}	15.20±2.90^{bc}	15.56±4.18^{bc}	18.91±3.02^{a}	9.20±1.88^{cd}	9.50±1.70d^{e}
	LED	14.56±2.64b^{c}	8.50±1.27d^{e}	10.40±0.97^{c}	11.60±1.81^{c}	6.90±1.28^{c}	18.00±3.95^{a}	4.91±0.76^{d}	10.73±2.91^{cde}

Different letters (a, b, c, d, e) in the same column are significantly different (P<0.05), Data is presented as mean±SD ***LDH,*** *lactate dehydrogenase;* ***GGT,*** *gamma-glutamyl transferase;* ***AST,*** *Aspartate aminotransferase;* ***NC,*** *negative control;* ***PC1:*** *positive control for FE and LE;* ***PC2,*** *positive control for FED and LED;* ***FE,*** *free enrofloxacin;* ***FED,*** *free enrofloxacin+dexamethasone;* ***LE,*** *liposomal enrofloxacin;* ***LED,*** *liposomal enrofloxacin+dexamethasone*

compared the MLVs and LUVs, we found that the LUVs were approximately two times bigger than the MLVs. This particle size difference affects the drug encapsulation capacity. The particle size of the LUVs was approximately 4.65±0.37 mm, and they had the highest encapsulation capacity *(Table 1)*. We suggest that increasing the drug encapsulation capacity produced the slow release of ENR. This result was similar to the results found in the literature [3,13,14]. The optimized LUVs with the appropriate particle size and distribution, high surface charge and drug release properties used in the *in vitro* studies were chosen for use in the *in vivo* experiments, and the treatment efficacy on rats with experimental pneumonia was partially investigated.

In a previous study, in rats in which pneumonia was induced by *K. pneumoniae*, the clinical therapeutic effect of liposome-encapsulated gentamicin was found to be higher than the free form [24]. In this study, while clear clinical findings were not observed in infected rats with normal immune systems and those with immune systems that were depressed by dexamethasone after L-ENR treatment in the 2nd, 3rd, and 4th weeks, the fact that clinical symptoms arose in the 4th week in rats given F-ENR suggested that experimental infection reoccurred. For that reason, liposome encapsulation increased the effectiveness of ENR. Studies showed that liposome-encapsulated CPR provided full protection after aerosol inhalation when compared to free CPR in mice in which respiratory infection was induced by *F. tularensis* [25].

In rats with weakened immune systems, the therapeutic effectiveness was found to be higher with one dose of

Table 6. *Urea and microprotein concentrations in the BAL fluids of the control and experimental groups (n=8)*
Tablo 6. *Kontrol ve deney gruplarının BAL sıvılarındaki üre ve mikroprotein konsantrasyonları (n=8)*

Parameter	Group	Day 1	Day 2	Day 3	Day 4	Day 5	Week 2	Week 3	Week 4
UREA (mg/dl)	NC	3.11±0.42^{b}	3.11±0.42^{b}	3.11±0.42^{b}	3.11±0.42^{b}	3.11±0.42^{c}	3.11±0.42^{b}	3.11±0.42^{b}	3.11±0.42^{c}
	PC1	3.10±0.36^{b}	3.10±0.35^{b}	3.10±0.35^{b}	3.10±0.35^{b}	3.10±0.35^{c}	3.10±0.35^{b}	3.10±0.35^{b}	3.10±0.35^{c}
	PC2	3.20±0.36^{b}	3.20±0.36^{b}	3.20±0.36^{b}	3.20±0.36^{b}	3.20±0.36^{c}	3.20±0.36^{b}	3.20±0.36^{b}	3.20±0.36^{c}
	FE	3.22±0.32^{b}	3.20±0.29^{b}	4.40±0.37^{b}	3.80±0.39^{b}	3.50±0.37^{bc}	3.80±0.39^{b}	4.40±0.43^{b}	3.50±0.34^{c}
	FED	3.30±0.37^{a}	3.60±0.31^{b}	3.20±0.36^{b}	3.60±0.52^{b}	3.55±0.41^{bc}	3.80±0.42^{b}	3.90±0.53^{b}	4.20±0.29^{bc}
	LE	3.20±0.57^{b}	4.30±0.52^{a}	4.40±0.43^{b}	4.50±0.37^{b}	4.56±0.24^{b}	5.00±0.40^{b}	4.50±0.60^{b}	5.70±0.93^{ab}
	LED	4.56±0.56^{a}	5.30±0.82^{a}	8.10±1.19^{a}	7.20±1.01^{a}	7.00±0.65^{a}	13.36±5.30^{a}	6.73±1.45^{a}	6.09±0.69^{a}
MP (mg/dl)	NC	179±98^{b}	179±98^{b}	179±98^{b}	179±98^{b}	179±98^{cd}	179±98^{b}	179±98^{bc}	179±98^{bc}
	PC1	225±27^{b}	225±27^{b}	225±27^{b}	225±27^{b}	225±27^{bcd}	225±27^{b}	225±27^{b}	225±27^{bc}
	PC2	242±34^{b}	242±34^{b}	242±34^{b}	242±34^{b}	242±34^{bcd}	242±34^{b}	242±34^{b}	242±34^{bc}
	FE	136±18^{b}	202±26^{b}	171±27^{b}	179±26^{b}	141±18^{d}	162±23^{b}	69±14^{c}	80±20^{c}
	FED	202±35^{b}	201±33^{b}	176±26^{b}	165±49^{b}	316±45^{abc}	516±123^{a}	415±54^{a}	370±55^{ab}
	LE	374±48^{a}	496±53^{a}	404±81^{a}	455±44^{a}	337±48^{ab}	259±41^{b}	248±45^{b}	348±51^{ab}
	LED	517±71^{a}	419±25^{a}	477±38^{a}	393±48^{a}	426±49^{a}	316±61^{b}	217±29^{b}	483±124^{a}

Different letters (a, b, c) in the same column are significantly different (P<0.05), Data is presented as mean±SD ***MP,*** *microprotein;* ***NC,*** *negative control;* ***PC1:*** *positive control for FE and LE;* ***PC2,*** *positive control for FED and LED;* ***FE,*** *free enrofloxacin;* ***FED,*** *free enrofloxacin+dexamethasone;* ***LE,*** *liposomal enrofloxacin;* ***LED,*** *liposomal enrofloxacin+dexamethasone*

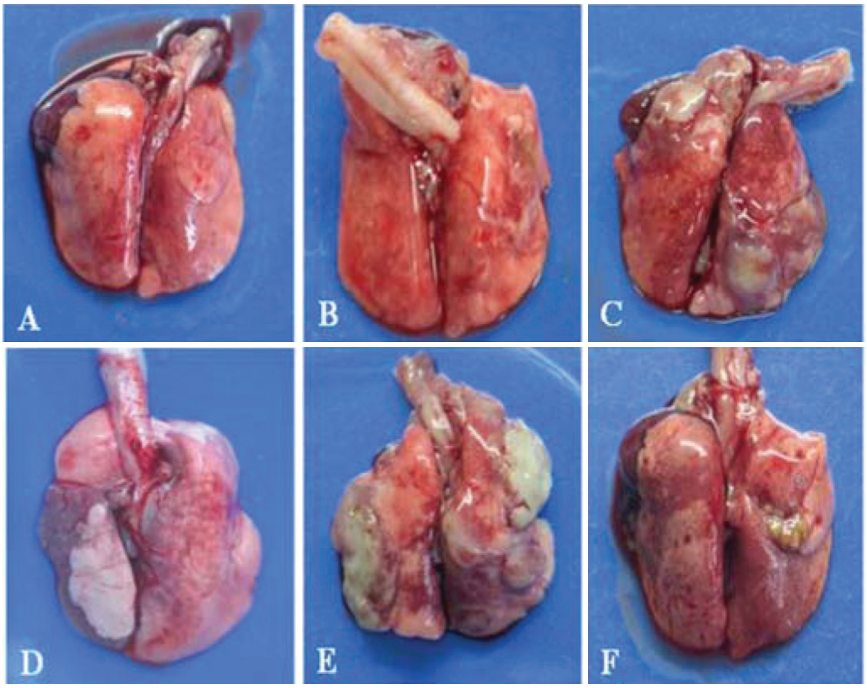


Fig 2. A- Mild congestion, NC, **B-** Pleuritis, PC1, **C-** Abscess containing yellow-green pus, LED-3rd day, **D-** Band shape hepatization in the left caudal lobe, SE-4th day, **E-** Pleuritis and abscess containing yellow-green pus, SED-4th day, **F-** Abscess containing yellow-green pus in the right cranial lobe, SED-2nd week

Şekil 2. A- Hafif konjesyon, NC, **B-**Pleuritis, PC1, **C-** Sarı-yeşil irin içeren abse, LED-3. Gün, **D-** Sol arka lobta kenarları keskin hepatizasyon, SE-4. Gün, **E-** Pleuritis ve sarı-yeşil irin içeren abse, SED-4. Gün, **F-** Sağ ön lobta sarı-yeşil irin içeren abse, SED-2. hafta

liposomal gentamicin rather than multiple doses of free gentamicin [26]. After F-ENR treatment in this study, in rats with a suppressed immune system, re-isolation of the bacteria was found in lung samples collected up to the 4th week, but in the BAL samples, re-isolation was found only on the 5th day. In the 3rd and 4th weeks, re-isolation could not be performed. However, after treatment with L-ENR, bacteria isolation was found in the lung samples, except during the 3rd and 4th weeks, and no bacterial reproduction was observed in the BAL samples in the 2nd, 3rd and 4th weeks. Nevertheless, there was less bacterial production in the BAL and lung samples of the LED group according to the FED group. However, in rats with depressed immune systems, the therapeutic effectiveness of L-ENR was found to be limited, even though L-ENR has better therapeutic effectiveness than F-ENR. Many studies [27,28] have stated that even with the improved therapeutic effectiveness of drugs encapsulated with liposomes, 100% of the bacteria could not be eliminated from the body. In addition to, the fact that death was not observed in the rats in all of the experimental groups is important in terms of showing the bactericidal effect of the drug. The fact that the morbidity rate was lower in the LE group than in the other groups can be explained by the fact that the

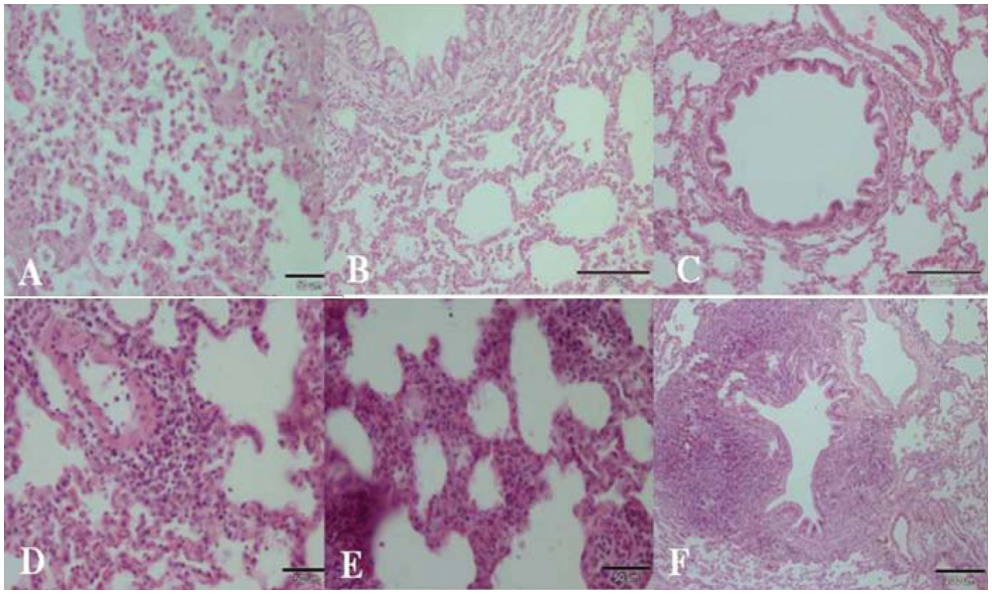


Fig 3. A- Alveolar macrophages and neutrophils in the alveolar lumen, SED-3rd day, H&E, Bar: 50 µm, **B-** Increases in the number of goblet cells, alveolar macrophages and neutrophils in the alveolar lumen, SED-3rd day, **C-** Mild perivascular mononuclear cell infiltrations, LE-4th day, H&E, Bar: 100 µm, **D-** Perivascular mononuclear cells and eosinophil infiltrations, LE-1st week, H&E, Bar: 50 µm, **E-** Thickness of interalveolar septa, LE-2nd week, H&E, Bar: 50 µm, **F-** Perivascular and peribronchiolar mononuclear cell infiltrations and lymphoid hyperplasia, SED-3rd week, H&E, Bar: 200 µm

Şekil 3. A- Alveol lumeninde alveoler makrofajlar ve nötrofiller, SED-3. gün, H&E, Bar: 50 µm, **B-** Alveol lumeninde goblet hücre, alveoler makrofaj ve nötrofil sayılarında artış, SED-3. Gün, **C-** Hafif perivasküler mononükleer hücre infiltrasyonları, LE-4. gün, H&E, Bar: 100 µm, **D-** Perivasküler mononükleer hücre ve eozinofil infiltrasyonları, LE-1. hafta, H&E, Bar: 50 µm, **E-** Alveoller arası septumda kalınlaşma, LE-2. hafta, H&E, Bar: 50 µm, **F-** Perivasküler ve peribronşiyal mononükleer hücre infiltrasyonları ve lenfoid hiperplazi, SED-3. hafta, H&E, Bar: 200 µm

drug was carried into the cell, increasing its therapeutic effectiveness.

In this study, when the cellular composition of the BAL fluid was evaluated compared to the control groups, the macrophage number decreased significantly compared to healthy rats (NC), and an increase in the neutrophil number was observed. These changes were evaluated as an indicator of experimental bacterial infection [9]. The increase in macrophage number in the FED and LED groups compared to the PC is explained as a classical leukocyte response (neutrophillia, lymphopenia, monocytosis and eosinopenia) to the corticosteroid application [29]. When the cellular composition of the BAL fluid was evaluated according to the differences in drug formations without taking the control groups into consideration, there was no difference between the FE and LE groups with the FED and LED groups, showing that free and liposomal forms of the drug did not affect cellular formation. Similarly, corticosteroid action was not effective in these two different drug treatments.

The increase in the enzyme activities in the BAL fluid (LDH, AST and GGT) reflects cell injury [11,21,22]. In this study, there were significant differences in the LDH and GGT enzyme activities of the BAL fluid between groups *(Table 5)*. It has been determined that the LDH enzyme in the BAL fluid can only be found extracellularly in the case of cell membrane injury, and can be a useful parameter for the diagnosis of injury in lung tissue [30]. Similarly, it has been reported that the increase in GGT enzyme activity in the BAL fluid can be an indicator of pulmonary endothelial cell injury [31]. Thus, the increase in enzyme activities of LDH, a cytoplasmic enzyme [32] and GGT, located on the brush-like edges of cell membranes [31] were evaluated as indicators of pulmonary injury. In this study, there were also significant decreases in AST enzyme activity of the BAL fluid between groups *(Table 5)*. AST is a cytoplasmic enzyme similar to LDH, yet AST tissue levels are lower than those of LDH [33]. Dere and Polat [34], in their study on paraquat toxicity in rats, found a significant increase in LDH enzyme activity and a significant decrease in AST enzyme activity at 2 and 16 h. In this study, the reasons for the differences in the AST enzyme level compared to the GGT and LDH enzyme levels can be attributed to low tissue levels and excessive variations within the groups.

Many studies [12,22,31] have shown that the BAL fluid protein and urea measurements in lung diseases can show vascular and respiratory permeability defects. In this study, when the increases in the enzyme activities were considered, the changes found in the MP and urea levels confirm the existence of respiratory and vascular permeability deformities and pulmonary damage. The high MP and urea concentrations in the LE and LED groups versus the FE and FED groups *(Table 6)* indicate that liposomal drug formations are more effective on vascular and respiratory membrane permeability.

In conclusion, ENR was successfully encapsulated into the LUVs and MLVs. The molar ratios of the DPPC, cholesterol and ENF in the liposome formulation are important for the drug encapsulation, vesicle size, electrophoretic mobility and drug release from the liposomes. The highest encapsulation capacity was obtained in LUVs. In the *in vivo* study, the data show that cytologic and enzymologic diagnoses of the BAL fluid are not meaningful in evaluating the treatment of the experimental pneumonia in rats. We concluded that the use of ENR in liposomal form at a 7.5 mg/kg dosage for five days was more effective than the free form both in the treatment of *K. pneumoniae* infections and in the prevention of recurrent infections. The pharmaceutical form of the antimicrobial agent, in addition to the choice of the agent, drastically affects the prognosis of infection. The liposome-encapsulated antimicrobial agents should provide another choice in antimicrobial therapy in the future, and further investigation is necessary before clinical use.

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Kafkas Univ Vet Fak Derg
19 (2): 191-197, 2013
DOI: 10.9775/kvfd.2012.7283

Journal Home-Page: http://vetdergi.kafkas.edu.tr
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RESEARCH ARTICLE

Kars İli Sığır İşletmelerinde Barınakların Mevcut Durumu ve Yetiştirici Talepleri: II. Yetiştirici Talepleri [1]

Muammer TİLKİ * Erol AYDIN ** Mehmet SARI * Ali Rıza AKSOY * Kadir ÖNK ***

[1] *Kars İl Gıda, Tarım ve Hayvancılık Müdürlüğü tarafından hazırlanan TRA2-11-DFD-009 nolu proje Serhat Kalkınma Ajansı (SERKA) tarafından desteklenmiştir*

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Makale Kodu (Article Code): KVFD-2012-7283

Özet

Bu araştırmada; Kars ili merkez ilçe, Akyaka, Arpaçay, Digor, Kağızman, Sarıkamış, Selim ve Susuz ilçeleri ve bağlı köylerinde yer alan sığır işletmelerinin genel yapısı incelenmiş, işletmelerin modernize edilmesi ve/veya yeni yapılması durumunda yetiştiricilerin talepleri değerlendirilmiştir. Araştırmanın verilerini Kars Merkez ilçe dâhil toplam 8 ilçesi ve bağlı 189 köylerindeki 412 süt sığırı işletme sahibi ile yüzyüze uygulanan anket çalışması oluşturmuştur. Araştırma kapsamında incelenen işletmelerin toplam sığır varlığı 16.066 baş olup, bu değer 2010 yılı TÜİK verilerine göre Kars ili sığır varlığının yaklaşık %4'ünü oluşturmaktadır. Anket yapılan işletmelerde kullanılan ahır tipleri içerisinde en yaygın "*kapalı bağlı ahır*"lar olduğu tespit edilmiştir. Yetiştiriciler gelecekte de "*kapalı bağlı ahır*" tipini tercih edeceklerini bildirmişlerdir. Kapalı bağlı ahırları tercih edenlerin oranı %79.13 olarak tespit edilmiştir. Ancak burada mevcut durumdan farklı olarak kapalı serbest dolaşımlı ahır talebinin (%16.26), ahır tipleri arasındaki oranının %14.32 puan yükseldiği belirlenmiştir. Bölgede ve ilde eski ahırların geliştirilmesi, modernize edilmesi ve tespit edilecek olan aksaklıkların giderilmesi, yeni ahırların yapılması amacıyla; sektör paydaşları ile ortak çalışmalar yapılması faydalı olacaktır. Ayrıca bölgede oldukça yaygın olan kapalı bağlı ahırlarda belirlenen olumsuzluklar ve yetersizliklerinden dolayı, yeni yapılacak ya da modernize edilebilecek kapalı bağlı ahırların yerine, serbest duraklı ahırların yapılmasının daha uygun olacağı kanaatine varılmıştır.

Anahtar sözcükler: *Kars, Sığır barınağı, İşletme yapısı, Yetiştirici talepleri*

Current Status of Cattle Shelters in Livestock Enterprises and Breeder Demands in Kars: II. Breeder Demands

Summary

In this study, the overall structure cattle shelters in the towns and villages of the Kars Central District and the districts of Akyaka, Arpacay, Digor, Kağızman, Sarıkamış, Selim and Susuz were investigated and the needs of animal producers on the ground of reconstruction or modernization of livestock shelters were evaluated. Data was collected from questionnaires conducted face to face with 412 livestock producers inhabited in 189 villages scattered through all eight districts. The surveyed cattle enterprises collectively had to a total of 16.066 head which corresponded to approximately 4% of cattle in Kars province according TUIK 2010 statistics. Among the housing types surveyed, the most commonly used shelter style was found to be "*closed and connected barn system*". According to the survey, this system would be chosen by animal producers since it has been seen as the most suitable, if a new barn is going to be constructed. The survey also revealed that closed and connected barn systems have been preferred most commonly (79.13%). However, the demand for a free circulation closed barn system (16.26%) is increasing and this increase is estimated to be around 14.32%. The animal husbandry in the Kars province and the entire region would benefit from modernization of the animal shelters and overcoming deficiencies identified in current barn systems. To achieve this goal, a close cooperation is required among the all affiliated organizations. Furthermore, due to the shortcomings and deficiencies of the "*closed binded system barns*", it is advisable that the new constructions or remodeling of the preexisting ones be done based on an "*open barn system*".

Keywords: *Kars, Cattle shelter, Livestock structure, Breeder demands*

GİRİŞ

Kars ili Doğu Anadolu Bölgesi'nin kuzeyinde yer almakta olup, Türkiye'de kışların en soğuk geçtiği illerden birisidir. Kars ilinin iklimi kışları uzun ve sert, yazları ılıman hatta serin geçmektedir. Bu durumun temel nedenleri; yüksek dağ sıra-

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larıyla denizlerin ılımanlaştırıcı etkisinden ayrılması, yüksekliğin fazla olması, kış mevsiminde Büyük Asya Kara Kütlesi üzerinde yerleşen soğuk ve ağır hava kütlesi (Sibirya yüksek basınç merkezi)'nin buraya kadar sokulmasıdır. İlde don vakalarına sıklıkla rastlanmaktadır. Yıl içerisinde donlu gün sayısı 160 günü bulabilmektedir. Kışın sıcaklığın -40°C'nin altına düştüğü dahi görülmektedir [1-3].

Kars ili toplam yüzölçümünde 351.959 ha olan tarımsal alan %34.7 ve 396.031 ha'lık çayır-mera alanı ise %39.1'lik orana sahiptir. Ekili alanların %90'dan fazlası tahıl üretimine ayrılmıştır. Tahıl türleri arasında buğday başta gelmekle birlikte, bazı yıllar arpa üretiminin buğday üretimini geçtiği belirlenmiştir [1,2].

Kars İl Gıda, Tarım ve Hayvancılık Müdürlüğü verilerine göre, 2011 yılında ildeki toplam sığır varlığı 477.502 baştır. Müdürlük kayıtlarına göre il toplam sığır varlığı ırk kompozisyonu içerisinde kültür ve melezi ırkların oranı %71'dir. Son yıllarda ilde kültür ırkı sığır sayısında belirgin bir artış görülmüştür. TÜİK'in 2010 yılı verilerine göre Kars, Türkiye genelinde en fazla sığır bulunan iller sıralamasında Erzurum, Balıkesir, Konya ve İzmir'den sonra 5. sırada yer almaktadır. Sığır varlığının en yüksek olduğu ilçe, merkez ilçe olup bunu Sarıkamış, Selim ve Arpaçay ilçeleri takip etmektedir [1,4,5].

Kars ilinde hayvancılık en önemli geçim kaynağıdır. Büyükbaş hayvansal üretim ve ticareti (et, süt ve ürünleri) ilin ekonomik faaliyetleri arasında önemli yer tutmaktadır. Kars ilinde 2011 yıllında süt, et ve kaşar üretim miktarları sırasıyla 169.720, 30.895 ve 8.500 tondur [6].

Kars ili ve çevresinde bulunan hayvancılık işletmelerinin büyük bir kısmı, küçük ölçekli aile işletmeleri şeklindedir. Bu işletmelerde ölçeklerinin küçük olmasından ve modern teknolojiye sahip olmamalarından dolayı üretim maliyetleri yükselmekte ve kâr marjı azalmaktadır. Bölgede hayvancılıkla uğraşan yetiştiricilerin çoğunluğu aileden gördükleri şekilde geleneksel yöntemlerle hayvancılık yapmaktadırlar. Bazı yetiştiriciler Kars ili ve çevresine son zamanlarda gelen kültür ırkı sığırlara bile geleneksel yöntemlerle bakım besleme uygulamaktadırlar [4,6].

Sığır yetiştiriciliğinde verimleri genetik yapının yanı sıra birçok çevresel faktör de etkilemektedir. Bu faktörlerin başında barınaklar gelmektedir. Hayvan barınakları konusunda bölgede ciddi sorunlar bulunmaktadır. Barınakların yanı sıra verimleri; iklim, coğrafik özellikler, tarım politikaları, çiftçilik gelenekleri, arazi yapısı, doğal otlaklar, yem kalitesi, su kalitesi ve genel sağlık bilgisi, durak yeri, yataklık malzemeleri, yer döşemesi, yem dağıtımı, sağımla ilgili idare ve sağım malzemeleri gibi çevresel faktörler etkilemektedir [7-11].

Bir işletmenin kapasitesi, hayvan sayısı, ahır kalitesi, üreticinin tecrübe ve eğitimi, kullanılan işgücü kalitesi ve sayısı, rasyondaki kesif yem miktar ve bileşimi gibi faktörler de, süt sığırcılığı işletmelerinin verimli çalışmasını etkiler. Hayvan materyali ve yönetim kalitesi sonucunda ortaya konulan faaliyet birimlerinin üretim değeri ve işletmenin tarımsal geliri önemli ekonomik başarı kriterlerindendir [10,12-14].

Bu araştırma, Kars ili ve ilçelerinde sığır işletmelerinin genel özelliklerinin ortaya konulması, eski ahırların geliştirilmesi, modernize edilmesi ve/veya tespit edilecek olan aksaklıkların giderilmesi ile yeni ahırların yapılması durumunda yetiştiricilerin taleplerinin değerlendirilmesi amacıyla yapılmıştır.

MATERYAL ve METOT

Bu araştırmanın materyalini işletme sahipleri veya sorumlu kişilerle yüzyüze anket yapılarak elde edilen veriler oluşturmuştur. Bu amaçla Kars ili Merkez, Akyaka, Arpaçay, Digor, Kağızman, Sarıkamış, Selim ve Susuz ilçeleri araştırma kapsamına alınmıştır. Çalışmada; Kars ili Merkez ilçeden 87, Akyaka'dan 50, Arpaçay ve Digor'dan 44'er, Kağızman'dan 40, Sarıkamış'tan 49, Selim'den 47 ve Susuz'dan 51 anket olmak üzere toplamda 412 anket yapılmıştır. İşletmelerin seçiminde tesadüfü örnekleme yönteminden yararlanılmıştır. Anket çalışmaları yapılırken İl Gıda, Tarım ve Hayvancılık Müdürlüğü personelinden de faydalanmıştır. Bunun için anket yapacak personelle önceden toplantı yapılmış, anket soruları ile ilgili kapsamlı bilgiler verilmiştir.

İlçelere göre anket yapılan köy sayısı; Kars ili Merkez ilçeden 49, Akyaka'dan 19, Arpaçay'dan 26, Digor ve Kağızman'dan 21, Sarıkamış'tan 27, Selim'den 25 ve Susuz'dan 20 köy olmak üzere toplamda 189 köyde anket yapılmıştır. Anket uygulanan 8 ilçe ve 189 köyde toplam sığır sayısı 16.066 baş olup, ilçelere göre dağılım ise şu şekildedir. Kars Merkez'de 3.560, Akyaka'da 2.493, Arpaçay'da 1.568, Digor'da 1.790, Kağızman'da 1.601, Sarıkamış'ta 1.589, Selim'de 1.789 ve Susuz'da 1.676 baş sığır işletmelerde belirlenmiştir.

Araştırma boyunca köylerde yapılan incelemeler ve yapılan anketlerle özellikle bölgedeki mevcut barınakların genel yapısı ve yetiştirici taleplerini ortaya konulmaya çalışılmıştır. Bunun yanı sıra ankette ifade edilemeyen bazı gözlemlerde sonuçlara yansıtılmıştır.

Araştırma sonucu elde edilen verilerin değerlendirilmesinde karşılaştırılmalı istatistiki analizlerin kullanılmasına gerek görülmemiştir. Elde edilen verilerin tanımlayıcı istatistikleri, frekansları ve yüzde dağılımları hesaplanmıştır. Verilerin elektronik ortama girilmesi ve hesaplamalarda Microsoft Office 2010 Excel ve SPSS for Windows (16.0) programları kullanılmıştır.

BULGULAR

Yetiştiricilere göre bölgeye en uygun ahır tipine ait bulgular *Tablo 1*'de gösterilmiştir. Bu soruya en çok verilen cevap şu anda işletmelerdeki mevcut ahır tiplerinde olduğu gibi kapalı bağlı sistem olmuştur.

İşletme sahiplerinin arazi varlığı *Tablo 2*'de gösterilmiştir.

Tablo 1. *Yetiştiricilere göre en uygun ahır tipi*
Table 1. *The type of most appropriate barn according to the breeder*

İlçeler	Açık Serbest Dolaşımlı		Yarı Açık		Kapalı Serbest Dolaşımlı		Kapalı Bağlı	
	Frekans	%	Frekans	%	Frekans	%	Frekans	%
Merkez	2	2.30	1	1.15	6	6.90	78	89.66
Akyaka	0	0.00	0	0.00	8	16.00	42	84.00
Arpaçay	1	2.27	5	11.36	2	4.55	36	81.82
Digor	0	0.00	1	2.27	2	4.55	41	93.18
Kağızman	3	7.50	0	0.00	10	25.00	27	67.50
Sarıkamış	3	6.12	3	6.12	10	20.41	33	67.35
Selim	0	0.00	0	0.00	19	40.43	28	59.57
Susuz	0	0.00	0	0.00	10	19.61	41	80.39
Kars İl Toplamı	9	2.18	10	2.43	67	16.26	326	79.13

Tablo 2. *İşletme sahiplerinin arazi varlığı*
Table 2. *The presence of land of livestock owners*

İlçeler	1-20 (da)		21-50 (da)		51≥ (da)	
	Frekans	%	Frekans	%	Frekans	%
Merkez	6	6.74	27	30.34	56	62.92
Akyaka	4	8.00	4	8.00	42	84.00
Arpaçay	4	10.26	8	20.51	27	69.23
Digor	1	2.44	6	14.63	34	82.93
Kağızman	1	2.50	7	17.50	32	80.00
Sarıkamış	2	4.35	9	19.57	35	76.09
Selim	4	9.52	7	16.67	31	73.81
Susuz	5	11.63	14	32.56	24	55.81
Kars İl Toplamı	27	6.92	82	21.03	281	72.05

Tablo 3. *Yetiştiricilerin kooperatifleşme hakkında görüşleri*
Table 3. *The views of the breeders about establishing cooperatives*

İlçeler	Olumsuz		Olumlu	
	Frekans	%	Frekans	%
Merkez	83	95.40	4	4.60
Akyaka	49	98.00	1	2.00
Arpaçay	44	100.00	0	0.00
Digor	44	100.00	0	0.00
Kağızman	39	97.50	1	2.50
Sarıkamış	47	95.92	2	4.08
Selim	44	93.62	3	6.38
Susuz	51	100.00	0	0.00
Kars İl Toplamı	401	97.33	11	2.67

Tablo 4. *Yetiştiricilerin barınak yapımında görüş alma yönelimi*
Table 4. *Tendency of breeders about consultative process for construction of shelter*

İlçeler	İl Gıda, Tarım ve Hayvancılık Müdürlüğü		Veteriner Fakültesi		Veteriner Hekim ve Ziraat Mühendisi		Birlik		Köy-Koop	
	Frekans	%	Frekans	%	Frekans	%	Frekans	%	Frekans	%
Merkez	68	78.16	3	3.45	8	9.20	4	4.60	4	4.60
Akyaka	44	88.00	0	0.00	6	12.00	0	0.00	0	0.00
Arpaçay	31	70.45	2	4.55	11	25.00	0	0.00	0	0.00
Digor	20	45.45	5	11.36	16	36.36	1	2.27	2	4.55
Kağızman	17	42.50	2	5.00	15	37.50	3	7.50	3	7.50
Sarıkamış	19	38.78	0	0.00	17	34.69	8	16.33	5	10.20
Selim	28	59.57	2	4.26	13	27.66	0	0.00	4	8.51
Susuz	42	82.35	2	3.92	6	11.76	1	1.96	0	0.00
Kars İl Toplamı	269	65.29	16	3.88	92	22.33	17	4.13	18	4.37

İlde bulunan tarımsal işletmelerin arazi büyüklüğünün büyük kısmının 51 dekardan daha fazla olduğu (%72.05) tespit edilmiştir. En yüksek oran Akyaka, Digor ve Kağızman ilçeleri için belirlenmiştir.

Hayvancılıkta yetiştiricilerin kooperatifleşme hakkında görüşleri *Tablo 3*'te sunulmuştur. Yetiştiricilerin bölgede örgütlü üreticilik ve üretim biçimini benimsemediği ve önemli oranda (%97.33) ayrı barınaklara sahip olmayı istedikleri tespit edilmiştir.

Yetiştiricilerin yeni barınak yapımında kimden fikir alırsınız sorusuna verdileri cevaplar *Tablo 4*'te gösterilmiştir. Buna göre yetiştiriciler tarafından en fazla fikir alınacak kurumun

İl Gıda, Tarım ve Hayvancılık Müdürlüğü olduğu görülmüştür.

Bölgede yeni bir ahır yapılması durumunda kaç başlık planlanması gerektiği konusunda sorulan soruya verilen cevaplar *Tablo 5*'te verilmiştir. Buna göre ilde ideal bir ahır kapasitesinin 40 baştan daha fazla olmasının gerektiğini düşünenlerin oranı %61.89 olarak tespit edilmiştir.

Yetiştiricilere yeni bir ahır yaparsanız en uygun yapı malzemesinin ne olacağı sorulmuş ve alınan cevaplar *Tablo6*'da özetlenmiştir. Buna göre yetiştiriciler en uygun ahır yapı malzemesinin betonarme, bunu takibende taş olduğunu ifade etmiştir.

Araştırmada en uygun ahır çatı ve taban malzemesinin ne olduğu sorusuna yetiştiricilerin vermiş olduğu cevaplar

Tablo 5. *Yetiştiricinin ideal ahır kapasitesine ilişkin görüşü*
Table 5. *View of breeder about ideal capacity of a barn*

İlçeler	≤10 (Baş)		11-20 (Baş)		21-30 (Baş)		31-40 (Baş)		41-50 (Baş)		51≥ (Baş)	
	Frekans	%	Frekans	%	Frekans	%	Frekans	%	Frekans	%	Frekans	%
Merkez	1	1.15	6	6.90	19	21.84	20	22.99	23	26.44	18	20.69
Akyaka	0	0.00	0	0.00	3	6.00	4	8.00	30	60.00	13	26.00
Arpaçay	0	0.00	3	6.82	9	20.45	10	22.73	17	38.64	5	11.36
Digor	0	0.00	5	11.36	5	11.36	4	9.09	15	34.09	15	34.09
Kağızman	2	5.00	3	7.50	6	15.00	7	17.50	11	27.50	11	27.50
Sarıkamış	0	0.00	3	6.12	6	12.24	6	12.24	22	44.90	12	24.49
Selim	0	0.00	3	6.38	10	21.28	6	12.77	21	44.68	7	14.89
Susuz	0	0.00	2	3.92	11	21.57	3	5.88	30	58.82	5	9.80
Kars İl Toplamı	3	0.73	25	6.07	69	16.75	60	14.56	169	41.02	86	20.87

Tablo 6. *Yetiştiriciye göre en uygun ahır yapı malzemesi*
Table 6. *The construction material for most suitable barn according to the breeder*

İlçeler	Betonarme		Toprak		Taş		Ahşap		Kerpiç-Briket	
	Frekans	%	Frekans	%	Frekans	%	Frekans	%	Frekans	%
Merkez	54	62.07	1	1.15	26	29.89	4	4.60	2	2.30
Akyaka	27	54.00	0	0.00	20	40.00	0	0.00	3	6.00
Arpaçay	37	84.09	0	0.00	5	11.36	0	0.00	2	4.55
Digor	31	70.45	1	2.27	11	25.00	0	0.00	1	2.27
Kağızman	30	75.00	1	2.50	5	12.50	1	2.50	3	7.50
Sarıkamış	16	32.65	2	4.08	22	44.90	4	8.16	5	10.20
Selim	31	65.96	1	2.13	14	29.79	0	0.00	1	2.13
Susuz	46	90.20	0	0.00	4	7.84	1	1.96	0	0.00
Kars İl Toplamı	272	66.02	6	1.46	107	25.97	10	2.43	17	4.13

Tablo 7. *Yetiştiriciye göre en uygun ahır çatı malzemesi*
Table 7. *The roof material for most suitable barn according to the breeder*

İlçeler	Saç		Toprak		Kiremit		Ahşap	
	Frekans	%	Frekans	%	Frekans	%	Frekans	%
Merkez	77	88.51	3	3.45	0	0.00	7	8.05
Akyaka	48	96.00	0	0.00	1	2.00	1	2.00
Arpaçay	44	100.00	0	0.00	0	0.00	0	0.00
Digor	41	93.18	3	6.82	0	0.00	0	0.00
Kağızman	39	97.50	0	0.00	1	2.50	0	0.00
Sarıkamış	44	89.80	2	4.08	0	0.00	3	6.12
Selim	46	97.87	0	0.00	0	0.00	1	2.13
Susuz	49	96.08	0	0.00	2	3.92	0	0.00
Kars İl Toplamı	388	94.17	8	1.94	4	0.97	12	2.91

ise *Tablo 7* ve *Tablo 8*'de gösterilmiştir.

Yetiştiriciye ideal bir hayvan barınağı nasıl olmalıdır şeklinde çeşitli sorular sorulmuş ve verilen cevaplar *Tablo 9*'da özetlenmiştir. Sorulan sorulara yüksek oranda evet cevapları verilmiştir. En düşük evet oranı işletmede soğutma tankının gerekli olup olmayacağı ile ilgili soruda verilmiştir (%85.68). Bunu, ahırların evden uzak olması gerekip gerekmeyeceği ile ilgili soruya verilen cevap takip etmektedir (%90.78).

Tablo 8. *Yetiştiriciye göre en uygun ahır taban malzemesi*
Table 8. *The base material for most suitable barn according to the breeder*

İlçeler	Beton		Taş		Diğer	
	Frekans	%	Frekans	%	Frekans	%
Merkez	75	86.21	9	10.34	3	3.45
Akyaka	49	98.00	1	2.00	0	0.00
Arpaçay	42	95.45	2	4.55	0	0.00
Digor	42	95.45	2	4.55	0	0.00
Kağızman	34	85.00	6	15.00	0	0.00
Sarıkamış	43	87.76	5	10.20	1	2.04
Selim	44	93.62	3	6.38	0	0.00
Susuz	49	96.08	2	3.92	0	0.00
Kars İl Toplamı	378	91.75	30	7.28	4	0.97

Bununla birlikte kültür ırkı sığır sayısının son yıllarda arttığı da görülmektedir. Kültür ırkı sığırlar içerisinde en yaygın ve bölgeye en iyi adapte olmuş olanları Esmer ve Simental ırklarıdır.

Yetiştiriciye göre yeni yapılması durumunda en uygun ahır tipinin hangisi olduğu sorulmuş ve buna en çok verilen cevap şu anda işletmelerdeki mevcut ahır tiplerinde olduğu gibi "kapalı bağlı sistem" olmuştur (%79.13). Ancak burada mevcut durumdan farklı olarak kapalı serbest dolaşımlı ahır talebinin (%16.26), ahır tipleri arasındaki oranının %14.32 puan yükseldiği belirlenmiştir. Bölgede serbest dolaşımlı ahırların tercih edilmeye başlanmasının nedenleri arasında yetiştiricilerin başka illerde bu tip ahırları görmesi, bu ahırların diğer ahırlara göre hayvanların süt ve et verimi üzerine olumlu etkilerinin olduğunun öğrenilmesi, ilde ve bölgede örnek olabilecek bu tip ahırların sayısının artmaya başlaması sayılabilir. Bunlara ek olarak başka illerden ve yurt dışından getirilen kombine verimli sığırların serbest dolaşımlı sisteme daha iyi adapte oldukları da gözlemlenmiştir.

İlde kapalı bağlı ahır tiplerinin yetiştirici gözünde yaygın olmasının çeşitli nedenleri vardır. Bunlar arasında ilde yetiştirilen hayvanların bir kısmının yerli ve melez olması, bu hayvanların serbest dolaşımlı pozisyonda bulunması durumunda birbirlerine zarar vereceklerine inanılmasındandır. Bir diğer bağlı sistem nedeni de bölgede kış mevsimi uzun sürmesinden dolayı, hayvanların serbest dolaşımlı pozisyon-

Tablo 9. *Yetiştiricinin hayvan barınaklarının nasıl olması gerektiği konusunda görüşleri*
Table 9. *Views of breeders about how it should be animal shelter*

Değerlendirme	Durak Demiri Olmalıdır	Kaşıntı Fırçası Olmalıdır	Süt Soğutma Tankı Olmalıdır	Süt Sağım Ünitesi Olmalıdır
Evet (%)	95.15	92.96	85.68	92.96
Hayır (%)	4.85	7.04	14.32	7.04
Değerlendirme	**Her Gün Temizlenmelidir**	**10-15 Metre Evden Uzak Olmalıdır**	**Yanında Gübre Çukuru Olmalıdır**	**Saman Yeri, Yem Deposu Olmalıdır**
Evet (%)	95.87	90.78	91.99	96.84
Hayır (%)	4.13	9.22	8.01	3.16
Değerlendirme	**Aydınlatma Olmalıdır**	**Havalandırma Olmalıdır**	**Suluk Olmalıdır**	**Drenaj/Atık Su Kanalı Olmalıdır**
Evet (%)	100.00	99.76	96.60	95.63
Hayır (%)	0.00	0.24	3.40	4.37
Değerlendirme	**Doğum-Buzağı Bölümü Bulunmalıdır**	**Güvenlik Cihazı Bulunmalıdır**	**Hayvanlar Sigortalı Olmalıdır**	**İçinde Veya Yanında Mutlaka Su Kaynağı Olmalıdır**
Evet (%)	94.42	92.23	94.66	97.57
Hayır (%)	5.58	7.77	5.34	2.43

TARTIŞMA ve SONUÇ

Hayvansal üretimde verimliliği artırmak için hayvanların genetik kapasitelerinin yüksek olmasının yanı sıra, bu genetik potansiyelin verime dönüşmesine imkân sağlayacak özellikte çevreye de sahip olunması gerekmektedir. Yani, hayvancılıkta verimi artırmak için yüksek verimli hayvanların uygun çevre koşullarında barındırılması şarttır. Kars ilinde daha ziyade yerli ve melez sığır ırkları bulunmaktadır. da olması durumunda fazla enerji harcayacakları ve daha fazla yem tüketeceklerinin düşünülmesidir. Uğurlu ve Şahin [15] Kayseri'de 24 adet süt sığırı işletmesi üzerinde yaptıkları çalışmada, işletmelerin %75'inin kapalı bağlı sistem yetiştiricilik yaptığını ve barınakların büyük bir kısmının yapı alanları, havalandırma ve aydınlatma yönünden yetersiz olduğu bildirmiştir. Kaygısız ve Tümer [16] ise Kahramanmaraş ilinde bu oranı %65 olarak hesaplanmıştır. Burdur ilinde yapılan bir araştırmada 677 işletmenin 184'ünün (%27.2) yarı açık-

serbest gezinmeli ahır, 142'sinin (%20.9) kapalı-serbest gezinmeli ahır ve 351 (%51.9) işletmenin ise kapalı sabit bağlamalı ahırlardan yapıldığı saptanmıştır [10].

Kars ilindeki küçük ve orta ölçekli hayvancılık işletmeleri genellikle kaba yemi kendi arazilerinden temin etmekte, büyük ölçekli işletmeler ise daha çok satın almaktadırlar. Üreticiler hayvan beslemede kaba yemin yanında, tane yem ve kepeği de birlikte vermektedir. Kars ilinde besin değeri yüksek ve ekonomik olmasına rağmen, mısır silajı kullanımının yeterli düzeyde değildir [17].

Anket uygulamasına katılan toplam 22 (%5.34) işletme sahibinin arazi varlığının olmadığı ve tarımsal üretim faaliyetinde bulunmadığı tespit edilmiştir. Diğer taraftan 390 (%94.66) işletme sahibinin ise çeşitli büyüklükte arazisi bulunmaktadır. İlde bulunan tarımsal işletmelerin arazi büyüklüğünün büyük kısmının 51 dekardan daha fazla olduğu (%72.05) tespit edilmiştir. Şahin ve Yılmaz [18] Van ilindeki tarım işletmelerinin ortalama arazi varlığını 52 dekar olarak belirtmiştir.

Bölgede aynı ahırda ya da organize hayvancılık bölgelerinde birlikte yetiştiricilik yapma fikri fazla kabul görmemektedir. İlde hayvancılık sektöründe üretim alt sektörleri itibariyle ihtisaslaşmış örgütlere yönelmek en bilimsel ve akılcı yol olacaktır. Bu sayede üreticiler girdilerini daha ekonomik elde ederken, ürünlerini de eder fiyattan satma imkânı yakalayacaklardır. Ülkemizde çoğu hayvansal ürünün pazarlanmasında görülen oligopson yapı böylece kırılmış olacaktır [19,20].

Ankette dikkat çeken en ilginç cevaplardan birisi mevcut ahırların büyütülmesine yönelik verilen cevaptır. İşletme sahiplerinin %86.89'u mevcut ahırlarını büyütmeyi düşündüklerini belirtmiş olup, bu oranda bölgede hatta başka illerde yapılmış ya da görülmüş olan modern ahırların katkısının büyük olduğu gözlemlenmiştir.

Yeni bir ahır yaptıracak olsanız kimden fikir alırdınız sorusuna, en fazla fikir alınacak kurumun İl Gıda, Tarım ve Hayvancılık Müdürlüğü olduğu belirlenmiştir. Bunu bölgedeki Veteriner Hekim ve Ziraat Mühendisleri takip etmektedir. İlde bulunan Veteriner Fakültesi ise 4. sırada gelmektedir. İl Müdürlüğü'nün ilk sırada yer almasının nedeninin, yetiştirici ile en fazla diyalog içerisinde olan kurum olmasından kaynaklandığı düşünülmektedir.

İlde yeni bir ahır yapılması durumunda ideal bir ahır kapasitesinin 40 baştan daha fazla olmasının gerektiğine inananların oranı %61.89 olarak tespit edilmiştir. İşletmelerde mevcut hayvan sayısının fazla olması ve yetiştiricilerin modern barınaklarda ekonomik bir yetiştiricilik yapabilmek için bu tür düşüncelere sahip oldukları gözlemlenmiştir. Özellikle 20 baştan küçük işletme yapmak isteyen yetiştirici sayısının son derece düşük olması da bunu desteklemektedir. Bitlis ilinde yapılan bir çalışmada mevcut işletmelerde ahır kapasitesinin oldukça düşük olduğu ve 10 baştan az hayvanı olan işletme oranının %60.80 olduğu belirlenmiştir [21].

Kars ili ve çevresinde yeni bir ahır yapılması durumunda, en uygun ahır yapı malzemesinin betonarme, bunu takiben de taş olduğu belirlenmiştir. Betonarme yeni yapılan ahırlarda oldukça yaygın bir şekilde kullanılmakta, eskiden ahırlarda en yaygın olarak kullanılan taş ve betonarmenin yerini, yalnızca betonarmenin aldığı görülmektedir. İlçeler arasında Sarıkamış'ta betonarme (%32.65) yerine taş kullanımını tercih edenlerin oranının (%44.90) yüksekliği dikkat çekicidir. Yine Akyaka ilçesinde taş kullanım oranı oldukça yüksek bulunmuştur (%40.00). Kaygısız ve Tümer [16] Kahramanmaraş ilinde yaptıkları çalışmada süt sığırı barınaklarında duvar malzemesi olarak işletmelerin %33'ünün taş, %40'ının briket, %26'sının kerpiç ve %1'inin ahşap kullandığını, barınak tabanının %67 beton, %2 ahşap ve %31 topraktan oluştuğu bildirmiştir.

Araştırmada yetiştiricilerin en uygun çatı malzemesi olarak %94.17 ile sacı ilk sırada belirtmiştir. Mevcut durum içerisinde %46.36 toprak olan çatı malzemesini yerini yeni yapılması planlanan ahırlar için saç almış görülmektedir. Yeni yapılması planlanan ahırlarda en uygun taban malzemesi olarak betonarme görülmektedir (%91.75). Mevcut ahırlar içerisinde bu oran düşük olmakla birlikte yine beton ilk sırada yer almaktadır.

Yetiştiriciye ideal bir hayvan barınağı nasıl olmalıdır şeklinde sorulan sorulara verilen cevaplar genel olarak toplam üzerinden değerlendirilmiş ve ilçelere göre ayrıca bir değerlendirme yapılmamıştır. Sorulan sorulara yüksek oranda evet cevapları verilmiştir. Sorulan soruların bir kısmı mevcut durumla karşılaştırıldığında, yetiştiricilerin modern bir ahır yapması durumunda bazı eksikliklerini görmüş oldukları ve bunları giderebilecekleri söylenebilir.

Yörede bulunan küçük ölçekli geçimlik aile tipi işletmelerde elbette süt sağım ünitesinin kurulması rasyonel olmayacaktır. Ancak 10 baş ve üzeri sağmal ineği olan işletmelerde seyyar sağım makinesinin kullanımı gerekmektedir. Seyyar sağım makinesinin kullanımı sütün hijyenik sağılmasına yardımcı olacaktır. Diğer taraftan 50 baş ve üzeri sağmal inek bulunduran işletmelerde süt sağım ünitesinin kurulması rasyoneldir. Zira bu sayede işçilik maliyeti azalacak, hayvanlar günde en az 2 kez sağılarak hayvan başına süt verimi artacak ve hayvanların verimleri ile kayıtlarının tutulması kolaylaşacaktır.

Kars ilinde süt üretiminde rastlanılan en önemli sorunlardan biriside sütün pazarlanma sorunudur. Halen ilde avans sistemi hâkimdir. Kars ilinde avans sisteminden dolayı süt fiyatları Türkiye genelinden daha düşüktür [22]. Bu sistem kaliteli ve hijyenik süt elde etmeye de engel olmaktadır. İlde sütün eder fiyattan satılmasıyla süt sığırcılığı ve bunun paralelinde besicilik gelişecek, böylece üreticilerin refahı da artacaktır [20].

Bölgede çiftlik tipi hayvancılıktan (entansif) ziyade mera ve yaylacılığa dayalı hayvancılık yapılmaktadır. Bu şekilde yapılan yetiştiriciliğe; örgütlenme ve sermaye yetersizliği, pazarlama olanaklarının eksikliği, genelde kâr marjının düşük oluşu gibi nedenler eklenince, büyük ölçekli hayvancılık yapan işletmelerin yaygınlaşması zorlaşmaktadır.

İlde ve bölgede eski ahırların geliştirilmesi, modernize edilmesi ve tespit edilecek olan aksaklıkların giderilmesi, yeni ahırların yapılması amacıyla Üniversitelerin Veteriner ve Ziraat Fakülteleri, İl Gıda Tarım ve Hayvancılık Müdürlükleri, Kalkınma Ajansları, Yetiştirici Birlikleri ve Kooperatifler, Veteriner Hekim Odaları, Ziraat Mühendisi Odaları ve hayvancılık sektörü ile ilgili yetkililerin ortak çalışmalar yapması faydalı olacaktır. Ayrıca bölgede oldukça yaygın olan kapalı bağlı duraklı sistem ahırların belirlenen olumsuzlukları ile yetersizliklerinden ve serbest duraklı ahırların avantajlarından dolayı, bölgede yeni yapılacak ya da modernize edilebilecek ahırlarda serbest duraklı ahırların yapılmasının daha yararlı olacağı kanaatine varılmıştır.

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Kafkas Univ Vet Fak Derg
19 (2): 199-202, 2013
DOI: 10.9775/kvfd.2012.7430

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Türk Toplumunun Hayvan Hakları Kavramına Yaklaşımının Belirlenmesine Yönelik Bir Araştırma: II. Hayvan Sevgisi, Hayvanları Tanımlama, Hayvan Sahipliği ve Beslenme Alışkanlıklarına Yönelik Tutum Analizi [1]

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Makale Kodu (Article Code): KVFD-2012-7430

Özet

Bu çalışma, Türk toplumunun hayvan hakları konusunda bilinç düzeyi ve algısına yönelik saptamalar yapmak amacıyla gerçekleştirildi. Bu amaçla hazırlanan anket, Türkiye'nin coğrafi bölgelerini temsil ettiği kabul edilen yedi ilde 2016 kişiye yüz yüze görüşme tekniği ile uygulandı. Anket verilerinin değerlendirilmesinde; hayvan sevgisine, hayvanları tanımlamaya, hayvan sahipliğine, beslenme alışkanlıklarına yönelik sorulara verilen yanıtlar kendi içlerinde gruplandırılarak SPSS istatistik programı ile analizi yapıldı. Analiz sonucunda: hayvan sevgisi, evde hayvanlarla temas, vejetaryenlik ve hayvanları birey olarak kabul etme düzeyi değişkenleri ile hayvan haklarına yönelik tutum arasında pozitif bir ilişki olduğu (P<0.001) sonuçlarına ulaşıldı.

Anahtar sözcükler: *Türk toplumu, Hayvan hakları, Tutum, Hayvan sahipliği, Vejetaryenlik*

A Survey to Identify the Turkish People's Approach on Animal Rights Concept: II. Attitude Analysis Related to Animal Love, Animal Identification, Animal Ownership and Eating Habits

Summary

The present study was undertaken to determine the Turkish people's cognitive level and perception on the concept of animal rights. For this aim, A questionnaire was developed for collecting data and was applied to participants in seven provinces which are considered to represent geographical regions of Turkey. The survey was applied by interviewing with 2016 participants individually. The responses to the questions of animal love, identification of animals, animal ownership and eating habits are grouped themselves after the evaluation of survey data and analysed statistically. As a results, positive proportion was determined by the parameters of animal love, contact with the animals at home, accepting the animals as an individual and being vegetarian (P<0.001).

Keywords: *Turkısh society, Animal rights, Attitude, Animal ownership, Vegetarianism*

GİRİŞ

Canlılara-hayvanlara haklar tanınması ve onların korunmasına yönelik hareketlerin kökleri Antik Çağ'a kadar uzanmaktadır. Osmanlı Devletinde ve Batı Ülkelerinde hayvanları korumaya yönelik olarak gerçekleştirilen bir takım öncü yasal düzenlemelerin yanında ilk müstakil hayvanları Koruma Yasası 1822 yılında İngiltere'de çıkartıl-

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mış ve 1960'larda modern hayvan hakları hareketi karşılığını bulmaya ve hayvan hakları çok sayıda ülkede çıkartılan yasal metinlerle güvence altına alınmaya başlanmıştır [1-4].

Yabancıların, Osmanlı Dönemine ilişkin kaleme aldıkları seyahatnamelerde, sadece sokaktaki kedi köpeklere verilmek üzere et satan seyyar satıcıların bulunduğunu bildirmesi, padişah fermannamelerinde ve ihtisab (belediye) kanunlarında hayvanları korumaya yönelik çeşitli düzenlemelerin yer alması Türkiye'de hayvanların korunması düşüncesini eski bir tarih ve geleneğe bağlıyor olsa da sokak hayvanlarının korunmasına yönelik ilk kapsamlı Kanunun çıkartılması için 2004 yılına kadar beklenmiştir [5,6]. Hayvanların korunması düşüncesi yalnızca sokaktaki hayvanların hakları ile sınırlandırılmamış, günümüze kadar çıkartılan çok sayıda yönetmelikle gıda için ve deneysel amaçlarla kullanılan hayvanların da refahlarının sağlanmasına yönelik adımlar atılarak üretici ve tüketicilerin konuya bakışlarına yönelik yeni bir pencere açılmıştır [7].

Yurt içinde ve yurt dışında yürütülen çalışmalarda [8-11] hayvanlarla temas halinde olmanın, hayvanları kişisel olarak tanımlamanın (birey, vs.), beslenme alışkanlıklarının ve diğer bazı parametrelerin hayvanlara yönelik gösterilen tutumu etkilediği ortaya konulmuştur. Belirli meslek gruplarının hayvan haklarına yönelik tutumlarını belirlemek için yapılan çalışmalar olmakla birlikte, Türk toplumunun konuya bakışına ve bilinçlilik düzeyine yönelik kapsamlı bir çalışma saptanamamıştır. Bu çalışma ile Türk toplumunun hayvan hakları konusundaki tutum ve yaklaşımlarının ortaya konması ve ulaşılan sonuçların uluslararası verilerle karşılaştırılması amaçlanmaktadır.

MATERYAL ve METOT

Çalışmanın örneklemi ve veri toplama aracına ilişkin bilgi "*Türk toplumunun hayvan hakları kavramına yaklaşımının belirlenmesine yönelik bir araştırma: I. Demografik özelliklere göre tutum analizi*" başlıklı makalede sunuldu [12]. Bu araştırmada veri toplama aracı olarak geliştirilen anketin parametrelerinden, hayvan sevgisi, hayvanları tanımlama, hayvan sahipliği ve beslenme alışkanlıkları ile ilgili olanlar kullanıldı.

Çalışma kapsamında, yüz yüze görüşme yöntemi kullanılarak toplam 2016 kişiye anket uygulandı ve elde edilen veriler SPSS 13.0 for Windows (Chicago, IL) istatistik paket programı ile analiz edildi. Hayvan haklarına yönelik sergilenen tutum düzeyleri için elde edilen puanların değişken gruplarıyla (ikiden çok grup için) karşılaştırılmalarında Kruskal Wallis Testi; iki gruplu değişken karşılaştırılmasında ise Mann Whitney U Testi kullanıldı. Tüm analizlerde anlamlılık düzeyi $P<0.05$ olarak kabul edildi. Tutum ölçeğinden elde edilen puanların değerlendirilmesinin yanı sıra, bağımlı değişkenler ile bağımsız değişkenler arasındaki ilişkileri belirleyebilmek için Pearson χ^2 (kikare) testi yapıldı.

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

BULGULAR

Katılımcıların büyük bölümü (%87.8) "*Hayvanları seviyor musunuz*?" şeklindeki soruya *"evet"* yanıtı verirken, bu soruyu *"hayır"* şeklinde yanıtlayanların oranı %7.1, *"emin değilim"* şeklinde yanıtlayanların oranı ise %5.1 oldu. *"Hayvanları seviyor musunuz?"* sorusuna verilen yanıtlara göre oluşturulan grupların hayvan hakları konusundaki tutumları incelendiğinde, bu soruya "*evet*" yanıtı verenlerin, "*emin değilim*" ve "*hayır*" yanıtı verenlere kıyasla daha pozitif tutum ($P<0.001$) sergiledikleri gözlendi *(Tablo 1)*.

"Evinizde hayvan (kedi, köpek, kuş vb.) beslediniz mi ya da besliyor musunuz?" sorusuna katılımcıların yarıdan fazlası (%60.6) *"evet"* cevabını verirken, %39.4'ü beslemediğini belirtti. Evde hayvan besleme değişkenine göre yapılan tutum değerlendirilmesinde evlerinde hayvan besleyenlerin beslemeyenlere oranla hayvan hakları konusunda daha pozitif tutum sergiledikleri gözlendi ($P<0.001$) *(Tablo 1)*.

"Çiftlik hayvanı yetiştirdiniz mi? Ya da yetiştiriyor musunuz" sorusuna katılımcıların %28'i *"evet"*, %72'si *"hayır"* cevabını verdi. Hayvan haklarına yönelik tutum ile verilen cevaplar arasında anlamlı farklılığın olmadığı belirlendi ($P=0.818$) *(Tablo 1)*.

Anketteki "*Vejetaryen misiniz?*" şeklindeki soruya, katılımcıların büyük çoğunluğu (%95.6) *"hayır"*, %4.4'ü *"evet"* cevabını verdi. Vejetaryenlik değişkenine göre yapılan tutum değerlendirilmesinde vejetaryen olanların, olmayanlara göre hayvan hakları konusunda daha pozitif tutum sergiledikleri belirlendi ($P<0.001$) *(Tablo 1)*.

"Kişisel olarak hayvanları nasıl tanımlarsınız?" sorusuna *"Birey"* olarak tanımladıkları yönünde yanıt veren katılımcıların oranı %15.3, *"Herhangi bir varlık"* olarak tanımlayanların oranı %7.9, *"Doğanın parçası"* olarak kabul edenlerin oranı %69.2, "*İnsanların kullanabilmesi için yaratılmış varlıklar"* olarak tanımlayanların oranı %6.4, *"Düşünemeyen değersiz varlıklar"* olarak kabul edenlerin oranı ise %1.2 olarak belirlendi. Katılımcıların hayvanları tanımlamalarına ilişkin tutumlarının analizi sonuçlarına göre en pozitif tutumu, hayvanları '*birey*' olarak veya '*doğanın parçası*' olarak görenler sergilerken; hayvanları '*düşünemeyen değersiz varlıklar*' olarak görenlerin ise negatif tutum sergiledikleri saptandı ($P<0.001$) *(Tablo 1)*.

"Hayvanları seviyor musunuz?" ve *"Evinizde hayvan (kedi, köpek, kuş vb) beslediniz mi ya da besliyor musunuz?"* sorularına verilen cevaplar ile cinsiyet ($P<0.001$), meslek ($P<0.001$) ve yaş ($P<0.05$) değişkenlerinin grupları arasında anlamlı düzeyde farklılık olduğu saptandı. Bu farklılıklar ayrıntılı olarak *Tablo 2*'de sunuldu.

TARTIŞMA ve SONUÇ

Araştırma sonuçları hayvan sevgisi değişkeni yönünden değerlendirildiğinde, hayvanları sevdiğini ifade eden katılım

Tablo 1. *Katılımcıların hayvan sevgisi, evde hayvan besleme, çiftlik hayvanı yetiştirme, vejetaryenlik ve hayvanları kişisel olarak tanımlama değişkenlerine göre tutum değerlendirmesi*

Table 1. *Assessment of participants' attitude according to the variables of animal love, feeding animals at home, animal husbandry, vegetarianizm and identification of animals as individual*

Soru	Seçenekler	n	%	X±Sx	Medyan	Minimum Değer	Maksimum Değer	p
S1	Evet	1768	87.8	4.33 ± 0.01	4.29	0.00	6.90	<0.001
	Hayır	143	7.1	2.97 ± 0.06	2.59	1.51	5.25	
	Emin Değilim	103	5.1	3.84 ± 0.05	3.86	1.10	5.14	
S2	Evet	1220	60.6	4.39±0.01	4.37	0.08	6.90	<0.001
	Hayır	792	39.4	3.92±0.03	3.95	0.00	6.41	
S3	Evet	560	28.0	4.24±0.03	4.18	0.00	6.69	0.818
	Hayır	1440	72.0	4.20±0.02	4.23	0.08	6.90	
S4	Evet	89	4.4	4.55±0.09	4.39	1.10	6.90	<0.001
	Hayır	1920	95.6	4.19±0.01	4.20	0.00	6.66	
S5	Birey	307	15.3	4.72±0.03	4.71	3.39	6.90	<0.001
	Herhangi bir varlık	159	7.9	3.74±0.05	3.76	1.80	5.51	
	Doğanın bir parçası	1389	69.2	4.22±0.01	4.22	0.00	6.41	
	İnsanların kullanabilmesi için yaratılmış varlıklar	129	6.4	3.67±0.06	3.71	2.07	5.95	
	Düşünemeyen değersiz varlıklar	24	1.2	2.86±0.19	2.62	1.10	5.47	

n: *Frekans;* ***X±Sx*** *Ortalama±Standart Hata,* ***S1:*** *"Hayvanları seviyor musunuz?";* ***S2:*** *"Evinizde hayvan (kedi, köpek, kuş vb.) beslediniz mi ya da besliyor musunuz?";* ***S3:*** *"Çiftlik hayvanı yetiştirdiniz mi? Ya da yetiştiriyor musunuz?";* ***S4:*** *"Vejetaryen misiniz?";* ***S5:*** *"Kişisel olarak hayvanları nasıl tanımlarsınız?"*

Tablo 2. *Hayvan sevgisi ve evde hayvan beslemeye ilişkin soruların bazı demografik özelliklere göre dağılımı*

Table 2. *Distribution of questions relating to animal love and feeding animals at home according to some demographic traits*

Demografik Özellikler		Soru 1							Soru 2				
		Evet		Hayır		Emin Değilim		P	Evet		Hayır		P
		n	%	n	%	n	%		n	%	n	%	
Cinsiyet	Erkek	1020	86.6	106	9.0	52	4.4	<0.001	653	55.5	523	44.5	<0.001
	Kadın	743	89.9	34	4.1	50	6.0		563	68.1	264	31.9	
Yaş Grubu	20-30	774	84.9	81	8.8	57	6.3	0.003	559	61.4	352	38.6	0.041
	31-40	462	87.2	44	8.3	24	4.5		298	56.2	232	43.8	
	41-50	278	91.7	16	5.3	9	3.0		189	62.6	113	37.4	
	51-60	126	95.5	2	1.5	4	3.0		87	65.9	45	34.1	
	60 ve üstü	40	93.0	0	0	3	7.0		32	74.4	11	25.6	
Meslek	Sağlık Personeli	176	86.3	14	6.9	14	6.9	<0.001	136	67.3	66	32.7	0.001
	Mühendis	137	89.0	8	5.2	9	5.8		94	61.0	60	39.0	
	Öğretmen	173	91.5	4	2.1	12	6.3		122	64.6	67	35.4	
	İşçi	215	79.6	43	15.9	12	4.4		134	49.8	135	50.2	
	Çiftçi	108	95.6	3	2.7	2	1.8		80	70.8	33	29.2	
	Hukukçu	72	90.0	5	6.3	3	3.8		41	51.3	39	48.7	
	Memur	181	95.8	7	3.7	1	0.5		128	64.3	71	35.7	
	Serbest Meslek	320	85.8	36	9.7	17	4.6		217	58.3	155	41.7	
	Öğrenci	221	87.4	18	7.1	14	5.5		155	61.3	98	38.7	
	Diğer*	132	94.3	1	0.7	7	5.0		93	66.4	47	33.6	

Soru 1: *"Hayvanları seviyor musunuz?";* ***Soru 2:*** *"Evinizde hayvan (kedi, köpek, kuş vb.) beslediniz mi ya da besliyor musunuz?",* * *Arkeolog, Bankacı (15), Bilgi İşlemci, Bilişim Teknolojileri Uzmanı, Ekonometri, Ekonomist (2), Ev Hanımı (53), Futbolcu, Finasman Uzmanı (2), Haberleşme, Hostes, İktisatçı (3), İşletmeci (6), Kimyacı, Mimar (2), Muhasebeci (7), Müzisyen (3), Özel Güvenlik (2), Psikolog, Radyo-Tv, Satış Yöneticisi, Sekreter (5), Sosyolog, Sporcu, Tasarımcı (2), Tiyatro Yönetmeni (2), Turizm (22), Uluslarası İlişkiler Uzmanı (2)*

cıların (4.33) pozitif; hayvanları sevmediğini bildirenlerin (2.97) ise negatif tutum sergiledikleri anlaşılmaktadır. Yine evlerinde hayvan besleyenlerin (4.39) beslemeyenlere (3.92) oranla daha pozitif tutum sergiledikleri, ayrıca iki gruptan elde edilen ortalama değerler arasındaki farkın anlamlı olduğu (P<0.001) araştırma verileri ile ortaya konulmuştur *(Tablo 1)*. Bütün bu verilerin, literatürde yer alan pet sahipliğinin, hayvanlara yönelik tutum ve davranışlar üzerinde olumlu etki yarattığına yönelik görüşleri [8,9,11,13] desteklediği ileri sürülebilir.

Katılımcılar arasında az sayıda yer alan vejetaryenlerin (et yemezlerin), vejetaryen olmayanlara göre hayvan hakları konusunda daha olumlu tutum sergiledikleri, bu iki gruptan elde edilen ortalama değerler arasındaki farklılığın anlamlı olduğu (P<0.001) görülmektedir *(Tablo 1)*. Bu sonuçların Hills [10] tarafından yürütülen çalışmanın sonuçlarıyla paralel olduğu söylenebilir. Söz konusu tutumun Singer'in [3] *"Vejetaryenlik gerek insandışı varlıkların öldürülmesine, gerekse onlara acı çektirilmesine son verme yönünde atılacak, pratik değeri ve etkinliği son derece yüksek bir adımdır"* şeklindeki ifadelerini doğrulayan bir yönünün olduğu da düşünülebilir.

Araştırmadan elde edilen bulgulara göre katılımcılar içerisinde, hayvanları *"birey"* veya *"doğanın parçası"* olarak görenler hayvan hakları konusunda pozitif tutum sergilerken; *"insanların kullanabilmesi için yaratılmış varlıklar"*, *"düşünemeyen değersiz varlıklar"* veya *"herhangi bir varlık"* olarak görenler ise negatif tutum sergilemişlerdir *(Tablo 1)*. Hayvanların düşünce süreçleri ile bilinçlerini incelemeyi hedefleyen son araştırmalar [14,15]; memelilerin, muhakeme ve bellek gerektiren bir '*temel bilince*' ve '*kayda değer zekâya*' sahip olduklarını; bilgiyi gelişkin ve karmaşık yollarla işleyebildiklerini, alternatifleri ya da sonuçlardan elde edilen kazanımları değerlendirmek suretiyle muhakeme yapabildiklerini ortaya koymaktadır. Buradan hareketle hayvanları düşünemeyen varlıklar olarak tanımlayan katılımcıların sergilemiş oldukları negatif tutumun bilgi eksikliğinden kaynaklanmış olabileceği söylenebilir. Hayvanların akıl sahibi olduğuna inanma düzeyi ile hayvanlar yerine empati kurma düzeyi arasında pozitif bir ilişki olduğu yönünde sonuçlar içeren çalışmanın [10] bulgularının da, hayvanları *"düşünemeyen varlıklar"* olarak tanımlayan katılımcıların tutumlarındaki negatifliği açıklar nitelik taşıdığı ileri sürülebilir. Öte yandan Doğan ve arkadaşları [11] tarafından yürütülen bir çalışmada, katılımcılara köpeklerde bilişsel özellikler olup olmadığına yönelik sorular sorulmuş ve alınan yanıtlar doğrultusunda, köpek psikolojisini önemseme düzeylerinin, köpeklerde zekâ, akıl yürütme yeteneği ve muhakeme yeteneğinin varlığını kabul eden katılımcıların, kabul etmeyenlere göre anlamlı düzeyde yüksek bulunduğu dikkate alındığında, hayvanları *"birey"* olarak tanımlayanların gösterdikleri pozitif tutumun onlara atfettikleri bilişsel özelliklerle ilişkilendirilebilineceği söylenebilir.

Bayan katılımcıların, 41-50, 51-60 ve 61 ve üstü yaş grubundaki bireylerin "*Hayvanları seviyor musunuz?*", "*Evinizde hayvan (kedi, köpek, kuş vb.) beslediniz mi ya da besliyor musunuz?*" sorularına daha fazla olumlu yanıt verdikleri gözlenmiştir *(Tablo 2)*. Bu durum gerek yaş gerekse cinsiyet parametresi açısından, "*Türk Toplumunun Hayvan Hakları Kavramına Yaklaşımının Belirlenmesine Yönelik Bir Araştırma: I. Demografik Özelliklere Göre Tutum Analizi*" başlıklı araştırmanın [12] verileri ile uyum göstermektedir. Öte hayvan sevgisi açısından çiftçilerin yüksek oranda (%95.6) olumlu cevap verdiği görülmektedir *(Tablo 2)*. Bu durum çiftçilerin hayvan haklarına yönelik daha az pozitif tutum sergilediği araştırmanın [12] verileri ile uyuşmamaktadır. Bu uyumsuzluk çiftçilerin söz konusu araştırmadaki tutum ölçeği ile ilgili soruları anlamamalarından kaynaklanmış olabilir.

Evde hayvan besleyen ve hayvanları sevdiğini beyan eden katılımcılar içerisinde, en büyük grupların bayanlardan ve 51 yaş üstü katılımcılardan oluşması Rollin'in [16]; günümüz şehir yaşamında büyük ailelerin yerini çekirdek ailelere bırakması, boşanma veya vefat gibi olaylarla yalnızlaşan bayanların ve yaşlı bireylerin gerek kendini güvende hissetme gerekse yalnızlıklarını paylaşmak için arkadaş hayvanları sahiplendiklerine yönelik saptamaları ile açıklanabilir.

Sonuç olarak, hayvan sevgisi, hayvan besleme, vejetaryenlik ve hayvanları birey olarak tanımlayabilme parametrelerinin hayvan hakları konusundaki yaklaşımları pozitif yönde etkilediği ileri sürülebilir. Bunlara ek olarak, hayvanların bilişsel özellikleri konusunda bilgilendirme amaçlı girişimlerin ve hayvanlarla temasın arttırılması amaçlı etkinliklerin hayvan hakları konusunda tutumları pozitif yönde değiştireceği sonucuna varılabilir.

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Kafkas Univ Vet Fak Derg
19 (2): 203-206, 2013
DOI: 10.9775/kvfd.2012.7470

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Characterization of Calpastatin Gene in Purebred and Crossbred Turkish Grey Steppe Cattle

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Makale Kodu (Article Code): KVFD-2012-7470

Summary

Calpastatin (CAST) is a specific inhibitor of calcium-dependent neutral protease μ-calpain found in mammalian tissues. The genetic variants in the bovine CAST gene were analysed by using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The animal material of the study consisted of 132 bulls and heifers in Turkish Grey steppe cattle and Turkish Grey steppe x Brown Swiss crossbreds. C and G alleles are characterized by CAST/RsaI polymorphism were detected in the sample of animals were studied. Allele frequency C was significantly frequent in the crossbred group compared to the pure Turkish Grey steppe animals ($P<0.05$). In the total samples of animals, the average allele frequency C was 56.1%. Genotypic frequencies were estimated as 0.257, 0.499 and 0.243 in the purebred Turkish Grey steppe, and 0.388, 0.470 and 0.142 in the Turkish Grey steppe x Brown Swiss crossbred population for genotypes CC, CG and GG, respectively. As a result, genotypic distributions were equilibrium in both pure and crossbred examples of Turkish Grey steppe cattle.

Keywords: *PCR-RFLP, Grey steppe cattle, Calpastatin (CAST), Genetic marker, Polymorphism*

Safkan ve Melez Türk Boz Irk Sığırlarda Kalpastatin Geninin Karakterizasyonu

Özet

Kalpastatin (CAST), memeli dokularında bulunan ve kalsiyuma bağımlı doğal bir proteaz olan μ-kalpain'in spesifik bir inhibitörüdür. Sığır CAST geni içindeki genetik varyantlar, polimeraz zincir reaksiyonu-restriksiyon parça uzunluk polimorfizmi (PCR-RFLP) metodu kullanılarak analiz edildi. Çalışmada hayvan materyali olarak, Boz ırk ve Boz ırk x İsviçre Esmer melezlerinden oluşan 132 boğa ve düve kullanıldı. Çalışılan hayvan örneklerinde CAST/RsaI polimorfizminin C ve G alleleri karakterize edildi. C allel frekansı, saf Türk Boz ırklara nazaran melez grup içinde önemli bulunmuştur ($P<0.05$). Hayvan örneklerinin tümünde, C allel frekansı ortalaması %56.1'dir. Genotipik frekanslar, CC, CG ve GG genotipleri için sırasıyla, safkan Türk Boz ırkta; 0.257, 0.499 ve 0.243 ve Türk Boz ırk x İsviçre Esmer melezi popülasyonda; 0.388, 0.470 ve 0.142 olarak tahmin edildi. Sonuç olarak, genotipik dağılımlar Türk Boz ırk sığırı saf ve melez örneklerinin her ikisinde de dengededir.

Anahtar sözcükler: *PCR-RFLP, Boz ırk sığır, Kalpastatin (CAST), Genetik belirteç, Polimorfizm*

INTRODUCTION

According to the theoretical basis [1], the Grey steppe breed originated from the *Bos taurus primigenius*. There are similar and relative breeds to the Grey steppe in different countries such as Greece, Ukraine, Romania, Hungary, Serbia, Bulgaria and Italy [2,3]. This breed is named the Boz step or Plevne breed in Turkey. It is called Podolion in Italy, Iskar in Bulgaria and Grey steppe in English literature [3,4]. In Turkey, the breed was seen widely in the Trakya region and was used as a powerful draught animal for many years. Grey steppe breeds are preferred in rural regions because of their tolerance to cold and heat, and resistance to ecto- and endoparasites. This native breed was unprocessed beef in Turkey and was used extensively by poor farmers to produce beef in Trakya [4,5].

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Meat tenderness has a major impact on consumer satisfaction is one of the most important issues in beef cattle production on consumer satisfaction [6]. Among the issues that have been identified as responsible for the post-mortem meat tenderisation process is the calpain proteolytic system. Calpastatin inhibits μ- and m-calpain activity and, regulates post-mortem proteolysis. Increased post-mortem calpastatin activity has been correlated with reduced meat tenderness [7]. Three enzymes responsible for this process are the μ-calpain (CAPN1) and m-calpain (CAPN2) and their inhibitor is calpastatin (CAST). A number of studies [8] have shown that the calpain-calpastatin system is also important in normal skeletal muscle growth.

There are different methods for the identification of the genetic structure of breed animals. The analysis of PCR is one of the effective methods to determine the genetic characterization of a population [9,10]. In *Bos taurus* and *Bos indicus*, two single nucleotide polymorphisms (SNPs) have been identified in the CAST gene in intron 5 between exons 5 and 6: a G/C SNP (known as UoG-CAST) [11-13], and an A/G SNP (known as CAST-T1) in the 3´UTR region in exon 30 [14,15]. These SNPs have been shown to be associated with meat tenderness and three commercially available genetic marker panels are currently used GeneSTAR Quality Grade, GeneSTAR Tenderness, and Igenity *Tender*-GENE. The UoG-CAST polymorphism was associated with shear force across days of post-mortem aging; genotype CC yielded beef that was more tender than GG, and CG had intermediate tenderness [13,14,16].

This study aims to determine the allele frequency of CAST/RsaI polymorphism related to meat tenderness in the calpastatin gene in the purebred and crossbred examples of Turkish Grey steppe cattle.

MATERIAL and METHODS

Animals: Samples of Turkish Grey steppe cattle (n=71) and first-generation (F_1) crossbreeds of Turkish Grey steppe x Brown Swiss (n=61) cattle were studied. The samples are taken from these cattle were bred in the rural feedlot sector from villages of the Edirne and Çanakkale in 2009 and 2010 and originated from the commercial rural herds (free range) of 20 farms. An extensive system is used in the rural feedlot sector. The 132 animals (32 females and 100 males) were bred according to the conditions in Turkey, and were slaughtered at 24 and 36 months of age are used in the study.

The research was carried out at Trakya rural feedyards, the slaughterhouse of Keşan, the Food Control Laboratory of Keşan and the laboratories of Keşan Vocational High School in Trakya University in Edirne in Turkey.

DNA Isolation: The sources of DNA were frozen steaks stored in the Keşan Vocational High School Laboratory at Trakya University. The DNA was isolated within the muscle tissue using the Fujifilm QuickGene mini-80 application tool and tissue kit (QuickGene, UK) as the manufacturer's instructions in the Food Control Laboratory of Keşan.

Amplifications of DNA Fragments: The CAST genotypes and gene polymorphisms were determined by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), a technique described by Maeda *et al.*[17]. For identification of alleles C and G of the calpastatin gene (GenBank Accession No. AY_008267.1:g.282C>G), a fragment of 523 base pair (bp) located in intron 5 was released with the primers 5′–cct cga ctg cgt acc aat tcc gaa gta aag cca aag gaa ca–3′ (forward) and 5′–att tct ctg atg gtg gct gct cac t–3′ (reverse) and cut with R*sa*I according to Schenkel *et al.*[13].

The PCR amplification reaction solution was performed in total volume of 25 μl containing 12.5 μl master mix 2X (Fermentas life sciences, Rott./Germany), 4.5 μL of distilled H_2O, 2 μL of forward primer (10 pmol/μL), 2 μL of reverse primer (10 pmol/μL) and 4 μL of DNA template (approximately 50 ng/μL).

The PCR cycling condition was 95°C (10 min) for one cycle; then 94°C (30 s); then from 69 to 62°C (30 s) and 72°C (30 s) for 8 cycles; then 94°C (30 s), 61°C (30 s) and 72°C (30 s) for 27 cycles; and finally 72°C (5.0 min) for one cycle, maintaining 4°C thereafter. The PCR reactions were performed on the My Genie 96 Thermal Block Bioner. The corresponding PCR products were amplified 523 bp fragments.

PCR-RFLP Genotyping: The PCR products were cut with the restriction endonuclease R*sa*I. 257 bp and 266 bp the specific allele fragments were amplified. In each PCR-RFLP reaction, a mixture containing 1 μL (3 units) of R*sa*I enzyme, 1 μl of restriction buffer, 2 μL of distilled H_2O and 6 μg of PCR product was incubated at 37°C for 4 h to ensure complete digestion.

DNA-fragments run on agarose gel to separate: the DNA fragments from the cutting PCR products were separated at a concentration 2% agarose gel containing ethidium bromide (0.4 μg/mL). Electrophoresis was performed in a 1xTBE buffer, at 125V for about 60 min. The genotype for each individual was read under ultraviolet light by using an appliance (DNR Bio-Imaging Systems MiniBİS Pro) and software was used for molecular analysis (Image Aide from Spectronics Corporation).

Statistical Analysis: The allelic and genotypic frequencies were calculated for each of the polymorphisms, according to Schenkel *et al.*[13]. The allele and genotype frequencies of each poly-morphism were calculated for deviations from the Hardy-Weinberg equilibrium by X^2 tests (significance based on $P<0.05$). Differences in allele frequencies between groups were evaluated by using probability tables [18].

RESULTS

In the studied genetic groups, two genetic variants (C and G) were found for the CAST/R*sa*I polymorphism. Three

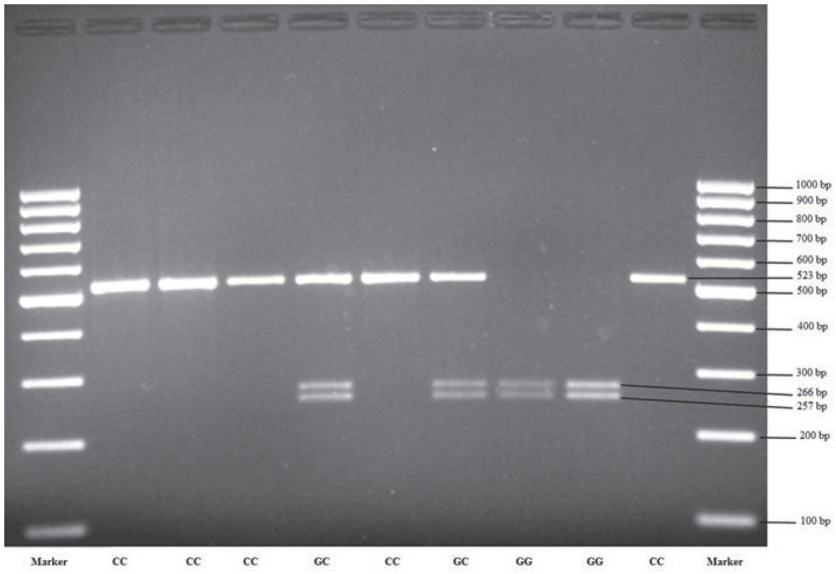


Fig 1. Genotyping for the bovine CAST gene polymorphism by the polymerase chain reaction restriction fragment-length polymorphism (PCR-RFLP) analysis. Samples were amplified by PCR followed by digestion with R*sa*I

Şekil 1. Sığır CAST geninin polimeraz zincir reaksiyonu-sınırlı parça uzunluk polimorfizmi (PCR-RFLP) ile genotiplendirilmesi

Table 1. *Allele and genotype frequencies of the CAST/RsaI polymorphism*
Tablo 1. *CAST/RsaI polimorfizminin allel ve genotip frekansları*

Breeds Genetic Groups	Number of Animals	CAST SNP/R*sa*I Allele frequency		Genotype Frequency *		
		C	G	CC	CG	GG
Greysteppe	71	0.507[b]	0.493[a]	0.257	0.499	0.243
Crossbred	61	0.623[a]	0.377[b]	0.388	0.470	0.142
Total (overall)	132	0.561	0.439	0.315	0.492	0.193

a, b; *Allele frequencies that differ significantly between genetic groups (P<0.05), * Genotype frequency was significantly different within breeds (P<0.05)*

unique banding patterns were identified, and either one or a combination of two different patterns was observed for individual cattle *(Fig. 1)*, which are consistent with homozygous or heterozygous genotypes, respectively, at the CAST locus. Genotype CC was characterised by the presence of single fragment of 523 bp, whereas genotype GG showed two fragments –257 and 266 bp. The heterozygotes (GC) were characterised by three fragments of 523, 257 and 266 bp.

The genotypic and allele frequencies reported for each of the SNPs analysis in the study are summarised in *Table 1*. Most of the animals were purebred, and so the allele frequencies for each breed could be thoroughly estimated. Allele frequencies for CAST were significantly different ($P<0.05$) between the Grey steppe and crossbred group. The G allele seemed lower among crossbred breeds.

Allele Frequencies were calculated according to the rule of the Hardy-Weinberg equilibrium *(Table 1)*. The allele frequency was significantly different ($P<0.05$) between purebred and crossbred groups. A greater frequency showed allele G of to the purebred Grey steppe than the crossbred. The present results show a higher frequency of allele C for the CAST/R*sa*I polymorphism in both pure and crossbred examples of Turkish Grey steppe cattle. This actuality indicates that the frequencies of alleles C and G were found to differ significantly between the purebred and crossbred genetic groups of Grey steppe cattle.

The overall results were observed *(Table 1)* of genotype frequencies (0.315, 0.492 and 0.193 for CC, CG and GG, respectively) which are in conformity with the Hardy-Weinberg equilibrium. Genotypic distributions were equilibrium (P>0.05) in examples of total cattle group. Genotype frequency was not significantly different within overall animal groups ($P>0.05$). Genotypic frequencies were identified as 0.257, 0.499 and 0.243 in the purebred Grey steppe breed, and 0.388, 0.470 and 0.142 in the crossbred population for genotypes CC, CG and GG, respectively. As a result, genotypic distributions were equilibrium in both pure and crossbred examples of Turkish Grey steppe cattle.

DISCUSSION

Schenkel *et al.*[13] reported allele frequencies for the CAST/R*sa*I SNP were 0.629 and 0.371 for allele C and G respectively, in *Bos taurus* cattle (Angus, Limousin, Charolais, Simmental), which were generally similar to frequencies found within the crossbreed cattle group at Trakya. In addition, Quaas *et al.*[19], Van Eenennaam *et al.*[14], Gill *et al.*[11], Curi *et al.*[20] and Reardon *et al.*[12] found average frequencies of the favourable allele C, as 72%, 72%, 64%, 69.3%, and 75% in crossbred populations of *B. taurus* breeds, respectively. On the other hand, in the present study the proportion of the allele C (50.7%) for the Grey steppe breed was lower than those were calculated in *B. taurus* breeds in other studies.

Van Eenennaam *et al.*[14] reported allele C frequencies ranging from 74% to 79% for genetic groups of *B. taurus* animals, and 79% for Brangus (5/8 *B. Taurus* + 3/8 *B. indicus*). The frequency of allele C on CAST /Rsal locus was reported as 0.64 in the Aberdeen Angus cattle by Gill *et al.*[11] and as 0.623 in the purebred Nellore cattle by Curi *et al.*[21]. These rates were similar to the Turkish Grey steppe crossbred

cattle group (0.623) in this study.

Gill *et al.*[11] reported genotypic frequencies as 0.41, 0.47 and 0.12 for CAST/R*sa*I SNP genotypes CC, CG and GG, respectively, in Aberdeen Angus cattle. Schenkel *et al.*[13] calculated genotypic frequencies 0.430, 0.398 and 0.172 for CC, CG, and GG SNP in a sample of *Bos taurus* cattle, respectively, and concluded that the frequencies are different between *Bos indicus* and *Bos taurus* cattle. In contrast, Curi *et al.*[21] estimated CAST genotypic frequencies as 0.377, 0.491 and 0.132 for CC, CG and GG, respectively in the purebred Nellore breed in *Bos indicus* cattle. In the present study, a proportional loss in the CC genotype frequency of the purebred Grey steppe was observed compared to the research results of the previous researchers.

SNP at the calpastatin and calpain loci appear to be associated with major gene effects for tenderness of meat according to Smith *et al*.[15]. The AY_008267.1:g282C>G SNP is corresponding with cross location change between cytosine and guanine in intron 5 of the bovine CAST gene, which was associated with meat tenderness in *Bos taurus* populations. Thus, Schenkel *et al*.[13] reported that the most favourable allele for CAST was C. Subsequently, CAST/R*sa*I polymorphism became part of a commercial test panel for the identification of animals that are superior in terms of meat tenderness [14]. Most of the published reports were indicated that CAST gene polymorphisms are associated with sheer force in the longissimus muscle [13,16,20,22,23].

In brief, Genetic marker technology is a promising tool for genetic improvement in livestock [24]. The findings of this study will contribute to the literature on the effect of CAST gene in the *Bos taurus* breed. CAST gene might be used as a candidate gene in the Grey steppe and its hybrid populations. Since CC genotype from CAST gene is related with meat tenderness, the meat quality in the Grey steppe might be improved by using Marker Assistant Selection (MAS).

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Kafkas Univ Vet Fak Derg
19 (2): 207-213, 2013
DOI: 10.9775/kvfd.2012.7475

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Genetic Diversity of Three Native Goat Populations Raised in the South-Eastern Region of Turkey [1]

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[1] *This work was supported by Harran University Scientific Research Projects Coordination (HUBAK-65)*

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Makale Kodu (Article Code): KVFD-2012-7475

Summary

The aim of this study was to investigate the genetic variability of three goat populations raised in the Southeastern region of Turkey by using microsatellite loci defined in gazelle *(Gazella granti)*. DNA was isolated from 120 blood samples collected from goats raised in Sanliurfa (n = 40), Kilis (n = 40) and Siirt (n = 40) provinces. Eight microsatellite loci (33HDZ8, 33HDZ290, 33HDZ433, 33HDZ496, 33HDZ593, 33HDZ692, 33HDZ749, 33HDZ974) were used for amplification with polymerase chain reaction (PCR). Fragment lengths of the amplifed loci were analysed using a capillary electrophoresis system. Six of the eight loci (75%) were successfully amplified and four (66.7%) of them were polymorphic. Three of the polymorphic loci (33HDZ496, 33HDZ749 ve 33HDZ974) were used for estimating genetic parameters. A total of 40 alleles were detected. Number of alleles and polymorphism information contents (PIC) for the loci 33HDZ496, 33HDZ749 and 33HDZ974 were 13, 15 and 12 and 0.836, 0.821 and 0.817, respectively. The mean number of alleles was 10.33±1.15, 10.67±2.08 and 11.33±0.58 and effective number of alleles was 6.131±1.42, 5.823±0.23 and 5.870±0.75 for Sanliurfa, Kilis and Siirt populations, respectively. The mean observed and expected heterozygosities for Sanliurfa, Kilis and Siirt populations were 0.827±0.083, 0.808±0.044 and 0.895±0.069 as well as 0.843±0.043, 0.841±0.007 and 0.838±0.023 respectively. For the 33HDZ496, 33HDZ749 and 33HDZ974 loci FIS values were 0.0164, 0.0245 and 0.0240, respectively. The FST values (0.009) showed that about 99% of the genetic variation was due to variation among individuals. The results have suggested that the three populations studied have a high genetic variability and the loci, 33HDZ496, 33HDZ749, 33HDZ974, can be used as markers due to their high number of alleles (≥12) and PIC (>0.81) values.

Keywords: *Goat, Microsatellite, Genetic diversity*

Güney Doğu Anadolu Bölgesi'nde Yetiştirilen Üç Keçi Populasyonunda Genetik Çeşitlilik

Özet

Bu çalışma bir ceylan türünde *(Gazella granti)* tanımlanmış sekiz mikrosatelit lokusunu kullanarak Güneydoğu Anadolu Bölgesinde yetiştirilen üç keçi populasyonunu genetik çeşitlilik yönünden değerlendirmek amacıyla yapılmıştır. Bu amaçla, Şanlıurfa (n = 40), Kilis (n = 40) ve Siirt (n = 40) illerinde yetiştirilen üç keçi populasyonundan toplam 120 adet kan örneği alınmış ve bu örneklerden DNA izole edilmiştir. İzole edilen DNA'lar sekiz mikrosatelit lokusuna (33HDZ8, 33HDZ290, 33HDZ433, 33HDZ496, 33HDZ593, 33HDZ692, 33HDZ749, 33HDZ974) ait primerler kullanılarak PCR ile çoğaltılmış ve PCR ürünleri kapillar elektroforeze tabi tutularak fragman uzunluğu analizi gerçekleştirilmiştir. Sekiz lokustan altısı (%75) PCR ile amplifiye olmuş ve bunlardan dördü (%66.7) polimorfik bulunmuştur. Polimorfik lokusların üçü (33HDZ496, 33HDZ749 ve 33HDZ974) genetik parametrelerin hesaplanmasında kullanılmıştır. Çalışmada toplam 40 allel tespit edilmiştir. 33HDZ496, 33HDZ749 ve 33HDZ974 lokusları için sırasıyla 13, 15 ve 12 allel gözlenmiş olup, herbir lokus için hesaplanan polimorfizm bilgi içeriği (PIC) aynı sırayla 0.836, 0.821 ve 0.817 olarak belirlenmiştir. Şanlıurfa, Kilis ve Siirt populasyonları için ortalama allel sayıları sırasıyla 10.33±1.15, 10.67±2.08 ve 11.33±0.58, etkili allel sayıları ise yine aynı sıra ile 6.131±1.42, 5.823±0.23 ve 5.870±0.75 olarak bulunmuştur. Şanlıurfa, Kilis ve Siirt populasyonları için ortalama gözlenen heterozigotluk oranları sırasıyla 0.827±0.083, 0.808±0.044 ve 0.895±0.069, beklenen heterozigotluk oranları ise yine aynı sıra ile 0.843±0.043, 0.841±0.007 ve 0.838±0.023 olarak belirlenmiştir. 33HDZ496, 33HDZ749 ve 33HDZ974 lokusları için hesaplanan FIS değerleri sırasıyla 0.0164, 0.0245 ve 0.0240 olarak bulunmuştur. Tüm populasyonlar için hesaplanan FST değeri (0.009) incelenen populasyonda gözlenen genetik çeşitliliğin %99'unun bireyler arasındaki farklılıktan kaynaklandığını göstermiştir. Çalışma sonuçları incelenen keçi populasyonunun oldukça yüksek bir genetik çeşitliliğe sahip olduğunu göstermiştir. Çalışma sonuçları ayrıca, sahip oldukları yüksek allelik zenginlik (≥12) ve PIC (>0.81) değerleri nedeniyle incelenen sekiz mikrosatelit lokusundan üçünün (33HDZ496, 33HDZ749, 33HDZ974) keçilerde moleküler belirteç olarak kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Keçi, Mikrosatelit, Genetik çeşitlilik*

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INTRODUCTION

Goats were domesticated about 10.000 years ago in the region defined as Fertile Crescent [1-4] partially including the Southeastern Anatolia. Goats are able to utilize roughage of lower quality and are adapted to various environmental conditions [5]. Although goat breeding has a lower proportion in the food production of animal origin in the world, still preserves its importance, especially for developing countries [6,7]. While presence of goats increased approximately three-fold between 1970 and 2010 in the world, the number of goats in Turkey decreased from 20 to 5 million [8,9]. On the other hand extensive goat breeding in South-eastern Anatolia still plays an important role in the regional economy.

Using a limited number of animals with high breeding values has resulted in a decrease of the number of local breeds or their extinction. This leads to a dramatic decline in the genetic variablity within local breeds. Therefore programs of national and international levels have been developed in order to maintain the genetic variability of livestock and several strategy papers have been published by the Food and Agriculture Organization [10,11]. The first step towards maintaining the genetic variability of local genetic resources is to assess their genetic potential in terms of genetic diversity [12]. Microsatellites are most common genetic markers used for determining genetic variabiliy, due to their high polymorphism, abundance in the genome and quasi neutral nature in terms of selection [13].

Primer binding regions of some microsatellite loci are highly conserved, which allows the use of same microsatellite markers in closely related species such as cattle, sheep and goat. For example, about 70-73% and 60% of the bovine derived microsatellites can be amplified with 60% and 40% of these are polymorphic in sheep and goat, respectively [14-17].

The purpose of this study was to investigate the utility of eight microsatellite loci defined in gazelle [18] *(Gazella granti)* as a molecular marker of genetic diversity in three goat populations raised in the Southeastern Anatolia Region of Turkey.

MATERIAL and METHODS

Sampling of Animals

A total of 120 blood samples were collected from goats raised in Kilis (n = 40), Sanliurfa (n = 40), and Siirt (n = 40) provinces of Turkey. Kilis goat has been developed by crossing native Hair goats with Aleppo goats and by subsequent interbreeding among the crossbred generations. This goat has been considered a separate breed. Kilis goats are distributed especially in the Kilis province and also raised in the provinces along the Syrian border of Turkey. They are kept in small flocks of 2 to 10 goats primarily for milk production. The samples collected from the Sanliurfa province included Aleppo goats, hair goats and the crossbred animals of these two breeds. The samples from the Siirt province included native hair goats and their crossbred animals with Angora goats.

DNA Analysis

DNA was isolated using Proteinase-K digestion and phenol-chloroform extraction according to Sambrook et al.[19]. DNA was purified using ethanol precipitation and the concentration of DNA was measured spectrophotometrically. The DNA samples were diluted to an end concentration of 100 ng/µl.

Eight microsatellite loci were amplified using primer pairs designed by Huebinger et al.[18] in *Gazella granti*. Polymeraze chain reaction (PCR) conditions were optimized by changing $MgCl_2$ concentrations and annealing temperatures. One of the primers of the loci successfully amplified was labeled with a fluoresecent dye for fragment length analysis. Fragment lengths of the PCR products were analyzed by using a capillary electrophoresis (Applied Biosystems, 3130xl Genetic Analyzer, Foster City CA). For detecting the alleles, peak scanner software v1.0 (Applied Biosystems) was used.

Data Analysis

Number of alleles (NA), allele frequencies, number of population specific alleles, observed (Ho) and expected heterozygosities (He) and probability of exclusion (PE) were calculated by using GenAlEx.6 software package [20]. Polymorphism information content (PIC) was calculated according to Botstein et al.[21]. Expected heterozygosities [22] (He), effective allele numbers [23] (NE), F statistics [24], exact test for deviations from Hardy-Weinberg equilibrium (HWE), genetic differentiation between populations and pair-wise linkage disequilibrium were estimated using GENEPOP [25] package (version 3.1). The program performs a probability test using a Markov Chain (dememorization 1000, batches 100, iterations per batch 1000) method. Presence of putative null alleles was tested by using Expectation Maximization (EM) algorithm [26] in FreeNA [27] program. Nei's original measures of genetic distance among populations [28] and gene flow (Nm) were computed by POPGENE [29] (version 1.31). Analysis of molecular varians (AMOVA), and pairwise F_{ST} differences were computed using ARLEQUIN [30]. FSTAT [31] (version 1.2) was used for calculating allelic richness (AR) (number of alleles in sample of standardized size was 31).

RESULTS

Using PCR method six loci were successfully amplified while two loci (33HDZ290 and 33HDZ433) yielded no PCR product. The optimized PCR conditions, size ranges of the PCR products and the fluorescent dye used for each loci are shown in *Table 1*.

Table1. *Details of microsatellite marker loci and PCR conditions*
Tablo 1. *Mikrosatelit belirteçleri ve PCR koşulları*

Loci	Template DNA (µl)	$MgCl_2$ (mM)	dNTP (mM)	Primers (µM)	Taq DNA Pol. (IU)	Annealing Temp. (°C)	Size Range (bp)	Florescent Label
33HDZ8	1	2	0.2	0.4	1.0	58	150-190	(NED)
33HDZ496	2	2	0.2	0.4	0.5	58	230-300	(FAM)
33HDZ593	1	2	0.2	0.4	0.5	58	200-210	(FAM)
33HDZ692	1	2	0.2	0.4	0.5	58	200-250	(NED)
33HDZ749	1	2.5	0.2	0.8	1.0	50	180-190	(HEX)
33HDZ974	1	2	0.2	0.4	0.5	58	270-290	(HEX)

One of the loci (33HDZ593) was monomorphic. For the 33HDZ8 locus two fragments of 165 and 169 bp length were observed, while for the 33HDZ692 locus PCR products of four different fragment lengths ranging from 267 to 270 bp were observed. Since 33HDZ8 and 33HDZ692 loci exhibited numerous non specific PCR products they were not included in the further analyses.

A total of 40 different alleles were detected for the three polymorphic loci in the three populations studied *(Table 2)*. Observed allele numbers and effective allele numbers for the loci 33HDZ496, 33HDZ749 and 33HDZ974 were 13, 15 and 12 as well as 6.69, 6.168 and 6.096 respectively. The mean number of alleles per locus was 13.33. Values of PIC and PE varied from 0.817 to 0.836 and from 0.679 to 0.710, respectively *(Table 3)*. The lowest heterozygosity was observed for the 33HDZ974 locus in Sanliurfa (0.7429) and the highest was observed for the same locus in Siirt population (0.9474). The lowest and highest expected heterozygosities were estimated for the 33HDZ974 (He = 0.795) and 33HDFZ496 (He = 0.8778) , in the Sanliurfa population, respectively *(Table 3)*.

Kilis population exhibited a heterozygosity deficiency for the 33HDZ749 locus (F_{IS} = 0.051, P<0.05), while in Siirt population an excess of heterozygotes for the 33HDZ974 locus (F_{IS} = -0.108, P<0.05) was observed. When the three populations were evaluated together the F_{IS} values were not diffferent from zero (P>0.05).

Genetic parameters in the three populations estimated by considering the three loci were shown in *Table 4*. A total of 31, 32 and 34 alleles were detected in Sanliurfa, Kilis and Siirt populations, respectively. Mean number of alleles and effective allele numbers per locus were 10.33, 10.67 and 11.33 as well as 6.13; 5.82 and 5.87 Sanliurfa, Kilis and Siirt populations, respectively.

Observed and expected heterozygisities varied from 0.808 to 0.895 and from 0.838 to 0.843 respectively. Deviation from the expected heterozygosity was only observed in Siirt population due to an excess of heterozygotes. However, the overall F_{IS} value in this population was not significantly different from zero. Deviations from Hardy-Weinberg equilibrium were observed in the Kilis population for the loci 33HDZ496 and 33HDZ749 due to a deficiency of heterozygotes and in the Siirt population for the locus 33HDZ974 due to an excess of heterozygotes.

The mean F_{ST} values for the loci 33HDZ496, 33HDZ749 and 33HDZ974 were 0.003, 0.007 and 0.016 respectively *(Table 5)*. The mean F_{ST}, F_{IT} and F_{IS} values were 0.009, 0.004 and 0.006 respectively. Number of migrants estimated based on F_{ST} values was estimated to be 16.57.

Genetic differentiation among three populations was found to be significant (P<0.05) for the 33HDZ974 locus which was estimated using exact G test. F_{ST} values estimated for population pairs varied from 0.0062 to 0.0139 *(Table 6)*. In exact G test significant differentiations were oserved between Sanliurfa and Kilis for the 33HDZ496, Sanliurfa and Siirt for the 33HDZ974 and Kilis and Siirt for the 33HDZ749 loci (P<0.05). Differentition between population pairs estimated by including the three loci was insignificant (P>0.05). AMOVA results showed that 0.9% of the total variance was explained by the variation among populations *(Table 7)*. Significant linkage disequilibrium was observed only in Siirt population between the loci 33HDZ496 and 33HDZ749. Presence of null alleles was not detected.

DISCUSSION

In this study six of eight loci (75%) were successfully amplified and four of these six loci (66.6%) were polymorphic. These findings are similar to those reported by other researchers who used microsatellite loci in different species [14,16,17]. Fragment lengths of the loci 33HDZ496, 33HDZ749 and 33HDZ974 in *Gazella granti* varied from 231 to 247, from 187 to 237 and from 253 to 255, respectively [18]. Number of alleles and the heterozygosities for 33HDZ496, 33HDZ749 and 33HDZ974 loci in *Gazella granti* have been reported to be 8, 12 and 2 as well as 0.815, 0.79 and 0.224, respectively [18]. The differences can be attributed to species differences.

The PIC values obtained in this study were similar to or higher than those reported for most of the loci [32-37]. Therefore the loci examined in this study were highly informative. The mean number of alleles per loci (13.33) were higher than those reported by Saitbekova et al.[38], Agha et al.[39], Araujo

Table 2. *Allele frequencies*
Tablo 2. *Alel frekansları*

Loci	Allele (bp)	Population			
		Sanliurfa (N=33)	Kilis (N=34)	Siirt (N=38)	All (N=105)
HDZ496	232	0.076	0.000	0.026	0.033
	234	0.045	0.176	0.145	0.124
	236	0.045	0.015	0.053	0.038
	238	0.121	0.059	0.066	0.081
	240	0.076	0.118	0.105	0.1
	242	0.258	0.279	0.316	0.286
	244	0.106	0.206	0.105	0.138
	246	0.121	0.029	0.079	0.076
	248	0.106	0.088	0.079	0.090
	250	0.015	0.000	0.013	0.01
	252	0.000	**0.015**	0.000	0.005
	254	0.030	0.000	0.013	0.014
	256	0.000	**0.015**	0.000	0.005
HDZ749	**Allele (bp)**	**(N=35)**	**(N=31)**	**(N=38)**	**(N=104)**
	139	0.000	**0.016**	0.000	0.005
	141	0.271	0.339	0.276	0.293
	143	0.114	0.113	0.184	0.139
	147	0.000	0.000	**0.013**	0.005
	149	0.057	0.000	0.079	0.048
	151	0.171	0.113	0.276	0.192
	153	0.171	0.081	0.053	0.101
	155	0.043	0.048	0.013	0.034
	157	0.000	**0.032**	0.000	0.01
	159	0.029	0.081	0.026	0.043
	161	0.029	0.065	0.013	0.034
	163	0.043	0.048	0.039	0.043
	165	0.043	0.016	0.013	0.024
	167	0.000	**0.032**	0.000	0.01
	169	0.029	0.016	0.013	0.019
HDZ974	**Allele (bp)**	**(N=35)**	**(N=34)**	**(N=38)**	**(N=107)**
	193	0.043	0.015	0.053	0.037
	197	0.200	0.103	0.105	0.136
	199	0.000	0.000	**0.013**	0.005
	203	0.343	0.265	0.145	0.248
	205	0.057	0.147	0.066	0.089
	207	0.086	0.074	0.079	0.079
	209	0.000	0.074	0.171	0.084
	211	0.214	0.235	0.276	0.243
	213	0.029	0.044	0.053	0.042
	215	0.014	0.044	0.026	0.028
	219	**0.014**	0.000	0.000	0.005
	227	0.000	0.000	**0.013**	0.005

Private alleles are given in bold

et al.[40], Fatima et al.[41], Oliveira et al.[42], Qi et al.[43], lower than those reported by Serrano et al.[33], Cañón et al.[44] and similar to those reported by Missohou et al.[37], Barker at al.[45], Chenyambuga et al.[46], Hassen et al.[47] and Li et al.[48]. The mean number of alleles/locus/breed found in this study (between 10.33 and 11.33) were similar to those found by Bozkaya et al.[49], Korkmaz-Ağaoğlu and Ertuğrul [50] and higher than those reported by Behl et al.[35], Saitbekova et al.[38], Agha et al.[39], Barker et al.[45], Li et al.[48], Gour et al.[51], Jandurova et al.[52], Ganai et al.[53] and Di et al.[54].

Several studies have indicated that allele numbers per population are generally lower in European breeds [44,46]. The reason for the higher allelic diversity observed in this study might be due to the lack of in the studied populations any selection program and that they were raised in a region considered to be the center of domestication. Thus, a higher genetic diversity was expected [1,3].

Barker [55] and Takezaki and Nei [56] have stated that the loci used for genetic diversity studies should have at least four alleles in order to reduce the standard error. Botstein et al.[21] have reported that the loci having a PIC value over 0.5 can be considered informative. The mean number of alleles (13.33±1.52) and PIC (0.809±0.03) values in the present study are indicative of adequate polymorphism and their appropriateness of selected loci for assessing genetic variation.

Observed and expected heterozygosities estimated in this study were higher than those found in most studies [12,38,39,52,53,57-59]. The values were also higher than those reported for Abaza (0.772), Angora (0.750), Gurcu (0.751) and Hair goat breeds (0.722) raised in Turkey [43]. However the values found for Kilis population in this study were similar to those reported for Kilis goats by Korkmaz-Ağaoğlu and Ertugrul [50]. Bozkaya et al.[49] have reported an expected heterozygosity of 0.70 for Kilis goats. On the other hand Bozkaya and Gurler [60] have investigated diversity of a microsatellite locus in *CSN1S1* gene and reported expected heterozygosities of 0.687, 0.721 and 0.497 in Sanliurfa, Kilis and Siirt populations respectively. Therefore the higher values of heterozygosities obtained in this study might be due to loci used. The F_{IS} values estimated for Sanliurfa (0.019), Kilis (0.040) and Siirt (-0.068) populations were similar to those reported by Korkmaz-Agaoglu and Ertugrul [50] for some Turkish goat breeds.

When the observed allele numbers and heterozygosity values are considered it can be suggested that Sanliurfa, Kilis and Siirt populations possessed similar genetic variability. The populations studied were raised in provinces near the south eastern border of Turkey and a higher migration rate was expected due to a nomadic system.

The F_{ST} value (0.009) estimated by including the three loci and the results of AMOVA indicated that less than 1% of the total variance was explained by the variance among the populations and more than 99% was explained by the

Table 3. *Genetic parameters for the three microsatellite loci in Sanliurfa, Kilis and Siirt populations.*
Tablo 3. *Şanlıurfa, Kilis ve Siirt populasyonlarında üç mikrosatelit lokusuna ait genetik parametreler*

Populations	Loci	N	NA	NE	AR	PIC	PE	Ho	He	F_{IS}	HWE	Null
Sanliurfa	33HDZ496	33	11	7.408	10.936	0.852	0.734	0.9091	0.8778	-0.036	0.3753	0.0000
	33HDZ749	35	11	6.380	10.962	0.826	0.694	0.8286	0.8559	0.032	0.1428	0.0470
	33HDZ974	35	9	4.605	8.759	0.753	0.588	0.7429	0.7950	0.066	0.1213	0.0244
Kilis	33HDZ496	34	10	5.612	9.729	0.799	0.651	0.7647	0.8351	0.084	0.0446*	0.0497
	33HDZ749	31	13	6.063	13.00	0.821	0.964	0.8065	0.8495	0.051	0.0188*	0.0490
	33HDZ974	34	9	5.794	8.911	0.807	0.662	0.8529	0.8396	-0.016	0.8638	0.0143
Siirt	33HDZ496	38	11	6.119	10.599	0.821	0.689	0.9211	0.8467	-0.088	0.2346	0.0000
	33HDZ749	38	12	5.031	11.041	0.775	0.617	0.8158	0.8119	-0.005	0.7595	0.0202
	33HDZ974	38	11	6.461	10.598	0.829	0.698	0.9474	0.8553	-0.108	0.0488*	0.0000
All	33HDZ496	105	13	6.690	10.600	0.836	0.710	0.8666	0.8527	-0.0163	NS	0.0029
	33HDZ749	104	15	6.168	11.942	0.821	0.709	0.8173	0.8379	0.0246	NS	0.0462
	33HDZ974	107	12	6.096	9.635	0.817	0.679	0.8505	0.8306	-0.0240	NS	0.0072

N: *Sample size,* ***NA:*** *Number of alleles,* ***NE:*** *Effective number of alleles,* ***AR:*** *Allelic richness per locus and per population based on minimum sample size of 31,* ***PIC:*** *Polymorphism Information Content,* ***PE:*** *Probability of Exclusion,* ***Ho:*** *Observed Heterozygosity,* ***He:*** *Expected Heterozygosity,* ***F_{IS}:*** *Fixation indices of subpopulation,* ***HWE:*** *Deviations from Hardy-Weinberg equilibrium (Exact P value by the Markov Chain method for heterozygosity deficit or excess),* ***Null:*** *Frequency of null alleles,* ***NS:*** *Not significant (P>0.05), * P<0.05*

Table 4. *Basic parameters of genetic diversity for three goat populations.*
Tablo 4. *Üç keçi populasyonu için temel genetik çeşitlilik parametreleri*

Parameters	Populations			All
	Sanliurfa	Kilis	Siirt	
N ±SD	34.33±1.15	33±1.73	38±0.00	35.11±2.47
TNA	31	32	34	40
MNA±SD	10.33±1.15	10.67±2.08	11.33±0.58	10.78±1.30
NEA± SD	6.131±1.42	5.823±0.23	5.870±0.75	5.941±0.82
AR±SD	10.22±1.26	10.55±2.16	10.75±0.26	10.50±1.28
Ho± SD	0.827±0.083	0.808±0.044	0.895±0.069	0.843±0.071
He± SD	0.843±0.043	0.841±0.007	0.838±0.023	0.841±0.025
F_{IS}	0.019	0.040	-0.068	-0.0055
PA	1	5	3	9

N: *Mean sample size,* ***SD:*** *Standard daviation,* ***TNA:*** *Total number of alleles,* ***MNA:*** *Mean number of alleles,* ***NEA:*** *Mean number of effective alleles,* ***AR:*** *Mean number of allelic richness,* ***Ho:*** *Observed Heterozygosity,* ***He:*** *Expected Heterozygosity,* ***F_{IS}:*** *Fixation indices of subpopulation,* ***PA:*** *Privte alleles*

Table 5. *Wright's F-statistics and gene flow (Nm) for each locus across the all populations.*
Tablo 5. *Wright'ın F-istatistikleri ve gen akış değerleri (Nm)*

Loci	F_{IS}	F_{IT}	F_{ST}	Nm
HDZ496	-0.016371	-0.013142	0.003178	20.826
HDZ749	0.024517	0.031747	0.007412	16.633
HDZ974	-0.024006	-0.007203	0.016409	12.276
All	-0.0055	0.0036	0.009096	16.578

Table 6. *Genetic distances between populations (Above diagonals Nei's standard genetic distances. below diagonals pairwise F_{ST} values)*
Tablo 6. *Populasyonlar arası genetik mesafeler (üst diagonal: Nei'nin standart genetik mesafesi, alt diagonal: popuasyon çiftleri için F_{ST} değerleri)*

Region	Sanliurfa	Kilis	Siirt
Sanliurfa	-	0.1122	0.1447*
Kilis	0.0062	-	0.1068
Siirt	0.0139*	0.0067	-

** P<0.05*

Table 7. *AMOVA design and results (average over 3 loci)*
Tablo 7. *Moleküler varyans analizi sonuçları (tüm lokuslar için)*

Source of Variation	Degree of Freedom	Sum of Squares	Variance Component	Percentage of Variation
Among populations	3	4.117	0.01141	0.89743
Within populations	107	261.776	1.26054	99.10257
Total	110	265.893	1.27196	

variance within individuals. The mean F_{ST} value estimated in this study was similar to those reported by Bruno-de-Soussa et al.[36], Araújo et al.[40], Rout et al.[59], Martinez et al.[61], Tadlaoui-Ouafai et al.[62], and lower than that reported by Oliviera et al.[42], Hassen et al.[47], Li et al.[48], Gour et al.[51], Dixit et al.[63] and Kumar et al.[64]. Luikart et al.[1] and Naderi et al.[3] have stated that a lower F_{ST} value generally observed in goat populations results from a higher mobility of goats between different regions, which reduces a genetic differentiation between populations. The higher number of migrants (Nm = 16.58) estimated in this sutudy was in accordance with this suggestion.

The differentiation between population pairs was not significant when the three loci were considered. Bozkaya and Gürler [60] have reported that Siirt population significantly differentiated from both Sanliurfa and Kilis populations, while Sanliurfa and Kilis populations did not significantly differentiated, in accordance with the geographical distance between the populations. However Bozkaya and Gürler [60] have used a single microsatellite locus in *CSN1S1* gene, which showed a mean allele number of 4.33. In the present study each population pair showed a significant differentiation for different loci. Therefore the differences might be due to the loci used. The results indicated a different evolutionary history of different loci.

In conclusion, eight microsatellite locus origined from *Gazella granti* were tested first time in goats with this study. Three of them can be used as genetic markers in goats. The optimal PCR conditions for each of the locus were described. In addition, populations of goats reared in the Southeastern Anatolia Region were evaluated in terms of genetic diversity regarding these microsatellite loci. Despite declining numbers, studied goat populations still carry a high genetic diversity. They have significant potential in the protection of indigenous genetic resources.

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Kafkas Univ Vet Fak Derg
19 (2): 215-220, 2013
DOI: 10.9775/kvfd.2012.7536

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Karayaka ve Bafra (Sakız x Karayaka G_1) Koyun Irklarının Ayrılmasında Diskriminant Analizinin Kullanılması

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Makale Kodu (Article Code): KVFD-2012-7536

Özet

Bu çalışmada, canlı ağırlık ve vücut ölçüleri kullanılarak Karayaka ve Bafra (Sakız x Karayaka G_1) koyunlarına ait kanonik ayırma fonksiyonu belirlenmiştir. Araştırmada, Tarım İşletmeleri Genel Müdürlüğü'ne bağlı Gökhöyük Tarım İşletmesi'nde yetiştirilen değişik yaşlardaki 223 Karayaka ve 462 Bafra koyununa ait canlı ağırlık ve vücut ölçüleri kullanılmıştır. Uygulanan aşamalı diskriminant analizinde, dokuz ölçü içerisinden ayırmada önemli bulunan beşinin (vücut uzunluğu, sırt uzunluğu, baş uzunluğu, cidago yüksekliği ve baş genişliği) genotiplerin sınıflandırılmasında yeterli ölçüler olduğu saptanmıştır. İki grup karesel diskriminant analizi sonucu elde edilen kanonik ayırma fonksiyonu ile koyun ırklarının %100 doğruluk oranıyla belirlendiği gözlenmiştir.

Anahtar sözcükler: *Diskriminant analizi, Koyun ırkları, Vücut ölçüleri, Karayaka, Bafra*

The Usage of Discriminant Analysis in Separation of Karayaka and Bafra (Chios x Karayaka B_1) Sheep Breeds

Summary

In this study, canonic discriminant function of Karayaka and Bafra (Chios x Karayaka B_1) sheep were determined by live weight and body measurements. In research, live weights and body measurements of 223 Karayaka and 462 Bafra sheep in various ages reared at Gökhöyük Agricultural Enterprise that belongs to General Directorate of Agricultural Enterprises were used. In stepwise discriminant analysis, 5 measurements (body length, back length, head length, wither height, head width) significant in discrimination from 9 measurements were found as sufficient in genotype classification. Sheep breeds were 100% correctly classified with canonic discriminant function gathered from two group quadratic discriminant analysis.

Keywords: *Discriminant analysis, Sheep breeds, Body measurements, Karayaka, Bafra*

GİRİŞ

Irk, cinsiyet, verim yönü ve yaş gibi faktörlere göre değişiklik gösteren canlı ağırlık ve vücut ölçüleri, hayvanların morfolojik yapısı ve gelişme kabiliyeti hakkında önemli bilgiler vermektedir. Bu nedenle, damızlık seçiminin iyi yapılmasında vücut yapısının göz önünde bulundurulması hayvan yetiştiriciliğinde önemlidir [1,2]. Özellikle koyun yetiştiriciliğinde canlı ağırlık ve vücut ölçülerinin belirlenmesi, bir ırkın tanımlanmasıyla birlikte ırklar arasındaki benzerlik ve/veya farklılıkların saptanması bakımından önem arz eder.

Bafra koyunu, süt ve döl verimi yüksek bir ırk olan Sakız ırkı ile verim özellikleri (süt, döl, canlı ağırlık) düşük Karayaka ırkının melezlenmesi (Sakız x Karayaka G_1) sonucunda elde edilen bir koyun ırkıdır [3-10].

Karayaka ve Bafra koyunları gibi birbirlerine akraba olan ırkların incelenen özellikler bakımından benzerlik ve/veya farklılık düzeylerinin saptanmasında farklı istatistiksel yöntemlerin yanı sıra diskriminant (ayırma) analizinden de yararlanılmaktadır. Diskriminant analizi, literatürde farklı koyun [11,12], keçi [13,14] ve arı [15] ırklarının morfolojik özelliklerine göre tanımlanması, farelerin her bir cinsiyet ve ırk için ağırlık ve bazı vücut özellikleri kullanılarak yaşlarının belirlenmesi [16] ve insan iskelet veya parmak izi ölçümlerinden cinsiyet tayini [17-19] gibi çeşitli amaçlar doğrultusunda uygulanan önemli bir yöntemdir.

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Çok değişkenli analiz yöntemlerinden biri olan diskriminant analizi, X veri setindeki değişkenlerin iki veya daha fazla gerçek gruplara ayrılmasını sağlayan ve birimlerin incelenecek p tane özelliğini ele alarak doğal ortamdaki gerçek gruplarına optimal düzeyde atanmalarını sağlayacak ayırma fonksiyonları türeten bir yöntemdir [20]. Veri setinin çok değişkenli normal dağılım göstermesi ve değişkenler arasında çoklubağlantı olmaması gibi temel varsayımları olan diskriminant analizi ile p değişken içerisinden sınıflamaya önemli düzeyde etki eden değişkenler belirlenir ve grup sayısına göre bu değişkenlere ait ayırma fonksiyon veya fonksiyonları tanımlanır [21]. Diğer bir ifade ile diskriminant analizi ile bir araştırmada, incelenen özellikler bakımından iki veya daha fazla grubun sınıflandırılmasında etkili değişkenleri saptayarak bu değişkenlere göre ayırma fonksiyon/fonksiyonlarını bulmak ve yeni birimleri en az hata ile bir gruba atamak amaçlanır. Diğer taraftan, bir araştırmada ele alınan değişkenler bakımından diskriminant analizinin uygulanabilmesi, veri matrisinin çok değişkenli normal dağılım göstermesi ve değişkenlerin varyans ve kovaryanslarının homojen olması gibi çeşitli varsayımları gerektirmektedir. Bununla birlikte, diskriminant analizinin, analize dahil edilen her bir bağımsız değişken için örneklem hacminin 30'dan yüksek olması durumunda daha güvenilir sonuçlar verdiği bildirilmektedir [22-24].

Bu araştırmada, diskriminant analizi ile canlı ağırlık ve vücut ölçüleri kullanılarak Karayaka ve Bafra (Sakız x Karayaka G_1) koyun ırklarının sınıflandırılmasında etkili değişkenlerin belirlenmesi ve bu değişkenlere ilişkin ayırma fonksiyonunu aracılığı ile bireylerin hangi düzeyde sınıflanabileceğinin saptanması amaçlanmıştır.

MATERYAL ve METOT

Bu araştırmada kullanılan veriler Akçapınar ve ark.[7] tarafından yürütülen bir projeden temin edilmiştir. Araştırmada, Tarım İşletmeleri Genel Müdürlüğüne bağlı Gökhöyük Tarım İşletmesi'nde yetiştirilen; bir (87), iki (12), üç (44), dört (61) ve beş ve üzeri (19) yaş gruplarındaki 223 Karayaka ile bir (45), iki (140), üç (152), dört (82) ve beş ve üzeri (43) yaş gruplarındaki 462 Bafra (Sakız x Karayaka G_1) koyununun kırkım sonundaki canlı ağırlık ve vücut ölçüleri (cidago yüksekliği, göğüs derinliği, göğüs genişliği, göğüs çevresi, vücut uzunluğu, baş uzunluğu, baş genişliği ve sırt uzunluğu) kullanılmıştır. Canlı ağırlıklar (kg) baskül, vücut ölçüleri (cm) ise ölçü bastonu ve ölçü şeridi ile belirlenmiştir.

Araştırmada, her iki genotip için değişkenlerin kaç temel boyut altında toplandığını belirlemek amacıyla varimax rotasyonu ve temel bileşenler (principal components) yöntemi kullanılarak açıklayıcı faktör analizi uygulanmıştır. Küresellik için faktör analizinin uygulanabilirliği Bartlett testi, örneklem hacminin yeterliği ise Kaiser-Meyer-Olkin (KMO) değeri ile kontrol edilmiştir.

Araştırmada, farklı yaşlardaki Karayaka ve Bafra koyunları, canlı ağırlık ve vücut ölçüleri yönünden diskriminant analizi ile incelenmiş ve genotiplere ait ayırma fonksiyonu ve yeni üyelerin gruplamasında alternatif olarak kullanılabilecek sınıflandırma fonksiyonları elde edilmiştir.

Doğrusal (linear) ve karesel (quadratic) ayırma analizi olarak iki temel gruba ayrılan diskriminant analizinin, ayırma (belirtici diskriminant analizi) ve sınıflama (tahminleyici diskriminant analizi) şeklinde iki işlevi bulunmaktadır. Ayrıca, iki (iki grup diskriminant analizi) veya daha fazla (çoklu diskriminant analizi) grubu ayırmak için modele alınması gereken ve ayırmaya önemli düzeyde katkıda bulunacak değişkenleri belirlemek amacıyla öncelikle aşamalı (stepwise) diskriminant analizi uygulamak gereklidir. Diskriminant analizinde doğrusal ve karesel ayırma fonksiyonları iki grup için aşağıda verilen formüller aracılığı ile elde edilir [20]:

İki grup doğrusal diskriminant analizinde grupların kovaryans matrisleri eşittir ($S_1=S_2$) ve ortak kovaryans matrisi (S);

$$S = \frac{(n_1 - 1)S_1 + (n_2 - 1)S_2}{n_1 + n_2 + 2} \qquad (1)$$

formülü ile hesaplanır. Her gruba ilişkin sınıflama fonksiyonu (Y_i);

$$Y_i = b_{i0} + b_{i1}X_1 + b_{i2}X_2 + \ldots + b_{ip}X_p \qquad (2)$$

şeklinde yazılır. Eşitlikte i=1,2 grup sayısını, b_{i0} sabit değeri (constant), b_{ij} (j=1,2…p) doğrusal bileşenleri (kanonik değişkenleri) ve p ise değ şken sayısını ifade eder. $\overline{X}_i$ i. grup ortalama vektörü olmak üzere b_{i0} sabit değeri ve b_{ij} katsayılar vektörü;

$$b_{i0} = -(1/2)\overline{X}_i' S^{-1} \overline{X}_i \qquad (3)$$

$$b_j = S^{-1} \overline{X}_i \qquad (4)$$

formülleri ile bulunur. İki grup için ortak doğrusal ayırma fonksiyonu ise;

$$Y = b_0 + b_1X_1 + b_2X_2 + \ldots + b_pX_p \qquad (5)$$

şeklinde yazılır ve b_j (j=1,2…p) doğrusal bileşenleri, ortalama fark vektörü (X_1-X_2) aracılığı ile aşağıdaki eşitlikle bulunur:

$$b_j = S^{-1}(\overline{X}_1 - \overline{X}_2) \qquad (6)$$

Grupların kovaryans matrisleri eşit değil ($S_1 \neq S_2$) ise iki grup karesel diskriminant analizi uygulanır ve ortak kovaryans (S) yerine grupların kovaryans matrislerinin farkları (S_1-S_2) alınarak ortak karesel ayırma fonksiyonu için b_j katsayılar vektörü aşağıdaki eşitlikle hesaplanır:

$$b_j = (S_1^{-1} - S_2^{-1})(\overline{X}_1 - \overline{X}_2)' \qquad (7)$$

Yukarıdaki bilgiler çerçevesinde, bu araştırmada öncelikle önemli düzeyde ayırma özelliğine sahip değişkenleri tespit etmek için aşamalı (stepwise) diskriminant analizi uygulanmış ve doğrusal (linear) sınıflandırma fonksiyonu ile birlikte

grup kovaryans matrislerinin homojenlik durumu dikkate alınarak iki grup karesel (quadratic) diskriminant fonksiyonu belirlenmiştir. Uygulanan aşamalı diskriminant analizinde değişkenlerin ayırmadaki önem veya etki derecesini belirlemek için (F önem düzeyine karşılık gelen) Wilks' Lambda yöntemi kullanılmıştır. Diğer taraftan, Karayaka ve Bafra koyunlarının canlı ağırlık ve vücut ölçülerine yönelik uygulanan varyans analizinde bazı vücut ölçüleri için ırk ve yaş arasında önemli interaksiyon tespit edildiği için yaşa göre düzeltme yapılmış ve düzeltilmiş değerlere ayrıca diskriminant analizi uygulanmıştır. Bu çerçevede, gerçek (düzeltilmemiş) ve yaşa göre düzeltilmiş değerlere yönelik hem normal (standardize edilmemiş) hem de standart (standardize) kanonik ayırma fonksiyonu elde edilmiştir. Canlı ağırlık ve incelenen vücut ölçülerinin yaşa göre düzeltilmesinde En Küçük Kareler (Least Squares) Yöntemi [25] kullanılmış olup, gerçek ve düzeltilmiş değerler genotipe göre bağımsız örneklemler için t testi ile karşılaştırılmıştır. Araştırmada kullanılan veriler SPSS 14.01 programı ile analiz edilmiştir.

BULGULAR

Araştırmada, öncelikle değişkenler arası ilişkiler incelenmiş ve çoklu belirleme (determinasyon) katsayısının (R^2) yüksek düzeyde olmamasından dolayı çoklubağlantı tespit edilmemiştir. Bununla birlikte, bazı değişken çiftleri arasında yüksek düzeyde korelasyon saptanmış olup, değişkenlere ait grupların belirlenmesi için açıklayıcı faktör analizi uygulanmıştır *(Tablo 1)*. Buna göre, Kaiser-Meyer-Olkin (KMO) değeri örneklem hacminin yeterliğini (KMO=0.886), Bartlett testi ise faktör analizinin uygulanabilirliğini (c^2=1114.32 ve P<0.001) ortaya koymuş ve değişkenlerin Karayaka'da toplam varyansın %67.68'ini açıklayan 2 faktör, Bafra'da ise toplam varyansın %70.85'ini açıklayan 3 faktör altında toplandığı görülmüştür. Faktör yükleri incelendiğinde her iki genotip için birinci boyut veya faktörde yer alan canlı ağırlık ve göğüs çevresi değişkenlerinin söz konusu faktör ile en yüksek düzeyde ilişkili değişkenler olduğu tespit edilmiştir. Bu bulgu canlı ağırlık ve göğüs çevresi arasındaki yüksek ilişkinin de bir göstergesidir.

Karayaka ve Bafra koyunlarının canlı ağırlık ve vücut ölçülerine ilişkin gerçek (düzeltilmemiş) ortalama ve en küçük kareler yöntemi ile yaşa göre düzeltilmiş beklenen ortalama değerlerleri *Tablo 2*'de verilmiştir. Koyunlara ait tüm canlı ağırlık ve vücut ölçüleri ortalamaları arasındaki farklılıklar genotipe göre önemli bulunmuştur (P<0.001). Bafra genotipi canlı ağırlık, cidago yüksekliği, göğüs derinliği, vücut uzunluğu, göğüs çevresi ve sırt uzunluğu, Karayaka genotipi ise göğüs genişliği, baş uzunluğu ve baş genişliği ölçüleri bakımından daha üstün bulunmuştur.

Karayaka ve Bafra koyunlarının gruplara ayrılmasında veya ırklarının belirlenmesinde önemli olan/olmayan değişkenlerin saptanması için uygulanan aşamalı (stepwise) diskriminant analizi sonucunda *(Tablo 3)*; göğüs genişliği, göğüs çevresi, göğüs derinliği ve canlı ağırlık ölçüleri ayırmada önemli bulunmamıştır. *Tablo 3*'te verilen ve değişkenlerin ayırmadaki önem veya etki derecesini belirten Wilks' Lambda değerlerine göre, genotiplerin sınıflandırılmasında önemli düzeyde katkısı olan ölçüler önem sırasına göre vücut uzunluğu, sırt uzunluğu, baş uzunluğu, cidago yüksekliği ve baş genişliğidir. Yaşa göre düzeltilmiş değerler için ayırmada baş genişliği ölçüsü cidago yüksekliğinden daha önemli bulunmuştur.

Karayaka ve Bafra koyunlarının sınıflandırılmasında önemli bulunan 5 vücut ölçüsü (VU, SU, BU, CY, BG) kullanılarak grup kovaryans matrisleri homojen olmadığı (Box's M=168.84 ve 97.94; P<0.001) için uygulanan iki grup karesel (quadratic) diskriminant analizi sonucunda elde edilen normal (standardize edilmemiş) ve standart (standardize) kanonik ayırma fonksiyonları ile yeni değerlerin sınıflanmasında kullanılabilecek sınıflama fonksiyon katsayıları *Tablo 4*'te verilmiştir. Grup merkezleri ve sınırlarını yansıtan kononik ayırma fonksiyon grafikleri ise *Şekil 1*'de verilmiştir. Toplam varyansın %100'ünü açıklayan ve gerçek ve yaşa göre düzeltilmiş değer için belirlenen kanonik ayırma fonksiyonlarının ırkları

Tablo 1. *Faktör analizi sonuçları*
Table 1. *Results of factor analysis*

Değişkenler		Karayaka İçin Faktör Yükleri		Bafra İçin Faktör Yükleri		
		Faktör 1	Faktör 2	Faktör 1	Faktör 2	Faktör 3
Canlı ağırlık	CA	**0.847**	0.342	**0.877**	0.220	0.190
Cidago yüksekliği	CY	**0.761**	0.380	0.043	**0.831**	0.219
Göğüs derinliği	GD	**0.838**	0.218	**0.810**	0.298	0.124
Göğüs genişliği	GG	**0.744**	0.241	**0.859**	-0.038	-0.061
Göğüs çevresi	GÇ	**0.862**	0.276	**0.887**	0.059	0.172
Vücut uzunluğu	VU	0.169	**0.814**	0.282	**0.834**	-0.024
Baş uzunluğu	BU	**0.677**	-0.150	0.020	0.264	**0.674**
Baş genişliği	BG	**0.693**	-0.190	0.201	-0.148	**0.751**
Sırt uzunluğu	SU	0.028	**0.815**	0.061	0.462	**0.564**
Özdeğer		*4.26*	*1.83*	*3.08*	*1.83*	*1.47*
Varyans %		*47.37*	*20.31*	*34.16*	*20.36*	*16.33*

***KMO** = 0.886 Bartlett testi: c^2=1114.32 ; P<0.001*

Tablo 2. *Karayaka ve Bafra koyunlarının canlı ağırlık ve beden ölçülerine ait gerçek (ham) ve düzeltilmiş ortalama değerleri*
Table 2. *Raw and adjusted mean values for live weight and body measurements of Karayaka and Bafra sheep*

Değişkenler	Gerçek Ortalama		P	Düzeltilmiş Ortalama		P
	Karayaka n=223	Bafra n=462		Karayaka n=223	Bafra n=462	
	X±Sx	X±Sx		X±Sx	X±Sx	
CA	48.97±0.54	61.42±0.37	*	51.05±0.39	61.28±0.31	*
CY	57.53±0.27	68.47±0.15	*	58.43±0.25	68.43±0.17	*
GD	29.85±0.16	32.50±0.11	*	30.42±0.13	32.60±0.12	*
GG	22.61±0.13	21.60±0.12	*	22.93±0.13	21.69±0.13	*
GÇ	92.76±0.39	100.24±0.30	*	94.21±0.37	100.42±0.30	*
VU	58.38±0.19	70.64±0.15	*	58.50±0.25	70.78±0.17	*
BU	22.11±0.10	20.63±0.05	*	22.31±0.12	20.68±0.06	*
BG	12.35±0.06	11.02±0.03	*	12.41±0.07	11.07±0.04	*
SU	61.84±0.20	75.30±0.18	*	62.10±0.26	75.47±0.21	*

* *P<0.001*

Tablo 3. *Aşamalı diskriminant analizi sonuçları*
Table 3. *Results of the stepwise discriminant analysis*

Sıra	Gerçek Değerler İçin				Düzeltilmiş Değerler İçin			
	Değişkenler	Tolerans 1-r	Wilks' Lambda	P	Değişken	Tolerans 1-r	Wilks' Lambda	P
1	VU	0.692	0.222	*	VU	0.695	0.212	*
2	SU	0.838	0.171	*	SU	0.832	0.162	*
3	BU	0.830	0.142	*	BU	0.910	0.134	*
4	CY	0.638	0.131	*	BG	0.950	0.123	*
5	BG	0.880	0.118	*	CY	0.675	0.117	*

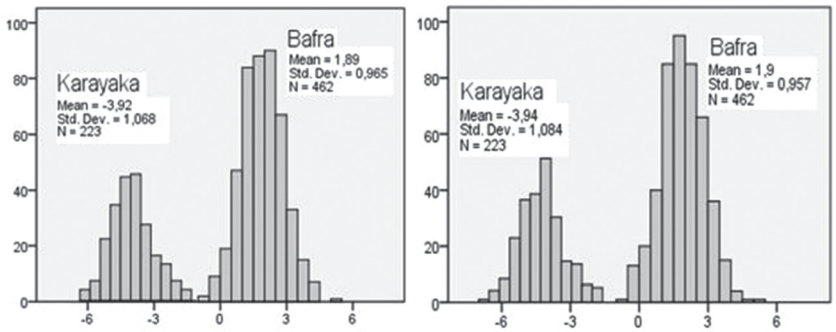
* *P<0.001*

Tablo 4. *Diskriminant fonksiyon katsayıları*
Table 4. *Discriminant function coefficients*

Değişkenler	Gerçek Değerler İçin				Düzeltilmiş Değerler İçin			
	Kanonik Ayırma Fonksiyon Katsayıları		Sınıflama Fonksiyon Katsayıları		Kanonik Ayırma Fonksiyon Katsayıları		Sınıflama Fonksiyon Katsayıları	
	Normal	Standart	Karayaka	Bafra	Normal	Standart	Karayaka	Bafra
VU	0.120	0.369	4.429	5.128	0.140	0.419	4.368	5.189
SU	0.141	0.515	2.239	3.058	0.130	0.539	2.949	3.743
BU	-0.344	-0.423	8.829	6.830	-0.356	-0.410	11.262	9.178
CY	0.125	0.431	0.288	1.014	0.151	0.289	2.035	2.594
BG	-0.432	-0.352	12.435	9.922	-0.426	-0.321	17.176	14.684
Sabit	-13.891	-	-381.884	-456.777	-13.855	-	-479.175	-554.234
Box's M = 168.84 P<0.001, Wilks' Lambda = 0.125; P<0.001, Kanonik korelasyon (r) = 0.927, Grup merkezleri: Karayaka = -3.923, Bafra=1.894					Box's M = 97.94; P<0.001, Wilks'Lambda = 0.117; P<0.001, Kanonik korelasyon (r) = 0.940, Grup merkezleri: Karayaka = -3.944, Bafra = 1.904			

saptamada önemli ayırma özelliğine sahip olduğu (Wilks' Lambda=0.125 ve 0.117; P<0.001) tespit edilmiştir. Kanonik korelasyon değerleri ise gerçek değerler için r=0.927 ve yaşa göre düzeltilmiş değerler için r=0.940 olarak hesaplanmıştır.

Karayaka ve Bafra koyunlarına ait ele alınan ölçümlerin tek tek ve (*Tablo 3*'teki ayırmada önemli ve yeterli görülen ölçülerin önem düzeyine göre) değişken kombinasyonları kullanılarak elde edilen doğru sınıflandırılma oranları *Tablo 5*'te verilmiştir. Buna göre, tek tek analizde sadece vücut uzunluğu kullanılarak en yüksek (gerçek değerler için ortalama %97.4 ve düzeltilmiş değerler için ortalama %97.5), sadece göğüs derinliği kullanılarak en düşük (gerçek değerler için ortalama %62.2 ve düzeltilmiş değerler için ortalama %63.4) doğru sınıflandırma oranı elde edilmiştir. Değişken kombinasyonlarına göre vücut uzunluğu ve sırt uzunluğu ölçüleri ile yaklaşık %99 oranında doğru sınıflandırma yapılırken, bu değişkenlere baş uzunluğu ve cidago yüksekliği eklendiğinde %100'e yakın doğru sınıflandırma yapılabilmektedir. Bununla birlikte 5 vücut ölçüsü (VU, SU, BU, CY, BG) kullanılarak *Tablo 4*'te verilen kanonik ayırma fonksiyonları ile bireylerin tamamı doğru sınıflandırılmaktadır *(Tablo 5)*.



Şekil 1. Gerçek *(solda)* ve düzeltilmiş *(sağda)* değerlere ait kononik ayırma fonksiyon grafikleri

Fig 1. Canonical discriminant function graphics for raw *(left)* and adjusted *(right)* values

Tablo 5. *Her bir değişken ve değişken kombinasyonları kullanılarak elde edilen doğru sınıflandırılma oranları*
Table 5. *Calculated correctly classified percentages using combined and individual variables*

Değişken(ler)	Gerçek Değerler İçin			Düzeltilmiş Değerler İçin		
	Karayaka	Bafra	Ortalama	Karayaka	Bafra	Ortalama
	*%	*%	*%	*%	*%	*%
VU	***97.8***	***97.2***	***97.4***	***96.9***	***97.8***	***97.5***
SU	***98.2***	***96.5***	***97.1***	***97.3***	***97.4***	***97.4***
BU	***94.6***	***95.5***	***95.2***	***96.0***	***95.5***	***95.6***
CY	***82.5***	***75.8***	***78.0***	***87.9***	***84.2***	***85.4***
BG	***67.3***	***82.0***	***77.2***	***70.0***	***82.0***	***78.1***
GG	*73.5*	*78.6*	*76.9*	*87.4*	*63.4*	*71.2*
GÇ	*74.4*	*67.5*	*69.8*	*61.4*	*75.8*	*71.1*
CA	*76.2*	*64.5*	*68.3*	*78.9*	*64.5*	*69.2*
GD	*51.6*	*67.3*	*62.2*	*80.3*	*55.2*	*63.4*
VU, SU	***98.2***	***99.1***	***98.8***	***98.7***	***99.4***	***99.1***
VU, SU, BU	100.0	99.1	99.4	100.0	99.4	99.6
VU, SU, BU, CY	100.0	99.4	99.6	100.0	99.8	99.9
VU, SU, BU, CY, BG	100.0	100.0	100.0	100.0	100.0	100.0

* *Doğru sınıflandırılma oranları*

TARTIŞMA ve SONUÇ

Araştırmada, canlı ağırlık ve vücut ölçüleri arasındaki ilişkileri tanımlamak üzere uygulanan açıklayıcı faktör analizinde, Karayaka genotipi için vücut uzunluğu ve sırt uzunluğu ikinci bir faktörde, Bafra genotipi için ise cidago yüksekliği ve vücut uzunluğu ikinci, baş uzunluğu, baş genişliği ve sırt uzunluğu da üçüncü bir faktörde yer almıştır. Gürcan ve Akçapınar [26] tarafından Alman Et ve Karacabey Merinosu üzerinde yapılan çalışmada, her iki genotip için cidago yüksekliği, sırt uzunluğu, vücut uzunluğu ve canlı ağırlık ilk grupta; baş uzunluğu, baş genişliği ve göğüs genişliği ikinci grupta ve göğüs çevresi ve göğüs derinliği ise üçüncü bir grupta toplanmıştır.

Hayvancılık alanında canlı ağırlık ile vücut ölçülerinin belirlenmesi ve vücut ölçülerinin canlı ağırlık üzerindeki etkisi üzerine pek çok çalışma [27-36] yapılmıştır. Bu çalışmalar içerisinde canlı ağırlık ve vücut ölçüleri arasındaki ilişkiler üzerine yapılan çalışmalarda [27-33], canlı ağırlık ile en yüksek düzeyde ilişkisi bulunan vücut ölçüsünün göğüs çevresi olduğu bildirilmiş olup, bu araştırmada da her iki genotip için aynı sonuca ulaşılmıştır.

Araştırmada, gerçek ve yaşa göre düzeltilmiş canlı ağırlık ve vücut ölçüleri kullanılarak Karayaka ve Bafra koyun ırklarını belirleyen diskriminant fonksiyonu elde edilmiştir. Literatürde, Karayaka ve Bafra koyunlarına ilişkin diskriminant analizi çalışması bulunmamakla birlikte, gerek farklı koyun ırkı gerekse farklı türler için ırk [11-15], yaş [16] ve cinsiyet [17-19] gibi özelliklerin belirlenmesi amacıyla yapılmış araştırmalar bulunmaktadır. Salako ve Ngere [11] Güney Batı Nijeryada'ki Batı Afrika bodur (cüce) ve Yankasa koyunlarını morfolojik özellikleri bakımından çoklu diskriminant analizi ile incelemiş ve genotiplerin sınıflandırılmasına ilişkin diskriminant fonksiyonlarını belirlemişlerdir. Traore ve ark.[12] tarafından yapılan çalışmada, Burkina Faso bölgesinde yer alan Sahel'de Burkina-Sahel, Sudan'da Djallonke ve Sudan-Sahel'de Mossi genotipinden 6440 koyun yedi vücut ölçüsüne (cidago yüksekliği, göğüs derinliği ile burun, baş, kuyruk, kulak ve vücut uzunluğu) göre diskriminant analizi ile incelenmiş ve Burkina-Sahel için %89.46, Mossi için %77.86 ve Djallonke için %38.82 oranında doğru sınıflandırma yapıldığı, doğru sınıflanma oranı düşük olan Djallonke koyunlarının %60.85'inin ise yakın özelliklere sahip Mossi koyunları olarak sınıflandırıldığı ve koyunların sınıflandırılmasında en önemli özelliğin vücut uzunluğu olduğu bildirilmiştir.

Bu araştırmada, uygulanan aşamalı diskriminant analizi sonucunda, ırkları belirlemede önemli ayırma özelliğine sahip olan normal ve standardize diskriminant fonksiyonları ile Karayaka ve Bafra koyunlarından oluşan bir popülasyonda, önem derecesine göre sırasıyla vücut uzunluğu, sırt uzunluğu, baş uzunluğu, cidago yüksekliği ve baş genişliği ölçüleri kullanılarak %100 doğrulukta ayırım yapılabileceği tespit edilmiştir. Bununla birlikte, sadece vücut uzunluğu veya sadece sırt uzunluğu ölçüsü ile ortalama yaklaşık %97, her iki değişken ile birlikte yaklaşık %99 oranında doğru sınıflama yapılabileceği belirlenmiştir. Diğer taraftan, elde edilen kanonik ayırma fonksiyonları üzerinde baş uzunluğu ve baş genişliği ölçülerinin negatif etkisinin bulunmasını, Karayaka genotipinin bu ölçülere ilişkin ortalamalarının Bafra'ya göre daha yüksek olması ile açıklamak mümkündür.

Diskriminant analizi ile Karayaka ve Bafra koyunlarında olduğu gibi birbirlerine akraba olan genotiplerin minimum değişken veya ölçüm kullanılarak bir popülasyon içerisinde yüksek doğruluk oranıyla tanımlanabilmesi ve elde edilen fonksiyonlar ile yeni bireylerin doğru bir şekilde sınıflandırılabilmesi, gerek ırklar arasındaki benzerlik ve/veya farklılık düzeylerinin bilinmesi gerekse diskriminant analizinin bu amaca yönelik araştırmalardaki rolü açısından önemli görülmektedir.

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Kafkas Univ Vet Fak Derg
19 (2): 221-224, 2013
DOI: 10.9775/kvfd.2012.7576

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Effects of CO_2 Narcosis on the Onset of Oviposition and Colony Founding Success of Post Diapausing Bumblebee (*Bombus terrestris*) Queens [1]

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[1] *This study was supported by Akdeniz University Scientific Researches Projects (Project number of "2010.02.0121.042")*
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Makale Kodu (Article Code): KVFD-2012-7576

Summary

In year-round rearing of bumblebees, rapid and successful colony production is the major criterion to reduce production costs. In this study, the effect of CO_2 narcosis on the oviposition and colony founding success of post diapausing Bombus terrestris queens were investigated. Mated queens that were kept in the refrigerator at 4.0±0.5°C for two months were randomly allocated to three groups for control, single and double narcosis. The queens in control group were not narcotized, while the queens in single narcosis group were treated once with CO_2 for 30 min and the queens in double narcosis group were treated with CO_2 for 30 minutes two times in consecutive days before introducing in starting boxes. All queens and colonies were kept in standard rearing conditions (27±1°C and 60±5% RH). There were significant differences between the treatment groups in terms of the duration of colony initiation (day) and the percentage of queens that laid eggs. The queens exposed to single and double narcosis started to lay eggs approximately 4.5 and 5 days earlier than the non narcotized queens, respectively. Similarly, 80% of single and double narcotized queens laid eggs, whereas only 45% of non narcotized queens laid eggs. The numbers of workers in the first brood batch, queen mortality rates at the first week and first month (%) were not significantly different between groups. The results showed that single CO_2 narcosis treatment to post diapausing queens can become a useful tool in the mass rearing of *B. terrestris*.

Keywords: *Bumblebee, Bombus terrestris, CO_2 treatment, Colony development*

Bombus Arısı (*Bombus terrestris*) Ana Arılarının Yumurtlamaya Başlaması ve Koloni Oluşturması Üzerine Diyapoz Sonrası CO_2 Uygulamasının Etkileri

Özet

Bombus arılarının yıl boyu yetiştiriciliğinde hızlı ve başarılı koloni üretimi, üretim maliyetini düşürmede temel kriterdir. Bu çalışmada diyapozdan çıkmış Bombus terrestris ana arılarına CO_2 uygulamasının yumurtlamaya başlama ve koloni oluşturma üzerine etkileri incelenmiştir. Çiftleşmiş ana arılar 4.0±0.5°C de iki ay muhafaza edildikten sonra rasgele üç gruba dağıtılmışlardır. Kontrol grubunu oluşturan ana arılar CO_2 narkozu uygulanmaksızın başlatma kutularına yerleştirilirken, bir grup ana arıya 30 dak. süre ile bir kez, diğer grup ana arıya ise 30 dakika süre ile iki kez CO_2 narkozu uygulanmıştır. Bütün ana arılar ve koloniler standart yetiştirme koşullarında tutulmuşlardır (27±1°C ve %60±5 nem). Ana arıların koloni başlatma süreleri (gün) ve yumurtlama oranları (%) bakımından gruplar arasındaki farklılıklar önemli bulunmuştur. Tek ve çift CO_2 narkozu uygulanan ana arılar CO_2 narkozu uygulanmayan ana arılardan sırayla yaklaşık 4.5 ve 5 gün daha erken yumurtlamaya başlamışlardır. Benzer şekilde, CO_2 uygulanmayan ana arıların sadece %45'i yumurtlamışken, hem tek hem de çift CO_2 narkozu uygulanan ana arıların %80'i yumurtlamıştır. İlk kuluçkadaki işçi arı sayısı, ilk hafta ve ilk aydaki ana arı ölüm oranları (%) bakımından gruplar benzer değerler almıştır. Sonuçlar *B. terrestris* arısının kitlesel üretiminde diyapoz sonrası ana arılara bir kez CO_2 uygulamasının faydalı bir yöntem olabileceğini göstermiştir.

Anahtar sözcükler: *Bombus arısı, Bombus terrestris, CO_2 uygulaması, Koloni gelişimi*

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INTRODUCTION

Most bumblebee species are annual social insects and are widely distributed in the temperate regions of the world [1]. Towards the end of the colony cycle in late summer, sexuals (young queens and males) are produced. After mating, the young queens go into diapause while the founding queen, the workers, and the males of the colony die. Finally, colony life cycle is restricted to a season. The following spring, the queens that survived hibernation give rise to the next generation. Thus, queens enter diapause during the summer, which means a diapause duration of six to nine months depending on the spring temperature [2]. However, bumblebees show great ecological flexibility, particularly in terms of diapause responses and duration [3,4]. Diapause is an adaptive strategy for survival in unfavorable environmental conditions and offers obvious advantages to insects that can only utilize small portions of the year for development and reproduction [5].

Bumblebees are important pollinators of many crops and wild flowers. Currently five species of bumblebees are reared commercially on a large scale. Due to its economic importance for pollination of greenhouse crops, the Eurasian bumblebee *Bombus terrestris* L. is the most prominent species reared under controlled conditions [4]. Although the development of management techniques has been rapid since its start in 1987, there is still a problem in commercial rearing to maximize the quality and profitability of commercial colonies [6]. It is considered that the relatively high labor costs and often low success rate are major problems for commercial rearing [7]. Colony initiation, queen rearing, mating and breaking of diapause are the major stages in the commercial rearing of bumblebees [6,7].

The long hibernation period, the single colony cycle per year, the variability in the social structure of the colonies and the lack of product shelf life are major obstacles in the year-round rearing of bumblebees [6]. *Bombus terrestris* producers today have developed their own rearing systems and have used several methods to rear colonies year-round, despite the long ovarian diapause of this species [7,8]. The storage of hibernating queens at low temperature for several months to mimic diapause has been widely used in the commercial rearing. Another important finding by Röseler [9] was that a CO_2 treatment could also be used to inhibit diapause as well as to activate overwintered queens. In bumblebee mass-rearing, mated queens are stored at temperatures between 1-5°C for different durations based on the demand by producers. After this process, the queens may receive CO_2 narcosis [7,9]. However, there has been limited research on the effects of CO_2 narcosis on the onset of oviposition and colony founding of post diapausing *B. terrestris* queens. In this study hibernated queens were divided into three groups; non-narcotized queens, queens exposed to a single narcosis with CO_2 for 30 min and queens exposed to a double narcosis with CO_2 for 30 min. Our aim was to compare the effects of narcotization on the onset of oviposition and development of colonies founded by post diapausing *B. terrestris* queens to find out the best method for mass rearing.

MATERIAL and METHODS

A total of 70 queens were collected from *Bombus terrestris* colonies kept in Animal Science Laboratory at Akdeniz University. Approximately 7 days old queens and 12 days old males were put together in plastic cage with mash walls (queen to male ration: 1:1.5). After successful matings, the queens were kept in the refrigerator at 4.0±0.5°C for two months. After that, hibernated queens were placed in flight cage for 5 days by providing sugar syrup and pollen. Then, the queens were randomly allocated to three groups of 20 queens each for control, single and double narcosis. The queens in single narcosis group were treated once with CO_2 for 30 min, while the queens in double narcosis group were treated with CO_2 for 30 min two times in consecutive days before introducing in starting boxes. The queens in control group were not narcotized. We narcotized the queens as described by Röseler [9]. The queens were put in a glass jar and exposed to CO_2 until they became immobilized. Then, the all queens were kept altogether in the closed jar for 30 min.

After narcotization process, each queen was placed separately in the small starting box and kept in captivity under standard rearing conditions (27±1°C and 60±5% RH). The queens and the colonies were fed with sugar syrup (approximately 1:1 water: sugar) and fresh pollen collected from honeybee colonies ad-libitum through the experimental period. Each queen was attended by two newly emerged honey bee workers to stimulate egg laying [10]. Honey bee workers were changed every week until the emergence of the first *B. terrestris* workers. When the workers from the first brood batch emerged, the colonies were transferred to larger plastic boxes. Clean conditions were maintained inside the boxes. Each nest box was observed daily for the duration of the experiment. The date of onset of oviposition of each queen in a starting box was recorded in order to determine the duration of colony initiation of queens (interval from installing a queen into starting box to onset of oviposition). The numbers of workers emerged from first brood batch in every starting box were also recorded. The colony founding success (%) was defined as the ability of a queen to produce at least 10 adult workers. Therefore, the queens that produced fewer than 10 workers were not considered to be successful colony producers [11]. In each group, percentage of queens that laid eggs, queen mortality rates at the first week and month were calculated as follows;

Percentage of queens that laid eggs (%): The number of queens that produced eggs/total number of queens x 100.

Queen mortality rate at first week (%): The number of dead queens during the first week after introducing in starting boxes/total number of queens x 100.

Queen mortality rate at first month (%): The number of dead queens during the first month after introducing in starting boxes/total number of queens x 100.

Statistical Analysis

Proportional data were analyzed by the Chi-square test. Data on the duration of colony initiation and the number of workers in the first brood were analyzed by ANOVA and the mean values were compared by Duncan multiple comparison test (SPSS version 12.01).

RESULTS

The means of the queen and colony development characteristics of control group and experimental groups are shown in *Table 1*. Our results revealed several similarities and differences between the non narcotized, single narcotized and double narcotized groups. The first important difference between groups is the interval from installing a queen into starting box to onset of oviposition (the duration of colony initiation). Single and double narcotized queens started to lay eggs 6.69 and 6.06 days, respectively, whereas non-narcotized queens (11.22 days) started to lay eggs about 4.5 days and 5 days later than single and double narcotized queens, respectively ($F=21.03$, $df=2$, $P=0.0001$). The second important difference we found between groups is the percentage of queens that laid eggs (%). Similarly, 80% of single and double narcotized queens laid eggs, whereas only 45% of non narcotized queens laid eggs ($\chi^2=7.548$, $df=2$, $P=0.023$). Colony founding successes of single and double narcotized queens were higher (50%) compared with non narcotized queens (35%), but the difference was not significant ($\chi^2=1.212$, $df=2$, $P=0.054$). The numbers of workers emerged from first brood batch did not vary significantly between groups. Queen mortality rates at the first week and at the first month were also similar between groups.

DISCUSSION

In commercial rearing of bumblebees, rapid colony initiation and successful colony production are the major criteria to reduce production costs. Several methods have been described to rear colonies in captivity [12,13]. Although *B. terrestris* queen can produce a colony without a period of cold storage (diapause) or CO_2 narcosis [4,14], this method has not been used in mass rearing, because non-diapausing queens do not establish high quality colonies for pollination purpose. In commercial bumblebee rearing industry, it is vital to plan the production within tight limits to avoid both over and underproduction [6]. A substantial prerequisite for year-round rearing is, therefore, to control diapause in captivity and to carry out an optimal diapause process suitable for the mass rearing of *B. terrestris*.

Under laboratory conditions, many researchers stored their queens at a temperature between 1-5°C to simulate hibernating conditions [3,4,15,16]. Previous studies also showed that queen survival during diapause (cold storage conditions) is strongly determined by the weight of the queen and the diapause duration [3,15]. Röseler [9] found another successful method that a CO_2 treatment could also be used to circumvent or break diapause. Tasei [8] reported that the timing of CO_2 narcosis after mating did not affect the delays of egg-laying within the range of 5-30 days. Yoon et al.[17] investigated the effect of CO_2 treatment on interrupting diapause, oviposition and colony development of *Bombus ignitus* and reported that CO_2 treatment showed a positive effect on the oviposition and colony development. They found that the days needed to first oviposition shortened to 17-18 days in CO_2 treated queens, comparing to 30 days in CO_2 untreated queens and CO_2 treatment at the second day after mating was appropriate to the oviposition and colony development. Yoon et al.[18] also found that CO_2 narcosis time favorable for colony development was 11 days of adult emergence in *B. terrestris*. Our results are not directly comparable with these results because queens were not narcotized with CO_2 after cold storage in these previous studies.

Röseler [9] also recommended that CO_2 narcosis induces egg formation in bumblebee queens not only prior to hibernation but also after hibernation. There are a few studies about cold storage in combination with CO_2 treatment [9,12,15]. Our finding that CO_2 narcosis of post diapausing queens was

Table 1. *The characteristics of non-narcotized, single narcotized and double narcotized queens and the colonies founded by these queens*
Tablo 1. *Narkozlanmayan, bir kez narkozlanan ve iki kez narkozlanan ana arıların ve bu ana arıların kurdukları kolonilerin özellikleri*

Queen and Colony Characteristics	Non Narcotized Queens	Single Narcotized Queens	Double Narcotized Queens
Duration of colony initiation of queens (day; mean ± s.e.)	11.22 ± 1.29^{A} (n=9)	6.69 ± 0.28^{B} (n=16)	6.06 ± 0.23^{B} (n=16)
Number of workers in first brood batch (mean ± s.e.)	9.29±1.35 (n=7)	10.10±1.49 (n=10)	10.20±1.08 (n=10)
Percentage of queens that laid eggs (%)	45^{a} (n=20)	80^{b} (n=20)	80^{b} (n=20)
Colony founding success of queens (%)	35 (n=20)	50 (n=20)	50 (n=20)
Queen mortality rate at first week (%)	10 (n=20)	10 (n=20)	10 (n=20)
Queen mortality rate at first month (%)	20 (n=20)	20 (n=20)	20 (n=20)

Different letters denote significant differences between means within a row (A, B: P<0.01; a, b: P<0.05)

effective to stimulate oviposition and colony initiation is consistent with these earlier studies [9,12,15]. Gosterit and Gurel [15] reported that hibernating queens for 45 days at 4.5°C and then anesthetizing them with CO_2 appears to be the best diapause regime for the mass rearing of *B. terrestris*. Similarly, Gretenkord and Drescher [12] found that after a short hibernation period (up to 3 month), CO_2 treatments of 30 min gave the best result.

In this study, if non-narcotized queens are compared with narcotized queens, there are differences in queen and colony characteristics whereas no difference is found between single and double narcotized queens. According to our results, single narcosis is sufficient to shorten the duration of onset of oviposition. It seems that second CO_2 narcosis is not necessary. In conclusion, our results have shown that single CO_2 narcosis of post diapausing queens can become a useful tool in the mass rearing of *B. terrestris* colonies.

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Kafkas Univ Vet Fak Derg
19 (2): 225-232, 2013
DOI: 10.9775/kvfd.2012.7606

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Determination of Phenotypic, Serotypic and Genetic Diversity and Antibiotyping of *Yersinia ruckeri* Isolated from Rainbow Trout [1]

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[1] *This study was supported by Scientific Research Administration of Uludag University (UAP (V) 2009/13) and carried out with a report by Local Ethic Committee of Uludag University (2009-07/1)*

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Makale Kodu (Article Code): KVFD-2012-7606

Summary

In this study, 15 *Yersinia ruckeri* isolates that had been isolated from rainbow trout farms and 2 reference strains (serotype 1 and serotype 2) were examined in terms of phenotypic, serotypic and genotypic characteristics. Conventional microbiological and API 20 E tests were used to determine phenotypic characteristics of *Y. ruckeri* isolates and it was determined that the *Y. ruckeri* showed significantly homogenous profile by microagglutination test performed by using serotype 1 and serotype 2 immunsera, and 11 of 15 isolates were serotyped as serogroup 1 while the rest 4 were serotyped as serogroup 2. In Random Amplified Polymorphic DNA (RAPD) analysis, *Y. ruckeri* isolates were genotyped within 2 separate clusters according to 70% similarity coefficient index and it was detected that first cluster includes 2 genotypes (YR1 and YR2) and second cluster includes 3 genotypes (YR3, YR4 and YR5). Furthermore, antibiotic resistance profiles of *Y. ruckeri* strains were determined and it was found that they were resistance to florfenicol, erythromycin, oxytetracycline and trimethoprim-sulphamethoxazole which have been licensed for fish health in Turkey. It is considered that findings obtained will form a basis to develop diagnosis kit and/or vaccination for *Y. ruckeri*.

Keywords: *Oncorhynchus mykiss, Yersinia ruckeri, API 20 E, Microagglutination, RAPD-PCR*

Gökkuşağı Alabalıklarından İzole Edilen *Yersinia ruckeri* Suşlarının Fenotipik, Serotipik ve Genetik Farklılıklarının Belirlenmesi ve Antibioyotiplendirilmesi

Özet

Bu çalışmada gökkuşağı alabalığı işletmelerinden izole edilmiş olan 15 adet *Yersinia ruckeri* izolatının 2 adet referans suşla (serotip 1 ve serotip 2) karşılaştırmalı olarak fenotipik, serotipik ve genotipik özellikleri bakımından incelenmesi amaçlanmıştır. *Y. ruckeri* izolatlarının fenotipik özelliklerinin belirlenmesinde klasik mikrobiyolojik ve API 20 E testleri kullanılmış ve bu testlerde bakterinin oldukça homojen bir yapı gösterdiği belirlenmiştir. Serotip 1 ve serotip 2 immunserumlar kullanılarak yapılan mikroaglütinasyon testinde ülkemizden izole edilmiş olan 15 suştan 11'nin serotip 1, 4'ünün ise serotip 2 özellikte olduğu belirlenmiştir. Rastgele Çoğaltılmış Polimorfik DNA (RAPD) analizinde *Y. ruckeri* izolatları %70 benzerlik katsayısına göre 2 ayrı küme içerisinde gruplanmış, kümelerden birincisinin 2 (YR1 ve YR2), ikincisinin ise 3 genotip (YR3, YR4 ve YR5) içerdiği saptanmıştır. Ayrıca bu çalışmada *Y. ruckeri* izolatlarının antibiyotik duyarlılıkları belirlemiş ve bu izolatların ülkemizde balıklarda ruhsatlı olan florfenikol, eritromisin, oksitetrasiklin ve trimetoprim-sulfamethoxazole karşı direnç geliştirmiş oldukları saptanmıştır. Çalışma sonucunda elde edilen bulguların *Y. ruckeri* için teşhis kiti ve/veya aşı geliştirme çalışmalarına temel teşkil edeceği düşünülmektedir.

Anahtar sözcükler: *Oncorhynchus mykiss, Yersinia ruckeri, API 20 E, Mikroaglütinasyon, RAPD PCR*

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INTRODUCTION

Yersinia ruckeri is the ethiological agent of the diseases known as yersiniosis or "enteric red mouth" which causes significant economical losses in fish production [1]. Although the disease is detected on salmonid fish in particular, it also effects other fish species [2]. The disease is now widely distributed and it has been reported in North America, Australia, South Africa and European countries [1]. In Turkey, *Y. ruckeri* was first isolated from a rainbow trout farming facility in İzmir in 1990 by Cagirgan and Yurekliturk [3].

Although there are serological differences between *Y. ruckeri* strains, it is mentioned that they have very similar serotypes with regards to biochemical characteristics [4-6]. However, it is reported that differences may be detected on methyl red (MR), voges proskauer (VP), lysine decarboxylase, arginine dihydrolase and lactose fermentation tests results in some strains [7]. It is specified that the ability to fermentate sorbitol can vary among *Y. ruckeri* strains and serotype 2 strains can be differed from other strains by the ability to fermentate sorbitol [1,2].

Conventional tests, API 20 E test kit and selective medium are used for identification and to determine the phenotypic characteristics of *Y. ruckeri* strains [5,6,8]. Nowadays, rapid and easy diagnosis of *Y. ruckeri* can be performed by Polymerase Chain Reaction (PCR) and this method is used as alternative to traditional identification methods [9,10]. The RAPD-PCR method which is a DNA based technique are used frequently to determine differences among the strains due to high discriminatory power, reproducibility, and low cost relatively [11-13].

In this study, it were determined the phenotypic and serologic properties of *Y. ruckeri* isolates isolated from rainbow trout in different geographical regions of Turkey and genetic diversity among the isolates by RAPD-PCR. Efficiency of the technique was also evaluated for epidemiological studies.

MATERIAL and METHODS

Bacterial Strains and Phenotypic Characterizations

Fifteen *Y. ruckeri* isolates obtained from different locations in Turkey and 2 reference strains belonging to serotype 1 (Genk 3281) and serotype 2 (Denmark 1850621-1116B) were used in this study. Origins and sources of the strains used in the study were presented in *Table 1*.

Isolates were identified as *Y. ruckeri* by gram staining, oxidation-fermentation (O/F), Simmons citrate, gelatin hydrolysis, cytochrome oxidase, catalase, growth in 1%, 3% and 7% NaCl, motility at 22°C and 37°C, indole, MR, VP, acid production from sorbitol, maltose, glucose [5,8]. Additionally, tween 80 hydrolysis ability was determined by examining the colony morphology on Shotts-Waltman Agar (SWA) [14].

Table 1. *Yersinia ruckeri strains in the study*
Tablo 1. *Çalışmada kullanılan Y. ruckeri suşları*

Strain No	Origin	Source	Isolation Year
1	Isparta	Rainbow Trout	2009
2	Isparta	Rainbow Trout	2009
3	Denizli	Rainbow Trout	2009
4	Afyon	Rainbow Trout	2009
5	Sakarya	Rainbow Trout	2010
6	Bursa	Rainbow Trout	2010
7	Bilecik	Rainbow Trout	2010
8	Kütahya	Rainbow Trout	2010
9	Denmark 1850621-1116B	Rainbow Trout	-
10	Muğla	Rainbow Trout	2010
11	Muğla	Rainbow Trout	2010
12	Bursa	Rainbow Trout	2010
13	Kütahya	Rainbow Trout	2010
14	Muğla	Rainbow Trout	2010
15	Kütahya	Rainbow Trout	2010
16	Bilecik	Rainbow Trout	2010
17	Genk 3281 (Belgium)	Rainbow Trout	-

Isolates were inoculated on strips according to API 20 E (Biomerieux) rapid diagnostic kit directory and incubated at 26°C for 24 h and identification was performed on API WEB.

PCR

Y. ruckeri isolates identified conventionally were confirmed by PCR. For this purpose, DNA was extracted with a commercial kit (Omega Bio-tek, Inc.) according to the manufacturer's instructions. DNA concentrations were measured on 260 nm and 280 nm and equalized to 50 ng/µl.

PCR was performed by using ruck1 (5'-CAGCGGAAAGTA GCTTG-3') and ruck2 (5'-TGTTCAGTGCTATTAACACTTAA-3') primers according to the method described by Lejune and Rurangirwa [10]. After amplification, 409 bp PCR products were identified as *Y. ruckeri*.

Genotyping

RAPD method was used for genotyping of the isolates. For this purpose, ERIC-2 primer (5'-AAGTAAGTGACTGGGGT GAGCG-3') was used. Amplification step was performed by modifying the method reported by Versalovic et al.[15]. At this stage, 25 µl RAPD master mixture including DEPC-treated water, 1XPCR Buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 2.5U Taq DNA polymerase, 25 pmol primer and 5 µl DNA was prepared. After initial denaturation of DNA at 95°C for 1 min, 25 amplificates cycles compose denaturation at 94°C for 1 min, annealing at 40°C for 1 min and from extension at 72°C for 1 min were followed. Amplification products were visualised by electrophoresis in 1.5% agarose gel including ethidium bromyde (2 mg/ml).

The phylogenetic relatedness of isolates was evaluated with dendogram of RAPD patterns. The dendogram was drawn by UPGMA (Unweighted Pair Group Method with Arithmetic Averages) with CHEF-DR® III, Quantity One® software (Bio-Rad Laboratories, Hercules, CA). To determine reproducibility of RAPD analysis, 5 strains from each bacteria species were selected randomly and RAPD analysis were repeated for 3 times subsequently.

Serological Characterization

- *Preparation of Immunsera:* In this study, 3 month-old, 8 healty New Zelland rabbits (2 positive and 2 negative controls for each serotype) taken from Uludag University Experimental Animals Breeding and Research Center were used. *Y. ruckeri* reference strains Genk 3281 (*Y. ruckeri* serotype 1) and Denmark 1850621-1116B (*Y. ruckeri* serotype 2) were inoculated into Triptic soy broth, incubated at 22°C for 48 h, centrifuged and inactivated by addition of formol (final concentration 0.3%). The suspension was kept for 2 h at room temperature and subsequently over-night at 4°C. Thereafter, the bacteria were washed 3 times in phosphate buffer saline (PBS) and the bacterial concentration was calibrated to McFarland no: 7 [16]. Prepared inoculums were inoculated subcutaneously to each test groups animals on 1st, 5th, 9th and 13th day in the quantity of 0.2, 0.4, 0.8 and 1.0 ml respectively. For negative control group, the same amount of steril PBS was inoculated on the same intervals. On the 15th day of the last inoculation, blood samples (20 ml) were collected from ear veins of rabbits and the sera preparated and aliquoted before freezing at -20°C. Microplate agglutination test was applied to detect antibodies [17,18].

- *Preparation of Antigen:* To prepare the antigen; before the isolates were incubated at 22°C for 48 h and collected in Phosphate Buffer Solution (PBS) then autoclaved at 100°C for 30 min to obtain the heat-stable O antigen. After, bacterial cultures were centrifuged at 5.000 rpm for 15 min. The sediment was removed and diluted with PBS to have suspension (antigen) at Optic Density (OD) of 0.65 at 525 nm wavelength on the spectrophotometer. This antigen was stored at -20°C until used for slide and microplate agglutination tests as somatic O antigen [19,20].

- *Microplate Agglutination Test: Y. ruckeri* isolates were serotyped by microplate agglutination test. For this purpose, an antiserum diluted with PBS on 2:1 proportion (50 ml PBS:25 ml antiserum) was put to wells of microplate. Then, antigen solution was added into these wells (25 µl) and mixed well. They were left at room temperature for 2 h and then at 4°C for overnight [21-23]. Positive and negative sera were put into the last and penultimate wells of each microplate for test control. Test results and agglutination titers were assessed according to $\log_2$ base [21,23,24].

Antimicrobial Susceptibility Test

All the isolates were tested for antimicrobial susceptibility by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar according to Clinical and Laboratory Standards Institute (CLSI). The following antibiotic discs were used: neomycin (10 µg, Oxoid), gentamicin (120 µg Oxoid), oxytetracycline (30 µg Oxoid), florfenicol (30 µg Oxoid), erythromycin (15 µg, Oxoid), sulfamethoxazole+trimethoprim (25 µg, Oxoid), doxycycline (30 µg, Oxoid), lincomycin (2 µg, Oxoid) and amoxicillin (25 µg, Oxoid). At 24th and 48th h of the incubation, incubation zone diameters were measured and evaluated [25].

RESULTS

Phenotypic Characteristics of Y. ruckeri Strains

Conventional microbiological tests and API 20 E test kits were used to determine phenotypic characteristics of *Y. ruckeri* isolates and results were presented in *Table 2* and *3*. It was observed that *Y. ruckeri* strains has created green colonies on SWA and a zone with an ice glass appearance due to precipitation of calcium salts and hydrolysis of tween 80 around these colonies.

By API 20 E and conventional tests, phenotypic characteristics of *Y. ruckeri* isolates were found significantly similar. It was determined that 5, 6, 8, 9 and 10 numbered isolates were positive for sorbitol test (both on API 20 E and conventional tests).

PCR was used for molecular confirmation of *Y. ruckeri* isolates. All of isolates and reference strains gave the final PCR product of 409 bp *(Fig. 1)*.

Genotyping

By RAPD analysis, *Y. ruckeri* isolates gave 5 different patterns. Isolates were grouped within 2 separate clusters according to 70% similarity index and it was detected that first cluster includes 2 genotypes (YR1 and YR2) and second cluster includes 3 genotypes (YR3, YR4 and YR5). Genotype YR2 was predominant and included 5 isolates (29.4%); and each of the genotypes YR3, YR4 and YR5 composed of 3 isolates. Furthermore, it was detected that isolates within genotypes YR4 and YR5 were closely related species.

Serological Characterization

It was detected that isolates 5, 6, 8, 9 and 10 numbered isolates have the characteristics of serotype 2 by microplate agglutination tests and other isolates have the characteristics of serotype 1. It was observed that antigenic structures of 15 isolates and two reference strains were not shown cross reaction against to serotype 1 and serotype 2 immune serums. Furthermore, 1:256 dilution of antigens prepared from isolates either serotype 1 or serotype 2 reacted with specific immunsera.

Antimicrobial Susceptibility Profiles

Seventeen *Y. ruckeri* isolate were examined for antibiotic sensitivity against to 9 different antibiotics and results were presented in *Table 4*. It was determined that all *Y. ruckeri*

Table 2. *Phenotypic characteristic of Y. ruckeri isolates with conventional microbiological tests*
Tablo 2. *Y. ruckeri izolatlarının konvansiyonel mikrobiyolojik testler kullanılarak belirlenen fenotipik özellikleri*

Phenotypic Characters	Isolates No																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Gram Staining	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Simons Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O/F	F*	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
Growth in 3% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in 7% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 30°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility at 22°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility at 37°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SWA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid from																	
Mannitol	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

** **F:** fermentative*

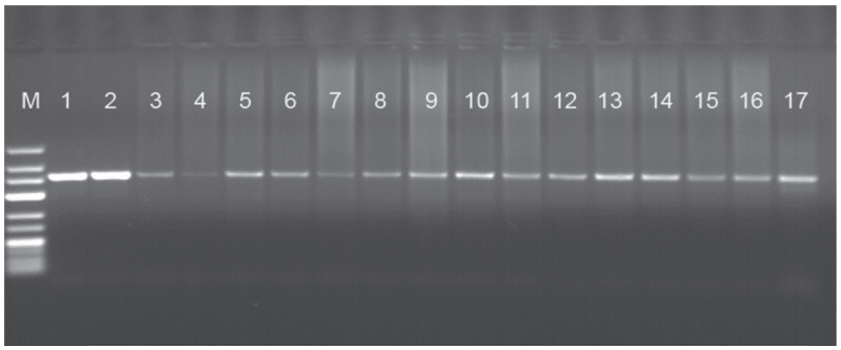


Fig 1. *Y. ruckeri* specific PCR, 409 bp. **M;** Molecular weight standard (25-700 bp), 9 (Denmark 1850621-1116B) and 17 (Genk 3281 [Belgium]) reference strains, others are sample strains

Şekil 1. *Y. ruckeri* spesifik PCR, 409 bp. M; Moleküler ağırlık standardı (25-700 bp), 9 (Denmark 1850621-1116B) ve 17 (Genk 3281 [Belgium]) referans suşlar, diğerleri saha suşları

isolates were resistant to neomycin, lincomycin, amoxicillin (except isolate 11) and erythromycin, 5 (33.33%) isolates to gentamicin and doxycyclin, 2 (13.33%) isolates to oxytetracycline, 3 (20%) isolates to florfenicol, and 1 (6.66%) isolate to sulphamethoxazole-trimetoprim.

DISCUSSION

In this study, it has shown that *Y. ruckeri* have serologic differences among isolates and such these strains are found very similar in terms of phenotypes [2,5,8,26].

Many typing strategies have been performed until today based on the phenotypic characteristics of *Y. ruckeri* strains. *Y. ruckeri* strains have been divided into two biotypes according to sorbitol fermentation ability [1], and five biotypes according to heat-stable O antigen [27].

In this study, no difference was detected between the strains in terms of gram staining, O/F, cytochrome oxidase, catalase, H_2S production, VP, indol production, glucose, inositol,

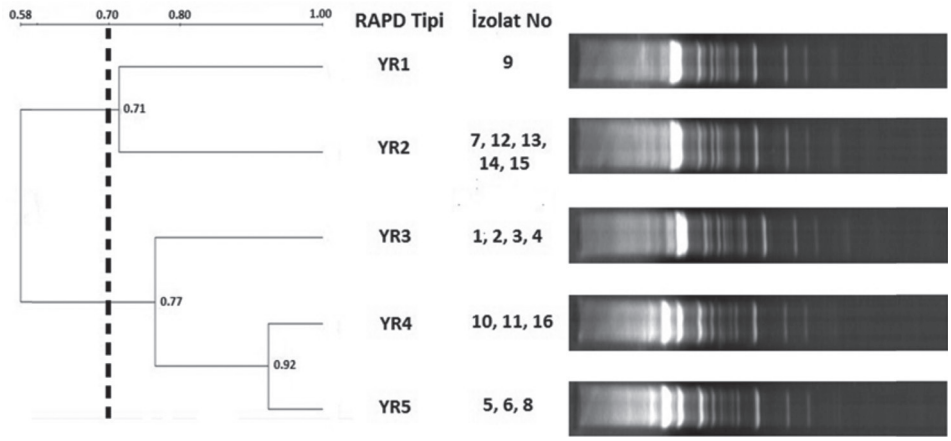


Fig 2. The RAPD band profile of *Y. ruckeri* isolates and phylogenetic tree of genotypes

Şekil 2. *Y. ruckeri* isolatlarının RAPD bant profilleri ve genotiplere ait filogenetik ağaç

Table 3. *Phenotypic properties of Y. ruckeri isolates with API 20E tests*
Tablo 3. *Y. ruckeri izolatlarının API 20E kullanılarak belirlenen fenotipik özellikleri*

Phenotypic Characters	Isolates No																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
ONGP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ADH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LDC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ODC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TDA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin Hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol*	-	-	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-
Rhamnose*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amygdalin*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sitokrom Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

ONPG: *b - Galaktosidaz,* ***ADH:*** *Arginine dihydrolase,* ***LDC:*** *Lysine decarboxylase,* ***ODC:*** *Ornithine decarboxylase,* ***TDA:*** *Tryptophan deaminase,* ***VP:*** *Voges proskauer reaction, * acid from*

maltose, mannitol, citrate utilization, gelatin hydrolysis, growth in 1%, 3% and 7% of NaCl, motility at 22°C and 37°C and pigment production. Although there were differences among the isolates on sorbitol fermentation test, a homogenous structure was seen between all studied *Y. ruckeri* isolates. These results are found similar *Y. ruckeri* isolates isolated from Turkey [6,28,29].

Some *Y. ruckeri* strains could not hydrolyse tween 80 [4,26,28]. Davies and Frerichs [26] announced that nonmotile *Y. ruckeri* strains cannot hydrolyse tween 80 and Bush [4] reported that there were no relation between motility and tween 80 hydrolyse. Çağırgan and Tanrıkul [28] provided evidence that all *Y. ruckeri* strains that he studies with were motile, however some of these strains could not hydrolyse tween 80. In our study, all 15 *Y. ruckeri* strains were motile and able to hydrolyse tween 80.

API 20 E test kits are one of the most common methods used to determine phenotypic characteristics of bacterial

Table 4. *Antibiotic susceptibility profiles of Y. ruckeri isolates*
Tablo 4. *Y. ruckeri izolatlarının antibiyotik duyarlılık profilleri*

Antibiotic Disc	Isolates No																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Gentamicin	I	R	I	I	R	R	R	R	I	I	I	I	I	I	I	I	I
Neomycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Lincomycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Oxytetracycline	I	R	I	I	R	I	I	I	I	I	I	I	I	I	I	I	I
Amoxicillin	R	R	R	R	R	R	R	R	R	R	I	R	R	R	R	R	R
Florfenicol	R	I	R	I	R	I	I	I	I	I	I	I	S	I	I	I	I
Erythromycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Doxycycline	R	R	R	I	I	I	I	I	R	I	I	I	R	I	I	I	I
Sulfamethoxazole-Trimethoprim	S	I	S	I	I	R	S	I	I	I	I	S	I	S	S	I	S

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

agents. The researchers reported that *Y. ruckeri* strains might be shown variety in terms of arginin dihydrolase production, gelatin hydrolysis, VP and sorbitol fermentation due to incubation periods (24, 48, 72 h) 20°C, 22°C and 25°C by API 20 E test kits [20,26,29,30]. Also, many researchers mentioned that there was a risk to confuse with *Hafnia alvei* for diagnosis with API 20 E [5,8,20,26,29-31]. While no difference was observed among *Y. ruckeri* strains for arginin dihydrolase test, mannitol fermentation, citrate utilization, gelatin hydrolysis and VP as a result of incubation at 26°C for 24 h in our study, the differences were determined in sorbitol tests. Differences in sorbitol fermentation were detected in only serotype 2 isolates. Candan and Yazıcı [29] have reported isolates negative gelatin hydrolysis (2 isolates), positive VP (9 isolates), negative citrate utilization (2 isolates) after an incubation at 22°C for 48 h in their research. They have studied in 15 *Y. ruckeri* strains to determine the most suitable temperature and time for diagnosis of *Y. ruckeri* by using API 20 E test. Observation of differences in gelatin hydrolysis tests differently from conventional microbiological tests in our research results related with API 20 E test complies with the results of other researchers who had studied on this subject [29,31-33].

Although it has been reported that *Y. ruckeri* might be confused with *H. alvei* in identifications by using API 20 E test, it was observed that while *Y. ruckeri* was positive β-galactosidase, gelatin hydrolysis and negative rhamnose, arabinose at 37°C, *H. alvei* was negative β-galactosidase, gelatin hydrolysis and positive rhamnose, arabinose results at 37°C [5,20,34]. Furthermore, *Y. ruckeri* may be differed from other bacteria easily by help of specific colony morphologies on SWA [6].

Serotypic scheme of *Y. ruckeri* has continuously modified by introduction of new antisera [35,36] or creation of new antigenic schemes [7,20,27,37] since first occurrence of yersiniosis. All these studies revealed that *Y. ruckeri* presented a complex structure for serology and antigenic characteristics. When serotyping of 15 *Y. ruckeri* strains in our country was performed by using microplate agglutination technique, it was detected that isolates 5, 6, 8, 10 and reference strain 9 presented serotype 2 and other ten strains presented serotype 1 characteristics. During agglutination test, "O" antigen, serotype 1 and serotype 2 hyperimmune (rabbit) sera were used. It was reported in studies performed with European isolates that *Y. ruckeri* strains have presented serotype O1 characteristics in common [6,20,26,36].

Diagnosis of *Y. ruckeri* has been performed successfully since 1992 by PCR [38]. Besides, PCR based genotyping methods such as PCR-RFLP ribotyping, REP-PCR, ERIC-PCR, AP-PCR and DNA amplification fingerprint has played important role on bacterial typing scheme for the last twenty years [39]. However, these techniques have been used very rarely for fish pathogens in compared with human and animal pathogens [40]. Plasmid profile analysis, ribotyping [41,42], PFGE [40], PCR based fingerprinting [40,43,44] have been used successfully as alternative to phenotypic techniques of *Y. ruckeri* strains. RAPD typing is one of the techniques where single oligonucleotides selected optionally [45]. ERIC2 and M13 primers are used featly as universal primers for typing various bacteria [46]. RAPD-PCR is advantageous since molecular preliminary information of the species is not necessary and clonal relatedness may be determined rapidly and cheaply. In this study, ERIC2 primer was used in RAPD-PCR analysis of *Y. ruckeri* isolates [15,40,47-49]. In this study, *Y. ruckeri* isolates were classified within 5 groups according to 70% similarity index by RAPD typing with using ERIC 2 primer. It was determined that *Y. ruckeri* strains grouped within YR1 and YR 2 have presented a 71% similarity among themselves and strains grouped within YR 3, YR 4 and YR 5 have presented 77% similarity among themselves. Onuk et al.[12] have grouped *Y. ruckeri* strains within 6 groups by RAPD method and reported that strains grouped as RE1 and RE2 have presented 83% genetic similarity and RAPD types that were specified within the same cluster except RE1 and RE2 have presented a genetic similarity between 27% and 53%. Same researchers have reported that strains which cause

epidemics within Central Anatolia and Black Sea regions were dominant types and isolates within RA, RB, RC and RC groups isolated from Mediterranean and Aegean regions were significantly different from isolates within Black Sea and Central Anatolia regions. In our research, in line with Onuk et al.[12], it was observed that *Y. ruckeri* isolates typed with RAPD were distributed among regions. This situation shows that there might be fish transfer among fish farms.

Y. ruckeri strains were classified by performing an antibiotyping according to their resistance to antibiotic [12,50]. Determination of antibiotic resistance profiles of bacteria can provide important information to take specific control preventions. De Grandis and Stevenson [50] have determined antibiotic susceptibilities of 50 *Y. ruckeri* strains and reported that all these strains have presented uniform susceptibility profile against many antibiotics. Onuk et al.[12] reported that 97 *Y. ruckeri* strains had presented similar antibiotic susceptibility profile against oxytetracyclin and trimethoprim while weak resistance to erythromycin and De Grandis and Stevenson [50] have reported that *Y. ruckeri* had presented an acquired resistance against oxytetracycline and sulphanomide although it is resistant to many antibiotics. Kırkan et al.[51] reported that 17 *Y. ruckeri* isolates were resistant to tetracycline with 30% and 40% by agar dilution and E- test, respectively. Furthermore, Kırkan et al.[52] found that all 8 *Y. ruckeri* strains were resistant to erythromycin and ampicillin. While in the present study all 17 *Y. ruckeri* isolates were resistant to neomycin, lincomycin and erythromycin, 16 strains were resistant to amoxicillin, 5 strains were resistant to gentamicin, 3 strains were resistant to florfenicol and doxycyclin, 2 strains were resistant to oxytetracycline and 1 strain was resistant to trimetoprim-sulphamethoxazole. Florfenicol, erythromycin, oxytetracycline and sulphamethoxazole-trimetoprim are antibiotics which have been licensed for bacterial fish diseases and used most frequently in Turkey. According to this study, it may be concluded that a resistance against oxytetracycline, florfenicole and sulphamethoxazole-trimetoprim has developed or is being developed (because of susceptibility of strains intermediately according to antibiogram results) among *Y. ruckeri* isolates.

Consequently, it was found that phenotypic characteristics of *Y. ruckeri* strains were very similar both of conventional microbiological tests and API 20 E rapid diagnosis kit and SWA agar has provided successful results for identification of *Y. ruckeri* strains and control of purity of the colonies, in this study. It was determined that 4 isolates have serotype 2 characteristics similarly with reference 9 (serotype 2) and other strains have presented a serotype 1 characteristics as a result of serotyping of *Y. ruckeri* strains with microplate agglutination test by using reference serotype 1 and serotype 2 strains. It was detected that *Y. ruckeri* has developed resistance to oxytetracycline, florfenicol and sulphamethoxazole-trimethoprim which have been used to treat bacterial diseases in Turkey (licensed for fish). RAPD analysis was discriminative for determination of genetic diversity among *Y. ruckeri* isolates and has provided rapid and safe results. This result showed that RAPD analysis is a useful epidemiological method for determination of genetic differences on fish pathogens. We consider that results obtained in this study can be useful for preparation of effective vaccination formulations against yersiniosis and for further studies that will be performed related with this subject.

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Kafkas Univ Vet Fak Derg
19 (2): 233-237, 2013
DOI: 10.9775/kvfd.2012.7608

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Hasat Zamanının Arı Sütünün Kimi Biyokimyasal ve İz Element Kompozisyonları Üzerine Etkisi

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Makale Kodu (Article Code): KVFD-2012-7608

Özet

Bu çalışmada, larva transferinden 24, 48 ve 72 saat sonra hasat edilen arı sütlerindeki bazı biyokimyasal değişimlerin ortaya konulması amaçlanmış ve arı sütünde temel şeker bileşenleri (fruktoz, glikoz ve sakkaroz), 10-HDA, nem ve kimi iz element analizleriyle bu değişimlerin arı sütü kalitesine olan etkileri araştırılmıştır. Araştırmada arılı, yavrulu ve ballı çerçeve sayısı bakımından eşitlenmiş (7 çerçeve kapalı kuluçka, 13-14 çerçeve ergin arı, 2 çerçeve bal ve polen) 24 adet bal arısı kolonisi kullanılmış, her biri 8 kovandan oluşan rastgele 3 grup oluşturulmuştur. Larva transferinden sonra 1.grupta yer alan kolonilerde 24 saat, 2. grupta yer alan kolonilerde 48 saat ve 3. grupta yer alan kolonilerde 72 saat sonra arı sütleri hasat edilmiştir. Deneme gruplarında hasat edilen arı sütleri için yapılan fruktoz, glikoz, sakkaroz, 10-HDA ve nem düzeyleri arasındaki fark önemli ($P<0.05$) bulunmuştur. Larva transferinden 72 saat sonra hasat edilen arı sütlerinde analiz edilen şeker bileşenlerinin, 10-HDA ve nem düzeylerinin, transferden 24 saat sonra hasat edilenlere göre artış gösterdiği belirlenmiştir. Denemede arı sütünde yapılan iz element analizinde K, Na ve Cu elementleri için gruplar arasında önemli ($P<0.05$) fark bulunmuştur. K, Cu ve Fe iz elementleri için en yüksek değerler, larva transferinden 48 saat sonra hasat edilen arı sütü örneklerinde saptanmıştır. Araştırma sonuçları arı sütünün biyokimyasal yapısının ve bazı iz elementler bakımından içeriğinin hasat zamanına göre değiştiğini ortaya koymuştur. Bu değişimlere göre, gelişen Dünya arı ürünleri pazarında arı sütüne ait uluslararası bir standardın geliştirilmesi için daha yoğun, özellikle "hasat zamanı" ibaresini de içeren standardizasyon çalışmaları yapılmalıdır.

Anahtar sözcükler: *Arı sütü, Şeker bileşenleri,10-HDA, İz element, Hasat zamanı*

The Effect of Harvesting Time on Some Biochemical and Trace Element Compositions of Royal Jelly

Summary

In this research, some biochemical changes in royal jelly that harvesting after 24, 48 and 72 hours had been purposed and effects of change on basic sugars (fructose, glucose and sucrose), 10-HDA, humidity and some trace element in quality of royal jelly had been discussed. In the research, 24 colonies were selected randomly had equalized (7 frames pupae, 13-14 frames bees, 2 frames pollen and honey) and 3 groups were designed as contained 8 hives in each of them. After larvae transferring, royal jelly harvested from the 1st group after 24h, from 2nd group after 48h and from 3rd group after 72 h. Differences of groups for fructose, glucose, sucrose, 10-HDA and humidity levels in harvested royal jelly were found important ($P<0.05$), statistically. Sugar compositions, 10-HDA and humidity levels of royal jelly harvested after 72 h of larvae transferring were found higher than that of 24 h of the treatment. Differences of groups for K, Na and Cu trace elements in royal jelly were found important ($P<0.05$), statistically. The highest values for trace elements of K, Cu and Fe were determined in royal jelly harvested in 48h after larvae transferring. Results obtained from the treatment proved that biochemical compositions and some trace elements contents of royal jelly had been changed by harvesting time. On based of these changes, intensive standardization studies should be made with expression of "harvesting time" in royal jelly for improving international standards of it for marketing of rising world bee product.

Keywords: *Royal jelly, Sugar compositions,10-HDA, Trace element, Harvesting time*

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GİRİŞ

Arı sütü 5-15 günlük yaştaki işçi arıların yutak üstü (hipofaringal) ve çene (mandibular) bezlerinden salgılanan, ana arı ve genç yaştaki larvaların beslenmesinde kullanılan bir besin maddesidir. Rengi açık kremden, koyu sarıya kadar değişen arı sütü; asidik yapıda ve kekrek bir tada sahiptir. Arı sütü, larvanın gelişimi için gerekli olan tüm besin maddelerini içermektedir. Protein, yağlar, şekerler, hormonlar, vitamin ve mineral maddeler bakımından oldukça zengindir [1]. Koloniyi oluşturan ana arı, işçi arı ve erkek arının larval dönemde aldıkları arı sütünün içerikleri farklıdır. Ticari anlamda üretilen ve pazarlanan arı sütü ise ana arı üretimi için işçi arılar tarafından özel üretilmektedir. Arı sütü genellikle 0-24 saatlik larva transferinden 72 saat sonra, ana arı yüksüğü içerisinde en yüksek miktara ulaştığı kabul edilerek hasat edilmektedir [2-4] Ana arı 3 günlük yoğun arı sütü beslemesi sonucunda 4-5 yıllık yaşam süresine, özellikle kuluçka veriminin yüksek olduğu bahar aylarında günde ortalama 2000 yumurta bırakabilme yetisine sahip olmakta, arı hastalık ve zararlılarına karşı yüksek direnç kazanabilmektedir [5] İnsan sağlığı açısından da önemli bir gıda maddesi olarak ilgi gören arı sütünün içeriğindeki maddelerin bir kısmı analiz edilebilse de, karmaşık kimyasal yapısında halen çözümlenememiş, "tanımlanamayan maddeler" yer almaktadır [6,7].

Yapısında ortalama olarak %60-70 su, %12-15 ham protein, %3-6 yağlar, %6-18 şekerler, %0.5-2 esensiyel aminositler, vitaminler, mineraller, %0.8-4 tanımlanamayan maddeler bulunan arı sütü viskoz bir yapıya sahip ve 2/3'ü sudan oluştuğundan, viskozitesi su içeriğine ve larva yaşına bağlı olarak değişir [1,8,9]. Arı sütündeki şeker içeriği arttıkça, arı sütü daha sıvı bir yapı kazanır ve nem düzeyi artar [10,11]. Bu viskozitedeki değişimlerin, arı kolonisinde kast farklılığının oluşmasında etkili olduğu varsayılmaktadır [10]. Kolonide bulunan bütün bireylerin (ana arı, işçi arı ve erkek arı) larval dönemde farklı içerikteki arı sütleriyle beslendiği, ancak ana arı larvasının beslenmesinde kullanılan arı sütü kompozisyonunun en nitelikli özelliğe sahip olduğu olduğu bilinmektedir. Ayrıca kast farklılığının düzeyi ile üretilen arı sütü miktarı ve kompozisyonu; koloni populasyonunun büyüklüğüne, hasat mevsimine, besleme şekline, arılığın bulunduğu coğrafi koşullara, iklime, nektar kaynağına ve arı genotipine bağlı olarak değişebilmektedir [2,12]. Arı sütünde şeker içeriği önemli ölçüde glikoz ve fruktozdan oluşmakta, daha düşük düzeyde sakkaroz da bulunmaktadır [3]. Larvada kas gelişiminin tamamlanmasında arı sütü içerisinde bulunan glikoz düzeyi büyük önem taşımaktadır. Genç ana arı larvalarının beslenmesinde kullanılan arı sütündeki şeker düzeyinin daha yüksek olması, ana arıların diğer arı ailesi bireylerinden daha güçlü yapısal özellikler kazanmasına neden olmaktadır. Arı sütünde bulunan şeker içeriği, besleyici arılar için uyarıcı etki yapmakta ve daha fazla arı sütü üretimi için teşvik edici rol oynamaktadır [13]. Koloni başına aşılanan larva sayısı arttıkça, yüksük başına üretilen arı sütü miktarı azalmaktadır. Ana arı yüksük sayısı arttıkça, arı sütünde yer alan önemli yağ asitlerinden 10-HDA (10-hidroksi-2-dekenoikasit) düzeyi azalmaktadır [14]. Arı sütü kalitesi, içeriğindeki 10-HDA oranına göre değerlendirilmekte ve kaliteli bir arı sütünde %1.4 ile %1.8 düzeyinde yer alması beklenmektedir. Bu değer, arı sütünün elde edildiği bölgenin vejetasyonuna ve arı sütü hasadında uygulanan teknik uygulamalara göre farklılık gösterebilmektedir [15,16]. Arı sütünde en fazla bulunan iz elementler; K, Na, Cu, Fe, Mn ve Zn'dur. Baldan farklı olarak, arı sütündeki mineral madde kompozisyonu, coğrafik yapı ve bitki örtüsünden fazla etkilenmemektedir. İz elementler, larvanın ihtiyacına göre homeostatik olarak, bir başka deyişle kendi iç dengesine göre düzenlenmektedir [17].

Arı sütü ticari olarak 72 saatte hasat edilse de, kimi üreticiler larva aşılamasından sonraki 24 saat ve 48 saatlerde arı sütü hasadı yaparak 72 saatte elde edilen arı sütüyle aynı miktarda ürün elde etmeye yönelmektedirler [2]. Bu durum üretilen arı sütü miktarı açısından mümkün olsa bile, 72 saatten önce hasat edilen arı sütünün kalite ve kompozisyonuna nasıl etki ettiği konusunda sınırlı sayıda çalışma bulunmaktadır. Dünya'da henüz arı sütü ile ilgili genel bir standardın geliştirilmemesi, ülkesel arı sütü standartlarının bile ticari anlamda yeterli olmaması konunun araştırılmaya gereksinim duyduğu görüşünü geliştirmiştir. Anılan gerekçelere dayanarak, bu çalışmada, larva transferinden 24, 48 ve 72 saat sonra hasat edilen arı sütlerindeki biyokimyasal değişimlerin ortaya konulması amaçlanmış ve arı sütünde toplam şeker bileşenleri (fruktoz, glikoz ve sakkaroz), 10-HDA, nem ve kimi iz element analizleriyle (K, Na, Cu, Fe, Mn, Zn) bu değişimlerin arı sütü kalitesine olan etkileri tartışılmıştır.

MATERYAL ve METOT

Deneme Aydın İli, Çine-Madran Dağı'nda (rakım 900 m) bulunan özel bir arıcılık işletmesinde 10 Haziran - 10 Temmuz 2010 tarihleri arasında yürütülmüştür. Arı sütü elde edilmesi amacıyla deneme arılığında eşit güce sahip (7 çerçeve kapalı kuluçka, 13-14 çerçeve ergin arı, 2 çerçeve bal ve polen) 24 koloni seçilerek, araştırma sırasında kolonilerin çevresel değişimlerden (iklim, sıcaklık değişimleri, yağış vb) en az etkilenmesi amacıyla her biri 8 koloniden oluşan rastgele 3 grup oluşturulmuş ve üretimler aynı dönemde yapılmıştır. Arı sütleri üreticinin kolonilerine uygulamakta olduğu yöntemle (çift katlı, analı kolonide, ana arı ızgarası ile ayrılmış kovan sistemiyle) elde edilmiştir. Bu yöntemde başlatıcı ve bitirici koloniler olarak aynı koloniler kullanılmıştır. Deneme kolonilerine balmumundan hazırlanmış 24'er ana arı yüksüğüne 6-12 saatlik larva aşılanmış çerçeveler verilmiştir [5]. Denemede 24 saatlik arı sütü hasat edilecek 1. gruptaki 8 koloniye 24 ana arı yüksüğü verilerek 8 kez aşılama ile toplam 1536 adet (24 yüksük x 8 koloni x 8 kez) aşılama, 48 saatlik arı sütü hasat edilecek 2. gruptaki 8 koloniye 24 ana arı yüksüğü verilerek 4 kez aşılama ile toplam 768 adet (24 yüksük x 8 koloni x 4 kez)aşılama ve 72 saatlik arı sütü hasat edilecek 3. gruptaki 8 koloniye 2 kez aşılama ile toplam 384 adet (24 yüksük x 8 koloni x 2 kez) aşılama yapılmıştır. Larva aşılaması sonrasında toplam 2688 adet ana arı yüksüğünün, 2240 adeti kabul

edilmiş ve arı sütü hasatı gerçekleştirilmiştir. Deneme sonucunda yapılacak analizler için gerekli olan arı sütü miktarı, her grubun aşılama sayısını belirlemiştir. Larva aşılamasından sonraki 24 saatlik sürede, 48 ve 72 saatlik süre sonunda hasat edilen arı sütü miktarından daha az arı sütü elde edileceğinden, aşılama tekrarı sayısı 48 ve 72 saatlik gruplardan daha fazla olmuştur (24 saatlik grupta 8 tekrar, 48 saatlik grupta 4 tekrar ve 72 saatlik grupta 2 tekrar). Deneme süresince üretim kolonilerine düzenli olarak gün aşırı 1 litre, 1:1 oranında (şeker:su) şerbet verilmiş, şerbetin hazırlanmasında şekerpancarından üretilmiş sofra şekeri (sakkaroz) kullanılmıştır.

Arı sütü hasatında tahta kaşık kullanılmıştır. Kolonilerden her hasatta elde edilen arı sütleri ayrı eppendorf tüplerine konularak, dış yüzeyine kovan numarası ve hasat günü etiketlenerek belirtilmiştir. Arı sütü tüpleri +4ºC koşulunda buzluk termoslara yerleştirilerek laboratuvara getirilmiş ve soğuk zincir bozulmadan analiz gününe dek -18ºC derin dondurucuda bekletilmiştir. Denemede aynı gruba ait kolonilerden elde edilen arı sütü örnekleri kendi içerisinde harmanlanarak, üç farklı hasat zamanına ait toplam 24 adet örnek oluşturulmuştur. Elde edilen arı sütü örnekleri analiz için laboratuvara gönderilmiştir.

Arı sütünde şeker analizi HPLC'de Agilent 1100 Serisi (Degasser, pompa, otomatik örnekleyici, kolon fırını, florimetrik dedektör) EC Nucleosil (150 mm x 4.6 mm, 5 µm, NH_2) (Macherey Nagel) kolonla yapılmıştır (Mobil faz: Su: Asetonitril (25:75), Akış hızı 1 ml/dak., Kolon ısısı 30ºC, Dedektör : Reflektör indeks (RI) , Enjeksiyon miktarı: 20 µl, Analiz süresi 15 dak). Fruktoz, glikoz ve sakkaroz'un her birinden 0.1 g tartılarak 10 ml ultra saf su içerisinde çözdürülmüş(final yoğunluk 10 mg/ml), bu çözeltilerden 2.5 ve 6.6 mg/ml olacak şekilde su ile dilüsyonlar yapılarak standart eğri oluşturulmuştur. Takiben 0.25 g arı sütü 2 ml'lik kapaklı tüplere tartılmış, üzerine 1.5 ml asetonitril: su (50:50) ilave edilerek 20 saniye vorteksle karıştırılmış ve tüpler 12.000 devirde 5 dak. santrifüj edilmiştir. Sıvı kısım 0.20 µ'luk filtreler ile süzüldükten sonra, süzüğün 20 µl'si HPLC'ye uygulanmış ve şeker düzeyleri belirlenmiştir [18].

10-hidroksi-2-dekenoikasit analizi (10-HDA) için yine HPLC cihazı *Agilent 1100 Serisi*, (Degasser, pompa, otomatik örnekleyici, kolon fırını, florimetrik dedektör) Luna C_{18} (150 mm x 4.6 mm, 3 µm) (Phenomenex, Cheshire, UK) kolon kullanılmıştır (Mobil faz: Su (%0.1 fosforik asit, pH: 2.5): Asetonitril (70:30), Akış hızı 1 ml/dak, Kolon ısısı: 30ºC, Dedektor: DAD (225 nm), Enjeksiyon miktarı: 20 µl, Analiz süresi 8 dak). 10-HDA analitik standardından 0.01 g tartılarak 50 ml (final yoğunluk 200 µg/ml) su:asetonitril (50:50) içerisinde çözdürülmüş ve bu çözeltiden 1, 5, 10, 20, 50 ve 100 µg/ml olacak şekilde dilüsyonlar yapılarak standart eğri oluşturulmuştur. Daha sonra 0.20 g arı sütü 50 ml'lik kapaklı tüplere tartılarak üzerine 50 ml asetonitril: su (50:50) ilave edilmiş ve 20 saniye vorteksle karıştırılmıştır. Karışım 30 dak. ultrasonik su banyosunda tutulduktan sonra, tüpler 6.000 devirde 5 dak. santrifüj edilmiş ve 0.20 µ'luk filtre ile süzülmeyi takiben 20 µl'si HPLC'ye uygulanmıştır [19,20]. Arı sütünde nem miktarını belirlemek amacıyla kuru madde analizi yapılmış ve bu amaçla darası alınmış porselen krozeler içerisine 1 g arı sütü örneği tartılmış ve kurutma fırını içerisinde 65°C'de 1 gece kurutulmuştur. Bir gece sonunda ardışık iki tartım arasında fark kalmayınca (örnek sabit tartıma geldiğinde) kurutma işlemine son verilmiştir. Desikatör içerisinde soğutulan krozeler tartılmış ve kuru örnek ağırlıkları tespit edilerek kuru madde oranı tespit edilmiştir. Denemede başlangıçta alınan arı sütü ağırlıklarından kuru maddenin düşülmesi ile arı sütündeki nem miktarı hesaplanmıştır [21].

Arı sütü örneklerinde iz element analizi yapmak amacıyla, kuru madde analizi yapılan örnekler kül fırınına konulmuş ve sıcaklık kademeli olarak 550°C'ye çıkartılmıştır. Kül fırınında 1 gece yanan örnekler beyaz grimsi renkte kül haline gelmişlerdir. Çeker ocak altında krozelere 5'er ml nitrik asit (%65) ve sonrasında da bidistile su eklenmiştir. Krozeler içerisinde çözündürülmüş olan örnekler whatman 42 filtre kağıdı ile süzülmüş ve bidistile su ile 100 ml'ye tamamlanmıştır. Elde edilen çözelti iz element analizi yapmak amacıyla Atomik Absorbsiyon Spektrofotometre (Perkin Elmer, AA 400 model) cihazına hazırlanmıştır. Bu amaçla öncelikli olarak standart seriler ve eğri oluşturulmuştur. Standart seriler hazırlanırken K için Merck 170230 , Na için Merck 170238, Cu için Merck 170314, Fe için Merck 170326, Mn için Merck 170332, Zn için Merck 170369 standart çözeltileri kullanılmıştır. Örneklerin konsantrasyonu mg/kg (ppm) olarak tespit edilmiştir [22,23].

Deneme gruplarına ait verilerin istatistik değerlendirilmesinde tesadüf parselleri deneme desenine göre tek yönlü varyans analizi yapılmış, bu amaçla SPSS istatistik programından yararlanılmıştır [24]. Grupların birbiri ile farkını belirlemek amacıyla Duncan testi uygulanmıştır [24-26].

BULGULAR

Toplam Şeker Bileşenleri (Fruktoz, Glikoz ve Sakkaroz), 10-HDA ve Nem

Deneme gruplarında arı sütü için yapılan fruktoz, glikoz, sakkaroz, 10-HDA ve nem düzeyleri arasındaki fark önemli bulunmuştur ($P<0.05$). Genel olarak denemede analiz edilen şeker bileşenleri ve 10-HDA düzeylerinin hasat zamanına göre artış gösterdiği, özellikle fruktoz ve 10-HDA düzeylerinin larva transferini izleyen ilk 24 saatten itibaren giderek artan bir seyir izlediği belirlenmiştir. Arı sütünde glikoz düzeyinde larva transferinden 24 ve 48 saat sonra; sakkaroz düzeyinde ise 24,48 ve 72 saat sonraki gruplar arasında belirgin bir farklılık olduğu saptanmıştır. Denemede larva transferinden 24 saat sonra hasat edilen arı sütündeki nem düzeyi, 48 ve 72 saat sonra hasat edilen arı sütündekilerden önemli düzeyde düşük bulunmuştur ($P<0.05$). Arı sütünde larva transferinden 24 saat sonra %58.43±3.22 olan nem düzeyi artarak, 48 saat sonra %66.79±2.09 ve 72 saat sonra ise %68.06±1.83 düzeylerine yükselmiştir *(Tablo 1)*.

İz Elementler

Denemede arı sütünde yapılan iz element analizinde K, Na ve Cu elementleri için gruplar arasında önemli fark bulunmuştur (P<0.05). Deneme grupları arasında arı sütünde Fe, Mn ve Zn iz elementleri için önemli bir fark saptanmamıştır. Araştırmada K, Na ve Cu iz elementlerinin, larva transferinden 48 saat sonra hasat edilen arı sütlerinde artış gösterdiği, ancak larva transferinden 72 saat sonra hasat edilen arı sütlerinde önemli düzeyde gerilediği belirlenmiştir (P<0.05) *(Tablo 2)*.

24 saat sonra %7.506±0.40, 48 saat sonra %8.683±0.38 ve 72 saat sonra %9.121±0.41 olarak bulunmuştur. Bu değerler, Zheng ve ark.[2] tarafından aynı hasat zamanları için sırasıyla %5.5±0.8, %5.5±0.9 ve %5.9±0.8 olarak bildirilen 10-HDA düzeylerinden daha yüksektir. Bu durum, besleyici arıların yaşı, besleme durumu, doğal nektar kaynakları, kabul edilen yüksük sayısı ile ilişkilendirilebilir. İşçi arılarda yaşla 10-HDA üretim düzeyi artmakta ve bu artış, besleyici kolonilerde arı sütüne yansımaktadır. Kolonin kaliteli polen ve nektarla beslenmesi de, arı sütünde 10-HDA düzeyini artırmaktadır. Kolonide kabul edilen yüksük sayısı arttıkça, arı sütünde

Tablo 1. *Deneme gruplarına (24. saat, 48. saat ve 72. saat) ait 24 adet arı sütü örneğine ait fruktoz, glikoz, sakkaroz, 10-HDA ve nem düzeyleri*
Table 1*. Fructose, glucose, sucrose, 10-HDA and humidity levels in 24 royal jelly samples of treatment groups (24, 48 and 72 h)*

Deneme Grupları	Fruktoz (%)	Glikoz (%)	Sakkaroz (%)	10-HDA (%)	Nem (%)
24. saat	7.021±0.33b*	10.100±0.81 b	2.269±0.18 b	7.506±0.40 b	58.43±3.22b*
48.saat	9.682±0.30 a	13.119±0.72 a	1.613±0.15 b	8.683±0.38ab	66.79±2.09 a
72.saat	10.331±0.28 a	11.782±0.77ab	4.750±0.18 a	9.121±0.41 a	68.06±1.83 a

** Aynı sütunda farklı harfleri taşıyan değerler birbirinden farklıdır (a,b; P<0.05)*

Tablo 2. *Deneme gruplarına (24. saat, 48. saat ve 72. saat) ait 24 adet arı sütü örneğine ait mineral madde düzeyleri*
Table 2. *Mineral levels in 24 royal jelly samples of treatment groups (24, 48 and 72 h)*

Deneme Grupları	K (ppm)	Na (ppm)	Cu (ppm)	Fe (ppm)	Mn (ppm)	Zn (ppm)
24.saat	9848.05±5.09 a*	36.02±3.22b	12.24±0.43b	62.36±7.73	1.35±0.26	136.06±4.85
48.saat	10170.17±5.02a	48.18±3.18a	14.29±0.39a	83.63±7.49	1.16±0.24	132.88±4.76
72.saat	7850.16±5.11b	45.43±2.99ab	11.73±0.40b	39.03±7.02	0.75±0.27	145.98±4.88

** Aynı sütunda farklı harfleri taşıyan değerler birbirinden farklıdır (a,b; P<0.05)*

TARTIŞMA ve SONUÇ

Denemede arı sütlerinde yapılan şeker bileşenleri analizinde, fruktoz düzeyinin larva transferinden 24 saat sonra %7.021±0.33 değerinde iken, larva transferinden 72 saat sonra %10.331±0.28 düzeyine ulaştığı belirlenmiştir. Benzer şekilde glikoz düzeyi larva transferinden 24 saat sonra %10.100±0.81 iken, larva transferinden 48 saat sonra %13.119±0.72 değerine yükselmiştir. Bu değerler, Sesta ve ark.[11] tarafından arı sütünde yapılan şeker analizlerinde larva transferinden 24 saat sonra %2.4 ve 72 saat sonra %6.9 düzeylerinde belirlenen fruktoz ve larva transferinden 24 saat sonra %3.7 ve 72 saat sonra %8.2 olarak saptanan glikoz değerlerinden oldukça yüksektir. Araştırmada bulduğumuz fruktoz, glikoz ve sakkaroz düzeyleri, aynı araştırıcı [18], tarafından bir diğer çalışmasında larva transferinden 72 saat sonra hasat edilen arı sütü örneklerinde sırasıyla %6.39, %5.94 ve %1.26 olarak saptadığı değerlerden de yüksektir. Çalışmada arı sütünde larva transferinden 72 saat sonra belirlenen fruktoz ve glikoz değerleri, Balkanska ve Kashamov [27] tarafından sırasıyla %11.85±0.45 ve %9.90±0.94 olarak bulunan değerlerle uyumlu bulunmuştur. Sakkaroz düzeyi, Lercker ve ark.'nın[3] sonuçları ile uyumlu olarak, fruktoz ve glikozdan daha düşük düzeyde bulunmuştur.

Çalışmada 10-HDA düzeyleri sırasıyla larva transferinden 10-HDA içeriği azalmaktadır [2,5,15]. Araştırmada arı sütünde belirlenen nem düzeyleri larva transferini izleyen 24 saatten itibaren artan bir seyir izlemiş; 24 saat sonra %58.43±3.22, 48 saat sonra %66.79±2.09 ve 72 saat sonra %68.06±1.83 olarak belirlenmiştir. Arı sütündeki şeker düzeyleri ile nem düzeyleri arasında benzer bir ilişki saptanmış, hasat günlerine bağlı olarak arı sütünde şeker içeriği ve nem düzeyinin arttığı görülmüştür. Bu bulgu taze arı sütünün büyük bir bölümünün sudan oluştuğu [1] ve şeker bileşenleri ile nem düzeyi artışının paralellik gösterdiği [10] görüşleriyle benzeşmektedir.

Denemede arı sütünde analiz edilen K, Na, Cu ve Fe iz elementlerinin larva transferinden 48 saat sonra, 24 saat sonraki hasat zamanına göre sırasıyla 322.12 ppm, 12.16 ppm, 2.05 ppm ve 21.27 ppm arttığı, ancak larva transferinden 72 saat sonra ise azaldığı tespit edilmiştir. Arı sütünde elde edilen en yüksek iz element miktarının K ve Zn'da, en düşük ise Mn'da belirlenmesi, Stocker ve ark.'nın [17] sonuçları ile uyumludur. Aynı araştırıcı, arı sütündeki iz element kompozisyonunun baldaki gibi coğrafik yapı ve vejetasyona bağlı olarak değişmediğini, homeostatik (organizmanın gereksinimi ve iç dengesine göre) olarak belirlediğini öne sürmektedir. Hatta bunu memelilerin anne sütünde yavru ihtiyacına göre sütün kompozisyonunun şekillenmesi temeline dayandırarak, böcek düzeyinde "laktasyon" olarak ifade etmektedir. Bu vurgu, arı sütü içerisinde halen işlevselliği bilinmeyen

ve tanımlanamayan mekanizma ile özdeşleştirilebilir.

Arısütü için henüz uluslararası bir standart yoktur. Farklı ülkelerin kendi ulusal standartları vardır. TSE'ye göre [9] arı sütünde 10-HDA, en az %1.4 ve nem düzeyi %70'in altında olmalıdır. 10- HDA düzeyinin %1.8'den daha fazla olması durumunda, arı sütü "yüksek kaliteli" olarak kabul edilmektedir. Dünya'da üretilen arı sütünün ancak %15-20'sinde 10-HDA düzeyi %1.8'in üzerindedir. Arı sütü üretimindeki en önemli kalite kriterleri; arı sütündeki şeker bileşenleri ve 10-HDA düzeyleridir. Bu parametrelerin belirlenmesi, ayrıca hasat gününe bağlı olarak şeker bileşenleri ve 10-HDA düzeylerinde meydana gelen değişimlerin bilinmesi, arı sütünün kalitesi ve pazarlanabilirliği konularında belirginlik sağlamakta, hileli arı sütü üretimini engellemektedir. Ancak farklı hasat zamanlarında elde edilen arı sütü kompozisyonlarının biyokimyasal yapısının farklılık gösterdiği ve arı sütü standartlarının altında veya üstünde değer alabildiği görülmektedir. Bu değişimlere göre, gelişen Dünya arı ürünleri pazarında arı sütüne ait uluslararası bir standardın geliştirilmesi için daha yoğun, özellikle "hasat zamanı" ibaresi de belirtilerek standardizasyon çalışmaları yapılmalıdır.

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Kafkas Univ Vet Fak Derg
19 (2): 239-244, 2013
DOI: 10.9775/kvfd.2012.7609

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Etlik Piliç Dokularındaki Levamizol Kalıntıları Üzerine Çeşitli Pişirme ve Dondurma İşlemlerinin Etkisinin Araştırılması [1]

Emine SEVER * Emine BAYDAN **

[1] *Aynı başlıklı doktora tez çalışmasından özetlenmiştir*

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Makale Kodu (Article Code): KVFD-2012-7609

Özet

Bu çalışma Türkiye'de paraziter hastalıklarının sağaltımı amacıyla kullanılan levamizolün kanatlı dokularındaki kalıntıları üzerine çeşitli pişirme ve soğukta muhafaza işlemlerinin etkisi olup olmadığını belirlemek amacıyla yapıldı. Bu amaçla 10 adet Ross PM-3 ırkı etlik civcivlere levamizol oral gavaj ile 36 mg/kg/2 ml olacak şekilde verildi. Tavuklar ilacın verilmesini takiben 12 saat sonra kesilerek sağ but, sağ göğüs ve karaciğer dokusu alındı. Numuneler çiğ, ızgara, haşlama, derin dondurucuda (-18ºC) 15 ve 30 gün bekletmek için beş eşit parçaya ayrıldı. Daha sonra dokular ekstrakte edilerek yüksek basınçlı sıvı kromotografisi yöntemiyle (HPLC) analiz edildi. Buna göre but, göğüs ve karaciğer dokularındaki levamizol miktarı sırasıyla çiğ dokuda µg/kg olarak 0.258±0.019, 0.178±0.021, 0.188±0.011; ızgara işlemine tabii tutulan dokularda 0.152±0.019, 0.094±0.011, 0.123±0.011; haşlama işlemine tabii tutulan dokularda 0.079±0.010, 0.089±0.093, 0.088±0.005; 15 gün -18ºC'de dondurma işlemine tabii tutulan dokularda 0.117±0.015, 0.119±0.009, 0.169±0.014; 30 gün -18ºC'de dondurma işlemine tabii tutulan dokularda ise 0.087±0.008, 0.089±0.093, 0.128±0.011 şeklinde bulundu. Yapılan bu çalışmada dokularda levamizolün kaybolmadığı, sadece uygulanan işleme göre konsantrasyonunun azaldığı, analizi yapılan dokuların çeşidine göre de dağılımının homojen olmadığı, aksine sırasıyla çiğ ve ızgarada but, karaciğer, gögüs haşlamada ise göğüs, karaciğer, but sırasını izleyerek azalma gösterdiği sonucuna varıldı.

Anahtar sözcükler: *Levamizol, Etlik piliç eti, Pişirme, Dondurma, HPLC*

The Effect of Various Cooking and Freezing Processes on the Levamisole Residues in Broiler Tissues

Summary

This study was carried out to investigate the effect of various cooking and freezing processes on the levamisole residues in poultry tissues, which is one of most commonly used antiparasiter drug, has been frequently using in Turkey as well. For this purpose, 10 of Ross PM-3 breed chicks were given levamisole by oral gavage at 36 mg/kg/2 ml. After 12 h administration of drug, chicken were sacrified, after that, their right legs, breasts and livers were cut out. The samples were divided into 5 equal parts in order to asses different processing such as raw, grilled, boiled and deep frozen (-18ºC) for 15 and 30 days storage. After processes, the extraction of tissues was done and analyzed by high pressure liquid chromatography (HPLC) method. Therefore, the levamisole amount in leg, breast and liver tissues were determined as follows, respectively: In raw tissues (µg/kg): 0.258±0.019, 0.178±0.021, 0.188±0.011; in grilled tissues 0.152±0.019, 0.094±0.011, 0.123±0.011; in boiled tissues 0.079±0.010, 0.089±0.093, 0.088±0.005; in frozen tissues for 15 days (-18ºC) 0.117±0.015, 0.119±0.009, 0.169±0.014; in frozen tissues for 30 days (-18ºC) 0.087±0.008, 0.089±0.093, 0.128±0.011. Finally, this study showed that levamisole did not clean from the tissues totally, but its amount reduced, depends on the kinds of processing, also its distribution regarding analyzed tissues was not homogeneous, on contrary, it showed a decline as follows respectively; legs, livers, breasts in raw and grilled samples and breasts, livers, legs in boiled samples.

Keywords: *Levamisole, broiler meat, cooking, freezing, HPLC*

GİRİŞ

Hayvanlarda hastalıkların sağaltımı ve önlenmesi, gelişmenin hızlandırılması ve yemden yararlanmanın artırılması, paraziter hastalıkların kontrolü ve beslenmenin desteklenmesi amacıyla çok sayıda ilaç, hormon, vitamin ve mineral

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madde kullanılmaktadır. Özellikle etlik piliçlerde olduğu gibi hayvanlardan bazıları son derece kısa süreli olan yaşamlarını tümüyle ilaçlı olarak geçirmektedirler [1,2]. Bir imidazol türevi olan levamizol bu ilaçlardan biridir. Bugün için birçok hayvan türünde geniş bir antelmentik spektruma sahip olması, aynı zamanda sindirim ve solunum sistemi nematodları üzerinde etkili olabilmesi ve ağızdan ve parenteral yollarla da verilebilmesi gibi üstünlükleri nedeniyle bu alanda en fazla kullanılan ilaçlardan biridir [3-12]. Benzimidazol bileşikleri ile karşılaştırıldığında levamizol ile tetramizolün çok geniş bir güvenlik eşiğine sahip olduğu bildirilmektedir. Levamizol kanatlı hayvanlara genellikle içme sularında çözünmüş olarak verilir. Sağaltım dozlarında 36-48 mg/kg levamizol alımının sağlanabilmesi için hayvanların su tüketimi dikkate alınarak %0.01 yoğunlukta levamizol içeren suların 12 saat süreyle verilmesi öngörülür [13].

İlacın tüketilmeden önce besinlerin pişirilme veya konserveleme gibi herhangi bir işlemden geçirilmesiyle mevcut kalıntıların kalış ve kaybolma durumunu açıklayacak konulara ilişkin yeterli bir bilgi yoktur. Pişirme esnasında proteinlerde yıkımlanma, sıcaklığa ve pişirilme şekline bağlı olarak rutubet ve yağ kaybı ile pH'da değişme olabilir. Bu faktörlerin bir veya bir kaçında görülen değişikliklere bağlı olarak ilaç kalıntılarının çözünebilirliği veya miktarında değişme görülebilir [14]. Keza çok sayıda olmamakla birlikte yapılan bazı araştırmalar hayvansal ürünlere uygulanan haşlama, kızartma, konserveleme gibi pişirme işlemleri, soğukta (+4°C) ve dondurma (-20,-76°C) ile depolamanın ilaç kalıntılarında azalmaya sebep olacağını göstermiştir [15].

Rose ve ark.[16] bazı ithal sığır ve domuz etlerindeki levamizol kalıntıları üzerine bazı pişirme işlemlerinin etkisini araştırmışlardır. Yapılan araştırma sonucunda levamizolün 100°C'de kaynatmaya dayanıklı olduğu ancak 260°C'de yağda yaklaşık 5 dak. kızartma ile ilacın yarı yarıya kayba uğradığı gözlenmiştir. Günümüze kadar hayvan dokularındaki levamizol kalıntıları üzerine yapılan araştırmalar çoğunlukla çiğ dokudaki düzeyleri üzerine olmuştur. Ancak çok sayıda olmasa da domuz ve sığır etlerindeki levamizol kalıntıları üzerine pişirme işlemlerinin etkileri değerlendirilmiştir [14,16]. Fakat bu araştırmalarda kanatlı dokularındaki levamizol kalıntıları ile ilgili olarak ne pişirmenin ne de soğukta muhafazanın etkisi değerlendirilmemiştir. Bu araştırmada, Yüksek Basınçlı Sıvı Kromotografisi (HPLC) yöntemiyle tavuk dokularında bulunan levamizol kalıntıları üzerine kömürde ızgara ve haşlama gibi pişirme yöntemleri ile dondurucuda (-18°C) saklamanın etkisi değerlendirilmeye çalışılmıştır.

MATERYAL ve METOT

Kimyasal Maddeler

Çalışmada kullanılan antelmentik standardı Levamizol HCl (%99 L-9756 Lot 97H3459) Sigma (Sigma-Aldrich Chemical Co., St. Louis, Missouri, USA), diğer kimyasal maddeler ise ticari firmalardan temin edildi.

Hayvan Materyali

Materyal olarak Abalıoğlu Tavukçuluk A.Ş.'den temin edilen kuluçkadan yeni çıkmış 10 adet Ross-PM3 ırkı etlik civcivler kullanıldı. Civcivler 1.5-2.0 kg ağırlığa ulaşıncaya kadar 45 gün süreyle ilaç kalıntısı ihtiva etmeyen 1-9. günler arası etlik civciv başlangıç granül yemi; 10-26. günler arası etlik civciv granül yemi; 27-38. günler arası etlik piliç pelet yemi; 39-42. günler arası piliç bitirme pelet yemi ile beslendi. Bu yemlerin ham protein miktarları sırasıyla %23, %22, %19-20, %19-20'dir. Metabolik enerji miktarları ise kcal/g/gün cinsinden 3050, 3150, 3200, 3150'dir. Ortamın ısısı ilk günden son güne kadar 34°C'den 22°C'ye kadar giderek azalan oranlarda günlük olarak değişim gösterirken, nem oranı ise %56'dan %78'e kadar giderek artan oranlarda günlük değişim göstermiştir. Ayrıca çalışma ile ilgili Ankara Üniversitesi Veteriner Fakültesi Etik Kurulu'ndan onay alınmıştır (18.01.2002/2).

Deneysel Aşama

Çalışmaya alınan tavuklara canlı ağırlıkları göz önünde tutularak levamizol hidroklorür teknik standardı 36 mg/kg/2 ml olacak şekilde ince bir plastik sondaya bağlı enjektör yardımı ile doğrudan kursağa verildi. İlaç verilme işleminden 12 saat sonra levamizolün kanatlılardaki kinetiği göz önünde tutularak tavuklar kesildi [17].

Kesim işlemleri sırasında her hayvandan sağ but, sağ göğüs ve karaciğer dokuları alınarak laboratuvara getirildi. Daha sonra laboratuvarda bekletilmeden çiğ, ızgara, haşlama, derin dondurucuda (-18°C) 15 ve 30 gün bekletme için beş eşit parçaya ayrıldı. Dondurulacak dokular alüminyum folyolara sarılarak derin dondurucuya yerleştirildi ve günü geldiğinde analiz işlemine tabii tutuldu. Izgara işlemleri meşe kömürü ile 7 dakikalık bir pişirme süresi içerisinde gerçekleştirildi. Haşlama işlemleri bir balon içersinde dokunun üzerine 25 ml'lik su ilavesi ile 100°C'de 20 dak. kaynatılarak yapıldı; ısının kontrollü olması için de ayarlı elektrikli ocak kullanıldı.

Ekstraksiyon

Ekstraksiyon işlemi Bukanski ve ark.'nın [18] bildirdiği yönteme göre yapıldı. Sağ but ve sağ göğüs doku örneklerinden 10 g, karaciğerden 7 g alınarak 100 ml'lik santrifüj tüpüne konuldu. Sağ but ve sağ göğüs doku örneklerinin üzerine 30 ml etil asetat, %50'lik 1ml potasyum hidroksit ve 5 g sodyum sülfat, karaciğer doku örneğine 21 ml etil asetat, 0.7 ml %50'lik potasyum hidroksit, 3.5 g sodyum sülfat ilave edildi. Numuneler 1 dak. vortekste çalkalandı. 15 dak. santrifüj tüpünde beklettikten sonra 10 dakika daha çalkalanarak karışım 3.000 devirde 5 dak. santrifüj edildi.

100 ml'lik santrifüj tüplerine but ve göğüs dokusu bulunan tüplerdeki etil asetat fazından ayrı ayrı 20 ml, karaciğer dokusu bulunan santrifüj tüpünden de 14 ml aktarıldı. But ve göğüs dokusu bulunan tüplere 5 ml 0.5 mol hidroklorik asit, karaciğer dokusu bulunan tüpe 3.5 ml 0.5 mol hidroklorik asit ilave edildi. Tüpler elle kuvvetlice çalkalandıktan

sonra iki dakika boyunca da vortekste çalkalandı ve 3.000 devirde 5 dak. santrifüj edildi. Üst tabakadaki etil asetat kısmı atılarak alttaki but ve göğüs için 5 ml, karaciğer için 3.5 ml 0.5 mol hidroklorik asit 10 ml'lik santrifüj tüplerine boşaltıldı. Sulu asit solüsyonlarından levamizolü ekstrakte etmek için ortam alkali yapıldı. Bunun için tüplere 1 ml %50'lik potasyum hidroksit solüsyonu ilave edilerek karıştırıldı ve oda ısısında soğumaya bırakıldı. 500 µl kloroform ilavesinden sonra karışımlar elle çalkalanarak 2 dak. vorteks cihazında karıştırıldı ve 10 dak. bekletildi. İki tabakanın ayrılması için tüpler 2.500 devirde 5 dak. santrifüj edildi. Tüplerin üstünde bulunan sıvı tabaka atıldı ve kloroform tabakası başka bir santrifüj tüpüne aktarıldı. Bu aktarma işlemi hareketli (mobil) fazdaki mevcut kirlilikleri bertaraf etmek için yapıldı. Kloroform 50-55°C'de ısıtılan buharlaştırıcıda azot gazı akımı altında uçuruldu ve kalıntı 500 ml mobil fazda (0.03 mol/litre sodyum asetat/metanol, %35 + %65 hacimle) tekrar çözdürüldü. Elde edilen bu ekstrakt bir steril enjektörle mikrofiltreden geçirilerek şişelere aktarıldı. Bunun 100 ml'si cihaza uygulandı.

Kromotografi Cihazının Hazırlanması

Numunelerin kromotografik analizinde HPLC (Agilent 1100, Agilent Technologies, Germany) cihazı kullanıldı. Cihazın hazırlanması işlemi için sodyum asetat (0.03 mol/litre) ve metanol 0.45 ml'lik kağıt filtrelerden süzülerek viallere alındı. Hazırlanan mobil faz sıvı kromotografisi sistemiyle bağlanarak dakikada kolondan (C-18) 1ml geçecek şekilde ayarlandı. DAD dedektörün sig=250.100 ref=360.100 dalga boyu seçildi. Analiz süresi 11 dakika olarak belirlendi. HPLC'ye 100 ml enjekte edildi. Mobil fazın akışı 1 ml/dak. olacak şekilde ayarlanarak yaklaşık 1 saat süresince kolondan geçiş sağlandı ve kolon analiz edilecek maddeyi belirlemesi yönünde şartlandırıldı.

Numunelerin Analizi

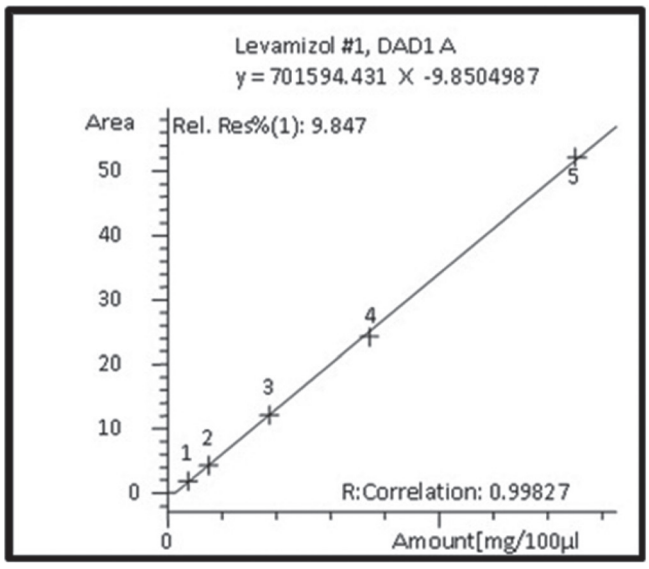
Ekstraksiyon işleminden geçirilerek toplanan ve viallere konulan numune eluatından 100 ml hacimde kromotografiye enjekte edildi. Analiz edilen numunede aynı yerde pik alanı oluşturup oluşturmadığı, eğer pik alanı mevcut ise kalibrasyon eğrisine göre hangi yoğunluğa tekabül ettiği belirlenerek değerlendirildi. Ayrıca çalışmada 7.5 mg/ml'lik stok solüsyonundan 250 ml (1.875 mg) ve 500 ml (3.75 mg) miktarlar (n=3) geri kazanım çalışmasında kullanıldı *(Şekil 2, 3* ve *4)*.

İstatistiksel Analiz

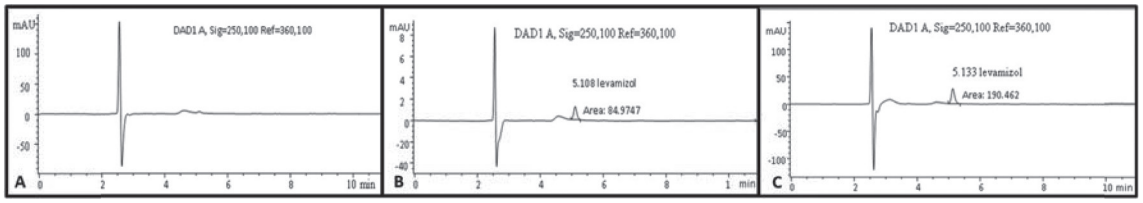
İstatistiksel analiz için SPSS paket programı kullanıldı. Analizde tek yönlü varyans analizi ve gruplar arası değerlendirmeler ise Duncan metoduna göre yapıldı. Çalışmadan elde edilen veriler aritmetik ortalama ve standart hata ile minumum ve maksimum değerler şeklinde verildi. İstatistiksel olarak önemlilik P<0.05 şeklinde ifade edildi.

BULGULAR

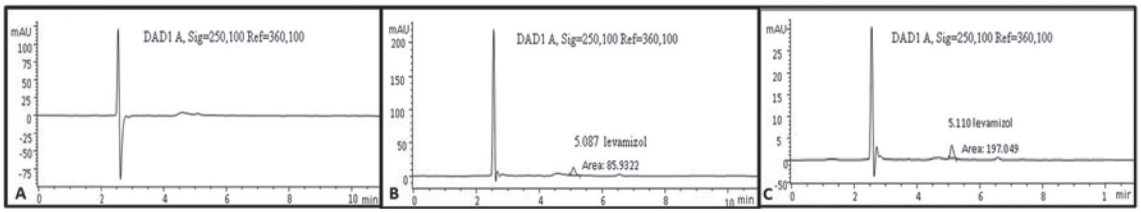
Araştırmada her çalışma başlangıcında numune analizinden önce 7.5 mg/ml olacak şekilde hazırlanan stok solüsyondan ayrı ayrı 2 defa enjekte edilerek levamizol alıkonma süresi (dakika) belirlendi. Yapılan 16 deneme sonucunda levamizolün ortalama alıkonma süresi 5.051±0.141 dak olarak tespit edildi. Yapılan denemeler ve hesaplamalar sonucunda levamizolün cihazda tespit edilebilir en alt düzeyi 9.15 ng/kg olarak belirlendi. Levamizol çalışma standart çözeltisinin kalibrasyon eğrisi *Şekil 1*'de verilmiştir. Çalışmada levamizol çalışma standart çözeltisinin 0.37; 0.75; 1.875; 3.75 ve 7.5 mg/ml'lik yoğunluklarına karşılık ölçülen alanlar sırasıyla 18.08; 42.74; 121.07; 231.45 ve 527.32 olarak belirlenmiştir. Her bir dokuya ait kalıntısız, 1.875 mg ve 3.75 mg levamizol eklenmiş kromotogram analiz sonuç grafikleride sırasıyla *Şekil 2, 3* ve *4*'te gösterilmiştir. Tavuklara ağızdan 36 mg/kg/2 ml miktarında verilen levamizolün çiğ, haşlama, ızgara, 15 günlük ve 30 günlük (-18°C) dondurma işlemlerine tabii tutulmuş sağ buttaki, sağ göğüsteki ve karaciğer doku ekstrakt kromotografileri sırasıyla *Şekil 5, 6* ve *7*'de gös-



Şekil 1. Levamizolün kalibrasyon eğrisi
Fig 1. Calibration curve of levamizol application

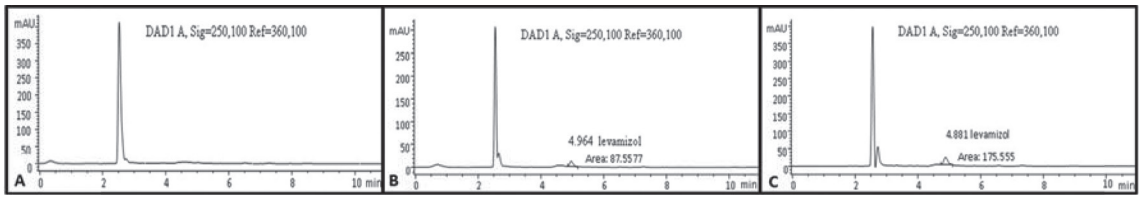


Şekil 2. Kalıntısız (A), 1.875 mg (B) ve 3.75 mg (C) levamizol eklenmiş tavuk but dokusundaki kromotogram analiz sonuçları
Fig 2. Chromatography analysis results of nonresidue (A) and added levamisole at 1.875 mg (B), and 3.75 mg (C) in chicken leg tissue



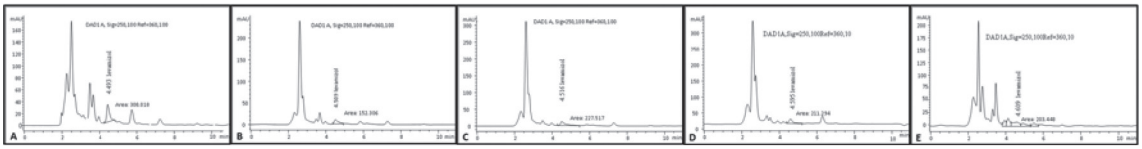
Şekil 3. Kalıntısız (A), 1.875 mg (B) ve 3.75 mg (C) levamizol eklenmiş tavuk göğüs dokusundaki kromotogram analiz sonuçları

Fig 3. Chromatography analysis results of nonresidue (A) and added levamisole at 1.875 mg (B), and 3.75 mg (C) in chicken breast tissue



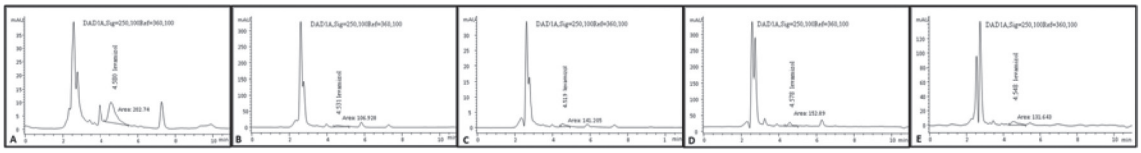
Şekil 4. Kalıntısız (A), 1.875 mg (B) ve 3.75 mg (C) levamizol eklenmiş tavuk karaciğer dokusundaki kromotogram analiz sonuçları

Fig 4. Chromatography analysis results of nonresidue (A) and added levamisole at 1.875 mg (B), and 3.75 mg (C) in chicken liver tissue



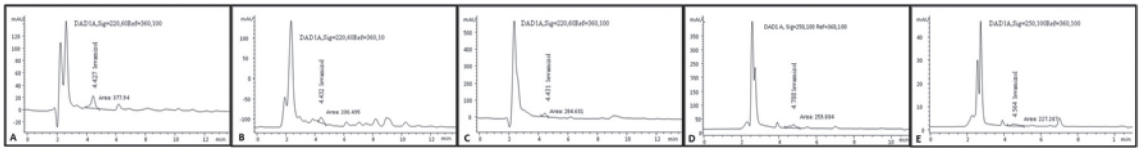
Şekil 5. Ağızdan 36 mg/kg/2 ml miktarında verilen levamizolün çiğ (A), haşlama (B), ızgara (C), 15 (D) ve 30 (E) günlük (-18°C) dondurma işlemlerine tabii tutulmuş but dokusundaki kalıntı kromotogram sonuçları

Fig 5. Leg tissue residue chromatography results given perorally 36 mg/kg/2 ml levamisole to animals in raw (A), boiled (B), grilled (C), 15 (D), and 30 (E) freezing (-18°C) days



Şekil 6. Ağızdan 36 mg/kg/2 ml miktarında verilen levamizolün çiğ (A), haşlama (B), ızgara (C), 15 (D) ve 30 (E) günlük (-18°C) dondurma işlemlerine tabii tutulmuş göğüs dokusundaki kalıntı kromotogram sonuçları

Fig 6. Breast tissue residue chromatography results given perorally 36 mg/kg/2 ml levamisole to animals in raw (A), boiled (B), grilled (C), 15 (D), and 30 (E) freezing (-18°C) days



Şekil 7. Ağızdan 36 mg/kg/2ml miktarında verilen levamizolün çiğ (A), haşlama (B), ızgara (C), 15 (D) ve 30 (E) günlük (-18°C) dondurma işlemlerine tabii tutulmuş karaciğer dokusundaki kalıntı kromotogram sonuçları

Fig 7. Liver tissue residue chromatography results given perorally 36 mg/kg/2 ml levamisole to animals in raw (A), boiled (B), grilled (C), 15 (D), and 30 (E) freezing (-18°C) days

Tablo 1. *Farklı pişirme işlemlerinin etlik piliç dokularındaki levamizol kalıntısı üzerine etkisi (µg/kg)*

Table 1. *The effect of different cooking procedures on the levamisole residues in broiler tissues (µg/kg)*

Doku	Çiğ	Izgara	Haşlama
Sağ But	0.258±0.019 ^{a,A}	0.152±0.019 ^{a,B}	0.079±0.010 ^{a,C}
Sağ Göğüs	0.178±0.021 ^{b,A}	0.094±0.011 ^{c,B}	0.089±0.093 ^{b,B}
Karaciğer	0.188±0.011 ^{b,A}	0.123±0.011 ^{b,B}	0.088±0.005 ^{b,C}

^{A,B,C}: Büyük harfler sütunlar arası farklılığı belirtir; ^{a,b,c}: Küçük harfler satırlar arası farklılığı belirtir. Ortalama ± Standart sapma, ***n:*** *10, P<0.05*

Tablo 2. *Farklı sürelerde soğukta (-18ºC) bekletmenin etlik piliç dokularındaki levamizol kalıntısı üzerine etkisi (µg/kg)*

Table 2. *The effect of cold waiting in different periods (-18ºC) on the levamisole residues in broiler tissues (µg/kg)*

Doku	1. gün	15. gün	30. gün
Sağ But	0.258±0.019 ^{a,A}	0.117±0.015 ^{b,B}	0.087±.0.008 ^{a,C}
Sağ Göğüs	0.178±0.021 ^{b,A}	0.119±0.009 ^{a,B}	0.089±0.093 ^{a,C}
Karaciğer	0.188±0.011 ^{b,A}	0.169±0.014 ^{b,B}	0.128±0.011 ^{b,C}

^{A,B,C}: Büyük harfler sütunlar arası farklılığı belirtir; ^{a,b,c}: Küçük harfler satırlar arası farklılığı belirtir. Ortalama ± Standart sapma, ***n:*** *10, P<0.05*

terilmiştir. Ayrıca 7.5 µg/ml'lik levamizol stok solusyonundan 250 ml (1.875 mg) ve 500 ml (3.75 mg) miktarında ilavesiyle elde edilen geri kazanım sonuçları, sırasıyla, sağ butta 52.62±0.028 ve 61.71±0.014 mg; sağ göğüste 53.14±0.042 ve 63.89±0.070 mg, karaciğerde 54.24±0.056 ve 56.93±0.070 mg olarak tespit edilmiştir.

Yapılan bu çalışmada 10 adet tavuğa ait sağ but, sağ göğüs ve karaciğer dokularına ızgara, haşlama gibi pişirme işlemleri ve soğukta 15-30 gün bekletme işlemleri yapılarak elde edilen 150 adet numune analiz edilmiştir. Çalışmada farklı pişirme işlemlerinin levamizol kalıntısı üzerine etkisi *Tablo 1*'de verilmiştir. Buna göre, levamizol kalıntısı içeren sağ but dokularına uygulanan ızgara işlemleri sonucunda, kalıntı miktarı çiğ dokuya göre %41.1 oranında, haşlama işlemi sonucunda ise % 69.4 oranında azalma göstermiştir ($P<0.05$). Sağ but dokusundaki pişirme işlemleri incelendiğinde haşlama yapılan dokulardaki ilaç kalıntı miktarları ızgaraya göre daha fazla azalmıştır ($P<0.05$). Sağ göğüs dokularında ızgara ve haşlama işlemi sonucunda bulunan levamizol kalıntı değerleri çiğ dokuya göre sırasıyla %47.2 ve %50'lik bir azalmaya yol açmıştır ($P<0.05$). Izgara ve haşlama dokuları incelendiğinde yine haşlama yapılan dokulardaki ilaç kalıntılarının ızgaraya göre azaldığı görülmüştür ($P>0.05$). Çiğ, ızgara ve haşlama yapılan karaciğer dokularındaki ilaç kalıntı miktarları incelendiğinde de ızgara ve haşlama dokularındaki ilaç kalıntısı çiğ dokuya göre sırasıyla %34.6 ve %53.2 azalma göstermiştir ($P<0.05$). Izgara yapılan ve haşlama işlemine tabi tutulan dokulardaki ilaç miktarları karşılaştırıldığında haşlama sonucu ilaç miktarının ızgaraya göre daha çok azaldığı belirlenmiştir ($P<0.05$).

Çalışma sonunda elde edilen verilerle ilgili olarak, farklı sürelerde soğukta (-18°C) bekletmenin levamizol kalıntısı üzerine etkisi *Tablo 2*'de gösterilmiştir. Soğukta bekletme sonucu levamizol kalıntısının çiğ dokuya göre sağ butta; 15. gün -18°C'de bekletmede %55, 30. gün -18°C'de bekletildiğinde ise %66.3 azaldığı, çiğ dokuya göre sağ göğüste 15. gün -18°C'de bekletmede %33.1, 30. gün -18°C'de bekletmede %50, çiğ dokuya göre karaciğerde 15. gün -18°C'de bekletmede %10.1, 30. gün -18°C'de bekletmede %32 azaldığı ($P<0.05$) görülmüştür.

TARTIŞMA ve SONUÇ

Veteriner ilaç kalıntılarının et ve et ürünlerinde bulunması tüketicilerin sağlığı açısından önemli sakıncalar oluşturmaktadır [19]. Bu durum, giderek bilinçlenen, kaliteli ve sağlıklı ürün satın almak isteyen tüketicilerde kaygı uyandırmaktadır [20]. Bu araştırmada Türkiye'de paraziter hastalıklarının sağaltımı amacıyla kullanılan levamizolün kanatlı dokularındaki kalıntıları üzerine çeşitli pişirme ve soğukta muhafaza işlemlerinin etkisinin olup olmadığı amaçlanmıştır. Yetmişbeş kanatlı ürününde veteriner ilaç kalıntısının araştırıldığı bir araştırmada oksitetrasiklin, sülfadiazin, neomisin ve gentamisin kalıntısı ince tabaka kromotografisi ile araştırılmıştır. Bunlardan 39 örnekte oksitetrasiklin ve sülfadiazin yönünden pozitiflik belirlenmiş, 25'nin karaciğer, göğüs ve but, 7'sinin ise karaciğer ve göğüs et örneklerinde sülfadiazin ve oksitetrasiklin, 7'sinin but etinde ise sadece oksitetrasiklin tespit edilmiştir. Neomisin ve gentamisinin ise tüm et örneklerinde tespit edildiği bildirilmiştir [21]. Yapılan bir diğer çalışmada da çiğ et materyallerinden tüketim için hazırlanan ürünlerde antibiyotik kalıntılarının %88-92 arasında kaldığı belirlenmiştir. Çeşitli işlemlere tabi tutulan bu materyallerde etin yıkamasıyla antibiyotiklerin %12-25, kaynatılmasıyla %5-10, fermentasyonuyla %10 azalma meydana geldiği tespit edilmiştir. Sonuçta ilaç veya metabolitlerinin insan sağlığını tehdit eder nitelikte ürünlerde kaldığı ve etlerin kalitesinin bozulduğu sonucuna varılmıştır [22].

Levamizol kalıntılarına yönelik yapılan çalışmalarda [23-25] geri kazanım düzeyleri %63-75 arasında bulunmuştur. Mevcut araştırmada tespit edilen geri kazanım değerlerinin bu araştırmalarla uyumlu olduğu görülmüştür. Sadece karaciğer dokusundaki geri kazanım düzeyinin daha düşük olmasındaki nedenin dokunun daha fazla bağlayıcı özelliğinin olmasından ileri geldiğini akla getirmektedir.

Mevcut araştırmada levamizol kalıntısı içeren sağ but, sağ göğüs ve karaciğer dokularına uygulanan ızgara ve haşlama işlemleri sonucunda, kalıntı miktarının azaldığı belirlenmiştir. Bu dokulardaki pişirme işlemleri incelendiğinde haşlama yapılan dokulardaki ilaç kalıntı miktarlarının ızgaraya göre daha fazla azaldığı görülmektedir. Haşlama işlemindeki bu azalmanın ısı etkisinin yanında, suya geçmesiyle ilgili olduğu düşünülmektedir [26]. Haşlamanın ilaç kalıntıları üzerine etkisine yönelik yapılan diğer çalışmalara göre de çiğ dokudaki ilaç kalıntılarının bir kısmı ısı etkisiyle yıkımlanmakta, bir kısmı da suya geçerek haşlanmış dokudaki ilaç miktarının azalmasına yol açmaktadır [27]. Rose ve ark.[16] bazı ithal edilen sığır ve domuz etlerindeki levamizol kalıntılarını araştırmışlardır. Çalışmada 7.5 mg/kg dozunda levamizol domuza verilmiş ve 48 saat sonra kesilerek örnekler alınmıştır. Yapılan araştırma sonucunda levamizolun 100°C'de kaynatmaya dayanıklı olduğu, ancak 260°C'de yağda yaklaşık 5 dak. kızartma ile ilacın yarı yarıya kayba uğradığını göstermişlerdir. Yapılan çalışmada levamizol düzeyinin haşlama işlemi sonucunda dokuda Rose ve ark.'nın [16] aksine düşük bulunmasının nedeni çalışılan hayvan türünün farklılığından dolayı ilacın suya kolayca geçmesi şeklinde değerlendirilebilir.

Sonuçlar 15 ve 30. günlerdeki ilaç kalıntıları açısından incelendiğinde karaciğerdeki ilaç kalıntılarının göğüs ve buta göre daha yüksek olduğu görülmektedir. Soğukta bekletme sonucu levamizol kalıntısının çiğ dokuya göre sağ butta, sağ göğüste ve karaciğerde azaldığı görülmüştür. Dondurma işleminin dokulardaki ilaç kalıntılarına yönelik etkilerinin incelendiği kimi çalışmaların [28] aksine bu çalışmada; but, göğüs ve karaciğer dokusundaki levamizol kalıntılarının 15 ve 30 günlük dondurma işlemi sonunda önemli oranlarda azaldığı görülmüştür.

Sağ but, sağ göğüs ve karaciğer dokularından elde edilen sonuçlar incelendiğinde çiğ, ızgara ve haşlama yapılan doku-

larda ilaç kalıntısının en fazla bulunduğu bölgenin sağ but olduğu görülmektedir. Bu durum vücudun diğer bölgelerine göre bacakta hareketliliğin fazla olması, dolayısıyla da dokuda kan dolaşımının daha fazla olmasıyla açıklanabilir. Çiğ ve ızgara yapılmış dokularda ilaç kalıntısı yoğunluğu but > karaciğer > göğüs; haşlama yapılan dokularda ise göğüs > karaciğer > but şeklindedir.

El-Kholy ve Kemppainen [29] levamizolün tavuk dokularında ve yumurtalarında kalıntı durumunu araştırdıkları çalışmada, oral olarak 40 mg/kg dozda verilen levamizolün 9. günden sonra yumurtada tolere edilebilir limitin (≤ 0.1 µg/kg; MRL-Amerika Birleşik Devletleri) altına düştüğünü ve tedaviden sonraki 18. günden sonra ise kesilen hayvanların etlerinin tüketici sağlığı için kalıntı bakımından güvenli olduğunu bildirmişlerdir. Çalışmada sağ but, sağ göğüs ve karaciğer dokularındaki ilaç kalıntı miktarları çiğ, ızgara, haşlama işlemleri sonucunda ve geri kazanımdaki kayıplar göz önüne alınarak da değerlendirildiğinde, Türk Gıda Kodeksi [30] ve EMEA'da [17] belirlenen 10 µg/kg olan tolerans düzeylerinin bir hayli altında kalmaktadır. Buradan yola çıkarak ağızdan 36 mg/kg/2 ml dozunda tavuklara verilen levamizol kalıntılarının kesim öncesi en az 12 saatlik bir bekletmeden sonra tüketilmesinin insan sağlığı açısından önemli bir risk oluşturmayacağı söylenebilir.

Sonuç olarak, yapılan araştırma kapsamında levamizolün etlik piliç dokularındaki kalıntıları üzerine ızgara, haşlama ve soğukta 15-30 gün bekletme işlemlerinin etkisi incelenmiş ve bu işlemlere rağmen levamizol kalıntısının tamamen yok olmadığı sadece miktarının azaldığı, azalmanın özellikle haşlama ile daha fazla olduğu görülmüştür. Analizi yapılan dokuların çeşidine göre de levamizol kalıntı düzeyinin aynı olmadığı aksine yoğunluğa göre sırasıyla çiğ ve ızgarada but, karaciğer, göğüs; haşlamada ise göğüs, karaciğer, but sırasını izleyerek azaldığı sonucuna varılmıştır. Ayrıca gerek çiğ dokularda gerekse haşlama ve dondurma işlemine tabi tutulmuş dokulardaki levamizol kalıntısının Türk Gıda Kodeksinde belirtilen limitin çok altında olduğu ve 36 mg/kg/2 ml dozda levamizol verilen tavuklarda ilaç verildikten en az 12 saatlik bekletmeden sonra yapılacak kesim işlemiyle etlerinin insan sağlığı açısından çok fazla bir risk taşımayacağı sonucuna da varılmıştır.

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Kafkas Univ Vet Fak Derg
19 (2): 245-252, 2013
DOI: 10.9775/kvfd.2012.7616

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Detection of Animal Species in Some Meat and Meat Products by Comparatively Using DNA Microarray and Real Time PCR Methods

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Makale Kodu (Article Code): KVFD-2012-7616

Summary

Determination of animal species in the meat and meat products is one of the interest of food science, it is also important for consumer rights and food safety. Increasing world population has also remarkably impacted the demand for meat and meat products. Based on this fact, issues related to safety and quality in the meat products have brought up in that manner as well. Through DNA based molecular methods are improved in food analysis, it is preferred increasingly in the control of food safety. In this study, a total of 73 samples of the meat and meat products sold in stores, meat selling markets and public bazaars located in different districts of İstanbul province were analyzed for the detection of animal species notified on the label by using Chipron LCD Array Analysis System. The results showed that 39 samples (53.4%) were labelled incorrectly. Randomly selected eleven samples were corrected by Iontek Fluorion Meat Species Identification Kit and FDS Detection System (Real Time PCR). Hence, it was found that the results obtained by DNA Microarray and Real Time PCR methods were identical (100%) with each other, and both methods should extensively be promoted for the detection of animal species in the meat and meat products.

Keywords: *DNA Microarray, Animal Species Detection, Food Safety, Real Time PCR*

Karşılaştırmalı DNA Mikroarray ve Real Time PCR Yöntemi Kullanılarak Bazı Et ve Et Ürünlerinde Tür Tayini Saptanması

Özet

Et ve et ürünlerinde tür beyanı uygunluğunun tespiti gıda biliminin ilgi alanlarından birisi olup; tüketici hakları ve güvenli gıda temini bakımından önem taşımaktadır. Dünya nüfusunda sürekli artış nedeniyle et ve et ürünlerine talep artırmaktadır. Artışın bu hızda devam etmesi gıda güvenliği ve kaliteyle ilgili sorunları gündeme getirmektedir. Gıda analizlerinde DNA tabanlı moleküler yöntemlerin hızla gelişmesiyle, bu yöntemler gıda güvenliği denetiminde artan oranda tercih edilmektedir. Bu çalışmada İstanbul' un farklı semtlerinde yerleşik marketler, et şarküterileri ve semt pazarlarında satışa sunulan 73 adet et ve et ürünü örneğinde DNA Mikroarray Teknolojisi tabanlı Chipron LCD-Array Analiz Sistemi kullanılarak tür beyan uygunluğu incelenmiştir. İncelenen örneklerin 39'unda (%53.4) tür beyanı uygunsuzluğu tespit edilmiştir. Rastgele seçilen 11 örnek için İontek Multipleks Fluorion Et Tür İdentifikasyon Kiti ve İontek FDS Tespit Sistemi (Real Time PCR) kullanılarak doğrulama yapılmıştır. Ayrıca, DNA Mikroarray ve Real Time PCR yöntemleri ile elde edilen sonuçlar arasında %100 uygunluk olduğu; bu iki yöntemin et ve et ürünlerinde tür tayini için yaygınlaştırılarak kullanılabileceği görülmüştür.

Anahtar sözcükler: *DNA Mikroarray, Et Tür Tayini, Gıda Güvenliği, Real Time PCR*

INTRODUCTION

Protein as a macro nutrient and energy source received from meat and meat products has important building and regulatory functions in the body. It is recommended that at least 1/3 of the daily protein requirement in a well and balanced diet should be taken from foods of animal origin. Protein synthesises hormons, enzymes and immune-related species as well as it protects homeostatic balance [1,2]. WHO reports that there is a positive correlation between the level in terms of development and the nutritional fact of protein rich of animal-origin in the report of the Global and

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Regional Food Consumption Patterns and Trends in 2011 [3]. According to this report it is estimated that the annual production of meat and meat products will increase up to 376 millions of tonne in 2030 whereas it was 218 millions of tonne between 1997 and 1999. The statitistics give the consumption rate of meat in Turkey to be about 28 kg per capita [4].

Hygiene and right labelling notified on the label of any food stuff are very important criterias especially for public health. Food safety covers all the preventive measures for the delivery of food in healthy and hygienic conditions to the consumer by protecting it from denaturation, microbiological and chemical contamination and adulteration. Inadequate management of food safety causes serious health problems [5]. There are many infectious diseases called zoonosis transmitted from foods of animal origin such as cyst hydatid, toxoplasma, leptospirosis and brucellosis [6-10]. According to the existing acts the suitable meat for human consumption is defined as the parts from carcasses of animal fitting for slaughter, especially blended mixtures of meat with no adulteration, and the processed ones [11]. The inedible parts of a meat animal are skin, glands, reproductive organs excluding, testicular parts, eyes and eyelids, urinary organs excluding kidney, larynx, trachea, cornea, ears, nails, horn, head, esophagus, craw, intestines, genitals and offals, respectively [12]. The studies have shown that inedible parts of carcases may be infected with some pathogens such as *Brucella melitensis*, *Brucella abortus*, Hepatitis E and coliforms which are potentially risk of zoonosis [13,14]. For instance, bovine spongiform encephalopathy (BSE) known as mad cow disease affected the United Kingdom in 1986. The animals being fed with meat and bone meal as feed additive and the human consuming offals and infected carcasses in the food chain caused this infection to spread out fast [15,16].

In meat and meat products the variety of animal species on the label should be inspected regularly. These Species are pig, chicken, turkey, goat, buffalo, deer, ruminants, sheep, duck, goose, camel, ostrich, kangaroo, quaill, pheasant and equines. The regulations to protect the public health against adulteration and zoonoses strictly prohibit the inedible and lower quality meat either to be directly launched or to be processed in the food chain [17-21]. Hence, detection of meat species by fast and accurate methods should routinely be carried out for the quality control as well as a public task to secure the food safety all over the world [22-28]. A study conducted in İzmir province showed that meat and meat products were mixed with meat belonging to various animal species by 15.5% and detected pork and equine species that were different from the notifications on the label [22]. Another study carried out in Bursa and İstanbul provinces indicated that 22.0% of the samples of fermented sausage, cooked salami, frankfurter, raw meat, ground meat, meat ball, pastramis, ham, bacon, cooked meat and canned product were not in compliance with the Turkish Food Codex violating consumer rights and presenting a potential public health risk whereas another study in the same region showed that the adulteration in meat ball, sausage and was 19.2% [24]. The results arising from the studies in USA also indicated the adulteration in meat and meat products so that 62% had one species, 36% had two species and 2% had three species, respectively [25,26]. A similar outcome in the States reported that adulteration was 46.4% in this category of food [29]. On the other hand, commercial samples of swine hamburgers marketed in Brasil showed no adulteration with bovine, chicken or horse meats, and expectation of hamburger adulteration was not confirmed [28].

Detection of adulteration has become a challenging area in the food science [30-32]. Recent developments in food additives as well as novel foods have remarkably changed the food matrix lowering the reliability of analytical methods based on sensorical, anatomical, morphological and histological differences in detection of adulteration [33-35]. Some testing characteristics like becoming fast, accurate, sensitive, selective, user friendly and capable of simultaneously detection of more than one species in only one reaction are commonly requested for acceptance of a new analytical method [36]. The detection of adulteration in meat and meat products can be done by using different methods such as HPLC, ELISA and PCR [29,36-43].

A new technique called DNA Microarray has been increasingly used to express the impacting mechanisms of neutraceuticals and functional foods in metabolism, to determine the microorganisms related to food safety studies [44-47]. It has also opened up new challenges for food analysis of adulteration in seafoods, meat and meat products [48-51]. Nowadays, DNA based molecular techniques are preferred in many disciplines like taxonomy, epidemiology, forensic, archeology, environmental sciences and food science [30,52-55]. The conventional methods of molecular biology including PCR and RNA blotting do usually examine only one gene in a reaction resulting in poor understanding of the whole of the picture of gene functions [56] whereas DNA Microarray makes possible the whole genome to be displayed on a chip and to express the interaction of thousands of genes with each other simultaneously [56-60].

In this study, 73 samples of the meat and meat products sold in stores, meat selling markets and public bazaars located in different districts of İstanbul province were analyzed to detect the existing animal species as notified on the label by using Chipron LCD Array Analysis System; and randomly selected 11 samples were controled for the verification of the previously found results by Iontek Fluorion Meat Species Identification Kit and FDS Detection System.

MATERIAL and METHODS

Material

In this study 33 of fermented sausages, 16 of grilled meatball, 11 of ground meat, 7 of salami and 6 of sausages sold

in stores, meat selling markets and public bazaars located in different districts of İstanbul province were collected. All the samples were examined for notification on the label and asessement of adulteration by DNA Microarray method

Method

The collected samples were placed in sterile sampling bags, and transported inside a refrigerated container kept at 4°C for sample preparation and DNA isolation. The pieces taken by means of lancet and spatula were homogenized in a blender. 0.20 gram of the homogenized sample was put into Eppendorf tubes.

DNA was extracted by following up the procedure given in Eurofins GeneScan GENE*Spin* DNA Isolation Kit (Catalog no: 5224400605) as outlined in *Fig. 1*. The extracted samples of DNA were stored at -20°C [61].

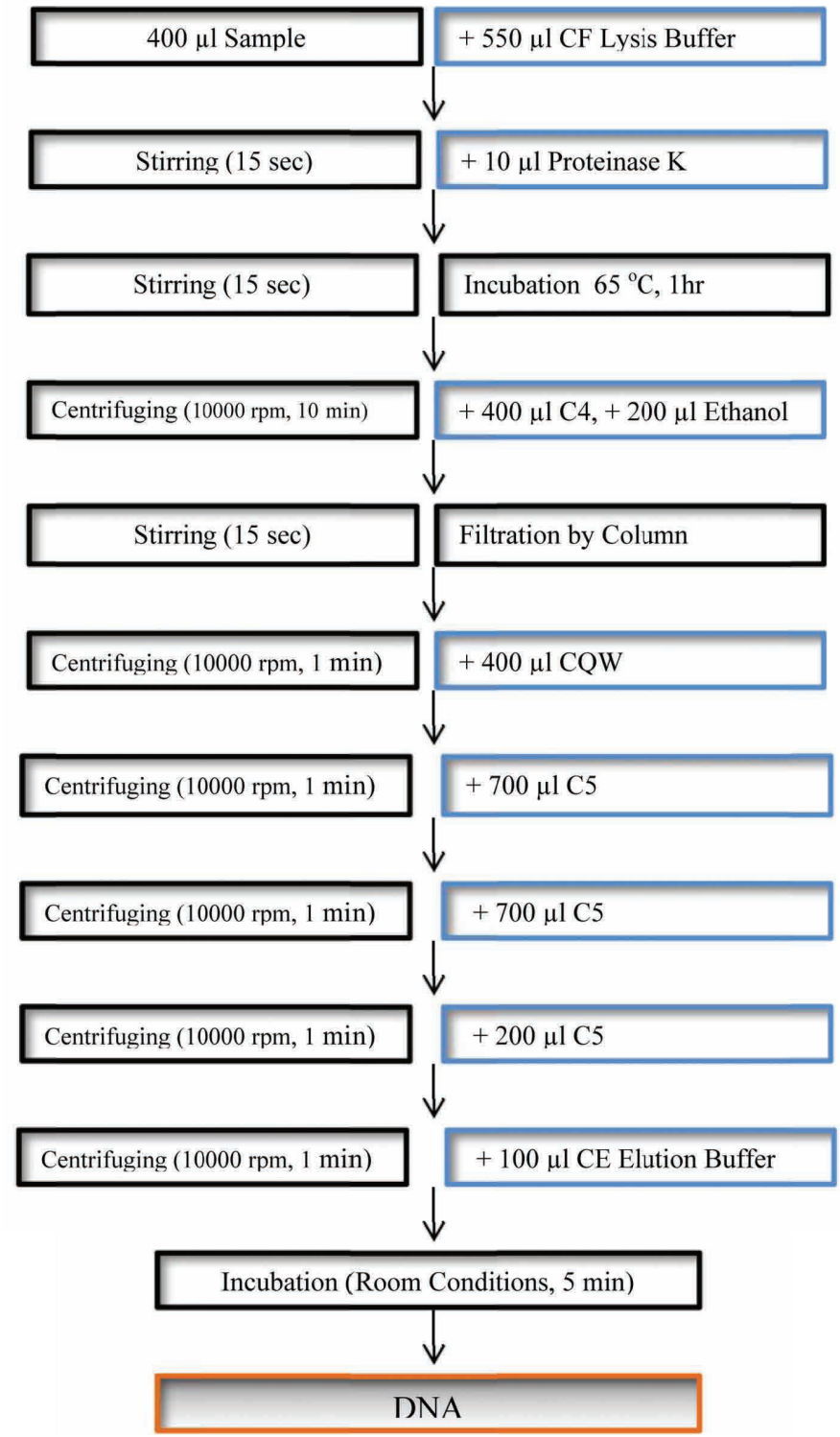


Fig 1. DNA isolation procedure

Şekil 1. DNA izolasyonu prosedürü

The extracted DNA samples were amplified by Real Time PCR (Agilent Stratagene Mx3000P) using the procedure given in Chipron LCD Array Meat Species 1.6 Kit (Chipron GmbH, Germany). Since the kit is ready to use, 12.5 µl of Chipron 2x all in one master mix, 1.5 µl of primer mix and 8 µl of sterile water were put into an Eppendorf tube, respectively. This prepared solution of 22 µl was pipetted to each of the plate wells following addition of 3 µl of DNA template. The plate was closed and was installed in Real Time PCR [62]. Thermal processing was given as 1 cycle at 96°C for 3 min, then 30 cycles at 94°C for 30 sec, 57°C for 45 sec and 72°C for 45 sec, and finally 72°C for 3 min [63].

Twenty two microliter of hybridization solution and 2 µl of modulator solution were added into an Eppendorf tube. This 24 µl of mix was pipetted to each of the plate wells following the addition of 10 µl of extracted DNA samples. Chip in the kit was placed in the chip box. 30 µl of each of the plate wells was pipetted onto the lower left hand corner of each of the eight patterns *(Fig. 2)*. Chip box was closed, allocated to standby at 35°C for 30 min, washed, dried, and then placed in the box again.

Putting the dilution solution into the Eppendorf tube 30 µl of annealing solution was pipetted into each of the patterns of the chip and allowed to standby for 5 min. After the incubation completed washing procedure was done, and chip was centrifuged for 15 sec, allowed to dry, and placed in the box again.

Thirty microliter of staining solution was put into each of the patterns of the chip, and the chip was allowed to standby for 3 min in room conditions. Following staining procedure, it was kept in washing box for 15 sec, and then centrifuged for 15 sec for drying.

Evaluation of the Results

Chipron LCD Array System can detect cattle, buffalo, pig, sheep, goat, horse, donkey, rabbit, hare, chicken, turkey, goose, and two duck varieties in food sample. The detection in this system is based on specific sites within 16S rRNA mitochondrial locus of all meat species in the analyzed food sample. A dark precipitate is formed by the enzyme substrate provided in the test kit, and it indicates a positive hybridization reaction. After staining procedure completed the chip was read with the scanner, and analysis was done by the software from the "Analysis-Package" provided by Chipron. Three different spots on the chip are called the control points (C) to detect a positive reaction which are located in upper-left, upper right and lower right corners, respectively. If no darker visualization occurs, the test should be repeated. The animal species was identified according to *Fig. 2* and *Table 1*.

Verification by FDS Detection System (Real Time PCR)

Randomly selected 11 samples which analysed by DNA Microarray method were verified by Iontek FDS

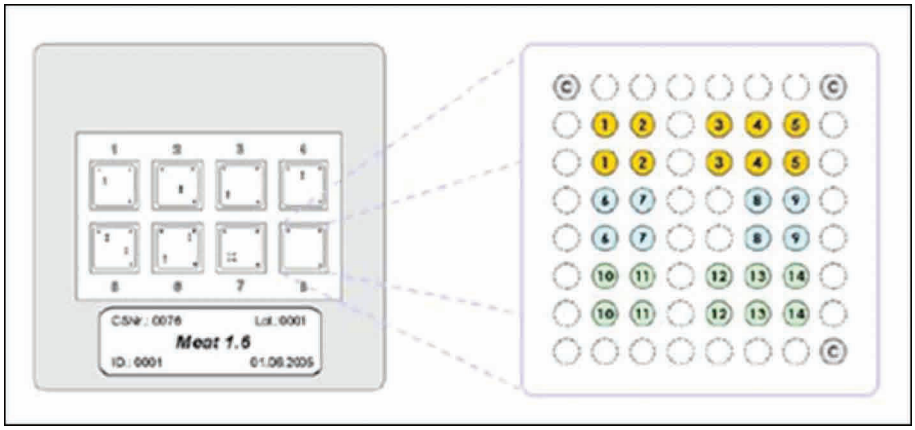


Fig 2. Spotting points of LCD-array meat 1.6

Şekil 2. Çip noktaları ve et tür eşlemeleri

Table 1. *Capture probes*
Tablo 1. *Çip noktalarına karşılık gelen et türleri*

Well No	Probe	Specificity	Well No	Probe	Specificity
01	Beef	*Bos taurus*	08	Rabbit	*Oryctolagus cuniculus*
02	Buffalo	*Bubalus bubalis*	09	Hare	*Lepus europaeus*
03	Pork	*Sus scrofa*	10	Chicken	*Gallus gallus*
04	Sheep	*Ovis aries*	11	Turkey	*Meleagris gallopavo*
05	Goat	*Capta hircus*	12	Goose	*Ansa albifrons*
06	Horse	*Equus caballus*	13	Mall. Duck	*Anas platyrhyncos*
07	Donkey	*Equus asinus*	14	Musc. Duck	*Cairina moschata*
			C	Hyb-Cont.	Functional controls (Hybridisation + Stain)

Detection System (Real Time PCR) method. The DNA which previously isolated by using Eurofins GeneScan DNA Isolation Kit (Catalog No: 5224400605) stored at -20°C were used. The procedure given by Iontek Fluorion Meat Species Identification QLP 1.0 Kit (Catalog No: F0560102) was followed up. Positive and negative controls were run in duplicate whereas DNA samples were run in triple. All the solutions and materials in the kit were dissolved before use. 23 µl of PCR master mix including 12.5 µl of PCR mix, 4 µl of detection mix and 6 µl of sterile water was pipetted into each of the plate wells. Two microliter of previously extracted DNA was added onto each. The tubes were closed off tightly and placed in Iontek FDS Real Time PCR System. The thermal processing was given as one cycle at 95°C for 15 min, then 40 cycles at 95°C for 25 sec and 62°C for 20 sec. The analysis was done by the FDS software from the "Analysis-Package" provided by Iontek [63].

RESULTS

The results obtained by DNA Microarray indicated that 39 out of 73 samples (53.4%) were labelled incorrectly, and adulteration was made in contrary to the notifications on the label. The adulteration was detected mostly in meat balls (87.5%), ground meat (72.7%), salami (57.1%), sausages (50%) and fermented sausages (30.3%), respectively. The results are presented in *Table 2*. It was mostly seen that meat balls and ground meat have significantly potential risk for adulteration. Following them fermented sausage samples showed incorrect labelling with the range of 30%. On the other hand, these three types of food were having a claim of 100% beef on the labels. Hence, mostly detected meat species in meat ball, ground meat and fermented sausage samples were chicken, turkey and sheep species. No pig and equine species were detected in 79 samples. Randomly selected 11 samples out of 79 were verified by Iontek FDS Detection System (Real Time PCR). The results obtained by both of DNA Microarray and Real Time PCR were identical to each other with the range of 100%. The verified results are also given in *Table 3*.

DISCUSSION

The inspection of the declared composition of food stuff as notified on its label is officially an obligatory task order to protect the public benefits and health against adulteration and infectious diseases caused by zoonoses. In this study, we compared DNA Microarray (Chipron, Germany) method to Iontek FDS (Real Time PCR) System for routine use.

A variety of animal species present in the meat and meat products were examined in the past. In Turkey many studies related to the animal species detection were carried out by official authorities and academic research institutes. In Izmir province totally 116 samples of joint meat, ground meat, sausage pulp, meat ball, hamburger, canned meat, Turkish Doner, fermented sausage, salami, roasted meat and sausage were reviewed for the notifications declared on the label. The results showed that 18 samples (15.5%)

Table 2. *Results of DNA microarray*
Tablo 2. *DNA mikroarray sonuçları*

Description of Sample	No of Sample	No of Samples Eligible	No of Samples İneligible
Meat ball	16	2	14 (87.5%)
Ground meat	11	3	8 (72.7%)
Salami	7	3	4 (57.1%)
Sausage	6	3	3 (50.0%)
Fermented sausage	33	23	10 (30.3%)
Total	73	34	39 (53.4%)

Table 3. *Compared results between DNA microarray and real time PCR*
Tablo 3. *Real time PCR ile karşılaştırmalı sonuçlar*

No	Description of Sample	Chipron LCD Array Meat Species Kit		Iontek Fluorion Meat Species QLP Kit	
		Beef	Chicken	Beef	Chicken
1	Fermented Sausage, 100% Beef	+	-	+	-
2	Fermented Sausage, 100% Beef	+	-	+	-
3	Fermented Sausage, 100% Beef	+	-	+	-
4	Fermented Sausage, 100% Beef	+	-	+	-
5	Fermented Sausage, 100% Beef	+	-	+	-
6	Fermented Sausage, 90% Chicken + 10% Beef	+	+	+	+
7	Fermented Sausage, 100% Beef	+	-	+	-
8	Fermented Sausage, 100% Beef	+	-	+	-
9	Fermented Sausage, 78% Chicken + 22% Beef	+	+	+	+
10	Salami, 30% Beef + 70% Chicken	+	+	+	+
11	Sausage, 95% Chicken + 5% Beef	-	+	-	+

were labelled incorrectly and containing animal species other than the declared ones like horse flesh (9.5%), pork (9.5%), chicken (23.8%) in the sliced meat; pork and beef (4.8%) whereas chicken and beef mix (9.5%) in ground meat. A hundred percent of sausage pulp samples were containing chicken and beef together. It was reported that 4.8% of meat balls had pork meat, and chicken was also detected in Tas Kebap, Turkish Döner, salami, roasted grill and sausages in contrary to the notification on the label. Consequently, meat and meat products produced in Izmir were mixed with meat belonging to various animal species [22]. Some other studies carried out in Istanbul and Bursa provinces totally 100 samples composed of 28 fermented sausages, 25 salami, 9 raw meat, 16 ground meat, 3 pastrami, 7 ham, 7 grilled meat, 5 canned meat were tested. The obtained results indicated that 11 fermented sausages (39.2%), 8 sausages (62.9%), 2 raw meat (22.2%) and 1 ground meat were contrary to the declarations on the label, and 22% of all collected samples were carrying potentially high risk for health [23]. Another study reported that 65 of ground meat, 35 of meat ball pulp, 50 of fermented sausage pulp, 125 of fermented sausage, 75 of salami and 60 of sausage totally making 410 samples the adulteration ratio was determined to be 19.2% (79 samples) [24]. A study done in USA indicated that 62% of the meat and meat products had only one foreign species, 36% had two, and 2% had three. A similar study in the States also showed that the adulteration ratio has increased up to 46.4% [25,26,29]. In Brasil commercial samples of swine hamburgers showed no adulteration with bovine, chicken, swine or horse meats, and expectation of hamburger adulteration was not confirmed [28]. It was found that our study and those carried out in Turkey and at abroad have delivered identical results. It is understood that the adulteration is a key tool in reducing the costing in the production of meat and meat products, preferably tried in contrary to the notifications on the label poultry, and especially encountered in processed meat products. This fact could somewhat explain the risk of zoonosis harmful to public health.

The detection of animal species in meat and meat products have been done by a variety of analytical methods. Each method has relatively advantages and disadvantages as compared to each others. Recently, Real Time PCR, a DNA based molecular technique, has been very popular in food analyses as a futher step of the conventional PCR. It brings away the demand for immunological and electrophoretic methods, and minimizes the risk of contamination during the testing [63]. Real Time PCR has a sensitivity in detection of meat species by 0.1% whereas ELISA can do it less sensitive by 2% [21,32,35,64-71].

DNA Microarray and Real Time PCR methods differentiate from each other in simultaneously detection of animal species in one reaction. The only common similarity between them is the step of DNA isolation. Microarray Analysis can enable us for detecting more than one species in one reaction only whereas Real Time PCR requires specially designed primers and probes needed to simultaneously amplify the specially selected regions of DNAs belonging to different species. This difference means longer time needed in the optimization step of primers and probes [30]. DNA Microarray can deliver the results faster and more sensitive using amplified DNA by conventional PCR technique [62]. Therefore, DNA Microarray method has been widely preferred for understanding mechanisms, detection of foodborne microbial pathogens and food safety studies, nutreaceuticals and functional foods as well as following up the different expression levels of DNA in bacteria, yeasts, plants and human; genetic and mutation analyses; environmental studies; identification of antimicrobial genes, proteomics, protein-nucleic acids, protein-protein interactions, biochemical analysis of protein functions and drug development [44-47,52,72-75]. In the recent years studies in the literature related to DNA Microarray have focused on the detection of adulteration in seafoods and meat and meat products [48-51]. In our study, DNA Microarray was used to determine adulteration in some selected meat products by making verification by Real Time PCR method. It was found that both of the methods delivered the identical results. Therefore, it was seen that DNA Microarray method is fast, accurate and safe by introducing this technique firstly to Turkey for detection of foreign animal species. DNA Microarray was preferred for higher capacity of data analysis, suitability for species detection, re-usability of the results, higher analysis throughput and becoming user-friendly.

In conclusion, adulteration is a serious food safety and quality issue with an increasing prevalance in meat and meat products all over the world. Regular controls for adulteration in meat and meat products should be frequently and intensively done due to the significant increasing demand for the meat. It was found that the results obtained by DNA Microarray and Real Time PCR assays were identical with each other, and both methods should extensively be promoted for the detection of animal species in the meat and meat products.

Acknowledgements

We are thankful to Hürriyet Gazetecilik A.Ş for supporting this study.

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Kafkas Univ Vet Fak Derg
19 (2): 253-258, 2013
DOI: 10.9775/kvfd.2012.7620

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Effects of Losartan on Glycerol-induced Myoglobinuric Acute Renal Failure in Rats

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Makale Kodu (Article Code): KVFD-2012-7620

Summary

Myoglobinuric acute renal failure (mARF) is an uremic syndrome which develops due to damage of skeletal muscle. It was demonstrated that free radicals and nitric oxide (NO) play an important role in pathogenesis of mARF. Our aim was to investigate the effect of losartan, a drug known for its antioxidant effect, on mARF. In our study, a total of 34 male Spraque Dawley rats were divided into four groups. 1st and 2nd groups were injected with saline, 3rd and 4th groups were injected with intramuscular glycerol. One and 24 hours later, 1st and 3rd groups received saline orally and 2nd and 4th groups have taken 10 mg/kg losartan. Urine was collected; the blood samples and kidneys of the rats were taken under the anesthesia. The levels of NO, arginine, asymmetric dimethylarginine (ADMA), glutathione (GSH) and malondialdehyde (MDA) were determined, renal functions and histopathological changes examined. In our study, we found that levels of urea, creatinine, and potassium, in serum samples and MDA and ADMA in renal tissue were increased in 3rd group when it's compared with the 1st group. Levels of sodium, arginine in serum samples and arginine in renal tissue were reduced 3rd group when compared with the 1st group. When 3rd and 4th groups were compared, serum creatinine was higher in the latter group whereas ADMA level in renal tissue was lower in the same group. We think that there is no positive effect of losartan on the pathogenesis of mARF.

Keywords: *Losartan, Myoglobinuric Acute Renal Failure, Free radicals, Nitric oxide, Asymmetric dimethylarginine*

Ratlarda Gliserol ile İndüklenen Miyoglobinürik Akut Böbrek Yetmezliği Üzerine Losartanın Etkileri

Özet

Miyoglobinürik akut böbrek yetmezliği (mABY) iskelet kaslarının hasarlanmasıyla oluşan üremik bir sendromdur. Nitrik oksit (NO) ve serbest radikallerin mABY patogenezinde önemli rol oynadığı gösterilmiştir. Çalışmamızda antioksidan etkileri olduğu bildirilen losartanın mABY'deki etkilerini araştırmayı amaçladık. Çalışmamızda 4 grupta toplam 34 adet erkek Spraque Dawley sıçan kullanıldı. 1. ve 2. gruba fizyolojik serum, 3. ve 4. gruba gliserol intramüsküler enjekte edildi. 1 ve 24 saat sonra 1. ve 3. gruba distile su, 2. ve 4. gruba 10 mg/kg losartan oral yolla verildi. İdrarları toplandı, anestezi altında kan ve böbrekleri alındı. NO, arginin, asimetrik dimetilarginin (ADMA), glutatyon (GSH), malondialdehit (MDA) düzeyleri, histopatolojik değişiklikler ve böbrek fonksiyonları incelendi. Çalışmamızda 1. grup ile karşılaştırıldığında 3. grupta serumda üre, kreatinin, potasyum, böbrek dokusunda MDA ve ADMA düzeylerinde artma; serum sodyum, arginin, böbrek dokusunda arginin düzeylerinde azalma gözlendi. 3. grup ile karşılaştırıldığında 4. grupta serum kreatinin düzeyinde artış, böbrek dokusunda ADMA düzeyinde azalma saptandı. Bu sonuçlar ışığında losartanın mABY patogenezi üzerinde olumlu etkilerinin olmadığını düşünüyoruz.

Anahtar sözcükler: *Losartan, Miyoglobinürik Akut Böbrek Yetmezliği, Serbest radikaller, Nitrik oksit, Asimetrik dimetilarginin*

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INTRODUCTION

Rhabdomyolysis is the clinical and laboratory condition caused by extracellular distribution of intracellular elements from striated muscle due to traumatic and non-traumatic reasons [1]. Rhabdomyolysis causes tubular obstruction, tubular necrosis and renal vasoconstriction which results in acute renal failure, a potential complication of rhabdomyolysis [2,3].

The most common method of setting up experimental mARF is intramuscular injection of hypertonic glycerol solutions in rats [4]. Intramuscular glycerol injection results in myolysis and hemolysis. Iron released from the degradation of myoglobin and hemoglobin catalyses Haber-Weiss and Fenton reactions and thus resulting in free radical formation, lipid peroxidation and detorioration of renal functions [3,4].

Nitric oxide is an intermediate product synthesized from L arginine [5]. NO is reported to have a role in normal renal function [6], reducing the rate of renal interstitial fibrosis [7] and in higher concentrations, it is known to participate in proximal renal tubular injury [6]. ADMA is a methylation product of arginine. Studies have shown that ADMA is a good indicator of increased oxidative stress and that it can be a potential marker during the progress of acute renal failure [8]. ADMA, as an inhibitor of nitric oxide syntase, contributes to reduced production of NO [9]. Free radicals and reduced NO levels have important roles in pathogenesis of experimental ARF [10]. Endothelial disfunction is correlated with ADMA levels in patients with ARF [11].

Losartan is a non-peptide angiotensin II receptor antagonist [12]. Losartan is reported to increase NO release [13]. Losartan is known to decrease the production of reactive oxygen species and supress lipid peroxidation [14]. Losartan is also reported to prevent progression of many chronic renal diseases such as diabetic nephropathy [12]. In rats with renal ablation, losartan administration has decreased blood pressure levels and proteinuria [15].

The aim of this study is to evaluate the effects of losartan on oxidative stress, renal functions, NO, arginine, ADMA, histopathological changes of renal tissue.

MATERIAL and METHODS

This study was approved by Trakya University School of Medicine Animal Care and Use Committiee (2009/065). The laboratory conditions were standardized as 22+/- 1°C and 12 h light-dark cycles. Male Spraque Dawley rats weighting 370-450 g were fed by standard rat chow and tap water. Rats were randomly devided into 4 groups. There were 7 rats in 1st and 2nd groups, 10 rats in the 3rd and 4th groups. The rats were deprived of water 24 h prior to saline injection for 1st and 2nd groups and im glycerol injection for the other groups.

One and 24 h after the im injection of saline, distiled water was administered to the 1st group (Control) via gavage and 10 mg/kg losartan (Cozaar® tablets, Merck Sharp & Dohme) was administered to the 2nd group (Control+ Losartan) via oral gavage after being dissolved in distiled water. 1 and 24 h after the glycerol injection, distiled water was administered to 3rd group (ARF) and 10 mg/kg losartan was administered to 4th group (ARF+Losartan).

After 24 h urine collection (48 h after glycerol injection) 10 mg/kg xylazine and 50 mg/kg ketamine were used to anesthetize rats. Blood sample were collected by cardiac puncture. Serum and urine samples stored at -80°C. Serum urea, creatinine, Na^+, K^+, urine creatinine and urine Na^+ levels were measured using automated analyzer.

Bilateral nephrectomy was performed to rats and then kidneys were cut longitudinally. One half of the right kidney is fixed in 10% formaline solution. The other half of the right kidney and left kidney were stored at -80°C until tissue analysis.

Renal tissues samples were homogenized in buffer solutions (0.15 M KCl solution for MDA and GSH; 50mM phosphate buffer (pH 7.4) for NO; phosphate buffer (pH 7.0) for arginine and ADMA). Homogenates were centrifuged (1.500xg for 10 min, at 4°C), supernatant of the homogenates were collected for the MDA, GSH, NO, arginine, ADMA measurements.

Protein content of the tissue samples was samples determined acording to the method of Lowry [16]. Spectrophotometric detection of the color produced by the interaction of free SH groups in the tissue homogenates with Ellman reagent was used to note GSH content [17]. Nitrate and nitrite detection was done by the method described by Cortes and Wakid [18]. Arginine and ADMA measurements were made by the method described by Teerlink [19].

Kidney slides were stained by Hematoxylin eosin and were blindly analyzed twice by the same pathologist under light microscope. Proximal tubular necrosis was identified and was conveyed as a percent of the total sum of all proximal tubules in x10 magnification. Similarly, proteinaceous casts in the distal tubules were analysed and tubules containing casts were conveyed as a percent of total number of distal tubules.

Statistical Analysis

Statistical analyses were performed using Statistica 7.0 (Licence code: 31N6YUCV38) programme. Quantitative data was given as mean±SD. Normal distribution of variances was determined using Kolmogorov-Smirnov test. Since normality assumption is not satisfied, Kruskal Wallis test was used to investigate inter-group differences. To determine which group was causing the difference, Mann-Whitney U test were used. $P<0.05$ was considered as statistical significance value.

RESULTS

Renal function markers of the study groups are shown in *Table 1*. Urine could not be collected in ARF and ARF+Losartan groups. In regards to urine sodium, creatinine, creatinine clearance and fractional sodium excretion no statistically significant difference was detected between the control and losartan groups. Compared to control group, serum urea levels were significantly elevated in ARF and ARF+Losartan groups (P=0.017 and P=0.007, respectively). No significant difference in serum urea levels was detected between ARF and ARF+Losartan groups. In a similar fashion, serum creatinine levels were significantly elevated in ARF and ARF+Losartan group (P=0.016 and P=0.008, respectively). Serum creatinine level was higher in ARF+Losartan group than ARF group which was statistically significant (P=0.034). Compared to control group, serum sodium levels were significantly decreased in ARF and ARF+Losartan groups (P=0.016 and P=0.029, respectively). No significant difference in serum sodium levels was detected between ARF and ARF+Losartan groups. Compared to control group, serum potassium levels were significantly elevated in ARF and ARF+Losartan groups (P=0.016 and P=0.008, respectively). No significant difference in serum potassium levels was detected between ARF and ARF+Losartan groups (P=0.157).

Biochemical parameters of the study groups are shown in *Table 2*. No statistically significant difference was detected between study groups in renal tissue GSH levels. Compared to control group, renal tissue MDA levels were significantly increased in ARF and ARF+Losartan groups (P=0.017 and P=0.008, respectively). No significant difference in renal tissue MDA levels was detected between ARF and ARF+Losartan groups. No significant difference was detected among groups in regard to serum NO, renal tissue NO, urine NO and serum ADMA levels. When ADMA levels in renal tissue were compared between groups, an increase in ARF group and a decrease in ARF+Losartan group were detected in comparison to control group, both of which were statistically significant (P=0.030 and P=0.014, respectively). Compared to control group, renal tissue arginine levels were significantly decreased in ARF and ARF+Losartan groups (P=0.017 and P=0.006, respectively). No significant difference in renal tissue arginine levels was detected between ARF and ARF+Losartan groups. When serum arginine levels were compared between groups, a decrease in ARF group was detected in comparison to control group (P=0.017). There was no significant difference in serum arginine levels between the control, ARF and ARF+Losartan groups.

Histopathological findings of the study groups are shown in *Table 3*. In our study, no change in glomeruli or tubular necrosis was seen in HE stained kidney slides of control and losartan groups. Tubular necrosis was present in HE stained kidney slides of ARF and ARF+Losartan groups. There was also evident dilatation of the tubules with accumulation of dense, proteinaceous casts in distal tubules. Hydropic cytoplasmic swelling and vacuolisation in distal tubule cells was prominent. Peritubular stroma showed edema and vascular congestion. Tubular cellular regeneration was noted. Glomerular congestion was more pronounced in ARF group *(Fig. 1)*.

Table 1. *Serum renal function markers of the study groups*
Tablo 1. *Çalışma gruplarının serum renal fonksiyon belirteçleri*

Renal Function Markers	Control (n=7)	Losartan (n=7)	ARF (n=3)	ARF+Losartan (n=4)
Urea (mg/dl)	22.71±2.56	24.29±4.19	595.33±31.56*	586.75±48.49*
Creatinine (mg/dl)	0.40±0.06	0.39±0.05	6.87±0.44*	8.23±0.21*#
Na^+ (mmol/L)	139.86±4.06	136.71±10.01	123.00±7.93*	132.00±3.92*
K^+ (mmol/L)	4.83±0.59	4.89±0.72	8.80±0.92*	7.85±0.76*

*Data are given as mean±SD, * P<0.05 compared with control group, # P<0.05 compared with ARF group*

Table 2. *Serum and kidney tissue biochemical parameters of the study groups*
Tablo 2. *Çalışma gruplarının serum ve böbrek dokusu biyokimyasal parametreleri*

Biochemical Parameters	Control (n=7)	Losartan (n=7)	ARF (n=3)	ARF+Losartan (n=4)
Serum				
NO (µmol/mg)	2.25±0.63	2.35±0.43	1.55±0.48	4.22±2.66
ADMA (µM)	1.42±0.31	1.64±0.30	3.87±3.97	0.87±0.47
Arginine (µM)	87.97±10.10	95.30±9.90	38.81±32.51*	79.64±12.36
Kidney Tissue				
MDA (nmol/g doku)	0.58±0.12	0.47±0.05	1.37±0.20*	1.20±0.12*
GSH (µmol/g doku)	62.56±10.17	58.89±7.76	59.85±6.02	70.47±9.06
NO (µmol/mg)	97.14±49.00	62.37±28.77	41.21±37.69	44.94±33.02
ADMA (µmol/mg protein)	1.25±0.29	0.72±0.57	3.62±2.84*	0.57±0.25*
Arginine (µmol/mgprotein)	190.10±18.98	181.38±22.37	67.87±25.01*	70.57±10.42*

*Data are given as mean±SD, *P<0.05 compared with control group*

Table 3. *Renal histopathological findings of the study groups*
Tablo 3. *Çalışma gruplarının renal histopatolojik bulguları*

Histopathological Findings	Control (n=7)	Losartan (n=7)	ARF (n=7)	ARF+Losartan (n=7)
Necrosis (%)	0.00±0.00	0.00±0.00	78.00±5.29*ǂ	80.00±7.16*ǂ
Cast (%)	1.42±0.53	1.29±0.49	77.67±2.51*ǂ	77.50±2.08*ǂ

*Data are given as mean±SD, * P<0.05 compared with control group, ǂ P<0.05 compared with losartan group*

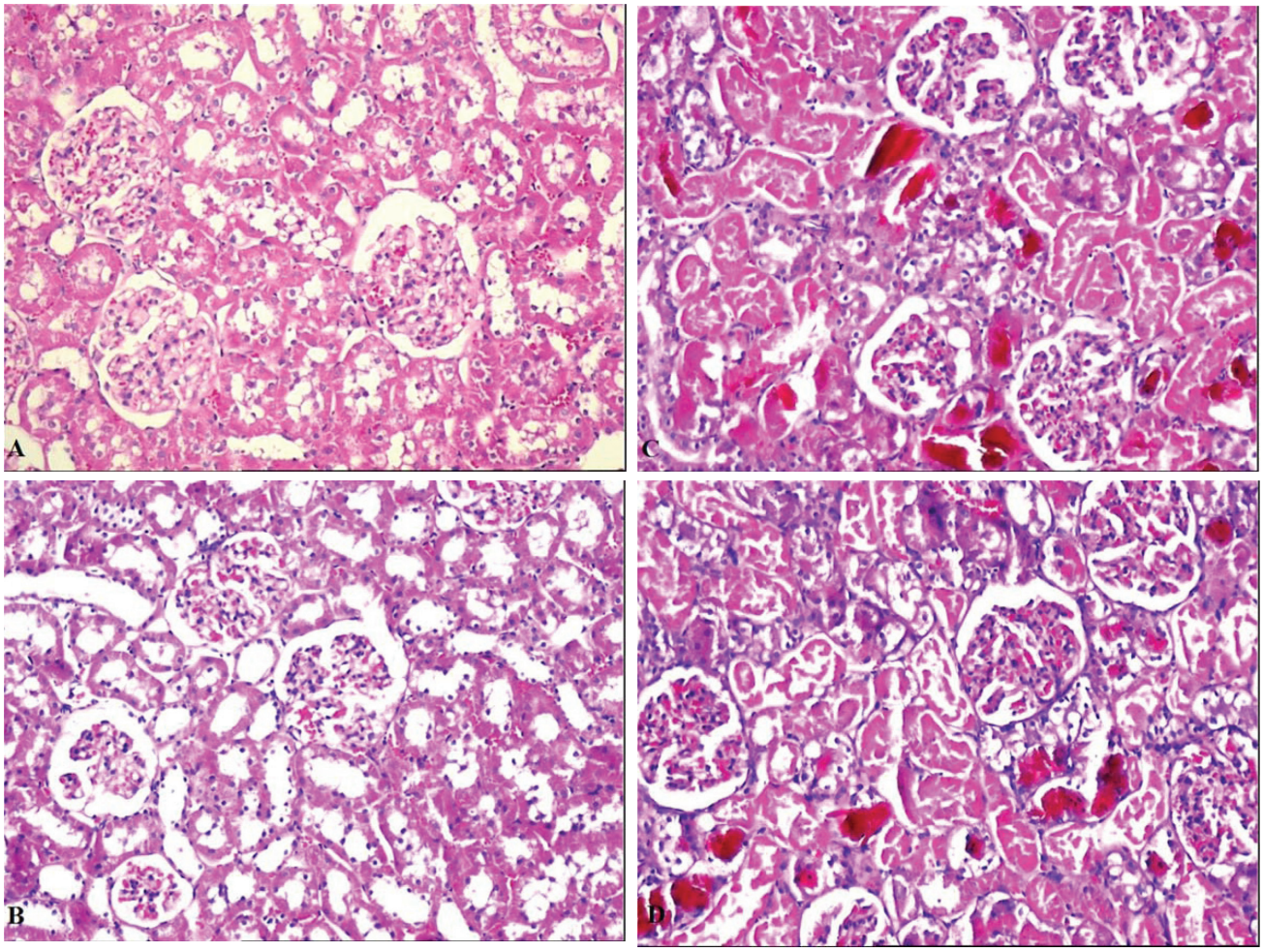


Fig 1. Histopathological appearance of the study groups
A) Control group, **B)** Losartan group, **C)** ARF group, **D)** ARF + Losartan group
Şekil 1. Çalışma gruplarının histopatolojik görünümü
A) Kontrol grubu, **B)** Losartan grubu, **C)** ABY grubu, **D)** ABY + Losartan grubu

DISCUSSION

Oxidative stress and NO play important roles in ARF due to myoglobinuria [3]. Losartan is a non-peptide angiotensin II receptor antagonist. Losartan has been shown to increase NO release and decrease oxidative stress [20]. As far as our knowledge, this is the first study in which the effect of losartan on experimental mARF is studied. It is also the first study to investigate ADMA levels in mARF pathogenesis. In our study, rhabdomyolysis was shown to cause renal tubular injury, decrase in renal functions, elevate oxidative stress and ADMA levels and diminish arginine levels. While losartan was shown to decrease ADMA levels, it also increased serum creatinine. Losartan seemed to have no effect on other findings.

In our study, MDA levels were significantly reduced in healthy subjects after administration of losartan. In a study performed by Saleh et al.[21], investigating the renoprotective effects of losartan, it was reported that in healthy rats given i.p. losartan either as a single dose of 60 mg/kg or in divided doses (10 mg/kg daily for 6 days), losartan showed no significant effect on renal MDA levels. The decrease of MDA in our study may be due to the differences in dose, timing, method and period of administration.

Seven of the rats in our ARF group died within the first 48 h due to complications. Previous studies reported lower mortality rates in mARF model [22-24]. Rats used in these studies are reported to weigh between 160 and 300 g whereas the rats in our study weighed between 370 and 450 g. High mortality rate of our study may be a result of high dose administered i.m. glycerol due to higher animal weight. High dose glycerol injection intramuscularly causes muscle damage which results in the release of K^+, myoglobin and other cytokines into systemic circulation. We believe increased serum K^+ levels result in cardiotoxicity and released myoglobin promote tubular necrosis and cast formation, thus precipitating renal failure. In our laboratory, the rate of tubular necrosis and cast accumulation was higher than any ARF groups formed in previous studies [22,25].

The increase in blood urea and creatinine levels in ARF in control groups is consistent with previous studies performed on this model [26-28]. Elevated K^+ is known to be a result of decreased urine output in ARF and severe muscle damage [29]. Decreased blood Na^+ level may be caused by tubular disfunction which is consistent with previous studies [25,30].

The significant increase in renal MDA levels in ARF group is consistent with previous studies [28,31]. Free iron ions released from the degredation of significant amount of myoglobin cause free radical formation and lipid peroxidation which may contribute to the increase in MDA levels.

A significant decrease in GSH has been reported in experimental studies on mARF [4,32]. However, no significant decrease in GSH was noted in our study which may be a result of low number of samples due to high mortality in this group.

Nitric oxide is an intermediate product synthesized from L arginine [5]. NO plays a key role in the regulation of renal blood flow and glomerular filtration, production of renin and angiotensin II and tubular reabsorption [33]. Heme proteins produced in mARF model consume NO and cause renal vasoconstriction via cytokines [34].

Previous studies stated that L-arginine treatment normalised renal oxidative stress and structural damage and reduced renal function loss [32,35]. When ARF is formed by myoglobin infusion, L-arginine treatment may prevent renal vasoconstriction and disfunction via NO. In our study, decrease of L-arginine may have exacerbated renal ischemia and thus aggravated tubular damage [32,36]. Previous studies performed by this model showed a decrease in renal NO levels [37]. It has been reported that decrease of NO levels and inhibition of NOS worsens pathogenesis [38]. There being no significant difference in NO levels may be attributed to high mortality rate seen in this group.

ADMA is a potent endogenous NOS inhibitor. Elevated ADMA levels have been linked to oxidative stress and it is also reported to be a marker for renal failure [35,39].

In the experimental model of Volti et al. rats underwent to 45 min of renal ischemia fallowed by 30 min, 1 h, and 3 h of reperfusion [40]. ADMA levels showed significant difference in the groups which 60 and 180 min reperfusion were performed. This result is consistent with renal ADMA levels of the present study.

Six of the rats in ARF+Losartan group died in 48 h because of the complications. When we reviewed the literature we could not find any study analysing the effects of losartan on experimental mARF models. On the experimental mARF models decreased renal perfusion due to increased renal angiotensin levels was reported. Increased angiotensin II, as a vasoconstrictor in renal vascular bed is known to reduce renal function [41]. Losartan used as a therapeutic agent of hypertension and minimal renal changes in renal transplant recipients, leads to elevated serum creatinine levels. Elevated levels of serum creatinine can be considered a result of low glomerular pressure [42]. In the present study elevated serum creatinine levels can be considered as a result of losartan related to low intraglomerular pressure and hemodynamic effects.

Yavuz et al.[43] performed a diabetic nephropathy model on rats. They administered 10 mg/kg losartan by gavage for 8 weeks and reported decreased MDA levels. In the study of Khattab et al.[44] rats were subjected to hypertension by administering L-NAME. In this study high level of MDA in renal tissue was decreased by a 10 mg/kg losartan treatment lasting 6 weeks. Rashchizadeh et al.[45] reported that losartan significantly decreased MDA levels in renal transplant recipients. Additionally creatinine levels were detected to be increased in the group is treated by losartan for 6 weeks. Preventive effect of losartan on lipid peroxidation reported in these studies is not correlated with our results. In the study of Raschizadeh et al.[45], a group of rats were treated with losartan and creatinine levels were increased. This is consistent with our study.

In the study of Ito et al.[46] untreated patients with essential hypertension were treated with perindopril, losartan, bisoprolol and they reported that perindopril and losartan reduced the ADMA levels. In the study serum MDA levels decreased significantly only in the group treated with losartan.

As mentioned above previous studies suggest that losartan has a protective role in many experimental models. In the present study, losartan is considered to worsen the pathogenesis of mARF and elevated serum creatinine levels are a sign of this entity.

It may also be due to different factors affecting experimental mARF pathogenesis which is not in correlation with other experimental studies. In previous studies, losartan was shown to increase NO levels by decreasing ADMA, which results in fluid accumulation in damaged muscles. We believe this condition exacerbates hypovolemia. We believe that hypovolemia causes renal vasoconstriction which worsens mARF pathogenesis.

We believe the effect of losartan on mARF model should be thoroughly investigated using variations in dosage, administration route, timing and period of administration.

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Kafkas Univ Vet Fak Derg
19 (2): 259-262, 2013
DOI: 10.9775/kvfd.2012.7637

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

In Vitro Antimutagenicity of *Allium Tuncelianum* Ethanol Extract Against Induction of Chromosome Aberration by Mutagenic Agent Mitomycine C

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Makale Kodu (Article Code): KVFD-2012-7637

Summary

Allium Tuncelianum is a garlic species which is locally produced in Tunceli, Turkey. Garlic contains several biologically active compounds known as organosulfur compounds which are reported to have anticarcinogenic and antimutagenic effects. Present study examined the *in vitro* antigenotoxic activity of ethanolic extracts of *A. Tuncelianum* in human lymphocyte cells by chromosome aberration test. Samples of *A. Tuncelianum* were extracted by ethanol using soxhlet extractor. The extract was tested on human lymphocyte cell culture *in vitro* chromosome aberration test. The effect of ethanol extract of *A. Tuncelianum* at concentrations of 0.10, 0.15, 0.20, 0.25 µl/mL were screened for chromatid and chromosome breaks, chromosome exchange as well as chromatid union and polyploid cells against negative acetone and positive control Mitomycine C. A significant decrease in the frequency of chromosomal aberration was observed for all treatments with *A. Tuncelianum* ethanol extract at 24 h. In conclusion, ethanol extract of *A. tuncelianum* significantly reduced the chromosomal aberration rate as compared with the culture treated with MMC. The results could also indicate that *A. Tuncelianum* could be protective against mutagenic and carcinogenic compounds when consumed through the diet.

Keywords: *Allium Tuncelianum, Antimutagenic, Chromosomal aberration, Toxicity*

Mutajenik Ajan Mitomisin C ile Uyarılmış Kromozom Aberrasyona Karşı *Allium Tuncelianum* Etanolik Ekstraktının *İn Vitro* Antimutajenik Etkinliği

Özet

Allium Tuncelianum, Tunceli/Türkiye'de lokalize olmuş bir sarımsak türüdür. Sarımsak bitkisi organosülfür bileşikler adı verilen, antikarsinojenik ve antimutajenik etkileri olduğu daha önceden bildirilmiş olan biyolojik yönden aktif komponentler içermektedir. Bu çalışmada *A. Tuncelianum* ethanolik ekstraktının *in vitro* antigenotoksik aktivitesi, kromozom aberrasyon testi ile insan lenfosit hücre kültüründe çalışılmıştır. *A. Tuncelianum* ekstraktı etanol ile soxhlet kullanılarak elde edilmiştir. Bu araştırmada *A. Tuncelianum*'un etanolik ekstraktı insan lenfosit hücrelerinde kromozom aberrasyon testi ile antigenotoksik aktivitesi çalışılmıştır. *A. Tuncelianum*'un etanolik ekstraktı, 0.10, 0.15, 0.20 ve 0.25 µl/mL konsantrasyonlarda aseton (negative kontrol) ve Mitomisin C (pozitif kontrol)'ye karşı kromatid ve kromozom kırıkları, kromozom değişimi, kromatid birleşimi ve poliploidi bakımından taranmıştır. Uygulamadan 24 saat sonra, kromozomal aberrasyon frekansı bakımından *A. Tuncelianum*'un etanolik ekstraktı tüm konsantrasyonlarda önemli bir düşüş ortaya koymuştur. Sonuç olarak *A. Tuncelianum*'un etanolik ekstraktı MMC uygulanan kültür hücreleri ile karşılaştırıldığında kromozomal aberrasyonda önemli bir düşüşe neden olmuştur. Bu sonuçlara gore *A. Tuncelianum*'un mutajenite ve karsinojeniteye karşı koruyucu gıda olarak diyetle tüketilebileceği düşünülmektedir.

Anahtar sözcükler: *Allium Tuncelianum, Antimutajenik, Kromozomal aberrasyon, Toksisite*

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INTRODUCTION

It is known that diet rich in vegetables and fruits has the potential to lower the risk of cancer [1]. Recently, there has been a great deal of interest about the anti-mutagenic and anti-carcinogenic potential of compounds derived from plants and natural food stuff [2]. Garlic which is produced and consumed worldwide has drawn attention for its protective effects against a number of disorders [1,3]. Epidemiological studies showed that garlic contains many biologically and pharmacologically active compounds [3]. It has been used for medical purposes since the ancient times, and its use for cancer treatment dates back to 3500 years ago [3]. Garlic has antimutagenic, anticarcinogenic, antithrombotic, antineoplastic and antioxidant properties [2,4]. Experimental and epidemiological studies have shown that there is a correlation between garlic consumption and lower rate of cancer incidences [1-3]. It is reported that organosulfur compounds found in garlic confer its antitumor action [4]. Furthermore, anti-mutagenic, anti-carcinogenic and antitoxic effects of garlic are also attributed to its active components including sulphydryil groups and organosulfur compounds as diallyl sulphide, diallyl disulphide, ajoene, allixin, allyl mercaptans and allyl methylsulphides found in the plant [5]. Components of garlic selectively inhibit tumor proliferation by a number of mechanisms including control of DNA repair, chromosomal stability and regulation of cell cycle [3]. Recent evidences suggest that inhibitory effect of garlic extract on adenosine deaminase and cyclic adenosine monophosphate cAMP phosphodiesterase enzymes play an important role in its biological and pharmacological actions [3]. *Allium tuncelianum* is grown in the eastern part of Turkey, and it is an endemic genus which is peculiar to this region [6]. It was reported that the biological activity of species varies with respect to the several factors including geographical location in which the plant grows, extraction method and section of the plant [7].

Genotoxic effects of chemical agents on human could be investigated through the screening tests which are utilized in genetic toxicology. These tests include chromosomal aberration test (CA), micronucleus test and sister chromatide exchange test (SCE) at the chromosomal level, Ames test at the gene level and single cell gel electrophoresis COMET assay at the molecular level [8]. Treatment of cells with a DNA-damaging agent could result in irreparable lesions in both strands of DNA chain. Chromosomal breaks due to this type of insults can be determined in cells during metaphase stage. Although various cell lines could be used for this test, use of human lymphocytes is mostly preferred [9]. The purpose of *in vitro* chromosomal aberration test in human lymphocytes is to determine structural chromosomal aberrations [10].

Although there are some studies about the various effect of garlic plant, *A. Tuncelianum* is an endemic genus which is specific to Tunceli district. There is no previously performed study on the effect of this genus for the anti-mutagenic effect.

In this study, the aim was to evaluate the antigenotoxic activity of crude ethanolic extracts of *A. Tuncelianum* on human lymphocyte cell culture *in vitro* by using chromosome aberration test.

MATERIAL and METHODS

Collection of Plant Material

Samples of *A. Tuncelianum* used in the study were collected from rural areas in Ovacik district of Tunceli province, Turkey in July 2011. The plants were dried in a room without receiving direct sun light. The plants were identified by biologist Mehmet N. Yilmaz Kafkas University, Faculty of Art and Sciences, Department of Biology.

Extraction of Garlic

The extraction procedure was performed by the method of Jabar and Mossawi [11]. Briefly, a sample of 100 g minced garlic was allowed to stand in 500 ml of ethanol at room temperature for 20 h, and then the mixture was filtered. The liquid portion was taken and evaporated at 30ºC in a rotary evaporator [11].

Chromosome Aberration Test with Human Lymphocytes

Human peripheral blood cells were used as a test system. Peripheral venous blood was collected from healthy, non-smoking adults aged 18-22 years old. Donors provided written, informed consent at the time of donation that the blood samples collected will be used in this study. Whole blood 0.4 mL was added to 5 mL chromosome medium Biochrome. Because longer exposure times caused considerable inhibition of cell division, the essential oil of *A. Tuncelianum* was added after 48 h of culture initiation. Human lymphocytes were exposed to different concentrations 0.10, 0.15, 0.20, 0.25 µl/mL of ethanol extract. Negative acetone and positive control [mitomycine-C MMC, 0.3 µg/mL] was included in each experiment. Colchicine 0.06 µg/mL was added to each culture at 70 h. Cells were collected by centrifugation 377 X g, 10 min, and resuspended in a hypotonic KCl solution 0.4% for 30 min at 37ºC. Following this process, cells were centrifuged again and fixed in a cold methanol: acetic acid 3:1 mixture for 35 min at +4ºC. At the end of this procedure, cells were treated with fixative twice. Then, slides were made by dropping concentrated cell suspensions. Air-dried slides were stained for 15-20 min with 5% Giemsa stain pH 6.8 prepared in Sorensen buffer. One hundred metaphases per culture were analyzed for the presence of chromosomal aberrations (CA). The number of CAs was obtained by calculating the percentage of metaphases at each concentration and

treatment period that showed structural and numerical chromosome aberrations. Chromatid and chromosome breaks, chromosome exchange and chromatid union, and polyploid cells were screened at all treatment concentrations [12].

Statistical Analysis

All statistical analyses were performed using Graph Pad In Stat version 3.05 for Windows 95 Graph Pad Software, San Diego California USA. The frequency of chromosomal aberrations in the cell cultures was analyzed using *Fisher's* exact test. Differences between the negative control and a series of treated groups were determined with Dunnett's *t*-test.

This study was approved by Kafkas University, Faculty of Medicine Ethics Committee with no. B.30.2.KAU.0.20.71. 100/15.

RESULTS

A significant decrease in the frequency of chromosomal aberration was observed for all treatments with *A. Tuncelianum* ethanol extract at 0.10, 0.15, 0.20, 0.25 µl/mL at 24 h compared with the negative control Acetone and mitomycine C (MMC: 0.3 µg/mL), which was used as a positive control *(Table 1)*. Examination of lymphocyte chromosomes revealed that chromosome exchange and chromatid union as well as chromatid and chromosome breaks in both the positive control and in treatment groups at all concentrations were present. Polyploidy was observed at concentrations of 0.20 and 0.25 µl/mL for the extract, and upon treatment with the positive control. Statistical analysis demonstrated significant increases in CA frequency in a dose dependent manner 24 h after addition of ethanol extract as compared with the negative control (r =0.95). Mitomycine treatment also caused a significant increase P<0.001 in the frequency of aberrant cells. Ethanol extract of *A. Tuncelianum* at all concentrations clearly inhibited the mitotic index in human lymphocytes *Table 1*.

Treatment of ethanol extract of *A. Tuncelianum* significantly reduced the chromosomal aberration rate as compared with the culture treated with MMC.

DISCUSSION

A significant difference in the frequency of chromosomal aberration was observed between ethanol extract of *A. Tuncelianum* at all concentrations; 0.10, 0.15, 0.20, 0.25 µl/mL and MMC group as well as negative control P<0.05. Bhattacharya *et al.* reported that garlic causes micronuclei induction in the mouse bone marrow cells [4]. In this study, the high aberration frequency observed as a result of applying *A. Tuncelianum* ethanol extract at 0.20 and 0.25 µL/ml could be linked to the toxic effect of the plant type as reported. Antigenotoxic effect of different garlic species was reported previously. The protective effect of water extract of garlic against asbestos Rhodesian Chrysotile-induced mutagenicity on human peripheral lymphocytes was reported *in vitro*. Simultaneous treatment of Rhodesian Chrysotile and garlic extract caused a significant decrease in micronucleus formation, sister chromatid exchange and chromosome aberration frequency [4]. Furthermore, Bhuvaneswari *et al.*[2] studied the protective effect of water extract of garlic and tomatoes against 7,12-dimethylbenz[a]anthracene DMBA-induced buccal pouch carcinogenesis in hamsters. It was observed that mean tumor load and incidence were effectively suppressed in group treated with garlic and tomatoes. In another study, the antigenotoxic effect of garlic organo-sulfur compounds OSCs including allicin DADSO, diallyl

Table 1. *Frequencies of Chromosome Aberrations and Mitotic Index in Cultured Human Lymphocytes Treated with A.tuncelianum ethanol extract and A.tuncelianum plus MMC*

Tablo 1. *A. tuncelianum etanol ekstraktı ve A.tuncelianum etanol ekstraktı ile MMC uygulanmış insan lenfosit hücrelerinde kromzom aberrasyon ve mitotik indeks*

Test Substance	Treatment		Structural Aberrations				Numerical Aberrations	Frequency of Aberrant Cell ± SEM(%)	Mitotic Index ± SEM(%)
	Period (h)	Doses (µL/ml)	ctb	csb	cse	cu	p		
Negative Control	24	1	1	-	-	-	-	0.2±0.2	4.3±0.3
Mitomycine C	24	0.3	17	2	3	8	1	6.2±2.9*	1.7±1.2*
Allium tuncelianum ethanol extract	24	0.10	1	-	1	2	-	0.8±0.3	3.2±0.5*
		0.15	2	-	1	1	-	0.8±0.3	2.7±0.4*
		0.20	3	-	2	-	-	1.0±0.6	2.4±0.6*
		0.25	2	1	3	4	1	2.2±0.5	2.1±0.4*
Allium tuncelianum ethanol extract + MMC	24	0.10	14	3	3	5	-	5.0±2.3*	3.3±0.5*
		0.15	13	1	3	5	-	4.4±2.3	3.6±0.2*
		0.20	9	-	4	6	-	3.8±1.7	3.9±0.3*
		0.25	5	-	2	4	-	2.2±1.0	4.1±0.4*

ctb: *chromatid break,* ***csb:*** *chromosome break,* ***cu:*** *chromatid union,* ***cse:*** *chromosome exchange, negative control (1% distilled water),* ***MMC:*** *(0.3 µg/ml mitomycine-C (24 h),* ***p:*** *polyploidy, * P<0.05 as compared to negative control (Dunnett's test). Mitotic index*

sulfide DAS, diallyl disulfide DADS, S-allyl cysteine SAC and allyl mercaptan AM was tested in the human hepatoma cell line HepG2 using comet assay. The organosulfur compounds at test concentrations ranging from 5 to 100 µM did not affect cell viability and caused no DNA damage. SAC and AM significantly reduced DNA break in dimethylnitrosamine treated Hep G2 cells. In addition, garlic organosulfur compounds decreased the genotoxicity of hydrogen peroxide and methyl methanesulfonate. These studies indicate that garlic organosulfur compounds have antigenotoxic effects [1]. Choudhury *et al.*[8] studied the protective effect of garlic and mustard oil against clastogenic activity of sodium arsenite by evaluating clastogenic activity in the bone morrow cells of rats with respect to the chromosomal aberration. The aqueous extract of dietary garlic and mustard oil applied for 30 days was reported to decrease the clastogenic effect of sodium arsenite.

Environmental pollution and accumulation of toxic chemicals due to widespread use of chemical agents as in pesticides result in increased cancer rates in humans [13]. It was reported that anti-genotoxic compounds consumed within the diet could decrease the increased number of these cases [1]. Plant extracts rich in polyphenols have antimutagenic potentials and DNA-protecting effects [14]. The mechanism of protection against cancer by *Allium* species and OSCs were reported to involve inhibition of mutagenesis, inhibition of adduct formation, free-radical scavenging and effects on cell proliferation and tumor growth [3]. Mutagenic and carcinogenic compounds such as polycyclic aromatic hydrocarbons exert their effects primarily through the production of electrophilic intermediates leading to mutations in microsomal enzymatic reactions. Antigenotoxic agents found in natural substances affect various cellular pathways including endogenous sequestration of mutagens via various enzymes [9]. Anticancer effects of garlic are reported to be due to the presence of OSCs which are released during processing [15]. In addition, previous studies showed that the constituents of garlic especially sulfhydryl compounds have anticarcinogenic and antimutagenic effects. The protective effect of garlic is also attributed to scavenging of potentially toxic and mutagenic electrophiles and free radicals as well as modification of phase I and II enzymes involving in detoxification pathways. Furthermore, the ally group of garlic constituents increases GST level which helps the detoxification of mutagens and carcinogens [9]. The apparent effect of *A. Tuncelianum* on anticarcinogenic effect observed *in vitro* chromosomal aberration test could involve all of these mechanisms.

In conclusion, increasing number of different environmental pollutants, toxins and chemicals pose a great risk for human health in terms of increasing cancer ratios in today worlds. It is important to reduce or alleviate the adverse effects of environmental carcinogens and mutagens through daily diet. The results of this study showed that *A. tuncelianum* could be protective against mutagenic and carcinogenic compounds by preventing mutagenic effects.

Acknowledgements

The authors thank Dr. K. Metiner for suplying the plant samples.

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Kafkas Univ Vet Fak Derg
19 (2): 263-269, 2013
DOI: 10.9775/kvfd.2012.7645

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Influence of the Route of Administration on Therapeutic Efficacy of Ivermectin in Saanen and Damascus Goats Naturally Infected with *Trichostrongylidae* spp.

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Makale Kodu (Article Code): KVFD-2012-7645

Summary

The aims of this study were to compare the therapeutic efficacy of ivermectin after subcutaneous, per os and pour-on administration in Saanen goats naturally infected with *Trichostrongylidea* spp. and to evaluate the efficacy of ivermectin pour-on in Damascus goats with similar gastrointestinal nematodes. After body weighing and faecal sample collection on day 0 (pre-treatment day), 45 Saanen goats were weighed, faecal sampled and allocated on the basis of day -3 faecal egg counts (FEC) to four treatment groups (n=9 in each group). Saanen goats were then treated with a single dose of ivermectin at a dose rate of 0.2 mg/kg by subcutaneous injection or oral administration and 0.5 mg/kg or 1 mg/kg by topical application. Nine Saanen goats were kept as non-medicated control. Fifteen Damascus goats were divided two groups. The first group (n=9) received ivermectin pour-on as a single dose of 0.5 mg/kg and the second group (n=6) served as a non-medicated control. The efficacy was measured on the basis of the reduction of the egg output and the evaluation of the results from larval differentiation on 14 days post-treatment. In Saneen goats, ivermectin provided equally excellent (100%) therapeutic efficacy after subcutaneous or oral administration whereas ivermectin pour-on treatment at a dose of 0.5 mg/kg and 1.0 mg/kg reduced pretreatment FEC by 96.5% and 99.9%, respectively. No statistically significant difference was found between 1.0 mg/kg of ivermectin by topical application and the ivermectin subcutaneous and oral treated groups for post treatment FEC whereas the efficacy of ivermectin pour-on at 0.5 mg/kg was significantly lower than that for each of the other 3 treatment groups. In Damascus goat, the therapeutic efficacy of 0.5 mg/kg ivermectin pour-on was recorded as only 30.7%.

Keywords: *Goat, Saanen, Damascus, Trichostrongylidae, Ivermectin*

Trichostrongylidae spp. ile Doğal Enfekte Saanen ve Damascus (Halep) Keçilerinde İvermektinin Terapötik Etkinliği Üzerine Uygulama Yolunun Etkisi

Özet

Bu çalışmada, *Trichostrongylidea* spp. ile doğal enfekte Saanen keçisinde subkutan, oral ve dökme (pour-on) ivermektin uygulanmasının terapötik etkinliğini karşılaştırılması ve benzer gastrointestinal nematodlarla enfekte Halep keçisinde pour-on ivermektin uygulamasının etkinliğini değerlendirilmesi amaçlanmıştır. Kırk beş Saanen keçisi tedavi öncesi (0. gün) tartılıp, dışkı örnekleri alınmış ve 3 gün öncesi dışkı yumurta sayıları (FEC) temel alınarak 4 tedavi grubuna ayrılmıştır (her grupta n=9). Daha sonra keçilere bir kez 0.2 mg/kg ivermektin subkutan veya oral yolla ve 0.5 mg/kg ya da 1.0 mg/kg topikal olarak uygulanmıştır. Dokuz Saanen keçisi ilaçsız kontrol olarak bırakılmıştır. İki gruba ayrılan 15 Halep keçisinden birinci gruba (n = 9) 0.5 mg/kg tek doz ivermektin pour-on uygulanırken, ikinci grup (n = 6) ilaçsız kontrol olarak tutulmuştur. Etkinlik, tedavi sonrası 14. günde yumurta atılımında azalma ve dışkı kültürü sonuçları baz alınarak değerlendirilmiştir. Saneen keçilerinde subkutan veya oral ivermektin uygulaması eşdeğer tam (%100) terapötik etkinlik sağlarken, 0.5 mg/kg ve 1.0 mg/kg, pour-on ivermektin uygulaması tedavi öncesi dışkı yumurta sayılarını sırasıyla %96.5 ve %99.9 azaltmıştır. Tedavi sonrası FEC açısından 1.0 mg/kg ivermektin pour-on uygulanan grupla, subkutan ve oral ivermektin uygulanan gruplar arasında önemli bir farklılık bulunmazken, 0.5 mg/kg pour-on ivermektin uygulamasının etkinliği, diğer 3 tedavi grubunun her birinden düşük olduğu belirlenmiştir. Halep keçilerinde ise 0.5 mg/kg tek doz ivermektin pour-on uygulamasının etkinliği sadece %30.7 olarak saptanmıştır.

Anahtar sözcükler: *Keçi, Saanen, Damascus, Trichostrongylidae, İvermektin*

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INTRODUCTION

The goat is a high-yield species, which can transform low quality vegetable food into high quality protein sources such as milk and meat. Its husbandry is increasing in importance both in developing countries, mainly as a source of protein, and in developed countries as a product for special markets [1]. Goat husbandry of dairy breeds is increasingly attracting interest as a result of increased demand for goat milk and government support for breeding projects. In this context, the intensive breeding of both the Damascus goat, still raising in Turkey, and the Saanen goat, which could be successfully raised in several regions, has been extending in recent years.

Nematode infections in goats cause important economic losses, directly by the related deaths and indirectly as a result of growth retardation, lost yield, increased sensitivity to other infections and treatment expenses [2,3]. Dairy breed goats are considered to be the most sensitive to nematode infection among domestic animals; this increased sensitivity compared to cattle and sheep is thought to be due to a physiologic character, feeding behavior or natural selection [4]. The progressive increase in intensive breeding of dairy goats in our country is accompanied by an increase in the size of economic losses due to nematode infections. Epidemiological investigation [5-7] showed that, out of 58 helminth species identified in goats in Turkey, five were trematodes, nine cestodes and 44 nematodes, characterizing gastrointestinal nematodes from the *Trichostrongylidae* as the most frequently encountered pathogens in the country as they are worldwide.

Endectocides (avermectins/milbemycin) are among the most important groups of anthelmintic drugs used today to control nematode infections; their use in goats is increasing due to the relatively frequent development of benzimidazole resistance [4]. The frequency of goat nematodes resistant to benzimidazoles is reported to be 70 - 100% in French farms [8,9], while no resistance is mentioned yet to avermectin - milbemycin (endectocide) in contrast to reports from Australia and South Africa. Ivermectin, a *Streptomyces avermitilis* fermentation product, is an avermectin which has been frequently used since its introduction in 1981. The main considerations for the rational use of ivermectin as an anti-nematodal agent are the dose, the route of administration, the yearly treatment schedule, periodicity and the implementation of selective treatment in the animals with severe disease [4,10]. Ivermectin is approved for use in cattle and sheep in many other countries besides Turkey; in goats it is used at the standard dose of 0.2 mg/kg, frequently also recommended for the subcutaneous (SC) treatment of nematode infections of cattle and sheep [4,11-14]. Phamacokinetic studies, however, have shown important differences in ivermectin absorption and elimination as compared to cattle and sheep [4,15,16]. It has therefore been proposed to use a dose of 0.3 mg/kg [1] or 0.4 mg/kg [10] for effective and rational treatment, observing that the use of the cattle or sheep standard dose for nematode infections of the goat may lead to pathogen resistance and impair therapeutic efficacy [4].

Different formulations of ivermectin (injectable, oral and pour-on) are used for nematode infections; the oral and pour-on administration is superior to the SC considering the ease of administration. In the absence of an ivermectin formulation for oral use in the goat, it has been shown that the standard dose for sheep of 0.2 mg/kg is active against goat gastrointestinal nematodes in 90 - 100% of cases, both in experimental [11-13] and natural [14] infections. It was determined that this efficacy of 0.2 mg/kg ivermectin given orally is equivalent to [13] or better than [17-20] that of the same dose administered by SC injection.

Beside the animal species and breed and the strain and biologic phase of the infectious agent, the efficacy rate of ivermectin in the nematode infections of ruminants also varies among different countries and regions, due to the degree of resistance induced by repeated low-dose administration, the frequent repetition of treatment and the time of administration [4,10]. There is, as far as we know, no study in our country comparing the efficacy of SC and oral dosing of ivermectin in Saanen goats naturally infected by *Trichostrongylidae* spp. The activity of the pour-on formulation of ivermectin in gastrointestinal nematode infections, which has been confirmed in several studies performed in cattle [21-23], has not yet been evaluated in goats [4]. The aim of this study was therefore to compare the efficacy of SC *versus* oral ivermectin given at the same dose (0.2 mg/kg) and that of two different doses (0.5 mg/kg *vs* 1 mg/kg) of the pour-on formulation of the same drug in Saanen goats, and to evaluate the efficacy of pour-on ivermectin, 0.5 mg/kg, in Damascus goats.

MATERIAL and METHODS

Animals

This study was planned in the aftermath of the clinical and laboratory evaluation of two Saanen goats, submitted to the Adnan Menderes University Veterinary School Internal Disease Clinic from a farm in the Tire (Izmir) area with growth retardation, diarrhea, weight loss and loss of appetite. It was stated that five other goats in the same farm had died with similar signs, while the others were also sick. An endoparasite infection was suspected on clinical evaluation of these two patients; examination of the feces showed intense infestation by *Trichostrongylidae* spp. A total of 60 female goats breeding on the same farm, 45 of the Saanen and 15 of the Damascus breed, with body weights of 27-55 kg for the former and 30-46 kg for the latter, were thought to be possibly also infected. These two breeds were housed in separate stalls on the farm and the goats have not been grazed on pasture. The animals on the farm had been obtained approximately one year earlier

from different areas of the country (Foça, Izmir for the Saanen and Adana for the Damascus goats) and that they had not been kept together at the same place.

Each of the two breeds of animals was divided into groups according to their count of eggs per gram (EPG) of feces in samples obtained three days before ivermectin treatment (on day 3). Particular attention was paid to dividing the study population, and more particularly the treated animals, in groups with similar EPG counts, while the control groups were comprised of patients with low EPG counts to avoid deaths due to severe *Trichostrongylidae* spp. infection. The Damascus breed included a single treatment group (n=9), because of the small number of total animals, and a control group (n=9), while the Saanen breed animals were divided into five equal groups (n=9 each) of which one was the control group.

Fecal Examination

Fecal samples were collected from each animal by digital rectal examination into individual bags; EPG counts for *Trichostrongylidae* spp. were determined by the McMaster technique [24]. Fecal samples were collected for EPG counts and fecal cultures for identification of parasite strain species, three days prior to treatment (day 3), before the start of treatment on its first day (day 0) and after the end of treatment (day 14). The feces of goats showed the presence of eggs were pooled by groups (two groups each for the Saanen and Damascus breeds); after thorough mixing they were made into a slurry with the addition of water and fine wood shavings (3 parts feces for 1 part wood shavings) to allow for egg development into third stage larvae, collected in plastic containers and incubated one week at 26-28°C [25-28]. During this period, the feces cultures were taken out of the incubator daily for mixing in order to keep them aerated; water was added if the water level was low. The growing larvae were collected by the Baermann-Wetzel method at the end of the incubation time and the larvae diagnosed morphologically according to the related keys [29,30].

Ivermectin Administration

The body weight of each goat was determined by automatic appliances before treatment and drug administration was performed according to individual body weight. The four ivermectin treatment groups into which the Saanen breed goats were divided were as follows: 0.2 mg/kg SC; 0.2 mg/kg orally (O) (both with Baymec® injectable solution 1%, Bayer, Turkey), ivermectin pour-on (Ivomec® pour-on, 0.5% w/v, Merial, Germany) at the standard dose of 0.5 mg/kg (P); finally the same formulation at double the standard dose, i.e. 1 mg kg (Px2), all as a single treatment. The single treatment group in the Damascus breed animals received the pour-on formulation at the dose of 0.5 mg/kg (P) as a single treatment. The control group in either breed was not given any treatment.

The anthelmintic efficacy was determined by the "fecal egg count reduction test" (FECRT) according to the following formula [16].

$$\text{Effect (\%)} = \frac{\text{EPG (pre–treatment)} - \text{EPG (post-treatment)}}{\text{EPG (pre –treatment)}} X100$$

Statistical Evaluation

Statistical evaluation of the data was performed with the SPSS 15.0 software package. The distribution of EPG in both the control and treatment groups on days 0 and 14 was not normal, as indicated by the Kolmogorov-Smirnov test; therefore the comparative statistical evaluation used non-parametric tests. The significance of differences in EPG counts among groups on days 0 and 14 was assessed by the Kruskal-Wallis H test; the Mann-Whitney U test was used to compare the groups and the Wilcoxon test to assess in-group change from day 0 to day 14. The significance limit was defined as a p-value <0.05. Pre and post-treatment (day 0 and 14) EPG counts were expressed as arithmetic mean, standard deviation X±s) and median (interquartile range) *(Table 2)*.

RESULTS

Clinical examination at the first visit to the facility to determine the groups (day 3) and on the first day of treatment (day 0) showed weight loss, irregular hair growth, pallor of mucosas and diarrhea in most animals. The mentioned findings were markedly reduced or had disappeared on day 14 in the treatment groups of the Saanen breed, while the control group animals had no noticeable change in their general condition. The findings were less severe in the Damascus as compared to the Saanen breed goats; most of the clinical findings on day 0 in the latter were still present on day 14. No side effect that could be related to the use or mode of administration of ivermectin was observed in any of the treatment groups in goats of either breed.

Table 1. *Fecal culture results before and after ivermectin treatment*
Tablo 1. *İvermektin tedavisi öncesi ve sonrası dışkı kültürü sonuçları*

Goat Breed	Day 0	%	Day 14	%
Saanen	*Haemonchus contortus*	67		
	Oesophagostomum spp.	19		
	Trichostrongylus spp.	8	*Trichostrongylus* spp.	100
	Cooperia spp.	3		
	Cooperia onchophora	2		
	Ostertagia spp.	1		
Damascus	*Haemonchus contortus*	60	*Haemonchus contortus*	100
	Cooperia onchophora	20		
	Ostertagia spp.	16		
	Cooperia spp.	4		

Table 2. *EPG counts, FECR results and mean EPG change before (day 0) and after treatment (day 14) in Saanen and Damascus breed goats*
Tablo 2. *Saanen ve Damascus keçilerinde oluşturulan gruplarda tedavi öncesi (0. gün) ve tedavi sonrası (14. gün) EPG değerleri, FECR sonuçları ve ortalama EPG değişimleri*

Goat No	Saanen														Damascus				
	SC (0.2 mg/kg) (n=9)			O (0.2 mg/kg) (n=9)			P (0.5 mg/kg) (n=9)			Px2 (1 mg/kg) (n=9)			Control (n=9)		P (0.5 mg/kg) (n=9)			Control (n=6)	
	Day 0	Day 14	FECR %	Day 0	Day 14	FECR %	Day 0	Day 14	FECR %	Day 0	Day 14	FECR %	Day 0	Day 14	Day 0	Day 14	FECR %	Day 0	Day 14
1	21500	0	100	19500	0	100	11350	350	97	10250	0	100	2300	550	3350	2450	27	200	100
2	6450	0	100	7050	0	100	7300	500	93	8750	0	100	1200	450	2850	2800	2	150	100
3	4800	0	100	4900	0	100	5000	50	99	5700	0	100	900	1050	2150	1350	37	50	150
4	4000	0	100	4000	0	100	4400	0	100	4600	0	100	750	2400	1350	1050	22	50	50
5	3000	0	100	3400	0	100	3400	50	99	3600	0	100	300	250	1300	50	96	50	50
6	2650	0	100	2800	0	100	2850	0	100	3000	0	100	200	300	650	1000	-54	50	0
7	1350	0	100	1650	0	100	1650	0	100	1750	50	97	200	1350	550	150	73		
8	950	0	100	950	0	100	1400	350	75	1200	0	100	200	300	500	550	-10		
9	400	0	100	500	0	100	800	50	94	850	0	100	150	150	300	200	33		
X± s	5011.1± 8478.8	**0**		4972.2 ± 5817.3	0		4238.9 ± 3349.7	150.0 ± 193.6		4411.± 3304.1	5.6 ± 16.6		688.9 ± 712.7	755.6 ± 734.6	1483.3 ± 1079.4	1027.8 ± 1003.2		91.7 ± 66.5	75.0 ± 52.4
Median (inter quartile range)	3000 (4475)	**0**[a]		3400 (4675)	**0**[a]		3400 (4625)	**139**[b] (350)		3600 (5750)	**0**[a]		300 (850)	**450**[c] (925)	**1300** (1575)	**650*** (1725)		**50** (112.5)	**75** (75)
	***Mean Effect* %**		**100**	***Mean Effect* %**		**100**	***Mean Effect* %**		**96.5**	***Mean Effect* %**		**99.9**			***Mean Effect* %**		**30.7**		

SC: *subcutaneous administration,* ***O:*** *oral administration,* ***P:*** *pour-on administration,* ***Px2:*** *double dose pour-on administration,* [a,b,c]***:*** *The difference among the day 14 values on the same horizontal line, marked by different letters, is significant,* * *The EPG count pre-treatment (day 0) is significantly different from the one post-treatment on day 14.*

Parasite larvae of the *Trichostrongylidae* found in the feces cultures sampled before treatment in both Saanen and Damascus goats are shown in *Table 1*; *Trichostrongylidae* spp. EPG counts and the statistical evaluation of feces samples of both breeds, pre-treatment on day 0 and after treatment on day 14, are presented in *Table 2*. While six different nematodes were found in the pre-treatment fecal samples of the Saanen and four in the Damascus breed goats, the parasites which were still present in the post-treatment feces of the two breeds were, respectively, *Trichostrongylus* spp. and *Haemonchus contortus (Table 1)*.

Differences among the four different treatment groups in the Saanen goats (SC, O, P and px2) as to EPG counts were not statistically significant (P=0.968), while highly significant differences among groups (P<0.001) appeared on day 14. The median EPG count in this breed was lower than that of the control group in the SC, O and Px2 groups with a p-value <0.001, while the significance level was still P<0.01 in the P group. Median EPG was comparable among the SC, O and Px2 groups (P>0.05), while that of day 14 in the P group remained significantly higher (P<0.01) than in the other three treatment groups. When comparing the pre-treatment day 0 EPG to those on day 14 for the control group and the four treatment groups, the latter all showed an important reduction (P<0.001), while no significant change was seen in the controls. Efficacy, quantified by using the FECR test post-treatment by both SC and oral ivermectin in the Saanen goats naturally infected by *Trichostrongylidae* spp., was 100%; it amounted to 96.5% for the pour-on formulation at a dose of 0.5 mg/kg and 99.9% for double this standard dose by the same route *(Table 2)*.

As for the Damascus breed goats, comparison of the pre-treatment day 0 to day 14 EPG showed a significant decrease in the P group (P<0.01) but no significant change in the control group. Therapeutic efficacy for pour-on treatment with ivermectin, 0.5 mg kg, was determined as being 30.7% according to FECR test results *(Table 2)*.

DISCUSSION

The standard dose of ivermectin administered for different indications in cattle and sheep is 0.2 mg/kg for SC and oral treatment and 0.5 mg/kg for pour-on application. Even though ivermectin has not received registration approval for use in goats, in which its results have been considered suboptimal [1,8,10], it is frequently used in practice by the animal farmers and the veterinarians. This study characterized the efficacy of ivermectin, determined with the help of the FECR test, as being 100% for SC and oral administration at the dose of 0.2 mg/kg, and 96.5% and 99.9%, respectively, for pour-on treatment with 0.5 mg/kg and 1 mg/kg in the Saanen breed goats naturally infected by *Trichostrongylidae* spp. (*Haemonchus contortus, Oesophagostomum* spp., *Trichostrongylus* spp., *Cooperia* spp., *Cooperia onchophora, Ostertagia* spp.); the efficacy of pour-on treatment with ivermectin, 0.5 mg/kg, was 30.7% in the Damascus goat *(Table 2)*.

The antinematode activity of ivermectine varies significantly according to the drug route, dose and formulation, the patient's feeding, physiologic condition and amount of fat tissue, finally the nematode species, biologic phase and resistance status [4]. Compared to oral and pour-on administration, SC injection was shown to be the most efficient route of administration, in goats as well as cattle or sheep, from the bioavailability viewpoint [4,31]. It has been considered that low bioavailability of oral and pour-on administration could be one of the causes of the low efficacy of treatment by these routes [4]. Experimental as well as field studies in goats have shown, however, that oral ivermectin treatment for intestinal nematodes provides an efficacy equivalent to, or better than, that of SC treatment [4,17,18]. The efficacy level of oral ivermectin, 0.2 mg/kg, reported in studies that consider the total parasite load in evaluating treatment efficacy against gastrointestinal nematodes has been reported as being >99% [4,11-13]. In the same context, oral administration of oral ivermectin at the mentioned dose obtained 99.9% efficacy compared to 98.7% for SC administration in goats experimentally infected by *Trichostrongylus colubriformis (T. colibriformis)*. In field studies evaluated by the FECR test, the efficacy of 0.2 mg/kg ivermectin SC in *Trichostrongylus* spp. infection was 94% versus 100% when given orally at the same dose). Borgsteede [32] has shown a difference in efficacy, in experimental infection models in sheep, between SC and oral ivermectin adminis-tration of, respectively, 92% *vs* 100% in *T. vitrinus* and 85.7 *vs* 98% in *Cooperia curticei*. In this study, the efficacy of 0.2 mg/kg SC and oral ivermectin administration was found equivalent in Saanen breed goats naturally infected by *Trichostrongylidae* spp. according to FECR results (100%); this result was compatible with earlier reports showing equivalence of standard dose oral ivermectin with the same dose given SC [4,13]. While it is possible to observe a poorer efficacy of oral ivermectin administration, compared to SC injection, against migrating larvae or tissue forms of nematodes due to lower bioavailability of the oral drug, the oral route can also provide a high gastrointestinal drug concentration, more active against nematodes in the intestinal lumen [16]. While food intake is a negative factor for oral administration, lactation and infectious agent resistance negatively influence both oral and subcutaneous routes [4]. In the Saanen breed goats naturally infected by *Trichostrongylidae* spp. in our study, a 12-hour fast preceding the ivermectin administration, the fact that the animals were not lactating, the absence of previous treatment by a drug of the avermectin group and the optimal individual dosing by body weight measurement for each animal may have contributed to a maximal efficacy (100%) by both routes of administration.

Pour-on drug administration is a preferred method, thanks to its ease of administration necessitating less involvement,

the absence of different sources of possible infective contamination in contrast to parenteral administration, the absence of residual drug at the site of treatment and the possibility of avoiding contact with potentially dangerous animal species. Ivermectin can be applied as a pour-on in addition to SC and oral administration; pour-on ivermectin preparations recommended for administration at a dose of 0.5 mg/kg are available and approved for use in cattle only. The sex-related plasma distribution of ivermectin following pour-on application [33] and the effects of age and dose on ivermectin plasma distribution and hair concentration [34] have been evaluated; no study in the therapeutic efficacy of this route of adminis-tration was hitherto available [4]. Therefore the efficacy of the pour-on administration to Saanen goats naturally infected with *Trichostrongylidae* spp., 96.5% at the recommended standard dose for cattle (0.5 mg/kg) and 99.9% at 1 mg/kg, *(Table 2),* could not be compared to other results. The efficacy of ivermectin at a dose of 0.5 mg/kg (96.5%) was found significantly lower ($P<0.01$) than that of SC and oral administration (100%). The efficacy of pour-on applications of ivermectin for similar purposes in cattle at a dose of 0.5 mg/kg was reported as 80-100% [21-23]. This leads to the idea that bioavailability may be poor following pour-on application of 0.5 mg/kg ivermectin to the Saanen goat.

This study was unable to compare the efficacy of all three routes of administration (SC, oral and pour-on) in the Damascus breed goat because of a small number of animals, insufficient to constitute groups. The efficacy characterized in the Damascus goat infected by *Trichostrongylidae* species similar to those of the Saanen goat was 30.7%, significantly poorer than the 96.5% determined in the latter breed *(Table 2)*. This difference in pour-on administration may have an explanation in anatomical or physiological peculiarities of the breed. In fact, the effect of anatomical and physiological differences of animal species on avermectin pharmacodynamics has been documented [35], as has been the marked difference in iver-mectin plasma levels between Damascus and Kilis goats given the same SC doses [36]. Also, cases of anthelmintic activity falling under 90% by calculation based on the FECR test are characterized as "resistance positive" [37]. The low FECR (30.7%) observed in Damascus goat indicates the presence of gastrointestinal nematodes resistant to ivermectin *(Table 2)*. The faecal cultures examined 14 days after treatment showed that resistance to ivermectin based on the low FECR results from *H. contortus (Table 1),* the most common trichostrongylid of goats in many parts of the World [1-5]. Factors that are of the greatest importance for anthelmintic resistance include generally indiscriminate use and overuse of anthelmintics, a general lack of biosecuriiy on the farm, frequent movement of animals off of and onto the farm, insufficient quarantine procedures for new arrivals, and a failure to treat new arrivals with effective anthelmintics during the quarantine period [1,4]. One or more of these factors may have played a role in resistance to ivermectin in Damascus goat of this study. In particular, frequent and indiscriminate use of ivermectin is commonly practiced by farmers because of its high efficacy, easy application and wide safety indices. As a consequence, there has been an increase in farms reporting the insufficient effect of ivermectin. Damascus goats on the farm in Tire/İzmir have not been previously treated with ivermectin; therefore, resistant *H. concortus* due to possibility of overuse and/or misuse of ivermectin may initially have been bought in with breeding stock from Adana.

In conclusion, it was determined that the administration of either SC or oral ivermectin at the dose of 0.2 mg/kg to Saanen goats naturally infected by *Trichostrongylidae* spp. resulted in full efficacy for both routes, that the efficacy of the pour-on preparation at a dose of 0.5 mg/kg was lower than that of the two other routes of administration, finally that the efficacy when administered by the latter route and dose was very limited in the Damascus goat.

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Kafkas Univ Vet Fak Derg
19 (2): 271-276, 2013
DOI: 10.9775/kvfd.2012.7659

Journal Home-Page: http://vetdergi.kafkas.edu.tr
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RESEARCH ARTICLE

The Research of Clonal Relationship Among *Aeromonas* Strains Isolated from Human, Animal and Drinking Water by PFGE

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Makale Kodu (Article Code): KVFD-2012-7659

Summary

Aeromonads infect human through potable water and causes various infections. Their existence in animal are being assessed as potential risk for human health. The aim of this study was to investigate clonal relationship among 52 *Aeromonas* strains isolated from human with diarrhea (14 strains), healthy food workers (2 strains), animals (24 strains) and drinking water (12 strains) by pulsed-field gel electrophoresis (PFGE). Clonal relation was determined between one diarrheic human isolate and one cattle isolate. No clonal relation was determined between drinking water and human isolates. Two fish isolates, *A. caviae* and *A. sobria*, were not distinguished PFGE patterns. Consequently no predominant clone was determined while clonal related strains were determined. Particularly, it is necessary to elicit the epidemiological importance of animals in respect of human *Aeromonas* infections and extensive studies are required for identification of environmental isolates.

Keywords: *Aeromonas spp., Human, Animal, Drinking Water, PFGE*

PFGE ile İnsan, Hayvan ve İçme Sularından İzole Edilen *Aeromonas* Suşları Arasında Klonal İlişkinin Araştırılması

Özet

Aeromonaslar insanlarda çeşitli enfeksiyonlara neden olmakta, içme suları ile insanlara bulaşabilmekte ve hayvanlardaki varlığı, insanlar için potansiyel risk olarak değerlendirilmektedir. Bu çalışmayla pulsed-field gel electrophoresis (PFGE) ile diyareli insanlardan (14 suş), sağlıklı gıda işçilerinden (2 suş), hayvanlardan (24 suş) ve içme sularından (12 suş) izole edilen 52 *Aeromonas* suşu arasındaki klonal ilişkinin araştırılması amaçlandı. Bir diyareli insan izolatı ve bir sığır izolatı arasında klonal ilişki tespit edildi. İnsan ve su izolatları arasında klonal ilişki tespit edilmedi. İki balık izolatı arasında (*A. caviae* ve *A. sobria*) ayırtedilemez PFGE paterni tespit edildi. Sonuç olarak, klonal ilişkili suşlar tespit edilmesine rağmen baskın bir klon tespit edilmedi. Özellikle hayvanların, insan *Aeromonas* infeksiyonları yönünden epidemiyolojik önemini aydınlatmaya, ayrıca çevresel izolatların identifikasyonuna yönelik kapsamlı çalışmalara ihtiyaç olduğu görüldü.

Anahtar sözcükler: *Aeromonas spp., İnsan, Hayvan, İçme Suyu, PFGE*

INTRODUCTION

Motile *Aeromonas* species may cause various infections in human mainly such as gastroenteritis, wound infection and systemic infection [1]. *Aeromonas* species exist in water, soil, food, human and animal feces [2]. It is possible that certain *Aeromonas* clones in the environment may predominate and cause human diseases more frequently than other clones [3].

Aeromonads are being transferred to food and other surfaces through the contaminated hands of food workers during manual processing and preparation [4]. Either the routes of contamination of food by *Aeromonas* or the role of contaminated food in the formation of *Aeromonas* infections has not been fully elicited. While it is being specified that

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food can be the source in respect of *Aeromonas* gastroenteritis by the determination of DNA hybridization group (HG) 4 and HG8 strains in food [5].

Aeromonads are a fairly heterogenous regarding to their biochemical, structural, and genetic features. The difficulties of correct identification of traditional *Aeromonas* biotypes are causing problem in epidemiological studies, in determination of disease spectrum and in determination of distribution of species in environment [6]. Especially, there are difficulties in phenotypic identification of environmental strains [7]. Pulsed-Field Gel electrophoresis (PFGE) is a very effective method to characterize clonal relationships among *Aeromonas* strains [6,8].

The aim of this study is to research of clonal relation in among *Aeromonas* strains isolated from human with diarrhea, healthy food workers, animals and potable water by PFGE.

MATERIAL and METHODS

Aeromonas Isolates

A total 52 epidemiologically unrelated *Aeromonas* strains that 14 isolates were from previous studies of Berktaş et al.[9] were investigated in this study. These 14 strains were isolated from stools of animals brought to Van municipality slaughterhouse and from stools of diarrheal patients samples collected from the laboratory Research Hospital of Yuzuncu Yil University. Other isolates, 24, were isolated from stools of diarrheic human of the same laboratory, gull strains from stools of gulls which inhabited in coast of Van Lake, fish strains from fish purchased from fisherman hunted in near the coast which inhabited of gulls and finally chicken strain from specimens collected from different hencoops in Van region. Also, drinking water and food worker isolates, 14, were isolated from specimens sent to Van Public Health Laboratory for analysis. Source, name and number of *Aeromonas* strains are given *Table 1*. Stock cultures were maintained frozen at -80°C in peptone water (1% peptone, 0.5% NaCl, [pH 7]) with 30% (vol/vol) glycerol.

Isolation and Identification of Motile Aeromonas spp. from Human and Animals

Fecal samples obtained from human (diarrheic and non-diarrheic), chickens and gulls via sterile swabs, and intestinal contents of fish (*Chalcalburnus tarichi* PALLAS 1811) were inoculated into alkaline peptone water (APW, pH 8.4). After incubation of samples overnight at 28°C within APW, 0.1 ml of inoculums was inoculated into 7% defibrine sheep blood agar (Blood Agar Base No.2, Oxoid CM0271) containing 10 µg/mL ampicillin. Oxidase test (Whatman filter papers no 1 soaked with N,N-Dimethyl-p-phenylen diammonium dichloride are used) was performed for suspicious colonies growing in medium. The passage of oxidase positive bacteria to Tryptone Soy Agar (TSA, Oxoid CM0131) was carried out and Gram staining of test microorganisms growing on TSA was performed. Catalase, motility, oxidation/fermentation test (O/F), growth in broth containing 6% NaCl, growth in saltless broth and acid from inositol tests were performed for Gram negative bacillus. When bacteria is motile, ferments glucose (O/F:+/+), no growth in broth including 6% NaCl, growing in saltless broth, not composing acid from inositol and resistant to vibriostatic agent O/129 (150 µg, Oxoid DD0015) were identified as *Aeromonas* spp. Test bacteria are identified by using BD phoenix™ (Becton, Dickinson and Company, Sparks, Maryland 21152 USA) panels at species level.

Isolation and Identification of Motile Aeromonas spp. from Drinking Water

Total 200 water samples (6 natural springs, 5 well waters, 189 public drinking water supply distribution systems) were collected in sterile colored bottles and then they were transferred to laboratory under cold chain and used to isolate *Aeromonas* bacteria. 100 ml from each water sample were filtered by vacuum through cellulose nitrate filters (Sartorius) with pore size 0.45 µm. Filters were placed on *Aeromonas* Medium Base (RYAN) (Oxoid) and incubated for

Table 1. *Resources of Aeromonas strains typed by PFGE in this study (*These strains were isolated at 2002 and other strains were isolated at 2005)*
Tablo1. *Bu çalışmada PFGE ile tiplendirilen Aeromonas suşlarının kaynakları (*Bu suşlar 2002 yılında, diğerleri 2005 yılında izole edildi)*

Resource	*A. hydrophila*	*A. caviae*	*A. sobria*	*A. veronii*	*A. media*	Total
Diarrhoeal patients	-	9*+5	-	-	-	14 (27%)
Nondiarrheic food workers	-	-	2	-	-	2 (3.8%)
Drinking waters	1	8	3	-	-	12 (23.1%)
Cattles	-	1*	1*	-	-	2 (3.8%)
Sheeps	-	2*	1*	-	-	3 (5.8%)
Fishes	-	1	2	3	1	7 (13.5%)
Chickens	-	-	1	-	-	1 (1.9%)
Gulls	-	11	-	-	-	11 (21.1%)
Total	1 (1.9%)	37 (71.2%)	10 (19.2%)	3 (5.8%)	1 (1.9%)	52 (100%)

24 h at 37°C. As it is mentioned above, dark green, opaque with darker centres suspicious colonies were identified at genus and species levels on RYAN (Oxoid) isolation plates.

Pulsed-Field Gel Electrophoresis

Stock cultures were inoculated on TSA plates and incubated for overnight at 37°C. A single colony of each isolate was inoculated in 2 ml tryptone soy broth and incubated at 37°C until blurring. Then, inoculated TSA plates via sterile swab were incubated for overnight at 37°C. Later, 1.5 mL inoculum inside 1.5 mL sterile cold PIV (10 mM Tris [pH 7.6]), 1 M NaCl) buffer were centrifuged in 1100 g for 15 min at 4°C. The pellet was diluted by PIV buffer and turbidity was set to 4 McFarland standard. 100 µL from the mixture, containing equal volume 3% agarose of low heat melting was distributed on plug molds placed previously on ice bars and kept for 30 min in order to provide solidification. Plugs were kept waiting inside 3 mL lysis buffer (6 mM Tris [pH 7.6], 1 M NaCl, 100 mM EDTA [pH 7.6], 0.5% Brij 58, 0.2 % sodium deoxycholate, 0.5% sodium lauryl sarcosine, 1mg/mL lysozyme) for 4 h at 37°C within shaking water bath. It was incubated for overnight at 50°C by placing proteinaze K solution (0.5 M EDTA [pH 8], 10% sodium lauryl sarcosine, 1 mg/mL proteinaze K) instead of lysis buffer. It was kept waiting for 5 min at room temperature, after placing 3 ml sterile distilled water instead of proteinase K. After aspiration of distilled water, plugs were washed four times with Tris-EDTA buffer (10 mM Tris [pH 7.6], 0.1 mM EDTA [pH 7.6]) and kept at 4°C. Genomic DNA was digested by adding 50 U *Xba*I as being incubated for overnight at 37°C. Electrophoresis was performed in 1%Saekem agarose (pulsed-field certified agarose, Bio-Rad Laboratories) by using the CHEF-DRII system (Bio-Rad Laboratories, Nazareth, Belgium) in 0.5x TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8.0]) at 14°C. The running parameters were 6 V/cm^2 for 8 h with 20-20 sec pulses and 12 h with 5 to15 sec pulse times. One *A. caviae* isolate (isolate GAC8) was run in multiple lanes of each gel owing to DNA global reference for standardizing run. DNA banding patterns were visualized with 0.5% ethidium bromide and digitally photographed. GelCompar software (version 3.0; Applied Maths, Sint-Martens-Latem, Belgium) was used to compare genetic similarity among isolates and construct a similarity dendogram by using Dice coefficient and the UPGMA algorithm (unweighted pair-group method with aritmetic mean) with a position tolerance of 1%.

RESULTS

Total 38 *Aeromonas* strains were isolated and identified in this study. The percentage, count and source of isolates were as follows; 5% (5 *A. caviae*) from stool sample of 100 diarrheic patient, 2% from the non diarrheic samples of food workers (2 *A.sobria*), 6% from the drinking water samples (1. *A. hydrophila*, 8 *A. caviae*, 3 *A. sobria*), 7% from the intestinal content of fish (1 *A. caviae*, 2. *A. sobria*, 3 *A. veronii*, 1 *A. media*), 1% from the feces samples of chickens (1 *A. sobria*), 11% from the feces samples of gulls (11 *A. caviae*).

Digestion of genomic DNA's of 52 strains (38 new strains isolated in the current study, 14 strains isolated in previous study) with *Xba*I enzyme show that no PFGE pattern has been obtained for 7 strains (1 human *A.caviae*, 1 sheep *A. caviae*, 1 water *A. caviae*, 1 gull *A. caviae*, 2 water *A. sobria*, 1 chicken *A. sobria*) while PFGE profile was obtained for 45 strain out of 52 *(Fig.1)*. PFGE profiles show that 4-12 well-resolved genomic DNA bands were observed from test strains and PFGE profiles of 45 isolates were shown 44 distinct patterns. Particularly, two identical isolates (*A. caviae* [FAC] and *A. sobria* [FAS1]) were grouped showing one identical pattern.

The result of one-to-one comparison of DNA patterns of forty five isolates exhibited that one pair was indistinguishable (*A. caviae* and *A. sobria* fish isolate, band difference 0), two pairs were closely related (*A. caviae* human and cattle isolate, *A. caviae* well water and gull isolate; band differences respectively 3, 2) and one pair was possibly related (cattle *A. sobria* isolate and gull *A. caviae* isolate, band difference 4), and no clonal relation determined among 37 strains. Eight strains were determined to have clonal relation according to assessment criteria of Tenover et al.[10].

Two genetically indistinguishable fish isolates (*A. caviae* and *A. sobria*) were isolated at same time period from the same fish population. Drinking water isolate isolated at same period from a well close to the same location while two gull isolate, having close relation, were isolated from the gulls living in coast of Van Lake.

Genetically close related human-cattle strains and possible related gull-cattle strains were isolated at different isolation periods. However it determined that here is no clonal relation among strains isolated at same isolation period from gulls living in same population. Moreover, no clonal relation were determined among human strains (diarrheic human and healthy food worker isolates) isolated previously in our region and isolated in this study. Briefly, no predominant clone was determined in human and animal populations and in drinking water.

DISCUSSION

It is being thought that various enzymes of *Aeromonas* such as lipase, protease, β-lactamase, amilase, kitinase and nuclease significantly contribute to their distribution in nature and their adaptation to environmental changes [11]. Having ability to remain alive in chlorinated water [1] and determination of these bacteries in animals [12,13], reveals the difficulty of epidemiological characterization of *Aeromonas*.

Finding our study show that no clonal relation was determined between strains isolated from human and drinking water. Results of our study are in good agreement with Stephenson et al.[14] research that comparison of

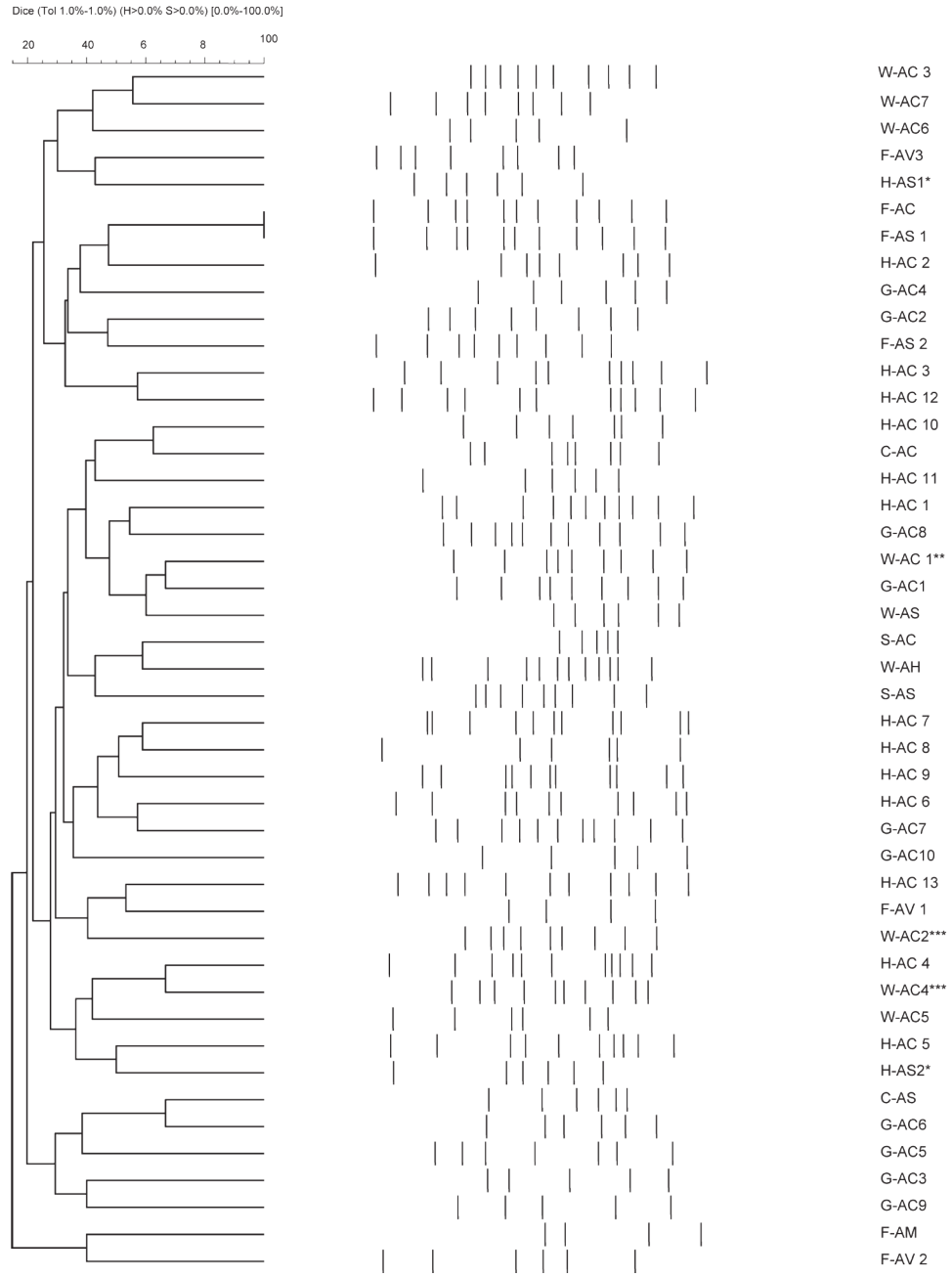


Fig 1. Pulsed-field gel electrophoresis patterns and similarity dendrogram of genomic DNA from *Aeromonas* spp. isolates from cattle (C), fish (F), gull (G), human (H), sheep (S) and water (W). AC: *A. caviae*; AV: *A. veronii*, AH: *A. hydrophila*, AS: *A. sobria*, AM: *A. media*. * nondiarrheic food worker isolates, ** well water isolate, *** natural spring isolates, other water isolates were isolated from public drinking water supply distribution system

Şekil 1. Sığırlardan (C), balıklardan (F), martılardan (G), insanlardan (H), koyunlardan (S) ve sulardan (W) izole edilen *Aeromonas* spp. izolatlarının genomik DNA'larının benzerlik dendogramı ve PFGE patternleri. AC: *A. caviae*; AV: *A. veronii*, AH: *A. hydrophila*, AS: *A. sobria*, AM: *A. media*. * Diyareli olmayan gıda çalışanı izolatları, ** Kuyu suyu izolatı, *** Doğal kaynak suyu izolatları, diğer su izolatları şebeke suyundan izole edilmiştir

protein profiles of 91 *Aeromonas* strains by the SDS-PAGE, were not similar human isolate stool and water isolates. Hanninen and Siitonen [15] reported that clinically significant *Aeromonas* hybridization groups are less in potable and soft water and there was a difference between *Aeromonas* hybridization groups isolated from potable water, chicken and cattle meat. Same investigators claim that contamination source of chicken and meat may not be water. However, Borchardt et al.[16] compared DNA patterns of 17 *Aeromonas* strains isolated from stools of diarrheic patients and 54 *Aeromonas* strains isolated from underground potable water by the PFGE. They stated that comparing stool and water of studied population were genetically irrelevant and gastrointestinal infections caused by *Aeromonas* were not due to exposure to underground water. Also, they clarified that it is necessary to determine at least a few stool and water isolates having closer relation than four band difference in order to claim that potable water is the source of *Aeromonas* infections in a population. Some researchers [17,18] suggested that the existence of epidemiological relation between *Aeromonas* infections and potable water are based on weak evidences. Burke et al.[17], found an epidemiological result owing to same biotypes *Aeromonas* bacteria isolated from children, causing infection, with well water. Millership and Want [18] compared protein profiles of 60 of *Aeromonas* strains isolated from human, food and water in London region, and data show that they might be similar strains in infected population, water and other environmental samples. These findings are supported by recent studies [3,19]. Bauab

et al.[19] ribotyped 88 *Aeromonas* strains isolated from human and environmental sources and extraintestinal infection isolates are within similar ribotypes by water isolates. Also they have informed that this finding verifies information regarding source of extraintestinal infections being water. However, Khajanchi et al.[3] searched 11 virulence gene and clonal relation of *Aeromonas* strains isolated from clinical samples and water of various regions of USA and world. In addition, they determined three pulsotypes, having same virulence genes and composed of epidemiologically relevant and irrelevant isolates. Humans are contaminated at least by *A. caviae-A. media* group living in water and due to exposing human to *Aeromonas* in water by virtue of these findings may cause gastroenteritis. Another research group reported that two *A.caviae* potable water isolate isolated in the same region at same period and one *A. caviae* isolate isolated from diarrheic human having indistinguishable PFGE patterns and identical virulence pattern indicated infection through water [20]. The same researchers reported that transmission through contaminated water of strains of the *A. caviae* group that can produce disease in humans at other their study [21].

There is no sufficient data based on strong evidences that the animals are reservoir for human infections as the existence of *Aeromonas* in animal being assessed as potential risk in respect of human infection. Maiti et al.[22] determined genetic variation between water, fish and clinical *Aeromonas* isolates by Random amplification of polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR. However Rahman et al.[23] asserted that specific fenotypic and genotypic stabile *Aeromonas* clones persisted for long time in treatment system and it may contaminate fishes produced for human consumption from hospitalized diarrheic children through sewage water treatment system. In our study, a clonal relation determined between strains isolated from two fish of same population (FAC and FAS1 isolate, indistinguishable, band difference 0), but no clonal relation found between human isolates and fish isolates. Moreover, no clonal relation determined between human isolates and gull isolates. Interestingly clonal relation determined between human and cattle isolates that are isolated at different period (HAC10 and CAC isolate, closely related, band difference 3).

Gulls are widespread coastal bird species and are being thought that they can contaminate coastal and lake water in world by their feces which is a major source of contamination [24]. In our study, a clonal relation determined between gull *A. caviae* strains living on the coast of Van Lake and well water *A.caviae* strain isolated from a close region (WAC1 and GAC1 isolate, close relation, band difference 2). Found from in a previous study in our region [25] that four *A. caviae* isolates (strains are not available now) isolated from gulls were determined to be identical by analyzing of result of typing with SDS-PAGE and concluded that these isolates might from same source and no genetic relation were determined between *A. caviae* isolates isolated from gulls living in same population. Moreover, clonal relation determined between gull isolate and cattle isolate isolated from same region at different time periods (CAS and GAC6 isolate, possible relation, band difference 4). Our findings are in parallel with Khajanchi et al.[3] study informing that isolates having weak epidemiological relation and isolated at different periods are same pulsotype and virulence group. They argued from this finding that very similar strains circulate in population and environment for a long period. On the contrary, Szcuka and Kaznowski [26] specified that they could not determine pathogenic clones among human living in different geographical regions despite clonal structure indication of *Aeromonas* species, and also they didn't determine reservoir of pathogenic strains in environment.

In our study, *A. caviae* ve *A. sobria* strains isolated from fish were grouped within same genotype. Our results are in good agreement with Bauab et al.[19] who specified that three different phenotypic types were within same ribotype. Øyvind et al.[7] compared the genetically and biochemical identification results of clinical and environmental *Aeromonas* isolates. Also, Øyvind et al.[7] claim that biochemical identification scheme being applied is developed based on data obtained from clinical isolates including only seven species, and thus that it is necessary to be careful while applying clinical data to environmental isolates and to use tests provided functional criteria for all *Aeromonas*. Moreover, same researchers reported that environmental isolates were more heterogenous than human stool isolates and it is accurate that biochemical profiles of environmental isolates is recognized less compared to clinical isolates, and consequently biochemical identification scheme being used in their study is not convenient for identifications of *Aeromonas* species, and that there is no sufficient current data to apply a full phenotypic identification for environmental strains.

Consequently, no predominant clone determined in this study. Findings of our study show that more extensive studies are required at three points. Firstly, more extensive studies, especially, on identification of environmental strains are required. Secondly, determination of clonal relation between a cattle isolate and a human isolate indicates that extensive studies are required regarding spread of these pathogens from animals to human. And third point is the determination of clonal relation between well water and a gull isolate being an indication of contamination of other water sources with swimming water by these birds.

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Kafkas Univ Vet Fak Derg
19 (2): 277-281, 2013
DOI: 10.9775/kvfd.2012.7664

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Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Effect of Different Conservation Conditions on the Active Compounds and Pharmacovigilance Screening of Different Florfenicol Preparations [1]

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[1] *This research was summarized of PhD thesis supported by the Scientific and Technological Research Council of Turkey (Project No: 110O033)*

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Makale Kodu (Article Code): KVFD-2012-7664

Summary

This study was aimed to evaluate the state of utilization of florfenicol, along with the investigation of adverse drug reactions and to evaluate the active substance content in different storage conditions. In this study, three different preparations were kept in different storage conditions and active substance content along with physical composition was determined at the beginning, 3rd, 6th and 9th months. No physical difference was observed for different condition storage preparations. In pharmacovigilance surveys; adverse drug declarations in which veterinarian reported were mostly from the second drug, following the third drug and the first drug lastly. Overall, active substance content lost was seen for the first drug groups. No significant difference of florfenicol levels (P>0.05) was observed for the second and the third drug groups stored both in room temperature and +4°C for the initial, 3rd, 6th and 9th month periods.

Keywords: *Florfenicol, Pharmacovigilance, Stability, Poultry, Veterinary drugs*

Florfenikol İhtiva Eden Preparatlara Farklı Saklama Şartlarının Etkileri İle Farmakovijilans Taramaları

Özet

Bu çalışma, Türkiye'deki florfenikol kullanım durumu ve florfenikolden kaynaklanan ters ilaç tepkimelerinin olup olmadığını araştırmak; florfenikolün farklı saklama koşullarında tutulması ve bu koşullardaki etkin madde miktarlarındaki değişimi belirlemek amacı ile yapıldı. Çalışmada, 3 farklı müstahzar farklı saklama koşullarında muhafaza edildi, başlangıçta, 3, 6 ve 9. aylarda etkin madde miktarları yönüyle analizleri yapıldı ve fiziki yönden de değerlendirildi. Farklı depolama şartlarında saklanan preparatlarda hiçbir fiziksel değişiklik gözlenmedi. Farmakovijilans araştırmalarında; veteriner hekimler tarafından ters ilaç etkisi bildirimi en fazla ikinci ilaç için bildirildi, bunu üçüncü ilaç ve son olarak ilk ilaç takip etti. Genel olarak, etken madde kaybı birinci ilaç grubu için görüldü. İkinci ve üçüncü ilaç gruplarının hem oda ısısında hem de +4°C'de muhafaza edilenlerinde başlangıç, 3., 6. ve 9. aylarda hesaplanan değerler arasında (P>0.05) önemli bir fark görülmedi.

Anahtar sözcükler: *Florfenikol, Farmakovijilans, Dayanıklılık, Kanatlı hayvan, Veteriner ilaçları*

INTRODUCTION

The antibacterial drugs used in veterinary medicine are often used for the treatment of the illnesses, the protection from the illnesses and for increasing the productivity of animals. For this reason, it is very important to follow the production, storage and market control conditions for the benefit from these drugs at the most level. Likewise the stability and pharmacovigilance works for drugs are done [1,2].

The Affects of Different Storage Conditions

Like the human drugs, the veterinary drugs and growing products are also the products made through the scientific studies and technological works. Consequently, the great usage of economical resources at the all stages of delivering and producing of these drugs are always relevant to the public

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care, the knowledge-ability experience at the most level, the practices on veterinary medicine and the contemporary growing principles [3,4].

Stability means the ability of an active substance or a pharmaceutical product to protect its specifications within definite limitations thorough the shelf life. Stability tests are the tests made for determining the shelf life and usage period of an active substance or a pharmaceutical product in its packing and on its storage conditions. Which is essential to stability is that the active substance be stable in specialties prepared. In the course of time, as a result of internal and external effects degradation products begin to form because the active substance changes. Besides, stability is not only valid for the active substance. The stability of helping substances used in formulations at the same time is also important [2,5].

Pharmacovigilance

Drugs may cause toxic effects even in normal usage conditions. Drug safety is researched at the all levels of developing preclinical and clinic drugs in priority and detail. However, doing these things perfectly and presenting great safety proofs often can not even prevent the adverse effects that may happen by being used by great majority after drug marketing. Consequently, drugs must be followed for safety during the period after marketing and there must be awareness about their potential toxicity [6-9].

Veterinary pharmacovigilance is a study of discipline which searches whether it is about taking drugs as a result of the adverse affects of the usage of veterinary medical drugs or not and also searches the degree of this. Following the veterinary medical drugs after marketing includes evaluating and preventing the probable adverse affects on animals which are treated and the effects on human health and environment at the same time [1,7,10].

Veterinary pharmacovigilance involves great scope. It has a priority to clinical safety. It includes the adverse affects of the product on animals treated in normal conditions or also human beings; because the person using it is in close contact with the animals treated [1,11-13].

It is aimed to search the usage of florfenicol in Turkey and the adverse affects of drugs, to keep florfenicol in different storage conditions and to determine the changes in quantity of the active substance in these conditions.

MATERIAL and METHODS

Reagents and Instruments

All reagents and solvents were analytical grade unless otherwise specified. Florfenicol standard (Fluka, Sigma-Aldrich, St. Louis, MO), Ethyl acetate (Sigma-Aldrich, St. Louis, MO), Acetonitrile (Merck, Darmstadt, Germany), Polyethylene glycol (PEG, Sigma-Aldrich, St. Louis, MO), Ultra-pure water was obtained from a Millipore system (Millipore, Molfheim, France). First drug: 300 mg/ml florfenicol; oral solution, 200 ml/bottle, second drug: 300 mg/ml florfenicol; oral solution, 1 L/bottle, third drug: 300 mg/ml florfenicol; oral solution, 1 L/bottle.

Shimadzu LC-20A, the high pressured liquid chromatography (HPLC, Shimadzu, Tokyo, Japan) system has a foto diode-array dedector and inertsil ODS-3 column (GL Sciences, Eindhoven, The Netherlands). The column heat 30°C, mobile phase; acetonitrile:water (27:73; v/v), wavelenght 223 nm and flow speed 1 ml/min.

Storage Conditions

The changes in quantity of the active substance of the preparation found in different storage conditions (room temperature and +4°C fridge) were followed. For this reason, 3 preparations including florfenicol as an active substance are determined. 5 drugs having the same serial number for each preparation were preserved in room temperature and +4°C, at the beginning these preparations were analyzed with their quantities of the active substance in the 3rd, 6th and 9th months and determined physically (turbidity, particulate substance, precipitation and color change).

The active substance in preparation was analyzed by means of HPLC parameters in the methods explained by Shen et al.[14].

Pharmacoviligance

Adeverse effects of the preparations including florfenicol were determined. The forms prepared for this reason were directly caused to reach the veterinarians working in the sector of animals having poultry. The information which was useful for veterinarians and presenting the adverse drug reactions was included in the forms.

Statistics

"SPSS 15.0 for Windows" statistic packet programme was used for the statistical calculations. The preparation including florfenicol for stability works were applied to HPLC and their quantities were determined with the peak areas obtained. The data were explained by arithmetic mean ± standard deviation at the most and least values. The variance analysis with the one direction (ANOVA) for the beginning, 3rd, 6th and 9th months of the drugs were applied and the importance of the differences between the groups was determined with the Duncan test. T- test was applied for the data of each drug in room temperature and +4°C in the beginning, 3rd, 6th and 9th months.

RESULTS

The Findings of Storage Conditions

Before first drugs, second drugs and third drugs (5 of each) were stored in room temperature and +4°C, each drug were

determined with the turbidity, particulate substance, precipitation, color change and the results were recorded. The same process was repeated in the following 3rd, 6th and 9th months. According to this, turbidity, particulate substance, precipitation, color change were not determined in each drug. However, white sediments were seen in the shell of the first drugs after they were taken for the analysis from the 3rd months. This was not seen in the second and third drugs.

Twenty µl polyethylene glycol (PEG) was applied to the HPLC before the determination of the quantities of the active substance of the preparation including florfenicol. In this way, PEG was controlled about whether it caused peak or not. Florfenicol standard was placed in autosampler after preparing the first, second and third drugs with the definite intensity (0.5 mg/ml and 2 mg/ml); the autosampler was ensured to use 20 µl from this mixture and the times of the peak were determined by controlling whether it causes proper peak or not *(Fig. 1)*. The results obtained are presented in *Table 1*.

Then in order to determine the quantity of the active substance of the first, second and third drugs at the beginning, 2 mg/ml of the density for each of them was diluted by considering the quantities stated in their prospectuses and they were placed in the autosampler by being put in vials. These samples enabled the autosampler to use 20 µl. All these works were repeated in the drugs stored in +4°C and in the room heat from the first month to the 3rd, 6th, and 9th months. The peak areas obtained were recorded.

By considering the peak area as 100% which was obtained from 2 mg/ml of the florfenicol active substance; the peak areas in the first, second, third drugs at all times and in different storage conditions were transferred into mg/ml according to this value. The comparison of the arithmetic mean±standard

Table 1. *The florfenicol active substance, first, second and third drugs HPLC output times*

Tablo1. *Florfenikol etkin maddesi, ilk, ikinci ve üçüncü ilaçların YBSK'da çıkış zamanları*

Effective Substance	Mean Peak Time (Minute)
Florfenicol Standard	13.25
First drug	13.35
Second drug	13.50
Third drug	13.40

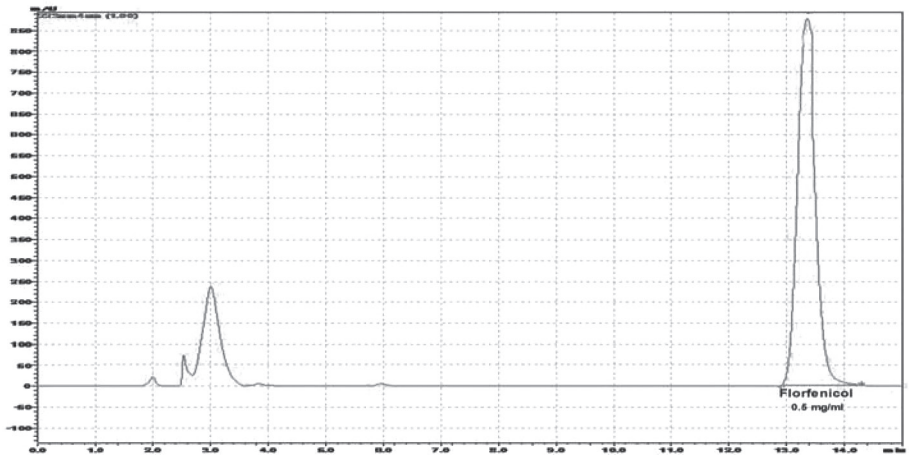


Fig 1. The chromatogram of the 0.5 mg/ ml density of the first drug in HPLC

Şekil 1. İlk ilacın 0.5 mg/ml'lik yoğunluğunun YBSK'deki kromatogramı

Table 2. *The comparison of the quantities of the preparation including florfenicol in point of the room temperature and +4°C in the beginning, 3rd, 6th, and 9th Months (arithmetic mean±standard deviation, the lowest-the highest values)*

Tablo 2. *Florfenikol ihtiva eden preparatların başlangıçta, 3., 6. ve 9. aylarda oda sıcaklığında ve +4°C'deki etkin madde miktarlarının karşılaştırılması (aritmetik ortalama±standart sapma, en alt-en üst değerleri)*

Drugs	Storage Conditions	Beginning (mg/ml)	3rd Months (mg/ml)	6th Months (mg/ml)	9th Months (mg/ml)
First drug	room temperature	1.917±0.081 [a] (1.784-1.991)	1.917±0.081 [a] (1.783-1.990)	1.804±0.017 [b] (1.778-1.827)	1.70±0.055 [c] (1.645-1.773)
	+4°C	1.731±0.161 [a] (1.585-1.998)	1.679±0.072 [a] (1.585-1.747)	1.576±0.070 [ab] (1.487-1.679)	1.483±0.135 [b] (1.270-1.615)
Second drug	room temperature	1.769±0.238 (1.435-2.00)	1.768±0.238 (1.434-1.998)	1.767±0.238 (1.433-1.996)	1.766±0.238 (1.432-1.994)
	+4°C	1.620±0.173 (1.448-1.897)	1.619±0.173 (1.448-1.896)	1.618±0.173 (1.447-1.895)	1.617±0.173 (1.446-1.893)
Third drug	room temperature	1.489±0.155 (1.272-1.643)	1.488±0.156 (1.270-1.642)	1.486±0.156 (1.268-1.642)	1.485±0.156 (1.265-1.641)
	+4°C	1.782±0.177 (1.589-2.00)	1.780±0.177 (1.587-1.998)	1.779±0.177 (1.584-1.996)	1.777±0.177 (1.581-1.994)

a,b,c, *Mean within in the same line with different letters are statistically significant (P<0.05)*

deviation, the lowest-the highest values, all drugs in the individual data in +4°C and room temperature are given in *Table 2*.

Pharmacovigilance Findings

80 questionnaire forms were used for the evaluation of the pharmacovigilance in 17 cities of Turkey. The questionnaires were filled by the veterinarians working in the service for poultry. According to the data obtained from all the questionnaires, it was observed that the preparation including florfenicol applied to poultry can be taken easily. The information about the areas where the preparations were stored is given in *Table 3*. The information given by veterinarians surveyed about adverse drug reactions are given in *Table 4*. By considering the notes about the problem of "the drugs not being dissolution completely" explained by the veterinarians in the choice of "the other "of the "WHAT ARE YOU GOING TO GIVE INFORMATION ABOUT?" part of the questionnaire; it was observed that the first and second drugs could not dissolution completely in normal water used for drinking but they could dissolution completely in warm water. It was stated that the third drugs could not dissolution completely in both normal water for drinking and warm water and the drug particles floated in the water.

Table 3. *The storage conditions of the preparation including florfenicol (n: 80)*
Tablo 3. *Florfenikol içeren preparatların saklama koşulları (n: 80)*

Storage Conditions	Number (n) and percent (%)
In Clinics	n: 17 - 21.25%
In refrigerator	n: 24 - 30%
In Room temperature	n: 19 - 23.75%
The dark warehouse	n: 20 - 25%

Table 4. *The pharmacovigilance notifications about the preparation including florfenicol (n: 80)*
Tablo 4. *Florfenikol içeren preparatlara ilişkin farmakovijilans bildirimleri (n: 80)*

Advers Drug Reactions	Number (n) and Percent (%)
There was no negativity in animals	n: 14-17.5%
The ineffectiveness of the drug/lack of sufficient activity	n: 16-20%
Drug did not dissolution in the drinking water of animals	n: 50-62.5%

DISCUSSION

Much quality control was applied to the preparation presented in the market during the period of their production. The stability tests done in both short and long duration are the most attractive ones. These tests are done to determine the shelf lives of the preparation. However, the works done to determine the quantity of the active substance after the presentation to the market are in limited numbers. Although the quantity of the active substance and the shelf life of the preparation are determined by means of the quality control and the stability tests during the period of their productions, it can be observed that the quantity of the active substance may diminish from the production date to the expiry date and then the products may be spoilt before the expiry date.

According to the study of Canefe et al.[15] on the stabilities of the drugs in the structure of liquid dispersion in Turkey's climate conditions; the results of the physical stability tests in long duration presented much more mutability than the results of the chemical stability tests and in spite of the fact that there was no chemical defeat in the active substance of the preparation during the period of observation, physical defeats came into being and some drugs could not be used.

By means of the study of Hismiogullari and Yarsan [2] on the contents of the active substance of the some authorized veterinary antibacterial drug specialities (aminoglycosides and sulfonamides) in Turkey and the effects of some storage conditions on these levels; it was revealed that the prior factors about the defeat in the speciality were the storage condition and the storage period and furthermore the chemical characteristics of the active substance in the speciality were important in this point of view. This study is similar to the one done by Hismiogullari and Yarsan [2] in this point of view.

The formulation of 11 including 5% PEG and the formulation of 12 including 5% PEG subjected to the stability tests for 3 months in the study of Bekmezci [16]. It was stated that the dissociation speed of the Formulation11 and Formulation 12 formulations decreased at the end of the 3rd month. According to the results of the study on the stability in long term of the chosen antibiotic standards done by German et al.[17]; it was seen that excessive defeat did not reveal during all the storage period and an important change didn't happen at the level of dirtiness.

It was understood that the drug was usually preferred for the respiration infections in poultry according to the pharmacovigilance questionnaires. The number of the studies on the concept "pharmacovigilance" is extremely limited in our country. There are extremely few studies on this concept in veterinary medicine. Doğan [1] determined that the vitamin-mineral group caused mostly the adverse drug reaction by means of pharmacovigilance scanning and he also stated that the local reactions in the anaphylaxis and injection area are the mostly stated adverse drug reactions.

4474 adverse drug reactions, 4250 of which are related to animals and 224 of which are related to human beings were stated in the report published by EMA [18]. 143.000 sick poultry were declared in the same report and it was emphasized that all these animals were affected and two urgent reports were published about all of them. In the same time in this report, more than 50.000 adverse drug reactions about veterinary pharmacovigilance were reported until 2010. In this report, it was stated that the usage of the injection and oral preparation including florfenicol for the eradication of

A. pleauropneumoniae off-label use caused the illnesses in productivity system and so this hadn't to be used in pregnant pigs in Denmark in 2009 and all the countries which are the members of the council were informed about this event.

The users must be informed about the fact that there is a close relationship between the effiency of the drugs they have bought and their storage conditions and that they should store the drugs in suitable conditions in order to get maximum benefit from the drug. The concept pharmacovigilance must be given more importance in Veterinary Medicine Education. Besides, following and controlling the veterinary adverse drug reaction made by authorized person in our country must be done by the State like the other countries for the sake of animal health and economic managership.

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Kafkas Univ Vet Fak Derg
19 (2): 283-289, 2013
DOI: 10.9775/kvfd.2012.7678

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

The Effects of Different Breeding Methods on Fattening Performance and Parameters Related to Slaughter, Carcass and Some Meat Quality in Broiler Chickens

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Makale Kodu (Article Code): KVFD-2012-7678

Summary

The present study was aimed at the comparison of floor-based growth (FBG) and caged-based growth (CBG) systems in broiler production for growth performance and parameters related to slaughter, carcass and meat quality. Ninety one-day-old male Ross-308 chicks constituted the material of the study. It was determined that, in Groups FBG and CBG, the differences between body weight and cumulative body weight values were statistically very significant (P<0.01). The final body weights measured at the end of the trial and the mean body weight gain values in Groups FBG and CBG were determined as 2530 g - 2046 g and 2389.16 g - 1904.77 g, respectively. The end-trial final feed conversion rates of Groups FBG and CBG were determined as 1.82 and 1.88, respectively, and it was ascertained that the difference between the values of the two groups was statistically insignificant (P>0.05). While the hot and cold dressing percentages and the neck, wing, drumstick and tail percentages did not statistically differ between Groups FBG and CBG (P>0.05), values pertaining to breast meat displayed significant differences (P<0.05). It was determined that the L*, b* and C* values pertaining to breast meat and the L*, a*, b* and C* values pertaining to chicken drumsticks displayed statistically significant differences (P<0.05, P<0.01) between the trial groups.

Keywords: *Broiler, Floor-based breeding, Caged-based breeding, Carcass, Meat quality*

Farklı Yetiştirme Metotlarının Etlik Piliçlerde Besi Performansı, Kesim ve Karkas Özellikleri İle Bazı Et Kalite Parametreleri Üzerine Etkisi

Özet

Bu araştırma altlıklı yer sistemi (FBG) ve kafeste (CBG) yapılan etlik piliç yetiştiriciliğinin besi performansı, kesim, karkas ve bazı karkas kalite parametrelerinin karşılaştırılması amacıyla yürütülmüştür. Hayvan materyali olarak 90 adet bir günlük Ross-308 erkek broyler civciv kullanılmıştır. FBG ve CBG gruplarında canlı ağırlık ve kümülatif canlı ağırlık değerleri arasındaki farklılığın istatistiksel olarak çok önemli (P<0.01) olduğu saptanmıştır. Deneme sonu canlı ağırlık değerleri ile ortalama canlı ağırlık artışları FBG ve CBG gruplarında sarasıyla 2530 ve 2046 g ile 2389.16 ve 1904.77 g olarak tespit edilmiştir. Deneme sonu kümülatif yemden yararlanma değerleri FBG ve CBG gruplarında sırayla 1.82 ve 1.88 olarak bulunmuş ve aralarındaki farklılığın istatistiksel olarak önemsiz (P>0.05) olduğu tespit edilmiştir. FBG ve CBG gruplarında sıcak ve soğuk karkas randımanı ile boyun, kanat, but ve kuyruk oranlarına ait değerler arasında istatistiksel olarak farklılığın olmadığı (P>0.05), göğüs oranına ait değerler bakımından ise önemli (P<0.05) farklılıkların olduğu belirlenmiştir. Göğüs etinden elde edilen L*, b* ve C* değerlerinin ve but etinden elde edilen L*, a*, b* ve C* değerleri bakımından deneme grupları arasında önemli farklılıkların olduğu (P<0.05, P<0.01) tespit edilmiştir.

Anahtar sözcükler: *Etlik piliç, Yerde yetiştirme, Kafeste yetiştirme, Karkas, Et kalitesi*

INTRODUCTION

Broiler production has a privileged position in the livestock sector, owing to the very short period of production, the availability of intensive breeding, the low feed conversion rate of broiler chickens, the lower labour force required

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for broiler farms compared to other types of agricultural holdings and the nutritional value of chicken meat as a good protein source. When applying conventional broiler production methods, generally floor-based growth systems are preferred. In view of emerging demands related to the new animal welfare rules implemented in European Union Member States, some modifications have been made in floor-based growth systems (i.e. free range systems). Cage-based systems are aimed at increasing broiler production quantitatively and are of economic interest.

Numerous studies have been conducted to demonstrate the advantages and disadvantages of floor-based and cage-based growth systems in broiler production. The assessment of the results of these studies has revealed significant contradictions between the findings obtained in different trials. Abrahamsson and Tauson [1] reported the main advantages of the use of cage-based growth systems in broiler production as high productivity, no contact with manure, and less aggressiveness and cannibalism among animals. On the other hand, Rodriguez et al.[2] and Santoso [3] indicated that the body weight gain and feed conversion rates (FCR) of broiler chickens raised in cages were poorer compared to broilers raised on floor. Furthermore, Swain et al.[4] suggested that neither cage-based nor floor-based growth systems had any effect on body weight gain, feed consumption or carcass traits in broiler production. While some researchers have claimed that cage-based growth systems enable better growth performance and a higher survival rate, and thus, provide economic advantage when compared to floor-based growth systems [5,6], some other researchers have reported that floor-based growth systems are more economical [7]. Duncan [8] has highlighted the inadequacy of physical space and behavioral restrictions as the major disadvantages of cage-based growth systems.

When evaluating the effects of cage-based and floor-based growth systems on broiler chickens, generally growth performance parameters are taken into consideration. The present study, in addition to growth performance parameters, also makes a comparison of slaughter and carcass traits, some stress and carcass quality parameters for the evaluation of floor-based and cage-based growth systems in broiler production.

MATERIALS and METHODS

Animals, Diets, and Experimental Design

The trial was conducted at the poultry unit of the Research Farm of Atatürk University, Faculty of Veterinary Medicine. Ninety one-day-old Ross-308 male broiler chicks constituted the material of the study. All chicks were raised in brooders (cages) between days 1-7 of the trial. After day 7, the animals were allocated to two trial groups, one which was subjected to a floor-based growth (FBG) system and the other to a cage-based growth (CBG) system. In Group CBG, the broiler chickens were maintained in cages until the end of the trial, whilst in Group FBG, as from day 7, the animals were raised on floor covered with wood chips of a depth of 10 cm. In both Group CBG and Group FBG, the stocking density was 0.083 m^2/broilers. Accordingly, Group FBG was divided into 5 subgroups, each of which was comprised of 12 broilers, whilst Group CBG was divided into 6 subgroups, each of which was comprised of 5 broilers. The animals included in the present study were fed on broiler chick feed between days 1-21 and were fed on broiler chicken feed between days 22-42. The laboratory analysis results and nutrient compositions of the starter and finisher feed rations are presented in *Table 1*. The chemical composition of the feeds used in this study was determined using the official method of the Association of Official Analytical Chemists [9]. The groups were subjected to 24 h of light, in other words, to a continuous lighting programme, until the end of the trial.

Determination of Body Weight, Feed Consumption, Uniformity and Survival Rate

Parameters related to body weight and feed consumption were determined by weekly weight measurements. Mortalities were recorded on a daily basis. In order to preserve the stocking density, in case of any mortality, a broiler chicken of the same age, selected from the stock, was introduced into the group. With an aim to determine uniformity, on day 42, each chicken was weighed individually and the measurements were recorded. The mean weights of the trial groups and variation coefficients were also calculated.

Determination of Tibial Dyschondroplasia

At the end of the trial, two chickens from each trial group were slaughtered and the left tibial bone was

Table 1. *The laboratory analysis results and nutrient compositions of the starter and finisher feed rations*

Tablo1. *Başlangıç ve bitirme yemlerinin besin madde kompozisyonları ve laboratuar analiz sonuçları*

Composition	Starter Diet	Finisher Diet
Dry matter (%)	91.86	91.84
Crude protein (%)	23.98	20.02
Crude fiber (%)	3.68	3.75
Crude ash (%)	7.91	8.19
Ether extract	7.23	9.56
Nitrogen-free extract (%)	49.06	50.32
Calcium (%)	1.50	1.50
Phosphorus (%)	0.70	0.65
Sodium (%)	0.30	0.30
NaCL (%)	0.35	0.35
Lysine (%)	1.20	1.00
Methionine (%)	0.50	0.40
Methionine + Cystine (%)	0.90	0.75
Metabolic energy (Kcal/kg)	3075	3200

incised longitudinally until the end of the epiphysis for the determination of its status for tibial dyschondroplasia. A millimetric calliper was used for the measurement of the lesions. The severity of tibial dyschondroplasia was scored as 0 if there were no lesions, 1 if the distal distribution area of the lesions was smaller than 0.5 cm, 2 if the size of the distal distribution area of the lesions ranged between 0.5 and 1 cm, and 3 if the distal distribution area of the lesions was larger than 1 cm [10].

Sample Collection and Measurements

At the end of the trial, the birds were held for 10-12 h without food and water prior to determining their final body weights. Two birds from each subgroup were randomly chosen as having body weights nearest to the average body weight of their own groups, slaughtered via a neck cut, bled for 120s, and semi-scalded 54°C for 30s before mechanical plucking. The birds were eviscerated manually, washed, and allowed to drain for 10 min [11]. After eviscerating, carcasses were stored at 3°C for 24 h, and then the carcasses were dissected [12-14].

Analysis for pH and Thiobarbituric Acid Reactive Substances (TBARS)

The pH values of the samples were determined using a pH meter (SCHOTT L 6880, Lab StarpH). The pH value was measured using a direct probe by thrusting the probe into the breast fillets. To determine TBARS values, 1 g of ground breast meat sample was taken from each breast fillet of each treatment, and 6 mL TCA solution was added (7.5% TCA, 0.1% EDTA, 0.1% 1-propyl gallate). The mixture was homogenised for 20-30s by Ultra-Turrax and was then filtered over Whatman one. Next, 1 mL 0.02 M ThioBarbituric acid solution was added to the 1mL filtrate. This mixture was kept in a boiling water bath for 40 min. It was then cooled and centrifuged at 2.000 rpm for 5 min. Finally, absorbance was measured at 532 nm (Shimadzu, UV 160), and TBARS values were determined using the standard coefficient as µmol malonaldehyde/kg.

Determination of Colour Values

A Minolta model colorimeter (CR-200, MinoltaCo, Osaka, Japan) was used for the colour measurement of the breast fillet samples, with a white tile as a reference [L* = 0, darkness; L* = 100, lightness (darkness/lightness); a*; + a* = red, -a* = green and b*; + b = yellow, -b = blue; hue = h* = $\tan^{-1}(b^*/a^*)$; and chroma = C* = $(a^{*2} + bb^{*2})^{1/2}$].

Statistical Analysis

The data obtained in the present study was analysed using the SPSS [15] software, such that a comparison of the use of the two different growth systems in broiler production was made for growth performance and parameters related to slaughter, carcass and meat quality. Differences between the trial groups were determined using the t test. For the assessment of the differences observed in survival rate and tibial dyschondroplasia, the Kruskal-Wallis test, which is a nonparametric statistical method, was employed.

RESULTS

The body weight and cumulative body weight values of Groups FBG and CBG are shown in *Table 2*. The evaluation of the values presented in *Table 2* demonstrate that, the body weight and cumulative body weight values of the trial groups, which were determined by weekly measurements, significantly differ from each other (P<0.01).

The feed consumption, feed conversion, cumulative feed consumption and cumulative feed conversion values of Groups FBG and CBG are shown in *Table 3*. The final cumulative feed consumption values of Groups FBG and CBG were determined as 4339.56 g and 3585.61 g, respectively. The investigation of weekly feed consumption and cumulative feed consumption values revealed that, excluding the first week of the trial, throughout the remaining time period, the broilers included in Group FBG consumed a greater amount of feed than those included in Group CBG, and it was ascertained that this difference was statistically very significant (P<0.01).

In Groups FBG and CBG, the maximum and minimum body weights and variation coefficients were determined as 2960.00 - 2790.00 g, 2000.00 - 1250.00 g and 7.68% - 16.55%, respectively.

Parameters pertaining to the carcass traits of Groups FBG and CBG are presented in *Table 4*. With an aim to determine slaughter and carcass traits, broiler chickens with

Table 2. *The live weight and cumulative body weight values of groups FBG and CBG, measured each week*
Tablo 2. *FBG ve CBG gruplarının haftalık ve kümülatif canlı ağırlık değerleri*

Weeks	Live Weight (g)				P<[1]
	Floor Breeding		Cage Breeding		
	Mean	SD	Mean	SD	
1	141.00	0.00	141.33	2.94	NS
2	350.40	5.68	282.83	9.13	**
3	770.00	21.05	677.50	26.77	**
4	1186.20	13.70	985.66	70.55	**
5	1827.20	49.65	1431.50	103.91	**
6	2530.16	74.28	2046.11	62.68	**
1-2	209.40	5.68	141.50	9.13	**
1-3	629.00	21.05	544.50	26,77	**
1-4	1045.20	13.70	852.66	70.55	**
1-5	1686.20	49.65	1298.50	103.91	**
1-6	2389.16	74.28	1904.77	61.00	**

[1] *Statistical significance, ** P<0.01,* ***NS:*** *Non-significant,* ***SD:*** *Standard Deviation*

Table 3. *The weekly feed consumption, feed conversion rate, cumulative feed consumption and cumulative feed conversion ratio values of groups FBG and CBG*
Tablo 3. *FBG ve CBG gruplarının haftalık yem tüketimi, yemden yararlanma, kümülatif yem tüketimi ve kümülatif yemden yararlanma değerleri*

Weeks	Feed Consumption (g)				P<[1]	Feed Efficiency				P<[1]
	Floor Breeding		Cage Breeding			Floor Breeding		Cage Breeding		
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
1-2	244.14	15.95	222.28	7.13	NS	1.16	0.06	1.57	0.20	**
2-3	707.28	31.01	504.93	16.59	**	1.69	0.11	1.26	0.11	**
3-4	723.29	54.46	560.36	46.83	**	1.74	0.15	1.86	0.30	NS
4-5	1198.28	21.61	970.36	41.44	**	1.87	0.09	2.20	0.29	*
5-6	1466.57	43.82	1327.74	82.18	**	2.09	0.15	2.18	0.27	NS
1-2	951.42	19.50	727.21	8.72	**	1.52	0.07	1.34	0.11	**
1-3	1674.71	53.95	1287.51	69.97	**	1.60	0.05	1.52	0.14	NS
1-4	2872.99	64.91	2257.87	84.86	**	1.70	0.03	1.75	0.16	NS
1-5	4339.56	104.46	3585.61	122.75	**	1.82	0.05	1.88	0.07	NS

*[1] Statistical significance, ** P<0.01, * P<0.05,* ***NS:*** *Non-significant;* ***SD:*** *Standard Deviation*

Table 4. *Parameters pertaining to the carcass characteristics of groups FBG and CBG*
Tablo 4. *FBG ve CBG gruplarının karkas özelliklerine ait parametreler*

Parameters	Carcass Characteristic				P<[1]
	Floor Breeding		Cage Breeding		
	Mean	SD	Mean	SD	
Pre slaughter weight (g)	2435.50	106.31	2048.50	167.30	**
Hot carcass weight (g)	1791.60	77.91	1504.70	141.04	**
Cold carcass weight (g)	1775.00	78.92	1486.20	141.14	**
Hot carcass yields (%)	73.57	1.33	73.49	1.69	NS
Cold carcass yields (%)	72.89	1.27	72.49	1.69	NS
Ratio of neck (%)	5.65	1.00	5.78	0.50	NS
Ratio of wing (%)	10.91	0.69	11.36	0.87	NS
Ratio of whole legs (%)	38.30	1.67	38.93	2.55	NS
Ratio of tail (%)	1.08	0.21	1.25	0.23	NS
Ratio of whole breast (%)	44.06	1.13	42.68	2.23	*

*[1] Statistical significance, ** P<0.01, * P<0.05,* ***NS:*** *Non-significant;* ***SD:*** *Standard Deviation*

Table 5. *Parameters pertaining to slaughter characteristics of groups FBG and CBG*
Tablo 5. *FBG ve CBG gruplarının kesim özelliklerine ait parametreler*

Parameters	Slaughter Characteristic				P<[1]
	Floor Breeding		Cage Breeding		
	Mean	SD	Mean	SD	
Ratio of blood (%)	4.34	0.45	3.74	0.51	**
Ratio of feather (%)	5.52	0.44	5.15	0.48	NS
Ratio of head (%)	2.52	0.29	3.15	0.32	**
Ratio of noneatable organ weights (%)	5.01	0.59	5.24	0.98	NS
Ratio of gizzard (%)	2.14	0.33	2.31	0.46	NS
Ratio of liver (%)	1.79	0.15	1.93	0.22	NS
Ratio of foot (%)	4.09	0.22	4.66	0.40	NS
Ratio of heart (%)	0.56	0.09	0.69	0.10	**

*[1] Statistical significance, ** P<0.01,* ***NS:*** *Non-significant;* ***SD:*** *Standard Deviation*

Table 6. *The tibial dyschondroplasia scores and survival rate values of groups FBG and CBG*
Tablo 6. *FBG ve CBG gruplarının tibial diskondroplazi skoru ve yaşama gücü değerleri*

Treatment Groups	TD[1]		P<[3]	SR[2] (%)		P<[3]
	Mean	SD		Mean	SD	
Floor Breeding	0.44	0.72	NS	100.00	0.00	*
Cage Breeding	0.33	0.50		94.40	23.20	

*[1] Tibial Dyschondroplasia, [2] Survival rate, [3] Statistical significance, * P<0.05,* ***NS:*** *Non-significant;* ***SD:*** *Standard Deviation*

values closest to the final mean body weight were selected from each trial group. Excluding baseline body weights, values pertaining to Group FBG were greater than those pertaining to Group CBG for all time points. Therefore, the differences observed between the two groups for the mean values of preslaughter body weight, hot carcass weight and cold carcass weight were statistically very significant (P<0.01).

Parameters pertaining to slaughter traits of Groups FBG and CBG are shown in *Table 5*. It was ascertained that, the differences between the two trial groups for feather, inedible organ weights, and gizzard, liver and feet percentages were statistically insignificant (P>0.05).

The tibial dyschondroplasia scores *(Table 6)* of Groups FBG and CBG were 0.44 and 0.33, respectively, and the groups did not statistically differ from each other for this parameter (P>0.05). In terms of survival rate *(Table 6)*, it was observed that no mortality occurred in Group FBG until the end of the trial, whilst the survival rate of Group CBG was determined as 94.4%.

The L*, a*, b*, C* and H* values of the skinless leg and breast meat of Groups FBG and CBG are shown in *Table 7*.

Table 7. *The L*, a*, b*, C* and H* values of the skinless leg and breast meat of Groups FBG and CBG*
Tablo 7. *FBG ve CBG gruplarının derisiz göğüs ve but etlerinin L*, a*, b*, C* ve H* değerleri*

CP[1]	Breast				P<[2]	Thigh				P<[2]
	Floor Breeding		Cage Breeding			Floor Breeding		Cage Breeding		
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
L*	54.49	4,35	51.81	3.35	**	58.20	2.37	53.11	8.29	**
a*	3.12	1.48	3.53	1.26	NS	4.05	1.54	6.11	5.68	*
b*	6.48	2.36	7.86	2.19	**	5.54	2.28	7.75	2.85	**
C*	7.28	2.45	8.91	1.95	**	6.90	2.06	9.62	2.17	**
H*	64.35	10.99	66.40	7.86	NS	53.09	14.73	54.23	13.99	NS

*[1] Colour Parameters, **L* :** lightness (0 = black; 100 = white), **a* :** redness (– = green; + = red), **b* :** yellowness (– = blue; + = yellow), **H* :** hue, **C* :** liveliness, [2] Statistical significance, ** P<0.01, * P<0.05, **NS:** Non-significant; **SD:** Standard Deviation*

Table 8. *The pH and thiobarbutiric acid reactive substances values of breast meat in groups FBG and CBG*
Tablo 8. *FBG ve CBG gruplarının göğüs etinden elde edilen pH ve tiabarbütirik asit reaktif substans değerleri*

Treatment Groups	pH		P<[2]	TBARS[1]		P<[2]
	Mean	SD		Mean	SD	
Floor Breeding	6.14	0.11	**	13.79	1.22	NS
Cage Breeding	5.95	0.21		13.77	2.05	

*[1] Thiobarbutiric acid reactive substances (mmol malonaldehyde kg^{-1}), [2] Statistical significance, ** P<0.01, **NS:** Non-significant; **SD:** Standard Deviation*

The differences observed between the trial groups for the a* and H* values of breast meat were statistically insignificant (P>0.05), whilst the differences observed for the L*, b* and C values were statistically very significant (P<0.01).

The pH values of breast meat in Groups FBG and CBG (*Table 8*) were determined as 6.14 and 5.95, respectively.

The TBARS values determined in Groups FBG and CBG are presented in *Table 8*. Accordingly, the TBARS values of Groups FBG and CBG were determined as 13.79 and 13.77 mmol malondialdehyde kg^{-1}, respectively, and the two groups did not differ from each other statistically.

DISCUSSION

The final body weights and mean body weight gains of Groups FBG and CBG were determined as 2530 g - 2046 g and 2389.16 g -1904.77 g, respectively. It was ascertained that, the body weight and mean body weight values of Group FBG were significantly higher than those of Group CBG. The review of previous literature reports has revealed that a consensus has not been reached on this issue, and that to date; very different results have been obtained. In agreement with the findings of the present study, Hypes et al.[16] (CBG 1.832 - FBG 1.911 g), Türkyılmaz et al.[17] (CBG 2168.4 - FBG 2261.1 g) and Fouad et al.[7] (CBG 1616.27 - FBG 1862.52 g) reported that the final body weight of broiler chickens was higher in Group FBG compared to Group CBG and that this difference was statistically significant. On the other hand, Elibol [18] (CBG 1792 - FBG 1745 g), Reece et al.[19] and Andrews et al.[20] indicated that the final body weights of Group CBG were higher than those of Group FBG and that the two groups differed from each other significantly. Furthermore, Andrews [21] (CBG 1572 - FBG 1573 g), Andrews et al.[22] (CBG 1513 - FBG 1485 g), and Athar et al.[23] (CBG 2000 - FBG 1910 g) indicated that Groups FBG and CBG did not display any statistically significant difference from each other in terms of final body weights.

Contradictory to the findings of the present study, Fouad et al.[7] reported the total feed consumption values of broiler chickens included in Groups FBG and CBG as 3096.48 g and 3139.46 g, respectively, and indicated the absence of any statistical difference between the trial groups. In parallel with the findings of Fouad et al.[7], Athar et al.[23] reported the feed consumption values of Groups CBG and FBG as 4.46 kg and 4.43 kg, respectively, and noted that the difference between the two groups was statistically insignificant. In a study conducted by Zahoa et al.[24], in which the effect of three different types of cage floor (wire, plastic and bamboo) on the growth performance of broiler chickens was investigated, the differences between the feed consumption values of the trial groups was found to be statistically insignificant, and these values were reported as 3.41 kg, 3.28 kg and 3.21 kg for the groups raised in cages with wire, plastic and bamboo floors, respectively.

In the present study, the feed conversion rates in Groups FBG and CBG during the time intervals between days 7-14, 14-21 and 28-35 were determined as 1.16-1.57, 1.69-1.26 and 1.87- 2.20, respectively, and it was ascertained that the difference between the groups was statistically significant. Furthermore, the cumulative feed conversion rates of Groups FBG and CBG were determined as 1.82 and 1.88, respectively, and the difference between the groups was statistically insignificant (P>0.05). In agreement with the findings of the present study, the final mean feed conversion rates of broiler chickens included in Groups CBG and FBG were reported as 2.18 and 2.14, respectively, by Welch et al.[25]; 1.71 and 1.71, respectively, by Türkyılmaz et al.[17]; 1.97 and 1.72,

respectively, by Fouad et al.[7]; and 2.23 and 2.32, respectively, by Athar et al.[23]. These researchers indicated that no statistical difference was observed between the groups for this parameter. Reece et al.[19] reported that the feed conversion rates of broiler chickens raised on floor were better than those of broilers raised in cages. Akpobome and Fanguy [26], on the other hand, reported that the feed conversion rates of broiler chickens raised in cages were better than those of broilers raised on floor. Furthermore, Hypes et al.[16] determined that, until day 42, the feed conversion rates of male broilers raised in cages and on floor were 2.047 and 2.115, respectively, and that the difference between the two groups was statistically significant. Zahoa et al.[24] indicated that the effect of three different types of cage floors (wire, plastic and bamboo) on the feed conversion rates of broiler chickens was statistically insignificant.

Group FBG showed a higher uniformity level in terms of final body weights, in comparison to Group CBG.

Hot and cold dressing percentages and neck, wing, drumstick and tail percentages did not differ statistically between Groups FBG and CBG (P>0.05). The breast meat percentages of Groups FBG and CBG were determined as 44.06% and 42.68%, respectively, and the difference between the two groups was found to be statistically significant. Türkyılmaz et al.[17] reported the hot dressing percentages of broiler chickens raised on floor and in cages as 69.6% and 71.4%, respectively, while Athar et al.[23] reported the same values as 68.9% and 67.60%, respectively. In both studies, the values of the different groups did not differ from each other statistically.

Blood, head and heart percentages of Groups FBG and CBG were 4.34% - 3.74%, 2.52% - 3.15% and 0.56% - 0.69%, respectively, and the differences between the trial groups were statistically very significant (P<0.01). The rate values belonging heart and liver were found to be similar as the results reported by Yildiz et al.[27]

In terms of survival rate *(Table 6)*, it was observed that no mortality occurred in Group FBG until the end of the trial, whilst the survival rate of Group CBG was determined as 94.4%. The difference observed between the trial groups for survival rate was statistically significant. Moreover, Andrews and Goodwin [28], Elibol [18], Türkyılmaz et al.[17] and Fouad et al.[7] reported that the difference between the mortality rates of broiler chickens raised in cages and on floor was statistically insignificant.

The assessment of the values obtained for chicken drumsticks revealed that, excluding the H* value, the two groups differed from each other significantly for the L*, a*, b* and C* values. The pH values of breast meat in Groups FBG and CBG were determined that the difference between the groups for this parameter was statistically very significant. Wariss et al.[29] reported that meat pH values were affected, to a greater extent, from applications employed prior to slaughter. The pH value of meat is the most significant parameter, which affects the shelf life of this food product. Meat with high pH values has a shorter shelf life, as microbial activity reaches a maximum level at high pH levels. In this respect, lower pH values having been determined for the meat of broilers included in Group CBG can be considered as an advantage. Yetişir et al.[30] reported the pH value of breast meat of broiler chickens as 5.837. Furthermore, the L*, a* and b* values they determined for skinless breast meat were 55.765, 4.334 and 7.204, respectively. For breast meat, Quio et al.[31] reported the L*, a* and b* values as 49.6, 3.3 and 4.9, respectively, Goksoy et al.[32] reported the L*, a* and b* values as 62.23, 3.41 and 5.60, respectively, whilst Ponsano et al.[33] reported the L*, C* and H* values as 60.6, 11.25 and 68.5, respectively. Northcutt [34] reported that the colour of chicken meat was affected by multiple factors, including age, sex, genotype, feed, intramuscular fat distribution, water content of meat, preslaughter conditions and processing techniques, while Fletcher [35] noted sex, age, breed, management techniques, chemical processing and deep freezing conditions as factors influential on meat colour. Based on the findings obtained in the present study, it is suggested that the particular growth system used for production also affects the colour parameters of poultry meat. Petracci et al.[36] reported for poultry meat that, dark meat colour and meat pH value were significantly correlated with each other, and also indicated that lower pH values were correlated with lighter meat colour. The assessment of the parameters obtained for chicken drumsticks and breast meat in the present study demonstrated that the L* values of Group FBG were higher and thus, it was observed that the meat of this group was of darker colour. This was attributed to Group FBG having higher pH values than those of Group CBG. The TBARS values determined in Groups FBG and CBG are presented in *Table 8*. Analyses performed for the detection of thiobarbituric acid-reactive substances (TBARS) are frequently employed for the detection of the malondialdehyde content of meat, which is an indicator of bad taste in meat. Owing to the high level of polyunsaturated fatty acids it contains, poultry meat is very sensitive to oxidative damage. Aksu et al.[37] reported the TBARS value for male Ross 308 broiler chickens as 14.11 mmol malondialdehyde kg^{-1}. The TBARS value previously reported by Aksu et al.[37] is higher than the TBARS values determined in the present study for Groups FBG and CBG.

The present study, which was aimed at the comparison of floor-based and cage-based growth systems in broiler production for growth performance and parameters related to slaughter, carcass and carcass quality, demonstrated that, in terms of growth performance, broiler chickens raised using the floor-based growth system reached a mean body weight of 2530.16 g at the end of the trial by consuming a mean feed amount of 4339.56 g, while broiler chickens raised using the cage-based growth system reached a mean body weight of 2046.11 g by consuming a mean feed amount of 3585.61 g. The present study demonstrated

that, in view of current feed and poultry meat prices alone, although the cage-based and floor-based growth systems did not statistically differ from each other in terms of feed conversion rates, the profitability of the floor-based growth system was 15.65% greater than that of the cage-based growth system. Furthermore, the breast meat percentages of the broilers raised on floor were greater than those of the broilers raised in cages. In the present study, it was determined that the pH values of meat obtained from broiler chickens raised on floor were higher. This is also considered as a major reason for the difference observed in the present study between the two groups for the colour of chicken leg and breast meat.

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Kafkas Univ Vet Fak Derg
19 (2): 291-297, 2013
DOI: 10.9775/kvfd.2012.7710

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Yonca Silajlarında Katkı Maddesi Olarak Gladiçya Meyvelerinin (*Gleditsia Triacanthos*) Kullanılma Olanakları

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Makale Kodu (Article Code): KVFD-2012-7710

Özet

Bu araştırma, yonca (*Medicago sativa* L.) silajında karbonhidrat kaynağı olarak kullanılan gladiçya meyvesi (GM)'nin laboratuvar koşullarında yapılan silajların fermantasyon, aerobik stabilite, *in vitro* gaz üretimi, mikrobiyolojik özellikleri üzerindeki etkilerinin saptanması amacı ile yapılmıştır. Araştırmada kullanılan yonca tam çiçeklenme döneminde hasat edilmiş ve yaklaşık 1.5-2.0 cm boyutunda doğranan taze materyale 0 (kontrol), 20, 40, 60, 80 ve 100 g/kg KM düzeyinde GM katılmıştır. Yoncaya GM ilavesi, elde edilen silajların ham besin maddeleri bileşimini etkilemiştir (P<0.01). Yoncaya katılan GM düzeyinin artışına bağlı olarak silajların ham protein (HP), ham yağ (HY), ham kül (HK), nötr deterjan lif (NDF) ve asit deterjan lif (ADF) içeriği azalmış, suda çözünebilir karbonhidrat (SÇK) ve toplam tanen (TT) içeriği artmıştır (P<0.01). Gladiçya meyvesi ilavesi silajların pH, asetik asit, bütirik asit ve amonyak azotu (NH_3-N) konsantrasyonlarını düşürürken, laktik asit ve propiyonik asit düzeylerini artırmıştır (P<0.01). Aynı şekilde GM ilavesi silajların *in vitro* gaz üretimi, sindirilebilir organik madde (SOM), metabolik enerji (ME) ile laktik asit bakterileri (LAB) ve maya sayılarını artırmış, küf sayısını ise düşürmüştür (P<0.05). Diğer yandan GM silajlardaki CO_2 üretimini düşürerek silajların aerobik stabilitelerini geliştirmiştir. Araştırma sonucunda, yoncada karbonhidrat kaynağı olarak 80 ile 100 g/kg KM düzeyinde GM kullanılabileceği belirlenmiştir.

Anahtar sözcükler: *Yonca silajı, Gladiçya meyvesi, Silaj fermantasyonu, İn vitro gaz üretimi, Aerobik stabilite*

The Use of Honey Locust Pods as A Silage Additive for Alfalfa Forage

Summary

The aim of current experiment was to determine the effects of honey locust pods (HLP) as carbohydrate source on the fermentation, aerobic stability, *in vitro* gas production, microbiological characteristics of alfalfa (*Medicago sativa* L.) silages in laboratory conditions. Honey locust pods was applied at 0 (control), 20, 40, 60, 80 and 100 g/kg DM levels to alfalfa forage harvested at full bloom stage and chopped to about 1.5-2.0 cm length. Supplementation of HLP had a significant (P<0.01) effect on the chemical composition of alfalfa silage depending on the level of HLP supplementation. The supplementation of HLP significantly (P<0.01) decreased the crude protein (CP), ether extract (EE), crude ash (CA), neutral detergent fiber (NDF) and acid detergent fiber (ADF) whereas the water soluble content (WSC) and total tannin (TT) increased with increasing level of HLP. The supplementation of HLP decreased the pH, acetic acid, butyric acid and ammonia-N (NH_3-N) whereas the supplementation of HLP increased the lactic acid and propionic acid content of alfalfa silage (P<0.01). The supplementation of HLP significantly (P<0.01) increased the *in vitro* gas production, digestible organic matter (DOM), metabolizable energy (ME), lactic acid bacteria and yeast whereas mould decreased. On the other hand the supplementation of HLP improved the aerobic stability of alfalfa silage decreasing the CO_2 production. As a conclusion the HLP can be used as a silage additive in the level of 80 and 100 g/kg DM when alfalfa plant is ensiled.

Keywords: *Alfalfa silage, Honey locust pods, Silage fermentation, In vitro gas production, Aerobic stability*

GİRİŞ

Baklagil kaba yemleri, önemli yem kaynaklarından olup dünyada yaygın olarak ruminant ve diğer hayvanların beslenmesinde kullanılmaktadır. Bu yemlerden en önemlisi ise yoncadır (*Medicago sativa* L.) [1]. Yonca kurutularak kullanımı yanında silo yemi olarak da kullanılmaktadır. Silaj olarak yonca; protein ve mineral madde düzeyinin yüksek olması,

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suda çözünebilir karbonhidrat (SÇK) içeriğinin düşük olması ve tampon kapasitesinin yüksekliği ile silolanması zor yemler sınıfına girmektedir [2,3]. Bu nedenle, protein ve mineral madde bakımından zengin, karbonhidrat bakımından fakir olan silajlık yem bitkilerinin silolanması sırasında, fermantasyonun güvence altına alınabilmesi için katkı maddelerinin kullanılması bazen zorunlu hale gelmektedir [3-5]. Bu amaçla, farklı katkı maddelerinden yararlanılmakla birlikte daha çok ortamda yetersiz düzeyde bulunan karbonhidrat açığını kapatmaya yönelik katkı maddelerinden yararlanılmaktadır. Karbonhidrat kaynağı olarak en fazla tahıl daneleri, melas, üzüm posası, şeker v.b. kullanılmaktadır [2,6-9]. Öte yandan bunlara alternatif olarak şeker içeriği yüksek ve ucuz olan bazı ağaçların meyveleri de bu amaçla ön plana çıkmaktadır [5,9,10].

Bu ürünlerden birisi de ülkemiz doğal florası içerisinde yer alan ve karbonhidrat içeriği yüksek gladiçya (*Gleditsia triacanthos*) bitkisidir. Gladiçya suda çözünür karbonhidrat (122.3-152.2 g/kg KM) ve tanen (27.8-148.2 g/kg KM) bakımdan zengin bir bitkidir [11]. Olgun bir gladiçya bitkisinin yılda 87 kg civarında meyve verebildiği bildirilmiştir [5]. Keçiboynuzuna benzeyen gladiçya meyveleri ruminant hayvanlar tarafından, şeker içeriği yüksek olduğundan sevilerek tüketilmektedir [11]. Ayrıca yüksek düzeyde (27.8-148.2 g/kg KM) tanen içermesi [11,12] nedeniyle silolardaki yemlerin yapısında bulunan proteinleri bağlamak suretiyle amonyak azotu (NH_3-N) şeklinde azot kaybını önlediği ve bu yolla silajlarda protein kaybını azalttığı bildirilmektedir [5,13-18]. Bazı silaj katkı maddelerinin sağlık riski nedeniyle kullanımlarının yasaklanması çevre, insan ve hayvan sağlığına zararlı olmayan alternatif ve doğal silaj katkı maddelerinin kullanımını gündeme getirmiştir. Bu bağlamda SÇK ve tanen içeriği yüksek olan gladiçya meyvesinin silaj katkı maddesi olarak kullanımını ön plana çıkarmıştır [5].

Bu çalışmanın amacı, tam çiçeklenme döneminde hasat edilen yoncaya SÇK ve tanen içeriği yüksek olan gladiçya meyvesi (GM) ilavesinin elde edilen yonca silajlarının fermantasyonuna, mikrobiyolojik özelliklerine, aerobik stabilitesine ve *in vitro* gaz üretimi üzerine olan etkilerini saptamaktır.

MATERYAL ve METOT

Yem Materyali ve Silolar

Araştırmanın silaj materyalini Uludağ Üniversitesi Ziraat Fakültesi Araştırma ve Uygulama Merkezi (ZFAUM)'nde yetiştirilen yonca oluşturmuştur. Gladiçya meyvesi ise Uludağ Üniversitesi yerleşkesinde bulunan ve doğal olarak yetişen gladiçya ağaçlarından hasat edilerek sağlanmıştır. Hasat edilen gladiçya meyveleri 65°C'de 12 saat süreyle kurutulmuştur. Daha sonra 2.5 mm elek çapına sahip değirmende öğütülmüş ve bu formda yoncaya katılmıştır. Yoncaların silolanması için ise yalnızca gaz çıkışına olanak tanıyan 1.5 l kapasiteli laboratuvar tipi özel anaerobik cam silolar (Weck®, Germany) kullanılmıştır.

Silajların Hazırlanması

Araştırmada kullanılan yonca, tam çiçeklenme döneminde (%27.8±0.28 KM) hasat edilmiş ve silaj makinesinde yaklaşık 1.5-2.0 cm boyutlarında parçalanmıştır. Daha sonra yoncaya 0 (kontrol), 20, 40, 60, 80 ve 100 g/kg KM düzeyinde GM ilave edilmiş ve böylece 6 farklı silaj grubu oluşturulmuştur. Yoncaya GM uygulaması sırasında her defasında 8 kg taze yonca temiz bir yere yayılarak üzerine GM serpilmiş ve homojen bir şekilde karıştırıldıktan sonra anaerobik silolara her biri 5 tekerrür olacak şekilde silolanmıştır. Silolar 60 gün boyunca laboratuvar koşullarında (24±4°C) tutulmuştur. Silolama dönemi sonunda (60. gün) açılan silajların kimyasal analizleri, mikrobiyolojik özellikleri, aerobik stabiliteleri ile *in vitro* gaz üretimi, sindirilebilir organik madde (SOM) ve metabolik enerji (ME) içerikleri saptanmıştır.

Hayvan Materyali

Araştırmada hayvan materyalini 1.5-2 yaşlarında 3 baş Holstein erkek sığır oluşturmuştur. Hayvanlar deneme süresince mısır silajı ve yoğun yem karması (%17 ham protein, 2.700 kcal ME/kg KM) ile yemlenmişlerdir. Rasyonlarda kaba ve yoğun yem oranı kuru madde temeline göre 60/40 olacak şekilde düzenlenmiş ve çalışma sırasında etik kurallara titizlikle uyulmuştur.

İn Vitro Gaz Üretim Özellikleri

Yonca silajlarının *in vitro* koşullarda sindirilebilirlik ve ME düzeyinin saptanmasında Menke ve Steingass [20] tarafından bildirilen *in vitro* gaz üretim tekniği kullanılmıştır. Silajların *in vitro* gaz üretim miktarları ile ME ve SOM'lerinin saptanmasında 100 ml hacimli özel cam tüplere (Model Fortuna, Häberle Labortechnik, Lonsee-Ettlenschieb, Germany) üç paralel olarak, yaklaşık 200±10 mg, kurutulmuş silaj konmuştur. Daha sonra üzerine Menke ve ark.[21] tarafından bildirilen yönteme göre hazırlanan rumen sıvısı/tampon çözeltisinden 30 ml ilave edilmiştir. Bu işlemden sonra tüpler 39°C' deki çalkalamalı su banyosunda inkübasyona alınmışlar ve sırasıyla 24 ve 96. saatlerde fermantasyonla oluşan gaz miktarları saptanmıştır.

Silajların ME ve SOM'leri Menke ve Steingass [20] tarafından bildirilen eşitliklerle saptanmıştır.

SOM, % = 15.38 + 0.8453 x GÜ+ 0.0595 x HP + 0.0675 x HK

ME, MJ/kg KM= 2.20 + 0.1357x GÜ + 0.0057 x HP+ 0.0002859 x HY^2

(ME: metabolik enerji, SOM: sindirilebilir organik madde, GÜ: 200 mg kuru yem örneğinin 24 saatlik inkübasyon süresi sonundaki net gaz üretimi, HP: % ham protein, HY: % ham yağ ve HK: % ham kül).

Kimyasal Analizler

Taze yonca ve yonca silajları 65°C'de etüvde 48 saat süreyle kurutularak GM ise elde edildiği şekliyle 1 mm elek

çapına sahip değirmende öğütülerek kimyasal analizlerde kullanılmıştır. Yemlerin kuru madde (KM) içeriği 105°C'de etüvde 3 saat kurutularak, ham kül içeriği 550°C'de 4 saat kül fırınında yakılarak, ham yağ analizi eter ekstraksiyonu yöntemi ile belirlenmiştir [22]. Ham protein analizi Kjeldahl metoduna göre yapılmıştır [22]. Yemlerin NDF, ADF ve ADL içerikleri ise Van Soest ve ark.[23] tarafından bildirilen yöntemlere göre ANKOM 200 Fiber Analyzer (ANKOM, USA) ile belirlenmiştir. Silajların tanen içerikleri Folin-Denis yöntemine göre saptanmıştır [22].

Silajların pH'sı dijital pH metre cihazı (Sartorius PB-20, Goettingen, Germany) ile amonyak azotu (NH_3-N) içerikleri AOAC'a [22] göre yapılmıştır. Asetik, propiyonik ve bütirik asit içerikleri gaz kromotografi cihazı (Agilent Technologies 6890N, kolon özellikleri: Stabilwax-DA, 30 m, 0.25 mm ID, 0.25 um df. Max. temp: 260°C. Cat. 11023) ile laktik asit analizi ise sperofotometrik yöntemle [24], suda çözünebilir karbonhidrat (SÇK) içerikleri fenol sülfürik asit yöntemine [25] göre belirlenmiştir. Silajlarda aerobik stabiliteleri Ashbell ve ark.[26] tarafından geliştirilen yöntemle, silajlardaki görsel küflenme ise Filya ve ark.[27] tarafından geliştirilen değerlendirme yöntemi ile belirlenmiştir.

Silajların yapısındaki laktik asit bakterileri, maya ve küf sayımları Seale ve ark.[28] tarafından bildirilen yöntemler doğrultusunda gerçekleştirilmiştir. Buna göre ekim ortamı olarak LAB için MRS agar, maya ve küfler için Malt Ekstrat agar kullanılmıştır. Örneklere ait LAB, maya ve küf sayımları 30°C sıcaklıkta 3 günlük inkübasyon dönemlerini takiben yapılmıştır. Örneklerde saptanan LAB, maya ve küf sayıları logaritma koliform ünite (cfu)/g'ye çevrilerek verilmiştir.

İstatistik Analizler

Araştırmadan elde edilen verilerin istatistiki olarak değerlendirilmesinde ortalamalar arasındaki farklılıkların saptanmasında varyans analizi (General Linear Model) Statistica [29], görülen farklılıkların önem seviyelerinin belirlenmesinde ise Duncan çoklu karşılaştırma testinden yararlanılmıştır [30].

BULGULAR

Araştırmada kullanılan Gladiçya meyvesi ile taze ve silolanmış yoncaya ait kimyasal analiz sonuçları saptanmış *Tablo 1*'de verilmiştir. Tablo incelendiğinde yonca silajına GM katılmasının yonca silajlarının kimyasal bileşimini önemli düzeyde etkilediği saptanmıştır (P<0.01). Gladiçya meyvesi ilavesi ile silolanan yonca, silolamanın 60. gününde açılarak fermantasyon özellikleri saptanmış ve elde edilen sonuçlar *Tablo 2*'de verilmiştir. Yonca silajına GM ilavesi silajların uçucu yağ asitleri düzeyi ile amonyak azotu (NH_3-N) üretimi ve pH'sını önemli düzeyde geliştirmiştir (P<0.01). Yonca silajlarına ilave edilen GM'nin silaj mikrobiyolojisi üzerine etkisi saptanmış *Tablo 3*'te verilmiştir. Yonca silajına GM eklenmesi silajların LAB ve maya sayısını önemli düzeyde artırmış, küf sayısını ise azaltmıştır (P<0.01). Silolama dönemi sonunda (60. gün) açılan silajlara 5 gün süre ile aerobik stabilite testi uygulanmış ve elde edilen sonuçlar *Tablo 4*'te verilmiştir. Ayrıca GM katılmış yonca silajlarının *in vitro* gaz üretimi, sindirilebilir organik madde ve metabolik enerji içerikleri saptanarak *Tablo 5*'te verilmiştir. Yonca silajına GM katılması silajların aerobik stabilitesini, in vitro gaz üretimini SOM ve ME içeriğini önemli düzeyde geliştirmiştir (P<0.01).

TARTIŞMA ve SONUÇ

Yem Ham Maddeleri ve Silajların Kimyasal Bileşimleri

Gladiçya meyvesi ile taze ve silolanmış yoncaya ait kimyasal analiz sonuçları saptanarak *Tablo 1*'de verilmiştir. Karbonhidrat kaynağı olarak yonca silajına ilave edilen GM'nin bileşiminde yer alan ve silajda homolaktik fermantasyon

Tablo 1. *Taze yonca, gladiçya meyvesi ve yonca silajlarının kimyasal analiz sonuçları, %*
Table 1. Chemical composition of *fresh alfalfa, honey locust pods* and alfalfa *silages, %*

Ana Yemler	KM	OM	HP	HY	HK	NDF	ADF	ADL	SÇK	TT
Taze yonca	27.80	94.03	17.57	5.73	5.97	49.32	32.00	8.07	1.38	0.80
GM	88.60	96.72	10.16	3.11	3.28	39.58	25.88	9.38	15.90	9.39
Yonca Silajı										
Kontrol	28.27^{e}	94.08^{c}	15.77^{a}	5.59^{a}	5.92^{a}	48.49^{a}	30.62^{a}	8.36^{a}	1.04^{b}	0.76^{e}
20 g/kg GM	29.07^{e}	94.16^{c}	15.37^{ab}	5.48^{ab}	5.84^{a}	46.97^{b}	29.89^{ab}	8.53^{a}	1.13^{ab}	0.88^{d}
40 g/kg GM	30.07^{d}	94.19^{c}	15.03^{bc}	5.38^{ab}	5.81^{a}	45.85^{bc}	28.29^{bc}	8.74^{a}	1.15^{ab}	1.03^{c}
60 g/kg GM	31.50^{c}	94.24^{bc}	14.87^{bc}	5.25^{bc}	5.76^{ab}	45.91^{bc}	27.66^{c}	8.81^{a}	1.14^{ab}	1.11^{c}
80 g/kg GM	32.83^{b}	94.40^{b}	14.53^{c}	5.03^{cd}	5.60^{bc}	45.32^{cd}	26.77^{c}	8.87^{a}	1.17^{ab}	1.39^{b}
100 g/kg GM	34.23^{a}	94.48^{a}	14.50^{c}	4.84^{d}	5.52^{c}	44.47^{d}	26.33^{c}	8.82^{a}	1.28^{a}	1.61^{a}
SS*	***0.213***	***0.032***	***0.115***	***0.015***	***0.038***	***0.274***	***0.424***	***0.147***	***0.037***	***0.025***

*^{a,b,c,d,e}Aynı sütunda farklı harfler ile gösterilen ortalamalar arasındaki farklılıklar önemlidir (P<0.01). ***SS:** standart sapma; **KM:** kuru madde; **OM:** organik madde; **HK:** ham kül; **SÇK:** suda çözünebilir karbonhidrat; **NDF:** nötr deterjanda çözünmeyen lif; **ADF:** asit deterjanda çözünmeyen lif; **ADL:** asit deterjanda çözünmeyen lignin; **HP:** ham protein; **HY:** ham yağ; **TT:** toplam tanen*

Tablo 2. *Silolamanın 60. gününde açılan yonca silajlarının fermantasyon özellikleri*
Table 2. *Fermentation profiles of* alfalfa *silages after 60 days of fermentation*

Yonca Silajı	pH	g/kg KM				NH_3-N
		Laktik Asit	Asetik Asit	Propiyonik Asit	Bütirik Asit	
Kontrol	5.37[a]	32.57[e]	32.71[a]	0.10[c]	0.55[a]	6.11[a]
20 g/kg GM	5.12a	34.42[d]	29.90[a]	0.12[c]	0.47[ab]	5.88[b]
40 g/kg GM	4.82[b]	37.40[c]	25.35[b]	1.09[b]	0.36[bc]	5.39[c]
60 g/kg GM	4.61[c]	43.51[b]	23.30[bc]	1.75[a]	0.31[bcd]	4.93[d]
80 g/kg GM	4.37[d]	46.61[a]	21.60[c]	1.88[a]	0.23[cd]	4.53[e]
100 g/kg GM	4.17[e]	47.99[a]	22.36[bc]	1.98[a]	0.14[d]	3.54[f]
SS*	***0.039***	***0.423***	***0.668***	***0.016***	***0.038***	***0.031***

*[a,b,c,d,e,f]Aynı sütunda farklı harfler ile gösterilen ortalamalar arasındaki farklılıklar önemlidir (P<0.01) * **SS:** standart sapma, **NH_3-N:** amonyak azotu (NH_3-N toplam N'in %'si olarak verilmiştir)*

Tablo 3. *Yonca silajlarının mikrobiyolojik özellikleri*
Table 3. *The microbiological characteristics of alfalfa silage*

Ana Yem	LAB (log10 cfu/g TM)	Maya (log10 cfu/g TM)	Küf (log10 cfu/g TM)
Taze Yonca	2.14	0.76	0.20
Yonca Silajı			
Kontrol	4.04[c]	0.99[c]	1.02[a]
20 g/kg GM	4.45[c]	1.55[b]	0.68[c]
40 g/kg GM	4.69[c]	1.12[bc]	0.58[c]
60 g/kg GM	5.85[b]	2.13[a]	0.80[b]
80 g/kg GM	6.34[b]	2.42[a]	0.40[d]
100 g/kg GM	7.38[a]	2.43[a]	0.68[c]
SS*	***0.184***	***0.159***	***0.108***

*[a,b,c,d] Aynı sütunda farklı harfler ile gösterilen ortalamalar arasındaki farklılıklar önemlidir (P<0.01) * **SS:** standart sapma, **LAB:** laktik asit bakterileri; **TM:** taze materyal*

Tablo 5. *Yonca silajların in vitro gaz üretimleri (ml/200 mg KM), sindirilebilir organik madde ve metabolik enerji içerikleri*
Table 5. *In vitro gas production (ml/200 mg DM), organic matter digestibility and metabolizable energy ingredient of* alfalfa *silages*

Yonca Silajı	İnkübasyon Süresi, Saat		SOM, %	ME, MJ/kg KM
	24	96		
Kontrol	52.43[c]	69.13[d]	61.04[c]	9.41[c]
20 g/kg GM	54.37[c]	69.63[d]	62.65[c]	9.67[c]
40 g/kg GM	53.20[c]	71.40[c]	61.64[c]	9.51[c]
60 g/kg GM	56.83[b]	73.73[b]	64.69[b]	10.00[b]
80 g/kg GM	58.13[ab]	74.50[b]	65.77[ab]	10.18[ab]
100 g/kg GM	59.57[a]	76.60[a]	66.97[a]	10.37[a]
SS*	***0.486***	***0.327***	***0.410***	***0.066***

*[a,b,c,d] Aynı sütunda farklı harfler ile gösterilen ortalamalar arasındaki farklılıklar önemlidir (P<0.01); * **SS:** standart sapma, **SOM:** sindirilebilir organik madde; **ME:** metabolik enerji*

Tablo 4. *Yonca silajlarının 5 gün sonunda saptanan aerobik stabilite test sonuçları*
Table 4. *Aerobic stability of* alfalfa *silages after exposure to air for 5 days*

Yonca Silajı	pH	CO_2 (g/kg KM) **	Görsel Küflenme ***
Kontrol	6.02[a]	33.28[a]	3.00[a]
20 g/kg GM	5.90[b]	32.34[ab]	2.33[ab]
40 g/kg GM	5.73[c]	30.70[b]	1.33[abc]
60 g/kg GM	5.42[d]	27.66[c]	1.33[abc]
80 g/kg GM	5.16[e]	23.72[d]	0.66[bc]
100 g/kg GM	5.10[e]	20.45[e]	0.33[c]
SS*	***0.016***	***0.419***	***0.384***

*[a,b,c,d,e]Aynı sütunda farklı harfler ile gösterilen ortalamalar arasındaki farklılıklar önemlidir (P<0.01), * **SS:** standart sapma, ** **CO_2:** karbondioksit (g/kg KM), *** Silajların küflenme durumlarının görsel olarak 1'den 5'e kadar olan sayılarla değerlendirilmesidir. 1: hiç küf içermeyen bir silaj, 2: noktalar halinde çok çok az düzeyde küf içeren bir silaj, 3: noktalar halinde yüzeye yayılmış bir şekilde küf içeren bir silaj, 4: yüzeyi kısmen küf ile kaplı, bölge bölge küflenmiş yüzeyleri olan bir silaj, 5: yüzeyi tamamen küf ile kaplı, ağır bir kokuya sahip ve partikülleri birbirine yapışmış bir silaj. Bu değerlendirmeler üç kişi tarafından yapılmakta ve daha sonra üçünün ortalaması alınmaktadır*

gelişiminin sağlanması bakımından önem taşıyan SÇK miktarı %15.90 olarak tespit edilmiştir. Araştırmada saptanan GM'nin SÇK içerikleri Kamalak ve ark.'nın [5] bildirdikleri (%12.2-15.2) değerlerden daha yüksektir. Araştırmada kullanılan GM'nin SÇK miktarının yüksek olmasının tür ve yöre farklılığından kaynaklanmış olabilir. Gladiçya meyvesinin tanen içeriği %9.39 olarak saptanmıştır. Bu çalışmada kullanılan GM'nin besin madde bileşimi Bruno-Soares ve Abreu'nın [13] bildirdikleri sonuçlardan yüksek, Kamalak ve ark.'nın [11] bildirdiği sonuçlarla uyum halindedir.

Gladiçya meyvesi katkısı yonca silajlarının KM ve SÇK içeriğini artırmış, HP içeriğini ise düşürmüştür (P<0.01). Söz konusu değişiklikler taze yonca ile GM arasındaki KM, SCK ve HP farklılıklarından kaynaklanmaktadır *(Tablo 1)*.

Yoncaya ilave edilen GM oranının artışıyla paralel olarak silajların NDF ve ADF içeriklerini önemli düzeyde düşürmüştür (P<0.01). Burada GM'nin NDF ve ADF içeriğinin düşük olması ve ayrıca GM'nin bir karbonhidrat kaynağı olarak ortamdaki laktik asit bakteri faaliyetini hızlandırması sonucu silajlardaki hücre duvarı bileşenlerinin parçalanmasının

etkili olduğu düşünülmektedir. Nitekim, Bolsen ve ark.[31] silajların NDF ve ADF içeriklerindeki düşüşe karbonhidrat kaynaklarının silaj ortamındaki laktik asit bakterileri ile birlikte bazı anaerobik bakterilerin sayılarını artırarak, silajların NDF, ADF ve ham sellüloz parçalanabilirliğini hızlandırmasının da neden olduğunu bildirmişlerdir. Benzer bulgular Canbolat ve ark.[9] ve Fisun ve ark.'nın [32] çalışmalarında da görülmüştür. Silaja GM katılması silajların tanen içeriklerini de önemli düzeyde artırmıştır (P<0.01). Tanen düzeyindeki artış ise 2.01 kat olmuştur. Tanen düzeyindeki bu artış yonca silajında üzüm posası ile çalışan Canbolat ve ark.'nın [9] bulguları ile paralellik göstermiştir.

Silajların Fermantasyon Özellikleri

Yonca silajlarının fermantasyon özelliklerinin verildiği *Tablo 2*'den de görüldüğü gibi GM ilavesi silajların UYA içeriğini önemli derecede iyileştirmiştir (P<0.01). Başka bir deyişle GM silaj fermantasyonunu olumlu yönde etkilemiştir. Gladiçya meyvesinin kullanım oranının artmasıyla birlikte asetik ve butirik asit miktarı azalmış, laktik ve propiyonik asit miktarları ise artmıştır. Yonca silajına %10.0 oranında GM katıldığında, üretilen asetik asit miktarında 1.46 kat, butirik asit miktarında ise 3.92 katlık bir azalma olmuştur. Diğer taraftan laktik asit üretimindeki artış ise yaklaşık olarak 1.53 kat olmuştur. Gladiçya meyvesi içerisinde yüksek miktardaki SÇK olması laktik asit üretimini artırmıştır. Silaj içerisindeki bakteriler yeterli miktarda enerji kaynağı buldukları için proteinleri enerji kaynağı olarak kullanmamıştır. Bunun en büyük göstergesi silajın butirik asit içeriğinin 3.92 kat oranında azalmasıdır. *Tablo 2*'de de görüldüğü gibi, GM'nin katıldığı bütün silajların laktik asit içeriği kontrol grubu silajından önemli derecede yüksek saptanmıştır (P<0.01). En yüksek laktik asit içeriği %10.0 oranında GM'nin katıldığı silajda elde edilmiştir. Bilindiği gibi silaj pH'nın düşürülmesinden sorumlu olan asitlerden en önemlisi laktik asittir. Laktik asit içeriği yüksek olan silajların pH'sı da daha düşük bulunmuştur (P<0.01). En fazla düşüş ise 100 g/kg KM düzeyinde GM katılan yonca silajında gerçekleşmiştir. Kontrol grubuna ait silajın laktik asit içeriği Wang ve ark.'nın [33] bildirdiği değere (19.5 g/kg kuru madde) yakın bulunmasına rağmen, asetik asit ve butirik asit içeriği daha yüksek bulunmuştur. Diğer taraftan bu çalışmada kontrol grubu silaja ait butirik asit içeriği Salawu ve ark.[19] yaptığı çalışmada elde ettiği yonca silajına ait butirik asit içeriğinden daha düşük bulunmuştur.

Gladiçya meyvesi laktik asit bakteri fermantasyonu için gerekli kolay fermante edilebilir karbonhidrat sağlaması [5] ve yüksek tanen içeriği [5,11] ile fermantasyon sırasında ortamdaki proteinlerin amonyağa dönüşmesini engelleyerek de, yonca silajlarının pH düzeylerinin düşmesine neden olduğu bildirilmektedir [5]. Çiftçi ve ark.[8] tarafından yoncaya elma püresi ilavesi ile Canbolat ve ark.[9] tarafında yonca silajına üzüm posası ilavesi ile elde edilen silaj pH dereleri araştırma sonuçları ile benzer bulunmuştur.

Yonca silajlarının NH_3-N içeriği toplam azot miktarı içerisinde 6.11 ile 3.54 g/kg KM arasında değişmiş ve en düşük 100 g/kg KM GM katılan yonca silajında saptanmıştır (P<0.01). Yoncaya GM ilavesi silajların laktik asit içeriğini artırmış, asetik ve butirik asit içeriğini düşürerek proteolisisi önlemiş ve bunun sonucu olarak silajların NH_3-N içeriğini azalttığı yönünde birçok araştırma sonucu bulunmaktadır [5,9,34]. Ayrıca GM'nin yapısında bulunan tanen, nötr ortama yakın olan ortamlarda (silolamanın başı), çözünebilir proteinler ile kompleks bileşikler oluşturmakta ve böylece proteinlerin mikroorganizmalar tarafından parçalanmasını önleyerek [35,36], silajda amonyak konsantrasyonunun düşmesine neden olmaktadır. Bu yolla silajlarda azot kaybı önlenmekte ve silaj kalitesi yükselmektedir.

Silolanan materyal içerisindeki proteinlerin parçalanması, bitkide bulunan proteaz ile silo ortamında bulunan mikroorganizmaların üretmiş olduğu enzimlere bağlıdır. Bitkideki proteaz enzimlerin aktivitelerinin pH 6 civarında optimum düzeyde olduğu, düşük pH'larda enzim aktivitesinin önemli düzeyde düştüğü bildirilmektedir [37,38]. Kısaca özetlenecek olursa, yonca materyalinin GM ile birlikte silolanmasıyla, laktik asit içeriği yüksek, bütirik asit ve amonyak azotu içeriği düşük kaliteli silajlar elde edileceği söylenebilir.

Silajların Mikrobiyolojik Özellikleri

Yonca silajlarının mikrobiyolojik özellikleri saptanmış *Tablo 3*'te verilmiştir. *Tablo 3* incelendiğinde GM yonca silajlarının LAB üretimini etkilemiş ve LAB sayısı 4.04 ile 7.38 (log10 cfu/g TM) arasında değişmiştir. Yonca silajlarının üretmiş oldukları LAB arasındaki farklılıklar önemli bulunmuştur (P<0.01). En yüksek LAB 7.38 (log10 cfu/g TM) ile 100 g/kg KM GM ilave edilen yonca silajında, en düşük ise kontrol grubu olan yonca silajında saptanmıştır. Artış oranı ise 1.83 kat olmuştur. Yoncaya katılan GM'nin kullanım dozunun artması silajların SÇK içeriğini artırmış, NDF ve ADF içeriğini düşürmüştür *(Tablo 1)*. Bu durum LAB için daha fazla besin sağlamış ve bu yolla silaj pH'sı *(Tablo 2)* LAB için uygun hale gelmiş buda silo ortamında LAB üretimini artmıştır. Yonca silajının *in vitro* LAB miktarları, benzer kaba yem ve GM ile çalışan Kamalak ve ark.'nın [5] bulgularından daha yüksek bulunmuştur. Bunun nedeni yoncaya ilave edilen GM'nin çeşidi ve kullanım dozu farklılığı olabilir. Laktik asit bakterileri yonca silajı ile çalışan Filya ve ark.'nın [39] bildirdikleri değerlerden daha düşük saptanmıştır.

Yonca silajlarına GM katılması silajların maya sayısı artarken küf sayısında önemli düzeyde azalma olmuştur (P<0.01). Yonca silajlarındaki maya sayısı 0.99 ile 2.43 (log10 cfu/g TM) arasında değişmiş ve en yüksek 100 g/kg KM GM ilave edilen yonca silajında saptanmıştır. Artış oranı ise 2.45 kat olmuştur. Maya oranındaki bu artışın GM'sinin silaj ortamına sağladığı SÇK'ın mayalar için besin madde sağlamasından kaynaklandığı [39] söylenebilir. Küf sayısında ise önemli düzeyde azalma olmuş ve en düşük küf miktarı 80 g/kg KM GM ilave edilen yonca silajında saptanmıştır. Silaja ilave edilen GM'nin silaj ortamında pH'yı düşürmesi ve ayrıca tanen içeriğin artırması küf sayısının azalmasına

neden olmuştur [5]. Araştırmadan elde edilen maya ve küf sayıları yonca silajı ile çalışan Filya ve ark.[39] ile Kamalak ve ark.'nın [5] bildirdikleri değerlerden daha düşük saptanmıştır.

Silajların Aerobik Stabilite Özellikleri

Silolama dönemi sonunda (60. gün) açılan silajlara 5 gün süre ile aerobik stabilite testi uygulanarak elde edilen veriler incelendiğinde *(Tablo 4),* beş gün boyunca doğrudan havanın oksijenine maruz bırakılan silajların pH değerlerinde bir miktar yükselme görülmüştür. Ancak GM katılan silajların pH değerleri yine de kontrol silajınkinden önemli düzeyde düşük bulunmuştur (P<0.01). Beş günlük bu dönem sonucunda özellikle 20, 40, 60, 80 ve 100 g/kg KM düzeyinde GM katılan silajlarda daha düşük bir CO_2 üretimi görülmüş olup, bu silajlar ile diğer silajlar arasında görülen farklılıklar önemli bulunmuştur (P<0.01). Beş gün boyunca hava ile temas eden silaj materyalinin ürettiği CO_2 miktarı 20.45 ile 33.28 g/kg KM arasında değişmiştir. Gladiçya meyvesi ilavesine bağlı olarak CO_2 üretimi 1.63 kat azalmıştır. Diğer yandan aerobik stabilite testi sonunda yoncaya katılan GM düzeyinin artması küflenmeyi de önlemiştir. Silajların bu dönemdeki küf düzeylerinin düşmesinin nedeni araştırmada kullanılan GM'nin hem silo içerisinde asidik bir ortam oluşturması [5] hem de GM'nin antimikrobiyal [5,9] özelliğe sahip olmasından (tanen) kaynaklandığı söylenebilir.

Araştırmada özellikle 60, 80 ve 100 g/kg KM düzeyinde kullanılan GM'nin yonca silajlarının aerobik stabilitelerini geliştirdiği saptanmıştır. Bu araştırmadan elde edilen sonuçlar yonca silajına üzüm posası ilave eden Canbolat ve ark.'nın [9] bildirdikleri sonuçlarla benzer saptanmıştır. Bu çalışmada elde edilen veriler pratik açıdan anlamı şudur. Beş gün süreyle havayla temas eden katkılı silajlar, kontrol silajına göre daha fazla bozulmadan kalmıştır. Küflenme düzeyi de düşmüştür. Yani bu silajların fazla besin madde kaybına maruz kalmadan uzun süre kalacağı anlamına gelmektedir.

Silajların in Vitro Gaz Üretimi Özellikleri

Gladiçya meyvesi katılmış yonca silajlarının *in vitro* gaz üretimi, sindirilebilir organik madde, metabolik enerji içeriklerinin verildiği *Tablo 5* incelendiğinde de görüldüğü gibi yonca silajlarının 96 saatlik *in vitro* gaz üretimi 69.13 ile 76.60 ml arasında değişmiştir. Yonca silajlarının üretmiş oldukları gaz miktarları arasındaki farklılıklar önemli bulunmuştur (P<0.01). En yüksek gaz üretimi 76.60 ml ile 100 g/kg KM GM ilave edilen yonca silajında, en düşük ise kontrol yonca silajında saptanmıştır. Yoncaya katılan GM, silajların SÇK içeriğini artırarak, NDF ve ADF içeriğini düşürmüş ve bunun sonucunda *in vitro* gaz üretimi de artmıştır. Yonca silajının *in vitro* gaz üretim miktarları, benzer kaba yemlerle çalışan Canbolat ve ark.[9] ile Kamalak ve ark.'nın [5] bulguları ile benzerlik gösterirken, Polat ve ark.'nın [40] bildirdikleri bulgulardan daha yüksek bulunmuştur. Bu çalışmada elde edilen 24 saatlik gaz üretim değeri Tabacco ve ark.[41] tarafından bildirilen değerden (43.0 ml) daha yüksek bulunmuştur.

Yonca silajlarının SOM içerikleri %61.04 ile 66.97 arasında değişmiş ve silajlar arasında görülen farklılıklar önemli bulunmuştur (P<0.01). En yüksek %66.97 ile 100 g/kg KM düzeyinde GM katılan yonca silajında saptanmıştır. Yemlerin 24. saatteki gaz üretim ve SÇK miktarındaki artış ile NDF ve ADF gibi rumende çözünmesi zor olan bitki hücre duvarı bileşenlerinin GM ilavesi ile azalması silajların SOM miktarını artırmıştır. Araştırmada saptanan SOM düzeyi, Öztürk ve ark.[42] ile yonca silajına üzüm posası ilave eden Canbolat ve ark.[9] ile yonca silajına GM ilave eden Kamalak ve ark.'nın [5] bulgularıyla benzerlik göstermiştir.

Yonca silajlarının ME içerikleri ise 9.41 ile 10.37 MJ/kg KM arasında değişmiş ve en yüksek ME değeri 100 g/kg KM düzeyinde GM içeren yonca silajında saptanmıştır (P<0.01). Yoncaya katılan GM düzeyinin artışına bağlı olarak silajların ME içeriklerinin artması, SOM'de olduğu gibi *in vitro* gaz üretiminin artması ile NDF ve ADF düzeyinin azalmasına bağlanabilir. Yonca silajlarının ME içerikleri yonca silajına üzüm posası ilave eden Canbolat ve ark.[9] ile yonca silajına GM ilave eden Kamalak ve ark.'nın [5] sonuçları ile uyum içerisinde bulunmasına karşın, Polat ve ark.'nın [40] bulgularından daha yüksek bulunmuştur. Bunun sebebi ME ve SOM hesaplanmasında kullanılan 24 saatlik gaz üretimin biraz yüksek olmasından kaynaklanmaktadır.

Sonuç olarak, bu çalışmada silolanması güç olan yoncadan kaliteli silaj elde edebilmek için ortamdaki kolay çözünebilir karbonhidrat düzeyinin yükseltilmesi amacıyla, GM'nin alternatif bir karbonhidrat kaynağı olarak kullanılabileceği sonucuna varılmıştır. Araştırmadan elde edilen bulgulara göre yonca silajlarına ilave edilecek en uygun GM miktarının 80 ile 100 g/kg KM arasında olduğu tespit edilmiştir. Araştırma sonuçlarına göre yonca bitkisinin silolanmasını önemli düzeyde geliştiren GM'nin gelecekte bu amaçla kullanımının yaygınlaştırılması için yeni çalışmaların yapılması gerekmektedir.

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Kafkas Univ Vet Fak Derg
19 (2): 299-304, 2013
DOI: 10.9775/kvfd.2012.7716

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Clinical and Endocrine Short-Term Effects of GnRH Analogue Deslorelin in Prepubertal Bitches: Does A "Flare-up" Occur? [1]

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[1] *This study was supported by Kafkas University, Scientific Research Projects Coordination Unit (BAP Project number: 2010-VF-72) and VIRBAC (France)*

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Makale Kodu (Article Code): KVFD-2012-7716

Summary

Long-acting GnRH agonists have been used both in canine estrus induction and prevention. The objective of the study was to investigate the clinical and endocrine short-term effects of a deslorelin implant (4.7 and 9.4 mg) in terms of initial "*flare-up*" in prepubertal bitches. Fourteen healthy, cross-breed prepubertal female dogs, aged 4 months, were used in the study. An implant containing 9.4 mg (G1, n=5) and 4.7 mg (G2, n=4) deslorelin (Suprelorin®) or a placebo (sodium chloride 0.9%, G3, n=5) was inserted subcutaneously. Estrus was monitored once daily by physical and sexual behavioral changes. Vaginal cytology, serum progesterone (P4) and estradiol 17β (E2) concentrations were monitored weekly for the first five weeks throughout the treatment. None of the bitches in the treatment (G1 and G2) and control (G3) group showed any signs of estrous throughout the study period. No local reactions were observed at the implantation site. Two bitches showed an increase in serum P4 concentrations (>1.0 ng/ml) in G1 (2/5; 40%), however, only one of these two animals (P4=6.37 ng/ml) showed an increase in serum E2 concentrations up to 37 pg/ml. No marked increase in serum P4 and E2 concentrations were observed during the first 13 weeks of treatment in G2. These data demonstrate that in prepubertal bitches, insertion of a deslorelin implant does not cause a "*flare-up effect*" which commonly occurs in anestrus adult bitches during the first month after implantation. However, further studies are needed to demonstrate that the "*flare-up*" effect of deslorelin implant in bitches especially during later prepubertal stages.

Keywords: *Prepubertal bitches, Suprelorin®, Flare-up effect*

Prepubertal Köpeklerde GnRH Analoğu Deslorelinin Kısa Dönem Klinik ve Endokrinolojik Etkileri: "Flare-up" Gözlenir mi?

Özet

Uzun etkili GnRH agonistleri köpeklerde hem östrusun uyarılmasında hem de baskılanmasında kullanılmaktadırlar. Bu çalışmada, pubertas öncesi dönemdeki dişi köpeklerde deslorelin implant (4.7 ve 9.4 mg) kullanımının "*flare-up*" dönemi olarak değerlendirilebilecek klinik ve endokrinolojik kısa dönem etkilerinin varlığı araştırılmıştır. Bu amaçla pubertas öncesi dönemde olduğu belirlenen ortalama 4 aylık yaşta on dört adet sağlıklı melez dişi kopek kullanıldı. Köpeklere deri altı yolla 9.4 mg (G1, n=5) ve 4.7 mg (G2, n=4) deslorelin (Suprelorin®) etken maddesi içeren implantlar ya da plasebo (sodyum klorür %0.9; G3; n=5) uygulandı. Östrus belirtileri günlük olarak fiziksel değişiklikler ve davranış değişiklikleri yönünden takip edildi. Ayrıca çalışma süresince vaginal sitoloji, serum progesteron (P4) ve östradiol 17β (E2) konsantrasyonları haftalık olarak izlendi. Tedavi (G1 ve G2) ve kontrol grubundaki (G3) hayvanların hiçbirinde östrus bulgusu belirlenmedi. İmplantın yerleştirildiği bölgede herhangi bir lokal yan etki gözlenmedi. Grup 1'de iki köpekte (2/5; %40) serum P4 düzeyinde bir artış gözlenirken, bu köpeklerden yalnızca birinde (P4=6,37 ng/ml) serum E2 düzeyinin 37 pg/ml'ye kadar yükseldiği belirlendi. Grup 2'de 13 haftalık tedavi süresince serum P4 ve E2 düzeylerinde önemli bir değişiklik gözlenmedi. Bu çalışmadan elde edilen bulgular, pubertas öncesi köpeklerde deslorelin implantın, yetişkin köpeklerde implante edildikten sonraki ilk bir ay içerisinde sıklıkla gözlenen "*flare-up etkisine*" neden olmadığını göstermiştir. Bununla birlikte deslorelin implant uygulamalarının özellikle pubertasa yakın dönemlerde oluşturabileceği "*flare-up*" etkisine dair yeni çalışmalara ihtiyaç vardır.

Anahtar sözcükler: *Prepubertal köpek, Suprelorin®, Flare-up etkisi*

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INTRODUCTION

Gonadotropin-releasing hormone (GnRH) or luteinizing hormone-releasing hormone is a decapeptide hypothalamic hormone that acts upon GnRH receptors in the pituitary. It is secreted in a pulsatile manner into the hypothalamo-hypophyseal portal system and has a short half-life of 2-5 min due to rapid cleavage by proteases. In the pituitary, GnRH stimulates production and secretion of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that in turn act on the gonads regulating steroid production, spermatogenesis, ovarian follicular development and ovulation [1,2].

Long-acting GnRH agonists can be used for indications such as preventing puberty in both sexes; controlling of estrus cycle and treating post-spaying urinary incontinence [3-6] and controlling the risk of recurrence of mammary neoplasia, prevention of mammary tumour metastatic disease and the termination of pregnancy in bitches [7-9].

GnRH agonists have been used both in canine estrus induction and prevention [3,5,6,10]. Chronic administrations of GnRH agonists result in downregulation and desensitation of pituitary GnRH receptors and, therefore, in a complete suppression of gonadal function [5,6,11]. A significant advance for their clinical use was obtained by the development of slow-release agonists that can be easily administered intramuscularly or implanted subcutaneously thus delivering constant doses of GnRH for 3-12 months depending on the formulation [5,12-14].

In dogs, GnRH agonist administration is an effective, safe and reversible method to prevent estrus cycles for a long period exceeding one year [2,11]. Bitches exhibit different responses to administration of deslorelin implants depending on the stage of the estrus cycle. A sharp, initial stimulatory gonadotropin response to GnRH agonist treatment known as '*flare-up*' occurs after implant insertion which results in an increased release of FSH and LH [15,16]. In dogs, estrus can be induced within 3 to 10 days after treatment [2,6,17]. Trigg et al.[12] noted the same phenomenon for animals aged seven months or more. In these animals, estrus systematically induced in the 1-2 months following implantation. Likewise, Inaba et al.[17] reported induction of estrus after use of a sustained-release formulation of a GnRH agonist (leuprolide acetate) in one year old prepubertal bitches; however, estrus induction has not been observed after implant insertion in prepubertal bitches with an average age of 4 months [5,12].

The objective of this study was to investigate the short-term effects of the deslorelin implant Suprelorin® (4.7 and 9.4 mg; Virbac®, Vienna, Austria) on vaginal cytological and hormonal changes and as a safe and reversible contraceptive in prepubertal bitches with special regard to the occurrence a flare-up effect.

MATERIAL and METHODS

Animals and Treatments

Fourteen healthy, crossbred prepubertal female dogs (mean age: 4.25±0.32 months; body weight range: 6-15 kg) were used in the study. The dogs were housed in indoor-outdoor runs and were fed with a standard commercial dog food. Water was available *ad libitum*. After the first vaginal cytological and blood sampling, an implant containing 9.4 mg (G1, n=5) and 4.7 mg (G2, n=4) deslorelin (Suprelorin®; Virbac, Vienna, Austria) or a placebo (2 ml sodium chloride 0.9%; G3, n=5) was inserted subcutaneously in the inter-scapular region by using a single use applicator. This study was approved by Kafkas University, Animal Local Ethics Committee (KAÜ-HADYEK; 2010/30).

Clinical Observation, Vaginal Cytology and Blood Sampling

Following implant insertion, animals were examined for presence local or systemic reactions (e.g. edema, suppuration at the implantation site or anaphylactoid reactions) and estrus signs were monitored once daily by physical (vulvar appearance, the length of the vulva and swelling, sero-sanguineous vaginal discharge) and sexual behavioral changes. Furthermore, vaginal cytology and serum progesterone (P4) and estradiol 17β (E2) concentration were monitored weekly for the first five weeks, and then every three weeks throughout the treatment period. The vaginal smears were obtained using a cotton swab and were stained with Papanicolaou staining technique [18] and examined microscopically to identify the cycle stage.

Hormone Assays

The concentrations of P4 and E2 in peripheral blood plasma were measured using "Electrochemiluminescence Immunoassay" (ECLIA) with the fully automated Cobas Modular E170 Analyzer® (Roche Diagnostics, Mannheim, Germany) in a special laboratory (Düzen Laboratories Group, Ankara, Turkey; internationally certified: TURKAK, TS EN ISO/IEC 17025:2005 laboratory of analysis).

Statistical Analyses

Changes in serum P4 and E2 concentrations were analyzed using Repeated Measures Define Factors with the General Linear Model procedure of PASW Statistics 18. All data presented are means±S.D. Values of $P<0.05$ were considered statistically significant.

RESULTS

Local/Systemic Side Effects

No clinically detectable local or systemic side effects associated with the treatment were observed in any of the treated (0/9) or control (0/4) bitches.

Behavioral and Physical Changes

Sexual behavioral changes were not observed in treatment and control animals during the daily examinations for the study period. The length of the vulva was unaffected by treatment. The changes in vulva size *(Fig. 1)* were not significantly different between treatment and control groups for 13 weeks after start of treatment (P>0.05).

Hormonal and Vaginal Cytological Changes

Serum concentrations of P4 *(Fig. 2-A,B)* and E2 *(Fig. 3-A, B)* varied throughout the study period. An increase in serum P4 (>1.0 ng/ml) concentrations was observed in two animals in G1 (2/5; 40%) during the first three weeks of treatment (6.37 ng/ml and 1.48 ng/ml, *Fig. 2, A*). However, only one of these two animals (1/5; 20%; P4=6.37 ng/ml) showed an increase in serum E2 concentrations up to 37 pg/ml *(Fig 3-A)*. Although one animal (1/4; 25%) from G2 (b8) showed an increase in E2 concentrations up to 41 pg/ml in the 10th week of treatment *(Fig. 3-B)*, similarly to G1, there was no significant increase in serum P4 concentrations during 13 weeks of treatment in this animal *(Fig. 2-B)*.

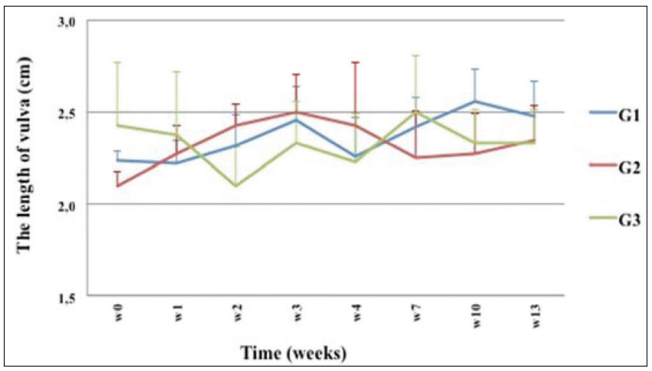


Fig 1. Changes in the length of the vulva (cm) in treatment and control groups (G1:G2:G3=P>0.05)

Şekil 1. Uygulama ve kontrol gruplarında vulva büyüklüklerindeki (cm) değişiklikler (G1:G2:G3=P>0.05)

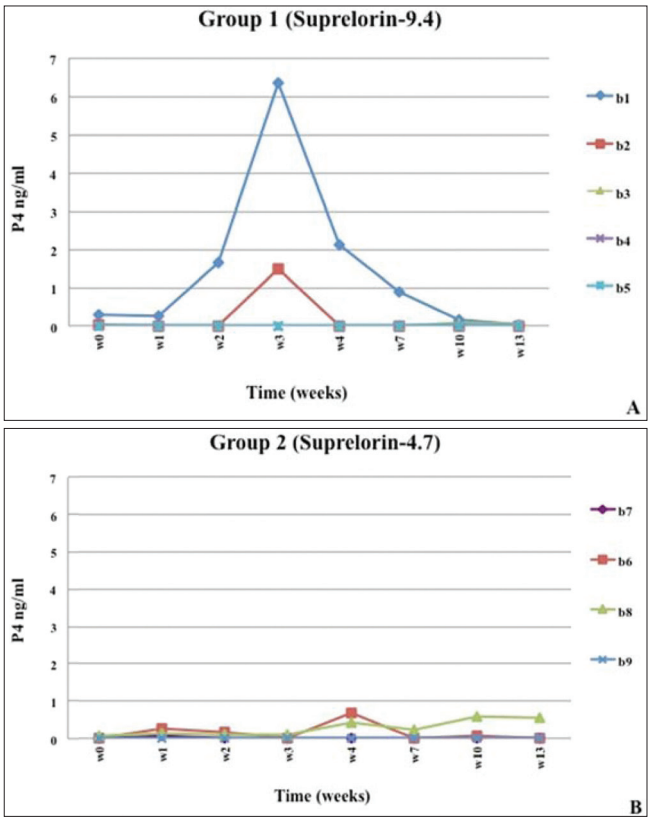


Fig 2. (A, B) Changes in individual P4 levels of treatment groups (G1 and G2)

Şekil 2. (A, B) Uygulama gruplarında (G1 ve G2) P4 düzeylerindeki bireysel değişiklikler

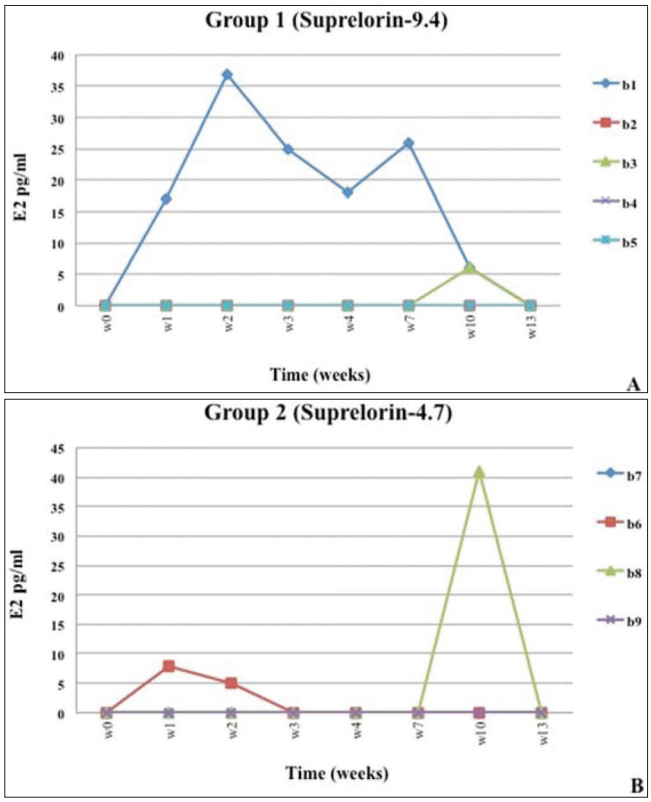


Fig 3. (A, B) Changes in individual E2 levels of treatment groups (G1 and G2)

Şekil 3. (A, B) Uygulama gruplarında (G1 ve G2) E2 düzeylerindeki bireysel değişiklikler

Two out of five (2/5; 40%) dogs in G1 and half of the dogs (2/4; 50%) in G2 showed an increase in superficial cell index (SCI, 15-20%) accompanied by the increase in serum E2 concentrations (6-41 pg/ml) during the first eight weeks after placement of the implant *(Fig. 4)*. Dogs in the control group (G3) showed neither increased SCI nor P4 and E2 concentrations during this period (0/5; 0%). Furthermore, there was no increase in SCI after implant insertion in the control group *(Table 1)*.

DISCUSSION

The present study was designed to evaluate the short-term effects of deslorelin implant Suprelorin® (4.7 and 9.4 mg) on vaginal cytological and hormonal changes and its

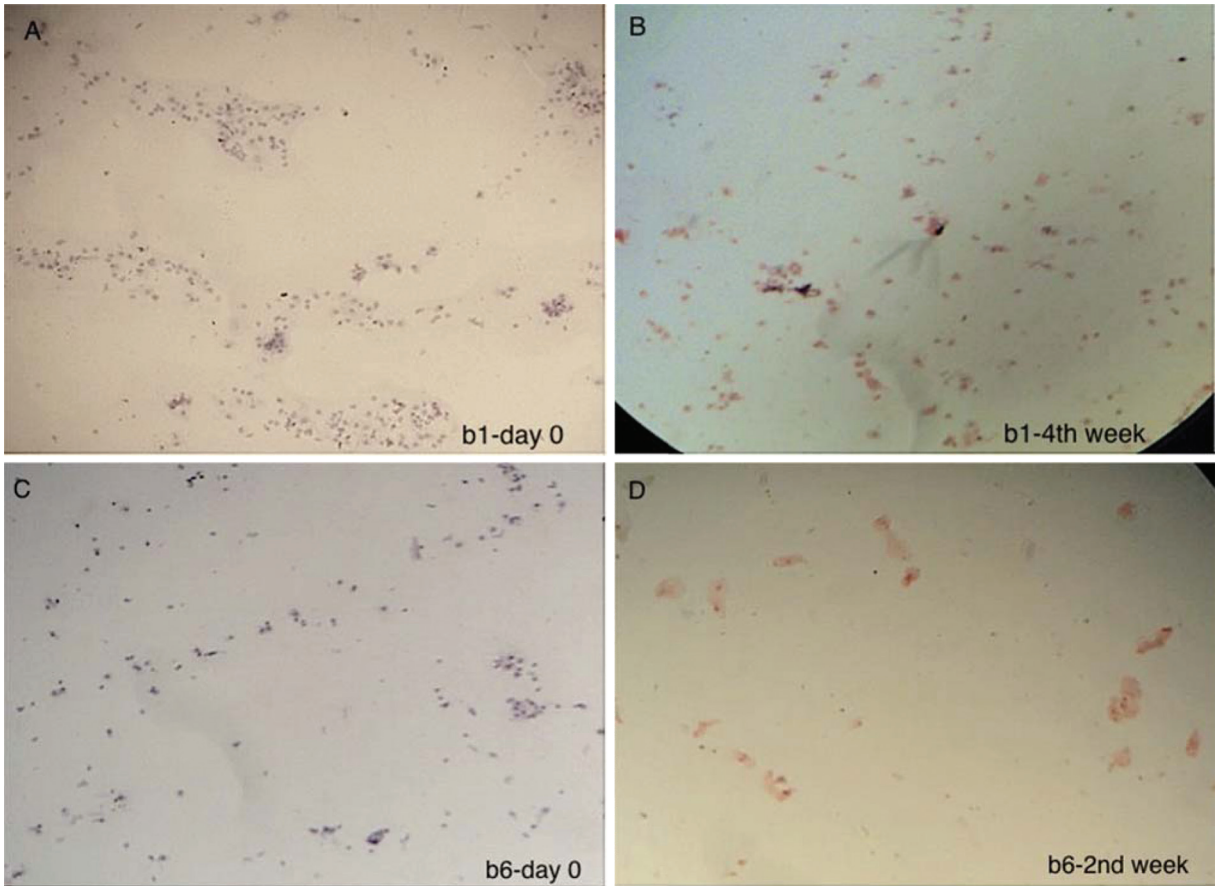


Fig 4. (A-D) Individual changes in superficial cell index of treatment groups (A, B: b1 from G1 and C, D: b6 from G2)
Şekil 4. (A-D) Uygulama gruplarında süperfisyel hücre indeksindeki bireysel değişimler (A, B: G1'den b1 ve C, D: G2'den b6 numaralı hayvan)

Table 1. *Follow-up of individual parameters from treatment and control dogs*
Tablo 1. *Uygulama ve kontrol gruplarındaki köpeklerde takip edilen bireysel parametreler*

Number of the Dog	Groups	Local/Systemic Side Effects	SCI within 13 wk After the Start of Treatment (%)	Clinical Signs of Estrus
b1	G1-9.4 mg	none	5	none
b2	G1-9.4 mg	none	not observed	none
b3	G1-9.4 mg	none	10	none
b4	G1-9.4 mg	none	20	none
b5	G1-9.4 mg	none	20	none
b6	G2-4.7 mg	none	15	none
b7	G2-4.7 mg	none	10	none
b8	G2-4.7 mg	none	20	none
b9	G2-4.7 mg	none	10	none
b10	G3-control	none	not observed	none
b11	G3-control	none	not observed	none
b12	G3-control	none	not observed	none
b13	G3-control	none	<5	none
b14	G3-control	none	not observed	none

effect on the occurrence of a flare-up effect, and as a safe and reversible contraceptive in prepubertal bitches.

GnRH agonists have been shown to be an effective and reversible alternative to surgical castration in dogs. Prolonged administration of GnRH agonists leads to desensitization of the pituitary gland and effectively inhibited the pituitary-gonadal axis [2,16]. However, treatment with GnRH agonists initially induces a large increase in the concentrations of LH and FSH *(flare-up effect)* that is sometimes associated with estrus induction, with or without ovulation [6,12]. This agonist-induced estrus is generally regarded as an inherent disadvantage when the implant was used to postpone puberty or for suppression of estrus.

A limited number of studies have been conducted on the efficacy of long-term release GnRH agonists to postpone puberty and its physiological effects on ovarian function and body development in prepubertal female dogs [5,19,20]. Although insertion of a GnRH agonist device did not induce estrus in prepubertal bitches [5,19,20], which commonly occurs in anestrus adult bitches (flare-up) within one month following implantation [2,6], it was also reported that the age at implantation is important for the response of animals to GnRH agonist [20]. According to the mentioned studies bitches implanted at four months of age showed no sign of estrus, however, all dogs implanted at seven months of age exhibited estrus within 1-2 weeks after implant insertion. In the present study, we observed the same findings for bitches aged four months inserted with deslorelin (4.7 and 9.4 mg) implant (Suprelorin®, Virbac, Austria). None of the bitches in the treatment groups showed any flare-up sign during the first 13 weeks after implant insertion.

Cytological evaluation of the vaginal mucosa and determination of increasing percentage of superficial cells as a bioassay for estrogen influence is even more reliable than a single plasma E2 determination [21]. It was confirmed that two-thirds (4/6) of prepubertal bitches implanted at seven months of age also showed an increase in SCI and increased P4 concentrations. However, an increase in SCI and P4 concentrations was not reported for bitches implanted at four months of age [20,22]. Similarly, Swangchan-Uthai et al.[19] suggested that the majority of vaginal cells were basal and parabasal for four weeks after deslorelin implant insertion in prepubertal bitches. In disagreement with these results, in the present study, we observed an increase in the average rate of 15-20% of SCI accompanied by a moderate increase in serum E2 concentrations in four of the nine treated bitches during the first eight weeks after the implant placement. Similar to our findings, Lanna et al.[23] reported an increase in the proportion of superficial cells, reaching a high average of 44±15% without clinical signs of estrus in adult bitches received a single intramuscular injection of 2 mg deslorelin. In our study, dogs in the control group (G3) did not show an increase in SCI or P4 and E2 concentrations which comparable to anestrus during the observation period.

It has already been described that GnRH agonist implants can be easily implanted and removed without local or systemic side effects [5,6,15]. In agreement with these results, in the present study, we did not observe any local inflammatory reactions and pain symptom or systemic side effects after implant insertion.

In sexually intact bitches, serum E2 concentration increases from 5-15 pg/ml initially to reach a peak of 40-120 pg/ml during proestrus [24]. Although it was shown that administration of GnRH implants led to induction of estrus in adult animals [6,15], lower P4 concentrations were determined after estrus induction with deslorelin implants than during spontaneous estrus in bitches [25]. In the present study, two of the treated prepubertal animals showed an increase in serum E2 concentrations from <5 to 37-41 pg/ml throughout the study period. However, values were decreased to <5 pg/ml thereafter in treated animals of both G1 and G2 without estrus signs.

In conclusion, our results clearly demonstrate that deslorelin treatment (4.7 and 9.4 mg) did not cause a "flare-up" and can be used safely without any clinical short-term side effect in prepubertal bitches when inserted at a mean age of four months. Although GnRH agonists appear to be a promising alternative for the reversible control of ovarian activity in domestic carnivores, further studies are needed to understand the hormonal changings and the long term effects on the development and health of bitches especially during later prepubertal stages.

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Kafkas Univ Vet Fak Derg
19 (2): 305-310, 2013
DOI: 10.9775/kvfd.2012.7771

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Effect of Vit E on Secretion of HSP-70 in Testes of Broilers Exposed to Heat Stress [1]

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[1] *This study was supported by Ataturk University, Scientific Researches Projects (Project number: BAP-2007/184)*

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Makale Kodu (Article Code): KVFD-2012-7771

Summary

The objective of this experiment was to investigate the ameliorative effect of vitamin E (Vit E) on tissue damage caused by heat stress in the testes of broilers, and to correlate this effect with the secretion of the HSP-70 protein. In the study totally 30 broilers (breed Ross 308) were used. Animals were divided into three groups, including the control group. Group Control (C); these broilers (24°C) were not administered with Vit E in the diet and not subjected to heat stress. Group Stress (S); these broilers not administered with Vit E in the diet but subjected to heat stress (34°C). Group Stress +Vitamin E (Group S + vit E); broilers subjected to heat stress (34°C) and administered with vitamin E. Histopathological examination demonstrated the presence of degenerative alterations in testes tissue of the group S. However, there has been seen diminishing the severity of degeneration in the group S+Vit E. While secretion of HSP-70 was determined in the testes of all groups in varying degree, the highest secretion level of HSP-70 was in the group C, the lowest secretion level of HSP-70 was in the group S. It is concluded that heat stress reduces the secretion of HSP-70 in testes of broilers, when vitamin E is administered, the secretion of HSP-70 increases again and reduces tissues damage.

Keywords: *Heat stress, Broiler, Testis, Vit E, HSP-70*

Sıcaklık Stresine Maruz Bırakılan Broilerlerin Testislerinde Vit E' in HSP-70 Sekresyonu Üzerine Etkisi

Özet

Bu çalışmanın amacı broilerlerin testislerinde ısı stresinin sebep olduğu doku hasarı üzerine vitamin E (Vit E)'in iyileştirici etkisini araştırmak ve bu etkinin HSP-70 proteininin sekresyonu ile ilişkisini ortaya koymaktır. Çalışmada toplam olarak 30 adet (Ross 308 ırkı) broiler kullanıldı. Hayvanlar kontrol grubu da dahil olmak üzere üç gruba ayrıldı. Grup kontrol (C); sıcaklık stresine maruz bırakılmayan (24°C) ve diyetlerine Vit E eklenmeyen broiler. Grup stress (S); diyetlerine Vit E eklenmeyen ancak sıcaklık stresine maruz bırakılan (34°C) broiler. Grup Stres +Vitamin E (Grup S + vit E); sıcaklık stresine maruz bırakılan (34°C) ve diyetlerine vitamin E eklenen broiler. Histopatolojik inceleme S grubunun testislerinde dejeneratif değişiklikler gösterdi. Ancak S + Vit E grubunda dejenerasyonun şiddetinin azaldığı gözlendi. Grupların tamamının testislerinde farklı seviyede HSP-70 sekresyonu saptanırken, HSP-70'in en yüksek seviyesi grup C'de en düşük seviyesi grup S'deydi. Isı stresinin broilerlerin testislerinde HSP-70 sekresyonunu azaltığı, vitamin E uygulandığında, HSP-70 sekresyonunun yeniden artığı ve doku hasarını azalttığı sonucuna varıldı.

Anahtar sözcükler: *Isı stresi, Broiler, Testis, Vit E, HSP-70*

INTRODUCTION

Heat stress is one of the most challenging environmental conditions affecting commercial poultry production and causes significant economic losses in the poultry industry [1,2]. Compared to other species of domestic animals, poultry

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are more sensitive to high ambient temperatures [3]. Because of the poultry have no sweat glands, a rapid metabolism and high body temperature, heat stress is highly affected this species [4].

Heat stress reduces several physiological and metabolic factors in poultry such as food consumption, growth rate, feeding efficiency, eggshell quality, survivability [5,6] and serum parameters [6]. High stress also causes oxidative stress and thus weakness the *in vivo* antioxidant defence system [2,7]. Dietary manipulations are among the methods to alleviate these negative adverse effects of heat stress. The role of dietary supplements such as vitamins [7-10] and minerals [10,11] for alleviating the effects of heat stress in poultry has been reviewed extensively.

Vitamin E (Vit. E) has been reported an excellent biological functions as a natural antioxidant prevent the oxidation of unsaturated lipid materials within cells, thus protecting the cell membrane oxidative damage [12-15]. Vit. E is not synthesized by poultry, and its availability is through exogenous administration only [10]. Vit E plays an important role in poultry industry because of its beneficial effect on the cell membrane integrity, immune system and reproduction performance [8,12,14,16].

Heat shock proteins (HSP) are a group of highly conserved proteins that are constitutively expressed in most cells under normal physiological conditions in every organism from bacteria to man. They perform important functions in the folding/unfolding and translocation of proteins as well as in the assembly/disassembly of protein complexes and the refolding of damaged proteins [17,18]. They also involve in both intracellular and extracellular immune functions [19] and apoptosis [20]. The HSP superfamily includes a number of different molecular-weight-class families: HSP110, HSP90, HSP70, HSP60, HSP47, and a group of small HSP ranging from 16 to 40 kDa. They make up approximately 5-10% of the total protein content of cells under conditions of normal, healthy growth [19] and are expressed or increased in response to various biological stressors, such as heat [2,8,9], malignant tumors [21], inflammation [22], viral infections and exposure to toxic chemicals [17,18]. These processes are also involved in the production of ROS, suggesting that oxidative stress is a key mechanism mediating HSP induction [23]. Of all the HSP families, HSP-70 has been most widely studied as a biomarker of stress [24]. However, only a limited number of immunohistochemical studies are available regarding the production of the HSP-70 in the testes of poultry subjected to heat stress.

In this study, our objective is to investigate the relationship between the protective effect of vit E and HSP-70 secretion on the tissue damage occurring in the testes of broilers exposed to heat stress by using histopathological and immunohistochemical methods.

MATERIAL and METHODS

Animals and Experimental Design

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine in Ataturk University (Decision No: 2007/5f). The trial was conducted at the Research and Practice Farm of Atatürk University, Faculty of Veterinary Medicine in 3 groups of animals (broilers of the commercial breed Ross 308). There were 3 cages in total; each cage had 10 broilers. Animals in each group were kept within the cages 100 x 55 x 35 cm in size and fed from day 1 to day 35. The trial groups were designed as follows: Group Control (C); these broilers (24°C) were not administered with antioxidants in the diet and not subjected to heat stress (n=10). Group Stress (S); these broilers not administered with antioxidants in the diet but subjected to heat stress (34°C) (n=10). Group Stress + Vitamin E (Group S + vit E); broilers subjected to heat stress (34°C) and administered with vitamin E (n=10). All groups were exposed to a comfortable temperature until day 14 of the trial. As of day 15, Groups S, and group S+Vit E were subjected to a temperature of 34°C between 8:00 and 16:00 hours, and to a temperature of 24°C for the rest of the 24 h day. Group C subjected to a temperature of 24°C throughout the trial. All groups were exposed to a daily regime of 17 h light and 7 h darkness. While all groups is fed with the basal ration during the study, only group S+ Vit E is given vitamin E (DL α Tocopheryl acetate: 150 mg/kg) in addition to basal ration *(Table 1)*. Dietary nutrient proportions were determined in compliance with the recommendations of the National Research Council [10].

At the end of the trial (Day 35), broilers were killed by cervical dislocation for necropsy and testes tissue were collected for histopathological and immunohistochemical evaluations.

Histopathology

The tissues were kindly removed and half of them were fixed in 10% neutral buffered formalin immediately. After dehydration in a graded ethanol series and clearing with xylene, the samples were embedded in paraffin and 4-µm-thick sections were stained with Hematoxylin-Eosin (H.E) for obser-vation under the light microscope.

HSP-70 Immunostaining

Four µm sections from all the tissue samples were cut and processed for immunohistochemical examination by a standard avidin-biotin-peroxidase method as described by the producer. Mouse monoclonal antibodies that react with human HSP-70 (Clone: BRM-22, Sigma, St. Louis, MO, USA) were used at dilution of 1:5000 for 60 min, respectively. Secondary antibody (horseradish peroxidaseconjugated goat antimouse) was added and incubated for 1 h. 3,3-Diaminobenzidine (DAB) was added an sections were kept in the dark for 10 min. Then, the sections were stained with

Table 1. *Ingredients of crude nutrient proportions in the basal ration*
Tablo 1. *Bazal rasyonda besin içeriklerinin ham madde oranları*

Ingredients	Nutrient Proportions %	
	1-10 day	11-35 day
Corn	56.99	58.74
Corn glutein	20.00	20.00
Wheat short	7.00	7.00
Soybean oil	0.78	3.72
Soybean meal	11.53	7.14
Calcium carbonate	1.36	1.23
Dicalciumphosphate	1.06	0.91
L-lysine	0.40	0.42
Salt	0.26	0.27
Vitamin-mineral premix	0.20	0.20
Toxin binder	0.10	0.10
Anticoccidial	0.10	0.10
Sodium bicarbonate	0.10	0.09
Growth factor	0.05	0.05
Phyzymexptpt	0.03	0.03
DL-Methionine	0.04	-
Nutritional levels		
Metabolic energy kcal/kg	3000	3200
Crude protein %	23	21
Ether extract	3.46	6.37
Crude fiber	2.79	2.68
Methionine	0.5	0.43
Lysine	1.10	1.0
Calcium	1.00	0.90
Phosphorus	0.7	0.65

Mayer's hematoxylin for 5-8 min as counter stain. Finally, the sections were mounted with cover slips using neutral balsam, viewed.

Image Analysis

Tissue sections were evaluated by high power light microscope (Olympus Bx52 with DP72 camera system). All immunohistochemical staining were estimated with an image processing system (Olympus, DP72-BSW). For morphometry of testes, ten tubulus of testes from each broiler (300 tubulus for each group) were chosen randomly. The intensity of staining with HSP-70 immune reactivities were scored semiquantitatively as i) A = non-reactivity, ii) B = weak, individualized cell reactivity in ≤ 25% of tubul, iii) C = mild to moderate reactivity in ≤ 50% of tubul, iv) D = strong reactivity in ≤ 75% of tubul, and v) E = very strong reactivity in >75% of tubul. The average staining intensity was calculated by the formula: [(Ax1) + (Bx2) + (Cx3) + (Dx4) + (Ex5)]/(A + B + C + D + E) and reported, as follows: i) – = 0.00, ii) + = 0.01 – 1.00, iii) ++ = 1.01 – 2.00, iv) +++ = 2.01–3.00, and v) ++++ = 3.01 – 4.00.

Statistical Analysis

Data were presented as means ± standard error (SEM). Values from semi-quantitative analysis of HSP-70 immune positive cells were analyzed separately by using one-way analysis of variance (ANOVA). For determining differences between the groups (C, S and S + Vit E), the Duncan test was used. Differences were considered significant when $P<0.05$.

RESULTS

There was no macroscopic finding in animals of group C, whereas testes were swollen and quite soft consistency in all animals of group S and in 8 animals of group S + Vit E.

Histopathological Findings

Histopathological examination of the control animals (Group C) showed normal testis tissue histology *(Fig. 1a)*. In the S group, thickening, desquamation, degeneration, necrosis in basal layer of some seminiferous tubules, and atrophy in the some seminiferous tubules were observed. It was remarkable of vacuolization in Sertoli cells and spermatogonia in the seminiferous tubules and the loss of spermatogonic cells *(Fig. 1b)*.

Although there were similar findings in testes of animals in group S + Vit E, it has been seen diminishing the severity of these findings, beginning to turn into a normal appearance of seminiferous tubulus, and the presence of spermatogonic cells *(Fig. 1c)*. Whilst there were interstitial connective tissue proliferation and hyperplasia in Leydig cells in most animals of group S, these findings were seen in only 3 animals of group S + Vit E.

Immunohistochemical Findings

In all groups the immunoreactivity of HSP-70 was positive in spermatogonia, spermatocytes, spermatids, elongated spermatids, Leydig cells and also a few Sertoli cells *(Fig. 2a, b,c)*. Although HSP-70 secretion was determined in the testes in all groups, the marked secretion levels were observed in the group C whilst the weak secretion levels in the group S were observed ($P<0.05$). The intensive secretion of HSP-70 was mainly in the cytoplasm of the spermatogenic cells in testes of group C ($P<0.05$), whereas in the group S the most intensive secretion of HSP-70 was in Sertoli cells.

DISCUSSION

Heat stress is one of the most important environmental stressors. For broiler chickens, the environmental thermo-neutral (comfort) (TN) zone covers a temperature range between 18 and 24°C. When environmental temperature increases above the TN zone and exceeds the upper critical temperature, the animals are considered to be heat stressed [1].

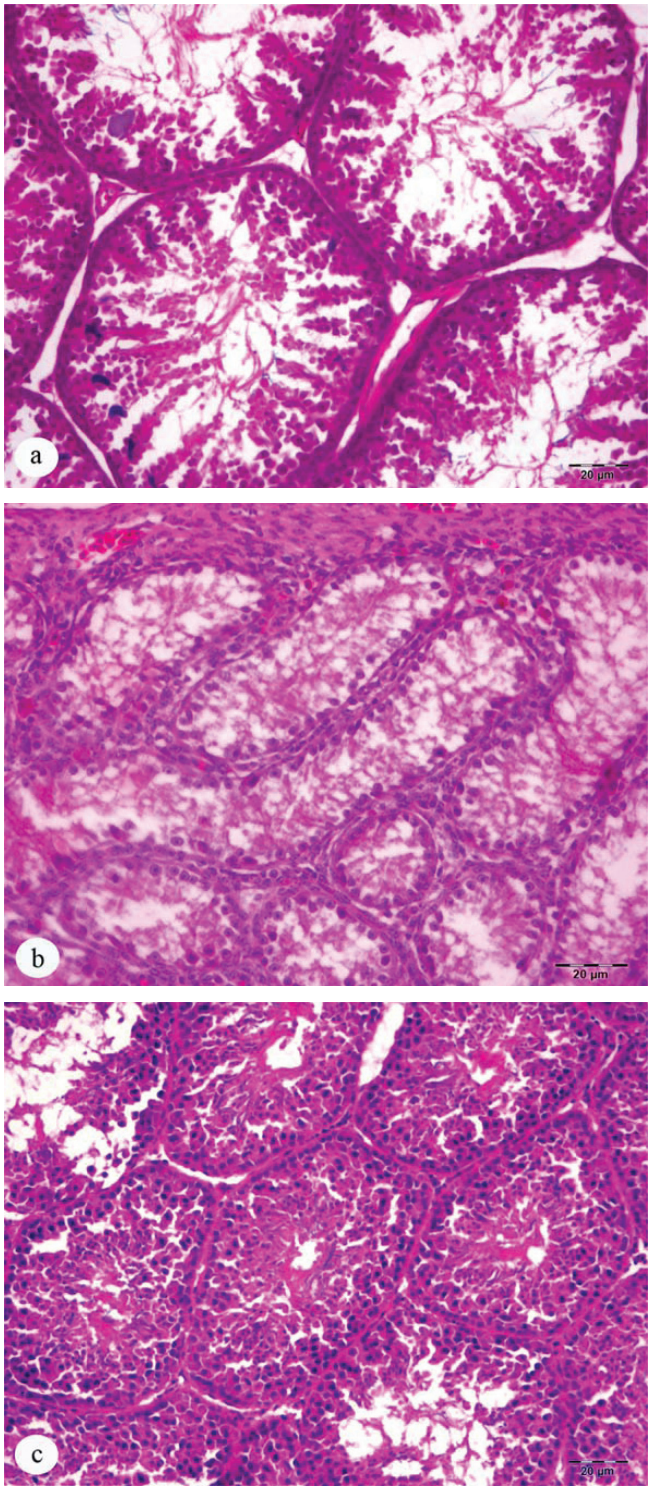


Fig 1. a- Normal appearance of seminiferous tubulus and the presence of spermatogenic cells (Group C), H.E., Bar: 20 µm, **b-** Intensive degenerative alterations in seminiferous tubules and loss of the spermatogenic cells (Group S), H.E., Bar: 20 µm, **c-** Diminishing degenerative alterations in seminiferous tubules and the presence of spermatogenic cells (Group S+Vit E), Bar: 20 µm

Şekil 1. a- Seminifer tubulusların normal görünümü ve spermatojenik hücre varlığı (Group C) H.E., Bar: 20 µm, **b-** Seminifer tubullarda yoğun dejeneratif değişiklikler ve spermatogenik hücre kaybı (Grup S), H.E., Bar: 20 µm, **c-** Seminifer tubullarda azalan dejeneratif değişiklikler ve spermatojenik hücre varlığı (Grup S+Vit E), H.E., Bar: 20 µm

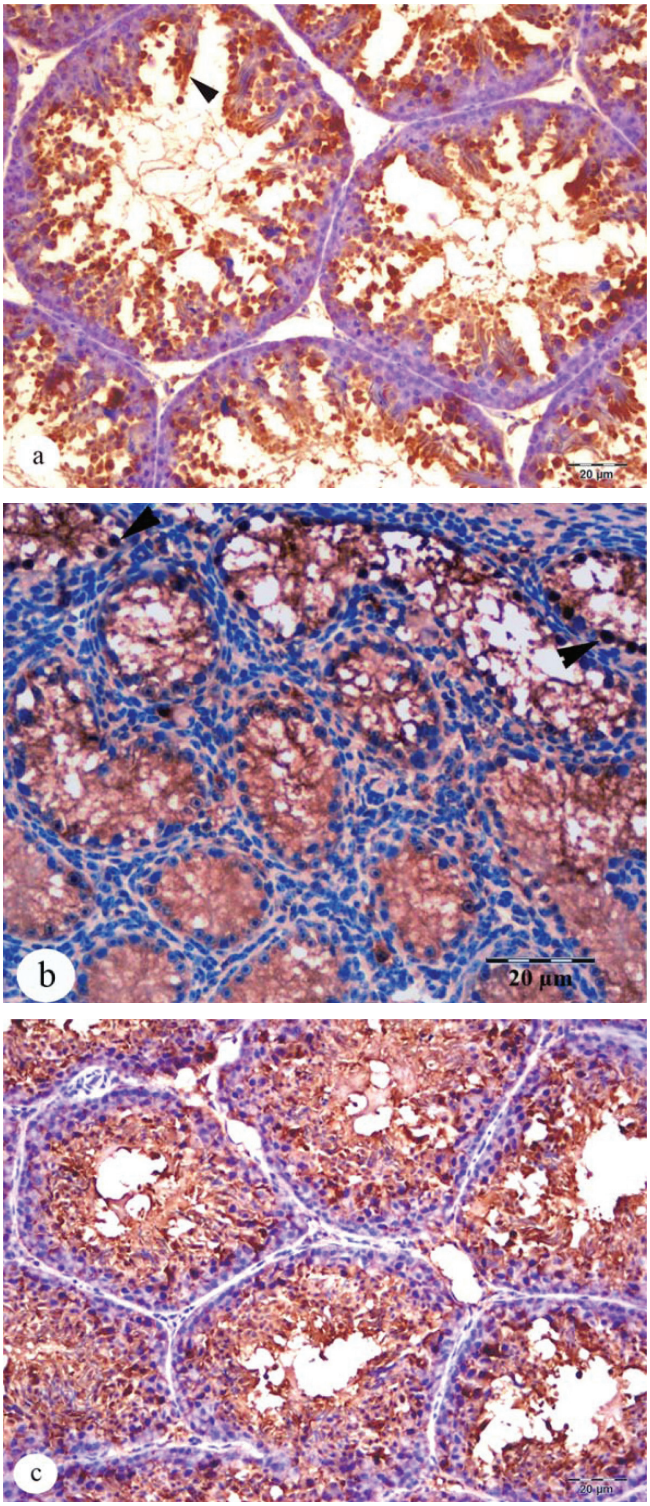


Fig 2. Immunohistochemstry stain, **a-** Intensive secretion of HSP-70 in spermatogenic cells (Group C) *(arrow head)*, Bar: 20 µm, **b-** Secretion of HSP-70 in sertoli cells (Group S), *(arrow head),* Bar: 20 µm, **c-** Moderate secretion of HSP-70 in testis tissue (Group S + Vit E), Bar: 20 µm

Şekil 2. Immunohistokimyasal boyama, **a-** Spermatojenik hücrelerde yoğun HSP-70 sekresyonu (Group C) *(ok başı)*, Bar: 20 µm, **b-** Sertoli hücrlerinde hafif HSP-70 sekresyonu (Grup S) *(ok başı),* Bar: 20 µm, **c-** Spermatojenik hücrelerde orta şiddette HSP-70 sekresyonu (Grup S+Vit E), Bar: 20 µm

For many years, researchers have been investigating the effect of high environmental temperature on the variety of tissues of poultry, such as liver, kidney, brain, heart, and have found that high environmental temperatures have destructive effects on tissues [9,12,25-27]. Previous studies showed that the fertility of male broilers was greatly reduced due to high ambient temperature [28] However, there are a limited number of studies on the effects of heat stress to testis tissues in poultry. There have been some studies regarding the negative effects of heat stress to testis tissue in different kinds of animals. In these studies, it has been reported there was degenerative changes in the seminiferious tubulus and the arrest in spermatogenesis [29-31].

Histopathological examination performed in the present study demonstrated the loss of spermatogonic cells and degenerative alterations in testes tissue in broilers subjected to heat stress. These findings were in compliance with previous studies.

During heat stress, the antioxidant defence of reproductive system downplays and generates an oxidative stress that may impair testis function and affect negatively semen characteristics. Because of high content of polyunsaturated membrane lipids, testicular tissue becomes one of the targets for oxidative stress [13]. Dietary manipulations are therefore among the methods used to alleviate these negative adverse effects of environmental stress. Several studies have shown that antioxidant nutrient supplementation is effective in preventing the deleterious effects of heat stress and that these types of nutrients could be included in diets to alleviate the negative effects of heat stress [2,8,9,12]. Vitamin E has been shown to be curative for reproductive tissues in several species. Those study reported that the supplementation of vitamin E during the devastating effects facilitated the recovery of the structure of the seminiferous tubules indicating normal epithelium with a regular arrangement of germinal cells with most phases of spermatogenesis process included differentiation phase of spermatids [13,15,32].

The results demonstrated that the tissue damage observed in testes such as degenerative alterations and sperm loss caused by heat stress could be minimized with the administration of Vit E.

As living organisms, chickens have protective measures against environmental challenges. HSPs are either constitutively expressed or their expression is induced by heat shock and other stresses which suggest their role in repair and protective mechanisms in various tissues [8,9,22,33,34]. The HSP-70 family is the most conserved and the most important constituent of the HSP proteins [24]. HSP-70 is involved in cellular repair and protective mechanisms. Previous studies have shown that heat stress is associated with HSP-70 induction, and an increase in the expression of HSP-70 occurred when poultry were kept at high ambient temperatures [8,9,25,26].

Recently, several HSPs have been demonstrated to be present and have important functions in the male chickens testis. HSPs are constitutively expressed in specific spermatogenic cell types during spermatogenesis [33,34].

In this study, it has been determined that HSP-70 was secreted in all groups. While secretion of HSP-70 was at the highest level in the group C, it was at the lowest level in the group S. It has been also seen that secretion of HSP-70 was at the moderate level in Group S + Vit E due to use of vit E. It is found that loss of spermatogenic cells in group S is directly proportional to decrease of secretion of HSP-70. This is the first study that importance of HSP-70 for spermatogenesis in testes of the poultry has been shown immunohistochemically.

Several studies demonstrated that heat stress causes an increased free radical production [7,11,14] and lowers the concentrations of antioxidant vitamins and minerals such as E, C, A, and Zn in serum. A strong relationship is now recognized between free radical production and HSP-70 synthesis in stressed cells [23]. Previous studies have demonstrated that levels of vit E are correlated with the secretion of HSP [35,36].

In another study, Sahin et al.[8] reported that serum HSP-70 levels increased in poultry exposed to heat stress, but also indicated that the HSP-70 levels of the groups administered with antioxidants did not differ from the levels measured in the stress group.

In the study performed by Terim et al.[9] it has been shown immunohistochemically that alpha- lipoic and ascorbic acid added to ration of the broilers exposed to heat stress decrease tissue damage in the liver and kidney and increase secretion of HSP-70.

In the present study, it has been observed that an increase secretion of HSP-70 in testes of broilers administered to vit E and a decrease in tissue damage occurred in heat stress.

Previous studies have reported that with increasing temperature increased secretion of HSP proteins in poultry. However, it has not been explored this increase and relationship with the vitamin E by using histopathological and immunohistochemical methods in the testes of poultry. This study has indicated the relationship between healing effect of vitamin E in tissue damage occured in testes due to heat stress and secretion of HSP-70.

In conclusion, today, HSP proteins used in cancer immunotherapy owing to the nature of biological adjuvant will take an important place in preventing of bacterial and viral disease in poultry, in supporting of immune system against to stress, in vaccine producing, and in poultry producing.

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Kafkas Univ Vet Fak Derg
19 (2): 311-317, 2013
DOI: 10.9775/kvfd.2012.7779

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Effects of Chitosan and Lactic Acid Immersion on the Mussels' Quality Changes During the Refrigerated Storage

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Makale Kodu (Article Code): KVFD-2012-7779

Summary

The objective of this study was to determine the effects of different pretreatment agents such as chitosan (0.5% (w/v), pH 2.9-3.2) and lactic acid (0.5% (v/v), pH 2.5-2.7) on the chemical and sensory qualities of mussels stored at 4ºC. Mussels were dipped in 100 mL of 0.5% solution of lactic acid (v/v) and chitosan (w/v) at room temperature (22ºC) for 15 min. Mussels from the control group were dipped in 100 mL of sterile distiled water (2% NaCl) without chitosan and lactic acid. Treatment of mussels with lactic acid and chitosan at the beginning of the experiment (day 0) for 15 min reduced bacterial counts of total aerobic mesophilic bacteria (0.53-1.07 log) psychrotrophic bacteria (0.11-0.13 log), *Lactobacillus* spp. (0.46-1.30 log), Enterobacteriaceae (0.43-0.48 log) and coliform bacteria (0.52-0.66 log). Total volatile basic nitrogen (TVB-N), thiobarbituric acid (TBA), trimethylamine nitrogen (TMA-N) and histamine values of control group mussels were increased from (day 0) 13.1 mg N 100 g^{-1}, 1.37 mg MA kg^{-1}, 1.97 mg N 100 g^{-1} and 4 ppm to 39.9 mg N 100 g^{-1}, 3.02 mg MA kg^{-1}, 4.86 mg N 100 g^{-1} and 7.75 ppm at the end of the storage period (day 11), respectively (P<0.05). The results indicated that shelf-life of mussels stored at 4ºC were limited to 4 days in the control group. However, mussels pretreated with lactic acid and chitosan were stored for 6-7 days and the shelf-life of mussels was extended for ca. 2-3 days, as compared with the control group (P<0.05).

Keywords: *Chitosan, Lactic acid, Mussel, Quality, Shelf life*

Laktik Asit ve Kitosanın Soğukta Muhafaza Edilen Midyelerdeki Kalite Değişiklikleri Üzerine Etkileri

Özet

Bu çalışma +4ºC'de muhafaza edilen midyelerde kitosan (%0.5 (w/v), pH 2.9-3.2) ve laktik asit (%0.5 (v/v), pH 2.5-2.7) uygulamalarının midyenin kimyasal ve duyusal kalitesi üzerine etkisini belirlemek amacıyla yapıldı. Midyeler 100 mL, %0.5'lik laktik asit (v/v) ve kitosan (w/v) solusyonları içine daldırıldı oda ısısında 22ºC'de 15 dk. bekletildi. Kontrol grubu midyeler ise içinde kitosan ve laktik asit olmayan 100 mL steril distile su (%2 NaCl) içinde bekletildi. Midyeler başlangıç gününde (0. gün) 15 dk. süreyle laktik asit ve kitosanda muamele edilmesi sonucu toplam aerobik mezofilik bakteri (0.53-1.07 log), psikrotrofik bakteri (0.11-0.13 log), *Lactobacillus* spp. (0.46-1.30 log), Enterobacteriaceae (0.43-0.48 log) ve koliform bakteri (0.52-0.66 log) sayılarında düşüş sağlandı. Kontrol grup midyelerinde sıfırıncı günde total volatile basic nitrogen (TVB-N), thiobarbituric acid (TBA), trimethylamine nitrogen (TMA-N) ve histamin değerleri sırasıyla 13.1 mg N 100 g^{-1}, 1.37 mg MA kg^{-1}, 1.97 mg N 100 g^{-1} ve 4 ppm iken depolamanın sonunda (11. günde) 39.9 mg N 100 g^{-1}, 3.02 mg MA kg^{-1}, 4.86 mg N 100 g^{-1} ve 7.75 ppm'e yükseldi (P<0.05). Elde edilen sonuçlara göre +4ºC de muhafaza edilen kontrol grup midyelerin raf ömrü 4 günle sınırlı kaldı. Buna karşın muhafaza öncesi laktik asit ve kitosan ile muamele edilen midyelerin raf ömrü 6-7 gün olarak belirlendi, kontrol grup ile karşılaştırıldığında raf ömrünün 2-3 gün daha fazla uzadığı görüldü (P<0.05).

Anahtar sözcükler: *Kitosan, Laktik asit, Midye, Kalite, Raf Ömrü*

INTRODUCTION

In the Black Sea region of Turkey, fishing is an important industry. In Turkey, average consumption of fishery products per person is 6.9 kg in the year 2010. The Turkish Statistical Institute [1] has reported that the amount of fish and mussels caught in Turkey was 399. 656 and 27.968 tons, respectively in 2010.

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In Turkey, mussels are in general freshly consumed and often cooked with rice called "Stuffed Mussels" or they are vacuum packaged for marketing purposes. The high level of microorganisms in the mussels are probably due to their filter-feeding activities. Furthermore, heat treatment could not satisfactorily penetrate to the centre of mussels because of their thick shells. The consumption of raw or undercooked mussels containing pathogenic microorganisms such as *Vibrio parahaemolyticus*, Hepatitis A, *Shigella* spp. and Norwalk viruses results in serious public health problems [2].

When mussels stored under inappropriate conditions, trimethylamine nitrogen (TMA-N), ammonia and other basic nitrogenous compounds, and total volatile basic nitrogen (TVB-N) are produced in a shorter time. Many methods have been developed to prolong the shelf-life of fishery products to reduce bacteria counts and to maintain quality of products such as cold shock, freezing, ultraviolet irradiation, salt treatment, storing at low temperatures, modified atmosphere packaging, decontaminating by using chitosan, chlorine, organic acids, sodium lactate, sodium acetate, nisin, ozone and chloroform [3-5]. The most widely used chemical decontaminants in the meat industry are organic acids [6]. The antimicrobial activity of lactic acid has been demostrated in a number of foods including chicken, meat and seafood [7]. Lactic acid inhibits the growth of microorganisms such as gram negative Enterobactericeae and *Pseudomonadaceae* spp [8]. The other decontaminant that is used in the seafood to reduce bacterial count is chitosan. Chitosan is a $\beta(1-4)$ linked N- acetyl-D-glucosamine polymer. It is derived by deacetylation of chitin, a major component of the shells of crustaceans such as crab, shrimp and crawfish [9,10]. Chitosan has been shown to have antibacterial activities against gram-positive and gram-negative bacteria [10-12]. The present study was undertaken to investigate the effects of different pretreatment agents such as chitosan and lactic acid on the shelf-life and quality of mussels stored at 4°C.

MATERIALS and METHODS

Sample

A total of 400 fresh mussels *(Mytilus galloprovincialis)* (approximately 3 kg) were obtained from the Samsun region in the middle Black Sea coast of Turkey in May 2010. Mussels were transported in containers with ice bags to the Department of Food Hygiene and Technology, Ondokuz Mayis University, within 1-2 h after harvesting. The samples were washed in sterile distilled water, disinfected with alcohol (70%) and opened aseptically by using sterilised scalpel and pincers.

Chemicals and Reagents

Chitosan (catalogue number: 44887-7, medium molecular weight, 75-85% deacetylated, viscosity 200-800 cps) was purchased from the Aldrich (Milwaukee, WI, USA) and lactic acid (catalogue number: 100366.2500, 90%) purchased from Merck (Darmstadt, Germany). The media used for microbiological analysis were obtained from Oxoid (Basingstoke, UK).

Decontamination with Lactic Acid and Chitosan

Lactic acid solution (0.5% (v/v), pH 2.5-2.7) was prepared by diluting in sterile distilled water. Medium molecular weight chitosan solution (0.5% (w/v), pH 2.9-3.2) was prepared by dissolving in a 0.5% (v/v) lactic acid solution with magnetic stirring. All solutions were autoclaved for 15 min at 121°C. Each mussel was then dipped in 100 mL of 0.5% solution of lactic acid (v/v) and chitosan (w/v) at room temperature (22°C) for 15 min. Control group of mussels was dipped in 100 mL of sterile distiled water (2% NaCl) without chitosan and lactic acid. After dipping, mussel samples were stored at 4°C in aerobic condition and chemical and sensory analyses were performed in duplicate at 0, 2, 4, 7, 9 and 11 days of storage.

Chemical Analyses

pH measurements: Ten gram of mussel tissue was homogenised with 20 mL of distilled water. The pH of the homogenate was measured using a pH meter (Inolab pH730, Germany) [13].

Determination of histamine: The histamine analysis in mussel samples was carried out by using Ridascreen Histamine ELISA test kit (R-Biopharm A.G. Kit, Art. No. R 1604, Darmstad, Germany). According to the ELISA test kit procedure, one gram of sample was taken and 200 mL distilled water was added. Then it was homogenized for 15 min in a blender and the mixture was centrifuged for 5 min at 2500 *g* at room temperature (20-25°C). The supernatant was diluted appropriately, and analysis was carried out according to the method described by the manifacturer. The extracts were read at 405 nm in an ELISA reader (Digital. Analog Systems, DAS RS 232, Rome, Italy).

Determination of total volatile basic-nitrogen (TVB-N): Total volatile basic-nitrogen was determined using the TVB-N quantification kit of reagents (Kit 66662) (Fluka, Italy) according to the Official Method for the European Union [14]. Briefly, 10 g of mussel was homogenised with 90 mL of perchloric acid solution (0.6 M) in a stomacher (Interscience-BagMixer 400, St Nom, France) for 1 min. The homogenates were filtered through a filter paper (Whatman No. 1) and 50 mL of filtrate was made alkaline with sodium hydroxide 20%. The filtrates were distilled by steam distillation for 10 min and the volatile base components were absorbed by an acid receiver (boric acid solution). After distillation, filtrates were titrated with 0.01 mol L HCl. The results were expressed as mg TVB-N per 100 g of sample.

Determination of thiobarbituric acid (TBA): The thiobarbituric acid (TBA) was determined by the distillation method of Tarladgis et al.[15] Ten gram of the mussel sample was blended with 50 mL distilled water for 2 min. The mixture was transferred into a Kjeldahl flask, one drop of silicone anti-foaming agent

was added plus 2.5 mL HCl (4N) and the jar was rinsed with an additional 47.5 mL of distilled water. This sample was then distilled and 50 mL of distillate was collected. Five mL of distillate were added to 5 mL 0.02 M thiobarbituric acid (Merck, Germany) and heated in a boiling water bath for 35 min and then cooled in an ice bath. Absorbance of the solutions was determined at 538 nm on a UV spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). The TBA values were expressed as milligrams of malonaldehyde per kilogram. The Standard curves were prepared by making appropriate dilutions of the $1x10^{-4}$ mol L 1,1,3,3-tetraethoxy-propane (TEP) (Sigma, USA) standard solutions.

Determination of trimethylamine nitrogen (TMA-N): Determination of TMA-N was carried out according to the method proposed by the AOAC [16] based on the colorimetric method. Briefly, ten gram of homogenised samples were weighed and blended with 90 mL of 7.5% trichloracetic acid solution. The blended solution was centrifuged at 2.000-3.000 rpm until the supernant was practically clear. Four mL of extract was transferred into test tubes and 1 mL formaldehyde (20%), 10 mL anhydrous toluene, and 3 mL potassium carbonate (K_2CO_3) solution (100 g%) were added. The tubes were shaken and the toluene phase was then transferred into a tube containing 0.2 g of anhydrous sodium sulfate and shaken to obtain a dehydrated extract. Five mL of the water-free toluene layer was pipetted and 5 mL of picric acid solution (0.02%) was added. The solution was mixed and transferred into a spectrophotometric cell. Absorbance was measured using a spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK) at a wavelength of 410 nm. At the same time, blank and standards were prepared and measured. The amount of TMA-N in the samples was calculated from the optical densities by using the standard curve described. Results of TVB-N were expressed as mg N 100 g^{-1} sample.

Microbiological Analysis

For microbiological analysis 10 g of the mussel samples were transferred to a sterile bag with 90 mL of 0.1% sterile peptone water (Oxoid CM 0009, Hampshire, UK) and blended for 90 s in a stomacher (Interscience-BagMixer 400, St Nom, France). From this homogenate serial decimal dilutions were prepared in 0.1% peptone water according to standard method given by APHA [17]. Total aerobic mesophilic and psychrotrophic bacterial counts [18,19] were determined by Plate Count Agar (PCA, Oxoid CM 325) with the incubation at 37°C for 1-2 days and 7°C for 10 days, respectively. *Lactobacilli* counts [20] were enumerated on de Man Rogosa Sharpe agar (MRS, de Man Rogosa Sharpe Agar, Oxoid CM 0361) incubated at 30°C for 2 days. Enterobacteriaceae [21] were enumerated in Violet Red Bile Dextrose Agar (VRBG, Oxoid CM 0485) incubated at 37°C for 2 days. Coliforms [22] were enumerated on Violet Red Bile Agar (VRBA, Oxoid CM 0107) incubated at 37°C for 1-2 days.

Sensory Analyses

The sensory quality of raw mussels was evaluated by a sensory panel consisting of ten experienced judges. The sensory characteristics of mussels were evaluated using the 9-point hedonic scale according to the characteristics shown in *Table 1*. Each panelist evaluated approximately 20 g of mussel sample for appearance, odour, colour and texture. Four categories were ranked: scores between 9 and 8 indicated "excellent quality (E)"; scores between 8 and 6 indicated "good quality (A)"; scores between 6 and 4 indicated "fair quality (B)"; scores ≤ 4 indicated "unaccaptable (C)" samples [23,24].

Statistical Analyses

Statistical analysis was performed using SPSS [25] (Statistical Package for Social Sciences SPSS, Inc, Chicago) software package. All experiments were carried out in duplicate and the results were presented as means ± standard deviations. The results were evaluated by using ANOVA and any significant differences further evaluated using the Tukey Multiple Comparison Test. The level of significance was set at $P<0.05$.

RESULTS

The bacterial counts of total aerobic mesophilic bacteria, psychrotrophic bacteria, *Lactobacillus* spp., Enterobacteriaceae and coliform bacteria of control group mussels in initial day (0 day) were found $4.7x10^5$ cfu/g, $4.6x10^5$ cfu/g, $4.0x10^4$ cfu/g, $4.8x10^3$ cfu/g and $4.6x10^3$ cfu/g, respectively. Following treatment of mussels with lactic acid for 15 min, the bacterial counts of total aerobic mesophilic bacteria, psychrotrophic bacteria, *Lactobacillus* spp., Enterobacteriaceae and coliform bacteria were $1.4x10^5$ cfu/g, $3.6x10^5$ cfu/g, $1.4x10^4$ cfu/g, $1.8x10^3$ cfu/g and $1.4x10^3$ cfu/g, respectively. Following treatment of mussels with chitosan for 15 min, the bacterial counts of total aerobic mesophilic bacteria, psychrotrophic bacteria, *Lactobacillus* spp., enterobacteriaceae and coliform bacteria

Table 1. *Sensory analysis scale for mussels.*
Tablo 1. *Midyeler için duyusal analiz skalası*

Parameter	Excellent Quality (E) 9 - 8	Good Quality (A) 8 - 6	Fairly Good Quality (B) 6 - 4	Unaccaptable (C) ≤ 4
Appearance	Glossy	Moist	Less moist	Dull
Odour	Characteristically sweet and fresh	Non- specific/slightly sweet	Slightly ammonia- like	Ammonia
Colour	Bright	Orange	Opaque	Grey/discoloured
Texture	Very firm	Firm	Slightly firm	Soft

were $4.0x10^4$ cfu/g, $3.4x10^5$ cfu/g, $2.0x10^3$ cfu/g, $1.6x10^3$ cfu/g and $1.0x10^3$ cfu/g, respectively.

In the present study, following immersion of mussels to lactic acid for 15 min, the bacterial counts of total aerobic mesophilic bacteria, psychrotrophic bacteria, *Lactobacillus* spp., Enterobacteriaceae and coliform bacteria (0.53, 0.11, 0.46, 0.43 and 0.52 log cfu/g), decreased, respectively. Mussels treated with chitosan for 15 min, the bacterial counts of total aerobic mesophilic bacteria, psychrotrophic bacteria, *Lactobacillus* spp., Enterobacteriaceae and coliform bacteria (1.07, 0.13, 1.30, 0.48 and 0.66 log cfu/g) decreased, respectively.

Table 2. *Changes in chemical constituents of mussels pretreated with chitosan and lactic acid during storage at 4°C (Mean+SD).*

Tablo 2. *Kitosan ve laktik asitle muamele edilen midyelerde +4°C'de muhafaza sürecinde şekillenen kimyasal değişiklikler*

Stroge Time (Day)	Control	Lactic Acid	Chitosan
TVB-N (mg N 100 g^{-1})			
0	13.1±0.2 ^{a1}	12.0±0.2^{a1}	11.9±0.2^{a1}
2	16.1±0.2 ^{a2}	14.0±0.2^{b2}	12.3±0.2^{c1}
4	30.1±0.3 ^{a3}	24.0±0.3^{b3}	15.0±0.4^{c2}
7	33.6±0.6 ^{a4}	28.0±0.4^{b4}	25.9±0.5^{c3}
9	38.5±0.5 ^{a5}	35.0±0.3^{b5}	33.6±0.2^{c4}
11	39.9±0.2 ^{a6}	38.0±0.2^{b6}	37.0±0.5^{b5}
TBA (mg MDA kg $^{-1}$)			
0	1.37±0.02^{a1}	1.21±0.02^{b1}	1.14±0.02^{c1}
2	1.72±0.02^{a2}	1.33±0.03^{b2}	1.23±0.03^{c2}
4	2.51±0.01^{a3}	1.34±0.01^{b2}	1.30±0.01^{c3}
7	2.72±0.01^{a4}	2.10±0.01^{b3}	1.96±0.01^{c4}
9	2.90±0.02^{a5}	2.47±0.02^{b4}	2.34±0.01^{c5}
11	3.02±0.01^{a6}	3.01±0.01^{a5}	2.87±0.02^{b6}
TMA-N (mg N 100 g^{-1})			
0	1.97±0.03^{a1}	1.27±0.02^{b1}	1.26±0.02^{b1}
2	3.25±0.02^{a2}	2.98±0.03^{b2}	2.79±0.04^{c2}
4	4.45±0.05^{a3}	2.79±0.09^{b3}	2.60±0.05^{c3}
7	4.60±0.04^{a4}	3.90±0.03^{b4}	3.50±0.05^{c4}
9	4.75±0.05^{a5}	4.16±0.06^{b5}	3.90±0.05^{c5}
11	4.86±0.06^{a5}	4.39±0.03^{b6}	4.35±0.02^{b6}
Histamine (ppm)			
0	4.00±0.02^{a1}	3.79±0.03^{b1}	3.80±0.03^{b1}
2	4.34±0.03^{a2}	4.10±0.02^{b2}	4.05±0.02^{c1}
4	4.48±0.02^{a3}	4.20±0.01^{b3}	4.18±0.01^{b1}
7	5.29±0.02^{a4}	4.78±0.02^{b4}	4.55±0.03^{c2}
9	5.69±0.03^{a5}	5.20±0.04^{b5}	5.12±0.03^{c2}
11	7.75±0.04^{a6}	5.60±0.02^{b6}	5.40±0.02^{c2}
pH			
0	5.99±0.02^{a1}	5.01±0.01^{b1}	5.48±0.02^{c1}
2	5.30±0.02^{a2}	5.04±0.02^{b1}	5.19±0.01^{c2}
4	5.02±0.01^{a3}	4.88±0.01^{b2}	5.00±0.01^{c2}
7	4.88±0.01^{a4}	4.60±0.02^{b3}	4.67±0.01^{c3}
9	4.66±0.02^{a5}	4.54±0.01^{b4}	4.65±0.02^{a4}
11	4.54±0.01^{a6}	4.52±0.01^{a4}	4.55±0.02^{a5}

^{a,b,c} Mean values within a column with different superscript letters differ significantly (P<0.05), $^{1,2,3....6}$ Mean values within a line with different superscript letters differ significantly (P<0.05)

Chemical and sensory analyses were used to evaluate the effect of these treatments during storage at 4°C. The changes that occured in pH, TVB-N, TBA, TMA-N and histamine of mussel meat during storage are presented in *Table 2*. The results of sensory evaluation are shown in *Fig. 1*. At the beginning of the storage, fresh mussel had a characteristic fresh odour (sealike, seaweed-like), bright orange colour, slightly firm, elastic, tight texture and moist appearance. Sensory scores significantly ($P<0.05$) changed by time. The acceptable shelf-

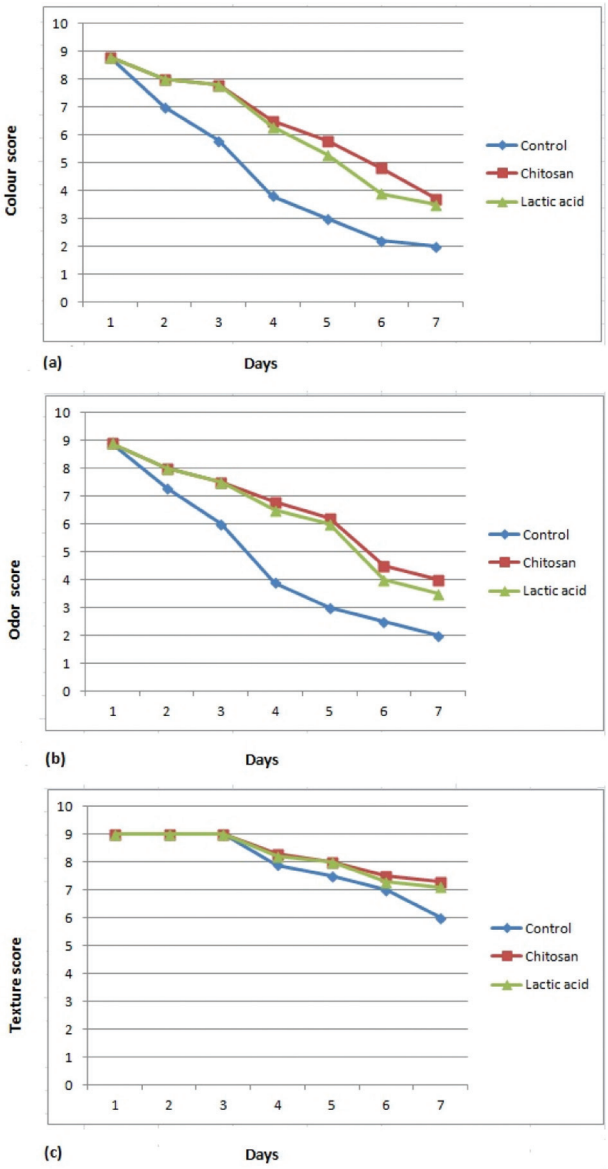


Fig 1. Sensory evaluation. Changes in (a) colour, (b) odor, (c) texture scores in mussels during storage at +4°C

Şekil 1. Duyusal değerlendirme. Midyelerde +4°C'de depolama süresince renk, koku ve tekstürdeki değişiklikler

life was considered from a sensory score of greater than 5.0. The results showed that sensory scores were greater than 5 for the non-treated mussels (control group) stored for 4 days at 4°C, while mussels pretreated with lactic acid and chitosan were stored for 6-7 days.

DISCUSSION

The initial pH of the mussels was 5.99 and gradually decreased in all treatments during storage ($P<0.05$). Similar results on the initial pH of mussels were reported as 5.96 by Erkan [24]. Mussels pretreated with lactic acid had lower pH than those pretreated with chitosan. The decrease in the pH of mussels may be due to the postmortem changes, degradation of muscle components and conversion of glycogen to lactic acid during the long term storage [26]. Pottinger [27] proposed the following pH scale as a basis for determining the freshness of mollusks: pH=6.2-5.9 good, pH=5.8 off, pH=5.7-5.5 musty, pH=5.2 and pH < 5.2 sour or putrid. The present study showed that pH value of control group mussels was unacceptable as 5.02 at the 4th day and was 4.54 at the end of the storage. On the other hand, Manousaridis et al.[4] reported that an attempt to correlate changes in pH to the sensory quality of mussels was unsuccessful and that pH is not useful as a quality index.

The TVB-N of seafood is an indicator of the freshness of the raw material. In this study the initial TVB-N value of mussel samples ranged from 11.9 to 13.1 mg N 100 g^{-1}. Thereafter it increased with time reaching 39.9 mg N 100 g^{-1} for the control group, whereas for mussels pretreated with lactic acid and chitosan it was 38 and 37 mg N 100 g^{-1}, respectively, on the eleventh day. The TVB-N value for the control group was found to be higher than the proposed acceptability limits of 35 mg N 100 g^{-1} of fish muscle set by the Commission Decision 95/149/EC [14] after 9 days of storage. On the other hand, when the mussels were pretreated with lactic acid and chitosan, the TVB-N value was found to be lower than the acceptability limits after 9 days of storage.

Erkan [24] reported that the TVB-N value of fresh mussel was initially 12.38 mg N 100 g^{-1}, reaching 22.55 mg N 100 g^{-1} after the sixth day. Goulas et al.[28] found that the TVB-N value was initially 11.48 mg N 100 g^{-1} in mussel samples and it was 36.72 mg N 100 g^{-1} after 15 days of storage for the control group. They stated that when mixtures of (M1: 50%/50% (CO_2/N_2), M2: 80%/20% (CO_2/N_2) and M3: 40%/30%/30% (CO_2/N_2/O_2) were used, the TVB-N values remained significantly lower ($P<0.05$) than the acceptability limit after 15 days of storage. TVB-N content was found higher in the control mussels when compared with acid pretreated mussel samples throughout storage ($P<0.05$) by Masniyom and Benjama [29]. They indicated that TVB-N content increased rapidly and reached 20 mg N 100 g^{-1} after 9 days of storage for the control and all samples dipped in acids had TVB-N contents less than 20 mg N 100 g^{-1} within 27 days of storage.

TMA is produced by the reduction of trimethylamine oxide (TMA-O) by TMA-O reductase producing organisms and enzymatic activity [30]. TMA is considered as a good quality indicator for shrimp; it only reached 3 mg per 100 g^{-1} TMA-N, which is below the suggested 5 mg per 100 g^{-1} TMA-N limit for spoiled shrimp [31]. A level of 10-15 mg per 100 g^{-1} was suggested by Connell [30] as the maximum limit of acceptability to indicate fish freshness. Ruiz-Capillas et al.[32] cited 5 mg per 100 g^{-1} as the acceptable limit of TMA-N in shrimp and lobster. In the present study, TMA-N values of mussels were ranged from 1.26 to 1.97 mg N 100 g^{-1} on the first day of storage. At the end of the storage period of 11 days, TMA-N values increased to 4.86 mg N 100 g^{-1} for the control group and in the samples pretreated with lactic acid and chitosan TMA-N values were 4.36 and 4.35 mg N 100 g^{-1}, respectively. Masniyom and Benjama [29] found the highest concentration of TMA-N in the control mussel samples (0.06 mg/g), followed by those treated with citric, acetic and lactic acids (from 0.02 mg/g to 0.04 mg/g), respectively after 9 days of storage. They indicated that even after 27 days of storage, samples pretreated with acids had a TMA-N less than 5 mg N 100 g^{-1}. Similarly, in the present study control group samples and samples pretreated with lactic acid and chitosan had TMA-N less than 5 mg N 100 g^{-1} after 11 days of storage. Manousaridis et al.[4] reported that TMA-N values of all mussel samples remained relatively low throughout the entire storage period, attaining values of 7.5, 6.0 and 6.4 mg N/100 g for the control and samples ozonated for 60 and 90 min, respectively, on day 12 of storage.

The TBA assay is one of the most commonly used indicators for monitoring lipid oxidation. Lipid oxidation is responsible for undesirable effects such as the formation of off-odours and off-flavours in edible oils and fat-containing foods, called oxidative rancidity, and the loss of fat-soluble vitamins and palatability problems [33,34]. As can be seen in *Table 2*, the TBA values of the control and samples pretreated with lactic acid and chitosan were significantly ($P<0.05$) increased from 1.37, 1.21 and 1.14 (day 0) to 3.02, 3.01 and 2.87 mg MDA kg^{-1}, respectively, at the end of the storage period of 11 days. Similar results have been found by Masniyom and Benjama [29], who found that TBA value increased in all mussel samples when the storage time increased ($P<0.05$), indicating the lipid oxidation. TBA values found in the mussel samples by Caglak et al.[35] were between 2.16 to 3.7 mg MDA kg^{-1} at the end of the storage period of 12 days. In this study, TBA values of control mussels were 2.51 mg MDA kg^{-1} on the 4th day, exceeding the value of 1-2 mg MDA kg^{-1}, regarded as the limit beyond which fish will normally develop an objectionable odour/taste [30], whereas samples treated with lactic acid and chitosan did not exceed the limit after 4 days of storage. TBA values for samples pretreated with chitosan were lower than for those treated with lactic acid. On the other hand, Masniyom and Benjama [29] found that samples pretreated with citric acid showed lower TBARS than those treated with lactic and acetic acids.

Histamine is formed through the decarboxylation of

specific free amino acids by exogenous decarboxylases released from microbial populations associated with seafood [36]. Fish species belonging to the family Scombridae (e.g. tuna, bonito and mackerel), which contain high levels of free histidine in their muscle, are often implicated in scombroid poisoning incidents when not properly processed and stored [37]. Microbial contamination, temperature, proteolytic enzymes, pH and oxygen are the factors affecting the formation of histamine in foods [38]. In the present study, histamine level in the mussel samples was between 3.80-4.00 ppm on day 0. Afterwards, the histamine level was found to be 7.74, 5.60 and 5.40 ppm for the control group and samples treated with lactic acid and chitosan, respectively, at the end of the storage period of 11 days. The histamine level was increased gradually during the storage period at 4°C. However, these concentrations were lower than 50 ppm of histamine, the allowed limit of the US Food and Drug Administration (FDA) for scombroid fish and/or products [39] Similarly, very low or negligible histamine concentrations were found in calamari, prawn and mussel samples by Auerswald et al.[40]. They found a histamine value of 5.1 ppm in black mussels *(Mytilus galloprovincialis*) purchased from various outlets.

Crapo and Himelbloom [41] stated that "histamine value was 6 ppm in pacific herring fish after two days storage at 10°C. In fish between six and ten days, histamine levels were slightly less than 50 ppm. Only the fourteen day sample exceeded the regulatory action level." Olley and Baranowski [42] pointed out that low-temperature enzyme activity by microorganisms that grow at warm temperatures is important in histamine formation, provided sufficient bacterial numbers have been reached before cold storage. It is estimated that the difference between these histamine levels may be caused by the differences in storage methods used, bacterial load in sample, storage temperature and type of fishes.

The results showed that non-treated mussels (control group) could be stored for 4 days at 4°C, whereas mussels pre-treated with lactic acid and chitosan could be stored for 6-7days. Mussels treated with lactic acid and chitosan had an extended shelf-life of ca. 2-3 days, as compared with the control samples. The highest sensory scores were observed for mussel samples treated with chitosan. These results are in agreement with other researchers who reported a shelf-life of 3-4 days for mussels stored in a refrigerator [24,43,44]. However Goulas et al.[28] reported a shelf-life of 8-9 days for mussels at +4°C. On the other hand, Gokoglu [45] states that the samples were not safe to consume after 5 days.

In the present study, treatment of mussels with lactic acid and chitosan for 15 min reduced bacterial counts of total aerobic mesophilic bacteria (0.53-1.07 log) psychrotrophic bacteria (0.11-0.13 log), *Lactobacillus* spp. (0.46-1.30 log), Enterobacteriaceae (0.43-0.48 log) and coliform bacteria (0.52-0.66 log). Chitosan was more effective for reducing bacterial loads of mussels than that of lactic acid. The superiority of the chitosan versus lactic acid was originated from that of chitosan was not only effective against both gram (+) ve gram (-) bacteria but the use of lactic acid and acetic acid for the dissolving of chitosan further contributed to the antimicrobial effectiveness of the chitosan.

Chemical and sensory analyses revealed that mussel samples (control group) were still "acceptable" on day 4; however, on day 5, they were no longer acceptable. On the other hand, mussels treated with lactic acid and chitosan had an extended shelf-life of ca 2-3 days, as compared with the control group ($P<0.05$). The TVB-N value exceeded the acceptable limit of 35 mg N 100 g^{-1} on the 9th day. The TBA value exceeded the limit of 2 mg MDA kg^{-1} on the 4th day. Sensory quality limits were exceeded on day 4. However, TMA-N and histamine values did not exceed the acceptable limits at the end of the storage (day 11) in the control group mussels stored at 4°C. The highest sensory scores were obtained for mussel samples treated with chitosan. In conclusion, the results of this study suggest that the lactic acid and chitosan can be used to extend the shelf-life and maintain the quality of mussels.

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Kafkas Univ Vet Fak Derg
19 (2): 319-323, 2013
DOI: 10.9775/kvfd.2012.7810

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Türkiye'de *Theileria annulata* Suşlarının Karakterizasyonu ve Tür İdentifikasyonunda Glukoz Fosfat İzomeraz İzoenzim Elektroforezi [1]

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Makale Kodu (Article Code): KVFD-2012-7810

Özet

Theileria annulata omurgalı konakçılarda kan ve dolaşım sistemini etkileyen ve *Hyalomma spp* keneleri tarafından nakledilen protozoan parazittir. Suş farklılıkları hastalığın şiddeti üzerine etkili olduğu bilindiğinden tayini önem arz etmektedir. Parazit izoenzim elektroforezi *T. annulata* yanında diğer bazı protozoa'ların tür ve suş tespitinde de kullanılmaktadır. Parazit enzimlerinin elektroforezi; tür tespiti, attenue canlı aşılarda kullanılmak üzere suş seçimi ve konakçı-parazit etkileşimi ile ilgili önemli bilgiler sağlamaktadır. Bu çalışmada *T. annulata* suşları arası farklılık nişasta jel glikoz fosfat izomeraz izoenzim elektroforezi tekniği aracılığı ile ortaya konmaya çalışılmıştır. Çalışma sonucunda Türkiye'de farklı bölgelerden elde edilen suşlarda farklılık olduğu ve ortaya konan farklılıkların çalışmaların daha da genişletilerek aşı geliştirme ve benzeri proflaktik uygulamalarda yararlanılmasında faydalı olabileceği kanaatine varılmıştır.

Anahtar sözcükler: *Theileria annulata, Glikoz fosfat izomeraz*

Characterization of *Theileria annulata* Strains in Turkey and Usage of Glucose Phosphate Isoenzyme Electrophoresis in Strain Identification

Summary

Theileria annulata is a protozoan parasite of which is transmitted via *Hyalomma spp* ticks is affecting the blood and circulatory system. It is essential to determine the strain infecting the animal as the severity of the disease closely related with it. Parasite isoenzyme electrophoresis is frequently used to identify type and strain of the many protozoa as in Theileriosis. Parasite isoenzyme determination gives clues in choosing the right strain for attenuated vaccine candidates and parasite-host interaction. In this study, it was aimed to demonstrate and characterize the variety of the serotypes of *T. annulata* with the biochemical technique called Glucose Phosphate Isomerase (GPI) starch gel electrophoresis. In our study, it is revealed that various serotypes of *T. annulata* exist in different regions of Turkey and this study can further be expanded to provide substantial data for various prophylactic measures such as vaccine development.

Keywords: *Theileria annulata, Glucose phosphate izomerase*

GİRİŞ

Theileria annulata (*T. annulata*) omurgalı konakçılarda kan ve dolaşım sistemini etkileyen protozoan parazittir. Doğada artropod konakçılar tarafından nakledilirler. Bu parazitler, tropik ve yarı-tropik bölgelerde önemli sığır hastalıklarına yol açmaktadırlar. Parazitin konakçı vertebrattaki gelişimi hem lenfosit hem de eritrositlerde görülür [1,2].

T. annulata Türkiye'de çok yaygın olarak görülen, Hyalomma keneleri tarafından nakledilen, parazitlerdir. Bu kenelerin aktif olduğu dönem ilkbahar ve yaz aylarıdır, bu yüzden neden oldukları hastalık sıklıkla Mayıs-Eylül ayları arasında görülür [1].

T. annulata tropikal Theileriosis etkeni olup, başta enzootik

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ve melez sığırlarda etkilidir. Endemik bölgelerdeki sığırlar (yerli sığırlar) daha dayanıklıdırlar, hatta kısmi bağışıklığa sahiptirler [1,2]. *T. annulata* sığır ithalinde büyük sorunlara yol açtığından Türkiye'de sığırcılığın gelişmesine engel olarak görülmektedir [3,4].

Theileriidae familyasında bulunan parazit hemoprotozoa birçok ülkede önemli sığır hastalığının etkenidirler. *Theileria annulata* tarafından oluşturulan Tropikal Theileriosis Akdeniz sahillerinden Orta Asya'ya kadar uzanan büyük bir alanda etkilidir. *Theileria parva* ise Doğu Afrika'da çok önemli bir sığır hastalığı olan Şark Sahili Humması'na yol açar [1,2].

Suş farklılıkları hastalığın şiddeti üzerine etkili olduğundan Theileria türlerinde suş tayini önem arz etmektedir [5-7].

Parazit izoenzimlerinin elektroforezi *T. annulata* yanında diğer bazı protozoa'ların tür ve suş tespitinde de kullanılmaktadır. Parazit izoenzim veya enzim elektroforezi Plasmodia, Trypanosoma, Eimeria, Babesia ve Entamoeba spp. identifikasyonu için de kullanılmıştır [4,8-11]. Parazitlerdeki enzimlerin elektroforezi ile tür tespiti yapılarak [12], attenue canlı aşılarda kullanılmak üzere suş seçimi yapılmakta [2,4,13] ve konakçı-parazit etkileşimi [7,14,15] ile ilgili önemli bilgiler sağlanmaktadır.

Doğada bazı enzimler bir, bazıları ise birden fazla polipeptid zincirine sahiptir. Birden fazla polipeptid zincirine sahip olan enzimlerde bu zincirler birbirlerine disülfid bağları veya kovalan olmayan bağlarla bağlıdırlar [16-18]. Bu kombinasyonların herbirine İZOENZİM adı verilir [17]. Her izoenzimin aminoasit kompozisyonu diğerlerinden az da olsa farklı olduğundan her izoenzimin belli bir pH'da taşıdığı net yükün özgüllüğü elektroforetik desende farklılık meydana getirir [8,16,18].

Bir parazitin enzimlerine bakılarak genetik yapısı, dolayısıyla suş veya türü hakkında bilgi edinilebilir, çünkü parazit enzim veya izoenzimlerdeki aminoasit kompozisyon farklılıkları, DNA yapısındaki değişiklikleri temsil eder [4,8].

T. annulata suşları arasındaki polimorfizmin gösterilmesinde Glukoz Fosfat İzomeraz biyokimyasal fenotipik belirteç olarak kullanılabilmektedir [19]. Bu çalışma ile *T. annulata* suşlarının tespiti, "iç karakterleri"ne bakılarak veya başka bir deyişle parazitlerin izoenzim elektroforezi (GPI izoenzim elektroforezi) aracılığı ile ortaya konulması amaçlanmıştır.

MATERYAL ve METOT

Araştırma materyalini *T. annulata* ile enfekte sığırlardan alınan kan örnekleri oluşturdu. Klinik olarak hastalık belirtileri gösteren hayvanlardan alınan kan örneklerinden sürme preparat hazırlandı ve preparatlar giemsa ile boyanarak mikroskopta incelendi. *T. annulata* ile enfekte olduğu tespit edilen kan örnekleri piroplazm izolasyonunda kullanıldı.

En az %10 düzeyinde parazitemili olduğu belirlenmiş sığırların kan örnekleri defibrine edildikten sonra +4°C'de 1500xg ve 20 dak. santrifüj edildi. Çöken hücreler saklanarak, süpernatant atıldı. Daha sonra hücreler 3 defa PBS pH 7.2 ile yıkanıp +4°C'de santrifüj edildi. En sonunda hücreler PBS ile sulandırılarak içinde yaklaşık 35 g. sellüloz tozu bulunan cam kolondan geçirildi. Bu safhada eritrositler kolondan kolaylıkla geçerken, beyaz kan hücrelerinin çoğu kolon tarafından alıkonmaktadır [8,18]. Kolonu terk eden kan santrifüj edildikten sonra (+4°C, 1.500xg., 15 dak.) süpernatant atıldı ve PBS ile tortudaki hücreler karıştırıldı. Bu süspansiyonun üzerine %0.83 NH_4Cl w/v ilave edildi. Bu işlem sonucunda sığır eritrosit membranlarının eriyerek parazitlerin açığa çıkması sağlandı. Santrifüj sonrası çökeltide yer alan parazitler üzerine %5 Triton X-100 ilave edildi. Karışım ardı ardına hızlı bir şekilde 3 defa -80°C'ye kadar dondurulup çözdürüldü. Bu şekilde parazit membranlarının iyice parçalanması ve böylece parazitteki protein, enzim, organel vb. maddelerin dışarı çıkması sağlandı. En son santrifüj işlemi ile (13.000-15.000xg, +4°C, 15 dak.) membran parçaları çöktürülerek enzimlerce zengin süpernatant elde edildi [8].

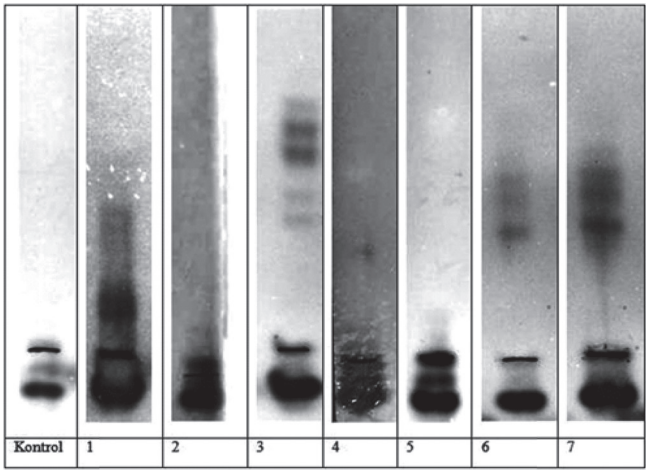
Enzim izoformlarının gösterilmesi için nişasta jel elektroforezi kullanıldı. Bu elektroforez tekniğinde adından anlaşılacağı gibi destek ortamı olarak, istenen pH'sı tamponla ayarlanmış nişasta jel kullandı. Elektroforez +4°C, 350 Voltta 3 saat sürdürüldü [18].

Elektroforezden sonra nişasta jel üzerinde birbirinden ayrılan enzim bantlarının gösterilebilmesi için enzim spesifik reaksiyona neden olan karışım ilave edildi. Enzimin kataliz ettiği reaksiyon, $NADP^+$ (nikotinamid adenin dinükleotid fosfat)' in redüklenmesini sağlayan başka bir reaksiyon ile birleştirildi.

Elektroforezden sonra, jelin ortasına bir çerçeve yerleştirildi ve bu çerçeve içerisine enzim reaksiyon karışımı dökülerek enzimin bulunduğu alanların mor renkte gözlenmesi sağlandı [8].

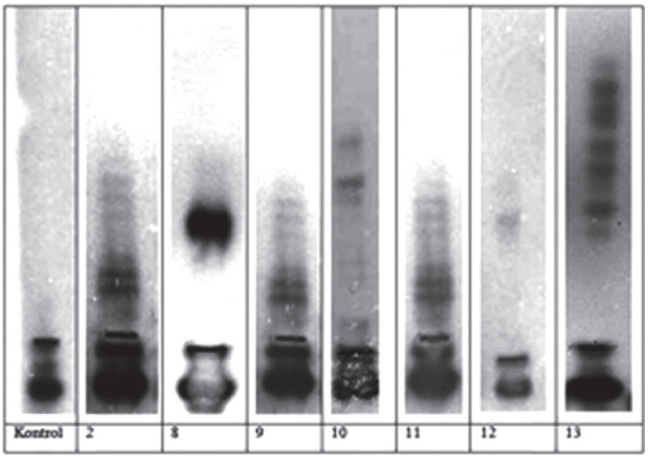
BULGULAR

T. annulata ile enfekte 13 sığırın eritrositlerinden izole edilen piroplazm lizatlarının glukoz fosfat izomeraz izoenzimlerinin nişasta jel elektroforezleri *Şekil 1* ve *2*'de görülmektedir. Kontrol örnekteki enzim bantları konakçıya aittir ve örnek uygulama yerine (orijine) yakın bulunmaktadır. Parazit izoenzim bantları ise anoda doğru hareket etmekte ve orijinden en az 1-2 cm uzaklıkta görülmektedir *(Şekil 1, 2)*. *Şekil 1*'de 1 ve 2 no'lu hayvanlar aynı ahırdaki hasta hayvanlardır. Parazitemi 1 no'lu hayvanda %70, 2 no'lu hayvanda ise %7' dir. *Şekil 1*'de 2 no'lu hayvandan elde edilen piroplazm lizatında bant gözlenmezken, *Şekil 2*'de daha yoğun örnek uygulaması sonrası izoenzim bantları görülmüştür. 4 ve 5 no'lu hayvanlardan elde edilen piroplazm lizatlarında ise bant gözlenmemiştir. 1 ve 6 no'lu örnekler aynı ilçenin farklı köylerinden alınmıştır, birbirine çok yakın olmakla birlikte farklı suşlar olduğu görülmektedir. Bununla birlikte farklı ilçelerden alınan örneklerde aynı sayıda benzer bantlar gözlenmiştir, *Şekil 1*'de 3 ve 6 no'lu örneklerde olduğu gibi. 8 no'lu örnekte ise tek bant görülmektedir (*Şekil 1* ve *2*).



Şekil 1. Farklı hayvanlara ait glukoz fosfat izomeraz izoenzimi elektroforez desenleri

Fig 1. Glucose phosphate isomerase isoenzyme electrophoretic patterns of different animals



Şekil 2. Farklı hayvanlara ait glukoz fosfat izomeraz izoenzimi elektroforez desenleri

Fig 2. Glucose phosphate isomerase isoenzyme electrophoretic patterns of different animals

TARTIŞMA ve SONUÇ

T. annulata enfeksiyonlarından iyileşen sığırlar premünizedir. *T. annulata*, *T. parva* ve *T. mutans* türleri arasında çapraz immunite görülmemektedir.

Kuzey Afrika ve İsrail'de virulansı düşük *T. annulata* suşları ile aşılar hazırlanmış ve oldukça başarılı olunmuştur. Virulansı olmayan veya daha az virulansa sahip *T. annulata* suşları ile aşılamada immunizasyon başarılı bir şekilde yapılamamaktadır, çünkü bu aşılar ile ancak kısmi premünisyon sağlanmakta ve yüksek virulanslı suşlara karşı koruyucu olmamaktadır. Bundan dolayı buzağı çağında aşılanan sığırlar, hayatı boyunca immun kalamamaktadır [1,2].

Hindistan'da yapılan bir araştırmada bazı *T. annulata* suşlarının immunolojik olarak farklı olabilecekleri öne sürülmüştür [20].

Piroplazma parazitlerinin sınıflandırılmasında enzim analizi, antijen analizi ve nükleoprotein analizinin önemli yeri vardır. *T. annulata* üzerinde yapılan bu tür analizler sınırlıdır. Çeşitli *T. parva* suşlarında (Muguga, Kiambu, Kilifi ve Marikebuni) GPI enzimine bakılmış ve her suşun 5 izoenzimi olduğu görülmüştür. Ancak, bu şekilde suşlar arası farklılıklar görülememiştir, çünkü elektroforez sırasında tüm proteinler aynı yere hareket etmektedir. *T. parva*'ya ait LDH izoenzimleri nişasta jel elektroforezi ile bulunamamıştır [6,14], ancak izoelektrik odaklama (isoelectric focussing) ile *T. parva* LDH'sı tespit edilmiştir [4,6,12,21,22].

Yapılan farklı araştırmalarda *T. annulata*'nın birçok enzimi incelenmiştir. Örneğin bir çalışmada LDH, MDH, G-6-PDH, 6-GPD, İzositrat Dehidrojenaz (ICD), Gliserildahid-3-fosfat dehidrojenaz (GALDH) ve GPI enzimleri *T. annulata* piroplasmalarında çalışılmış ve bunların içinden sadece GPI ve GALDH enzim aktiviteleri tespit edilebilmiştir [14].

Başka araştırmalarda ise *T. annulata*'ya ait GPI izoenzimleri nişasta jel elektroforezi ile gösterilmiş, hatta bu enzime ait elektroforetik motifin çeşitli suşlarda farklı olduğu ortaya konulmuştur [6,12,14,23]. İran, Türkiye ve Hindistan'dan izole edilen *T. annulata* suşları arasında GPI enzimleri açısından önemli farklılıklar olduğu görülmüştür. Bu şekilde suş tespitinin mümkün olduğu bildirilmiştir [6,12].

T. annulata'nın sporozoit safhasındaki enzimleri nişasta jel elektroforezi ile incelenmiş ve bunun sonucunda Ankara (Türkiye) ve Hissar (Hindistan) suşlarının GPI izoenzimlerinin farklılık gösterdikleri görülmüştür [23].

Yapılan çeşitli araştırmalarda, aynı *T. annulata* suşunun kene tükrük bezindeki şekli (sporozoit), sığır lenfositlerindeki şekli (şizont) ve sığır eritrositlerindeki şekli (piroplasm) arasında GPI izoenzimleri açısından az da olsa bazı farklılıklar görülmüştür. Bu farklılığın sebebi bilinmemektedir, ancak parazitin değişik hücrelerde adaptasyona uğradığı şeklinde yorumlanabilir [23].

Theileria sporozoitlerinde G-6-PD ve 6-PGD enzimlerinin düşük düzeyde oluşu ve ICD ve MDH enzimlerinin bulunmayışı, *T. annulata* sporozoitlerinde Pentoz fosfat yolunun işlemediğini gösterir. Elektron mikroskobu ile yapılan bir çalışmada Theileria sporozoitlerinde mitokondri görülememiştir [23]. Bazı araştırıcılar ise Theileria şizont ve merozoitlerinde kristalsiz mitokondriler görmüşlerdir [23].

T. annulata ve *T. parva*'da GPI enziminin çok yüksek oluşu glikolizis yolunun çok işlek olduğunu gösterir. Ancak diğer glikolizis enzimleri (örneğin Heksokinaz) düşük aktiviteye sahiptirler. Bu yüzden GPI'nin önemi henüz tam olarak bilinmemektedir [23].

T. annulata suşları arasındaki polimorfizmin tespitinde GPI biyokimyasal fenotipik markır olarak kullanılmaktadır. *T. annulata*'nın Ankara suşlarında üç alt-populasyonu temsil eden 3 izoenzim tipi bulunmaktadır [19].

Türkiye'de *T. annulata'*nın Eryaman, Hıdırlı, Ankara ve Gülseren suşlarının GPI izoenzimlerine bakılmış ve GPI izoenzim bantlarında önemli farklılıklar görülmüştür [4].

Şizont ve piroplazm formundaki *T. annulata'*nın İspanya izolatlarında izoenzim elektroforezi yapılmış ve *T. annulata'*nın İspanya izolatları arasında orta derecede polimorfizm görülmüştür [21]. Sudan'da izole edilen altı *T. annulata* suşlarının GPI izoenzim bantlarında büyük varyasyonlar gözlenmiştir. Aynı çalışmada *T. parva* suşlarının enzim bantları arasında hiçbir değişim görülmemiştir [6].

Üç farklı coğrafik bölgeden alınan *T. annulata'*nın beş suşunun GPI izoenzim elektroforezinde de farklı coğrafik bölgeler arasında büyük farklılıklar gözlenirken aynı coğrafik bölgelerde fark tespit edilmemiştir [12].

*T. annulata'*nın hücre kültürü pasajlarında 50. pasajda kısmi atenüasyon görülmekte, 130. pasajlamada ise avirulant hale gelmektedir. Klonlama ile GPI'ın 3 izotipi olduğu gösterilmiştir. Atenüasyonun görüldüğü 50. pasaj ile avirulant hale geldiği 130. pasajlarda sadece tek bir izotip görülmektedir [24].

T. parva piroplazmları içeren sığır kanından elde edilen lizatlarda 13 farklı enzime izoelektrofokusing ile bakılmış ve GPI hariç diğer enzimlerin bantları görülmemiştir. Parazitemiye bağlı olarak bir suşa ait 4'e kadar bant görülürken diğer bir suşta 3'e kadar bant görülmüştür [22].

Bu çalışmada nişasta jel elektroforez görüntülerindeki bant desenleri incelendiğinde parazit enzimlerinin anoda göç ederken konakcı enzimlerinin ise örnek uygulama yerinden uzaklaşmadığı görülmüştür.

Türkiye'de 3 alt-populasyonu temsil eden 3 GPI izoenzimi bulunmaktadır [19]. Bir sığırın, aynı anda birkaç parazit populasyonu ile enfekte olması mümkündür. Böylece üst üste 3, 6, 9 bant görülebilir. Bu sayıların haricinde görülen bant desenleri (4, 5, 7, 8 gibi), bantların birbiri üzerine düşmesi sonucu görülebilir.

*T. annulata'*daki polimorfizmin tesadüfen şekillenmediği ve parazitin sığıra ya da keneye transmisyonu sırasında birçok faktörden etkilendiği bildirilmektedir. Bu faktörler; enfekte olan hayvanla, parazitin kendisiyle veya paraziti taşıyan hücreler ile ilgili olabilir. Eğer tüm *T. annulata'*nın suşları aynı transmisyon kabiliyetinde ve/veya kenede aynı şekilde gelişme kabiliyetinde olsa idi, suşlar arasında polimorfizmin olmadığı ve tüm izolatların aynı olduğu görülürdü. *T. annulata'*daki polimorfizm genetik değişim ve rekombinasyonun görülmesi sonucu olduğu şeklinde açıklanmaktadır [13].

Eritrositlerde piroplazmların bulunuşu, hücre membranında poliunsature yağ asitlerinin peroksidasyonuna ve eritrositlerin oksidatif stresine neden olmaktadır [25-28]. Bu etkiler de polimorfizmin şekillenmesinde etkili olabilir.

Sonuç olarak *T. annulata*'nın çeşitli suşların tespitinde nişasta jel elektroforezi ile GPI izoenzimi farklarına bakmanın oldukça kolay ve çok güvenilir bir metod olabileceği ve bu sayede, suşların izoenzim patternleri sayesinde takip edilebileceği kanaatine varıldı.

Birçok suşa karşı etkinliği belirlemede glukoz fosfat izoenzim tekniğinden faydalanılabilir. Örneğin rekombinant aşı üretiminde, aşının birçok suşa karşı etkili olduğunu göstermek gerekir. Bunu da GPI izoenziminin farklılıkları aracılığı ile göstermek mümkündür.

Daha kesin sonuçlar için parazit suş tespitinde, izoenzim çalışmalarının yanında, bulguların diğer biyokimyasal (Blotting teknikleri gibi) ve immunolojik (IFAT gibi) tekniklerle desteklenmesinin faydalı olabileceği kanaatindeyiz.

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Kafkas Univ Vet Fak Derg
19 (2): 325-330, 2013
DOI: 10.9775/kvfd.2012.7822

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Salmonella Türlerinin Hızlı Tanısında Kromojenik Besiyeri İle Birlikte "Matrix-Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry" Yönteminin Phoenix Otomatize Sistemi ile Kıyaslanması

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Makale Kodu (Article Code): KVFD-2012-7822

Özet

İnsan ve hayvanlarda gıda ile bulaşan hastalıklara yol açan etkenlerin en başta gelenlerinden biri olan *Salmonella* identifikasyonu genellikle 2-3 günden fazla sürmektedir. Kütle spektrometrisi esasına dayanan bir yöntem olan MALDI-TOF ile rutin mikrobiyoloji laboratuvarlarında kullanılan otomatize sistemlere göre daha kısa sürede, güvenilir ve maliyet etkin sonuçlar elde edilebilmektedir. Bu çalışmanın amacı, "Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF) yönteminin Phoenix otomatize identifikasyon sistemi ile karşılaştırmasını yaparak bu patojen organizmaların yol açtığı gastrointestinal enfeksiyonlarda kısa sürede sonuç veren, pratik, maliyet etkin ve güvenilir bir yöntem olduğunun değerlendirilmesidir. Ağustos 2007 ve Ağustos 2011 tarihleri arasında toplanan 11186 hasta örneği çalışmaya dahil edilmiştir. Hasta örnekleri rutin olarak CHROMagar *Salmonella* plus ve Mac Conkey agar besiyerlerine ekilmişlerdir. 347 *Salmonella* türü hem Phoenix otomatize sisteminde hem de MALDI-TOF cihazında çalışılmıştır. 2 yöntemin sonuçları birbiri ile %100 uyumludur. Her iki cihazda da tür düzeyinde tanımlama yapılabilmekte, alttür tanımlamaları için konvansiyonel olarak *Salmonella* antiserumları ile serolojik tiplendirmeye gereksinim duyulmaktadır. CHROMagar *Salmonella* plus besiyerinin MALDI-TOF ile birlikte kullanımı identifikasyon için gereken zamanı azaltmış, *Salmonella* türlerinin kolaylıkla isimlendirilmelerini sağlamıştır. MALDI-TOF'un rutin klinik mikrobiyoloji laboratuvarında kullanımı *Salmonella* türlerinin tek bir besiyeri ile ve 48 saat içerisinde antibiyotik duyarlılıkları ile birlikte maliyet-etkin ve güvenilir bir şekilde saptanabilmesini sağlamaktadır.

Anahtar sözcükler: *Salmonella, Kültür ortamları, Matriks yardımlı-lazer salmalı-iyonizasyon kütle spektrometrisi*

Comparison of "Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry" Plus Chromogenic Media with Phoenix Automated System in the Rapid Diagnosis of *Salmonella* Species

Summary

The identification of *Salmonella* which is a major food-borne pathogen generally takes more than 2-3 days. More reliable, rapid and cost effective results can be achieved with MALDI-TOF which is based on mass spectrometry principles in comparison with the automated systems in routine microbiology laboratories. The aim of this study is to evaluate the use of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF) in gastrointestinal infections caused by those pathogen microorganisms by using practical cost-effective and reliable methods within a less time period. Between August 2007 and August 2011, 11186 stool specimens of patients were included to the study. Patient samples were routinely inoculated onto CHROMagar *Salmonella* plus and Mac Conkey agar media. 347 *Salmonella* species were run with both Phoenix automated system and MALDI-TOF. The results of both of the methods are 100% concordant with each other. Using Chromogar *Salmonella* plus media together with MALDI-TOF identification shortened the time, helped to detect *Salmonella* species easily. Species identifications are being performed in both of the systems, but identification of subspecies still require conventionally serological typing with *Salmonella* antiserums. MALDI-TOF will help the routine clinical microbiology laboratory to detect *Salmonella* rapidly with only one media and in 48 h together with their antibiotic susceptibility testings in a cost-effective and reliable manner.

Keywords: *Salmonella, Culture media, Matrix assisted laser desorption ionization mass spectrometry*

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GİRİŞ

Salmonella, insan ve hayvanlarda gıda ile bulaşan hastalıklara yol açan ve tüm dünyada sporadik olgulara ve gastroenterit salgınlarına neden olan mikroorganizmaların başında gelmektedir. Her yıl 16 milyar olgu görülmekte ve bunların 600.000'i ölümle sonuçlanmaktadır. *Salmonella* türleri gram-negatif fakültatif hücre içi anaerop bakteriler olup çok geniş bir hastalık spektrumuna sahip bakterilerdir. Bu spektrum gastroenteritten, enterik ateşe, bakteriyemiye, fokal enfeksiyonlara, konvalesan yaşam boyu taşıyıcı konumuna dek değişebilmektedir. Enfeksiyonun türü *Salmonella*'nın serotipine ve konak faktörlerine göre değişiklik göstermektedir. *Salmonella* enfeksiyonlarının yüzde doksan beşi gıda kökenlidir [1]. *Salmonella* türleri birçok hayvanın gastrointestinal sisteminde bulunmaktadır. Bu nedenle hayvanlarla ya da hayvan kaynaklı gıdalarla temas sıklıkla insanlarda salmonellozise yol açmaktadır. *Salmonella* enfeksiyonlarının önlenmesi kümeslerde ve endüstriyel üretim alanlarında iyi bir örnekleme planı ile birlikte hızlı, duyarlı ve güvenilir izleme ve tarama programları ile elde edilebilir [2]. Bir kez enfeksiyon geliştikten sonra salmonellozis belirgin bir morbidite ve mortaliteye yol açar. Tedavi edilmeyen hastaların üçte birinde komplikasyonlar gelişir ve bu olgular salmonellozise bağlı ölümlerin dörtte üçünden sorumludurlar. Tüm dünyada 2.500 serotip tanımlanmıştır. İnsanlar en fazla *Salmonella enterica, typhi ve choleraesuis* alttürleri ile enfekte olmaktadırlar [3]. *Salmonella* türleri çevrede çok fazla miktarlarda bulunmakta ve belli bazı konak faktörleri insanları enfeksiyona karşı duyarlı kılmaktadır. Tüm dünyada prevalansı, virülansı ve çevreye uyum sağlayabilirliği ile birlikte antibiyotiklere karşı direnci de artış göstermektedir [4].

Klinik örneklerden koliform gram negatif bakterilerin ve diğer enterik patojenlerin izolasyonu ve ayırdedici tanısında en fazla kullanılan besiyerlerinin başında Mac Conkey ve Drigalski agar gelmektedir. Ancak bu besiyerlerinin *Salmonella* açısından ayırdedici olma özelliği pek fazla yoktur, bunun nedeni yalnızca laktoz kullanımının saptanmasına bağlı olmasıdır [5]. *Salmonella* da dahil patojenik mikroorganizmaların hızlı ve güvenilir identifikasyonları gıda kökenli hastalıkların sürveyans, korunma ve kontrolunda çok önem kazanmaktadır. Klinik mikrobiyoloji laboratuvarında bakterilerin identifikasyonunda kullanılan yöntemler sıklıkla zahmetli ve zaman kaybettirici olabilmektedir. Bakterinin saflaştırılabilmesi için zaman gerekmekte, patojenik bakterilerin biyokimyasal tiplendirilme prosedürleri için de uzun inkübasyon sürelerine gereksinim duyulmakta ve bu nedenle de bakterinin isimlendirilmesinde gecikmeler yaşanmaktadır. Uygulanan işlemlerin sonuçlarının değerlendirilmesi de deneyim gerektirmekte, böylelikle bu yöntemler sübjektiflik ve kullanılan yöntemin özgüllüğünün düşük olması ile sınırlanmaktadır. Mikroorganizmaların hızlı ve güvenilir bir şekilde tanımlarına olan gereksinim gittikçe artmaktadır [4]. *Salmonella* taramasında kullanılmak üzere *Salmonella*'ya özgü enzimleri saptayabilecek kromojenik substratlar içeren besiyerleri geliştirilmiştir [6]. Bu besiyerleri ile yapılan çalışmalarda diğer konvansiyonel besiyerleri ile kıyaslandığında doğrulama için gereken biyokimyasal ve serolojik testlerle birlikte gereken sürenin yaklaşık olarak 24 saat kısaldığı gösterilmiştir [7]. Bu besiyerleri kullanılarak *Salmonella* türlerinin tanısında yüksek oranda duyarlılık ve özgüllük sağlandığı, ayrıca tanının konması için gereken sürenin azalmasının yanısıra yapılan maliyet analizinde de kullanılan besiyerleri ve teknisyen zamanı hesaplandığında maliyetin de %26 azaldığı gösterilmiştir [8].

Biz de laboratuvarımızda rutin olarak dışkı kültürlerinin incelenmesi sırasında *Salmonella* CHROMagar plus (CHROMagar Microbiology, Fransa) besiyerinden yukarıda belirtilen tüm nitelikleri nedeniyle yaklaşık 5 yıldır yararlanmaktayız.

Son yıllarda, "Matrix-Assisted Laser Desorption Ionization (MALDI)—Time of Flight (TOF) mass spectrometry (MS) yöntemi ile mikroorganizmaların tanımlanmaları uygunluğu açısından gittikçe daha fazla uygulanan bir yöntem olmaya başlamıştır [9,10]. MALDI-TOF MS yönteminde tipik "parmak izi" görüntüsü tüm hücre kullanımı ile gerçekleşmekte ve önceden biyobelirteç ile ayırım, parçalama, farklı şekillerde ayırma ya da temizlik gerektirmeksizin elde edilebilmektedir. Yöntem çok hızlı sonuç vermesi, çok az biyolojik malzemeye gereksinim göstermesi (alınan koloni miktarı) ve çok fazla örneği bulunan rutin laboratuvarlarda kullanıma uygun olması nedeniyle klinik mikrobiyoloji ve gıda ve laboratuvarları uygulamaları için büyük bir potansiyele sahiptir. Gözlenen protein biyobelirteçleri tipik olarak incelenen mikroorganizmaya özgü proteinlere aittir, bunlar arasında ribozomal ya da nükleik asit bağlayan proteinler bulunmaktadır [11].

Çalışmamızda *Salmonella* türlerinin saptanmasında birçok laboratuvarda kullanılan otomatize sistemlerden biri olan Phoenix (Becton Dickinson, New Jersey, ABD) ile MALDI-TOF MS (Bruker Daltonics, Bremen, Almanya)"yönteminin kıyaslanarak rutin laboratuvar uygulamalarında hız, uygulama kolaylığı, doğru saptama ve maliyet-etkinlik açısından yararlılığının değerlendirilmesini amaçladık. Ayrıca birlikte kullandığımız *Salmonella* Plus CHROMagar besiyeri ile de klasik besiyerlerine göre yaklaşık olarak 24 saat gibi bir süre kazanımını hedefledik.

MATERYAL ve METOT

Materyal

Ağustos 2007 ve Ağustos 2011 tarihleri arasında kurumumuza bağlı 16 hastaneden karın ağrısı, bulantı, kusma ve diyare bulguları ve genel olarak orta şiddette rahatsızlıkları bulunan 11186 hastadan toplanan dışkı örnekleri çalışmaya dahil edilmiştir. Çalışmada iki besiyeri kullanılmıştır. Bunlardan bir tanesi dışkıda *Salmonella* ve *Shigella* varlığını araştırmada rutin olarak kullanılan klasik besiyerlerinden bir tanesi olan Mac Conkey agardır. Bu besiyerinde laktoz negatif koloniler şeffaf olarak görülmekte ve değerlendirmeye alınarak ileri işlemler uygulanmaktadır. Bir diğeri ise son yıllarda birçok çalışma yapılarak güvenilirliği kanıtlanmış kromojenik

bir besiyeri olan CHROMagar *Salmonella* plus besiyeridir. Besiyerinde üremiş pembe koloniler *Salmonella* şüphesi ile ileri işleme alınmaktadır. Bu besiyeri *Salmonella* üretebilmek için kullanılmaktadır, klasik olarak dışkı kültüründe araştırılması gereken *Shigella* türlerini üretememektedir. *Shigella* üretebilmek amacıyla yukarıda belirtilen Mac Conkey besiyeri kullanılmıştır. Bu çalışmada örneklerle birlikte referans suş *Salmonella Enterica subsp. enterica* ATCC 13076 da aynı şekilde tüm işlemlerden geçirilmiştir.

Metot

Çalışma iki kısımda gerçekleştirilmiştir: Bunlardan birincisinde örnekler 2007-2011 yılları arasında *Salmonella* CHROMagar plus ile birlikte Mac Conkey agar besiyerleri ve Phoenix otomatize cihazı ile çalışılmıştır. Bu örneklerden izole edilmiş ve *Salmonella* olarak tanımlanmış mikroorganizmalar -80°C'de saklanmıştır. 2011 yılında daha önce *Salmonella* olarak tanımlanmış ve saklanmış olan bu suşlar tekrar *Salmonella* CHROMagar plus ve Mac Conkey besiyerlerine ekilerek canlandırılmış ve MALDI-TOF yöntemi ile tekrar çalışmaya alınarak Phoenix otomatize sistemi ile MALDI-TOF yöntemi karşılaştırılmıştır. Çalışma için Acıbadem Üniversitesi Tıbbi Araştırmaları Değerlendirme Komisyonundan 04.01.2010 tarihinde 2010/132 karar numarası ile Etik Kurul onayı alınmıştır.

I. Phoenix Otomatize Sistemi ile Çalışma

Çalışmanın birinci kısmında *Salmonella* türleri *Salmonella* CHROMagar plus ile birlikte Mac Conkey agara ekim yapılarak, *Salmonella* CHROMagar plusda pembe koloniler ve Mac Conkey agarda laktoz negatif şeffaf koloniler değerlendirilerek Phoenix otomatize cihazında NMIC paneli (Becton Dickinson, New Jersey, ABD) kullanılarak çalışılmıştır. *Salmonella* olarak tanımlanan ve biyokimyasal reaksiyonlar ile doğrulanan mikroorganizmaların antibiyotik duyarlılıkları da yine Phoenix otomatize sistemine göre Clinical Laboratory Standards Institude(CLSI)'a uygun olarak rapor edilmiştir [12].

II. Biyokimyasal Reaksiyonlar ile Doğrulama

Salmonella şüpheli olan bu koloniler aynı zamanda triple sugar iron (TSI) agar ve üreaz besiyerine de ekilmişlerdir. 24 saat inkübasyon sonrasında TSI'de dip kısmının sarı olması glikozun kullanımı; siyah olması hidrojen sülfür oluşumu; yüzeyin kırmızı olması laktoz ve sakkarozun kullanılmaması; besiyerinde gaz delikleri ve yarıklarının olması veya dip kısmından sonra yukarı doğru itilmesi glikozdan gaz oluşması *Salmonella*'yı doğrulamıştır. *Salmonella* analizinde tipik reaksiyonlardan birisi olan "hidrojen sülfür (H_2S) oluşumu" reaksiyonu *Salmonella*'da pozitif, *Escherichia coli*'de negatiftir. Shigella besiyerinin eğri yüzeyinde alkali, dip kısmında asit reaksiyon oluşturur. Tüpün dip kısmında değişiklik yapmaz, H_2S ve gaz üretmez. *E. coli* ise tüpün eğri yüzeyinde ve dip kısmında asit reaksiyona neden olur. H_2S negatif ve gaz pozitiftir. TSI agarda *Proteus Salmonella*'ya benzer reaksiyon verdiği için kesin ayırım için üreaz besiyerine de ekilmişlerdir. Üreaz testi negatif olanlar *Salmonella*, pozitif olanlar ise *Proteus* olarak kabul edilmişlerdir. Saptanan *Salmonella*'ların alttür tanımlamaları ise antiserumlar (Denka-Seiken, Tokio, Japan) yardımı ile Kaufmann-White şemasına göre yapılmıştır [13].

Çalışma sonucunda CHROMagar *Salmonella* besiyerinin duyarlılığının %100, özgüllüğünün ise %85 olduğu saptanmıştır. Mac Conkey besiyerinin duyarlılığı ise %70 ve özgüllüğü de %60 olarak saptanmıştır. CHROMagar *Salmonella* besiyeri ile yalancı pozitif olarak saptanan suşlar hem Phoenix otomatize sistemi hem de MALDI-TOF ile çalışılmış ve *Salmonella* olmadıkları gösterilmiştir. Mac Conkey besiyerinde şeffaf koloni oluşturan, ancak *Salmonella* olarak tanımlanmayan koloniler ise *Shigella* varlığı açısından araştırılmış, TSI ve üre besiyerlerindeki görüntüleri incelenmiş ve *Shigella* antiserumları (BD Difco Shigella Antisera, Sparks, ABD) ile tiplendirmeleri yapılmıştır.

III. MALDI-TOF Yöntemi ile Çalışma

Çalışmanın ikinci kısmında daha önce yukarıda anlatıldığı gibi çalışıldıktan sonra -80°C'de dondurularak saklanan bu suşlar tekrar canlandırılarak MALDI-TOF ile incelenmişlerdir.

MALDI-TOF MS yöntemi rutin laboratuvarlarda kullanılan biyokimyasal reaksiyonların incelenmesi esasına dayanan otomatize sistemlerden farklı olarak çalışmaktadır. Otomatize sistemlerde mikroorganizmaya ait çeşitli biyokimyasal reaksiyonlar değerlendirilerek tanımlama yapılmaktadır. Oysa ki bir kütle spektrometrisi yöntemi olan MALDI-TOF yönteminde mikroorganizmaya ait protein profili incelenmektedir. Bu sistem çalışması sırasında kendisine ait kalibrasyon kontrolü (myoglobin), pozitif kontroller: bir gram negatif (örn. *E.coli*), bir gram pozitif (örn. *S. aureus*) referans suş ve bir de negatif kontrol (örn.distile su) kullanılmaktadır.

MALDI-TOF Çalışma Prensibi: MALDI-TOF MS yönteminde *Salmonella* şüpheli koloniden alınan az bir miktar "target" adı verilen cihaza ait metal bir tepsi üzerinde belirlenmiş alanlara yerleştirilip kuruması beklenir. 1'er µL matriks daha sonra örnekler üzerine eklenir, tekrar havada kurutmaya bırakılır. Matriks solüsyonu içerisinde %50 "acetonitrile" ve %2.5 "trifluoroacetic acid" içinde doymuş "α-cyano-4-hydroxycinnamic acid" bulunmaktadır. Örnekler hazırlandıktan sonra target olarak isimlendirilen plak "Bruker Autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany)" cihazına yüklenir ve işlem başlatılır. 4.000-10.000 Da kütle aralığında 20 Hz'lik bir lazer vuruş sıklığı ile lineer pozitif iyon modeli olarak elde edilen spektrum otomatik olarak kaydedilir. Her bir spektrum için örnek alanına yapılan 100 kısa aşamada 600 uygun vuruş elde edilir. Kullanılan programa özel ayarlanmış lazer atışları yardımı ile matriks ışığı emer ve örneğin molekülleri iyonize hale geçip cihazın tüpü içerisinde molekül ağırlığına göre uçmaya başlar. Dijital hale çevrilen veriler TOF(time of flight- uçuş zamanı) oluşturmak üzere biriktirilir ve cihaz içerisinde bulunan bir detektör yardımı ile saptanır. Çalışmamızda "Autoflex II Smartbeam MALDI-TOf MS (Bruker Daltonics, Bremen, Almanya)" kullanılmıştır. Mikroorganizmalara ait protein moleküllerinin pik-

leri "Biotyper" (Bruker Daltonics, Bremen, Almanya) ile tanımlanmıştır. Skor değerleri güvenli okuma sınırı olan 2.00'nin üzerinde olan sonuçlar doğru olarak kabul edilmişlerdir. Bu skor değerine ulaşmamış sonuçlar daha hassas bir sonuç elde etmek amacıyla formik asit asetonitril ekstraksiyonu yapılarak tekrar çalışmaya alınmışlar ve 2.00'nin üzerinde bir skor değeri elde edilmiştir. Kütle spektrometrisi "FlexAnalysis software version 3.0" (Bruker Daltonik, Bremen, Almanya) programı ile değerlendirilmiştir. Elde edilen verilerin analizi "MALDI Biotyper software version 3.0" (Bruker Daltonik GmbH, Almanya) ile analiz edilerek çalışılan mikroorganizmalar isimlendirilmişlerdir.

BULGULAR

Salmonella CHROMagar Plus besiyerinde saptanan pembe/leylak rengi koloniler şüpheli olarak kabul edilip araştırılmıştır. Bununla birlikte kullanılan Mac Conkey agar besiyerinde de şeffaf koloni varlığı gözlenerek laktoz negatif bakteri varlığı araştırılmış, rutin dışkı kültürlerinde *Salmonella* ile birlikte mutlaka araştırılması gereken *Shigella* da araştırılmıştır.

37°C'de 24 saatlik inkübasyon sonrasında *Salmonella* CHROMagar Plus besiyerinde pembe/leylak rengi *Salmonella* kolonileri en iyi şekilde gözlenebilmiştir. Çalışmada kullanılan referans suş *Salmonella enterica subsp. enterica* ATCC 13076 da diğer *Salmonella* türleri gibi *Salmonella* CHROMagar plus besiyerinde pembe koloniler oluşturmuştur. 4 yıllık bir süre içerisinde 347 *Salmonella* türü saptanmıştır. Saptanan 347 *Salmonella* türü 7 farklı serovardan oluşmaktadır. *Salmonella* türlerinin antiserumlar ile saptanan alttürleri ve sayıları *Tablo 1*'de belirtildiği gibidir.

Her bir *Salmonella* için MALDI-TOF MS'de tipik olarak 2.000 ve 25.000 Da arasında ve genellikle 300 pikten daha fazla pik saptanmıştır. Bunlar arasında *Salmonella*'ya özgü pikler de bulunmaktadır. Bu nedenle de MALDI-TOF cihazıyla

Tablo 1. *Çalışmada incelenen Salmonella türleri ve alt türleri*
Table 1. *Salmonella species and subspecies in the study*

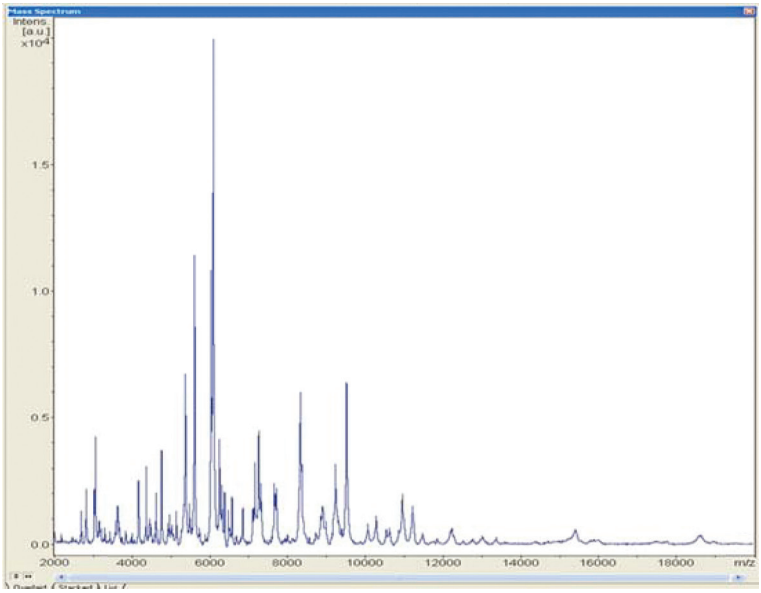
Mikroorganizma	**Sayı/(%)**
Salmonella(S) enteriditis	190/(54.8)
S. gallinarum/pullorum	144/(41.5)
S. paratyphi B	5/(1.4)
S. typhimurium	4/(1.2)
S. paratyphi A	2/(0.6)
S. typhi	1/(0.3)
S. paratyphi C	1/(0.3)

birlikte kullanılan ve içerisinde *Salmonella* türlerinin de bulunduğu bir veritabanı içeren ve mikroorganizmalara özgü piklere dayanarak mikroorganizma tiplendirmesi yapan "Biotyper" ile isimlendirilebilmişlerdir. *Salmonella* türlerinin MALDI-TOF MS'de "parmak izi" görünümü *Şekil 1*'de verilmiştir. Çalışma sırasında hiç Shigella saptanmamıştır.

TARTIŞMA ve SONUÇ

Yaptığımız çalışmanın sonuçlarına göre Phoenix otomatize sistemi ile *Salmonella* spp. Olarak tanımlanan tüm suşlar MALDI-TOF'da da güvenli okuma sınırı olan 2'nin üzerinde skorlar ile *Salmonella* olarak tanımlanmışlardır.

MALDI-TOF'un başarısı Phoenix otomatize cihazı ile *Salmonella* olarak tanımlanan, biyokimyasal reaksiyonları ile kontrol edilen ve antiserumlar ile alttürleri saptanan suşlarda tür saptamada %100 iken alttür saptama açısından %54.8'de kalmıştır. Alttürlerde antiserumlarla yapılan tiplendirme esas alınmıştır. Buna göre *Salmonella* türlerinden MALDI-TOF ile ancak *Salmonella enteritidis* alttür düzeyinde tanımlanabilmiş, diğer alttürlerin tanımı yapılamamıştır. Phoenix otomatize sistemi ile alttür hiçbir şekilde saptanamaz iken avantaj olarak MALDI-TOF ile en azından klinik örneklerden



Şekil 1. *Salmonella* türlerinin MALDI-TOF MS'de "parmak izi" görünümü

Figure 1. "Fingerprinting" image of *Salmonella* species at MALDI-TOF MS

en sık izole edilen *Salmonella enteritidis* alttürü saptanabilmektedir. Süre açısından sonuçlarımız incelendiğinde CHROMagar *Salmonella* besiyerinin kullanılması ile saflaştırma işlemine gerek kalmadığı için en azından 24 saat buradan kazanılmaktadır. Çalışmanın tür saptama için gereken süresi Phoenix otomatize sisteminde yaklaşık 18-24 saat, MALDI-TOF sisteminde ise yaklaşık olarak 20 dak. civarındadır. Yani MALDI-TOF ile otomatize sisteme göre çok daha çabuk sonuç alınmakta ve yaklaşık olarak 24 saatlik bir kazanç da buradan elde edilmektedir. Bir başka dikkat çekici nokta ise de, MALDI-TOF yöntemi Phoenix otomatize sisteminin %10'u kadar bir maliyete sahip olmasıdır.

MALDI-TOF MS yöntemi ile mikroorganizmaların tanımlanmaları son yıllarda gittikçe daha fazla tercih edilen bir yöntem olmuştur. Bu yöntem ile örneğin tüm bakteri hücrelerinden protein profilleri çıkarılabilmektedir. Bu profillerin burada kullanılan "Biotyper" programı gibi referans bir spektra ile karşılaştırılmaları sonucu ile bakteriler kolaylıkla tanınabilmektedirler [14]. Son yıllarda bu yöntem ile *Escherichia coli* ve *Enterobacteriaceae* ailesinin diğer üyeleri gibi gram negatif basiller, *Staphylococcus aureus* ve streptokoklar gibi gram-pozitif koklar; *Bacillus cereus* ve *Listeria* türleri gibi bazı gram-pozitif basiller üzerinde çalışılarak farklı bakteri türlerinin tanımlama nitelikleri üzerinde çalışılmıştır. 2009 yılında da Seng ve arkadaşları tarafından klinik örneklerden izole edilen bakteriler üzerinde yapılmış ilk çalışma yayınlanmıştır [15-21]. Günümüzde rutin mikrobiyoloji laboratuvarlarına giren bu yöntem yapılan araştırma çalışmaları ile de gittikçe daha fazla yarar sağlamaktadır. Hızlı tanı konabilmesinin yanı sıra maliyet açısından da avantaja sahiptir. Kullanılan malzemeler otomatize sistemlerin sarf malzemelerine göre yaklaşık olarak 10 kat daha ekonomiktir. Gastroenterit etkenleri arasında önemli bir yere sahip olan *Salmonella* türlerinin hızlı tanısında MALDI-TOF MS kullanımı yarar sağlayan bir yöntemdir. Bu konuda yapılmış bazı çalışmalar bulunmaktadır. Sparbier ve arkadaşları yaptıkları çalışmada öncelikle Selenit F besiyerine ardından Hektoen enterik agara ekim ile birlikte burada birinci günde üreyen *Salmonella* şüpheli bakterileri MALDI-TOF MS yöntemi ile tanımlamışlardır. 4847 örnek üzerinde çalışmış ve 108 örnekte *Salmonella* tanımlamışlardır [22].

Biz çalışmamızda klasik besiyerleri ile daha önce karşılaştırma çalışmaları yapılmış, güvenilirliğini kanıtlamış bir kromojenik besiyeri olan CHROMagar *Salmonella* plus besiyerini kullanmayı tercih ettik [23-26]. *Salmonella* türü bakterilerin bu besiyerinde yaptıkları tipik koloni görünümü ve rengi (*S. Typhi, S. Paratyphi A* ve laktoz pozitif *Salmonella* bu besiyerinde pembe/leylak rengi olarak görülür*)* nedeni ile ayrıca zaman kaybettirici saflaştırma işlemlerine gerek kalmamaktadır [6]. Klasik olarak kullanılan konvansiyonel besiyerlerinde şüpheli olarak görülen kolonilerin saflaştırma işlemine alınması ve sonuçlarının değerlendirilmesi için yaklaşık olarak 1-2 güne gereksinim vardır. Bizim de çalışmamızda, CHROMagar *Salmonella* plus besiyerinde kolaylıkla saptanan bakteriler, saflaştırma işlemine gerek duyulmaksızın tür tayini için kullanılabilmişlerdir. Mac Conkey besiyerinde laktoz negatif koloni oluşturan suşların saflaştırma için işleme alınmaları ve isimlendirilmeleri için geçen sürenin CHROMagar *Salmonella* plus besiyerinde üremiş *Salmonella* şüpheli kolonilerin tür tayini yapılmasına kıyasla 24-48 saat daha fazla olduğu ve bu durumun daha geç raporlamaya yol açabileceği gözlenmiştir. *Salmonella* türlerinde MALDI-TOF MS ile tür saptanması son derece başarılı iken tüm alttürlerin tanısında henüz çok güvenli değildir. Bu konuda çalışmalar halen sürmektedir. Bizim de çalışmamızda benzer sonuçlar elde edilmiştir. MALDI-TOF ile tür düzeyinde %100 başarılı sonuç elde edilmesine karşın alttür düzeyinde yalnızca *Salmonella enteritidis*' de başarı sağlanmıştır. Diğerlerinin okumaları güvenli okuma sınırının altında altında olduğu ve antiserumlarla elde edilen alttür sonuçlarıyla da uyum göstermediği için başarılı okuma olarak kabul edilmemişlerdir. Dieckman ve ark. en çok rastlanan alttürlerden olan *S. enterica subsp. enterica* izolatlarının bu yöntemle güvenli bir şekilde tanımlanabileceğini göstermiş ve böylelikle antiserum kullanımına daha az başvurulabileceğini savunmuşlardır [26]. Bu çalışmada da en fazla saptanan *S. enteritidis, Salmonella enterica* grubu içerisindedir ve saptanan Salmonella'ların %55'lik bir kısmını oluşturmaktadır. 2012 yılında yapılan yeni bir çalışmada ise yine MALDI-TOF kullanılarak *S.Typhi*'nin ayırımının tüm diğer *Salmonella* serotiplerinden oluşturulan bir "ortalama delta skoru" ile yapılabileceği gösterilmiştir. Bu çalışmada enfeksiyonlara yol açan *Salmonella enterica* alttürlerinin en önemli grubunun MALDI-TOF ile saptanabildiği belirtilmektedir [27]. Bu konuda çok daha fazla çalışma yapılmasına gereksinim olmakla birlikte bu çalışmaların sevindirici olan bir yönü en fazla hastalık etkeni olan *Salmonella* türlerini saptayabilmeleri ve dolayısı ile daha hızlı *Salmonella* tanımlanmasına izin vermeleridir. Ülkemizde de *Salmonella* türlerinin gastroenterit salgınlarından çoklu ilaç dirençli hastane enfeksiyonlarına dek uzanan geniş bir yelpazede enfeksiyonlara yol açabildikleri gösterilmiştir [28,29]. Güvenilir ve daha hızlı yöntemler kullanılması erken tanı konmasını, erken tedaviye başlanmasını ve olası salgınların kontrol altına alınmasını sağlayacaktır.

Sonuç olarak, *Salmonella*'ların hızlı tanısında maliyet etkin ve güvenilir bir yöntem olarak CHROMagar *Salmonella* plus besiyeri ile birlikte MALDI-TOF MS yönteminin kullanılmasının önerilebilir. Klasik yöntemlere, besiyerlerine ve Phoenix otomatize sistemine göre ortalama olarak en az 24-48 saat erken sonuç alınabilecek bu yöntem ile ilk 24 saat içerisinde *Salmonella* tür tayini yapılabilir, antiserum kullanımı ile de alttür tanımı verilebilir. Antibiyotik duyarlılık testi de çalışılarak hastaya hastaneye başvurduktan sonra 48 saat içerisinde hızlı, güvenilir ve maliyet etkin bir şekilde raporlama yapılabilmesi sağlanabilir.

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Kafkas Univ Vet Fak Derg
19 (2): 331-335, 2013
DOI: 10.9775/kvfd.2012.7842

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Transglutaminaz Enziminin Tavuk Köftesinin Kalite Özelliklerine Etkisi

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Makale Kodu (Article Code): KVFD-2012-7842

Özet

Transglutaminaz enzimi katılarak işlenmiş gıdaların, başta tekstür olmak üzere çeşitli kalite özelliklerinde gelişmeler olduğu farklı kaynaklarda belirtilmektedir. Bu çalışmada, tavuk göğüs etinden köfte üretimi yapılmıştır. Yapılan köfteler biri kontrol olmak üzere üç gruba ayrılmıştır. 1. grup kontrol grubu olup, enzim kullanılmamıştır. 2. grup köftelerde %0.5, 3. grup köftelerde ise %1 enzim ilavesi yapılmıştır. Bu köftelerin fiziksel, kimyasal ve duyusal özelliklerinin belirlenmesi amacıyla analizler yürütülmüştür. Bulgulara göre, enzim ilave edilen ve edilmeyen köftelerde kül, yağ, protein miktarı, renk özellikleri (L, a, b değerleri) ve duyusal açıdan farklılık olmadığı ($P>0.05$); %1 enzim ilavesi yapılan köftelerde, diğer köftelere göre kurumadde miktarının yükseldiği ($P<0.05$) ve enzim katkılı köftelerde kontrol grubuna göre pH değerinde düşme olduğu ($P<0.05$) saptanmıştır. Bununla birlikte enzim katkısının köftelerde pişirme kaybını azalttığı ($P<0.05$) tespit edilmiştir. Ayrıca, köftelerde yapılan tekstür analizi sonuçlarına göre, enzim katkısının köftelerin tekstürünü önemli ölçüde arttırdığı ($P<0.01$) gözlenmiştir.

Anahtar sözcükler: *Transglutaminaz, Tavuk köftesi, Kalite*

Effect of Transglutaminase on the Quality Properties of Chicken Breast Patties

Summary

Transglutaminase enzyme is known to improve the textural and several other quality properties of food. In this study, patties were produced from chicken breast and dividend into three groups. The first group was not treated by transglutaminase and called as control samples. The second and third group patty samples were treated by 0.5% and 1% transglutaminase, respestively. Physical, chemical and sensory properties of the control and transglutaminase treated patty samples were studied.The transglutaminase treatment did not significantly affect the ash, lipid, protein content, colour properties (L, a, b values) and sensory properties of chicken breast patties compared to the control samples ($P>0.05$). However, dry matter content of the 1% enzyme treated samples was significantly ($P<0.05$) higher than the control and 0.5% transglutaminase treated samples. On the other hand, the pH of enzyme treated patties was found to be lower than the control samples. In addition, the transglutaminase treatments were significantly ($P<0.05$) effective in reducing the cooking loss and significantly improved the textural properties of the chicken breast patties ($P<0.01$).

Keywords: *Transglutaminase, Chicken breast patty, Quality*

GİRİŞ

Kanatlı hayvanların gerek hayvancılık gerekse beslenme açısından dünyadaki önemi gün geçtikçe artmaktadır. Ayrıca bağ dokudan fakir oldukları için çiğnenebilirlik ve sindirilebilirlik özellikleri, kasaplık hayvan etlerine oranla daha fazla tercih edilmelerine neden olmaktadır. Kanatlı etlerinin bir taraftan proteinden zengin ve az yağlı oluşu, diğer taraftan ucuz olması tüketim talebinin artmasında önemli rol oynamaktadır [1].

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Son zamanlarda gıda maddeleri üretiminde gıda işleme yardımcı maddelerinin kullanılması, vazgeçilmez uygulamalar arasında yer almaktadır. Çeşitli ürünlere belirli işleme yardımcı maddeleri ilave edilerek, ürünün fiziksel, kimyasal ve duyusal özellikleri istenilen yönde değiştirilebilmektedir.

Gıdaların özelliklerinin geliştirilmesinde içerdikleri proteinlerin fonksiyonel özellikleri oldukça önemlidir. Önemli oranda protein içeren gıdaların su tutma kapasitesi, jel oluşturma kabiliyetleri, besinsel özellikleri ve daha birçok özellikleri, içerdikleri proteinlerin özellikleri ile yakından ilgilidir. Dolayısıyla gıdaların özelliklerinin geliştirilmesinde proteinlerin modifiye edilmesi, ürün özelliklerini önemli derecede geliştirmektedir [2].

Gerek kırmızı et ve gerekse de piliç eti ürünlerinin üretiminde çeşitli katkı maddeleri kullanılmaktadır. Bu amaçlar doğrultusunda gıdalara katılan işleme yardımcı maddelerinin bir grubunu enzimler oluşturmakta ve transglutaminaz enzimi de bu grup içerisinde önemli bir yer teşkil etmektedir. Bu enzim, et ürünleri, süt ürünleri, tahıl ürünleri ve su ürünleri gibi geniş yelpazede gıda ürünlerine katılabilmektedir. Bu enzimin en önemli özelliği proteinler arasında çapraz bağ oluşumunu arttırarak, ürünün jel yapısını ve tekstürünü geliştirmesidir. En iyi sonuçlar verdiği gıda grubunu ise et ürünleri oluşturmaktadır.

Transglutaminaz çoğunlukla gıda proteinlerinin fonksiyonel özelliklerini geliştirmek için kullanılır. Enzim, protein içindeki birincil aminleri, indirgenmiş glutaminin γ-karboksilamid grubu ile değişik birincil aminler arasında katalizleyerek birleştirir. Substrat olarak amin olmadığı durumlarda TGaz, su moleküllerini açil yakalayıcılar olarak kullanıp, indirgenmiş glutaminin deaminasyonunu katalizler. E-lisindeki çapraz bağlar molekül içi ve molekül dışı olabilir ve özellikle lisin ve glutamin içeren proteince zengin gıdalarda fiziksel değişikliklere neden olabilmektedir [3]. TGaz aynı zamanda proteinlerdeki diğer bağlara da etki edebilmektedir (örneğin et ve soya proteinleri, kazein ile gluten arasındaki bağlar). Bunun yanında TGaz, polifenoloksidaz ve lipoksigenaz eklendiğinde enzimlerin sülfidril ve disülfit bağlarına etki ederek çapraz bağ dizilimi oluşturabilmektedir [4].

Transglutaminazlar, çeşitli hayvan dokularından, bitkilerden ve bazı mikroorganizmalardan elde edilebilmektedirler. Genel olarak TGaz'ı endüstriyel alanda geliştirmek üzerine 3 tane yaklaşım söz konusudur. İlk yaklaşım; enzimin gıdanın sıvı kısmından veya dokusundan ekstrakte ederek ve süzerek elde edilmesidir (hayvanlarda sığır, balık kullanılabilir). Avrupa'da TGaz, ticari olarak kesilen domuz ve sığır etinden ekstrakte edilir. İkinci yaklaşıma göre enzim, *E. coli*, *Bacillus*, *Aspergillus* gibi mikroorganizmalardan genetik özellikleri değiştirilmek suretiyle elde edilir. Birçok araştırmacı yüksek miktarda TGaz'ı düşük maliyetle elde etmeye çalışmıştır. Ancak bunların hiçbiri bazı ticari faktörler nedeniyle hayata geçirilememiştir (örneğin gıda üretimi ve müşteri memnuniyeti). Üçüncü ve son yaklaşım ise TGaz üreten mikroorganizmalardır. Bu yaklaşıma göre; TGaz üreten mikroorganizmalar bulunursa TGaz geleneksel fermantasyon yöntemiyle üretilebilecektir.

Daha kolay ve ekonomik olarak elde edilebilmesinin yanında ticari üretiminin gerçekleştirilmesi nedeniyle, gıdalarda daha çok mikroorganizmalardan elde edilen transglutaminazlar (MTG) tercih edilmektedir. Enzim üretim zinciri için binlerce mikroorganizma kolektif olarak çalışır. Çoğu mikroorganizmaların "Hidromate Yöntemi" ile TGaz ürettiği gözlenmiştir. Mikroorganizmalar TGaz'ı metabolizma artığı olarak atarlar. Bu enzimin protein zincirlerini, G formundan L formuna dönüştürebilme gibi özel bir yeteneğe sahiptir [5]. Mikroorganizma olarak ise özellikle *Streptoverticillium* (*S. mobaraense ve S. griseocameun*) cinsi bakteriler etkin bir şekilde kullanılmaktadır [6].

Et ürünleri yüksek oranda protein içermekte ve bu proteinler arasında myofibriler proteinler et ürünlerinin tekstürünü önemli ölçüde etkilemektedirler. Myofibriler proteinlerin büyük bir kısmını aktin ve myosin oluşturmaktadır. Aktin ve myosin TGazlar için oldukça önemli substratlar olup, TGazlar'ın ilavesiyle polimerleşebilmektedirler. Bu durum jel yapıdaki et ürünlerinin jel ağlarının özelliklerini geliştirebilmektedir [7]. Yapılan araştırmalarda TGaz ve kazeinatın aynı anda kullanımı ile et bağlama sistemlerinin geliştiği tespit edilmiştir. TGaz ve kazeinat birlikte uygulandığı zaman gıda bileşenlerini bir arada tutmak için yapıştırıcı gibi davranırlar. Bu yöntemler yüksek miktarda domuz eti, biftek veya balık filetoları, bunların küçük parçalarından hazırlanabilmektedir.

Et ürünlerinde TGaz kullanımı üzerine birçok çalışma mevcuttur. Pek çok durumda enzimin düşük sıcaklıkta (10°C) kullanılabildiği durumların yanı sıra, daha iyi ürün karakteristikleri sağlamak amacıyla yüksek sıcaklıklarda (40-50°C) kullanıldığı durumlar da bulunmaktadır. Yapılan birçok araştırma, TGaz ilavesi ile et ürünlerinde jel kuvvetinin artış gösterdiğini ve karakteristik özelliklerin olumlu yönde geliştiği kaydedilmiştir [8-12].

Bu araştırmada, bir gıda işleme yardımcı maddesi olarak dünyada yaygın bir şekilde kullanılmaya başlanan transglutaminaz enzimi ilave edilerek üretilen tavuk köftelerinin, üretim sonrası kalite kriterleri ile duyusal özelliklerinin incelenmesi amaçlanmıştır.

MATERYAL ve METOT

Materyal

Üretimde taze, derilerinden arındırılmış piliç göğüs etleri hammadde olarak kullanılmıştır. Transglutaminaz enzimi (Activa EB) ise Ajinomoto firmasının Fransa şubesinden temin edilmiştir. Piliç göğüs etleri kıyma makinasından çekildikten sonra içerisine tuz, baharatlar ve iki farklı konsantrasyonda enzim (%0.5 ve %1) ilave edilmiş ve bir grup ta kontrol grubu olarak enzim katılmadan hazırlanmıştır.

Hazırlanan köfteler ayrı olarak paketlenmiş ve buzdolabında 1 gün bekletildikten sonra analizlere alınmıştır.

Metotlar

Köftelerin; kurumadde, kül, Kjeldahl yöntemiyle ham protein ve Soxhlet yöntemi kullanılarak yağ analizleri AOAC'a göre yapılmıştır [13]. pH ölçümlerinde, homojenize edilmiş örneklerden 10 g örnek alınmış ve 100 ml destile saf su içerisinde homojenizatör kullanılarak 1 dk homojenize edildikten sonra pH-metre (WTW Inolab Level 2) probu daldırılmıştır ve değerler kaydedilmiştir [14].

Köftelerin CIE L* (parlaklık), a* (kırmızılık) ve b* (sarılık) değerleri ColorFlex HunterLab Renk Ölçüm Sistemi (Hunter Associates Laboratory, Inc.) kullanılarak belirlenmiştir. Eşit boylarda kesilen her örnekten beş ölçüm yapıldıktan sonra aritmetik ortalamaları hesaplanmıştır [15].

Köfte örneklerinin Share Force (Warner-Bratzler) değerleri tekstür ölçüm cihazı (Instron, Model 3343) ile belirlenmiştir [16]. Örnekler eşit boylarda kesilerek 200°C'deki fırında 5 dk ısıl işleme tabi tutulmuşlar ve daha sonra cihaza yerleştirilerek tekstür değerleri kgf olarak cihazda okunup kaydedilmiştir.

Pişirme kaybının belirlenmesi amacıyla köfteler elektrikli ızgarada yaklaşık 180°C'de 5 dk süre ile pişirilmiştir. Köftelerde pişirme kaybı, pişirmeden önceki köfte ağırlığının pişmiş köfte ağırlığından çıkarılıp, çiğ köfte ağırlığına bölümünün yüz ile çarpımı sonucu tespit edilmiştir [17].

Analizlerde elde edilen sonuçlara varyans analizi uygulanmış ve önemli bulunan varyasyon kaynaklarından farklı etkide bulunanı belirlemek amacıyla ortalamalar Duncan Çoklu Karşılaştırma Testi ile karşılaştırılmıştır [18].

BULGULAR

Çalışmamızda enzim katılmayan kontrol grubu ile %0.5 ve %1 enzim ilave edilen tavuk köftelerine ait araştırma bulguları *Tablo 1* ve *Tablo 2*'de verilmiştir. Araştırma sonuçlarına göre enzim ilavesi köftelerin kurumadde içeriklerinde daha fazla olmakla birlikte, kül ve protein içeriklerinde yine az da olsa artışa; yağ içeriğinde de azalmaya neden olduğu tespit edilmiştir.

Tablo 1. *Tavuk köftelerine ait kimyasal analiz bulguları* [1]
Table 1. *Chemical analysis findings of chicken breast patties*

Analiz	Örnek		
	Kontrol	%0.5	%1
Kurumadde (%)	33.69±0.33 b	34.54±0.43 ab	35.41±0.57 a
Kül (%)	1.93±0.31 a	2.09±0.03 a	2.11±0.01 a
Yağ (%)	11.26±0.43 a	11.12±0.10 a	11.16±0.09 a
Protein (%)	17.36±0.25 a	17.99±0.03 a	17.52±0.26 a

[1] Değerler ± standart sapmayı ifade etmektedir, Kolonda aynı harflerle işaretlenen ortalamalar istatistiksel olarak birbirinden farklı değildir (P>0.05)

Tablo 2. *Tavuk köftelerine ait fiziksel analiz bulguları* [1]
Table 2. *Physical analysis findings of chicken breast patties*

Analiz		Örnek		
		Kontrol	%0.5	%1
pH		5.90±0.007 a	5.78±0.01 b	5.77±0.02 b
Pişirme Kaybı (%)		13.80±1.03 a	12.10±0.66 ab	11.48±0.19 b
Tekstür (kgf)		0.20±0.14 c	0.57±0.25 b	0.92±0.23 a
Renk	L	41.81±0.80 a	43.10±1.03 a	42.15±1.86 a
	a	7.42±0.43 a	6.98±0.25 a	6.96±0.54 a
	b	24.47±0.27 a	24.42±1.12 a	23.07±0.48 b

[1] Değerler ± standart sapmayı ifade etmektedir, Kolonda aynı harflerle işaretlenen ortalamalar istatistiksel olarak birbirinden farklı değildir (P>0.05)

Fiziksel analiz sonuçlarına bakıldığında enzim ilavesi köftelerin pH değerlerinde ve pişirme kayıplarında düşüşe neden olduğu gözlenmiştir. Yine enzim ilavesi köftelerin parlaklık düzeyinde artışa neden olurken, en belirgin artış tekstür değerlerinde belirlenmiştir.

Enzim ilavesi köftelerin renk değerlerinde fark oluşturmazken (P>0.05), pişirme değerinde azalmaya ve tekstür değerinde de artışa neden olmuştur.

TARTIŞMA ve SONUÇ

Araştırma sonucu elde ettiğimiz bulgulara göre örneklerin kül, yağ ve protein içerikleri açısından, enzim katılan ve katılmayan gruplar arasında istatistiki olarak fark tespit edilmemiştir (P>0.05). Cofrades ve ark.[19], transglutaminaz enzimi ve deniz yosunu kullanarak tavuk göğüs etlerinden tavuk biftek yapmışlar ve bu bifteklerin kalite özellikleri incelemişlerdir. Araştırma sonuçları çalışmamız ile benzerlik göstermiş olup; enzim katılmayan grup ile enzim ilaveli gruplar arasında kül, yağ ve protein içerikleri bakımından istatistiki bir fark gözlememişlerdir.

Enzim ilavesi köftelerin pH değerlerinde düşüşe neden olmuş ve enzim ilave edilen grup, kontrol grubu örneklerine göre farklılık göstermiştir (P<0.05). Enzim miktarının artışı ise pH değerinde değişikliğe neden olmamıştır (P>0.05). Trespalacios ve Pla [8], tavuk eti jelinin fonksiyonel özelliklerini geliştirmek için basınçla eşzamanlı olarak transglutaminaz enzimi (%1) kullanmışlar ve uygulama sonucunda enzim eklenmeyen örneklerin pH değerini 6.89 bulurlarken; enzim ilave edilen örneklerin asitliğinde küçük bir artış (6.84) tespit etmişlerdir. Çalışmamızda elde ettiğimiz bulgulara benzer olarak tüm örneklerin pH değerlerinde istatistik olarak fark olmadığını (P>0.05) bildirmişlerdir.

Transglutaminaz enzimi, et ürünlerinde pişirme ve çözündürme kayıplarını azaltarak, su bağlama özelliğini arttırmaktadır [20]. Araştırmamızda elde ettiğimiz bulgular da bu tezi destekler niteliktedir. Enzim ilavesi köftelerin pişirme kayıplarında azalmaya neden olmuş ve bu azalma enzim katılmayan grup ile %1 enzim ilaveli grup arasında

istatistik olarak farklılık sağlamıştır (P<0.05). Kontrol grubu ile %0.5 enzim katkılı örnekler ve %0.5 ile %1 enzim katkılı gruplar kendi içerisinde istatistik olarak fark yaratmamıştır (P>0.05). Transglutaminaz enzimi ile yapılan çalışmalarda elde edilen araştırma verileri, enzim ilave edilen örneklerde ilave edilmeyenlere göre en belirgin farklılıklardan birinin, pişirme kayıplarında meydana gelen azalma olduğunu açıkça ortaya koymaktadır. Pietrasik [21] ve Basaran ve ark.[22] %0.5 MTGaz ilavesinin ticari pişirme yöntemleri kullanılarak pişirilmiş sığır ve tavuk jellerinin nem ve ağırlık kayıplarını önemli düzeyde azalttığını bildirmişlerdir.

TGaz enzimi üzerine yapılan çalışmaların sonucunda elde edilen en önemli bulgular, ürünlerin tekstür ve jel kuvvetindeki meydana gelen artışlardır. TGaz ilavesinin, moleküller arası ε (γ-glutamil)-lisil çapraz bağlarının oluşumu yoluyla daha sıkı jel ağ yapısına neden olduğu birçok araştırmada bildirilmektedir [9,23-26]. Araştırmamızda da bu durum net bir şekilde ortaya konmuştur. Enzim katkısı köfte örneklerinin tekstür değerlerinde kayda değer artışa neden olmuş; enzim ilave edilmeyen ve %0.5 ile %1 enzim ilaveli köfteler arasında istatistik olarak önemli fark bulunmuştur (P<0.01). Yapılan araştırmalar da çalışmamızla paralellik göstermektedir. Tseng ve ark.[9], transglutaminaz katkısının tavuk köftelerinin jel kuvveti ve tekstürünü arttırdığını bildirmişlerdir. Lantto ve ark.[27] lakkaz ve TGaz'ın pişirilmiş tavuk homojenat jellerinin sıkılığı ve ağırlık kaybı üzerine etkisi üzerine yaptıkları çalışmada, düşük tuz içerikli (%1) ve düşük etli (%65), fosfat içermeyen jellerin sıkılığını azalttığını; bir miktar transglutaminaz ilavesinin ise düşük etli, düşük tuzlu ve fosfatsız ürünlerde, enzim içermeyen kontrol gruplarına göre mukavemeti önemli ölçüde geliştirdiğini bulmuşlardır.

Enzim ilavesi az da olsa köftelerin L değerlerinde artışa, a değerlerinde azalmaya neden olurken; bu değerlerin istatistik olarak enzim katılan ve katılmayan gruplar arasında bir fark oluşturmadığı gözlenmiştir (P>0.05). b değeri ise sadece %1 enzim katkılı örneklerde, diğer örneklere göre azalma göstermiş ve istatistik olarak farklı bulunmuştur (P<0.05). Bu konu üzerine yapılan araştırmalarda renk açısından benzer bulgular bulunmaktadır. Enzim ilave edilerek üretilen tavuk köftelerinde [9], sığır jellerinde [21], sosislerde [28] ve tavuk döner kebapta [29], kontrol gruplarına göre renk parametrelerinde istatistik olarak fark meydana gelmediği (P>0.05) bildirilmiştir.

Transglutaminazlar, ucuz ve oldukça kolay olarak üretilebilen kitle enzimlerdir. Çeşitli moleküllerin çapraz bağlanmalarını kataliz ederler ve daha birçok özelliklere sahiptirler. Benzer amaçlarla kullanılan diğer kimyasallara kıyasla daha güvenilir olup, kullanımları daha pratiktir. Bu yüzden, özellikle gıdaların üretiminde kullanımları önemli bir potansiyele sahiptir. Son yıllarda bu enzim ile yapılan çalışma sayısında önemli ölçüde artış görülmektedir. Yurt dışına bakıldığında bu enzimin hemen hemen her gıda prosesinde kullanıldığı, özellikle de çalışmaların et ürünlerinin üretimi ve çeşitlendirilmesinde yoğunlaştığı görülmektedir. Ülkemizde ise bu enzim ile yapılan çalışmaların henüz araştırma aşamasında olduğu, gıda endüstrisine tam olarak aktarılamadığı söylenebilir.

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Kafkas Univ Vet Fak Derg
19 (2): 337-342, 2013
DOI: 10.9775/kvfd.2012.7846

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

The Study of Histogenesis of Sheep Fetus Iris

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Makale Kodu (Article Code): KVFD-2012-7846

Summary

In this study, for determination of histogenesis of iris in sheep, 32 samples of sheep fetuses at different and certain ages were used. After sample preparation and fixation, their age were determined and histological and anatomical studies were done. In histological studies, the H&E staining and two other specific staining (verhoeff and Masson's Trichrome) were used. Obtained results demonstrated that a two-layered cup formed in first month but iris separation from the anterior and ceca part of retina occurred in second month. The separation of iris from ciliary body was completed in third month. From the beginning of third month, the iris formed changes and its circular shape changed to oval form so that its internal angle was larger than its external angle and this condition was retained until the end of pregnancy. General shape of pupil conformed to general shape of iris. It was histologically revealed that iris was formed from the anterior part of the optic cup just at the connection place of outer and inner layers in second month and thereafter a part of superficial ectoderm and choroid was added to it. The first signs of elastic fibers in iris as well as smooth muscles appearance were significant in second month. The posterior cells are pigmented from the beginning of the iris cell formation. Anterior epithelial tissue of the iris was simple squamous and two layer cuboidal cells formed its posterior epithelium which were stabilized in third month. The number of collagen and elastic fibers and smooth muscles and their size increase with age.

Keywords: *Iris, Sheep fetus, Histogenesis*

Koyun Fetus Irisinin Histogenesisi Üzerine Bir Çalışma

Özet

Bu çalışmada, koyunlarda iris histogenezinin belirlenmesi için farklı ve belirli yaşlarda 32 adet koyun fetus örneği kullanıldı. Numune hazırlama ve tesbit sonrası, yaş durumları belirlenerek histolojik ve anatomik çalışmalar gerçekleştirildi. Histolojik çalışmalarda, H & E boyama ve diğer iki spesifik boyama (Verhoeff ve Masson's Trichrome) kullanıldı. Elde edilen sonuçlar, iki katmanlı çanak formasyonunun ilk ayda oluşmasına rağmen retinanın anterior ve ceca bölümlerinden iris ayrılmasının ikinci ayda meydana geldiğini gösterdi. İrisin siliyer cisimden ayrılması üçüncü ayda tamamlandı. Üçüncü ayın başından itibaren, iris değişiklikleri oluşmuş ve onun iç açısının dış açısından daha büyük olabilmesi için sirküler şekli oval forma değiştiği ve bu durumun gebeliğin sonuna kadar muhafaza edildiği gözlemlendi. Pupilin genel şekli iris genel şekli ile uyumlu bulundu. Histolojik olarak ortaya çıkan bulguda, iris ikinci ayda dış ve iç katmanların bağlantı yerindeki optik çanağın anterior bölümünü oluşturduğu ve bundan sonra yüzeysel ektodermin bir kısmının ve koroidin buna eklendiği belirlendi. Iris elastik liflerinin yanı sıra düz kasların görünümünün ilk belirtileri ikinci ayda anlamlı bulundu. Posterior hücreler iris hücre formasyonu başlangıcından itibaren pigmentli halde gözlemlendi. Irisin anterior epitel dokusu, üçüncü ayda stabilize olan onun posterior epitelini oluşturan basit skuamöz ve iki katmanlı kübik hücreler şeklinde olduğu belirlendi. Kollajen ve elastik lifler ve düz kasların sayısı ve onların büyüklüğünün yaş ile arttığı sonucuna varıldı.

Anahtar sözcükler: *Iris, Koyun fetusu, Histogenez*

INTRODUCTION

Eye is one of important and sensory organs of the body and has many roles in relationship between animal and environment. Because of the importance and sensitiveness of eye, some researchers have worked about this organ in many years [1-7].

The eyeball has three thin tunics that, being in close apposition, form a laminated sheet that surrounds the partly liquid, partly gelatinous center [8]. The three tunics are an external fibrous tunic that gives form to and protects the eye-ball, it is the only complete tunic, a middle vascular

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tunic that consists largely of blood vessels and smooth muscles and is concerned with the nutrition of the eyeball and the regulation of the shape of the lens and size of the pupil, and an internal tissue and is the layer most directly concerned with vision [9].

The vascular tunic consists of three zones: choroid, ciliary body and iris. The third and smallest part of the vascular tunic is the iris, which is suspended between the cornea and lens. It is a flat ring of tissue attached at its periphery to the sclera and to the ciliary body the opening in the center is the pupil [9]. Iris is susceptible to various injuries because it is anterior part of eye. The normal structures of eye in sheep fetus have not been showed clearly yet so before studying about any abnormal cases, it seems helpful to evaluate the normal conditions of this organ. Therefore, this study proposed to demonstrate the histogenesis of iris in sheep fetus.

MATERIAL and METHODS

The present study was conducted on 32 samples of sheep fetus which were gathered from slaughterhouse in Tabriz city. The age of gathered fetuses were determined by the following formula [10];

X = 2.1(17+y), y = Fetus length, X = fetus age

Understudied groups included 30, 40, 50, 60, 80, 110, 135, and 150-day old fetuses (n=4 for each group). The fetuses with length less than 7 cm were fixed completely in 10% formalin and the eyes of others (fetuses with length more than 7 cm) were placed in 10% formalin for 48 h. For completing the fixation for internal parts of eye, 10% formalin was injected to internal part of the eye. Also, the dimension measurements were done by ruler and caliper. Tissue samples were prepared by usual histological method. Histological sections were stained with three staining methods; H&E (for general examination of tissue), Verhoeff (for examination of elastic fibers), Masson's tricrome (for examination of collagen fibers and muscle cells) [11]. Finally the samples were studied under light microscope.

RESULTS

The Results of the First Month Group or 30-Day Old Fetuses

Histological findings suggested that two-layer optic cup was form in this stage and its two inner and outer layers were separated from each other by intra retinal space. Outer layer of optic cup was clear with its small granules and formed retinal pigmented layer.

However, the anterior fifth of the inner layer and the pars ceca retinae of optic cup which was the separated place of iris and other tissues were seen circular and there was no sign of iris. The retinal granular layer was obvious clearly in Verhoeff staining and there was no sign of elastic fibers. In Masson's tricrome staining, the fine collagen fibers and mesenchyme cells were observable clearly and most of them were not differentiated *(Fig. 1)*.

Results of 40-Day Old Fetuses

In the early of second month, there was no clear sign of iris. The anterior fifth of the inner layer, the pars ceca retinae, remained one-layer thick. The pars ceca retina was not divided to iris and ciliary body yet. At this stage, retinal pigmented layer producing the iris pigmented layer was seen and the anterior part of choroid originated plenty of iris blood vessels. In addition, the influence of superficial ectoderm in forming of iris muscles was obvious. In Verhoeff staining, little signs of elastic fibers in undifferentiated part of iris was observable. These fibers were concentrated mostly in superficial ectoderm. In Masson's tricrome staining, the pigments continued from retina and collagen fibers accompanied with muscular cells differentiated from superficial ectoderm were obvious. Separation of iris from superior parts of eye has not been done yet and this demonstrated that the differentiation of this part was done in the middle of the second month.

The Results of 50-Day Old Fetuses

In the middle of second month, the separation of iris and ciliary body was very little and has not been separated completely. Iris had squamous epithelium in anterior part and unclear two-row epithelium in posterior part. The posterior layer cells were completely pigmented and their granules were clear. There was loose connective tissue among epitheliums consisted of fibrocytes, fibroblasts, blood

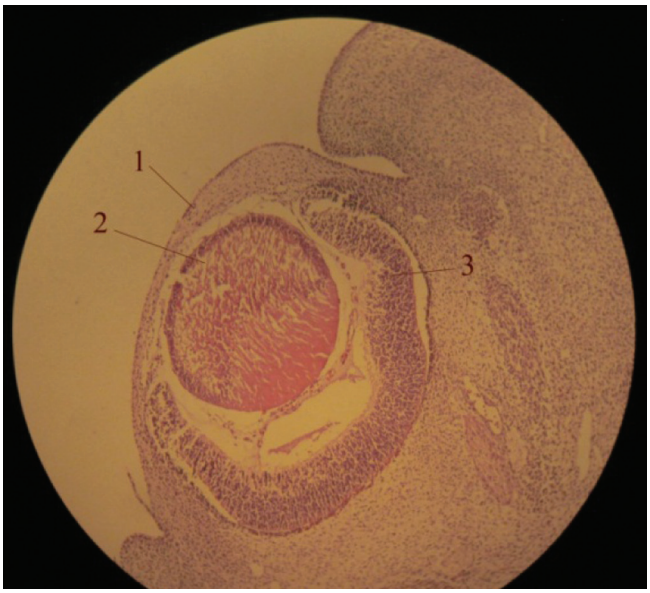


Fig 1. Histological findings in the first month group or 30-day old fetuses (H&E, ×100); **1**- Cornea, **2**- Lens, **3**- Optic cup

Şekil 1. Birinci ay veya 30-günlük fetus gruplarında histolojik bulgular (H&E, × 100); **1**- Kornea, **2**- Lens, **3**- Optik çanak

vessels and muscular cells. In Verhoeff staining, elastic cells were very weak and limited to vascular wall. The pigments of epithelium cells were obvious. In Masson's tricrome, blue-colored fine collagen fibers in connective tissue and flat muscle cells were significant under the epithelium *(Fig. 2)*.

The Results of 60-Day Old Fetuses

Cornea was oval, iris was dark and pupil was relatively circular. The average length and transverse of external diameter of iris and were 0.46±0.081 cm and 0.30±0.051 cm respectively and the average length and transverse diameter of pupil were 0.44±0.071 cm and 0.4±0.31 cm respectively *(Table 1)*.

In the late of this month, the iris existed but less separated. Iris was seen in the form of a very fine pigmented layer in anterior part of the eye. Iris was separated from other tissues slowly and grew like a small mass between the lens and cornea. Epithelium cells of anterior part were as simple squamous but posterior part of epithelium cells was stratified and much pigmented. The border among posterior epithelial cells was not clear due to compression. Fibrocytes were seen in middle position and fine muscular cells were visible. In Verhoeff staining, elastic fibers were very limited and posterior layer pigments were visible strongly. In Masson's tricrome staining, the fine collagen fibers in loose connective tissue among epithelium tissues were seen. Also smooth muscular cells in iris were significant. The general result showed that the iris was separated from ciliary body slowly in the late of second month *(Fig. 3)*.

The Results of 80-Day Old Fetuses (Third Month)

In this period, iris color was dark brown. The iris followed cornea shape and its internal angle was larger than external angle. The average length of external diameter and transverse of iris were 1.0±0.51 cm and 0.7±0.51 cm respectively and the average of length and transverse diameter of pupil were 0.7±0.032 cm and 0.3±0.24 cm respectively *(Table 1)*.

In this month, the iris was fine and separated from choroid. The anterior part of iris is smoother and covered with squamous epithelium. There was a loose connective tissue under the epithelium. There were fibroblasts, fibrocyts and smooth muscles under the epithelium. Blood

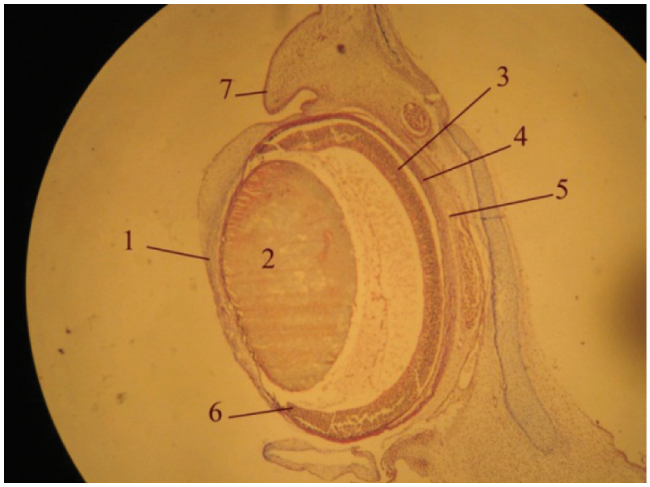


Fig 2. Histological findings in the middle of second month or 50-day old fetuses (Masson's Trichrome, ×40); **1-** Cornea, **2-** Lens, **3-** Retina, **4-** Choroid, **5-** Sclera, **6-** Root of iris and ciliary body, **7-** Eyelid

Şekil 2. İkinci ayın ortasında veya 50 günlük fetuslarda histolojik bulgular (Masson Trichrome, × 40); **1-** Kornea, **2-** Lens, **3-** Retina, **4-** Koroid, **5-** Sklera, **6-** İrisin kökü ve siliyer cisim, **7-** Gözkapağı

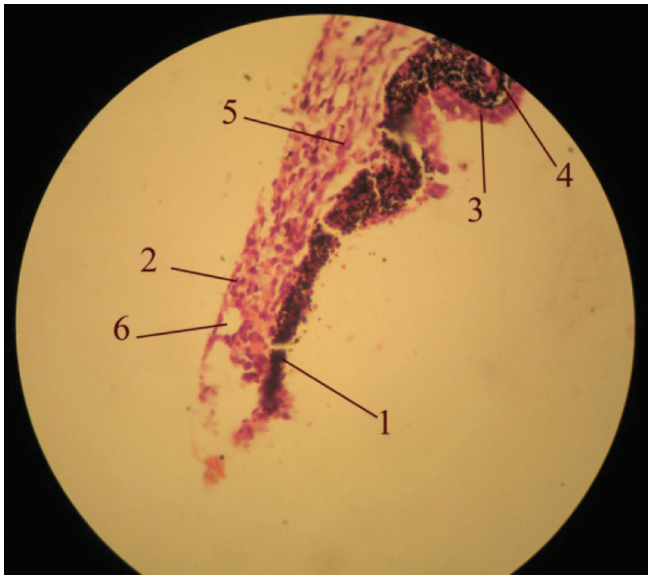


Fig 3. Histological findings in 60-day old fetuses (H&E, ×100); **1-** Posterior pigment epithelium of iris, **2-** Anterior epithelium of iris, **3-** Superficial cells of ciliary body, **4-** Deep cells of ciliary body, **5-** Smooth muscles, **6-** Blood vessels

Şekil 3. Otuz günlük fetuslarda histolojik bulgular (H & E, × 100); **1-** İrisin posterior pigment epiteli **2-** İrisin anterior epiteli, **3-** Siliyer cisim yüzeysel hücreleri, **4-** Siliyer cismin derin hücreleri, **5-** Düz kaslar, **6-** Kan damarları

Table 1. *Average length and transverse of external diameter of iris and average length and transverse of diameter of pupil in sheep fetuses at different ages*
Tablo 1. *Farklı yaşlardaki koyun fetuslarında iris dış çapının ortalama uzunluk ve eni ile pupil dış çapının ortalama uzunluk ve eni*

Fetus Age	Iris		Pupil	
	Average Length	Average Transverse of External Diameter	Average Length	Average Transverse of Diameter
Second month	0.46±0.081 cm	0.30±0.051 cm	0.44±0.071 cm	0.4±0.31 cm
Third month	1.0±0.51 cm	0.7±0.51 cm	0.7±0.032 cm	0.3±0.24 cm
Fourth month	1.3±0.51 cm	0.8±0.51cm	0.7±0.32 cm	0.3±0.77 cm
Fifth month	1.6±0.41 cm	0.9±0.72 cm	0.8±0.22 cm	0.4±0.35 cm

vessels were more than the previous months. The superior epithelium was relatively smooth and formed of stratified cuboid cells. Internal cells had more pigments. Masson's tricrome staining showed fine blue collagen fibers in connective tissue. Also, there were smooth red muscles under epithelium and in the center of iris *(Fig. 4)*.

The Results of 110-Day Old Fetuses (Fourth Month)

The 110-day old fetuses eyelids were semi open and iris was dark brown. The iris had oval shape and followed the shape of cornea; internal angle of iris was larger than its external angle. The pupil dimension in internal angel was larger than the external one too. The average length and transverse of external diameter of iris was 1.3±0.51 cm and 0.8±0.51cm respectively and the average length and transverse diameter of pupil were 0.7±0.32 cm and 0.3±0.77 cm respectively *(Table 1)*. Also, the third eyelid was clear in internal angle of the eye. The separation of ciliary body and iris from the choroid was clear completely in fourth month and they separate from each other in the limbus region. The iris was thin and fine and moved between cornea and lens. Iris had an anterior irregular simple squamous epithelium. The loose connective tissue consisted of fibrocytes, fibroblasts and blood vessels were seen under this tissue. Melanocyte cells were low in connective tissue. Epithelium cells of posterior layer were relatively irregular and consisted of one or two rows of cuboid cells with plenty of melanocytes. There were a lot of melanocytes with not observable cytoplasm. In the center of iris, there were significant muscular cells placed in a row behind each other. In Verhoeff staining, there was no important sign of elastic fibers in iris connective tissue and there were elastic fibers only in vessels wall. In Masson's tricrome staining, the dispersed blue collagen fibers in loose connective tissue were seen especially under the anterior epithelium. In addition, the existence of many muscular cells in red ribbon form at the center of iris was confirmed. The muscular cells adjacent to choroid were greater *(Fig. 5)*.

The Results of 135-Day and 150-Day Old Fetuses (Fifth Month)

In fifth month, the eyelids were open and the third eyelid was seen in internal angle. Iris was dark brown and rested between cornea and lens. Iris resembled cornea and its internal angle was larger than external one. Pupil was oval and its internal angle was larger than external one. Two small nodes were seen in lower edge of pupil in the

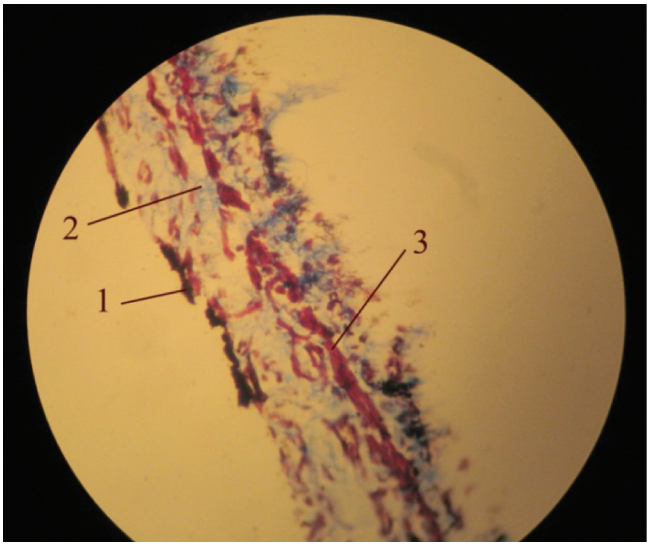


Fig 5. Histological findings in the fourth month (Masson's Trichrome, ×400); **1**- Posterior pigment epithelium of iris, **2**- Collagen fibers, **3**- Smooth muscle cells

Şekil 5. Dördüncü ayda histolojik bulgular (Masson Trichrome, × 400); **1**- İrisin posterior pigment epiteli, **2**- Kolajen lifler, **3**- Düz kas hücreleri

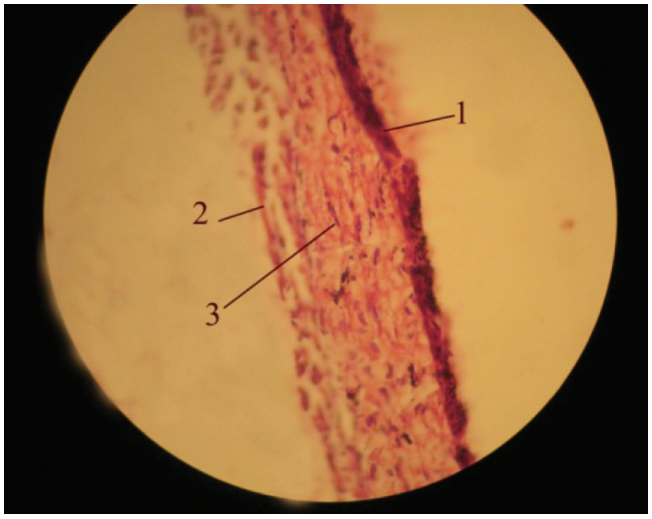


Fig 4. Histological findings in the third month (H&E, ×400); **1**- Posterior pigment epithelium of iris, **2**- Anterior epithelium of iris, **3**- Connective tissue with fibrocyte

Şekil 4. Üçüncü ayda histolojik bulgular (H & E, × 100); **1**- İrisin posterior pigment epiteli, **2**- İrisin anterior epiteli, **3**- Fibrositli bağ doku

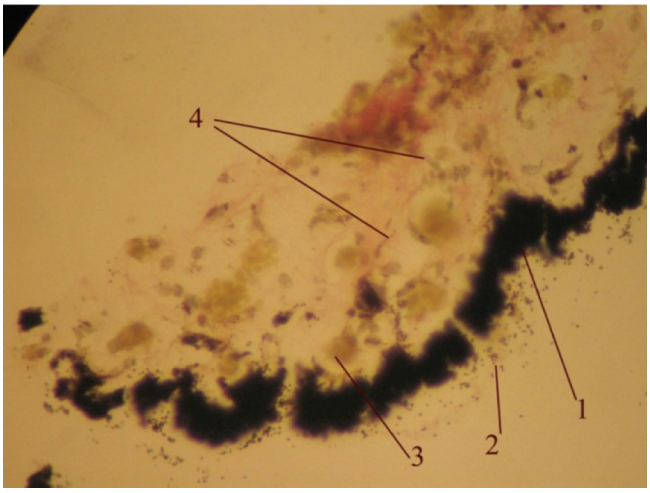


Fig 6. Histological findings in the fifth month (verhoeff, ×400); **1**- Posterior pigment epithelium of iris, **2**- Pigments, **3**- Blood vessels, **4**- Elastic fibers

Şekil 6. Beşinci ayda histolojik bulgular (Verhoeff, × 400); **1**- İrisin posterior pigment epiteli, **2**- Pigmentler, **3**- Kan damarları, **4**- Elastik lifler

late of fifth month. The average length and transverse of external diameter of iris were 1.6±0.41 cm and 0.9±0.72 cm respectively and the average of length and transverse diameter of pupil were 0.8±0.22 cm and 0.4±0.35 cm respectively *(Table 1)*. A part of choroid had inclined to the eyeball adjacent to limbus and divided to two parts; anterior part (iris) and superior part (ciliary body). Iris was seen finer than cornea and had two different epitheliums. Anterior epithelium of iris was irregular and covered with simple squamous or cuboid cells. There was a loose connective tissue with plenty of blood vessels or accumulated red blood cells under this tissue. Fibrocytes, fibroblasts and melanocytes were very low in connective tissue. In connective tissue, plenty of vessel sections were significant because iris was member of vascular layer of eye. The smooth muscle cells and fine myoepithelial cells for iris contractions were seen in connective tissue. Most of these muscular cells exist under the iris epithelium. The posterior epithelium of iris was relatively smooth and covered with two rows of irregular cuboidal cells. These cells were completely pigmented and had more intracellular black granules. The muscular and myoepithelial cells under the epithelium became greater at the adjacent to free edge of iris. In Verhoeff staining, there were not any signs of elastic fibers but the pigmented cells and plenty of blood vessels in internal layer of iris and smooth muscular cells in connective tissue were significant. In Masson's tricrome staining, the collagen fibers of connective tissue and red smooth muscles with myoepithelial cells were observable. The muscular cells were concentrated under the epitheliums and blood vessels gathered in the center of iris *(Fig. 6)*.

DISCUSSION

Anatomical examinations weren't possible in one-month old or 30-day old samples due to smallness of samples so only histological examination was conducted. As it was mentioned in results section, in this month optic cup was formed relatively and was clear as circle mass under microscope. These optic cups grew bilaterally from the head of fetus. But there was not any sign of optic cup and mesenchymal differentiation to choroid, sclera, iris and ciliary body in sheep fetus in this stage.

In human fetus the developing eye appears in the 22-days embryo as a pair of shallow grooves on the sides of the forebrain [12]. Also it has been reported that in human fetus with closure of the neural tube these grooves form outpocketings of the forebrain, the optic vesicles. Shortly thereafter the optic vesicle begins to invaginate and forms the double-walled optic cup. The inner and outer layers of this cup are initially separated by a lumen, the intra retinal space [12]. In 16-18 days old dogs the optic vesicles are formed while it is formed on 5-30 days old cattle [13]. Based on this report the optic cup is formed on 19-day old in dogs and on 30-day old in cattle [14]. The reports suggest that in most animals the optic cup is formed relatively in first month of pregnancy.

In the early of the second month's samples of sheep fetus it was clear that optic cup were deepen slowly and were formed double walled completely. The outer layer of the optic cup is known as the pigmented layer of the retina. Retina was the most clear and thick eye layer. This issue suggested that retina follows this rule that nervous part of the body is differentiated earlier than the other parts. Also, outer layer of optic cup which forms external part of retina has small pigmented granules and seen as a dark pigmented layer. In human fetus the optic vesicle moves to internal part and forms a two-layer cup on its 42-day old [15]. At first there is a space between these two layers but the space disappears after a short time and the two layers of cup appose each other [16]. In human fetus in this stage the outer layer of the optic cup which is characterized by small pigment granules, is known as the pigment layer of the retina. This issue added that development of the inner (neural) layer of the optic cup is more complicated [12]. The posterior four-fifth, the pars optica retina, differentiates into light-receptive elements. The anterior fifth of the inner layer, the pars ceca retinae, remains one cell layer thick. It later divides into ,the pars iridica retinae, which forms the inner layer of the iris, and the pars ciliaris retinae, which participates in formation of the ciliary body [12].

As it was mentioned the outer layer of optic cup in one-month old fetus was clear with its small pigmented granules and the pigmented layer is called retina [17]. This pigmented layer will form the pigmented layer of iris and ciliary body on fifth of anterior part of optic cup [13]. In this stage (first month) the bending part of optic cup's anterior layer which will divide to ciliary body and iris is seen circular yet [18]. Also, there is not any clear sign of iris and ciliary body in the early of the second month. In other words, one fifth of anterior part of optic cup; ceca part of retina has not been separated to iris and ciliary body. Moreover, in this period, the participation of choroid anterior part in forming of iris and ciliary body is seen and produces plenty of blood vessels in the structure of these parts. Meanwhile, in this stage the influence of superficial ectoderm on forming of iris and ciliary body's muscles is observable. In human fetus the region between the optic cup and the overlying surface epithelium is filled with loose mesenchyme [12,16]. The sphincter and dilator pupillae muscles form in this tissue. These muscles develop from the underlying ectoderm of the optic cup. In the adult the iris is formed by the pigment-containing external layer with the unpigmented internal layer of the optic cup, and a layer of richly vascularized connective tissue that contains the pupillary muscles [19,20].

In the middle of the second month, the separations of iris from ciliary body is very low but in the late of this month (near 60-day old) iris and ciliary body are separate from each other so that they have common root but they

are separated in anterior part. In general in the late of second month iris is separated from ciliary body slowly.

Complete separation and relative differentiation is done in third month and final specifications of these two parts are apparent in this month. In human fetus, iris and ciliary body is formed from one fifth of internal part of retina's ceca part [12].

Superior part of iris consists of granular epithelium cells which extend from anterior edges of optic cup to lens [18]. These conditions conformed to sheep samples. Furthermore, connective tissues of choroids, sclera, iris and some other parts of eye are formed from neural crest and small participation of mesoderm [21,22]. Also the iris muscles which are produced from pigmented epithelium cells, are placed in the edge of optic cup earlier [23,24].

In general, it is concluded that in third month (90-day old) the eyelids are opened. The iris is like a dark mass. The iris and pupil shape are horizontal oval and their sizes become larger by the time. These results confirm with the results about other animals and human [9].

Furthermore, histological examinations revealed that elastic and collagen fibers are clear slowly during fetus maturation. Based on the findings of this research, number and size of smooth muscle increases with evolution of fetus which confirms with the fetus growth rules [11]. It is worth to note that blood vessels and cell pigmentation increase significantly with fetus growth.

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Kafkas Univ Vet Fak Derg
19 (2): 343-346, 2013
DOI: 10.9775/kvfd.2012.7847

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Pathological Investigations of Winter-like Disease in Cultured Sea Breams (*Sparus aurata*)

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Makale Kodu (Article Code): KVFD-2012-7847

Summary

During the January 2008, an outbreak occurred in 15 sea bream farms located in Güllük gulf of Bodrum, Turkey and approximately two million sea breams (*Sparus aurata*) were died in three weeks. When the outbreak was took place, surface temperature and dissolved oxygen of sea water was measured as 10ºC and 7.9 mg/l on January 10, 2008. For pathological examinations, a total of 65 fish (8-9-month-old) were collected from 9 of 15 farms. At necropsy, extreme abdominal distention, milk-like discharge around the anus and depigmentation of skin were observed in all fish. The liver was swollen and yellowish-white in color. Microscopically, degeneration, severe fatty changes and multifocal necrotic areas were observed in the liver. The similar necrotic areas were also noted in the kidney and spleen. Severe verminous enteritis due to *Myxidium spp.* was seen in two fish farms. The gills were generally edematous and hyperemic. According to result of bacteriological examinations, *Vibrio spp.* (farm no: 1) and *Staphylococcus spp.* (farms no: 1, 8) were isolated and identified in some farms. In conclusion, it is thought that the outbreak occurred in sea breams might be winter disease associated with sudden decreases in water temperature together with induced fatty liver.

Keywords: *Winter disease, Sea bream, Histopathology, Liver*

Kültür Çipuralarında (*Sparus aurata*) Winter-benzeri Hastalık Üzerinde Patolojik İncelemeler

Özet

Bodrum Güllük körfezinde bulunan 15 farklı balık çiftliğinde, 10 Ocak 2008 döneminde, kültür çipuralarında (*Sparus aurata*) görülen bir salgın sonucu üç hafta içinde yaklaşık 2 milyon balık ölümü meydana geldi. Ölümlerin meydana geldiği dönemde, deniz suyunun yüzey sıcaklığı 10ºC ve çözünmüş oksijen miktarı 7.9 mg/l olarak ölçüldü. Çalışmada, salgının görüldüğü 15 işletmenin dokuzundan alınan toplam 65 adet çipura (8-9 aylık) incelendi. Dış bakıda, tüm balıklarda karında aşırı şişkinlik, anus etrafında süt benzeri akıntı ve deride depigmentasyon görüldü. Karaciğerler şişkin ve sarımsı-beyaz renkteydi. Mikroskobik incelemede, karaciğerde dejenerasyon, şiddetli yağlanma ve multifokal nekroz alanları dikkati çekti. Benzer nekroz alanlarına böbrek ve dalaklarda da rastlandı. İki işletmede barsaklarda *Myxidium spp.*ye bağlı şiddetli paraziter enteritis saptandı. Solungaçların sekunder lamellerinde ödem ve hiperemi oldukça belirgindi. Bakteriyolojik inceleme sonucunda, bir işletmede *Vibrio spp.* (işletme no: 1), iki işletmede ise *Staphylococcus spp.* (işletme no: 1, 8) izole ve identifiye edildi. Sonuç olarak, çipuralarda görülen bu ölümlerin, deniz suyu sıcaklığında meydana gelen ani düşüşün neden olduğu karaciğer yağlanmasıyla karakterize Winter hastalığına bağlı olarak oluşabileceği düşünüldü.

Anahtar sözcükler: *Winter hastalığı, Çipura, Histopatoloji, Karaciğer*

INTRODUCTION

"Winter syndrome" or "winter disease" (WD) is a seasonal, multifactorial disease and found primarily in sea breams *(Sparus aurata)* [1-3]. In recent years, it has caused significant mortality rates in sea bream farms along the Mediterranean coast such as Spain, Italy, Greece, Croatia, Malta and Portugal, during the winter months [2,4]. Etiology of WD has not been certain because of determining association between the disease and any specific etiological agent [4-6]. Whereas, some environmental stress factors such as decrease in salinity of sea water, low water temperature (<13ºC), fish density, immunosuppression of fish and nutritional factors can be effective on occurrence of WD and particularly the

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lower water temperature may be initial reason of all these factors [4,5,7,8]. The purpose of this study was to investigate pathologic changes of an outbreak of winter-like disease affecting cultured sea breams in Turkey.

MATERIAL and METHODS

The total of 65 dead sea breams (8-9-month-old) was collected from 9 of 15 farms (Farms no: 1 - 9). However, healthy 10 sea breams were sampled and used as control group. These control fish were euthanasied by overdose of anesthesia. Necropsies of all fish were performed. Tissue samples of liver, kidney, heart, gill, pyloric caeca, stomach, intestines, swim-bladder, brain, skin and skeletal muscle of all fish were fixed in 10% neutral-buffer formalin solution. Then, they were embedded in paraffin, sectioned at 4 to 5 µm and stained with haematoxylin and eosin (HE), Brown-Brenn and PAS stains for routine light microscopic examination. In addition to determine the presence of lipid in the liver, Oil Red O staining method was used in the frozen liver sections [9]. Bacteriological analysis was performed in the University of Ege, Faculty of Fisheries and Bornova Veterinary Control and Research Institute, Izmir, Turkey.

RESULTS

On January 2008, an outbreak was detected on the Güllük gulf of Bodrum which affected 15 fish farms. Approximately two million sea breams died because of this outbreak. But death was not seen in sea bass *(Dicentrarchus labrax)* in these farms. Salinity rate, surface temperature, pH and dissolved oxygen of sea water of Güllük Gulf were measured as 0.1%, 10°C, 7.77, 7.9 mg/l respectively on January 10, 2008. For protection to outbreak, sea bream cages of many fish farms transported from Güllük Gulf to open sea and Akbük Gulf (surface water temperature of Akbük gulf: 16-17°C). So, the fish deaths were stopped after the moving of these fish cages.

Macroscopic Findings: Macroscopically, extreme abdominal distention, milk-like discharge around the anus and depigmentation of skin were generally observed in fish. At necropsy, abdominal cavity was completely filled with testicles and enlarged swim-bladders. The stomach was greatly enlarged and their lumens contained many undigested foods. The liver was swollen, yellowish-white in color and soft to touch with blunt edges in all fish *(Fig. 1)*. In the some fish, the gills were pale, swollen and covered by amount of mucus. Petechial hemorrhages were also observed in the gills. At four fish farms, white spots with different sizes were seen on the liver, kidney and spleen. At two fish farms, distention of the stomach and intestines was observed. Stomachs were filled with white fibrin content and intestines were filled with clear green content.

Microscopic Findings: The most common microscopic finding observed in all fish was severe and diffuse lipid infiltration and degeneration in the liver (hepatic steatosis). The hepatocytes were swollen and contained varying size of lipid droplets *(Fig. 2A)*. These droplets were detected as fat droplets with Oil Red O stain method *(Fig. 2B)*. In the control fish, slight lipid infiltration in the liver was detected *(Fig.2C)*. At four farms (farms no: 2, 4, 6, 7) multifocal well encapsulated necrotic areas were diagnosed in the macroscopically white spotted areas of liver, kidney *(Fig. 3A)* and spleen *(Fig. 3B)*. Any causative agent was not found in the necrosis areas with *special staining* techniques such as Brown-Brenn and PAS. Degeneration together with hyaline droplet accumulation was detected in some renal tubules of the kidneys. At two farms (farms no: 1, 2), *verminous enteritis due* to *Myxidium spp*. was observed *(Fig. 3C)*. In the intestines of these fish, abundant intraepithelial myxosporeans with inflammatory cells infiltrations were seen in propria mucosa. The observed main microscopical lesions in the gills were marked edema, hyperemia, telangiectasia, adhesions in the secondary lamellae and increased numbers of eosinophilic granular cells (EGC). In addition, at farm no- 4, there were eggs of *Sanguinicolid* trematodes in the lumens of arterioles of fish *(Fig. 4A-B)* and at three farms (farms no: 3, 4, 5), *Monogenean* parasites were detected in gills of fish. Vascular and cellular reactions were more severe in the gills of these fish.



Fig 1. Macroscopic appearance of liver was swollen and yellowish-white in color *(arrows)*

Şekil 1. Sarımsı-beyaz renkte ve şişkin karaciğerin makroskobik görünümü *(oklar)*

At the bacteriological examinations, bacterial agents such as *Vibrios spp.* (farm no: 1) and *Staphylococcus spp*. (farms no: 1, 8) were isolated and identified in some affected fish.

DISCUSSION

Outbreaks of WD have been occurred along the coasts of Spain, Italy, Greece, Croatia, Malta and Portugal in sea breams [2,4]. In some outbreaks, mortality rates reached as high as 30-50% and it caused severe economic losses [2,10]. In this study, during the outbreak, approximately two million dead fish were found and it was resulted from serious economic losses in all affected farms.

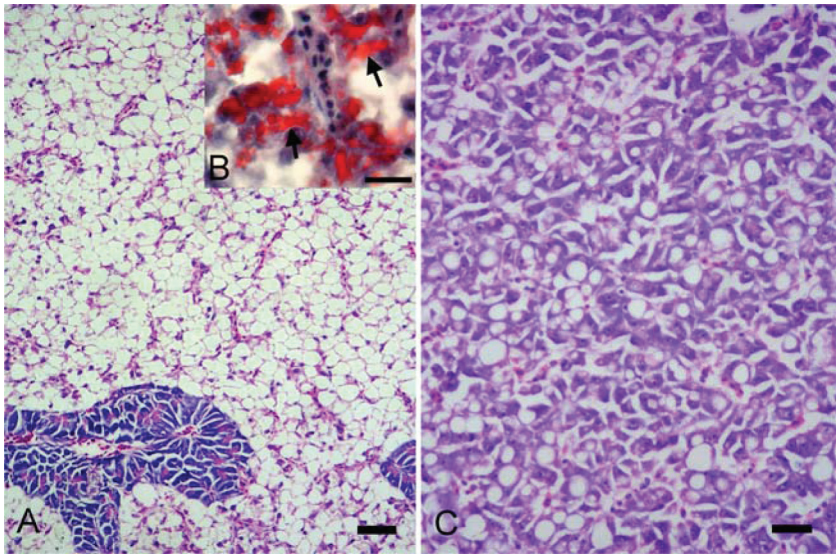


Fig 2. Severe and diffuse infiltration of lipid in the liver of diseased fish. **A-** HE. Bar, 100 µm, **B-** Oil Red O *(arrows)*, Bar, 10 µm, **C-** Mild infiltration of lipid in the liver of control fish. HE, Bar, 50 µm

Şekil 2. Karaciğerde şiddetli ve yaygın lipid infiltrasyonu. **A-** HE. Bar, 100 µm, **B-** Oil Red O *(oklar)*, Bar, 10 µm, **C-** Karaciğerde hepatosit sitoplazmalarında az sayıda bulunan yağ damlaları, kontrol grubu. HE. Bar, 50 µm

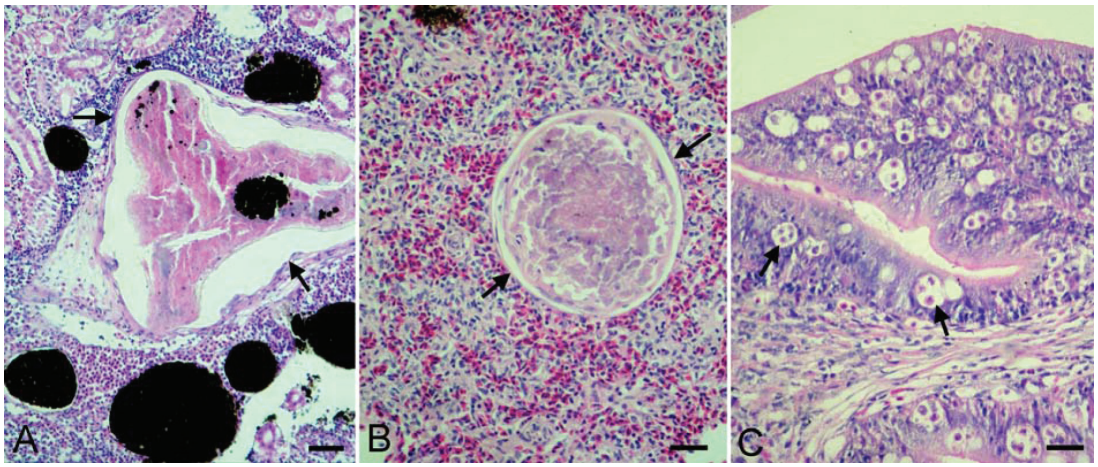


Fig 3. A- Necrotic area in the kidney of diseased fish *(arrows)*. HE. Bar, 100 µm, **B-** Necrotic area in the spleen of diseased fish *(arrows)*, HE. Bar, 50 µm, **C-** Verminous enteritis of diseased fish due to *Myxidium spp.* *(arrows)*, HE. Bar, 50 µm

Şekil 3. A- Böbrekte fokal koagulasyon nekroz alanı (oklar). HE. Bar, 100 µm, **B-** Dalakta fokal koagulasyon nekroz alanı *(oklar)*. HE. Bar, 50 µm, **C-** Hasta balıklarda *Myxidium spp*'in neden olduğu verminöz enteritis *(oklar)*, HE. Bar, 50 µm

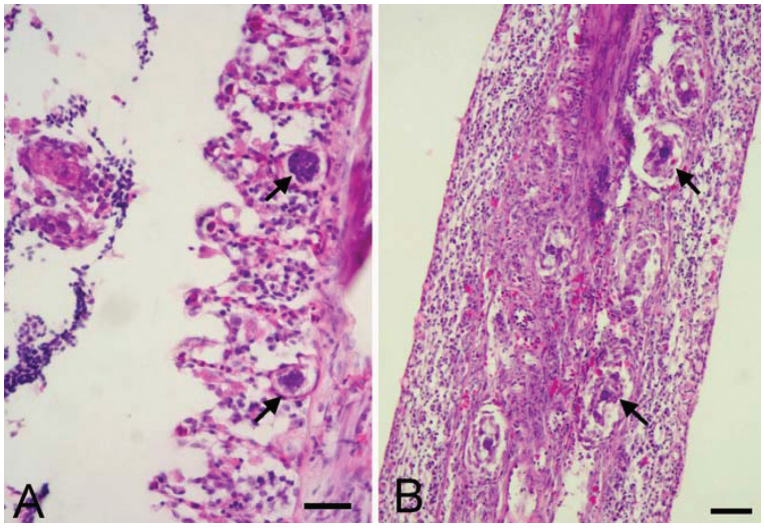


Fig 4. Eggs of *Sanguinicolid* trematodes in the lumens of arteries of gills *(arrows)*, HE.
A- Bar, 50 µm, **B-** Bar, 100 µm

Şekil 4. Solungaç damar lümenlerinde *Sanguinicolid* trematod yumurtaları *(oklar)*, HE.
A- Bar, 50 µm, **B-** Bar, 100 µm

The controversial etiology of WD is still unknown because there is no correlation between the disease and any specific etiological agent [5,6,8]. However, in previous studies, some bacterial agents such as *Aeromonas hydrophila,*

Enterobacteriaceae [5,6], and *Pseudomonas anguilliseptica* [11-13], have been isolated and some parasites such as *Sanguinicolid* trematodes [14] have been reported during the outbreaks of WD. In the present study, according to bacteriological examinations, bacterial agents such as *Vibrio spp. and Staphylococcus spp*. were isolated in three farms. In addition, eggs of *Sanguinicolid* trematode and *Monogenean* parasites were seen in the gills of some fish. It is thought that these isolated agents and detected parasites are not responsible for outbreak because of these etiologic factors is not seen in all affected farms.

It has been reported that WD outbreaks are appeared during the winter seasons and several environmental stress factors such as decrease in salinity and temperature (<13ºC) of sea water influenced the formation of WD [5,8,10,13]. Hepatic steatosis is accentuated in sea bream affected by WD and a polifactorial, metabolic-based pathology which causes high mortality when the water temperature drops below 11-13ºC [2,4]. In this study, in the winter of 2008, some environmental stress factors such as sudden decreases in salinity and water temperature were occurred in Güllük gulf of Bodrum, Turkey. Salinity rate and temperature of sea water were measured at 0, 1% and 10ºC respectively.

Clinically and pathologically, hyposensitivity, erratic swimming, atrophy of the exocrine pancreas, pale and friable liver related to hepatic steatosis and necrosis in the muscles are reported as characteristic findings of WD [2,4,8]. In the present study, necrosis in the muscles and atrophy of the exocrine pancreas were not seen. Pathological changes may occur in direct response to the stress factors and this state may lead to increased respiration, oxygen consumption and depletion of stored body lipids [15]. Previous studies on WD indicated that macroscopically friable and whitish or yellowish liver associated with hepatic steatosis is noted in all fish [3,8,16]. It has been argued that lipid infiltration in the liver is a rapid lipid accumulation and an adaptive mechanism for the response to decrease in water temperature [3,17]. It was thought that, in this study, hepatic steatosis in many fish was occurred in liver tissue for the response to decrease in water temperature as an adaptive mechanism. In addition, there has been no previous report about multifocal well encapsulated necrotic areas in the liver, kidney and spleen for WD. But, these necrotic areas were observed at four farms (farms no: 2, 4, 6, 7) in this study. Furthermore, in many experimental and natural fish disease, pathological findings of gills have been reported [18-20]. In the present study, lesions were found in gills consisted of edema, hyperemia, telangiectasia, adhesions in the secondary lamellae and increased numbers of EGC in accordance to literatures, and they were recorded more severe in fish with gill parasites. Although, these gill findings have not been reported in WD, it was considered that they could be nonspecific and could not relate to WD.

In the present study, a common etiologic pathogen agent was not detected in affected fish farms. In addition, reductions in fish deaths with carriage of the affected fish cages where the water temperature was measured as 16-17ºC in the gulf supported that cause of outbreak in sea breams might be WD associated with decreases in water temperature.

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Kafkas Univ Vet Fak Derg
19 (2): 347-350, 2013
DOI: 10.9775/kvfd.2012.7161

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

SHORT COMMUNICATION

Concentrations of Total Serum Immunoglobulin E, A, G and M in Stray Dogs with Healthy and Dermatological Problems

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Makale Kodu (Article Code): KVFD-2012-7161

Summary

In this study, clinical and laboratory findings along with serum IgE, IgA, IgM and IgG concentrations were determined in stray dogs (healthy and with dermatological problems). It was determined that skin lesions were localized in the rate of 69.8% (n=44) and generalized in the rate of 30.2% (n=19) of sick animals. Serum IgE concentration in stray dogs with dermatologic problems was significantly higher (P<0.001) than that of healthy dogs whereas serum IgM and G concentrations were significantly lower (P<0.05). It was evaluated that, serum total immunoglobulin concentrations could be useful in making contribution to differential diagnosis of skin diseases in stray dogs.

Keywords: *Skin disease, Immunoglobulin E, A, G and M, Dog*

Sağlıklı ve Dermatolojik Problemli Başıboş Köpeklerde Total Serum İmmunoglobulin E, A, G ve M Konsantrasyonları

Özet

Bu araştırmada sağlıklı ve deri problemleri bulunan başıboş köpeklerde serum IgE, IgA, IgM ve IgG konsantrasyonları ile birlikte klinik ve laboratuar bulgular belirlendi. Köpeklerde gözlenen tüm deri lezyonlarının %69.8 (n=44) oranında lokalize, %30.2 (n=19) oranında generalize lezyonlardan oluştuğu belirlendi. Deri problemleri bulunan köpeklerde sağlıklı gruba göre IgE konsantrasyonunun önemli düzeyde (P<0.001) yüksek, IgG ve IgM konsantrasyonlarının ise önemli düzeyde (P<0.05) düşük olduğu tespit edildi. Sonuç olarak, serum total immunoglobulin konsantrasyonlarının deri hastalıklarının ayırıcı tanısına katkı sağlayabileceği değerlendirildi.

Anahtar sözcükler: *Deri hastalığı, İmmunoglobulin E, A, G ve M, Köpek*

INTRODUCTION

Most skin diseases are diagnosed by detailed anamnesis, physical examination and able to be treated [1]. Immunoglobulin E (IgE)-mediated hypersensitivity against environmental allergens is associated with atopic diseases in both humans and dogs [2]. Significant increase in serum IgE concentration has been observed in flea-bite allergic and atopic dermatitis. IgE is also known to be principal antibody class in food allergy [3]. Fadok [4], has suggested that measurement of IgE with ELISA was a useful tool in the diagnosis of food allergy. Similarly, it has been suggested that measurement of IgE using ELISA was a reliable method (93-97%) in the diagnosis of atopic dermatitis in dogs [5]. Day [6], has suggested that markedly low serum IgA and subnormal serum IgG concentrations was found in Rottweiler pups with inflammatory skin diseases. In the majority of canine cases of bacterial diseases, hypersensitivity disorders, endocrine dermatosis, dermatomycosis, parasitic disease, cutaneous Leishmaniasis and non-specific dermatopathies of uncertain aetiology were found to show intercellular deposits of IgG, M and A in skin sections [7].

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Stray dogs are susceptible to many diseases due to life conditions. Stray dogs live in dirty environments, eat spoiled food remains in dump, sleep in dirty areas and are highly susceptible to a wide variety of fatal diseases. In a study, low body condition score (70%), and skin problems (69%) were the most common health problems in stray dog population [8]. Therefore, this study was carried out in the stray dogs.

The aim of this study was to determine IgE, A, G and M concentrations, and to evaluate their clinical value in the differential diagnosis of skin disease in stray dogs by comparing healthy and sick individuals.

MATERIAL and METHODS

A total of 84 stray dogs [21 healthy (control group) and 63 with dermatologic problem (experimental group)] of both sexes, and different ages (from 1 to 3 years old) were used as materials. All dogs admitted to the Animal Hospital of Faculty of Veterinary Medicine for treatment from the Animal Facilities of Konya Municipality. The study was approved by the Ethic Committee of Selcuk University Faculty of Veterinary Medicine (code: 2012/052).

All dogs were examined for the presence of dermatological disorders including pruritis, erythema, papula, pustule, seborrhoea, crusted lesions, hyperpigmentation, erosion, ulcer and complications (pyoderma, dermatitis etc.) were evaluated in the dogs. Endo- and ecto-parasitic examinations were performed in all dogs.

Blood samples were collected from each dog by cephalic venipuncture into plain and anticoagulated Vacutainer tubes (Venoject® - Terumo Corp. Belgium) in the morning following 12 h of fasting. Blood samples with anticoagulant were kept for complete blood count. Serum was separated by centrifugation at 3.000 rpm for 15 min and stored at -20°C until biochemical analyses. Serum Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Creatinine phosphokinase (CPK) enzyme activities, total protein (TP), albumin (ALB), triglyceride (TRI), cholesterol (COL), urea nitrogen (BUN) and creatinine (CR) concentrations were determined using a commercial enzyme immunoassay kit (Biocon®) on a Schimadzu spectrophotometer (UV-Vis 2100 Model). Serum total immunoglobulin concentrations (IgE, A, M and G) were measured by use of a commercially available Sandwich ELISA kit (E40-125, E40-104, E40-118 ve E40-116; Bethyl Lab. Inc., Montgomery, TX). Complete blood count (erythrocyte (RBC), leukocyte (WBC), haemoglobin (Hb) and packed cell volume (PCV) was performed in all dogs.

Data were analyzed by the two-sample *t* test in order to detect statistically significant differences between the results of control and experimental groups (SPSS 10, Statistical Package of Social Science, SPSS Inc., USA).

RESULTS

The local and general skin lesions were determined in 69.8% (n=44) and 30.2% (n=19) dogs, respectively. Local lesions were observed in neck, thorax, extremities, tail, gluteal areas, periocular and perioral areas of the face, abdomen, inguinal areas and ears. *Table 1* shows the extent and rates of these lesions. Non of the dermatologic lesions were observed in healthy dogs.

Serum IgE concentration was significantly higher (P<0.001) while serum IgG and M concentrations were significantly lower (P<0.05) in dogs with dermatological problems *(Table 2)*. The biochemical and haematological parameters measured for each group and the results of statistical analyses are summarized in *Table 3* and *Table 4* respectively.

Table 1. *The extent and rates of skin lesions in stray dogs with dermatological problems (n=63)*
Tablo 1. *Deri problemleri bulunan başıboş köpeklerde deri lezyonlarının kapsamı ve oranları*

Lesions	%	Lesions	%
Pruritis	75.7	Hyperkeratosis	35.1
Hyperpigmentation	45.9	Seborrhoea	21.6
Papula	24.3	Erosion	18.9
Pustule	18.9	Ulceration	10.8
Alopecia	86.5	Pyoderma	18.9
Crustacea	45.9		

Table 2. *Total serum concentrations of IgE, A, G and M (g/L) in healthy stray dogs and stray dogs with dermatological problems*
Tablo 2. *Sağlıklı ve deri problemleri bulunan başıboş köpeklerde total serum IgE, A, G ve M (g/L) konsantrasyonları*

Parameters	Healthy Dogs Mean±SD (n: 21)	Dogs with Dermatological Problems Mean±SD (n: 63)	P
IgE	0.029±0.028 (0.005-0.122)	0.092±0.056 (0.027-0.203)	0.001***
IgA	0.78±0.38 (0.26-1.45)	0.62±0.33 (0.22-1.58)	0.127-
IgG	16.35±2.74 (10.57-19.98)	14.33±2.67 (10.29-19.09)	0.015*
IgM	1.45±0.53 (0.79-2.6)	1.10±0.39 (0.59-2.08)	0.015*

*-: P>0.05, * P<0.05 ** P<0.01 *** P<0.001*

DISCUSSION

In this study, clinical and laboratory findings along with serum IgE, IgA, IgM and IgG concentrations were determined and their contribution to differential diagnosis of skin diseases in healthy and dermatologically sick stray dogs. Serum IgE concentration in stray dogs with dermatologic problem was significantly higher (P<0.001) than that of

healthy dogs whereas serum IgM and G concentrations were significantly lower (P<0.05).

The significant increases of IgE concentrations were observed in the cases of flea-bite dermatitis, atopic dermatitis and food allergy [6,9]. Taszkun [10] found polysensitization in 98.6% of dogs with atopic dermatitis in intradermal skin tests. Hence, the differential diagnosis of dermatologic disease is difficult problem for veterinary clinician. Hill et al.[11] have found that increases of total serum IgE and IgG concentration and decrease of total serum IgA concentration in atopic and parasitized dogs. In a study [12], IgE and IgG response to different food antigens have found indicating significant variation between dogs with atopic dermatitis (78.0-92.3 µg/ml, 2.3-4.01 µg/ml), gastrointestinal diseases (21.9-98.4 µg/ml, 2.6-4.09 µg/ml), and healthy (55-100 µg/ml, 1.9-4.12 µg/ml). A variety of factors may influence antigen-specific serum IgE production. These include possible genetic variation within and between breeds, the type of antigen, and the dose, route, frequency and interval of administration. Previous or concurrent gastrointestinal, parasitic and microbial infections may influence immune responses [11]. Even age and gender may be associated with variation in total serum IgE concentrations [13]. In our study, mean total serum IgE concentrations significantly increased (P<0.001) in stray dogs with dermatological problems. So, it could be said that increases observed in the serum IgE concentrations may be indicator of any allergic diseases in stray dogs.

Day [6], has reported that markedly low serum IgA and subnormal serum IgG concentration was found in Rottweiler pups with inflammatory skin disease. IgA concentrations in serum and skin washings have been compared in a study [14] and showed that there was no significantly difference in the mean serum IgA concentration in normal dogs versus atopic animals. When skin washings from all sites in both groups were compared, atopic dogs had significantly greater concentrations of IgA in their skin washings than normal dogs. In a study [14] further investigations that need to determine whether the greater concentrations were caused by nonspecific inflammation or by secretion of allergen-specific IgA onto the skin surface were emphasized. Similarly, skin sections from 71 dogs and 10 cats with bullous autoimmune skin diseases and various non-autoimmune dermatopathies have been studied for the presence of immunoglobulins (canine IgG, IgM, IgA; feline IgG) using the direct immunofluorescence and indirect immunoperoxidase methods. Positive reactions have been found in canine cases with hypersensitivity disorders,

Table 3. *Biochemical parameters in healthy stray dogs and stray dogs with dermatological problems*
Tablo 3. *Sağlıklı ve deri problemleri bulunan başıboş köpeklerde biyokimyasal parametreler*

Parameters	Unit	Healthy Dogs Mean±SD (n: 21)	Dogs with Dermatological Problems Mean±SD (n: 63)	P
ALT	U/L	31.9±14.88	16.9±11.3	0.004**
AST	U/L	28.6±12	27±22.17	0.833-
ALP	U/L	111.2±42.39	208.38±157.55	0.067-
CPK	U/L	162.1±66.54	210.95±137.07	0.300-
TP	g/dl	5.89±0.677	6.05±1.10	0.683-
ALB	g/dl	3.73±0.298	3.114±0.292	0.001***
TRI	mg/dl	97.1±79.25	32.95±20.8	0.001***
KOL	mg/dl	169.2±55.71	162.43±32.97	0.673-
BUN	mg/dl	20.1±6.82	59.57±23.91	0.001***
CR	mg/dl	0.75±0.23	0.51±0.39	0.096-

*-: P>0.05, * P<0.05 ** P<0.01 *** P<0.001*

Table 4. *Hematological parameters in healthy stray dogs and stray dogs with dermatological problems*
Tablo 4. *Sağlıklı ve deri problemleri bulunan başıboş köpeklerde hematolojik parametreler*

Parameters	Unit	Healthy Dogs Mean±SD (n: 21)	Dogs with Dermatological Problems Mean±SD (n: 63)	P
WBC	$x10^3$ /µl	19.42±9.49	13.44±2.11	0.115-
RBC	$x10^6$ /µl	5.52±1.4	7.12±0.721	0.010*
HGB	g/dl	11.59±2.66	15.23±1.26	0.002**
HCT	%	35.06±8.566	46.34±4.74	0.003**
MCV	fL	64.51±5.11	65.57±4.44	0.634-
MCHC	g/dl	33.36±1.65	33.91±0.96	0.417-

*-: P>0.05, * P<0.05 ** P<0.01*

endocrine dermatosis, dermatomycosis, parasitic disease, cutaneous Leishmaniasis and in cases with non-specific dermatopathies of uncertain aetiology [15]. Furthermore, it was suggested that the determination of anti-canine IgG, IgM and IgA using an immunoperoxidase method in skin sections from dogs with autoimmune skin disease were useful [9]. In our study, it could be considered that insignificant decreases (P>0.05) in serum IgA concentrations and significant increases (P<0.05) in serum IgG and IgM concentrations might be resulted from local accumulation in skin surface.

Serum ALT, ALB and TRI concentrations in dogs with dermatologic disease significantly decreased (P<0.01) when compared with healthy dogs in our study. The BUN concentration significantly increased (P<0.01) in dogs with dermatologic disease *(Table 3)*. It was determined that these parameters were including reference range except BUN concentration which was interpreted as prerenal azotemia and dehydration considered along with haematological parameters. The significant increase of RBC, Hb and PCV concentrations were found in dogs with dermatologic disease in this study *(Table 4)*. This might be attributed to hemoconcentration and dehydration on the basis of clinical and laboratory findings. When management and feeding conditions for these dogs were evaluated, it was concluded that these dogs were fed insufficiently by an inappropriate diet.

Skin disease of uncertain aetiology is common in the stray dogs and generally complicated by allergic, parasitic, and inflammatory conditions. Therefore clinical diagnosis of stray dogs with dermatologic disease is challenging cases. In our study, it was also concluded that serum total immunoglobulin concentrations (IgE, A, G and M) could be useful in making contribution to differential diagnosis of allergic or inflammatory skin diseases in stray dogs. However, the measurement of specific immunoglobulin formations could be recommended in the definitive diagnosis of allergic skin disease in stray dogs.

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Kafkas Univ Vet Fak Derg
19 (2): 351-353, 2013
DOI: 10.9775/kvfd.2012.7613

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

SHORT COMMUNICATION

Techniques to Increase Queen Production in *Bombus terrestris* L. Colonies [1]

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[1] *This study was supported by Akdeniz University Scientific Researches Projects (Project number of "2010.02.0121.042") and was presented at "First International Biology Congress" held on 24-26 September 2012, Bishkek/KYRGYZSTAN*

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Makale Kodu (Article Code): KVFD-2012-7613

Summary

To increase the production of queens in *B. terrestris* colonies, after the emergence of the first workers, colonies were randomly allocated to four groups as comprised 4 colonies in each of them. Colonies in control group (group 1) were formed without adding workers or pupae from a different colony, while colonies in group 2, group 3 and group 4 were designed by adding 60 pupae from a different colony, adding 60 workers from a different colony and placing two colonies (queens) together in the nest box, respectively. The two- queen colonies could not be developed because of aggressiveness towards each other. The average number of queens produced per colony in control, group 2, and group 3 colonies were 56.3±35.9, 72.5±25.0 and 145.3±61.8, respectively. The results showed that adding workers from a different colony can be used as a useful method in the rearing of bumblebee queens.

Keywords: *Bombus terrestris, Queen breeding, Colony management*

Bombus terrestris L. Kolonilerinde Ana Arı Üretimini Artırma Teknikleri

Özet

B. terrestris kolonilerinde ana arı üretimini artırmak için ilk işçi arıları çıktıktan sonra koloniler, her birinde dört adet koloni olacak şekilde rast gele dört gruba ayrılmıştır. Kontrol grubunu (grup 1) oluşturan kolonilere farklı bir koloniden işçi arı veya pupa eklenmemişken, 2. gruptaki kolonilere başka bir koloniden 60 adet pupa, 3. gruptaki kolonilere farklı bir koloniden 60 adet işçi arı eklenmiştir. 4. gruptaki koloniler ise tek bir yuvada iki ana arının (kolonin) birleştirilmesiyle oluşturulmuştur. Birbirlerine karşı saldırgan davranışlardan dolayı iki ana arılı koloniler geliştirilememiştir. Koloni başına üretilen ortalama ana arı sayısı kontrol grubu, 2. grup ve 3. grup kolonilerinde sırasıyla 56.3±35.9 adet, 72.5±25.0 adet ve 145.3±61.8 adet bulunmuştur. Elde edilen sonuçlar, farklı bir koloniden işçi arı ilavesinin bombus arısı ana arı yetiştiriciliğinde faydalı bir yöntem olarak kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Bombus terrestris, Ana arı üretimi, Koloni yönetimi*

INTRODUCTION

Recently commercially reared bumblebee colonies are increasingly used in greenhouses and orchards for pollination of different crops. Commercial bumblebee rearing has expanded rapidly since 1987 and almost one million colonies are produced annually. Currently five species of bumblebees are reared commercially. The main species is the Eurasian *Bombus terrestris* L. Bumblebee producers today have reared colonies under standardized breeding conditions [1]. These producers have developed their own rearing systems which have become independent from field collected queens, but commercial breeders have not published any comparative data. One of the major problems of the breeding bumblebees is the production of queens for continuing the next rearing cycle [2]. *B. terrestris* colonies show much variation in the number of workers, males, and queens produced [3]. Some colonies produce only males or only queens, some colonies produce both males and queens, while some colonies produce neither males nor queens, even if colonies are maintained under similar controlled conditions [4-6]. In commercial rearing, one of the main criteria is how many

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of 100 queens can establish colonies of saleable quality (including around 50 workers). Early male and progeny queen producing in the colonies, mating, managing diapause and the founding of new colonies are major problems during the year round rearing of bumblebees. Major losses occur during these stages [1-3]. Although accurate information is not available, this ratio is estimated to be not more than 50% in commercial companies. Therefore, main difficulty for commercial *B. terrestris* rearing has been the production of young queens in sufficient numbers to meet the demand of a rapidly increasing market for colonies [7]. The objective of this study was to compare three different colony models; colonies with additional workers, colonies with additional pupae and two-queen colonies in order to improve queen production in *B. terrestris* colonies.

MATERIAL and METHODS

Experimental queens were obtained from *Bombus terrestris* L. colonies reared in the Animal Science Laboratory, Akdeniz University. After the mated queens had hibernated for two months in refrigerator at 4.0±0.5°C, they were anesthetized with CO_2 once for 30 min and placed separately in the small starting box with two callow honey bee workers to stimulate egg laying. All queens and colonies were kept in captivity in standard laboratory rearing conditions (27±1°C and 60±5% RH) and fed with the same sugar syrup and fresh pollen collected from honeybee colonies ad-libitum. After the emergence of the first workers, when the colonies comprised 10-20 workers and many diploid larvae, experimental colonies were randomly divided into four groups. The following groups were tested: (group 1) the control group (without adding workers or pupae from a different colony), (group 2) adding 60 pupae from a different colony, (group 3) adding 60 workers from a different colony and (group 4) placing two colonies (queens) together in the nest box. A total of four colonies were used for each group. Before the workers adding in group 3, workers were kept in a room without any queen and brood for 24 hours. In two queen group, to prevent aggressiveness towards each other, queens and workers from two different colonies were exposed to CO_2 for 5 min. Immediately afterwards, queens and workers from two different colonies were placed together in the nest box. Colony development was tracked by daily observation. During observations, produced new queens (gynes) were removed colonies and noted to calculate the total number of queens produced by each colony. Descriptive statistics relating to the total number of queens (gynes) produced by each colony were calculated by using MINITAB statistical program.

RESULTS

In this study, various methods have been tested to improve the production of queens in *B. terrestris* colonies. One of them is the two- queen method. A total of eight colonies were used for this method. As soon as the queens and workers from two different colonies were exposed to CO_2 for 5 min, two different colonies were placed together in the nest box. However, because of aggressiveness towards each other, one of the two queens died in the each nest box. Therefore two- queen colonies could not be obtained. *Table 1* shows the comparison of the other three groups. All the colonies that were added workers produced queens, but only %50 (2/4) of control colonies produced queens. Colonies that were added workers produced a total of 581 queens, with an average of 145.3 per colony, whereas colonies that were added pupae produced a total of 290 queens, with an average of 72.5 per colony. Control colonies produced a total of 225 queens, with an average of 56.3 per colony.

Table 1. *Comparison of the numbers of queens produced in colonies*
Tablo 1. *Kolonilerde üretilen ana arı sayılarının karşılaştırılması*

Colony Number	Groups		
	Group 1 (Control)	Group 2	Group 3
1	0	0	36
2	0	79	64
3	75	105	173
4	150	106	308
Total numbers of queens produced	225	290	581
Means± SE	56.3±35.9	72.5±25.0	145.3± 61.8

DISCUSSION

Many factors such as split sex ratio, worker/larva ratio, food quality and quantity, diapause history of founder queen have an influence on male and queen production [4,8]. Previous studies showed that native *B. terrestris* populations tend to produce fewer queens than commercial colonies. Yeninar and Kaftanoglu [9] and Yeninar et al.[10] reported that the colonies founded by the queens collected from the field produced an averaged of 27.8±9.1 and 31.3±4.61 queens, respectively. Similarly, Ings et al.[11] found that commercial colonies produced significantly more queens than native colonies under identical field conditions. The high queen production of commercial colonies may be due to the short diapause and/or queen production process. This is because commercial companies are likely to minimize diapause length and have selected for high queen producing colonies to increase turnover [11]. Previous studies also demonstrated that queenlessness induced significantly earlier onset of queen production [5,6,8]. But Gosterit et al.[12] found that queen removal did not seem to have a direct influence on the number of gynes and males produced, although queenless colonies started to gyne production significantly earlier than queenright colonies. Queen and male production occur under several different social conditions; not only in the presence of the queen, but also

in the absence of queen [5]. Ruijter et al.[13] discovered that adding workers from different colony was a significant improvement in the rearing of bumble queens. Similarly in this study, colonies that were added workers reared more than twice as many queens as the control colonies. Therefore adding workers from different colony can become a useful method in the queen rearing of *B. terrestris*. But there is a notable variation among colonies in terms of the number of queen produced. To optimize queen production all the factors affecting queen production should be examined in detail.

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Kafkas Univ Vet Fak Derg
19 (2): 355-358, 2013
DOI: 10.9775/kvfd.2012.7808

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

CASE REPORT

A Case Report: Colic Due to Jejuno-jejunal Intussusception in A Mare

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Makale Kodu (Article Code): KVFD-2012-7808

Summary

This paper reports the clinical findings, the results of diagnostic imaging, the medical management, and the results of necropsy of a 2 year old Arabian mare suffering from jejuno-jejunal intussusception. Determining clinical parameters for the in-live diagnosis were the characteristic bull´s eye appearance of a part of the small intestine at ultrasonography, tubular structures found at transrectal palpation and the abnormal peritoneal fluid obtained by abdominocentesis.

Keywords: *Mare, Colic, Jejuno-jejunal intussusceptions*

Olgu Sunumu: Bir Kısrakta Jejuno-jejunal Invaginasyon Nedeniyle Oluşan Kolik

Özet

Bu olgu sunumunda, jejuno-jejunal invaginasyonlu 2 yaşlı Arap ırkı bir kısrakta klinik bulgular, tanısal görüntüleme, medikal yönetim ve otopsi sonuçları bildirildi. Anlık teşhis için belirlenen klinik parametreler; ultrasonografide ince barsakların bir kısmının karakteristik boğa gözü görünümünde olması, rektal palpasyonda tubuler yapıların bulunması ve abdominosentez ile elde edilen anormal periteneonal sıvının belirlenmesi idi.

Anahtar sözcükler: *Kısrak, Kolik, Jejuno-jejunal invaginasyon*

INTRODUCTION

Intestinal intussusceptions are uncommon in horses. But, it must be considered in the differential diagnosis of horses with acute or chronic pain [1]. The more common forms of intussusceptions in horses are jejuno-jejunal, ileal-ileal or ileocecal [2]. The length of intestine that case become invaginated (the intussusceptum) into the more distal segment of intestine (the intussuscipiens) ranges from a few centimeters to as much as a meter. There is no breed and sex predisposition but horses younger than 3 years are affected relatively more than older animals [3-5]. Diarrhea, worm infections and polyps may predispose the horses for this condition [1,2,6,7]. Horses may be predisposed to intussusception because of enteritis, rapid dietary changes, parasitism, mesenteric arteritis, abdominal surgery, intraluminal foreign bodies and intramural masses [1,2,8]. Jejuno-jejunal intussusception was found to be the cause of colic in only 7of 310 (2.26%) horses [9]. Horses with intestinal intussusceptions exhibit signs of acute intestinal obstruction or have a chronic disease, characterized by abdominal pain, reduced apetite, weight loss, intermittent fever, soft feces and pyrexia [10].

The diagnosis and treatment of this disease can be extremely difficult [10]. History, clinical and clinicopathologic examinations are important in the diagnosis of intussusceptions. A diagnosis may be made by ultrasonography or rectal palpation, but exploratory laparotomy is the predominant method [11]. Trans-abdominal ultrasonography is a potentially useful for diagnosing intussusceptions [12-14]. The

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main goals for treating horses with colic include relieving pain, correcting physiologic imbalance and stimulating or maintaining intestinal transit. Primary treatments are aimed at decompressing the gastrointestinal tract, treating dehydration or shock, correcting electrolyte imbalances, stimulating intestinal motility [15]. Treatment of intussusception always requires surgical intervention [16]. The prognosis of the small intestine intussusception depends on many factors, including the length of the bowel affected, duration of the lesion and variability of the bowel [17]. The longer the intussusception is present and the intestine compromised, the worse the prognosis [8,9].

This paper presents a case of a jejuno-jejunal intussusception in a mare that was diagnosed with the use of ultrasonography. This condition, that is relatively simple to diagnose by ultrasonography, should be taken into account in the examination and differential diagnosis of severe colic's symptoms in young horses.

CASE HISTORY

A 2 year old Arabian mare was presented at the equine clinic of Veterinary University of Vienna for severe colic symptoms. Clinical signs included episodes of mild to severe abdominal pain. The horse had a rectal temperature of 37.8°C, the respiratory rate was 20/min and the breathing pattern was costo-abdominal, heart rate was rhythmic but its frequency was increased to 82 beats per minute (bpm) *(Table 1)*, the mucous membranes were slightly cyanotic. Various small excoriations were present on head, the rump and legs. Severe abdominal distension was observed. Abdominal auscultation revealed reduced borborygmy indicating decreased peristaltic. Blood gas analysis showed a blood pH of 7.41 with base excess (BE) of 6.4 mmol/L, hypokalemia (3.2 mmol/L) and high plasma bicarbonate (31.7 mmol/L). These parameters are given in *Table 1*.

The mare was intravenously treated with 0.5-1.0 mg/kg BW xylazine (2%); metamizol-sodium 0.2 mg/kg BW; N-butylscopolammonium bromide 25 mg/kg BW and flunixin meglumine 1.1 mg/kg BW to reduce pain. Effort was made to restore the circulation with intravenous infusion of 0.9% NaCl (1 L/h for 1 h, IV).

Table 1. *Parameters of mare associated with clinical sings*
Tablo 1. *Kısrağın klinik bulguları ile ilgili parametreler*

Parameters	Value of Mare	Reference Range
Blood pH	7.41	7.32-7.44
Base excess (mmol/L)	6.4	-2.5-2.5
Potassium (mmol/L)	3.2	3.5-4.5
Plasma bicarbonate (mmol/L)	31.7	20-28
Rectal Temperature	37.8°C	37.3-38.2
Respiratory rate (min)	20	10-14
Heart frequency (bpm)	82	30-40

At rectal examination the characteristic loops of distended small intestine were identified, the invagination however could not be reached. The peritoneal fluid obtained by paracentesis in the midline was light orange-colored, very cloudy, had an elevated total protein levels (2.8 g/dL), a nearly normal specific gravity (1.022) and an elevated lactate level (2.4 mmol/L). These parameters are summarized in *Table 2*.

Trans-abdominal ultrasound was performed with a 3.5 MHz sector transducer. In the ventral abdomen, severe peritoneal effusion was observed. Several loops of distended small intestine with thickened walls and weak peristaltic were seen. The characteristic "bull eyed" like structure suggesting small bowel intussusception was seen *(Fig. 1)*.

The clinical and ultrasonographic findings combined with the results of the peritoneal fluid analysis supported the diagnosis of intussusception of the small intestine. Surgical treatment was declined by the owner and the mare was euthanized.

At gross pathology dilated jejunal loops and hemorrhagic spots on them were apperent. After the opening of the abdominal cavity, a jejuno-jejunal intussusception, 30 cm tract of small intestine, was noticed *(Fig. 2)*.

The portion of the invaginated jejunum revealed signs of transmural necrosis and large hemorrhagic areas *(Fig. 3)*. Intense hyperemia, acute catarrhal enteritis and severe

Table 2. *Parameters of peritoneal fluid obtained by paracentesis from mare*
Tablo 2. *Kısraktan parasentezis ile elde edilen periteonal sıvı parametreleri*

Parameters	Value of Mare	Reference Range
Total protein levels (g/dL)	2.8	<2.5
Specific gravity	1.022	<1.020
Lactate level (mmol/L)	2.4	≥1.2

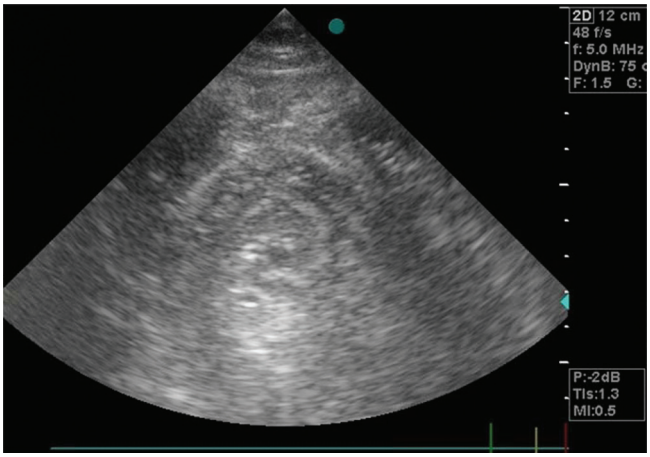


Fig 1. Ultrasonographic appearance of the jejuno-jejunal intussusception (Bull`s eye)

Şekil 1. Jejuno-jejunal invaginasyonun ultrasonografik görünümü (Boğa gözü)



Fig 2. The anatomical macroscopic view of the jejuno-jejunal intussusceptions

Şekil 2. Jejuno-jejunal invaginasyonun anatomik makroskobik görünümü



Fig 3. The invaginated part of the bowel

Şekil 3. İnvagine olan bağırsak bölümü

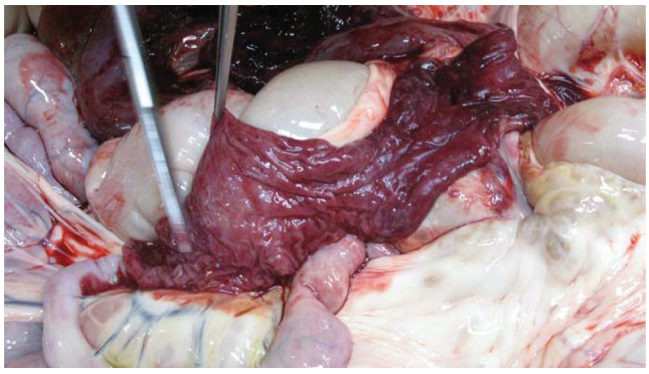


Fig 4. The macroscopic view of the lumen of the invaginated tract after the cross section

Şekil 4. Enine kesitten sonra invagine olan bağırsak lümeninin makroskobik görünümü

necrosis affected the portion of small intestine preceding the intussusception *(Fig. 4)*.

DISCUSSION

Different types of intussusception are recognized in horse such as enteric, ileoileal, ileocecocolic, cecocolic and colocolic. Usually the ileum is involved at or close to, the ileocaecal junction [9,18]. Jejuno-jejunal intussusceptions are less frequently encountered and hence its incidence is very low (2.26%) in horses [9].

Clinical signs are extremely variable depending on the degree of compromise or obstruction of the affected portion of the gut. Pain may range from intermittent mild to severe. Pain may be so uncontrollable that immediate surgical intervention is required [9,10,19,20]. In addition diarrhea and fever may be seen occasionally.

In this case, the horse had mild respiratory compromise, tachycardia, mild cyanosis likely an increased intra-abdominal pressure, a partial ileus and hypokalemic metabolic alkalosis. Hematological and blood biochemical data may be normal, as was shown by Vieitez et al.[21] in foal with jejuno-jejunal intussusceptions. On the other hand clear hematological and biochemical abnormalities are commonly seen. The findings of the current case are similar to those described by Albanese et al.[22] and Lin et al.[23]

In the present case, the young Arabian mare was presented at our clinic for severe colic symptoms. With the rectal findings and echography results the diagnosis was not difficult to make. Since intussusception is a relatively uncommon cause of colic in young or adult horses [1], this disorder may be overlooked if horses are not carefully examined.

Intussusceptions are believed to arise either as a result of segmental motility disorder or as a result of local changes in the intestinal wall [24]. Regarding the latter, a special case is the jejuno-jejunal intussusception that is reported as a complication of jejuno-jejunal anastomosis by researchers [25-27].

In humans and many animal species, transabdominal ultrasonography is an excellent diagnostic procedure to diagnose many abdominal disorders. Bell and Textor [28] suggested that transabdominal ultrasound was extremely useful for diagnosing horses with caeco-colic or caeco-caecal intussusceptions (10/12 and 4/5 horses that were scanned, respectively). The ultrasonographic characterization of intussusception has been given for men [29], foals [12], adult cattle [30], cats [31], and dogs [32]. Bell and Textor [28] could diagnose only 54% of the caecal intussusceptions by rectal palpation and/or by ultrasonography. So, in roughly half of the cases the diagnosis cannot be made by clinical examination and only at explorative surgery a definitive diagnosis can be made.

At a longitudinal sonographic view a sandwich-like appearance of the alternating loops of bowel, with a loop-within-a loop appearance is seen. Fluid distention of the more proximal small intestine may be detected, usually with normal or nearly normal wall thickness and little or no peristaltic activity [12,30,33]. If a "target" or "bull´s eye" lesion is observed, the diagnosis is definitive [12,13]. In this case, the ultrasonographic appearance of the intussusception corresponds to those previously described papers using a 3.5 MHz

transducer [30,33]. The rectal findings in this case were not conclusive but indicated a strangulative small intestinal problem and the final diagnosis was relative simply made by the pathognomic "bull´s eye" aspect seen at ultrasonography, which indicated small bowel intussusception.

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Kafkas Univ Vet Fak Derg
19 (2): 359-363, 2013
DOI: 10.9775/kvfd.2012.7909

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

REVIEW

Nükleotid Dizilerinin Aminoasit Formatına Dönüştürülmesi ve Dünya Veri Tabanlarındaki Verilerle Karşılaştırılması

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Makale Kodu (Article Code): KVFD-2012-7909

Özet

Moleküler biyoloji ve DNA sekans analizi çalışmalardaki gelişmeler sonucu elde edilen yeni bilgi ve verilerin hızlı ve hassas bir şekilde değerlendirilmesi ihtiyacı Biyoinformatik adlı bir bilimin kullanılmasını gerekli kılmıştır. Mikroorganizmalardan virüslere ait hedef alınan gen bölgesinin tüm veya kısmi sekans analizleri yardımıyla filogenetik analizler ve genotiplendirme çalışmaları yapılabilmektedir. Viral proteinleri kodlayan gen sekansları protein dizilerine çevrilebilmektedir. Bu sayede belli pozisyonlardaki nükleotid farklılıklarının aminoasit farklılığına da sebep olup olmadığı gibi durumlar değerlendirilebilmektedir. Nükleotid ve aminoasit sekans sonuçları veri tabanlarındaki diğer verilerle mukayese edilip, benzerlik ile ilgili değerlendirmeler yapılabilmektedir. Elde edilen nükleotid ve aminoasit sekans verilerinin veri tabanlarına girilmesi bilgilerin diğer araştırmacılar tarafından kullanılması açısından çok önemlidir.

Anahtar sözcükler: *Biyoinformatik, Veri tabanları, Sekans analizi*

Translation of Nucleotide Sequences to Amino Acids and Their Comparison with Data Stored in Databases

Summary

The advances in the field of molecular biology and DNA sequencing have been leading to obtain a large amount of new data and information. The rapid and precise evaluation of these data has made it imperative to use a new interdisciplinary science called Bioinformatics. Of microorganisms, viruses, whole or partial sequence analysis of the target gene can be used for genotyping and phylogenetic analysis studies. The viral DNA sequences encoding viral protein can be translated to amino acid sequences. In this way, whether or not certain mutation(s) on nucleotide sequences result in amino acid substitutions can be investigated. In addition, the obtained nucleic and amino acid sequence data can be compared with those stored in the databases by means of similarity researches. Thus, it is very important to store sequence results to databases.

Keywords: *Bioinformatics, Databases, Sequence analysis*

GİRİŞ

Moleküler biyoloji ve DNA sekanslama teknolojilerindeki hızlı gelişim sonucu araştırma merkezlerinde devamlı ve büyük miktarda yeni veriler elde edilmektedir. Bu verilerin hızlıca değerlendirilmesi, diğer araştırmacıların erişebilecekleri şekilde depolanması amacıyla uluslararası veri tabanları oluşturulmuştur [1]. Veri tabanları, elde edilen bilgi ve verilerin depolandığı, bünyelerinde bulunan yazılımlar sayesinde çeşitli analizlerin yapılabildiği, araştırmacıların kullanımına açık programlardır. Günümüzde Biyolojik sistemler ve özellikle sekans analizleri sonucu elde edilen kapsamlı verileri sağlıklı, doğru biçimde değerlendirme ihtiyacı; biyoloji, biyokimya, tıbbi bilimler, bilişim bilimleri, matematik ve istatistik gibi alanların işbirliği Biyoinformatik adı verilen yeni ve uygulamalı bir bilimin doğmasına sebep olmuştur [2,3].

Biyoinformatik, Amerikan Ulusal Biyoteknoloji Bilgi Merkezi (NCBI) tarafından biyoloji, bilgisayar bilimi ve bilgi teknolojilerinin birlikte kullanıldığı bir bilim dalı olarak tanımlanmaktadır [4].

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Bu derlemede, veri tabanları, nükleik asit sekanslarının aminoasit formatına çevrilmesi, dünya veri tabanlarına girilmesi ve veri tabanlarındakilerle karşılaştırılması uygulamaları açıklanacaktır.

VERİ TABANLARI

Dünyada çeşitli canlı ve organizmalara ait nükleotid dizi bilgilerinin organizasyon ve depolama işlemini üstlenen üç veri tabanı vardır. Bunları Amerika Birleşik Devletleri (ABD) merkezli 'NCBI-GenBank' (Amerikan Ulusal Biyoteknoloji Bilgi Merkezi (Gen Bankası; ABD-Maryland) [5,6], Avrupa orijinli 'EMBL' (Avrupa Moleküler Biyoloji Laboratuvarı; İngiltere-Hinxton) [7] ve Japonya merkezli 'DDJB' (Japonya veri tabanı; Japonya-Mishima) [8] oluşturmaktadır. Bu üç veri tabanı 'International Sequence Database Colloboration (INSDC) olarak işbirliği halinde olup, günlük veri alış verişi yoluyla sekansların üniform bir şekilde depolanıp erişime açık hale getirilmesine olanak sağlarlar [6].

GenBank ve Kullanımı

GenBank, nükleotide sekanslarının ve sekanslara ait biyografik ve biyolojik bilgilerin depolandığı en önemli veri tabanı özelliğini taşımaktadır. Bünyesinde 380.000'den fazla genus sınıfında veya daha alt sınıfta organizmaya, insan genom projesi gibi geniş kapsamlı büyük projeler, farklı çalışmalardan ve çevresel örnekleme metotları ile yapılan projeler sonucu elde edilen sekans bilgilerini kapsamaktadır. Nisan 2011 tarihi itibariyle GenBank üzerinde yaklaşık olarak 135.440.924 sekans kaydında 126.551.501.141 nükleotide ve "Whole genome shotgun sequences" (WGS) bölümünde de 62.715.288 sekans kaydında 191.401.393.188 nükleotide ait bilgi bulunmaktadır [6].

GenBank Veri Erişimi

GenBank üzerindeki sekans kayıtlarına "NCBI Entrez" erişim sistemi yoluyla erişilebilir [9]. Bir guruba veya filogenetik analize konu olan sekanslar topluca "Entrez Popset" ve kavramsal olarak translate edilen protein kodlayan sekanslar (CDS) "Entrez Protein" üzerinde depolanır. GenBank üzerine kayıtlı olan her bir veri bilimsel literatürlere "PubMed" veya "PubMed central" vasıtası ile bağlanır. Ayrıca, NCBI tarafından sunulan bir başka hizmet olan OMIM ile genler ve genetik rahatsızlıklar ile ilgili günümüze kadar belirlenmiş olan mutasyon ve ilgili bilgilere de erişilebilir [3].

Veritabanı Organizasyonu

Sekans kayıtları GenBank'da kaynak taksonomisi veya uygulanan sekans stratejisine göre gruplandırılır. Bu amaçla 12 adet taksonomik bölüm (BCT, ENV, INV, MAM, PHG, PLN, PRI, ROD, SYN, UNA, VRL, VRT) ve altı yüksek verimlilikte bölümler (EST, GSS, HTC, HTG, STS, TSA) bulunmaktadır. Bunlara ilave olarak, PAT bölümü patent ofislerince sağlanan verileri ve WGS bölümü de tüm genom projelerine ait sekansları bulundurmaktadır. Komple genomlar (www.ncbi.nlm.nih.gov/Genomes) halen GenBank'ın hızlı gelişen bölümünü oluşturmaktadır [5,6].

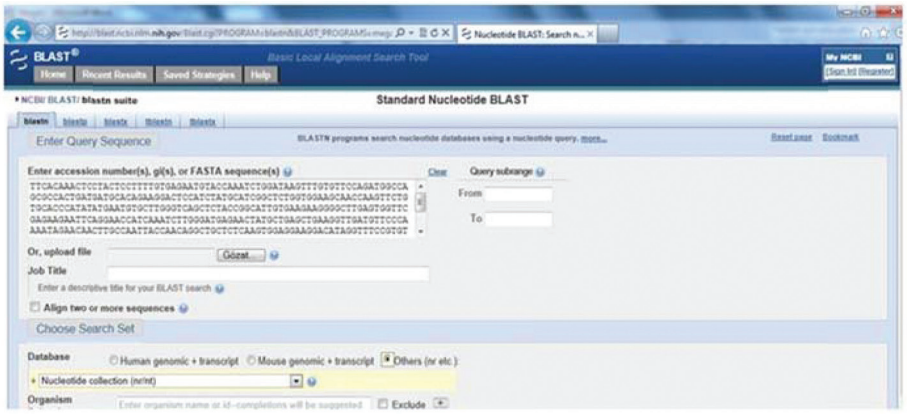
NÜKLEOTİD DİZİLERİNİN AMİNOASİT FORMATINA ÇEVRİLMESİ

DNA dizilerinin protein kodlayan bölümleri ilk olarak Messenger RNA (mRNA) dönüştürüldükten sonra proteini oluşturan aminoasit dizisine çevrilir. Bu aşamada translasyonun başlatılıp, sonlandırıldığı açık okuma alanının 'Open Reading frame' (ORF) belirlenmesi önemlidir. ORF, DNA molekülünden bir parça olup, kodladığı proteine ait aminoasitlere dönüştürüldüğünde stop kodon içermez [10]. Bir viral gen dizisine ait ORF'in belirlenmesi o genin kodladığı protein esas alınarak yapılacak antiviral veya aşı geliştirme çalışmaları açısından önemlidir [11].

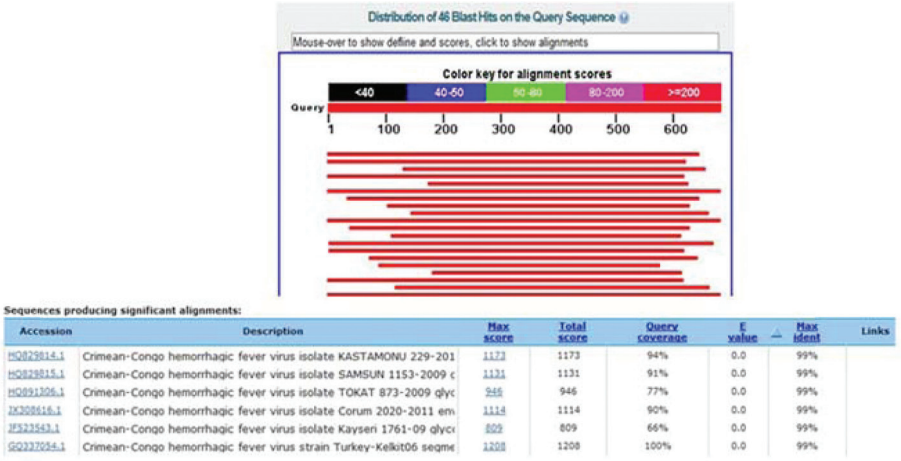
Uygulamada bir virüse ait herhangi bir genin kısmi veya tümü için sekans analizi yapıldığında doğru ORF belirlenmelidir. Bu amaçla çevrimiçi olarak "ExPASy Translate tool" (http://www.expasy.org/tools/dna.html) veya "NCBI ORF finder" (http://www.ncbi.nlm.nih.gov/gorf.html) programları kullanılabilir. Bir gene ait tüm veya kısmi bir DNA dizisi 5' ileri (forward) ve 3' ters (reverse) yönde üçer adet olmak üzere altı potansiyel ORF'e sahiptir. Bir ORF ATG nükleotidleri ve kodladıkları Metionin (M) ile başlar ve genellikle TAA, TAG veya TGA nükleotidlerinden oluşan bir STOP kodon ile sonlanır.

Örnek olarak Ülkemizde 2002 yılından itibaren özellikle Kelkit vadisi ve çevresinde Kırım Kongo Kanamalı Ateşi (KKKA) hastalığı görülmektedir. Bu hastalığa sebep olan Kırım Kongo Kanamalı Ateş virüsü (KKKAV) izolatlarının S ve/veya M, L gen segmentlerinin sekans analizleri yapılarak, ülkemizde ve dünyada daha önce elde edilmiş sekans verileri ile kıyaslanıp, virüslerin filogenetik analizleri yapılabilmektedir. Elde edilen nükleotid sekansları aminoasit formatına çevrilerek nükleotid dizlerindeki farklılıkların aminoasit farklılıklarına (mutasyon varyasyon) sebep olup olmadığı belirlenebilmektedir [12]. Bu durum virüsün antijenik yapısı ve izolatlar arasındaki antijenik farklılıkların araştırılması açısından önemlidir.

Uygulamada KKKAV izolatlarının S veya M segment bölgeleri polimeraz zincir reaksiyonuyla çoğaltılıp (PZR), sekanslanır. Elde edilen ileri (5' forward) ve ters (3' reverse) yönde nükleotide sekans verileri "BioEdit "(http://www.mbio.ncsu.edu/bioedit/bioedit.html) veya "Clustal W" (http:// www.ebi.ac.uk/Tools/msa/clustalw2/) gibi programlar kullanılarak karşılaştırılır [13,14]. Böylece hatalı olan veya iyi okunmamış bölgeler tespit edilir. Bu bölgeler sekans analiz sonucunu gösteren "Sequencher" (http://genecodes.com/) gibi programlar kullanılarak kontrol edilip, hatalar düzeltildikten sonra doğrulanmış sekans verisi elde edilir. Elde edilen sekans verisi 'ExPASy translate tool '(http://web.expasy.orgtranslate/) programı ile aminoasit formatına çevrilir.



a



b

Şekil 1. KKKAV Kastamonu 241-2012 izolatı 540 nükleotide uzunluğunda kısmi M segment sekansı BLAST analizi

a) BLAST sayfasına 540 nükleotide uzunluğunda KKKAV Kastamonu 241-2012 izolatı kısmi M segment envelope glycoprotein precursor geni (Gn) sekansın kopyalanması, **b)** Sonuçların grafik formatında gösterilmesi ve GenBank veri tabanında en yüksek oranda altı benzer eşleşmenin verilmesi, **c)** BLAST analizi yapılan Kastamonu 241-2012 izolatı (Query) ile 99% oranında sekans homolojisi gösteren Corum 2020-2011 izolatı (Subject) eşleşmesinin gösterilmesi

Fig 1. BLAST analysis of 540 nucleotide long partial M segment sequence of the KKKAV Kastamonu 241-2012 isolate

a) Initial BLAST input page with a 540 nucleotide long KKKAV Kastamonu 241-2012 isolate partial M segment envelope glycoprotein precursor gene (Gn) sequence, **b)** Results of the search are given in graphic format at the top, along with the first six hits from the GenBank, **c)** BLAST analysis of Kastamonu 241-2012 isolate (Query), which displayed 99% sequence homology with the isolate Corum 2020-2011 (Subject)

```
>gb|JX308616.1|  Crimean-Congo hemorrhagic fever virus isolate Corum 2020-2011
envelope glycoprotein precursor, gene, partial cds
Length=760

 Score =   976 bits (528),  Expect = 0.0
 Identities = 536/540 (99%), Gaps = 0/540 (0%)
 Strand=Plus/Plus

Query  1    GGGTGTTATACCATCAACAGGGTGAAGTCATTCAAGCTATGCGAAAACAGCGCCACAGGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  140  GGGTGTTATACCATCAACAGGGTGAAGTCATTCAAGCTATGCGAAAACAGCGCCACAGGG  199

Query  61   AAAACCTGTGAGATAGACAGCACTCCGGTTAAGTGCAGGCAAGGTTTCTGTTTAAAAATC  120
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  200  AAAACCTGTGAGATAGACAGCACTCCGGTTAAGTGCAGGCAAGGTTTTTGTTTAAAAATC  259

Query  121  ACCCAAGAGGGAAGGGGCCACGTAAAATTATCTAGAGGCTCAGAGGTTGTCCTGGATGCT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  260  ACCCAAGAGGGAAGGGGCCACGTAAAATTATCTAGAGGCTCAGAGGTTGTCCTGGATGCT  319

Query  181  TGCGACTCAAGCTGTGAAGTAATGATACCTAAAGGCACTGGAGATATCCTAGTGGACTGT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  320  TGCGACTCAAGCTGTGAAGTAATGATACCTAAAGGCACTGGAGATATCCTAGTGGACTGT  379

Query  241  TCAGGCGGGCAGCAACATTTCTTAAAAGACAACTTAATTGACCTAGGGTGCCCCCATATC  300
            ||||| |||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  380  TCAGGTGGGCAGCAACATTTCTTAAAAGACAACCTAATTGACCTAGGGTGCCCCCATATC  439

Query  301  CCGCTATTGGGTAAGATGGCTATTTACATTTGCAGAATGTCAAATCATCCTAGAACAACC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  440  CCGCTATTGGGTAAGATGGCTATTTACATTTGCAGAATGTCAAATCATCCTAGAACAACC  499

Query  361  ATGGCTTTTCTCTTCTGGTTCAGCTTTGGCTATGTTATCACTTGTCTATTTTGCAAGGCT  420
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  500  ATGGCTTTTCTCTTCTGGTTCAGCTTTGGCTATGTTATCACTTGTATATTTTGCAAGGCT  559

Query  421  CTTTTTTATTCATTGATAATTATTGGAACACTAGGGAAAAAGATCAAGCAATATAGGGAA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  560  CTTTTTTATTCATTGATAATTATTGGAACACTAGGGAAAAAGATCAAGCAATATAGGGAA  619

Query  481  CTGAAACCTCAAACCTGCACCATATGTGAGACAGCTCCTGTCAATGCAATAGATGCTGAA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  620  CTGAAACCTCAAACCTGCACCATATGTGAGACAGCTCCTGTCAATGCAATAGATGCTGAA  679
```

c

BLAST-NÜKLEOTİDE VE PROTEİN DİZİ EŞLEŞTİRME ANALİZİ

"Basic Local Alingment Search Tool" (BLAST) (http://blast.ncbi.nlm.nih.gov/) yazılımı NCBI tarafından geliştirilmiş ve biyoinformatik uygulamalarında araştırmacılar tarafından sıklıkla kullanılmaktadır. BLAST analizi ile hedef gen bölgesine ait kısmi veya tüm sekans dizisi (nükleotid- aminoasit) dünyanın değişik merkezlerinde çeşitli araştırıcılar tarafından GenBank veri tabanına girilen sekanslar ile karşılaştırılır. Aynı ve en yüksek benzerliğe sahip olan eşleşmelerdeki benzerlik oranı yüzde olarak verilir [15-17]. BLAST analizi kullanım kolaylığı ve araştırmacıya hızlıca bilgi vermesi açısından önemlidir. Böylece analiz edilen mikroorganizmaya ait sekans verisiyle genetik analiz veya genotiplendirme çalışmaları yapılabilmektedir. Bu durum özellikle ülkemizde dolaşımda olan KKKAV'lerinin genetik olarak takip edilmesi açısından önemlidir. Örnek olarak, 'Türkiye Halk Sağlığı Kurumu' (THSK), Moleküler Mikrobiyoloji Araştırma ve Uygulama Laboratuvarında sekansı yapılan KKKAV Kastamonu 241-2012 izolatının (KSTM241/12) 540 nükleotid uzunluğunda kısmi M segment envelope glycoprotein precursor (Gn) geni nükleotid sekansının BLAST analizi uygulaması *Şekil 1 (a,b,c)*'de gösterilmiştir. Bu uygulamada sekansı değerlendirilen KSTM241/12 izolatın ülkemizde daha önce sekans analizi yapılan izolatlarla yüksek oranda benzerlik gösterdiği belirlenmiştir.

NÜKLEOTİD VE AMİNOASİT DİZİ SEKANSLARININ GenBank VERİ TABANINA GİRİLMESİ

GenBank üzerine verilerin girişi elektronik ortamda çalışmayı yapan araştırmacılar tarafından "BankIt" veya "Sequin" programları kullanılarak yapılmaktadır (www.ncbi.nlm.nih.gov(GenBank/). Bilimsel dergilerin birçoğu gönderilen bir yayının yayınlanması için yayın içeriğinde kullanılan sekansların veri tabanına gönderilmesini şart koşmaktadır. Nükleotid ve aminoasit dizi bilgileri veri tabanlarına; ait oldukları organizma adı, kaynağı, izolasyon yeri, izolasyon tarihi, ait olduğu genin adı, veri tabanına giriş yapan araştırıcıların çalıştığı merkezlerinin adı ve adres bilgileri ile birlikte girilebilmektedir. Her bir gönderi için genelde iki iş günü içinde bir takip numarası verilmekte ve gönderilen bilgiler ve sekanslar veri tabanı tarafından kontrol edilmektedir. Eğer her hangi bir tarih belirtilmiş ise o tarihte veya tarih belirtilmemiş ise veri tabanı tarafından belirlenen bir tarihte veri erişime açılmaktadır. Bu süreçte veri tabanı tarafından veriye ait bilgiler mail ortamında yazara kontrol amacı ile gönderilir. Düzeltme ve ilave bilgiler yine mail ortamında veri tabanına iletilir. Kabul edilen ve erişime açılan her bir veri için bir kayıt numarası (accesion number) verilmekte ve bu numara diğer veri tabanları (DDJB ve EMBL) ile paylaşılmaktadır. Kayıt numarası GenBank veri tabanının üyelik hattı kısmında yer alıp, sekans verisinde ve/veya diğer bilgilerde değişiklikler olsa bile kayıt ömrü boyunca sabit kalmaktadır. Kayıt numarası bilimsel yayınlarda veriyi kaynak gösterme amacıyla da kullanılmaktadır. Sonuç olarak, her GenBank kaydı sekans ve sekansa ait bilgileri içermekte olup kayda özgü erişim numarasına sahiptir. Kabul edilen sekans verisi ile ilgili ilave bilgi ve düzeltmeler veriyi giren araştırmacı tarafından update@ncbi.nlm.nih.gov adresine iletilir [5,6].

GenBank'a ve diğer veri tabanlarına kayıt edilen sekans verilerine ait bilgiler iki bölümden oluşur. İlk bölümü; sekans verisini veritabanına kaydeden araştırmacılara ait bilgiler, laboratuvar adresi, sekansı yapılan organizmaya ve kaynağına ait bilgiler (Strain-izolat adı, ülke, izolasyon kaynağı, izole edilen canlı türü, tarih…), sekans dizisinin başlangıç ve bitiş kodonları, ait olduğu gen ve kodladığı protein ismi gibi bilgileri bulunduran sayfa oluşturur. İkinci bölümü; gene ait nükleotid ve aminoasit sekanslarını FASTA formatında içeren kısım oluşturur [5,6].

SONUÇ

Bilimsel gelişmelerdeki baş döndürücü gelişim ve araştırmaların sürekliliği bir yandan birçok problemin çözümüne katkıda bulunurken, diğer yandan da yeni araştırmalar için temel oluşturmaktadır. Elde edilen bilgi ve verilerin ileri düzeyde analiz ihtiyacı Biyoinformatik biliminin önemini ve kullanımını gittikçe artırmaktadır. GenBank, içeriğinde çeşitli canlı ve mikroorganizmalara ait sekans verilerinin depolandığı ve bünyesinde benzerlik analizlerinin yapılabildiği BLAST gibi programı bulunduran oldukça sık kullanılan kapsamlı bir veri tabanıdır. Bilginin paylaşılması ve diğer araştırmacıların faydalanması açısından elde edilen sekans verilerinin veri tabanlarına kaydedilmesi büyük önem taşımaktadır.

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YAZIM KURALLARI

1- Yılda 6 (Altı) sayı olarak yayımlanan Kafkas Üniversitesi Veteriner Fakültesi Dergisi'nde (Kısaltılmış adı: Kafkas Univ Vet Fak Derg) Veteriner Hekimlik ve Hayvancılıkla ilgili (klinik ve paraklinik bilimler, hayvancılıkla ilgili biyolojik ve temel bilimler, zoonozlar ve halk sağlığı, hayvan besleme ve beslenme hastalıkları, hayvan yetiştiriciliği ve genetik, hayvansal orijinli gıda hijyeni ve teknolojisi, egzotik hayvan bilimi) orijinal araştırma, kısa bildiri, ön rapor, gözlem, editöre mektup, derleme ve çeviri türünde yazılar yayımlanır. Dergide yayımlanmak üzere gönderilen makaleler Türkçe, İngilizce veya Almanca dillerinden biri ile yazılmış olmalıdır.
2- Dergide yayımlanması istenen yazılar Times New Roman yazı tipi ve 12 punto ile A4 formatında, 1.5 satır aralıklı ve sayfa kenar boşlukları 2.5 cm olacak şekilde hazırlanmalı ve resim, tablo, grafik gibi şekillerin metin içindeki yerlerine Türkçe ve yabancı dilde adları ve gerekli açıklamaları mutlaka yazılmalıdır.
Dergiye gönderilecek makale ve ekleri (şekil vs) *http://vetdergi.kafkas.edu.tr* adresindeki online makale gönderme sistemi kullanılarak yapılmalıdır.
Başvuru sırasında yazarlar yazıda yer alacak şekilleri (13 X 18 cm boyutlarından büyük olmamalı) online makale gönderme sistemine yüklemelidirler. Yazının kabul edilmesi durumunda tüm yazarlarca imzalanmış *Telif Hakkı Devir Sözleşmesi* editörlüğe gönderilmelidir.
3- Yazarlar yayınlamak istedikleri makale ile ilgili olarak gerekli olan etik kurulu onayı aldıkları kurumu ve onay numarasını Materyal ve Metot bölümünde belirtmelidirler. Yayın kurulu gerekli gördüğünde etik kurul onay belgesini ayrıca isteyebilir.
4- ***Makale Türleri***
Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır.
Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, giriş, materyal ve metot, bulgular, tartışma ve sonuç ile kaynaklar bölümlerinden oluşur ve toplam (metin, tablo, şekil vs dahil) 10 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 4 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.
Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 2 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.
Gözlem, uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri giriş, olgunun tanımı, tartışma ve sonuç ile kaynaklardan oluşmalı ve 4 sayfayı geçmemelidir.
Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 1 sayfayı geçmemelidir.
Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri giriş, metin ve kaynaklardan oluşmalı ve 10 sayfayı geçmemelidir.
Çeviri, makalenin orijinal formatı dikkate alınarak hazırlanmalıdır.
Yazarla ilgili kişisel ve kuruma ait bilgiler ana metin dosyasına değil, on-line başvuru sırasında sistemdeki ilgili yerlere unvan belirtilmeksizin eklenmelidir.
5- Makale ile ilgili gerek görülen açıklayıcı bilgiler (tez, proje, destekleyen kuruluş vs) makale başlığının sonuna üst simge olarak işaret konularak makale başlığı altında italik yazıyla belirtilmelidir.
6- Kaynaklar, metin içinde ilk verilenden başlanarak numara almalı ve metin içindeki kaynağın atıf yapıldığı yerde parantez içinde yazılmalıdır.
Kaynak dergi ise, yazarların soyadları ve ilk adlarının başharfleri, makale adı, dergi adı (orijinal kısa ad), cilt ve sayı numarası, sayfa numarası ve yıl sıralamasına göre olmalı ve aşağıdaki örnekte belirtilen karakterler dikkate alınarak yazılmalıdır.
Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.
Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.
Editörlü ve çok yazarlı olarak yayınlanan kitaptan bir bölüm kaynak olarak kullanılmışsa, bölüm yazarları, bölüm adı, editör(ler), kitap adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı sırası dikkate alınarak aşağıdaki örneğe göre yazılmalıdır.
Örnek: **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.
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.
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1- Kafkas Üniversitesi Veteriner Fakültesi Dergisi (Journal of the Faculty of Veterinary Medicine, Kafkas University) (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly, covers original papers, short communication and preliminary scientific reports, case reports, observation, letter to the editor, review and translation on all aspects of veterinary medicine and animal science (clinical and paraclinical sciences, biological and basic sciences, zoonoses and public health, animal feeding and nutritional diseases, animal breeding and genetics, hygiene and technology of food from animal sources, exotic animal science). Manuscripts submitted for publication should be written in Turkish, English or German.

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure, table and graphic must clearly be written in both Turkish and foreign language and their appropriate position should be indicated in the text.

The manuscript and its supplementary (figure etc.) should be submitted by using online manuscript submission system at the address of *http://vetdergi.kafkas.edu.tr/*

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4- Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments. Manuscripts written in Turkish consist of the title in Turkish, summary and keywords in Turkish, introduction, material and methods, results, discussion and references and it should not exceed 10 pages including text, tables and illustrations. Manuscripts written in a foreign language should follow the title in foreign language, summary and keywords in foreign language, title in Turkish, summary and keywords in Turkish and the remaining sections described above for the manuscripts written in Turkish.

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Preliminary scientific reports are short description (maximum 2 pages) of partially completed original research findings at interpretable level. These should be prepared in the format of full-length original articles.

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