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

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

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

(Yılda altı sayı yayımlanır)

(Published Bi-monthly)

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

Cilt / Volume : 22

Sayı / Number : 1

OCAK - ŞUBAT
JANUARY - FEBRUARY

Yıl / Year : 2016

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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36040 - Kars / TÜRKİYE
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Fax: +90 474 2426853
E-mail: vetdergi@kafkas.edu.tr

E-ISSN: 1309-2251

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

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

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

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

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Kafkas Univ Vet Fak Derg
22 (1): 1-5, 2016
DOI: 10.9775/kvfd.2015.13236

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

Research Article

Determination of Ochratoxin-A in Cattle Liver By HPLC-FD Method

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Article Code: KVFD-2015-13236 Received: 26.02.2015 Accepted: 09.09.2015 Published Online: 05.10.2015

Abstract

Ochratoxin-A (OTA) is a mycotoxin which is produced by several *Penicillium* and *Aspergillus* spp. OTA has carcinogenic, hepatogenic, nephrotoxic and immunosuppressive effects in human. The toxin accumulates in the animal tissues (kidney, liver and muscle) which could be potential harmful source for human health. Following results of previous investigations our aim was to validate HPLC-FD method for OTA determination in cattle liver which could be implemented in laboratory practise, complying with the European Union Commission Decision (2002/657/EC). Specificity, linearity of method, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision were evaluated. The linearity of the method was determined by preparation of eight standard solutions. The standard curve showed high correlation coefficient (r^2>0.9999). LOD and LOQ were determined as 0.088 ng/ml and 0.268 ng/ml, respectively. Accuracy and precision were established in three concentration levels: 1.0, 5.0 and 10.0 μg/kg. Recovery was in the range of 76.1 to 102.5% and repeatability (RSD_r%) was expressed through relative standard deviation which resulted in the range of 3.7 to 14.28%. Reproducibility results (RSD_R%) were found in the range of 0.16-11.8% for the first day and for the second day in the range of 2.15-17.88%. (Post-hock analysis of samples (n=15), only in one sample OTA was detected (0.2 μg/kg) as close to the LOQ value. As a result, we recommend this method for OTA detection and quantification in cattle liver, for concentrations which are lower than the maximum residue limits (MRL).

Keywords: *Validation, Ochratoxin-A, HPLC-FD, Cattle liver*

Sığır Karaciğerinde Okratoksin-A Varlığının HPLC-FD Metodu İle Belirlenmesi

Özet

Okratoksin- (OTA), *Penicillium* ve *Aspergillus* spp. tarafından üretilen bir mikotoksindir. OTA insanlar üzerinde karsinojenik, nefrotoksin ve immunosupresif etkilere sebep olmaktadır. Ayrıca toksin hayvan dokularında (böbrek, karaciğer, kas) birikerek, insan sağlığı için potansiyel tehlike kaynağı oluşturmaktadır. Bu çalışmadaki amaç, sığır karaciğerinde OTA varlığının HPLC-FD metodu ile araştırıldığı diğer çalışmaların sonuçlarını takiben, 2002/657/EC sayılı komisyon kararına uygun bir HPLC-FD metodu geliştirmektir. Bu amaç doğrultusunda; spesifite, doğrusallık, tespit limiti (LOD), tayin limiti, (LOQ), doğruluk ve kesinlik gibi parametreler değerlendirilmiştir. Doğrusallık; sekiz adet standart solüsyonun analizi ile belirlenmiştir. Korelasyon katsayısı (r^2)>0.9999, tespit limiti 0.088 ng/ml ve tayin limiti 0.268ng/ml değerinde bulunmuştur. Doğruluk ve kesinlik analizleri üç farklı konsantrasyon seviyesi (1.0, 5.0 and 10.0 μg/kg) denk alınarak uygulanmıştır. Geri kazanım, %76.1-102.5 arasında bulunmuştur. Tekrarlanabilirlik relatif standart sapma (RSD_r %) göre ifade edilerek; %3.7-14.28 arasında hesaplanmıştır. Tekrar üretilebilirlik sonuçları (RSD_R%); birinci gün %0.16- 11.8, ikinci gün %2.15-17.88 arasında bulunmuştur. Metot oluşturulduktan sonra, OTA varlığı 15 adet doku örneğinde araştırılmıştır. Sadece 1 örnekte tayin limitine yakın oranda (0.2 μg/kg) OTA tespit edilmiştir. Sonuç olarak, üzerinde çalıştığımız bu HPLC-FD metodunun sığır karaciğerinde maksimum kalıntı limitleri altındabulunan OTA miktarlarının belirlemesinde kullanılmasını önermekteyiz.

Anahtar sözcükler: *Validasyon, Okratoxin-A, HPLC-FD, Sığır karaciğeri*

INTRODUCTION

Ochratoxin - A (OTA) is a toxic metabolite which is produced by fungi *Aspergillus* and *Penicillium* spp. The molds grow on food products (such as; cereals, grains, coffee, beverages, cacao and spices) and produce the toxin under favorable conditions of temperature, moisture and humidity. For example, OTA originates from *Penicillium verrucosum* at 0.8a_w and below 30°C. Also, the toxin can be produced at lower temperatures (5°C) by some *Penicillium*

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spp.[1-3]. OTA contamination is a significant public food safety concern due to accumulation of its residues in animal tissues. Animals, especially pigs, when consume OTA contaminated animal food, the toxin accumulates in kidney, liver, muscle and fat. The researches found out that in German markets, among 620 meat and meat products; 77% of blood sausages and 68% of liver-type sausages were OTA contaminated. Human are exposed to OTA when the toxin contaminated animal products (such as salami, dry-cured ham, sausages) are consumed [3,4]. It has been reported that OTA has carcinogenic, teratogenic, nephrotoxic, neurotic, immunosuppressive, genotoxic and mutagenic effects on experimental animals [5]. International Agency for Research on Cancer (IARC) classified OTA in group 2B (possibly cancerogenic for humans) [6].

Kidneys are the target organs of OTA and nephrotoxicity is the most pronounced toxicity. The toxic effects of OTA in kidneys are mostly affective in non-ruminants like, pigs, birds, rodents, dogs and young ruminants [7,8]. It was observed that in Bulgaria, in several farms, pigs were prone to have Mycotoxic Porcine Nephropathy Disease (MPN) due to consumption of OTA contaminated feed. Additionally, the research revealed that morphological changes and damages in kidney during porcine nephropathy were similar to human kidney disease; endemic nephropathy. Endemic nephropathy is a chronic tubulointerstitial disease with unknown aetiological agent, which is mostly seen in Europe and Balkan countries (Bulgaria, Romania, Serbia, Croatia, Bosnia, Herzegovina, Slovenia, and Macedonia). The disease is also called Balkan Endemic Nephropathy (BEN), due to its common incidence in these regions. It has been reported that in these countries at least 20.000 people have suffered from the disease [9].

The Commission of the European Communities has not set maximum residue limits (MRL) for OTA in animal products. Hence, several countries, including Macedonia, have enforced their own regulations for animal origin foodstuff. MRL for each country were given as following: Denmark (pig kidney) 10 µg/kg, Italy (pork meat and derived products) 1 µg/kg, Romania (pig kidney, liver and meat) 5 µg/kg, Slovenia (milk and meat) 5 µg/kg and Macedonia (bovine liver) 10 µg/kg [10,11]. Determination of public exposure to OTA was evaluated by both international and internal bodies in different countries. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a Provisional Tolerable Weekly Intake (PTWI) of 112 ng/kg body weight (16ng/kg body weight per day). The European Commission's Scientific Committee for Food (SCF) estimated tolerable daily intakes (TDI) of 1.2 to 14 ng/kg body weight [12]. In France, regulations have been in force for pork meat and derived products for OTA since 1978 and in Italy since 1999 [13,14].

OTA analytical methods are based on three steps of analyses; extraction, clean-up and detection of the toxin; respectively. Since now, in several scientific articles, different techniques and equipment have been used for OTA detection in animal tissues. Extraction of toxin from tissues usually has been done with different solvent mixtures such as; ethyl acetate-phosphoric acid, chloroform-phosphoric acid or dichloromethane-ethyl acetate-phosphoric acid. Solid phase extraction (SPE) with immunoaffinity columns has been employed for the clean-up step. Chemical solutions such as methanol and dichloromethane have been used for OTA elution from the SPE columns. Immunochemical and chromatographic methods have been developed for OTA detection and quantification. ELISA is usually applied as a screening method especially in the laboratories which are dealing with big number of samples. HPLC-FD is enough sensitive, precise and mostly used method for the detection and the quantification of the toxin and LC-MS/MS is usually used for confirmation of positive samples or multi-toxin analysis [13-16].

In this study we worked on validation of HPLC-FD method for OTA determination in cattle liver tissues which could be implemented in laboratory practise. The validation parameters were conducted complying with the European Commission Decision (2002/657/EC) [17].

MATERIAL and METHODS

Samples

The validation procedure was done with OTA free cattle liver samples (previously determined with HPLC-FD). Liver samples were analyzed before the validation procedure to control OTA occurrence according to the methodology from the study [18] which were brought by food inspectors from all over Macedonia. After the method validation, cattle liver samples (n=15) were analyzed according to the validated method. Before the analysis, all the samples were stored in specimen containers at -18°C.

HPLC-FD Equipment Condition

HPLC system (λ_{ex} = 333 nm and λ_{em} = 460 nm) and analytical column (RP C18150 mm 4.6 I.D.5 mm) was used. The mobile phase consisted isocratic mixture of water: acetonitrile: glacial acetic acid (99:99:2 v/v/v) at a flow rate 1.0 ml/min. The sample injection volume was 100 µl and run time was 10 min.

Reagents

OTA standard solution was obtained with the concentration level about 50 µg/ml in benzene: acetic acid (99:1). Aliquot of OTA standard were dissolved in benzene: acetic acid 99:1 (*v/v*) in amber volumetric flask of 10 ml, in order to obtain OTA stock solution at 5.0 µg/ml. The stock solution was kept in refrigerator at-18°C and used for preparation of 1.000 ng/ml OTA standard solution. Intermediate solution with concentration of 100 ng/ml was made from OTA standard solution at level of 1.000 ng/

ml. Calibration (working) OTA solutions with concentration levels 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0 and 50.0 ng/ml were obtained from the intermediate solution at 100 ng/ml.

Sample Extraction and Clean-up

A 25 g of tissue sample was measured in glass beakers for extraction. 100 ml extraction solvent (dichloromethane: ethylacetate (1:3)) and 10 ml 0.5 M H_3PO_4 in 2M NaCl solution were added to the 25 g of tissue sample. The samples were blended for a few minutes using homogenizer and mixed for 30 min on a horizontal shaker. The mixture was filtered by using filter paper and an aliquot of the filtrate (10 ml) was evaporated under nitrogen evaporator until the liquid dried. The residue was dissolved in 2 ml methanol and in 30 ml PBS buffer and the new solution was filtered by using microfiber filter. 20 ml of the filtrate passed through the immunoaffinity column and the column was washed with 20 ml of distillate water. OTA was eluted with 4 ml of methanol in a glass tube and was evaporated under stream of nitrogen. The dry residue was redissolved in 1 ml of mobile phase in a glass vial. The samples were ready for HPLC-FD analyses [18].

Method Validation Parameters

Method validation was performed according to the European Union Commission Decision (2002/657/EC) [17]. Specificity, linearity of method, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision were evaluated as follows: Specificity measurement was done with one blank and one fortified tissue at concentration level of 5.0 µg/kg. Linearity of method was determined by preparation of eight standard solutions with concentration levels at 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0 and 50.0 ng/ml. Also, regression analyses are done. The standard solutions were replicated six times in order to obtain calibration curve. Standard deviation of the response and slope was calculated in order to estimate LOD and LOQ according to the formula [19]. Standard deviation (SD) was obtained by analysing 20 tissue samples which were fortified at the lowest concentration of the calibration curve (0.1 ng/ml).

Accuracy was determined by analyzing fortified samples at three levels (1.0, 5.0 and 10.0 µg/kg) with 6 times replication for each concentration. Accuracy was expressed through recovery (%) levels and precision of the method was performed through repeatability (RSD_r%) and reproducibility (RSD_R%) measurements. The same three concentration levels with 6 replicates were also used for reproducibility. The measurements were done using same sample, same method, personal and equipment in two different days as it written in the EU Directive (2002/657/EC) [17]. F-test (two-sample for variances) was done for the three level of concentration (1.0, 5.0 and 10.0 µg/kg). Also, t-test (two-sample assuming unequal variances) was applied only for concentration of 5.0 µg/kg.

Determination of uncertainty was aimed to estimate the errors associated with the various stages of the analysis. Variance of 5.0 µg/kg concentration in two days was calculated in order to obtain uncertainty for reproducibility. Precision of method was evaluated according to measurements from eight standards (0.1, 0.5, 2.5, 5.0, 1.0, 10.0, 20.0, 50.0 ng/ml). Standard error (sy/x) of the calibration curve was calculated according to regression analysis. Later, combined uncertainty of the calibration curve was calculated.

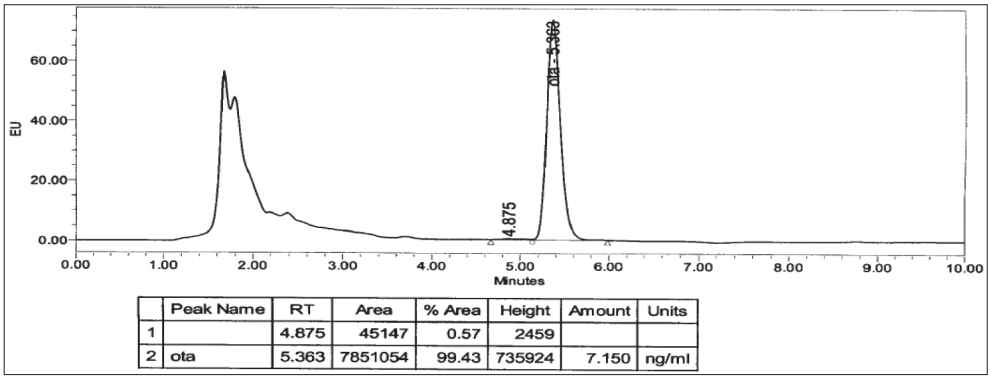
The uncertainty results of OTA Standard Solution 50 µg/ml, calibration curve, reproducibility; pipette (1000 µl) and balance were adjusted as a percentage according to the NIST Uncertainty Guideline [20]. In the respect of the guideline, expanded uncertainty was calculated with coverage factor of 2, at 95% confidence level. Maximum standard uncertainty was determined according to the 'Fitness-for-purpose' approach in the respect of European Commission Decision (401/2006) [21].

RESULTS

The method was found linear with coefficient correlation (r^2)>0.999 with the formula (y=1E+06x-152916). According to the regression analyses, F-value was significantly smaller, which indicated strong relationship between y and x values (P<0.05). The retention time for OTA was measured between 5-6 min in *Fig. 1*. Chromatography of blank sample was clean without any interferences at the retention time *(Fig. 2)*. LOD and LOQ values were found

Fig 1. HPLC-FD chromatogram of Ochratoxin -A (OTA) in cattle liver sample fortified at 5.0 µg/kg

Şekil 1. 5.0 µg/kg konsantrasyon seviyesinde Okratoxin-A (OTA) içeren sığır karaciğeri HPLC-FD kromotogramı



	Peak Name	RT	Area	% Area	Height	Amount	Units
1		4.875	45147	0.57	2459		
2	ota	5.363	7851054	99.43	735924	7.150	ng/ml

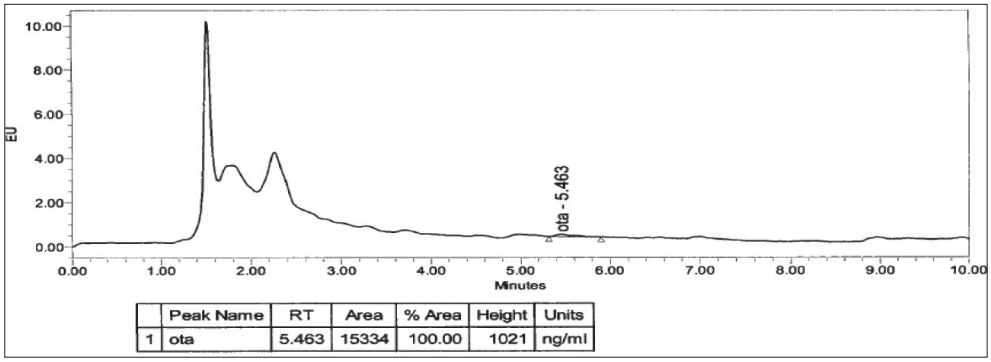


Fig 2. HPLC-FD chromatogram of Ochratoxin -A (OTA) in blank cattle liver sample

Şekil 2. Okratoxin -A (OTA) içermeyen sığır karaciğerinin HPLC-FD kromatogramı

0.088 ng/ml and 0.268 ng/ml, respectively. Accuracy and precision results for 1.0, 5.0 and 10.0 µg/kg concentration levels were presented in *Table 1*.

Reproducibility results for different two days were demonstrated in *Table 2*. F-test calculations were resulted as follows: For the concentration of 1.0 µg/kg; F=1.97 (F critical = 5.05 and P=0.23) and for the concentration of 10.0 µg/kg; F=2.63 (F critical = 5.05 and P=0.15). For the concentration of 5.0 µg/kg, F- test didn't provide equality but t-test did (t-stat = 0.063 and t-critical = 2.57).

Precision of the system was resulted as follows: Mean RSD% value was 0.62%. The uncertainty of predicted sample was estimated 0.11 µg/kg, (At the 95% confidence interval the predicted sample was calculated 11.3±0.26 µg/kg.). The uncertainty of reproducibility for fortified sample (concentration level 5 µg/kg) was 0.28 µg/kg (5±0.28 µg/kg) and for the standard solution was 0.407 µg/ml (50.46±0.407 µg/ml). Expanded uncertainty was resulted as 5.63±11.26% with coverage factor of 2 at the 95% confidence interval. Maximum standard uncertainty was 2.0 µg/kg. Only one of the analyzed samples (n=15) had OTA amount of 0.2 µg/kg which was close to the level of LOQ.

Table1. *Determination of mean, standard deviation (SD), repeatability (RSD_r%) and recovery (%) values for 1.0, 5.0 and 10.0 µg/kg concentration levels*

Tablo 1. *1.0, 5.0 ve 10.0 µg/kg konsantrasyon seviyelerindeki ortalama, standart sapma (SD), tekrarlanabilirlik (RSD_r) ve geri kazanım (%) değerlerinin belirlemesi*

Parameter	Concentration		
	1.0 µg/kg	5.0 µg/kg	10.0 µg/kg
Mean	0.76	5.12	9.36
SD	0.04	0.19	1.34
RSDr%	4.85	3.70	14.28
Recovery (%)	76.1	102.5	93.6

Table 2. *Reproducibility results (RSD_R% values)*

Tablo 2. *Tekrar üretilebilirlik sonuçları (RSD_R% değerleri)*

Period	Concentration		
	1.0 µg/kg	5.0 µg/kg	10.0 µg/kg
1 Day	2.76	0.16	11.18
2 Day	2.15	5.48	17.88

DISCUSSION

Linearity of method with the high coefficient determination showed reliable results. As can be seen from the *Fig. 2*, specificity was satisfactory. There were no potential interfering compounds around the OTA retention time. In several studies, OTA analyses in pig tissues with HPLC-FD method were successful enough to determine very low levels of LOD and LOQ [22-25]. Some of these studies were compared with our study and demonstrated in *Table 3*. Our study showed that the HPLC-FD method which we worked on was efficient and appropriate for detection of the toxin level at the low concentrations in cattle liver.

The uncertainty measurements for each variable were complying with the 'Fitness-for-purpose' approach. There

Table 3. *Demonstration of recovery (%), limit of detection (LOD) and limit of quantification (LOQ) levels from several studies (µg/kg)*

Tablo 3. *Diğer çalışmalarda elde edilen geri kazanım (%), tespit limiti (LOD) ve tayin limiti (LOQ) değerleri (µg/kg)*

Tissue	Recovery (%)	LOD	LOQ	References
Kidney	71±19	0.02	0.06	[18]
Meat	53±10	0.03	0.09	[18]
Kidney	86	0.05	0.16	[13]
All tissues	85±15	0.14	0.52	[22]
Kidney and liver	71	0.14	0.25	[25]
Liver	76.1±12, 102.5±12	0.088	0.26	Our Study

were not any higher uncertainty values than the maximum standard uncertainty (2 µg/kg). The results were equally suitable to be evaluated according to the performance criteria for OTA [21]. Our results for accuracy, repeatability, reproducibility-*(Table 1* and *Table 2)* were consistent with the values (for the concentrations between 1-10 µg/kg: Recovery %70-110, RSD_R 30%, RSD_r 20%) which were written in European Union Commission Decision (401/2006) [21].

Higher values of OTA in pig tissues were found in other studies. Especially, kidney was the most contaminated tissue with the levels of 15.0, 27.5, 52.5 and 23.8 µg/kg [18,22,23,25,26]. Beside kidney, in muscle 2.9 µg/kg, in spleen 0.5 µg/kg, in urinary bladder 11.5 µg/kg and in liver 5.3, 14.5 µg/kg were reported in different studies from several countries (Denmark, Italy, Serbia) [18,22,23,25].

As a result of this study we achieved to validate method for OTA detection and quantification in cattle liver according to European Union Commission Decision (2002/657/EC) [17]. The method was found applicable to analyze significantly lower concentrations than the maximum residue limits. Sample analyses from our study, did not show any significant contamination in liver samples. However, we recommend administration of regular OTA monitoring program in cattle liver samples in Republic of Macedonia.

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Kafkas Univ Vet Fak Derg
22 (1): 7-12, 2016
DOI: 10.9775/kvfd.2015.13479

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

Research Article

Influence of Ketoprofen Application on Lipid Mobilization, Ketogenesis and Metabolic Status in Cows during Early Lactation [1]

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Article Code: KVFD-2015-13479 Received: 31.03.2015 Accepted: 07.09.2015 Published Online: 08.09.2015

Abstract

Changes in metabolic functions in transition dairy cows represent a result of negative energy balance. This leads to increased lipid mobilization and ketogenesis, followed by increased concentrations of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB). Hence, high lipid mobilization and ketogenesis modulate inflammation response and vice versa. The aim of this study was to investigate correlations between ketoprofen administration, high lipid mobilization, ketogenesis and characteristics of metabolic adaptation in cows. Ketoprofen was administered intramuscularly in the concentration of 3 mg/kg, during three consecutive days in 15 postpartum cows. The control group included 15 cows which were not treated with ketoprofen. Blood samples were taken from coccygeal vein, after calving, in the first and second week of the postpartum period. When compared with control, ketoprofen administration decrease the levels of NEFA, BHB and total bilirubin, increase levels of glucose, albumin and cholesterol. Our results showed decreased activity of AST in ketoprofen treated cows in comparison with control group. There was an increase in the intensity of lipolysis and ketogenesis in 66.7% of cows, with NEFA and BHB values over the optimal results, because ketoprofen was not applied to these animals. Cows in the control group were 2 or 2.4 times more likely to come to a state of increased lipid mobilization and ketogenesis. We have found high concordance between NEFA and BHB, and metabolic parameters. This correlation was lower in experimental group of cows hence we can conclude that the use of ketoprofen immediately after calving reduces lipid mobilization and ketogenesis during early lactation and the metabolic adaptation dependence on the intensity of these two processes.

Keywords: *Ketoprofen, Dairy cows, Metabolic status, Early lactation*

Ketoprofen Uygulamasının Süt İneklerindeki Erken Laktasyon Döneminde Lipit Mobilizasyon, Ketogenez ve Metabolik Adaptasyon Üzerine Etkisi

Özet

Geçiş dönemindeki süt ineklerinin metabolik fonksiyonlarında değişiklikler negatif enerji dengesinin sonucudur. Bu da esterleşmemiş yağ asitlerinin (NEFA) ve beta-hidroksibütirat (BHB) düzeylerindeki artışı takiben lipit mobilizasyonu ve ketogeneze yol açar. Bu yüzden yüksek lipit mobilizasyonu ve ketogenez yangısal tepkimeler ile düzenlenir ve (veya?) tersi de söz konusu. Bu çalışmanın amacı, ineklerde ketoprofen uygulanması ile yüksek lipit mobilizasyonu, ketogenez ve metabolik adaptasyonun özellikleri arasındaki ilişkileri incelemektir. Ketoprofen doğum sonrası dönemde ineklerde art arda üç gün, 3 mg/kg dozunda intramüsküler olarak uygulandı. Kontrol grubu ketoprofen uygulanmayan 15 inekten oluşturuldu. Kan örnekleri doğum sonrası birinci ve ikinci haftada coccygeal toplardamardan alındı. Kontrol grubu ile karşılaştırıldığında, ketoprofen uygulaması NEFA, BHB ve total bilirubin düzeylerini azaltırken, glikoz, albümin ve kolesterol düzeylerinde ise artışa neden oldu. Bizim sonuçlarımız, kontrol grubuyla karşılaştırıldığında ketoprofen uygulanmasının AST aktivitesinde azaldığını gösterdi. İneklerin %66.7'si normalin üzerinde NEFA ve BHB değerine sahip, yüksek lipoliz ve ketogenez yoğunluğunda artış mevcuttu; çünkü bu hayvanlara ketoprofen uygulanmadı. Kontrol grubundaki inekler kuvvetle muhtemel 2 ila 2.4 kat daha fazla yüksek lipid mobilizasyonu ve ketogenez durumuna sahipti. Biz NEFA ve BHB ile metabolik parametreler arasındaki yüksek düzeyde uyum olduğunu bulduk. Bu ilişki deney grubu hayvanlarında daha düşüktü ve doğum sonrasında hemen ketoprofenin kullanımının lipit mobilizasyon ve ketojenezi erken laktasyon döneminde düşürdüğü ve metabolik adaptasyonun bu iki sürecin yoğunluğuna bağlı olduğu sonucuna varabiliriz.

Anahtar sözcükler: *Ketoprofen, İnek, Metabolik durum, Erken laktasyon*

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INTRODUCTION

The transition period for dairy cows is from 3 to 2 weeks prepartum to 2 to 3 weeks postpartum, when cows are undergoing numerous physiological adaptations, including endocrine and metabolic changes in order to meet increase in energy requirements that are necessary for the synthesis of milk [1-3]. These adaptive processes have resulted in increased lipid mobilization and ketogenesis, with increased concentration of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB). Hence, it contributes to the deposition of excess energy in the form of fats, which introduces body into a state of metabolic realignment, oxidative stress, inflammation and immunosuppression [1], and entails a number of consequences.

The correlation between inflammatory and metabolic response in cows is the subject of much contemporary research. Although the mechanism of action by which lipids induce inflammatory response is not known, there are several ways in which lipids trigger this response. The importance of fatty acids as modulators of inflammatory reaction is confirmed in many studies on humans and animal models [4-6]. Also, many studies have confirmed that excessive amounts of fat and elevated NEFA concentrations represent positive risk factors for the development of many pro-inflammatory peripartum diseases in dairy cows, including mastitis and metritis [7-9]. There is another important way in which fatty acids can affect the immune and inflammatory response, and that is through the biosynthesis of lipid mediators, including eicosanoids, lyso-phospholipids, sphingolipids, diacylglycerol, phosphatidic acid and ceramide [6]. Among these lipid mediators, eicosanoids are the regulators of the inflammatory response and they play a key role in the regulation of acute and chronic inflammatory reactions. Eicosanoids are formed from polyunsaturated fatty acids, which are metabolized by cyclooxygenase (COX) or lipoxygenase pathway [4]. There are two isoforms of cyclooxygenase; COX-1 and COX-2. The COX-1 isoform is present in the most tissues and synthesized by the action of low concentrations of prostaglandins and COX-2, which is associated with the biosynthesis of inflammatory mediators [4,10].

The clinical setting has proved that cows after calving were burdened by metabolic and inflammatory changes. It is important to find solution to reduce the incidence of inflammation and metabolic stress. Non-steroidal anti-inflammatory drugs (NSAIDs) are pharmacological group of drugs that equally affects both processes [11,12]. The use of NSAIDs can lead to reduced lipid mobilization and ketogenesis in the liver by inhibiting the action of epinephrine [13] and potentiating insulin action [14], reducing the concentration of pro-inflammatory cytokinesis whose value rises during early lactation and in ketosis [15-18].

As a NSAID, the primary mechanism of action of ketoprofen is a reversible inhibition of the cyclooxygenase enzyme, and a reduced biosynthesis of thromboxane A_2 and prostaglandin from arachidonic acid [19-21]. Ketoprofen inhibits both isoforms of the COX enzyme, although it is considered as a COX-1 selective drug [22,23] and it is used extensively in human and veterinary medicine. This NSAID has powerful anti-inflammatory, analgesic and antipyretic properties [24]. In veterinary practice, ketoprofen is used in the treatment of inflammatory and painful conditions of the bones and joints and muscular-skeletal systems in cattle, horses, dogs and cats, and in symptomatic treatment of colic in horses and cattle [25].

The aim of this study was to investigate the correlations between ketoprofen administration, high lipid mobilization, ketogenesis and metabolic adaptation in cows treated with ketoprofen immediately after calving.

MATERIAL and METHODS

Animals and Blood Collection

This study included 30 Holstein-Friesian cows divided in two groups, 15 cows in experimental and 15 cows in control group. The first group of cows was treated with ketoprofen (experimental group) and the second one was not treated with ketoprofen (control group). Ketoprofen was administered in therapeutic dose, intramuscularly, 3 mg *per kg* of body weight in the period of three consecutive days after parturition, starting at the first day postpartum. The mixture of vitamin C (vol. 10 ml, dose 1.000 mg) and rehydration agent (Saline solution 500 ml) was applied to all cows by parenteral route (slow i.v.) and thus, all of them were exposed to the same stress, due to application of the drug, and there was no need to apply a placebo to the control group in order to ensure an identical impact of stress for both groups. Administered volume and dose of saline solution and vitamin C not affect the metabolic adaptation or blood volume of cows and there is not interaction with ketoprofen.

Blood samples were taken three times, at the day of calving and during first and the second week after parturition. They were collected from the *coccygeal* vein using sterile vacuum tubes containing EDTA for bio-chemical analyses (BD Vacutainer® EDTA, BD Plymouth, UK).

This research was approved by the decision, number 01-90/11-4, of Ethical Committee of the University of Novi Sad, in order to safeguard the welfare of experimental animals.

Measurement of Metabolic Parameters

Metabolic parameters such as NEFA, BHB, glucose, total protein, albumin, AST, cholesterol, total bilirubin and calcium were determined using colorimetric reaction according to the manufacturer's instructions colorimetric kits (Randox, UK and Pointe Scientific, USA) and were

measured using a semi-automatic biochemistry analyzer (Analyzer Rayto RT- 1904cv, Rayto L.L.C. Rayto Electronics Inc., China).

Statistical Analyses

The effect of ketoprofen on the metabolic adaptation of cows has been tested using several statistical methods. The difference in the concentration of the metabolite (mean ± SD) in cows which were treated by ketoprofen, in comparison to the control group, was determined by t-test. Application of ketoprofen and its influence on intensive lipid mobilization and ketogenesis were evaluated using Chi-square test. All samples of the experimental and control group (45 samples, 15 cows x 3 weeks) were classified as those in which an optimum lipid mobilization was found (NEFA <0.6 mmol/L) and ketogenesis (BHB <1.1 mmol/L), and those in which we have found increased lipid mobilization (NEFA ≥0.6 mmol/L) and ketogenesis (BHB ≥1.1 mmol/ L).

We have also calculated positive predictive value (PPV), in order to determine the percentage of high lipid mobilization and ketogenesis that can be attributed to the fact that ketoprofen is not applied to cows, as well as likelihood ratio (LR) to determine the extent of the risk of intensive lipid mobilization and ketogenesis development in cows which were not treated with ketoprofen. Changes in the value of the metabolites in cows depended on the intensity of lipid mobilization and ketogenesis, and often showed a linear dependence. Differences in the correlation test between NEFA and BHB, and other blood parameters in experimental and control group of cows were determined by the Fischer r-to-z transformation test. Linearity is tested in all 45 samples of the experimental and control group (15 cows x 3 weeks). The data analysis was performed using SPSS, version 19.0, software package for Microsoft Windows (IBM, Armonk, NY, USA). Compared results with P<0.05 were considered as statistically significant.

RESULTS

When compared with control, ketoprofen administration decrease the NEFA and BHB levels (in the first and second week after calving, P<0.01), increase glucose levels (first week, P<0.05), increase albumin levels (second week, P<0.05), decrease AST levels (first and second week, P<0.05), increase cholesterol levels (the second week, P<0.01), decrease serum total bilirubin levels (second week, P<0.01). Difference in total protein and calcium concentration did not find between control and ketoprofen group. These results are provided in *Table 1*.

There was a statistically significant relation between application of ketoprofen and proportions of blood samples in early lactation with high lipid mobilization

Table 1. *Influence of ketoprofen application on metabolic status in cows in early lactation*
Tablo 1. *Erken laktasyon ineklerde metabolik durumu hakkında ketoprofen uygulamasının etkisi*

Metabolite (Mean±SD)	Group	Week 0	Week 1	Week 2
NEFA mmol/L	Control Ketoprofen P	0.38±0.13 0.32±0.11 >0.05	0.76±0.1 0.58±0.08 <0.01	0.6±0.12 0.49±0.09 <0.01
BHB mmol/L	Control Ketoprofen P	0.55±0.16 0.49±0.18 >0.05	0.88±0.12 0.63±0.15 <0.01	1.05±0.13 0.86±0.14 <0.05
Glucose mmol/L	Control Ketoprofen P	2.9±0.32 3.06±0.4 >0.05	2.15±0.43 2.65±0.49 <0.05	2.2±0.39 2.45±0.42 >0.05
Total protein g/L	Control Ketoprofen P	73±3.1 75±2.9 >0.05	69±3.3 72±3.2 >0.05	68±3.3 71±3.5 >0.05
Albumin g/L	Control Ketoprofen P	35±1.6 36±1.9 >0.05	30±1.9 34±1.8 >0.05	31±1.7 32±1.8 <0.05
AST IU/L	Control Ketoprofen P	92.1±10.5 87.5±10.1 >0.05	105.9±9.5 96.1±9.8 <0.05	112.7±9.9 101.3±11.1 <0.05
Cholesterol mmol/L	Control Ketoprofen P	2.5±0.3 2.2±0.4 >0.05	2.4±0.2 2.5±0.2 >0.05	1.9±0.2 2.6±0.3 <0.01
Total bilir. µmol/L	Control Ketoprofen P	5.1±1.6 5.9±1.7 >0.05	7.6±2.0 6.2±1.8 >0.05	9.5±1.8 7.1±1.6 <0.01
Ca mmol/L	Control Ketoprofen P	2.3±0.1 2.2±0.09 >0.05	2.2±0.12 2.1±0.11 >0.05	2.3±0.12 2.1±0.14 >0.05

(χ^2 = 16.2, P<0.01) and ketogenesis (χ^2 = 10, P<0.01), hence the proportion of cows with high lipid mobilization and ketogenesis were lower in ketoprofen treated cows. In the group of cows that had increased lipid mobilization and ketogenesis, 66.4% were from the control group (PPV value). The risk to develop high lipid mobilization and ketogenesis, when we did not apply ketoprofen, was 2 or 2.4 times higher than in the group of ketoprofen treated cows (LR). These results are provided in *Table 2*.

Linear relationship was significant between NEFA or BHB with glucose, cholesterol, AST and total bilirubin. Albumin showed linear relationship only with BHB. Total protein and Ca were not showed linear relationship with NEFA or BHB. The correlation coefficients were significantly lower in the experimental-ketoprofen group, which means that metabolic changes are not strongly defined by NEFA and BHB as in control group of cows. The results are provided in *Table 3*.

DISCUSSION

Testing of anti-inflammatory action of ketoprofen was carried out in cows in different conditions. It was found that the application of ketoprofen during fistula surgery on cows positively influences the postoperative period [26]. Also, one study indicates that ketoprofen promoted the contractions of rumen and decreased inflammatory responses to mastitis [11]. In both cases, it is shown that ketoprofen has a positive effect on food intake in cows, which is important because during the

Table 2. *Influence of ketoprofen application on the intensity of lipolysis and ketogenesis in cows in early lactation*
Tablo 2. *Erken laktasyon ineklerde lipoliz ve ketogenez yoğunluğuna ketoprofen uygulamasının etkisi*

Intense of Lipid Mobilization and Ketogenesis	Ketoprofen Aplication		Chi-square (P)	PPV	LR
	Yes (Ketoprofen)	No (Control)			
High NEFA ≥0.6 mmol/l	11	21	16.2 (P<0.01)	66.7%	2.4
Normal NEFA <0.6 mmol/l	34	14			
High BHB ≥1.1 mmol/l	15	18	10.0 (P<0.01)	66.7%	2.0
Normal BHB <1.1 mmol/l	30	17			

Table 3. *Influence of the application of ketoprofen on correlations between NEFA or BHB with metabolic parameters in cows during early lactation*
Tablo 3. *Erken laktasyon döneminde ineklerde metabolik parametreleri ile NEFA veya BHB arasındaki korelasyon üzerine ketoprofenin uygulamasının etkisi*

Metabolite	Group	NEFA	BHB
BHB mmol/L	Ketoprofen Control P	0.62 0.86 <0.01	/ / /
Glucose mmol/L	Ketoprofen Control P	-0.52 -0.81 <0.01	-0.59 -0.84 <0.01
Total protein g/L	Ketoprofen Control P	0.19 0.25 >0.05	0.27 0.29 >0.05
Albumin g/L	Ketoprofen Control P	0.26 0.33 >0.05	0.43 0.64 <0.05
AST IU/L	Ketoprofen Control P	0.49 0.76 <0.05	0.51 0.81 <0,01
Cholesterol mmol/L	Ketoprofen Control P	-0.37 -0.56 <0.05	0.51 -0.74 <0.01
Total bilirubin µmol/L	Ketoprofen Control P	0.32 0.65 <0.01	0.49 0.82 <0.01
Ca mmol/L	Ketoprofen Control P	0.26 0.48 <0.05	0.26 0.33 >0.05

perinatal period, food intake is being reduced and we can also see a negative energy balance.

Increased NEFA and BHB represent characteristic changes in the period after calving in cows due to increased lipid mobilization and ketogenesis. Also, excessive elevations in BHB concentrations are associated with poor health and production outcomes in dairy cattle [27]. The increased lipid mobilization and ketogenesis lead to numerous changes in metabolic adaptation in cows and to the emergence of various diseases, such as ketosis. The results show different limits for NEFA (0.5 to 0.7) and BHB (0.9 to 1.2), above which the risk for poor metabolic adaptations or disease increases significantly [28-32].

It is well known that NEFA concentration are increased in the week after calving as a result of energy deficit and changes in hormonal status of cows [33,34]. These changes lead to increased lipogenesis and ketogenesis in the liver, while high concentrations of BHB decrease rates of β-oxidation, gluconeogenesis and the citric acid cycle [27]. In our study, we have found a significant increase in the concentration of NEFA and BHB in the weeks after parturition, but it is less conspicuous in cows which are treated with ketoprofen. It is known that lipid mobilization is the most intensive in the first week after calving, while ketogenesis is the most intensive in the second week after calving, and our findings have confirmed that [35-37].

Fatty acids can be used as precursors for inflammatory eicosanoids and that is the reason why increased NEFA concentration in early lactation may promote inflammation and why it has an impact on the duration and magnitude of it [4]. In another study [13], the NSAIDs in isolated rat adipocytes, inhibited stimulated lipolysis by reducing the release of fatty acids from adipose tissue to the liver by inhibiting the epinephrine-stimulated lipolysis. This, in part, explains the protective action of NSAIDs and potentially cause of decrease in NEFA and BHB concentration. There is a strong correlation between concentration of NEFA, BHB and acute phase proteins in cows after parturition [38]. Decrease in concentrations of total proteins and albumin levels could be linked to the decreased liver synthesis of albumin during inflammation [13]. In contrast to these findings, there is an increase of albumin levels in cows treated with ketoprofen. It could be potentially linked with anti-inflammatory influence of ketoprofen.

It is well known that peripartal period is characterized by increased bilirubin concentration [40] and decreased cholesterol concentration [41]. The bilirubin concentration is increased as a result of puerperal ketosis [39]. The cholesterol concentration is decreased as a result of metabolic disorder [40] and hepatic lipidosis [27]. Low concentrations of the AST in the blood are also present in the healthy animals, but AST rises in the cows around calving [42]. In this study, there was an increase in cholesterol concentrations and decrease in AST and bilirubin concentrations in ketoprofen treated cows, since there is smaller influx of NEFA and BHB in the liver as a result of a lower degree of fatty liver. It is probably because of influence of ketoprofen. Examining the effects of NSAIDs administration in experimentally induced hyperlipidemia in rats [43], it was concluded that these drugs significantly reduce the total cholesterol, triglycerides and LDL concentrations in the plasma of hyperlipidemic rats. Their results potentially link anti-inflammatory activity with hypolipidemia. Trevisi et al.[44] demonstrated that in high yielding cow with high IL-6 concentration as inflammatory marker there is lower liver functionality index. Correlation coefficients between determined metabolic parameters and the values of NEFA and BHB were decreased and significantly lower in the group of cows treated with ketoprofen. NEFA and BHB showed influence on metabolic adaptation in early lactation [28], and connection between metabolic adaptation and lipolisis/ketogenesis. It depends on energy balance and period of lactation [45].

In conclusion, the use of ketoprofen immediately after calving reduces lipid mobilization and ketogenesis during early lactation, as well as metabolic adaptation dependence on the intensity of these two processes. Accordingly, the use of ketoprofen could be recommended in the prevention of ill effects of intensive homeorhesis and the adjustments on milk production during early lactation.

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Kafkas Univ Vet Fak Derg
22 (1): 13-19, 2016
DOI: 10.9775/kvfd.2015.13516

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

Research Article

Fuzzy Logic-Based Decision Support System for Dairy Cattle

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Article Code: KVFD-2015-13516 Received: 04.04.2015 Accepted: 23.10.2015 Published Online: 23.10.2015

Abstract

Various methods have been developed to achieve the most suitable solution in the face of challenges of constantly changing living conditions. Fuzzy logic is one of these methods and frequently preferred by researchers in recent years. Fuzzy logic is based on artificial intelligence modeling the information that includes uncertainty in the most appropriate mindset of people, especially in the decision making process. It also offers flexible and realistic perspectives to people. In this study, a fuzzy logic-based decision support system designed using reproduction and milk yield records of Holstein Friesians. Inputs of designed system are 305-day milk yield (305 DMY), calving interval (CI), service period (SP), artificial insemination (AI), dry period (DP). Output of the system is determined as classification decision. Similarities between expert and system decisions were investigated. Kappa statistics were used for this purpose and fitting value was found as 92.6% (P<0.05).

Keywords: *Decision support systems, Fuzzy logic, Fuzzy sets*

Süt Sığırlarında Bulanık Mantık Tabanlı Karar Destek Sistemi

Özet

Sürekli değişen yaşam koşullarının sorunları karşısında en uygun çözüme ulaşmak için çeşitli yöntemler geliştirilmektedir. Bulanık mantık bu yöntemlerden biridir ve son yıllarda araştırmacılar tarafından sıklıkla tercih edilmektedir. Bulanık mantık yöntemi, belirsizlik içeren bilgileri insanların düşünce yapısına en uygun biçimde modellemektedir. Özellikle karar alma süreçlerinde insanlara gerçekçi ve esnek bir bakış açısı sağlayan yapay zeka temelli bir yöntemdir. Bu çalışmada Siyah Alaca ırkı süt sığırlarına ilişkin üreme ve süt verimi kayıtları kullanılarak, bulanık mantık tabanlı bir karar destek sistemi tasarlanmıştır. Oluşturulan sistemin girdileri 305 günlük süt verimi, buzağılama aralığı (BA), servis periyodu (SP), aşım sayısı (AS), kuru periyodu (KP) bilgilerinden oluşmaktadır. Sistemin çıktısı ise sınıf kararı olarak belirlenmiştir. Sistemin performansını değerlendirmek amacıyla uzman kararları ve sistem kararları arasındaki uyum araştırılmıştır. Bu amaçla kullanılan Kappa istatistiği ile uyumun % 92.6 değerinde olduğu tespit edilmiştir (P<0.05).

Anahtar sözcükler: *Karar destek sistemleri, Bulanık mantık, Bulanık kümeler*

INTRODUCTION

Success of herd management in animal breeding directly effects the continuation of profitable production process. One of the major components of herd management is the right and rational pick of animals. A successful picking policy is the vital element needed to reap economic benefits from animals [1]. Dairy cow breeders focus on two basic factors when picking animals; voluntary culling causes (low efficiency level etc.) and involuntary culling causes (breeding problems etc.) [2-4].

Individuals that work in the field of herd management often seek consultation from experts for their management process, which helps them in raising high amounts of profit. However, when considering the high rates charged by consultants and availability issues, such options are not a viable or long-term solution for breeders in terms of make decisions promptly. Therefore, information technology options offer a way out. Today, especially artificial intelligence based software technologies and designed systems can provide information to individuals in many fields, including the one we are concerned with here - animal field and herd management [5-9].

Fuzzy logic-based decision support systems, is one of the methods developed to achieve the optimum solution against the problems of constantly changing living conditions. The Fuzzy Logic method is common for such study fields as medicine, engineering and biology

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due to its usage in the processing of uncertainty of data set; recently, it is also preferred in agriculture, too. For instance, this method is used in animal breeding [6,7], estrus detection [10-12], detection of sicknesses such as mastitis and lameness [13-16], animal nutrition [8,17], prediction of various production traits (milk, egg, body weight etc.) and quality classification of animal products [18-23].

Strasser et al.[6] study is one of the first studies on fuzzy logic and culling the animals. In this study, a fuzzy logic based decision support system is developed in order to investigate the use of fuzzy logic for dairy cow culling. They have looked animals to be culled according to the decision support system on dairy cows' monthly milk yield, developed via use of the fuzzy logic method. Research has also been mentioned that support the idea that the fuzzy logic method can be used in future animal culling related studies. In the study of Wade et al.[7] such elements as milk yield index from the dairy cow, reproduction activity and lactation numbers have been considered as input variations for decision support system and for taking animals to dry - all done via the use of the fuzzy logic method has. Researchers mentioned in the results part of their study that the system, which has been designed by them, would be beneficial for the producers in culling, classification of animals and other issues. Memmedova et al.[9] in their study formed a desired unified culling model by using the fuzzy logic method and used age at first calving, calving interval and lactation milk yields as input variables. The output variable was also the classification of cows in terms of these traits. In addition, in their unified culling model, as in this study, they successfully determined animals to the subject of culling and they mentioned that the fuzzy logic could be successfully useable in the animal field as well.

In this study a Fuzzy Logic based Decision Support System has been designed in order to provide solutions for the classification problem. For this purpose, class of animals was determined that using these input variables; 305 daily milk yield (305 DMY), calving interval (CI), service period (SP), the number of artificial insemination (AI), dry period (DP). Also system's output was determined as classification decision. The purpose of system was to assist to breeders in the identification of animals which elite and removed from the herd.

MATERIAL and METHODS

Material

Reproduction and milk yield records of 121 Holstein Friesian cows were the material of this study. The designed decision support system's input variables are determined using factor analysis in order to determine which associated of the 11 different variables. Because of factor analysis, the Kaiser-Meyer-Olkin value found 0.54. Varimax factor rotation method was used in the analysis. Factors that have eigenvalue greater than one are considered significant. As a result of factor analysis; 305 daily milk yield (305 DMY), calving interval (CI), service period (SP), the number of artificial insemination (AI), dry period (DP) variables were identified as common factors. Three different classes are defined as "good (class 1) -normal (class 2) -poor (class 3)" for output variable. Ideal values for the input variables are defined as average of cows' own herd. Analysis of data was carried out with Matlab (R2010b) and SPSS 20.0 statistical package programme [24].

Method

Fuzzy Logic theory recognized for the first time in the research of Zadeh [25]. In his Fuzzy Logic theory the truthfulness of statements, between absolute truth or absolute wrong infinite number of truthfulness degree values in the cluster or contrary to numeric meaning of classical logic as sole "1" or sole "0", its defined as a function related to values between [0,1] [25-27]. Compared to human experts, the Fuzzy Logic method can provide more objective and flexible perception in situations that reflects uncertainty. Nonetheless, taking information from the data of the Decision Support System, this method also provides vital benefits to users, which are in the process of taking decision. Fuzzy system basically consists of four components. These components are fuzzifier, fuzzy rule base, fuzzy inference engine (decision unit) and defuzzifier.

In the fuzzification stage, fuzzy system operation preliminary is done in order to processing the data from outside using information in system's inference mechanism and fuzzy rule base [28,29]. Firstly, all membership functions including these function positions are determined.

In practice, the types used most in terms of membership functions are triangular, trapezoidal, bell shape, Gaussian, Sigmoidal, S and π [29]. In this research, expert opinion and a detailed literature review [12,14,18,19,23] of fuzzification stage triangular and trapezoidal membership functions are used. In this stage, variables' minimum and maximum values and the average values of the h erd is considered for fuzzy sets. Triangular membership function with such as a, b and c three parameters are expressed as,

$$\mu_A = (x;a,b,c) = \begin{cases} a \le x \le b, & (x-a)/(b \\ b \le x \le c, & (c-x)/(c \\ x > c \quad or \quad x < a, & 0 \end{cases} \qquad [1]$$

In Equation 1, b parameter is core of the membership function and values between a and c parameters refers to the support of the function.

Trapezoidal membership function with such as a, b, c and d parameters are expressed as [21,27].

$$\mu_A = (x;a,b,c,d) = \begin{cases} a \le x \le b, & (x-a)/(b \\ b \le x \le c, & 1 \\ c \le x \le d, & (d-x)/(d \\ x > d \quad and \quad x < a, & 0 \end{cases} \quad [2]$$

At trapezoidal membership function, part between a-b and c-d parameters forms the boundaries of the function; and values between b-c forms core of the function.

The data that come to system in fuzzy rule base after ready to be processed is processed by inference mechanism according to defined by the form "if- then" rules. A structural learning takes place according to defined parameters [27,28]. The information is modeled via a variety of methods in fuzzy inference mechanism. The methods, that is, the inference methods; these are the Mamdani method, the Larsen method, the Tsukamoto method or the Tagaki-Sugeno-Kang method. Mamdani method is widely used in decision support system studies. The Mamdani and Tagaki-Sugeno-Kang fuzzy inference methods can be used in Fuzzy Logic Toolbox of Matlab Programme. In this research, the Mamdani method was used because output variables are fuzzy set. Tagaki-Sugeno-Kang method is not preferred because the output variable is not a fuzzy set, it is a linear function or a constant value in Sugeno method [19,30].

Mamdani method's rule structure is as follows:

If $X_1=A_1$ and $X_2=B_1$ then $Z_1=C_1$

If $X_1=A_2$ or $X_2=B_2$ then $Z_2=C_2$

In these rule structures, X_1 and X_2 represent input variables; Z_1 and Z_2 represent the output variables. A_1, B_1, A_2 and B_2 represent membership functions; C_1 and C_2 show that fuzzy result set at the end of each rule [19,29,30]. The fuzzy result set consists of the area under the point where separate thresholds that defined for each rule. Threshold value is determined by "and-or" processor. When "and" processor is used the threshold degree of membership is equal to the smallest on the basis of fuzzy sets in intersection feature. "Or" processor is used is equal to the threshold value based on the union transaction largest membership degree in fuzzy sets.

The fuzzy set that is obtained from fuzzy inference engine transformation process is taking place to the absolute value in the defuzzication stage. In practices maximum membership principle, the mean of maximum membership, weighted average, centroid, smallest of maximum and largest of maximum methods are used as defuzzification methods [27,28]. In this research, the centroid method is used as the defuzzification method. Centroid method is most prevalent and commonly used in many applications in animal science. Also this method is determined by the structure of problem's suitability and detailed literature review [12,19,23]. Defuzzification value is calculated as following [29].

$$y^* = \frac{\sum_{i=1}^{n} y_i . \mu_c(y_i)}{\sum_{i=1}^{n} \mu_c(y_i)} \quad [3]$$

In this equation y_i represents the defined output variable, $\mu_c(y_i)$ represents output variable's membership degree and y* represents the defuzzification value.

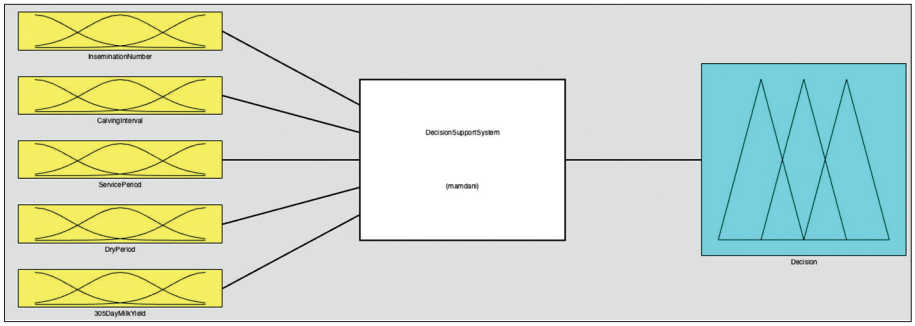
RESULTS

Decision support system has benefited from the work done previously on fuzzy logic [9,12,19,23], the knowledge and experience of experts, the textbooks on animal breeding [4,31] for decision support system of input variables, the formulation of rules and membership functions of creation. An overview of the system is located in *Fig. 1*.

In order to carry out the fuzzification process, not only was the membership function of each input determined but also their position on the x-axis has also been defined. According to the formed fuzzy logic based decision support system, for its input variables' number of artificial insemination "low, medium and high"; calving interval "short, normal and long"; service period "short, medium and high"; dry period "low, medium and high"; 305 day milk yield "low, medium, high and very high"; fuzzy sets have been determined. Designed fuzzy system that was created using Matlab fuzzy logic tool box for number of artificial insemination, calving interval, service period, dry period and 305 day milk yield membership function of each input variable and output variable for representing classification decision are presented in *Fig. 2*.

Fig 1. Designed fuzzy system structure

Şekil 1. Tasarlanan bulanık sistem yapısı



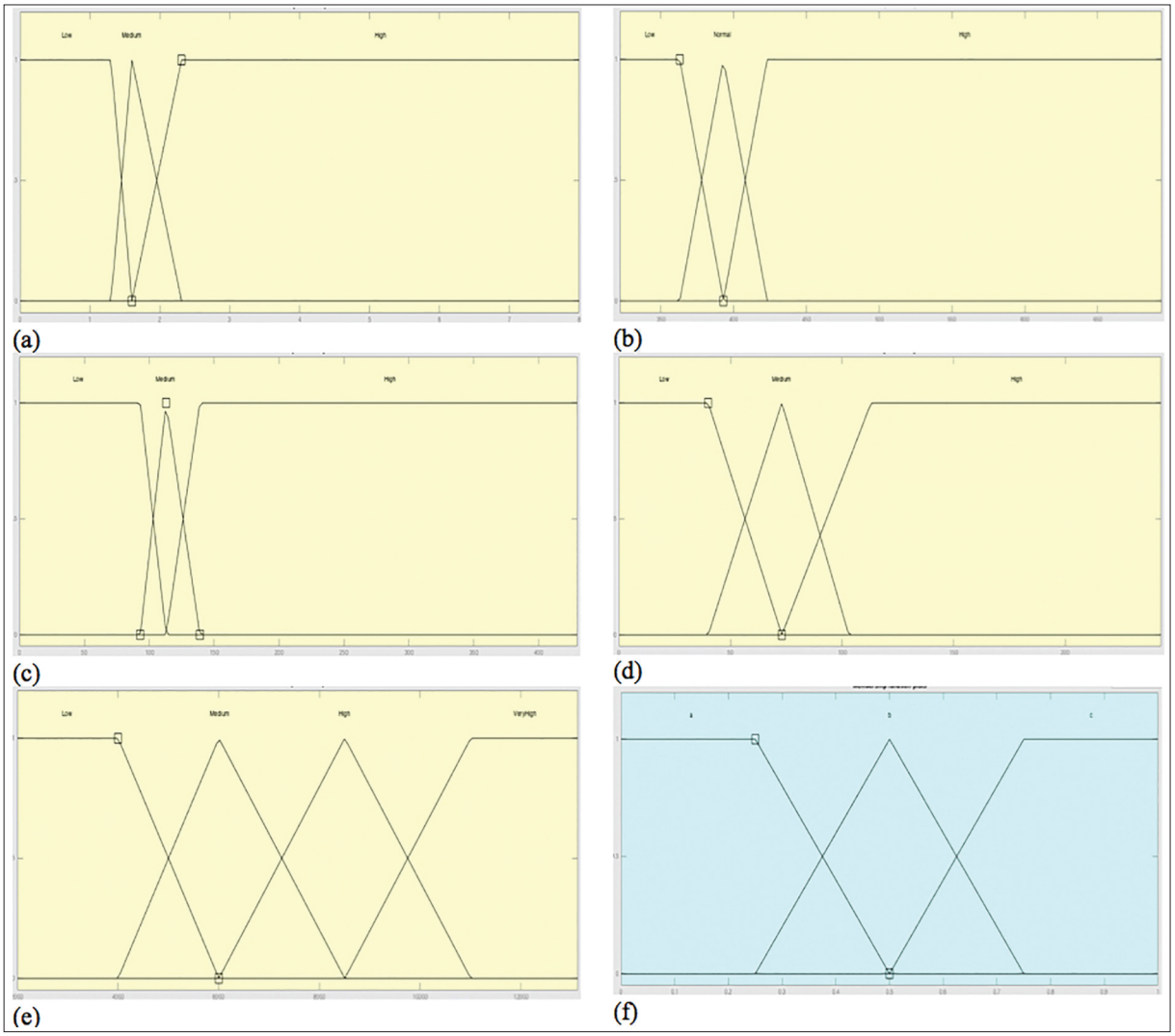


Fig 2. The input variables. The number of artificial insemination (a), calving interval (b), service period (c), dry period (d), 305 day milk yield (e); the output variable: classification decision (f)

Şekil 2. Girdi değişkenleri. Aşım sayısı (a), buzağılama aralığı (b), servis periyodu (c), kuru periyodu (d), 305 günlük süt verimi (e) girdi değişkenlerine ait üyelik fonksiyonları; sınıflandırma kararını temsil eden çıktı değişkeni (f)

In this research, the Mamdani method is used as an inference method and according to the information in fuzzification unit 328 fuzzy the "if-then" rules are written. Some of these rules are shown as follows:

Rule 163 If AI low and CI low and SP low and DP low and 305 DMY high Then Decision (1).

Rule 81 If AI high and CI high and SP high and DP high and 305 DMY low Then Decision (3).

Rule 272 If AI medium and CI low and SP low and DP medium and 305 DMY very high Then Decision (1).

In *Fig. 3* the "if-then" rules' prepared view with the fuzzy logic toolbox in the Matlab programme is located.

A three dimensional relation between 305 day milk yield and calving interval can be seen at *Fig. 4;* the relation between 305 day milk yield and classification decision can be seen at *Fig. 5*.

In *Fig. 4,* it can be noted that the increase in 305-day milk yield suggests that the decision to low class should not be taken. That means, the increase in milk yield indicates the animals, which will be identified as in the good class (Class 1). In *Fig. 6* the relation between calving interval and classification decision can be seen; in *Fig. 7* the relation between service period and classification decision can also be observed. An increase for both two variables can lead to a decision for the decision that animals can be taken in low class. In *Table 1,* input variables' value and some parts of system and expert decisions are located.

The similarities between expert decision and system decisions were researched using Kappa statistics to evaluate the performance of the system and 92.6% value has been found to fit ($P<0.05$). According to result of analysis in one hand, animals in Class 1 are the most productive cow in terms of milk yield and reproductive traits. On the other hands, animal in Class 3 are candidates

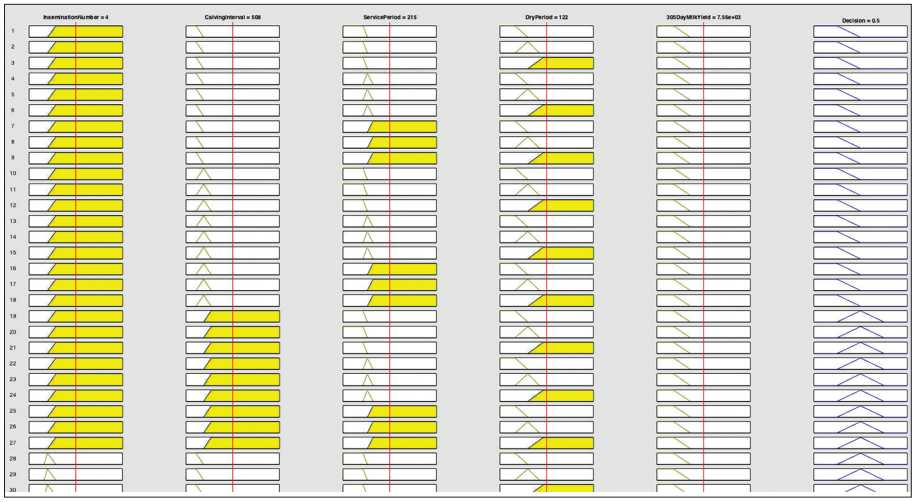


Fig 3. Rule viewer
Şekil 3. Matlab programında kural gösterim penceresi

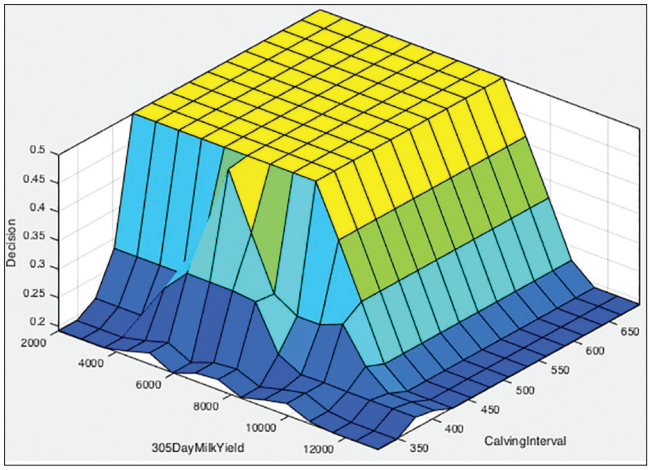


Fig 4. Surface viewer of study's input variables (305 Day milk yield, Calving interval) and output variable (Classification decision) in Matlab
Şekil 4. Matlab programında çalışmanın girdi değişkenleri (305 Günlük Verimi, buzağılama aralığı) ve çıktı değişkenine (sınıflandırma kararı) ilişkin yüzey gösterim penceresi

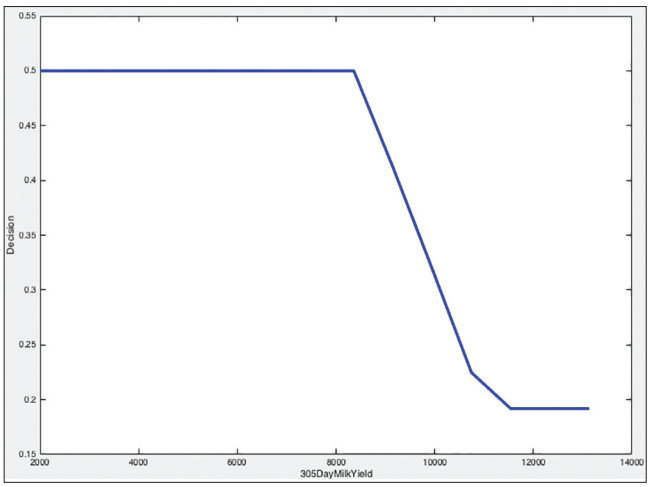


Fig 5. 305 Day milk yield input variable- Classification decision (System output)
Şekil 5. 305 günlük süt verimi girdi değişkeni ile sınıflandırma kararı (Sistem çıktısı) ilişkisi

to leave the herd and yield level can be interpreted as the lowest of animals.

DISCUSSION

In this study, it is aimed to classify animals using milk production and reproductive information for dairy cows. It refers to the output variable, which will take place in the class of animals that are detailed in viewing with five different input variables obtained factor analysis results. According to our results of analysis in examined data set, the distribution of the class of cows is as follows: 35 dairy cows in class 1, 171 dairy cow class 2 and 15 dairy cow in the class 3. As a result of the classification, it is observed that animals, which are located in class 1, can be chosen as breeding animals and the third class of animals can have the possibility of culling. In this study, 328 fuzzy rules written for designed fuzzy logic based decision support system. Thus, both milk yield and reproduction information could be evaluated simultaneously with different perspectives. The research results show that 305-day milk yield and calving interval are very important for the classification of animals. Besides from that, obtained results supported by literature information and expert opinion. The study analyzes indicates that even if the milk yield of examined dairy cows are high, calving interval and insemination numbers can be an obstacle against the selection as breeding animals due to high values.

There are limited number of studies in the literature that focuses on culling, classification etc. decision taken through usage of the fuzzy logic method. Strasser et al.[6] is one of these studies. Strasser et al.[6] used monthly production information in their study. The monthly production data as well as reproductive information is

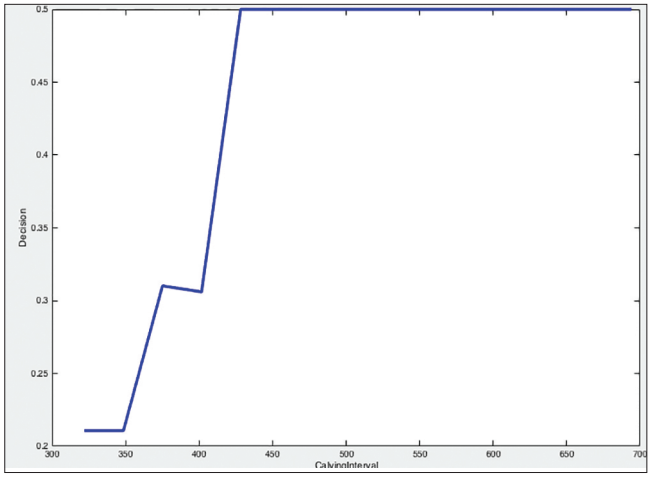


Fig 6. Calving interval input variable - Classification decision (System output)

Şekil 6. Buzağılama aralığı girdi değişkeni ile sınıflandırma kararı ilişkisi (Sistem çıktısı)

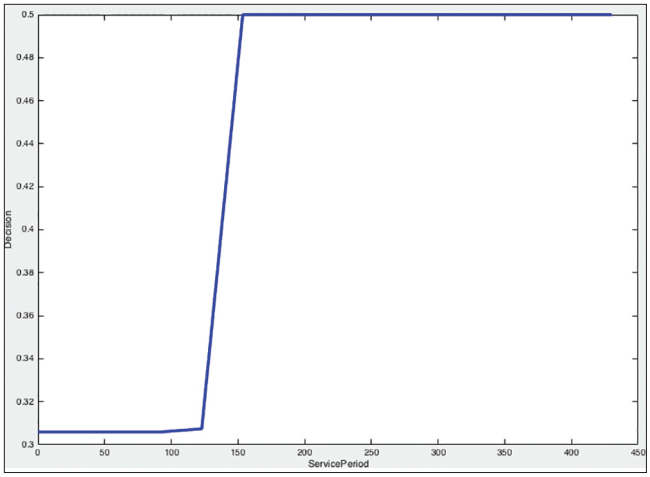


Fig 7. Service period input variable- Classification decision (System output)

Şekil 7. Servis periyodu ile sınıflandırma kararı ilişkisi (Sistem çıktısı)

Table 1. *The analysis results by using Matlab*
Tablo 1. *Matlab programı ile elde edilen analiz sonuçları*

305 DMY	Number of Insemination Number	Calving Interval	Service Period	Dry Period	System Decision	Expert Decision
9288	1	333	57	50	Class1	Class1
8591	2	363	83	76	Class1	Class1
8443	4	473	188	70	Class2	Class2
11121	4	430	157	58	Class2	Class2
9869	6	466	176	76	Class3	Class3

used also in our study. Similar to the results of our work, researchers reported that can be used successfully for the study of the fuzzy logic by culling animals. Wade et al.[7] is one of the other studies about culling and fuzzy logic. Different from Wade et al.[7], we looked at instead of milk yield index, the 305 day milk yield; and instead of reproduction activity, the calving interval, service period, dry period and number of artificial insemination information in our study. Memmedova et al.[9] formed a desired unified culling model by using the fuzzy logic method. Different from those studies, in this study (even though different input variables and number of data have been used) the results achieved were of a similar nature. The greater number of data and the number of variables are given place in our work with success rates similar values.

In this study, it is given to a greater number of input variables and If-Then rules. In this context, examining simultaneously in terms of both milk yield and the reproductive ability of the animals were provided according to examined works. It is aimed to gain much more detailed and flexible perspective to the solution of the classification problem in comparison with the other related studies. Our work has a similar success rate with studies in the literature. In our study, the elimination of errors in the solution of classification problems stemming from people and aimed to prevent cost increases.

Today, problems are becoming more complex and the systems, which emerged in the past in order to solve problems, could not respond to the problems of today by way of offering adequate solutions. The system variables mentioned may not bear enough absolute value for mathematical modeling; in these situations, expert consultation might be needed. Expert persons provide insights due to “very few, few, medium, many” like linguistic qualifier. Insights derived from experts can be transferred to a computer environment through fuzzy logic mathematical based systems and could provide solutions suitable for real life. Computer systems such as fuzzy logic that can mimic expert insights since they too hold ‘expert information’; moreover, the increasing use of these systems may mean lowering expert expenses. It can be said that this approach is an alternative perspective for traditional methods in animal science.

In this study, a decision support system that has been constructed via using the fuzzy logic method was used to provide help for researchers that study on animal field in terms of taking the decision to classification due to milk yields and reproduction data. Compared to expert insights and the system’s decisions, it is noted that there

is a 92.6% success rate of the classification. The designed system's success in terms of animal classification showed that fuzzy logic based decision support systems will be also successful in the animal field, too. Furthermore, it can be that in the future combined systems that use fuzzy logic and other artificial intelligence methods both might provide perspectives that are even more useful for researchers against uncertainties in the animal field.

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Kafkas Univ Vet Fak Derg
22 (1): 21-28, 2016
DOI: 10.9775/kvfd.2015.13586

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

Research Article

Origin Estimation of Honey Samples by Using Constant and Discriminative Function Coefficients of Pure Honey and Honey Produced by Colonies Feeding with Different Sugars

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Article Code: KVFD-2015-13586 Received: 14.04.2015 Accepted: 01.08.2015 Published Online: 04.08.2015

Abstract

In this study, 25 chemical characteristics of 60 pure and adulterated honey samples obtained from feeding honeybee colonies with different syrup levels (20 and 100 L/colony) of High Fructose Corn Syrup 85 (HFCS-85), High Fructose Corn Syrup 55 (HFCS-55) and sucrose (SS) were statistically analysed in order to determine their discriminative power using a Stepwise Method. Seven characteristics including C_4%, vitamin C, Fructose/Glucose (F/G), viscosity, invertase and the difference between the $\delta^{13}C$ value of honey and its protein ($\Delta\delta^{13}C_{p\text{-}h}$) were found to be discriminative. These seven characteristics allowed 60 honey samples to be grouped in their original groups with complete accuracy. The original sources of eight honey samples of unknown origin could be identified by using Standard Multivariate Canonical Discriminant Function and Constant Descriptive Coefficients (SMCDFCDC) belonging to the seven biochemical characteristics. It is possible to identify any honey sample of unknown origin taken from the market or brought to the laboratory for analysis as pure or adulterated by using these functions and descriptive coefficients.

Keywords: *Biochemistry, Colony, Commercial sugars, Discriminant analysis*

Saf ve Değişik Şekerlerle Beslenmiş Kolonilerden Üretilmiş Ballara Ait Sabit ve Ayrımsama Fonksiyonu Katsayıları İle Bal Örneklerinin Kaynağının Tahmini

Özet

Bu çalışmada, balarısı kolonilerinin farklı şurup seviyelerinde (20 l ve 100 l/koloni) Yüksek Früktoz Mısır Şurubu 85 (YFMŞ-85), Yüksek Früktoz Mısır Şurubu 55 (YFMŞ-55) ve Sukroz (SS) beslenmesi ile elde edilen 60 adet saf ve katkılı bal örneğinin 25 kimyasal karakteristiğinin ayrımsama gücünü belirlemek amacıyla adımsal yöntemle istatistiksel analize tabi tutulmuştur. %C_4, Vitamin C, Früktoz/Glikoz (F/G), viskozite, İnvertaz ve bal ve bal proteinine ait $\delta^{13}C$ değeri farkı ($\Delta\delta^{13}C_{p\text{-}h}$) başta olmak üzere yedi özellik ayrımsayıcı olarak belirlenmiştir. Bu yedi özellik 60 bal örneğinin tam doğrulukla orijinal gruplarına ayrılabilmesini sağlamıştır. Kaynağı bilinmeyen sekiz bal örneğinin orijinal kaynağı yedi biyokimyasal özelliğe ait Standart Çoklu Kanonik Ayrımsama Fonksiyonu ve Sabit Tanımlama Katsayısı (SMCDFCDC) kullanılarak tanımlanabilmiştir. Marketlerden alınan ya da analiz için laboratuvarlara getirilen kaynağı bilinmeyen balların katkılı olup olmadığı bu çalışmada ortaya konulan fonksiyonlar ve tanımlayıcı katsayılar ile belirlenebilir.

Anahtar sözcükler: *Biyokimya, Koloni, Ticari şekerler, Ayrımsama analizi*

INTRODUCTION

Honey is vulnerable to various adulterations at each stage of production and processing [1-3]. Honey can be adulterated by adding different industrial sugar syrups (glucose and fructose) obtained from starch by heat, enzyme or acid treatment to the honey [4] or by feeding the bee colonies with excessive amounts of these syrups during the main nectar flow period [5,6]. These practices not only deteriorate honey quality but also lead to losses for

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unadulterated honey producers and cheat consumers [7,8].

Different methods are used to determine the botanical and geographical origins of honeys and also to detect whether honey samples are adulterated or not [9]. Many characteristics have been evaluated for different purposes. These characteristics are as follows: amino acid content [9], carbon isotope ratio ($\delta_{13}C/_{12}C$) and C_4% rate [1,4,6,10], protein profile, aroma, melissopanalogic analysis [11-13], organoleptic characteristics [14] and biochemical characteristics [2,5,15]. There has been discussion as to which characters or methods are reliable in distinguishing adulterated honey produced by adding sugar syrup (direct adulteration) or by excessively feeding bee colonies with industrial sugar syrup (indirect adulteration). Although carbon isotope ratio ($\delta_{13}C/_{12}C$) and C_4% rate have been accepted as the most reliable characteristics, they are not sufficient for discriminating honeys adulterated using sugars originating from C_3 plants such as sugar cane and wheat [4,6,16].

Statistical methods such as Canonical analysis, Principal Component analysis [17,18] and Multivariate Discriminant Analysis [8,10,19,20] have been used with the aim of classifying pure and adulterated honey samples. Each of these methods has advantages and disadvantages. The Multivariate Discriminant Analysis method is used to determine whether the origins of different biological units are different or not [21,22]. Furthermore, this method offers the opportunity to determine the origin of unknown honey samples using Standard Multivariate Canonical Discriminant Function and Constant Descriptive Coefficients (SMCDFCDC). Thus, Guler *et al*.[14] showed that this method was able to discriminate unadulterated honey samples from sucrose-adulterated ones using organoleptic characteristics.

In addition, greater cost and time are required to determine whether honey samples produced from many different sources are adulterated or not using 25-30 chemical characteristics. For this reason, our aim was to determine whether Multivariate Discriminant Analysis Stepwise Method (MDASM) can be used to discriminate adulterated and unadulterated honey samples using fewer biochemical characteristics. In the present study, the aims were: 1) to determine SMCDFCDC for each biochemical characteristic of pure and adulterated honey samples produced from 20 and 100 L/colony levels of HFCS.85, HFCS.55 and sucrose (SS) sugar syrups by analyzing 25 biochemical characteristics via the MDASM method, 2) to determine whether it is possible to discriminate adulterated honey samples, and 3) to estimate the origin of unknown honey samples by using these coefficients.

MATERIAL and METHODS

Materials

This study was carried out between 2011 and 2013 at the Apicultural Research and Application Unit of the Agricultural Faculty of Ondokuz Mayis University, Samsun, Turkey. Types, origins, compositions, forms, proportions and company's names of the industrial sugars used in the study are summarized in *Table 1*.

Methods

Colony management and honey production: Colonies with two aged queen bees of the same genetic origin were used in the study. All of the environmental factors (frames covered with adult bees, frames covered with brood, foundation comb, drugs, transport) were equalized, and all maintenance and control procedures were performed by the same staff. Honeys from all treatments group were produced by the shaking method [14]. After settling bees in the empty hives, cake and syrup were not further provided to the colonies and veterinary drugs were not used for any honeybee diseases. Levels of 20 and 100 L/colony of HFCS.85, HFCS.55 and SS were used first in the study. Syrup was applied at different intervals (eight times for the 20 L/colony and forty times for the 100 L/colony). Before new syrup application, the amount of unconsumed syrup (g/colony) was recorded on each colony's card. Sources and characteristics of the Industrial sugars used in the study are summarized in *Table 1*. A total of 60 honey samples {(HFCS.85-20 L/colony = 6 + HFCS.85-100 L/colony = 6 + HFCS.55-20 L/colony = 6 + HFCS.55-100 L/colony = 6 + SS-20 L/colony = 6 + SS-100 L/colony = 12 + pure honey = 18) = 60)} were analyzed using the analytical methods described below.

Analytical Methods: Honey samples were analysed for the characteristics given as quality criteria by the International Honey Commission (IHC) [2,15]. Moisture was measured at 20°C by an Abbe Refractometer by a refractive method [23]. Fructose, glucose, maltose, and sucrose were

Table 1. *Types, origins, compositions, forms and proportions of the industrial sugars used in the experiment*
Tablo 1. *Denemede kullanılan ticari şekerlerin tip, kaynak, kompozisyon, form ve oranları*

Sugar Type	Origin of Sugar	Form	Composition	Usage Proportion (water:sugar; w:w)	Company Name
HFCS.85	Corn *(Zea mays)*	Liquid	84.9% fructose 12.8% dextrose	1:3	Cargill
HFCS.55	Corn *(Zea mays)*	Liquid	55.6% fructose 39.6% dextrose	1:3	Cargill
SS	Beet sugar *(Beta vulgaris)*	Crystalline	99.5% sucrose	1:1.5	Turhal Sugar Company (Turkey)

identified and determined by high performance liquid chromatography (HPLC) according to DIN 10758 [24]. Hydroxymethylfurfural (HMF) was determined spectrophotometrically as outlined by Harmonization methods of the International Honey Commission (IHC). The diastatic activity was based on starch hydrolysis [23] as 300/time to a value of absorbance of 0.235 at 660 nm. A weighed sample was ignited in a muffle furnace at 550°C to a constant weight for ash determination [23]. Potassium was determined using an Atomic Absorbance Spectrophotometer (AAS) according to AOAC [23] method 985.35. Proline was determined spectrophotometrically using ninhydrin in methyl cellosolve, and the absorbance was read at 512 nm. A standard curve using pure proline was constructed according to AOAC [23] method 979.20. After calibrating the conductimeter, the electrical conductivity of each honey solution at 20% dry matter was measured at 20°C according to the Harmonised methods of the IHC [2]. Free acidity was determined photometrically by AOAC [23] method 962.19, and vitamin C and vitamin B_5 were quantified by R-Biopharm Vitafast Panthotenic Acid, Microbiological microtiter Plate Test. For pure blossom honey (control), and adulterated honey samples: $\delta^{13}C$ values were determined by isotope ratio mass spectrometry (EA-IRMS) after complete sample combustion to carbon dioxide, as described by AOAC [23] method 991.41. The C_4% sugar contents in honey samples were determined using the AOAC (998.12) standard [1,4,7].

Statistical analysis: The Multivariate Discriminant Stepwise Analysis Method (MDASM), which determines differences and grouping levels in terms of biochemical characteristics between more than two biological sources, was used to determine the SMCDFCDC of pure and adulterated honey samples produced from 20 and 100 L/colony syrup levels of HFCS.85, HFCS.55 and SS [22]. The territorial regions of honeys in a Coordinate system were determined and standardized using these SMCDFCDC *(Fig. 1)*. Then, eight samples were randomly selected from a total of 60 honey samples. The origins of these eight samples were kept confidential. The real groups of these eight unknown samples were confirmed using the SMCDFCDC *(Table 4)*. To achieve this aim, the Score Function 1 (SF1) and Score Function 2 (SF2) were calculated [21,22].

Data were evaluated in two steps. First, MDASM was applied to the data to determine the differences between honeys produced with different commercial sugar syrup levels (20 and 100 L/colony) in terms of a great number of biochemical properties and to determine the descriptive SMCDFCDC of the biochemical properties of seven original honeys. Second, a model for predicting unknown honey samples was developed using the SMCDFCDC of biochemical properties of these original honeys [21]. All analysis was executed using SPSS [25] with licence of Ondokuz Mayis University.

RESULTS

The results of the ANOVA are presented in *Table 2*. Except for F+G, there were significant differences ($P<0.001$) between sugar types and syrup levels in terms of the investigated 25 biochemical characteristics. As shown in *Table 2*, it was rather difficult to discriminate adulterated honey samples from pure samples by assessing many biochemical characteristics according to ANOVA. For this reason MDASM was used for that purpose.

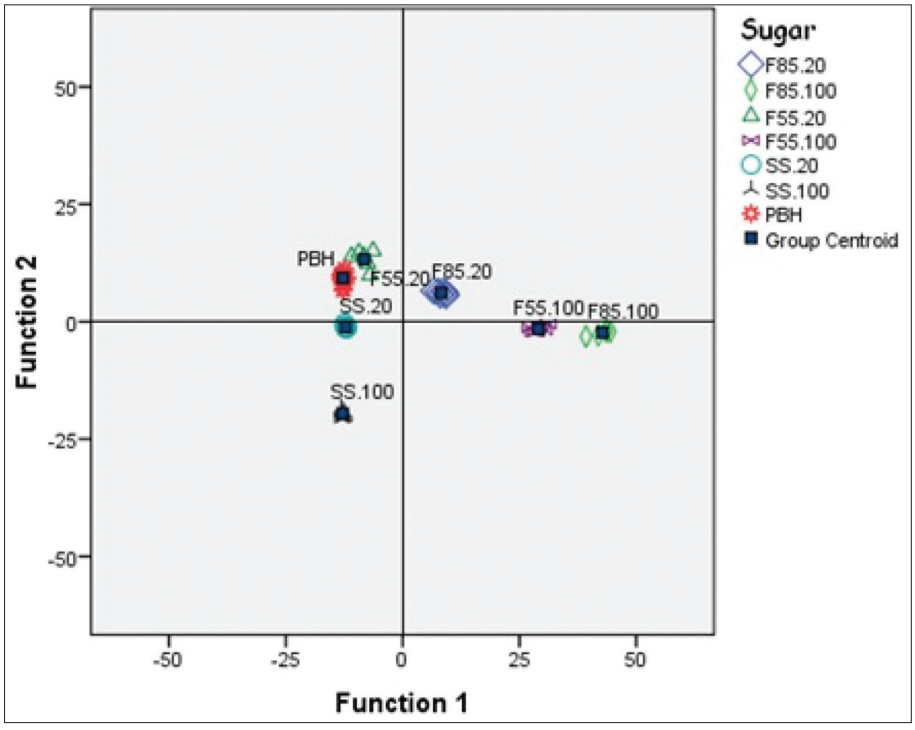

Fig 1. The distribution regions of 60 pure and adulterated honey samples in coordinate system according to the Multivariate Discriminant Stepwise Method. The horizontal axis, the vertical axis and square icon represent 1st canonical function, 2nd canonical function and the centre of each honey group, respectively

Şekil 1. Adımsal Çok Değişkenli Ayrımsama Yöntemine göre 60 adet saf ve katkılı bal örneğinin dağılım bölgeleri. Yatay eksen, dikey eksen ve kare simge sırasıyla 1. kanonik fonksiyonu, 2. kanonik fonksiyonu ve her bal grubunun merkezini göstermektedir

Table 2. *The means ($\bar{X}$) and pooled standard error (PSE) values of biochemical characteristics of pure (PBH) and adulterated honeys produced by feeding bee colonies with HFCS.85, HFCS.55 and sucrose syrups (SS)*

Tablo 2. *YFMŞ.85, YFMŞ.55 ve Sukroz şurubu (SS) ile beslenmiş balarısı kolonilerinden elde edilen saf ve katkılı ballara ait biyokimyasal özelliklerin ortalama ($\bar{X}$) ve bileşik standart hata (PSE) değerleri*

Sugar Syrup Level	HFCS.85		HFCS.55		SS		PBH	PSE
	20	100	20	100	20	100		
Water	19.20^{e}	16.77^{b}	17.07^{bc}	18.40^{d}	18.20^{d}	15.72^{a}	17.93^{c}	0.04
pH	14.7^{c}	9.2^{a}	16.0^{d}	11.0^{b}	15.5^{d}	8.0^{a}	16.8^{e}	0.02
HMF	6.27^{a}	10.68^{b}	6.27^{a}	10.93^{b}	4.67^{a}	4.68^{a}	3.71^{a}	0.99
Proline	530.00^{c}	279.17^{a}	618.17^{d}	348.67^{b}	704.50^{e}	249.33^{ab}	768.2^{e}	7.98
EC	0.201^{cd}	0.117^{a}	0.203^{cd}	0.138^{b}	0.195^{c}	0.130^{b}	0.213^{d}	0.006
Diastase	7.70^{b}	7.70^{b}	7.14^{a}	7.70^{b}	7.70^{b}	7.70^{b}	7.70^{b}	0.00
Invertase	58.15^{ab}	57.65^{a}	70.20^{d}	62.57^{c}	60.23^{b}	58.53^{ab}	59.33^{b}	0.58
α- Glucosidase	27.43^{b}	27.64^{b}	31.23^{c}	26.94^{b}	26.40^{b}	22.38^{a}	27.36^{b}	0.83
Fructose	44.02^{b}	57.07^{c}	37.78^{a}	37.20^{a}	38.95^{a}	36.07^{a}	35.49^{a}	0.66
Glucose	24.00^{b}	17.08^{a}	29.35^{cd}	27.85^{c}	30.27^{de}	30.40^{e}	30.35^{e}	0.47
Sucrose	0^{b}	0 ^{b}	0 ^{b}	0 ^{b}	0 ^{b}	3.05^{a}	0 ^{b}	0.01
F+G	68.02^{a}	74.15^{b}	67.13^{a}	65.05^{a}	69.22^{a}	66.47^{a}	65.84^{a}	1.05
F/G	1.84^{c}	3.35^{d}	1.29^{b}	1.34^{b}	1.29^{b}	1.19^{a}	1.17^{a}	0.02
G/water	1.250^{b}	1.019^{a}	1.719^{de}	1.514^{c}	1.663^{d}	1.934^{f}	1.693^{e}	0.03
Vit C	1.89^{d}	0.20^{ab}	3.70^{f}	0.34^{b}	1.30^{c}	0.19^{a}	2.94^{e}	0.03
Vit B_5	0.077^{c}	0.062^{b}	0.084^{cd}	0.059^{b}	0.086^{d}	0.050^{a}	0.094^{e}	0.002
Ash	0.113^{c}	0.056^{a}	0.098^{bc}	0.055^{a}	0.082^{ab}	0.070^{a}	0.109^{bc}	0.006
Na	0.792^{d}	0.585^{ab}	0.809^{bcd}	0.465^{a}	0.699^{bcd}	0.746^{cd}	0.603^{bc}	0.05
K	16.88^{b}	6.81^{a}	20.9^{b}	7.6^{a}	15.05^{b}	7.34^{a}	18.11^{b}	0.42
K/Na	19.69^{b}	12.87^{c}	26.67^{a}	16.35^{c}	21.60^{b}	9.86^{c}	30.75^{a}	1.41
δ^{13}Cprotein	-24.82^{d}	-23.38^{e}	-25.07^{c}	-23.38^{e}	-25.4^{b}	-25.57^{a}	-25.97^{a}	0.06
δ^{13}C honey	-21.70^{c}	-15.87^{e}	-24.52^{b}	-17.2^{d}	-25.75^{a}	-25.75^{a}	-26.07^{a}	0.09
$\Delta\delta^{13}C_{p\text{-}h}$	-3.12^{c}	-7.52^{a}	-0.55^{d}	-6.18^{b}	0.35^{e}	0.18^{e}	0.10^{e}	0.09
C_4%	20.62^{c}	54.77^{e}	3.67^{b}	45.2^{d}	0^{a}	0^{a}	0.09^{a}	0.39
Viscosity	5111.17^{a}	14605.42^{d}	15650.08^{d}	7611.08^{b}	8888.83^{b}	33111.0^{e}	10773.2^{c}	312.2

HFCS: *High Fructose Corn Syrup,* ***$\Delta\delta^{13}C_{p\text{-}h}$:*** *Difference between the δ^{13}C value of honey and its protein,* ***HMF:*** *Hydroxymethylfurfurol,* ***EC:*** *Electrical conductivity,* ***δ^{13}C:*** *Carbon, ●values within rows with different superscripts differ significantly at P<0.05*

Determination of Discriminating Biochemical Characteristics Using the Stepwise Method

The SMCDFCDC of seven biochemical characteristics that were found to be significant (P<0.001) in classifying honey samples according to step order are given in *Table 3*. The C_4%, Vit C, F/G, viscosity, invertase, $\Delta\delta^{13}C_{p\text{-}h}$ and proline were found to be significant (P<0.001) in discriminating honey samples. In addition, 60 honey samples were classified in their original groups with 100% accuracy when they were evaluated according to these seven biochemical characteristics *(Fig. 1)*.

In total, 6 functions were found to be significant in classification. However, while the 1st Discriminant Function defined the 66.4% of the total variance, the 2nd and 3rd functions defined 21.7 and 8.4%, respectively. These functions altogether defined 96.5% of the total variance. The 4th, 5th and 6th functions defined only 2.9, 0.4 and 0.3% of the total variance, respectively. Furthermore, while C_4% and $\Delta\delta^{13}C_{p\text{-}h}$, which were successful in 1st step, were represented by the 1st discriminant function, Vit C, F/G ratio, viscosity, invertase and proline were represented by the 2nd, 3rd, 4th, 5th and 6th functions, respectively.

Determination of Standard Original Distribution Areas of Honeys Produced with Different Sugar Syrups in the Coordinate System

The projection and intersection regions of pure and adulterated honey samples are shown in coordinate system *(Fig. 1)*. The first discriminant function was able to differentiate the adulterated honey samples produced by the 100 L/colony of HFCS.55 from; i) pure honey samples, ii) 20 L/colony of HFCS.55 and iii) 20 and 100 L/colony of SS. This function represented C_4% and Δδ13Cp-h. This

Table 3. *The unstandardised canonical discriminant functions and constant coefficients of biochemical characteristics to be used for classification of pure and adulterated honeys*

Tablo 3. *Saf ve katkılı balların sınıflandırılması için kullanılan biyokimyasal özelliklerin standardize edilmemiş kanonik ayrımsama fonksiyonları ve sabit katsayıları*

Characters	Canonical Discriminant Function Coefficients					
	1	2	3	4	5	6
C_4%	0.819	0.345	-0.538	0.386	-0.183	1.046
Vit C	-0.104	6.652	1.321	3.375	-2.269	0.077
F/G	2.975	0.914	15.588	-3.329	2.821	-0.370
Viscosity	0.000	0.000	0.000	0.000	0.000	0.000
Invertase	0.097	0.104	-0.006	0.304	0.634	0.016
$\Delta\delta^{13}C_{p\text{-}h}$	-0.399	1.241	-1.605	0.751	-0.142	6.697
Proline	-0.003	0.007	0.004	0.000	0.001	0.011
Constant coefficients	-18.867	-19.218	-27.185	-30.988	-33.969	-11.309

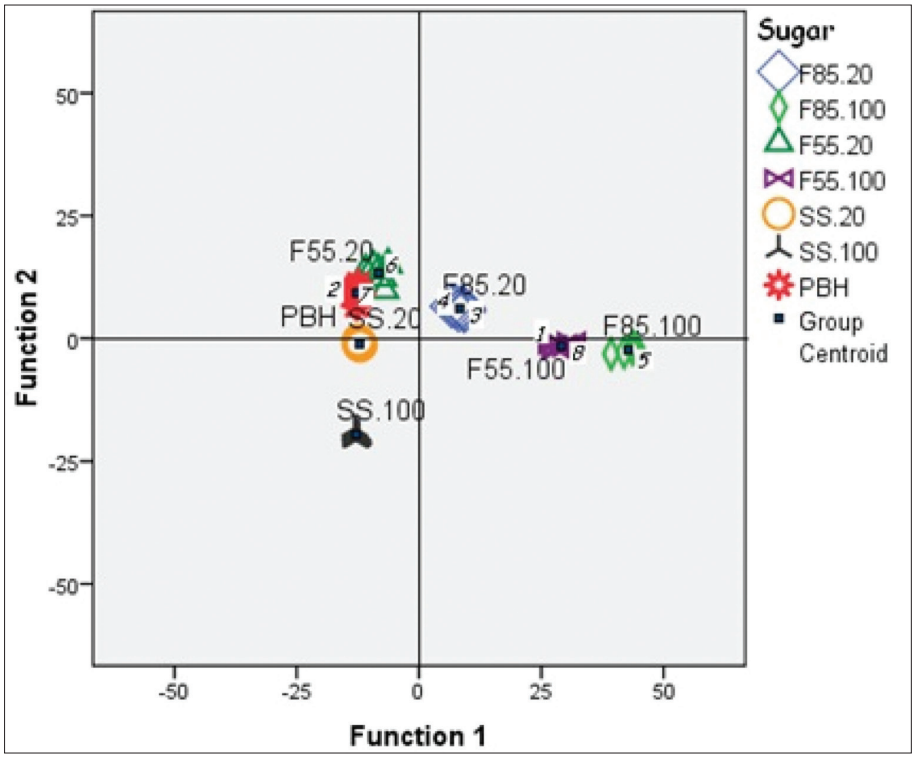

Fig 2. Real grouping regions of unknown eight honey samples in coordinate system

Şekil 2. Koordinat sisteminde kaynağı bilinmeyen sekiz bal örneğine ait gerçek gruplama bölgeleri

function was also accepted as a differentiation function that can differentiate adulterated honey samples produced from C_3 and C_4 plants.

Furthermore, the 2nd discriminant function was found to be effective in discriminating adulterated honey samples produced using the 100 L/colony of HFCS.85 and HFCS.55 and the 20 and 100 L/colony of SS from pure honey samples. Also, the honey samples produced from the 20 L/colony of HFCS.55, SS and PBH were grouped in the same coordinate axis although they all were completely different from each other. The adulterated honey samples produced from the 100 L/colony of HFCS.85, HFCS.55 and SS were grouped in the farthest region of the coordinate axis. Furthermore, adulterated honey samples produced using the 100 l/colony of HFCS.85 and HFCS.55 were located along the same axis.

Verification Test

For the verification test eight honey samples were selected randomly from the 60 samples. The production method, honey type (pure or adulterated) and number of these 8 samples were kept secret and the source of these 8 samples was unknown during analysis. Prior to analysis the samples were coded as UnS_1, UnS_2,...,UnS_8. The region of each unknown honey sample was determined using SMCDFCDC *(Table 4)*. Two score functions (SFs) were calculated with the aim of determining the groups. While calculating these functions, the standard first discriminant

Table 4. *Standardised canonical classification functions and constant descriptive coefficients for seven biochemical characteristics to be used for classification of pure and adulterated honey samples, and calculation of score functions related to the unknown samples*

Tablo 4. *Saf ve katkılı bal örneklerinin sınıflandırılmasında kullanılan yedi biyokimyasal özellik için standardize edilmiş kanonik sınıflama fonksiyonu ve sabit tanımlama katsayıları ve bilinmeyen örnekler için skor fonksiyonlarının hesaplanması*

Characteristic	Canonical Classification Coefficients						Unknown Sample (X_i)	SCORE Func.1	SCORE Func.2
	F1(α_1)	F2(α_2)	F3(α_3)	F4(α_4)	F5(α_5)	F6(α_6)			
C_4	0.819	0.345	-0.539	0.386	-0.183	1.046	44.5	36.4455	15.3525
Proline	-0.003	0.007	0.004	0.000	0.001	0.011	341	-1.023	2.387
Viscosity	0.000	0.000	0.000	0.001	0.000	0.000	9500	0	0
$\Delta\delta^{13}C_{p\text{-}h}$	-0.399	1.241	-1.605	0.751	-0.142	6.697	-6.1	2.4339	-7.5701
Vit C	-0.104	6.652	1.321	3.375	-2.269	0.077	0.34	-0.03536	2.26168
Invertase	0.097	0.104	-0.006	0.304	0.634	0.016	63.5	6.1595	6.604
F/G	2.975	0.914	15.588	-3.329	2.821	-0.370	1.35	4.01625	1.2339
Constant (α_0. β_0)	-18.87	-19.22	-27.19	-30.99	-33.97	-11.31		48.00	20.27
Coordinate scores of sample								29.13	1.05

$\Delta\delta^{13}C_{p\text{-}h}$: *The difference between the $\delta^{13}C$ value of honey and its protein*

Table 5. *Analysis results of eight unknown honey samples related to the seven biochemical characteristics*

Tablo 5. *Yedi biyokimyasal özelliğe bağlı olarak sekiz bilinmeyen bal örneğine ait analiz sonuçları*

Characters	Samples of Unknown Origin							
	UnS_1	UnS_2	UnS_3	UnS_4	UnS_5	UnS_6	UnS_7	UnS_8
$\%C_4$	44,50	0.00	21.20	0.00	54.20	3.90	0.00	44.10
Vit C	0.34	2.86	1.97	1.37	0.22	3.84	0.16	0.31
F/G	1.35	1.16	1.87	1.29	3.19	1.29	1.21	1.32
Viscosity	9500.0	11266.5	5300.0	8900.0	15066.5	16733.5	32166.5	8700.0
Invertase	63.50	59.60	57.4	58.4	58.20	69.3	57.20	62.40
$\Delta\delta^{13}C_{p\text{-}h}$	-6.10	0.10	-3.20	0.50	-7.70	-0.60	0.00	-6.00
Proline	341,00	748.00	516.00	736.00	282.00	642.00	247	358.00

$\Delta\delta^{13}C_{p\text{-}h}$: *The difference between the $\delta^{13}C$ value of honey and its protein*

function coefficient (a_i) of each property was multiplied by the value of this property given by the analysis of (X_1, X_2,..., X_n) additional samples. Then this value added to the constant coefficient of Function 1 and so SF1 was calculated. SF2 was calculated in a similar way *(Table 4)*. In the coordinate system *(Fig. 1)* SF1 is the apsis and SF2 is the ordinate [21,22]. For each sample two score functions were calculated using equations 1 and 2 given below.

Score Function 1=$a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 + a_6x_6 + a_7x_7$ (1st correlation)

Score Function 2=$\beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_4x_4 + \beta_5x_5 + \beta_6x_6 + \beta_7x_7$ (2nd correlation)

The calculated values of these SFs were located in places of F1 and F2 in the standard clustering diagram *(Fig. 1)* and so the real group of this sample was determined. The SF1 and SF2 were calculated for samples of unknown origin and then the clustering regions of these samples were determined in the coordinate system *(Fig. 2)*. Furthermore, the Excel Programme was used for easy calculation of SF1 and SF2 and then the method was standardized *(Table 4)*.

The 1st and 2nd SFs of 8 randomly selected honey samples from 60 of unknown origin were calculated as follows:

UnS_1: SF1=29.13; SF2=1.05

UnS_2: SF1= -12.22; SF2=12.43

UnS_3: SF1=9.15; SF2=8.52

UnS_4: SF1=11.91; SF2=2.92

UnS_5: SF1=42.86; SF2=2.33

UnS_6: SF1= -7.20; SF2=19.81

UnS_7: SF1= -10.48; SF2= -9.37

UnS_8: SF1=28.52; SF2=0.81

When the SF1 and SF2 values were inserted in the coordinate system *(Fig. 2)*, the UnS1 coded sample overlapped with the 100 L/colony of HFCS.55. Similarly, UnS_2 overlapped with pure honey (PBH), UnS_3 with the 20 L/colony of HFCS.85, UnS_4 with the 20 L/colony of HFCS.85, UnS_5 with the 100 L/colony of HFCS.85, UnS_6 with the 20 L/colony of HFCS.55, UnS_7 with pure honey (PBH) and UnS_8 with the 100 L/colony of HFCS.55. Thus, the origins

of all 8 unknown origin honey samples were determined by using a confirmation test. More importantly, there was no overlap among the samples. The analysis results for the 8 unknown origin honey samples in relation to the 7 biochemical characteristics are shown in *Table 5*.

DISCUSSION

Many biochemical characteristics of pure and adulterated honey samples produced by feeding bee colonies with different syrup levels of various industrial sugars are significantly different. These differences are greater in syrup level when compared to sugar type. The biochemical characteristics of pure and adulterated honey samples determined in the present study are compatible with previous studies, and also with international standards [1,3,6,9].

The Stepwise method could group the 60 honey samples with 100% accuracy. The honey samples were clustered in different regions in the coordinate system. The grouping levels, clustering regions and importance of discriminant functions all indicated that the honey samples originated from different sugar sources. Thus correct classification level of the 60 samples was 100% and values of Wilks' λ indicating the importance of the first and second discriminant functions were found to be λ=0.002 and λ=0.000, respectively. The high (100%) classification ability of the Stepwise method has been reported previously by many authors [8,10,20,26,27].

The C_4%, vitamin C, F/G ratio, viscosity, invertase, $\Delta\delta^{13}C_{p\text{-}h}$ were found to be successful in discrimination of honey samples in the Stepwise method, which was applied to 25 biochemical characteristics. The C_4% ranked first (1st step) and the classification ability of this characteristic is evident from its relationship with the 1st discriminant function, because the relationship of this function with total variation was found to be very high (r=0.995). Furthermore, this function could define the differences among 60 honey samples at a 66.4% level. The C_4% ratio was determined to be the most important criterion for determining whether sugar or syrups originating from C_4 plants were added to honey directly or indirectly by bee feeding as in the present study. This ratio has been reported to not be higher than 7% [1,16]. For this reason, this characteristic, which is considered a formal method, creates the basis for many standards [2,15,28]. The finding that the C_4 ratio ranked first in the Stepwise method confirms the importance of this characteristic as mentioned in previous studies [1,4,16]. Similarly, the difference between the $\delta^{13}C$ value of honey and its protein, which is used to determine the C_4 plant-derived adulteration, was found to be significant in the Stepwise method.

In the present study, discriminative biochemical characteristics (C_4%, Vitamin C, F/G, Viscosity, Invertase, $\Delta\delta^{13}C_{p\text{-}h}$ and proline) were different from those reported by Devillers *et al*.[27], Ruoff *et al*.[10], and Guler *et al*.[5]. These differences might be attributed to the differences in sugar types and research methods used in these studies. In the present study, we evaluated adulterated honey samples produced from different syrup levels (20 and 100 L/colony) of HFCS (derived from corn) or sucrose (derived from sugar beet). However, other researchers evaluated pure honey samples produced using different plants [10], monofloral honey samples [9] and polyfloral honey samples [5,10,29]. Consequently, the inconsistent results between the studies are not surprising. For instance, the fructose and glucose ratios of HFCS.85 used in the present study were 84.9% and 12%, respectively. However, many plant nectars do not contain fructose at this level.

So far, proline [5,11], K/Na ratio [10,11], electrical conductivity [5,26] and sugar contents [2,15,28] have been among the characteristics used to discriminate adulterated honey samples produced by using sucrose. Whereas in the present study, electrical conductivity and any sugar did not present in the Stepwise, and proline was found significant only in the 7th step. Vitamin C, viscosity and invertase have taken their place in the upper row in the Stepwise. The inefficiency of proline might be attributed to the fact that the sugars (SS, HFCS.85 and HFCS.55) used to produce adulterated honey samples were derived from C_3 (sugar beet) and C_4 (corn or sugar cane) plants. Thus, the average C_4% sugar content of HFCS.85 originating from corn, and SS originating from sugar beet were found to be significantly different (54.77±0.71 and 0.0±0.0, respectively). However, the average proline contents of these adulterated honey samples (100 L/colony of HFCS.85 and SS) were close to each other *(Table 2)*.

When we evaluated only sucrose-adulterated (20 and100 L/colony of SS) and pure honey samples by stepwise discriminant analysis in terms of 24 biochemical characteristics, proline ranked first in the Stepwise. The relationship among the 24 biochemical characteristics underlines the importance of proline. There were significant relationships ($P<0.001$) between proline and acidity (r=0.969), $\Delta\delta^{13}C_{p\text{-}h}$ (r=0.662), electrical conductivity (r=0.906), vitamin C (r=0.823), vitamin B5 (r=0.966), and K/Na ratio (r=0.742). In addition, there were negative relationships between proline and characteristics causing loss of quality such as the $\delta^{13}C$ value of honey (r=- 0.659), the $\delta^{13}C$ value of protein (r= - 0.588), C_4% ratio r= -0.641, and sucrose (r= - 0.589). This is confirmed by the high multiple regression coefficient of this relationship (R^2=0.922). All these findings showed that proline is more efficient in discriminating adulterated honey samples produced by using sugars originating from C_3 plants compared to C_4 plants.

Similarly, the lack of discriminating effect of sucrose and glucose sugars in discriminating pure and adulterated honeys produced using strong syrups might be attributed to the fact that some biochemical characteristics have extremely different values depending on the type of sugar

(C_3 or C_4). For example, the C_4% ratio of honey coming from the 100 L/colony of HFCS.85 (54.77±0.71%) was significantly different from the value coming from the same syrup level of SS (0.0±0.0%). Similar findings have been reported by other researchers [7,9,11,29]. However, F/G ratio was found to be significant in the 3rd step. The fact that the highest F/G ratio was determined for the 100 L/colony of HFCS.85 (3.35±0.07) confirms this finding. All these results indicated that distinctive biochemical characteristic(s) can change depending on the plant source, honey production method, sugar type (C_3 or C_4), sugar content and amount of sugar syrup given to the colony.

In the present study, the origin of 8 unknown origin honey samples was estimated with 100% accuracy by using biochemical characteristics SMCDFCDC. Hence, through this method it is possible to determine; i) whether honeys found in the market are pure or not, ii) whether they are produced from HFCS.85, HFCS.55 and sucrose (SS), and iii) the syrup levels with which they are produced.

Acknowledgements

This study was funded by TUBITAK (The Scientific and Technological Research Council of Turkey) with project number 111 O 268 TOVAG and the title "Determination of the effect of some industrial sugars on honeybee *(Apis mellifera* L.) colony productivity, behavior and honey quality". The authors are grateful to TUBITAK for financial support and Dr. Semiha Guler for her contribution to the manuscript.

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Kafkas Univ Vet Fak Derg
22 (1): 29-35, 2016
DOI: 10.9775/kvfd.2015.13639

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

Research Article

The Potential of Microarray Databases to Identify Tissue Specific Genes

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Article Code: KVFD-2015-13639 Received: 24.04.2015 Accepted: 16.09.2015 Published Online: 01.10.2015

Abstract

Tissue specific genes play important roles in development and metabolism. Currently, GenBank has 3363628 GEO Profiles and 397988 microarray data related to various tissues. To evaluate the huge amounts of data and identify tissue specific genes, it is necessary to develop and use new strategies. To that end, this study discusses if microarray and microarray related GEO Profiles are a useful tool to identify new tissue specific genes. In the current study, adipose tissue sellected as a target tissue in order to find new tissue specific genes. Therefore, the human and mouse microarray data were analyzed comparatively. To support the microarray data, adipose tissue related GEO Profiles were selected from PubMed. Subsequently, adipose tissue related microarray and GEO Profiles were analysed simultaneously. According to analysis of microarray and GEO Profiles, Chrdl1 (Chordin-like 1) gene was hypothesized as a novel adipose specific gene. In order to test the hypothesis, RT-PCR analysis were performed for the bovine tissue distribution. As a result, the hypothesis was successfully tested and Chrdl1 gene was found highly specific in bovine adipose tissue than in various other tissues. Thus, it is concluded that microarray and microarray related GEO Profiles are a useful tool to identify new tissue specific genes.

Keywords: *Adipose tissue, Adipose specific gene, Chordin-like 1, Microarray*

Mikroarray Veritabanlarının Doku Spesifik Genlerin Belirlenmesindeki Potansiyeli

Özet

Doku spesifik genler gelişim ve metabolizma da önemli roller oynamaktadır. Günümüzde, GenBankasında farklı dokularla ilgili 3363628 gen ekspresyon profili ve 397988 mikroarray verisi bulunmaktadır. Büyük miktardaki verileri değerlendirmek ve doku spesifik genleri ortaya çıkarabilmek için yeni stratejilerin geliştirilmesi ve kullanılması gerekmektedir. Bu amaçla, bu çalışma mikroarray ve mikroarraylerle ilişkili gen ekspresyon profillerinin yeni doku spesifik genlerin belirlenmesinde kullanışlı bir araç olup olmadığını tartışmaktadır. Bu çalışmada, adipoz doku yeni doku spesifik genlerin belirlenmesi için hedef doku olarak seçilmiştir. Bu amaçla, insan ve fare mikroarray verileri karşılaştırmalı olarak analiz edilmiştir. Mikroarray verilerini desteklemek için, adipoz dokuyla ilgili gen ekspresyon profilleri GenBankasından seçilmiştir. Daha sonra, adipoz dokuyla ilgili mikroarray ve gen ekspresyon profil verileri eş zamanlı olarak değerlendirilmiştir. Mikroarray ve mikroarraylerle ilişkili gen ekspresyon profillerinin analiz sonuçlarına göre, Chrdl1 (Chordin-like 1) geninin yeni bir adipoz spesifik gen olduğuyla ilgili hipotez kurulmuştur. Hipotezi test etmek için, sığır dokularında RT-PCR analizleri gerçekleştirilmiştir. Sonuç olarak, hipotez başarılı bir şekilde test edilmiş ve Chrdl1 geni sığır adipoz dokusunda diğer dokulara göre yüksek derecede spesifik bulunmuştur. Böylelikle, mikroarray ve mikroarraylerle ilişkili gen ekspresyon profillerinin yeni doku spesifik genlerin belirlenmesinde kullanışlı bir araç olduğu sonucuna varılmıştır.

Anahtar sözcükler: *Adipoz doku, Adipoz spesifik gen, Chordin-like 1, Mikroarray*

INTRODUCTION

Since invented in 1995, microarray technologies have been widely used to compare the expression profiles of thousands of genes simultaneously under different biological conditions [1]. Nowadays, high throughput microarray technologies have numerous applications, including the study of gene expression, genotyping, proteomics, cell biology, recognizing infectious diseases, cancer biology, pharmaco genomics, detecting the existence of a pathogen in food samples and identification of microbial isolates [2].

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Obesity is a result of excess adipose development. There is a growing recognatiton that obesity is a metabolic disorder and can cause serious health problems such as heart disease, insulin resistance, hyperglycemia, dyslipidemia, hypertension, obstructive sleep apnea, and certain types of cancer [3,4]. Thus, obesity is approved as one of the most serious public health concerns in the 21st century [5].

Adipose development is significantly influenced by adipose specific genes [6]. To date, approximately 20 genes have been identified that are highly expressed in adipose tissue [7] It is well known that there have been strong relationship between adipose specific genes and obesity both human and animals. One of the most important adipose specific genes leptin and adiponectin plasma levels are decreased in both obese humans and animals [8,9]. Therefore, discoveries of new adipose specific genes will be valuable to the understanding of genetic mechanisms underlying adipose development and obesity and will also contribute to improved animal production by reducing fat accretion. In order to clarify adipose development, it is necessary to develop new methods for finding and understanding the functions of adipose specific genes.

Currently, GenBank has 1674749 GEO Profiles and 19928 microarray data related to adipose tissue [10]. These huge amounts of data are important resource to identify adipose specific genes. Thus, the main objective of this study was elucidate microarray and microarray related GEO Profiles can be used to identify new adipose specific genes. Therefore, human and mouse microarray data were analyzed comparatively. The information from GEO Profiles and literature were also used to support microarray analysis. According to the analysis, Chrdl1 was identified as a novel adipose specific candidate gene. Then, RT-PCR experiments were performed in bovine tissue distribution. The RT - PCR analysis confirmed that Chrdl1 gene was highly specific in bovine adipose tissue. As a result, this study proved that comparative analysis of microarray and microarray related GEO Profiles are very effective tool to identify new tissue specific genes.

MATERIAL and METHODS

Data Mining

To investigate the adipose specific genes, the keywords (adipose tissue) and (adipocyte) were used to search gene expression omnibus (GEO) database from PubMed [10]. Therefore, 1674749 GEO Profiles analysed in (GEO) database from PubMed [10]. Due to tissue distribution similarity GDS3142 various tissues *(Mus musculus)* and GDS596 Large-scale analysis of the human transcriptome (HG-U133A) *(Homo sapiens)* microarray data were selected in GEO database [10]. The GDS3142 [11] microarray consists of 22 different tissues from 10 to 12 wk old C57BL/6 mice and the GDS596 [12] microarray includes 79 physiologically normal tissues obtained from various sources. The dataset file of each microarray was downloaded from GEO database [10]. The GDS3142 [11] dataset file is composed of gene expression profiles. Each tissue except for muscle has 3 biological replicates. Muscle has 4 biological replicates of gene expression profiles. Tissue biological replicates of spleen (GSM252067, GSM252068, GSM252069), muscle (GSM252070, GSM252071, GSM252072, GSM252073), adipose (GSM252093, GSM252094, GSM252095), heart (GSM252113, GSM252114, GSM252115), lung (GSM252080, GSM252081, GSM252082), liver (GSM252074, GSM252075, GSM252076) and kidney (GSM252083, GSM252084, GSM252085) were collected from the GDS3142 [11]. A new Excel file was formulated for the dataset obtained from the GDS3142 [11]. Next, triplicate data from the various tissues were averaged. To find the adipose specific genes, the adipose tissue gene expression value was divided by the various tissue gene expression values. Then, values were averaged again in order to elucidate the common ratio of the various tissues. One common ratio was found which represents the ratio of various tissue expression values versus to adipose tissue. Finally, this ratio was sorted smallest to largest to identify the adipose specific genes.

The GDS596 [12] dataset file also includes gene expression profiles. Each tissue has 2 biological replicates from various sources. Heart (GSM18951, GSM18952), lung (GSM18949, GSM18950), liver (GSM18953, GSM18954) adipocyte (GSM18975, GSM18976), skeletal muscle (GSM19013, GSM19014), and kidney (GSM18955, GSM18956) gene expression profiles were selected from the GDS596 dataset [12]. All of the procedures were repeated for the GDS596 dataset as were used for GDS3142 and the common ratio of the various tissue expression values according to adipocyte were detected. The flow chart of the study illustrated in the schematic diagram shown in *Fig. 1*. To support and predict reliability of microarray data, different GEO profiles were selected from GEO database [10].

Tissue Collection

Adipose tissue, heart, muscle, spleen, lung, liver, and kidney of cattle (Angus; n = 5) were harvested after slaughter of animals at Yılet Meat Company located in Konya province. In this procedure, different equipments are used for each tissue to prevent contamination during the removal of the tissues. Finally, 300 mg tissues put into eppendorf tubes and snap frozen in liquid nitrogen.

RNA Isolation and RT-PCR Detection of Total Gene Expression

To prepare snap frozen animal tissues for RNA extraction, tissues are ground into fine powder to obtain a high yield of RNA by using a tissue homogenizer. Total RNA from the adipose tissue, heart, muscle, spleen, lung, liver, and kidney

of cattle were isolated using GF-1 Total RNA extraction kit (Vivantis) following the manufacturer's instructions. RNA quality was assessed by agarose gel electrophoresis. To make cDNA for various tissues, RT-PCR was performed using 1 µg of total RNA and M-MLV reverse transcriptase (Moloney murine leukemia virus RT, Vivantis). Gene expression was quantified using SYBR Green RT-PCR. The RT-PCR was performed using Quantitect SYBR PCR Master Mix (Qiagen), and SYBR green was used as the detection dye. The Cyclophilin gene was used as a housekeeping gene for normalization of RT-PCR calculation. Chrdl1 gene mRNA expression was normalized to Cyclophilin mRNA. The forward and reverse primers for Cyclophilin (BC102462) were 5`- TTCCATCGTGTGATCAAGGA -3`; 5`- TTAAGCTTGAAGTTCTCATCGG -3`, respectively. The forward and reverse primers for Chrdl1 (540275) were 5`- ACT CCATCACTTCAAGCTGGTGA -3`; 5`- TGCAGTCCAGCT GCAGCTT -3`, respectively. Reactions were performed in duplicate 25µL volumes on Fluorion Real-Time PCR Instrument. Conditions for RT-PCR were 95°C for 15 min, and then 50 cycles of 95°C for 30 s, 60°C for 35 s, and 72°C for 40 s. Finally, Melting curve step was performed in RT-PCR. Relative expression was calculated using the comparative ΔΔCt method for relative quantification [13].

Statistical Analysis

Comparisons of gene expression in various tissues of the mouse and human microarray were performed by one-way ANOVA, respectively. If the P-value was <0.05 in the ANOVA, Duncan multiple range test was performed. All statistical analyses were performed using GLM procedure of SAS statistical software [14].

RESULTS

In this study, statistical analysis revealed that Chrdl1, FABP4 (Fatty Acid Binding Protein 4) and ATGL (Adipose Triglyceride Lipase) genes are highly expressed in human adipocyte and mouse adipose tissue than various tissues as shown *Table 1* and *Table 2* (P<0.0001). Since, there has not been any study that showed adipose specific gene expression of Chrdl1, Chrdl1 gene was selected for further investigation. Therefore, we hypothesize that Chrdl1 gene expression is highly specific in adipose tissue than other tissues. We supported our hypothesis with further information collecting from different GEO profiles. Moreover, our hypothesis was also successfully tested by RT-PCR experiment. To test the hypothesis, RT-PCR performed for bovine tissue distribution. Results demonstrate that Chrdl1 is more highly expressed in bovine adipose tissue than in other tissues (P<0.05) as shown in *Fig. 2*. Considering the literature, Chrdl1 shows a broad expression pattern and functions in many tissues [15-19]. However, our study which represents to our knowledge

Table 1. *Gene expressions of Chrdl1, FABP4 and ATGL in tissue distributions of mouse microarray*
Tablo 1. *Chrdl1, FABP4 ve ATGL genlerinin fare mikroarrayinin doku dağılımındaki gen ekspresyonları*

Gene	Adipose	Muscle	Heart	Lung	Liver	Kidney	Spleen	P Value
FABP4	2924.9±443.9^{a}	198.6±5.9^{b}	296.9±34.9^{b}	86.1±7.7^{b}	82.3±0.9^{b}	118.0±16.0^{b}	74.7±5.6^{b}	P<0.0001
ATGL	4526.4±388.3^{a}	785.7±82.6^{bc}	1165.2±78.8^{b}	453.9±36.5^{bc}	327.2±37.4^{c}	410.2±34.4^{c}	195.8±8.9^{c}	P<0.0001
CHRDL1	825.2±129.8^{a}	99.8±6.9^{b}	76.7±2.0^{b}	110.2±6.8^{b}	64.9±1.2^{b}	97.8±12.0^{b}	92.9±3.4^{b}	P<0.0001
GSM NO	GSM252093	GSM252070	GSM252113	GSM252080	GSM252074	GSM252083	GSM252067	
	GSM252094	GSM252071	GSM252114	GSM252081	GSM252075	GSM252084	GSM252068	
	GSM252095	GSM252072	GSM252115	GSM252081	GSM252076	GSM252085	GSM252069	
		GSM252073						

GSM NO represents the biological replicates of adipose, muscle, heart, lung, liver, spleen and kidney tissues. Biological replicates number for adipose, heart, lung, liver, spleen and kidney tissues (n=3) and muscle tissue (n=4). Results are shown as average ± standard error. Different letters in the same row show significant differences between the averages (P<0.05)

Table 2. *Gene expressions of Chrdl1, FABP4 and ATGL in tissue distributions of human microarray*
Tablo 2. *Chrdl1, FABP4 ve ATGL genlerinin insan mikroarrayinin doku dağılımındaki gen ekspresyonları*

Gene	Adipocyte	Muscle	Heart	Lung	Liver	Kidney	P Value
FABP4	13364400±503100^{a}	446400±130800^{bc}	317400±153700^{bc}	1099850±275350^{b}	123700±74000^{c}	189500±58100^{bc}	P<0.0001
ATGL	2838150±111150^{a}	367800±92800^{c}	853150±260250^{b}	274250±54750^{c}	299600±105500^{c}	357850±76950^{c}	P<0.0001
CHRDL1	1702350±86950^{a}	303450±191450^{b}	130900±80600^{b}	315400±41500^{b}	58550±27050^{b}	240550±24550^{b}	P<0.0001
GSM NO	GSM18975	GSM19013	GSM18951	GSM18949	GSM18953	GSM18955	
	GSM18976	GSM19014	GSM18952	GSM18950	GSM18954	GSM18956	

GSM NO represents the biological replicates of adipocyte, muscle, heart, lung, liver and kidney tissues. Biological replicates number for adipocyte, muscle, heart, lung, liver and kidney tissues (n=2). Results are shown as average ± standard error. Different letters in the same row show significant differences between the averages (P<0.05)

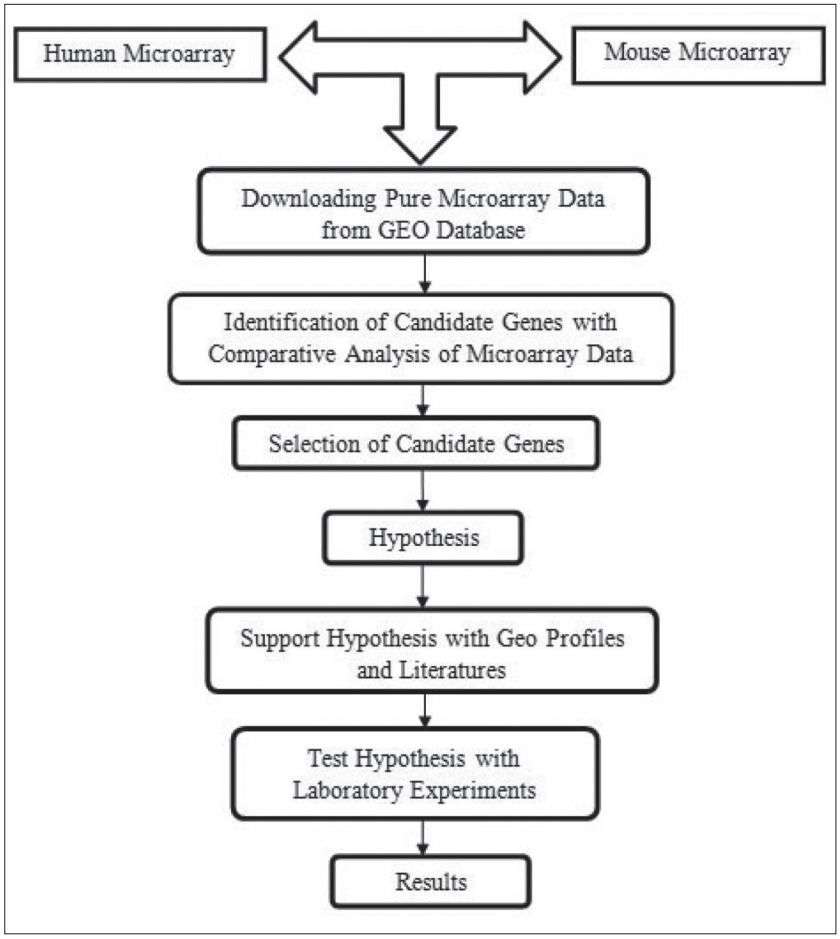

Fig 1. The flow chart of the study

Şekil 1. Çalışmanın akış şeması

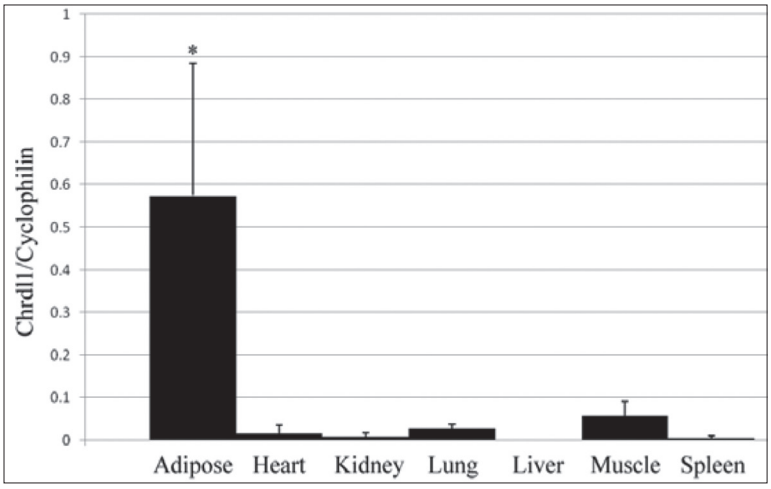

Fig 2. Chrdl1 gene expression in bovine tissue distribution . The y axis shows the relative expresion of Chrdl1\Cyclophilin. The bar graphs represent average ± standard error. (*) sign is shown the gene expression of Chrdl1 in bovine adipose tissue statistically important than other tissues according to the results of one-way ANOVA followed by Duncan multiple range test (P<0.05)

Şekil 2. Sığır doku dağılımında Chrdl1 gen ekpresyonu. Y ekseni Chrdl1\Cyclophilin genlerinin karşılaştırmalı gen ekspresyonunu göstermektedir. Grafikte görülen barlar ortalama ± standart hatayı temsil etmektedir. (*) tek yönlü varyans analizini takiben yapılan Duncan çoklu karşılaştırma testinin sonuçlarına göre Chrdl1 geninin adipoz dokudaki ekspresyonunun diğer dokulara göre istatistiksel olarak önemli olduğunu göstermektedir (P<0.05)

the first report that Chrdl1 gene expression is highly specific in adipose tissue versus other tissues.

DISCUSSION

Currently, microarray technology is used extensively for scientific research. This technology allows screening of thousands of genes simultaneously under different biological conditions. However, the challenging part is how to select the target genes from thousands of genes. As aforementioned, we identified that Chrdl1, FABP4 and ATGL genes are highly specific in adipose tissue by comparative analysis of microarray database. Among these genes, FABP4 and ATGL genes are well known adipose specific genes [20,21]. According to analysis, the FABP4 and ATGL genes were more highly expressed in mouse adipose tissue than in other tissues in the mouse microarray data (P<0.0001) as shown *(Table 1)*. Similarly, the FABP4 and ATGL genes were predominantly expressed in human adipocytes as compared to other tissues in the human

microarray data (P<0.0001) as shown *(Table 2)*. The FABP4 and ATGL genes were selected as examples for how to we analyzed microarray and microarray related GEO Profiles related to adipose tissue.

Fatty Acid Binding Protein 4 (FABP4)

Searching for FABP4 in the GEO Profiles revealed 5788 microarray analyses that contained the FABP4 gene and searching for "adipocyte" as a key word in the GEO Datasets showed 3919 microarray analyses dealing with adipocytes under different developmental, hormonal, nutritional, genetic, and pathological conditions [10]. As shown in GEO Profiles obtained from PubMed [10], GDS2659 demonstrated that FABP4 gene expression was increased during adipocyte differentiation [22]. Li et al.[20] reported that FABP4 was highly expressed during 3T3-L1 adipocyte cell differentiation. This finding was also verified by previous studies [23-25]. The GDS2818 data demonstrated that adipocytes had higher levels of FABP4 gene expression than do preadipocytes [26]. It`s previously reported that FABP4 was more highly expressed in adipocytes than in preadipocytes [27,28]. GDS3688 showed that obese children had higher FABP4 gene expression in adipose tissue [29]. Ma et al.[30] demonstrated that the FABP4 and PPAR-γ genes were more highly expressed in obesity groups than in obesity resistant groups. GDS734 [31] clarified the PPAR-γ induced expression of FABP4 in adipocytes which was confirmed the previous finding of Shin et al.[32] and suggested that PPAR-γ binds to the FABP4 promoter and activates transcription of FABP4. In addition, PPAR-γ agonist rosiglitazone treated cultures induced FABP4 and appeared earlier than in control cultures [33]. Overall, GEO Profiles and literatures demonstrated that FABP4 is an important adipose specific marker.

Adipose Triglyceride Lipase (ATGL)

Currently, there are 5484 ATGL GEO Profiles relative to microarray analyses in PubMed [10]. As shown in the GEO Profiles obtained from PubMed [10], GDS2818 showed that ATGL was more highly expressed in adipocytes than in preadipocytes [26]. This finding was confirmed by previous studies. Previous studies reported that porcine ATGL was more highly expressed in adipocytes than in the stromal vascular fraction which was rich in preadipocytes. In addition, ATGL was predominantly expressed in chicken fractionated adipose cells as compared to the stromal vascular fraction [34-36]. GDS2366 showed that ATGL gene expression was induced by adipocyte differentiation [37]. Kim et al.[21] stated that ATGL gene expression was dramatically increased during 3T3-L1 adipocyte differentiation. The ATGL gene was highly induced during porcine adipocyte differentiation [34]. GDS3688 illustrated that obese children had lower ATGL expression in adipose tissue [29]. Steinberg et al.[38] demonstrated that obese subjects had significantly reduced ATGL mRNA expression (P<0.05). Jocken et al.[39] reported that insulin resistant subjects have decreased ATGL mRNA expression compared to insulin sensitive subjects (P<0.05). GDS1298 showed that over expression of peroxisome proliferator-activated receptor (PPAR) gamma 2 induced ATGL gene expression during the differentiation of NIH-3T3 embryonic fibroblasts into adipocytes [40]. Kershaw et al.[41] reported that PPAR-γ played a significant role in regulation of ATGL mRNA expression in adipocytes under both *in vivo* and *in vitro* conditions. The GEO Profiles and literatures draw attention to ATGL adipose specificity and developmental changes in ATGL gene expression in adipose tissue.

A novel adipose specific candidate gene Chrdl1 (Chordin-like 1)

Now, there are 4366 Chrdl1 GEO Profiles related to microarray analyses in PubMed [10]. However, there are very limited numbers Chrdl1 GEO Profiles as to adipose tissue [10]. GDS2813 showed that Chrdl1 was highly expressed in white adipose tissue than brown adipose tissue [42]. GDS3102 stated that caloric restriction decreased Chrdl1 gene expression [43]. GDS2366 mentioned that Chrdl1 was specifically highly expressed in differentiated preadipocytes than undifferentiated preadipocytes [37]. GDS2319 indicated that high weight gainer individuals had much more Chrdl1 gene expression than low weight gainer [44].

Taken together, these GEO Profiles give informations about Chrdl1 and adipose tissue. In the current study, we gathered these informations from GEO Profiles and microarray database and revealed that the expression of Chrdl1 is highly specific both adipose tissue and adipocyte (P<0.0001). These findings are also supported with laboratory experiments. However, there is no previous study confirm these microarray data and GEO Profiles. Therefore, this paper is the first assessment of these microarray data and GEO Profiles to confirm Chrdl1 adipose specific gene expression.

In conclusion, this study aimed to put forward the potential of microarray and microarray related GEO Profiles to identify new adipose specific genes. Therefore. the depth research and detailed analysis of microarray database were performed to identify adipose specific genes. Here, we successfully tested how to effectively use microarray and GEO Profiles information to predict new tissue specific genes before conducting laboratory experiments. We have proof of concept to discover a new adipose specific gene is called Chrdl1. The primary positive impact of our study will be the future identification of a new set of adipose specific candidate genes that will provide a new platform for functional studies of these genes to enhance human health and improve livestock production efficiency. Moreover, this study also suggests that various tissue specific genes can be easily identified by detailed analysis of microarray and microarray related GEO Profiles.

Acknowledgements

We thank to Yılet and Selet meat companies for providing tissue samples.

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Kafkas Univ Vet Fak Derg
22 (1): 37-41, 2016
DOI: 10.9775/kvfd.2015.14364

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

Research Article

Estimation of Breeding Values with Heterogeneous Residual Variances by Random Regression Models [1]

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[1] *This study was financially supported by The Scientific and Technological Research Council of Turkey (Project No: 109O336)*

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Article Code: KVFD-2015-14364 Received: 10.09.2015 Accepted: 03.11.2015 Published Online: 03.11.2015

Abstract

In this study the third order random regression models (RV1 and RV10) including the fixed, random additive genetic and permanent environmental effects were used. In the RV1 model residual variance was constant and in the RV10 model all test day records were taken as different groups. The predicted error variance was found 6.83 in RV1 model and this variance was changed between 5.30 and 9.19 in RV10 model. Heritability values were estimated 0.12-0.53 for RV1 and 0.04-0.18 for RV10 models. Spearman rank and Kendall rank correlations between estimated breeding values of test day milk yields estimated from RV1 and RV10 models within cows (0.79, 0.61) were found almost same with within sires (0.82 and 0.63). Consequently, these correlations indicate that breeding values estimated from RV1 and RV10 models were highly correlated. Although the association between two set of breeding values estimated from RV1 and RV10 models were high, ranking of cows and sires by breeding values were different for two models. The shift in rank of first 100 cows was found 22% same and 78% different, also the shift in rank of first 50 sires was found 30% same and 70% different on RV1 and RV10 models breeding values rank list. Overall, the RV10 model was found better than RV1 model in estimation of breeding value.

Keywords: *Residual variance, Random regression, Breeding value, Turkish Holstein*

Şansa Bağlı Regresyon Modelinde Heterojen Hata Varyansları ile Damızlık Değerinin Tahmin Edilmesi

Özet

Bu çalışmada sabit, şansa bağlı genetik ve kalıcı çevre etkilerine sahip üçüncü dereceden şansa bağlı regresyon modelleri (RV1 ve RV10) kullanılmıştır. RV1 modelinde hata varyansı sabit, RV10 modelinde ise denetim günlerinin her biri farklı kabul edilmiştir. Hata varyansı RV1 modelinde 6.83 ve RV10 modelinde 5.30 ile 9.19 arasında tahminlenmiştir. Kalıtım dereceleri RV1 modelinde 0.12-0.53 iken, RV10 modelinde 0.04-0.18 arasında tahminlenmiştir. RV1 ve RV10 modelleri ile tahminlenen denetim günü süt verimi damızlık değerlerine ait Spearman ve Kendall sıra korelasyonları inek (0.79, 0.61) ve boğalarda (0.82, 0.63) birbirine benzer bulunmuştur. Sonuç olarak, her iki modelle elde edilen damızlık değerleri arasındaki ilişkinin yüksek olduğu belirlenmiştir. Sığırların damızlık değerlerinin sıralamasına yönelik korelasyonların yüksek olmasına karşın, sıralama bakımından modeller arasında farklılıklar saptanmıştır. Her iki modele ait damızlık değeri listesinde ilk 100 ineğin sıra değişimleri %22 oranında ortak ve %78 oranında farklı iken, ilk 50 boğanın sıra değişmeleri ise %30 oranında ortak ve %70 oranında farklı bulunmuştur. Genel olarak RV10 modeli damızlık değer tahmininde RV1 modelinden daha iyi bulunmuştur.

Anahtar sözcükler: *Hata varyansı, Şansa bağlı regresyon, Damızlık değeri, Siyah Alaca*

INTRODUCTION

Breeding values for all traits are used to rank and select animals in order to achieve intended genetic improvements**.** As a matter of fact; breeding values from a random regression model are not equivalent to the breeding values from the more traditional models [1]. Last decade therefore, many countries have implemented random regression models for genetic evaluation of test day records. In random regression models, residual effect is generally assumed constant throughout the trajectory. However, recent studies have shown that the residual variance (RV) is changing over time [2,3] because of herd management, weather conditions, lactation number, age at calving, month of calving, days in milk, pregnancy status, medical treatments and milking times etc. [4,5].

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Homogeneous residual variance assumption leads to lower or higher impact on the evaluation for different parts of the lactation [6]. Therefore, the information coming from each part of the lactation where the residual variance is actually larger than the assumed homogeneous value will has lower weight than it really has [7]. In addition, Olori et al.[8] conveyed that constant residual variance assumption causes residual variances to be underestimated and heritability values to be overestimated in early stage of lactation. Instead of constant residual assumption, lactation can be divided into different classes with assuming homogeneity of residual variance within the classes and heterogeneity between them [7-9]. This approach is easy to implement and useful to provide the information on the expected pattern of residual variance which changes over lactation.

There are few studies on heterogeneity of residual variance in random regression model for the estimation of genetic parameters for test day milk records [7-10]. However, there is not enough research in which examines different residual variance structures on test day milk yield estimated breeding values (EBV) by random regression models. Fujii and Suzuki [11] reported genetic parameters and EBV's for milk yield using random regression models under homogeneous and heterogeneous residual variances, larger permanent environmental variances than additive genetic variances and there was no difference between EBV's from homogeneous and heterogeneous residual variance models.

In this study, it is aimed to compare effect of homogeneity and heterogeneity residual variance on breeding values of test day milk records for Turkish Holsteins using random regression model.

MATERIAL and METHODS

Data

Test day milk records obtained from different farms who are the members of Isparta province Cattle Breeders Association in Turkey were the material of this study. First lactation test day milk yields were collected at monthly periods (TD1-TD10) from 2001 through 2011. Last test day milk records less than 5 kg were excluded and records used from monthly milk between 5th days and 307th days. Age at first calving of cows was also limited between 20 and 51 months. In final data set total of 43206 test day milk records from 6085 Turkish Holstein cows in 248 herds were analyzed. The descriptive statistics of the final data set were given in *Table 1*.

Table 1. *Structure of the final data*
Tablo 1. *Veri setinin yapısı*

Item	Statistics
Number of records	43206
Mean of milk yields per TD	18.78
Number of herds	248
Number of herd-year-season level	3809
Number of Animals with records	6085
Number of Sires with progeny records	667
Number of Dams with progeny records	4241

Method

Third order random regression model using legendre polynomial was preferred due to the best fit [10,12-17] as follows:

$$y_{ijk} = HYS_i + \sum_{m=1}^{5}\beta_m X_m(t_{jk}) + \sum_{m=1}^{3}\alpha_{jm}\phi_m(t_{jk}) + \sum_{m=1}^{3}p_{jm}\phi_m(t_{jk}) + e_{ijk}$$

where y_{ijk} is the kth test day milk yield of the cow j at ith herd -year-season, HYS_i is the ith herd-year-season effect, β_m is the mth fixed regression coefficients associated with the mth covariate, t_{jk} is the kth test day of the cow j, $X_m(t_{jk})$ is the mth covariates (X_1: Age at first calving, c=305, X_2=DIM/c, $X_3=(X_2)^2$, X_4=ln(c/DIM), $X_5=(X_4)^2$ depending on DIM=t evaluated at t_{jk}), α_{jm} is the mth additive genetic random regression coefficients for cow j, p_{jm} is the mth permanent environmental random regression coefficients for cow j, ϕ_m is the mth polynomial and e_{ijk} is the random residual effect with $e_{ijk} \sim N(0,\sigma^2 e_{ijk})$. In this model homogeneity and heterogeneity residual variance assumptions were tested. In RV1 model, the residual variance was assumed constant throughout lactation. On the contrary to RV1, residual variance was assumed different for each test day in RV10 model.

DXMRR option of the DFREML statistical package [18] was used for fitting of the models. The goodness of fits for the models with different error variances were examined using as Akaike's information criterion-AIC [19]. This likelihood based criterion has been calculated as: AIC= -2*LogL +2*p where p denotes the number of parameters estimated. The model which gives the lowest AIC values was chosen as the better approximating model [20,21]. Furthermore, two error structures were compared by Likelihood ratio test-LRT [22]. LRT for model i and j was $LRT_{ij}=2*(LogL_i-LogL_j)$. In the LRT, the Log Likelihood (LogL) differences were tested using Chi-square test with the degree of freedom determined as the number of the parameter differences between the models [23]. Spearman rank and Kendall rank correlations between EBV of test day milk yields obtained from RV1 and RV10 models were also calculated for cows and sires. Shifts in rank of animals according to EBV were determined to show the changes in ranks of cows and sires between models.

RESULTS

The comparison of RV1 and RV10 models were presented in *Table 2*. The differences in LogL values of the

RV1 and RV10 models were found significant (P<0.05). A decrease in AIC values with increased numbers of parameters was noticed between models *(Table 2)*.

The residual, additive genetic, permanent environmental variances and heritability estimates of test day milk yields from the RV1 and RV10 models were summarized in Table 3. Two models had different tendency for estimation of variance components. The residual variance was 6.83 from the RV1 model and changed between 5.30 and 9.19 from RV10 model. In RV10 model, the predicted residual variances were lower at the beginning of the lactation but higher at end of the lactation. Estimates of additive genetic variances were varied from 1.83 to 14.28 for RV1 and 0.91 to 3.77 for RV10 models. Changes of the permanent environmental variances were found between 5.24 to 8.94 for RV1 and 6.39 to 14.45 for RV10 models. Heritability estimates of test day milk yields from the RV1 and RV10 models were ranged from 0.12 to 0.53 and 0.04 to 0.18, respectively *(Table 3)*.

The Spearman rank correlations between EBV's from RV1 and RV10 models were statistically significant (P<0.01) and found 0.79 for cows and 0.82 for sires. The difference between the probabilities in the same and different orders of animal according to EBV with Kendall rank correlations were also statistically significant (P<0.01) and determined 0.61 for cows and 0.63 for sires.

Mean and standard deviations for test day milk yield EBV's from RV1 and RV10 models were illustrated in *Table 4*. Number and percent of cows and sires were detected to indicate the rank changes of Turkish Holsteins sorted by their EBV's from RV1 and RV10 models. Moreover, largest rank shift of cows and sires was also determined according to various top lists EBV's from both models rank list in *Table 4*.

The mean of EBV's for cows ranged from 8.98 to 6.79 for RV1 model and changed from 1.05 to 1.38 for RV10 model. The mean of EBV's of sires changed from 5.34 to 3.35 and from 1.03 to 0.63 for RV1 and RV10 models, respectively. Mean of EBV's were decreased when increased number of animals in top lists as expected. On the other hand, when only the first 10 cows are considered, there are 2 cows (20%) on both lists. The percentages of cows on both lists were 30% when the first 20 and 50 cows are considered. When only the first 100 cows are considered, there are 22 cows (22%) same and 78% different on RV1 and RV10 models EBV's rank list. However, the first 5, 10, 25 and 50 sires are considered, the percentages of sires on both lists were ≥25%. 50% sires (5 sires) are on both lists when the first 10 sires are considered. Largest shifts in rank for the first 10, 20, 50 and 100 cows, and for the first 5, 10, 20 and 50 sires are presented based on the ranking for milk yield EBV's. As shown in *Table 4*, when the first 10 cows are considered, the cow in the 4th rank on RV1 list appeared in the 9th rank on RV10 list, which was the largest rank shift in this group. When the first 5 sires are considered, the sire in the 4th rank on RV1 list appeared in the 3th rank on RV10 list. Considering largest rank shifts, the order for RV10 is generally higher than the orders for RV1. The largest rank shifts for only first 10 cows and 50 sires showed opposite direction. This shows each two model are different in estimation of breeding value.

Table 2. *LogL and AIC values for RV1 and RV10 models*
Tablo 2. *RV1 ve RV10 modellerinde LogL ve AIC değerleri*

Models	Number of Parameters	AIC	Log Likelihood Values	LRT
RV1	13	140325	-70150	-
RV10	22	140143	-70043	107*

AIC: *Akaike's information criterion;* ***LRT:*** *Likelihood ratio test;* * *LRT values between RV1 and RV10 models are significant (P<0.05)*

Table 3. *Estimations of additive genetic (G), permanent environmental (PE), residual variances (RV) and heritability estimates (h^2) of test day milk yields from the RV1 and RV10 models*
Tablo 3. *RV1 ve RV10 modellerinde denetim günü süt verimleri için eklemeli genetik, kalıcı çevre, hata varyansları ve kalıtım derecesi (h^2) tahminleri*

Model	Parameters	Test Day									
		1	2	3	4	5	6	7	8	9	10
RV1	G	5.81	3.47	2.40	1.97	1.83	1.94	2.55	4.20	7.73	14.28
	PE	8.94	7.40	6.96	6.98	7.00	6.78	6.27	5.64	5.24	5.64
	RV	6.8									
	h^2	0.27	0.20	0.15	0.12	0.12	0.12	0.16	0.25	0.39	0.53
RV10	G	3.77	3.14	2.65	2.17	1.74	1.40	1.16	1.00	0.92	0.91
	PE	12.17	8.49	7.65	6.80	6.39	6.63	7.60	9.25	11.56	14.45
	RV	5.30	7.88	6.26	5.50	7.46	6.84	5.94	6.82	7.86	9.19
	h^2	0.18	0.16	0.16	0.15	0.11	0.09	0.08	0.06	0.05	0.04

Table 4. *Shifts in rank of cows and sires ranked by EBV's from RV1 and RV10 models*
Tablo 4. *Sığırların RV1 ve RV10 modelleri ile tahminlenen damızlık değerleri sıralamasındaki değişimler*

Animals Considered	X±S for EBV's		Number of Animals on Both Lists	% of Animals on Both Lists	Largest Rank Shift	
	RV1 Model	RV10 Model			RV1 Model	RV10 Model
Cows						
first 10	8.98±0.59	1.38±0.08	2	20	4	9
first 20	8.44±0.69	1.31 ±0.06	6	30	17	13
first 50	7.59±0.86	1.17±0.13	15	30	50	22
first 100	6.79±1.02	1.05±0.15	22	22	94	8
Sires						
first 5	5.34±0.81	1.03±0.13	2	40	4	3
first 10	4.76±0.82	0.94±0.13	5	50	10	5
first 20	4.16±0.85	0.81±0.16	5	25	16	2
first 50	3.35±0.87	0.63±0.18	15	30	47	76

DISCUSSION

In this study constant residual variance (RV1) and heterogeneous residual variances (RV10) for each test day periods were compared. The RV1 model was differ from RV10 model for the goodness of fit test. The RV10 model was fit better than RV1 model due to lower AIC value. The predicted residual variances were lower at beginning and had fluctuating tendency in middle but higher at the end of the lactation under RV10 model. Fujii and Suzuki [11] observed similar tendency for beginning and end of lactation but had generally lower estimates of residual variances. Estimates of Olori et al.[8] are not similar to those in this study with higher estimates at beginning and lower estimates at the end of the lactation. Moreover, Olori et al.[10] had highest estimates at the beginning of lactation and relatively constant in mid lactation with lower values than this study. The fluctuating estimates of residual variances might clarify with number of test day records used and the models in the analysis.

It can be seen that RV1 model was appeared with higher genetic variance estimates at the beginning and end of lactation, but lower at the middle of the lactation. Genetic variances for RV10 model follows lower estimates at the both side of the lactation than RV1. In our study, estimates of additive genetic variances (1.83-14.28 for RV1 and 0.91-3.77 for RV10) were lower than the estimates of Rekaya et al.[9]. These estimates were higher than the estimates obtained from Fujii and Suzuki [11] for the early and late part of lactation. The differences in genetic variations were the reason of genetic differences among cows since variance components for test day milk yields are change according to lactation stages, parity, year of calving and generally populations etc. Also inconsistent environmental factors in early and late stages of lactation may partly explain the discrepancy in estimates in our study.

Furthermore, permanent environmental variances (6.39-14.45) had higher values at the beginning and end of lactation, but lower at the middle of the lactation under RV10 model. The estimates of permanent environmental variances (5.24-8.94) tended to slightly increase at the beginning and decrease at the end of lactation under RV1 model. Olori et al.[8], Rekaya et al.[9], Fujii and Suzuki [11] have also found similar findings but reported higher estimates than our results.

The heritability estimates from RV1 model (0.39-0.15) were found lower at middle of the lactation and higher early and late part of the lactation because of lower environmental but higher genetic variances. Heritability estimates from RV10 model (0.3-0.11) were found lower for early and late part of the lactation as expected due to lower genetic variances at these stages. When compared these results to Olori et al.[8] and Olori et al.[10] have obtained opposite trend and higher estimates because of higher estimates of genetic variances. Heritability estimates only reflect the proportion of genetic variance of test day milk yields, but they cannot say anything about causes of variances of the test day milk yields. Constant residual variance assumption caused lower permanent environmental and higher genetic variance estimates and therefore may be induced over estimated heritability values at late of lactation stages (TD9 and TD10) in our study.

In this study, spearman rank correlations that clarify correlations of cows and sires EBV's ranking for RV1 and RV10 models were found high and significant. The difference between the probability of same order EBV's of two models and probability of different orders EBV's of two models were also high and significant. These correlations explain that, estimated breeding values of animals from RV1 and RV10 models are highly correlated. Moreover, ranking of sires was less affected than ranking of cows with heterogeneous residual variance model. When the

differences of largest rank shifts for models are examined, it can be seen that more extreme changes in ranks occurred for the both lists. A possible explanation of this finding could be reason of lower accuracy for cows EBV's. The drastic changes in ranks of EBV's in the cow and sire lists and also different model lists seemed to may be associated with differences on lactation curves that deviated from the standard lactation curve.

Consequently, our results indicate that type of residual variance assumption in random regression models might have significant effect on the variance components at any stages of the lactation. This effect is also available in EBV's as on variance components. In this study, it can be seen that heterogeneous residual variance assumption is more effective than constant residual assumption. Therefore, heterogeneous residual variance effect will be more informative for detecting reliable breeding values.

Acknowledgement

The authors would like to thank the Cattle Breeders Association of Turkey for providing data.

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Kafkas Univ Vet Fak Derg
22 (1): 43-48, 2016
DOI: 10.9775/kvfd.2015.13847

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

Research Article

The Effect of Cellulase Enzyme Treatment on Digestibility of Rice Straw [1]

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[1] *This study was conducted by supplied chemicals and infrastructure established by Ondokuz Mayis University Research Fund (Project number: PYO VET 1906.11.004)*

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Article Code: KVFD-2015-13847 Received: 11.06.2015 Accepted: 23.10.2015 Published Online: 24.10.2015

Abstract

The aim of this study was to determine the effects of different levels of cellulase enzyme treatments and incubations on digestibility of rice straw. Rice straw was treated by using cellulase from *Trichoderma reseei* at 0 (Control, C), 1 (RS+CEL1), 1.5 (RS+CEL1.5) and 2% (RS+CEL2) levels (dry matter basis, DM), and treated rice straw samples were ensiled in 6 glass jars for C, RS+CEL1, RS+CEL1.5 and RS+CEL2. For control and each level of cellulase treatment, 3 glass jars were incubated at room temperature (22ºC) in the dark for 30 days, 3 glass jars were incubated at 40±0.2ºC in an incubator for 30 days. After the treatment, the significant increases (P<0.05) were determined in treatment groups compared to control for the percentage of *in vitro* true digestibility as fed ($IVTD_{as\ fed}$), *in vitro* true dry matter digestibility (IVTDMD), *in vitro* true neutral detergent fiber digestibility in DM basis ($IVTNDFD_{DM}$) and *in vitro* true organic matter digestibility in DM basis ($IVTOMD_{DM}$) of rice straw. The best results obtained from the highest level of cellulase (RS+CEL2) treatment at 40ºC for $IVTD_{as\ fed}$, IVTDMD, $IVTNDFD_{DM}$ and $IVTOMD_{DM}$ were 62.99±0.22, 60.43±0.23, 30.70±0.23 and 63.58±0.34%, respectively. In conclusion, observed results showed that treatment of rice straw with cellulase improved the true digestibilitiy.

Keywords: *Fibrolytic enzyme, In vitro true dry matter digestibility, In vitro true neutral detergent fiber digestibility, Rice straw*

Sellülaz Enzimi Muamelesinin Çeltik Samanı Sindirilebilirliği Üzerine Etkisi

Özet

Bu çalışmanın amacı, farklı düzeylerdeki selülaz enzimi muamelesi ve inkübasyonunun, çeltik samanının sindirilebilirliği üzerine olan etkisini araştırmaktır. Çeltik samanı *Trichoderma reseei* kaynaklı selülaz ile %0 (Kontrol, K), 1 (ÇS+SEL1), 1.5 (ÇS+SEL1.5) ve 2 (ÇS+SEL2) düzeylerinde (kuru maddede, KM'de) muamele edildi. Muamele edilen çeltik samanları, kontrol ve her bir muamele grubu için 6'şar adet cam kavanoza silolandı. Kavanozların 3 tanesi karanlıkta oda sıcaklığında (22ºC) diğer 3 tanesi de bir inkübatörde 40ºC'de 30 gün süre ile inkübasyona bırakıldı. Çeltik samanının farklı düzeylerde selülaz ile muamelesi sonucunda kontrol grubuna göre çeltik samanı *in vitro* gerçek sindirilebilirliğinde ($IVGS_{yem}$), *in vitro* gerçek kuru madde sindirilebilirliğinde (IVGKMS), kuru madde bazında *in vitro* nötral deterjan lif sindirilebilirliğinde ($IVGNDFS_{KM}$) ve *in vitro* organik madde sindirilebilirliğinde ($IVGOMS_{KM}$) önemli artışlar (P<0.05) saptandı. Araştırmada çeltik samanının en yüksek düzeyde selülaz (ÇS+SEL2) ile muamele ve 40ºC'de inkübe edilmesiyle $IVGS_{yem}$, IVGKMS, $IVGNDF_{KM}$ ve $IVGOMS_{KM}$ değerleri sırasıyla %62.99±0.22, 60.43±0.23, 30.70±0.23 ve 63.58±0.34 olarak bulundu. Sonuç olarak, elde edilen bulgular selülaz enzimi ile çeltik samanı muamelesinin gerçek sindirilebilirliği artırdığını göstermiştir.

Anahtar sözcükler: *Çeltik samanı, Fibrolitik enzim, İn vitro gerçek kuru madde sindirilebilirliği, İn vitro gerçek nötral deterjan lif sindirilebilirliği*

INTRODUCTION

In many Asian and other developing countries, cereal straws are used in ruminant nutrition because they can be easily and cheaply provided for ruminant nutrition. Rice straw is a by-product of rice production. However, its nutritive value and digestibility are relatively low because its palatability is poor and it has high lignocellulosic complex, low crude protein value and high silica concentration resisting bacterial attachment to surface of rice straw in

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rumen [1]. Average dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin contents of different varieties of rice straw vary from 92.21 to 93.05%, 81.21 to 86.24%, 3.49 to5.10%, 72.16 to 77.57%, 41.38 to 46.32% and 4.3 to 6.97%, respectively [2].

Rice straw like other straws decribed low quality forages may be treated with different physical (ground, pelleting/ chopping for reducing particle size, soaking, steam or X-rays treatment, cooking under pressure etc.), chemical (sodium/calcium/potassium/ammonium hydroxide, urea/ ammonia treatments etc.) physico-chemical (reducing particle size and chemical treatment, sodium hydroxide treatment and pelleting, urea treatment and pelleting, chemical treatments and steaming etc.) or biological (enzyme/white rot fungi treatment etc) methods for improving its feeding value. Although application of chemical treatments like sodium hydroxide to improve of digestibility of straw increased degradability of straw [3], it can be a cause of enviromental pollution. Therefore, the interest is focused on the biological treatments due to the outgoing concerns on food safety in animal originated food.

Addition of exogenous enzymes obtained from fungi, white rot fungi and mushroom are a potential biological treatment to improve the digestibility of low quality forages because of being able to break down lignocellulose [4]. Recently, the degradation of cereal straw cell walls with fibrolytic enzymes has been reported to increase the digestibility and improve the nutritive value of cereal straws [5-7]. Beauchemin et al.[8] reports that the nutritive value of rice straw may be improved by using exogenous fibrolytic enzymes. However, use the fiber degrading enzyme as a feed additive and its treatment are important for increasing degradation of rice straw.

Harvested rice production in Turkey is around 900.000 tons [9] which is equivalent to 500.000 tons potential rice straw production capacity. Generally this high amount of rice straw has not been used efficiently for ruminant feeding in Turkey because of its low digestibility, therefore, rice straw has been ploughed, burned in the field or used for bedding of cattle. Only slight amount of rice straw has been used for ruminant nutrition mostly for buffaloes [10,11]. On the other side, there is a worldwide shortage of finding roughage for ruminant nutrition including Turkey. Hence, the aim of the study was to investigate effect of different levels of cellulase enzyme treatments on digestibility of rice straw.

MATERIAL and METHODS

Chemical analyses and *in vitro* digestibility of rice straw were conducted in the Ruminant Feed Evaluation Laboratory of Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine of Ondokuz Mayis University, Samsun, Turkey.

Animals

Rumen fluid was collected from three ruminally cannulated Karayaka rams, average weight 50 kg and two-year-aged (approved by Ondokuz Mayis University, the Local Ethics Committee on Animal Experiments, 18/12/2012, HADYEK 2012/70) were kept in the individually penned indoors at barn. The rams were fed twice daily (08:00 am and 17:00 pm) by 650 g of alfalfa hay and 350 g concentrate. These amounts were estimated to the level of 1.25 x maintenance requirements according to NRC [12] during the study. Fresh drinking water was freely supplied.

Chemical Analysis

Rice straws were milled for passing through a 1 mm sieve and analyzed according to AOAC methods [13]. ADF, NDF (with sodium sulfite) and acid detergent lignin (ADL) contents of rice straw used in the study were detected by Van Soest et al.[14].

Enzyme Treatments of Rice Straw

Rice straw was obtained from private dairy farm in Doganca Bafra, Samsun, Turkey. Rice straw was chopped to 5 cm length and treated with the cellulase enzyme (Farmazyme cellulase, FARMAVET International, Turkey, EC: 3.2.1.4, from *Tricoderma reseei* and activity ≥5.000 U/ kg; 1 unit of cellulase activity is described as the enzyme required to release 1 µg reducing sugar from 4 mg/ mL sodium cellulose glycolate in one min at 37ºC and pH 5.5) at the levels of 0 (C), 1 (RS+CEL1), 1.5 (RS+CEL1.5) and 2.0% (RS+CEL2) of dry matter (DM) basis of rice straw according to the method reported by Nakashima et al.[15]. The amount of water added to rice straw for enzyme treatment calculated to provide approximately 40% DM of rice straw. To ensure a homogeneous distribution of cellulase enzyme in rice straw, cellulase enzyme was dissolved in water and then was immediately sprayed on rice straw and mixed for absorption by the rice straw. After mixing, rice straw samples treated with cellulase enzyme were compressively filled into 6 glass jars each of which is a 1 L for each level of enzyme treatment. To prevent the air inlet to the jars, around of the lids of jars were coated with silicone. For each level of cellulase enzyme treatment, 3 glass jars were incubated at room temperature (22ºC) in the dark for 30 days, 3 glass jars were incubated at 40±0.2ºC in an incubator for 30 days. After incubation, all jars were opened and dried at 100±0.2ºC) in an incubator to stop the cellulase activity. Dried rice straws were ground for *in vitro* digestion technique and chemical analysis.

Preparation of Bags

The bags (Ankom F57 filter bag, Ankom Technology Corp., Fairport, NY, USA) were rinsed in acetone for 3 min

and then they were allowed to air dry. Once air dry, the bags were marked and placed in a drying oven set at 60ºC for 8 h. After the bags were dried, they were kept in a desiccator before being weighed. Ground rice straw samples were weighed into the bags (triplicate per treatment) at a mass of 0.5±0.01 g per bag, sealed by an impulse bag sealer (Ankom 1915/1920 Heat Sealer, Ankom Techonology Corp., Fairport, NY, USA). A bag without substrate was also used for a blank [16].

Ruminal Content Collection

Ruminal content was collected from different sites within the rumen at 3 h after morning feeding in thermos flasks and transported instantly to the laboratory. Ruminal content was strained through different layers of cheesecloth and held at 39ºC under a CO_2 atmosphere.

In vitro Digestion Method

The Ankom Daisy^{II} *in vitro* fermentation system (Ankom Technology Corp., Fairport, NY, USA) was used for the determination of *in vitro* true digestibility, and the procedure performed was according to that described by the manufacturer [16].

In this technique, buffer solutions were prepared described by the Ankom Daisy^{II} *in vitro* fermentation system. An amount of 1600 mL of the buffer solution was poured into each digestion jar of incubator, before the jars were placed into the incubator with the temperature set at 39ºC. The heat and agitation switches on the incubator were turned on, and the required time was allowed for the temperature of the digestion jars containing the buffer to equilibrate. Thereafter, 400 mL rumen fluid was added to each digestion jars individually. Each digestion jars were purged with CO_2 gas before activating of incubator for 48 h. After 48 h of incubation, jars were took out from their chambers, the incubation medium inside of jars was removed and bags gently washed under running cold water until they were completely clean. Thereafter, the bags were placed in the Ankom $^{200/220}$ Fiber Analyzer, and the NDF procedure was performed according to operating manual supplied by ANKOM. After the NDF procedure, the bags were dried at 105ºC for 12 h and ashed in a furnace at 550ºC for 6 h. The percentage of *in vitro* true digestibility as fed ($IVTD_{as\ feed}$), *in vitro* true dry matter digestibility (IVTDMD), *in vitro* true NDF digestibility in DM basis ($IVTNDFD_{DM}$) and *in vitro* true organic matter digestibility in DM basis ($IVTOMD_{DM}$) values of the samples were calculated with equations consisting the difference between the amount of incubated and the residue after NDF analysis for the different treatments [16]. Digestibilities were calculated with equations 1, 2, 3 and 4.

$$IVTD_{as\ feed}\ (\%) = 100 - [(W3 - (W1 \times C1)) \times 100]/(W2) \quad [1]$$

$$IVTDMD\ (\%) = 100 - [(W3-(W1 \times C1)) \times 100]/(W2 \times \%DM_{Feed}) \quad [2]$$

$$IVTNDFD_{DM}\ (\%) = 100 \times [(W2 \times \%NDF_{Feed}) - (W3-(W1 \times C1))]/(W2 \times \%DM_{Feed}) \quad [3]$$

$$IVTOMD_{DM}\ (\%) = 100 - [(W4) \times 100]/(W2 \times \%DM_{Feed}) \quad [4]$$

where W1 is weight of filter bag, W2 is weight of sample, W3 is final weight (filter bag + sample), W4 is organic material weight (calculated after inceration of filter bags contained sample), NDF_{Feed} is % of NDF contain in feed (%DM), DM_{Feed} is % of dry matter contain in feed and C1 is correction factor of blank filter bag value.

Statistical Analysis

Data were designed for appropriate of one-way classification with 2x4 factorial experimental design and analyzed with GLM procedure. Main effects were compared with Duncan's multiple range test. Mean differences of interaction effects were compared to Tukey test. All analyses and calculations were performed with SAS [17].

RESULTS

Dry matter, ash, crude protein, ADF, NDF and ADL contents of rice straw were 93.53, 16.68, 4.15, 41.15, 65.72 and 7.85%, respectively. The percentages of $IVTD_{as\ fed}$, IVTDMD, $IVTNDFD_{DM}$ and $IVTOMD_{DM}$ of rice straw untreated and treated with the different levels of cellulase enzyme, and incubated at room temperature (22ºC) and 40±0.2ºC for 30 d were given in *Table 1* and *Table 2*, respectively. There were increases (P<0.05) in the percentages of $IVTD_{as\ fed}$, IVTDMD, $IVTNDFD_{DM}$ and $IVTOMD_{DM}$ in group RS+CEL1.5 and RS+CEL2 incubated at room temperature (22ºC) for 30 d *(Table 1)*. Although the same parameters except for the percentage of $IVTOMD_{DM}$ increased in RS+CEL1, RS+CEL1.5 and RS+CEL2 incubated at 40ºC for 30 d *(Table 2)*. However, the percentage of $IVTOMD_{DM}$ was the highest (P<0.05) in RS+CEL2 incubated at 40ºC for 30 d. The effect of cellulase treatment and incubation temperature of rice straw on the percentages of $IVTD_{as\ fed}$, IVTDMD, $IVTNDFD_{DM}$ and $IVTOMD_{DM}$ of rice straw were presented in *Table 3*. The percentages of $IVTD_{as\ fed}$, IVTDMD, $IVTNDFD_{DM}$ and $IVTOMD_{DM}$ of rice straw were the highest in RS+CEL2 incubated at 40ºC for 30 d *(Table 3)*. The best results obtained from the highest level of cellulase (RS+CEL2) treatment (P<0.05) at 40ºC for $IVTD_{as\ fed}$, IVTDMD, $IVTNDFD_{DM}$ and $IVTOMD_{DM}$ were 62.99±0.22, 60.43±0.23, 30.70±0.23 and 63.58±0.34%, respectively.

DISCUSSION

The chemical composition of rice straw except for NDF content that was lower in the present study was similar to that of Rahman et al.[2]. Yadav and Yadav [18] stated that NDF value of rice straw was 64.94% which is similar to the value of this study. The lower NDF content may be resulted in improving of fiber digestion. The ADL content of rice

Table 1. *The percentages of in vitro true digestibility as fed (IVTD $_{as\ fed}$ %), in vitro true dry matter digestibility (IVTDMD%), in vitro true neutral detergent fiber digestibility in dry matter basis (IVTNDFD$_{DM}$%) and in vitro true organic matter digestibilityin dry matter basis (IVTOMD$_{DM}$%) of rice straw untreated and treated with cellulase enzyme + incubated at room temperature (22ºC) for 30 d*

Tablo 1. *Selülaz enzimi ile muamele edilen ve edilmeyen çeltik samanının oda sıcaklığında (22ºC) 30 gün inkübasyonu sonucu yemin in vitro gerçek sindirilebilirlik (%IVGS $_{yem}$), in vitro gerçek kuru madde sindirilebilirliği (IVGKMS),kuru maddede in vitro gerçek nötral deterjan lif sindirilebilirliği (IVGNDFS$_{KM}$), kuru maddede in vitro geçek organik madde sindirilebilirliği (%IVGOMS$_{Km}$)*

Digestibility, %	**C** **X ± Sx**	**RS + CEL1** **X ± Sx**	**RS + CEL1.5** **X ± Sx**	**RS + CEL2** **X ± Sx**
IVTD $_{as\ fed}$	59.07±0.36b*	60.16±0.35b	61.32±0.15a	61.87±0.44a
IVTDMD	56.24±0.39b	57.40±0.37b	58.64±0.16a	59.24±0.47a
IVTNDFD$_{DM}$	26.51±0.39b	27.67±0.37b	28.91±0.16a	29.50±0.47a
IVTOMD$_{DM}$	58.92±0.30b	59.78±0.38b	61.39±0.36a	61.96±0.52a

** Different letters within the same rows indicate differences among groups (P<0.05)*
C: *Control, 0%;* ***RS+CEL1:*** *Rice Straw + cellulase treatment of dry matter basis at the level of 1%;* ***RS+CEL1.5:*** *Rice Straw + cellulase treatment of dry matter basis at the level of 1.5%;* ***RS+CEL2:*** *Rice Straw + cellulase treatment of dry matter basis at the level of 2%*

Table 2. *The percentages of in vitro true digestibility as fed (IVTD $_{as\ fed}$ %), in vitro true dry matter digestibility (IVTDMD%), in vitro true neutral detergent fiber digestibility in dry matter basis (IVNDFD$_{DM}$%) and in vitro true organic matter digestibility in dry matter basis (IVOMD$_{DM}$%) of rice straw untreated and treated with cellulase enzyme + incubated at 40ºC for 30 d*

Tablo 2. *Selülaz enzimi ile muamele edilen ve edilmeyen çeltik samanının 40ºC'de 30 gün inkübasyonu sonucu yemin in vitro gerçek sindirilebilirlik (%IVGS $_{yem}$), in vitro gerçek kuru madde sindirilebilirliği (IVGKMS),kuru maddede in vitro gerçek nötral deterjan lif sindirilebilirliği (IVGNDFS$_{KM}$), kuru maddede in vitro gerçek organik madde sindirilebilirliği (%IVGOMS$_{Km}$)*

Digestibility, %	**C** **X ± Sx**	**RS + CEL1** **X ± Sx**	**RS + CEL1.5** **X ± Sx**	**RS + CEL2** **X ± Sx**
IVTD $_{as\ fed}$	58.99±0.26b*	62.25±0.23a	62.30±0.31a	62.99±0.22a
IVTDMD	56.16±0.28b	59.64±0.25a	59.70±0.33a	60.43±0.23a
IVTNDFD$_{DM}$	26.42±0.28b	29.90±0.25a	29.96±0.33a	30.70±0.23a
IVTOMD$_{DM}$	58.72±0.25c	61.90±0.23b	62.47±0.33b	63.58±0.34a

** Different letters within the same rows indicate differences among groups (P<0.05)*

Table 3. *The percentages of in vitro true digestibility as fed (IVTD $_{as\ fed}$ %), in vitro true dry matter digestibility (IVTDMD%), in vitro true neutral detergent fiber digestibility in dry matter basis (IVTNDFD$_{DM}$%) and in vitro true organic matter digestibility in dry matter basis (IVTOMD$_{DM}$%) of rice straw untreated and treated with cellulase enzyme + incubated atroom temperature (22ºC) and 40ºC for 30 d*

Tablo 3. *Selülaz enzimi ile muamele edilen ve edilmeyen çeltik samanının oda sıcaklığında (22ºC) ve 40ºC'de 30 gün inkübasyonu sonucu yemin in vitro gerçek sindirilebilirlik (%IVGS $_{yem}$), in vitro gerçek kuru madde sindirilebilirliği (IVGKMS),kuru maddede in vitro gerçek nötral deterjan lif sindirilebilirliği (IVGNDFS$_{KM}$), kuru maddede in vitro gerçek organik madde sindirilebilirliği (%IVGOMS$_{Km}$)*

Treatments	**IVTD $_{as\ fed}$** **X ± Sx**	**IVTDMD** **X ± Sx**	**IVTNDFD$_{DM}$** **X ± Sx**	**IVTOMD$_{DM}$** **X ± Sx**
C inc at 22ºC	59.07±0.36d*	56.24±0.39d	26.51±0.39d	58.92±0.30c
C inc at 40ºC	58.99±0.26d	56.16±0.28d	26.42±0.28d	58.72±0.25c
RS+CEL1 inc at 22ºC	60.16±0.35c	57.40±0.37c	27.67±0.37c	59.78±0.38c
RS+CEL1 inc at 40ºC	62.25±0.23ab	59.64±0.25ab	29.90±0.25ab	61.90±0.23b
RS+CEL1.5 inc at 22ºC	61.32±0.15b	58.64±0.16b	28.91±0.16b	61.39±0.36b
RS+CEL1.5 inc at 40ºC	62.30±0.31ab	59.70±0.33ab	29.96±0.33ab	62.47±0.33b
RS +CEL2 inc at 22ºC	61.87±0.44b	59.24±0.47b	29.50±0.47b	61.96±0.52b
RS+CEL2 inc at 40ºC	62.99±0.22a	60.43±0.23a	30.70±0.23a	63.58±0.34a

** Different letters within the same column indicate differences among groups (P<0.05)*
C inc at 22ºC: *Control incubated at 22ºC;* ***RS+CEL1 inc at 22ºC:*** *Rice Straw + cellulase treatment of dry matter basis at the level of 1% and incubated at 22ºC;* ***RS+CEL1.5 inc at 22ºC:*** *Rice Straw + cellulase treatment of dry matter basis at the level of 1.5% and incubated at 22ºC;* ***RS+CEL2 inc at 22ºC:*** *Rice Straw + cellulase treatment of dry matter basis at the level of 2% and incubated at 22ºC*
C inc at 40ºC: *Control incubated at 40ºC;* ***RS+CEL1 inc at 40ºC:*** *Rice Straw + cellulase treatment of dry matter basis at the level of 1% and incubated at 40ºC;* ***RS+CEL1.5 inc at 40ºC:*** *Rice Straw + cellulase treatment of dry matter basis at the level of 1.5% and incubated at 40ºC;* ***RS+CEL2 inc at 40ºC:*** *Rice Straw + cellulase treatment of dry matter basis at the level of 2% and incubated at 40ºC*

straw in the present study was lower than that of Akinfemi and Ogunwole [19] and higher than that of Rahman et al.[2] The differences for NDF and ADL contents may be due to diversity in agro-ecological condition, variety, soil fertility, climate, and other environmental events.

Rice straw cell wall like other cell walls of plants consists of cellulose, hemicellulose and lignin. Although these components are broken down with cellulase, hemicellulase and ligninase enzymes, these enzymes except for ligninase are produced by microorganisms in the reticulorumen of ruminants [20]. Theander and Aman [21] stated that rice straw has higher leaves compared to that of other cereal straws such as barley, oats and wheat. Vadiveloo [3] reported that IVDMD of the leaves and the stems was 50-51% and 61%, respectively. Phang and Vadiveloo [22] stated that IVDMD for the leaf and stem of rice straw in goats was 56.2% and 68.5%, respectively. In the present study, IVDMD of rice straw untreated and treated with cellulase were min 56.16±0.28 and max 60.43±0.23%. The difference in our findings may be atributed to cellulase treatment of whole rice straw including leaves and stem together.

The addition of fibrolytic enzymes in ruminant diets can increase digestibility of forages and improve productivity [23]. Wang et al.[7] reported that exogenous fibrolytic enzymes have potential effect on improving of *in vitro* fibre digestibility of barley straw. Wang et al.[7] and Salem et al.[24] mentioned that exogenous fibrolytic enzymes increase the DM and NDF digestibilities. Tang et al.[5] reported that fibrolytic enzyme supplementations improved the IVDMD and IVOMD of rice straw.

Beauchemin et al.[25] suggested that treatment of dried feeds with exogenous enzymes implemented in a liquid form was important for adsorption of enzyme to provide suitable attachment to feed material before feeding and protection against proteolytic degradation in rumen. In present study, while treatment with cellulase dissolved in water and sprayed on rice straw at the level of 1.5 and 2% and incubated at room temperature (22ºC) for 30 d improved IVTDMD and $IVTNDFD_{DM}$ *(Table 1)*, IVTDMD and $IVTNDFD_{DM}$ increased in treated groups incubated at 40ºC for 30 d *(Table 2)*. These results were compatible with those of Bowman et al.[26], Yang et al.[27] and Beauchemin et al.[8] who reported incorporating exogenous fibre degrading enzymes to diets improved digestion of DM and fiber in ruminants.

Treatment with the fungi for increasing feeding value of rice straw is difficult because of controlling the optimal conditions for fungal growth and when the fungi grow, they can produce some toxic metabolites. Therefore, new commercial products will have important role in future for ruminant nutrition [8]. Fazaeli et al.[28] and Rodrigues et al.[29] mentioned that enzyme treatment or its in combination with other treatments can increase the digestibility of cereal straw in ruminants. It was stated that IVDMD and IVOMD had improved in rice straw treated with fungi [30]. In the present study, treatments of rice straw with increasing level of cellulase significantly improved $IVTD_{as\ fed}$, IVTDMD, $IVTNDFD_{DM}$ and $IVTOMD_{DM}$ compared to untreated rice straw. This result was compatible with the statements of the some researchers [28-31]. $IVTOMD_{DM}$ was the highest in RS+CEL2 incubated at 40ºC for 30 d. This result may be attributed to the cellulase level and its activity resulted in changes in cell wall structure in incubation.

Based on our findings, it may be concluded that cellulase treated rice straw resulted in higher $IVTD_{as\ fed}$, IVTDMD, $IVTNDFD_{DM}$ and $IVTOMD_{DM}$ values compared to untreated rice straw. The best result was obtained with the highest level of cellulase treatment (RS+CEL2) at 40ºC when *in vitro* true digestibilities were considered. Although cellulase treatment improved the digestibility, further rice straw treatment experiments with exogenous enzymes alone and in combinations are required to improve digestibility.

Acknowledments

The authors would like to thank to the FARMAVET International and Kurtbey Türk for supplying cellulase enzyme and rice straw, respectively.

Declaration

The authors declare that they have no commercial relationship with the company.

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Kafkas Univ Vet Fak Derg
22 (1): 49-55, 2015
DOI: 10.9775/kvfd.2015.13850

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

Research Article

The Effect of Eugenol on Survival of *Listeria monocytogenes* Inoculated İnegöl Meatball [1] [2]

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[1] *This project was supported by the Scientific Research Projects Coordination Unit of Selcuk University with the project number 09202057*

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Article Code: KVFD-2015-13850 Received: 11.06.2015 Accepted: 02.10.2015 Published Online: 05.10.2015

Abstract

In this research, it was aimed to determine the effect of eugenol with concentrations of 0.5% and 1.0% on *L. monocytogenes* with amount of 10^3 cfu/g, 10^4 cfu/g, 10^5 cfu/g and on total aerobic mesophilic microorganisms. In the experimental samples, presence of *L. monocytogenes*, the total aerobic mesophilic microorganisms and pH values were determined in the beginning (day 0), 5th and 10th days of cold storage at 4°C and 30th and 60th days of frozen storage at -18°C. There were significant differences in the number of total aerobic mesophilic microorganisms at the beginning day (day 0) and 30th day of the experimental İnegöl meatballs between groups, statistically ($P<0.01$). Considering the storage period, it was observed that the number of initial total aerobic mesophilic microorganisms was reduced in experimental samples during whole storage time ($P<0.01$). As an overall evaluation it was determined that the initial number of *L. monocytogenes* untreated with eugenol was higher than the samples treated with eugenol 0.5% and 1% after 60 days of frozen storage. This difference probably indicates that eugenol has an inhibitory effect on *L. monocytogenes*.

Keywords: *Eugenol, İnegöl meatballs, L. monocytogenes*

Eugenolün İnegöl Köfteye İnokule Edilen *Listeria monocytogenes*'in Varlığını Sürdürmesi Üzerine Etkisi

Özet

Araştırma, %0.5 ve %1.0 eugenol uygulamalarının 10^3 kob/g, 10^4 kob/g, 10^5 kob/g *L. monocytogenes* 4b ile inokulasyon sonrası etkisi ve aynı oranlardaki eugenol uygulamalarının toplam genel canlı mikroorganizmalar üzerine etkisini belirlemek amacıyla yapıldı. Deneysel numunelerde başlangıç (0. gün), 4°C'de muhafazanın 5. ve 10. günlerinde, -18°C'de donmuş muhafazanın 30. ve 60 günlerinde *L.monocytogenes*, toplam canlı mikroorganizması sayısı ve pH değerleri belirlendi. Deneysel İnegöl köftelerin başlangıçta (0.gün) ve donmuş muhafazanın 30. gününde toplam mezofilik aerob mikroorganizma sayısında istatistiki bakımdan gruplar arası önemli farklılıklar gözlemlenmiştir ($P<0.01$). Muhafaza periyodu dikkate alındığında ise deneysel uygulamalar yapılan numunelerin başlangıçta belirlenen toplam canlı mikroorganizma sayısının muhafaza periyodu süresince azaldığı gözlemlenmiştir ($P<0.01$). Genel olarak değerlendirildiğinde eugenol uygulanmayan numunelerin başlangıçta tespit edilen *L. monocytogenes* sayısının 60. gün donmuş muhafaza sonrasında %0.5 ve %1 eugenol uygulanan numunelerde belirlenen *L. monocytogenes* sayısından daha yüksek olduğu belirlenmiştir. Bu farklılık muhtemelen eugenolün *L. monocytogenes* üzerine inhibe edici etkisinin olabileceğini göstermektedir.

Anahtar sözcükler: *Eugenol, İnegöl köfte, L. monocytogenes*

INTRODUCTION

Listeria monocytogenes, which is an important food-borne pathogen, cause widespread epidemics, pneumonia, septicemia, meningitis, central nervous system infections and approximately 30% result with deaths. Human listeriosis formed by three serotypes (4b, 1/2a, 1/2b) [1,2]. Meat and meat products are contaminated with *L. monocytogenes* in various stages of production. It was reported by various investigators that, minced meat widely

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contaminated with *L. monocytogenes*. It's stated that, up to 100 colonies of *L. monocytogenes* can be tolerated in a gram or milliliter of foods and may be inactivated with heat before consumption. However it is stated that foods with high numbers of *L. monocytogenes* needs to be removed from consumption [3].

Many methods are used against the risks created by pathogen microorganisms in foods. Heating, freezing, antimicrobial compounds and synthetic preservatives are the most common applications among these methods. However, these methods cause changes in organoleptic properties of food and loss of nutrients. Therefore, there has been increasing interest in the obtaining of the natural antimicrobial compounds which are effective, nontoxic, constant flavour and nondecremental of nutrient value of foods instead of synthetic preservatives which has negative effects on public health [4,5]. Although spices obtained from plants is usually used as flavoring agents, it is shown by numerious researches that essential oils (EO) of various spices has antimicrobial activity and can be used as natural preservative [6,7].

Essential Oils (EO) are known to have different biological effects since the MiddleAges [8]. In addition to antibacterial [9-12] effect of EO or EO compounds, antiviral [13,14], antimycotic [15-18], antioxidant [19-22], antitoxigenic [23-25], antiparasitic [26,27] effects have been reported by several researches.

This study was then conducted to investigate the effects of eugenol on total aerobic mesophilic bacteria (TAMB) and *L. monocytogenes* in İnegöl meatballs, which has widespread ready to consumption in Turkey and has low microbiological quality.

MATERIAL and METHODS

Experimental Materials

Meat and spices, used in this study were obtained from markets in Konya. The rib cap (lower portion, LP) of lamb and brisket region of veal meats were used.

Experimental Production of İnegöl Meatballs

In this study, İnegöl meatball samples were prepared according to conventional methods. Veal (70%), lamb (12%) and tallow (18%) were passed together through a meat grinder. Then 1.5% NaCl, 10% breadcrumbs, 0.05% ascorbic acid, 1.0% caseinate, 0.15% garlic, onion 5%, 1.2% spices (pepper powder, paprika, cumin, allspice) added to this main mixture and mixed thoroughly by adding 0.7% water and passed through a grinder again. 25 g meatballs were formed of rod from meatball dough. 12 İnegöl meatball production groups were carried out for experimental studies. Procedures applied to the production groups are shown in *Table 1*.

Table1. *Experimental samples and control groups used in the study*
Tablo 1. *Araştırmada kullanılan deneysel numuneler ve kontrol grupları*

Group	Procedures
I.	- Meatball + inoculation of 10^3 CFU/g *L. monocytogenes 4b* - Meatball + inoculation of 10^4 CFU/g *L. monocytogenes 4b* - Meatball + inoculation of 10^5 CFU/g *L. monocytogenes 4b*
II.	- Meatball + 0.5% Eugenol + inoculation of 10^3 CFU/g *L. monocytogenes 4b* - Meatball + 0.5% Eugenol + inoculation of 10^4 CFU/g *L. monocytogenes 4b* - Meatball + 0.5% Eugenol + inoculation of 10^5 CFU/g *L. monocytogenes 4b*
III.	- Meatball + 1.0% Eugenol + inoculation of 10^3 CFU/g *L. monocytogenes 4b* Meatball + 1.0% Eugenol + inoculation of 10^4 CFU/g *L. monocytogenes 4b* - Meatball + 1.0% Eugenol + inoculation of 10^5 CFU/g *L. monocytogenes 4b*
P1	Production of traditional Inegöl Meatballs
P2	Meatball + 0.5% Eugenol
P3	Meatball + 1.0% Eugenol

After production and applications, all experimental İnegöl meatball groups were porsioned and placed in foam plates and covered with stretch film. Packages were stored under cold and freezing conditions at +4°C and –18°C. Analysis were carried out at 0th, 5th and 10th days for +4°C storage and 0th, 30th and 60th days for –18°C storage. Production were carried out in 3 replications.

Experimental Methods

Detection of Total Mesophilic Aerobic Bacteria: Detection of Total mesophilic aerobic bacteria count were performed according to the method suggested by Maturin and Peeler [28] in FDA Bacteriological Analytical Manual.

Detecting and Enumeration of L. monocytogenes: Detecting and enumeration of *L. monocytogenes* was performed according to the methot suggested by Hitchins ve Jinneman [29] in FDA Bacteriological Analytical Manual.

Statistical Analysis

The data obtained from the survey were analyzed using SPSS software package 21.00. Variance analysis (One-way ANOVA) of the obtained data, was subjected in accordance with the experimental design, and Duncan test was applied to detect differences between groups ($P<0.05$).

RESULTS

In this study; it was investigated that the use of eugenol essential oil and its' antibacterial effect on the total aerobic bacteria and *Listeria monocytogenes* in İnegöl meatballs which is sold and consumed commonly in Turkey and reported to have a low microbiological quality. The first group of experimental production of İnegöl Meatballs

held for 10 days at the temperatures between 0-4°C and the samples of the other groups were stored at -18°C for 60 days. After the production of experimental samples (day 0), changes in the total mesophilic aerobic and *L. monocytogenes* count was determined on 5th and 10th days of cold storage at 4°C and 30th and 60th days of frozen storage at -18°C. It was confirmed that the experimental samples do not carry any *L. monocytogenes* with analyzing of control samples before the inoculation process.

The count of total aerobic mesophilic bacteria in experimentally manufactured meatball samples obtained with inoculation of 10^3 cfu/g *L. monocytogenes* 4b and different rates of eugenol is shown in *Table 2*.

There were major statistical differences in the number of the total mesophilic aerobic microorganisms between the groups at the beginning stage (day 0) and frozen storage at 30th day of the experimental İnegöl meatballs *(Table 2*; P<0.05). When considering the storage period, it was determined that the total number of aerobic mesophilic microorganisms decreased during the storage period. The minimum number of total aerobic mesophilic microorganisms count was observed in the experimental samples stored at -18°C. These differences were found to be statistically important *(Table 2*; P<0.01).

At the beginning stage, the total mesophilic aerobic number of viable microorganisms were found between 5:46 and 6:51 in the experimental samples. Considering the eugenol application the differences was determined only 30th day of frozen storage between groups. At this period, the lowest number of microorganisms was determined in the group that 0.5% eugenol application was performed. In general consideration, it can be stated that this effect is due to the low number of microorganisms of these groups of samples at the beginning stage rather than the effective application of eugenol. Furthermore, in view of storage period, the frozen storage of the samples were considered to be more effective on the growth of total viable microorganisms *(Table 2)*.

In general, a reduction is achieved in the microflora at varying rates by freezing of food. A quite large variation of the reduction in microflora may be caused by the contamination in production process and growth of microorganisms during the thawing stage.

This can be explained by the view of Yıldırım [30]. The researcher stated that the microflora in a certain period (lag phase) of cold stored food has been unchanged qualitatively and quantitatively. He stated that bacteria in food start to multiply at the end of the lag phase depending on storage conditions (cold or frozen), replication of microflora can be inhibited in the products which chilled in accordance with technological rules and has a high quality. He also indicated that, both mesophilic bacteria and psychrotrophic bacteria reduce during the cold storage for 3-5 days in meat and meat products. The microbiological results of this study support the views and ideas mentioned above.

The experimental meatball samples with different rates of eugenol which inoculated with 10^3cfu/g *L. monocytogenes* 4b displayed intergroup differences on 10th day of cold storage and 60th day of frozen storage with regard to the number of bacteria *(Table 3*; P<0.05). The highest value of bacteria on 10th day of cold storage and 60th day of frozen storage was determined in the Group I which the samples are untreated with eugenol. When considering the storage period, samples of Group III were only observed to be statistically different *(Table 3*, P<0.01).

The number of *L. monocytogenes* in the first experimental group (Group I) which is untreated with eugenol, was almost stable and maintain the level of initial stage (3.45 cfu/g). The number of the bacteria in the second experimental group (Group II) which is treated with 0.5% eugenol decreased 3.49 cfu/g to 2.92 cfu/g from the initial level to the 60th day of frozen storage, respectively. In the sample which is treated with 1.0% eugenol, it was determined that the initial count of *L. monocytogenes* detected as 3.52 cfu/g and decreased to 3.12 cfu/g on 60th day of frozen storage. Based on these results, it was thought that the application of the eugenol may have an inhibitory effect on *L. monocytogenes*. In addition, reduction in the number of *L. monocytogenes* in 1.0% eugenol treated

Table 2. *Total aerobic mesophilic bacteria count of experimental Inegol meatball obtained by the application of different rates of eugenol (log10/g)*
Tablo 2. *Farklı oranlarda eugenol uygulanan deneysel İnegöl köfte numunelerinde toplam mezofilik aerobik bakteri sayıları (log10/g)*

Application	Storage					
	4°C			-18°C		P
	Day 0	Day 5	Day 10	Day 30	Day 60	
A1	6.51±5.66^{aA}	5.49±4.53^{B}	5.47±4.60^{B}	5.35±4.21^{bB}	4.20±3.18^{B}	0.001
A2	5.46±4.86^{bA}	5.50±4.73^{A}	5.52±4.69^{A}	4.36±3.13^{aB}	4.20±3.35^{B}	0.001
A3	6.21±5.86^{bA}	5.54±4.65^{B}	5.51±4.57^{B}	5.35±4.18^{bB}	4.21±3.27^{B}	0.001
P	0.002	0.877	0.858	0.001	0.982	

a,b,c: *The differences between the mean values in the same column with different letters are important (P<0.05).* ***A,B,C:*** *The differences between the mean values with different letters on the same line are important (P<0.05).* ***A1.*** *Traditional Inegöl Meatball production,* ***A2.*** *Meatball + 0.5% Eugenol addition,* ***A3.*** *Meatball + 1.0% Eugenol addition*

samples were determined to have statistically significant (*Table 3*; P<0.01).

The experimental meatball samples with different rates of eugenol which are inoculated with 10^4 cfu/g *L. monocytogenes* 4b displayed intergroup differences only on 5th day of storage with regard to the number of bacteria. The number of *L. monocytogenes* of the groups II and III were found lower rather than the group I which was untreated with eugenol (*Table 4*, P<0.05) Considering the storage period, the groups II were found to contain lower numbers of *L. monocytogenes* only in 60th days of -18°C storage (*Table 4*, P<0.05).

The experimental meatball samples with different rates of eugenol which are inoculated with 10^5 cfu/g *L. monocytogenes* 4b displayed intergroup differences only on 10th day of storage with regard to the number of bacteria (*Table 5*, P<0.05). When the groups were compared the groups II and III which were treated with eugenol displayed a lower number of *L. monocytogenes* rather than the group I which was untreated with eugenol. Considering the storage period, the samples including group II and III were found to contain lower numbers of *L. monocytogenes* on 30th and 60th days.

On the 60th day of the experiment, the initial number of *L. monocytogenes* in the experimental samples which were untreated with eugenol, treated with 0.5% eugenol and treated with 1.0% eugenol was determined to decrease from 5.35 cfu/g to 5.11 cfu/g, 5.26 cfu/g to 4.30 cfu/g and 5.24 cfu/g to 4.69 cfu/g, respectively.

DISCUSSION

An overall evaluation of the results displayed that the number of *L. monocytogenes* in the experimental samples of group II and III which are treated with eugenol was determined to reduce at the duration of the storage period. This finding of the study was suggested that the eugenol may possess a growth inhibitory effect on *L. monocytogenes* as well as in other studies. While a large number of studies [31-47] have reported that eugenol has an inhibitory effect against *L. monocytogenes*, in contrast several researchers [48] claimed that the eugenol has no effect against *L. monocytogenes*.

It was observed that the eugenol was more effective on *L. monocytogenes* strains in comparison with other essential oils (cinnamaldeyhd, thymol, citral, citronellol, limonenes) by Balch and Deans [31]. Filgueiras and Vanetti [35]

Table 3. *Number of L. monocytogenes in experimental samples of Inegol meatballs which inoculated different rates of eugenol and 10^3 cfu/g L. monocytogenes 4b (log10/g)*

Tablo 3. *10^3 kob/g oranında L. monocytogenes 4b (log10/g) inokule edilen deneysel İnegöl köfte numunelerinde L. monocytogenes sayısı*

Application	Storage					P
	4°C			-18°C		
	Day 0	Day 5	Day 10	Day 30	Day 60	
I	3.45±3.10	3.55±2.85	3.72±3.10^{a}	3.62±2.97	3.83±3.20^{a}	0.292
II	3.49±2.72	3.32±3.11	3.33±3.10^{b}	3.33±3.11	2.92±2.24^{b}	0.102
III	3.52±2.50^{A}	3.31±1.96^{B}	3.45±2.19^{abA}	3.30±2.46^{B}	3.12±2.11b^{C}	0.001
P	0.828	0.122	0.028	0.069	0.002	

a,b,c: *The differences between the mean values in the same column with different letters are important (P<0.05).* ***I.*** *Meatball + Inoculation of 10^3 cfu/g L. monocytogenes 4b.* ***II.*** *Meatball + 0.5% Eugenol + Inoculation of 10^3 cfu/g L. monocytogenes 4b.* ***III.*** *Meatball + 1.0% Eugenol + Inoculation of 10^3 cfu/g L. monocytogenes 4b*

Table 4. *Number of L. monocytogenes in experimental samples of Inegol meatballs which inoculated different rates of eugenol and 10^4 cfu/g L. monocytogenes 4b (log10/g)*

Tablo 4. *10^4 kob/g oranında L. monocytogenes 4b (log10/g) inokule edilen deneysel İnegöl köfte numunelerinde L. monocytogenes sayısı*

Application	Storage					P
	4°C			-18°C		
	Day 0	Day 5	Day 10	Day 30	Day 60	
I	4.27±3.30^{a}	4.30±3.73^{a}	4.40±3.43	4.14±3.39	4.52±4.20	0.110
II	4.39±3.27^{A}	4.11±3.40^{bA}	4.23±3.82^{A}	4.24±3.86^{A}	4.17±3.95^{B}	0.009
III	4.35±3.48	4.13±3.18^{b}	4.19±3.38	4.10±3.22	3.71±3.22	0.236
P	0.116	0.046	0.067	0.251	0.087	

a,b,c: *The differences between the mean values in the same column with different letters are important (P<0.05).* ***I.*** *Meatball + Inoculation of 10^4 cfu/g L. monocytogenes 4b.* ***II.*** *Meatball + 0.5% Eugenol + Inoculation of 10^4 cfu/g L. monocytogenes 4b.* ***III.*** *Meatball + 1.0% Eugenol + Inoculation of 10^4 cfu/g L. monocytogenes 4b*

Table 5. *Number of L. monocytogenes in experimental samples of Inegol meatballs which inoculated different rates of eugenol and 10^5 cfu/g L. monocytogenes 4b (log10/g)*

Tablo 5. *10^5 kob/g oranında L. monocytogenes 4b (log10/g) inokule edilen deneysel İnegöl köfte numunelerinde L. monocytogenes sayısı*

Application	Storage					
	4°C			-18°C		P
	Day 0	Day 5	Day 10	Day 30	Day 60	
I	5.35±4.33	5.32±5.11	5.41±4.54^{a}	4.92±4.31	5.11±4.39	0.185
II	5.26±4.26^{A}	5.16±4.60^{A}	5.16±4.63^{bAB}	4.36±4.11^{AB}	4.30±4.10^{B}	0.035
III	5.24±4.33^{A}	5.16±4.52^{A}	5.17±4.55^{bA}	4.48±4.14^{B}	4.69±4.23^{B}	0.018
P	0.083	0.315	0.021	0.206	0.143	

a,b,c: *The differences between the mean values in the same column with different letters are important (P<0.05).* ***I.*** *Meatball + Inoculation of 10^5 cfu/g L. monocytogenes 4b.* ***II.*** *Meatball+ 0.5 %Eugenol + Inoculation of 10^5 cfu/g L. monocytogenes 4b.* ***III.*** *Meatball + 1.0% Eugenol + Inoculation of 10^5 cfu/g L. monocytogenes 4b*

investigated the growth of *L. monocytogenes* and listeriolysin O (LLO) production. They stated that eugenol promoted a delay on the growth of *L. monocytogenes* at concentrations of 100, 300 and 500 mg mL^{-1} and above 800 mg mL^{-1} the effect was bactericidal. In addition, they argued that production of LLO by *L. monocytogenes* was reduced 80-100% in the presence of eugenol.

It was suggested that Gram (-) bacteria are more resistant to volatile oils [45]. Indeed Bežić et al.[32] stated that lipopolysaccharide (LPS), a structure of Gram (-) bacteria cell wall, inhibited the interaction of the volatile oil cell to bacteria membrane. However, Kim et al.[42] argued that *L. monocytogenes* is more resistant to the volatile oil although it's Gram (+).

Blaszyk and Holley [33] stated that 500 ppm eugenol has an inhibitory effect on *L. monocytogenes*. Chen et al.[34] argued that the forms of eugenol and thymol are more effective on *L. monocytogenes* than other forms. The bactericidal activity of clove on food-borne pathogens such as *L. monocytogenes* had been also reported by Ting and Deibel [46].

Garcia-Garcia et al.[36] reported that 350 mg/kg^{-1} of eugenol did not inactivate *L. innocua*. However, a 450 mg /kg^{-1} concentration of this antimicrobial agent inactivated the microorganism in the first few hours, and this condition prevailed after 72 h. So they argued that 450 mg/ kg^{-1} eugenol concentration was the minimal bactericidal concentration for *L. innocua*.

Gaysınsky et al.[37] stated that Eugenol encapsulated in Surfynol 485W micelles was most efficient in inhibiting of the growth of the pathogens. They argued that 1.0% Surfynol 485W and 0.15% eugenol was sufficient to inhibit the growth of all strains of *E. coli* O157:H7 and three of four strains of *L. monocytogenes* (Scott A, 310 and 108).

Gill et al.[38] stated that, eugenol and carvacrol lead to degradation in *E. coli* and *L. monocytogenes*' in cell membrane, also they caused to increase extracellular ATP concentrations and reduce to intracellular ATP concentration. In addition, Gill et al.[39] stated that eugenol and carvacrol inhibited the membrane ATPase activity of *E. coli* and *L. monocytogenes*.

Gill and Holley [40] suggested that eugenol was a more effective bactericidal agent than Cinnamaldehyde in same concentration. They stated that eugenol has a dose-dependent bactericidal effect on log-phase cells of *L. monocytogenes* within 15 min.

Hao et al.[41] stated that eugenol was slow down the growth of *L. monocytogenes* in cooked beef while it was maintained at 5°C and 15°C. Smith-Palmer et al.[45] argued that the clove essential oil could be implemented to control of *L. monocytogenes* and it has low bacteriostatic and bactericidal effects at 4°C.

Perez-Conesa et al.[43] reported that *L. monocytogenes* strain Scott A was more sensitive to eugenol than to Carvacrol after 2 min of exposure, as eugenol led to a 3.3 log cfu/cm^2 reduction compared with the 1.9 log cfu/cm^2 reduction achieved by carvacrol and they observed that viable cells were below detectable levels for *L. monocytogenes* strain ScottA was exposed to 0.7% eugenol-loaded micelles for 10 and 20 min.

Santiesteban-L'opez et al.[44] evaluated the effects of antimicrobial agents on *S. aureus*, *L.innocua*, *E. coli* and *S. typhimurium* and they determined that the most effective antimicrobial agent was carvacrol followed by thymol and eugenol.

Upadhyay et al.[47] investigated that the effects of generally recognized as safe (GRAS), plant-derived anti-microbials (PDAs); trans-cinnamaldehyde (TC 0.50, 0.75 mM), carvacrol (CR 0.50, 0.65 mM), thymol (TY 0.33, 0.50 mM) and eugenol (EG 1.8, 2.5 mM) on *L. monocytogenes* (LM) biofilm formation. When applied at subinhibitory concentrations, they were considerably effective in killing mature LM biofilms and has an inhibitory effect on biofilm synthesis.

Despite advances in food technology, food poisoning continues to maintain an increasing importance in terms

of public health. Pathogenic microorganisms such as *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* found in meat products threaten public health even today.

L. monocytogenes is considered as an important pathogen that causes food-borne epidemia, pneumonia, septicemia, meningitis, central nervous system infections and death about 30% of cases. Meat and meat products are contaminated with *L. monocytogenes* at different stages of the production. Many methods are used in food against the risks generated to pathogenic microorganisms. Heating, freezing, preservatives and synthetic antimicrobial compounds are the most frequently used methods among these. However, these methods cause changes in the organoleptic properties of food and loss of nutrients. Synthetic preservatives are known to affect negatively to public health; because of this reason the natural antimicrobial compounds which are effective, non-toxic, constant flavour and nondecremental of nutrient value of the product are use in food production. It has been shown in the studies, spices obtained from plants are use as flavorer in food products, many spices has essential oils (EO) which can be used as a natural preservative for their antimicrobial activity.

Thus, using essential oils of plant origin as an alternative to chemical compounds in the manufacturing of the meat products, particularly İnegöl meatball would be beneficial for protecting the public health. It was concluded that eugenol can show inhibitory activity especially against *L. monocytogenes* and other pathogenic microorganisms, but for a certain opinion, new experimental models and new researches need to be done.

Conflict of Interest

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

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Kafkas Univ Vet Fak Derg
22 (1): 57-62, 2016
DOI: 10.9775/kvfd.2015.13887

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

Research Article

Evaluation of the Canine Epididymal Sperm Morphology with two Different Staining Methods, One Fixative Solution and Motile Sperm Organelle Morphology Examination (MSOME) [1]

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Article Code: KVFD-2015-13 887 Received: 19.06.2015 Accepted: 02.10.2015 Published Online: 02.10.2015

Abstract

The aim of this study was to compare the use and effectiveness of two different stains, one fixative solution and Motile Sperm Organelle Morphology Examination (MSOME) on morphological characteristics of spermatozoa in fresh dog semen samples from epididymis. After routine castration, cauda epididymides were collected from 20 dog testes. Morphological abnormalities were evaluated by using Hancock's buffered formol saline solution, the aniline blue stain, Diff-Quik stain and MSOME analysis in epididymal semen. Conventional semen analysies and MSOME were simultaneously performed on the same sample from each dog. Percentage of abnormal spermatozoa, head abnormality and acrosomal defects were significantly higher in samples with two different staining methods and one fixative solution than MSOME technique. It was concluded that the usage of the Aniline blue stain may be an efficient method for evaluating the sperm morphology of dog semen. Although no correlation was established amoung four different methods on sperm morphology assessment.

Keywords: *Canine, Morphology, MSOME, Semen, Stain*

Motil Sperm Organel Morfolojisi Muayenesi (MSOME) ve Fiksatif Solüsyon, İki Farklı Boyama Tekniği İle Köpek Epididimal Spermatozoa Morfolojisinin Değerlendirilmesi

Özet

Bu çalışmanın amacı, taze köpek sperması örneklerinde spermatozoonların morfolojik özellikleri üzerine Motil Sperm Organel Morfolojisi Muayenesi (MSOME), bir fiksatif solüsyon ve iki farklı boyanın kullanımını ve etkinliğini karşılaştırmaktı. Kastrasyon işleminden sonra, 20 adet köpek testisinden kauda epididimisler elde edildi. Epididimal spermada morfolojik bozukluklar, Hancock sıvı fikzasyon yöntemi, aniline blue boyama yöntemi, Diff Quik boyama yöntemi ve MSOME tekniği ile değerlendirildi. Her bir köpekten bir örnek alınarak, konvensiyonel semen analizi ve MSOME analizi aynı örnekte eş zamanlı olarak incelendi. Morfolojik olarak ortalama abnormal baş ve akrozom defekti MSOME tekniğine göre diğer üç farklı methodta önemli derecede yüksek bulunmuştur. MSOME tekniği ile 3 farklı yöntem karşılaştırıldığında, anilin boyama yönteminde baş bozukluk oranı bulunan anormal spermatozoa oranı önemli derecede yüksek bulunurken, köpek spermatozoa morfolojisi değerlendirilmesinde Aniline blue boyama yönteminin etkili bir yöntem olduğu sonucuna varıldı. Spermatozoa morfolojisinin değerlendirilmesinde üç farklı method ile MSOME tekniği arasında herhangi bir paralellik bulunmadı.

Anahtar sözcükler: *Boya, Köpek, Morfoloji, MSOME, Sperma*

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INTRODUCTION

The male factor is considered a major contributory factor to infertility [1]. Sperm morphology has become an area of great interest to assess of male infertility, since observation of normal and abnormal morphological sperm forms in semen samples [2]. Evaluation of sperm morphology displays a potential impact on male fertility [3] and has been recognized to be the best predictor of outcome of natural fertilization [4], intrauterine insemination [5], and conventional *in vitro* fertilization [6]. Nowadays, many conventional and advanced methods exist to assess semen quality [7]. To evaluate the morphology of mammalian spermatozoa, many stains and staining combinations have been used, for example, Papanicolaou, Hematoxiline, Toluidin blue-pironin, Giemsa and Nigrosin. But conventional light microscopic analysis of spermatozoa has limitations in evaluating the fine structures, such as the acrosome and nucleus [8,9]. New possibilities have arisen because the latest technical facilities improvements are available in the IVF-laboratories allow to assess the sperm morphology in details. The introduction of MSOME (Motile Sperm Organelle Morphology Examination) by Bartoov et al.[4] allows the examination of subcellular disorders like nuclear vacuoles at high magnification (6000-12500x) in real time on motile sperm. Oliveira et al.[10] evaluated the correlation between MSOME classification and sperm morphology classification according to the Tygerberg criteria in 97 semen samples from an unselected group of couples undergoing infertility investigation. The study showed a strong positive correlation between the percentage of normal sperm forms according to the Tygerberg criteria and MSOME. The main aim of this study was to test the effectiveness of two different staining techniques (Aniline blue, Diff-Quick), one fixative solution (Hancock's method), traditionally used for the assessment of sperm morphological analysis, and to determine their correlation with Motile Sperm Organelle Morphology Evaluation (MSOME).

MATERIAL and METHODS

This experiment was conducted to compare the effects of two stains, one fixative solution and MSOME analysis on morphological characteristics of spermatozoa in fresh semen samples. Testicles were obtained from 20 privately owned mixed-breed dogs (age range: 2-8 years, body weight<10 kg) after routine castration at local veterinary practices. Ethics committee approval for this study was given by Ethical Committee of Poland University Veterinary Faculty (Ike 72/2009).

Sample Collection

Samples were kept in phosphate buffered saline (PBS) at room temperature for transport to the laboratory. All tissue was processed within 2 h of collection. The cauda epididymis and vas deferens were dissected from each testis and placed in a clean and dry petri dish. After removal and dissection of the testicles, samples were obtained from the distal portion of the epididymis by cutting the tail of epididymis with a scalpel blade and placing it into 1 ml Human Tubal Fluid (HTF). The semen samples were washed with Sperm Washing Medium (Irvine Biologicals) by centrifugation at 800 g for 10 min and supernatant was discarded. Pellet was suspended in the same solution and thereafter centrifuged once more. The pellet was gently over-layered with medium in the tube which was sealed, inclined at 45°C and kept at 37°C for 60-90 min in 5% CO_2. A sterile Pasteur pipette was used to remove the supernatant containing actively motile sperms [11]. After diluted sperm samples, morphological abnormalities were evaluated by using Hancock's buffered formol saline solution, the aniline blue stain, Diff-Quik stain and MSOME analysis. Since the main objective of this experiment was only to compare the effects of the two staining solutions, fixative solution and ultramorphological analyses upon the morphological characteristics of spermatozoa, no additional control group was included. Conventional semen analysis (Hancock's method, aniline blue stain, Diff-Quik stain) and MSOME exploration were performed simultaneously on the same sample from each dog. Sperm abnormalities were categorized as abnormal heads (including pear shaped, small heads, narrow, heads alone, or large heads), acrosome defects, abnormal midpieces and proximal cytoplasmic droplets.

Diff Quick

A modified Diff Quick method was used as follows. Thin smears of the well-mixed diluted sperm samples were prepared in duplicate by placing 10 µL on clean slides. After air-drying, the slides were stained using Diff-Quik kit (Baxter Healthcare Corporation, Inc., McGaw Park, IL). Afterwards the smears were consecutively stained with solution 1 (10 min), then air-dried and stained with solution 2 (10 min). Finally, the slides were washed in running tap water to remove the excess stain (10 to 15 times). The stained slides were evaluated at x1000 magnification with oil immersion (Leica Microsystems). For each smear, at least 200 spermatozoa were examined [12].

Aniline Blue

Ten-microliter drops of diluted semen samples were spread onto glass slides and allowed to dry. These smears were then fixed at room temperature in buffered 3% glutaraldehyde in phosphate - buffered saline (PBS) for 30 min and air-dried. After fixation, the slides were stained with 5% aqueous aniline blue mixed with 2% acetic acid (pH = 3.5) for 5 min, washed with distilled water and air-dried. Briefly, the staining solution was prepared by adding 5 g of aniline blue (Water blue, Fluka, Buchs, Switzerland) to 100 mL of PBS, filtering, and adjusting the pH to 3.5 with

2% glacial acetic acid (Merck, Darmstadt, Germany). For each stained smear, 200 spermatozoa were evaluated with light microscope in oil immersion magnification (100x objective) [13].

Hancock's Method

According to the Hancock's method, 0.5 ml of each sample were added to an Eppendorf test- tube which containing 1 ml Hancock's solution [62.5 ml formalin (37%), 150 ml saline solution, 150 ml buffer solution and 500 ml double distilled water] [14]. One drop of the semen mixture was dropped on a slide and covered with a cover slip. Sperm morphology was determined by counting a total of 200 sperm cells under phase contrast microscope with an oil immersion objective.

Determination of Morphology by MSOME

An aliquot of 1 µL of diluted sperm suspension was transferred to a 5 µL microdroplet of modified HTF medium containing 7% polyvinylpyrrolidone (PVP medium; Irvine Scientific). This microdroplet was placed in a sterile glass dish (Fluorodish; World Precision Instruments, USA) under sterile paraffin oil (Light Mineral Oil for Embryo Culture, Irvine Scientific, USA). The sperm cells, which were suspended in the microdroplet, were placed on a microscope stage covered by a droplet of immersion oil. The examination was performed with Leica DMI 6000B inverted microscope equipped with DIC/Nomarski optics using a Leica objective HC χ PL FLUOTAR 100 χ/1.30, under oil immersion. Spermatozoa were analyzed at magnifications greater than or equal to 6600× Classification of four categories was done according to Vanderzwalmen et al.[15] for each sperm sample. MSOME involves the grading of spermatozoa according to the presence of nuclear vacuoles:

– Grade I, oval shaped sperm head without vacuoles.

– Grade II, oval shaped sperm head with 1-2 small vacuole < 4% of the head area

– Grade III, oval normal shape and size sperm head with one large vacuole > 4% of the head area or several small vacuoles

– Grade IV sperm head with abnormal morphology with or without vacuoles.

At least 200 motile spermatozoa per semen sample were evaluated and percentage of abnormal spermatozoa was determined [16]. *Fig. 1A* and *Fig. 1B* shows normal spermatozoa and spermatozoa with large vacuoles analysed by MSOME.

Statistical Analyses

All data were checked for normal distribution with Shapiro-Wilk and homogenity of variance with Levene's test. Data were not normally distributed. The non-parametric Kruskal Wallis test was used to determine the differences between the methods. Post hoc analysis of pairwise difference between methods was performed using Mann Whitney U test with Bonferonni correction. P values <0.05 were considered to be significant. The results were presented as the mean ± SEM.

RESULTS

The values of the morphological changes observed from the use of each technique are presented in *Table 1*. Significantly increased morphological head defects and abnormal acrosome were observed in the Aniline blue method when compared with Diff-Quik and Hancock's methods. Average percentages of morphologically abnormal head defects and abnormal acrosome were significantly higher in samples with 3 different methods than MSOME technique. For abnormal heads alteration,

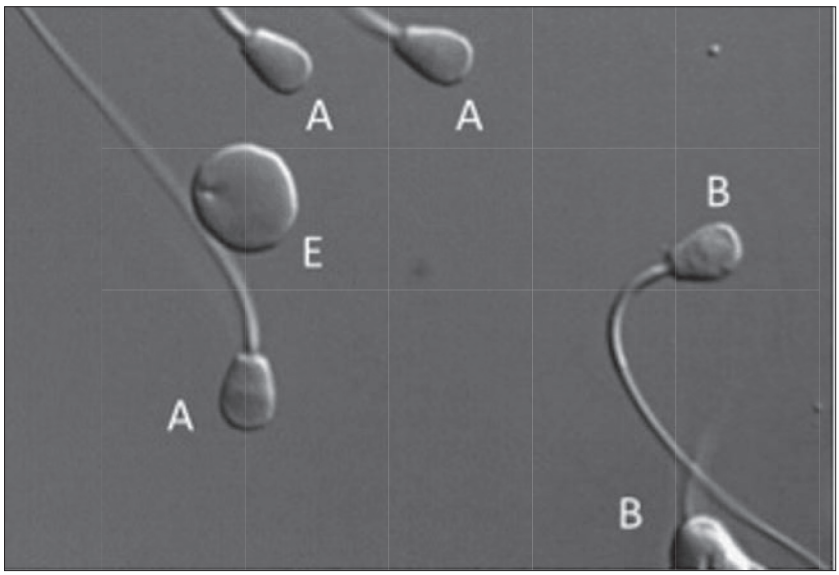

Fig 1. A- Normal spermatozoa observed at high magnification (×8400); **B-** Spermatozoa with large nuclear vacuoles observed at high magnification (×8400), **E-** Erythrocyte

Şekil 1. A- Yüksek büyütme (×8400) altında gözlenen normal morfolojiye sahip sperm hücresi, **B-** Yüksek büyütme (×8400) altında gözlenen geniş vakuole sahip sperm hücresi, **E-** Eritrosit

Table 1. *Percentage data (mean ± SEM) of morphological sperm defects of dog semen using different staining, Hancock's solution and Motile Sperm Organelle Morphology Examination (MSOME) methods*

Tablo 1. *Motil Sperm Organel Morfolojisi Muayenesi (MSOME) methodu, Hancock solüsyonu ve farklı boyalar kullanılarak köpek spermasında saptanan morfolojik sperm defektlerinin ortalama verileri (ortalalama ± SEM)*

Method (n=20)	Abnormal Acrosomal Rate (%)	Abnormal Head Rate (%)	Abnormal Middle Piece Rate (%)	Presence of Cytoplasmic Droplet (%)
Diff Quick	9.50±1.2^{c}	17.60±1.4^{b}	2.00±0.7^{a}	17.00±0.5^{a}
Aniline Blue	18.50±1.8^{a}	24.7±1.4^{a}	2.00±0.6^{a}	15.00±0.3^{a}
Hancock's	12.00±1.2^{b}	19.10±1.4^{b}	1.00±0.6^{b}	15.00±0.4^{a}
MSOME	1.00±0.6^{d}	1.60±0.8^{c}	1.02±0.5^{b}	1.00±0.4^{b}

Groups with different letters (a,b,c,d) in the same column are significant different (P<0.05)

an average of 24.7±1.4% was obtained in aniline blue, a value that was higher (P<0.05) than the one found when using Diff Quick and Hancock methods. For middle piece changes, a higher average was obtained when using the smear with the conventional Diff-Quik and aniline blue compared with Hancoock and MSOME methods. In this study, statistically significant difference was observed for cytoplasmic droplet alteration when using the four different methods.

DISCUSSION

In most mammalian species, conventional semen analysis is mainly based on the assessment of the sperm concentration, the motility characteristics and the morphological classification of spermatozoa in the evaluation of male factor infertility [17,18]. Assessment of sperm morphology can be influenced by many factors, such as the fixation and staining technique (e.g. Diff-Quik, nigrosin/eosin) [17], sperm preparation methods procedures, quality of the microscope and examiner's skills [18]. A number of studies of sperm staining procedures used to assess sperm morphology for several animal species have reported that the same fixatives and stains have different reactions with the sperm of individual species [19]. Therefore, it is important to find the most suitable staining technique for each species [20]. Our results indicate that it is not suitable for clearly defining and indicating the boundaries of the acrosome for evaluating canine semen morphology, although Diff-Quik stain method is simple and easy to evaluate. Normally, it is sufficient to fixate the smear in Diff-Quik fixative for 30 sec, but we recommend leaving smears in each solution for 10 min to achieve the best result and it can be effect the results. Therefore, Diff Quik stain is not to be a useful alternative method to evaluate for canine semen morphology [13]. But the aniline blue staining is suitable for clearly defining the main components of sperm and allowed good visualization of canine spermatozoa morphology [13]. The present study is the first to describe aniline blue staining of canine sperm for sperm morphology. Aniline blue staining can be used to examine two different sperm parameters as integrity of the DNA and sperm morphology. The assessment of both sperm morphology and chromatin on the same slide would be suitable for andrology laboratories. Also, Hancoock's method is not suitable for clearly defining the main components of sperm because the sperm samples are not fixed.

The resolving power offered by MSOME enables the identification of spermatozoa showing shape and size changes and intranuclear vacuoles as well, that would not be detected with conventional evaluation methods [21]. The presence of vacuoles on sperm head (size, number, localization and frequency) can be revealed during sperm movement [21,22]. Thus, the analysis of only motile spermatozoa by MSOME provides an advantage for morphological observation [21]. Higher magnification provided by the 100× DIC objectives are more appropriate to allow more detailed analysis of small cells [21]. We were the first to describe nuclear vacuoles in canine spermatozoa. In this study, the relationship between normal sperm morphology obtained by the conventional method and MSOME was assessed in 20 male dogs. No significant correlation was found between the frequency of morphologically normal spermatozoa as defined by MSOME and the frequency of morphologically normal spermatozoa using conventional method. The incidence of sperm normalcy by conventional sperm analysis was significantly lower than that by MSOME in this study. It should be stressed that MSOME focuses only on motile spermatozoa, unfixed motile sperm fraction, while the conventional morphological examination is applied to the entire semen sample post-fixation. The methods used (fixation and staining) do not allow the selective analysis of the motile sperm fraction alone [21]. Thus, the usage of MSOME might show a potential improvement in the morphological diagnosis of the sperm. But, a positive correlation has been observed between normal MSOME spermatozoa and normal spermatozoa using Tygerberg criteria (r 1⁄4 0.83, 0.0001) [10]. Oliveira et al.[10] evaluated the correlation between MSOME classification and sperm morphology classification according to the Tygerberg criteria [6] in 97 semen samples from an unselected group of couples undergoing infertility investigation. The study showed a strong positive correlation between the percentage of normal sperm forms according to the

Tygerberg criteria and MSOME (r=0.83; P<0.001). Conversely, the frequency of abnormal MSOME spermatozoa was negatively correlated with sperm concentration, sperm motility, and the percentage of spermatozoa with normal morphology [23]. The relationships between spermatozoa with size and number of nuclear vacuoles and conventional semen parameters have been more debated. Vacuoles in the sperm head have been reported to be associated with low sperm concentration, low sperm motility [24] or high teratozoospermia [25]. No correlation was reported between the rate of spermatozoa with large vacuoles and sperm morphology in the study. Also, Bartoov et al.[4] investigated the relationship between normal spermatozoa according to the WHO reference values [26] and MSOME in 20 patients. The authors found no significant correlation between the percentage of morphologically normal spermatozoa as defined by the WHO and the percentage of morphologically normal spermatozoa as defined by MSOME, since the incidence of sperm normalcy by routine sperm analysis was significantly higher than that by MSOME (26.1±7.2% and 2.9±0.5%, respectively). Perdrix et al.[27] analysed semen samples from 440 males, aged between 24 and 66 years, consulting for infertility investigation. The presence of vacuoles in the sperm head was significantly larger in poor semen samples (P<0.001). Relative vacuolar area (RVA), defined as vacuole area (l μm^2)/head area (l μm^2) X100, was the most discriminative MSOME criterion between normal and abnormal semen samples, and was negatively correlated with poor sperm morphology (r=0.53; P<0.001). It is noteworthy that conventional morphological examination is applied to semen sample including both alive and dead sperms, whereas the most remarkable feature of MSOME is the focused on motile sperm fractions, providing information about the sample fraction referred for ICSI (intra cytoplasmic sperm injection treatment) [4].

In the light of these findings, Aniline blue stain is an efficient method for evaluating the sperm morphology of canine semen. MSOME has been proposed as much stricter criterion of sperm morphology evaluation as compared to the conventional semen analysis [10]. But MSOME seems to be not a more strict technique for the classification of morphologically normal spermatozoa in this study. The sperm nuclear vacuoles evaluated at high magnification can be routine use of MSOME for ICSI as a criterion for semen analysis. It should be noted that more studies performed in greater number of infertile dogs are required to confirm the usefulness of MSOME in dog sperm morphology analysis.

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Kafkas Univ Vet Fak Derg
22 (1): 63-67, 2016
DOI: 10.9775/kvfd.2015.13895

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

Research Article

The Evaluation of Indirect Enzyme-Linked Immunosorbent Assay Using Antigens Prepared from *Brucella abortus* RB51 and *Brucella canis* M- Variant Strains for Serologic Diagnosis of *Brucella ovis* Infection

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KVFD-2015-13895 Received: 19.06.2015 Accepted: 16.09.2015 Published Online: 29.09.2015

Abstract

The aim of this work was to investigate the possible usage of hot saline extract antigens (HSE) of *Brucella abortus* RB51(HSE-RB51) and *B. canis* M- variant strains (HSE-M-) in ELISA by comparing the results with those of obtained from commercial I-ELISA kit for the serological diagnosis of *B. ovis* infection. In this study, a total of 183 serum samples collected from different cities (Şanlıurfa, Mardin, Gaziantep, Diyarbakır) in Southeastern Anatolia Region of Turkey were tested by using three ELISAs, one of which is from a commercial source, Rose Bengal plate agglutination tests prepared by rough (R-RBPT) and smooth strains (S-RBPT). Recombinant protein A/G conjugated with horseradish peroxidase (A/G-HRPO) was used as conjugate in the in house ELISAs. Seropositivity rate was 11% for HSE-RB51-ELISA and 3.3% for both HSE-M- and commercial ELISA. The percentage of positive results was 7.6% for S-RBPT and 2.7% for R-RBPT. Only 2 serum samples were positive for all the tests except S-RBPT. Because similar results were obtained from the same serum samples by both commercial ELISA and HSE-M-ELISA, these results may suggest that HSE-M- antigen could be used in ELISA for serologic diagnosis of *B. ovis* infection in sheep. Since 7.6% of the serum samples were found as positive by only HSE-RB51-ELISA, it was assumed that this test could be less specific or more sensitive than other tests used in the study. Although R-RBPT is a screening test, it showed the lowest seropositivity in the study. This could be explained by less mucoid nature of its antigen than other test systems using natural rough species.

Keywords: *Brucella ovis, ELISA, Serology*

Brucella abortus RB51 ve *Brucella canis* M- Varyant Suşlarından Hazırlanan Antijenlerin Kullanıldığı Enzim Bağlı İmmünosorbent Testinin Koyunlarda *Brucella ovis* İnfeksiyonunun Serolojik Tanısında Kullanılabilirliğinin Araştırılması

Özet

Bu çalışmada, biri ticari *Brucella ovis* ELISA kiti olmak üzere 3 ELISA ve smooth ve rough suşlardan hazırlanan Rose Bengal pleyt aglütinasyon testleri (S-RBPT ve R-RBPT) ile karşılaştırmalı olarak test edilerek, *B. ovis* infeksiyonunun serolojik tanısında ELISA'nın kullanılabilirliğinin araştırılması amaçlandı. Çalışmada, Güneydoğu Anadolu Bölgesinin çeşitli illerinden (Şanlıurfa, Mardin, Gaziantep, Diyarbakır) toplanan toplam 183 koyun serumu test edildi. ELISA antijeni olarak *B. abortus* RB51 ve *B. canis* M- varyant suşlarının sıcak tuzlu suda ekstraksiyonları (STE-RB51, STE-M-) hazırlandı. Konjugat olarak horseradish peroksidaz ile işaretli rekombinant A/G proteini (A/G-HRPO) kullanıldı. Seropozitiflik yüzdesi RB51 antijenini kullanan ELISA için %11 iken, diğer iki ELISA için %3.3 olarak bulundu. S-RBPT ile serumların %7.6'sı ve R-RBPT ile %2.7'si pozitif bulundu. Sadece 2 serum R-RBPT ve 3 ELISA ile pozitif reaksiyon verdi. Ticari *B. ovis* ELISA kiti ile STE-M-ELISA ile aynı sayıda ve aynı serumlardan pozitif reaksiyon alınması STE-M- antijeninin *B. ovis* infeksiyonunun serolojik tanısında ELISA için iyi bir aday antijen olabileceğinin kanısına varıldı. Kullanılan serolojik testlerden sadece STE-RB51-ELISA ile pozitiflik yüzdesinin %7.6 olması testin özgüllüğünün diğer testlere göre daha düşük olabileceği kanısı uyandırdı. Ayrıca R-RBPT' nin bir tarama testi olmasına rağmen en düşük seropozitiflik oranı göstermesinin M-varyant suşunun daha az mukoid bir yapı içermesi nedeni ile çapraz reaksiyonları azaltması sonucu olabileceği düşünüldü.

Anahtar sözcükler: *Brucella ovis, Seroloji, ELISA*

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INTRODUCTION

Brucella ovis causes a genital disease in sheep manifested by epididymitis in rams and placentitis in ewes producing reduced fertility in the flock. Clinical diagnosis is not sensitive enough because many other bacteria might cause same clinical picture and only about 50% of infected rams present epididymitis [1]. Bacterial isolation is not practical for detection of the disease in large numbers of animals and it also is not very sensitive because of the intermittent shedding of bacteria through semen by infected rams. Therefore indirect methods using serological testing are preferred for routine diagnosis. The most widely used serological tests are complement fixation (CFT), agar gel immunodiffusion (AGID), and indirect enzyme linked immunosorbent assay (I-ELISA). But only CFT is prescribed for international or intra-community trade. However, CFT has found limited application because of its complexity, incompability with anticomplementary, prozoning and hemolyzed sera. In addition, among chronically infected rams which show CFT negative results are not rare. On the other hand, AGID is a very labour intensive test and has low sample capacity [2-6]. Various I-ELISAs have been developed for detection of antibodies against *B. ovis* antigens with various results. According to literature data, most I-ELISAs appear more sensitive and less prone to problems than the CFT and the AGID [7-9].

Antigens used in the immunodiagnosis of *Brucella* infections consist of various somatic proteins and surface components. When rough *Brucella* cells are heat-extracted with saline (HS), they yield water-soluble antigenic extracts mainly composed of outer membrane proteins (OMPs) and rough lipopolysaccharides (R-LPS). Although OMP and R-LPS contain immunodominant epitopes, some cross reactivities have been described between *Brucella* OMPs and bacteria belong to the *Rhizobiaceae* [2,10-14].

Nielsen et al.[14] demonstrated that rough lipopolysaccharide of *B. abortus* RB51 could be used as antigen for detection of antibodies against *B. ovis, B. canis* and *B. abortus* RB51 by ELISA. More recently, other authors have reported that since *B. ovis* shares antigenic components with *B. canis*, it seems that this strain could be used as antigen to detect antibodies to *B. ovis* with the same results [15]. Since *B. ovis* shares antigenic components with *B. canis*, it would seem that either strain could be used as antigen with the same results. However, the advantage of the *B. canis* (M-) strain variant is that it can be used to develop a satisfactory antigen for agglutination tests because of less mucoid structure of its cell wall [16].

In this study, we aimed to investigate the possible usage of hot saline extract antigens (HSE) of *B. abortus* RB51 (HSE-RB51) and *B. canis* M- variant strains (HSE-M-) in ELISA by comparing the results with those of obtained from a commercial I-ELISA kit for the serological diagnosis of *B. ovis* infection.

MATERIAL and METHODS

Serum Samples

The study included 183 sheep sera collected from different cities in Southeastern Anatolia Region of Turkey. Blood samples were collected in tubes without anticoagulant by jugular venopuncture and kept at RT for 24 h. Sera were separated and stored at -20ºC until testing for detection of *B. ovis* antibodies.

Bacteria Cultures and Antigen Preparation

The M- strain of *B. canis* (kindly provided by Dr. Carmicheal, Cornell University, NY, USA) and *B. abortus* RB51 (kindly provided by Pendik Veterinary Control Institute, İstanbul, Turkey) were cultured in tryptic soy agar supplemented 10% sterile calf serum at 37ºC and harvested during the logarithmic phase of growth. For the antigen preparation, a hot saline extract antigen (HSE) was obtained by following the method described by Barrouin-Melo et al.[16], with minor modifications. Briefly, bacterial cells were harvested with 20 ml steril phosphate buffered saline (PBS; 150mM NaCl, 2.5 mM KCl, 1.5 mM KH_2PO4, 9mM $Na_2HPO_4.12\ H_2O$, pH 7.4) and inactivated by heat (1 h, 56ºC). Inactivated bacterial suspansions were washed three times by centrifugation (3.500xg, 10 min) in PBS. Finally the resulted pellets were then re-suspended in PBS and autoclaved at 121ºC for 20 min. The cells were then centrifuged at 12.000xg for 20 min, at 4ºC. The supernatants were collected and identified as HSE and stored in small aliquots at -20ºC until their use as the ELISA solid phase antigen.

Serological Tests

The serum samples were tested comparatively by three ELISAs, one of which is I-ELISA kit from a commercial source, and Rose Bengal plate agglutination tests prepared by rough (R-RBPT) and smooth strains (S-RBPT).

Rapid slide agglutination tests (S-RBPT/R-RBPT) were performed as described previously [17] using antigens prepared with *B. canis* M- and *B. abortus* S99.

Indirect ELISA (I-ELISA): Commercial I-ELISA kit (Chekit *B. ovis*, Idexx, France) was used according to the manufacturer's instructions and 2 in house ELISAs (HSE-M-) and HSE-RB51) were performed in paralel on all test and control sera. The working dilutions of the horseradish peroxidase conjugated protein A/G (ImmunoPure, Pierce Lab), HSE-M- and HSE-RB51 antigen preparations and positive and control sera were determined previous checkboard titrations to achieve the highest positive-to-negative ratio with the lowest background reading. The antigen diluted in 0.06 M sodium carbonate buffer (pH 9.6) was passively coated onto polystyrene plates (Nunc 269620, Denmark), 100 µl/well, incubated for overnight at 4ºC and then washed five times in 0.01 M phosphate

buffered saline containing 0.05% Tween 20, pH 7.2 (PBS/T). Control and test sera were added 1:100 in PBS/T, 100 µl/well, for 1 h at room temperature (RT). After five washes in PBS/T, protein A/G horseradish peroxidase conjugated was added, 100 µl/well, and incubated for 1 h at RT. Finally, after five washes in PBS/T, 100 µl of chromogenic substrate (4.0 mM hydrogen peroxide and 1.0 mM 2,2'-azino-bis (3- ethylbenz-thiazoline-6-sulfonic acid) diammonium salt in 0.05 M citrate buffer, pH 4.5) per well was added. The plates were shaken continuously on an orbital shaker for 15 min prior to reading at OD_{405} nm in a microplate reader (VERSAmax 3.13/B2573). Optimum antigen and conjugate working dilutions were established by making serial dilutions of both antigen and conjugate. Optimum dilution was considered as the one which gives the greatest differential between positive and negative sera.

Data Analysis

The triplicate mean optical density (OD_{405}) of the each positive, negative sera and test sera were calculated and the OD value of the test serum was substracted from the mean OD of negative sera. This figure was divided the difference between the mean OD of positive and negative sera and multiplied by 100. The results were expressed as a percent positivity value (%P). If the resulted figure was more than 50, the test serum was considered as positive.

RESULTS

Seropositivity rate was 11% for HSE-RB51 and 3.3% for both HSE-M- and commercial ELISA. The percentage of positive results was 7.6% for S-RBPT and 2.7% for R-RBPT *(Table 1)*. Only 2 serum samples were positive for all the tests except S-RSAT. Three serum samples were positive by only R-RSAT. Two of the serum samples were found positive by only 3 ELISAs while 2 serum samples were positive to all tests except R-RSAT *(Table 2)*.

Table 1. *Test serum samples that were positive to at least one serological test employed*
Tablo 1. *Uygulanan serolojik testlerden en az birine pozitif sonuç veren test serum örnekleri*

Serum No	Rapid Slide Agglutination Tests (S-RBPT/R-RBPT)		ELISAs		
	S-LPS	R-LPS	*B. ovis* (Idexx Commercial I-ELISA Kit) %P	*B. canis* M- (Home Made) %P	*B. abortus* RB51 (Home Made) %P
472	-	-	-	-	+ 69
468	-	-	-	-	+ 50
189	-	+	+ 61	+ 58	+ 51
474	-	-	-	-	+ 57
463	-	-	-	-	+ 91
420	-	-	-	-	+ 84
423	-	-	-	-	+ 68
493	-	-	-	-	+ 83
495	-	-	-	-	+ 54
15	-	-	-	-	+ 79
419	-	+	+ 72	+ 51	+ 88
461	-	-	-	-	+ 61
499	-	-	-	-	+ 71
460	-	-	-	-	+ 100
469	-	-	-	-	+ 59
478	-	-	-	-	+ 72
82	++	-	+ 65	+ 66	+ 52
446	+	-	+ 71	+ 100	+ 65
28, 98, 203, 355, 560, 600, 619	++	-	-	-	-
76, 135, 148, 580, 582,	+	-	-	-	-
471	-	-	+ 80	+ 67	+ 53
599	-	-	+ 78	+ 82	+ 61
108, 616	-	+	-	-	-
157	-	++	-	-	-
Total	14 (7.6%)	5 (2.7%)	6 (3.3%)	6 (3.3%)	20 (11%)

Table 2. *Comparison of serological results of RSAT and three ELISAs*
Tablo 2. *RBPT ve üç ELISA serolojik test sonuçlarının karşılaştırılması*

Number of Serum Samples	Rapid Slide Agglutination Test Using Smooth and Rough Strains		ELISA		
	S-LPS	R-LPS	*B. ovis* (Commercial, Idexx)	*B. canis* M-HSE Antigen	*B. abortus* RB51 HSE Antigen
14	-	-	-	-	+
12	+	-	-	-	-
2	-	+	+	+	+
3	-	+	-	-	-
2	-	-	+	+	+
2	+	-	+	+	+
148	-	-	-	-	-
Positivity rates	14 (7.6%)	5 (2.7%)	6 (3.3%)	6 (3.3%)	20 (11%)

DISCUSSION

Diagnosis of brucellosis based on clinical examination is not sensitive enough because similar symptoms are caused by other microorganisms or by trauma and almost half of the infected animals do not show clinical picture [6]. Bacteriological culture is not sensitive enough to detect all infected animals for practical reasons due to intermittent shedding of the agent by infected animals. Serological tests appear to be useful for routine diagnosis and control of the disease. The most widely used serological tests are CFT, AGID and ELISA. But only CFT is officially accepted test for international trade. However, CFT has some drawbacks like complexity, incompatibility with anticomplementary and hemolyzed sera, prozone phenomena and occasional false reactions. On the other hand, AGID is a very labour intensive test and has low number of test capacity [2-4,6,18,19]. Of the serological methods used to detect antibodies to *B. ovis*, I-ELISA has been shown to be the most sensitive and specific test. As a diagnostic serological method, the ELISA has important advantages over other serological tests commonly used for the diagnosis of ovine brucellosis, such as providing readily measurable results and being easy to perform and standardize [7,15,20,21].

This study compared the results of five serological tests. The percentage of positive results was 11% for ELISA with HSE-RB51, 3.3% for ELISAs with HSE-M as well as commercial indirect *B. ovis* ELISA kit, 7.6% for RSAT with *B. abortus* S99 and 2.7% for RSAT with *B. canis* M- strains *(Table 1)*. These results might showindicated that brucellosis caused by smooth speciesis more prevelant than those caused rough species These results are not surprising since small ruminant brucellosis caused by *B. melitensis* is endemic in Turkey [6,22]. Only two serum samples were positive for all the tests except S-RSAT. These two serum samples (189 and 419) might be from real *B. ovis* infected animals. Three serum samples were positive by only R-RSAT. RSAT is a screening test with which some false positives results might be seen [15,23].

Two of the serum samples were found positive by only 3 ELISAs. This was in agreement with the findings that ELISA has been proven to be more sensitive thanagglutination-based techniques [6,21].

Various ELISA-based methods for serodiagnosis of brucellosis have been proposed and used with various success rates depending on the antigens used in the assay. In this study, hot saline extract (HSE) was used as antigen for the ELISA, which has been shown to be a complex antigen, mainly composed of outer membrane proteins (OMPs) and rough lipopolysaccharide (R-LPS) [8,14,16]. In this study, two serum samples were positive to all tests except R-RSAT, this might suggest that OMPs can be shared between rough and smooth brucellae [1,21,24].

Among three ELISAs, HSE-RB51 ELISA showed the highest amount of seropositivity. This finding was not consistent with the findings of Nielsen et al.[14] in which *B. abortus* RB51 RLPS based ELISA gave the best spesificity and sensitivity results. This discrepancy suggests that the presence of OMPs in our HSE extract of *B. abortus* RB51 could explain why the HSE-ELISA was more sensitive than RLPS-ELISA.

In our study I-ELISA- *B. canis* and commercial I-ELISA-*B. ovis* kit detected the same serum samples. Since *B. ovis* shares antigenic components with *B. canis* [15,25,26], it would seem that both strains might be used as an antigen with the same results.

As conclusion, the similar results were obtained from the same serum samples by both commercial I-ELISA and HSE-M-ELISA. The results indicated that HSE-M-antigen could be used in ELISA for serologic diagnosis of *B. ovis* infection in sheep. Since 14 serum samples were found as positive by only HSE-RB51-ELISA, it was assumed that this test could be less specific than other tests used in the study. Although R-RBPT is a screening test, it showed the lowest seropositivity in the study. This could be explained by its less mucoid nature than other natural rough species. In

this context, we also concluded that R-RBPT using *B. canis* M(-) strain could be specific and practical screening test for serologic diagnosis of infection caused by rough strains.

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Kafkas Univ Vet Fak Derg
22 (1): 69-73, 2016
DOI: 10.9775/kvfd.2015.13902

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

Research Article

Morphological and Immunohistochemical Features of Interdigital Sinus in Kivircik Sheep

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Article Code: KVFD-2015-13902 Received: 22.06.2015 Accepted: 24.08.2015 Published Online: 24.08.2015

Abstract

Interdigital sinus secretions play a role in sexual behavior and reproductive process of sheep. In the present study, it was aimed to characterize the topographical, anatomical and immunohistological features of interdigital sinus in Kivircik sheep. The interdigital sinuses were dissected from 50 forefeet of female Kivircik sheep which were slaughtered in the slaughterhouse. The shape of sinus resembled a pipe and was located between the proximal and distal interphalangeal joints of two digits. The wall of interdigital sinus had three layers as epidermis, dermis and fibrous capsule. Epidermis consisted of a stratified squamous keratinized epithelium. Sebaceous glands, hair follicles, arrector pili muscles, and apocrine sweat glands were in the dermis. Furthermore, there were vessels and adipose tissue in the fibrous capsule. There was immunohistochemical reaction for estrogen receptor expressed in epidermis, apocrin sweat glands and sebaceous glands of interdigital sinus. In conclusion, our results about the topographical, anatomical and immunohistological features of interdigital sinus are very important to understand sexual behavior and reproductive process of sheep, and to make a new approach for its surgery. Furthermore, this study is the first to report the interdigital sinus of Kivircik sheep immunohistochemically.

Keywords: *Interdigital sinus, Kivircik sheep, Morphology, Immunohistochemistry*

Kıvırcık Koyunlarda *Sinus Interdigitalis*'in Morfolojik ve Immunohistokimyasal Özellikleri

Özet

Sinus interdigitalis salgıları koyunlarda seksüel davranış ve üreme sürecinde rol oynamaktadır. Bu çalışma ile Kıvırcık koyunlarda *sinus interdigitalis*'in, topografik, anatomik ve immünohistolojik özelliklerinin tanımlanması amaçlanmıştır. Çalışmada kullanılan *sinus interdigitalis* 'ler, mezbahada kesilen 50 adet Kıvırcık koyunun ön ayaklarından disseke edildi. Şekli bir pipoyu andıran *sinus interdigitalis*'in, iki parmak arasında articulatio interphalangea proksimalis ve distalis manus hizasında yerleştiği gözlendi. S*inus interdigitalis* duvarının epidermis, dermis ve fibröz kapsül olarak üç katmandan oluştuğu gözlendi. Epidermis'in, çok katlı yassı keratinize epitelden oluştuğu saptanırken, dermis katmanında yağ bezleri, kıl folikülleri, mm. arrectores pilorum ve apokrin ter bezlerinin varlığı görüldü. Ayrıca, fibröz kapsül içerisinde kapillar damarlar ve yağ doku bulunmaktaydı. Sinus 'un epidermis, apokrin ter bezleri ve yağ bezlerinde östrojen reseptörleri için immünhistokimyasal tepki olduğu gözlendi. Sonuç olarak, *sinus interdigitalis*'in topografik, anatomik ve immünohistolojik özellikleri hakkında elde ettiğimiz sonuçlar, koyunlardaki seksüel davranış ve üreme sürecini anlama ve *sinus interdigitalis*'in operasyonlarına yeni bir yaklaşım geliştirmesi bakımından önemlidir. Çalışma, Kıvırcık koyunlarda *sinus interdigitalis*'in immunohistokimyasal yapısı üzerine yapılan ilk çalışmadır.

Anahtar sözcükler: *Sinus Interdigitalis, Kıvırcık koyunu, Morfoloji, Immunohistokimya*

INTRODUCTION

In mammals, there are different kinds of skin glands such as circumoral, horn, infraorbital and interdigital glands. Shape, size and location of these glands are unlike between in species and even in race. Interdigital glands are located in space between the digits and calls interdigital sinus in sheep. While these sinuses are found on 4 limbs in sheep of both sexes, a rudimentary type is also present in goat [1-7].

Interdigital sinus resembles tobacco pipe shape [4,8]. This sinus excretes holocrine and apocrine secretion [1-8]. This chemical secretion plays an important role on bio-

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logical behaviors and territorial demarcation [9,10]. The scent material secreted from interdigital sinus is composed of evaporating elements such as pheromones, ketones, aldehydes and alkanes [11,12]. Pheromones are very important for sexual behavior and reproductive process of sheep and their secretion start especially in the estrus cycles. Estrogen receptors effect on sexual behaviors in mammals, and are very important for secretion of sexual pheromones [13]. Interdigital sinuses are also apt to be contaminated, therefore their location is very important for the surgical intervention.

Kivircik sheep are mainly raised in the northwestern part of Turkey and there is limited report about their interdigital sinus's topographical, anatomical and histological features [14]. Therefore in the present study it was aimed to investigate morphological, topographical and immunohistological features of interdigital sinus of Kivircik sheep to understand more their sexual behavior and reproductive process of sheep, and to make a new approach for its surgery.

MATERIAL and METHODS

In the current study, 50 Kivircik breed, which were slaughtered in the slaughterhouse of Çimet/Bursa in April, were used. Sinuses were dissected *(Fig. 1)* and collected from forefeet of 3 to 6 months aged female sheeps. The average weights of these sheep were 25 kg. After dissection, interdigital sinus was weighted and length and diameter of the sinus were measured with a digital compass (Mitutuyo Corparation, Kawasaki, Japan). Diameters of the sinus were showed in *Fig. 2*.

For histological procedures, tissues were fixed in 10% neutral buffered formalin solution for 24 h at room temperature and routine tissue processing procedures were applied. After dehydration in 70% ethanol, 80% ethanol, 96% ethanol, and absolute ethanol, the interdigital sinus tissues were embedded in paraffin wax and the sections are obtained at 5 µm. intervals. Tissue sections were mounted on polylysine-coated slides and incubated for 1 h at 37ºC in an oven. Slides were stained with triple Mallory staining modified by Crossman [15] for investigating the histological structure of interdigital sinus or kept until immunohistochemistry was performed.

The streptavidin biotin peroxidase complex technique was used for immunohistochemical staining. Briefly, sections were deparaffinized and rehydrated, then the slides were placed in 0.05% Saponin solution (Serva, Cat. No: 8047-15-2, Germany) for 20 min at room temperature. Sections were washed three times for 5 min each in phosphate buffered saline (PBS) and blocked with blocking serum-Reagent A (Histostain®-Plus Bulk Kit, 85-6743, Zymed Laboratories, USA). Then, sections were incubated with ready to use monoclonal rabbit anti-estrogen receptor primary antibody (Neomarkers, RM9101) overnight at 4ºC in a humidified chamber. After being rinsed three times each in PBS for 5 min, sections were incubated for 10 minutes with biotinylated antibody-Reagent B (Histostain®-Plus Bulk Kit, 85-6743, Zymed Laboratories, USA). Subsequently, slides were washed with PBS and incubated with streptavidin complex containing horseradish peroxidase-Reagent C (Histostain®-Plus Bulk Kit, Zymed Laboratories, USA) for 10 min at room temperature. After washing with PBS, 3, 3' dimethylaminoazobenzene (DAB, Zymed Laboratories, USA) was used as the chromogen which was prepared according to manufacturer's instructions and applied for 5 min. All sections were counterstained with Harris' heamatoxylin, in order to distinguish negative reactions from positive reactions. The slides were then rinsed in tapped water, dehydrated, and mounted with Entellan (Merck, Germany). Negative controls were performed using the same protocol but substituting the primary antibodies with PBS. Slides were examined under

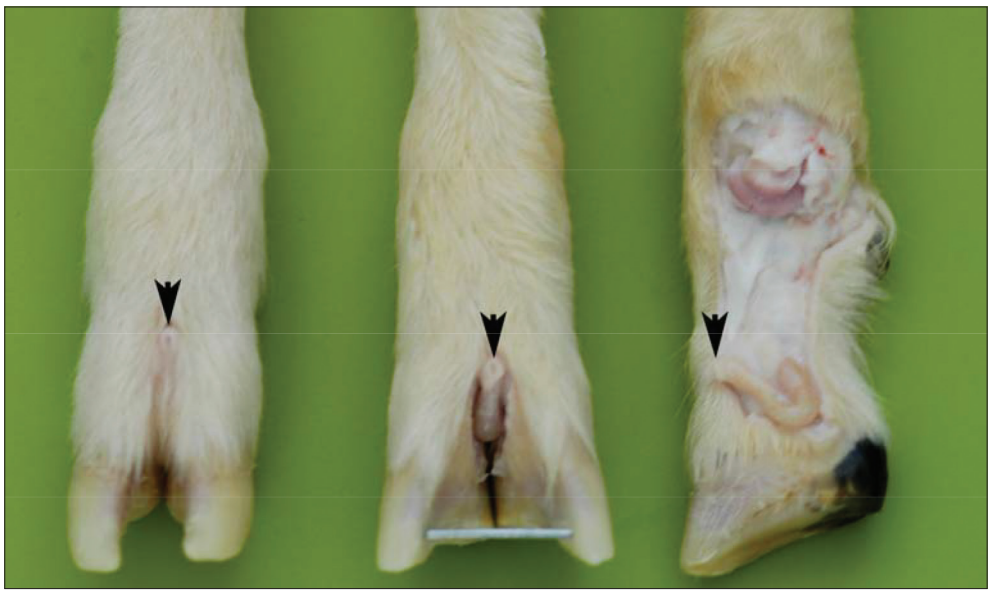

Fig 1. Interdigital sinus and it's oriface *(arrow)* in Kivircik Sheep

Şekil 1. Kıvırcık koyunda *sinus interdigitalis* ve açılışı *(ok)*

the light microscope (Nikon eclipse 80i Microscope, Tokyo, Japan). Photography was taken with Nikon Ds Camera Control Unit DS-L1 (Tokyo, Japan).

Immunohistochemical staining was scored in a semi-quantitative manner to determine differences between the slides in the distribution patterns of intensity of immuno-labelling of the epithelium, sebaceous glands, apocrine glands, and fibrous capsule. All slides were scored as no staining (-), weak (+), moderate (++), and strong (+++) by two researchers in a blind manner. This analysis was performed according to Adams et al.[16].

RESULTS

Topographically, the interdigital sinus was located between the proximal and distal interphalangeal joints of two digits *(Fig. 1)*.

Anatomically, the interdigital sinus resembled a pipe in shape. It was composed of a blind proximal end, a wide body and long-narrow neck *(Fig. 2)*. The body of the sinus was proximodorsal to distopalmar positioned in-between medial and lateral digits. Then the body was curled up at the level of the distal interphalangeal joints and formed the neck of the sinus. The body of gland was distinguishably had a notable flexure from the neck. Its oriface was determined from dorsal aspect at the level of the anterior part of the interdigital cleft *(Fig. 1, arrow)*. The interdigital sinus was connected to neighbor tissues with connective tissue. Morphometric values of the sinus were shown in *Table 1*.

The weight of interdigital sinus in Kivircik Sheep was 0.84±0.24 g. The ratio of sinus weight to body weight was found to be 0.003. The sinus' proximal and distal end diameters were close to each other although the body diameter is thicker than that value. The excretory duct of interdigital sinus was quite long. Wool fibers in the excretory duct were found.

Histologically, the wall of interdigital sinus had three layers: epidermis, dermis and fibrous capsule. Epidermis consisted of a stratified squamous keratinized epithelium. In the dermis, there were sebaceous glands, hair follicles, arrector pili muscles, and apocrine sweat glands. Sebaceous glands, hair follicles and arrector pili muscles were located in the upper portions of dermis however apocrine sweat glands were located in the deeper portions of dermis as seen in *Fig. 3*. Sebaceous glands were lobular with cluster of acini. Each acinus was composed of peripherally located small basal cells, which were surrounded by larger round cells that were filled the remainder of the acinus. Each hair follicle were surrounded by connective tissue and there were arrector pili muscles near hair follicles. The epithelium of apocrine sweat glands were simple columnar in the body however the epithelium of apocrine sweat glands were simple cuboidal in the neck. Moreover, myoepithelial cells were surround by the apocrine sweat glands. The outer part of the interdigital sinus was of fibrous capsule and had vessels and adipose tissue *(Fig. 3)*.

The cellular localization of estrogen receptor in the interdigital sinus was ascertained by streptavidin biotin complex method *(Fig. 4)*. In this protocol, a positive reaction is characterized by the deposition of a brown

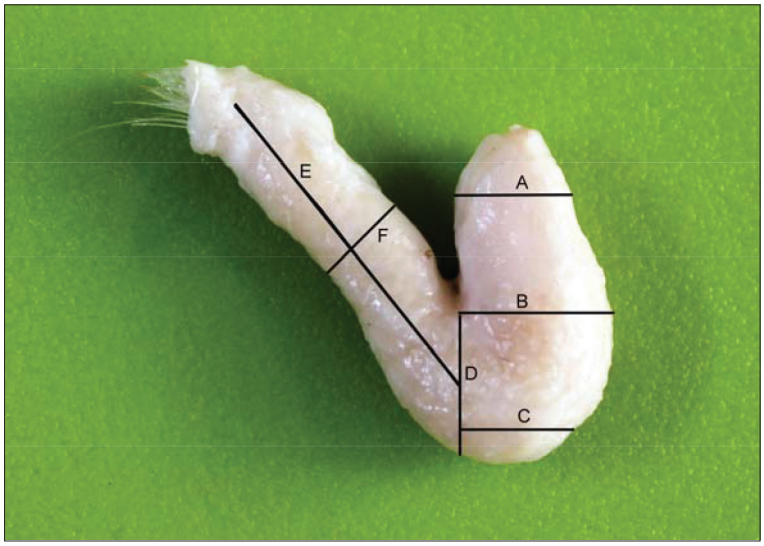

Fig 2. Interdigital sinus and its parts

A: Diameter of proximal end, **B:** Diameter of body, **C:** Diameter of distal end, **D:** Diameter of duct and sinus anostomose, **E:** Length of duct, **F:** Diameter of duct

Şekil 2. *Sinus interdigitalis* ve bölümleri

A: Proximal ucun çapı, **B:** Gövde çapı, **C:** Distal ucun çapı, **D:** Kanal ve sinus birleşme yerinde çapı, **E:** Kanalın uzunluğu, **F:** Kanalın çapı

Table 1. *Morphometric values of the interdigital sinus*
Tablo 1. *Sinus interdigitalis'in morfometrik değerleri*

Weight (g) $\bar{x} \pm S\bar{x}$	A (mm) $\bar{x} \pm S\bar{x}$	B (mm) $\bar{x} \pm S\bar{x}$	C (mm) $\bar{x} \pm S\bar{x}$	D (mm) $\bar{x} \pm S\bar{x}$	E (mm) $\bar{x} \pm S\bar{x}$	F (mm) $\bar{x} \pm S\bar{x}$
0.84±0.24	6.47±1.07	6.95±0.97	5.77±0.90	8.62±1.31	16.74±2.21	4.60±0.60

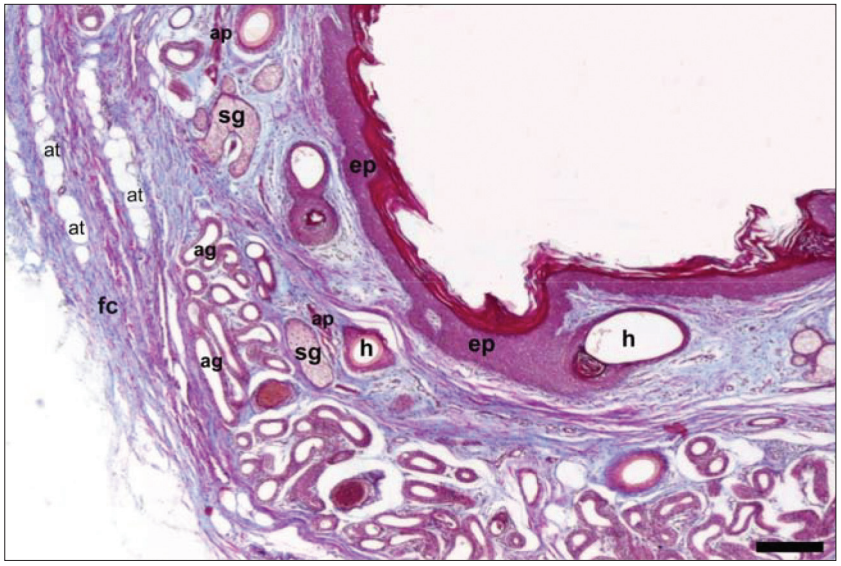

Fig 3. Transverse section of interdigital sinus. Stratified squamous keratinized epithelium (ep), sebaceous gland (sg), apocrine sweat gland (ag), arrector pili muscle (ap), hair follicle (h), adipose tissue (at), fibrous capsule (fc). Crossman's triple staining technique. Bar: 100 µm

Şekil 3. *Sinus interdigitalis*'in transversal kesiti. Çok katlı yassı keratinize epitel (ep), yağ bezi (sg), apokrin ter bezi (ag), m. arrector pili (ap), kıl folikülü (h), yağ dokusu (at), fibröz kapsül (fc). Crossman's üçlü boyama tekniği. Bar: 100 µm

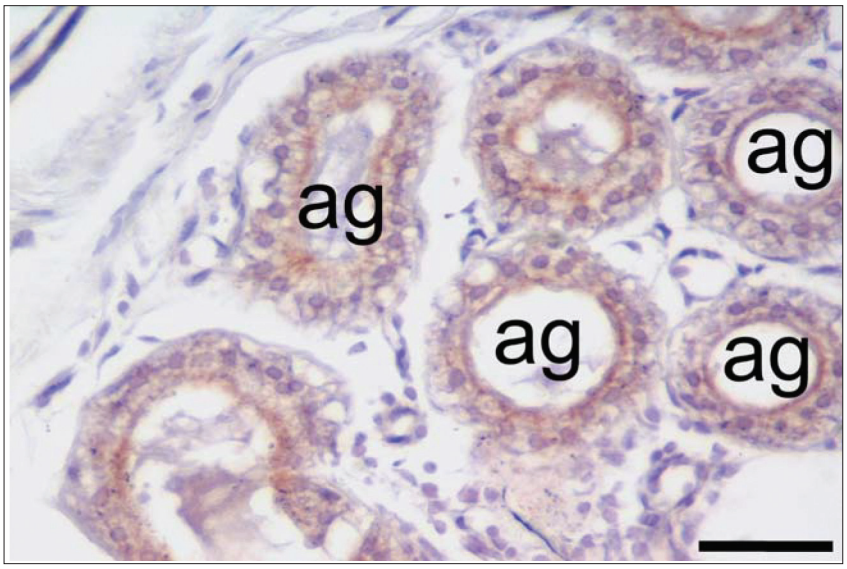

Fig 4. Immunostaining of estrogen receptor in apocrine sweat gland (ag). Bar: 25 µm

Şekil 4. Apokrin ter bezinde (ag) östrojen reseptörlerinin immun boyaması. Bar: 25 µm

precipitate at the site of the antigen-antibody interaction. It is notable that, in all positive immunoreactions were found in intracytoplasmic location and nuclear staining was not observed. Estrogen receptor immunoreactivity was only seen in epidermis, apocrin sweat glands and sebaceous glands. The expression of estrogen receptor was weak (+) in sebaceous gland, moderate (++) in epidermis and strong (+++) in apocrine sweat glands. In addition that, there was no immunostaining in fibrous capsule and no immunoreactivity in the negative control was observed.

DISCUSSION

The histomorphological structure of the interdigital sinus has been described in many animal species [1-4,11,12,17,18]. This study presents some characteristics of interdigital sinus in Kivircik sheep, which is usually raised in West Anatolia.

Embryologically, the interdigital sinus's epidermis and associated glands are formed by ectoderm and connective tissue of dermis and hypodermis are formed by mesoderm [5,19,20]. So, it contains sweat and sebaceous glands, hairs. In agreement with previous articles, the luminal surface of interdigital sinus in Kivircik sheep contained wool fibers. The localization of interdigital sinus was similar to the findings reported by the literature [2,4,5,20].

The interdigital sinus, a pipe shaped, contained a blind proximal end, a wide body and long-narrow neck, as documented by the literature [3-5,20]. The body diameter values of sinus had close values in sheep [21,22] and in Iranian Native Breed of sheep [4]. While the average length of duct in Kivircik sheep was 16.74 mm, the same parameter was 18-20 mm in sheep [22] and 10 mm. for Japanese serow [18]. While the diameter of duct in sheep was reported as 2-4 mm [22], we measured as 4.60 mm in Kivircik sheep, as in Iranian Native Breed of sheep [4]. Some minimal value

differences may result from breeding and non-breeding season of sheep.

We showed that the wall of interdigital sinus in the Kivircik sheep had similarities with other small ruminants' skin histological structures due to having three layers such as epidermis, dermis and fibrous capsule. Epidermis consisted of a stratified squamous keratinized epithelium. In the dermis, there were a great number of sebaceous glands, hair follicles, arrector pili muscles, and apocrine sweat glands. Also, there were vessels and adipose tissue in the fibrous capsule. Our findings are agree with the results of previous studies that showed the histological structures of Iranian Native Breed of sheep [4], Akkaraman breed sheep [5] and Tuj sheep [20].

The estrogen receptor belongs to the steroid hormone nuclear receptor superfamily [23], and it plays important roles on either regulation of various functions in skin, mainly hair growth, regulate sebaceous and sweat gland activity [24] or secretion of sexual pheromones [25]. Especially, apocrine sweat glands regarded as pheromone-producing scent glands [24] and their secretions play a role in demarcation of territorial area [6]. The interdigital sinus is a structure which is rich in apocrine glands.

In our study, estrogen receptor was detected only in epidermis, apocrine sweat glands and sebaceous glands. The immunoreaction of estrogen receptor was stronger in apocrine sweat glands. Estrogen receptor was observed in human skin and it was reported that there were immunoreaction of estrogen receptor in epidermis, sweat and sebaceous glands [24,26]. Our findings are consistent with these reports.

As a result, this study is the first to report the histological structures of the interdigital sinus including immunohistochemical localization of estrogen receptor in Kivircik sheep. Our immunohistochemical results showed that interdigital sinus's secretions may play a role on mammalian sexual behavior and reproductive process and also these results provide information for future investigation on the roles of steroid hormone either on skin functions or in sexual behaviors in sheep.

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Kafkas Univ Vet Fak Derg
22 (1): 75-78, 2016
DOI: 10.9775/kvfd.2015.13931

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

Research Article

Factors Related to the Frequency of Cat Ear Mites *(Otodectes cynotis)*

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Article Code: KVFD-2015-13931 Received: 25.06.2015 Accepted: 01.10.2015 Published Online: 01.10.2015

Abstract

Otodectes cynotis is an important, highly prevalent ectoparasite responsible for approximately 50% to 84% of otitis externa infestations in cats. This study investigates the factors related to the frequency of infestations in cats in Alanya, Antalya. A total of 105 cats underwent physical examination including ear examination with otoscope. *Otodectes cynotis* infections were found in 28 cats (27.7%). Cats were categorized by sex, age (<1 years, 1-4 years, >4 years), lifestyle (indoors alone, indoors with other pets, outdoors), and clinical symptoms (pruritus, erythema, ulceration, ear discharge, pain) to reveal the association of risk factors with using chi-square tests. Statistically significant differences were between lifestyle and infestation (P<0.05).

Keywords: *Cat, Ear mite, Otodectes cynotis, Risk factors, Turkey*

Kedi Kulak Uyuzu *(Otodectes cynotis)* Sıklığı İle İlgili Faktörler

Özet

Otodectes cynotis; kedilerde görülen otitis externa etiyolojisi içerisinde önemli bir yeri olan ve prevalansı (%50-84) oldukça yüksek paraziter bir enfestasyondur. Bu çalışmada, Türkiye'deki kedilerde görülen *Oc* enfestasyonunun sıklığını etkileyen faktörler araştırılmıştır. Toplamda 105 kedinin her iki kulak kanalında fiziksel ve otoskopik muayeneleri yapılmış ve 28 kedide *Oc* enfestasyonu tespit edilmiştir (%27.7). Kediler cinsiyet, yaş (<1 yaş, 1-4 yaş, >4 yaş), yaşam tarzları (evde yalnız, evde diğer pet hayvanları ile, dışarıda) ve klinik semptomlar (kaşıntı, eritem, ülserasyon, kulak akıntısı, ağrı) yönünden kategorize edilmiş ve ki-kare testleri kullanılarak bu risk faktörlerinin enfestasyon ile ilişkisi ortaya konmuştur. Yaşam tarzı ve enfestasyon arasında istatistiksel olarak anlamlı bir fark bulunmuştur (P<0.05).

Anahtar sözcükler: *Kedi, Kulak uyuzu, Otodectes cynotis, Risk faktörleri, Türkiye*

INTRODUCTION

Otodectes cynotis is an important, highly prevalent ectoparasite, responsible for 50%-84% of otitis externa in cats worldwide. Named by Sweatmean [1], *Otodectes cynotis* is highly contagious and which was reported to be with zoonotic character [2]. The mites live in the ear canals of cats, dogs, foxes, ferrets, and other carnivores and appear as small, white organisms within the ears or on swabs of material removed from ears. The mites feed on skin debris and tissue fluid from the epidermis. Transmission occurs by direct contact. The highest incidence has been noted in kittens. The life cycle, which is completed in the ear, includes larva, protonymph, and deutonymph stages over 18 to 28 days [3,4]. Despite these mites' importance as a cause of otitis externa in cats, information about their prevalence and the factors influencing their survival is lacking [5]. This study attempts to identify the factors related to the frequency of infestations in cats in Alanya, Antalya.

MATERIAL and METHODS

The study was carried out at a private animal clinic in 2013 in Alanya, Antalya. A total of 105 cats underwent physical examination and examination of both ear canals were done with an otoscope. Data on cats sex, age, breed, erythema, ulceration, pruritus, pain, secretion amount and type, and the findings of parasitological examination were recorded during the examinations. In all, 28 of 105 cats were found to be infected with *Otodectes cynotis.*

Waxy material from the ear canal was collected by ear cotton swap. This material was examined under a

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microscope by dropping 1-2 ml of mineral oil onto a glass microscope slide to determine the presence or absence of alive mites and the total mite count. Mites were identified using the diagnostic Pictorial Key to Arthropods of Centers for Disease Control, United States Health Education Centers for Disease [6].

Statistical Ananlysis

Statistical analysis was conducted to create a dataset using the packed statistic program. Cats were categorized into four groups: Sex (female, male), age (<1 years, 1-4 years, >4 years), lifestyle (indoors alone, indoors with other pets, outdoors), and clinical symptoms (pruritus, erythema, ulceration, ear discharge, pain) to reveal the association of risk factors with using chi-square tests. Associations were considered significant at $P<0.05$.

RESULTS

In this study of effective factors of *Otodectes cynotis* infestation, 28 (27.7%) of 105 cats (male: 49, female: 47, ages: <1->4) were found to be infected. Prevalence distributions by sex, age, and lifestyle are shown in *Table 1*. All exposure variables were identified with $P<0.05$. Significant difference was detected with lifestyle (indoor and outdoor) of cats ($P<0.05$) while no significant difference was detected with living alone or with other pets of indoor cats and with sex or age ($P>0.05$). Although no statistical differences were found for age, infestation prevalence tended to decrease with age (<1 years 31.3%; 1-4 years 28.2%; >4 years 11.1%) *(Table 1)*. There was an association between clinic symptoms (pruritus, ear discharge) and infestation ($P<0.001$) *(Table 2)*.

Table 1. *Factors affecting Otodectes cynotis infestation*
Tablo 1. *Otodectes cynotis enfestasyonunu etkileyen faktörler*

Factors			*Otodectes cynotis* Infestation					
			N	Positive	Prevalence (%)	Negative	Prevalence (%)	P Value
Sex	Male		49	12	24.5	37	75.5	0.637
	Female		56	16	28.6	40	71.4	
Age	<1 years		48	15	31.3	33	68.8	0.248
	1-4 years		39	11	28.2	28	71.8	
	>4years		18	2	11.1	16	88.9	
Life style	Indoor	Alone	63	11	17.5	52	82.5	0.310
		With other pets	27	8	29.6	19	70.4	
		Total	90	19	21.1	71	78.9	**0.003**
	Outdoor		15	9	60.0	6	40.0	

Table 2. *Association between clinic symptoms with infestation*
Tablo 2. *Enfestasyon ile klinik semptomlar arasındaki ilişki*

Clinical Symptoms		*Otodectes cynotis* Infestation					
		N	Positive	Prevalence (%)	Negative	Prevalence (%)	P Value
Pruritus	Yes	51	28	54.9	23	45.1	**0.000**
	No	54	0	0	54	100	
Erythema	Yes	62	16	25.8	46	74.12	0.811
	No	43	12	27.9	31	72.1	
Ulceration	Yes	64	12	18.8	52	81.3	0.022
	No	41	16	39.0	25	61.0	
Ear discharge	Yes	41	28	68.3	13	31.7	**0.000**
	No	64	0	0	64	100	
Pain	Yes	80	25	31.3	55	68.8	0.057
	No	25	3	12.0	22	88.0	

DISCUSSION

Otodectes cynotis is an extremely important external parasite that causes infestations in cats [3,7]. The prevalence of *Otodectes cynotis* in cats has been reported at 25.5%-29% in Greece and London and 22.5%-37% of feral cats in the United States [8-10]. In the present study, the prevalence of *Otodectes cynotis* infestation was 27.7%, similar to previous findings.

Although the parasite can affect all age groups of cats, it is seen more frequently in the kittens (<1 year old) due to transmission from infested mothers [7,11,12]. However, the present study found no association between age and the presence of mite infestation, though statistical analysis showed that infestation prevalence decreased with age. Some studies have shown that both male and female cats are susceptible [5,9,13,14]. Accordingly, no significant difference was found in the prevalence of *Otodectes cynotis* in male (24.5%) and female cats (28.6%).

There was an association between lifestyle and infestation, with a higher prevalence in outdoor cats (60%). Many authors have suggested that infestations are more common among animals living in poor environmental conditions and among street animals [5,14]. Degi et al.[15] have reported that outdoor cats (84%) are more affected than indoor cats (16%). However, these results do not agree with the claim of Sotiraki et al.[9] that lifestyle has not been proven to have a significant effect on risk of infestation.

The parasite is easily transmitted through contact with infected animals, whether of the same or a different species [9,11,16]. The present study found a significant difference associated with cats lifestyle. However the prevalence among cats which lived indoors alone (17.5%) was lower than that among indoor cats living with other pets (29.6%) there was no statisticaly significant difference. Also Degi et al.[15] have reported no infested cat observed that contact with other animals.

Otodectes cynotis might cause otitis externa in up to half 50% of cases worldwide, while 84% of cats with ear discharge have been diagnosed with the agent [9,17,18]. Clinical symptoms (pruritus, erythema, ulceration, ear discharge, pain) have been observed in infected animals depending on the level of parasitism [12,19,20]. This finding is in agreement with the results of the present study. Some researchers have observed otic pruritus in non-parasitized cats (9.2%) [7]. The infestation had a characteristic appearance like outer ear canal was filled with various amounts of a dry, dark, red-brown substance [9,11]. Approximately 85.4% of infested cats have been observed to have abnormal ear secretion, and 41.5% suffer from mechanical irritation caused by the parasite [9,11,21]. We found an association with symptoms (pruritus, ear discharge) and infestation, however rarely some cases can be asymptomatic [9,21,22].

In conclusion, we found that *Otodectes cynotis* is a highly common ectoparasite in cats. The potential risk of infestation varies by age and sex. Additionally, lifestyle influenced infestation prevalence. Clinical signs of ear mites were not always apparent, but higher rates of *Otodectes cynotis* infestation were found among cats with pruritus, erythema, ulceration, ear discharge, and pain.

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Kafkas Univ Vet Fak Derg
22 (1): 79-83, 2016
DOI: 10.9775/kvfd.2015.13944

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

Research Article

Occurrence of ESBL-Producing Enterobacteriaceae in Red Meat Samples

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Article Code: KVFD-2015-13944 Received: 30.06.2015 Accepted: 29.09.2015 Published Online: 01.10.2015

Abstract

Extended spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae is becoming a worldwide concern for the public health. They can adversely affect the treatment based on modern beta-lactam antibiotics against bacterial infections as well as possibly becoming a source for the spread of ESBL-encoding genes among different and/or the same bacterial species. The objective of this study was to determine the occurrence of ESBL-producing Enterobacteriaceae from a total of 110 red meat samples sold in İstanbul. The samples were initially homogenized in Enterobacteriaceae broth. Subsequently, the homogenized suspensions were exposed to pre-enrichment at 37°C for 18-24 h. After that, Chromogenic ESBL agar was used for selective enrichment at 37°C for 18-24 h again. The presumptive ESBL-producers were sub-cultured on Trypton Soy agar, followed by an overnight incubation at 37°C. The isolates were identified by Vitek®MS (bioMérieux). Finally, the identified isolates were subjected to agar disc diffusion testing, disc diffusion confirmation testing and MIC determination testing using a combination of cefotaxime (CTX), ceftazidime (CAZ), and cefpodoxime (CPD) discs ± clavulanic acid (CV) according to the guidelines by Clinical and Laboratory Standards Institute. The results revealed that a total of 23 isolates were confirmed to be positive for ESBL-production. Of 23 isolates, the most common species was determined as *E. coli* (30%), followed by *C. brakii* (22%), *E. cloacae* (17%), *K. pneumoniae* (9%), *C. freundii* (9%), *S. fonticola* (4%), *K. intermedia* (4%), and *M. wisconsensis* (4%). In conclusion, the results of this study provided that the red meat samples harbored ESBL-producing Enterobacteriaceae, and they may, therefore, hold a potential risk for the colonization of the consumers with ESBL-producers.

Keywords: *Antibiotic resistance, Enterobacteriaceae, ESBL, Red meat, Public health*

Kırmızı Etlerde GSBL-Üreten Enterobacteriaceae Suşlarının Varlıkları

Özet

Genişlemiş spektrumlu beta-laktamaz (GSBL)-üreten Enterobacteriaceae tüm Dünya'da halk sağlığı açısından ciddi endişelere yol açmaktadır. GSBL-üreten bakteriler, bakteriyel infeksiyonlara karşı kullanılan modern beta-laktam antibiyotikleri işlevselliklerini olumsuz şekilde etkilemekte ve aynı zamanda farklı ve/veya türdeş bakteri türleri arasında direnç kodlayan genlerin yayılmalarında rol oynamaktadırlar. Bu çalışmada, İstanbul ilinde satışa sunulan toplam 110 adet kırmızı et örneklerinde GSBL-üreten enterobakterlerin tespiti amaçlanmıştır. Örnekler, Enterobacteriaceae buyyonda homojenize edilmişlerdir. Homojenize edilen süspansiyonlar ilk olarak 37°C ve 18-24 saat ön zenginleştirme işlemine, devamında ise kromojen GSBL agarda tekrar 37°C ve 18-24 saat selektif zenginleştirme işlemine alınmışlardır. Şüpheli izolatlar saflaştırma için trypton soy agara ekimi yapılmış ve gece aşırı 37°C'de inkübe edilmişlerdir. İnkübasyon sonunda izolatlar Vitek®MS (bioMérieux) cihazı kullanılarak tiplendirilmiştir. Tiplendirilen şüpheli GSBL-üreten enterobakteri izolatlar Clinical and Laboratory Standards Institute talimatları takip edilerek ve sefotaksim (CTX), seftazidim (CAZ) ve sefpodoksim (CPD) ± klavulanik asit (CV) diskleri kullanılarak sırasıyla disk difüzyonu, disk difüzyon doğrulama ve MİK tespiti testlerine alınmışlardır. Sonuçlara göre toplam 23 adet enterobakteri izolatları kesin GSBL pozitif tespit edilmiştir. Aralarında en yaygın tip *E. coli* (%30) olarak belirlenirken, bunu *C. brakii* (%22), *E. cloacae* (%17), *K. pneumoniae* (%9), *C. freundii* (%9), *S. fonticola* (%4), *K. intermedia* (%4) ve *M. wisconsensis* (%4) izlemiştir. Sonuç olarak, kırmızı et örneklerinin GSBL-üreten enterobakteriler içerdikleri ve bu nedenle tüketicilerin GSBL-pozitif türler ile kolonize olmaları bakımından potansiyel risk taşıdıkları görülmüştür.

Anahtar sözcükler: *Antibiyotik direnci, Enterobacteriaceae, GSBL, Kırmızı et, Halk sağlığı*

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INTRODUCTION

Antibiotics are the chemical agents that were first developed in the early 1940 against bacteria-causing infections in humans. The antibiotics have been widely used in both human and veterinary medicine [1]. However, over- and improper-use of antibiotics have increasingly caused the dissemination of foodborne resistant-bacteria, not only in healthcare and community settings, thereby leading to higher resistant-bacteria-associated morbidity and mortality rates in the recent years. Therefore, the international health authorities are warning about the emergence of antibiotic resistance, including the non-medicinal use of antibiotics in the food animals [2].

Antimicrobial resistance is mainly caused by mechanisms of resistance. Within them, beta-lactam antibiotics are enzymatically inactivated by the specific enzymes, which are encoded by specific plasmid-mediated genetic materials. Especially, extended spectrum beta-lactamases (ESBL) are widely produced by the bacteria belonging to the family of Enterobacteriaceae. To date, more than 400 beta-lactamases were identified. ESBL-encoding genes can be transferred between the different and/or the same species through their plasmid-mediated characteristics [3]. Therefore, unpredictable and uncontrollable dissemination of ESBL-producing Enterobacteriaceae, including *E. coli*, *K. pneumoniae*, *Citrobacter* spp., *Salmonella* spp., and *Enterobacter* spp. are adversely affecting the human health [4-6].

Antibiotics are also widely used in the veterinary medicine, especially in the food animals for therapeutic purposes, not only in clinical and community settings [7,8]. The recent studies have showed that the foods of animal origin contain ESBL-producing enterobacteria and their ESBL-encoding *bla*-genes transferable to the human's intestinal microflora [9-11]. Except for the controlled therapeutic purposes, the antibiotics are, therefore, banned as growth promoters used in the farm animals by European Union, including Turkey. Also, the researchers are challenging on alternative antibiotic-replacers [12]. Probiotics and prebiotics, which are naturally occurring species and substances have been used for prevention of emerging resistant bacteria [7,8]. The leading international health and food organizations such as WHO and FAO are enhancing the surveillance programs for monitoring food, clinical, and community-related antibiotic resistance all over the [10,11].

The red meat is an important source in the human nutrition. It contains essential nutrients such as proteins and fatty acids, including minerals and vitamins [13]. For the human nutrition, slaughtering of the farm animals under unhygienic conditions threats the human health if contaminated with Enterobacteriaceae strains, especially with ESBL-producing enterobacteria. The recent studies have indicated that the red meat and the red-meat-derived foods may possibly be reservoirs for ESBL-producing Enterobacteriaceae, and their ESBL-encoding genetic elements [9-11].

The objective of this study was to determine the occurrence of ESBL-producing Enterobacteriaceae from a total of 110 red meat samples sold in İstanbul.

MATERIAL and METHODS

Sampling

A total of 110 red meat samples were randomly collected from butchers, supermarkets and slaughter-houses located in İstanbul from October 2014 to December 2014.

Reference Strains

ESBL-positive *K. pneumoniae* ATCC®700603 and ESBL-negative *E. coli* ATCC®25922 were used for control purpose in ESBL-screening testing.

Microbiological Evaluation of Samples

25 g of sample was added in 225 ml of Entero-bacteriaceae Enrichment Broth (LABM, England). The mixture was homogenized in a sterile blender-bag (Inter-science, France) for 2 min by stomacher (EasyMix, France), and exposed to aerobic incubation at 37°C for 18-24 h [11]. After that, 10 µl of the pre-enriched suspension was inoculated to Chromatic ESBL agar (Liofilchem, Italy). The inoculated plates were incubated again at 37°C for 18-24 h under aerobic conditions [14]. After that, the colonies were selected according to the manufacturer's instructions by a sterile loop, i.e. the blue-green one as *Klebsiella* spp., red-pink-purple one as *E. coli*, and white-yellowish colony as *Proteus* spp. The selected presumptive ESBL-producing colonies were subcultured on Tryptone Soy Agar (LABM), followed by an overnight incubation at 37°C [14]. The subcultures were identified by a mass spectrometer (Vitek®MS, bioMérieux, France).

Screening and Confirmation of ESBLs

Disc Diffusion Testing: The suspected ESBL-producers were subjected to disc diffusion testing according to the guidelines by Clinical and Laboratory Standards Institute (CLSI) [15]. The isolate was spiked in a sterile saline physiological solution to get a density expressed as 0.5 McFarland standard. Using a cotton swap, it was spreaded over Mueller Hinton Agar (MHA) (Merck, Germany) [15,16]. The ESBL screening was performed by cefotaxime (CTX; 30 µg), ceftazidime (CAZ; 30 µg), and cefpodoxime (CPD; 10 µg) discs (TurkLab, Turkey). The disc inserted plates were then incubated at 37°C for 18-24 h. After that, the zone measurements were taken by a milimetric ruler. The breakpoints with zone diameters were evaluated

according to the criteria by CLSI (2013), so that CTX ≤22 mm, CAZ ≤17 mm, and CPD ≤17 mm were considered to be positive for ESBL-production [15].

Disc Diffusion Confirmation Test: A combined combination of CTX, CAZ, and CPD ± clavulanic acid (CV; 10 µg) (Turklab, Turkey; Alkim, Turkey) was used for the confirmation of disc diffusion testing. The disc-inserted plates were incubated at 37ºC for 18-24 h. The zones of inhibition was evaluated according to the criteria by CLSI (2013) [16,17]. A difference of ≥5 mm between the zone measurements of the identical discs ± CV were considered to be ESBL-producing species [15,17,18].

Minimal Inhibitory Concentration (MIC) Determination

The MIC values of the previously conducted disc diffusion testing were finally obtained using Micronaut-S beta-lactamase VII kit (Merlin Diagnostika, Germany). A 50 µl of 0.5 McFarland suspension of the isolate was spiked in 10 ml of Mueller Hinton Broth (Merck, Germany), vortexed for a couple of seconds, and 100 µl of this suspension was inoculated into the plate. Following that, the inoculated plate was allowed for an incubation overnight at 37ºC. A Thermofischer Multiskan FC Spectrometer was used for readings. MIC analysis was automatically conducted by MCN6 Software (Sifin, Germany).

RESULTS

This study revealed that a total of 23 ESBL-producing Enterobacteriaceae was detected in a total of 110 red meat samples. The most common ESBL-producing species was determined to be *E. coli* (30%), followed by *C. brakii* (22%), *E. cloacae* (17%), *K. pneumoniae* (9%), *C. freundii* (9%), *S. fonticola* (4%), *K. intermedia* (4%), and *M. wisconsensis* (4%). All the ESBL-combined-disc screening and MIC confirmatory results were presented in *Table 1* and *Table 2*.

Table 1. *ESBL-screening results*
Tablo 1. *GSBL tarama sonuçları*

Type of Isolate	No of ESBL (+) Isolates
E. coli	7
C. braakii	5
E. cloacae	4
K. pneumoniae	2
C. freundii	2
S. fonticola	1
K. intermedia	1
M. wisconsensis	1
Total	23

DISCUSSION

Extended spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae is becoming a worldwide concern for the public health. The different profiles of antibiotic resistance are arised from use of diverse antibiotics [19]. They may adversely affect the treatment based on modern beta-lactam antibiotics against bacterial infections and possibly becoming possibly a source for spread of ESBL-encoding genes among the different and/or the same species. However, epidemiological data related to this biohazard situation are very limited in different geographical regions, including in Turkey. The Standart Commitee of European Doctors and the Federation of Veterinarians of Europe issued a common press release for keeping all the authorities on fighting against ESBL-producing Enterobacteriaceae in 2014. This study was, therefore, the first reporting from Turkey based on the methods used for determination of ESBL-producing enterobacteria occurring from red meats.

The foods sold in Turkey had widely poor microbial quality and represented a potential health risk to customers [20]. Especially, foodborne ESBL bacteria has not been well-examined in various foods from Turkey. Most of the studies related to this field have been limited to the clinical isolates and veterinary medicine. The food-related works were restricted with screening of ESBL-producing bacteria based on only standard disc-approximation testing, including a diverse combination of antibiotics. On the other hand, disc diffusion confirmation testing and MIC testing were not simultaneously conducted, and not supported by auto-analyzers such as Vitek® MS and BD Phoenix instruments.

In Turkey, a study showed that 22.3% of the red meats were positive for ESBL-producing *E. coli*, *C. youngae, E. cloacae* [21], another study determined 26.3% of the red meat samples harbored *E. coli*, *K. pneumoniae*, *K. oxytoca* [22], and the analyzed red meat samples mostly contained ESBL-producing *E. coli* in concordance with our study and other regional findings [23]. The transfer of ESBL-encoding genetic elements between resistant Enterobacteriaceae and non-resistant strains was proved in both animal and human intestinal tractus [24]. All these studies showed that disc-approximation testing was a golden method in detection of resistant bacteria [25]. However, they cannot be used for all species of Enterobacter [26,27].

In this study, ESBL-screening was performed by disc diffusion testing, combined-disc diffusion testing, and disc diffusion confirmation based on MIC determination, including auto-identification of the isolates by mass spectrometer. Our results showed that 60 pre-assumptive isolates were initially determined to be positive for ESBL-production. However, MIC analysis revealed that only 23 (38.3%) of the 60 presumptive isolates were actually real

Table 2. *Disc diffusion and MIC results*
Tablo 2. *Disk tarama ve MİK sonuçları*

Isolate no	Type of Isolate	Zone Diameter (mm)						MIC (µg/ml)						
		CAZ	CAZ CV	CTX	CTX CV	CPD	CPD CV	CAZ	CAZ Reference	CAZ Actual	CTX	CTX Reference	CTX Actual	Result ESBL +/-
1	*E. cloacae*	22	26	23	26	16	17	?	-	=8/4	?	-	=8/4	+
2	*S. fonticola*	26	27	27	28	20	23	S	<=1	<=0.25/4	R	=128	<=0.25/4	+
3	*E. coli*	18	25	13	28	0	21	S	32	≤0.25/4	R	>128	≤0.25/4	+
4	*K. pneumoniae*	21	30	15	31	13	21	R	16	=4/4	R	128	=4/4	+
5	*M. wisconsensis*	19	28	22	32	14	21	R	128	-	R	32	=0.5/4	+
6	*C. freundii*	24	25	24	25	16	20	S	2	1	R	16	=8/4	+
7	*C. braakii*	22	22	15	25	16	20	R	64	<=0.25/4	R	128	=16/4	+
8	*K. intermedia*	21	22	20	22	18	20	S	<=1	<=0.25/4	S	<=1	<=0.25/4	+
9	*E. coli*	15	27	10	28	10	16	R	128	<=0.25/4	R	>128	<=0.25/4	+
10	*E. coli*	11	29	25	27	10	13	R	128	<=0.25/4	S	<=1	<=0.25/4	+
11	*E. coli*	12	18	10	19	7	9	R	=64	=2/2	R	>128	=4/4	+
12	*C. freundii*	18	24	19	22	14	16	R	=64	=8/4	R	=32	=4/4	+
13	*E. cloacae*	20	12	10	9	-	-	S	=4	>32/4	R	=32	>8/4	+
14	*C. braakii*	9	18	17	24	14	16	R	>128	=8/4	R	=128	=8/4	+
15	*C. braakii*	22	14	25	26	16	18	R	=64	=8/4	R	=4	=1/4	+
16	*K. pneumoniae*	16	25	10	24	7	9	R	=32	-	R	>128	=4/4	+
17	*C. braakii*	23	31	10	28	13	16	R	=16	=2/4	R	>128	=8/4	+
18	*E. cloacae*	21	26	21	27	18	19	R	=128	=4/4	R	=128	>32/4	+
19	*E. coli*	15	25	9	25	7	10	R	>128	=0.5/4	R	>128	=0.5/4	+
20	*E. cloacae*	16	22	16	22	6	9	R	=64	-	R	>128	-	+
21	*E. coli*	19	14	27	23	17	19	R	=64	=16/4	R	32	=8/4	+
22	*E. coli*	22	31	21	24	17	18	R	=128	=32/4	R	=64	=4/4	+
23	*C. braakii*	20	27	25	31	18	20	R	=128	<=0.25/4	R	=64	-	+

R=Resistant, ***I***=Intermediate, ***S***=Suspected

ESBL-producers. Therefore, we concluded that there was not still a common agreement on which confirmatory test was the most sensitive. Fast and accurate detection of ESBL-producers are, therefore, important for epidemiological surveillance and infection control of ESBL-producing enterobacteria.

A study in Spain reported that the frequency rate of ESBL-producing bacteria was 25% in the beef samples, confirmed ESBL-producing *E. coli* as the most common species within the examined beef samples [28]. Also, some further studies mainly focused on understanding the dissemination ways of ESBL-producing bacteria from numerous sources by epidemiological research [24,25,29]. The epidemiological studies have been arised due to spreadable and transferable properties of ESBL-encoding genetic elements among the same and/or different bacterial species through a diverse of mechanisms of antibiotic resistance. That's why, the studies should be extended for further genotypic testing.

The red meat and red meat derived-products should be supplied to the customers under hygienic conditions because of their importance for the human nutrition as well as including food safety concerns. To date, the number of the studies related to the occurrence of ESBL-producing Enterobacteriaceae are still limited worldwide, including in Turkey.

In conclusion, the results of this study indicated that the red meat samples harbored ESBL-producing Enterobacteriaceae, and they may hold a potential risk for the colonization of the consumers with ESBL-producers. Therefore, antibiotic use in the veterinary medicine should be intensively monitored.

Conflicts of Interest

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

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22 (1): 85-91, 2016
DOI: 10.9775/kvfd.2015.13957

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

Research Article

Effect of Reusing Litter on Broiler Performance, Foot-Pad Dermatitis and Litter Quality in Chickens with Different Growth Rates

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Article Code: KVFD-2015-13957 Received: 01.07.2015 Accepted: 07.10.2015 Published Online: 12.10.2015

Abstract

This study examined the effect of reusing litter on performance, viability and foot pad dermatitis (FPD) rates of three meat-type chicken genotypes with different growth rates and slaughter weights. Caking and manure accumulation of reused litter were also measured. A total of 780 chicks of 3 genotypes were raised in compartments (26 chickens per compartment, 11.55 chickens/m^2). Wood shavings were used as litter, with 5 compartments containing new litter and 5 containing re-used litter for each genotype. Differences in live weight, feed consumption, feed conversion ratio, viability and carcass parameters were significant among genotypes ($P<0.05$), but insignificant between new and used litter groups. FPD, caking and manure scores were higher on used litter. Overall findings suggest that with sufficient ventilation, litter can be reused, thereby decreasing costs of poultry production.

Keywords: *Reused litter, Wood shavings, Foot pad dermatitis, Caking score, Manure score*

Farklı Gelişme Düzeyindeki Etlik Piliçlerde Altlığın Yeniden Kullanılmasının Broiler Performansı, Foot-Pad Dermatitis ve Altlık Kalitesine Etkileri

Özet

Bu çalışma farklı gelişme hızına ve kesim ağırlığına sahip üç etlik piliç genotipinde altlığın ikinci defa kullanımının performans, yaşama gücü ve foot pad dermatitis (FPD) gibi özelliklere etkisini ortaya koymak amacıyla yürütülmüştür. Kullanılmış altlıktaki kekleşme ve gübre seviyeleri de belirlenmiştir. Her genotip grubunda 5 yeni ve 5 ikinci defa kullanılan kaba rende talaşı altlık ve 8 nipel suluk bulunan bölmelerde, 3 genotipten toplam 780 civciv olacak şekilde (26 piliç; m^2'de 11.55 piliç) deneme yürütülmüştür. Genotipler arasında canlı ağırlık, yem tüketimi, yemden yararlanma oranı ve yaşama gücü ile karkas özelliklerinde farklılıklar önemli bulunmuştur ($P<0.05$), buna karşılık yeni ve kullanılmış altlıkta yetiştirme bu özellikler üzerinde farklılık yaratmamıştır. FPD skorları, kekleşme skoru ve gübreleşme skorları ile altlık nem düzeyi kullanılmış altlıkta daha yüksek bulunmuştur. Elde edilen sonuçlar, yeterli havalandırma ile altlığın ikinci defa kullanmanın uygun olacağını ve altlık giderlerini azaltmada etkili olabileceğini önermiştir.

Anahtar sözcükler: *Altlığın ikinci kullanımı, Kaba rende talaşı, Foot pad dermatitis, Kekleşme skoru, Gübreleşme skoru*

INTRODUCTION

Short production periods, negative effects of the cage system on carcass characteristics and concerns over animal welfare have contributed to the increased use of litter in broiler production. Litter materials consist mainly of post-harvest plant waste, wood industry residuals and various locally available products [1]. Common litter materials that have shown good results include wood shavings [2,3]; rice hulls and ash [4-7]; soft sawdust; corncob particles, fodder and stems of legumes and poaceae, sugarcane stems and peanut shells [8,9]; exsiccated tree leaves [10]; hazelnut husks [11,12]; composted municipal garbage [13]; recycled paper chips, shredded paper and pelleted newspaper [14-16]; and inorganic soil products, pumice, clay, zeolite and sand [3,17-20]. Other alternatives developed in recent years include pelleted industrial litter composed of various disinfected materials.

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All suitable litter material must possess adequate moisture-holding capacity and no toxic or other negative effects on animal health and physical characteristics. Other important litter properties include sustainability of ventilation and resistance to 'caking', the term used to describe the sealing of the litter surface that can occur if the moisture content of the litter reaches the maximum level. Moisture content and caking are related in that high moisture levels produce caking, and caking traps water, thereby increasing moisture levels. When the water that is saturating litter is unable to escape, the poultry are raised on a continually damp, slippery and sticky surface. Caking as well as slippery surfaces can lead to foot and carcass defects [17-19,21].

Based on these requirements, wood shavings have become the most common litter material throughout the world. However, due to increases in the use of wood shavings in industry and difficulties in finding alternative litter material, litter costs have come to represent a significant part of production costs. Furthermore, litter materials now account for a significant proportion of environmental pollution in regions with intensive broiler production [18]. For these reasons, the reuse of litter, particularly in healthy flocks, is worth examining. Several studies have indicated that reusing litter could increase the quality of litter manure and help to reduce some of the environmental problems associated with litter [18,22,23].

Bird performance is affected by litter conditions, which are in turn affected by stocking density, slaughter age and weight, house climate conditions and litter enrichments. Studies have shown that ammonia production starts earlier when litter is re-used, thus increasing ventilation and heating costs; however, costs of litter as well as costs related to cleaning and changing litter are minimized in good weather conditions [23,24]. This study investigated the effects of reusing litter on broiler performance, mortality, slaughter and carcass characteristics, incidence of foot pad dermatitis and litter moisture content, manure levels and caking. A new subjective scoring method developed by the authors was used to determine caking and manure levels.

MATERIAL and METHODS

This study was conducted at the Ondokuz Mayis University, Agricultural Faculty experimental farm between February-July 2013. All procedures were approved by the Ondokuz Mayis University Animal Care and Use Ethics Committee (2009/15). Animal material consisted of 3 different genotypes, as follows: SG1: slow-growing meat production ROSSx(ROSSxRIR); SG2: slow-growing meat production ROSSx(ROSSxBAR); FG: fast-growing ROSS-308 hybrids.

Chicken were reared using a floor system with 8-cm-thick wood shavings as litter in a 20×12×2.5 m windowed, artificially lighted, ventilated house containing 8 infrared heaters. For each genotype, a total of 260 mixed male/female chicks were randomly allocated among 10 1.5× 1.5 m×2 m wire-mesh compartments (n=26; 11.55 chicks per m^2) containing 1 round feeder and 8 nipple drinkers. In order to prevent litter material from escaping, the wire mesh was covered with plastic sheeting to a height of 15 cm from the compartment floor. Water and feed were provided ad libidum. Diet was formulated according to NRC [25] and purchased from a commercial mill and varied with age *(Table 1)*. Chicks were vaccinated against Newcastle disease, Gumboro disease and Infectious Bronchitis, and no health problems were observed. All chickens were slaughtered at 7 weeks of age.

Following slaughter, litter was removed from the compartments in which new litter was to be placed (5 compartments per genotype), and all compartments were cleaned, fumigated and ventilated for two days. For each genotype, 5 compartments were refilled with new litter (wood shavings to a height of 8 cm), whereas the used litter and manure in the remaining 5 compartments were dredged and redistributed. After these preparations, the house was heated, and the study was initiated under the conditions described above, with 5 new-litter compartments and 5 reused-litter compartments for each genotype.

Broiler performance traits measured included live weights, feed consumption, feed conversion ratios,

Table 1. *Nutritional content of feeds used in the study**
Tablo 1. *Çalışmada kullanılan yemlerin besin madde düzeyleri*

Nutrients	Broiler Chick Starter (1-7.days)	Broiler Chick (8-28.days)	Broiler Chicken (29-35.days)	Broiler Finisher (36 days- slaughter)
Crude Protein (%)	23.00	22.00	21.00	18.00
ME (Kcal/kg)	3000	3100	3100	3100
Crude cellulose (%)	4.00	4.00	4.00	6.00
Crude ash (%)	5.00	5.00	5.00	8.00
Ca (%)	1.00	0.95	0.80	0.80
Phosphorus (%)	0.50	0.50	0.45	0.60
Methionine (%)	1.00	0.45	0.40	0.40
Lysine (%)	1.35	1.20	1.10	1.00

** Calculated values*

mortality rates and slaughter-carcass characteristics. Feed conversion ratios were calculated weekly based on feed consumption (measured by compartment) and chicken weight. Chickens were counted daily, and overall mortality rates were determined for each genotype. At 49 days, after reaching slaughtering weights of 2-2.5 kg, 40 SG1 and 40 SG2 chickens were slaughtered (2 male and 2 female chickens from each compartment). In addition, 40 FG chickens were slaughtered at both 42 days, the common age for this commercial genotype, and at 49 days, to facilitate comparisons among genotypes. (FG birds slaughtered at 42 days were designated as 'FG6' and 'FG7', respectively). Slaughtering weight was determined before slaughter, and cold-carcass weight was determined after storage at +4°C for 24 h. Carcasses were cut into parts according to standard methods [26], and thigh and breast weights and ratios to carcass weights were recorded.

Foot pad dermatitis (FPD) scores were measured according to Mayne [27]; as follows: 0 = No external signs of FPD; 1 = raised central pad, reticulate scales are separated, with or without small, black necrotic areas; 2 = marked swelling of the foot pad, black reticulate scales forming scale-shaped necrotic areas, with necrosis evident on less than one-quarter of the total foot pad area; 3 = marked swelling and enlargement of the entire foot pad, necrosis extending up to one-half of the total foot pad area; 4 = marked swelling and enlargement of the entire foot pad, necrotic cells covering more than one-half of the total foot pad area.

Litter moisture content was determined following slaughter of chickens at 42 and 49 days. Litter samples were collected from 3 different places in each compartment and mixed together; 100 g of this mixture was dried at 60°C for 48 h, after which moisture contents were measured [28]. Litter caking was determined visually for each compartment at the end of the study and scored as follows: 0: No caking of litter; 1: caking in less than 1/4 of litter; 2: caking in 1/4-1/3 of litter; 3: caking in 1/3-1/2 of litter; 4: caking in more than 1/2 of litter. Litter manure level was similarly determined visually for each compartment as follows: 0: No manure on the litter; 1: manure observed on less than 1/4 of litter; 2: manure on 1/ 4-1/3 of litter; 3: manure on 1/3-1/2 of litter; 4: manure on more than ½ of litter.

Factorial analysis was conducted using a completely randomized design (3 x 2 x 5), with genotype and litter type as factors on the data of performance, carcass traits and litter moisture. Data recorded as percentages were subjected to arcsine square root transformation, and real mean values were calculated and are presented in the tables. Differences among genotypes were identified using Duncan's multiple comparison test. A difference of $P<0.05$ was considered statistically significant. Kruskal-Wallis test was used to determine correlations between food pad dermatitis and litter manure and caking scores. Kruskal-Wallis results showed the effects of genotype and litter on FPD, litter manure and litter caking scores as well as litter moisture content (%) to be significant, but the interaction between genotype and litter to be insignificant; therefore, the Mann-Whitney test was used for 2-way comparisons between genotype and litter type, with results given as means, medians and standard errors of means. Kendal's Tau test was used to determine correlations between caking and manure scores and live weights.

RESULTS

Significant differences were found in live weight, feed consumption, feed conversion ratio and mortality rates among genotypes at the end of the production period ($P<0.05$; *Table 2, 3, 4*). Mortality rates were significantly lower among SG1 (1.43%) and SG2 (1.76%) than FG (3.43%) chickens ($P<0.05$).

Table 2. *Live weight of chickens, by age, genotype and litter type*
Tablo 2. *Piliçlerin yaşa, genotipe ve altlık tipine göre canlı ağırlıkları*

Traits		Age (weeks)							
		Hatch	1	2	3	4	5	6	7
Genotype	SG1	42.85b	222.96b	462.89b	668.78b	1075.79b	1531.96b	2034.27b	2429.25b
	SG2	41.11b	216.11c	450.18b	646.17b	1041.21b	1499.05b	1985.79b	2411.43b
	FG	45.23a	233.93a	518.73a	829.41a	1407.80a	2028.66a	2759.26a	3452.72a
Litter Type	New	42.88	224.57	474.22	710.02	1175.95	1687.98	2272.90	2780.17
	Used	43.24	224.09	480.31	719.55	1173.92	1685.13	2247.64	2748.78
SEM		0.358	0.903	3.277	5.757	9.032	12.802	15.752	18.937
Effects									
Genotype		**	**	**	**	**	**	**	**
Litter		NS	NS	NS	NS	NS	NS	NS	NS
Genotype x Litter		NS	NS	NS	NS	NS	NS	NS	NS

SEM: *Standard Error of Means;* ** *P<0.01;* ***NS:*** *Insignificant, P>0.05;* ***a. b. c:*** *According to Duncan Test, different letters in the same column indicate significant differences*

Live weight, feed consumption and feed conversion ratios did not vary significantly between chickens reared on new and used litter. Mortality rates were higher with used litter (3.77%) than new litter (2.85%), but the difference was insignificant.

The present study found some slaughter and carcass characteristics varied significantly between genotypes of different growth rates (P<0.05; *Table 5*), but not among chickens raised on new or used litter. FG chickens slaughtered at 6 weeks had higher abdominal fat contents than SG chickens slaughtered at 7 weeks. Breast ratios of FG at 6 and 7 weeks were also higher than those of SG1 and SG2 genotypes at 7 weeks. Thigh ratios of SG1, SG2 and FG at 6 weeks were similar and higher than those of FG at 7 weeks (P<0.05).

Table 6 shows the findings of this study for FPD. Significant differences in FPD scores were found between chickens of all genotypes.

Caking scores of litter in our study were found to correlate with litter moisture content, with significant differences found between caking scores of chickens related to differences in live weights among genotypes (P<0.05; *Table 7*). Caking scores also varied between new (2.77) and used (3.17) litter, but this difference was insignificant. Manure levels on litter varied significantly by genotype, with manure levels of fast-growing chickens higher (3.40 at 6 weeks and 3.80 at 7 weeks) than those of slow-growing chickens (2.05 for SG1 and 2.55 for SG2 at 7 weeks).

Table 3. *Feed consumption of chickens, by age, genotype and litter type*
Tablo 3. *Piliçlerin yaşa, genotipe ve altlık tipine göre yem tüketimleri*

Traits		Age (weeks)						
		1	2	3	4	5	6	7
Genotype	SG1	201.1a	504.9a	984.7b	1726.9b	2607.9b	3673.6b	4703.6b
	SG2	191.2b	477.6b	952.8b	1670.6b	2556.1b	3584.4b	4643.3b
	FG	207.9a	526.7a	1092.1a	2032.6a	3140.4a	4579.7a	6041.9a
Litter Type	New	199.2	495.5	992.1	1790.1	2747.4	3937.0	5148.9
	Used	200.9	511.6	1027.8	1829.9	2788.8	3954.8	5110.4
SEM		1.408	4.588	8.599	15.260	19.891	25.902	35.048
Effects								
Genotype		**	**	**	**	**	**	**
Litter		NS	NS	*	NS	NS	NS	NS
Genotype x Litter		NS	*	NS	NS	NS	NS	NS

SEM: *Standard Error of Means; * P<0.05; ** P<0.01;* ***NS:*** *Insignificant, P>0.05;* ***a.b:*** *According to Duncan Test, different letters in the same column indicate significant differences*

Table 4. *Feed conversion ratio and mortality rates, by age, genotype and litter type*
Tablo 4. *Piliçlerin yaşa, genotipe ve altlık tipine göre yemden yararlanma oranı ve ölüm oranları*

Traits		Feed Conversion Ratio (age, weeks)							Mortality (%)
		1	2	3	4	5	6	7	
Genotype	SG1	0.902a	1.089a	1.472a	1.607a	1.702a	1.807a	1.937a	1.43b
	SG2	0.885a	1.061a	1.477a	1.606a	1.705a	1.804a	1.926a	1.76b
	FG	0.888a	1.016b	1.319b	1.446b	1.548b	1.660b	1.755b	3.43a
Litter Type	New	0.887	1.045	1.407	1.536	1.637	1.745	1.870	2.85
	Used	0.897	1.065	1.439	1.570	1.666	1.769	1.875	3.77
SEM			0.008	0.011	0.010	0.010	0.009	0.010	0.012
Effects									
Genotype		NS	**	**	**	**	**	**	**
Litter		NS	NS	NS	NS	NS	NS	NS	NS
Genotype x Litter		NS	NS	NS	NS	*	NS	NS	NS

SEM: *Standard Error of Means; * P<0.05; ** P<0.01;* ***NS:*** *Insignificant, P>0.05;* ***a.b:*** *According to Duncan Test, different letters in the same column indicate significant differences*

Table 5. *Slaughter and carcass traits of chickens*
Tablo 5. *Piliçlerin kesim ve karkas özellikleri*

Traits		Carcass Traits						
		Live Weight (g)	Carcass (g)	Carcass Yield (%)	Gizzard (g)	Abdominal Fat (%)	Thigh (%)	Breast (%)
Genotype	SG1	2481.8d	1822.2d	73.38	30.24b	2.70b	27.12a	36.02b
	SG2	2637.4c	1916.6c	72.69	28.86b	3.16a	27.19a	35.27c
	FG6 FG7	2842.5b 3532.7a	2126.6b 2644.5a	73.91 74.84	32.12a 32.60a	1.93d 2.41c	26.63a 25.77b	40.27a 40.35a
Litter Type	New	2863.9	2123.9	74.06	31.03	2.46	26.70	37.93
	Used	2883.8	2131.0	73.35	30.88	2.64	26.66	38.02
Gender	M	3185.6	2359.1	73.95	33.37	2.07	27.19	37.47
	F	2561.9	1895.8	73.46	28.54	3.04	26.18	38.48
SEM		14.810	11.820	0.318	0.329	0.050	0.105	0.134
Effects								
Genotype		**	**	NS	**	**	**	**
Litter Gender		NS **	NS **	NS NS	NS **	NS **	NS **	NS **
Genotype x Litter		NS	NS	NS	NS	NS	NS	NS
Genotype x Gender		NS	NS	NS	NS	NS	NS	NS
Litter x Gender		NS	NS	NS	NS	NS	NS	NS
Genotype x Litter x Gender		NS	NS	NS	NS	NS	NS	NS

SEM: *Standard Error of Means; ** P<0.01;* ***NS:*** *Insignificant, P>0.05;* ***a.b.c.d:*** *According to Duncan Test, different letters in the same column indicate significant differences*

Table 6. *FPD scores of chickens, by genotype and litter type*
Tablo 6*. Piliçlerin , genotipe ve altlık tipine göre FPD skorları*

Genotype and Traits	Left Foot FPD Score		
	X±Sx	Median	Min-Max
Genotype			
SG1	2.45±0.16 b	3	0-4
SG2	1.75±0.22 c	2	0-4
FG6	2.60±0.17ab	3	0-4
FG7	2.83±0.11 a	3	0-4
Litter Type			
New	2.39±0.10a	3	0-4
Used	2.64±0.14 b	3	0-4

a. b. c: *According to Kendal's Tau comparison test, different letters in the same column indicate significant differences*

DISCUSSION

SG1 and SG2 chickens were hatched from the eggs of slow-growing parents, so that a one-week difference in growing rates between these chickens and FG chickens was expected [25,29]. The mean final body weight of the FG group (2759.26 g) at 6 weeks was higher than that of the SG1 (2429.25 g) and SG2 (2411.43 g) groups at 7 weeks. Sarica and Cam [23] also reported no differences in live weight, feed consumption, or feed conversion ratios among chickens reared on new and second-use litter of different materials, but found mortality to be significantly lower with re-used litter. But, Cressman [30] found that live weight of chickens reared on re-used litter was 5.5% higher than the ones reared on new litter and there were not significant differences between the mortalities of chickens reared on new and used litter.

Overall carcass yields did not vary significantly among genotypes. In line with our findings, previous studies [3,12,31] reported re-use of litter did not significantly affect carcass characteristics.

FG chickens had significantly higher FPD scores than SG1 and SG2 genotypes at both 6 and 7 weeks (P<0.05). SG1 chickens also had significantly higher FPD scores than SG2 chickens, despite the similarity in live weights between these genotypes (P<0.05). FPD scores could have been affected by specific factors related to genotype, particularly live weight and growth rate. In line with our findings, a previous study examining FPD scores of chickens with different growth rates in a free-range production system found growth rate and FPD scores to be highly correlated, with scores of 0.44 and 2.35, respectively, for slow-growing and fast-growing chickens [32].

FPD scores in our study were also affected by litter reuse, with FPD scores for new litter significantly lower than those for reused litter (P<0.05). In contrast to our findings, Ruiz et al.[31] found no significant differences in FPD scores between new and used litter. Cressman [30] used FPD scores as a criterion of animal welfare and reported

Table 7. *Litter caking and manure scores and moisture ratios*
Tablo 7. *Altlık kekleşme ve gübreleşme skoru ile nem yüzdeleri*

Genotype and Traits	Caking Score			Manure Score			Moisture of Litter (%)	Effects	
	X±Sx	Median	Min-Max	X±Sx	Median	Min-Max			
Genotype									
SG1	2.95±0.17bc	3.0	2.0-3.5	2.55±0.19b	2.5	2.0-3.5	22.99 A		
SG2	2.35±0.27c	2.0	1.5-4.0	2.05±0.05c	2.0	2.0-2.5	21.63 AB		
FG6	3.40±0.12c	4.0	3.0-4	3.40±0.19a	3.5	3.0-4.0	27.15 BC		
FG7	3.80±0.24ab	3.0	3.5-4.0	3.80±0.20a	3.0	3.0-4.0	31.17 C		
Litter Type									
New	2.77±0.23	3.0	1.5-4.0	2.50±0.19	2.0	2.0-4.0	24.38	Genotype	**
Used	3.17±0.18	3.0	1.5-4.0	2.97±0.21	3.0	2.0-4.0	27.02	Litter	**
							SEM 0.734	Gen x litter	NS

a.b.c: *According to Kendal's Tau comparison test, different letters in the same column indicate significant differences;* ***SEM:*** *Standard Error of Means;* ** *P<0.01;* ***NS:*** *Insignificant, P>0.05;* ***A,B,C:*** *According to Duncan Test, different letters in the same column indicate significant differences*

that bird welfare was not affected by litter treatment. Litter moisture and ventilation have been found to be the most important factors affecting FPD [27,33,34]. Other factors include production system, water management and feed composition [32,35]. Yıldız et al.[19] showed that adding vermiculite to litter lowered FPD scores. Also, Garcia et al.[36] showed that FPD lesions were affected by litter material.

The differences in FPD scores found in our study could be related to differences in litter moisture content and caking levels *(Table 7)*. Litter moisture content was significantly higher for the FG chickens at 7 weeks (31.7%) than for the FG chickens at 6 weeks (27.15%) and for the SG1 and SG2 chickens at 7 weeks (22.99% and 21.63%, respectively). The high moisture levels of litter in the compartments of fast-growing chickens could be due to higher levels of feed and water consumption or because the high live weights of these chickens induce them to spend most of their time lying on the litter [32,34]. Litter moisture levels also varied significantly between new (24.38%) and used (27.02%) litter; however, in both cases, levels were within acceptable limits and were not considered to adversely affect performance [1,3,11,18,20,23].

This finding was expected, given that chickens with higher live weights produce more feces and urine. Differences in manure levels of SG1 and SG2 genotypes were also significant (P<0.05) and may be attributed to the higher live weights of the SG2 genotype. Differences in manure scores did not vary significantly between new and re-used litter.

In conclusion, the results of this study indicate that use of litter for a second time has no adverse effect on performance, viability, carcass traits, litter caking scores, litter manure scores, or litter moisture levels. However, FPD incidence increases with re-use of litter. These results suggest that the re-use of litter could be an advantage in terms of shortening the length of time between production periods and reducing litter costs, particularly in seasons when ventilation can be easily provided.

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Kafkas Univ Vet Fak Derg
22 (1): 93-99, 2016
DOI: 10.9775/kvfd.2015.13960

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

Research Article

Molecular Prevalence, Phylogenetic Characterization and Benzimidazole Resistance of *Haemonchus contortus* from Sheep [1]

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Article Code: KVFD-2015-13960 Received: 02.07.2015 Accepted: 18.08.2015 Published Online: 18.08.2015

Abstract

This study was conducted to determine the molecular prevalence and characterization of *Haemonchus contortus* from sheep along with the benzimidazole (BZ) resistance in *H. contortus* populations. Fecal samples were collected from a total of 300 sheep in research area and analyzed by fecal flotation. qPCR assays were utilized on trichostrongylid egg positive samples in order to identify *H. contortus*. Phylogenetic analyses were performed on ribosomal ITS-2 and mt-COI gene regions. BZ sensitive and resistant allele frequencies were determined by qPCR along with sequence analyses of the β-tubulin isotype 1 gene for single nucleotide polimorphizms (SNPs). *H. contortus* was identified in 36 (24.8%) out of 145 trichostrongylid egg positive samples. *H. contortus* isolates showed 100.0% identity to each other and 0.1% difference with the isolates available in the GenBank based on phylogenetic analyses of ITS-2 gene while mt-COI analyses of the isolates exhibited a mean of 97.4±0.5% identity to each other and 5.5±0.8% difference with the isolates in GenBank. BZ sensitive and resistant allele frequencies in *H. contortus* populations were determined as 87.1%±16.2 and 12.9%±16.2, respectively. SNPs were detected only in the codon 200 of the sequenced isolates belong to resistant allele. This study provides the first data on molecular prevalence, phylogenetic characterization and BZ resistance in *H. contortus* populations from sheep in Turkey.

Keywords: *Haemonchus contortus, Sheep, Molecular characterization, Benzimidazole resistance*

Koyunlarda *Haemonchus contortus*'un Moleküler Prevalansı, Filogenetik Karakterizasyonu ve Benzimidazol Dirençliliği

Özet

Bu çalışmada koyunlarda *Haemonchus contortus*'un moleküler prevalansı ve karakterizasyonu ile benzimidazol (BZ) dirençliliğinin belirlenmesi amaçlanmıştır. Araştırma yöresinde toplam 300 koyundan dışkı örnekleri toplanmış ve fekal yüzdürme yöntemiyle incelenmiştir. Trichostrongylid yumurtalarıyla pozitif belirlenen örneklerde *H. contortus* identifikasyonu için qPCR analizleri gerçekleştirilmiştir. Filogenetik analizler *H. contortus* izolatlarının ribosomal ITS-2 ve mt-COI gen bölgeleri üzerinde yürütülmüştür. BZ duyarlı ve dirençli allel sıklıkları β-tubulin izotip 1 gen bölgesindeki tek nükleotid polimorfizmi (SNPs) temelinde qPCR ile belirlenmiş, ayrıca ilgili genin sekans analizleri gerçekleştirilmiştir. Trichostrongylid yumurtalarıyla pozitif 145 örneğin 36'sında (%24.8) qPCR ile *H. contortus* identifiye edilmiştir. ITS-2 gen bölgesi filogenetik analizine göre *H. contortus* izolatlarının %100 identik oldukları ve GenBank'ta mevcut izolatlarla %0.1 genetik farklılık gösterdikleri belirlenirken mt-COI sekans analizleri izolatların ortalama %97.4±0.5 identiklik ve GenBank'ta mevcut izolatlarla da %5.5±0.8 farklılık gösterdiklerini ortaya koymuştur. *H. contortus* populasyonlarında BZ duyarlı ve dirençli allel sıklıkları qPCR ile sırasıyla %87.1±16,2 ve %12.9±16.2 belirlenmiştir. Dirençli allele ait izolatların sekans analizlerinde yalnızca 200. kodonda SNPs saptanmıştır. Bu çalışma ile Türkiye'de koyunlarda *H. contortus*'un moleküler prevalansı ve genetik karakterizasyonu üzerine ilk veriler sağlanmış ve *H. contortus* popülasyonlarında BZ dirençliliği ortaya konmuştur.

Anahtar sözcükler: *Haemonchus contortus, Koyun, Moleküler karakterizasyon, Benzimidazol dirençliliği*

INTRODUCTION

Gastrointestinal nematodes are the most prevalent and major parasites in domestic and wild ruminants in tropical, subtropical and temperate regions worldwide [1,2]. One of the highly dangerous and economically important of these nematodes is *Haemonchus contortus* which is also known as "barber pole worm" and an abomasal pathogen of sheep [3]. It causes anemia, stunted growth, weight loss, loss in protein, disorder in fertility, decrease in milk, meat and wool production and the last death if untreated [1].

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In recent years, DNA-based techniques have been implemented to specific identification and characterization of these parasites and also to determine the level of infections. For this purpose several PCR-based methods targeting mainly the nuclear and mitochondrial genes have been applied and used in the phylogenetic relationships among trichostrongylid nematodes [1,4-6]. However there have been no molecular-based studies conducted on *H. contortus* populations in Turkey up to date. The broad spectrum anthelmintic drugs including the benzimidazole (BZ) group has been widely used for several years in the control of trichostrongylid nematodes all over the world and as a result anthelmintic resistance began in these parasites [1]. Several molecular techniques especially target the intron I of the ß-tubulin gene of trichostrongylid nematodes including *H. contortus* have been developed to specifically detect and measure the frequency of the sensitive and resistant alleles for BZ [7,8]. The β-tubulin is highly conserved gene and is found in different trichostrongylid nematodes of small and large ruminants, horses, pigs and also dogs [9]. It has been revealed that BZ resistance in trichostrongylid nematodes is particularly associated with the alterations in the β-tubulin isotype 1 gene which has an importance as genetic marker and useful for predicting BZ resistance problems [10]. A point mutations also called as single nucleotide polymorphisms (SNPs) at the codons 167, 198 and 200 of β-tubulin isotype 1 gene have been explored and linked to BZ resistance up to date [11-13].

The present study was conducted to determine the molecular prevalence and phylogenetic characterization of *H. contortus* from sheep in Kayseri region based on sequence analyses of mitochondrial cytochrome oxidase subunit 1 (mt-COI) and nuclear ribosomal internal transcribed spacer 2 (ITS-2) gene regions. Potentially problems of BZ resistance and the frequency of BZ-resistance-associated β-tubulin SNPs in *H. contortus* populations in sheep were also investigated.

MATERIAL and METHODS

Sample Collection and Parasitological Examination

Ethics committee approval was received for this study from the ethics committee of Erciyes University Local Ethics Committee for Animal Experiments (Date: 09/05/2012, Document no: 12/61). The study was conducted on a total of 300 Akkaraman sheep (≤2 age group 78, >2 age group 222; male 12, female 288) raised in various farms in Kayseri and vicinity between 2012 and 2013. Fresh fecal samples were directly collected from the rectum of each sheep into the sterile plastic bags, transferred to the laboratory, and then kept at 4°C until the parasitological examination. Zinc sulphate flotation technique was utilized to investigate trichostrongylid eggs [14]. The specimens found positive for trichostrongylid eggs were further examined by modified McMaster technique for determining the EPG values [12].

Genomic DNA Isolation

A saturated sodium nitrate flotation method was used to isolate trichostrongylid eggs from infected fecal material according to the procedures described by Bott et al.[15]. Genomic DNA was extracted from the isolated trichostrongylid eggs using a commercial kit (Axygen Biosciences, USA) according to the manufacturer's instructions. The extracted genomic DNA's were stored at -20°C until molecular analysis.

TaqMan Probe Based Real Time PCR Analyses for Identification of H. contortus

Genomic DNA from the obtained isolates were analyzed by TaqMan probe based real time PCR with the primers ITS-2F, ITS-2R and the fluorogenic probe ITS-2P labelled with FAM-BHQ1 probe that amplified ribosomal ITS-2 gene region of *H. contortus* [16].

Amplification and Phylogenetic Analyses of Ribosomal ITS-2 and mt-COI Genes of H. contortus

For specific amplification of *H. contortus* ribosomal ITS-2 gene region (248bp) the primers ERU-HconITS2F (5'GTTAACCATATACTACAATG-3') and ERU-HconITS2R (5'G AG CTCAGGTTGCATTATAC-3') were originally designed from the published sequences of the related gene of *H. contortus* isolates available in GenBank with the assistance of Primer3Plus software. Primer specificity was confirmed by primer-BLAST analyses in GenBank. PCR amplifications were utilized in a 25 µL reaction volume containing 10X PCR buffer, 2.5 mM $MgCl_2$, 100 nM each primers, 200 mM each dNTPs, 50 ng genomic DNA and 2.5 U Taq polymerase. The cycling conditions were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 30s, 55°C for 30s and 72°C for 1 min, followed by 10 min at 72°C and 4°C to finalize. For the amplification of mt-COI region of *H. contortus* isolates, Nested PCR analyses were carried out following the described protocols [1].

The selected amplicons for both gene regions were further gel purified by a commercial kit (High Pure PCR product purification kit, Roche). Sequence analyses were performed on the obtained plasmids including the target gene regions after cloning procedures (CloneJET PCR Clonning Kit; Thermo Scientific, USA). Obtained sequences were aligned to homologues available from GenBank using the BLASTn algorithm with the default settings and edited in Geneious 6.1.6 software. Phylogenetic trees were constructed using the neighbor-joining (NJ) method based on the Kimura 2-parameter model in Mega 5.2 with 1000 boot-strap replicates for each tree [17].

Real Time PCR Amplification of β-tubulin Isotype 1 Gene for BZ Resistance and Sequence Analyzes for SNPs

TaqMan probe based qPCR assays targeting the

β-tubulin isotype 1 gene including the SNPs sites of BZ susceptible and resistant alleles of *H. contortus* were utilized as described by Walsh et al.[18]. The qPCRs were performed separately on the isolates that were determined as positive for *H. contortus* by ITS-2 real time PCR analyses. Allele frequencies were calculated by using the formula described by Germer et al.[19]. Allele frequencies of samples with no threshold cycle (Ct) value for benzimidazole resistant allele were evaluated as 100% susceptible for BZ.

β-tubulin isotype 1 gene including the SNPs sites from the isolates selected according to the determined allele frequencies in qPCR assays were further amplified by PCR with the primers HcTub-s5 and HcTub-as5 following the described protocols by Ghisi et al.[20] in order to explore the point mutations in the related gene region. The obtained amplicons were gel purified, cloned and plasmid isolations were performed. The plasmids were sequenced and the point mutations were analyzed in the obtained nucleotide sequences.

RESULTS

Fecal Examination and Real Time PCR Results

145 of the examined sheep were positive for trichostrongylid type eggs with a prevalence of 48.3%. The EPG values for trichostrongylid type are shown in *Table 1*. The mean EPG value in the trichostrongylid type positive samples was determined as 120.3±53.5. EPG values were determined as approximately two times higher in over 2 years age group than under ones and this difference was found statistically significant (P<0.05).

Of the 145 sheep with positive for trichostrongyle eggs, 36 (24.8%) were found to be infected with *H. contortus* by TaqMan qPCR assay. Parasitemia based on the mean Ct values *(Table 1)* were found to be higher in >2 year age group than ≤2 year age group and this difference was found statistically significant (P<0.001).

Sequence and Phylogenetic Analyses of Ribosomal ITS-2 and mt-COI Gene Regions

The ITS-2 gene regions of totally four *H. contortus* isolates (TrERUHcon04, TrERUHcon05, TrERUHcon06 and TrERUHcon07) were deposited into the GenBank database under accession numbers KJ188203 to KJ188206. The *H. contortus* Kayseri isolates showed 100% identity to each other and 0.1% genetic diversity with the available ITS-2 sequences of *H. contortus* in GenBank. Our isolates were also found 100.0% identical with the isolates obtained from the sheep in Brazil (JN128898, JQ342247), China (HQ844231), Iranian (HQ389229), Uzbekistan (KC503915), from the goat in USA (EU084691) and from the human stool in Australia (KC632567) and clustered together *(Fig. 1)*.

Mt-COI gene region of three *H. contortus* isolates (TrERUHcon01 to TrERUHcon03) were also deposited into the GenBank, with the accession numbers KJ188200 to KJ188202, respectively. *H. contortus* Kayseri isolates showed a mean of 97.4±0.5% identity to each other and a mean genetic diversity of 5.5±0.8% with the isolates available in GenBank. TrERUHcon01-03 isolates clustered together *(Fig. 2)* with the mt-COI sequences from the *H. contortus* isolates from sheep in Pakistan with a mean pairwise identity of 95.9±0.7% and showed a mean 7.7±1.6%, 7.8±1.4%, 8.4±1.6% genetic differences with the isolates from USA, Brazil and Australia, respectively which constituted the other major cluster.

BZ Resistance Allele Frequencies and Sequence Analyzes for SNPs

The qPCR analyses targeting the SNPs in β-tubulin isotype 1 gene region for BZ resistance in totally 145 *H. contortus* positive samples revealed a frequencies of 87.1±16.2% and 12.9±16.2% for sensitive and resistant alleles which were calculated by using the cycle threshold (Ct) values, respectively *(Table 2)*. Susceptible allele frequency was found as sevenfold higher than resistant allele frequency and this difference was found statistically

Table 1. *Distribution of EPG and Ct values over the age groups and gender of trichostrongyle type and H. contortus positive sheep*
Tablo 1. *EPG ve Ct değerlerinin trichostronhylid tip ve H. contortus pozitif koyunlarda yaş grupları ve cinsiyete göre dağılımı*

Factor	Number of Trichostrongylid Type Positive Sheep	EPG					Ct (dR)					
		Min-Max (95% Confidence)	Mean	St. Dev	F	P	Number of *H. contortus* Positive Sheep	Min-Max (95% Confidence)	Mean	St. Dev	F	P
Age Group												
≤2	22	51.6-80.2	65.9	32.3	32.744	0.000	10	25.3-33.5	29.2	1.96	86.93	0.000
>2	123	121.0-139.1	130.1	50.7			26	33.7-37.8	35.5	1.37		
Gender												
Male	8	69.1-168.4	118.7	59.4	0.007	0.931	3	25.3-36.8	31.0	5.78	0.000	0.998
Female	137	11.4-129.5	120.4	53.4			33	27.0-37.8	30.9	3.23		
Total	145	111.5-129.1	120.3	53.5			36	25.3-37.8	30.9	3.38		

F: *Anova Test;* ***EPG:*** *Eggs per gram of feces;* ***Ct:*** *Threshold cycle;* ***(dR):*** *Fluorescence;* ***St. Dev:*** *Standard deviation*

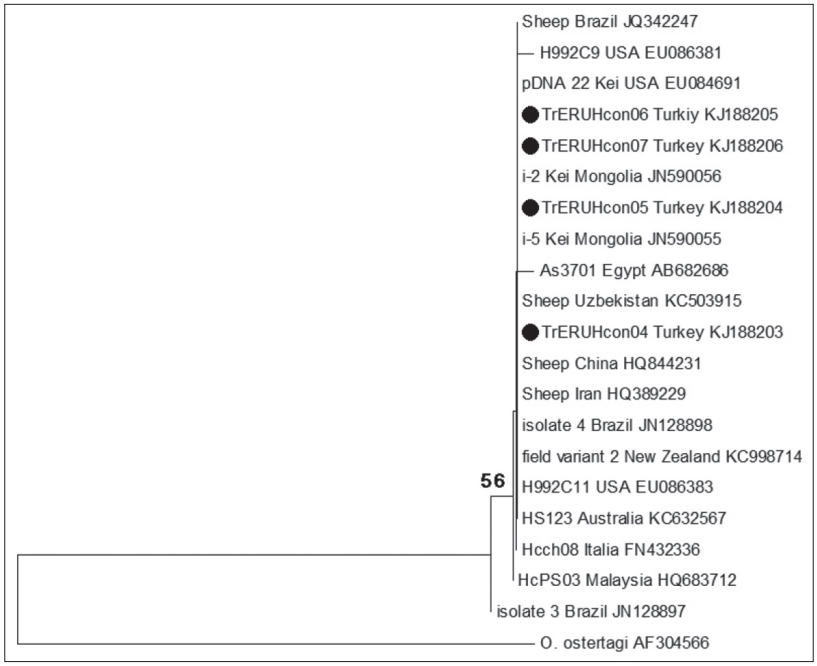

Fig 1. Phylogenetic relationship among the obtained *H. contortus* isolates (with the symbol ●) and some other *H. contortus* isolates from different countries with in the same or different genotypes as inferred from sequences of the ribosomal ITS-2 gene. The sequences were given as isolate name, country and GenBank accession number. The numbers above branches indicate neighbor-joining bootstrap supports (1000 replicates). Scale bar indicates number of nucleotide substitutions per site. *Ostertagia ostertagia* (AF304566) was used as an out group

Şekil 1. Ribosomal ITS-2 gen bölgesi sekanslarına göre elde edilen *H. contortus* izolatları (● sembolü ile işaretli) ile Dünyada çeşitli bölgelerden aynı veya farklı genotipteki diğer bazı *H. contortus* izolatları arasındaki filogenetik ilişki. Sekanslar izolat ismi, ülke ve GenBank aksesyon numarası olarak verilmiştir. Branşların önündeki rakamlar neighbor-joining bootstrap desteğini (1000 tekrar), ölçek çizgisi bölgeye göre nükleotid değişimini göstermektedir. Dış dal olarak *Ostertagia ostertagia* (AF304566) kullanılmıştır

Fig 2. Phylogenetic relationship among the obtained *H. contortus* isolates (with the symbol ●) and some other *H. contortus* isolates from different countries with in the same or different genotypes as inferred from sequences of the partial mt-COI gene. The sequences were given as isolate name, country and GenBank accession number. The numbers above branches indicate neighbor-joining bootstrap supports (1000 replicates). Scale bar indicates number of nucleotide substitutions per site. *Ostertagia ostertagia* (AB246112) was used as an out group

Şekil 2. Mt-COI gen bölgesi sekanslarına göre elde edilen *H. contortus* izolatları (● sembolü ile işaretli) ile Dünyada çeşitli bölgelerden aynı veya farklı genotipteki diğer bazı *H. contortus* izolatları arasındaki filogenetik ilişki. Sekanslar izolat ismi, ülke ve GenBank aksesyon numarası olarak verilmiştir. Branşların önündeki rakamlar neighbor-joining bootstrap desteğini (1000 tekrar), ölçek çizgisi bölgeye göre nükleotid değişimini göstermektedir. Dış dal olarak *Ostertagia ostertagia* (AB246112) kullanılmıştır

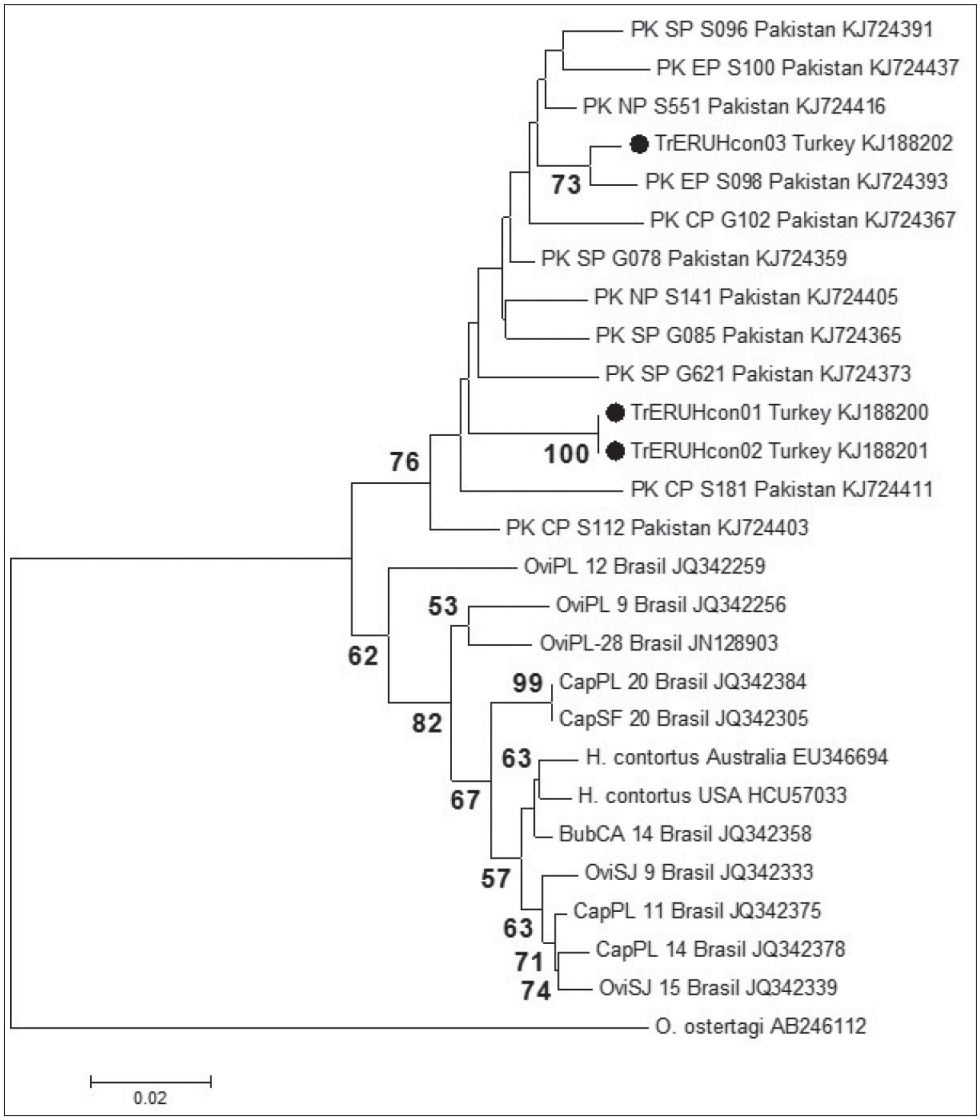

Table 2. *BZ sensitive and resistant allele frequencies in H. contortus populations according to age groups and gender of infected sheep*
Tablo 2. *Enfekte koyunların yaş grupları ve cinsiyetine göre H. contortus populasyonlarında BZ duyarlı ve dirençli allel sıklıkları*

Factor		Allele Frequency (%)					
		Sensitive		Resistant		F	P
		Min-Max (95% Confidence)	Mean+St.dev	Min-Max (95% Confidence)	Mean+St.dev		
Age group	≤2	55.2-100.0	83.2±18.5	0.0-44.8	16.8±18.5	0.820	0.372
	>2	56.9-100.0	88.6±15.3	0.0-43.1	11.4±15.3		
Gender	Male	55.2-100.0	81.8±23.6	0.0-43.1	18.2±23.6	0.349	0.559
	Female	56.9-100.0	87.6±15.8	0.0-44.8	12.4±15.8		
Total		55.2-100.0	87.1±16.2	0.0-44.8	12.9±16.2	378.404	0.000

F: *Anova Test;* ***St.dev:*** *Standard deviation*

significant (p<0.001). No statistically significant difference (P>0.05) was found in the susceptible and resistant allele frequencies over age groups and gender of sheep.

Totally 14 isolates in which 9 had a susceptible allele frequency of 100.0% and 5 had a resistant allele frequency of over 39.0% were subjected to the sequence analyses in order to screen the point mutations in β-tubulin isotype 1 gene. Sequence analyses of the 9 isolates with 100.0% susceptible allele frequency and 3 of 5 isolates with over 39.0% resistant allele frequencies revealed that phenylalanine (TTC), phenylalanine (TTC) and glutamine (GAA) amino acids were present in 167, 200 and 198 codons as in BZ susceptible populations. Whereas a point mutation [transformation of phenylalanine (TTC) to tyrosine (TAC)] only at the codon 200 was detected in both remaining two isolates from over 39.0% resistant allele frequencies. Two isolates belong to susceptible (TrERUHcon08) and resistant (TrERUHcon09) alleles were deposited to the GenBank under accession numbers KJ410522 and KJ410523, respectively.

DISCUSSION

The prevalence and intensity of gastrointestinal nematodes in sheep may vary in different regions. The present study reports the first molecular prevalence of *H. contortus* in Turkey with a rate of 24.8% in sheep, which indicates high prevalence of the parasite and insufficient control measurements in the study area. The number of nematode eggs in a fecal sample varies according to such factors as host and parasite species. Generally less than 500, 500-1500 and over 1500 EPG are considered as low, moderate and high infection levels, respectively [21]. In this study a mean of 120.3±53.5 EPG was determined in infected sheep in the study area which indicates a low infection level. This low level might be attributed to several factors such as host immunity, time of sampling and pasture status depending on climatic conditions and anthelmintic treatment process. However, age depending differences in EPG values was found statistically significant in the study and the sheep over 2 age group had higher EPG values than under ones. This result is also in accordance and supported with the *H. contortus* parasitemia levels which were indicated by Ct values in qPCR assays in our study. This could be related to the development of hypobiotic larvae (arrested at the early L4 stage), triggered by periparturient relaxation of immunity which is also indicated by several researchers [21-23].

Phylogenetic characterization and genome sequencing techniques on several gastrointestinal parasites have been contributed to improve knowledge of the biology and physiology of these parasites [1]. Genetic characterization of these parasites is also an important indicator for the diagnosis of drug resistance and formation of the control strategies for the parasite infections [6]. In this perspective, the current study evaluated the genetic diversity in *H. contortus* from sheep in Kayseri region for the first time in Turkey based on the sequences of a mitochondrial DNA marker (mt-COI) and a nuclear ribosomal marker (ITS-2) which have been widely used in phylogenetic characterization of trichostrongylid nematode species [1,4,5]. Genetic diversity among the Kayseri isolates based on the mt-COI gene was determined as higher than ITS-2 gene region which indicates the usefulness of mt-COI gene in the investigation of intraspecific variations and genetic variability in *H. contortus* populations as also indicated by Hussain et al.[5]. However the regional mt-COI sequences in GenBank are restricted with only the isolates from sheep, cattle and goats in USA, Australia, Brazil and Pakistan. As it has been seen in several nematodes, *H. contortus* has also a high biotic potential along with a high infection rate and direct life cycle which does not need an intermediate host. These feature gains this parasite a large effective population size with wide genetic variability [5,24]. In accordance with this situation on the characteristics of *H. contortus*, genetic variability based on mt-COI phylogeny among the Kayseri isolates and the other isolates from Pakistan, USA, Brazil and Australia was also found high with a mean rate of 5.5%. The mt-COI sequence analyses also indicates that *H. contortus* Kayseri isolates are genetically

more close to the Pakistan isolates than the isolates from USA, Brazil and Australia which might be attributed to geographical proximity of the Pakistan than the others.

BZs have been widely used as main drugs for the last five decades to control the trichostrongylid nematodes in different hosts and showed anthelmintic activity over β-tubulin, by leading to interference in microtubule polymerization dynamics. The wide usage of BZs has leaded to co-evolution of resistant parasite alleles across worldwide [1,25]. BZ resistance in *H. contortus* populations has been well explored and is a model for the studies on population genetics of anthelmintic resistance due to the availability of molecular tools and increasing knowledge of its genetics and population biology [26,27]. In general *in vivo* and *in vitro* methods such as fecal egg count reduction test (FECRT) and the egg hatch assay (EHA) has been widely used for BZ resistance in trichostrongylids [28]. However these techniques are insufficient for measuring the level of resistant and sensitive alleles within the population and also *in vivo* tests are rather slow and expensive. Thus, in recent years several rapid, reliable and sensitive molecular tools including real time PCR in order to explore resistant alleles have been developed [8,18,29]. The current study provides the first molecular data and allele frequencies on the BZ resistance in *H. contortus* populations from sheep in Turkey by utilizing highly sensitive qPCR assay targeting the β-tubulin isotype 1 gene as also indicated by several researchers [8,18,29]. A mean resistant allele frequency of over 12.0% determined with this study indicates an increasing risk for sustainably control of the parasites in the region in which the commercial deworming agents have been only choice for struggling with trichostrongylid nematodes.

Three non-synonymous SNPs in the isotype-1 β-tubulin gene have been described associated with benzimidazole resistance in *H. contortus* until today. A point mutation in codon 200 (TTC to TAC), causing a phenylalanine to tyrosine substitution, is the most common SNP and often at high frequency in several countries [1,26,27,30]. While the SNPs at codons 167 (TTC to TAC) and 198 (GAA to GCA) are less common and have been reported in a number of different countries [1,11,20,27,31]. In the present study sequence analyses of the isolates which had an over 39.0% resistant allele frequency revealed the existence of only the widest SNP at codon 200 in *H. contortus* populations in the study area which is in agreement with above studies [1,26,27,30]. However further studies should be conducted on large scale isolates from different regions in Turkey in order to explore true picture of the benzimidazole resistance in *H. contortus* populations from livestock which is essential for guiding and establishing effective control strategies.

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Kafkas Univ Vet Fak Derg
22 (1): 101-107, 2016
DOI: 10.9775/kvfd.2015.13975

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

Research Article

The Effects of Different Antioxidants on Post-thaw Microscopic and Oxidative Stress Parameters in the Cryopreservation of Brown-Swiss Bull Semen [1] [2]

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Article Code: KVFD-2015-13975 Received: 07.07.2015 Accepted: 05.08.2015 Published Online: 05.08.2015

Abstract

The aim of this study was to research the effects of four antioxidants that had been added to semen diluents, on spermatological parameters, anti-oxidant enzymes activities and DNA integrity after the freeze-thaw procedures in Brown-Swiss bull semen. A total of 24 ejaculates were collected from three bulls. Each ejaculate was divided into five equal parts and they diluted to the Tris + 25 mM trehalose base extender containing fetuin 1 mg/ml, dithioerithritol 1 mM, cysteamine 4 mM or linoleic acid 0.5 ml/50 ml, and no additives (control). Group dithioerithritol and linoleic acid showed the higher rates of CASA progressive motility, CASA sperm motility ($P<0.05$) compared with the other groups. Group linoleic acid provided the better protective effect for acrosome and total abnormalities ($P<0.01$, $P<0.05$; respectively). Also, group dithioerithritol showed the highest values for the HOS test ($P<0.01$). In the comet test, group dithioerithritol enhanced tail with lesser chromatin damage than the other groups ($P<0.05$). Supplementation of dithioerithritol significantly affected the GSH activity ($P<0.01$). Also groups dithioerithritol and linoleic acid gave higher CAT values than the other groups ($P<0.05$). Additionally, supplementation of fetuin and cysteamine showed the lowest total antioxidant activity value ($P<0.05$). In conclusion, we may said that, the addition of antioxidants, specially dithioerithritol and linoleic acid, to added with 25 mM trehalose Tris extender improve post-thaw sperm parameters.

Keywords: *Antioxidants, Catalase, Glutathione peroxidase, Spermatological charecteristics, Bovine*

Brown-Swiss Boğa Spermasının Dondurulmasında Çözündürme Sonrası Mikroskopik ve Oksidatif Stress Parametreleri Üzerine Farklı Antioksidanların Etkileri

Özet

Bu çalışmanın amacı Brown-Swiss boğa spermasında dondurma çözündürme işlemleri sonrasında, spermatolojik parametreleri, antioksidant enzim aktiviteleri ve DNA bütünlüğü üzerine sperma sulandırıcısına eklenen dört antioksidanın etkisini araştırmaktı. Üç boğadan toplam yirmidört ejakülat toplandı. Her bir ejakülat beş eşit kısma ayrıldı ve 25 mM trehaloz katılmış Tris temel sulandırıcısı içerisinde, fetuin 1 mg/ml, dithioerithritol 1 mM, sisteamin 4 mM veya linoleik asit 0.5 ml/50 ml, ve antioksidan içermeyen (kontrol) ile sulandırıldı. Grup dithioerithritol ve L diğer gruplarla kıyaslandığında daha yüksek CASA progressif motilite ve CASA sperma motilitesi gösterdi ($P<0.05$). Grup linoleik asit akrozom ve toplam abnormalitede daha iyi koruyucu etki sağladı (sırasıyla $P<0.01$, $P<0.05$). Keza, grup dithioerithritol, HOS test için en yüksek değeri verdi ($P<0.01$). Comet testte göre, dithioerithritol grubunda kuyruk kromatin hasarı diğer gruplara göre daha azdı ($P<0.05$). Dithioerithritol eklenmesi GSH aktivitesini önemli derecede etkiledi ($P<0.01$). Keza, dithioerithritol ve linoleik asit grupları diğer gruplara göre daha yüksek CAT değeri verdi ($P<0.05$). Buna ek olarak fetuin ve sisteamin eklenmesi endüşük total antioksidan aktivite değeri gösterdi ($P<0.05$). Sonuç olarak, 25 mM trehaloz eklenmiş Tris sulandırıcısına eklenen antioksidanların, özellikle de dithioerithritol ve linoleik asit'in, çözüm sonu sperma parametrelerini artırdığı söyleyebilir.

Anahtar sözcükler: *Antioksidanlar, Katalaz, Glutatyon peroksidaz, Spermatolojik özellikler, Boğa*

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INTRODUCTION

Animal breeding industry has been extensively used frozen bull semen in artificial insemination (AI). Achievement of AI is contingent upon the quality and quantity of fresh semen and its capacity for dilution and storage with minimum deprivation of fertilizing capacity [1]. Freezing-thawing processes conduct to the production of reactive oxygen substances (ROS) that weaken post-thaw motility, viability, intracellular enzymatic activity, fertility and sperm functions [2,3]. Spermatozoa consist of high concentrations of polyunsaturated fatty acids (PUFA) in the sperm plasma membrane exposed to peroxidation, and consequently are extremely responsive to lipid peroxidation (LPO), which leads to a following loss of motility, membrane integrity, fertilizing capability and metabolic changes of sperm cells [4,5]. When frozen semen is used for AI, spermatozoa are exposed to oxygen and light radiation which could irreversibly influence sperm functions. Under these conditions, it is declared that addition of antioxidants increased the post-thaw motility, viability, membrane integrity and fertility of boar [6], bull [1], ram [7] and goat [8] sperm cells. Oxidative stress is the result of an extreme production of ROS and/or diminishes in the antioxidant defence system, and these oxidative stress targets lipids, proteins and DNA [9]. Fetuin, which is a micro heterogeneous protein, appears in fetal calf serum and a protease inhibitor, and has been shown to inhibit zona pellucida hardening during the *in vitro* maturation of equine oocytes [10]. In additionally, fetuin is a commercially available protein, which improves sperm motility [11]. Dithiothreitol is known as an antioxidant and it decreases protamine disulfide bond [12]. It avoids the oxidation of sulfhydryl groups, and it has a mucolytic effect on mucoprotein disulfide bonds, which may likely damage the frozen membranes [13]. Deshpande and Kehrer [14] revealed that dithiothreitol provides a defensive effect against apoptosis and oxidative damage. It is declared that cysteamine stimulates glutathione (GSH) synthesis during the *in vitro* maturation of ovine oocytes, promoting embryonic growth [8]. Additionally, it improves post-thaw motility, reduces total abnormality rates and advances the antioxidant capacity of goat sperm [15]. Takahashi et al.[16] revealed that low molecular weight thiol compounds such as mercaptoethanol (P3-ME) and cysteamine supported to cell viability. Limited number of studies on the supplementation of unsaturated fatty acids for sperm production or cryopreservation provided positive findings. Fair et al.[17] stated that dietary addition of wide range of PUFA complements has shown to modify the sperm fatty acid profile. There are contradictory results about the effects of using PUFA on fresh and frozen-thawed sperm. Some authors suggest that supplementation of fatty acids on the preservation of sperm function after long-term liquid storage or freezing needs to be addressed [18].

The composition of the extender is highly important for semen cryopreservation. Sugar is utilized by spermatozoa as an energy source to support sperm motility and movement [19]. Trehalose is a non-reducing disaccharide which is able to protect the integrity of cells against a variety of environmental stresses such as dehydration, heat, cold and oxidation [20]. The extender containing trehalose improved antioxidant action and reduced the oxidative stress induced by cryopreservation [21]. So, we have added in 25 mM trehalose to tris extender. The novelty of this study was to evaluate the effects of four antioxidants added to this modified tris extender prior to cryopreservation on post-thawing microscopic sperm parameters (motility, acrosome and total abnormalities, HOS test), antioxidant activities (GPx, LPO, GSH, CAT and Total antioxidant activity) and DNA integrity of frozen-thawed Brown-Swiss bull semen.

MATERIAL and METHODS

Chemicals

All chemicals used in this study were obtained from Sigma-Aldrich Chemical Co. (Interlab Ltd., Ankara, Turkey).

Animals, Semen Collection and Semen Processing

The research materials belong to the Lalahan Livestock Central Research Institute (Ankara, Turkey) and they were fed under the same conditions. A total of 24 ejaculates were obtained from the three Brown-Swiss bulls (between 3-4 years of age) with the aid of an artificial vagina twice a week, according to AI standard procedures. Immediately after collection, the initial semen characteristics (ejaculate volume, sperm motility and concentration) were microscopically evaluated using routine laboratory procedures and methods described by Tuncer et al.[1]. The ejaculates comprising sperm cells which had more than 80% forward progressive motility and concentrations higher than 1.0 x 10^9 spermatozoa/ml were used in the study. The ejaculates were pooled in a warm water bath at 35°C until they were evaluated in the laboratory. A modified Tris-based extender (T) (189.5 mM of Tris, 63.2 mM of citric acid, 55.5 mM of fructose, 25 mM trehalose, 20% egg yolk (v/v) and 1000 ml of distilled water at a pH of 6.8) was used as diluents. Each ejaculate was divided into five equal parts. After that, they diluted to a final concentration of 60 x 10^6 ml^{-1} spermatozoa with the Tris + 25 mM trehalose base extender containing fetuin 1 mg/ml, dithioerithritol 1 mM, cysteamine 4 mM or linoleic acid 0.5 ml/50 ml, and no additives (control). Diluted semen samples cooled down to 4°C in 4 h and afterwards they were inserted in a digital freezing machine (Digitcool 5300 ZB 250; IMV) and were frozen at a programmed rate of 3°C/min from +4 to -10°C; 40°C/min from -10 to -100°C; and 20°C/min from -100 to -140°C. Subsequently the straws were put into liquid nitrogen. The straws were stored in liquid nitrogen at least 24 h and thawed in a water bath (37°C) during 20 s.

The experimental procedures were approved by the Animal Care Committee of Istanbul University, Faculty of Veterinary Medicine (number and date: 2006/172 and September 27, 2006).

Assessment of Sperm Quality

A computer-assisted sperm motility analysis (CASA; IVOS version 12; Hamilton-Thorne Biosciences, Beverly, MA, USA) was also used to analyse sperm motion characteristics. The method was described by Büyükleblebici et al.[22]. We used accurate identification of sperm is easy with use of the IDENT Stain - a specialized, DNA-specific, fluorescent dye that it is CASA analysis to avoid the effects of egg-yolk particles on sperm discernibility. This method was described in Hamilton Thorne website [23]. For the evaluation of sperm abnormalities, at least three drops of each sample were placed into Eppendorf tubes containing 1 ml Hancock solution [24]. One drop of this mixing was placed on a slide and covered with a coverslip. The percentages of total sperm abnormalities (acrosome and other abnormalities) were ascertained by counting a total of 200 spermatozoa under phase-contrast microscope (x1000, oil immersion, Olympus BX43, Tokyo, Japan). The integrity of the sperm membrane tail was evaluated using the hypo-osmotic swelling test (HOS test). A hypotonic solution containing fructose and sodium citrate was prepared with an osmolality of 100 mOsm/kg. A 30 µl of prewarmed (37°C) semen was mixed with 300 µl of the hypotonic solution and incubated at 37°C for 60 min. Smears were prepared from the incubated semen sample; a minimum of two hundred cells were counted under phase-contrast microscope (x400, Olympus BX43) [25]. The swelling is characterized by a coiled tail, indicating that the plasma membrane is intact. Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay, which was performed at high alkaline conditions. The method described by Tuncer et al.[26]. The images of 100 randomly chosen nuclei were examined using a fluorescent microscope at a magnification of 400x (Zeiss, Germany). The percentage of the total DNA in the comet tail was taken as a measure of DNA break frequency. Tail DNA (%) was assessed in 100 cells by using Comet Assay III image analysis system (Perceptive Instruments, UK). Analysis was done blindly by one slide reader.

Biochemical Assays

Semen samples were thawed in 37°C water for 20 s and they were centrifuged at 4°C at 1.000 x g for 15 min in order to separate spermatozoa. Pellet was washed 3 times with a 0.5 ml of PBS. This final solution was homogenized 5 times by sonication in cold for 15 s for the Lipid Peroxidation Analysis (LPO), 120 µl of homogenate was mixed with 10 µl 0.5 mM butyl hydroxyl toluene (BHT) and kept in –80°C until the analysis. The rest of the homogenate was centrifuged at 8.000 x g for 15 min and the supernatant was separated and kept in –80°C for the other enzyme analysis. The levels of lipid peroxidase (LPO) were measured with the commercial LPO-586TM Oxis research kit, glutathione peroxidase (GPx) levels with GPx-340TM Oxis research kit, superoxide dismutase with Sigma–Aldrich Fluka FL 19160 kit, catalase (CAT) with Oxisresearch TM Catalase-520TM kit, GSH (glutathione) with Oxisresearch-420TM kit and antioxidant capacity with Sigma-Aldrich Antioxidant assay CS 0790 kit with spectrophotometric analysis. The assessment of sperm biochemical assays was investigated using spectrophotometric analysis by the method of previously described by Taşdemir et al.[27].

Statistical Analysis

The study was replicated eight times. The results were expressed as mean ± SEM. Data set is normally distributed using the Shapiro Wilk normality test. Homogeneity of variances with Levene's test groups was compared. The test revealed that the variances were homogeneous. After that, comparisons between the groups were made variance with Duncan post hoc test using the SPSS/PC computer programme (version 14.1, Chicago, IL). The $P<0.05$ value was considered as significant.

RESULTS

The effects on the spermatological parameters of different antioxidant additives in the cryopreservation of Brown-Swiss bull semen are presented in *Table 1*, group dithioerithritol and linoleic acid resulted the higher rates of CASA progressive motility (21.88±1.29% and 22.75±1.35%; $P<0.05$), CASA sperm motility (47.25±2.58% and 49.75±2.80%; $P<0.05$), respectively compared to the other groups. Group linoleic acid provided the better protective effect for acrosome ($P<0.01$) and total abnormalities ($P<0.05$). Also, group dithioerithritol showed the highest values for the HOST (49.88±0.35%; $P<0.01$). In the comet test, group dithioerithritol enhanced tail length; group fetuin, dithioerithritol and linoleic acid also enhanced tail movement intensity and tail moment with lesser chromatin damage than the other groups ($P<0.05$).

As set out in *Table 2*, when fetuin or cysteamine was added to the extender, sperm motion characteristics such as VAP ($P<0.05$), VSL ($P<0.05$) and VCL ($P<0.01$) had significantly decreased. No significant differences were observed among the groups for ALH and STR values ($P>0.05$).

As shown in *Table 3*, there is no significant differences observed in the level of GPx and LPO activities among the groups ($P>0.05$). Supplementation of dithioerithritol significantly affected the GSH activity ($P<0.01$). Also dithioerithritol and linoleic acid groups gave higher CAT values than other groups ($P<0.05$). Additionally, supplementation of fetuin and Cys showed the lowest total antioxidant activity value ($P<0.05$).

Table 1. *Mean (±SE) sperm values in frozen thawed bull semen*
Tablo 1. *Dondurulmuş çözdürülmüş boğa spermasında ortalama spermatolojik değerler*

Groups	C	F (1 mg/ml)	D (1 mM)	Cys (4 mM)	L (0.5 ml/50) ml	P
Progressive Motility (%)	18.88±1.04^{b}	8.00±0.70^{c}	21.88±1.29^{a}	13.00±3.26^{c}	22.75±1.35^{a}	*
Motility (%)	40.00±2.28^{b}	34.50±4.52^{c}	47.25±2.58^{a}	29.38±4.92^{c}	49.75±2.80^{a}	*
Acrosome (%)	5.38±0.32^{c}	6.50±0.54^{d}	4.25±0.25^{b}	4.00±0.10^{b}	2.63±0.18^{a}	**
Total Abnormality (%)	16.50±0.33^{c}	15.13±0.97^{c}	14.38±1.07^{b}	15.50±0.82^{c}	12.63±0.37^{a}	*
HOST (%)	40.25±0.37^{c}	32.75±0.68^{d}	49.88±0.35^{a}	38.88±0.35^{c}	45.63±0.60^{b}	**
Tail length	74.93±7.28^{c}	55.31±6.28^{b}	48.07±9.24^{a}	73.73±8.85^{c}	62.33±5.90^{b}	*
Tail intensity (%)	19.06±2.24^{b}	14.86±2.76^{a}	13.27±0.66^{a}	18.22±2.34^{b}	14.56±0.65^{a}	*
Tail moment (µm/s)	8.29±2.14^{b}	4.77±2.00^{a}	3.70±0.57^{a}	8.37±1.99^{b}	4.86±0.77^{a}	*

*$^{a, b, c, d}$ Different superscripts within the same row demonstrate significant differences (** P<0.01, * P<0.05)*
C: *Control,* ***F:*** *Fetuin,* ***D:*** *Dithioerithritol,* ***Cys:*** *Cysteamine,* ***L:*** *Linoleic acid*

Table 2. *Mean (±SEM) CASA sperm motion charecteristics in frozen–thawed bull semen*
Tablo 2. *Dondurulmuş çözdürülmüş boğa spermasında ortalama CASA sperm hareket özellikleri*

Groups	C	F (1 mg/ml)	D (1 mM)	Cys (4 mM)	L (0.5 ml/50) ml	P
VAP (µm/sec)	99.63±2.34^{a}	85.83±5.15^{b}	99.00±2.20^{a}	77.00±4.81^{c}	98.31±3.14^{a}	*
VSL (µm/sec)	77.50±1.65^{a}	60.25±1.42^{b}	77.88±1.42^{a}	63.25±3.14^{b}	75.91±1.96^{a}	*
VCL (µm/sec)	155.63±4.49^{a}	143.75±8.82^{b}	158.25±3.53^{a}	108.25±8.10^{c}	157.71±6.07^{a}	**
ALH (µm)	6.50±0.27	8.88±6.31	6.63±0.18	5.75±0.25	6.86±0.18	-
BCF (Hz)	10.00±0.46^{c}	13.25±1.11^{a}	12.25±0.31^{abc}	12.63±1.69^{abc}	11.64±0.96^{abc}	*
STR	67.63±1.02	68.38±1.96	69.63±8.82	76.50±2.69	76.88±1.01	-
LIN (%)	51.63±1.19^{a}	42.75±1.07^{b}	51.00±0.54^{a}	49.75±2.45^{a}	50.00±1.09^{a}	*
Elongation	39.00±0.71^{b}	45.13±2.51^{a}	38.38±0.94^{b}	38.38±0.65^{b}	38.38±0.75^{b}	*

*$^{a, b, c}$ Different superscripts within the same row demonstrate significant differences (** P<0.01, * P<0.05)*
C: *Control,* ***F:*** *Fetuin,* ***D:*** *Dithioerithritol,* ***Cys:*** *Cysteamine,* ***L:*** *Linoleic acid*

Table 3. *Mean (±SE) glutathione peroxidase (GPx), lipid peroxidase (LPO), reduced glutathione (GSH), catalase (CAT) and total antioxidant activities in frozen thawed bull semen*
Tablo 3. *Dondurulmuş çözdürülmüş boğa spermasında ortalama glutatyon peroksidaz (GPx), lipit peroksidaz (LPO), redükte glutatyon (GSH), katalaz (CAT) ve total antioksidan değerleri*

Analysis	C	F (1 mg/ml)	D (1 mM)	Cys (4 mM)	L (0.5 ml/50) ml	P
GPx (mU/ml-10^{9} cell/ml)	9.99±0.09^{a}	10.00±0.10^{a}	13.00±0.19^{b}	12.00±0.60^{b}	13.00±0.65^{b}	*
LPO (µm/ml-10^{9} cell/ml)	0.50±0.27	0.75±0.37	0.63±0.32	0.13±0.13	0.48±0.22	-
GSH (µm/ml-10^{9} cell/ml)	15.75±0.88^{d}	20.13±2.84^{bc}	36.63±11.87^{a}	19.25±1.33^{bc}	24.33±4.97^{b}	**
CAT (µm/ml-10^{9} cell/ml)	8.88±2.82^{b}	2.13±0.61^{c}	11.50±5.14^{a}	2.50±0.65^{c}	11.92±3.05^{a}	*
Total antioxidant activities (mmol/ trilox/ml-10^{9} cell/ ml)	10.25±1.74^{bc}	6.63±1.41^{bc}	18.50±3.52^{a}	8.13±1.54^{bc}	13.88±2.75^{ab}	*

*$^{a, b, c, d}$ Different superscripts within the same row demonstrate significant differences (** P<0.01, * P<0.05*
C: *Control,* ***F:*** *Fetuin,* ***D:*** *Dithioerithritol,* ***Cys:*** *Cysteamine,* ***L:*** *Linoleic acid*

DISCUSSION

Cryopreservation procedures lead to cold shock, ice crystal formation, oxidative stress, osmotic changes and lipid-protein reorganisations within the cell membrane, which affect normal sperm functions and result in loss of motility, viability, fertilising ability, deterioration of acrosome and plasma membrane integrity and structural damage to DNA [2,28]. The sperm cryopreservation procedure, which involves the decrement in temperature, cause oxidative stress and impress the sperm surface. This results in irreversible damage to the sperm organelles and modifies in enzymatic activity, related with a decrease in sperm motility, membrane integrity and fertilizing ability [29]. In the current study, addition of different doses of antioxidants significantly increased the percentages of post-thaw sperm progressive and CASA motilities, except for fetuin and cysteamine. Contradict with our results; Başpınar et

al.[30] reported that when dithioerithritol was suplemented to semen extender, it did not produce a beneficial effect on sperm motility. Moreover, according to Çoyan et al.[31], the supplementation of dithioerithritol did not improve bull sperm motility during liquid storage. Its declared that, dithioerithritol had no significant improvement in motility characteristics, and CASA and progressive motilities of bovine sperm [32]. After freeze-thawing process, motility analysis did not give enough data for the evaluation of sperm. Sperm morphology has major importance for fertilizing capacity and the assessment of membrane functions. Non-motile sperm would have an intact plasmalemma and acrosome and morphology integrity [7]. In our study, acrosome abnormalities decreased in the presence of antioxidants in the extender, except for fetuin. Total abnormalities also decreased in the presence of dithioerithritol and linoleic acid. These results were in agreement with those reported by some researchers, suggesting that antioxidants could decrease the sperm acrosome and total abnormalities for bovine [27,32,33] and for goat [26]. The sperm plasma membrane is sensible to peroxidative damage with accompanying loss of membrane integrity, reduced sperm motility, and finally loss in fertility [34]. According to Büyükleblebici et al.[35], addition of 25 mM trehalose beneficially effected acrosome morphology and total abnormalities in Tris extenders. This study was assumed to determine which antioxidants, would afford the most effective protection against membrane damage during the freeze–thawing process. The results presented clearly showed that dithioerithritol and linoleic acid provided the strongest protective effect against cryodamage. Bucak et al.[36] showed that supplementing antioxidant to the diluent had positive effect on plasma membrane integrity. In contrast with our study, addition of 5 mM dithioerithritol did not have any positive effect on acrosome and total abnormality and plasma membrane integrity [22]. The COMET assay is a widely applied technique for measuring and analysing DNA breakage in individual cells [37]. In this study, by the addition of the antioxidants fetuin, dithioerithritol and linoleic acid, DNA integrity was well-kept, compared to the control group. These results were consistent to those reported in a previous research on bovine sperm [32]. On the other hand, the antioxidants which were used had no effects on upgrading at DNA integrity [27]. Adding cysteamine to the diluent had cryo-protective effect on goat [15] and ram [38] semen collected by artificial vagina. However, according to another study, there were no favorable effects of cysteamine on ram semen quality before or after freezing in which sperm was collected by electro-ejaculator [39]. On the basis of our results, we may hypothesize that the differences among our results may be associated with the types and amounts of antioxidants that were used. Several studies have been reported contradict with our findings. In terms of post-thaw sperm motion kinematic characteristics (VAP, VSL, VCL, ALH and BCF), our results were in accord with those reported by Taşdemir et al.[37] who demonstrated that using ethylene glycol and dimethyl sulfoxide as cryoprotectant instead of glycerol did not give marked effect on sperm motion kinematic characteristics. In contrast with our study, Tris-based extender containing sucrose reduced sperm motion kinematic parameters [40]. Based on our findings, we may hypothesize that these differences among the studies may be due to the turbidity of other substances in Tris based extender.

Oxidative stress commonly causes deprivation of motility, enlargement and the blebbing of the acrosomal membrane and disruption or gained permeability of the plasma membrane of spermatozoa [41]. Morphologic damage of the plasma membrane increases the susceptibility to LPO when high ROS was produced during the freeze-thawing process. This was declared for boar [6], buck [26], ram [30] and bull [37] sperm. When cells are frozen, they are subjected to various stresses such as cold shock and oxidative stress that arise through ice crystallization and LPO due to membrane changes [26]. Sperm cells and seminal plasma contain ROS scavengers, including the enzymes SOD, GSH-PX and catalase, which convert superoxide (O_2^-) and peroxide (H_2O_2) radicals into O_2 and H_2O. SOD and catalase also transfer (O_2^-) generated by NADPH-oxidase in living cells [42]. However, GSH-Px removes peroxyl radicals from different peroxides, including H_2O_2, to better sperm motility [43]. GPx is a selenocysteine containing antioxidant enzyme that participates in a role in the elimination of hydrogen peroxide and is also recognized to be involved in the detoxification of reactive lipids [8]. In the current study, supplementation of antioxidants had better GPx activity except for fetuin. According to Sarıözkan et al.[44], addition of 2.5 and 7.5 mM of cysteamine to the sperm diluent procured a higher rise in GPx antioxidant enzyme activities. It's declared that, addition of 2 mM dithioerithritol improved GSH and GPx activity [31]. In the present study, the supplementation of antioxidants elevated GSH levels after thawing. These results were consistent with the study of Bucak et al.[45], which they researched ram sperm in which an increase of total GSH level was reported in the presence of GSH for the frozen state or during storage. They were also discrepant with the studies in goat [8] and ram [38] sperm, in which total GSH levels were not elevated when, had been cryopreserved or stored with various antioxidants. Additionally, De Matos et al.[46], revealed that the supplementation of cysteamine in the maturation media concluded with elevate in GSH activity.

In conclusion, Group dithioerithritol and linoleic acid have result the higher rates of CASA progressive motility and CASA sperm motility. Group linoleic acid provided the better protective effect for acrosome and total abnormalities. Also, group dithioerithritol showed the highest values for the HOST. In the comet test, group dithio-erithritol enhanced tail length; group fetuin, dithioerithritol and linoleic acid also enhanced tail movement intensity and tail moment with lesser chromatin damage than

the other groups. Supplementation of dithioerithritol significantly affected the GSH activity. Also dithioerithritol and linoleic acid groups gave higher CAT values than the other groups. We may said that, the addition of antioxidants, specially dithioerithritol and linoleic acid, to added with 25 mM trehalose Tris extender improve post-thaw sperm parameters.

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Kafkas Univ Vet Fak Derg
22 (1): 109-113, 2016
DOI: 10.9775/kvfd.2015.13976

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Research Article

The Effect of Low Level Green LASER on Autologous Full-Thickness Free Skin Graft in Rats

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KVFD-2015-13976 Received: 03.07.2015 Accepted: 16.09.2015 Published Online: 29.09.2015

Abstract

The effect of green LED laser on free full-thickness autologous skin graft was tested in rats. Ten adult male Wistar rats were divided into two groups of in each group as test (Low level green LED laser irradiation) and the control group. A full-thickness square incision with the sides of three centimeters was created at the dorsal region of animals. Then, the skin was removed and replaced with a rotation of 90 degrees and sutured. Green LED irradiation (1 J/cm^2, 60 s, and 532 nm) was performed immediately in the test group daily over a period of 6 days post-operatively. Biopsy taking was carried out on 3, 5, 7 and 14 days after surgery and was sent to the pathology laboratory. The size of graft was recorded on determined days. The results showed that the laser with the amount of has ability to maintain tissue autograft in the surgical site. Macroscopic and microscopic studies showed that the graft tissue in the control group showed significantly (P>0.05) shrinkage and fibrosis.

Keywords: *Autologous, Full-thickness graft, Low level green LED LASER, Rat, Skin*

Sıçanlarda Tam-Katmanlı Otolog Serbest Deri Greftine Düşük Seviye Yeşil LAZER'in Etkisi

Özet

Tam katmanlı otolog deri grefti üzerine yeşil LED lazerin etkisi sıçanlarda test edildi. On adet yetişkin erkek Wistar sıçan düşük seviye yeşil LED lazer radyasyon grubu ve kontrol grubu olmak üzere iki gruba ayrıldı. Tüm kalınlıkta kenarları 3 cm olan bir kare insizyon hayvanların dorsal kısmında oluşturuldu. Sonrasında, deri uzaklaştırıldı ve 90 derece döndürülerek yerine tekrar dikildi. Yeşil LED radyasyonu (1 J/cm^2, 60 s, ve 532 nm) operasyon sonrası 6 gün boyunca her gün test grubuna uygulandı. Biyopsiler cerrahi işlemin 3, 5, 7 ve 14. günlerde alınarak patoloji laboratuarına gönderildi. Greftin boyutu aynı günlerde kaydedildi. Sonuçlar lazerin cerrahi müdahale bölgesinde doku otogreftini sağladığını gösterdi. Makroskopik ve mikroskopik çalışmalar kontrol grubunda greft dokusunun anlamlı derecede (P>0.05) çekme ve fibrozise uğradığını ortaya koydu.

Anahtar sözcükler: *Otolog, Tüm kalınlıklı greft, Düşük doz yeşil LED LAZER, Sıçan, Deri*

INTRODUCTION

Skin is the largest apparatus that its function is protection body from mechanical damage and chemical exposure. This organ experiences most damage such as loss of and regeneration throughout its life. Surgical treatment and plastic surgery has been used to resolve the defect and restoration its function and is the choice treatment. Besides acceptable results of each method, they are not free of complication. For many decades, researchers had various trail to achieve good results in resolving severe and large skin injury. As previously mentioned, the use of skin flap and free graft have a positive role in acceptable appearance. The two common main postoperatively complications are ischemia and necrosis for reason such unsuitable bed and incorrect prearation of graft [1,2]. Seroma formation (SF) and shrinkage of transplant tissue are the major surgical complications specifically on non-fresh recipient bed [1-4]. One of ways to bypass these likely post-operatively complications is trying to prepared meshed full-thickness and partial thickness skin grafts and placed on the bed with the aim of removing the negative role of

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SF between bed and grafts and help adhere graft on bed. More attempts in solving SF are stab incision in the center of skin graft. The scar has the lack acceptable appearance [1]. Another problem in the use of full-thickness skin graft is shrinkage and size reduction in the wound healing process which is highly inappropriate and unacceptable effect on aesthetic plastic surgery [2].

Positive role of laser with wavelengths lower than 800 nm on wound healing and acceleration of repair phase in animal model has been claimed by many investigators, but the effect of laser on autologous skin graft has not been studied so far. The stimulation of wound healing, anti-inflammatory and activation of vasodilation effects of low level laser known well in recent years [5]. Researcher of the present study profit by laser properties to investigate the role of green LED light laser radiation on autologous-graft in rat.

MATERIAL and METHODS

The study was approved by the Ethics Committee on Animal Research of the number of Ethics IAEC 3-12/5.

Ten male Wistar rats with the average weighting 430±25 g in the same life-style conditions of temperature, humidity, water and food were randomly divided into two groups of five animals tested (green LED laser) and control (no green LED laser radiation) and placed in separate cages. Rats were anesthetized by a combination of ketamine hydrochloride (75 mg/kg) (Alfasan, Woerden-Holland) and Medetomidine hydrochloride (10 mg/kg) (Syva, Avda-Spain) intraperitoneally. After clipping, surgical scrub and preparation of the surgical area, the rats were laid on sternal recumbency. After drawing a square with the sides of 3 cm by using sterile pen and ruler, the full-thickness incision was created using a sterile scalpel # 11 *(Fig. 1)*. The skin was separated from the bed by dissecting with Metzenbaum scissor and it was placed on the 90-degree rotation. It was sutured by simple suture pattern with nylon number 4-0 *(Fig. 1)*. All animals received a daily dose of Enrofloxacin (5 mg/kg, SC)(Hipra, Avda-Spain) as an antibiotic and Meloxicam (2 mg/kg, SC)(Razak, Karaj-Iran) as an analgesic subcutaneously for 3 days, postoperatively.

In the test group, green LED light (1 J/cm^2, 60 s, and 532 nm) Mustang 2000 (Russia) laser radiation was considered immediately post-operatively daily over a period of 6 days. Radiation pattern was circular from the edges of the autologous skin into the center *(Fig. 1)*. In control group, animals received no radiation. Under anesthetic and aseptic condition, biopsy samples taken from the center of each side of the transplanted tissue as a 0.4´ 0.8 cm rectangle shape on the days 3, 5, 7 and 14 after surgery in both control and experimental groups *(Fig. 1)*. The samples were fixed in 10% buffered formalin and prepared for microscopic evaluation. All four sides of the square were measured by ruler on days 3, 5, 7 and 14 and recorded in the respective tables to compare the macroscopic changes between two groups. At the end of the study, none of the rats were euthanized.

Statistical Analysis

Macroscopical study of graft: The statistical results of macroscopic changes according to the normal distribution and homogeneity of variance data was evaluated by non-parametric Mann-whitney U test with SPSS software version 22. The statistical analysis was performed between two groups in 5% significance level.

Histopathological study of graft: The statistical results of histopathology were performed with non-parametric Mann-whitney U test to comparison between groups using SPSS version 22.

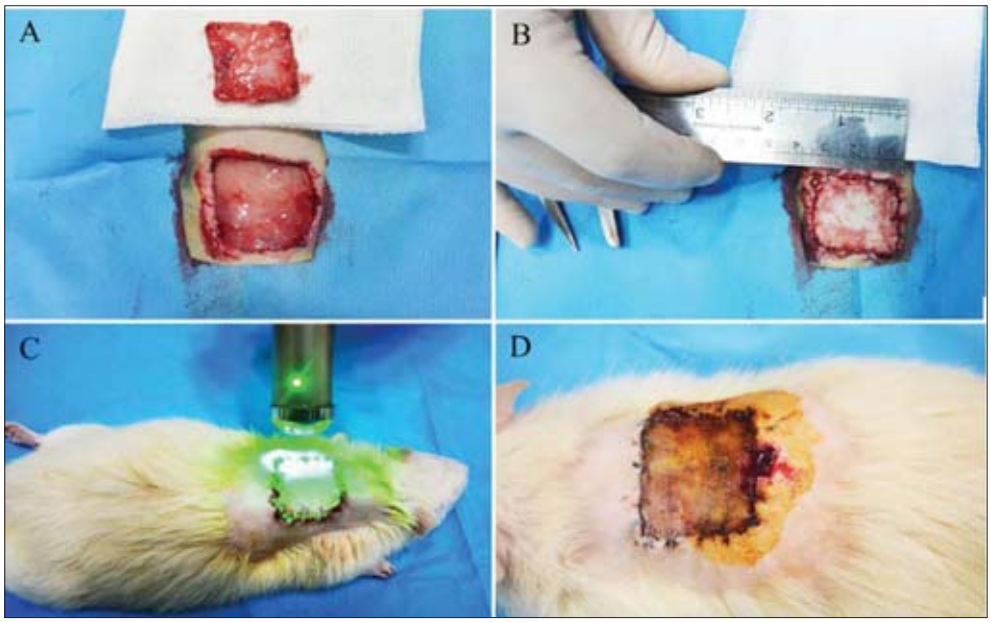

Fig 1. A- The full-thickness incision and removed skin; **B-** Skin suturing; **C-** Laser radiation; **D-** Sample biopsy

Şekil 1. A- Tüm katmanlı insizyon ve uzaklaştırılmış deri; **B-** Deri dikişi; **C-** Lazer radyasyonu; **D-** Örnek biyopsi

RESULTS

Macroscopical Results of Transplantation

Based on clinical and macroscopic evaluations, SF was not observed at the distance between the bed and graft. Transplantation area was calculated in the day of 3, 5, 7 and 14 in all rats in two groups. The difference of transplant sizes of day (3 and 5), (3 and 7), (3, 14) and (5 and 7) were analyzed. So that the difference in days of (3, 14), and (5, 7) after transplantation was significant between the groups (P =0.05). But the transplant level between days (3, 5) and (3, 7) did not show significant difference (P>0.05).

Histopathological Results of Transplantation

According to the reference table [6] and our pathological study, the amount of migration and thickness of keratinized layer at days 3 and 5 in the control group and at day 3 in test group were observed less than 50%. However, the migration of the tissue at days 5, 7 and 14 increased in the comparison with the control group but there was no statistically significant difference in the analysis (P>0.05). Infiltration of inflammatory cells on days 3 and 5 were reported average (between 50 and 75%) but at the end of the study on day 7 and 14 reduced to 25%. The rate of infiltration substantially on days 5, 7 and 14 had increased (more than 75%). Fibroblast was observed mild (surrounding tissue) on day 3, and in the granulation tissue on days 5 and 7 which increased in the control group on day 14. The presence of fibroblasts in granulation tissue was the same in the test group as compared to the control group on day 3. Despite of increasing of fibroblast on days 5, 7 and 14, the difference was not significant between two groups (P>0.05). Vascular regeneration increased on days 5, 7 and 14 in the test group and only on day 14 in the control group and was not significant (P>0.05). The amount of collagen maturation increased in the test group to control group on day 14, although the difference was not significant *(Fig. 2)*. Statistical analysis of pathological finding revealed that green LED lasers on fibroblast migration (P=0.06) and keratinized layer (P=0.07) not significant in the comparison to the control group, although it was very close to the significance level *(Fig. 3)*.

DISCUSSION

The healing process of large defect is very problematic and clinicians and researchers have paid attention to manage of this old and powerful competitor in plastic surgery field. This defect threats people and animals life and mortality rate is high in who suffer from the defect. Hence, for many years from past to now, the majority of researches have concentrated in solving and bypassing the problem in their investigations. In this way, using of full-thickness autografts are as one of the most practical and choice method of treatment of these lesions that accelerates healing of lesions and makes appropriate and acceptable appearance. Some factors such as SF affected on tissue adhesion and graft separation is predictable [1]. Surgeons used some methods like stab incision in the center of transplantation to diminish such post-operation complication of free skin graft. Formation of seroma is not only problem in the use of full-thickness and partial thickness skin graft, fibrosis and shrinkage of skin on its bed are other skin problems in literature [1]. So, seeking the ancillary method to optimize the result is important.

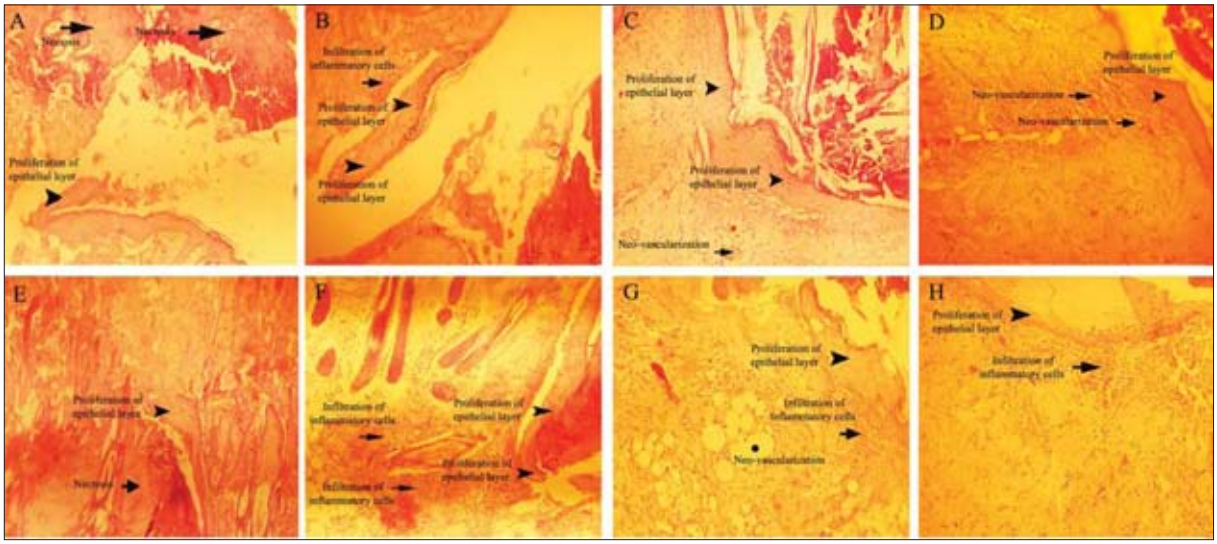

Fig 2. H&E staining of control group and test group. Representative histology from biopsy in days 3, 5, 7 and 14 after surgery. **A-** Day 3, control group, **B-** Day 5, control group, **C-** Day 7, control group, **D-** Day 14, control group, **E-** Day 3, test group, **F-** Day 5, test group, **G-** Day 7, test group, **H-** Day 14, test group (A-H ×10)

Şekil 2. Kontrol ve test gruplarında H&E boyanması. Cerrahi sonrası 3, 5, 7 ve 14. günlerde biyopsi örneklerinin histolojik görüntüsü. **A-** 3. gün, kontrol grubu, **B-** 5. gün, kontrol grubu, **C-** 7. gün, kontrol grubu, **D-**14. gün, kontrol grubu, **E-** 3. gün, test grubu, **F-** 5. gün, test grubu, **G-** 7. gün, test grubu, **H-** 14. gün, test grubu (A-H ×10)

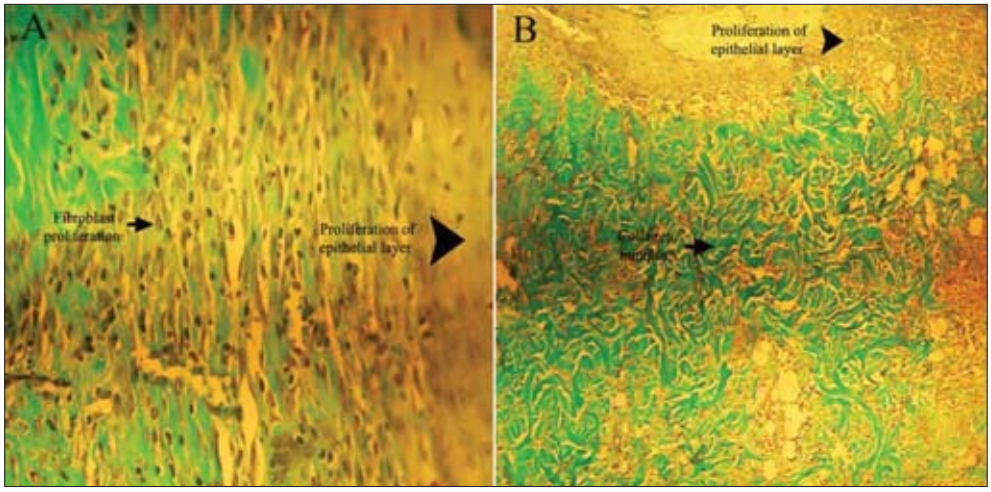

Fig 3. Trichrome staining of control group and test group. Representative histology from biopsy in day 14 after surgery. **A-** Day 14, control group, **B-** Day 14, test group (A, B ×10)

Şekil 3. Kontrol ve test gruplarında Trikrom boyanması. Cerrahi sonrası 14. günde biyopsilerin histolojik görüntüsü. **A-** 14. gün, kontrol grubu, **B-** 14. gün, test grubu (A, B ×10)

As noted earlier, laser it was used in the present study to preserve, survive and taking free skin autograft in its bed with the aim of finding a way in using full-thickness skin graft with green LED laser treatment. Tissue contamination and infection are two causes of SF and separate graft from its bed. Absence of SF between the bed and transplant tissue in present study in both control and test groups, skin graft was taken to the bed. In many patients of medical and veterinary hospital especially in burns, bed discharge and contamination following the necrotic tissue are as a barrier in connection of graft to bed [1,2]. In laser studies and its properties on wound healing, both factors including wave length and density are important role. The changes of these factors will have different results in prescribed time of laser. Shrinkage of wound with presence of granulation tissue and fibrosis has two edges condition of wound and scar tissue formation [1,2]. Many studies have focused on the role of laser on the fate of the transplanted skin. Machneva et al.[7] were reported 33% reduction in the size of the wound and preservation of transplant tissue with low level green LED laser in comparison with 39% reduction with low level red laser [7]. The authors of present study showed the role of green LED laser in prevention of wound size reduction and maintaining size of graft was significant which agrees with research of Machneva et al.[7] result. That also found that increasing the density twice of their previous study caused 20 percent reduction in wound and transplant tissue size on day 15 after transplantation and no further reduce in course of wound healing process that verified our present study.

Rodrigo et al.[8] have shown that using of infrared laser with wave length of 850 nm, have significant influence on increasing the chance of transplant acceptance on day 3 and however, its effects decreased in proceeding until day 14 compared to day 3 that agrees with Fekrazad et al.[5] results. Walsh et al.[9] showed that the amount of vascular regeneration and facilitating infiltration of white blood cells to the wound increases at a wave length of 830 nm [9]. The results of this investigation, contrary to research of Moore et al.[10] which reported that the wave length of 810 nm is an inhibitory role in proliferation of fibroblasts. They showed the effect of wave length of 665-675 nm as proliferative stimulator [10]. Another study by Nasirian et al.[11] showed that laser with a wave length of 630 nm and 0.5 J/cm^2 power can be significantly increased vascular regeneration process, the proliferation of fibroblasts and collagen compared to control group. The researchers of the study also showed that the infrared laser with the wave length of 850 nm and 0.5 J/cm^2 power have significant difference in comparison with wave length of 630 nm and 0.5 J/cm^2 power in terms of all parameters [11]. The researchers of current study revealed that green LED laser with a wave length of 532 nm increased the vascular regeneration, inflammatory cell infiltration, amount of keratinized layer formation and its thickness, fibroblast migration, amount of collagen and its maturation rate compared to the control group, but this increase is not significant. All data agrees to Fekrazad et al.[5] study. Another study was conducted by Fattahian et al.[12] showed that infrared laser with wave length of 850 nm and 1 J/cm^2 power was more effective compared to red laser with wave length of 635 nm and 1 J/cm^2 power on all mentioned factors such as new vascularization, proliferation of fibroblasts, production of collagen and keratinized layer in epithelial tissue [12], which agree with the results of present study and using of green LED laser with wave length of 532 nm and 1 J/cm^2 power. Our study cleared laser that suitable macroscopic and microscopic changes after full-thickness autologous transplantation.

Based on our study, it can be mentioned that green LED light laser therapy besides having significant prevention in wound and maintenance of transplant tissue size. Significant increase in new vascularization, proliferation of fibroblasts, collagen production, leukocyte migration and generation of keratinized layer in epithelial tissue were seen.

Therefore, it could be claimed that low level laser is likely practical solution in elimination of post-operative complication such as shrinkage and size reduction and viability and preserving of skin on bed in rat model.

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Kafkas Univ Vet Fak Derg
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DOI: 10.9775/kvfd.2015.13998

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Research Article

Xylazine-Ketamine Anesthesia Following Premedication of New Zealand White Rabbits with Vitamin C

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Article Code: KVFD-2015-13998 Received: 06.07.2015 Accepted: 24.08.2015 Published Online: 24.08.2015

Abstract

This study was aimed out to evaluate the effects of vitamin C premedication on xylazine-ketamine anesthesia in New Zealand white rabbits. Twelve New Zealand white rabbits were divided into two groups (Group XK and Group VCXK) that each had 3 males and 3 females. The animals in group XK were treated with 10 mg/kg Xylazine HCl and 50 mg/kg Ketamine HCl. The animals in group VCXK were pre-treated with 60 mg/kg Vitamin C prior to xylazine-ketamine combination. The onset of anesthesia, the length of surgical anesthesia and the recovery time were recorded. Body temperature, heart and respiratory rates were recorded at 0, 5, 10, 15, 30, 45, 60 min. In XK group onset of action and length of surgical anesthesia were 5.81±0.19 min and 55.00±2.81 min, respectively. Premedication of rabbits with 60 mg/kg vitamin C resulted in significant decrease in the onset of action (3.93±0.13 min) and increase in the duration of xylazine-ketamine anesthesia (77.50±6.15 min). The body temperature change between treatment groups were significant at 30, 45 and 60 min. This study shows that vitamin C administration prior to xylazine-ketamine anesthesia in New Zealand white rabbits decreases the time for onset of action and prolonged the length of surgical anesthesia.

Keywords: *Xylazine, Ketamine, Anesthesia, Vitamin C, Recovery, New Zealand white rabbit*

Beyaz Yeni Zelanda Tavşanlarında Vitamin C Premedikasyonu Sonrasında Ksilazin-Ketamin Anestezisi

Özet

Bu çalışma, beyaz Yeni Zelanda tavşanlarında xylazine-ketamine anestezisi üzerine vitamin C premedikasyonunun etkilerini değerlendirmeyi amaçladı. On-iki beyaz Yeni Zelenda tavşanı her grupta 3 erkek ve 3 dişi olacak şekilde iki gruba (Grup XK ve Grup VCXK) ayrıldı. Grup XK'daki hayvanlara 10 mg/kg Xylazine HCl ve 50 mg/kg Ketamin HCl uygulandı. Grup VCXK'daki hayvanlara Xylazine-Ketamine kombinasyonu öncesinde, 60 mg/kg Vitamin C verildi. Anestezi başlangıcı, cerrahi anestezi süresi ve uyanma zamanı kaydedildi. Vücut sıcaklığı, kalp frekansı ve solunum oranları 0, 5, 10, 15, 30, 45 ve 60. dakikalarda kaydedildi. XK grubunda anestezi başlangıcı ve cerrahi anestezi süresi sırasıyla 5.81±0.19 dk ve 55.00±2.81 dk'idi. Tavşanların 60 mg/kg vitamin C ile premedikasyonu anestezi başlangıcında önemli bir azalma (3.93±0.13 dk) ve ksilazin-ketamin anestezi süresinde artış (77.50±6.15 dk) ile sonuçlandı. Gruplar arasında vücut sıcaklığı değişimi 30, 45 ve 60. dakikalarda anlamlıydı. Bu çalışma beyaz Yeni Zelanda tavşanlarında ksilazin-ketamin anestezisi öncesinde vitamin C uygulamasının anesteziye giriş süresini kısalttığını ve cerrahi anestezi süresini uzattığını göstermektedir.

Anahtar sözcükler: *Xylazine, Ketamine, Anestezi, Vitamin C, Rekover, Beyaz Yeni Zelanda tavşanı*

INTRODUCTION

Anesthesia is loss of the sensations which allows medical and surgical procedures to be undertaken without causing discomfort. Premedication prior to the anesthesia provides reduction of anxiety, pain and the dosage needed for anesthetic agents [1]. Xylazine is one of the most commonly used premedicant drug in veterinary medicine. It is a sedative, analgesic, muscle relaxant and has been used safely with other drugs for anesthesia [2-4].

Ketamine is a drug in a group of cyclohexylamines and it is routinely used for induction and maintenance of anaesthesia. It is used in rabbits due to rapid onset

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of action with minimal respiratory and cardiovascular effects [3]. Because the sole use of ketamine is not sufficient for anaesthesia, it is commonly combinated with preanesthetic drugs such as xylazine. Xylazine and ketamine combination has been reported to be more effective in rabbits and used with wide margins of safety [4].

Vitamin C is a water-soluble vitamin that is necessary for a variety physiological reactions such as adrenaline production and collagen synthesis [5]. Previous animal studies have showed that there is a close relationship between extracellular vitamin C levels and recovery from anesthesia [6].

There are some studies in rabbits which combined vitamin C with xylazine [1] and ketamine [7] alone. Moreover, one research has been used vitamin C with xylazine and ketamine combination in rats [8]. However, to the authors' knowledge, there is no study about using vitamin C as a premedicant with xylazine-ketamine combination in New Zealand white rabbits. Based on this premise, this study was aimed to evaluate the effects of vitamin C on xylazine-ketamine anesthesia in New Zealand white rabbits.

MATERIAL and METHODS

Animals

Twelve New Zealand white rabbits, 6 males and 6 females, 8-11 months old and weighing between 2.15 and 3.50 kg, were used. The rabbits were healthy, according to physical examinations and biochemistry results within reference range for the species. The animals were divided randomly into two experimental groups (Xylazine-Ketamine: Group XK; Vitamin C-Xylazine-Ketamine: Group VCXK) that each had 3 males and 3 females. They were maintained in individual cages, where they were fed with commercial pellet food and water *ad libitum*. The rabbits were acclimatized for a period of 14 days prior to the start of the study. Animals were not fasted before anesthesia. Atatürk University Local Board of Ethics Committee for Animal Experiments has approved the study protocol of this research (HADYEK decision no: 2015/115).

Study Design

The baseline vital parameters including body temperature, respiratory rate and heart rate were taken prior to the treatment. In group XK; 10 mg/kg Xylazine HCl (2% Rompun, Bayer, Istanbul, Turkey) and 10 min later 50 mg/kg Ketamine HCl (10% Ketasol, Interhas, Richter Pharma, Austria) were administered intramuscularly. Whereas, in group VCXK; 60 mg/kg Vitamin C (20% Injacom C, Ceva, Istanbul, Turkey) was administered intramuscularly 20 min prior to xylazine-ketamine combination. All drugs were injected into the quadriceps femoris muscle. All injections were performed by the same anesthetist who was unaware of the experimental design and all recordings were taken by other researchers who were unaware of the performed injections.

The onset of anesthesia was evident by decrease respiratory rate, recumbency, loss of pedal reflexes and loss of pinprick sensation on skin. The length of surgical anesthesia was evaluated by loss of withdrawal and ear-pinch reflexes [9]. The recovery was assessed by the existence of righting reflex (able to return sternal position by own after being placed on its back) [9,10] and excitement signs (apperance of vocalization, growling, thumping and convulsion). The heart and respiratory rate values were recorded with a veterinary vital signs monitor (Cardell, 9404, Sharn Veterinary Inc, USA) and stethoscope, respectively. The body temperature was measured with a digital rectal thermometer from the rectum. All rates were recorded at preinduction (-1), immediately following induction (0 h) and at 5, 10, 15, 30, 45, 60 min. Complications such as bradypnoea, bradycardia and excessive secretion were recorded.

Statistical Analysis

The generated data were analyzed by analysis of variance (ANOVA). As the data were normally distributed, differences among mean values were treated with paired sample t-test. Statistical significance was considered at *P* value below 0.05. The results are presented as means±standard error (SE). All data were analyzed using the SPSS19 (IBM Company, Version 19.0, SPSS Inc, USA, 2010) statistical package.

RESULTS

The results of the effects of vitamin C administration on xylazine-ketamine anesthesia at different time intervals are presented in *Table 1*. There were no significant differences in heart and respiratory rates between two groups at all time intervals. The body temperature change between treatment groups were significant at 30, 45 and 60 min ($P<0.05$).

The mean heart rate change before and after xylazine-ketamine treatment showed a significant depression ($P<0.05$) at 0, 45 and 60 min. However, in VCXK group mean heart rate depression was significant at 0, 5, 10, 15, 30, 45 min. The mean respiratory rate was significantly decrease at 0, 5, 10, 15, 30, 45 min XK group. In VCXK group, significant respiratory decrease was observed at all time intervals. In XK group, there were no significant differences in body temperature at all time intervals. However, significantly increase were detected in VCXK group at all time intervals.

The effect of vitamin C on onset of action, length of surgical anesthesia and recovery time are shown in *Table 2*.

Table 1. *The mean heart rate, respiratory rate and body temperature (mean±SE) differences in XK (xylazine-ketamine) and VCXK (Vitamin C- Xylazine-ketamine) groups at different measurement times*

Tablo 1. *Farklı ölçüm zamanlarında XK (xylazine-ketamine) ve VCXK (Vitamin C- Xylazine-ketamine) gruplarındaki ortalama kalp frekansı, solunum sayısı ve vücut sıcaklığı farklılıkları (ortalama±standart hata)*

Paremeter	Groups	Measurement Time							
		-1thmin	0thmin	5thmin	10thmin	15thmin	30thmin	45thmin	60thmin
HR	XK	216.00±21.46	186.67±9.00**	192.67±24.71	184.00±24.39	186.00±20.04	183.33±31.94	171.33±24.96**	180.67±17.04**
	VCXK	216.00±21.46	186.00±12.58**	180.67±9.93**	180.67±10.55**	177.33±14.01**	183.33±12.75**	179.33±13.95**	186.33±14.50
RR	XK	144.66±36.52	46.66±10.93**	46.00±10.95**	44.00±10.43**	44.66±10.85**	57.33±12.56**	57.00±6.66**	111.00±9.27
	VCXK	146.00±26.13**	39.33±9.60**	38.00±9.38**	38.66±7.86**	39.33±9.93**	48.66±12.75**	53.33±12.56**	115.33±5.88**
BT	XK	38.93±0.51	39,38±0.29	39.25±0.34	39.20±0.47	39.08±0.45	38.92±0.53	38.75±0,65	38.75±0.65
	VCXK	38.85±0.26	39.81±0.43*	39.76±0.58*	39.71±0.59*	39.71±0.53*	39.80±0.52*	39.78±0.48*	39.86±0.39*

HR: *Heart rate,* ***RR:*** *Respiratory rate,* ***BT:*** *Body temperature,* ***-1:*** *Preinduction,* ***0:*** *Immediately following anesthesia induction, * Significantly increase (P<0.05), ** Significantly decrease (P<0.05)*

Table 2. *Effects of vitamin C on anesthesia parameters*

Tablo 2. *Vitamin C'nin anestezi parametreleri üzerine etkisi*

Groups	Onset of Action (min)	Length of Surgical Anesthesia (min)	Recovery Time (min)
XK	5.81±0.19	55.00±2.81	117.83±5.64
VCXK	3.93±0.13**	77.50±6.15*	110.50±3.93

XK: *Xykazine-ketamine;* ***VCXK:*** *Vitamin C-xylazine-ketamine; * Significantly increase (P<0.05); ** Significantly decrease (P<0.05)*

DISCUSSION

All rabbits were not experience any complications due to anesthesia. Anesthesia induction and recovery from anesthesia were smooth in both groups. The present study shows that vitamin C premedication prior to xylazine-ketamine anesthesia decreases the time needed to induce and increases duration of surgical anesthesia in New Zealand white rabbits. It has been reported in the literature that using vitamin C for premedication can accelerate the onset of action and increase the recovery time [11]. Although in this study we did not use different ratios of xylazine-ketamine combinations to detect whether the vitamin C administration could minimize the anesthetic needs, our results supported that administration of vitamin C can reduce the dose of anesthetics required for general anesthesia [7]. We hypothesized that using Vitamin C as a premedicant might accelerate the effects of xylazine and ketamine without prolonged the recovery time, which is desirable for anesthesia [12,13].

In this study, there were no significant differences in heart rates among groups, but heart rate decreased significantly in XK group at 0, 45 and 60 min, and in VCXK at all intervals except for 60 min. The current findings appear to be in agreement with Elsa and Ubandawaki [7] who reported that ketamine and vitamin C combination in rabbits cause decreasing in heart rate. The reason of this decrease is probably due to vitamin C induced central nervous system depression activity [8]. The main disadvantage of xylazine on the cardiovascular system may have contributed the decreased heart rate in anesthesia [14]. In current study, the heart rate also decreased in XK group at some time intervals. This could be the reason that although ketamine may increase the heart rate by stimulating the sympathetic activity and decreased vagal tone, xylazine overrides these effects by hypotension and decreased sympathetic and increased vagal activity [15]. Moens and Fargetton [16] also found decrease in the heart rate at 45 min in dogs. The same results in cats had been reported previously by Allen et al.[17].

Tachypnoea may observed due to xylazine administration. On the other hand, ketamine may produce mild respiratory depression [18]. A change in respiratory rate is usually an indicator to some physiologic changes [19]. In the current study, there were no significant differences in respiratory rates among groups. However, the mean respiratory rate was significantly decrease after anesthesia induction. Previous studies have reported that xylazine-ketamine anesthesia decrease the respiratory rate [20,21]. Furthermore, more recent study has showed that ketamine at 40 mg/kg in rabbits with or without vitamin C induced significant respiratory depression [7], which is similiar to our results.

Body temperature is expected to decline following the anesthesia by reduction of muscular activity and depression of thermoregulatory center [22]. In our study, however, xylazine-ketamine anesthesia, with our without vitamin C premedication, did not significantly reduce the body temperature. This is in agreement with Kul et al.[14] and Wyatt et al.[23]. On the contrary, in this study, vitamin C premedication prior to xylazine-ketamine anesthesia increased the body temperature. This increasement has

been observed in rabbits due to a possible modulating effect of vitamin C [1].

This study showed that vitamin C administration prior to xylazine-ketamine anesthesia in New Zealand white rabbits decreases the time for onset of action and prolonged the length of surgical anesthesia. Moreover, vitamin C administration did not cause any changement in recovery time. The exact mechanism of vitamin C on anesthesia will require further investigations, thus the anesthetics needed in general anesthesia could decrease and it minimizes their side effects on patients.

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Kafkas Univ Vet Fak Derg
22 (1): 119-125, 2016
DOI: 10.9775/kvfd.2015.13999

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
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Research Article

The Effect of Estrogen Treatment on Eccentric Exercise-Induced Damage in Rat Kidneys

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KVFD-2015-13999 Received: 07.07.2015 Accepted: 29.09.2015 Published Online: 01.10.2015

Abstract

The aim of this study was to examine the effects of anabolic steroids on eccentric exercise-induced kidney tissue, using biochemical and histological methods. Sprague Dawley male rats (n=36) were equally divided into the estrogen (17-β Estradiol, 10 mg/kg per day)-induced and non estrogen-induced groups. Both groups were subdivided into as rest groups (n=6), and as exposed to the eccentric exercise groups (n=12) that were also subdivided into decapitated one hour (n=6) and 48 hour (n=6) groups after eccentric exercise. Histopathological effects of estrogen treatment on eccentric exercise-induced rat kidney tissues were seen. The histopathological results revealed that hemorrhage, endothelium-dependent vasodilatations and degenerative changes in proximal tubules, glomeruli, and connective tissue in the estrogen-given and estrogen-given plus eccentric exercise groups. Distal tubule degenerations and dilatations with peritubular capillary dilatations and polymorphonuclear leucocyte (PMNL) infiltrations to connective tissue in eccentric exercise and eccentric exercise plus estrogen-given groups were detected but also it was noticeable decreases in PMNL infiltrations in estrogen-given groups. Biochemical results showed oxidative stress in experimental groups except Control group. It has been concluded that estrogen could not have been effective for preventing eccentric exercise-induced kidney damage and might have toxic effects on kidney tissues on clinical practice however anti-inflammatory effect on kidney tissue detected. It has been requested further investigation.

Keywords: *Eccentric exercise, Estrogen, Kidney, Rat*

Östrojen Tedavisinin Eksantrik Egzersiz Uygulanan Sıçan Böbrekleri Üzerinde Oluşturduğu Hasara Etkisi

Özet

Bu çalışmanın amacı, anabolik steroidlerin etkilerini eksantrik egzersiz uygulanan sıçanların böbrek dokusunda, biyokimyasal ve histolojik yöntemler kullanarak incelemektir. Sprague Dawley erkek fareler (n = 36), eşit şekilde östrojen uygulanan (17-β Estradiol, günde 10 mg/kg) ve östrojen uygulanmayan iki gruba ayrıldı. Bu iki grup da kendi aralarında eksantrik egzersiz uygulanan (n = 12) ve uygulanmayan (n = 6) olmak üzere iki alt gruba ayrıldı. Daha sonra eksantrik egzersiz uygulanan gruplarda egsersiz işleminde bir saat (n = 6) ve 48 saat (n = 6) sonra dekapite edilerek iki alt gruba ayrıldı. Östrojenin eksantrik egzersiz uygulanan sıçanların böbrek dokusunda histopatolojik etkileri olduğu görüldü. Östrojen ve östrojen+eksantrik egzersiz uygulanan grupların böbrek dokularında hemoraji, damarlarda endotel bağımlı vazodilatasyonlar ve proksimal tübüller, glomerüller ve bağ dokusunda da dejeneratif değişiklikler saptandı. Eksantrik egzersiz ve eksantrik egzersiz+östrojen uygulanan gruplarda distal tübüllerde dejenerasyonlar ve dilatasyonlar, peritübüler kapiller dilatasyonları ve bağ dokuya polimorfonükleer lökosit (PMNL) sızıntıların saptandı. Bununla birlikte östrojen uygulanan gruplarda PMNL infiltrasyonunda dikkat çekici bir azalma izlendi.. Biyokimyasal sonuçlar Kontrol grubu dışındaki diğer deneysel gruplarda oksidatif stress olduğunu gösterdi. Östrojenin eksantrik egzersize bağlı böbrek hasarı önlemede anti-inflamatuar etkisinin görülmesine rağmen yeterli derecede etkin olamayabileceği ve klinik uygulamalarda böbrek dokuları üzerinde toksik etkileri olabileceği sonucuna varılmıştır. Bu alanda ileriye yönelik daha ayrıntılı çalışmalar yapılabilir.

Anahtar sözcükler: *Böbrek, Eksantrik egzersiz, Östrojen, Sıçan*

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INTRODUCTION

Physical exercise is an activity that improves and maintains physical fitness, overall health and wellness. Regular moderate exercise has positive effects on the immune system, cardiovascular, nerve, endocrine and renal systems [1-5]. Beside this, doing too much exercise may be harmful. Without enough rest, striated or heart muscle tissue damages may occur that can cause dynamic upper respiratory tract or heart rhythm abnormalities and etc [6,7]. Otherwise, many negative effects like proteinuria, hematuria and changes on renal hemodynamics that may cause acute renal failure may occur [8,9].

Estrogens, a group of compounds, which are female sex hormones, have a major role for both menstrual and ovarian cycles. Estrogens bind to and activate the estrogen receptors (ER) which moderate the expression of many genes when they enter the cell [10-12]. The presence of the ER determines the actions of estrogens in the cell [13]. Estrogens play several important roles in protein synthesis, coagulation, lipid metabolism and structural changes like promoting development of female secondary sex characteristics, accelerating metabolism, increasing fat stores or stimulating endometrial growth [10,11,14,15]. There are also many benefits of estrogens such as preventing role in atherosclerosis via vascular protective action [14,15] and immunological role with anti-inflammatory properties providing migration of polymorphonuclear leucocytes (PMNLs) [15]. Beside this, some researches revealed that estrogen administration can cause various types of DNA damage in estrogen-responsive tissues via free radicals and lipid hydroperoxide-mediated DNA modifications [16]. Researches on the role of estradiol on eccentic exercise-induced rat kidney tissues are limited. The aim of the present study was to assess the role estrogen usage on eccentric exercise-induced rat kidney tissues.

MATERIAL and METHODS

Animals

Thirty six male, 12 weeks old Sprague Dawley rats, weighing 245±22.99 g were kept in facilities accredited by international guidelines. All experimental designs and trials were conducted with the approval of the Instutional Animal Care and Use Committee of Ataturk University (ATADEM-Approval No: B.30.2.ATA.0.23.71-514; 22.04.2011). The rats in groups of six per cage were housed in environmentally controlled room in the conditions of constant temperature/humidity with reversed a 12-hour light/dark cycles. They were allowed free access to food (AIN-93 puried rodent diet) and water. After one day of acclimatization, the animals were divided randomly into six groups, as shown in *Fig. 1*.

Drug Administration

Animals were injected daily subcutaneously with β-estradiol 3-benzoate (10 µg 0.1 ml of sunflower oil-1.100 g body wt-1) [17] for 30 consecutive days and following the final injection after 24 h the animals were acutely exercised [18,19].

Eccentric Exercise Protocol

A motorized rodent treadmill having an electric shock

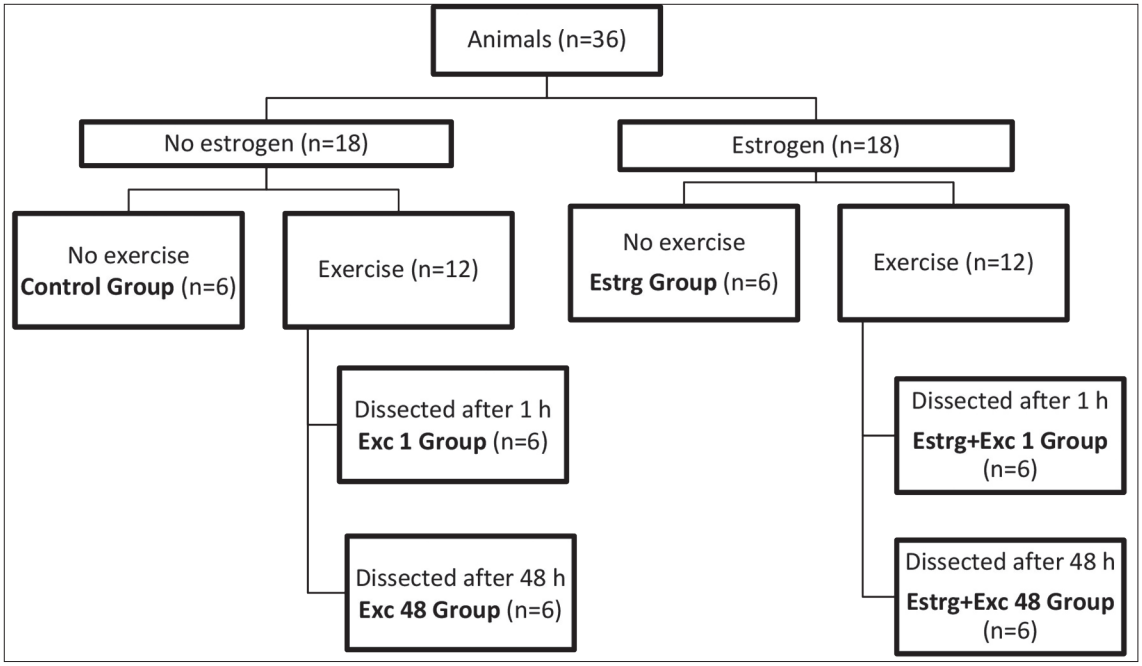

Fig 1. Experimental protocols of all groups of study
Şekil 1. Tüm gruplarda uygulanan deneysel protokoller

grid was used for modeling the acute exercise. Animals were forced to run on a 15% downhill grade at 20 m·min^{-1} speed for 90 min [20]. After 1 h or 48 h exercise management all rats were decapitated and dissected. Then their kidney tissues were removed and kept in proper conditions for histological procedures.

Biochemical Analyses

Rat kidney tissues were collected and homogenized with liquid nitrogen in a mortar and were kept at -80°C for the biochemical investigation. Measuring malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities were done weighing 15 mg kidney tissue for each group of rats and 1.5 ml of an appropriate buffer for treating. An Qiagen tissue lyser LT homogeniser was used to homogenize the mixtures on ice in eppendorf tubes with steel balls for 15 min. Then homogenates were filtered and centrifuged respectively in a refrigerated centrifuge at 4°C. The supernatants were used to evaluate MDA, SOD, CAT, GPx and GST activities. All analyses were carried out at room temperature.

MDA Level: MDA as an end outcome of lipid peroxidation that reacts with thiobarbituric acid manufacturing thiobarbituric acid reactive substance (TBARS) was measured. After the occurring of a pink chromogen reaction, MDA level can be measured at 532 nm spectrophotometrically, an MDA standard to create a standard curve against which the sample readings were plotted was used. MDA level was expressed as nmol/min/mg tissue [21].

SOD Activity: The SOD activity measurement was achieved according to the method proposed by Misra and Fridovich [22] that depends on the generation of superoxide radicals. Superoxide radicals react with nitroblue tetrazolium (NBT) to form formazan dye, produced by the xanthine and xanthine oxidase system. The SOD activities of kidney tissues were identified at 560 nm by the degree of inhibition of NBT reaction. The activity of SOD was expressed as mmol/min/mg tissue.

CAT Activity: CAT activity was defined as the amount of CAT required to decompose 1 nmole of H_2O_2 per minute, at 25°C and pH 7.8 and The kinetic analysis of the activity of CAT was started after the addition of H_2O_2 and at 240 nm the color reaction was measured [23]. CAT activity was expressed as mmol/min/mg tissue.

GPx Activity: GPx activity was measured according to the reaction between glutathione that remains after the action of 5, 5-dithiobis-(2-nitrobenzoic acid) and GPx to form a complex as IU/ g wet tissue that absorbs maximally at 412 nm [24]. The activity of GPx of 1 U/mg was defined as 1 µg of GSH consumed µmol/min/g.

GST Activity: The GST activity was assayed according to the method of Habig detected by at 340 nm spectrophotometrically by using 1-chloro-2,4-dinitrobenzene with reduced glutathione which produces a dinitrophenyl thioether and GSH level was expressed as IU/min/g [25].

Histological Analysis

The specimens were fixed in 10% buffered formalin solution for 72 h, tissue processing for the conventional light microscopic technique examination was applied. Tissues were get from water to paraffin wax and sectioned 5 µm thicknesses using a microtome (Leica RM2125RT, Germany). Sections were stained with Hematoxylin & Eosin and photographed under a light microscope with a camera attachment (Nikon Eclipse E600, Japan).

Statistical Analysis

Biochemical parameter results (MDA, SOD, and etc.) were analyzed statistically using SPSS (IBM SPSS Statistics 18.0, IBM Corporation, Somers, NY, USA). Using one-way analysis of variance (abbreviated as one-way ANOVA) followed by Duncan test (P value <0.05 was determined as significant) the numerical data of groups were analyzed. The values were stated as mean ± standard deviation.

RESULTS

Biochemical Results

Biochemical results revealed that there were significant differences between the control and the other experimental groups in the MDA levels of all groups (P<0.05) and the experimental groups did not have significant differences with each other. Control group had the lowest MDA value when compared to other experimental groups. The SOD values were found higher in Control group and lower in Estrg group than in eccentric exercise and eccentric exercise plus in estrogen-given groups. The values of CAT were found higher in Control group and lower in Exc 1 group than estrogen-given and estrogen-given plus eccentric exercise groups. There was not any significant difference in terms of GPx and GST parameters in the experimental groups *(Table 1)*.

Histopathological Results

Control Group: The medulla and cortex parenchyma and stroma were seen normal. The nephrons with Bowman's capsules and spaces, proximal and distal convoluted tubules, post tubular capillaries, Henle's loops, and collecting and papillary duct systems were seen in normal architecture. The connective tissue with its properties and the vessels' epithelium and their tunicas were determined normal *(Fig. 2)*.

Estrg Group: There were scattered hemorrhagic foci in this group. The congested veins and peritubular capillaries were conspicuous. Spaces were detected near the some hemorrhagic foci. Vasoconstrictions in some arteries

Table 1. *Statistical comparison between all studied biochemical parameters in the kidney tissues of all groups*
Tablo 1. *Tüm grupların böbrek dokularında çalışılan biyokimyasal parametrelerin istatistiki olarak karşılaştırılması*

Treatment	SOD (mmol/min/mg)	CAT (mmol/min/mg)	GST (µmol/min/g)	GPx (IU/min/g)	MDA (nmol/min/mg)
Control	881.65± 305.37^{a}	373.58±114.50^{a}	0.30±0.09^{a}	24.62±45.40^{a}	193.52± 31.96^{c}
Estrg	522.13±182.80^{c}	282.95±60.48^{b}	0.16±0.05^{a}	53.38±21.60^{a}	243.52±57.25^{b}
Exc 1	583.11±198.70^{b}	249.13±73.72^{c}	0.20±0.07^{a}	48.79±25.66^{a}	238.50±48.10^{b}
Estrg + Exc 1	563.52±306.78^{b}	312.11±104.93^{b}	0.28±0.12^{a}	52.63±25.24^{a}	482.64±127.22^{a}
Exc 48	608.60±205.85^{b}	293.21±95.45^{b}	0.24±0.17^{a}	80.61±54.46^{a}	421.88±110.99^{b}
Estrg + Exc 48	570.31±210.54^{b}	295.49±51.79^{b}	0.23±0.18^{a}	119.04±63.92^{a}	327.89±90.73^{b}

Means in the same column by the same superscript letter are not statistically significantly different under the LSD test (P<0.05). Results are mean±standard deviation of the mean

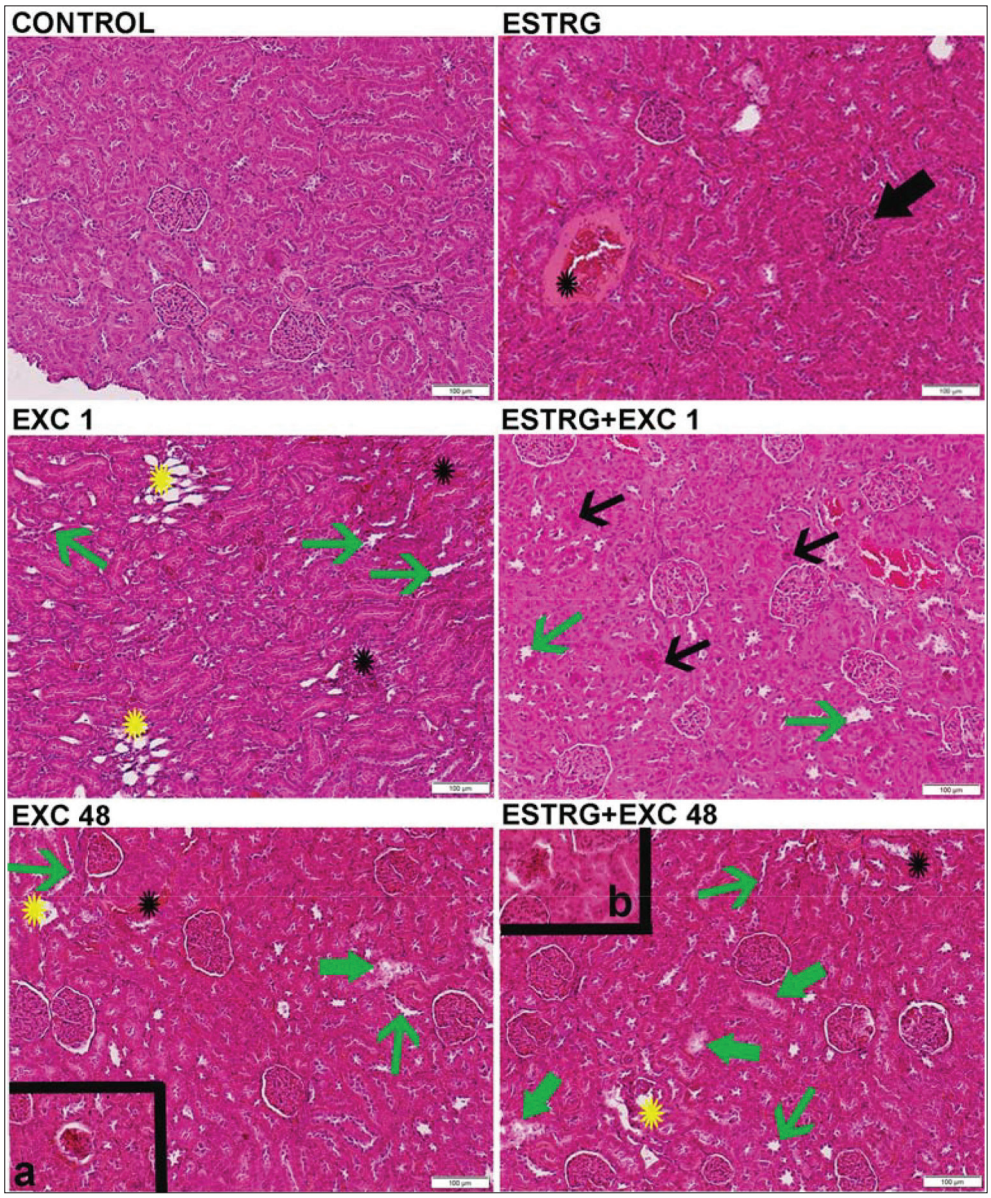

Fig 2. Micrograph of kidney tissue in lower magnification for all groups, *black star*- hemorrhage with fibrinated blood, *yellow star*- dilated peritubular capillaries, *thin green arrow*- dilated distal convoluted tubules, *thin black arrow*- degenerated proximal convoluted tubules, *thick black arrow*- narrowed bowman space that was hard to see, *thick green arrow*- degenerative areas in parenchyma, *a*- a necrotic glomerulus, *b*- a necrotic and hemorrhagic glomerulus, H&E staining

Şekil 2. Tüm gruplarda böbrek dokularının mikroskopik görünümü, *siyah yıldız*- fibrinleşme ile beraber hemoraji, *sarı yıldız*- dilate olmuş peritübüler kılcal damarlar, *ince yeşil ok*- dilate olmuş distal kıvrımlı tübüller, *ince siyah ok*- dejenere olmuş proksimal kıvrımlı tübüller, *kalın siyah ok*- daralmiş, zor görülebilen bowman boşluğu, *kalın yeşil ok*- parankimde dejeneratif alanlar, *a*- nekrotik bir glomerül, *b*- nekrotik ve hemorajik bir glomerül, H&E boyama

were distinguished and the walls of some veins were not conspicuous in which fibrinated blood was seen. Because of increasing in mesangium and dilatations and congestion in the capillaries of glomeruli, it was hard to see renal corpuscles and Bowman's spaces. Degenerative podocytes and proximal convoluted tubule cells were discriminated. The lumens of tubules were very narrow *(Fig. 2)*.

Exc 1 Group: It was very distinctive to see the increasing congestions in veins and peritubular capillaries and

degeneration areas in parenchyma. Renal corpuscles were seen with Bowman spaces. There were hemorrhagic foci like in the Estrg group, in that area, fibrinated blood was seen and there were increased fibrosis in peritubular area. There were necrotic podocytes, proximal convoluted cells and especially distal convoluted tubule cells with condensed nuclei. Distal convoluted tubule dilatations were detected, too. The most increased dilatations and disruptions in the epitheliums of peritubuler capillaries (they seem like clusters of spaces with dilated distal convoluted tubules) were distinguished in this group. PMNL infiltrations in the area of glomeruli and peritubular capillary dilatations were seen. The congestions and dilatations in the capillaries of glomeruli and increased mesangium were detected in this group, but also that was less than Group Estrg. There were degenerations in parietal layer cells and increasing in the connective tissue cells, too *(Fig. 2)*.

Estr + Exc 1 Group: There were some hemorrhagic foci like in the Estrg and Exc groups. The dilatations in distal convoluted tubule were distinctive like in the Exc 1 group but it was less. There were scattered post capillary tubules' dilatations. Renal corpuscles were seen with Bowman spaces. Degenerative cells with eosinophilic cytoplasms and condensed nuclei in proximal convoluted tubule cells were clearly seen in this group than in other experimental groups. Degenerative cells in glomeruli were discriminated. The hemorrhage in glomeruli and peritubular capillaries were less than Estrg and Exc 1 groups. Degenerative podocytes were noticeable. The congestion in and fibrosis around of vessels were seen. There was PMNL infiltration and it was less than the Exc 1 group *(Fig. 2)*.

Exc 48 Group: The Bowman spaces were seen. Many of the distal convoluted tubules were dispersed and post capillary tubules were dilated. A few necrotic glomeruli were detected differently *(Fig. 2a)* The hemorrhagic foci in this group were less and smaller than in Estrg and Esc 1 groups but also congestions and fibrinated blood were seen in some post capillary tubules. Disruptions in the epithelium of distal convoluted tubules and post capillary tubules were determined. Some degenerative areas were distinguished in parenchyma. The PMNL infiltrations in the area of glomeruli and peritubular capillary dilatations were seen like Exc 1 group *(Fig. 2)*.

Estrg + Exc 48 Group: There were increased glomerular hemorrhage and necrotic glomeruli in this group *(Fig. 2b)*. Dispersed hemorrhagic foci were seen, too. Degenerative areas in parenchyma like Exc 48 group were detected, but also decreased degenerations in tubular cells were distinguished. In addition, dilatations and fibrinated bloods in veins and post tubular capillaries were seen *(Fig. 2)*.

DISCUSSION

Regular exercise has been confirmed as a counter-measure to defend and control against cardiac injury, diabetes, mental health and etc [26-29]. On the other hand, acute, intense or prolonged exercises have been stated as a risk factor for infertility, damaging bone structure or others [30,31]. There are also many stated harmful effects of these types of exercises such as abruptly rise in blood plasma calcium concentrations, developing of fatal hyponatremia, reducing in the number of lymphocytes and suppressing the natural killer cells' functions in that immunodepression time micro-organisms can pass the first line of defence [31-33]. Variety of studies have revealed that acute physical exercise can trigger a stress response and significant pathological changes, comprising cell apoptosis in the tissues of the distant organs, such as kidney, liver and intestine as well as in working skeletal muscles. During intense exercise, some organs including kidney undergo partial ischemia that causes reducing in the blood supply, after that, tissue reoxygenation and excessive production of ROS occur [34,35]. Through the oxidative stress, apoptosis of various cell types can occur [36]. In previous studies it has been concluded that the apoptosis of the distal tubular and collecting duct cells occur in rat kidney tissues as a response to the acute exercise [37] and intense exercise cause proteinuria and biochemical changes in proximal convoluted tubules [38]. Several defence mechanisms have been demonstrated to decrease the ROS concentration, including both antioxidant enzymes (SOD, CAT, and GPx) and non-enzymatic agents (GSH) in intense exercise- induced kidney tissues [37]. Otherwise endothelium-dependent vasodilatations can occur via increasing production of oxidative stress in humans [39-41].

Estrogen is an important agent for the anti-inflammatory and antithrombotic roles. In addition, estrogen may cause vasomotor dysfunction that is a possible cause for triggering of plaque rupture [42]. It has been observed in as little as 15 min an acute improvement endothelium-dependent vasodilation occurred in vascular tissues after estrogen treatment that could be related with interaction of the classic steroid-hormone receptor [43]. Estrogen treatment has not only effects the endothelium but also involves extracellular matrix, vascular smooth muscle, and the establishment of collaterals [44,45]. Stumpf et al.[46] have detected estrogen receptors are in interstitial cells, glomeruli and proximal tubule cells in kidney tissues. Estrogen binds to specific cytoplasmic and nuclear receptors [16,47]. Faroqui et al.[48] reported that the management of estrogen to postmenopausal women is related with hypophosphatemia which is associated with a reducing in phosphate reabsorption in the proximal tubules.

In this study, the biochemical results revealed that decreases in antioxidant enzymes' activities in Exc 1 or Estrg groups. These results could be associated with acute effects of intense exercise that cause reducing in blood supply, partial ischemia, that give rise to the more production of ROS in Exc 1 group and degenerations in responsive tissues to estrogen like interstitial cells, glomeruli and proximal tubule cells via cytoplasmic or

nuclear receptors' interactions in Estrg group. The MDA levels in all experimental groups were significantly higher than in control group, and Estrg+Exc 1 group had the highest value. That could be related to the acute effect of estrogen and exercise together. In histological results in experimental groups except control group hemorrhage occurred but it was more in estrogen-given and estrogen-given plus eccentric exercise group. Hemorrhage could occur due to the antithrombotic effects of estrogen or an acute development of endothelium-dependent vasodilation and alterations in connective tissue due to the response to estrogen. The most dilatation in distal convoluted tubule neighboring peritubular capillaries dilatations that were seen like clusters of spaces and significantly degenerations in distal tubule cells occurred in the Exc 1 group. These results could be related with to the apoptosis of distal tubular cells rejection to acute exercise and the endothelium-dependent vasodilatations through the increased oxidative stress in peritubular capillaries in Exc 1. The most degenerative proximal tubules in Estrg + Exc 1 were discriminated that could be associated with estrogen receptors in proximal tubules triggering molecular alterations via oxidative stress. The PMNL infiltrations were less in Estrg + Exc 1 and Estrg + Exc 48 groups than in the Exc 1 and Exc 48 groups, respectively, and these results had accordance with SOD and CAT activities of Estrg and Exc 1 groups, respectively. Decrease in antioxidant enzymes' activities indicate oxidative stress process in which PMNL infiltration occur. These results had also revealed that the role of anti-inflammatory effect of estrogen. The other distinctive results; there were increased degenerative areas in parenchyma and necrotic renal corpuscles in Exc 48 and especially in Estrg + Exc 48 groups that showed prolonged effects of estrogen and exercise together on kidney tissues could cause damages in parenchyma tissue.

In conclusion, this current study was carried out to examine the role of estrogen on eccentic exercise-induced rat kidney tissue. The researches on this issue are limited. The results of this study indicated that estrogen (since it leads to decreases in PMNL infiltration in estrogen-given plus eccentric exercise groups) could play anti-inflammatory role on eccentric exercise-induced rat kidney tissues in short or long period. On the other hand, hemorrhage and endothelium-depended vasodilatations in estrogen given and estrogen-given plus eccentric exercise groups occurred. Dilatations of distal and peritubular capillaries in especially Exc 1 group revealed acute effect of hypoxemia via oxidative stress. Degenerations of distal and proximal convoluted tubule cells and renal corpuscles showed that prolonged effects of estrogen and exercise together on kidney. The current study's results force us to think about whether estrogen had any therapeutic effect or not on clinical practice. It has been requested further investigation to examine with other histological methods such as immunohistochemical and stereological methods and other biochemical methods such as studying related oxidative stress parameters.

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Kafkas Univ Vet Fak Derg
22 (1): 127-131, 2016
DOI: 10.9775/kvfd.2015.14029

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

Research Article

The Serological Study on Brucellosis of the Bulls and Comparison of the used Tests in the Northeast Anatolia Region of Turkey

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KVFD-2015-14029 Received: 10.07.2015 Accepted: 06.10.2015 Published Online: 07.10.2015

Abstract

The goal of this study was to use Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT) and Complement Fixation Test (CFT) to determine the prevalence of brucellosis in bulls raised in Turkey's Northeast Anatolian Region (the provinces of Kars, Ardahan and Iğdır), where bovine brucellosis is endemic and compare these tests. The study material consisted of blood samples from bulls raised on cattle farms operated by families using extensive farming methods in the provinces of Kars, Ardahan and Iğdır, where animals exhibited no clinical symptoms of disease. This is the first study to use sampling to determine the prevalence of brucellosis in bulls. Of 227 bulls that were evaluated in the study, 21 (9.25%) were found positive for RBPT, 19 (8.37%) were positive for SAT and 20 (8.81%) were positive for CFT. In animals, venereal transmission that is thought to play the least role in transmission of disease considering founction of nonspecific barrier of vagina to infection is an issue that should be taken into consideration besides ingestion of infected tissues or body fluids, contact with mucous membranes, direct inoculation, and fomites that are the most common ways in transmission of bovine brucellosis.

Keywords: *Brucellosis, Bull, Serological test, RBPT, SAT, CFT*

Türkiye'nin Kuzeydoğu Anadolu Bölgesi'nde Boğaların Brusellozisi Üzerine Serolojik Araştırma ve Kullanılan Testlerin Karşılaştırılması

Özet

Bu çalışmada ineklerde brusellozisin endemik olduğu Kuzeydoğu Anadolu Bölgesinde (Kars, Ardahan, Iğdır illeri) yetiştirilen boğalarda brusellozis prevalansının Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT) ve Complement Fixation Test (CFT) ile belirlenmesi ve bu testlerin karşılaştırılması amaçlanmıştır. Çalışmanın materyalini Kars, Ardahan ve Iğdır illerinde ailesel düzeyde ve ekstansif olarak yetiştirilen sığır işletmelerinde bulunan hastalığa ilişkin herhangi bir klinik belirti göstermeyen boğaların kan örnekleri oluşturdu. Örneklem alanında boğalarda brusellozis prevalansının belirlenmesine yönelik ilk olan bu araştırmada değerlendirilen 227 boğaya ait kan serumlarının 21'i (%9.25) RBPT, 19'u (%8.37) SAT ve 20'si (%8.81) CFT ile pozitif olarak saptanmıştır. Hayvanlarda, sığır brusellozisinin bulaşmasında en çok karşılaşılan doku ve vücut sıvılarının sindirimi, mukoz membranlarla temas, direkt inokülasyon ve fomitlerin yanı sıra vajinanın infeksiyonlara nonspesifik bariyer oluşturması göz önünde bulundurularak hastalığın aktarımında daha az rol oynadığı düşünülen veneral bulaşma da dikkate alınması gereken bir husustur.

Anahtar sözcükler: *Brusellozis, Boğa, Serolojik test, RBPT, SAT, CFT*

INTRODUCTION

Brucellosis is a disease, caused by *Brucella* species, that is characterized by abortions, infertility, joint inflammation and mastitis in cows and epididymitis, orchitis and seminal vesiculitis in bulls [1,2]. The disease is transmitted between animals via the digestive system, mating and direct contact with skin and conjunctiva, while it can spread to humans by direct contact with infected animals or the consumption of contaminated milk and milk products. For this reason, it is considered to be the most widespread zoonosis in the world. The disease is endemic in cattle populations in the Central Anatolia, Eastern Anatolia and Southeastern Anatolia regions of Turkey, which is

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geographically situated in a high risk area between Europe and the Middle East [3,4].

Although conclusive diagnosis of brucellosis requires isolation and identification of the causative agent, it is not practical for field and laboratory personnel to identify infected animals with cultural examinations when there are a large number of animals. Furthermore, these bacteria are delicate and grow slowly, and since isolating them requires a long incubation period, specific growth environments and subcultures, it may not always be possible to isolate the agent from the infected animal for a number of reasons [5]. Consequently, the control and eradication of brucellosis has been dependent on identifying reactors with serologic tests. Tests used for serological diagnosis of brucellosis include the Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT), Rivanol Agglutination Test (RAT), Complement Fixation Test (CFT), Agar Gel Immunodiffusion (AGID) and Enzyme Linked Immunosorbent Assay (ELISA) [6-11]. Serologic tests are preferable in the diagnosis of brucellosis because it is easy to collect the samples (from blood, blood serum, milk serum, vaginal flow, seminal plasma, etc.) and the results are obtained quickly and produce high specificity and sensitivity. However, the recommendation has been made to evaluate the results obtained from at least two tests when attempting serological diagnosis of brucellosis. There are a number of reasons for this, including the fact that each test identifies different immunoglobulins, false positives can be observed, chronically and actively infected animals can give inconsistent responses to serologic tests, and immunization can be confused with a natural infection [7-12]. Although RBPT is a good screening test, it is not sensitive enough to distinguish between individual cases and immunized animals. SAT is cheap and easy to administer, but does not have enough specificity. CFT is very sensitive and specific for diagnosing the disease [7,9]. It is very important for the necessary steps to be taken to control and eradicate brucellosis in the regions where it is endemic because the disease poses a threat to human and animal health [4,13].

In the Northeastern Anatolia region of Turkey, cattle farming is largely done on family farms and with extensive methods. Artificial insemination is rarely used to produce calves and instead bulls from the herd or from other herds are used in this region. The goal of this study was to use RBPT, SAT and CFT tests to determine the prevalence of brucellosis in bulls raised in Turkey's Northeast Anatolian Region (the provinces of Kars, Ardahan and Iğdır), where bovine brucellosis is endemic and compare these tests.

MATERIAL and METHODS

Serum Samples

The study material consisted of blood samples from bulls raised on cattle farms operated by families using extensive farming methods in the provinces of Kars, Ardahan and Iğdır, where the mentioned animals exhibited no clinical symptoms of disease. The samples were collected from January 2010 to March 2015 and brought by the cattle farmers to the Microbiology Department of the Veterinary Faculty of Kafkas University to be checked for brucellosis. The sera from the blood samples was extracted and stored at -20°C until they were obtained subjected to serologic testing. The samples were taken from 227 bulls in 87 farms (98 from Kars, 74 from Ardahan and 55 from Iğdır), where the bulls ranged from 1-9 years old. According to the information that was gathered, cases of abortion were occurring on 23 of the farms, while no abortion was reported on the other farms. The provinces from which the samples were obtained have been shown on the map in *Fig. 1*.

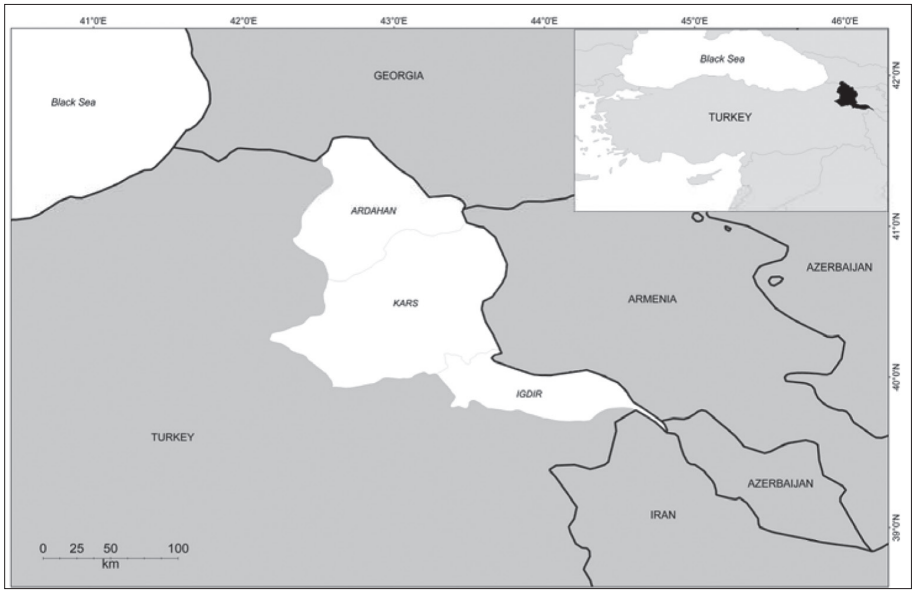

Fig 1. Provinces in the Northeast Anatolia region where the samples were obtained

Şekil 1. Kuzeydoğu Anadolu Bölgesinde örneklerin alındığı iller

Serological Tests

All serum samples were evaluated with the RBPT, SAT and CFT. In order to prevent incorrect test readings, positive and negative control serums were used that had been procured from Institut Pourquier-Montpellier (France). Antigens used for RBPT and SAT were provided by the Istanbul Pendik Veterinary Control Institute, while a commercial kit (Virion/Serion CFT Reagents, Germany) was used for CFT.

RBPT was conducted according to the procedure described by Alton et al.[12]. Equal volumes (30 µl) of serum and antigen were mixed on a clean plate and gently agitated. If agglutination occurred within 4 min, the test was considered to be positive.

For SAT, sera were double-diluted from 1/5 to 1/320 in sterile physiological salt solution. An equal amount of antigen was added to all tubes, which were shaken hard and then incubated at 37°C for 18-24 h. Agglutination that developed was evaluated at the end of this period, and sera producing a titer of 1/40 or more were considered to be positive [12,14].

CFT was conducted according to the procedure specified in a kit that was commercially obtained (Virion/Serion CFT Reagents, Germany). After the serum samples were diluted with veronal buffer, they were kept in a 62°C water bath for 30 min in order to prevent anticomplementary activity that might occur in the serum. Samples that titrated at 1/5 ++ or higher (100% inhibition of hemolysis) after these procedures were considered to be positive. In order to eliminate this characteristic of serum samples were exhibit anticomplementary activity, a 5% solution of Bovine Serum Albumin (BSA) fraction V was prepared in VB [12]. The serum dilution used in the test was mixed with the aforementioned solution (at the ratio of 0.2 ml serum + 0.6 ml solution) and incubated for 30-60 min at 37°C. The sera were then inactivated for 30 min at 62°C and the subsequent steps were followed exactly, after which they were evaluated.

RESULTS

Of 227 bulls that were evaluated in the study, 21 (9.25%) were found positive for RBPT, 19 (8.37%) were positive for SAT and 20 (8.81%) were positive for CFT. One serum sample found positive for RBPT and SAT was negative for CFT, while two samples found positive for RBPT and CFT were negative for SAT. The location in which the samples were taken, the number of samples and the results of the serologic tests have been shown in *Table 1*. Seventeen of the samples that were positive were from bulls on farms where abortions had been reported, and 16 of these 17 positive samples were positive for all three tests, while the remaining sample was positive for RBPT and CFT but negative for SAT.

DISCUSSION

Although Brucellosis has been eradicated in many developed countries in Europe as well as in countries like Australia, Canada, Israel, Japan and New Zealand, it is still one of the most significant problems in the world for animal farming and public health in Africa, Asia, Latin America, the Middle East and Mediterranean countries including Turkey [4,7]. The primary reasons that brucellosis is widespread and endemic in Turkey and in the Northeast Anatolia region in particular include the following: It is difficult to control the entry and exit of animals from the country because of its geographic location, there is a high volume of animal movement because they are on family-operated farms that use extensive farming methods, immunization programs are not fully implemented, the compensation paid for diseased animals is not sufficient or paid regularly, and calves are largely obtained by natural breeding.

Although many studies have been conducted that attempt to identify the prevalence of brucellosis in cattle in other countries, very few studies have been carried out with bulls. In a study conducted by Plant [15] that reported observations about two bulls infected with brucella, it was reported that although bull number 1 was found serologically positive for SAT and CFT from day 0 until day 141, the causative agent could not be isolated from its semen and the bull appeared clinically normal, while bull number 2 produced varying serologic results over a period of 203 days with the same tests and was found

Table 1. *The location in which the samples were taken, the number of samples and the results of the serologic tests*
Tablo 1. *Serum örneklerinin alındığı yerler, serum örneği sayısı ve serolojik test sonuçları*

Provinces	n	Serological Tests											
		RBPT				SAT				CFT			
		Positive	%	Negative	%	Positive	%	Negative	%	Positive	%	Negative	%
Kars	98	10	10.2	88	89.8	9	9.2	89	90.8	9	9.2	89	90.8
Ardahan	74	8	10.8	66	89.2	7	9.4	67	90.6	8	10.8	66	89.2
Iğdır	55	3	5.4	52	94.6	3	5.4	52	94.6	3	54.4	52	94.6
Toplam	227	21	9.3	206	90.7	19	8.4	208	91.6	20	8.8	207	91.2

negative for 74 days after that period. The researchers reported that orchitis followed by epididymitis developed in bull number 2 on day 122 and although no causative agent could be isolated from semen cultures conducted up until day 363, they isolated *B. abortus* biotype 1 from the seminal vesicle and ampulla obtained from an autopsy conducted on the animal. Based on the information they gathered, the researchers concluded that there are serious problems with diagnosing brucellosis in bulls, and that regardless of their serologic condition, all bulls in infected herds should be viewed with suspicion. Hill [16] conducted a histologic, bacteriologic and serologic investigation of 34 bulls and the serologic tests identified 17 of the 34 bulls as positive using CFT or the Indirect Hemolysis Test (IHLT). The author reported that *B. abortus* biotype 1 was isolated from various genital samples taken from 5 of the 17 bulls that were serologically positive. They reported that no causative agent could be isolated from genital samples of the remaining 12 bulls, and serologic testing of tissue fluids from these animals was also negative. Campos et al.[17] used the Huddleston and card tests to examine blood serum samples collected from 139 bulls on 60 farms. Two bulls were found positive with the Huddleston test, while the card test found all samples to be negative. The researchers reported that one of the bulls found positive was in a herd where abortions had been observed. Patel et al.[8] used RBPT and indirect-enzyme linked immunosorbent assay (i-ELISA) to investigate blood serum from 422 breeding buffalo bulls. They found 4 positive cases with RBPT and 12 positive cases with i-ELISA. The researchers found that seroprevalence can occur at different levels in studies that use various tests and that false positives and negatives can occur, so they recommend that i-ELISA be used together with other tests to identify brucellosis. Rhyan et al.[1] studied seven bison bulls that had been found serologically positive for brucellosis and isolated *B. abortus* biovar 1 from blood, lymph node, spleen and genital samples taken from six of the bulls. They reported that more studies need to be conducted to identify the role of the bulls in the spread of the causative agent.

In Turkey, brucellosis in cattle is endemic in the North-eastern Anatolian region, and many studies have discussed this situation. In a study conducted in the provinces of Kars and Ardahan, Genç et al.[18] employed Competitive Enzyme-Linked Immunosorbent Assay (C-ELISA), CFT, RBPT and SAT to evaluate the blood serum of 163 cattle that had not been immunized against brucellosis and had abortions, and found 68.1% (111), 65.6% (107), 58.9% (96) and 55.2% (90) of the animals tested positive for *B. abortus*, respectively. In a study carried out in the province of Kars, Sahin et al.[19] used RBPT, SAT and ELISA tests to investigate brucellosis in 626 blood serum samples collected between 2001 and 2006 from 27 herds with a history of abortion, finding positive results in 221 (35.30%), 206 (32.92%) and 247 (39.45%) of the samples. Otlu et al.[20] conducted a study from 2004 to 2006 in the province of Kars in which they used RBPT and SAT to examine 407 serum samples they collected from 27 cattle herds with a history of abortions and found 134 (32.92%) and 141 (34.64%) positive results, respectively. However, there are very few studies that have investigated the disease in bulls. Ours is the first study carried out in the same region as the aforementioned studies but which investigates the prevalence of brucellosis in bulls. Of 227 bulls that were evaluated in the study, 21 (9.25%) were found positive for RBPT, 19 (8.37%) were positive for SAT and 20 (8.81%) were positive for CFT. Not only do the findings obtained in this study show that brucellosis is also quite widespread among bulls, it also suggests that this plays a significant role in the spread of the disease when we consider the high degree of prevalence in the cattle. Another notable finding is that the bulls found positive did not have any clinical symptom and that they were frequently part of herds that were experiencing abortions. These data are congruent with the aforementioned studies. However, the data supports the conclusions of reports indicating that a single test is not sufficient to effectively identify brucellosis [7,21]. Furthermore, when we consider the results of the serologic tests used in our study, the data supports studies which recommend using a simple serologic test like RBPT, which offers high sensitivity and rapid screening for identifying brucellosis in a herd, in addition to a verification test with high specificity such as CFT [7,9,22].

In conclusion, this is the first study to use sampling to determine the prevalence of brucellosis in bulls found in the Northeastern Anatolia region of Turkey. Of 227 bulls that were evaluated in the study, 21 (9.25%) were found positive for RBPT, 19 (8.37%) were positive for SAT and 20 (8.81%) were positive for CFT. The findings show that the prevalence of brucellosis in bulls is quite high. In the region in question, cattle farming is largely done on family farms and with extensive methods. For this reason, it is critical that periodic serologic tests be used on bulls employed for natural breeding, whether the bulls are from the same herd or brought in from another herd. When we consider that venereal transmission is one of the least significant horizontal transmission modes, it is clear that bulls must be monitored, identified and isolated if we are to control and eradicate this disease. We concluded that the cattle farmers themselves wanted to participate in the fight against brucellosis and that because of their interest, efforts to educate the farmers were effective. Bulls that tested positive were removed from breeding in a controlled fashion according to government regulations. This prevents venereal transmission of the disease by these bulls. The role of the bulls in *Brucella* transmission in cattle farms operated by families using extensive farming methods can be questionable and monitored, if we are to control and eradicate this disease.

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Kafkas Univ Vet Fak Derg
22 (1): 133-138, 2016
DOI: 10.9775/kvfd.2015.14075

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

Research Article

Molecular Detection and Typing of Anaplasma Species in Small Ruminants in Thrace Region of Turkey [1]

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Article Code: KVFD-2015-14075 Received: 27.07.2015 Accepted: 25.08.2015 Published Online: 26.08.2015

Abstract

This study was conducted to determine the presence and distribution of *Anaplasma ovis* and *Anaplasma phagocytophilum* in small ruminants in Istanbul, Tekirdag, Edirne and Kirklareli provinces in Thrace region of northwestern Turkey during May-September in 2014. A total of 423 blood samples (216 sheep and 207 goats) were collected randomly from small ruminants regardless of the clinical symptoms. Species-specific polymerase chain reaction (PCR) assays, targeting the major surface protein 4 (msp4), were employed for identification of *A. ovis* and *A. phagocytophilum* and selected products were confirmed via sequencing. A total of 230 small ruminants (54.37%) were found to be infected with *A. ovis* and/or *A. phagocytophilum*. The rates of infected animals for *A. ovis* and *A. phagocytophilum* were 50.83% (215/423) and 8.51% (36/423) respectively. Coinfection rate in small ruminants was determined as 4.96% (21/423). Sequence diversity rates of 0-0.94% for *A. ovis* and 0.41-2.49% for *A. phagocytophilum* have been observed. This is the first detection of *A. ovis* and *A. phagocytophilum* in sheep and goats in Thrace region of northwestern Turkey via polymerase chain reaction and sequence characterization. Further researches are needed to determine the vectors, vector-host interactions and genotypic variants that may affect the presence and distribution of Anaplasma species in the region.

Keywords: *Anaplasma ovis, Anaplasma phagocytophilum, Sheep, Goat, msp4, Thrace, Turkey*

Türkiye'nin Trakya Bölgesindeki Küçük Ruminantlarda Görülen Anaplasma Türlerinin Moleküler Yöntemlerle Tespiti ve Tiplendirmesi

Özet

Bu çalışma, Türkiye'nin Trakya bölgesindeki küçük ruminantlarda *Anaplasma ovis* ve *Anaplasma phagocytophilum*'un varlığı ve dağılımını belirlemek amacı ile Mayıs-Eylül 2014 tarihleri arasında İstanbul, Tekirdağ, Edirne ve Kırklareli illerinde yürütülmüştür. Klinik semptom göstermelerine bakılmaksızın rastgele seçilen küçük ruminantlardan toplam 423 kan örneği (216 koyun ve 207 keçi) toplanmıştır. *A. ovis* ve *A. phagocytophilum* türlerinin identifikasyonu için major surface protein 4 (msp4) genini hedef alan tür-spesifik polimeraz zincir reaksiyonu (PZR) kullanılmış olup seçilen ürünler sekanslanarak doğrulanmıştır. Toplam 230 (%54.37) küçük ruminantın *A. ovis* ve/veya *A. phagocytophilum* ile enfekte olduğu bulunmuştur. *A. ovis* ve *A. phagocytophilum* yönünden pozitif hayvanların yüzdesi sırasıyla %50.83 (215/423) ve %8.51 (36/423) bulunmuştur. Her iki tür için pozitif hayvanların yüzdesi %4.96 (21/423) olarak tespit edilmiştir. Sekans farklılıklıkları *A. ovis* için 0-0.94% ve *A. phagocytophilum* için 0.41-2.49% oranlarında izlenmiştir. Bu çalışma, *A. ovis* ve *A. phagocytophilum*'un Türkiye'nin Trakya bölgesindeki koyun ve keçilerde varlığı ve dağılımı üzerine polimeraz zincir reaksiyonu ve sekans karakterizasyonu ile yapılan ilk araştırmadır. Bölgedeki Anaplasma türlerinin varlık ve dağılımını etkileyebilecek vektör, vektör-konak ilişkileri ve genotipik varyantlar konusunda yeni araştırmalara ihtiyaç duyulmaktadır.

Anahtar sözcükler: *Anaplasma ovis, Anaplasma phagocytophilum, Koyun, Keçi, msp4, Trakya, Türkiye*

INTRODUCTION

The family Anaplasmataceae belongs to order Rickettsiales of class α-Proteobacteria. The genus *Anaplasma* comprises six species; *Anaplasma centrale*, *A. marginale*, *A. bovis* (formerly *Ehrlichia bovis)*, *A. ovis*, *A. phagocytophilum* (formerly *Ehrlichia equi*, *E. phagocytophila* and Human Granulocytic Ehrlichiosis [HGE] agent) and *A. platys* [1]. *Anaplasma* species are Gram negative bacteria parasitizing in the blood cells of mammals. The life cycle of *Anaplasma* include the

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reproduction stages taking place in both vector ixodid ticks and vertebrate animals [2]. Ticks belonging to the genera *Ixodes, Dermacentor, Rhipicephalus* and *Amblyomma* are the main biological vectors of *Anaplasma* species [3].

Anaplasma ovis and *A. phagocytophilum* are medically-important species, pathogenic for small ruminants [3]. *Anaplasma ovis* causes ovine anaplasmosis in small ruminants, which is associated with significant morbidity and mortality, especially in goats [4]. Moreover, *A. ovis* can cause severe clinical disease in bighorn sheep as well as predisposing animals to other pathogens [5-7]. *A. ovis* infections have been reported to be endemic world-wide including Europe [8], China [9] and United States of America [10]. *A. ovis* has been reported from Turkey's neighboring countries Greece [11], Cyprus [12] and Iran [13].

Anaplasma phagocytophilum is the causative agent of tick borne fever in ruminants and granulocytic anaplasmosis in humans, equines and canines [1,14]. *A. phagocytophilum* can cause subclinical or severe infection in sheep and it is seldom fatal unless complicated by other infections. In addition to crippling, direct and production losses, *A. phagocytophilum* can cause abortion and impaired spermatogenesis in sheep [15-17]. *A. phagocytophilum* has been reported in China [9], United States of America, Europe, Asian part of Russia and north Africa [18]. *A. phagocytophilum* has also been reported from Turkey's neighboring countries Bulgaria [19], Greece [20] and Iran [21]. In Turkey, early records of *A. ovis* have been reported in small ruminants by using direct microscopy [22,23]. *A. phagocytophilum* in sheep [24,25], in goats [25], in ixodid ticks [26-28], in cattle [24,29,30] and in dogs [31] as well as *A. ovis* in sheep [25], in goats [25] and in ixodid ticks [28,32] have been reported by nucleic acid detection in various regions of Turkey.

Morphological and serological techniques are not reliable to differentiate *Anaplasma* and *Ehrlichia* species due to morphological similarities and antigenic cross reactions between species [33]. Detection of the bacterial nucleic acids via polymerase chain reaction (PCR) provide tools with high sensitivity and specificity and thus, are widely used in definitive diagnosis of *Anaplasma* species. These techniques also have the advantages of detecting the positive hosts in the early acute phase of the infection as well as the carrier stages [34,35].

This study was undertaken to investigate the presence and the distribution of *Anaplasma ovis* and *A. phagocytophilum* in sheep and goats in Thrace region by species-specific PCRs, where no previous information on *Anaplasma* is available.

MATERIAL and METHODS

Research Area and Sample Collection

The study was conducted between May and September 2014 in four representative provinces (Istanbul, Tekirdag, Edirne and Kirklareli) in Thrace region of northwestern Turkey.

Totally 423 blood samples (216 sheep and 207 goats) were collected randomly from 2-4 aged small ruminants regardless of showing any clinical symptoms. Ten ml blood sample was collected in tubes containing ethylene diamine tetra acetic acid (EDTA) in (K2E BD Vacutainer®) from each individual and transferred to laboratory in cold chain. Blood samples were stored in -20°C until DNA extraction.

PCR and Sequencing

Total genomic DNA extraction was performed by using a commercial kit (High Pure® PCR Template Preparation Kit Roche Diagnostics GMBH) according to the manufacturer's instructions.

For the identification of *A. ovis*, species-specific primer sets AovisMSP4Fw (5′-TGAAGGGAGCGGGGTCATGGG-3′) forward and AovisMSP4Rev (5′-GAGTAATTGCAGCCAGGG ACTCT-3′) reverse were used for amplification of the *A. ovis* major surface protein (msp4) gene 347-bp coding region [36]. For the identification of *A. phagocytophilum*, species-specific primer sets MAP4AP5 (5′-ATGAATTACA GAGAATTGCTTGTAGG-3′) forward and MSP4AP3 (5′-TTAAT TGAAAGCAAATCTTGCTCCTATG-3′) reverse were used for amplification of the *A. phagocytophilum* msp4 gene 849-bp coding region [37].

Protocols described by Torina et al.[36] and de la Fuente et al.[37] were optimized for PCR amplifications. The final PCR conditions were established as: reaction buffer 1x, 0.4 µM of each primer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 1.25 U of Taq DNA Polymerase (ThermoScientific, Waltham, M.A.). PCR reactions were performed in an automated PCR thermal cycler (Axygen, Corning, N.Y.). For *A. ovis*, the thermal profiles for PCR were optimized as: 2 min at 94°C for denaturation followed by 35 cycles with denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The final extension step was 5 min at 72°C. For *A. phagocytophilum*, 15 min at 95°C for denaturation followed by 40 cycles with denaturation at 94°C for 30 sec, annealing at 54°C for 45 sec and extension at 72°C for 1 min. The final extension step was 7 min at 72°C. The programs were terminated by storing the reaction mixtures at 4°C. PCR products were visualized via observtaion under UV light in a 1.5% agarose gel containing 0.1 µg/ml ethidium bromide.

For the confirmation of positive PCR results, randomly-selected PCR products for *A. ovis* and *A. phagocytophilum*, were cleaned up using High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany), and were sequenced via sense and antisense primers. employing an ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA, USA). Obtained sequences were handled using CLC

Main Workbench v7.5.2 (CLCBio, Aarhus, Denmark) and by MEGA software v5.2 [38].

Statistical Analysis

Chi square test was used for statistical analysis by SPSS v13 and P<0.05 was accepted statistically significant.

RESULTS

Among 423 small ruminants examined, 230 (54.37%) were found infected with *A. ovis* and/or *A. phagocytophilum*. The percentages of positive animals for *A. ovis* and *A. phagocytophilum* were 50.83% (215/423) and 8.51% (36/423) respectively. Coinfection rate of *A. ovis* and *A. phagocytophilum* in small ruminants was 4.96% (21/423).

The distribution of *A. ovis* and *A.phagocytophilum* in sheep and goats according to the sampling provinces in Thrace region of northwestern Turkey and the significance level of differences among sampling provinces were presented in *Table 1*.

According to *Table 1*, the percentages of *A. ovis* in sheep and goats were 58.8% (127/216) and 42.51% (88/207) respectively, whereas the percentages of *A. phagocytophilum* in sheep and goats were 11.11% (24/216) and 5.8% (12/207) respectively. There was a statistically-significant difference among the provinces in Thrace region of Turkey for prevalence of *A. ovis* in sheep (P<0.001), *A. ovis* in goats (P=0.028), *A.phagocytophilum* in goats (P=0.015) and *A. phagocytophilum* in goats (P=0.008) *(Table 1).*

Agarose gel electrophoresis of *A. ovis* and *A. phagocytophilum* PCR products extracted from sheep and goat blood samples were demonstrated in *Fig. 1*.

Representative sequences of the msp4 gene were obtained for *A. ovis* and *A. phagocytophilum* and submitted to GenBank (accession no. KT251211 for *A. ovis* and

Table 1. *The distribution of A. ovis and A. phagocytophilum in sheep and goats by sampling provinces in Thrace region of northwestern Turkey and the significance level of differences among sampling provinces*

Tablo 1. *Türkiye'nin Trakya bölgesindeki koyun ve keçilerde A. ovis ve A. phagocytophilum'un örneklem illerine göre dağılımı ve örneklem illeri arasındaki farklılık dereceleri*

Parasite (Host)	Total		Sampling Provinces								P Value
			Istanbul		Tekirdag		Edirne		Kirklareli		
	n	%	n	%	n	%	n	%	n	%	
A. ovis, (Sheep)	127/216	58.8	14/52	26.92^{c}	28/50	56.0^{b}	47/62	75.81^{a}	38/52	73.08^{ab}	<0.001
A. ovis (Goat)	88/207	42.51	14/52	26.92^{b}	21/50	42.0^{ab}	24/53	45.28^{ab}	29/52	55.77^{a}	0.028
A. phagocytophilum (Sheep)	24/216	11.11	3/52	5.77^{b}	5/50	10.0^{ab}	4/62	6.45^{b}	12/52	23.08^{a}	0.015
A. phagocytophilum (Goat)	12/207	5.8	1/52	1.92^{b}	1/50	2.0^{b}	2/53	3.77^{b}	8/52	15.38^{a}	0.008

a, b, c, *Differences among percentages represented with different letters in the same line is significant (P<0.05)*

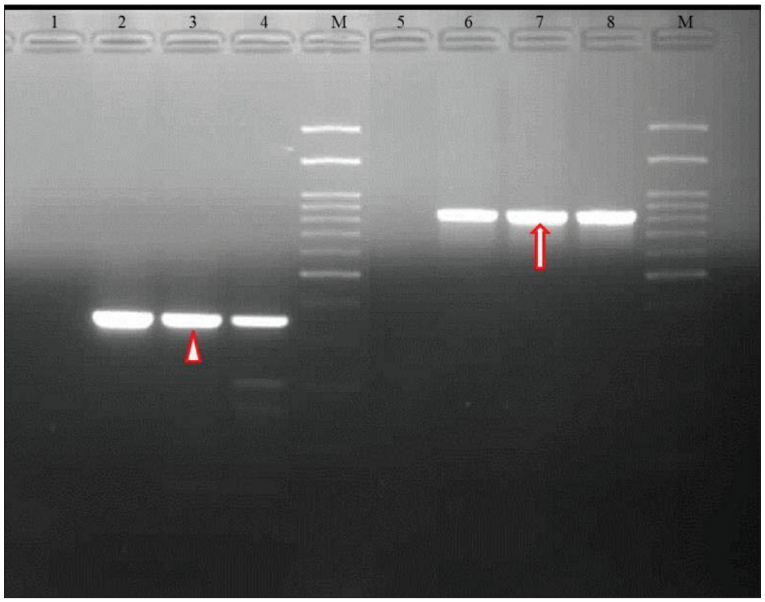

Fig 1. Agarose gel electrophoresis of *A. ovis* and *A. phagocytophilum* PCR products extracted from sheep and goat blood samples. Arrowhead indicates the position of 347-bp and arrow indicates the position of 849-bp PCR products. Lines: M: 100-bp DNA marker; 1: *A. ovis* negative control (PCR-grade water); 2: *A. ovis* DNA extracted from sheep; 3: *A. ovis* DNA extracted from goat; 4: *A. ovis* positive control; 5: *A. phagocytophilum* negative control (PCR-grade water); 6: *A. phagocytophilum* DNA extracted from sheep; 7: *A. phagocytophilum* DNA extracted from goat; 8: *A. phagocytophilum* positive control

Şekil 1. Koyun ve keçi kan örneklerinden elde edilen *A. ovis* ve *A. phagocytophilum*'a ait PCR ürünlerinin agaroz jel elektroforezi. Ok ucu 347-bp ve ok 849-bp PCR ürünlerini göstermektedir. Sıralar: M: 100-bp DNA işaretleyicisi; 1: *A. ovis* negatif kontrol (PCR-kalite su); 2: Koyun kan örneği *A. ovis* DNAsı; 3: Keçi kan örneği *A. ovis* DNAsı; 4: *A. ovis* pozitif kontrol; 5: *A. phagocytophilum* negatif kontrol (PCR-kalite su); 6: Koyun kanörneği *A. phagocytophilum* DNAsı; 7: Keçi kan örneği *A. phagocytophilum* DNAsı; 8: *A. phagocytophilum* pozitif kontrol

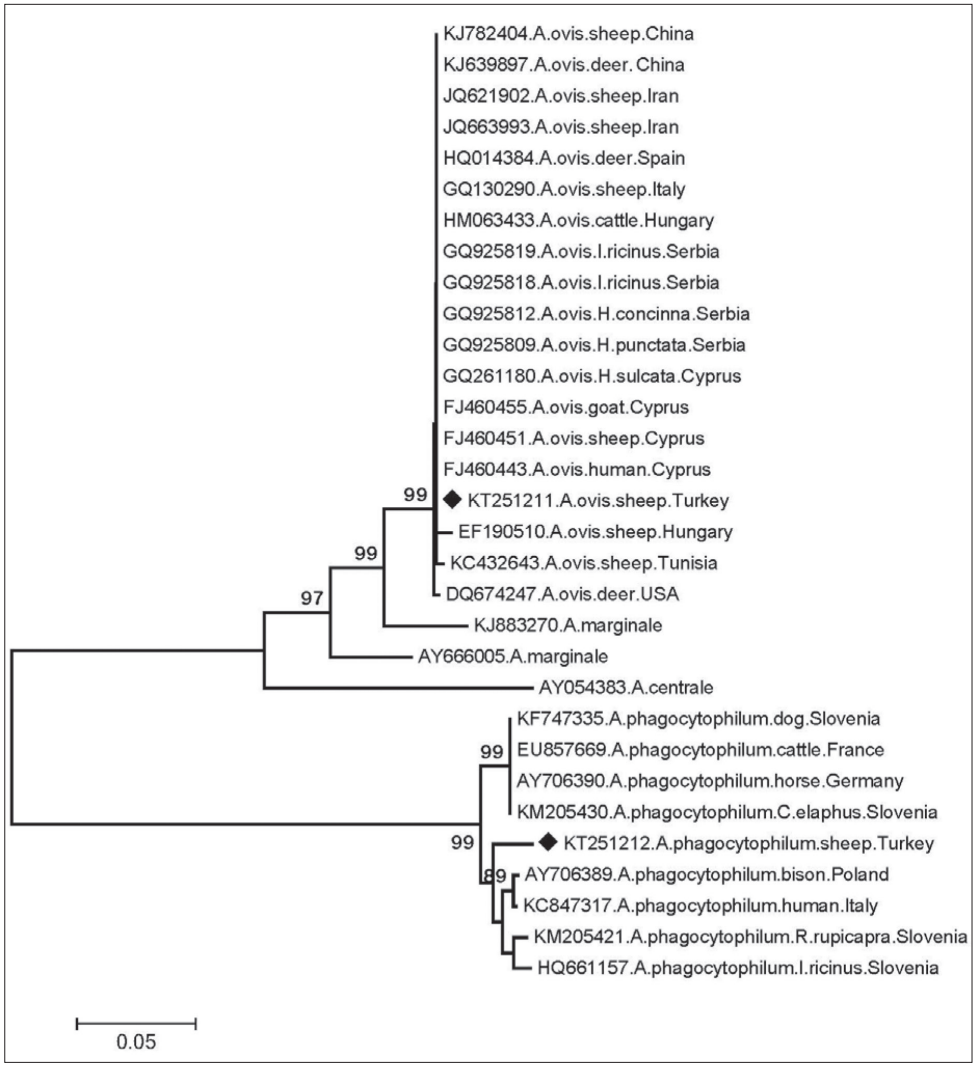

Fig 2. Neighbour-joining phylogenetic tree constructed via p-distance model, based on *Anaplasma* partial msp4 nucleotide sequences. Sequences included in the analysis are indicated with GenBank accession number, species, host and country of detection. Sequences characterized in this study are marked (black diamond). For the major branches, bootstrap values are indicated for 1.000 replicates

Şekil 2. Çalışmada elde edilen msp4 dizilerinin "p-distance" modeli ile oluşturulmuş "neighbour-joining" filogenetik ağacı. Analizde yer alan sekanslar GenBank erişim numarası, tür, konak ve tespit edilen ülkeler ile gösterilmiştir. Bu çalışmada karakterize edilen sekanslar işaretlenmiştir (siyah elmas). Ana dallanmalar için bootstrap değerleri 1.000 olarak alınmıştır

KT251212 for *A. phagocytophilum*). Comparison of *A. ovis* sequences with several selected homolog regions from various sources revealed very limited diversity, with 99.06-100% nucleotide similarities. However, *A. phagocytophilum* sequences demonstrated 0.41-2.49% divergence, and observed to constitute a distinct cluster, separated from sequences from Poland, Slovenia and Italy *(Fig. 2)*.

DISCUSSION

Anaplasmosis is a tick borne disease caused by various species of *Anaplasma* with a significant impact on animal breeding due to the economic burden resulting from morbidity and mortiality associated with the disease. Thus, the epidemiology, diagnosis and regional prevalence of Anaplasmosis remain as an important issue for mitigating the impact of the disease in the current practice of veterinary parasitology and microbiology [3].

The circulation of various *Anaplasma* species have been investigated in Turkey. Recent studies reported the presence of *A. phagocytophilum* nucleic acids in sheep [24,25], in goats [25] in ixodid ticks [26-28], in cattle [24,29,30] and in dogs [31], as well as *A. ovis* in sheep [25], in goats [25] and in ixodid ticks [28,32] in discrete regions. In East Black Sea region of Turkey, *A. phagocytophilum* seroprevalence by IFAT has been observed 14.86% (107/720) and specific DNA ratio by nested PCR has been found 12.35% (22/178) in sheep [24]. In East Anatolia region of Turkey, 71.32% (301/422) small ruminants have been reported to be infected by *A. ovis* and/or *A. phagocytophilum*. The percentages of positive animals for *A. ovis* and *A. phagocytophilum* have been reported 67.06% (283/422) and 19.66% (83/422) respectively. Coinfections of *A. ovis* and *A. phagocytophilum* have been reported in 15.40% (65/422) of analysed small animals. The percentages of *A. ovis* in sheep and goats were 67.35% (196/291) and 66.41% (87/131) respectively, whereas the number of *A. phagocytophilum* in sheep and goats were 18.90% (55/291) and 21.37% (28/131) respectively [25].

In this study, *A. ovis* and/or *A. phagocytophilum* infections were revealed in a total of 230 (54.37%) small ruminants investigated. The detection rates of *A. ovis*

and *A. phagocytophilum* in small ruminants were 50.83% (215/423) and 8.51% (36/423) respectively. Moreover, *A. ovis* and *A. phagocytophilum* coinfection frequency was noted as 4.96% (21/423). The prevalences of *A. ovis* in sheep and goats were 58.8% (127/216) and 42.51% (88/207) respectively, whereas the prevalences of *A. phagocytophilum* in sheep and goats were 11.11% (24/216) and 5.8% (12/207) respectively *(Table 1)*.

The results according to the study location demonstrated *A. ovis* to be the most abundant in Edirne (75.81%) and Kirklareli (55.77%) in sheep and goats respectively. *A. phagocytophilum* detection frequencies were highest in Kirklareli with 23.08% and 15.38% observed for sheep and goats respectively *(Table 1)*. The prevalence of *A. ovis* in sheep in Edirne was significantly higher compared to Istanbul and Tekirdag provinces, whereas it was significantly higher in Kirklareli than Istanbul. No statistically-significant difference was noted of *A. ovis* detection rates in sheep among Kisklareli, Edirne and Tekirdag provinces (P<0.05) *(Table 1)*. In Istanbul, prevalence of *A. ovis* in sheep was statistically lower than the other provinces while the prevalence of *A. ovis* in goats was only statistically lower than Kirklareli (P<0.05) *(Table 1)*.

A comparison of *A. phagocytophilum* detection rates revealed a significantly higher the prevalence of *A. phagocytophilum* in goats in Kirklareli province than the remaining provinces, while the prevalence of *A. phagocytophilum* in sheep was statistically higher than Istanbul and Edirne. *A. phagocytophilum* prevalence in sheep in Tekirdag was statistically similar to other provinces in the study (P<0.05) *(Table 1)*. In Europe, *Ixodes ricinus* (European sheep tick) acts as the main vector of *A. phagocytophilum* [39]. It has been reported that the *Ixodes ricinus* in Istanbul metropolitan area and in Kirklareli were infected with *A. phagocytophilum* at a rate of 2.7% and 17.5% respectively [27]. In our study, the prevalence of *A. phagocytophilum* in Istanbul in sheep and goats were 5.77% and 1.92% respectively while the prevalence of *A. phagocytophilum* in Kirklareli in sheep and goats were 23.08% and 15.38%, respectively.

The identities of the PCR products for *A. ovis* and *A. phagocytophilum* were verified by sequencing of the amplicons obtained from selected samples in the study. Despite the high level of similarity observed for *A. ovis, A. phagocytophilum* sequences demonstrated divergence up to 2.49% *(Fig. 2)*. Several genotypes and variant clusters, some of which are associated with the host species have been characterized for *A. phagocytophilum* [40,41]. Moreover, phylogenetic analyses of the msp4 region were reported to differentiate strains of *A. phagocytophilum* obtained from ruminants from those obtained from humans, dogs, and horses [37]. However, sequence data from several regions have been utilized for a more precise interpretation of phylogenetic relations among *A. phagocytophilum* isolates. Given that sequence data was available only from selected samples and employed for confirmatiory purposes, a thorough analysis of *A. phagocytophilum* sequence variations was not possible. Limited divergence was reported from various targets such as 16S rRNA and ankA sequences from Turkey [25,31].

So far, *Anaplasma* infections in small ruminants have not been documented in Thrace region of Turkey. Herein, we reported the presence and the distribution of *Anaplasma ovis* and *A. phagocytophilum* in sheep and goats in Thrace region of northwestern Turkey for the first time by using species-specific PCRs *(Table 1)*. Potential vectors of Anaplasmosis are known to be endemic in Thrace region of Turkey [42-44]. Therefore further researches are needed to determine the vectors, vector-host interactions and genotypic variants that may affect the presence and distribution of *Anaplasma* species in Thrace region of northwestern Turkey.

Acknowledgements

Authors are grateful to Prof.Dr. Munir AKTAS and Dr. Sezayi OZUBEK for technical assistance and providing positive control DNAs for *A. ovis* and *A. phagocytophilum*. Koray Ergunay is a recipient of the Georg Forster Research Fellowship (HERMES) for Experienced Researchers award of the Alexander Von Humboldt Foundation, 2015.

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Kafkas Univ Vet Fak Derg
22 (1): 139-145, 2016
DOI: 10.9775/kvfd.2015.14115

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

Research Article

The Effect of Cysteamine and Oviductal Cells in Different Culture Media on the Development of Sheep Embryos [1]

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Article Code: KVFD-2015-14115 Received: 28.07.2015 Accepted: 29.09.2015 Published Online: 02.10.2015

Abstract

Sheep is a very important source of wool, meat and milk all over the world. Oxidative stress during in vitro culture leads to defects in development of gametes and embryos. Several antioxidants such as cysteamine, L-ascorbic acid, beta mercaptoethanol, cysteine, glutathione, proteins, vitamins are used to supplement culture media to counter the oxidative stress. This study was aimed to detect the effect of cysteamine supplementation to the maturation medium and oviductal cell supplementation to culture medium on the subsequent development rates of sheep embryos with the control group. Oocytes were obtained from slaughtered sheep ovaries. Selected oocytes were incubated with or without 100 µM cysteamine in TCM-199 medium under 38.5-38.8ºC 5% CO_2 for 23 h. During IVF fresh semen was collected from ram by electroejaculation, they were washed in H-SOF medium and were fertilized in B-SOF medium with oocytes incubated for 18 hours under 38.5-38.8ºC 5% CO_2, 5% O_2 and 90% N_2. The oocytes were obtained from maturation medium with/without cysteamine (C+,-) and were cultured in SOF or CR1aa media with/without oviductal cells (Ov+,-) and were grouped as; Group Ia: SOF+(C+ Ov-), Group Ib: SOF+(C+Ov+), Group Ic: SOF+(C- Ov-) Group Id: SOF+(C- Ov+); Group IIa: CR1aa+(C+ Ov-), Group IIb: CR1aa+(C+ Ov+), Group IIc: CR1aa+(C- Ov-), Group IId: CR1aa+(C- Ov+). Embryos were incubated under 38.5-38.8ºC 5% CO_2, 5% O_2, 90% N_2 in culture medium for 7 days. Embryo developments were observed and recorded daily. GLM procedure found in SPSS packet program was used for statistical analysis in this study. In conclusion, the addition of cysteamine or oviductal cells in vitro culture media found to have any effect in terms of the capacity of reaching to blastocyst stage in SOF or CR1aa media and no statistical difference is detected between groups.

Keywords: *Cysteamine, Embryo, Oviductal cell, Medium, Sheep*

Kültür Medyumlarında Sisteamin ve Ovidukt Hücrelerinin Koyun Embriyo Gelişimi Üzerine Etkisi

Özet

Koyun yün, et ve süt kaynağı olarak dünya çapında önemli bir çiftlik hayvanıdır. İn vitro kültür esnasında oksidatif stres embriyo ve gametlerin gelişiminde defektlere neden olur. Sisteamin, L-askorbik asit, beta merkaptoetanol, sistein, glutatyon, proteinler, vitaminler gibi birçok antioksidanlar oksidatif stresi engellemek için kültür medyumunda kullanılır. Bu çalışmada maturasyon medyumuna sisteamin ve kültür medyumuna ovidukt hücreleri eklenen oositlerin kontrol grubu oluşturularak kültürleri sonucundaki gelişim aşamalarının değerlendirilmesi hedeflendi. Oositler mezbahada kesilen koyunların ovaryumlarından elde edildi. Seçilen oositler 100 µM sisteamin ilaveli ve ilavesiz TCM-199 medyumunda 38.5-38.8ºC'de %5 CO_2 altında 23 saat boyunca inkübe edildi. IVF aşamasında koçlardan elektroejakülasyon yöntemiyle elde edilen taze sperma H-SOF medyumunda yıkandıktan sonra fertilizasyon amacıyla B-SOF medyumunda bulunan oositler ile 18 saat 38.5-38.8ºC'de %5 CO_2, %5 O_2 ve %90 N_2'de inkübe edildi. Sisteaminli/sisteaminsiz (S +, -) maturasyon medyumundan çıkan oositler, SOF veya CR1aa medyumlarında ovidukt hücreli/ovidukt hücresiz (Ov +, -) şu şekilde gruplara bölündü; Grup Ia: SOF+(S+ Ov-), Grup Ib: SOF+(S+Ov+), Grup Ic: SOF+(S- Ov-) Grup Id: SOF+(S- Ov+); Grup IIa: CR1aa+(S+ Ov-), Grup IIb: CR1aa+(S+ Ov+), Grup IIc: CR1aa+(S- Ov-), Grup IId: CR1aa+(S- Ov+). Embriyolar 38.5-38.8ºC'de %5 CO_2, %5 O_2, %90 N_2 içeren inkubatörde 7 gün boyunca kültüre edildi. Kültür sonucunda embriyoların blastosit aşamasına kadar olan gelişimleri günlük olarak izlenerek kaydedildi. Bu çalışmada istatistik analiz için SPSS paket programında bulunan GLM prosedürü kullanıldı. Sonuç olarak, SOF ve CR1aa medyumlarında kültüre edilen koyun embriyolarının blastosiste ulaşma kapasitesi açısından in vitro üretim medyumlarında sisteamin veya ovidukt hücresi ilavesinin gelişimleri üzerine bir etkisinin olmadığı ve gruplar arasında istatistiksel bir önemin olmadığı tespit edildi.

Anahtar sözcükler: *Sisteamin, Embriyo, Ovidukt hücresi, Medyum, Koyun*

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INTRODUCTION

Sheep is a very important source of wool, meat and milk all over the world. In recent years, sheep population is significantly decreased in our country, the increase in the population of sheep can be achieved by the transfer of embryos obtained from *in vivo* and *in vitro* biotechnological methods. *In vitro* embryo production technologies in sheep and goats provide so many advantages; i) maintain to obtain plenty of embryo from the animals that have high genetic values as the oocytes can be harvested from the prepubertal, pregnant and even slaugtered sheep and goats ii) maintain a wonderful source for basic embryonic researches by its lower costs iii) provide a strategy for embryo transfer of some endangered animal species by interspecific embryo transfer [1,2]. The environment that supports the embryo development can be achieved by different culture conditions, oviduct cells and medium supplementation and gas atmospheres [3]. Embryo recovery and viability can be usually affected by different culture media and systems [4,5]. Culture media are species specific and there are many systems in the culture of *in vitro* fertilized oocytes. SOF medium is usually used for the development of sheep embryos *in vitro* when strengthen with amino acids supports the embryo development of sheep and goats [6], besides Hams-F-10, Thyrodes medium and CR1 media are used in *in vitro* sheep culture experiments [5-10]. TCM-199, Minimum essential medium (MEM) can be used for *in vitro* maturation of sheep oocytes and supplementing FSH and LH to maturation medium is essential for subsequent *in vitro* fertilization and culture [11,12]. Bovine serum albumin (BSA) can be used for fertilization medium to support the sperm capacitation [13]. Ewe serum, foetal calf serum, human serum and BSA can be used to support the culture media of sheep [10]. Aerobic metabolism causes the production of reactive oxygen species even under basal conditions [14]. The physiologic level of ROS; H_2O_2 and superoxide is produced especially during normal metabolism of mammal embryos [14]. During *in vitro* culture, embryos are exposed to inevitable suboptimal culture conditions and the production of ROS molecules exceeds the level of its *in vivo* production [14]. Oocytes and embryos are prevented against oxidative stress by oxygen scavengers present both in follicular and oviductal fluid. As the oocytes and embryos are moved away from their normal enviroment in *in vitro* production system, their defense system is lost [15]. Reactive oxygen species (ROS) has got so many harmful effects on the cells, these are DNA damage, lipid peroxidation, and oxidative changes of proteins [16]. Oxidative stress leads to mitochondrial damage, DNA, RNA and protein damage, reduction in sperm oocyte junction, especially embryo cell bloque during blastocyst period and cell death [17]. So many researchers have added various antioxidants to the culture medium in order to reduce the harmful effects of ROS on embryo development. Low molecular weight thiol compounds such as β-mercaptoethanol, cysteine, cystine and cysteamine are used to supplement oocyte maturation media and embryo culture media [18-24]. To develop *in vitro* embryo production oxidative stress during *in vitro* culture must be taken under control [25]. Glutathione is a natural antioxidant that is present in gametes and its level changes [26-29]. Cysteamin is a low molecular weight thiol compound which reduces cystine to cysteine and enhances oocyte glutathione synthesis [30]. de Matos *et al.*[31] and de Matos *et al.*[32] have reported the addition of cysteamine to the *in vitro* maturation media improves the rate of embryo development by increasing glutathione synthesis in cow and sheep. It was reported previously that oviduct cells in the oviduct play a major role in the embryo development [33-37]. Therefore, oviduct cell cultures are widely used during *in vitro* culture studies [6,38]. Usage of a co-culture passively influences the development of embryos at an early stage of development by reducing the negative effects of toxic substances within the culture media [39,40].

The aim of the present study is to detect the development competence of the embryos in which they were supplemented with cysteamine in maturation medium and oviductal cells in different culture media (Synthetic Oviduct Fluid-SOF and Charles&Rosencrans-CR1aa) by comparing them with controls.

MATERIAL and METHODS

Oocyte Recovery

Eight replica 604 ovaries were obtained from a local abattoir and transported to the laboratory in PBS supplemented with antibiotic combination at 30-35°C within 2-3 h of slaughter and total 2060 oocytes were collected out of the season. The cumulus oocyte complexes (COCs) were recovered by slicing method of antral follicles of 2-8 mm in diameter.

Preparation of Oviductal Cells

The oviducts that were brought to laboratory at the same time with ovaries were freed from surrounding fat and connective tissue without compromising the integrity of the channel and washed with PBS 3 times. The epithel cells in the canal squeezed from the begining of uterotubal junction with the help of forceps were transferred into the TCM-199 medium. The epithel cells were centrifuged 3.000 rpm for 5 min and the supernatant was throwed away and the fresh medium was added over the pellet at the bottom and centrifugation was repeated for 3 times and the remaining pellet at the bottom of the tube was transferred to the 80 cm^2 tissue culture flask and incubated for 48 hours under 5% CO_2. At the end of this process two different sample obtained from the culture suspension that contain oviductal cells were transferred to different tubes and centrifuged at 3.000 g for 2 min. The same process repeated again. After the supernatant was threw away 2 ml medium was added up the remaining oviduct

cell pellet at the bottom of the tube and were transferred at an appropriate proportion to the culture dishes to be used.

In vitro Maturation (IVM)

Selected oocytes were washed 3 times in TCM-199 supplemented with 10% FCS (v/v), 1 µg/mL FSH, 10 µg/mL LH, 0.3 mM Na +L-glutamine + 50 µg/ml gentamycine sulphate±100 µM cysteamine and without cysteamine then were transferred to the 4 well dishes that contains the same medium and were incubated 38.5-38.8°C under 5% CO_2 in humidified air for 23 h.

In vitro Fertilization (IVF)

After maturation the oocytes that had expanded cumulus oophorus cells were accepted as maturated and they were transferred in B-SOF medium that was recently incubated in a gas environment. BSOF medium was supplemented with 0.1 mM Na pyruvate, 1 mM glutamine, 2% oestrus sheep serum (v/v), 0.07 mM streptomycine, 0.14 mM kanamycine and 0.2 mM penicillin combination. Osmotic pressure of BSOF medium was 283±10 mOsm/kg and pH; 7.9. Fresh ram semen was collected from ram by electroejaculation and used for fertilization. Spermatozoa were incubated for 20 min for swim-up process in H-SOF medium, counted in Thoma slide and calculated as $4x10^5$-$1x10^6$/ml per oocyte for fertilization. HSOF medium was supplemented with 0.7 mM Na pyruvate, 2 mM glutamine, 3 mg/mL BSA, 0.14 mM streptomycine, 0.28 mM kanamycine and 0.4 mM penicillin combination. Then they were incubated for 18 h fertilization at 38.5-38.8°C in 5% CO_2, 5% O_2 and 90% N_2. Osmotic pressure of HSOF medium was 283±10 mOsm/kg and pH was 7.2-7.4.

In vitro Culture (IVC) and Blastocyst Controls

Maturated ovine oocytes in oocyte maturation medium (OMM) with or without cysteamine were tested in SOF and CR1aa culture media by addition of oviductal cells or not. Totally 8 culture groups were formed.

Group Ia: SOF+(C+ Ov-), **Group Ib:** SOF+(C+Ov+), **Group Ic:** SOF+(C- Ov-), **Group Id:** SOF+(C- Ov+);

Group IIa: CR1aa+(C+ Ov-), **Group IIb:** CR1aa+(C+ Ov+), **Group IIc:** CR1aa+(C- Ov), **Group IId:** CR1aa+(C- Ov+).

SOF medium supplemented with 5% FCS (v/v), 2% BME, 1% MEM, 0.3 mM glutamine, 4 mg/mL BSA, 0.35 mM Na pyruvate, 0.07 mM streptomycine, 0.14 mM kanamycine and 0.2 mM penicillin combination. Osmotic pressure of SOF medium was 283 mOsm/kg and pH; 7.2-7.4. CR1aa medium supplemented with 2% BME, 1% MEM, 0.2 mM glutamine, 3 mg/mL BSA, 5% FCS (v/v), 0.2 mM penicillin, 0.14 mM streptomycine. Osmotic pressure of CR1aa medium was 283 mOsm/kg and pH; 7.2-7.4. The presumptive zygotes were vortexed in tubes containing H-SOF to remove the cumulus cells and spermatozoa and they were cultured in 100 µl droplets SOF and CR1aa medium at 38.5-38.8°C in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 for 6-7 days. The development of embryos till blastocyst stage was evaluated and recorded daily. GLM procedure from SPSS packet program was used for this study.

RESULTS

Eight replica 604 ovaries were obtained and 2060 ovine oocytes were collected, from these oocytes 1626 were used for maturation, out of them 1056 oocytes were incubated in maturation medium with cysteamine whereas 570 oocytes were incubated in maturation medium without cysteamine. Total cleaved oocytes that were incubated in culture medium were 1034 and out of them 619 oocytes were obtained from maturation medium with cysteamine and 415 oocytes from maturation medium without cysteamine. At the end of the culture period totally 315 early blastocysts and blastocysts were obtained. In the used statistical model the main effects of SOF/CR1aa, cysteamine and oviductal cell status were taken part. These main effects bilateral interactions were found insignificant so these effects were not included in the statistical model. Average early blastocyst/blastocyst rate was detected as 376/1163=32.3% in all groups.

Embryo development cultured in SOF/CR1aa media with/without oviductal cells which were previously maturated with/without cysteamine is given in *Table 1* and *Table 2*.

DISCUSSION

In present study, development competence of the embryos were evaluated at the end of the culture period in which they were supplemented with cysteamine in maturation medium and oviductal cells in culture medium by comparing them with their controls.

It is well known that the addition of antioxidant substances to maturation medium is a critical point for to ensure the desired effect on the first phase of embryo development [41]. Gasparrini *et al.*[42] reported that the addition of cysteamine to the medium during buffalo oocyte maturation has increased the cytoplasmic glutathione content and subsequent embryo development. Cysteamine was added to medium during maturation period but the effect of addition of cysteamine on cytoplasmic glutathione content was not evaluated in current study.

Shabankareh and Zondhi [43] detected the blastocyst rate of bovine embryos 30.8% without cysteamine in maturation medium, wheras they found the same ratio as 35% when added cysteamine to maturation medium. In current study, the blastocyst rate (Group Ia) was found as 33.3% in the embryos that were maturated in medium

Table 1. *The effect of cysteamine and oviductal cells supplementation in different culture media on the development of sheep embryos*
Tablo 1. *Değişik kültür medyumlarında sisteamin ve ovidukt hücrelerinin eklenmesinin koyun embriyo gelişimi üzerine etkisi*

Groups	Group Ia %			Group Ib %			Group IIa %			Group IIb %			Total %		
	Oocyte Number	Clevaged Oocytes	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*
n1	35	15	4	41	18	3	36	12	2	41	12	2	153	57	11
n2	25	17	8	18	14	6	25	16	5	18	12	4	86	59	23
n3	29	16	3	24	16	4	29	16	4	24	7	1	106	55	12
n4	28	20	10	42	27	8	29	10	4	42	28	5	141	118	33
n5	52	30	7	60	42	10	60	30	5	60	38	7	232	140	29
n6	30	13	4	36	19	6	28	10	2	34	20	4	128	62	16
n7	25	19	12	19	15	6	20	16	6	24	19	13	88	69	37
n8	60	35	7	22	14	5	15	12	5	25	16	4	122	77	21
TOTAL	284 58.0%	165	55 33.3%	262 68.7%	180	48 26.6%	242 50.4%	122	33 27.0%	268 56.7%	152	40 26.3%	1056 73.1%	772	227 29.4%

Table 2. *The effect of cysteamine and oviductal cells supplementation in different culture media on the development of sheep embryos (continued)*
Tablo 2. *Değişik kültür medyumlarında sisteamin ve ovidukt hücrelerinin eklenmesinin koyun embriyo gelişimi üzerine etkisi (devam ediyor)*

Groups	Group Ic %			Group Id %			Group IIc %			Group IId %			Total %		
	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*
n1	6	4	2	5	3	1	6	3	2	5	3	1	22	13	6
n2	22	17	8	21	16	7	15	13	7	26	18	5	84	64	27
n3	26	18	7	20	16	6	20	17	6	23	17	5	89	68	24
n4	10	7	3	9	7	2	10	6	2	10	6	3	39	26	10
n5	9	6	2	55	42	18	51	18	9	64	43	15	179	109	44
n6	15	11	3	9	5	2	12	9	4	10	7	3	46	32	12
n7	17	12	4	14	11	3	11	9	3	12	9	4	54	41	14
n8	8	3	2	22	18	4	20	13	5	7	4	1	57	38	12
TOTAL	113 73.4%	83	31 37.34%	155 76.12%	118	33 27.9%	145 74.4%	108	38 35.1%	157 67.5%	106	37 34.9%	570 68.5%	391	149 38.1%

** Early blastocyst/blastocyst; % Clevaged oocyte/Oocyte number*

with cysteamine, whereas the same ratio was found similar as 37.34% in Group Ic and 35.1% in Group IIc without cysteamine supplementation during maturation. In this study addition of cysteamine to maturation medium and later addition of oviductal cells to culture medium was found to have no effect on embryo development. We think that this difference can be originated because of the marked variations in terms of the material and methods variations of these two studies and could be attributed to different animal species. Besides Shabankareh and Zondhi [43] was added EGF and IGF-I to maturation medium suggests us more effective for embryo development. Shabankareh and Zondhi [43] preferred SOF medium to fertilize the maturated oocytes in their study, in this study BSOF medium was used and indeed in terms of the culture periods there are important differences in these two studies. In present study, SOF and CR1aa media were compared for culture and higher proportion of embryo development was determined in SOF medium.

Balasubramanian and Rho [24] added cysteamine to the maturation medium of bovine oocytes and were assessed significantly higher blastocyst rate on day 7 and 8 than control group. Cysteamine addition showed no different blastocyst yield when compared to control group in this study indicates us to think this difference could have been arisen because of the different animal species.

The addition of thiol compounds like cysteamine or beta-mercaptoethanol in the medium during sheep and bovine oocyte maturation was reported to facilitate the induction of glutathione synthesis and reduces the hydrogen peroxide levels and increase the embryo development [31,44,45]. We think their higher amount of cysteamine addition to maturation medium in their studies could have been lead to higher rate of embryo development than this study. Wani *et al.*[46] added cysteamine to maturation medium of sheep oocytes and was aimed to detect the effect of cysteamine on the development competence of sheep embryos after culture period and they used TCM-199 medium in their study for maturation as we did. They added 200 µM/ml cysteamine to maturation medium and after culture with TCM-199 they found 36.7% embryo rate that reached to morula stage. In current study 100 µM cysteamine was added to maturation medium, and at the end of the culture period in SOF or CR1aa media early blastocyst/blastocyst rates were detected as 33.3% and 27%, respectively and this result was consistent with the findings of Wani *et al.*[46] study.

Rodriquez-Dorta *et al.*[47] investigated the effect of goat oviductal cells on *in vitro* goat embryo development in SOF medium supplemented with 3 mg/ml BSA and they detected the cleavage rate as 83%, this rate was detected as 76.12% in present study.

The usage of somatic cell was encouraged to support the *in vitro* mammal' embryo development in the past. Firstly mouse embryo culture was used in mouse ovary organ culture. Till than, embryo somatic co-culture was used in a large scale of animal specie including buffalo. Somatic cells have highly effective embryotrophic impact. These are faster cleavage, less fragmentation, increased blastocyst cell numbers, advanced morphologic image, low apoptosis rate, advanced prenancy rates and live births [48]. During *in vivo* development through somatic cells like oviductal epitel cells embryonic environment can be regulated metabolically. These cells take part in several tasks like reducing of oxygen pressure partially and provision of useful metabolites for embryos. So the usage of oviductal epitel cells can be helpful during *in vitro* embryo development [49].

Özdaş *et al.*[49] were used SOF and B2 media for culturing bovine embryos and were added oviductal cells to these culture media and they found a positive effect of oviductal cells on development competence of embryos besides they were detected this effect as statistically significant in their study. In current study, conversely the addition of oviductal cells to culture media of sheep embryos were not found statistically significant. The difference organized from the addition of oviductal cells suggests us that can be arisen because of the media types and conditions used in laboratory can affect in different ways on embryo development. As it is determined that There is no difference with about the addition of oviductal cells on subsequent embryo development during culture period of fertilized oocytes when compared to control group in this study. We think this difference could have been arisen because of the planning of two studies and during culture periods two culture media (SOF and CR1aa) were used in this study but only SOF medium was used as common in the other study.

Sandal and Özdaş [23] supports our hypothesis that they did not find any effect of cysteamine supplementation to maturation medium on subsuquent embryo development of bovine embryos.

In conclusion, it was found to have any impact effect on sheep embryo development supplemented with cysteamine or oviductal cells in terms of the capacity of reaching to blastocyst stage cultured either in SOF nor CR1aa media and no statistical significance was detected between the groups in present study. We tried to emphasize the importance of different systems on embryonic development capability with this study. Still today the addition of antioxidants such as cysteamine to maturation medium and the effect of it on development of embryos during culture period is a matter under investigation.

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Kafkas Univ Vet Fak Derg
22 (1): 147-154, 2016
DOI: 10.9775/kvfd.2015-14122

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

Research Article

Effect of Climate on the Epidemiology of Bovine Hypodermosis in Algeria

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Article Code: KVFD-2015-14122 Received: 29.07.2015 Accepted: 21.10.2015 Published Online: 21.10.2015

Abstract

In order to explore the effect of climate on cattle warble fly infestation, a total of 1.635 animals from 4 departments of Northern Algeria were examined visually and by manual palpation for the presence of warbles. Cattle were examined from March to June 2014, coinciding with the peak of emergence of warbles, and both the prevalence and intensity of infestation were recorded. The departments included in this study were located in the two different climatic areas in Northern Algeria: humid (Bejaia and Tizi Ouzou) and semi-arid (Tissemssilt and Ain Defla). The overall prevalence was 28.75%; the intensity of infestation ranged from 1 to 98 warbles per animal (mean 18.93±11.05). The prevalence and intensity of infestation within the departments with semi-arid climate (38.23%; 21.57±11.98) was significantly higher than in those with humid climate (20.74%; 14.84±7.86). The CHAID algorithm showed the climate as the most influencing factor for warble fly prevalence, followed by the husbandry system and breed. Logistic regression and multivariate ANOVA indicate that in addition to climate, other intrinsic (age, sex, breed) and extrinsic factors (husbandry system, treatment) included in the study also were associated with both, prevalence and intensity of infestation. Our results indicate that in semiarid areas of Northern Algeria environmental conditions are more favorable for the development of free stages (pupae and adult flies) of *Hypoderma* spp life-cycle than in humid areas.

Keywords: *Bovine hypodermosis, Myiasis, Cattle, Climate, Algeria*

Cezayir'de Sığır Hipodermosis'in Epidemiyolojisine İklimin Etkisi

Özet

Sığırlarlarda warble sineği (büvelek) istilası üzerine iklimin etkisini ortaya koymak amacıyla, Kuzey Cezayir'in 4 bölgesinden toplam 1.635 hayvan görsel ve el palpasyonuyla büveleklerin varlığı yönünden muayene edildi. Sığırlar büveleklerin ortaya çıkışının zirve (pik) yaptığı Haziran-Mart 2014 arası incelenerek, hem prevalans hem de enfestasyon yoğunluğu kaydedildi. Bu çalışma kapsamındaki bölgeler, Kuzey Cezayir'de iki farklı iklim alanında yer aldı: nemli (Bejaia ve Tizi Ouzou) ve yarı-kurak (Tissemssilt ve Ain Defla). Tüm prevalans %28.75 idi; hayvan başına istila yoğunluğu ise 1 - 98 büvelek arasında değişti (ortalama 18.93±11.05). Yarı-kurak iklime sahip bölgelerdeki yaygınlık ve istila yoğunluğu (%38.23; 21.57±11.98) nemli iklime sahip olanlara göre anlamlı derecede daha yüksek bulundu (%20.74; 14.84±7.86). CHAID algoritması, büvelek sineği prevalansını etkileyen başlıca faktörün iklim olduğunu, diğerlerinin ise yetiştirme sistemi ve ırk olduğunu gösterdi. Lojistik regresyon ve çok değişkenli ANOVA, iklime ek olarak, diğer iç (yaş, cinsiyet, ırk) ve çalışma kapsamındaki dış faktörlerin (yetiştiricilik sistemi, tedavi) hem yaygınlık hem de istila yoğunluğu ile ilişkili olduğunu göstermektedir. Bizim sonuçlarımız; Kuzey Cezayir çevre koşulları altındaki yarı-kurak alanların, *Hypoderma* spp. yaşam döngüsünün serbest aşamalarının (pupa ve ergin sinek) gelişimi için nemli alanlara göre daha uygun olduğunu göstermektedir.

Anahtar sözcükler: *Bovin hipodermozis, Miyazis, Sığır, İklim, Cezayir*

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INTRODUCTION

Cattle hypodermosis is an obligatory myiasis caused by larvae of *Hypoderma bovis* and *Hypoderma lineatum* (Diptera, Oestridae), characterized by the presence of subcutaneous warbles in the dorsal and lumbar region of the animals. For the last 50 years, cattle hypodermosis has represented one of the most significant parasitic diseases in many countries of the northern hemisphere. Warble fly infestation (WFI) greatly impairs livestock production not only by inducing mechanical damage to internal organs (esophagus, spinal column, rumen, lungs) or skin but also by down-regulating the host immune system [1-4].

The biology of *Hypoderma* is very influenced by weather conditions. Climate directly influences the development of the free stages of the parasite, pupae and adult flies, affecting the chronology of this myiasis and the intensity of infestation [5,6]. Adults are generally active from April to June in the case of *H. lineatum* and from mid-June to early September in the case of *H. bovis*, though precise periods of activity depend on seasonal and geographic differences in climate [7].

Algeria is the second largest country in Africa; it extends from the Mediterranean coastline to the Sahara desert. The slopes of Algeria's northern mountains and plateaus are used for grazing farming. Cattle are mainly limited to the north of the country with some enclaves elsewhere. Cow's milk and sheep's meat are the highest-earning agricultural products for domestic farmers.

Hypodermosis is an underestimated problem in Algeria. Algerian farmers are not aware of the economic losses caused by this parasitic disease and therefore, no specific control measures are undertaken against this myiasis. Moreover, the application of some anthelmintics having ectoparasitic action against *Hypoderma* spp. (i.e. ivermectin, moxidectin etc.) is widely used in some departments and scarcely employed in others. In Algeria, except the data provided by Benakhla *et al*.[8] and Benakhla *et al*.[9] during the 90s in the North East of the country, and more recently by Saidani *et al*.[10] in the North Central part, not any reliable data are available regarding the epidemiology of this infection. In order to cover this lack of information, the present clinical survey, including departments from humid and semi-arid areas of Algeria, was carried out.

The main objective of this study was to explore the effects of the climate, on the prevalence and intensity of infection of bovine hypodermosis in Algeria. In addition, our survey could provide some epidemiological data on bovine hypodermosis in the western part of northern Algeria giving that the previous studies on this topic have never covered this area.

MATERIAL and METHODS

Study Area

Northern Algeria is in the temperate zone with a mild Mediterranean climate. However, its broken topography provides sharp local contrasts in both prevailing temperatures and incidence of rainfall. This area is inhabited by more than ninety percent of Algeria´s population, because is the most fertile region in the country. The geographic distribution of cattle, very scarce in southern Algeria, follows almost the same pattern as human inhabitants The present study has been conducted in two bioclimatic areas of northern Algeria *(Table 1)*: in the humid area temperatures are mild and precipitations are around 1.000 mm annually; in contrast, in semi-arid areas differences between high and low temperatures are high and annual rainfall is scarce.

Fig. 1 shows the location of the four Departments included in this study. Two Departments were sampled in the humid area: Bejaia (n=419) and Tizi Ouzou (n=468) and another two in the semi-arid area: Ain Defla (n=325) and Tissemssilt (n=423).

Animals and Sampling

A total of 1.635 animals were randomly examined visually and by manual palpation for the presence of warbles, and both the prevalence and intensity of infestation were recorded.

All the animals were examined at monthly intervals from March to June 2014, which are the months of the year that correspond to the peak of emergence of warble flies in this region. To ovoid false results, neglecting the month effect, all the animals were examined at monthly intervals, and the peak value was taken into account (that of April)

In order to found out the relative distribution of the two species of *Hypoderma spp.*, naturally emerged larvae (n=152; 80 larvae from Tizi Ouzou and 72 from Ain Defla) were collected and preserved in ethanol 70% and subsequently identified by using the morphological keys

Table 1. *Mean weather conditions in two climatic areas of northern Algeria (2013)*
Table 1. *Kuzey Cezayir'in iki iklim bölgesindeki ortalama hava koşulları (2013)*

Climate	Temperature (°C)	Rainfall (mm)	Length of Dry Period (month)	Relative Humidity (%)
Humid	Min: 0-9; Max: 28-31	900-1.200	3-4	79.09
Semi-arid	Min: 2-4; Max: 33-38	300-600	5-6	62.25

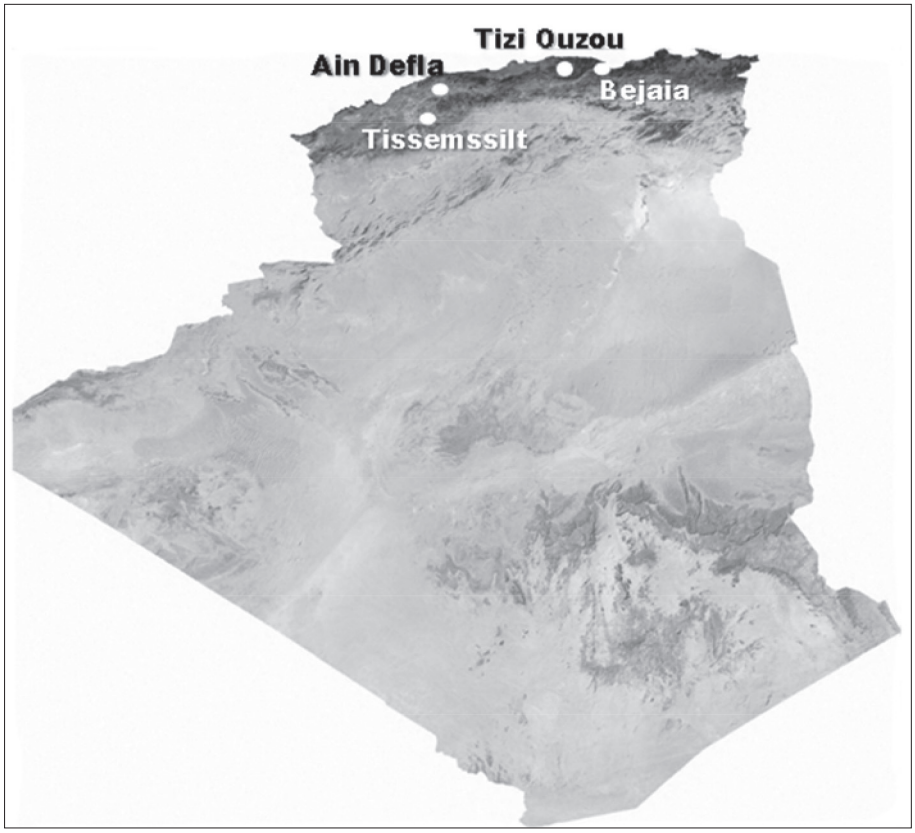

Fig 1. Geographic map of Algeria showing the location of the Departments included in this study

Fig 1. Cezayir'in bu çalışmaya dahil edilen Bölgelerin yerini gösteren coğrafi haritası

as described by James [11] and Zumpt [12].

Variables were grouped and categorized for statistical analysis as follows:

Age groups: 1 (≤ 12 months-old), 2 (13-36), 3 (>36),

Climatic area: 1 (Humid area), 2 (Semi-arid area),

Department: 1 (Bejaia), 2 (Tizi Ouzou) in humid area; 3 (Ain Defla), 4 (Tissemssilt) in semi-arid area,

Breed: 0 (Local breed), 1 (Frisian), 2 (Montbéliard), 3 (Flechvieh), 4 (Crossbreed),

Husbandry system: 1 (Intensive), 2 (Semi-extensive), 3 (Extensive),

Sex: 1 (Male), 2 (Female),

Treatment: 0 (Without treatment), 1 (Treated).

Statistical Analysis

In order to detect the influence of climate and other intrinsic (age, sex, breed) and extrinsic factors (husbandry system, treatment) in the prevalence of WFI, a Logistic Regression algorithm was applied. The dependent variable was the presence of warbles in each animal. Factors indicated previously were introduced in a backward conditional method and removed from the model one by one (on the basis of the highest p-value) until the best model was built. Next, all pairwise interactions that were biologically plausible were evaluated. Odds ratio were computed by raising e to the power of the logistic coefficient over the first category of each factor, not over the last.

Chi-squared automatic interaction detector (exhaustive CHAID) has been performed to stratify risk factors in order of importance. CHAID algorithm identified factors that divide cattle in subgroups with different positive/negative ratio [13]. CHAID is a tool to identify major factors using as criteria the significance of a Chi-squared test and successively splitting data in increasingly homogeneous nodes in relation to dependent variable (warble presence) until the classification tree is fully grown.

A multifactorial ANOVA over positive animals was used for the examination of the intensity of infestation; the dependent variable -number of nodules counted in animals- had been previously transformed (squared root of nodules plus 0.5) to normalized the variable. Tukey HSD post hoc test was used to detect the differences between pairs.

Statistical analyses were done using R statistical package v. 3.2.0 [14]. CHAID algorithm was performed with Answer Tree 3.1 (SPSS Inc., Chicago, IL USA) [10].

RESULTS

In the present study, the overall prevalence was 28.7%. Notable variations in prevalence were observed

Table 2. *Prevalence by Hypoderma in cattle from northern Algeria*
Table 2. *Kuzey Cezayir'deki sığırlarda hypoderma prevalansı*

Factor	Levels	Examined Animals	Non Infested Animals	Infested Animals	Prevalence
Climate	Humid	887	703	184	20.74%
	Semi-arid	748	462	286	38.23%
Age	<13 months	493	346	147	29.82%
	13-36 months	790	552	238	30.12%
	>36 months	352	267	85	24.14%
Sex	Male	309	236	73	23.62%
	Female	1326	929	397	29.94%
System	Intensive	129	117	12	9.30%
	Semi-extensive	611	464	147	24.06%
	Extensive	895	584	311	34.75%
Breed	Holstein	52	40	12	23.08%
	Montbéliard	222	165	57	25.68%
	Fleckvieh	25	5	20	80.00%
	Crossbreed	572	378	194	33.91%
	Local breed	764	577	187	24.48%
Medication	Medicated	769	619	150	19.50%
	Non-medicated	866	546	320	36.95%

Table 3. *Logistic regression results*
Table 3. *Lojistik regresyon sonuçları*

Factors	Estimate	S.E.	Z value	P	OR*	Lower 95% CI for OR	Upper 95% CI for OR
Climate	1.5112	0.1594	9.481	<0.001	4.53	3.32	6.19
Local breed							
Freisian breed	2.1550	0.4524	4.763	<0.001	8.63	3.55	20.94
Montbéliard breed	3.4894	0.3251	10.732	<0.001	32.77	17.32	61.97
Flechvieh breed	4.8749	0.5690	8.567	<0.001	130.96	42.93	399.49
Croosbreed	1.3806	0.1675	8.240	<0.001	3.98	2.86	5.52
< 13 months							
13-36 months	-0.1107	0.1397	-0.793	0.428	0.89	0.68	1.17
> 36 months	-0.4271	0.1751	-2.439	0.015	0.65	0.46	0.91
Sex	0.6358	0.2269	2.803	0.005	1.89	1.21	2.95
Intensive system							
Semi-extensive system	1.8659	0.3754	4.971	<0.001	6.46	3.10	13.49
Extensive system	4.6646	0.5684	8.207	<0.001	106.13	34.84	323.314
Treatment	0.8670	0.4013	2.161	0.031	2.38	1.08	5.22

** Odds ratio were computed by raising e to the logistic estimation over the first category of each factor*

between the climatic areas *(Table 2)*; the prevalence within the departments with a semi-arid climate was significantly higher than in those with humid climate. Logistic regression *(Table 3)* indicated that in addition to climate, the other intrinsic (age, sex, breed) and extrinsic factors (husbandry system, treatment) included in the study were associated with warble prevalence *(Table 4)*. According to this test, cattle in semi-arid areas have 4.53 times more risk to acquire this myiasis than cattle in humid areas. When considering other factors, Flechvieh cattle have the highest prevalence and local breed the lowest, young animals (≤12 months) are more frequently infested than old animals. The likelihood of being positive is 106.13 times higher in animals kept in an extensive husbandry system than in an intensive system.

Table 4. *Intensity of infection by Hypoderma spp and statistical result with multifactorial ANOVA*
Table 4. *Hypoderma spp enfeksiyonu yoğunluğu ve çok faktörlü ANOVA ile istatistiksel sonucu*

Factors	Mean±SD	F	P Value	Factors	Mean±SD	F	P Value
Climate		62.582	< 0.001	Sex		0.080	0.778
Humid	14.84±7.86			Male	16.03±9.76		
Semi-arid	21.57±11.98			Female	19.18±11.13		
Breed		2.417	0.048	Husbandry system		37.152	< 0.001
Local	20.65±12.96			Intensive	4.67±2.9		
Freisian	19.58±7.72*			Semi-extensive	14.38±6.55		
Montbéliard	13.75±6.68			Extensive	21.64±11.8		
Flechvieh	11.6±6.56*						
Croosbreed	19.52±9.88						
Age		16.074	< 0.001	Treatment		24.164	< 0.001
< 13 months	21.67±12.02			No treatment	21.64±11.8		
13-36 months	18.5±10.74			Treatment	13.16±6.6		
> 36 months	15.41±8.9						

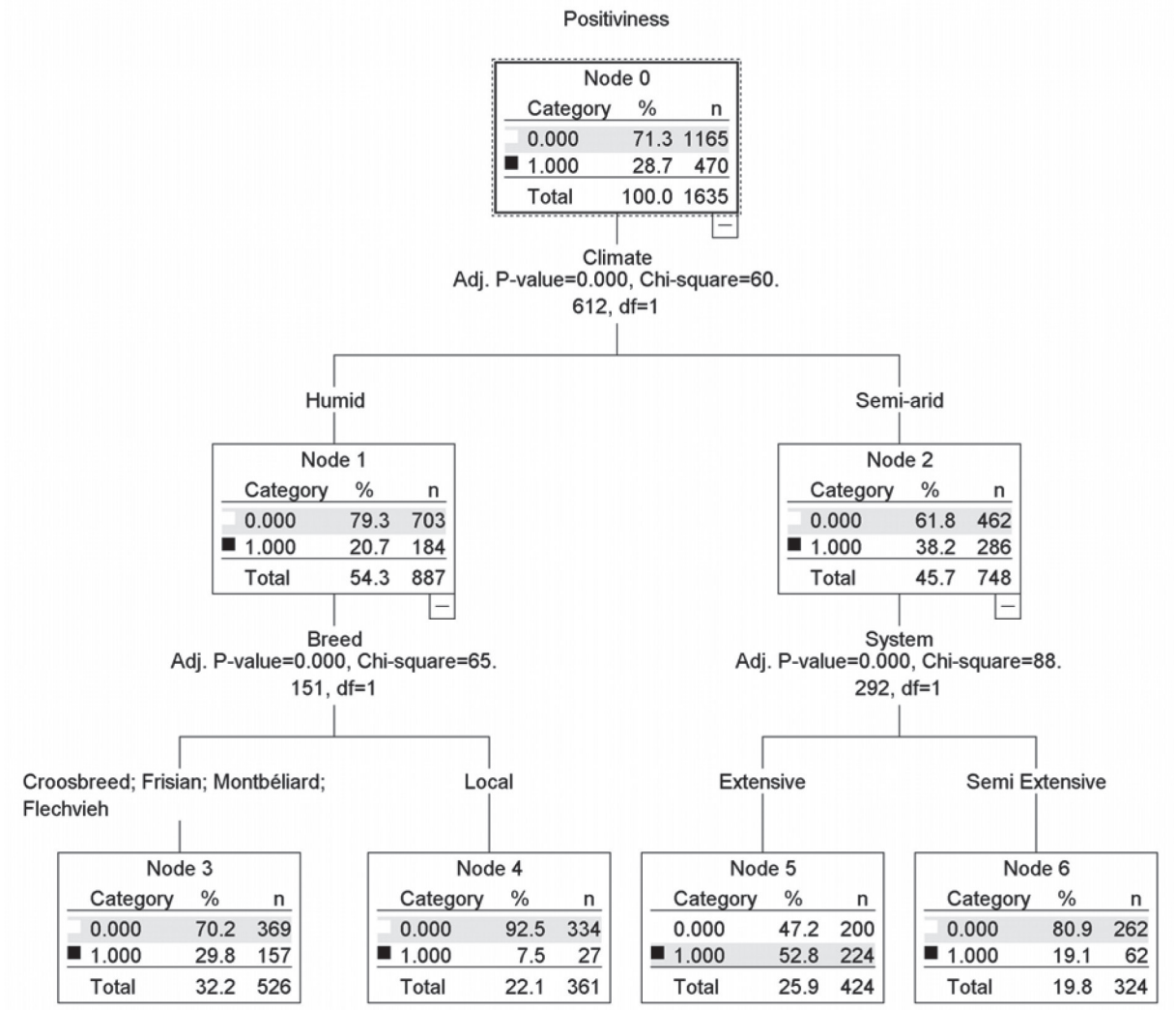

Fig 2. Classification tree produced by the CHAID algorithm when considering different factors
Fig 2. CHAID algoritmasıyla farklı faktörler dikkate alınarak üretilen sınıflandırma ağacı

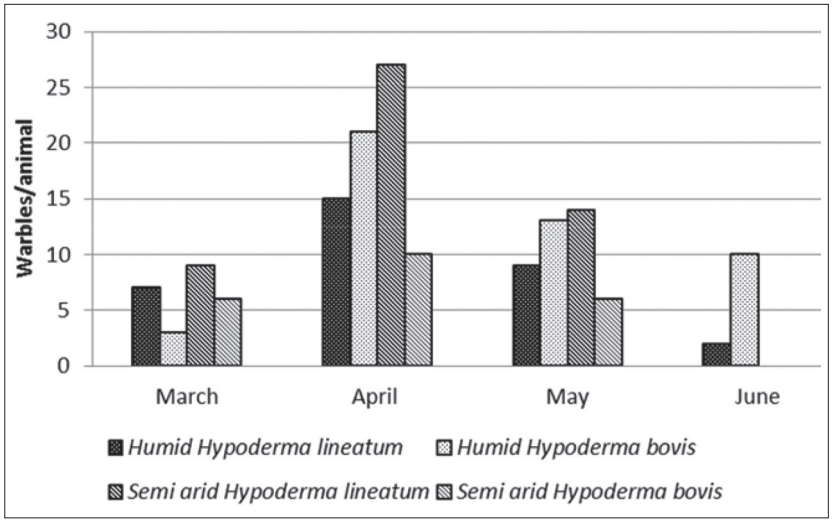

Fig 3. Monthly evolution of *H. bovis* and *H. lineatum* in two climatic areas in northern Algeria

Fig 3. Kuzey Cezayir'de iki iklim bölgesindeki H. bovis ve H. Lineatum'un aylık evrimi

The CHAID algorithm showed the climate as the most influencing factor for warble fly prevalence, followed by the husbandry system and breed *(Fig. 2)*.

The intensity of infestation ranged from 1 to 98 warbles per animal (mean 18.9±11.05). As occurred with the prevalence, the lowest intensity was registered in the humid area *(Table 3)*. In addition to climatic area, multifactorial ANOVA also showed significant differences in the intensity of infection when considering the breed and age of the animals; with local breed and young animals showing the highest rates. Management factors like husbandry system and treatment also have a significant influence in the number of warbles/animal.

Out of the 152 identified larvae, 83 (54.5%) were found to belong to *Hypoderma lineatum* and 69 (45.5%) to *H. bovis*. Under the humid climate, 47 out of 80 (58.7%) of larvae were *H. bovis* and the remaining part of *H. lineatum*. However, in the area from semi arid climate, 50 out of 72 (69.4%) belonged to *H. lineatum* species *(Fig. 3)*.

DISCUSSION

The overall prevalence of hypodermosis detected in northern Algeria can be considered as moderate (28.7%); this percentage was higher than the 18.1% observed by Saidani *et al.*[10] in cattle farms from North Central Algeria and the 3.7% found in an abattoir. However, this prevalence resulted very low as compared to the 76% recorded by Benakhla *et al.*[9] in the eastern part of the country.

When considering the department of origin of cattle the lowest prevalence was registered in Bejaia (16.21%), followed by Ain Defla (19.08), Tizi Ouzou (24.79%) and finally, the highest percentage was recorded in Tissemssilt (52.96%). Similarly, as occurred in our survey, Panadero *et al.*[5] found that Coastal areas, characterized by relatively high temperatures and by an important summer rainfall, had the lowest percentages of grub presence, whereas in the interior areas the infestation percentages were higher. The wide variations on prevalence and intensity of infection between humid and semiarid departments could be mainly explained by differences on rainfall and temperature values, as flies generally are not active on dark, cloudy days, at wind velocities above 8m/s, or during periods of rain or snow [15]. However, other factors affecting the prevalence and intensity of infection might include, breed, husbandry system and the use of insecticides [16-19]. Indeed, Chi-squared automatic interaction detector (exhaustive CHAID) revealed the climate as the most important factor followed by the management system and breed. The husbandry system is known to exert a major effect on both prevalence and intensity of bovine hypodermosis since the free grazing system is the most favorable for the occurrence of this myiasis, under which flies have more chances to contact the animal. This fact agrees with several previous studies [5,10,16-19]. Surprisingly, in this study local breeds mainly kept under extensive and semi-extensive husbandry systems showed the lowest prevalence but the highest parasite burdens. Moreover, multifactorial ANOVA and logistic regression also identified the use of drugs (avermectins) as a factor influencing, the intensity and prevalence of infection.

Both the prevalence and the intensity of bovine hypodermosis differ significantly from one area to another. It is usual to notice a wide variation in the prevalence of WFI among different parts of the world and even within the same country [16]. This variability might be due to the differences in the climatic factors that affect the developmental stages of the larvae [5,20]. Consequently, the lower prevalence and intensity recorded in the area from humid climate can be explained by the high relative humidity and rainfall, which are harmful to the free stages of warble flies according to Tarry [20]. Ahmed *et al.*[18] found that the high level of infestation were due to the climatic conditions, location, treatment procedures, topography of the area and extensive grazing. Despite

the moderate prevalence found in this study especially in Bejaia and Tizi Ouzou departments, the burden of infestation is generally heavy (up to 98 larvae), which indicate that the implementation of a control program for hypodermosis in Algeria is very indispensable.

In our survey, *H. bovis* and *H. lineatum* were found infesting cattle, although there was a slight predominance of *H. lineatum* (54.5%). This statement agrees with previous findings in the same country [10,21]. The identification of the species of *Hypoderma* implicated in a given area is of vital importance for several reasons. Firstly because *H. bovis* is currently believed to affect only cattle living in the Northern countries, and secondly because the impact of *H. bovis* on animal welfare and health is more important than *H. lineatum* due to the fact that if treatments are not carried out promptly, when first stage larvae are still in the peri-rachidian channel, paralysis of the hind quarters may occur. This finding is of relevance for the correct use of drugs against hypodermosis [22]. Thirdly, *Hypoderma bovis* is more harmful and frighten the host several times during laying because its eggs are glued singly on the flank or lower abdomen while *Hypoderma lineatum* lays in groups of 5 to 15 eggs onto the hair of the forelegs, breast or underside of the body [1], thus it causes less fear. It is thus obvious that the severity of gadding is different according to the *Hypoderma* species.

As regard to the distribution of the two species, our results were in accordance with what was found by Benakhla *et al*.[9], where the 2 species of genus *Hypoderma* were present in cattle in Algeria *(H. bovis* and *H. lineatum)* with a predominance of *H. lineatum* (63%) in the semi-arid area and a majority of *H. bovis* (75%) in humid area. Warbles appeared and disappeared earlier in the semi-arid area. Indeed, in the present study no larva was found in the semi arid area since the end of May. During March, *Hypoderma lineatum* was predominant in both climatic areas, which is in favor of the early emergence of this species [8,9]. The peak of emergence was reached during April regardless to the species, similarly as recorded for the 1635 animals clinically examined. On May, *H. lineatum was* slightly more frequent. Finally, on June the larvae collected exclusively from humid climatic with of a majority of *Hypoderma bovis (Fig. 3)*. This finding is once more in agreement to previous studies on this topic, where the warbles disappear one month later in humid area [8].

As conclusion, climatic area was identified as a significant factor affecting the percentage of positive animals and the intensity of parasitation by *Hypoderma* sp. Our results indicate that in semiarid areas of Northern Algeria environmental conditions are more favorable for the development of free stages (pupae and adult flies) of *Hypoderma* spp life-cycle than humid areas. However, the climate does not exert its effect alone, others intrinsic and extrinsic factors such as grazing pattern, breed and medication were also involved on the epidemiology of warble fly infestation.

Acknowledgements

This work was partially supported by a grant to K. Saidani (PNE2014/2015 MESRS Algérie) and by a grant for Consolidating and Structuring competitive research groups (R2014/005, Xunta de Galicia, Spain)

Conflict of Interest

The authors declare that they have no conflict of interest.

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Kafkas Univ Vet Fak Derg
22 (1): 155-158, 2016
DOI: 10.9775/kvfd.2015.14242

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

Short Communication

Complete Genome Sequence of Goose Parvovirus Isolated from *Anser cygnoides* in China

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KVFD-2015-14242 Received: 18.08.2015 Accepted: 13.10.2015 Published Online: 13.10.2015

Abstract

In the present study, we sequenced and analyzed the complete genome of goose parvovirus isolates derived from *Anser cygnoides*. Comparing with other GPV isolates, the results indicated that the event may cause by GPV direct infection to *Anser cygnoides*. These findings suggest that the *Anser cygnoides* could serve as a potential host for GPV and enable us to understand the molecular characteristics and evolutionary diversity of GPV.

Keywords: *Goose parvovirus, Anser cygnoides, Genome, Analysis*

Çin'de *Anser cygnoides*'ten Izole Edilen Kaz Parvovirusu'nun Tam Genomik Dizilimi

Özet

Bu çalışmada, *Anser cygnoides*'ten elde edilen kaz parvovirusu genomunun dizilimi yapıldı ve analiz edildi. Diğer GPV izolatlarıyla kıyaslandığında, sonuçlar olayın doğrudan *Anser cygnoides*'e bağlı bir GPV enfeksiyonuna bağlı olabileceğini gösterdi. Bu bulgulara göre, *Anser cygnoides*'in GPV için potansiyel bir konakçı (ev sahibi) olarak hizmet edebildiği ve GPV'nin moleküler özelliklerinin ve evrimsel çeşitliliğinin anlaşılmasını sağlayabildiği kanısına varıldı.

Anahtar sözcükler: *Kaz parvovirusu, Anser cygnoides, Genom, Analiz*

INTRODUTION

Goose parvovirus (GPV), also named Derzsy's disease virus, is the causative pathogen that results in high mortality and morbidity in domestic ducklings and goslings under the age of three weeks, however not cause fatal diseases with adult birds [1,2]. Goose parvovirus described as 20-22 nm in diameter with an icosahedral outer appearance, which belonging to the Anseriform dependoparvovirus 1 species of the dependoparvovirus genus, under the *Parvoviridae* family [3].

The goose parvovirus genome is approximately 5100 nucleotides long with single-stranded DNA and no helper viruses are required for virus replication in host cells. The GPV genome contains two major open reading frames (ORFs). The left ORF encodes the non-structural protein (NS), while the right ORF encodes for three capsid proteins VP1, VP2 and VP3, derives from the same gene by alternate splicing. The VP2 and VP3 genes shared the same carboxyl terminal portion of VP1 gene [4].

In this study, we sequenced and analyzed the complete genome of goose parvovirus strain FJ01 that was isolated from *Anser cyanide's*. Derivation of the genomic sequences of goose parvovirus from *Anser cygnoides* implied that *Anser cygnoides* might serve as a potential host for goose parvovirus, which provides insights with the genome characterization and etiology for goose parvovirus.

MATERIAL and METHODS

Case History

A commercial *Anser cygnoides* flock was experienced elevated mortality associated with lethargy, weight loss,

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dysphagia, ataxia, and watery ocular discharges at October 2012 in Fujian Province, China. Most of the sick *Anser cygnoides* goslings were younger than 21-day-old and the mortality was nearly 40%. To determine the pathogens which were responsible for the disease, we collected the liver, spleen and intestinal cavity from the succumbed *Anser cygnoides* goslings and subjected to PCR (RT-PCR) method to test all possible classical endemic and emerging viruses outbreaks in goose flocks such as goose parvovirus, goose herpesvirus, goose adenovirus, avian paramyxovims type I, avian influenza virus, avian Tembusu virus, goose circovirus and goose reovirus.

Virus Isolation and DNA Extration

The tissues were homogenized in sterile phosphate-buffered saline (PBS, pH7.2) and centrifuged at 8.000 rpm for 30 min at 4°C. Supernatants were filtered through 0.45 μm and 0.22 μm filters (Merck KGaA, Darmstadt, Germany) and stored at -80°C prior to virus isolation. The goose parvovirus isolated from *Anser cygnoides*, designated FJ01, was isolated by suspension into the allantoic cavities of 10-day-old goose embryos. The embryos died 84-120 h post inoculation and dead embryos were observed. All goose embryos were purchased from commercial goose farms, which had with no history of GPV exposure or vaccination with attenuated GPV vaccine. The viruses were harvested after three passages of infected goose embryos. DNA was extracted using the Viral DNA Kit (Omega Bio-Tek, GA, USA) according to the manufacture's instructions.

Genome Sequencing

The GPV strain FJ01 genome were amplified by polymerase chain reaction (PCR) according with the similar strategy described previously [3], with overlapped fragments encompassed the completely GPV genome. The PCR products were purified using the Gel Extraction Kit (Omega Bio-Tek, GA, USA) and then ligated into the pBackZero8-T vector with cloning kit (Takara, Dalian, China). In each case, five positive clones were randomly selected and sequenced (Sangon Biotech, Shanghai, China) to both directions using an ABI model 3730 automatic DNA sequencer (ABI, CA, USA). We connected the overlapped gene fragments into the FJ01 full-length genome with software Lasergene (DNAStar, v7.1, Madison, WI, USA).

Genomic Sequence Alignment and Phylogenetic Analysis

For comparative studies, the complete genome sequences of GPV virulent strains and attenuated vaccine strains were retrieved from GenBank *(Table 1)*. Sequence comparison and genomic homology was determined using the ClustalW method. Phylogenetic analysis was performed by MEGA 6.0 using the neighbor-joining method

Table 1. *Virus descriptions and GenBank accession numbers for sequences used in this study*
Tablo 1. *Çalışmada kullanılan dizilimler için virus tanımlamaları ve GenBank girişi*

Accession Number	Strains	Genome Size (nt)	ITR	Origin[a] (Province, Country)	Host	Date	Reference
EU583389	82-0321V	4980	381	TW, China	Goose	**A**	[5]
EU583390	82-0321	5050	416	TW, China	Goose	1982	[5]
EU583391	06-0329	5054	418	TW, China	Goose	2006	[5]
EU583392	VG32/1	5104	443	Germany	Goose	**B**	[5]
HQ891825	GDaGPV	5106	444	GD, China	Goose	1978	[6]
JF333590	SH	5106	444	SH, China	Goose	2009	NA
KC178571	Y	5106	444	AH, China	Muscovy duck	2011	[7]
KC184133	E	5125	443	AH, China	Goose	2012	NA
KC478066	SHFX1201	5050	416	SH, China	Swan	2012	[9]
KC996729	SYG61v	5102	442	JS, China	Goose	**C**	[8]
KC996730	YZ99-6	5046	414	JS, China	Goose	1999	NA
KM272560	LH	5047	414	JS, China	Goose	2012	[10]
KR029617	G7	5106	444	FJ, China	Muscovy duck	2013	[3]
KT232256	FJ01	5104	443	FJ, China	*Anser cygnoides*	2012	TS
U25749	B	5106	444	Hungary	*Anser anser*	-	[4]
U22967	FM	5132	457	Hungary	*Cairina moschata*	-	[4]

[a] *Origin abbreviations: Anhui, AH; Fujian, FJ; Guangdong, GD; Jiangsu, JS; Shanghai, SH; Taiwan, TW;* ***A:*** *82-0321V live vaccine strains, which was derived from 82-0231 after 64 passages in Muscovy duck eggs, two in geese fibroblasts, and four in duck embryos;* ***B:*** *means VG32/1 live vaccine strains, which can purchase from Impfstoffwerk Dessau-Tornau GmbH (Rodleben, Germany);* ***C:*** *means SYG61v live vaccine strains, which were used in mainland China;* ***TS,*** *this study;* ***NA,*** *not available;* ***-,*** *unknown*

with the maximum-likelihood model. Bootstrap scores were generated from 1.000 replicates.

RESULTS

Genomic Organization

The GPV strain FJ01 genome is 5104 nucleotides (nt) in length and has a basic structure similar to previously reported GPV genomes, belonging to a complete replication component virus with 29.12% A, 24.08% G, 23.55% T and 23.24% C. The genome is flanked on the 5′ and 3′ terminal ends by 443 nt inverted terminal repeats (ITRs) regions. The distal 405 nt of each repeat form a U-shaped hairpin structure consisting of a 181 base-pair double-stranded "stem" region and a 43 nt bubble region, which serve as the origin of GPV replication. The sequenced genome has a NS coding region of 1884 nucleotides and a VP1 coding region of 2199 nucleotides *(Fig. 1)*.

The complete genome of GPV strain FJ01 isolated from *Anser cygnoides* had been submitted to GenBank under accession number KT232256.

Comparison of the Genomic Sequences and Phylogenetic Analysis

To identify the nucleotide sequences of goose parvovirus isolated from *Anser cygnoides*, we compared strain FJ01 with the GPV virulent and attenuated strains which were retrieved from GenBank *(Table 1)*. The nucleotide homology of FJ01 to virulent strains varies from 94.2% to 99.9%. Compared with the attenuated strains SYG61v, 82-

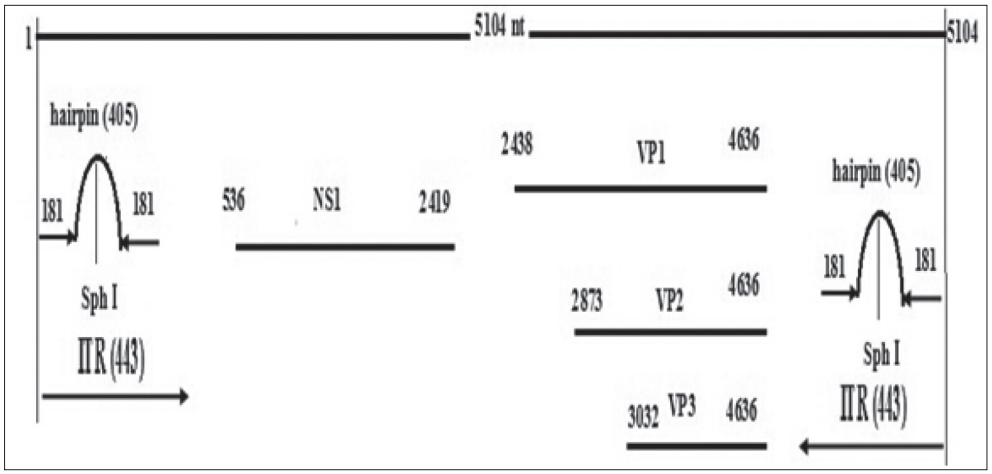

Fig 1. A graphical representation of the genome organization of FJ01 strain. ITR means inverted terminal repeat. Sph□means there has a Sph□ enzyme cutting site (GCATGC) in the bubble. Numbers in the graph mean the position in the genome

Şekil 1. FJ01 suşunun genom organizasyonunun grafik sunumu. ITR invert terminal tekrarını gösterir. Sph□, baloncukta Sph□ enzimi keskin tarafı (GCATGC)'ye sahip demektir. Grafikteki sayılar genomdaki pozisyonu tanımlamaktadır

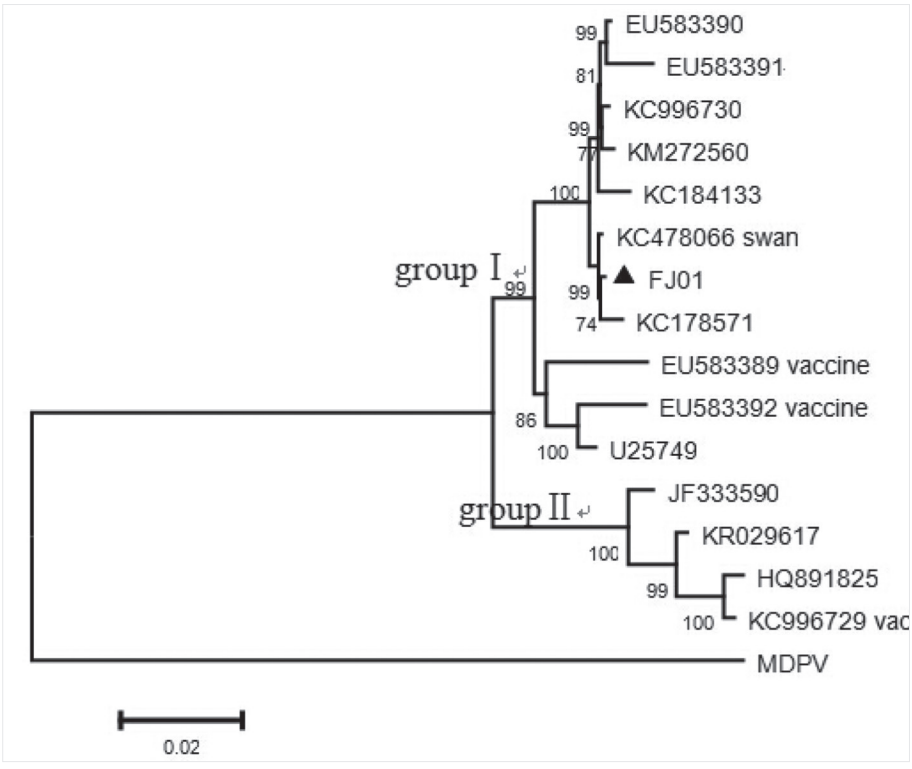

Fig 2. Phylogenetic tree based on the genome of GPVs isolates
The tree generated using the Neighbor-Joining method as described in Materials and Methods. Bootstrap scores were generated from 1000 replicates. MDPV denote the MDPV isolates FM

Şekil 2. GPV izolatları genomu esaslı filogenetik ağaç
Ağaç, Materyal ve Metot'ta tanımlanan Komşu-katılımı yöntemi kullanılarak oluşturuldu. Bootstrap skorları 1000 kez tekrarla oluşturuldu. MDPV, MDPV FM izolatları demektir

0321V and VG32/1, the FJ01 shares nucleotide identity of 94.3%, 96.9% and 96.9%, respectively.

A plylogenetic tree was generated based on the complete GPV genome nucleotide sequences retrieved from GenBank. The Muscovy duck parvovirus (MDPV) strain FM was used as MDPV respective strain (GenBank accession number U22967). The plylogenetic tree contains two groups, which indicates that at least two types of GPV virulent viruses are circulating in China. The GPV strain FJ01 appears closer to the GPV isolates SHFX1201, which was isolated from a swan in Shanghai, China *(Fig. 2)*.

DISCUSSION

The swan goose *Anser cygnoides* is confined to the Eastern Palearctic, which was bred in China, Mongolia and parts of Russia. There were very few researches about the virus disease outbreaks to the swan goose *Anser cygnoides* species reported previously. Previous observation and research indicated that the goose parvovirus can infect both goslings and ducklings. Then, goose parvovirus was isolated from swans in China in 2012 [9]. Earlier studies have confirmed that goose parvovirus genome remained genetically stable in the field. However, based on the phylogenetic analysis in the present study, the GPV isolates can be divided into two major groups (Group I and Group II), which means more genetic diversity between GPVs. In our study, the GPV strain FJ01 showed highest similarity to those of GPV strains, which suggested the emergence of GPV strain FJ01 was more likely resulted from a direct GPV infection.

Wang recently reported GPV strain MDGPV/PT shared genetic recombination with Muscovy duck parvovirus in the NSP gene [11], and their research indicated that inter-genotype recombination within the VP2 gene cluster contributes to the genetic diversity of the VP2 genes of Taiwanese GPV field strains [12]. Simplot program was used to analyze the recombinant event of the GPV strain FJ01 with the vaccine strain SYG61v and virulent MDPV strain FJM5 in China [3], with no recombination observed (data not shown).

In summary, this report presents the first evidence that goose parvovirus can infect *Anser cygnoides* directly. Bivalent attenuated vaccine against GPV had been used to prevent GPV infections in geese and duck flocks in China for decades, however whether attenuated vaccine against GPV can be used for *Anser cygnoides* flocks to prevent GPV infection needs further investigation.

Acknowledgments

This work was supported by the China Agriculture Research System (CARS-43), Special Fund for Agro-scientific Research in the Public Interest (201003012), the Chinese Natural Sciences Foundation (U1305212), the Hundred-Talent Program (Fujian Academy of Agriculture Sciences) (YC2015-12). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Kafkas Univ Vet Fak Derg
22 (1): 159-162, 2016
DOI: 10.9775/kvfd.2015.14082

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
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Case Report

Microbiological Analysis of Acute Mastitis in a Van Cat

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Article Code: KVFD-2015-14082 Received: 21.07.2015 Accepted: 29.09.2015 Published Online: 02.10.2015

Abstract

In this case report, a 7-year-old female Van cat of 2.3 kg weight, bred and given birth in the *Van Cat Research Center*, is presented. There was a two day history of inappetence and fatigue, along with lying without movement for long. Our physical examination showed an acute inflammation accompanied by swelling, severe pain and hardness in the left caudal thoracic mamma, in addition to a bruise starting from the nipple and extending to the rest of the tissue. The cat was diagnosed with mastitis. *Escherichia coli* and *Staphylococcus simulans* were identified after the microbiological analysis of the milk from the affected breast.

Keywords: *Van cat, Acute mastitis, Microbiology, mecA*

Bir Van Kedisinde Görülen Akut Mastitisin Mikrobiyolojik Analizi

Özet

Bu olgunun materyalini Van Kedisi Araştırma Merkezi'nde yetiştirilen 7 yaşında 2.3 kg ağırlığında yeni doğum yapmış bir Van kedisi oluşturmuştur. Kedinin son iki gün içerisinde iştahsız olduğu, keyifsiz ve halsiz bir şekilde uzun süre yattığı bilgisi ile birlikte yapılan muayenede sol ikinci memede akut seyirli bir yangının olduğu memenin şiştiği, serleştiği, şiddetli ağrı ve meme ucundan başlayarak dokuya doğru yayılmış tarzda morarmanın olduğu izlendi. Hastaya akut mastitis tanısı konuldu. Sütte yapılan mikrobiyolojik muayene sonucunda *Escherichia coli* ve *Staphylococcus simulans* izole ve identifiye edildi.

Anahtar sözcükler: *Van kedisi, Akut mastitis, Mikrobiyoloji, mecA*

INTRODUCTION

Mastitis is an uncommon reproductive disease in cats and dogs. Although it is encountered less frequently in carnivores, since it causes mortality of the offspring, it is considered as an important condition [1]. The disease may develop due to direct infection, trauma or presence in a contaminated environment [2]. The most common causative agents of mastitis in cats are *Escherichia coli, Staphylococcus* spp. and *Streptococcus* spp.[3]. These species can normally live in the cutaneous and mucosal floras of cats and dogs. *Staphylococcus* spp. are primarily found in the anterior nasal cavity and skin [4,5]. Nonetheless, it can also be isolated from the oral cavities [6,7]. In one study, authors isolated coagulase-positive *Staphylococcus intermedius* and *Staphylococcus aureus* from the skin of healthy cats [8]. However, the most commonly isolated coagulase-negative species in healthy cats is *Staphylococcus simulans* [6].

To our knowledge, there is not much information about feline mastitis in the literature. Feline mastitis occurs in the peracute, acute and mild chronic forms [9]. The most common symptoms of mastitis in cats are tissue hardness, redness, and swelling, as well as colorless mammary lobe and changes in milk secretion. Affected cats may exhibit unwillingness to breastfeed, fatigue, depression, fever and inappetence. The mammary tissue may develop abscess or gangrene [10].

The diagnosis of mastitis is reached based on the history, clinical and microbiological findings, as well as changes in the milk [11]. Many of the mastitis cases can be treated on an outpatient basis [12]. The efficacy of the selected antibiotic agent depends on factors such as susceptibility, transition to the mammary tissue, and milk concentration. Moreover, these antibiotics should not be harmful to the infants via breastfeeding [1]. In cases with necrotic mammary tissue, the infants should be distanced from the mother and fed elsewhere [9,12].

In this case report, it is aimed to contribute to the literature by providing information on clinical signs, microbiological analysis and treatment of mastitis in Van cats.

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CASE HISTORY

In this case report, a 7-year-old female Van cat of 2.3 kg weight, bred and given birth in the Van Cat Research Center, is presented. She gave birth to 4 kittens 10 days ago and has a 2-day history of inappetence and fatigue, along with lying without movement and showing reluctance to breastfeed her kittens. The first physical examination revealed mild depression and significant indifference to surroundings. There was an inflammation with acute course accompanied by severe pain in the left caudal thoracic mamma. The mamma was swelled, hardened, and having a bruise starting from the nipple and extending to the mammary tissue *(Fig. 1)*. The milk was not easily coming out and it was of yellowish color with purulent character. In the light of these findings, the Van cat was diagnosed with acute mastitis. The mammary tissue was cleaned with 70% alcohol for the micrologiological assessment and the purulent content sampled by 2 sterile swabs was sent to the Veterinary Microbiology Department under cold chain. Isolation and identification were carried out by culturing in 5% sheep blood agar (Salubris), MacConkey agar (Salubris), and eosin methylene blue agar (Salubris). The suspected colonies were tested for Gram staining, catalase and oxidase reactions.

The identification and antibiotic susceptibility testing were evaluated by the BD Phoenix automated Microbiology systems (Becton Dickinson, US), using Gram-positive and negative panels. *E. coli* and *S. simulans* were identified from the milk sample. The antibiotic susceptibility test results for these two bacteria are shown in *Table 1*.

The application of polymerase chain reaction (PCR) method using oligonucleotide primer series (F-5'-AAAATCG ATGGTAAAGGTTGGC-3'; R-5'-AGTTCTGCAGTACCGG

ATTTGC-3') revealed that *S. simulans* carried *mecA* gene. DNA extraction was performed with a colon-based ready-

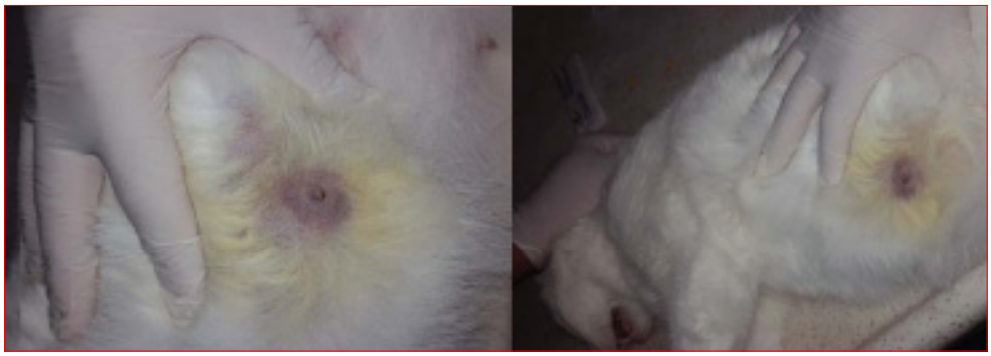

Fig 1. Acute mastitis in a Van cat

Şekil 1. Akut mastitis görülen Van Kedisi

Table 1. *Antibiotic susceptibility test results*
Tablo 1. *Antibiyotik duyarlılık testi sonuçları*

Agent	Antibiotics	ESCCOL MIC/Conc	SIR	Agent	Antibiotics	ESCCOL MIC/Conc	SIR
E. coli	Amikacin	≤ 8	S	*S. simulans*	Ampicillin		R
	Ampicillin-Sulbactam	≤ 4/2	S		Cefazolin	≤ 2	S
	Cefazolin	≤ 2	S		Clindamycin	≤ 0.25	S
	Cefepime	≤ 1	S		Daptomycin	≤ 0.5	S
	Cefoperazone-Sulbactam	≤ 0.5/8	S		Erythromycin	≤ 0.25	S
	Cefoxitin	≤ 4	S		Linezolid	≤ 1	S
	Ciprofloxacin	≤ 1	S		Oxacillin	≤ 0.25	S
	Ceftriaxone	≤ 1	S		Penicillin G		R
	Ertapenem	≤ 0.25	S		Rifampin	≤ 0.5	S
	Gentamisin	≤ 2	S		Teicoplanin	≤ 1	S
	Imipenem	≤ 0.5	S		Tetracycline	≤ 0.5	S
	Levofloxacin	≤ 1	S		Trimethoprim-Sulfamethoxazole	≤ 1/19	S
	Meropenem	≤ 0.5	S		Vancomycin	≤ 1	S
	Piperacillin- Tazobactam	≤ 4/4	S				
	Ticarcillin-Clavulanate	≤ 8/2	S				
	Trimethoprim-Sulfamethoxazole	≤ 1/19	S				

to-use kit (DNA mini kit, Qiagen, Hilden, Germany) for DNA extraction from the isolates growing from the tryptic soy agar (TSA, Plasmatec). Ready-to-use amplification mixture was employed in the PCR analysis and the primer binding temperature was kept at 55°C during the amplification process. The amplification products were subjected to gel electrophoresis, stained in 1xTBE buffer containing 0.5 µg/ml ethidium bromide, and the bands were viewed by UV transilluminator. As a result of PCR analysis, *S. simulans* was demonstrated not contain *mecA* gene.

The cat was put on intramuscular cefazolin therapy at 15 mg/kg dose for 5 days, to which *E. coli* and *S. simulans* are known to be highly susceptible. Sefapir® (Cephapirin sodium 200 mg, trypsin 10 mg, chymotrypsin 10 mg) was applied at 11 mg/kg dose twice every other day via intramammary route after squeezing the milk out of the related mammary tissue.

DISCUSSION

Van cats are acknowledged as a protected cat species. The university that this study accomplished has been carrying out protective projects for the Van cats in a research center. To protect them, we aim to perform a successful genetic breeding, as well as producing healthy generations. Therefore, it is important to both conserve the genetic material and fight with possible diseases of Van cats. In this regard, there are several studies on Van cats in the literature [13,14]. However, our patient is the first mastitis case in a Van cat. To our knowledge, the literature on feline mastitis cases is very scarce. Mastitis can be diagnosed by evaluating several clinical, bacteriological, cytological, hematological, and pathological parameters together [10]. It has been reported that feline mastitis may have an acute or chronic course, and that mortality can be seen in acute mastitis [9,10].

Moreover, despite being uncommonly seen, mastitis should be deemed as an important disease because the kittens may die eventually due to loss of the mother. Similar to the studies in the literature, we determined symptoms of mastitis such as hardness, swelling, and redness of the mammary tissue, as well as local pain [10,15]. Moreover, we observed behavioral symptoms such as inappetance, depression, and fatigue which are known to be common features encountered in feline mastitis cases. In addition, in a case report, Wilson (10) noted bruises turning into necrosis, as well. Similarly, Ververidis et al (3) induced experimental mastitis in female dogs and reported the same clinical symptoms along with a purulent milk secretion. This report stated that the pathogenesis of mastitis can be agreeable with the findings of previous reports.

The microbiological analyses showed that the causative pathogenes of the acute mastitis in this Van cat were *E. coli* and *S. simulans*. In his study, Wilson (10) isolated *E. coli and S. aureus* in a cat with gangrenous mastitis. Lilenbaum et al.[11] isolated *Staphylococcus* species in samples collected from the skin of healthy cats which are known to be the causative agents of certain infections. Sura et al.[16] reported *E. coli* as the causative agent in a cat with acute necrotizing pneumonia. In the light of these studies, the represented study is the first report of *E. coli* and *S. simulans* as the main causative agents of a feline mastitis case.

The susceptibility of the bacteria isolated from cats and dogs to certain antibiotics has been reported by various studies [11,17]. Lilenbaum et al.[11] observed varying degrees of resistance to different antibiotics in *Staphylococcus* spp. isolated from healthy cats. Moreover, 6 of the 11 isolated *S. simulans* were found to be methicillin-resistant. Similarly, Wilson (10) reported varying susceptibility to different antibiotics in a cat diagnosed with gangrenous mastitis. In our case, among the isolated pathogens, *E. coli* was susceptible to all the antibiotics, however, *S. simulans* was observed to be resistant to penicillin G and ampicillin. As a result of PCR analysis, *S. simulans* was demonstrated not contain *mecA* gene.

In parallel with the other studies in the literature, our case report shows that antibiogram and PCR analysis is a very important test in clinical mastitis cases.

In order to prevent bacteria from developing resistance to antibiotics in Van cats with mastitis, multicenter epidemiologic studies should be carried out to follow the antibiotic resistance status of pathogens and bacterial flora.

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22 (1): 163-164, 2016
DOI: 10.9775/kvfd.2015.14146

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

Letter to the Editor

Bronchial Foreign Body (Olive Pit) in a Puppy (Yavru Bir Köpekte Bronşial Yabancı Cisim [Zeytin Çekirdeği])

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Article Code: KVFD-2015-14146 Published Online: 24.08.2015

Dear Editor,

Many inhaled or aspired vegetal foreign bodies can be encountered in veterinary practice [1-4]. The most common ones are grass inflorescences that these foreign bodies migrate from trachea to the pulmonary parenchyma [1]. Although radiology and the other advanced techniques present diagnose of the foreign bodies [1,3,4], bronchoscopy is the best of all for diagnoses and removal of the foreign bodies [1] instead of the invasive approach by thoracotomy plus bronchotomy reported [2]. This letter reports to olive pit as an inhaled novel foreign body and its removal from the left main bronchus by bronchoscopy.

A 2 month-old, Belgium Malinois breed male puppy was presented with complaint of the coughing and vomiting reflexes following an olive eating. Clinically inspiratory dyspnea, thoracoabdominal respiration, intermittent vomiting and hemoptysis were determined. When the puppy laid down the lateral recumbent, dyspneic respiration was increased. Thus, respiration movements were closely monitored. The other vital parameters of the puppy were normal, and laboratory analysis results of the blood sample taken from peripheral vein were also within normal reference rates. Aspiration pneumony was suspected and thoracic radiographs were taken immediately. In lateral radiograph, an olive pit-shape foreign body back of the carina was clearly observed in the tracheal lumen *(Fig. 1A)*. Dexamethasone 0.25 mg/kg iv (Dekort®, Deva, Istanbul), amoxicillin clavulanate 15 mg/kg, im (Amoklovin®, Deva, Istanbul) and n-acetylcysteine 10 mg/kg, iv (Assist®, Bilim ilac, Istanbul) were given parenterally to relieve the respiration and prevent the secondary pulmonary infection. After sedation with xylazine HCl (Alfazine®, Egevet, İzmir) (1 mg/kg, im), 1/1 combination of the ketamine HCl (Alfamine®, Egevet, Izmir) (10 mg/kg) plus diazepam (Diazem®, Deva, Istanbul) (0.5 mg/kg) was administered intravenously for general anesthesia and maintenance. Intubation was provided 6.5 no endotracheal tube and 5.2 mm diameter flexible bronchoscope (Karl Storz®, Germany) was inserted through the inside of the intubation tube. Intraluminal endoscopy revealed the olive pit in the left main bronchus *(Fig. 2)*. The fluids in the luminal passage were aspirated and a cage-shaped foreign body forceps was inserted to the lumen. The olive pit was catched and then removed *(Fig. 3)*. The lateral thoracic radiograph was taken as control *(Fig. 1B)*. Antibiotherapy (amoxicillin clavulanate) and muco-lytic (n-acetylcysteine) applications were continued to prevent the pulmonary infections and bronchial fluids medically. The puppy was discharged from the clinics without any respiration complication at 3th days after removal of the foreign body.

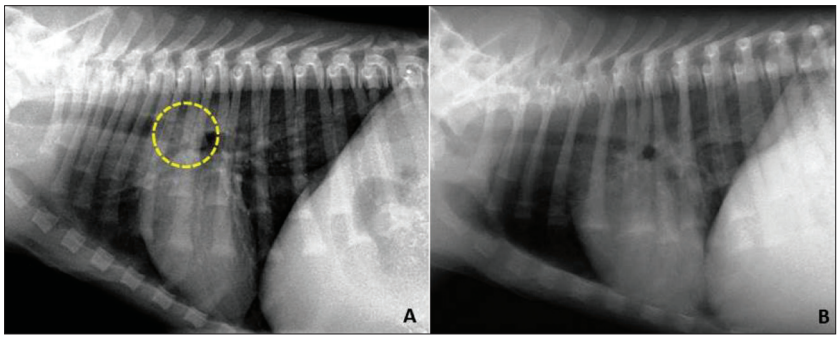

Fig 1. The lateral thoracic radiographs of the puppy. **A-** The yellow circle points out the localization of the olive pit in the tracheal lumen. **B-** Post-endoscopic control radiograph of the thorax shows the minimal bronchial pattern pulmonary influence

Şekil 1. Yavru köpeğin lateral toraks radyografisi. **A-** Sarı çember trakeal lumen içindeki zeytin çekirdeğinin lokalizasyonunu belirtmektedir. **B-** Toraksın endoskopi sonrası control radyografisi minimal bronşial desen pulmoner etkilenimi göstermektedir

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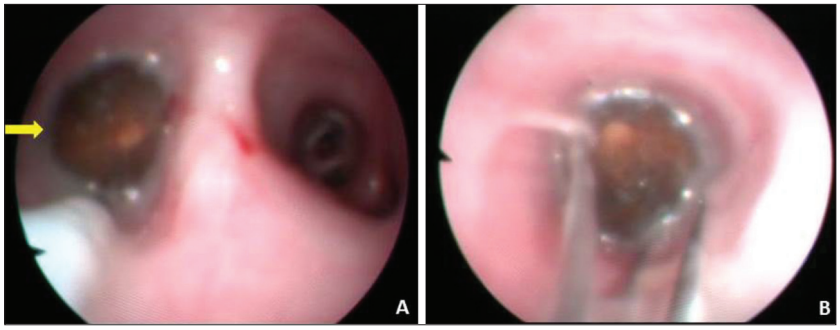
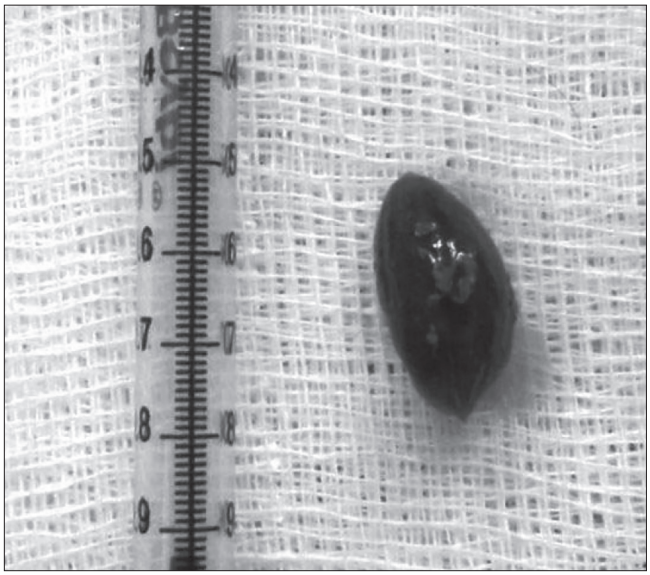


Fig 2. A- Bronchoscopic appearance of the olive pit *(arrow)* in the left main stem bronchus, and **B**- Its removal with cage foreign body forceps

Şekil 2. A- Sol ana bronş içindeki zeytin çekirdeğinin *(ok)* bronkoskopik görünümü ve **B**- kafes yabancı cisim forsepsi ile çıkarılımı

Fig 3. The removed olive pit

Şekil 3. Çıkarılan zeytin çekirdeği



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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.
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Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır. Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Giriş, Materyal ve Metot, Bulgular, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşur ve toplam (metin, tablo, şekil vs dahil) 12 sayfayı geçemez. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler dışında Türkçe makale yazım kurallarında belirtilen diğer bölümlerden oluşur. Türkçe ve yabancı dilde özetlerin her biri yaklaşık 200±20 sözcükten oluşmalıdır.
Kısa Bildiri, konu ile ilgili yeni bilgi ve bulguların bildirildiği fakat orijinal araştırma olarak sunulamayacak kadar kısa olan yazılardır. Kısa bildiriler, orijinal araştırma makalesi formatında olmalı, fakat özetlerin her biri 100 sözcüğü aşmamalı, referans sayısı 15'in altında olmalı ve 6 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.
Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 4 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.
Gözlem (Olgu Sunumu), uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri Giriş, Olgunun Tanımı, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşmalı ve 4 sayfayı geçmemelidir.
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