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Αριθμός Άδειας 1

Ε.Κ.Ε. Πατησίων 158, 112 57 Αθήνα
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Εκδότης
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Mailing address
J Hellenic Vet Med Soc (M. Zafiropoulou)
Hellenic Veterinary Medical Society
Patission 158, 11257 Athens, Greece

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Update: July 2019
Ενημέρωση: Ιούλιος 2019

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ΕΥΡΩΠΑΪΚΑ ΚΟΛΕΓΙΑ ΕΙΔΙΚΕΥΜΕΝΩΝ ΚΤΗΝΙΑΤΡΩΝ

				Number of specialist veterinarians active in Greece Αριθμός ειδικευμένων κτηνιάτρων εργαζόμενων στην Ελλάδα
1		ECAR	European College of Animal Reproduction	2
2		ECAWBM	European College of Animal Welfare and Behavioural Medicine	2
3		ECAAH	European College of Aquatic Animal Health	3
4		ECBHM	European College of Bovine Health Management	3
5		ECEIM	European College of Equine Internal Medicine	0
6		ECLAM	European College of Laboratory Animal Medicine	0
7		ECPHM	European College of Porcine Health Management	3
8		EPVS	European College of Poultry Veterinary Science	4
9		ECSRHM	European College of Small Ruminant Health Management	12
10		ECVAA	European College of Veterinary Anaesthesia and Analgesia	1
11		ECVCN	European College of Veterinary Comparative Nutrition	0
12		ECVCP	European College of Veterinary Clinical Pathology	1
13		ECVD	European College of Veterinary Dermatology	3
14		ECVDI	European College of Veterinary Diagnostic Imaging	2
15		ECVECC	European College of Veterinary Emergency and Critical Care	0
16		ECVIM-ca	European College of Veterinary Internal Medicine-companion animals	0
17		ECVN	European College of Veterinary Neurology	2
18		ECVO	European College of Veterinary Ophthalmology	0
19		ECVP	European College of Veterinary Pathology	0
20		ECVPH	European College of Veterinary Public Health	5
21		ECVPT	European College of Veterinary Pharmacology and Toxicology	1
22		ECZM	European College of Zoological Medicine	1
23		ECVS	European College of Veterinary Surgery	1
24		EVDC	European Veterinary Dentistry College	0
25		EVPC	European Veterinary Parasitology College	3

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ΕΛΛΗΝΙΚΗ ΚΤΗΝΙΑΤΡΙΚΗ ΕΤΑΙΡΕΙΑ (ΕΚΕ)
HELLENIC VETERINARY MEDICAL SOCIATY (HVMS)

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History of the Hellenic Veterinary Medical Society

The Hellenic Veterinary Medical Society (HVMS) is one of the oldest Scientific Societies in our Country. It was founded in 1924 and its first scientific journal was published in 1926. Prompter, Founder and Animating Spirit of HVMS was the General Ioannis D. Petridis (1870-1947), first President and for many years Honorary President of the HVMS. Among the 49 founding members of the HVMS there was also the memorable professor Konstantinos Livadas, the founder of the Veterinary School of the Aristotelian University in Thessaloniki. In spite of the disagreements, the HVMS contributed greatly to the foundation of Veterinary School.

During that time there was only one Scientific Society in Greece, the Medical Society of Athens, which was founded in 1835 and published its first scientific journal in 1922. The HVMS dealt not only with scientific but also with professional topics, like the establishment of the invoices for the veterinarians' payment, taxes, insurance etc. Also, at that period, the accession of the Veterinary Branch in the Hygienists' Pension and Self Insurance Treasury (TSAY) was achieved.

The first post-war assembly of the HVMS took place in the private medical office of Petros Kiappe, on Peta Street in Athens. With its post-war first president Konstantinos Melanidis, the HVMS has been working by implementing its old memorandum of association and has been located in the premises of the Veteri- nary Microbiological Institute of Votanicos, from where all mem- bers of the Governing Board and the Editorial Board of the Journal of the HVMS, were coming from. There, the first «nucleus» of the Library of the HVMS, has been created. That is the reason, this second period of the HVMS successor of the «Petridis period», used to be called «Votanikos period, 1944-1965».

Because HVMS's income was very small, it will remain homeless for many years. Looking for a meeting place the HVMS will find positive response from several services and societies (State Veterinary Offices, Greek Chemical Society, Hellenic Agricultural Society, Medical Society of Athens, Insti- tute of Agricultural Studies, State Veterinary Service of Athens, National Organization of Greek Handwork), which during the following years are going to offer its premises, while in the mid 1958 and for a short period, depending on its financing capabil- ities, the HVMS will rent its own room.

In 1944, the HVMS writes down its first post-war Member Book and in 1948 has already acquired its first 74 regular mem- bers. Also, HVMS is actively working with scientific subjects during regular meetings and public seminars, analyzing current veterinary issues, members' proposals and so on. On 29th May 1947 Mr Petridis presented in the Academy of Athens an issue for veterinary science and its contribution to the progress of the agricultural production and safeguard of Public Health. Also, it should be pointed out, that because there was no professional body, the HVMS is also dealing with issues related to the exe- cution of the veterinary profession.

Furthermore, the role of the HVMS has been determinative on the decision making of the Ministry of Agriculture on veter- inary legislation, on the organization of the Veterinary Service in the Ministry of Agriculture as well as on livestock topics. In the decade of 30s the Supreme Veterinary Advisory Council was created mainly dealing with scientific issues and other aims like promotion, publicity and consolidation of the veterinary science and the veterinary profession in our country and internationally.

The Hellenic Veterinary Medical Society publishes a quarterly scientific journal called Journal of the Hellenic Veterinary Medical Society (J Hellenic Vet Med Soc), as well as other scientific publications, organizes Congresses, Symposiums, Meetings, Lectures etc and generally and almost exclusively it has undertaken for life the Continuing Education of the Greek veterinarians and the students of the two Veterinary Schools.

Nowadays, the Hellenic Veterinary Medical Society is governed by a 9 member Governing Board which is elected every 3 years and has 3 branches:

- **Branch of Companion Animals**
- **Branch of Food Hygiene and Public Health**
- **Branch for Farm Animals**

The HVMS collaborates with the Supreme Educational Foundations, the Technological Educational Institutes, the Veterinary Services, and the Veterinary Associations as well as with Scientific Societies and the Greek and Foreign Chambers.

- **The HVMS is member of the:**
- **Worldwide Veterinary Society**
- **Worldwide Veterinary Society for Companion Animals**
- **Federation of European Veterinary Societies for Compan- ion Animals (founding member)**
- **Veterinary Society of the Balkan and the Black Sea (found- ing member)**

The HVMS has a total of 1220 members many of which have been distinguished in the scientific field (University Profes- sors, Researchers), in the Public Administration, in the Army as well as in the Professional Veterinary Societies and Chambers, in Greece and abroad.

Since 29 May 2001, having signed the contract and since 15 December 2002 the date on which the official opening celebration took place, the Hellenic Veterinary Medical Society is housed in its private premises in a beautiful and majestic one-floor apartment, on the 7th floor of a building in the centre of Athens at 158, Patission street, of 265m2 area, including main lobby (14m2), secretary (13m2), lecture room (91m2), the President's office (22m2), the Governing Board meeting room & library (44m2), the kitchen (18m2), two big baths, a storage room and a large veranda. Al the actions performed for possessing this new private office for the HVMS were performed during the presidency of Dr Theodoros Cl. Ananiadis and the following Governing Board:

President:	Theodoros Cl. Ananiadis†
Vice-President:	Veniamin Albalas
General Secretary:	Athanassios E. Tyrpenou
Spec. Secretary:	Konstantinos Chandras
Treasurer:	Olga Sabatakou
Member:	Emmanuel Archontakis
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Research article
Ερευνητικό άρθρο

Effects of the physical form of diet on growth performance, ascites and sudden death syndrome incidences in broiler chickens

M. Azizian, A.A. Saki*

Department of Animal Science, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, Iran

ABSTRACT: This experiment was conducted to determine the effects of the diet physical form, Mash (M), Pellet (P) and Extruded (E) on the growth performance, carcass characteristics and metabolic disorders ascites (AS) and as well as sudden death syndrome (SDS) in the broiler chickens. In this respect, feed intake (FI), weight gain (WG) and mortality were recorded throughout the experiment and biochemical parameters, hematology and carcass characteristics were tested at 35 and 42 days of age respectively. The results showed that with the increase of the average daily weight gain (ADWG) ($p<0.01$), the relative breast weight to the carcass weight ($p<0.05$), better feed conversion ratio ($p<0.01$), the lower relative cecum weight and gizzard to the carcass weight ($p<0.05$) were observed by applying the E diet form, as compared with the other treatments. A significant increase in the average daily feed intake (ADFI) was also observed by using the P diet form ($p<0.01$). Hematological parameters including hemoglobin (Hb), hematocrit (HCT), urea, uric acid, triglyceride, the ratio of low-density lipoprotein to high-density lipoprotein (LDL/HDL), very low-density lipoprotein (VLDL), enzyme activities of aspartate aminotransferase(AST) and alanine aminotransferase (ALT) of the blood serum were lowered by applying the M treatment, as compared with the other treatments ($p<0.05$). The results indicated that the performance and carcass characteristics were improved by the E and P diet forms; also, with raising the hematology parameters in these treatments, the mortality of ascites and SDS was increased.
***Keywords*:** Ascites, Feed form, Metabolic disorder, Sudden death Syndrome

Corresponding Author:
Ali Asghar Saki, Department of Animal Science, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, Iran
E-mail address: alisaki34@yahoo.com

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INTRODUCTION

The physical forms of the diet mainly include three physical forms; mash, crumble and pellets (Banerjee, 1998).Various feed forms are the most important factors which directly influence the cost of mixed feed and production performance in the broiler (Abdollahi et al., 2013).

Mash form diets can be easily prepared, requiring relatively less energy in the process of preparation (Banerjee, 1998). Mash feed is a form of complete feed that is milled and blended so the birds could not easily separate out the diet ingredients. Feed milled on a farm is typically a mash feed. Pelleted feed involves a finer ground feed processed with heat, pressure, moisture and the added ingredients to bind pellets together. A crumble is a pellet that has been broken down to the desired size, which is usually smaller than the pelleted feed. Making a pellet or crumble diet requires extra specialized processing and equipment and this could increase the processing diet costs (Jean and Trevidy, 2000). Several studies have reported that pellets increased the body weight,feed intake and feed efficiency (Munt et al., 1995;Asha Rajini et al., 1998b).Pelleted diet enhances feed intake and growth of animals probably related to reduce feed waste, decreases energy expended for consumption, improves palatability, and reduces dustiness of feed (Abdollahi et al., 2013). In recent years the extrusion technique is extensively used in animals feed because this process has many benefits such as high productivity, efficiency and high quality of the final product (Moritz et al., 2005).The extrusion process is a short-time by high temperature, starchy and/or proteinious feed or feed materialsare cooked with the help of moisture, temperature and pressure. The results of this processing showed molecular transformation and chemical reactions within the processed feed or ingredients. The extrusion process also has positive impact in term of denaturing of harmful enzymes, inactivation of anti-nutritional factors, such as tannin, phytate, trypsin inhibitor, haemagglutinin and also sterilizesthe final product of extrusion (Bjorck and Birkhed, 1984).

Ascites and sudden death syndromes are two types of the important metabolic disorders which caused by genetics, environmental and nutritional factors (Baghbanzadeh and Decuypere, 2008). The major factor regarding mortality is related to the fast growing birds in the poultry industry; these syndromes are related to the poor performance of the heart in fast growing broilers (Korte et al., 1999). Ascites is characterized by fluid accumulation in the abdominal cavity and SDS is a sudden death in good conditions in a healthy bird without any apparent causes within 1-2 minutes, such that broilers flip over on their backs and die (Olkowski et al., 2008; Saki and Hemati, 2011).

Feed form is one the factors influencing the performance and growth rate in the broiler chickens. A direct relationship between the broiler growth rate (a high metabolic rate) with susceptibility to AS and SDS, especially when the birds are fully fed, has been reported (Proudfoot and Hulan,1982; Olkowski et al., 2008; Siddiqui et al., 2009). Several researchers have shown that blood parameters change in the body with these syndromes (Kaul and Trangadia, 2003; Saki and Hemati, 2011). Our previous studies have revealed that blood profiles can be used as an to indicator to determine and prevent the metabolic disorders, AS and SDS (Saki and Hemati, 2011). In the light of these issues, the objective of this study was to investigate the effects of the physical form of feed (M, P, and E diet forms) on the performance, carcass, and organ characteristics and to find out the relationship between agents to metabolic disorders, AS and SDS in the broiler chickens.

MATERIALS AND METHODS

Birds and housing

The experiment was carried out at Karaj-Iran. A total of 936 day-old broiler chicken (Ross, 308) with approximately 44.0±2g body weight was assigned randomly into 3 treatments: mash (M), pellet (P) and extruded (E), included 12 replicate pens per treatment. The study was conducted during 0–42 days of age. Broiler chickens were raised in floor pens (160×300 cm). Access to feed and water was *ad libitum.* The lighting schedule was 23h light/ 1h darkness at temperature 32^{o} C the first day and then reduced by 3^{o}C each week until third week and thereafter it was constant. The vaccination program was done according to local veterinarian suggestion.

Experimental diet

The ingredient chemical composition as well as the calculated composition of the diets during starter (0–14 d), grower (15–28 d) and finisher periods (29–42 d) are presented in Table 1.

The average length of feed in pellet and extruded was 1-1.5 mm at starter periods, 1.5-2.5 mm at grower periods and 2.5-3.5 mm at finisher periods. Diets were supplied at Beyza Feed Mill. Nutritional requirements were provided based on the standard recommendations (Ross, 2014).

Table 1. Composition and estimated nutrient value of diets at 0-42 days of age.

Ingredient, (%)/days	0-14	15-28	29-42
Corn	44.3	49.4	51
Soybean meal 44%	39.4	33.1	28.9
Wheat	8	10	14
Soybean oil	1.77	1.9	1.85
Other Nutrients*	6.53	5.6	4.25
Chemical composition			
Crude Protein	23.58	20.8	18.8
ME, (kcal/ kg)	2850	2920	2960
Crude Fiber	3.74	3.61	3.5
Ether Extra	3.89	4.17	4.14
Lysine	1.24	1.1	0.97
Methionine	0.61	0.55	0.49
Methionine+Cystine	0.9	0.82	0.74
Threonine	0.9	0.75	0.7
Ca	1.02	0.98	0.94
Available P	0.49	0.47	0.45

*Other Nutrients: Dicalcium phosphate, Salt, Fish meal, Vitamin and mineral premix.

Vitamin and trace elements supplied per kg of diet: vit A, 16000 IU; vit D3, 5000 IU; vit E, 60 mg; vit K3, 60 mg; vit B1, 2 mg; vit B2, 3.5 mg; niacin, 35mg; calcium pantothenate, 12.8 mg; vit B3, mg; vit B12, 0.017 mg; choline chloride, 1000 mg; folic acid, 1 mg; biotin, 0.2 mg; Mn,85 mg; Zn:70 mg; Cu:18 mg; I, 1 mg; Co, 0.6 mg, Fe, 40 mg; Se, 0.07 mg; antioxidant, 100 mg.

Data collection

Feed intake, body weight and feed conversion ratio were measured weekly, as well as mortality was recorded daily and mortality was divided into three groups (Table 4).

The blood samples were taken at 35 days of age, due to a peak in blood and biochemical parameters at this age (Malan et al., 2007; Hosseini et al., 2014). Two birds in each replicate were selected and the blood samples were collected in two tubes; one tube including ethylenediamine tetra-acetic acid anticoagulation (EDTA) and other tubes without EDTA. The first tube was used to determine hematocrit (HCT) and hemoglobin (Hb) concentration by microhematocrit and cyanmethemoglobin methods respectively by kits Zist shimi (Zist shimi, Tehran, Iran). In the second tubes, obtain serum for the determine of glucose, triglyceride, cholesterol, uric acid, high-density lipoprotein (H.D.L), low-density lipoprotein(L.D.L),very low-density lipoprotein (VLDL), Ca, K were measured by diagnostic kits ParsAzmun (ParsAzmun Co, Tehran, Iran) and Spectrophotometer (Jenway Geneva MK3, UK). Enzymes activities ALT, AST, Alkaline Phosphatase (ALP) and lactate dehydrogenase (LDH) were considered by Vitros 350 autoanalyser (New York, USA; code 680-2153) and commercial kits (Vitros Chemistry Products, Ortho-Clinical Diagnostics, Johnson Company, New York, USA).

Broiler chickens were eviscerated and separated into different parts for assessment of carcass characteristics at 42 days of age. Weights were expressed as a percentage of the body weight, thus obtaining the relative weight of organs.

Statistical analysis

A completely randomized design (CRD) and the GLM procedure of SAS 9.1 software (SAS, 2009) by one-way analysis of variance (ANOVA) were used for each phase (1-14 d, 15-28 d and 29-42 d) and for the whole experimental period (1-42 d). Differences between treatments means were compared with Duncan's test. All significance was based on a P-value equal to 0.01 and 0.05. Mortality analyses were performed based on Chi-Square.

RESULTS

The effects of the diet physical form on the performance in the broiler chickens are shown in Table 2. The increase of the ADWG during different weeks ($P<0.01$) and better FCR at these weeks ($P<0.01$) have shown by the E diet form in the broiler chickens. Average daily feed intake was increased by the P diet form at 2- 5 weeks of age ($P<0.01$). This indicated that the performance of the broiler chickens was strongly influenced by the physical form of the diet. No significant differences were observed in ADWG at 2-5 weeks of age between the P and E diet form in the broiler chickens (Table 2).

The effects of the feed form on hematology parameters and enzyme activities are presented in Table 3. There were no significant differences in the levels of cholesterol, H.D.L, L.D.L, ALP, LDH, Ca, K and glucose serum in broilers. The highest values of the hemoglobin and the hematocrit and the serum level of the urea, uric acid, triglyceride, ratio of the Low-density lipoprotein to the high-density lipoprotein (LDL/HDL),very low-density lipoprotein (VLDL), enzymes of the Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) ($p<0.05$) were observed by the E and P treatments and also the lowest was shown by the M treatment (Table 3). In contrast, no significant differences were found between the E and P treatments.

Table 2. Average performance of broiler chicken by treatments at the different weeks

Variable	Dietary treatments *			SEM	P-Value
	Mash	Pellet	Extruded		
ADWG (g/d)					
1	13.81. ^{c}	17.05 ^{b}	17.71 ^{a}	0.115	0.0001
2	31.04 ^{b}	38.76 ^{a}	41.03 ^{a}	0.959	0.0018
3	52.77 ^{b}	74.20 ^{a}	75.66 ^{a}	1.094	0.0001
4	70.60 ^{b}	95.49 ^{a}	89.11 ^{a}	1.342	0.0001
5	85.80 ^{b}	95.55 ^{a}	93.64 ^{a}	1.168	0.0091
6	76.19 ^{a}	63.12 ^{b}	78.01 ^{a}	1.726	0.0059
ADFI (g/d)					
1	18.88	19.57	19.52	0.317	0.6171
2	44.04 ^{b}	53.16 ^{a}	51.48 ^{a}	0.719	0.0003
3	80.30 ^{b}	96.70 ^{a}	99.34 ^{a}	0.738	0.0001
4	116.52 ^{c}	144.92 ^{a}	136.97 ^{b}	1.341	0.0001
5	164.52 b	176.60 a	170.44 ab	1.567	0.0231
6	189.81	185.44	190.54	1.778	0.4676
FCR					
1	1.37 ^{a}	1.15 ^{b}	1.12 ^{b}	0.021	0.0004
2	1.24	1.18	1.11	0.021	0.0849
3	1.37 ^{a}	1.24 ^{b}	1.206 ^{b}	0.007	0.0001
4	1.48 ^{a}	1.36 ^{b}	1.33 ^{b}	0.008	0.0001
5	1.63 ^{a}	1.51 ^{b}	1.48 ^{b}	0.010	0.0001
6	1.84 ^{a}	1.82 ^{a}	1.70 ^{b}	0.018	0.0153

*The same superscript alphabets in the same row indicate a non-significant different at p<0.01, P<0.05.
(ADWG = Average Daily Weight Gain; ADFI= Average Daily Feed Intake; FCR= Feed Conversion Ratio)
*Treatments (n=3 Mash, Pellet, Extruded); replicates (Mash (n= 12), Pellet (n= 12), Extruded (n= 12), 26 bird in each pen

Table 3. Biochemical and hematology parameters and enzymes activities of blood broilers by treatments at 35 day of age

Variable	Dietary treatments*			SEM	P-Value
	Mash	Pellet	Extruded		
Hemoglobin, (g/dl)	11.825 ^{b}	13.475 ^{a}	12.725 ^{a}	0.157	0.0025
Hematocrit, (%)	32.450 ^{b}	36.400 ^{a}	34.625 ^{ab}	0.457	0.0107
Urea, (mg/dl)	2.333 ^{b}	2.458 ^{ab}	3.333 ^{a}	0.169	0.0057
Uric acid, (mg/dl)	4.900^{b}	7.083^{a}	6.533^{a}	0.241	0.2334
Cholesterol, (mg/dl)	111.667 ^{a}	115.667 ^{a}	122.500 ^{a}	2.496	0.2334
Triglyceride, (mg/dl)	102.17^{b}	131.50 ^{ab}	142.33 ^{a}	6.009	0.0409
H.D.L, (mg/dl)	82.500 ^{a}	75.500 ^{a}	76.167 ^{a}	1.811	0.2513
L.D.L, (mg/dl)	113.167 ^{a}	113.667 ^{a}	116.167 ^{a}	5.63	0.2122
LDL/HDL	1.371 b	1.505^{ab}	1.525^{a}	0.180	0.0311
VLDL, (mg/dl)	20.167 ^{b}	26.500 ^{a}	28.000 ^{a}	1.190	0.0387
AST, (IU/l)	272.17 ^{b}	481.83 ^{a}	503.67 ^{a}	26.708	0.0052
ALT, (IU/l)	11.000 ^{b}	22.667 ^{a}	27.000 ^{a}	1.933	0.0115
ALP, (IU/l)	91.390 ^{a}	93.017 ^{a}	95.132 ^{a}	18.195	0.7073
LDH, (IU/l)	1955.7 ^{a}	1975.5 ^{a}	2023.2 ^{a}	132.208	0.9773
Ca, (mg/dl)	10.50 ^{a}	10.87 ^{a}	11.32 ^{a}	0.152	0.119
K, (mmol/l)	5.28 ^{a}	5.38 ^{a}	5.66 ^{a}	0.100	0.2982
Glucose, (mg/dl)	241 ^{a}	253.33 ^{a}	248.167 ^{a}	2.388	0.1407

*The same superscript alphabets in the same row indicate a non-significant different at P<0.05.
(HDL: High-density lipoprotein, LDL: Low-density lipoprotein, VLDL: Very low-density lipoprotein, AST:Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline Phosphatase, LDH: Lactate dehydrogenase)
*Treatments (n=3 Mash, Pellet, Extruded); replicates (Mash (n= 12), Pellet (n= 12), Extruded (n= 12) with 26 bird in each pen.

There were significant differences in mortality among treatments ($p<0.05$) (Table 4). The higher mortality percentage was found in the E as well as the P treatment and a lower mortality percentage was observed in the M treatment during the experiment. The rate of mortality was 5.45% during the experiment period. The higher mortality percentage of AS and SDS were appeared in the broiler chickens, which received the E and P diet form rather than the M diet form (Table 4).

Table 4. Number and rate of mortality in broiler chicken by treatments at 0-42 days of age

Mortality/	Mash		Pellet		Extruded		Total	
Treatments	No	(%)	No	(%)	No	(%)	No	(%)
Ascites	0	0	12	1.28	9	0.96	21	2.24
SDS	2	0.21	3	0.32	4	0.43	9	0.96
Others	8	0.85	2	0.21	11	1.18	21	2.24
Total	10	1/07	17	1.82	24	2.56	51	5.45
p-value							0.0471	

*The same superscript alphabets in the same row indicate a non-significant different at $P<0.05$.
*Treatments (n=3 Mash, Pellet, Extruded); replicates (Mash (n= 12), Pellet (n= 12), Extruded (n= 12) 26 bird in each pen
Number: 936 day-old broiler chicken; Number (No), Percent (%)

The relative breast weight to the carcass was lower by the M diet form than the E and P treatments ($p<0.05$). The greater relative weight of the cecum and gizzard to the carcass weight were indicated than the E and P treatments at 42 days of age ($p<0.05$) (Table 5). No significant differences were monitored in carcass percentage, lung, abdominal fat, liver, intestine length and tibia bone yields in respect to all treatments.

Table 5. Average relative weights (%) of the viscera of the broilers by treatments at 42 day of age

Variable	Dietary treatments			SEM	P-Value
	Mash	Pellet	Extruded		
Carcass yield, %	68.49 [a]	71.33 [a]	71.38 [a]	2.429	0.0928
Breast, %	23.10 [b]	25.39 [a]	26.19 [a]	0.376	0.0118
Lung, %	0.46 [a]	0.36 [a]	0.44 [a]	0.022	0.1775
Liver, %	2.48 [a]	2.53 [a]	2.65 [a]	0.059	0.4918
Gizzard , %	1.84 [a]	1.21 [b]	0.84 [c]	0.061	0.0001
Abdominal fat, %	0.93 [a]	1.22 [a]	0.95 [a]	0.058	0.1136
Length of intestine, %/g	7.29 [a]	6.82 [a]	7.02 [a]	0.2221	0.107
Cecum, %	0.63 [a]	0.56 [a]	0.46 [b]	0.018	0.0054
Tibia bone, %	0.93 [a]	0.91 [a]	0.85 [a]	0.040	0.7032

*The same superscript alphabets in the same row indicate a non-significant different at $P< 0.01$, $P<0.05$.

DISCUSSION

With the increase of the average daily weight gain, a better FCR was found by applying E and P treatments, in comparison to the M diet form. The average daily feed intake was increased by the P diet form rather than the M one in the broiler chickens.

Atapattu et al. (2005) have found that broiler chickens spend more and less time in feeding and resting by M and P diet respectively. Therefore, better utilization of energy and nutrients in the case of P diets, may be increased. Nir et al. (1996) have also indicated a higher weight gain in those broiler chickens fed with an extruded soybean meal rather than those fed with a non-extruded soybean meal. Also, Asha Rajini et al. (1998b) have reported a heavier body weight in the chickens fed with the P form than the M diet.

Hamm and Stephenson (1959) have indicated that P diets could give a greater feed intake than the M form diet. Asha Rajini et al. (1998b) have stated that P diet could have a better feed efficiency than the M diet. Munt et al. (1995) observed that broilers fed by the P diet showed a better growth performance in comparison to those fed by the M diet. Increased body weight, feed intake and improved the feed: the gain ratio was shown by feeding the P, as compared with those birds fed by the M diets.

Many researchers have indicated the positive effect of E on the performance of the chicken (Moritz et al., 2005; Marsman et al., 1997). They have attributed this better performance to improved gelatinization, intake, digestibility, removal of antinutritional factors and the good quality of the extruded feed or ingredients. The different performance of poultry and nutrient digestibility could be mainly due to various processing techniques and extrusion conditions. To obtain the maximum nutrient digestibility and ensure the best performance of the poultry processing techniques and extrusion conditions should be maintained at the standard levels.

Moritz et al. (2005) observed that the E process of corn led to an increase in the body weight of broiler chickens when they were in 0 to 3 weeks of their age. The improved growth performance of the broilers fed by the extruded (E) SBM has been lighted by Marsman et al. (1997). In modern feed milling operations, E can be considered as the basic process to enhance the profitability of the feed. The E process can be useful in terms of the enhanced nutritional

value and efficiency of ingredients and feed, depending upon many factors such as the structure and chemical composition of the ingredients, processing conditions and the machinery used in processing (Rahman et al., 2015).

The nutritional value and, digestibility of feed ingredients, therefore, could influence the performance of the poultry based on the variations in temperature, moisture, screw speed, pressure, time along with extruded material chemical composition and structure. To attain the maximum results from the extrusion processing techniques, all conditions should be maintained at optimum levels (Lin et al*.,* 1997). In general, heating improves the digestibility of proteins by inactivating enzyme inhibitors and denaturing the proteins that may expose new sites for enzyme attacks (Camire et al*.,* 1990). Conditioning time can change gelatinization degree of starch. So, the degree of gelatinization can affect the growth rate and body weight in the poultry.

Several studies have also shown that AS and SDS could cause high mortality in the broilers which are in 3 to 5 weeks of their age (Gardiner et al., 1988; Wideman, 2001).Broilers in the M treatment had a significantly lower mortality rate due to ascites and SDS.

Genetic selection for growth and feed conversion ratio increased the final body weight and performance (Decuypere et al.,2005; Olkowski et al., 2008); this was accompanied by the insufficiency of oxygen supply to the tissues in rapidly growing broiler chickens (Huchzermeyer, 2012; Wideman et al., 2013; Hasanpur et al., 2015). This could be as a result of the enhanced cardiac output and the reduced oxygen content of blood, the increased production of red blood cells and hematocrit value; these were consistent with the results of the current study (Decuypere et al., 2005).

Feed form is one of the effective factors on the performance and growth rate in the broilers. Feeding by the P diet form, as compared to the M one, increased body metabolism, hence raising the incidence of SDS and ascites in the broiler chickens; so it could identified as the major causes of mortality related to the fast growth (Proudfoot and Hulan, 1982; Kaul and Trangdia, 2003; Druyan et al., 2009).

In the broilers susceptible to AS and SDS syndromes, the biochemical conditions, hematological parameters and enzymes, and hormones were changed. The P and E diet forms increased the feed intake and growth rate due to high metabolic demand; this was as a result of the increase in hemoglobin, hematocrit and enzymes such as AST, ALT and ALP values (Schindhelm et al., 2006). Broiler chickens fed with E and P diet forms showed increased hematological parameters, as well as higher serum levels including urea, uric acid, triglyceride, as well as the rise of the ratio of LDL/HDL, VLDL, AST and ALT, as compared with those fed by the M diet form. The changes in enzymes activities and lipid metabolism could be related to the metabolic abnormalities and the high metabolic demand in the liver. This could lead to hepatocellular degeneration and destruction of the liver cells, which, in turn, result in the leakage of these enzymes into the blood stream; so, liver damage could occur (Hung et al., 2008; Saki and Hemati, 2011;Parmar et al., 2012; Shen et al., 2014; Senanayake et al., 2015). There is, however, little information regarding the changes in biochemical parameters and enzymes activities that can cause AS and SDS in different forms of feed.

In this experiment, carcass characteristics such as the relative breast weight were increased and the relative weight of cecum and, gizzard was decreased by E and P diet forms. The results, therefore, showed that feeding the mash form had a limited effect on carcass characteristics.

Amerah et al*. (*2007) also observed that the weight of the breast was significantly increased by the P diet than the M diet form in broiler chickens; this was in agreement with our study. Ahmed and Abbas (2013) indicating that the decrease in the relative weight of gizzard could be realized by the P diet rather than the M one in chickens. Gizzard weight was influenced by the diet form and size, resulting in a higher weight and yield in the broilers fed by M, as compared to the P diets. The gizzard function might have been affected by the M diet, resulting in a significant muscle development. Detailed results are reported in the literature (Lopez and Baiao, 2004), demonstrating that the gizzard relative weight of the broilers fed by the M diets was greater than of those fed by the P feeds, which was in agreement with the current study.

CONCLUSION

The results of this study, therefore, showed that the diet form played an important role in the feed efficiency, carcass characteristics and mortality of the broiler

chickens. The P and E diet forms increased the performance and carcass characteristics; so the increase in these parameters was associated with the mortality of ascites and SDS in the broiler chickens. However, the association between mortality (AS and SDS) and changes in hematology and enzymatic parameters needs to be further investigated in the future studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Investigation of antimicrobial resistance in pigeons (*Columba livia domestica*) using indicator bacteria

Ö. Aslantaş*, N. Gövce

Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Microbiology, 31060, Hatay, Turkey

ABSTRACT: The aim of this study was to determine the prevalence of antibiotic resistance as well as presence of resistance-associated genes in *Escherichia coli* and *Enterococcus* spp. strains isolated from pigeons. One hundred and fifty cloacal swabs were collected from apparently healthy pigeons in Hatay, Turkey, between March 2014 and June 2014. Antimicrobial susceptibilities of the isolates were tested with disc diffusion method, and resistance genes were investigated by polymerase chain reaction (PCR). *E. coli* were isolated from 94.7% (142) of the examined cloacal swab samples. *E. coli* isolates revealed higher resistance rates to tetracycline (51.4%) and ampicillin (50%), followed by nalidixic acid (19.7%), streptomycin (12.7%), amoxycillin-clavulanic acid (15.5%), trimethoprim-sulfamethoxazole (10.6%), cephalothin (7.0%), ciprofloxacin (6.3%), kanamycin (4.9%), gentamicin (4.2%), tobramycin (4.2%), ceftazidime (4.2%), cefotaxime (4.2%), chloramphenicol (2.8%), aztreonam (2.8%), and cefoxitin (0.7%), respectively. Twentyeight (%19.7) *E. coli* isolates were susceptible to all tested antimicrobials. A total of 136 (90.7%) *Enterococcus* spp. were isolated and species distribution of the isolates was determined by species-specific PCR. The isolates were identified as 64 (47.1%) *E. hirae*, 17 (12.5%) *E. faecium*, 8 (5.9%) *E. faecalis*, 4 (2.9%) *E. columbea*, and 2 (1.5%) *E. durans*. The rest of the isolates (30.1%) were identified as *Enterococcus* spp. with the used primers. *Enterococcus* spp. were resistant to tetracycline (67.6%), erythromycin (23.5%), rifampicin (17.6%), chloramphenicol (6.6%) and ciprofloxacin (5.9%). By contrast, 38 (27.9%) *Enterococcus* spp. were sensitive to all tested antimicrobials. The data obtained in the study showed that pigeons were carriers of antimicrobial resistant *E. coli* and *Enterococcus* spp. in their intestinal microbiota, and may pose public health risk due to not only transmission of these resistant bacteria to humans but also contamination of the environment. The current status of antimicrobial resistance in different animal species should be continuosly monitored and control measures should also be taken.

Keywords: Pigeon, antimicrobial resistance, *Escherichia coli*, *Enterococcus* spp.

Corresponding Author:
Ö. Aslantaş, Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Microbiology, 31060, Hatay, Turkey
E-mail address: ozkanaslantas@yahoo.com, aslantas@mku.edu.tr

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INTRODUCTION

Bacterial resistance to antimicrobials are growing problem in both human and veterinary medicine worldwide. The main risk factor for the emergence of resistant bacteria is misuse and overuse of antibiotics (van den Bogaard and Stobberingh, 2000). Pigeons can not only play an important role for the dissemination of zoonotic agents such as chlamydiosis, cryptococcosis, aspergillosis and can also host antimicrobial resistant bacteria such as *Escherichia coli*, *Campylobacter* spp., *Salmonella* spp. and *Enterococcus* spp. (Vasconcelos et al., 2018; Perez-Sancho et al. 2020). Oral administration of various antibiotics for prophylactic and therapeutic purposes causes selective pressure on the microbiota and leads the selection of resistant bacteria (Mehdi et al. 2018). Tetracyclines and beta-lactam antibiotics are widely used for the treatment of poultry infections due to its low cost, efficacy, and lack of side effects (Filazi et al. 2017)

E.coli and *Enterococcus* spp. are commensal inhabitants of gastrointestinal flora of animals, and have been used as a indicator bacteria not only for faecal contamination of environment and but also of food, in particular, monitoring antimicrobial resistance in different animal species (Kojima et al., 2009; Persoons et al., 2010; Radimersky et al., 2010). In additon to being a potential reservoir for resistance genes, indicator bacteria are of particular importance because they can transfer resistance genes to other bacterial populations either with in the same or other any host. Indicator bacteria have also important role for giving an overview of the resistance load of the ecosystem in which they are in (Wray and Gnanou, 2000). Antimicrobial resistance in bacteria occured by intrinsic or acquired mechanisms. Acquired resistance occurs due to different mechanisms in bacteria: (i) target mutation, (ii) acquisition of resistance genes located on mobile transmissible elements such as plasmids, transposoons, and integrons via conjugation, transduction and transformation (Munita and Arias, 2016).

Recent studies have shown that both free-living pigeons and domesticated pigeons are potential reservoirs of resistant bacteria (Radimersky et al., 2010; Aşkar et al., 2011; Blanco-Peña et al., 2017). Due to the fact that pigeons are close proximity to humans and its impact on public health, it is important to investigate the antimicrobial resistance in pigeons using indicator bacteria. In Turkey, pigeon keeping and breeding on the roof of the houses are a common hobby. However, the data on carriage of antimicrobial resistance in their gastrointestinal flora is very limited (Aşkar et al., 2011). Therefore, the objectives of this study were to investigate the occurence of antimicrobial resistance in indicator bacteria in faeces of pigeons and the mechnanisms mediating resistance.

MATERIAL AND METHODS

Ethical statement

The study was approved by the Animal Ethical Committee of Hatay Mustafa Kemal University (2013-7/7).

Sampling

A total of 150 cloacal swab samples were collected from the houses belonging to people dealing with pigeon breeding as a hobby in three locations in Hatay, Turkey, between March 2014 and June 2014. For this purpose, five pigeon premises from each settlement were sampled, and the cloacal swab samples were taken from 10 pigeons from each premises.

Isolation of *E. coli* strains

Individual cloacal swab samples were taken by Stuart Transport Medium and transported to laboratory in cold chain. For *E. coli* isolation, cloacal swab samples were directly inoculated onto Eosin Methylene Blue (EMB) agar and incubated at 37 ^{o}C for 24 h. Following biochemical tests, the isolates were confirmed by polymerase chain reactions (PCR) using *E. coli* species specific primers E16S-F 5'-CCC CCT GGA CGA AGA CTG AC-3 'and E16S-R 5'-ACC GCT GGC AAC AAA GGA TA-3' (Wang et al., 2002).

Antimicrobial susceptibility testing and detection of resistance genes of *E. coli* isolates

Antimicrobial susceptibilities of *E. coli* isolates to nineteen antimicrobials were determined by disk diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI, 2012) guidelines. The antimicrobial disks (Bioanalyse, Turkey) used were: ampicillin (AMP, 10 μg), amoxycillin-clavulanic acid (AMC,20/10 μg), nalidixic acid (NA, 30 μg), ciprofloxacin (CIP, 5 μg), cefpodoxim (CPD, 10 μg), ceftriaxone (CRO, 30 μg), cefepime (FEB, 30 μg), cefoxitin (FOX, 30 μg), cefuroxime (CXM, 30 μg), cephalothin (KF, 30 μg), aztreonam (ATM, 30 μg), imipenem (IMP, 10 μg), chloramphenicol (C, 30 μg), gentamicin (CN, 10 μg), tobramycin (TOB, 10 μg), amikacin (AK, 10 μg), kanamycin (K, 30 μg), tetracycline (TE, 30 μg), and

sulphamethoxazole-trimethoprim (SXT, 1.25/23.75 µg). *E. coli* ATCC 25922 strain was used as control strain for antimicrobial susceptibility testing. The isolates showing resistance to three or more antimicrobials from different classes were defined as multidrug resistant (MDR). Penicillins and cephalosporins were considered as separate classes. The isolates showing resistance to 3rd generation cephalosporins were confirmed as extended spectrum beta-lactamase (ESBL) producer by double disk synergy (Jarlier et al., 1988) and disk combination method according to guidelines of CLSI (2012).

The isolates showing resistance to particular antibiotics were screened for the presence of antibiotic resistance genes in *E. coli* by PCR using the primers listed in Table 1.

Table 1. Primers used for detection of antibiotic resistance genes in *E. coli* isolates

Antibiotics	Gene	Sequence (5'-3')	Product Size (bp)	Reference
Tetracyclines	*tet*(A)	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	210	Ng et al. (2001)
	tet(B)	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	659	
	tet(C)	CTTGAGAGCCTTCAACCCAG ATGGTCGTCATCTACCTGCC	418	
	tet(D)	AAACCATTACGGCATTCTGC GACCGGATACACCATCCATC	787	
	tet(E)	AAACCACATCCTCCATACGC AAATAGGCCACAACCGTCAG	278	
	tet(G)	GCTCGGTGGTATCTCTGCTC AGCAACAGAATCGGGAACAC	468	
Chloramphenicol	*catI*	AGTTGCTCAATGTACCTATAACC TTGTAATTCATTAAGCATTCTGCC	547	Maynard et al. (2004)
	catII	ACACTTTGCCCTTTATCGTC TGAAAGCCATCACATACTGC	543	
	catIII	TTCGCCGTGAGCATTTTG TCGGATGAGTATGGGCAAC	286	
Trimethoprim	*dhfrI*	AAGAATGGAGTTATCGGGAATG GGGTAAAAACTGGCCTAAAATTG	391	Maynard et al. (2004)
	dhfrV	CTGCAAAAGCGAAAAACGG AGCAATAGTTAATGTTTGAGCTAAAG	432	
	dhfrVII	GGTAATGGCCCTGATATCCC TGTAGATTTGACCGCCACC	265	
	dhfrIX	TCTAAACATGATTGTCGCTGT C TTGTTTTCAGTAATGGTCGGG	462	
	dhfrXIII	CAGGTGAGCAGAAGATTTTT CCTCAAAGGTTTGATGTACC	294	
Aminoglycosides	*aadA*	GTGGATGGCGGCCTGAAGCC AATGCCCAGTCGGCAGCG	525	Kozak et al. (2009)
	strA/*strB*	ATGGTGGACCCTAAAACTCT CGTCTAGGATCGAGACAAAG	893	
	aac(3)IV	TGCTGGTCCACAGCTCCTTC CGG ATGCAGGAAGATCAA	653	
	aadB	GAGGAGTTGGACTATGGATT CTTCATCGGCATAGTAAAAG	208	
	aphA1	ATGGGCTCGCGATAATGTC CTCACCGAGGCAGTTCCAT	600	
	aphA2	GATTGAACAAGATGGATTGC CCATGATGGATACTTTCTCG	347	

Sulphanamid	*sul1*	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433	Kozak et al. (2009)
	sul2	CGGCATCGTCAACATAACCT TGTGCGGATGAAGTCAGCTC	721	
	sul3	CAACGGAAGTGGGCGTTGTGGA GCTGCACCAATTCGCTGAACG	244	
β-lactams	bla_{SHV}	ATGCGTTATATTCGCCTGTG TGCTTTGTTATTCGGGCCAA	747	Monstein et al. (2007)
	bla_{TEM}	TCGCCGCATACACTATTCTCAGAATGA ACGCTCACCGGCTCCAGATTTAT	445	
	bla_{CTX}	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAAYCAGCGG	593	
	bla_{CMY-2}	GACAGCCTCTTTCTCCACA TGGAACGAAGGCTACGTA	1015	Zhao et al. (2001)
Quinolones	*qnr*A	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA	516	Kim et al., 2009
	*qnr*B	GATCGTGAAAGCCAGAAAGG ATGAGCAACGATGCCTGGTA	416	
	*qnr*C	GGGTTGTACATTTATTGAATCG CACCTACCCATTTATTTTCA	307	
	*qnr*S	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	428	
	aac(6')-Ib-cr	TTGCGATGCTCTATGAGTGGCTA CTCGAATGCCTGGCGTGTTT	482	Park et al., 2006

***Enterococcus* spp. isolation and species determination using PCR**

Cloacal swab were firstly inoculated into Enterococcosel Broth (BD, USA) and incubated at 37 °C for 24 h. In case of colour change, a loopful of culture was plated onto VRE agar. Plates were incubated at 37 °C for 24 h, and then one typical colony was selected and passaged to blood agar plates supplemented with 5% defibrinated sheep blood in order to obtain pure culture. The isolates were identified on the genus level by Gram staining, catalase tests. Determination of *Enterococcus* spp. on genus and species level were done by using primers and method described by Layton et al. (2010), except *E. columbae*, which was examined as previously described by da Silva et al. (2012).

Antimicrobial susceptibility testing and detection of resistance genes of *Enterococcus* spp.

Antimicrobial susceptibilities of the isolates to eight antimicrobials were determined by disk diffusion method in accordance with CLSI (2012) criteria, and the used disks were as follow: ampicillin (AMP, 10 μg), vancomycin (VA, 30 μg), erythromycin (E, 15 μg), tetracycline (TE, 30 μg), teicoplanin (TEC, 30 μg), ciprofloxacin (CIP, 5 μg), and chloramphenicol (C, 30 μg). For the phenotypic determination of high level gentamicin resistance (HLGR), 120 μg gentamicin containing disks were used. The isolates showing resistance to particular antibiotics were screened for the presence of antibiotic resistance genes in enterococci by PCR using the primers listed in Table 2.

Table 2. Primers used for detection of antibiotic resistance genes in enterococci

Antibiotic	Primer	Sequence (5'-3')	Product size (bp)	Reference
Macrolides	*erm*(A)	CCCGAAAAATACGCAAAATTTCAT CCCTGTTTACCCATTTATAAACG	590	Malhotra-Kumar et al. (2005)
	erm(B)	TGGTATTCCAAATGCGTAATG CTGTGGTATGGCGGGTAAGT	745	
	mef(A/E)	CAATATGGGCAGGGCAAG AAGCTGTTCCAATGCTACGG	317	
Tetracycline	*tet*(K)	GATCAATTGTAGCTTTAGGTGAAGG TTTTGTTGATTTACCAGGTACCATT	155	
	tet(M)	GTGGACAAAGGTACAACGAG CGGTAAAGTTCGTCACACAC	406	
	tet(O)	AACTTAGGCATTCTGGCTCAC TCCCACTGTTCCATATCGTCA	515	
	tet(L)	TGGTGGAATGATAGCCCATT CAGGAATGACAGCACGCTAA	229	
Aminoglycosides	*aac(6)-Ie-aph(2)-Ia*	CAGGAATTTATCGAAAATGGTAGAAAAG CACAATCGACTAAAGAGTACCAATC	369	Vakulenko et al. (2003)
	aac(6)-Ie-aph(2)-Ia	CAGAGCCTTGGGAAG ATG AAG CCTCGTGTAATTCATGTTCTGGC	348	
	aph(2)-Ib	CTTGGACGCTGAGATATATGAGCA C GTTTGTAGCAATTCAGAAACACCCTT	867	
	aph(2)-Ic	CCA CAATGATAATGACTCAGTTCCC CCA CAGCTTCCGATAGCAAGAG	444	
	aph(2)-Id	GTG GTTTTTACAGGAATGCCATC CCCTCTTCATACCAATCCATATAACC	641	
	aph(3)-IIIa	GGCTAAAATGAGAATATCACCGG CTTTAAAAAATCATACAGCTCGCG	523	
	ant(4)-Ia	CAAACTGCTAAATCGGTAGAAGCC GGAAAGTTGACCAGACATTACGAACT	294	
Chloramphenicol	*Cat*pIP 501-159-	GGATATGAAATTTATCCCTC CAATCATCTACCCTATGAAT	505	Aerestrup et al. (2000)
Vancomycin	*vanA*	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	Depardieu et al. (2002)
	vanB	ACGGAATGGGAAGCCGA TGCACCCGATTTCGTTC	647	
	vanC1/2	ATGGATTGGTAYTKGTAT TAGCGGGAGTGMCYMGTAA	815/827	
	vanD	TGTGGGATGCGATATTCAA TGCAGCCAAGTATCCGGTAA	500	
	vanE	TGTGGTATCGGAGCTGCAG ATAGTTTAGCTGGTAAC	430	
	vanG	CGGCATCCGCTGTTTTTGA GAACGATAGACCAATGCCTT	941	

RESULTS

E. coli isolation and antimicrobial testing

One hundred and forty two (94.7%) *E. coli* were isolated from 150 cloacal swab samples. Various rates of resistance among *E. coli* isolates were observed to tetracycline (73, 51.4%), ampicillin (71, 50%), nalidixic acid (28, 19.7%), amoxycillin-clavulanic acid (22, 15.5%), streptomycin (18, 12.7%), trimethoprim-sulfamethoxazole (15, 10.6%), cephalothin (10, 7.0%), ciprofloxacin (9, 6.3%), kanamycin (7, 4.9%), gentamicin (6, 4.2%), tobramycin (6, 4.2%), ceftazidime (6, 4.2%), cefotaxime (6, 4.2%), chloramphenicol (4, 2.8%), aztreonam (4, 2.8%), and cefoxitin (1, 0.7%), respectively (Figure 1). Twentyeight (19.7%) isolates were found susceptible to all antimicrobials tested. Twentyseven (19%) isolates showed MDR phenotype. Among the isolates showing MDR phenotype, resistance to 6, 5, 4, and 3 isolates were observed in 2, 3, 8, and 14 isolates, respectively (Table 3).

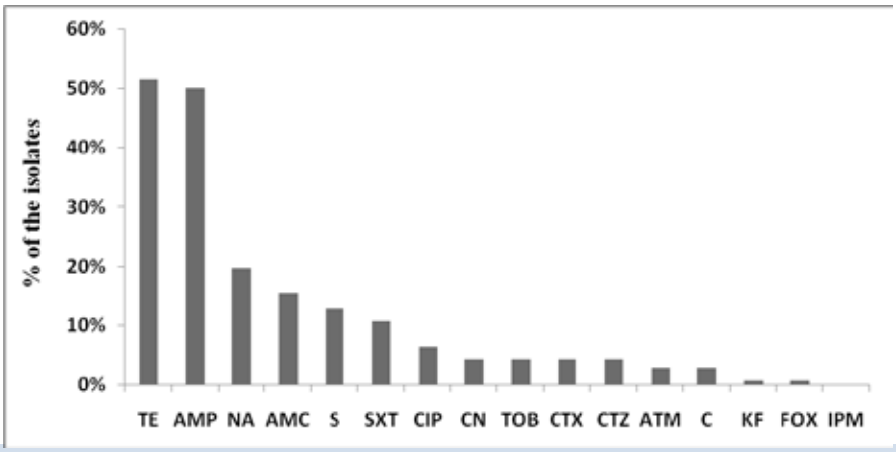

Figure 1. Antibiotic susceptibilities of 142 *E. coli* isolates

Table 3. Antibiotic resistance phenotypes among the *E. coli* isolates

Phenotype	Number of the isolates
AM, AMC, KF, TE, CN, S, K, TOB, SXT	1
AM, KF, TE, K, TOB, SXT, CIP NA, C	1
AM, TE, K, TOB, SXT, CIP, NA, C	1
AM, TE, CN, S, K, TOB, SXT	1
AM, TE, CN, TOB, CIP, NA, C	1
AM, AMC, TE, CN, S, K, SXT	1
AM, AMC, TE, S, K, SXT	1
AM, AMC, KF, TE, NA	2
AM, TE, SXT, CIP, NA	2
CN, TOB, CIP, NA, C	1
AM, TE, S, SXT, NA	2
AM, TE, CN, K, SXT	1
AM, AMC, TE, S, K	1
AM, AMC, KF, TE	1
AM, SXT, CIP, NA	2
AM, KF, TE, NA	1
AM, TE, S, SXT	1
AM, AMC, TE	6
AM, AMC, KF	3
AM, TE, S	4
AM, KF, TE	1
TE, S, NA	3
AM, AMC	1
AM, CIP	1
TE, SXT	1
CIP, NA	1
AM, TE	20
TE, NA	8
AM, S	1
TE, S	1
AM	15
TE	18
NA	7
KF	1
S	1
Susceptible	28

Table 4. Antibiotic resistance and resistance mechanisms of *Enterococcus* spp.

Phenotype	Resistance Genes	Species (n)
C, CIP, E, RA, TE	*cat*, *tet*M, *tet*L, *erm*B	*E. faecium* (1)
CIP, E, RA, TE	*tet*M, *tet*L, *erm*B	*Enterococcus* spp. (1)
C, E, RA, TE	*cat*, *tet*M, *tet*L, *erm*B	*E. faecium* (1)
CIP, E, RA	*erm*B	*E. faecium* (1)
CIP, E, TE	*tet*M, *tet*L, *mef*A/E	*E. columbea* (1), *E. faecium* (2)
E, RA, TE	*tet*M, *erm*B	*Enterococcus* spp. (2)
E, RA, TE	*tet*M, *tet*L, *mef*A/E	*Enterococcus* spp. (1)
E, RA, TE	*tet*M, *tet*L, *erm*B	*E. hirae* (2)
C, E, TE	*tet*M, *tet*L, *erm*B	*Enterococcus* spp. (2), *E. hirae* (1)
C, E, TE	*tet*M, *tet*L	*E. hirae* (1)
RA, TE	*tet*M, *tet*L	*Enterococcus* spp. (5), *E. faecalis* (1), *E. faecium* (1), *E. hirae* (4)
RA, TE	*tet*M	*E. faecium* (1), *E. hirae* (1)
CIP, E	-	*E. columbea* (1)
C, TE	*tet*M, *tet*L	*Enterococcus* spp. (1)
C, TE	*tet*M	*E. hirae* (1)
E, TE	*tet*M, *tet*L, *erm*B	*Enterococcus* spp. (3)
E, TE	*tet*M, *erm*B	Enterococcus spp. (1)
E, TE	*tet*L	*Enterococcus* spp. (1), *E. faecium* (1), *E. hirae* (1)
E, TE	*tet*M, *tet*L, *erm*B	*Enterococcus* spp. (1), *E. faecium* (1), *E. hirae* (2)
E, TE	*tet*M, *tet*L, *mef*A/E	*Enterococcus* spp. (1), *E. faecium* (1), *E. hirae* (2)
TE	*tet*M, *tet*L	*E. hirae* (2)
TE	*tet*M, *tet*L	*Enterococcus* spp. (4), *E. columbea* (2), *E. faecium* (1), *E. hirae* (3)
TE	*tet*M	*Enterococcus* spp. (6), *E. hirae* (26)
TE	*tet*L	*E. hirae* (1)
TE	-	*E. hirae* (2)
RA	-	*E. faecalis* (2)
CIP	-	*E. faecium* (1)
C	-	*E. hirae* (1)
Sensitive	-	*Enterococcus* spp. (12), *E. durans* (2), *E. faecalis* (5), *E. faecium* (5), *E. hirae* (14)

Distribution of resistant genes among resistant *E. coli* isolates

Tetracycline resistance was only associated with *tet*A and *tet*B genes, which were found in 77 (95.1%) of 81 tetracycline resistant *E. coli* isolates. The distribution of resistance genes were as follows: 62 (80.5%) *tet*A, 14 (18.2%) *tet*A and *tet*B, and one (1.3%) *tet*B. All isolates were negative for *tet*C, *tet*D, *tet*E and *tet*G.

Among ampicillin resistant isolates, bla_{TEM} was found in 66 (91.7%) isolates. PMQR genes were detected in four ciprofloxacin resistant isolates, of which three isolates carried *aac(6')-Ib-cr*, and one carried *qnr*A. Among trimethoprim-sulfamethoxazole resistant isolates (n=15), the distribution was determined as follows: *sul*1-*sul*2 in four isolates, *sul*1-*sul*2-*dhfr*1 in two isolates, *sul*1-*dhfr*1 in two isolates, *sul*2-*dhfr*5 in two isolates, *sul*1 in two isolates, *sul*1 in two isolates, and *sul*1-*sul*2-*dhfr*5 in one isolate. While all ESBL producing *E. coli* isolates carried bla_{CTX-M}, bla_{C-MY-2} gene was only detected in one cefoxitin isolate.

Of 18 streptomycin resistant isolates, 15 (83.3%) carried *str*A/B. Three isolates didn't carry any of the genes examined. Out of four chloramphenicol resistant isolates, only 3 (75%) carried *cat*I. Of kanamycin resistant eight isolates, *aph*A1 was only detected in 6 (75%) isolates. The *aad* and *aac*(3)IV genes were not detected in any tobramycin and gentamicin resistant isolates.

Isolation, species determination and antimicrobial susceptibility of *Enterococcus* spp.

Enterococcus spp. were isolated 136 (90.7%) from pigeon's cloacal swabs. Based on species spesific PCR, distribution of enterococci were as follow: 64 (47.1%) *E. hirae*, 17 (12.5%) *E. faecium*, 8 (5.9%) *E. faecalis*, 4 (2.9%) *E. columbea*, and 2 (1.5%) *E.*

durans. However, 41 (30.1%) isolates were only detected as *Enterooccus* spp. with current primers used.

Antibiotic resistance rates of 136 enterococci were 67.6% (92) to tetracycline, 23.5% (32) to erythromycin, 17.6% (24) to rifampicin, 6.6% (9) to chloramphenicol, and 5.9% (8) to ciprofloxacin. Thirty-eight (27.9%) isolates were sensitive to all tested antimicrobials. Resistance phenotypes and resistance-mediated genes in enterococcal isolates are shown in Table 4. MDR phenotype was observed in 16 (11.8%) isolates. Among the isolates showing MDR phenotype, resistance to 5, 4, and 3 antimicrobials was observed in one, two and thirteen isolates, respectively.

DISCUSSION

Pigeons not only freely lives in urban and rural areas, but also they were raised by people as a hobby. In addition, pigeons are in close contact with humans in different public locations, such as historical places, parks, and squares. These birds may pose possible risks to public health due to carriage of different zoonotic microorganisms (bacteria, fungi, viruses, and protozoa) and antimicrobial resistant bacteria (Vasconcelos et al., 2018; Perez-Sancho et al. 2020).

In this study, 80.3% of the *E. coli* isolates were resistant to one or more antimicrobials tested. In other conducted studies on the occurence of antimicrobial resistant *E. coli* isolates in pigeons, low or lower rates of resistance in *E. coli* isolates have been reported by Radimersky et al. (2010) in Czech Rebuplic (1.5%) and da Silva et al. (2009) in Brazil (37.9%), respectively.

Nineteen percent (n=27) of *E. coli* isolates showed MDR phenotype. MDR bacteria are an increasing an healthcare problem because the presence of pathogens with MDR phenotype, making treatment options very limited. The fact that co-existence of resistance genes on transmissible genetic elements such as plasmid and transposon, facilitate horizantal transfer of resistance genes to susceptible bacteria and lead to an expansion in MDR bacteria population. Therefore, continuous surveillance of antimicrobial resistance in different animal species and environments are important for taking timely necessary measures (Frye and Jackson, 2013)

Resistance to tetracycline (51.4%) and ampicillin (50%) were the most prevalent among the isolates in this study, which are consistent with the findings of Kimpe et al. (2002), who reported resistance rates of 65% and 42%, respectively. However, in Poland, Stenzel et al. (2014) reported a higher resistance rate for amoxicillin (63%) and oxytetracycline (75%), respectively. The *tet*A was the most common resistance gene in comparison with other resistance genes in the study. High prevalence of *tet*A among the tetracycline resistant isolates also indicates that the main resistance mechanism is the active efflux system (Blake et al., 2003). There are few studies on prevalence of antimicrobial resistance genes in pigeons around the world. Blanco-Pena et al. (2017) found *sul*1 and *cat*1 as the most common gene by real time PCR from directly enema samples of pigeons from Public Parks in Costa Rica. In Iran, Ghanbarpour et al. (2020) reported phenotypically the prevalence of tetracycline resistance as very high (98%), but detected a lower prevalence of *tet*A (6.5%) and *tet*B (6.5%) genes.

Nearly all ampicillin resistant isolates carried bla_{TEM} gene (91.7%, 66/72), which was the second most common gene found in the study. In contrast, in Iran, bla_{TEM} was reported to be the most common gene (52.6%) by Ghanbarpour et al. (2020). Similarly, the TEM type beta-lactamase has also been reported as main resistance mechanism of ampicillin resistance in *E. coli* isolates from different origin of animals in previously conducted studies (Radhouani et al., 2012; Santos et al., 2013; Aslantaş, 2018).

Sulfanamids and trimethoprim are folate patway inhibitors, and main resistance mechanisms to these antimicrobials are due to mutations in target enzymes, encoded by *sul* and *dhfr* genes (Skold, 2001). Trimethoprim-sulfamethoxazole resistant isolates had a combination of *sul* and *dhfr* genes, except four isolates which carried only *sul*1 and *sul*2 genes. None of the isolates harbored *sul*3, *dhfr7, dhfr9* and *dhfr13*. Recently, Aslantaş (2018) reported not only high sulfanamid and trimethoprim resistance but also high frequency of these resistant genes among commensal *E. coli* isolates from broilers in Turkey. Widespread dissemination of the resistance genes in *E. coli* could be explained by localization of these genes on plasmids, integrons, or insertion elements (Frye and Jackson, 2013).

Aminoglycoside resistance in *E. coli* strains are mainly related with aminoglycoside modifying enzymes, which is encoded by genes located on plasmids (Frye and Jackson, 2013). Low rate of aminoglycoside resistance is not surprising, because these drugs are not widely used in veterinary field in Turkey. Similarly, Ghanbarpour et al. (2020) reported a

low prevalence of resistance (11%) for gentamicin. Occurence of low resistance might be originated from contaminated feeds and their environments of the pigeons (Radimersky et al., 2010).

Low level of ciprofloxacin resistance was observed in this study. This is important due to the fact that fluoroquinolones are critically important antimicrobials used for the treatment of *E. coli* infections (WHO, 2012). The ciprofloxacin resistance rate is consistent with previous studies conducted by Radimersky et al. (2010) and Aşkar et al. (2002), who reported resistance rates of 2% and 0%, respectively.

Resistance to 3rd and 4th generation cephalosporins mediated by ESBL have clinical importance for both human and veterinary medicine (WHO, 2012). Prevalence of ESBL producing *E. coli* isolates was found to be low in this study. It should be cautiously approached to low rate of resistance. Because selective isolation methods are needed to determine the true prevalence of these bacteria in different animal species (Aslantaş, 2018).

Although 41 (30.1%) isolates were assigned as *Enterococcus* spp. with current primers used in this study. The most common species were identified as *E. hirae* (47.1%), followed by *E. faecium* (12.5%), and *E. faecalis* (5.9%), respectively. *E. columbea* (2.9%) and *E. durans* (1.5%) were detected only in small number of the isolates. In Belgium and Brazil, *E. columbea* was reported as the most frequent species by Baele et al. (2002) and da Silva et al. (2012), respectively. Radimersky et al. (2010) reported that *E. faecalis* and *E. faecium* were as the most frequent species among enterococci isolated from feral pigeons in Czech Republic. Aşkar et al. (2011) reported *E. avium* as most prevalent species among enterococci from domestic pigeons. In a recent study, *E. faecium* and *E. durans* were reported as dominant species in pigeons in Egypt by Osman et al. (2019). Species distribution of enterococci in pigeon in different geographies could be explained by dietary habits of pigeons, which leads colonization of pigeon with different enterococci (Beale et al., 2002).

Although enterococci can exhibit intrinsic resistance to different classes of antimicrobials at low or high levels, they can frequently acquire antimicrobial resistance to different class of antimicrobials such as high-level aminoglycoside resistance (HLAR), fluoroquinolones, glycopeptides, and beta-lactams (ampicillin), via mutations or acquisition of resistance genes (Marothi et al., 2005). The prevalence of antimicrobial resistance in enterococci (72.1%, 98/136) was higher in comparison with previous studies in pigeons, and tetracycline resistance were the most prevalent type of resistance, and were mainly associated with *tet*M. Similar resistant rate (78%) and resistance determinant were also reported by Radimersky et al. (2010) in Czech Republic. Recently, Zigo et al. (2017) found both higher prevalence of antimicrobial resistant enterococci and high resistance rate to tetracycline (75.2%) in Slovakia. In this study, the high observed tetracycline resistance can be attributed to empirical use of this antibiotic for many years by pigeon owners.

The second most common resistance observed was to erythromycin (23.5%), mainly associated with *erm*B gene (79.2%). In contrast, Aşkar et al. (2011) and Zigo et al. (2017) reported higher resistance rate for erythromycin (52%) and 52.2%, respectively. However, a low resistance rate was reported by Radimersky et al. (2010) in Czech Republic, who found a resistance rate of 9% for erythromycin. Interestingly, Osman et al. (2019) found resistance rates ranging from 63.4% and 100% for antibiotics tested, except linezolid (17.1%), in enterococci in Egypt.

Low rate resistance to chloramphenicol (6.6%) among enterococci in this study is not surprising. Since the use of chloramphenicol was banned in food producing animals in Turkey (Regulation No: 2002/68 of 19 December 2002). Low rate resistance to this drug could be explained by the persistence of chloramphenicol resistant strains in the environment (Persoons et al., 2010) or co-existence of chloramphenicol resistance genes with other resistance genes on the same mobile genetic elements (Harada et al., 2006). However, in contrast with this study, da Silva et al. (2009) reported a higher resistance rate (21.7%) in Brazil.

Main resistance mechanism to fluoroquinolones in enterococci is characterized by mutations in the quinolone determining regions of *gyr*A and *par*C genes. The level of resistance to fluoroquinolones varies according to the intensity and duration of use of these antimicrobials. Indeed, in countries where the use of fluoroquinolones is prohibited in food-producing animals, no or low resistance rates can be accepted as an indication of this view (Cheng et al., 2012). Ciprofloxacin resistance rate (5.9%) observed in this study was consisted with previous studies conducted by da Silva et al. (2012) in Brazil and Radimersky et

al. (2010) in Czech Rebuplic, who reported resistance rates of 8.4% and 5%, respectively. But, Aşkar et al. (2011) found higher resistance rate (37%) in Kırıkkale, Turkey. The low resistance rate observed in this study was due to low level empirical use of this drug by pigeon owners for the treatment or prevention of infectious diseases.

One of the striking results of the study was no resistance against high level gentamicin and vancomycin. Gentamicin is one of the antimicrobials having clinical importance. Because combination of this drug with beta-lactams have been widely used for the treatment of enterococcal infections. However, this combination is ineffective in the treatment of infections caused by enterococci with HLGR resistance (del Campo et al., 2000). Vancomycin is a last resort antibiotic to be used for the treatment of nosocomial infections caused by Gram positive bacteria. Similarly, no vancomycin resistance was reported by Silva et al. (2012) in Brazil, Blanco-Peña et al. (2017) in Costa Rica and Aşkar et al. (2011) in Turkey. However, Radimersky et al. (2010) in Czech Rebuplic reported vancomycin resistance in three *E. faecalis* isolates (2%) carrying *van*A gene. In a study conducted in Egypt, Osman et al. (2020) reported higher level (40/41, 97.6%) of VRE colonization and detected frequency of *van*A, *van*B and *van*C genes as 17.1%, 24.4%, and 22%, respectively

CONCLUSIONS

In conclusion, various rates of resistance to different classes of antimicrobials in *E. coli* and *Enterococcus* spp. isolates from the faeces of pigeons were observed in this study. These findings are important not only due to spreading of resistant bacteria to environment and susceptible animals, but also transfer of resistance genes to pathogenic bacteria. Based on the results of this study, there is an urgent need to investigate the antimicrobial resistance in different animal species, and to promote prudent use of antimicrobials for the treatment and control of bacterial infections.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest

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Research article
Ερευνητικό άρθρο

Evaluation of serum homocysteine and nitric oxide concentrations compared with other biochemical parameters in sheep naturally infected with *Fasciola hepatica*

V. Denizhan[1], S. Kozat*[2]

[1]*Department of Parasitology, Faculty of Veterinary Medicine, Van Yuzuncu Yil University, Zeve Campus, Van-Turkey*

[2]*Department of Internal Diseases, Faculty of Veterinary Medicine, Van Yuzuncu Yil University, Zeve Campus, Van-Turkey*

ABSTRACT: This study aims to determine the changes in serum homocysteine (Hcy) and nitric oxide (NO) concentrations in sheep naturally infected with *F. hepatica*. The animal material of the study consisted of a total of 50 sheep: 40 sheep with fascioliasis and 10 healthy sheep.

The statistical analysis indicated that serum homocysteine concentrations, folate and vitamin B_{12} levels of the sheep infected with *F. hepatica* were higher than those of the control group ($P<0.001$ $P<0.001$ and $P<0.05$, respectively), whereas the nitric oxide levels of the sheep infected with *F. hepatica* were significantly lower than those of healthy sheep ($P<0.001$).

In conclusion, it is thought that vitamin B_{12} and folate are not used sufficiently for the conversion of homocysteine to methionine in the remethylation cycle due to the damage in the liver tissue of sheep naturally infected with *F. hepatica.* This results in the increase of homocysteine which in turn inhibits the formation of nitric oxide.

Keywords: *F. hepatica*, sheep, homocysteine, nitric oxide

Corresponding Author:
Süleyman Kozat, Department of Internal Diseases, Faculty of Veterinary Medicine, Van Yuzuncu Yil University, Zeve Kampus, 65080 Van, Turkey
E-mail address: skozat@hotmail.com

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INTRODUCTION

Turkey has about 31.507.934 sheep (Tuncer et al., 2017; Tuncer et al., 2018; Tuncer 2019) and is the 7th greatest sheep raiser country in the world (Tuncer, 2019). *F. hepatica* is a severe disease causing significant economic losses in the countries across the world where sheep, goats, buffalo (Alvarez et al., 2009), and cattle are raised (Yang et al., 1998). This parasitic disease may cause failure to thrive, decrease in meat and milk production, and even death in animals (Souza et al., 2002; Alcala-Canto et al., 2007; Hodžić et al., 2013). Young forms of the parasite destroy the parenchymal tissue, and mature forms destroy the bile ducts (Benzer and Ozan, 2003). Many researchers have focused on changes in serum enzyme activities to determine the level, prognosis, and diagnosis of liver damage caused by *F. hepatica* (Kozat and Denizhan, 2010).

Homocysteine is an intermediate in methionine metabolism, which takes place mainly in the liver (Still and McDowell, 1998; García-Tevijano et al., 2001; Kozat and Okman, 2017). Hcy is a sulfuric amino acid formed during the methionine metabolism and does not enter the primary structure of proteins (García-Tevijano et al., 2001; Fischer et al., 2003; Kozat and Okman, 2017). 70% of total homocysteine in plasma is bound to protein. One-fourth of it is present as disulfide homocysteine by binding to each other, the rest as cysteine-homocysteine or homocysteine thiolactone (Kozat and Okman, 2017). Hcy is metabolized in two major metabolic pathways: the remethylation and transsulfuration cycle (Blom and Smulders, 2011). Hcy is converted to methionine with the addition of a methyl group in a reaction catalized by vitamin B_{12}-dependent methionine synthase. The methyl donor can be either 5,10-methyltetrahydrofolate (MTHF) or betaine. The reaction of MTHF takes place in all tissues and is dependent on vitamin B_{12}. The reaction in which the betaine is the methyl donor takes place in the liver and kidney and is independent of vitamin B_{12}. Methionine is converted to S-adenosyl methionine (SAM) in the presence of ATPand S-adenosyl methionine synthetase. SAM acts as a methyl donor in many reactions. This methylation reaction results in S-adenosyl homocysteine (SAH). SAH is converted into Hcy by a reaction catalyzed by the enzyme hydrolase, and all reactions occur in the liver (Kozat and Okman, 2017). The liver plays a crucial role in sulfur amino acid metabolism. Therefore, Hcy metabolism may be impaired in chronic liver diseases (Ventura et al., 2005).The sulfur-containing amino acid methionine and its derivatives play a central role in the metabolism of homocysteine and cysteine. Chronic liver diseases and especially cirrhosis cause abnormalities in the methionine metabolism, which reflects disorders in multiple enzyme levels. Disorders in methionine demethylation, transsulfuration and remethylation of homocysteine, as well as cystathionine synthesis and hydrolysis, have been described both at the functional and genetic levels throughout the natural course of chronic liver diseases (Ventura et al., 2005; Kozat and Okman, 2017). Increases in total homocysteine levels, that is, the role of homocysteine in atherogenesis, atherosclerosis and thrombosis in hyperhomocysteinemia, cause cardiovascular system disease (Cayir and Kozat, 2016). Hyperhomocysteinemia leads directly to vascular endothelial damage; endothelin changes the anticoagulant effect to procoagulant and causes proliferation of smooth muscle cells (Temel and Ezerol, 2002). Some researchers have reported that the release of nitric oxide (NO), a potent vasodilator and platelet aggregation inhibitor, from bovine endothelium is inhibited by Hcy and inhibition and/or reduction of NO release may cause thrombotic events in hyperhomocysteinemia (Kerkeni et al., 2006).

This study was carried out to determine whether the Hcy remethylation mechanism was affected in sheep that were naturally infected with *F. hepatica*. Moreover, it aimed to determine whether there is a relationship between serum homocysteine values and NO changes in liver disorders.

MATERIALS AND METHODS

Animals

This study was conducted on all sheep that came from one farm: 40 sheep with fascioliasis and 10 healthy sheep. This research was approved (07/03/2017 and 27552122-604.01.02-E.16823) by the Animal Research Ethics Committee of Van Yuzuncu Yil University in Van, Turkey.

At the beginning of the study, all the animals were subjected to general clinical examination and the body temperature, respiratory rate, and heart rate of all sheep were recorded. At the end of the clinical examinations of sheep, approximately 30-50 g of faeces were taken from the rectum of each sheep and placed in the faeces collection containers. Samples were numbered according to the pedigree chart. Faecal samples were taken to the laboratory and kept at -20 °C until examinion. Benedek's sedimentation method

was used to examine the samples (Toparlak and Tuzer, 1994) and determine determine which sheep were infected with *F. hepatica* and which were healthy.

Homocysteine measurements

Sera from blood samples were collected and homocysteine levels in serum healthy sheep and the sheep infected with fascioliasis were determined by ELISA device (ELISA reader®-DAS).

Nitric oxide measurements

Suitable Nitrate/nitrite for sheep was determined by ELISA device (ELISA reader®-DAS) using the colourimetric assay kit (Cayman Chemical Company, catalogue No. 780001/USA).

Vitamin B_{12} and Folate measurements

Vitamin B_{12} and Folate levels in serum samples were determined by autoanalyser (Elecyc 2010 Roche Hitachi-Japan).

Statistical Analysis

Descriptive statistics for the measured parameters are expressed as Mean, Standard Deviation. To compare the groups, Student's t-test was performed. The statistical significance level was taken as 5% and SPSS (ver: 21) statistical package program was used for calculations.

RESULTS

Table 1 shows the statistical analysis of the measurements of serum Hcy, NO concentrations, folate and vitamin B_{12} levels of healthy sheep and sheep infected with *F. hepatica*. Homocysteine concentrations, folate and vitamin B_{12} levels of the sheep infected with *F. hepatica* were higher than those of the control group ($P < 0.001$, $P < 0.001$ and $P<0.05$, respectively), whereas the nitric oxide levels of the sheep infected with *F. hepatica* were significantly lower than those of healthy sheep ($P < 0.001$).

Table 1. Serum homocysteine, nitric oxide, folate and vitamin B12 levels in healthy sheep and the sheep infected with *F. hepatica*

Parameter	Control (n=10) (Mean±SD)	Infected with *F. hepatica* (n=40) (Mean±SD)	P<
Hcy (pg/ml)	5.83±0.93	9.99±2.99	.001
Vit B_{12} (pg/ml)	1227.25±431.16	1552.75±450,74	.05
Folate (ng/ml)	1.56±0.31	2.34±0.54	.001
NO (μmol/L)	53.19±7.20	34.15±15.82	.001

DISCUSSION

The purpose of this study was to investigate the changes in serum Hcy and NO concentrations of sheep infected with *F. hepatica.* The liver plays a key role in the synthesis and metabolism of Hcy, which is an essential intermediate metabolite of methionine metabolism. Methionine is largely metabolized in this organ. The liver has activated genes involved in the methionine and homocysteine metabolism. MAT (Methionine adenosyltransferases), which plays a role in the homocysteine metabolism is found only in the liver, whereas the majority of BHMT (Betaine-homocysteine methyltransferase) and CBS (Cystathionine β-synthetase) are synthesized in the liver ((Finkelstein, 1990; James et al., 1999). For these reasons, damage of the liver affects the metabolism of homocysteine (Paxton et al., 1986; Ciftci and Yuce, 2013). It has been reported that the mean plasma homocysteine levels in human patients with cirrhosis were much higher than in healthy controls, and there was a statistically significant difference between the homocysteine plasma values in patients with cirrhosis and healthy subjects (Ćulafić et al., 2013). In another study a disorder in the liver is thought to cause disorders in Hcy metabolism. Plasma Hcy levels in fatty liver patients are higher than in nonalcoholic and healthy individuals (de Carvalho et al., 2013). It is thought that basal hyperhomocysteinemia in cirrhosis is due to impaired transsulfuration and remethylation mechanisms (Duce et al., 1988; Look et al., 2000; Bosy-Westphal et al., 2001). In human studies it has also been reported that homocysteine metabolism is impaired in chronic liver diseases and leads to an increase in baseline hyperhomocysteinemia in hepatitis patients by 34%, in the fatty liver by 50%, in patients with cirrhosis by 54% and in 52% after orthotopic liver transplantation (Bosy-Westphal et al., 2003). When we examined the Hcy results from sheep infected with fascioliasis in this study (Table 1), serum homocysteine levels were significantly higher ($P < 0.01$) than in the control group. For these reasons, significant changes occur in the metabolism

of Hcy when the liver is damaged. These results are consistent with literature findings indicating that homocysteine levels are high in living organisms with liver damage (Paxton et al., 1986; Ćulafić et al., 2013; James et al., 1999; Ciftci and Yuce, 2013). There is a reported relationship between serum / plasma Hcy levels and folate and vitamin B_{12} levels. (Klee, 2000; Blom and Smulders, 2011; Kozat and Okman, 2017). Hcy is increased in the plasma of patients with deficiency in vitamin B_{12} or folate (Klee, 2000). In this study, Hcy concentrations, folate and vitamin B_{12} levels of the sheep infected with *F. hepatica* were higher than those of the control group ($P < 0.001$, $P < 0.001$ and $P<0.05$, respectively). High concentrations of Hcy are consistent with increases in serum folate and vitamin B_{12} levels. A significant increase in homocysteine concentrations of sheep infected with *F. hepatica* can be attributed to the deterioration of the mechanism of remethylation due to inadequate use of folate and Vitamin B_{12} as a result of liver damage

Some researchers have reported that the release of nitrite oxide (NO), a potent vasodilator and platelet aggregation inhibitor, from bovine endothelial cells is inhibited by homocysteine (Danishpajooh et al., 2001). Several studies have confirmed that the bioavailability of NO is decreased in hyperhomocysteinemia (Fisher et al., 2003; Stanger and Weger, 2003), which might be attributable to diminished NO production or to alternative mechanisms such oxidative stress or nitrosylation (Fisher et al., 2003; Stanger and Weger, 2003, Dayal et al., 2004; Kerkeni et al., 2006). Many studies have been conducted on changes in NO levels in liver disorders (Gupta et al., 1998; Cervi et al., 1998; Clemens, 1999; Benzer and Ozan, 2003; Chen et al., 2003). In a study investigating the functional role of vascular endothelium on increased vascular tone in intrahepatic microcirculation in rats with experimental cirrhosis; a decrease was reported in endothelial dysfunction and NO production in intrahepatic microcirculation of cirrhotic rats (Gupta et al., 1998). Another study investigated the proliferative responses of spleen cells against mitogens in *F. hepatica* -infected rats and reported a decrease in the amount of NO produced, and this decrease was partly associated with extra secretory antigens of *F. hepatica* (Cervi et al., 1998). A study examining lipid peroxidation, antioxidant enzymes and nitric oxide levels in the sheep infected with *F. hepatica* compared the results with those of healthy sheep and reported that serum NO levels of the sheep infected with *F. hepatica* were not affected but there was a significant decrease in NO levels in the liver disease (Benzer and Ozan, 2003). Some experimental and clinical studies have reported that hyperhomocysteinemia causes vascular oxidative stress and disrupts the vascular response to NO, indicating endothelial dysfunction in rats (Gupta et al., 1998). It has also been reported that homocysteine reacts with NO and inhibits not only the biological activity of endothelium-derived NO but also the biological activity of exogenously supplied NO (Nappo et al., 1999; Fu et al., 2002). In this study, serum NO levels of sheep with fascioliasis were found to be significantly lower than serum NO levels of healthy sheep. The decrease in NO levels in the infected sheep is consistent with the data reported by the researchers (Cervi et al., 1998; Gupta et al., 1998). Furthermore, in the sheep infected with *F. hepatica,* homocysteine concentrations were found to be significantly higher ($P < 0.001$) while nitric oxide levels were found to be lower than in healthy sheep ($P < 0.001$). Hyperhomocysteinemia and decreased NO concentration in the diseased group support the data of the researchers (Still and McDowell, 1998; Bosy-Westphal et al., 2003; Fisher et al., 2003; Stanger and Weger, 2003; Dayal et al., 2004; Kerkeni et al., 2006; Ćulafić et al., 2013).

In conclusion, homocysteine, folate and vitamin B_{12} changes can be caused by the following reasons: 1) It is thought that *F. hepatica* infection causes severe damage in the liver, which, in turn, leads to the deterioration of functions and disruption in the conversion of homocysteine to methionine, and, as a result, causes hyperhomocysteinemia, 2) Folate and Vitamin B_{12} levels were found to be high in the fascioliasis group because folate and Vitamin B_{12}, used as cofactors in the conversion of homocysteine to methionine due to functional disorders in the liver, are not used sufficiently, 3) Low NO level in the diseased group may be due to intrahepatic vascular endothelial disorder or may be due to the inhibition of NO synthesis due to the increase of Hcy.

The present study concludes that there is a negative correlation between serum Hcy values and NO values in liver disorders caused by *F. hepatica*. Besides, since the present study is the first study to present evidence with regards to hyperhomocysteinemia in liver damage, it is thought that it will promote future research on liver damage.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Molecular profile of avian pathogenic *Escherichia coli* (APEC) from poultry associated with colibacillosis in Algeria

M. Lounis[1], G. Zhao[2], Y. Li[2], Y. Gao[2], J. Wang[2], M. Oumouna[3], K. Oumouna[3]

[1] *Département des Sciences Agrovétérinaires, Faculté des Sciences de la Nature et de la Vie, Université Ziane Achour, Djelfa 17000, Algérie.*

[2] *Laboratory of Quality and Safety Risk Assessment for Animal Products, China Animal Health and Epidemiology Center, Qingdao, China.*

[3] *Faculté des Sciences, Université Yahia Fares, Médéa, Algérie.*

ABSTRACT: The objective of the present study was the detection of virulence-associated genes of *E. coli* isolated from chicken with colibacillosis. Seventeen (17) APEC isolates were examined by two panels of PCRs for the presence of 11 genes described for avian pathogenic (*hlyF, iroN, iss, ompT, iutA* and *fimC*) and diarrheagenic (*eae*, *stx*, *est*, *elt*, *ipaH* and *aggR*) *E. coli*. Results revealed that none of the APEC isolates harbored the genes *eae*, *stx*, *est*, *elt*, *ipaH* and *aggR*. In another hand, 88.2% of the isolates were positive for 3 or more of the virulence genes *hlyF, iroN, iss, ompT, iutA* and *fimC*. Also, 35.3% of the isolates harbored all the six genes. Genes *fimC* (88.2%), *iss* (82.3%) and *ompT* (76.5%) were the most prevalent while genes *hylF, iutA* and *iroN* which were present with the same frequency (52.9%) were mostly associated with highly pathogenic strains.

Key words: *E. coli*, APEC, virulence factors, avian colibacillosis, Algeria

Corresponding Author:
Lounis M., Département des Sciences Agrovétérinaires, Faculté des Sciences de la Nature et de la Vie, Université Ziane Achour, BP 3117, Route de Moudjbara, Djelfa 17000, Algérie.
E-mail address: lounisvet@gmail.com

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INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) are *E. coli* strains that can cause a localized or a systemic disease in birds of all ages named colibacillosis (Guabiraba and Schouler, 2015) one of the most important bacterial diseases in the poultry industry throughout the world (Kunert filho et al., 2015, Paixao et al., 2016). Avian colibacillosis is responsible for significant economic losses due to decreased egg production and hatching rates, morbidity, mortality, lowered production, carcass total or partial condemnation at processing and antibiotic treatment costs (Ewers et al*.,* 2004). Furthermore, the potential for zoonotic transmission must be considered, since poultry serves as the main host for APEC and the consumption of undercooked poultry may infect humans, which can serve as a reservoir of this pathotype (Kunert filho et al., 2015).

Long considered secondary pathogen, APEC has become in recent years accepted as a primary pathogen rather than a consequence of respiratory or immunosuppressive viral or mycoplasmal infections (Vandekerchove et al., 2004; Collingwood et al., 2014). This pathogen seems to be mainly restricted to a few O-serogroups where O1, O2, and O78 are the most common in epidemiological studies (Jeong et al., 2012; Kunert Filho et al., 2015)

Recently, multiple virulence factors were described in APEC including adhesins, toxins, iron uptake systems, invasins, autotransporters and resistance to the host serum (Ewers et al., 2004; Schouler et al., 2012).

However, no single common virulence factor has been identified in all APEC strains (Collingwood et al., 2014). Furthermore, some APEC isolates carry few, if any, of the most common APEC virulence factors and they are all rarely present in the same isolate (Collingwood et al., 2014; Guabiraba and Schouler, 2015).

Due to a lack of definitive consensus of classification APEC pathotype, multiple studies have attempted to define common associated virulence genes of APEC using essentially multiplex PCRs (Ewers et al., 2005; Johnson et al., 2008; Jeong et al., 2012; Schouler et al., 2012; Dissanayake et al., 2014). However, one of the most adopted studies is the work of Johnson et al (2008) based on the presence of five genes located on ColV plasmid and considered as potential markers for differentiation and identification of highly pathogenic APEC that has a strong potential of causing extra-intestinal diseases in birds (Johnson et al., 2008). These genes include the episomal outer membrane protease (*ompT*) that cleaves colicins, the outer membrane siderophore receptor gene (*iroN)* and the aerobactin gene *iutA (iron uptake transporter)* implicated in iron acquisition, the increased serum survival gene (*iss*) which has a role in the complement resistance and the new class of avian haemolysin gene (*hlyF)* implicated in the production of outer memebrane vesiscule, toxin releasing and contribute to iron uptake (Morales et al., 2004; Murase et al., 2016).

Other virulence factors like the type 1 fimbriae *fimC* are also highly associated with APEC (Ewers et al., 2004; Jeong et al., 2012). This gene is implicated in the adherence to host epithelial cells of the respiratory tract and colonization (Jeong et al., 2012).

On the other hand, it has been shown that APEC can harbor a number of virulence genes described for diarrheagenic *E. coli* like *eae*, *stx*, *elt*/*est*, *ipaH* and *aggR* (Hughes et al., 2009; Ramadan et al., 2016) suggesting its zoonotic potential and its possible risks to humans.

Little literature is available on molecular characterization of APEC strains isolated from Algeria. This study was carried out in order to provide more information on the virulence factors of APEC strains isolated from chicken with colibacillosis in Algeria.

MATERIALS AND METHODS

Bacteria

Seventeen (17) isolates were obtained from a diagnosis veterinary laboratory located in the department of Tizi Ouzou, Algeria. These isolates were previously isolated from birds (turkey, layer, breeders) with clinically symptoms of colibacillosis from different departments. The isolates were subcultured on Mac Conkey agar (Celmed Company, Algeria) at 37 °C for 18 to 24 h. One suspected colony was picked and subcultured on nutrient agar (Institut Pasteur Algeria) overnight at 37 °C. Isolates with typical characteristics were identified biochemically using API20E® system (Biomerieux, France). All *E. coli* isolates were stored at 4 °C until use. The biochemical identification was performed at the Laboratoire d'Hygiène Intercommunal, Draa El Mizan, Algeria.

DNA extraction

For the molecular detection of the genes, DNA of the *E. coli* isolates was extracted by boiling method

as described by Blanco et al (2004). *E. coli* isolates were, subcultured overnight at 37 °C in Trypticase Soy Broth (TSB) agar. A bacterial suspension was obtained by adding 200 μL of sterile water. Bacteria were boiled for 10 min to release the DNA and centrifuged at 10,000 rpm/5 min. The supernatant containing DNA was poured into a new microtube and stored at -20 °C until use for PCR analysis.

Polymerase chain reaction (PCR)

All *E. coli* isolates were analyzed by PCR for the presence of the virulence-associated genes. The detection of *hlyF*, *iroN*, *iss*, *ompT*, *iutA* and *fimC* was analyzed by simplex PCR as previously described (Jeong et al., 2012) (Table 1). The PCR reaction was carried out in 25 μL volumes using 200 ng of DNA, 12.5 μL of GO Taq® Green Mix (Promega), 10.5 μL nuclease-free water (Sigma-Aldrich) and 0.5 μL of each primer (10 μM). The cycling conditions consisted of a 5 min activation step at 95 °C followed by 35 cycles of 95 °C for 30 s, annealing temperatures (Table 1) for 30 s, an elongation step at 72 °C for 1 min followed by the final extension step at 72 °C for 10 min.

The prevalence of genes *eae*, *stx*, *est*, *elt*, *ipaH* and *aggR* were determined by multiplex PCR as described by Toma et al (2003) (Table 1). The amplification was performed in 25 μL volumes with 200 ng of DNA, 12.5 μL of GO Taq® Green Mix (Promega), 10.5 μL nuclease-free water (Sigma-Aldrich) and 1 μL of mixed primer (10 μM).

The PCR program consisted of a 5 min activation step at 95 °C, followed by 35 cycles of 95 °C for 30 s, annealing at 56 °C for 30 s, an elongation step at 72 °C for 1 min and a final extension step at 72 °C for 10 min.

The amplified products were separated by 1% ethidium bromide-stained agarose gel electrophoresis along with a 100-bp ladder (BIOWEST, Hong Kong, China) and visualized under UV light. An *E. coli* isolate was considered positive for the gene of interest if it produced an amplicon of the expected size (Table 1).

Statistical analysis

Statistical analysis was performed using Fisher's exact test. Data were considered as significant when the p value was ≤ 0.05.

Table 1. Primer sequences and annealing temperature for the targeted virulence genes

Gene	Primers sequences (3'-5')	Annealing (°C)	References
hlyF	For GGCGATTTAGGCATTCCGATACTC	59	Jeong et al., 2012
	Rev ACGGGGATCGCTAGTTAAGGAG		
iroN	For AAAGTCAAAGCAGGGGTTGCCCG	61	Jeong et al., 2012
	Rev GACGCCGACATTAAGACGCAG		
iss	For AGCAACCCGAACCACTTGATG	57	Jeong et al., 2012
	Rev TAATAAGCATTGCCAGAGCGG		
ompT	For ATCTAGCCGAAGAAGGAGGC	57	Jeong et al., 2012
	Rev CCCGGGTCATAGTGTTCATC		
fimC	For GGAAATAACATTCTGCTTGC	51	Jeong et al., 2012
	Rev TTTGTTGCATCAAGAATACG		
iutA	For GGCTGGACATCATGGGAACTGG	61	Johnson et al., 2008
	Rev CGTCGGGAACGGGTAGAATCG		
eae	For CCCGAATTCGGCACAAGCATAAGC	56	Toma et al., 2003
	Rev CCCGGATCCGTCTCGCCAGTATTCG		
stx	For GAGCGAAATAATTTATATGTG	56	Toma et al., 2003
	Rev TGATGATGGCAATTCAGTAT		
est	For TTAATAGCACCCGGTACAAGCAGG	56	Toma et al., 2003
	Rev CCTGATCCTCAAAAGAGAAAATTAC		
elt	For TCTCTATGTGCATACGGAGC	56	Toma et al., 2003
	Rev CCATACTGATTGCCGCAAT		
IpaH	For GTTCCTTGACCGCCTTTCCGATACCGTC	56	Toma et al., 2003
	Rev GCCGCTCAGCCACCCTCTGAGAGTAC		
aggR	For GTATACACAAAAGAAGGAAGC	56	Toma et al., 2003
	Rev ACAGAATCGTCAGCATCAGC		

Table 2: Presence or absence of virulence genes and genotype of APEC isolates

Strains	*iss*	*hlyF*	*ompT*	*iroN*	*iutA*	*FimC*	Genotype
1	-	-	-	-	-	+	*fimC*
2	+	+	+	+	-	+	*iss, hylF, ompT, iroN, fimC*
3	-	+	+	-	+	+	*hylF, ompT, iutA, fimC*
4	+	+	+	-	-	+	*iss, hylF, ompT, fimC*
5	+	-	+	+	+	+	*iss, ompT, iroN, iutA, fimC*
6	+	+	+	+	+	+	*iss, hylF, ompT, iroN, iutA, fimC*
7	+	+	+	+	+	+	*iss, hylF, ompT, iroN, iutA, fimC*
8	+	-	-	-	-	-	*iss*
9	+	+	+	+	+	+	*iss, hylF, ompT, iroN, iutA, fimC*
10	+	-	+	-	+	-	*iss, ompT, iutA*
11	+	-	-	+	-	+	*iss, iroN, fimC*
12	+	+	-	+	-	+	*iss, hylF, iroN, fimC*
13	+	+	+	+	+	+	*iss, hylF, ompT, iroN, iutA, fimC*
14	+	+	+	+	+	+	*iss, hylF, ompT, iroN, iutA, fimC*
15	+	-	+	+	+	+	*iss, ompT, iroN, iutA, fimC*
16	-	+	+	-	+	+	*hylF, ompT, iutA, fimC*
17	+	+	+	+	+	+	*iss, hylF, ompT, iroN, iutA, fimC*
Total	14	9	13	9	9	15	
Frequency (%)	82,4	52,9	76,5	52,9	52,9	88,2	

RESULTS

Prevalence of virulence-associated genes

In the present study, seventeen 17 APEC isolates were examined for the presence of 11 virulence-associated genes described as APEC specific (*hlyF, iroN, iss, ompT, iutA* and *fimC)* and diarrheagenic *E. coli* genes (*eae*, *stx*, *est*, *elt*, *ipaH* and *aggR*). The prevalence of each gene in APEC isolates is shown in Table 2. PCR analysis revealed that all the isolates had at least one of the APEC specific virulence factor while none of them harbored the diarrheagenic *E. coli* specific gene. Genes *fimC* (88.2%), *iss* (82.3%) and *ompT* (76.5%) are the most prevalent. The genes *hylF, iutA* and *iroN* were present with the same frequency (52.9%).

Association of the virulence genes:

Results show that 88.2% of the isolates had 3 or more virulence genes. Furthermore, 23.5%, 17.6% and 35.3% harbored 4, 5 and 6 genes (Table 3).

Table 3. Percentage of associations between the detected virulence-associated genes in APEC isolates

	iss	*ompT*	*hlyF*	*iroN*	*iutA*	*fimC*
iss	-					
ompT	70.6	-				
hlylF	52.9	58.8	-			
iroN	64.7	52.9	59.2	-		
iutA	58.8	64.7	47.1	47.1	-	
fimC	70.6	70.6	64.7	64.7	58.8	-

Regarding genes of the association of Johnson et al (2008), results show that 35.3% were positive for the five genes and all the isolates also harbored the gene *fimC* while 63.6% of the negative strains (strains without the combination of the five genes) harbored this gene.

Different combinations were also tested. Results show that the association *iss-ompT*, *iss-fimC* and *fimC-ompT* were the most prevalent (70.6%) while *iutA-hylF* and *iutA-iroN* were the less prevalent among APEC strains (Table 4).

Table 4. Number and frequency of virulence genes among APEC isolates

Number of genes	Number of positive strains	Percentage (%)
6	6	35,3
5	3	17,6
4	4	23,5
3	2	11,8
2	0	0,0
1	2	11,8

All the strains were farther classified according to the study of Johnson et al, (2008) on highly pathogenic APEC strains for those possessing the five genes. Results show that *hylF*, *ompT* and *iutA* were statistically associated with highly pathogenic strains (Table 5).

Table 5. Frequency of virulence genes among highly and moderate APEC isolates

	Highly pathogenic APEC (%)	Moderate pathogenic APEC (%)
iss	100	72,7
hlyF	100	36,4*
ompT	100	63,6
iroN	100	36,4*
iutA	100	45,5*
fimC	100	63,6

Results were compared using the Fisher's exact test *p < 0.05

DISCUSSION

E. coli is present in the normal microflora of the intestinal tract and in the environment of poultry; certain strains must possess specific virulence attributes to cause disease. APEC is a particular pathotype of *E. coli* that carries specific virulence genes which induce avian colibacillosis; an extraintestinal syndrome commonly encountered which has a major economic impact in the poultry industry through the world (Colingwood et al., 2014; Guabiraba and Schouler, 2015).

In this study, seventeen *E. coli* isolates were obtained from birds (turkey, layer, breeders) with confirmed cases of colibacillosis and were screened for 11 virulence genes.

Results show that *fimC,* which encodes a periplasmic chaperone that directs assembly of type 1 fimbriae was the most frequent gene detected in APEC isolates (88.2%) which is in accordance of the results of different studies in the world describing a prevalence exceeding 90% (Ewers et al., 2004; Won et al., 2009; Jeong et al., 2012; Dou et al., 2016; Paxiao et al., 2018). This gene however, has been also detected with high prevalence in non-pathogenic isolates (McPeak et al., 2005; Lounis et al., 2018; Paxiao et al., 2018) suggesting that *fimC* may not play an important role in the pathogenesis of avian colibacillosis.

The *iss* (increased serum survival) gene usually located on large ColV and ColBM plasmids encodes a protein that plays a role in serum resistance, protecting against the actions of complement, and contribute to increase in *E. coli* virulence in one day old chicks (Binns et al., 1979). Gene *iss* was one of the most prevalent genes (82.8%) in this study. Similar observations were also reported (McPeack et al., 2005; Hussein et al., 2013; Ahmed et al., 2013; de Oliveira et al., 2015; Dou et al., 2016 ; Lounis et al., 2018 ; Paxiao et al., 2018; Varga et al., 2018).

All these data suggests that *iss* may be critically important in the pathogenesis of avian colibacillosis. Several trials were done using this gene as a potential vaccine target in the protection of this infection (Lynne et al, 2006, Lynne et al, 2012).

The outer episomal membrane protein encoded by the gene *ompT*, was also detected with high prevalence (76.5%) in this study. *ompT* could play a role in adherence to eukaryotic cells and cleaves antimicrobial peptides, protamine, plasminogen and colicins and may be implicated in the pathogenesis of avian colibacillosis (Stumpe et al., 1998; Hejair et al, 2017). Results obtained in our study are consistent with other reports describing high prevalences of this gene (Johnson et al. 2008; Ahmed et al, 2013; De Carli et al., 2015; de Olivera et al., 2015; Sola-Gines et al. 2015; Dissanayake et al., 2016; Chalmers et al., 2017; Varga et al, 2018). Lower prevalence were also described by Li et al (2015) and Mbanga and Nyararai (2015). This gene has been also isolated among commensal-fecal strains with prevalences that can reach 60% (Jeong et al., 2012; Hussein et al., 2013; Mohsenifard et al., 2016; Lounis et al., 2018).

Genes *HlyF*, *iroN* and *iutA;* all implicated in iron uptake were detected with the same prevalence (52.2%). These prevalences are generally lower than those described in several publications (Johnson et al., 2008; Ahmed et al., 2013; Hussein et al., 2013; Li et al., 2015 ; Wang et al., 2015; Mohsenifard et al., 2016; Lounis et al, 2018).

However, these genes are more frequent in highly pathogenic isolates than the others isolates determined by the association of Johnson et al (2008). This suggests that these genes may play critical role in the avian colibacillosis pathogenesis.

It has been reported that highly pathogenic APEC lead to primary infections while less pathogenic strains only cause disease when the poultry are under severe stressful conditions such as other diseases and environmental stress factors. In this study, 88.2% of the APEC isolates harbored 3 or more of the virulence factors which are in accordance with the available literature (Ahmed et al., 2013). In another hand, only 35.3% of the isolates are positive for the combination of the five genes *iss-ompT-hylF-iutA-iroN.* Higher frequencies of this combination were reported

in APEC isolates through the world with prevalence from 57.6% to 91% (De Carli et al., 2015; De Olivera et al., 2015, Hussein et al., 2013; Lounis et al., 2018) while Li et al (2015) found that four isolates (4.6%) only among 87 APEC harbored these combination. Our results revealed that not all the APEC isolates are equally virulent.

Regarding the specific genes of diarrheagenic *E. coli*, our results shows that all the 17 APEC isolates were negative the genes *eae, stx, elt/est, ipaH* and *aggR*. Similar to the results of current study, none of the isolates from septicemic broilers and quails harbored *ipaH*, *stx1*, *stx2*, and *eaeA* genes (Ghanbarpour et al., 2010; Salehi and Ghanbarpour, 2010). In a previous study, Lounis et al (2018) reported that all the 92 APEC strains tested are negative for *eae* and *aggR* genes while 5.4%, 2.1% and 2.1% were positive for the genes *est/elt, stx* and *ipaH* respectively. These results suggest that APEC strains have a lower potential to cause diarrhea in human.

CONCLUSIONS

In conclusion and despite the potential biases related to the relatively small sample size of APEC isolates; this study could contribute to the molecular characterization of APEC in Algeria.

Results of the prevalence of the virulence associated genes of APEC in this study are generally lower to those reported in several countries.

Concerning the specific genes of diarrheagenic *E coli*, our results show that APEC isolates have a low potential in inducing diarrhea to humans

Other studies using a large sample size are needed which could provide more informations and definitive conclusions about the molecular profile of APEC.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Occurrence of black spot disease induced by *Posthodiplostomum cuticola* (Nordmann, 1832) (Digenea: Diplostomatidae) in endemic and native fish of Turkey: seven new host records

D. Innal[1], F. Özdemir[2], M. M. Stavrescu-Bedivan[3], O. Ozmen[4], M. O. Özturk[5]

[1]*Mehmet Akif Ersoy University, Department of Biology, Burdur, Turkey*

[2]*Hacettepe University, Department of Biology, Ankara, Turkey*

[3]*University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Agriculture, Romania*

[4]*Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Pathology, Burdur, Turkey*

[5]*Afyon Kocatepe University, Department of Molecular Biology and Genetics, Afyon, Turkey*

ABSTRACT: This study was conducted to determine parasite infection with *Posthodiplostomum cuticola* in eight fish species collected from five Rivers Basins of Turkey (Aras, Çoruh, Sakarya, Marmara and Susurluk River Basins). Five of eight fish species are endemic to Turkey. The highest values of prevalence and mean intensity (60.87%; 4.86) of cysts with metacercaria of *P. cuticola* were recorded in *Alburnus filippii*, a native fish species in Ağıl Creek. At the gross examination numerous black lesions were observed at the skin of the fishes. Microscopically black-spot disease was revealed by melanin pigmentation and atrophy of fish muscles in lesioned areas. Parasites were covered by thing connective tissue capsule. *Alburnus escherichii*, *Alburnus filippii*, *Capoeta tinca*, *Chondrostoma angorense*, *Chondrostoma colchicum*, *Squalius pursakensis* and *Squalius turcicus* were found as new host records for *P. cuticola*.

Keywords: black-spot disease, cyprinid, histopathology, *Posthodiplostomum cuticola*, Turkey

Corresponding Author:
D. Innal, Mehmet Akif Ersoy University, Department of Biology, Burdur, Turkey
E-mail address: innald@yahoo.com

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INTRODUCTION

Posthodiplostomum cuticola (Nordmann, 1832) is a digenean parasitic fluke, responsible for black-spot disease, a pathological condition manifested by an intensive pigmentation of the cyst wall located in the skin, muscle tissue and fins of freshwater fish hosts throughout the world (Ondračková et al., 2004; Zrnčić et al., 2009; Kvach et al., 2017).

In the complex life-cycle of *P. cuticola* involving piscivorous birds as definitive hosts and planorbid snails as first intermediate host, various fishes belonging mainly to Cyprinidae and Cobitidae families play the role of the second intermediate host (Ondračková et al., 2004; Markovic and Krsmanovic, 2008; Zrnčić et al., 2009). Members of Escoidae, Percidae and Salmonidae could also represent hosts for *P. cuticola* (Mierzejewska et al., 2004).

Fish reaction to the parasite presence consists in dark pigment produced by decomposition of hemoglobin and chromatophores disposed around metacercaria, the penultimate development stage of *P. cuticola* (Markovic and Krsmanovic, 2008; Markovic et al., 2012). Although the black-spot disease is not deadly to fish hosts in general, symptoms such as body deformation, muscle fibers necrosis, kidney and liver dystrophy may occur and could be harmful to fry (Rolbiecki, 2004; Markovic and Krsmanovic, 2008; Iqbal et al., 2014).

To date, occurrence of *P. cuticola* infection was reported only in six cyprind species from ichthyofauna of Turkey: *Alburnus chalcoides*, *Blicca bjoerkna*, *Cobitis* cf. *turcica*, *Cyprinus carpio*, *Scardinius erythropthalmus* and *Vimba vimba* (Öztürk, 2005; Kırankaya and Ekmekçi, 2011; Öktener, 2014; Altan and Soylu, 2018). Therefore, the aim of this paper was to assess new host-parasite relationships in native and endemic fish species from Turkish inland waters in which the causal agent of black-spot disease is spread.

MATERIALS AND METHODS

Overall, 153 individuals from eight fish species belong to 2 families (Cyprinidae and Cobitidae) and 5 genera were analysed: *Alburnus escherichii* Steindachner, 1897 (Caucasian bleak); *Alburnus filippii* Kessler, 1877 (Kura bleak); *Capoeta tinca* Heckel, 1843 (Anatolian khramulya); *Chondrostoma angorense* Elvira, 1987 (Ankara nase); *Chondrostoma colchicum* Derjugin, 1899 (Colchic nase); *Cobitis taenia* Linnaeus 1758 (Spined loach); *Squalius pursakensis* Hankó, 1925 (Sakarya chub) and *Squalius turcicus* De Filippi, 1865 (Transcaucasian chub). The study was carried out during 2010-2018 years. Fishes were collected from seven different locations (Seydisuyu Creek, Pasinler Creek, Süvari Creek, Düzköy Creek, Çerpeş Creek, Karaçaltı Creek, Ağıl Creek) (Figure 1) capturing five different river basins of Turkey: Aras, Çoruh, Sakarya, Marmara and Susurluk.

Figure 1. Map of sampling localities

For parasitic isolation, the skin and fins of the host fish specimens were taken into Petri dishes with physiological water. The parasites were separated by a scalpel. Subsequently, the parasites were fixed in 70% ethyl alcohol medium between the lamella for 24 hours. The parasite samples were then stained with hematoxylin (Pritchard & Kruse, 1982) and species identified, according to Bykhovskaya et al (1962).

During the necropsy, clinically lesioned and abnormal pigmented samples of analyzed fish species were collected and fixed in 10% neutral formalin solution for histopathological examination. After fixation tissue samples were routinely prepared by automatic tissue processing equipment (Leica ASP300S; Leica Microsystem, Nussloch, Germany). The samples were embedded in paraffin, and 5 μm serial sections taken from sections were prepared using a Leica RM 2155 rotary microtome (Leica Microsystem, Nussloch, Germany). Then sections were stained with hematoxylin and eosin (HE) and examined under the 40X a light microscope. Morphometric evaluation and microphotography were performed using the Database Manual cellSens Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan).

Prevalence (Pr %), as the percentage of hosts infected with a particular parasite species or taxonomic group and intensity (Int), as the number of individuals of a parasite species in/on a single infected host were calculated following Bush et al. (1997).

RESULTS

Among eight host species (Table 1) examined for infestation with *Posthodiplostomum cuticola* (Nordmann, 1832), the highest prevalences (60.87%) of infection were reached in *Alburnus filippii*, a native fish from Ağıl Creek, followed by *A. escherichii* (57.14%) and *Squalius pursakensis*, endemic fish in Seydisuyu Creek (55%) (Table 2). With respect of mean intensity, the highest values were recorded in *A. filippii* (Ağıl Creek), *A. escherichii* (Seydisuyu Creek) and *Capoeta tinca* (Süvari Creek) (4.86; 4.75; 3.75 parasites/fish, respectively).

At the histopathological analysis, most of the black spots were composed of parasites surrounding by thick fibrous capsule around the encysted parasite developmental stages with periphery of the capsule containing numerous melanocytes. Most of the lesions contained one or more metacercariae of the parasites.

Table 1. Host fish species and their status

Fish Species	Family	Locality	River Basin	Status	Length (cm) (Min-Max)
Squalius pursakensis	*Cyprinidae*	Seydisuyu Creek	Sakarya	Endemic	8.7-19.4
Alburnus escherichii	*Cyprinidae*	Seydisuyu Creek	Sakarya	Endemic	5.2-15.1
Chondrostoma angorense	*Cyprinidae*	Seydisuyu Creek	Sakarya	Endemic	8.2-11.5
Squalius turcicus	*Cyprinidae*	Pasinler Creek	Aras	Endemic	10.2-14.9
Alburnus filippii	*Cyprinidae*	Pasinler Creek	Aras	Native	8.9-12.3
Capoeta tinca	*Cyprinidae*	Süvari creek	Sakarya	Endemic	16.2-24.0
Chondrostoma colchicum	*Cyprinidae*	Düzköy Creek	Çoruh	Native	7.6-13.4
Cobitis taenia	*Cobitidae*	Çerpeş Creek	Marmara	Native	7.0-9.4
Capoeta tinca	*Cyprinidae*	Karaçaltı Creek	Susurluk	Endemic	10.4-18.6
Alburnus filippii	*Cyprinidae*	Ağıl Creek	Aras	Native	8.2-11.6

Table 2. Occurence of *Posthodiplostomum cuticola* infestation in fish community of study area (N = total number of hosts examined; N'= number of infected fishes; Pr = prevalence)

Fish Species	Locality	N	N'	Pr%	Total parasite	Mean Intensity
Squalius pursakensis	Seydisuyu Creek	20	11	55	15	1.36
Alburnus escherichii	Seydisuyu Creek	14	8	57.14	38	4.75
Chondrostoma angorense	Seydisuyu Creek	11	4	36.36	7	1.75
Squalius turcicus	Pasinler Creek	11	4	36.36	11	2.75
Alburnus filippii	Pasinler Creek	12	4	33.33	9	2.25
Capoeta tinca	Süvari creek	9	4	44.44	15	3.75
Chondrostoma colchicum	Düzköy Creek	16	1	6.25	2	2.00
Cobitis taenia	Çerpeş Creek	22	10	45.45	32	3.20
Capoeta tinca	Karaçaltı Creek	15	3	20	7	2.33
Alburnus filippii	Ağıl Creek	23	14	60.87	68	4.86

The melanin capsules often presented an opening on the surface of cuticle layer. Amount of the melanocytes was different in each nodule. Either not observed at all or slight inflammatory reaction was observed near the lesions (Figure 2). The lesions were usually associated with migration of developmental stages of the parasite within the host tissues. Atrophy of the muscle near the parasitic lesion was commonly noticed. Melanocytes were also observed between the muscles near the cysts.

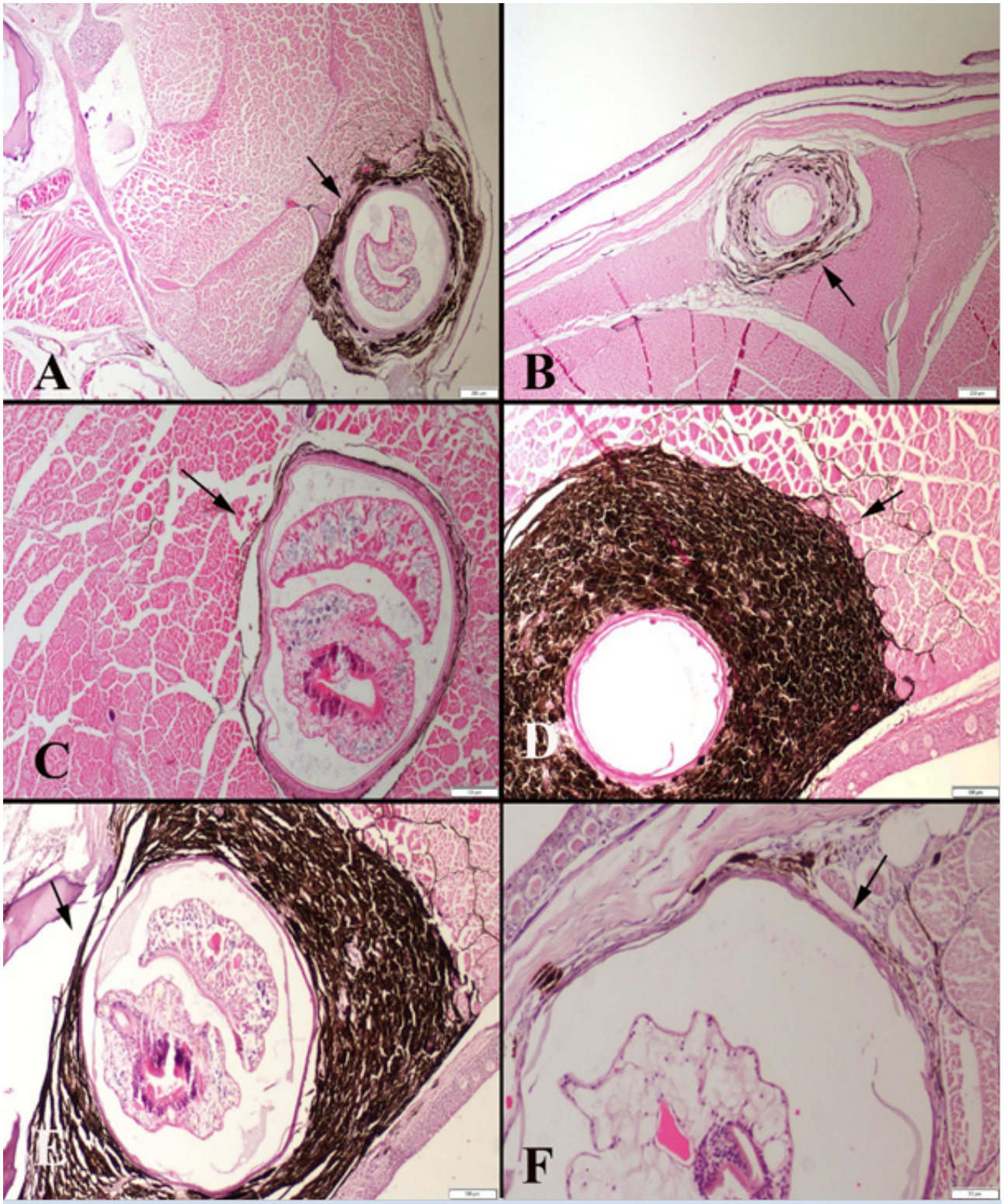

Figure 2. Histopathology of the black spot lesions in fish muscle related to parasite developmental stages, with different severity of melanocytic reaction. HE, Bars=200μm (A, B), 100 μm (C, D, E) and 50 μm (F).

Figure 3. Black spot disease found in selected fishes in the present study

Grossly affected fishes exhibit multifocal black spots in the skin which were different in shape and diameter. Black spots were localized in all skin regions from mouth to caudal fin (Figure 3). In some fish hyperemia was observed around the lesions.

DISCUSSION

Black-spot disease induced by *P. cuticola* was reported before in freshwater fish of Turkey, however there was no histopathological information available up to date. Worldwide, there is still a paucity of data concerning histological examination of fish diagnosed with posthodiplostomatosis. Nevertheless, few observations similar to ours on melanin deposits were made on: *Ctenopharyngodon idella* from a fishpond in Serbia (Markovic et al., 2012) and *Blicca bjoerkna*, *Rutilus rutilus*, *Scardinius erythrophthalmus* from a freshwater lake from Romania (Negrea et al., 2015). Parasitological data have been reported just for four species including *Alburnus escherichii*, *Capoeta tinca, Squalius pursakensis* and *Cobitis taenia* (Öktener, 2014; Innal et al., 2016). On the other hand, *P. cuticola* was reported only in *Cobitis taenia* of eight host species. The prevalence and intensity levels of infection among host species differed by host and area. The prevalence of *P. cuticola* infection in all host species ranged from 6.25% to 60.87%. The difference in the prevalence is likely due to abiotic parameters of systems and also biotic factors such as host health status, age and size and fish community structure.

Bykhovskaya-Pavlovskaya et al (1962) stated that this parasite is found in the most common cyprinids, rarely seen in sturgeon, catfish and freshwater perch. Fish specimens which were examined in this study and recorded with *P. cuticola* infection support this view. Öztürk (2005) which recorded *P. cuticola* infection in skin and fins of *Cyprinus carpio* stated that the occurrence of parasite decreases in autumn and winter reaching its highest level during the summer period. The same research determined that *P. cuticola*

infection was found in carp fishes of 15-20 cm in size, but it was not found in fishes of 13 cm and smaller. In contrast to Öztürk (2005), in the present study small sized fish individuals were found infected by *P. cuticola*.

There is a little knowledge about pathology of the black spot disease in fishes. Histopathological observations revealed that the black spots on the fishes are melanized nodules formed against metacercaria of *P. cuticola.* In addition, inflammatory cell infiltration and atrophy of the skeletal muscle around the metacercaria of the parasite were common findings indicating inflammatory reaction. The parasites were penetrated under the skin of the fish and formed encysted metacercaria and covered by a thick fibrous capsule these findings being in agreement with classical knowledge (Lane and Morris, 2000; Bush et al., 2001).

As a result, in this study, *P. cuticola* was determined to be a parasite species found in various fish species in different regions of Turkey. In addition, histopathological effects of this parasite species on host fish tissues were identified. In future studies, water birds in the study areas where parasitized fish live may be investigated whether they are infected with *P. cuticola*.

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CONFLICT OF INTEREST

None declared

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Research article
Ερευνητικό άρθρο

Evaluation of metabolic profiles of Saanen goats in the transition period

F. Akkaya[1], S. Senturk[2], Z. Mecitoğlu[2], S. Kasap[2], S. Ertunc[2], C. Kandemir[1]

[1]Department of Animal Science, İzmir Ege University, 35040, İzmir, Turkey

[2]Department of Internal Medicine, Faculty of Veterinary Medicine, Bursa Uludag University, 16059, Bursa, Turkey

ABSTRACT: Healthy Saanen goats (n=30) in periparturient period were used in the present study. Blood samples were collected 21, 14 and 7 days before parturition, at the time of birth and postpartum days 7, 14 and 21. Non-esterified fatty acids (NEFA), betahydroxy butyric acid (BHBA), Total protein (TP), albumin (ALB), blood urea nitrogen (BUN), creatinine (CREA) aspartate amino transferase (AST), gamma glutamyl transferase (GGT), sorbitol dehydrogenase (SDH), glucose (GLU), cholesterol (CHOL), triglyceride (TG), calcium (Ca), phosphorus (P), and magnesium (Mg) levels were evaluated. During the study period, NEFA, SDH, CREA levels increased and CHOL and TG levels decreased at time of parturition. When the changes of parameters in prepartum and postpartum period were compared, the concentrations of NEFA, CHOL, GLU, TG, Ca were higher ($p < 0.001$) in prepartum period;however BHBA, Mg, ALB, GGT, AST, TP, P, BUN, SDH and CREA concentrations were detected to be higher ($p < 0.001$) in postpartum period. Metabolic Profile Test based on biochemical parameters evaluated in our study would be beneficial for diagnosis, prevention and control of diseases such as pregnancy toxemia, hypocalcemia, infertility in goats.

***Keywords*:** BHBA, Metabolic profile test, NEFA, Saanen goat, Transition period

Corresponding Author:
Sezgin Senturk, Department of Internal Medicine, Faculty of Veterinary Medicine, Uludag University, 16059, Bursa, Turkey
E-mail address: sezsen@uludag.edu.tr

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INTRODUCTION

Milk production levels in excess of the metabolic reserve capacity of the animal leads to many metabolic diseases in periparturient period(Gilbert et al., 1998). The transition period, 3 weeks before and 3 weeks after birth, is a period in which many metabolic changes occur. If this period is not well managed and the nutritional needs of animals are not met, metabolic diseases will inevitably occur (Caldeira et. al.,2007, Soutor et.al., 2013, Araujo et al., 2014)Metabolic profile test is widely used evaluate andtake early preventive measures in periparturient period problems of cattle. Unfortunately, in small ruminants these studies are mostly conducted on sheep and studies conducted with goats are rare.

Metabolic profile test (MPT) is a quantitative test to typically performed to assist in the early diagnosis of many metabolic diseases and to monitor animal health status on a flock basis (Ghergariu et al, 1984; Boginet al., 1988; Batmaz et al., 1992; Kida, 2002a, 2002b).MPT scores when used in conjunction with environment, nutrition, and body condition evaluationcould be employed for the assessment of yield characteristics, as well as in the improvement of milk quality and quantity, elimination of fertility problems, and prevention of many subclinical diseases. In sum, protection of herd health and reduction of possible economic losses could be achieved by regular MPT implementation (Ghergariu et al, 1984; Boginet al., 1988; Ivanov et al., 1993).

In order to check MPT in goats; parameters such as glucose (GLU),beta hydroxybutyrate (BHBA), non-esterified fatty acid (NEFA),cholesterol (CHOL), total protein (TP), albumin (ALB), globulin, blood urea nitrogen (BUN), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), sorbitol dehydrogenase (SDH), triglyceride (TG), calcium (Ca), magnesium (Mg) and inorganic phosphorus (P) could be evaluated(Borges et al., 1997; Kida, 2002a, 2002b). By measuring these, it is possible to comprehensively evaluate the nutritional status ofanimals, especially during the transition period (Batmaz et al., 1992; Ivanov et al., 1993).

Although there have been many studies on metabolic profile testing in cattle, especially in the Holstein cattle (Cozzi et al., 2010; Samanc et al., 2011), limited research has been conducted on high milk producing Saanen goats. This gap in the extant knowledge has been motivated the present study, the aim of which is to obtain a preliminary reference for the biochemical parameters and allow monitoring of changes in metabolic profiles of the Saanen goats during the transition period.

MATERIALS AND METHODS

Animal material: The study sample consisted of 30 dairy Saanen goats in second and third lactation from a flock of 300 animals. All goats were from the sameherd and yield group, and the management andfeeding conditions were same for all.They ranged in age from two to four years. Lactation milk yield average was about 850lt per goat in the previous lactation.It was stated that the rate of twins in the previous pregnancy wasabout 64%.Further, the previous average lactation period was reported as 273 days.

Routine clinical examinations (body temperature, pulsation and respiration rates, lymph nodes, tracheal palpation, lung auscultation and percussion) were performed, and only clinically healthy animals were selected for inclusion in the study and these routine clinical examinations were repeated before each sampling(Diffay et al., 2005). Animals suffering from any diseases that cause such as ketosis, pneumonia, enteritis, mastitis, metritis, lameness that might affect the biochemical parameters were excluded from the study.

After birth, the kids were left with their mother. They were allowed to suckle their mothers freely. Goats were milked twice a day during and after the study period.All animals included in the study were fed with the same rations (Table 1) during the study, and their housing and environmental conditions were identical. All goats were provided with 1 kg of hay per animal daily. A concentrate containing 12% protein was also offered each morning (0,9 kg for each goat). All animals had unlimited access to water and salt blocks. Animals included in the study were given antiparasitic treatment one month before conception.

Sample collection and evaluation

Blood samples were collected from the animals 21, 14, and 7 days before expected parturition, at the time of parturition, as well as 7, 14 and 21 days postpartum. While all blood samples for the measurement of BHB were taken in the morning, approximately 6 hours after the morning feed, for other biochemical parameters including albumin, phosphorus, magnesium, AST, GGT, TP, BUN, and NEFA were taken before the morning feeding.

Table 1. Ingredient of the experimental diets for Saanen goats in prepartum and early lactaiton periods

Period	Forage	Quantity (gr)	Participation Rate (%)	DM (%)	Energy (Mcal/ kg)	Protein (%)
Pre-partum	Corn Silage	1.500	0.52	34.10	2.35	8.90
	Alfaalfa	400	0.14	89.20	1.82	16.20
	Straw	250	0.09	92.70	1.34	4.70
	Concentrate feed	500	0.17	91.20	2.55	18.60
	Barley	250	0.09	90.65	2.89	11.90
Early lactation	Corn Silage	2.000	0.53	34.10	2.35	8.90
	Alfaalfa	600	0.16	89.20	1.82	16.20
	Straw	100	0.03	92.70	1.34	4.70
	Concentrate feed	1100	0.29	91.20	2.55	18.60
	Barley	-	-	90.65	2.89	11.90

The blood samples were collected by jugular venipuncture,with 25x8mm needles, using vacuum tubes with clot activator and gel for serum separation (Becton Dickinson and Company, Franklin Lakes, NJ) Serum samples were separated off by centrifugation for 15 minutes at 3000 rpm (for BHBA testing within 1/2 h of collection. Haemolysed samples were excluded from the study. Serum samples were stored at – 20 ºC until analyzed. Samples were then centrifuged again after thawing before the analysis

Serum biochemical parameters: Serum levels of albumin, phosphorus, magnesium, AST, GGT, TP and BUN were determined by Vet Scan-VS2® device (Abaxis, Inc.Union City, CA 94587) in Uludag University Veterinary Faculty Animal Hospital Central Laboratory. Glucose, triglyceride, calcium, cholesterol and creatinine levels were determined spectrophotometrically using an Abbott c16000 (Abbott ARCHITECT c16000, Abbott Park, Abbott Laboratories, Illinois-USA) in Uludag University, Faculty of Medicine Central Laboratory which is an accredited laboratory. BHBA levels were measured using on farm ketone test kit and corresponding reading device (Ketosite® BHBA test card, Ketosite® instruments, Stanbio Laboratory Texas-USA). NEFA levels were determined spectrophotometrically in Balikesir System Laboratory, using a commercial NEFA kit (NEFA-HR (2) Wako Chemicals GmbH, Germany). SDH levels were measured in Istanbul Bilim Laboratory, using a Goat SDH ELISA kit (Goat SDH ELISA kit, SunRed, Cat. No: 201-07-3106, Epoch microplate spectrophotometry SN:242136, Biotek Winooski, VT, USA).

Statistical analysis

The normality of the data was determined by Shapiro–Wilk test. The one-way repeated measures (RM) ANOVA test was used to determine whether there was a difference between the values of thesame parameters on different days, using Sigma Plot 12 software. For the all pairwise multible comparison procedures, Holm-Sidak test was used for the data that was normally distributed and Tukey test was selected for the data with normality lower than <0.05. For all analyses, $P < 0.05$ was accepted as significant.

The study was approved by Uludag University Animal Experiments Local Ethics Committee (HADYEK), Bursa, Turkey (Decision no: 2014-16/04).

RESULTS

During the study, routine clinic examinations including body temperature, heart rate, respiratory rate, mucosal membranes, auscultation, and percussion of lungs of animals were normal in all animals. Also, the appetite of goats was very good during the working period. No animals were excluded from the study due to any disease during the study.Goats in farm in the scope of work were milked two times a day. The average daily milk production were determined as 3.8 ±0.324 liters during the study period.In this study, In this study, while 24 out of 30 pregnant goats bore twin kids, one pregnant goat bore triplets kids. Each of the remaining 5 pregnant goats bore a kid.The average live weight of the goats in the 21 days before birth was 51.45±4.1kg.On the 21st postnatal day, their mean live weight was47.1±5.4 kg.

NEFA concentrations were within normal range in the prepartum period, reaching the peak level (0.53 mmol/L) at the day of parturition,whereasthe lowest level (0.15 mmol/L) was detected 21 days postpartum (Table 2). The difference between prepartum and postpartum NEFA levels was not statistically significant.

However, NEFA levels were significantly higher on the day of delivery when compared to prepartum levels (0.58 mmol/L). The mean BHBA (as well as measured levels) concentrations were found to be within the reference limits during the entire study period (Table 2). However, while the BHBA levels steadily decreased in the periparturient period, increased significantly at the time of parturition (0.38 mmol/L) to reach the peak levels on day 21 (0.55 mmol/L) (Table 2).

Table 2. Levels (Mean± SEM) of non esterified fatty acids (NEFA; mmol/L), betahydroxybutyric acid (BHBA; mmol/L), total protein (TP; g/L), albumin (Alb; g/L), blood urea nitrogen (BUN; mmol/L), creatinine (Crea; mg/dL), aspartate aminotransferase (AST; IU/L), gamma glutamyl transferase (GGT; IU/L), sorbitol dehydrogenase (SDH; IU/L), Glucose (Glu; mmol/L), cholesterol (Chol; mg/dL), triglycerides (TG; mg/dL), calcium (Ca; mg/dL), phosphorus (P; mg/dL) and magnesium (Mg; mg/dL) in prepartum,at time of parturition and postpartumSaanen Goats.

	Day of pregnancy						
Paremeters	-21	-14.	-7.	0.	7.	14.	21.
NEFA	0.30±0.03^{b}	0.17±0.01^{b}	0.29±0.07^{b}	0.53±0.05^{a}	0.29±0.04^{b}	0.26±0.05^{b}	0.15±0.02^{b}
BHBA	0.39±0.04^{a}	0.31±0.04^{a}	0.20±0.03^{b}	0.38±0.05^{a}	0.23±0.03^{b}	0.45±0.07^{a}	0.55±0.04^{a}
TP	6.56±0.13^{b}	6.52±0.07^{b}	6.69±0.10^{a}	6.71±0.11^{a}	6.92±0.12^{a}	6.62±0.10^{b}	6.79±0.09^{a}
Alb	4.05±0.27^{a}	4.19±0.25^{a}	3.47±0.21^{b}	3.71±0.20^{b}	3.74±0.22^{b}	4.57±0.20^{a}	4.73±0.19^{a}
BUN	12.7±0.85^{b}	13.6±0.94^{a}	9.0±0.63^{b}	11.1±0.68^{b}	13.9±0.75^{a}	16.4±0.85^{a}	16.0±0.96^{a}
CREA	0.56±0.01^{b}	0.63±0.01^{a}	0.64±0.01^{a}	0.68±0.01^{a}	0.63±0.01^{a}	0.59±0.01^{b}	0.60±0.01^{b}
AST	45.1±3.76^{b}	52.7±3.08^{b}	42.0±2.78^{b}	62.7±3.30^{a}	58.7±3.64^{a}	63.6±3.38^{a}	69.8±4.38^{a}
GGT	27.3±2.76^{b}	27.0±2.64^{a}	29.3±2.06^{b}	29.3±1.85^{b}	30.3±2.24^{b}	34.9±1.72^{a}	39.8±2.95^{a}
SDH	15.1±3.05^{b}	13.3±2.52^{b}	18.7±3.59^{b}	29.9±4.43^{a}	24.0±3.73^{a}	25.4±3.73^{a}	20.8±2.61^{a}
Glu	45.5±3.1^{b}	60.4±1.4^{a}	62.8±1.5^{a}	57.2±1.7^{a}	53.7±1.2^{b}	50.2±1.0^{b}	54.1±1.4^{b}
Chol	97.7±2.26^{a}	92.4±2.36^{a}	88.4±1.94^{b}	83.8±2.06^{b}	92.3±2.48^{a}	88.6±2.79^{b}	94.4±3.20^{a}
TG	32.9±2.0^{a}	34.9±2.2^{a}	36.0±2.6^{a}	9.4±0.4^{b}	15.6±1.3^{b}	13.2±0.9^{b}	14. 3±1.1^{b}
Ca	9.38±0.09^{a}	8.98±0.14^{a}	9.15±0.09^{a}	8.68±0.14^{b}	8.7±0.12^{b}	8.33±0.12^{b}	8.67±0.13^{b}
P	5.46±0.4^{a}	6.29±0.4^{a}	4.59±0.3^{b}	4.66±0.3^{b}	5.31±0.3^{a}	6.59±0.4^{a}	5.63±0.3^{a}
Mg	2.23±0.1^{b}	2.21±0.1^{a}	1.94±0.1^{b}	2.08±0.1^{a}	1.92±0.1^{b}	2.74±0.1^{a}	2.56±0.1^{a}

$^{a, b}$: There is statistical significance between values expressed in different letters on the same line ($p < 0.001$).

The total protein concentrations between days of the study were found to be very close to each other and these values were also found at the reference range (TP reference range = 3.5-13gr/dL). It was determined that the TP levels peaked (6.92 g/dL) at the first week postpartum and was lowest (6.52 g/dL) at the second week prepartum (Table 2). Again serum albumin levels were detected within reference range during the study period (albumin reference range = 0.5-5 g/dL), lowest albuminlevelswere detected at the week before parturition (3.47 g / dL) and the highest level was detected at postpartum 3rd week (Table 2).

BUN levels were detected to decrease till the delivery and steadily increased after parturition. BUN levels were lowest (9 mmol/L) at the week before parturition and highest in postpartum week 2 (16.4 mmol/L), respectively (Table 2). The level of creatinine gradually increased until the day of delivery, reached the peak level at the time of delivery (0.68 mg/dL), and gradually decreased in the postpartum period and remained within the reference range (Table 2).Serum AST values were found to be within normal limits throughout the study period (AST reference range = 2- 75 IU/L). AST levels were lowest birth 1 week before the parturition (42 IU/L) and highest at week 3 postpartum (69.8 IU/L) (Table 2).

It is known that the serum GGT in goats is 0-30 IU/L (Batmaz, 2013). According to that reference value, GGT levels were found to be slightly higher in our study especially at 2. and 3 weeks after parturition (Table 2). Serum SDH values gradually increased until the birth, reached to peak level during delivery (29.9 IU/L) and gradually decreased after birth. Serum glucose values of the animals used in the study were at the peak level (62.8 mmol/L) at the 7th day preparum and the lowest levels (45.5 mmol/L) were detected three weeks before parturition(Table 2). It was also detected that the lowest level value is slightly below the reference values (Glucose reference range = 50-75 mg/dL).

The serum cholesterol levels were within reference range (cholesterol reference range = 17-210 mg/dL) during the study period. Lowest cholesterol levels

(83.8 mg/dL) were detected at the day of parturition and the highest (97.7 mg/dL) levels were detected on the third week before parturition (Table 2). Triglyceride levels were significantly higher in prepartum period when compared to postparum period. Along with that triglyceride levels reach the lowest point (9.4 mg/dL) which is very close to the lowest reference value (triglyceride reference range = 6 - 200 mg/dL) at time of parturition. Serum calcium levels were also detected within reference range in all samplings during the study period (reference = 8.9 -11.7 mg/dL) (Batmaz, 2013). Calcium levels were highest at week 3 prepartum (9.38 mg/dL) and lowest on the second week after parturition (8.33 mg/dL)(Table 2).

Serum phosphorus levels in our study were found to be lowest (4.59 mg/dL) in the week before parturition (Table 2), and peaked (6.59 mg/dL) in the second week after delivery. Along with that phosphorus levels were detected within reference values in all samplings during the study period (Phosphorus reference range = 4.2-9.1 mg/dL). Magnesium levels were lowest on day 7 postpartum and highest on day 14 postpartum (Table 2).

DISCUSSION

This study was conducted in order to determine whether some significant biochemical blood parameters in Saanen goats vary between prepartum and postpartum periods.

Increased lipolysis and decreased lypogenesis around parturition causes elevation of blood NEFA levels and BHB levels (Mc Namara, 1994). Sadjadianet al. (2012) reported that serum concentrations of NEFA in Saanen goats gradually increased from day 30 before parturition to the highest level on day of delivery and then gradually decreased until the 35th day postpartum. In our study we detected similar results as NEFA levels were highest at day of parturition and afterwards decreased steadily (Table 2). The increased concentration of NEFA during delivery could be related lipolysis and hormonal changes triggered by both the energy that the animal will spend for labor, the energy needed for milk production after birth, in order to meet the energy required for the development of the fetus and mammary gland in the last period of pregnancy (Vazquez-Anon et al., 1994; Grummer, 1995; Cheng et al., 2007). According to Herdt(1988, 2000), the rise of plasma NEFA in the end of pregnancy may not end with an increase in TG in the liver however but acute NEFA increases at time of delivery may trigger liver TG infiltration. Vazquez-Anon et al.(1994) and Herdt (2000) have concluded that, the concentration of NEFA reaching peak levels during delivery decreased in the first weeks of lactation. During these events, hormonal changes are also very important.Before parturition, the insulin/glucagon ratio is reduced in favor of glyconeogenesis and lipolysis (Vazquez-Anon et al., 1994).In the present study, it was observed that the level of the NEFA gradually began to descend to the basal level in postpartum period.In addition, reductions in NEFA can be interpreted as a sign of reduced fat mobilization or the use of NEFA for VLDL synthesis in the liver. On the other hand, highest plasma concentrations of NEFA were detected at birth and the highest BHBA concentrations were detected at third week postpartum. This lag may be explained by the fact it can be used in the synthesis of BHBA in the liver following the increase of NEFA firstly due to lipolysis (Cheng et al., 2007).

The BHBA is the most important indicator of energy status in the transition period (Duffield, 2003; Inal et al., 2007). Navarre and Pugh (2002) suggested that the concentration of BHBA in the range 0.8 to 1.6 mmol / L was indicative of NEB in sheep. In another study conducted by Moghaddam and Hassanpour (2008) the BHBA concentrations of sheep was higher in the goats in the prepartum period.Sadjadianet al. (2012) reported that the BHBA concentrations increased from 15 days prepartum till the third week postparum and than tended to decrease in Saanen goats. In our study BHBA levels decreased as parturition approached, increased during labor and gradually increased in the following weeks after birth.The reason for this may be increased energy demand with the onset of lactation.

Similar to other studies (Tanrıtanır et al.,2009; Sadjadianet al. (2012), Serum total protein levels in our study were lower before parturition than the postpartum period. This reduction may be due to the fact that protein synthesis required for the development and growth of the fetus, is carried out using maternal amino acids and that requirement is highest at the end of pregnancy.

As reported by Shetawi et al. (1992), in the present study, albumin levels gradually decreased until parturition and significantly increased 1 week after delivery(Table 2). As in humans (Ogbodo et al., 2012), the decrease in albumin level may be associated with decreased albumin synthesis in pregancy, also and increased loss in urine in pregnancy.

As Sadjadian et al (2012) found, BUN concentration in our study were lower in the prepartum period, tended to increase after parturition (Table 2). The reason for lower BUN levels around parturition may be related to reduced feed intake and dry matter consumption, associated with increased stress and hormonal changes.

AST activity is an important indicator for fatty liver disease in cows (Kaneko 1989, Cebra et al., 1997; Herdt, 2000, Seifi et al., 2007, Herdt, 2009). Studies conducted on sheep by Taghipour et al. (2011) was found a significantly higher AST concentration in the postpartum period, and this elevation was explained to be indicative of fat infiltration in the liver due to NEB. Despite the fact that the postpartum AST levels were higher than the prepartum levels in our study, the fact that the AST levels in the pregnancy were within the reference ratecould indicate that the animals used in our study were not in the NEB(Table 2).

It has been reported that serum GGT levels are high in negative energy balance associated fatty liver of ruminants, and this parameter may be used as a diagnostic tool (Senturk, 2013). Sevinc et al.(1999) found no statistically significant difference in serum GGT levels between the 7th month of pregnancy and 2nd month after birth in a study conducted in cattle. In the present study, no significant change was observed in GGT levels.

SDH enzyme activity is very important in determining acute hepatocellular damage in ruminants. (Senturk, 2013). SDH levels in our study gradually increased until birth and reached peak levels during delivery and then gradually began to fall after birth(Table 2). Although this short-term rise in SDH reaching peak levels at birth strengthens the likelihood of fatty liver, but this elevation also be related to hormonal changes during labor.

According to Khan et al.(2009), blood glucose concentrations are one of the most important parameters that can reveal the nutritional status of animals during pregnancy. Gurgoze et al. (2009) assessed blood glucose levels in sheep during to transition period, according to it is reported that blood glucose levels of pregnant goats are lower than those of non-pregnant animals. On the other hand, Al-Dewachi (1999) reported that plasma glucose levels are increasing in pregnancy. In our study, blood glucose levels reached peak levels during the parturition, gradually decreased in the postpartum period, and a statistically significant difference between the postpartum glucose level and prepartum glucose level were detected. Decreased glucose levels at the beginning of lactation detected in our study are in accordance with Seifi et al. (2007), and are probably caused by high milk yield.

Cholesterol levels are reported to decrease at the final stages of pregnancy (Ozpinar et al., 1989, Azab and Abdel-Maksoud, 1999; Krajnicakova et al., 2003). Mbassa and Poulsan (1991) reported that plasma cholesterol levels increased during lactation. Similarly in the present study, cholesterol levels decreased during pregnancy and signigicantly increased after parturition.The cause of decreased cholesterol during to give birth; It is thought that it was caused by mothers giving colostrum and using storage oils for feeding their offspring.

Fatty liver is associated with elevated serum fatty acids and decreased VLDL production. Findings in our study are similar to previous studies (Hamadeh et al., 1996; Rukkwamsuk et al. 1999, Balıkcı et al., 2007) that reported an increase in serum triglyceride levels during the last months of pregnancy in sheep and goats. In contrast, Obidike et al.(2009) reported elevated triglyceride levels after parturition. In the present study, we determined that the triglyceride levels were significantly increased as the parturition approached, reaching peak level in the last week of pregnancy, decreased at birth, and gradually increased again in the postpartum period (Table 2). These changes could be related with increased energy uptake in order to supplemet the energy requirement of the fetus by producing glycerol. Increased triglyceride levels after birth may be caused by trglyceride synthesis of mammary glands for milk synthesis.

Krajnikova et al.(2003) reported that serum calcium levels began to fall in the near term and were at the lowest level (1.73 mmol / l) on the third day postpartum and concluded that this decrease was a characteristic of the puerperal period. In the present study, the lowest calcium levels were detected in the second week after birth, in accordance with pervious reports (Krajnikova et al.,2003) this could probably related with the milk yield of the lactating animal(Table 2).

Karapınar et al.(2007) reported a marked hypophosphatemia in ketotic animals, compared to healthy animals and concluded that this could be related with appetite loss due to disease, as well as excessive loss of phosphorus in the milk after parturition. The lowest phosphorus concentration in our study was detected

one week before parturition, slightly elevated at birth, and gradually increased after birth to reach prenatal levels(Table 2). The explanation of the decreased phosphorus levels near term may be due to the fact that as the birth approaches, consumption of dry matter decreases due to stress.

In the present study, the magnesium concentration was found to be lowest in the last week before and the first week after parturition(Table 2). It is possible to relate the decline in the last week near term to the reduction of feed intake due to stress and the postpartum decline with hormonal adaptation or the onset of postpartum hypocalcemia (Senturk, 2017).

Diseases such as pregnancy toxemia, hypocalcemia, infertility, low milk yield cause serious economic loss in Saanen goats.It is necessary to conduct prenatal and postnatal metabolic profile testing in Saanen goats susceptible to metabolic diseases such as pregnancy toxemia and ketosis due to their genetic characteristics. In the present study, we aimed to investigate the presence of these diseases in Saanen goats by metabolic profile test. During the study, the values of NEFA, SDH and CREA increased and CHOL and TG values decreased with parturition. When the values of 3 weeks prepartum and 3 weeks postpartum were compared, NEFA, CHOL, GLU, TG, Ca levels were higher before and BHBA, Mg, ALB, GGT, AST, TP, P, BUN, SDH and CREA were higher after parturition. Not any of the animals used in the study suffered a metabolic or infectious diseases thus results of our study may be considered as pioneering study for reference biochemical parameters for Saanen goats. On the other hand, in order to clarify metabolic profiles changes during the transitional period in Saanen goats, it is necessary conduct studies with higher study population also evaluating more detailed hormonal and biochemical parameters.

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CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Seroprevalence and associated risk factors of *Toxoplasma gondii* infection in stray cats in Algiers urban area, Algeria

A. Mohamed-Cherif, K. Benfodil, S. Ansel, K.H. Ait-Oudhia

Higher National Veterinary School, Oued Smar, Algiers, Algeria

ABSTRACT: Toxoplasmosis is a zoonotic parasitic disease caused by the protozoan *Toxoplasma gondi*i. Human infections are common and generally asymptomatic, but they can become very dangerous in immunosuppressed and HIV-positive patients. The infection can also be serious if it is transmitted to the fetus during pregnancy. Infection in untreated mothers can lead to abortion, birth defects and blindness of the fetus. The aim of this study is to determine the seroprevalence of Toxoplasma IgT antibodies in cats in the urban area of Algiers. From December 2017 to August 2018, blood samples from 184 stray cats were collected and analyzed for IgG antibodies against T. gondii using an ELISA method. Overall, the prevalence of T. gondii infection in stray cats was 58.15% (107/184). There was no statistically significant difference between male and female cats. The rate of seropositivity of T. gondii increased with age ($p < 0.05$). There was no statistically significant difference between the different regions from which the samples were taken. The results of the present study showed the high seroprevalence of toxoplasmosis in Algiers rather than in other countries. Due to the high seroprevalence of Toxoplasma IgG antibodies in cats, it is recommended to include in the prenatal evaluation, together with the adoption of a screening test and the determination of the IgG antibody titer in the high-risk populations (young girls, pregnant women) public information programs on the disease and measures that can contribute to prevention

Keywords: *Toxoplasma gondii*, seroprevalence, cat, ELISA, Algiers, Algeria

Corresponding Author:
Khatima Ait-Oudhia, ENSV, PB 161 Rue Issad Abbes, Oued Smar, Algiers, Algeria.
E-mail address: khatima.aitoudhia@gmail.com

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INTRODUCTION

Toxoplasmosis is a worldwide zoonotic infestation caused by *Toxoplasma gondii*, which is transmitted to humans through ingestion of oocysts in contaminated water, soil and oocysts in raw and undercooked meat (Fayer et al., 2004).

The prevalence of toxoplasmosis in humans has increased significantly due to the high incidence of immunosuppressive infections such as AIDS. It can infect all warm-blooded animals and about 30% of the human population is a carrier of the parasite (Petersen et al., 2001).

Cats are considered the key to the transmission of *T. gondii* to humans and other animals because they are the only hosts that can excrete environmentally resistant oocysts in their feces (Dubey et al., 1995; Dubey, 2010). Compared to companion cats, stray cats are particularly important for public health, since they are considered to be the best sentinels of the level of *T. gondii* in the environment (Wang et al., 2012).

Serological prevalence data are important in determining the epidemiological significance of *T. gondii* infection in cats, since oocysts are rarely found in cat feces (Dubey et al., 1995). Since toxoplasmosis is a major concern for pregnant women, sero-epidemiological studies in young girls are useful for designing prevention policies before pregnancy.

The prevalence of *T. gondii* differs from country to country depending on the socioeconomic model of cat accommodation, their feeding behavior and the education level of people (Al-Kappany et al., 2010). Infection rates in stray cats are an indirect indication of *T. gondii* in the environment (Wang et al., 2012).

Although *T. gondii* infections are generally asymptomatic, toxoplasmosis can be a serious disease in humans (Montoya and Liesenfeld, 2004). Infection with *T. gondii* can cause toxoplasmic encephalitis in immunocompromised patients, blindness, abortion, fetal abnormalities or even prenatal death in congenital cases (Cook et al., 2000).

The ingestion of ecologically robust stages (sporozoites in oocysts), the consumption of raw or undercooked meat or meat products containing tachyzoites or bradyzoites are the main routes of transmission of *Toxoplasma* to humans (Tenter et al., 2000; Dubey et al., 2009).

In Algeria, cats mainly live outside like stray dogs where they hunt for food or live on waste. Even indoor cats are allowed to roam. Thus, the environment is likely to be contaminated by oocysts excreted by these cats. In recent years, the cat population has increased due to the accumulated attention paid to animal welfare in cities. On the other hand, animals are highly respected in Algerian culture, so stray cats live freely in our environment. The aim of this study was to determine the seroprevalence of *Toxoplasma* IgG antibodies in cats in the urban area of Algiers, Algeria.

MATERIALS AND METHODS

Ethic statement

Risk assessment was submitted to and approved by the ethics committee and decision board of Hygiène Urbaine d'Alger (HURBAL). HURBAL is an institution affiliated with the Algerian Ministry of the Interior, the Local Government and the Algerian Ministry of Agriculture and Rural Development. By decision of the Ministry of the Interior and in the context of the National Program for Rabies Control, HURBAL captured stray cats and dogs from Algiers area. Once captured, the stray animals were housed in cages, euthanized after the expiration of the legal waiting period (7 days, to allow owners to claim their pets). To facilitate the work in the field, the veterinary surgeons and their assistants working in this establishment collaborated with us.

Samples collection

Samples were taken from December 2017 to August 2018. A total of 184 blood samples were taken from stray cats (109 males, 75 females) living in various places and neighbors (12 in stables, 15 in zoos, 17 in hatcheries, 19 in slaughterhouses, 21 in farms and 100 in a canine furrier in the city of Algiers (Algeria), located in the north of Algeria (latitude 36 ° 42 '00' 'N, longitude 3 ° 13 '00' 'E) . Sampling was performed in a room devoted to veterinary activities. Blood samples were taken from the heart immediately after the animals were euthanized.

The approximate age of each cat was estimated based on the teeth. Cats were classified as juveniles (<2 years) and adults (> 2 years) (García-Bocanegra et al., 2010). Sex was also recorded for each animal. The serum was separated from the blood clot by centrifugation for 10 min at 1900 x g and was stored at -20 ° C until analysis.

Serological examination

The presence of anti-T.gondii antibodies was test-

ed using an ELISA test (ID Screen® Toxoplasmosis Indirect ELISA Multi-species, ID.VET. Innovative Diagnostics. Montpellier, France) according to the manufacturer's instructions. The sensitivity of this ELISA test reaches 100% while the specificity has been determined at 96% (manufacturer's data).

The results were expressed in optical density (OD); the absorbance was read at 450 nm (wavelength) with an EL-800 ELISA plate reader (Biotek Instruments Inc., USA). The 96-well plate is coated with P30 T. gondii antigen, and the antigen-antibody complex is formed using the peroxidase conjugate which is added later. Positive and negative controls were provided by the manufacturer and used to validate each test.

The samples were considered positive if they had a value ≥ 50%; doubtful for values between 40% and 50% and negative if ≤ 40%. This percentage was calculated as follows: Percentage of positivity = 100 * OD of the sample / OD of the PC. The sensitivity and specificity of this ELISA test are 100% and 96% respectively (information provided by the manufacturer).

Statistical analysis

Statistical analysis as performed using SPSS Statistics 22.0. The degree of significance of the correlation between seroprevalence, age and sex was achieved by the χ test. These correlations were considered significant for $p < 0.05$. Confidence intervals (95%) were calculated according to Toma et al., 1996.

RESULTS

Out of 184 serum samples, 58.15% (107/184) were positive and 41.84% (77/184) were negative for the presence of antibodies to *T. gondii*. The percentages of seropositivity of the samples taken at different sites were as follows: 75% of the 12 samples from the stables, 46.6% from the 15 samples from zoos, 64.7% from the 17 samples from hatcheries, 73.6% from the 19 samples from the slaughterhouses, 61.9% of the 21 farm samples and 53% of the 100 canine fur samples. (Table 1)

There was no statistically significant difference between male and female cats. The seropositivity rate of *Toxoplasma gondii* increases with age (**p** = 0.05), but there is no statistically significant difference between the age groups (Table 1).

Table 1. Analysis of risk factors related to *T. gondii* seroprevalence in stray cats (*n = 184*) in Algiers urban area

Variables		N	No. of positive	Percentage (%)	95% CI	*P-value*
Age group (year)	Young	32	12	37.5	20.7 – 54.3	0.0589
	Adult	152	97	63.8	56.2 – 71.4	
Gender	Male	109	66	60.5	51.3 – 69.5	0.0656
	Female	75	44	58.6	47.5 – 69.7	
Region (sample collect location)	Stables	12	09	75	50.5 – 99.5	0.3130
	Zoos	15	07	46.6	22.3 – 71.7	
	Hatcheries	17	11	64.7	42 – 87.4	
	Slaughterhouses	19	14	73.6	53.8 – 93.4	
	Farms	21	13	61.9	11.55 – 29.45	
	Canines furrier	100	53	53	43.2 – 62.8	

N: number of animals tested; No.: number; CI: Confidence Interval;

DISCUSSION

The size of the cat population has increased in recent years due to the improvement of the human lifestyle and the awareness of animal welfare. Stray cats live freely in the environment and feed on waste thrown around the houses during the night. The cat plays an important role in the spread of toxoplasmosis because it is the only animal to excrete oocysts in the environment (Silva et al., 2002).

Due to the close contact of cats with humans and the fact that children play outside on the ground, cats can be an important potential source of transmission of zoonotic parasites such as Toxoplasma as they are the only hosts that can excrete resistant *T. gondii* oocysts in the environment (Asthana et al., 2006; Dubey et al., 2006; Pas and Dubey, 2008)

Toxoplasmosis is a zoonotic parasitic disease caused by the protozoan *T. gondii*. Human infections are common and generally asymptomatic, but they can become very dangerous in immunocompromised

and HIV-positive patients. The infection can be serious if it is transmitted to the fetus during pregnancy. In unexposed mothers, it can lead to abortion, birth defects and blindness of the fetus.

Toxoplasmosis is one of the most important zoonotic diseases in Algeria and anti-Toxoplasma antibodies have been detected in humans, cattle, goats, sheep, wild and domestic birds (Keshavarz Valianand Ebrahimi, 1970) (Ghazaei, 2006; Sharif et al., 2010; Mohamed-Cherif et al., 2015; Mohamed-Cherif et al., 2019). The seroprevalence of *T. gondii* in cats varies according to their type (stray or domestic), age, test method and geographic location (Dubey et al., 2002; Silva et al., 2002; Mohan et al., 2002).

In the present study, 58.15% of the stray cats were positive for *T. gondii*. The estimated seroprevalence is comparable to the seroprevalences in other countries, for example 55% in an urban cat population in Germany (Tenter et al., 1994b) and 64.6% in Iran (Tehrani-sharif et al., 2015). The results of the present study showed a higher seroprevalence of *T. gondii* in the urban area of Algiers rather than in other countries, for example 18.6% in a population of urban cats in France (Afonso et al. , 2006), 23.1% in Japan (Nishikawa et al., 2003), 25.0% in domestic cats in Belgium (De Craeye et al., 2008), 30.5% in northern Italy (Spada et al., 2012), 32% in German (Tenter et al., 1994a; Tenter et al., 1994b), 36.9% in Spain (Miró et al., 2004), 40% in Sari-Iran (Sharif et al., 2009) and 50% in Algiers (Yekkour et al., 2017). Our results are lower than those observed in other studies: 70.2% in Belgium (Dorny et al., 2002), 85.4% in Addis Ababa, Ethiopia (Tiao et al., 2013), 90% in Tehran (Haddadzadeh et al., 2006) and 95.5% in Egypt (Al-Kappany et al., 2011). It is difficult to compare different serological surveys in felines because the seroprevalence of *T.gondii* varies between wild and domestic animals, between cat age groups, the serological test method, the sample size and the geographical location. The difference in seroprevalence in the areas where the samples are located could be explained by differences in the local reservoirs of the parasite, both in prey and in the environment, which serve as local sources of infection for cats. In Algiers, cats are generally kept outside in urban areas and often roam more freely, which increases their access to parasites.

In addition, the higher seroprevalence of *T. gondii* found in cats is likely due to the carnivorous behavior of cats living outdoors and the consumption of prey animals such as rodents and birds. The higher seropositivity with the age of cats increases the hypothesis that most cats acquire a *T. gondii* infection after weaning (Torrey and Yolken, 2013; Ahmad et al., 2014; Must et al., 2015), even if in our study, there is no significant statistical difference between the age groups.

CONCLUSION

In conclusion, the results of the present study revealed that *T. gondii* infection is highly prevalent (58.15 %) in stray cats, with 75% of them are from stables, 46.6% from zoos, 64.7% from hatcheries, 73.6% from Slaughterhouses, 61.9% from farms and 53% of 100 from canine furrier. In order to protect public health, more measures should be taken. The proper disposal of cat litter, keeping cats indoors to minimize their acquisition of infection from prey or the environment, and reducing the feral cat population are recommended.

Minimizing close contact with cats and protecting the play areas of children might potentially reduce the oocyst burden (Zhang et al., 2009). Moreover, children, pregnant women, and immunosuppressed people should adhere to hygiene principles after contact with soil and cats, as well as the good handling of meat, meat products and fruits and vegetables.

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Research article
Ερευνητικό άρθρο

HER-2 positivity rate in dogs with mammary carcinoma: a systematic review and meta-analysis

E. Uzabaci[1*], M.O. Ozyigit[2], I. Ercan[3], O. Arda[4]

[1]*Department of Biostatistics, Faculty of Veterinary Medicine, Bursa Uludag University, Bursa, Turkey*

[2]*Department of Pathology, Faculty of Veterinary Medicine, Bursa Uludag University, Bursa, Turkey*

[3]*Department of Biostatistics, Faculty of Medicine, Bursa Uludag University, Bursa, Turkey*

[4]*Department of Pathology, Uludag University Institute of Health Sciences, Bursa, Turkey*

ABSTRACT: Human epidermal growth factor receptor 2 (HER-2) plays an essential role in cell growth and survival. HER-2 overexpression occurs in 20-30% of human breast tumors and has prognostic value as it is associated with disease progression. HER-2 overexpression is also associated with tumor progression and metastasis in malignant mammary tumors of the canine. However, in the literature, different positivity classifications/scoring were used in the evaluation of HER-2 status, and there is no consensus in terms of scoring of HER-2 expression in canine mammary tumors. In this study, it was aimed to estimate the HER-2 positivity rate by evaluating the results of the study using different positivity classifications by meta-analysis. In this context, by using ”HER-2 canine mammary tumor” keywords, Pubmed and Web of Science electronic databases were scanned until February 2019, and a total of 97 related studies were found. However, 20 of these studies were used for the analysis. Two different meta-analyses were performed to evaluate the HER-2 positivity status with “2+ and 3+” and “3+” scores. As a result, HER-2 positivity rates were determined at 25.87% and 25.99% for the studies using “2+ / 3+” scores and “3+” respectively for HER-2 positivity. Therefore, this result suggests that the rate of HER-2 positivity is similar between humans and dogs.

Keywords: HER-2, meta-analysis, canine, mammary tumor.

Corresponding Author:
Ender Uzabaci, Bursa Uludag University, Faculty of Veterinary Medicine, Department of Biostatistics, 16059 Gorukle/Bursa Turkey
E-mail address: euzabaci@gmail.com

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INTRODUCTION

Changes in growth factors, growth factor receptors, and other regulators of cellular proliferation play a role in tumorigenesis and therapeutic response in both canine and human mammary carcinomas (Araújo et al., 2016). Human epidermal growth factor receptor 2 (HER-2), a member of the epidermal growth factor receptor (EGFR) family with tyrosine kinase activity, plays an important role in the regulation of cell growth, survival, and differentiation (Ressel et al., 2013). HER-2 overexpression is seen in 20-30% of human breast tumors and has a prognostic value, as it is associated with disease progression and shorter survival (Kim et al., 2011; Ressel et al., 2013). In addition to its prognostic value, analysis of HER-2 status in human medicine is important for the selection of the patient who will undergo treatment (Ressel et al., 2013). HER-2 overexpression is associated with tumor progression and metastasis in canine malignant mammary tumors as well. However, the relevance of HER-2 overexpression and tumor progression or prognostic factors has not been clearly determined so it is controversial in canine mammary tumors, mainly due to variations in methods that are used in the evaluation of HER-2 expression status (Kim et al., 2011). One of the most important reasons for this situation is the low level of accuracy obtained with the comparison of results from different scoring systems. In studies concerning the role of HER-2 in canine mammary tumor, there is no consensus in terms of scoring. Another important issue concerns the use of small sample sizes in studies making them of inadequate strength and consequently of limited validity and acceptability (Tas et al., 2018).

Meta-analysis is a method that can be used to solve such problems, and by combining similar study results, more general information about the population parameter can be obtained by increasing the sample size (Avci, 2018). The aim of this study was to estimate the HER-2 positivity rate by analyzing the results of the studies with different scoring systems used in the examination of HER-2 positivity by meta-analysis method.

MATERIALS AND METHODS

For researching the effects of different HER-2 expression scoring in immunohistochemical analysis of malignant canine mammary tumors, Pubmed and Web of Science electronic databases were scanned up to February 2019 using the keywords “HER-2 canine mammary tumor” and 97 studies were accessed. The referees were blinded to the author and institution of the studies under investigation in determining the studies to be included in this research. A total of 20 studies were found suitable for analyses (Fig. 1, Table 1).

Table 1. Studies and frequency distribution of score values for meta-analysis

Study	Frequency of score value (Score value:n)	Sample Size
Nguyen et al., 2017	**0**: 262; **1**$^+$: 71; **2**$^+$:17; **3**$^+$:0	*350*
Damasceno et al., 2016	**0**: 1; **1**$^+$**:**14; **2**$^+$:22; **3**$^+$:6	*43*
Campos et al., 2015	**0**: 9; **1**$^+$:10; **2**$^+$:9; **3**$^+$:0	*28*
Silva et al., 2014	**0**: 2; **1**$^+$: 1; **2**$^+$:4; **3**$^+$:8	*15*
Dutra et al., 2004	**0**: 12; **1**$^+$:19; **2**$^+$:11, **3**$^+$:6	*48*
Ressel et al., 2013	**0, 1**$^+$: 25; **2**$^+$**,3**$^+$: 10	*35*
Im et al., 2012	**0, 1**$^+$: 103; **2**$^+$**,3**$^+$:36	*139*
Oh et al., 2012	**0, 1**$^+$: 56; **2**$^+$**,3**$^+$: 6	*62*
Kim et al., 2011	**0, 1**$^+$: 34; **2**$^+$**,3**$^+$:18	*52*
Kurilj et al., 2011	**0, 1**$^+$: 46; **2**$^+$**,3**$^+$: 5	*51*
Millanta et al., 2010	**0, 1**$^+$: 36; **2**$^+$**,3**$^+$: 10	*46*
Hsu et al., 2009	**0, 1**$^+$: 64; **2**$^+$**, 3**$^+$: 27	*91*
Gama et al., 2008	**0, 1**$^+$: 79; **2**$^+$**,3**$^+$: 21	*100*
Martín de las Mulas et al., 2003	**0, 1**$^+$: 14; **2**$^+$**, 3**$^+$: 3	*17*
Abadie et al., 2018	**0, 1**$^+$**, 2**$^+$: 350; **3**$^+$: 0	350
Araujo et al., 2016	**0, 1**$^+$**, 2**$^+$: 67; **3**$^+$: 6	73
Shin et al.,2015	**0, 1**$^+$**, 2**$^+$: 53; **3**$^+$: 34	*87*
Burrai et al., 2015	**0, 1**$^+$**, 2**$^+$: 7; **3**$^+$**:** 2	*9*
Muhammednejad et al., 2012	**0, 1**$^+$**, 2**$^+$: 23; **3**$^+$:12	*35*
Bertagnolli et al., 2011	**0, 1**$^+$**, 2**$^+$**:**61**; 3**$^+$**:**10	*71*

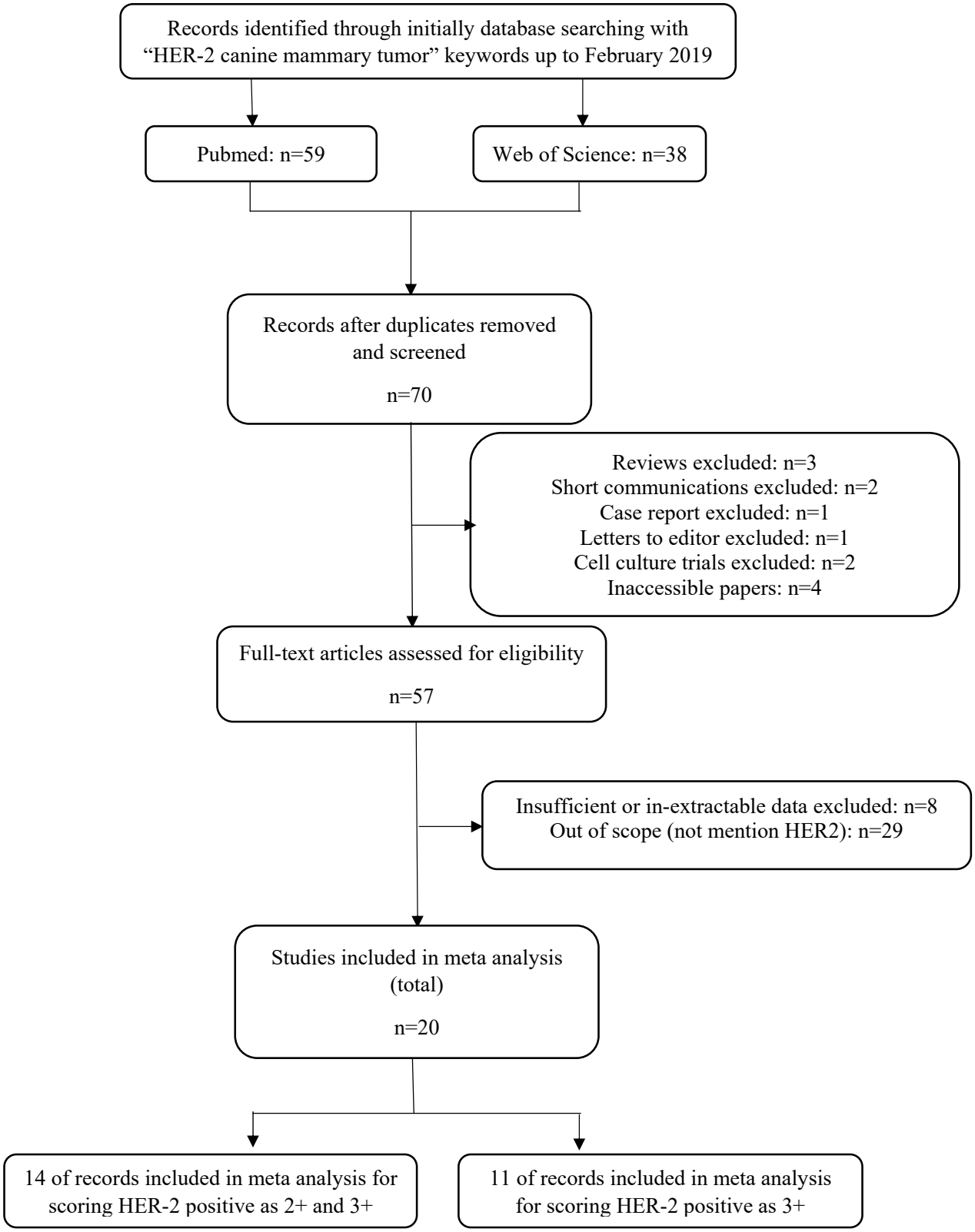

Figure 1. Flow chart showing the selection of studies for meta-analysis

Studies in which HER-2 expression status was indicated separately as a score (0, 1+, 2+, 3+) or was classified with scores clearly as "positive" and "negative" were included in the meta-analysis. Number of HER-2 positive and total number of cases were extracted from studies and used to calculate proportions (number of HER-2 positive/total number of cases) in the analysis. Different scoring systems were used in the studies evaluating HER-2 positivity. Studies that use three types of scoring systems were considered in this research. In the first type of studies, frequencies of scores were reported for 0, 1+, 2+ and 3+ scores separately (n=5). Furthermore, in the second type of studies, results were classified as negative for scores 0 and 1+ and as positive for scores 2+ and 3+ (n=9). Finally in the third type of studies, only 3+ score was used to determine HER-2 positivity, whereas 0, 1+ and 2+ scores indicate HER-2 negativity (n=6). In order to increase the number of publications included in analyses and thus, to obtain as much detailed information as possible, two different meta-analyses were performed considering particularly two different scoring systems. These meta-analyses were performed for second type of studies, indicating HER-2 positivity with 2+ and 3+ scores (n=14), and third type of studies indicating HER-2 positivity with 3+ score (n=11). Studies that use "0, 1+, 2+, 3+" scoring system (first type of studies) were used in both meta-analyses.

Statistical Analysis

The publication bias of the studies was examined before the meta-analysis with Begg's and Egger tests. Even if one of these tests is significant, it indicates that there is a publication bias; in this case, the trim and fill method was applied. The heterogeneity of the studies was evaluated with the Cochran Q test. In the evaluation of homogeneity and publication bias, α=0.10 was taken (Erdoğan and Kanık, 2011). Since heterogeneity was determined with the Cochran Q test, then DerSimonian Laird method was performed using the random effects model. For statistical analysis, "metafor" and "meta" packages were used in R version 3.5.3 software (R Development Core Team, 2014).

RESULTS

Evaluation of the studies that use 2+ and 3+ scores for HER-2 positivity

In dogs with mammary tumors, 14 studies classify HER-2 positivity based on immunohistochemical staining value of "2+ and 3+". There was no publication bias according to the Egger test (p = 0.630) and Begg's test (p = 0.956). Cochran Q test showed heterogeneity among studies ($p<0.001$; $I^2=89.4\%$). The meta-analysis results of the studies in which HER-2 positivity is scored as 2+ and 3+ were given in Table 2, and the related forest plot was shown in Fig. 2.

Table 2. Results of meta-analysis for studies in which HER-2 positivity is expressed with 2+ and 3+ scores

Study	Positive	Total	Positivity Percent of HER-2 (%)	95% C.I.	Weight (%) REM
Damasceno et al., 2016	28	43	65.12	49.07-78.99	7.4
Campos et al., 2015	9	28	32.14	15.88-52.35	7.0
Ressel et al., 2013	10	35	28.57	14.64-46.30	7.2
Im et al., 2013	36	139	25.90	18.85-34.01	8.0
Oh et al., 2014	6	62	9.68	3.63-19.88	6.8
Kim et al.,2011	18	52	34.62	21.97-49.09	7.6
Millanta et al., 2010	10	46	21.74	10.95-36.36	7.2
Nguyen et al., 2018	17	350	4.86	2.85-7.66	7.8
Gama et al., 2008	21	100	21.00	13.49-30.29	7.8
Dutra et al., 2004	17	48	35.42	22.16-50.54	7.5
Silva et al., 2014	12	15	80.00	51.91-95.67	5.6
Kurilj et al., 2011	5	51	9.80	3.26-21.41	6.6
Hsu et al.,2009	27	91	29.67	20.55-40.16	7.9
Martín de las Mulas et al.,2003	3	17	17.65	3.80-43.43	5.6
Random Effects	219	1077	**25.87**	17.24-36.88	100

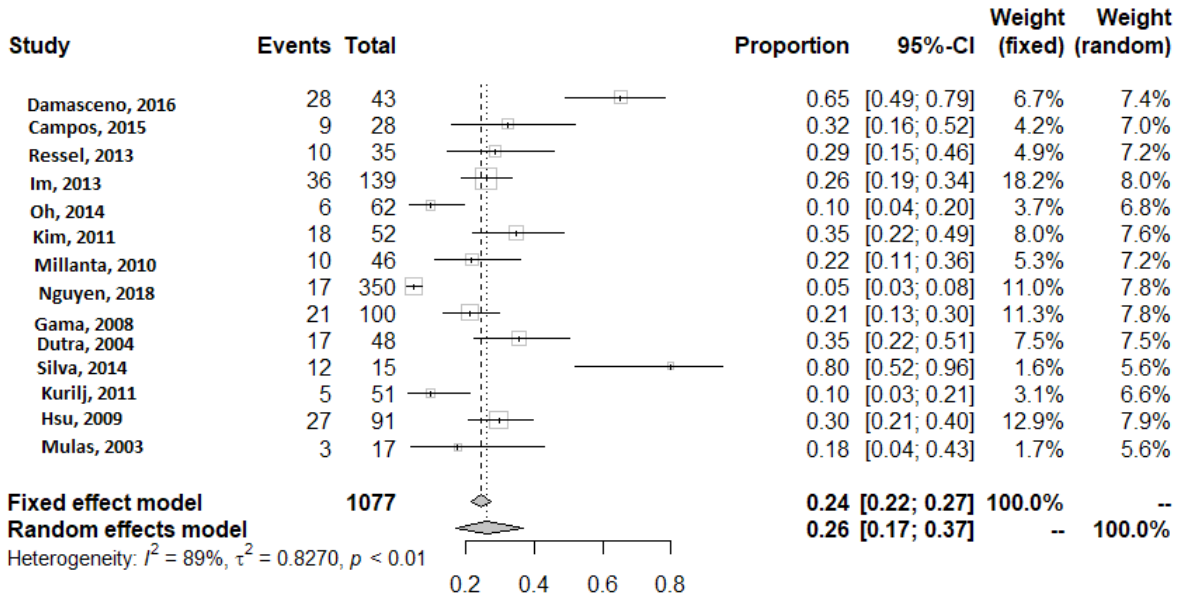

Figure 2. Forest plot for studies in which HER-2 positivity is expressed with 2+ and 3+ scores

Evaluation of the studies that use 3+ score for HER-2 positivity

In dogs with mammary tumors, 11 studies classify HER-2 positivity based on immunohistochemical staining value of "3+". Unlike studies that use "2+ and 3+", there was publication bias in studies that use 3+ score for HER-2 positivity according to the Egger test (p = 0.014) and Begg's test (p = 0.158). Because of the observed publication bias, trim and fill method was applied. However, the Cochran Q test demonstrated heterogeneity among studies (p<0.001; I^2=88.4%). The meta-analysis results of the studies in which HER-2 positivity is scored as 3+ were given in Table 3 and the related forest plot was given in Fig. 3.

Table 3. Results of meta-analysis for studies in which HER-2 positivity is expressed with 3+ scores

Study	Positive	Total	Positivity Percent of HER-2 (%)	95% C.I.	Weight (%) REM
Damasceno et al., 2016	6	43	13.95	5.30-27.93	10.8
Araujo et al., 2016	6	73	8.22	3.08-17.04	10.9
Campos et al., 2015	0	28	0.00	0.00-12.34	4.7
Bertagnolli et al., 2011	10	71	14.08	6.97-24.38	11.4
Burrai et al., 2015	2	9	22.22	2.81-60.01	8.3
Muhammednejad et al.,2012	12	35	34.29	19.13-52.21	11.3
Shin et al., 2015	34	87	39.08	28.79-50.13	12.0
Nguyen et al., 2018	0	350	0.00	0.00-1.05	4.7
Dutra et al., 2004	6	48	12.50	4.73-25.25	10.8
Abadie et al., 2018	0	350	0.00	0.00-1.05	4.7
Silva et al., 2014	8	15	53.33	26.59-78.73	10.4
Random Effects	84	1109	12.96	6.47-24.27	100
Random Effects (Trim fill was applied)	84	1109	**25.99**	13.49-44.16	100

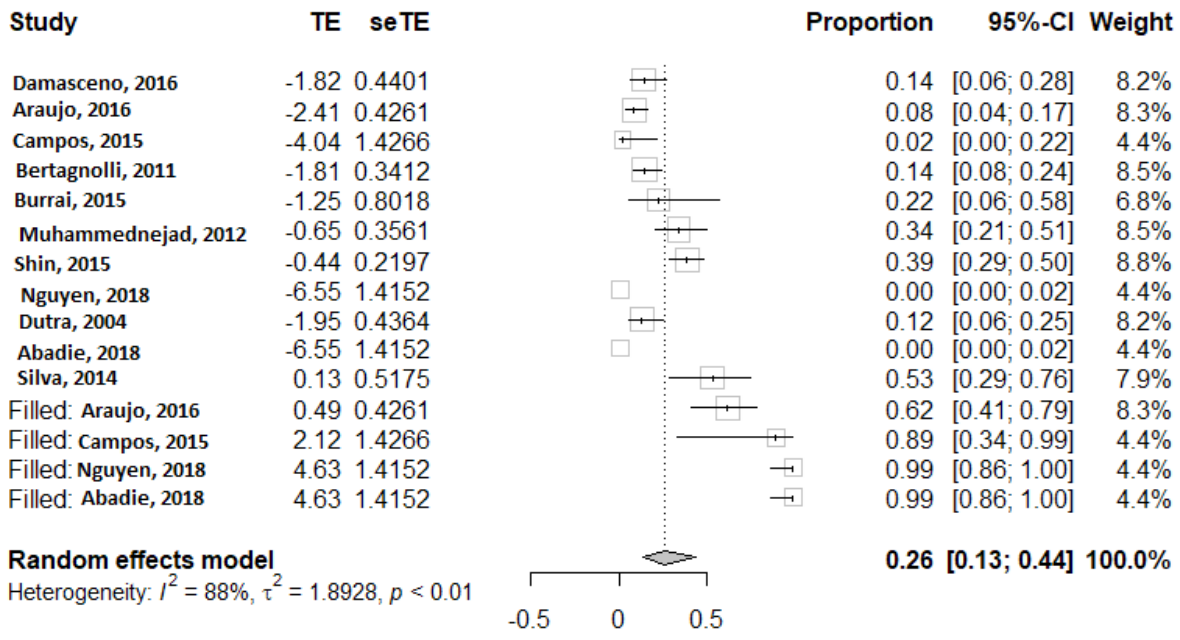

Figure 3. Forest plot for studies in which HER-2 positivity is expressed with 3+ score

DISCUSSION

There are many restrictions on research planning and implementation. The most effective of these constraints are cost and time. Cost and time constraints may prompt studies that use a relatively small number of cases. In addition to a large number of studies with a small number of cases, there also exist a few studies that use a large number of cases. Both of these situations may lead to conclusions with inadequate strength about population. To eliminate this concern and to ensure generalization in science, meta-analysis studies are used.

In this study, 20 out of 97 publications retrieved from Pubmed and Web of Science were found suitable for meta-analysis. Fourteen out of 20 studies employed 2+ and 3+ scores whereas 11 out of 20 employed 3+ score for HER-2 positivity. Five studies used "0, 1+, 2+ and 3+" scoring system for HER-2 expression status; therefore, these studies were included in both groups. Because two different scores were used for HER-2 positivity in the studies, two meta-analyses were performed to evaluate HER-2 positivity in both situations.

Since the 2+ and 3+ scores were considered as HER-2 positive, the pooled proportion was 25.87%, and when the 3+ score was considered as HER-2 positive, the pooled proportion was 25.99%. These two ratios can be considered as lower and upper limits in estimating the population parameter. However, HER-2 positivity proportions of studies with 2+ and 3+ scores ranged between 4.86% to 80.00%, and for 3+ score ranged from 0.00% to 53.33%. This result indicates that HER-2 positivity rate varies considerably in individual studies using different scoring systems. Therefore, the need for a meta-analysis of HER-2 positivity was justified. Moreover, given the relatively fewer number of studies (20 studies) that give detailed information about HER-2 positivity, a meta-analysis was needed to evaluate HER-2 positivity across studies better.

Canine malignant mammary neoplasms are common tumors, and the prevalence varies from 26% to 73% in female dogs and, in terms of morphology and biological behavior, represent a remarkably heterogeneous group of cancers (Burrai et al., 2015; Campos et al., 2015). Identification of diagnostic, prognostic, and therapeutic biomarkers is an urgent need to evaluate and manage this disease more effectively (Burrai et al., 2015). In addition to the estrogen and progesterone receptors, HER-2 is an essential biomarker for human breast cancer prognosis (Charpin et al., 1997; Ross and Fletcher, 1998). HER-2 overexpression is observed in 20–30% of human breast cancers (Slamon et al., 1989; Almasri and Al Hamad et al., 2005) but characterization of HER-2 expression and its association with histologic type and tumor grading is controversial in canine mammary tumors (Kim et al.,

2011; Kurilj et al., 2011).

HER-2 protein overexpression is observed in different percentages in canine malignant mammary tumors (19 to 74%) and this is related to a number of factors, including number of cases, different immunohistochemical methods, the sensitivity of the detection method, antibody used, the level of gene expression or the stages of tumor samples (Martín de las Mulas et al., 2003; Gouvea et al., 2006; Hsu et al., 2009; Kurilj et al., 2011; Oh et al., 2012). The most frequently used scoring system for HER-2 expression status is based on Hercept Test, but this system gives a significant number of false positives (Bertagnolli et al., 2011). Other methods (ASCO and CAP) established cut-off points for defining HER-2 status. A threshold was adopted to avoid false positives, which is at least 30% of tumors (rather than the originally specified 10%). This ratio (%30) concerns the degree of membrane staining. If more than 30% of the total number of the neoplasmatic cells have their membrane uniform and intense stained immunohistochemically with the HER-2, then this neoplasm will be considered as positive for HER-2 (Bertagnolli et al., 2011). Moreover, Ressel et al. (2013) reported that they could not compare their result, which was observed by another researcher due to the different scoring system. The prognostic significance and clinical importance of HER-2 status remain unclear in canine mammary tumors (Bertagnolli et al., 2011). No relationship was identified between HER-2 overexpression and tumor progression or prognostic factors (Martín de las Mulas et al., 2003; Hsu et al., 2009; Kim et al., 2011; Ressel et al., 2013; Burrai et al., 2015). Moreover, Peña et al. (2014) reviewed that implication of HER-2 expression in canine mammary carcinogenesis was controversial and inconclusive. Thus, a standardized method is needed to obtain objective results for HER-2 overexpression status (Oh et al., 2012). Alternatively, the meta-analysis method may be used as a more suitable technique to evaluate the results of different studies that use different scoring systems for HER-2 positivity.

CONCLUSIONS

In conclusion, according to the results obtained by the meta-analysis of studies that use two different scorings for HER-2 positivity, HER-2 positivity rate in dogs with mammary tumors ranges from 25.87% to 25.99%. Therefore, this result suggests that the rate of HER-2 positivity is similar between humans and dogs.

CONFLICT OF INTEREST

There is no conflict of interest to declare.

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Research article
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Effect of whey fat content on the properties and yields of whey cheese and serum

S. Kaminarides*, H. Zagari, E. Zoidou

Laboratory of Dairy Science and Technology, Department of Food Science and Human Nutrition, Agricultural University of Athens, Athens, Greece

ABSTRACT: The objective of this research was to study the effect of cheese whey fat content on the yield, physico-chemical, textural and sensory properties of cheeses made from sheep's whey. Four types of whey cheese were made from skimmed whey (cheese A), whole whey (cheese B) and whey with added cream of 2.5% and 5% fat (cheeses C and D respectively) and evaluated. Significant differences in yield, sensory properties, total solids, moisture, fat, fat in dry matter, protein, calcium, hardness, modulus of elasticity, gumminess and chewiness were observed between the different types of cheeses. No significant differences were observed in pH, lactose, ash, lactic acid, citric acid, galactose, glucose, Mg, K, Na and cohesiveness. The increase in fat in whey cheeses improved yield, sensory and textural properties. Cheese D was the most preferred of all the experimental cheeses in sensory analysis: it had the highest level of fat in dry matter (77.3%) and the lowest moisture content (51.6%) and according to Greek Food Legislation is characterized as an excellent quality whey cheese. Cheese A had the lowest fat content (3.94%), scored of 61.2% overall in sensory analysis and was characterized as a new reduced-fat whey cheese, particularly suitable for customers who prefer reduced-fat cheese. Serum from whey cheese production should not be considered as a waste but might be exploited as a valuable source of carbohydrate, nitrogenous compounds and minerals. Serum resulting from whey cheese production indicates that it should not be treated as waste-pollutant but as a valuable source of carbohydrate, nitrogenous compounds and minerals from which usable products may be obtained.

***Keywords*:** whey cheese, serum, biochemical characteristics, texture, sensory evaluation.

Corresponding Author:
Stelios Kaminarides, Laboratory of Dairy Science and Technology, Department of Food Science and Human Nutrition, Agricultural University of Athens Iera Odos 75 - 11855 Athens, Greece
E-mail address: skamin@aua.gr

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INTRODUCTION

Cheese whey is the liquid portion that remains after milk coagulation and removal of the curd during the cheese-making process. Cheese whey is a major by-product of the dairy industry, produced in ever increasing quantities (Peter, 2006) and usually discarded, resulting in a loss of milk constituents of excellent nutritional value in addition to environmental pollution (Gonzalez, 1996). Cheese whey contains about half of milk dry matter and its chemical composition differs according to the types of cheese produced, in particular, the kind of milk used. Sheep's cheese whey is richer in fat, proteins and ash than cow's and goat's cheese whey (Anifantakis, 1991) and is of higher biological and nutritional value. Cheese whey is an exellent source of readily available proteins and essential amino acids comprise 37.6% of the total amino acids of sheep whey proteins (Hejtmankova *et al*., 2012). Whey cheeses are manufactured all over the world and bear distinct names depending on the country of origin. In the Mediterranean region whey cheese manufactured mainly from sheep's cheese whey, which contains high percent of the water soluble milk proteins β-lactoglobulin and α-lactalbumin. In Greece, approximately 250,000 tons of whey cheeses are produced, mainly from sheep's cheese whey. The best known traditional fresh Greek whey cheeses are Myzithra and two cheeses with a protected designation of origin (PDO) fresh Manouri and mature Xynomyzithra Kritis cheese.

The novelty and aims of this study were: (a) the effect of cheese whey fat content on the yield, physicochemical, textural and sensory properties of whey cheeses made from sheep's cheese whey, (b) the collection of data on the constituents of serum after the manufacture of whey cheese in relation to the fat content of the cheese whey.

MATERIALS AND METHODS

Whey cheese

Whey was obtained from sheep's milk after 'Graviera' cheese manufacture in the pilot plan of the Dairy Laboratory of the Agricultural University of Athens. The main constituents of whole whey (B-whole whey, control) determined by the Milkoscan apparatus (model 255 A/B, type 25700, Fosselectric, Denmark), were as follows (mean values ± the standard errors of the means): 6.73±0.12% total solids, 0.61±0.08% fat, 1.74±0.30% protein, 3.93%±0.14% lactose and 0.45±0.06% ash. The remaining whole whey was skimmed to a fat content of 0.19% (A-skimmed whey) from which two additional whey forms (samples C and D standardized to 1.65% and 3.15% fat content, respectively were produced by the addition of cream of 2.5% and 5% fat to whole whey). These, four types of sheep's whey with 0.19%, 0.61%, 1.65% and 3.15% fat content were then used to make whey cheeses A, B, C and D, respectively.

Cream

Sheep's cream with 50% fat was used.

Cheesemaking and sampling

Four types of whey cheese were produced at the Dairy Laboratory of the Agricultural University of Athens according to the following procedure:

-Collection of whey after the production of Graviera cheese from sheep milk.

- Filtration of the cheese whey in order to remove any existing curd grain.

- Whey standardization to 0.19%, 0.61%, 1.65% and 3.15% fat and transfer to four circular cheese vats.

-Heating of the whey gradually under continuous stirring to 65°C.

-At 65°C, cream was added to two vats of whey in the amounts stated in the experimental plan for C and D whey cheeses.

-Heating continued under gentle stirring up to 85°C. When small curd particles of whey proteins appeared, the temperature was increased to 90°C, and the rate of stirring reduced, finally stopping when a very thin layer of coagulum formed on the surface of the cheese whey.

-The curd was held at 90°C for 30 min; then transferred gradually by a perforated ladle to cheese cloth to facilitate drainage.

-Finally, the curd was transferred to a well-ventilated room at 20°C to drain until the next day.

Four replicates of each type of whey cheese were produced. The cheeses from each treatment were weighed, sampled and analyzed.

Physico-chemical analysis

The pH of all the whey cheeses was determined using a pH-meter (632 Metrohm, Herisau, Switzerland).

Total solids (TS) were determined according to IDF (1982). Fat was analyzed according to the volumetric method of Gerber- Van Gulic (Schneider, 1954). Ash was determined by drying at 550°C to constant weight (IDF, 1964). Total nitrogen (TN), was determined by the Kjeldahl method according to IDF (1993). Water-soluble nitrogen (WSN) was obtained by homogenizing 25g cheese with 100 ml H_2O according to the method for WSN extraction, cited in Butikofer and Ruegg (1993) and determined by the Kjeldahl method (IDF 1993). Lactose, galactose, glucose, lactic acid and citric acid were analyzed by high performance liquid chromatography (HPLC). An HPLC system, GBC (Scientific Equipment, Dandenorg, Victoria, Australia) equipped with a pump GBC, LC 1150 and refractive index detector (RI) GBC, LC 1240 was used. Separation was carried out in a column Aminex HPX-87H cation exchange 300 mm x 7.8 mm (Bio-Rad, Hercules, CA, USA) following the procedure described by Kaminarides *et al*., (2007). Ca, Mg, K and Na contents were determined by Atomic Absorption Spectrometry (IDF, 2007). All analyses were performed in triplicate.

Textural evaluation

The textural properties of the whey cheese were measured with an Instron Universal Machine (Angstrom 1011, Buckingham HP 12 3SY, UK). A plunger with a diameter of 6 mm was attached to the moving crosshead. The speed of the crosshead was set at 2.5 cm min^{-1}. The cheese sample was placed on a flat holding plate at 15°C and the plunger inserted 20 mm below the cheese surface. Two bites were taken per sample. The following textural characteristics were calculated from the resulting force-deformation curve obtained:

Hardness: Perception of required force (N) to achieve a deformation in the first bite.

Modulus of elasticity: The modulus of elasticity was also calculated at the part of the force-deformation curve and defined as the ratio of stress to strain and is the slope of the force-deformation curve.

Cohesiveness: Defined as the ratio of the positive area under the curve during the second compression to that during the first compression.

Gumminess: Is the product of hardness X cohesiveness and is the energy required to disintegrate a cheese to a state ready for swallowing.

Chewiness: Is the product of gumminess X elasticity and is the energy required to masticate a cheese to a state ready for swallowing.

Sensory evaluation

The cheese sensory characteristics were graded by a ten-member taste-panel of the Dairy Laboratory of the Agricultural University of Athens. Panel members, who were familiar with this type of cheese, evaluated each sample 1 day after its manufacture for appearance, texture and flavour on a 10-point scale, from one signifying the worst to ten signifying the best quality. More importance was given to flavour and to texture than appearance of the cheese, as advised by the IDF Standard 99C (1997), by multiplying their scores by 5 and 4 respectively. The total score was obtained by adding the scores based on the three attributes. An excellent cheese obtained a total score of 100. The panel was asked to note any defects.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using the statistical program Statgraphics Plus for Windows v. 5.2 (Manugistics, Inc., Rockville, MO, USA). Paired comparisons of means were made using the Duncan test with a 95% confidence level and 5% significance ($P \leq 0.05$).

RESULTS AND DISCUSSION

Physicochemical characteristics of whey cheeses

The physicochemical characteristics of the experimental whey cheeses A, B, C and D are shown in Table 1. There were statistically significant differences (*P>0.05*) in moisture content, which decreased as the percentage of fat in whey increased. The moisture content ranged from 51.57 to 75.27% (w/w) and in the experimental cheeses B, C and D remained within the upper limit (maximum 70%) set for whey cheese by the Greek Codex Alimentarius (2014). In contrast, the moisture content of A-cheese (75.27%) was significantly higher than that of whey cheeses B, C and D ($P < 0.05$). In the present study, as expected, the fat content of the four types of whey cheese differed significantly (*P>0.05*) and it was observed that the increase in fat content in cheeses B,C and D compared with that of A resulted in a decrease in moisture content. This could be due to the presence of fat globules in the network protein creating wider pores in the curd (Walstra *et al.,* 1999) and thus facilitating drainage. Lteif *et al.* (2009) reported a similar finding for Haloumi cheese where low fat content resulted in a high-

er moisture level. The total solids content followed a trend that was the reverse of that relating to moisture.

The fat content of whey cheeses was highest (37.33%) in D-cheese produced from whey + 5.0% cream, followed by 24.61% in C-cheese produced from whey + 2.5% cream. Cheese B made from whole whey without the addition of cream had a significantly lower fat content (11.79%) than C and D, while A-cheese (made from skimmed whey had the lowest fat content of all (3.94%). Overall whey cheeses produced from whey with 4 different fat contents (0.19- 3.15), contained from 3.94 to 37.33% fat or 15.80-77.48% fat in dry matter (FDM). We classify A as a new reduced-fat whey cheese, while the cheeses B and C are classified as Myzithra cheese on the basis of maximum moisture 70% (Greek Codex Alimentarius, 2014). Type Cheese-D is characterized as an excellent quality whey cheese based on maximum moisture content of 60% and minimum fat in dry matter 70% and is classified as Manouri cheese (Greek Codex Alimentarius, 2014).

The protein content ranged from 11.37% to 15.77% (w/w) and decreased as the amount of fat in the whey increased. According to Sánchez-Macías et al. (2010), in traditional cheese produced from fresh cow's milk fat reduction resulted in increased protein.

The mean lactose content among the different types of whey cheese ranged from 4.06% (cheese-A) to 3.56% (cheese D) which was similar to that reported by Kalantzopoulos (1993) for Myzithra and Riccotta cheese. Although the lactose content did not differ to a statistically significant level between the cheeses, the lower value for cheese D is likely to have been resulted from due to the addition of 5% cream, which had lower lactose content than that of the whole whey itself.

The percentage of ash among cheeses did not differ (P> 0.05), but appeared to be highest in cheese A, which had the highest Ca content (Table 1). The concentrations of the inorganic elements in whey cheeses are shown in Table 1. Ca content ranged between 61.43 mg/100g cheese in cheese D and 162.40 mg/100g cheese in cheese A. The high Ca content of cheese A was probably due to its high percentage of serum proteins (Table 1), including α-lactalbumin (α-La), in each molecule of which 2 mole Ca is strongly bound (Walstra and Jenness 1984). The Mg, K and Na content of the cheese samples ranged from 11.31-24.31, 85.84-139.44 and 39.38-112.69 mg/100g cheese respectively without statistically significant differences between them.

Galactose and glucose, the monomers of lactose, produced via lactose catabolism by the starter cultures used, ranged from 0.15% to 0.23% and 0.03% to 0.05%, respectively. The greater proportion of galactose in relation to glucose can be attributed to the fact that *Streptococcus thermophilus,* present in the starting culture used in the production of Graviera cheese, does not metabolize galactose. A correspondig result was observed in Mozzarella cheese where *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were used and the whey content of galactose and glucose was 0.12% and 0.01%, respectively (Gernigon et al., 2009). Citric acid was detected at low concentrations in the whey cheeses, ranged from 0.17% to 0.27, and did not differ significantly between the four types of cheese. There were also no significant differences ($P>0.05$) in pH values and lactic acid. Lactic acid, which ranged from 0.15–0.20 %, constitutes the main metabolic product of lactose by starter cultures and lowers the pH of whey cheese (Table 1).

Texture profile analysis of whey cheeses

Table 1 shows the data obtained from texture profile analysis carried out on cheese made from sheep's whey with different fat percentages. Statistically significant differences were recorded for hardness ($P<0.05$), which decreased as fat content increased. The increase in fat globules within the network protein creates wider pores in the curd (Walstra *et al.,* 1999), which weaken the protein matrix and give a softer texture to the cheese (Walstra and Jenness, 1984). Also, this phenomenon related to the protein and Ca content of the samples, since cheese-A with a high protein and Ca content exhibited greater hardness than cheese-D with a low percentage of these constituents (Kaminarides and Stachriaris, 2000). The calcium lever acts as cement within the cheese body and the increase of cheese protein leads to a harder texture (Adda *et al.,* 1982). Statistically significant differences were also found ($P<0.05$) in modulus elasticity. Elasticity decreased with fat content and was lowest in cheese-A, produced from skimmed whey. According to Adda *et al.* (1982), a higher fat content in cheese leads to a less elastic body. No significant difference ($P > 0.05$) was observed in cohesiveness between the different types of whey cheese. Cheese-A, produced from skimmed whey, exhibited the highest values for gumminess and chewiness due to its greater hardness. As the fat content increased, less strength was required to chew because the body became softer.

Cheese yield and recovery of whey constituents in cheese

Cheese yield is one of the most economically important aspects of cheese manufacture and the factor that most affects cheese yield is its chemical composition, especially the fat and protein content, which accounts for more than 90% of the solid ingredients. The yield of whey cheeses affected significantly by the whey fat content: The more fat within the whey higher the yield. From the results (Table 1), it appears that average yield was highest in cheese D (7.09%) derived from whey to which 5% cream was added, followed in decreasing order by the types C (5.02%) derived from whey to which 2.5% cream was added, B (4.27%) derived from whole whey and A (2.73%) derived from skimmed whey. The mean yield of cheese B was similar to that reported by Kaminarides *et al.* (2013) for Myzithra whey cheese (4.30%with 68.04% moisture) produced from sheep's whole whey without the addition of milk or cream to the whey.

The recovery of milk constituents (i.e. the percentage of milk constituents converted into cheese) was computed from the following equation:

$$Z \text{ component recovery (\%)} = \frac{\text{Cheese weight (kg) x \% Z content in cheese x 100}}{\text{Milk weight (kg) x \% Z content in milk}}$$

Table 1. Yield, physicochemical and textural characteristics of whey cheese made from whey differing in fat content. (Means of 4 trials ± standard error of mean).

Parameters	Whey cheeses			
	A	B	C	D
Moisture (%)	75.27^{d}± 1.99	69.07^{c}± 2.67	57.50^{b}± 3.02	51.57^{a}± 3.62
Total solids (%)	24.73^{a}± 1.99	30.93^{b}± 2.67	42.50^{c}± 3.02	48.43^{d}± 3.62
Fat (%)	3.94^{a}± 1.00	11.79^{b}± 1.20	24.61^{c}± 4.67	37.33^{d}± 0.58
Fat in dry matter (%)	15.80^{a}± 2.78	38.15^{b}± 2.83	58.14^{c}± 11.65	77.48^{d}± 6.90
Total protein (%)	15.77^{b}± 0.39	14.21^{b}± 1.34	13.87^{b}± 1.75	11.37^{a}± 1.45
Lactose (%)	4.06 ^{a}± 0.36	3.68^{a}± 0.31	3.62^{a}± 0.32	3.56 ^{a}± 0.10
Ash (%)	0.71^{a}± 0.12	0.65^{a}± 0.10	0.57^{a}± 0.07	0.55^{a}± 0.06
Lactic acid (%)	0.15 ^{a} ± 0.06	0.20^{a} ± 0.05	0.19 ^{a} ± 0.06	0.16^{a} ± 0.07
pH	6.00 ^{a} ± 0.09	5.36 ^{a} ± 0.81	5.69 ^{a} ± 0.67	5.88 ^{a} ± 0.88
Galactose (%)	0.15^{a}± 0.11	0.23^{a}± 0.11	0.19^{a}± 0.15	0.17 ^{a}± 0.09
Glucose (%)	0.03^{a}± 0.01	0.05^{a}± 0.04	0.05^{a}± 0.04	0.05^{a}± 0.03
Citric acid (%)	0.27^{a}± 0.07	0.21^{a} ± 0.04	0.23^{a} ± 0.04	0.17^{a} ± 0.01
Ca (mg/100g cheese)	162.40^{b}± 54.03	95.52^{ab}± 10.46	69.80^{ab}± 7.93	61.43^{a}± 51.68
Mg (mg/100g cheese)	24.31^{a}± 5.98	17.81^{a}± 3.86	14.68^{a}± 4.85	11.31^{a}± 1.05
K (mg/100g cheese)	139.44^{a} ± 34.34	133.01^{a} ± 18.00	100.66^{a} ± 47.66	85.84^{a}± 6.69
Na (mg/100g cheese)	81.13^{a}± 34.05	112.69^{a} ± 49.34	58.55^{a}± 17.01	39.38^{a}± 14.57
Hardness (**N**)	7,60 ^{c} ±0,40	5,73 ^{b} ±0,45	5.30^{b}± 0.30	4.40^{a}± 0.14
Modulus of elasticity (N/mm)	1.17 ^{b} ±0.08	0.84 ^{a} ±0.12	0.83 ^{a} ±0.13	0.79 ^{a} ±0.12
Cohesiveness (**N** mm)	0.56 ^{a} ±0.01	0.60 ^{a} ±0.05	0.61 ^{a} ±0.10	0.55 ^{a} ±0.13
Gumminess (**N** mm)	4.27 ^{b} ±0.27	3.19 ^{a} ±0.47	2.67 ^{a} ±0.35	3.10 ^{a} ±0.53
Chewiness ((**N** mm)	4.95 ^{b} ±0.01	2.63 ^{a} ±0.02	2.07 ^{a} ±0.01	2.61 ^{a} ±0.80
Yield cheese (%)	2.73^{a}± 0.27	4.27^{b}± 0.70	5.02 ^{b}± 0.73	7.09^{c}± 0.63
Coefficient of recovery:				
-of whey total solids into cheese (%)	10.55^{a}± 0.98	19.72^{b}± 1.54	27.66^{b}± 2.39	38.26^{c}± 1.56
-of whey fat into cheese (%)	59.57^{a}± 2.14	62.53^{a}± 3.00	75.07^{b}± 6.95	84.23^{b}± 7.58
-of whey protein into cheese (%)	24.78^{a}± 2.93	34.28^{ab}± 7.94	39.39^{b}± 3.05	43.54^{b}± 6.82
-of whey lactose into cheese (%)	2.52^{a}± 0.13	4.04 ^{b}± 0.77	4.46 ^{b}± 0.91	4.81 ^{b}± 0.87

Means in the same row followed by different letters are significantly different (Duncan test, P<0.05).
A: skimmed whey; B: 100% whole whey; C: 97.5% whole whey + 2.5% cream; D: 95% whole whey + 5% cream.

Fat recovery was the highest of the four main solid components because milk fat is transferred almost entirely to the cheese mass. Protein recovery was lower than fat recovery because the proteose-peptone is a fraction of the whey proteins which is not rendered insoluble by heating the whey during whey cheese manufacture and is therefore not transferred to the cheese mass. Total solids recovery was lower than protein recovery because a significant part of the water-soluble ingredients of the solids such as lactose, soluble salts, the proteose-peptone fraction and other soluble constituents remained in serum. Lactose recovery was still lower because the bulk of the water-soluble lactose remained in serum. There were statistically significant differences ($P<0.05$) in the recovery of fat, protein, total solids and lactose from the different types of whey used to whey cheeses production and this significantly affected whey cheese yield. Cheese D, produced from 95% whole whey + 5% cream, had the highest cheese yield as it had the highest recovery of total solids, fat and protein this led to better incorporation of each main whey constituent into cheese.

Physicochemical characteristics of different serums

Serum is the liquid residue that remains after whey cheese production. Since to our knowledge the composition of serum after whey cheese production of whey components and the rate of transfer of whey components to the serum has not been previously published, a second objective of this study was to examine the physicochemical characteristics of serum after the preparation whey cheese varying in fat content and to calculate the coefficients of total solids, fat, protein and lactose of remain in the liquid residue following removal of whey cheese. The results obtained are shown in Table 2. Serum is composed of 5.88-6.25% total solids, of which around 69.9-75.5% is lactose, 18.2-19.7% is crude proteins and 7.7-8.5% is ash. The remaining constituents determined quantitatively were lactic acid, citric acid, galactose, glucose and the elements Ca, Mg, K and Na. The coefficient of lactose transfer into serum was particularly high and ranged from 88.66% in the serum D to 93.7% in the serum A. These compositional characteristics render whey serum a serious pollutant if disposed of as waste. On the other hand, with appropriate treatment serum but might be exploited as a valuable source of carbohydrate suitable for incorporation into products for animal feed (biomass), human food (alcohol, other organic chemicals, lactose extraction, glucose-galactose syrup) and as an industrial fuel (methane).

Table 2. Physicochemical characteristics of serum after the manufacture of whey cheese from whey differing in fat content. (Means of 4 trials ± standard error of mean).

Parameters	Serum			
	A	B	C	D
Total solids (%)	6.13 ^{a} ±0.41	5.88 ^{a} ±0.42	5.97 ^{a} ±0.42	6.25 ^{a} ±0.43
Fat (%)	0.02 ^{a} ±0.02	0.07 ^{a} ±0.02	0.13 ^{ab} ±0.04	0.25 ^{b} ±0.16
Crude protein (%)	1.21 ^{a} ±0.05	1.12 ^{a} ±0.14	1.15 ^{a} ±0.10	1.14 ^{a} ±0.12
Lactose (%)	4.63 ^{b}± 0.20	4.21 ^{a}± 0.09	4.18 ^{a} ± 0.07	4.37 ^{ab} ± 0.21
Ash (%)	0.47^{a}± 0.02	0.48^{a}± 0.05	0.51^{a}± 0.02	0.49^{a}± 0.03
Lactic acid (%)	0.10 ^{a} ± 0.05	0.21^{a} ± 0.07	0.15 ^{a} ± 0.09	0.15 ^{a} ± 0.05
pH	6.18 ^{a} ± 0.25	6.03 ^{a} ± 0.46	6.12 ^{a} ± 0.40	6.10 ^{a} ± 0.47
Galactose (%)	0.16^{a}± 0.08	0.31^{a}± 0.11	0.27^{a}± 0.13	0.22 ^{a}± 0.09
Glucose (%)	0.02^{a}± 0.01	0.05^{a}± 0.04	0.05^{a}± 0.03	0.06^{a}± 0.03
Citric acid (%)	0.21^{a}± 0.03	0.21^{a} ± 0.01	0.19^{a} ± 0.04	0.22^{a} ± 0.01
Ca (mg/100g)	49.67^{a}± 1.58	48.41^{a}± 8.78	45.25^{a}± 5.20	49.03^{a}± 9.96
Mg (mg/100g)	13.61^{a}± 1.58	14.41^{a}± 2.67	12.86^{a}± 0.67	13.01^{a}± 1.15
K (mg/100g)	153.54^{a} ± 44.23	133.53^{a} ± 11.47	180.72^{a} ± 29.02	172.33^{a}± 41.03
Na (mg/100g)	79.83^{a}± 17.40	86.98^{a} ± 33.36	83.31^{a}± 22.46	78.36^{a}± 14.22
Coefficient of transfer :				
-of whey total solids into serum (%)	85.84^{d}± 1.51	76.93^{c}± 3.07	68.75^{b}±4.96	56.57 ^{a} ± 2.89
-of whey fat into serum (%)	9.67^{a}± 2.53	7.29^{a}± 9.83	7.01^{a}± 4.85	6.47^{a}± 4.11
-of whey protein into serum (%)	61.49^{a}± 2.88	56.10^{a}± 7.09	54.88^{a}± 5.35	50.31^{a}± 5.69
-of whey lactose into serum (%)	93.70^{a}± 11.83	93.11^{a}± 1.92	90.07^{a}± 2.67	88.66^{a}± 3.53

Means in the same row followed by different letters are significantly different (Duncan test, P<0.05).
A: skimmed whey; B: 100% whole whey; C: 97.5% whole whey + 2.5% cream; D: 95% whole whey + 5% cream.

Table 3. Sensory evaluation of whey cheese made from whey differing in fat content. (Means of 4 trials ± standard error of mean).

Sensory characteristics	Whey cheeses			
	A	B	C	D
Appearance (1-10)	7.04[a] ±0.63	8.05[b] ±2.56	8.73[bc] ±2.68	9.25[c] ±2.64
Texture (1-40)	23.72[a] ±0.89	31.76[b] ±2.48	33.36[bc] ±2.50	35.40[c] ±2.56
Flavour (1-50)	30.4[a] ±0.82	37.45[b] ±2.37	40.55[bc] ±2.43	42.55[c] ±2.45
Total (1-100)	61.16[a] ±8.25	77.26[b] ±1.97	82.63[bc] ±0.49	87.21[c] ±4.98

Means in the same row followed by different letters are significantly different (Duncan test, P<0.05).
A: skimmed whey; B: 100% whole whey; C: 97.5% whole whey + 2.5% cream; D: 95% whole whey + 5% cream.

The lactose content of the serum varied significantly between 4.18 and 4.63% and it is necessary either to develop an effective method to recover lactose by evaporation, crystallization, washing, centrifuging and drying for crystalline lactose, or by fermentation of serum lactose by different microorganisms and the production of valuable products such as: a) ethyl alcohol and single cell protein (SCP) using yeast strains (e.g. *Saccharomyces fragilis*, *Kluyveromyces marxianus, Torula cremoris*, *Candida pseudotropicalis*), b) microbial lipid that contains "rare" poly-unsaturated fatty acids (PUFAs) of nutritional and pharmaceutical importance, e.g. g-linolenic acid (GLA–D6,9,12C18:3) by various oleaginous microorganisms, c) lactic acid by lactic acid bacteria such as *Lactobacillus bulgaricus*, d) butyric acid by butyric bacteria such as *Clostridium butyricum*, e) propionic acid by propionic bacteria such as *Propionibacterium shermanii*, f) the enzyme β-galactosidase or lactase by *Kluyveromyces marxianus* or *Candida pseudotropicali and g)* methane, vitamins, amino acids, penicillin, polysaccharides as food gums, hydrolysed lactose syrup, beverages etc.

Crude protein transfer rates from cheese whey to serum were low and varied from 1.12- 1.21% with no significant differences in relation to the fat content of the whey. The term 'crude protein' in Table 2 indicates that the nitrogen content is converted to protein content using the conversion factor 6.38. However, nitrogen in serum consists mainly of non-protein nitrogen and true proteins are mainly proteose-peptones, which are heat-resistant (Walstra and Jenness, 1984) and remain soluble by heating the whey during the cheese-making process, and fine casein granules which were not incorporated in the cheeses during drainage. Salvatore *et al.* (2014) reported that the crude protein content of serum after the manufacture of Ricotta cheese was about 1%, which was similar to that in our experiments.

Fat content of the sera varied significantly between 0.02-0.25%. The highest fat transfer from cheese whey to serum occurred in serum A (9.67%) and the lowest in serum D (6.47%).

Other serum components, which did not differ significantly in relation to the fat content of the whey were: 0.47-0.51 % ash; 0.10-0.21 % lactic acid; 0.16-0.31 % galactose; 0.02-0.06% glucose; 0.19-0.22 % citric acid; 133.53- 180.72 mg/100g K; 78.36-86.98 mg/100g Na; 45.25-49.67 mg/100g Ca; 12.86- 14.41 mg/100g Mg.

3.5. Sensory characteristics of whey cheeses

Table 3 shows the results for the sensory evaluation of the four cheeses. The highest total scores were received by D-cheese, which was characterized as the most flavorful, soft and aromatic, possibly due to its increased fat content resulting from the greater amount of cream added to the whey during cheese production. D-cheese is characterized by the Greek Codex Alimentarius (2014) as Manouri, which is a PDO cheese and is considered the most delicious of whey cheeses with higher fat content (>70% of its dry substance) and lower moisture content than other types of whey cheese. C-cheese, which contained less fat than D-cheese, was considered better than A and B cheeses which were produced from whey without added cream. C and B cheeses are characterized by the Greek Codex Alimentarius (2014) as Myzithra. B-cheese, produced from whole whey, it was frangible and had a slightly granular texture. Finally, A-cheese, which was made by skimming the cheese whey to 0.2% fat, was hard, frangible and granular, due to it having the lowest fat and highest protein and calcium content (Table 1): Cheese-A is characterized as a new reduced-fat whey cheese. The above are in agreement with Sanchez-Macias et al. (2012) who stated that the fat content positively modifies sensory properties and consumer acceptance.

CONCLUSION

The fat content significantly influenced physico-chemical parameters such as moisture content, protein, Ca and cheese yield. The increase in fat in the cheese whey affected the physico-chemical composition and improved the rheological, sensory characteristics and yield of whey cheese. The addition of cream reduced hardness, modulus of elasticity and led to higher yield and cheese quality (soft texture and rich flavor) compared to cheese made from whey without the addition of cream. Also, the use of cream in the production of whey cheese will help the valorization of cream. In contrast, the skimming of whey caused a significant deterioration in whey cheese quality. The recovery of fat, protein, total solids and lactose in whey cheeses was proportional to the fat content of cheese whey and was significantly higher in D-cheese. In serum, during the manufacture of whey cheese 90-93% of cheese whey lactose, 50-61.49% of crude protein and 6.47-9.67% of fat was transferred and if this serum is irresponsibly disposed of, it will cause environmental pollution due to its high organic load. In contrast, we propose that serum from whey cheese production can be processed in various ways to obtain usable products or be used as a substrate by microbial species to produce metabolic compounds of added value.

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CONFLICT OF INTEREST STATEMENT

We have no conflict of interest to declare.

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Research article
Ερευνητικό άρθρο

Recombinant erythropoietin treatment does not alter the blood pressure despite elevated haematological parameters in normotensive Wistar rats

F. Sultan[1], B. A. Ganaie[2], D. M. Mir[3], J. A. Dar[4]

[1]*Division of Pharmacology, Indian Veterinary Research Institute, UP, India*

[2]*Department of Veterinary Gynaecology, SKUAST-Kashmir, J&K, India*

[3]*Department of Animal Nutrition, SKUAST-Kashmir, J&K, India*

[4]*Division of Pathology, Indian Veterinary Research Institute, UP, India*

ABSTRACT: Use of erythropoietin (EPO) is believed to be associated with adverse cardiovascular events, especially high blood pressure. Also, its illegal use in blood doping is thought to result in detrimental events both in humans and equines. To test this hypothesis, normal Wistar rats were treated with recombinant erythropoietin (rEPO @ 400 i.u/kg s.c) or normal saline one day apart for one week. Heart rate, systolic, diastolic, mean arterial pressure and blood count were determined. Rats were also observed for their behaviour during the study period. rEPO significantly (P<0.001) increased the erythrocyte count (RBC), haemoglobin (Hb), hematocrit (HCT) and platelet count (PLT) in comparison to control animals. Despite such an increase in hematocrit which in turn increases blood viscosity, the systemic blood pressure and heart rate did not differ between the groups. rEPO treatment did not cause any untoward behavioural change in animals. In conclusion, despite the profound effect on haematological parameters (especially hematocrit), rEPO was without any effect on blood pressure and heart rate and the hypothesis of short-term erythropoietin-induced alterations in cardiac parameters was not verified.

***Keywords*:** Rats, Blood doping, Erythropoietin, Hypertension, Hematocrit, Plethysmography.

Corresponding Author:
Faheem Sultan, Division of Pharmacology, Indian Veterinary Research Institute, UP-243122, India
E-mail address: faheemsltn@gmail.com

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INTRODUCTION

Erythropoietin is a glycoprotein which is primarily secreted from the kidney. It acts on erythroid progenitor cells in the bone marrow and stimulates their proliferation and differentiation (Jelkmann, 1992). Clinical applications of erythropoietin include severe anaemic hemodialysis patients, predialysis patients and patients in which anaemia is caused by a tumour or chemotherapy. In veterinary practice, rEPO is used in canine and feline patients suffering from non-regenerative anaemia secondary to chronic renal failure (Mikiciuk *et al*., 1990; Giger, 1992; Cowgill *et al*., 1998). Erythropoietin is illegally used in "blood doping" which is defined as an artificial increase in Hb/ hematocrit to enhance performance. Jelkmann (2000, 2001) has reviewed the use of rEPO as an anti-anaemic and performance-enhancing drug. EPO is used by top endurance athletes such as long-distance runners and cyclists on a systemic basis (Pommering, 2007). Blood doping in racehorses is done to increase red blood cell count and thus enhancing oxygen-carrying capacity (Ungemach, 1985; Toutain, 2010).

Therapy with rEPO is associated with the development of high blood pressure or else its aggravation (Eschbach *et al*., 1989; Abraham *et al*., 1991). EPO therapy completely prevented the chronic renal failure (CRF)-associated anaemia but led to a marked rise in arterial blood pressure which began one week after the onset of EPO administration (Ni *et al*., 1998). It was also shown that in normotensive subjects there was a significant increase in resting mean arterial pressure (MAP) of +6mmHg (Lundby *et al*., 2007). Even it has been shown that rEPO produces a direct pressor effect on bilateral rat renal resistance vessels (Heidenreich et al., 1991) and human placental vessels (Resch *et al*., 2003). It was previously seen that rEPO impaired nitric oxide production and eNOS mRNA expression in pulmonary vasculature (Sultan *et al*., 2017).

In order to investigate the effect of recombinant erythropoietin on cardiovascular function and haematology, normal rats were treated with rEPO or saline for one week. Heart rate, systolic blood pressure, diastolic blood pressure, mean arterial blood pressure and haematological parameters were determined. We hypothesised that erythropoietin induces excessive erythrocytosis which can cause increased blood viscosity and increased systemic blood pressure. If this hypothesis was verified, more cautious use of erythropoietin is warranted.

MATERIALS AND METHODS

Animals

Male Wistar rats (150-250g, 6-9 weeks of age) were acclimated for ten days before the experiments, placed in transparent polycarbonate cages and housed in an environmentally controlled room. They were kept under 12-h dark/light cycle and allowed free access to feed and water. A diet of following composition was provided to the rats: crude wheat 61 %, crushed maize 30 %, wheat bran 7 %, common salt 1 % and mineral mixture 1 %. The rats were divided into two groups of eight animals each. During the acclimatization, we measured the blood pressure of rats at least three times using the tail-cuff method (NIBP system, CODA, Kent Scientific Corporation, Torrington, CT, USA). We didn't get any animal that was hypertensive thus ensuring that we are using normotensive animals. According to "resource equation method" eight animals per group in our study is an acceptable sample size. The protocol was approved by the Institutional Animal Ethics Committee of Indian Veterinary Research Institute, Izzatnagar (Approval No. F1-53/2012-13/JDR).

Experimental design

Animals were divided into two groups i.e. Control group and Test group. The test group was administered recombinant erythropoietin subcutaneously at 400 IU/ kg (Sultan et al, 2017) on every other day (i.e. day1,3,5 and 7) for one week. In a similar fashion, the control group received normal saline. On the 8th day, the blood pressure was taken by tail-cuff plethysmography. After measuring the blood pressure rats were anaesthetized with urethane (1.2 g/kg body weight i.p). Blood was collected from the retro-orbital plexus with the help of a capillary tube.

Blood pressure and heart rate

Initially, the rats were trained for ten days to make them friendly while taking blood pressure and heart rate. Systolic, diastolic and mean arterial pressures (MAP) were measured at the end of the treatment by tail-cuff plethysmography (IITC, mode 31, Woodland Hills, CA, USA) following established procedures. This method is analogous to sphygmomanometry in humans. Animals were adjusted to the experimental cages by bringing them into measuring chamber 3-4 times before the start of the experiment for a period of 30–60 min. To measure blood pressure, a tubular inflatable cuff was placed around the base of the tail and a piezoelectric pulse detector was positioned dis-

tal to the cuff. The systolic pressure was detected and subsequently recorded on a polygraph. Each measurement was repeated at least three times and the mean of these values was used for further calculations. All the recordings and data analysis was done using a computerized data acquisition system (BPMon, version 1.32).

Haematological parameters

On the 8th day, blood was collected using EDTA containing vacutainers from retro-orbital plexus after proper anaesthesia. The collected blood was used for haematological assessment viz. haemoglobin (Hb), red blood cells (RBC), platelets (PLT), white blood cells (WBC), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). These haematological parameters were determined by using automated haematology analyser MEK-6450 (Nihon Kohden, Japan).

Statistical analysis

GraphPad Prism version 4.00 (San Diego, California, USA) software was used for statistical analysis. Student's *t*-test was used in comparing data from two groups. For statistical comparisons, a *P*-value <0.01 was considered significant. All data given are as means ± standard error of the mean (SEM).

RESULTS

Systolic (SAP), diastolic (DAP), mean arterial pressure (MAP) and heart rate were measured at the end of the treatment by tail cuff-plethysmography (IITC, mode 31, Woodland Hills, CA, USA). No significant differences were observed in these parameters as shown in Table 1. Animals were also observed for any abnormal behaviour during the whole study and were found active and normal.

The effect of one-week EPO-treatment on various haematological parameters is presented in Table 2 and Figure 1. Erythropoietin significantly ($p<0.001$) increased the erythrocyte count (RBC) $8.83 \pm 0.27\times10^6$/µl in comparison to control ($7.44 \pm 0.13\times10^6$/µl); hemoglobin (Hb) 16.3 ± 0.38 g/dL in comparison to control (13.3±0.20 g/dL); hematocrit (PCV/HCT) 51.2 ± 1.09 % in comparison to control (40.1 ± 0.56 %) and platelet count (PLT) $903 \pm 54\times10^3$/µl in comparison to control $669 \pm 46\times10^3$/µl. There was no significant effect on leukocyte count (WBC), mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH). There was a significant decrease in mean corpuscular haemoglobin concentration (MCHC).

Table 1. Effect of EPO-treatment on hemodynamic parameters. Data are presented as mean ± SEM; Pressure is in mmHg and Heart rate in Beats/min

Group	Systolic pressure (mmHg)	Diastolic pressure (mmHg)	MAP (mmHg)	Heart rate (BPM)
Control	146 ± 1.77	112 ± 1.82	122.8 ± 1.67	485.6 ± 10.68
rEPO-treated	146 ± 1.73	112 ± 2.13	122.9 ± 1.92	468.6 ± 9.46

Table 2. Effect of one-week rEPO-treatment on various haematological parameters. Data are presented as mean ± SEM, ***P*<0.001 and ****P*<0.0001 in comparison to control animals.

Parameter	Control	rEPO-treated
WBC (10^3/µl)	13 ± 1.2	15 ± 1.4
RBC (10^6/µl)	7.44 ± 0.13	8.83 ± 0.27***
Hb (g/dL)	13.3 ± 0.20	16.3 ± 0.38***
HCT (%)	40.1 ± 0.56	51.2 ± 1.09***
PLT (10^3/µl)	669 ± 46	903 ± 54**
MCV (fL)	53.9 ± 0.51	58.1 ± 0.83
MCH (pg)	17.9 ± 0.19	18.6 ± 0.26
MCHC (g/dl)	33.1 ± 0.22	31.9 ± 0.15***

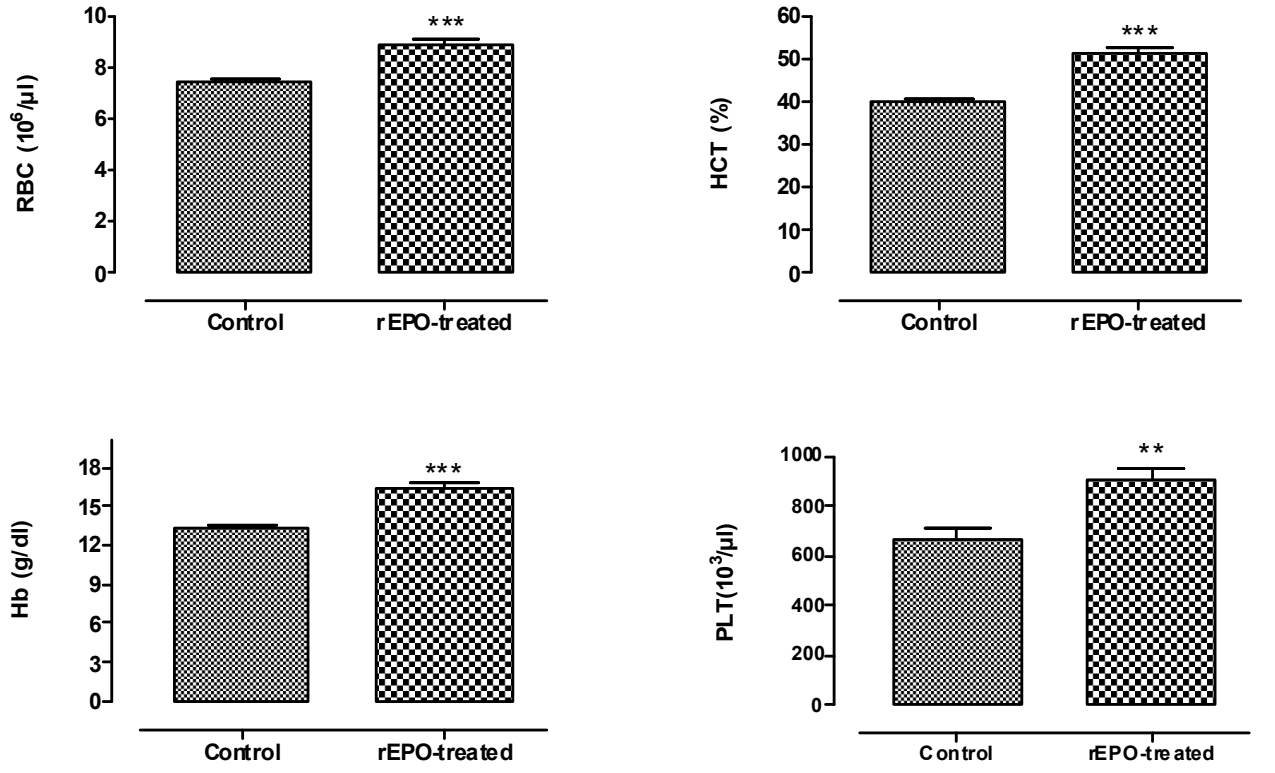

Figure 1: Bar diagrams showing the effect of EPO-treatment on various haematological parameters. ***P*<0.001 and ****P*<0.0001 in comparison to control animals

DISCUSSION

An expanding increase in the clinical use of erythropoietin is witnessed. Apart from the clinical use, its illegitimate use has reached the point where various athletes have relied on its systemic use (Pommering, 2007). The annual prescription sales of EPO and erythropoiesis-stimulating agents (ESA) are expanding (Steinbrook, 2007). The EPO doping is dangerous because of excessive erythropoiesis which can result in high blood viscosity, thromboembolism, and death. A meta-analysis showed an increased risk of venous thromboembolism and increased mortality rates after EPO administration (Bennet, 2008). The mechanisms of such events are not fully elucidated but EPO-induced arterial hypertension is a leading candidate (Fishbane, 2007). A cause-effect relationship was hypothesized in the present study.

We got profound changes in some haematological parameters in short term rEPO use in otherwise normal Wistar rats. Hematocrit was highly elevated from 40% to 51% which is a profound increase. Haemoglobin level jumped from 13g/dl to 16 g/dl within a week. We assumed that an increase in the hematocrit will result in increased blood viscosity and thus may result in hypertension since peripheral resistance is governed by blood viscosity apart from blood vessel diameter and total vessel length. Blood viscosity is a major determinant of cardiac work and tissue perfusion (Levy et al., 2008). The more viscous the blood, the greater the resistance it encounters and the higher the blood pressure. A direct correlation between blood pressure and viscosity has been shown in both normotensive and hypertensive subjects (Letcher et al., 1981).

However, despite the increase in HCT the normal rats did not develop hypertension. No significant difference in blood pressure was found between the rEPO treated and control rats. It is unlikely that the dose of EPO in our study was too low to have any effect as we used a high dose over one week. Athletes associated with endurance sports possibly use EPO for short durations as in our study. EPO treatment was without effect on heart rate, blood pressure and behaviour except haematology variables in normal rats. The short-term changes described for these parameters in normal animals are therefore unlikely to

cause changes in cardiovascular function. Some authors found no alteration of blood pressure, heart rate, or cardiac output, in transgenic mice over-expressing EPO (Vogel et al., 2003). They explain this by discussing an adaptive mechanism that involves the increased activity of endothelial nitric oxide synthase and increased flexibility of transgenic erythrocytes.

CONCLUSION

Acute and high-dose administration of rEPO instigated a profound effect on haematological parameters (especially hematocrit) but it was without any effect on heart rate or blood pressure. Our study does not provide evidence for pronounced cardiovascular side-effects of rEPO treatment over a period of one week.

CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

Correlation between *ESβL Salmonella* Serovars Isolated from Broilers and their Virulence Genes

M. Rady[1, 2]*, N.A. Ezz-El-Din[1], K.F. Mohamed[1], S. Nasef[2], A. Samir[1], W.K. Elfeil[3]

[1]*Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt*

[2]*Molecular Biology Unit, Reference Laboratory for Veterinary Quality Control on Poultry Production (NLQP), Animal Health Research Institute, Giza, Egypt.*

[3]*Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt*

ABSTRACT: *Salmonell*a is considered to be one of the major poultry bacterial pathogens. The poultry species are one of the main reservoirs for the human types, thus serving as public health hazards. The development of drug resistant genes and multidrug resistant types from *Salmonell*a has increased recently. This current study was undertaken to estimate the correlation between extended spectrum beta-lactamase multidrug resistant (*ESβL*) *Salmonella* serovars isolated from broilers and their virulence genes. Two hundred and forty samples were collected from clinically diseased broilers chicks (showed disorders of the intestinal tract) and examined for the presence of *Salmonella* isolates according to ISO 6579: 2002 and ISO. 6579-3:2014 Fifty *Salmonella* isolates were isolated with an incidence of 20.8%. Isolates of *Salmonella* were serotyped as follows: 25 *S.* Kentucky, 9 *S.* Infantis, 6 *S.* Enteritidis, 4 *S.* Heidelberg, and one isolates per serovars *S.* Labadie, *S.* Typhi, *S.* Agona, *S.* Pullorum, *S.* Newport and *S.* Virginia. AST (antimicrobial susceptibility testing) showed that high percentage of isolates were resistant to all Ampicillin (90%), Nalidixic acid (88%), Sulfamethoxazole + Trimethoprim (82%) and Tetracycline (82%). Approximately 86% of the isolates demonstrated multiple resistance, of which 18.75% and 25% were resistant to three and four antimicrobial types, respectively. Phenotypic detection of *ESβL*s by using screening test (*Cefinase*®) and confirmatory test by using combined disk diffusion test revealed that 32% of isolates were positive for both tests with 20% similarity and 12% diversity between the two tests. Molecular characterization of some *ESβL*s genes (bla_{TEM}, bla_{CTX}, bla_{OXA}, bla_{CMY} and bla_{SHV}) and some virulence genes (*inv*A, *avr*A, *sop*B, *bcf*C, *stn* (was done using PCR. The results showed that all the *ESβL*s positive serovars were positive for amplification of all tested virulence genes and noticed that all the isolates were negative for bla_{CMY} gene. The present study suggests that virulent *ESβL Salmonella* serovars could infect broilers and should be taken into consideration as an important bacterial pathogen affecting poultry.

***Keywords*:** *Salmonella,* virulence, ESBL, Broilers, genes

Corresponding Author:
Mohamed Rady, Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
E-mail address: dr.m.rady26@gmail.com

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INTRODUCTION

The poultry industry is exposed to several threats such as of viral origin like avian Influenza, Newcastle disease, infectious bronchitis, infectious bursal disease, avian adenovirus, or of bacterial origin like the Salmonellae species, Escherichia coli, Clostridium spp. (Ayoub *et al.* 2019, Diab *et al.* 2019, Elhady *et al.* 2018, Fawzy *et al.* 2020, Sedeik *et al.* 2018, Sultan *et al.* 2019a, Sultan *et al.* 2019b, Sultan *et al.* 2020). Salmonella is one of the most serious bacterial pathogens as it is associated with economic losses in poultry farms in addition to the zoonotic effects. There are more than 2700 serotypes from *Salmonella* species according to the White Kauffmann classification*; Salmonella* species are responsible for a variety of acute and chronic diseases in poultry (Grimont . and Weill 2007). Avian Salmonellosis may occurs as a result of infection with poultry-specific serovars, *Salmonella* Gallinarum and *Salmonella* Pullorum, causing systemic illness in birds as well as other *Salmonella* serotypes, including *Salmonella* Typhimurium and *Salmonella* Enteritidis and many others, which contribute to paratyphoid infections (Gast *et al.* 2003)**.** *Salmonella***e** are recognized by the specific host that belongs to pathogen recognition patterns (PRR) in birds and bony fish (Abouelmaatti *et al.* 2013, Elfeil *et al.* 2012, Elfeil *et al.* 2016).Since the 1990s, several reports highlighted that some *Salmonella* strains developed resistance to a range of antimicrobials agents and the range of developed resistance increased since then; whereas, this resistance developed due to improper use of antimicrobials agents in humans and animals' husbandry**,** either using subtherapeutic doses, use using antimicrobial agents as growth promoters and incomplete antimicrobial courses...etc (Eid *et al.* 2019, Enany *et al.* 2018, M. Algamma *et al.* 2016). Multi drug resistant (MDR) bacteria (bacteria resistant to more than four different types of Antimicrobial agents' families) increased dramatically during the last decade. They have a high impact in developing several disease conditions in humans and birds and are associated with elevated levels of morbidity and mortality caused by those pathogens in addition to high economic costs of therapy (cost of medication) and real risks of the spread of resistant strains which transmit acquired Multidrug resistant (MDR) genes to other bacteria among animals and humans (Chen *et al.* 2004, Eid *et al.* 2019, Elfeil *et al.* 2020, White *et al.* 2001). Thus, the MDR bacteria have a high impact on public health issues. The rapid development of resistance to extended-spectrum Cephalosporin in different serovars of *Salmonella* subspecies enterica has been observed worldwide and is predominantly linked to plasmid-mediated production of β- lactamases-producing bacteria (*ESβL*) (Authority 2009, EFSA 2018, EFSA. 2008). The widespread of *ESβL*-producing organisms and related treatment failures may be associated with failure in detection of the complex resistant phenotypes and may reflect to uncontrolled spreading of such pathogens (Sinha *et al.* 2008).The rapid spread of *ESβL*-producing bacteria in different poultry farms provoke a serious risk to livestock and humans, especially with cross spreading of the of *ESβL*-producing bacterial strains from poultry farms to livestock farms and humans which vice versa spread the MDR pathogens that cause wide-spread population infections (Brinas *et al.* 2003, Winokur *et al.* 2000).Some bacterial virulence genes are associated with main pathogenic behavior of the bacteriał (as ability of bacteria to make adhesion to specific host cells as mucosal cell, ability for invasion cells and/ or ability to triggering fluids secretions which leads to diarrhea symptoms). Those genes are associated with the pathogenesis of *Salmonella,* as it reflects on its ability to survive and replicate inside host cells. This virulence trait is associated with the ability to develop systemic infections and a large number of genes are required to enable *Salmonella* to cope with nutritional limitations, to avoid clearance by the host defense mechanisms and immune system or overcome damage effect by antimicrobial peptides and radicals on bacterial cell (Hegazy . and Hensel 2012).

MATERIALS AND METHODS

- All methods were carried out in accordance with Cairo University (Egypt) guidelines and regulations.
- All experimental protocols were approved by Cairo University (Egypt) Ethical committee.

***Samples*:** Two-hander and forty samples were collected randomly from broilers clinically diseased chicks (showing disorders of the intestinal track.). These were 45 liver, 60 yolk sacs, 40 lung, 50 caecum and 45 spleen. The samples were then brought to the Microbiology department, faculty of Veterinary Medicine, Cairo University, in sterile wide-mouth screw capped bottles under cooling conditions and then analyzed for the presence of *Salmonella.*

***Isolation of Salmonella*:** Under complete sterile condition broilers' internal organs were examined for the presence of *Salmonellae* according to the ISO 6579: 2002/ Amd. 1: 2007 (Standardization 2007). The isolates were serotyped according to ISO 6579-3: 2014 in the Central Laboratory for Quality Control on Poultry Production

(CLQP) in Dokki, Giza, Egypt (Standardization 2014).

The Antibiogram disk diffusion technique was adapted according to CLSI manual (NCCLS 2015).

β-lactamase detection using Nitrocefin disks (Cefinase®): According to the Manufacturer's instruction, The Cefinase disc is impregnated with the chromogenic cephalosporin, Nitrocefin. Those disks exhibit quick color change from yellow to red due to hydrolysis of the amide bond in the beta-lactam ring by a beta-lactamase enzyme. When a bacterium produces certain amount of beta-lactamase enzyme the disc colour changed from yellow to red colour in the area where the isolate is smeared.

***β-lactamase detection using Combined Disc Diffusion Test (CDD)*:** The test inoculums (compared to 0.5 McFarland turbidity) were spread by sterile cotton swabs on Mueller-Hinton agar plates. The used discs were either Cefotaxime and Ceftazidime, separate and in combination with Clavulanic acid (cefotaxime 30 µg, cefotaxime/clavulanic acid 30/10 µg) and (Ceftazidime 30 µg, Ceftazidime/Clavulanic acid 30/10 µg). The plate was incubated at 35±2°C for 16-18 hours. The organisms were considered to be producing *ESβL* when A ≥ 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with Clavulanic acid, versus the zone diameter of the agent when tested alone, equals *ESβL*.(NCCLS 2015).

Molecular Detection of ESβLs Salmonella serovars and its virulence genes by PCR: This was carried out by using primers for detection of *ESβL*s *Salmonella* serovars and some of its virulence genes as presented in tables (1/2).

Table 1. Primers Used for Molecular Detection of *Esβl's Salmonella Serovars and Its Virulence Genes by PCR*

Primer		Sequence	Amplified product	Reference
Stn	F	TTG TGT CGC TAT CAC TGG CAA CC	617 bp	Murugkar*et al*., (2003).
	R	ATT CGT AAC CCG CTC TCG TCC		
invA	F	GTGAAATTATCGCCACGTTCGGGCAA	284 bp	Oliveira*et al*., (2003).
	R	TCATCGCACCGTCAAAGGAACC		
sopB	F	TCA GAA GRC GTC TAA CCA CTC	517 bp	Huehn*et al*., (2010).
	R	TAC CGT CCT CAT GCA CAC TC		
avrA	F	CCT GTA TTG TTG AGC GTC TGG	422 bp	
	R	AGA AGA GCT TCG TTG AAT GTC C		
BcfC	F	ACC AGA GAC ATT GCC TTC C	467 bp	
	R	TTC TGC TCG CCG CTA TTC G		
bla_{TEM}	F	ATCAGCAATAAACCAGC	516 bp	Colom *et al*., (2003).
	R	CCCCGAAGAACGTTTTC		
bla_{SHV}	F	AGGATTGACTGCCTTTTTG	392 bp	
	R	ATTTGCTGATTTCGCTCG		
Bla_{OXA-1}	F	ATATCTCTACTGTTGCATCTCC	619 bp	
	R	AAACCCTTCAAACCATCC		
CMY-2	F	TGG CCA GAA CTG ACA GGC AAA	462 bp	Pérez-Pérez and Hanson, (2002).
	R	TTT CTC CTG AAC GTG GCT GGC		
Bla_{CTX}	F	ATG TGC AGY ACC AGT AAR GTK ATG GC	593 bp	Archambault *et al*., (2006).
	R	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		

Table 2. Thermal cycle condition Used for Molecular Detection of *Esβl's Salmonella Serovars*

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Stn	94˚C\10 min.	94˚C\45 sec.	59˚C \45 sec.	72˚C\45 sec.	35	72˚C\10 min.
invA	94˚C\5 min.	94˚C\30 sec.	55˚C\30 sec	72˚C\30 sec	35	72˚C\5 min.
SopB	94˚C\10 min.	94˚C\30 sec.	58˚C \30 sec.	72˚C\30 sec.	35	72˚C\10 min.
avrA	94˚C\10 min.	94˚C\30 sec.	58˚C \30 sec.	72˚C\30 sec.	35	72˚C\10 min.
bcfC	94˚C\10 min.	94˚C\30 sec.	53˚C \30 sec.	72˚C\30 sec.	35	72˚C\10 min.
bla_{TEM}	94˚C\10 min.	94˚C\45 sec	54˚C\45 sec	72˚C\45 sec	35	72˚C\10 min.
bla_{SHV}	94˚C\10 min.	94˚C\45 sec	54˚C\45 sec	72˚C\45 sec	35	72˚C\10 min.
bla_{OXA-1}	94˚C\10 min.	94˚C\45 sec	54˚C\45 sec	72˚C\45 sec	35	72˚C\10 min.
CMY-2	94˚C\10 min.	94˚C\45 sec	55˚C\45 sec	72˚C\45 sec	35	72˚C\10 min.
bla_{CTX}	94˚C\10 min.	94˚C\45 sec	60˚C\45 sec	72˚C\45 sec	35	72˚C\10 min.

RESULTS

Results of *Salmonella* isolation from 240 samples of clinically diseased chicks revealed that 50 *Salmonellae* were isolated with an incidence of 20.8 % as shown in Table (3) with high recovery rate from liver 31% (14\240) followed with yolk sac 20% (12\240). Serotyping of the *Salmonella* isolates revealed that there were 25 *S.* Kentucky (10.4%), 9 *S.* Infantis (3.7%), 6 *S.* Enteritidis (2.5%), 4 *S.* Heidelberg (1.6%), and one isolate for the following serovars *S.* Labadi, *S.* Typhi, *S.* Agona, *S.* Pullorum, *S.* Newport and *S.* Virginia in percentage (0.4%) for each serovar. Results of antimicrobial susceptibility testing (AST) in table (4), showed that there were two pan susceptible *Salmonella* isolates (i.e. susceptible to all antimicrobial agents under test). There was one *Salmonella isolate* susceptible to one type of antimicrobial agents under test and three *Salmonella* isolates were susceptible to two types of antimicrobial agents under the test. A large percentage of isolates were resistant to all antimicrobials i.e. ampicillin (90%), Nalidixic acid (88%), Sulfamethoxazole-trimethoprim (82%) and tetracycline (82%). Out of 50 *Salmonella* serovars, 13 were *ESβL* when detected phenotypically by Cefinase with a rate of 26 % while when combined with the disc diffusion test, again, 13 serovars were *ESβL* with a rate of 26 %. The comparison between the screening and confirmatory methods revealed that 10 isolates had similar response and 6 isolates responded differently. Genotypic detection of *ESβL*-producing *Salmonellae* by using PCR revealed that 16 isolates which were tested for the presence of 5 genes that are responsible for *ESβL* production, CTX (Cefotaxime), TEM (Beta-lactamase), SHV (sulfhydryl variable active site), OXA (oxacillinase) and CMY (class C carbapenemase); as showed in Table (5-6) with a comparison between results of phenotypic and genotypic detection of *ESβL*-producing *Salmonellae*. The PCR amplification result in this study shows that a total of 10 isolates of phenotypic positive isolates were positive for bla_{TEM} gene type using universal primers, 5 isolates were positive for bla_{CTX} gene type, 2 isolates were positive for bl_{OXA-1} gene type and one isolate was positive for bla_{SHV} gene type but no amplification was found encoding the gene of bla_{CMY-2} noticed that all the *ESβL*s positive serovars were positive for amplification of all tested virulence genes and also noticed that all the isolates were negative for bla_{CMY} gene.

Table 3. Result of *Salmonella* Isolation from different broilers chicks' organs

Organ	No. of samples	No. of positive	percentage	Percentage to all
Liver	45	14	31%	5.8%
Yolk Sac	60	12	20%	5%
Lung	40	4	10%	1.6%
Caecum	50	12	24%	5%
Spleen	45	8	17.7%	3.3%

Table 4. Collective resistance pattern of *Salmonella* serovars to the antibiotics used

Antimicrobial agents	Resistance patterns					
	R	%	I	%	S	%
Sulphamethaxole-Trimethoprim (SXT)	41	82%	-	0	9	18%
Amikacin 30 µg	-	0%	2	4%	48	96%
Imepenem 10 µg	-	0%	3	6%	47	94%
Tetracycline 30 µg	41	82%	1	2%	8	16%
Ampicillin 10 µg	45	90%	1	2%	4	8%
Nalidixic acid 30 µg	44	88%	1	2%	5	10%
Chloramphenicol 30 µg	21	42%	2	4%	27	54%
Gentamicin 10 µg	1	2%	3	6%	46	92%
Ciprofloxacin 5 µg	29	58%	17	34%	4	8%
Azetreonam 30 µg	8	16%	3	6%	39	78%
Ampicillin +Sulbactam	12	24%	4	8%	34	68%
Cefepem	8	16%	2	4%	40	80%
Ceftriaxone 30 µg	7	14%	5	10%	38	76%
Cephalothin 30 µg	16	32%	12	24%	22	44%
Cefotaxime 30 µg	4	8%	-	0%	46	92%
Ceftazidem 30 µg	4	8%	-	0%	46	92%

Table 5. Comparison between Cefinase® and combined disk diffusion test as phenotypic methods for detection of ESβLs.

	Cefinase®	CDD
Positive isolates	13	13
Negative isolates	37	37
Shared isolates	10	10
Different isolates	3	3
Cefinase® +ve / Genotypic -ve	(1/16) 6.25%	----------
Cefinase® -ve / Genotypic +ve	(3/15) 20%	----------
Cefinase® +ve / Genotypic +ve	(12/15) 80%	----------
CDD +ve / Genotypic -ve	----------	0
CDD –ve / Genotypic +ve	----------	(3/15) 20%
CDD +ve/ Genotypic +ve	----------	(13/15) 86.6%

Table 6. Genotypic results of the 6 isolates different in ESβLs pattern detected by phenotypic methods.

Isolate	Cefinase®	CDD	Genotypic resistance genes
S. Kentucky	Negative	Positive	bla_{CTX}
S. Kentucky	Negative	Positive	bla_{TEM}
S. Heidelberg	Negative	Positive	bla_{SHV}+bla_{OXA-1}
S. Infants	Positive	Negative	bla_{TEM}
S. Infants	Positive	Negative	bla_{TEM}
S. Agona	Positive	Negative	bla_{TEM}

DISCUSSION

Salmonellosis in poultry is a worldwide spread infection, both for poultry as a disease causative agents and as a vehicle for human infection (Mohammed *et al.* 1999). Data from current study showed; *Salmonella* different serotypes incidence rate 20.8 % with high recovery rate from liver31% (14\240) followed with yolk sac20% (12\240),the serotyping of recovered *Salmonella* isolates revealed that there were 25 *S.* Kentucky (10.4%), 9 *S.* Infantis (3.7%), 6 *S.* Enteritidis (2.5%),4 *S.* Heidelberg (1.6%), and one isolates for the following serovars *S.* Labadi, *S.* Typhi, *S.* Agona, *S.* Pullorum, *S.* Newport and *S.* Virginia in percentage (0.4%) for each serovar. The emergence of antimicrobial agents' resistance is a matter of concern. People infected by antimicrobial resistant *Salmonella* spp., particularly Nalidixic acid–resistant *Salmonella* spp., are more likely to die, are more likely to be hospitalized, and are hospitalized for longer periods than patients with infections caused by susceptible to antimicrobials strains of *Salmonella* spp. (Helms *et al.* 2004, Helms *et al.* 2002, Lee *et al.* 1994). Antimicrobial susceptibility tests described in this study; show a high percentage of isolates that were resistant to ampicillin (90%), Nalidixic acid (88%), Sulfamethoxazole-trimethoprim (82%) and tetracycline (82%) and out of 50 *Salmonella* serovars, 13 were found to be *ESβL* when detected phenotypically by Cefinase with a rate of 26% while when use combined disc diffusion test 13 serovars were *ESβL* with a rate of 26%. The comparison between the screening and confirmatory methods revealed that 10 isolates expressed similar results and 6 isolates expressed different results. All *Salmonella* isolates were positive by polymerase chain reaction assay for *inv*A gene; which agreed with previous studies about the existence of the invA in *Salmonella* around the world. The detection of those genes in huge number of *Salmonella* isolates from different geographical location maybe associated with the ability of the isolates for cell invasion (Amini *et al.* 2010, Campioni *et al.* 2012, Crăciunaş *et al.* 2012). PCR is a useful and rapid tool for *Salmonella* spp detection in clinical samples., where *inv*A and the *sop*B genes may be a target gene for the detection of this genus, as both genes associated with the virulence of S*almonella* enteritis in birds (Hughes *et al.* 2008, Wood *et al.* 1998). The *avr*A gene prevalence showed 100% detection in all *salmonella* isolates similar to the detection rate found in previous report focused on *Salmonella* Enteritidis (Hopkins . and Threlfall 2004), while in other reports lower detection rate for this gene of *Salmonella* Enteritidis (Liu *et al.* 2012, Rahman *et al.* 2004, Streckel *et al.* 2004, Zou *et al.* 2010) was reported. The variation in detection rate maybe associated with the variation of recombination location of these genes (Hopkins . and Threlfall 2004). These findings are important, since variation in the repertoire of those genes, such as *avr*A and *sop*E, can be associated with the variation of these serovars ability to adapt to new

host cells and consequently provoke the emergence of novel virulent strains (Prager *et al.* 2000).The obtained data from this study showed a high percentage (100%) of *Salmonella* Enteritidis isolates that had the *avr*A gene; while, only 17.1% and 9.7% of *Salmonella* Hadar isolates possessed the *avr*A and *sop*E genes, respectively, which showed difference in the pattern of those genes among different *Salmonella* serovars. (Cesco *et al.* 2009)**.** Some reports associated the high frequency of *avr*A gene in serovars that are considered as the most pathological agents of salmonellosis in poultry farms (Ben-Barak *et al.* 2006).The obtained data from the current study showed significant association between *inv*A, *avr*A, *sop*B, *stn* and *bcf*C virulence genes and resistance to the commonly used antibiotics in Egypt (Table-4/5) as previously observed in Senegal and Gambia but with different virulence genes and antimicrobials resistant genes (Dione *et al.* 2011)**.** These correlations could be explained based on the mechanisms involved in the pathogenicity of bacteria and the acquisition of resistance genes by *Salmonella* species. In the pathogenic bacterial isolates the vast majority of molecular pathogenicity determinant fragments located specific bacterial plasmid (virulence-associated plasmids) or chromosome (Groisman . and Ochman 1996, Hacker *et al.* 1997). The MDR genes may be located on bacterial chromosome either segmented inside chromosome or located as extra chromosomal genetic elements within the chromosome that originate from other genomes (Carattoli 2003)**.** The simultaneous detection of both types of genes either different types of virulence gene or antimicrobial resistance genes has been frequently reported in different *salmonella* species (Carattoli 2003).

CONCLUSION

The data obtained from this study, suggests that broilers play a potential role as a reservoir of multi drug resistant *Salmonella* serovars with special reference to *ESβL* serovars; which contain many virulence genes that magnify the disease condition and imply failure of control.

CONFLICT OF INTEREST

All authors declare no conflict of interest

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Research article
Ερευνητικό άρθρο

Isobolographic analysis of analgesic interactions of silymarin with ketamine in mice

A.S. Naser[1*], Y. Albadrany[2], K.A. Shaaban[2]

[1]*Department of physiology, biochemistry and pharmacology, Faculty of Veterinary Medicine, University of Mosul, Mosul, Iraq*

[2]*Department of physiology, biochemistry and pharmacology, Faculty of Veterinary Medicine, University of Mosul, Mosul, Iraq*

ABSTRACT: The present study was undertaken to explore the analgesic effect of silymarin and ketamine alone or in combination in mice. Analgesia was measured by using a hot plate and the writhing test. The up-and-down method was used to determine the median effective analgesic dosages (ED_{50s}) of silymarin and ketamine administered intraperitoneally (ip) either alone or together. The ED_{50s} of both drugs were analyzed isobolographically to determine the type of pharmacological interaction between them. The analgesic ED_{50s} for silymarin and ketamine in mice were 57.22 and 1.96 mg/kg, *ip*, respectively. Concomitant administration of the silymarin and ketamine at fixed ration (0.5:0.5) of their individual ED_{50s} was 38.4 mg/kg and 1.28 mg/kg, ip, respectively. Silymarin and ketamine at fixed ration (1:1) of their individual ED_{50s} were 47.54 mg/kg and 1.58 mg/kg, ip, respectively . Depending on the isobolographic analysis and calculating Y value, the type of pharmacological interaction between silymarin and ketamine at a ratio of 0.5:0.5 and 1:1 of their analgesic ED_{50} values of each drug , was antagonistic .In the writhing test the concomitant administration of silymarin and ketamine at 120mg/kg and 4mg/kg , ip, respectively reduce significantly the numbers of writhing in compare with silymarin120 mg/kg,ip and ketamine 4mg/kg, ip separately. The results suggest that the co-administration of silymarin and ketamine was ineffective to reduce the central pain while the concomitant administration of silymarin and ketamine was effective to reduce the visceral pain.

Keywords: silymarin, ketamine, analgesia, isobolographic, hotplate, mice.

Corresponding Author:
S. Senturk, Department of Internal Medicine, Faculty of Veterinary Medicine, Uludag University, 16059, Bursa, Turkey
E-mail address: sezsen@uludag.edu.tr

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INTRODUCTION

Ketamine is an anesthetic and pain killeragen-tused in human and animals for anesthesia which characterized by hypnosis and good analgesia with muscle rigidity(Carter & Story, 2013; Persson, 2013; Tawfic, 2013). Ketamine inhibits the polysynaptic actions of the excitatory neurotransmitter acetylcholine (Ach) and glutamate in the spinal cord and block the N-methyl-D-aspartate receptor complex (Hsu, 2013). Ketamine used in human(Aiello & Mays, 1998)and veterinary clinic (Rang, Dale, Ritter, & Moore, 2003) as a general anesthetic agent usually in combination with sedatives, tranquilizers and analgesics. Ketamine combinations with sedatives, tranquilizers and analgesics are also used as balanced anesthetics and restraining agents in wild animals(Carter & Story, 2013; Coetzee, 2013; Short, 1992). Further, ketamine have anticonvulsant activity in mice (Tricklebank, Singh, Oles, Preston, & Iversen, 1989) and chicks (Reder, Trapp, & Troutman, 1980)

Silymarin (milk thistle), a medical herb belonging to the Asteraceae family(Ottai& Abdel-Moniem, 2006), is home Southern Europe, Southern Russia, Asia Minor and Northern Africa (Abenavoli, Capasso, Milic, & Capasso, 2010).Silymarin is mixture of silibinin A and B (silybin A and B), silydianin and silychristin. Other flavonolignans include isosilybin A and B, isosilychristin and taxifolin(Ottai & Abdel-Moniem, 2006; Stolf, Cardoso, & Acco, 2017).

Silymarin was used for the management of numerous liver disorders including cirrhosis, hepatitis (Herz, Heywood, Harborne, & Turner, 1977; Luper, 1998) Except hepatoprotective effects, it has cardioprotective, neuroprotective, cytoprotective, anti-inflammatory , analgesic and anti-carcinogenic effects (Manna, Mukhopadhyay, Van, & Aggarwal, 1999; Naser & Amin, 2019; Škottová et al., 2003)A useful practice is a combination of two drugs with the same therapeutic effect where in each agent is administered to obtain additive, synergistic or antagonism interaction in a fixed ratio. If the combination resulted in addition or synergism, the doses employed by each drug are reduced, then the side effects are decline; this type of study is calledisobolographic analysis (Raffa, 2001).

The purpose of the presentstudy was to explore the analgesic effect of silymarin and ketamine combination in mice. The models of pain induction used were the hot plateand chemical-induced writhing, which are predictive of acute pain responses with involvement of central and peripheral systems, respectively(Acharya et al., 2011; Barrot, 2012; de Campos Buzzi et al., 2010; Jain, Kulkarni, & Singh, 2001).

MATERIALS AND METHODS

46 Male and female Swiss albino mice weighing 28–33 g were housed at a temperature of 20 ± 2°C and 10/14 h light/dark cycle, with water and food ad libitum. All experiments complied with institutional regulations addressing animal use, and the mice received suitable attention and humane care. The Scientific Committee of the physiology, biochemistry and pharmacology of the College of Veterinary Medicine at the University of Mosul has reviewed and approved the protocol of this study. The required doses of The commercial powder of silymarin (175mg, 21ST Century HealthCare, Inc.) was dissolved in propylene glycol(Sigma chemical CO. 99%) . Ketamine (10 % injectable solution, DOPHARMA Netherland.) was further diluted in saline solution (Pharmaceutical Solution Industry, Saudi Arabia) to obtain the desired concentrations of the drug. The volume of administration of each drug was at 5 ml/kg body weight given intraperitoneally (ip). Two experimenters simultaneously observed the responses of the mice during the experiments which were conducted between 9–12 a.m.

Experiments

The up-and-down method (Dixon, 1980)was used to determine the median effective analgesic dosages (ED_{50s}) of silymarin and ketamine (administered either alone, or concomitantly – silymarin followed immediately with ketamine) in mice. The initial dose of silymarin was at 100 mg/kg, *ip*whereas that of ketamine was at 2 mg/kg, *ip*. In the combination experiment, the initial dosages of silymarin and ketamine were 30 and 1 mg/kg, *ip*, respectively (50:50 of their individual ED_{50} values) and the initial dosages of silymarin and ketamine were 60 and 2 mg/kg, *ip*, respectively (1:1 of their individual ED_{50} values) . We based our choices for silymarin and ketamine dosages on preliminary experiments in mice, as well as on previous studies (G. Jadhav & Upasani, 2009; Mohammad, Al-Baggou, & Naser, 2012; Sabiu, Sunmonu, Ajani, & Ajiboye, 2015; Vahdati Hassani et al., 2015) . Analgesia was measured by the thermal method using a hot plate (Panlab, S.I.U., Cornella, Spain) held at a temperature of 56°C. Mice were individually placed on the hot plate 15 min after the drug administration and the latency time to the first hind paw removal/

licking and/or jumping response was measured (Barrot, 2012; Le Bars, Gozariu, & Cadden, 2001)The cutoff point latency for the induction of analgesia was equal to or more than 6 s (i.e., positive analgesic response), and the maximum time allowed for the animal to stay on the hot plate was 20 s to prevent tissue damage .. The ED_{50s} of both drugs were subjected to isobolographic analysis to determine the type of interaction involved in the administration of silymarin and ketamine concomitantly (Gonzalez, Zegpi, Noriega, Prieto, & Miranda, 2011; Mohammad, Al-Zubaidy, & Alias, 2007; Tallarida, 1992)A straight line was drawn for the isobolographic analysis between isoeffective analgesic doses (ED_{50}) of silymarin and ketamine given to the mice either alone or in combination. The ED_{50} points of silymarin and ketamine given alone are represented on the x- and y-axes, respectively. The straight diagonal line indicates the line of additivity (zero interaction) at the ED50 values, and the location of the combination points on the left (below) and right (above) sides of the additive line indicates synergistic and antagonistic interactions, respectively (Gonzalez et al., 2011; Mohammad et al., 2007; Tallarida, 1992) The interaction index was calculated by the equation da/Da + db/Db(Tallarida, 1992)Da and Db are the individual ED_{50s} of silymarin and ketamine for the induction of analgesia, respectively, whereas da and db are their combined ED_{50s} for causing analgesia. An interaction index of 1 means additively (no interaction), <1 synergism and > 1 antagonism (Gonzalez et al., 2011; Mohammad et al., 2007; Tallarida, 1992).

To further examine the potential analgesic effect of silymarin and ketamine coadministration (by doublingthe ED_{50s} of both drugs given to mice), additional experiments were conducted to measure the analgesic effect of the combination 15 min after the administration by the thermal and chemical methods (Acharya et al., 2011; Le Bars et al., 2001).Acetic acid-induced writhing test in the mice,Thistest was conducted employing (Koster, 1959) method. Mice were divided into 4 groups of 6 mice each. The first group served as control and was given 10ml/kg i.p normal saline to act as control group. Groups II, received ketamine at 4mg/kg i.p , group III received silymarin at 120 mg/kg i.p and the group IV received ketamine 4mg/kg and silymarin 120 mg/kg i.p concomitantly . 15 minutes later, each mouse was injected with (0.06% acetic acid of 1ml per 100g i.p). The number of abdominal constriction for each mouse was counted 30 minutes after injection of acetic acid . Percentage inhibition of writhing was calculated using the formula. Inhibition % = Mean no of writhes (control) – mean no of writhes (test) / Mean no of writhes (control) × 100.

Statistical Analysis

Data are expressed as mean + SEM. Statistical analysis was done by using one way analysis of variance (ANOVA).$P<0.05$were considered significant.

RESULTS

Hot plate test

The ED_{50s} for silymarin - and ketamine induced analgesia in mice, as determined by the up and- down method, were 57.22 and 1.96 mg/kg, *ip*, respectively (Table 1). The silymarin-treated mice appeared to be slightly sedated. However, Combined administration of silymarin and ketamine at fixed ratio (50:50) of their individual ED_{50} values were 38.4 and 1.28 mg/kg, *ip*, respectively(Table 2) whereas , Combined administration of silymarin and ketamine at fixed ratio (1:1) of their individual ED_{50} values were 47.54 and 1.63 mg/kg, *ip*, respectively (Table 3). Isobolographic analysis of these ED50s for both drugs (either alone or in combination) at fixed ration either 0.5:0.5 or 1:1 revealed that combined administration of the drugs has antagonistic effect on the induction of analgesia in mice (Fig. 1). This antagonistic effect was indicated by the location of the points representing the combined analgesic ED50s of silymarin and ketamine above the diagonal line that connect their isoeffective analgesic doses (ED50s) given alone (Fig. 1). Further, the calculated interaction index for analgesia at fixed ration either 0.5:0.5 or 1:1 was 1.32 and 1.63 respectively indicating an antagonistic interactions between both drugs (an index of > 1indicates antagonism). The animals did not reach the point of tissue damage, so at the end of the experiments, we didn't have to give an analgesic dose.

Acetic acid-induced writhing response in mice

We found that ketamine and silymarin at 4 mg/kg and 120mg/kg significantly reduced the writhing number as compared to mice treated with normal saline (control),silymarin 120mg/kg and ketamine 4mg/kg (Table 4) . The percentage of inhibition of writhing was 62.1, 63.6 and 84.8 in ketamine at 4mg/kg ,silymarin 120mg/kg and concomitant silymarin 120mg/kg +ketamine 4mg/kg treated mice, respectively (Table 4) (Fig 1).

Table 1. Median effective doses (ED50) of silymarin and ketamine administered ***ip*** alone for induction of analgesia in the hot plate test in mice

Variable	Silymarin	Ketamine
ED50	57.22	1.96
Range of the doses used	100-40= 60mg/kg	2-1.5=0.5mg/kg
Initial dose	100mg/kg	2mg/kg
Last dose	40mg/kg	2mg/kg
Number of mice used	6(xxoxxo)	5(xoxoo)
Increase or decrease in the dose	20mg/kg	0.5mg/kg

aX – analgesia; O – no analgesia. The ED50s were determined by the up-and-down method (Dixon, 1980)

Table 2. Median effective doses (ED50) of silymarin and ketamine administered *ip* concomitantly for induction of analgesia in the hot plate test in mice at fixed ratio (0.5:0.5)

Variable	Silymarin + Ketamine	
ED50	38.4mg/kg	1.28mg/kg
Range of the doses used	45-30= 15mg/kg	1.5-1=0.5mg/kg
Initial dose	30mg/kg	1mg/kg
Last dose	45mg/kg	1.5mg/kg
Number of mice used		5(oxoox)
Increase or decrease in the dose	7.5mg/kg	0.25mg/kg
Y		1.32

Table 3. Median effective doses (ED50) of silymarin and ketamine administered *ip* concomitantly for induction of analgesia in the hot plate test in mice at fixed ratio (1:1)

Variable	Silymarin + Ketamine	
ED50	47.54mg/kg	1.58mg/kg
Range of the doses used	60-30= 30mg/kg	2-1=1 mg/kg
Initial dose	60mg/kg	2mg/kg
Last dose	45mg/kg	1.5mg/kg
Number of mice used		6(xxooxo)
Increase or decrease in the dose	15mg/kg	0.5mg/kg
Y		1.63

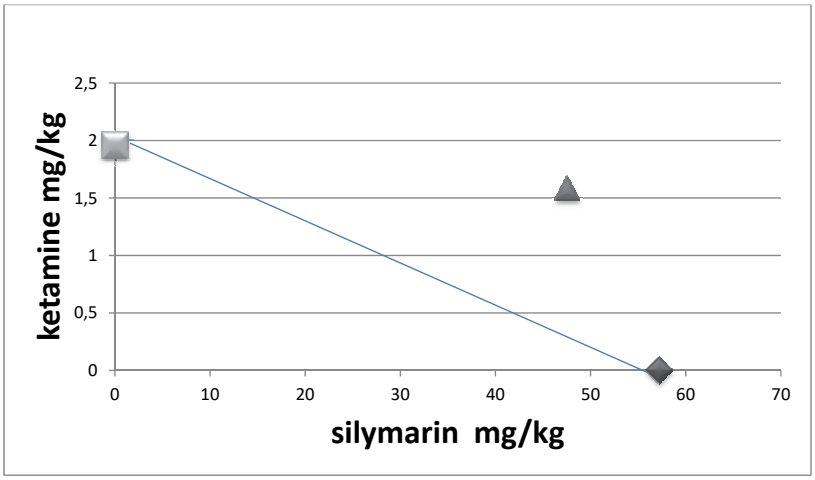

Figure 1. Isobolographic analysis of the analgesic interaction of silymarin and ketamine in mice.silymarin and ketamine was injected intraperitoneally. Points on x- and y-axes represent median analgesic doses (ED50s, mg/kg) of the drugs given alone, whereas the triangular point represents 1:1 of ED50 combinations of both drugs. The diagonal line between the individual ED50s of silymarin and ketamine is antagonistic and the triangular point indicates synergistic interaction.
n=5–6 mice/each ED50 experiment.

Table 4. The analgesic activity of Silymarin and ketamine in visceral pain model-writhing method following concurrent administration in mice

Groups	Onset of writhing	Numbers of writhing	Percentage of inhibition
Normal saline	31.5±3.7	13.2±1.7	0
Ketamine 4mg/kg	30.0±3.7	5.0±0.5*	62.1%
Silymarin 120mg/kg	37.5±3.7	4.8±0.5*	63.6%
Ketamine 4mg/kg+ Silymarin 120mg/kg	25.8±4.7	2.0±0.5*ab	84.8%

n = 6 for each group.The observations are mean ± SEM.
* p<0.05, as compared to control
a p<0.05, as compared to ketamine 4mg/kg .
b p<0.05, as compared to silymarin at 120mg/kg.

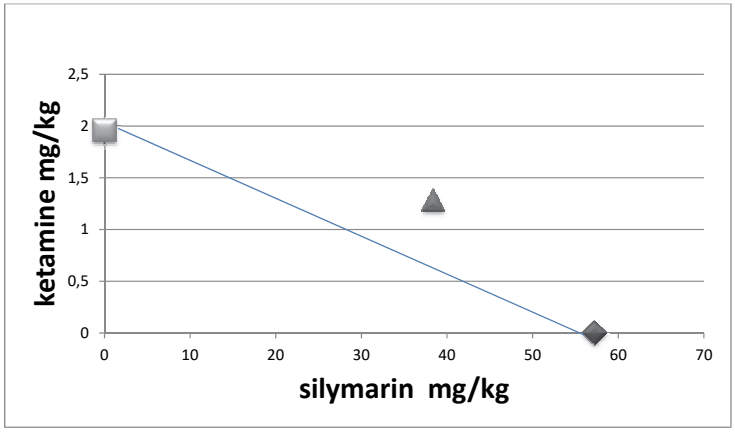

Figure 2. Isobolographic analysis of the analgesic interaction of silymarin and ketamine in mice . silymarin and ketamine was injected intraperitoneally. Points on x- and y-axes represent median analgesic doses (ED50s, mg/kg) of the drugs given alone, whereas the triangular point represents 0.5:0.5 of ED50 combinations of both drugs. The diagonal line between the individual ED50s of silymarin and ketamine is antagonistic and the triangular point indicates synergistic interaction.
n=5–6 mice/each ED50 experiment.

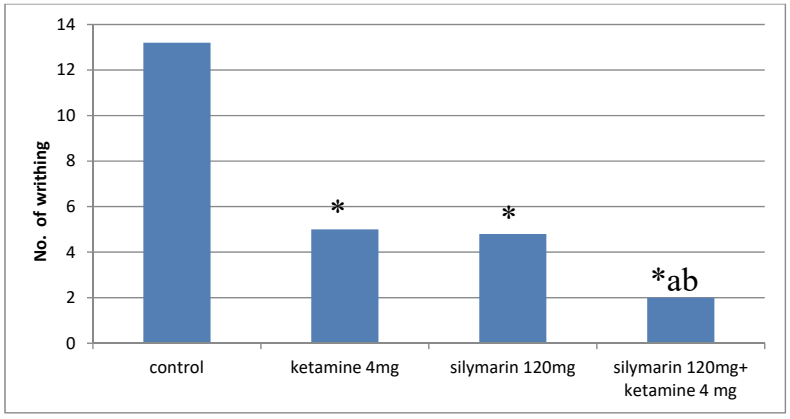

Figure 3. The analgesic activity of Silymarin and ketamine in visceral pain model-writhing method following concurrent administration in mice treated with normal saline , ketamine 4mg , silymarin 120mg and ketamine 4mg+ silymarin 120mg were injected i.v. 30 min before 0.6% acetic acid i.p. After 15 min the no. of abdominal constrictions was counted during 30 min. Results expressed as mean S.E.M. n=6for each group.

DISCUSSION

A large number of herbal medicinal products are reputed to have excellent medicinal value and are used to treat multiple ailments. In herbal medicine, different indigenous drugs are used with considerable success in single and/or combined forms to treat different types of inflammatory and arthritic illnesses(Hussain, Jassim, Numan, Al-Khalifa, & Abdullah, 2009). Silymarin produced considerable analgesic effects (on the writhing test) in mice either alone or in combination with ketamine while the combination of silymarin and ketamine was antagonistic in the hot plate test. Silymarin's individual action is reliable with the reported analgesic effect of the drug on mice, rats and chicks(Amin & Arbid, 2015; Hassani et al., 2015; G. B. Jadhav, Upasani, & others, 2009; Sahib, 2011)

In the acetic acid induced writhing tests, the administration of silymarin and ketamine combinations produced antinociceptive effects that were greater than would be achieved for individual drug administration alone.

The method of hot plate and tail immersion originally described by (Woolfe & Macdonald, 1944) was found to be suitable for the evaluation of analgesics that act centrally but not peripherally. Acetic acid induced writhing is a sensitive approach for the testing of compounds ' peripheral analgesic effect. Peritoneal nociceptor stimulation is indirect and occurs by releasing endogenous substances that stimulate nerve endings (Bhutia, Vijayaraghavan, & Pathak, 2010; Gawade, 2012). silymarin has cardioprotective, neuroprotective, cytoprotective, anti-inflammatoryandanti-carcinogenic effects, analgesic effect and hepatoprotective effects(Corchete, 2008; Fanoudi, Alavi, Karimi, & Hosseinzadeh, 2018; Kren & Walterová, 2005; Semalty, Semalty, Rawat, & Franceschi, 2010) . Silymarin is a free radical scavenger that uses cyclooxygenase and lipoxygenase pathways to various steps in arachidonic acid cascade (O. P. Gupta et al., 2000). Silymarin showed inhibitory effects on macrophage production of IL-1β and PGE2 and blocked IL-1β and cyclooxygenase-2 expression of mRNA in LPS-stimulated RAW 264.7 cells (Mateen, Raina, & Agarwal, 2013). There is a marked increase in the concentration of PGE2 and PGF2a in the peritoneal fluid following injection of acetic acid(Ricciotti, E., & FitzGerald, 2011), and the analgesic effect of silymarin similar to aspirin may be due to the Inhibition of prostaglandin synthesis (Fiebrich & Koch, 1979; Soon, Young, Song-Kyu, Kyu-Hwan, & Mook, 2004).

Ketamine acts to induce analgesia and anesthesia in humans and animals by antagonizing N-methyl-D-aspartate receptors(Aiello & Mays, 1998; Cornick-Seahorn JL:, 2001; Rang et al., 2003) By interacting with the supraspinal μ-opioid receptors, ketamine also induces analgesia(Nieuwenhuijs et al., 2004)In this context, by modulating phosphorylation in cells that express μ-opioid receptors endogenously, ketamine enhances opioid-induced analgesic signaling(Gupta, A., Devi, L. A., & Gomes, 2011).

This is the first study to provide an isobolographic analysis of the analgesic interaction between silymarin and ketamine. These data suggest that silymarin and ketamine in combination may have greater than additive effects in the treatment of visceral hyperalgesic conditions.

In the present study, the analgesic effect of combined administration of silymarin and ketamine was found to be antagonistic in the hotplate test as substantiated by the isobolographic analysis of the individual and combined ED50s of both drugs for the induction of analgesia at a fixed ratio of 0.5:0.5 and 1:1 of their individual ED50s, resulting in an interaction index of >1 (1.32 and 1.63) respectively .The antagonistic effect at level of central nociception may be due to the different mechanism between two drug and may be due to the distribution of silymarin in the CNS.Most flavonoids are metabolized in the gastro-intestinal tract and liver, are absorbed into the bloodstream and can reach the CNS by crossing the blood brain barrier (BBB)(Rodriguez-Mateos et al., 2014). To date, there is little data on the bioavailability of flavonoids and their capacity to reach the CNS.Detection of certain polyphenols and their metabolites indifferent brain regions after oral administration of polyphenolic extracts in murine models indicates that at least some flavonoids are able to cross the BBB(El Mohsen et al., 2002; Ferruzzi et al., 2009).

The pain eliciting models used in this study vary from one another and reflect the central (hot plate test) and peripheral (writhing test) mechanisms involved in pain generation and recognition(Acharya et al., 2011; Dambisya YM, 1994; de Campos Buzzi F, Fracasso M, Filho VC & del Olmo E, 2010). Different research groups have used acetic acid induced writhing test primarily to evaluate antinociceptive of natural compounds worldwide(Danion, Diemunsch, & Brandt, 2000; V. K. Gupta, 2006). Several endogenous noxious mediators such as bradykinin, serotonin, histamine, substance P(Danion et al., 2000; Irifune et

al., 1998) were released by acetic acid. The resulting pain is symbolized by abdominal muscle contraction accompanied by forelimb extension and elongation of the body. Our study did not investigate the mechanisms involved in the synergy between silymarin andketamine. The synergy observed with the writhing test may be associated with a pharmacokinetic or pharmacodynamic interaction. Silymarin may induce a decrease in the glomerular excretion of ketamine and its active metabolite (i.e., norketamine) through prostaglandin synthesis inhibition..

A pharmacodynamic interaction is more likely, as indicated by the peak amplitude synergistic effect. Silymarin acts as analgesic primarily through peripheral inhibition of the cyclooxygenase enzyme (Hussain et al. 2009). . Ketamine has mainly a central site of action through the block of NMDA receptors . A sensible hypothesis to explain our outcomes may be that silymarin decreased nociceptive inputs reaching the central nervous system, thereby improving the effectiveness of ketamine's central action. A reduced frequency of nociceptive inputs is probable to require less blockage of the NMDA receptor to induce an analgesic effect.however, some researcher indicate that repeated administration of silymarin prevents the formalin – induced pain behavior but it is ineffective in the treatment of sciatic neuropathic pain in mice (Hassani et al., 2015) . Another proposed mechanism involves pharmacodynamic interactions between the two drug-affected receptors (Yaksh, 1994)However, in the present study, the combination of silymarin and ketamine not only induced synergistic antinociceptive effects, but also reduced side effects by reducing each drug's dose.

In conclusionsilymarin and ketamine have antagonistic antinociceptive effects at the central pain, but both drugs have synergeticantinociceptive effects at the peripheral pain .These results could be of clinical importance in the species of rodents or could be extended to mammals after further research.

CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

In-vitro efficacy of *Arachis hypogaea* (Peanut) peels extract against *Haemonchus contortus*

Tahir A.[1], Akhtar R.[2*], Lateef M.[1], Sattar F.[1], Hafeez M. A.[1], Omer M. O.[3], Zahid B.[4], Javed A.[2]

[1]*Department of Parasitology, University of Veterinary and Animal Sciences, Lahore, 54000. Pakistan*

[2]*Department of Pathology, University of Veterinary and Animal Sciences, Lahore, 54000. Pakistan*

[3]*Department of Pharmacology, University of Veterinary and Animal Sciences, Lahore, 54000. Pakistan*

[4]*Department of Pathology, College of Veterinary and Animal Sciences, Narowal, 51600. Pakistan*

ABSTRACT: Synthetic anthelmintics are becoming ineffective due to development of parasitic resistance. For this reason, traditional medicinal plants are being explored for their anthelmintic potential. The aim of this study was to evaluate the *in-vitro* anthelmintic activity of *Arachis hypogaea* L. (peanut) peels against *Haemonchus contortus*. To assess the anthelmintic effect of *Arachis hypogaea* L. on 3 life cycles of *Haemonchus contortus,* egg hatch assay (EHA), larval developmental assay (LDA) and adult motility assay (AMA) were conducted. In these tests, methanol and n-hexane extracts of the plant were used in three concentrations of 10, 15 and 20 mg/ml. Levamisole and PBS were used as positive and negative control groups respectively. Results of these tests showed that methanol extract of *Arachis hypogaea* L. had higher anthelmintic effect than that of n-hexane extract. Overall both extracts exhibited a significant ($p<0.05$) dose and a time dependent anthelmintic effect. At 20 mg/ml, methanol extract and n-hexane extract showed 87% and 80% egg hatching inhibition respectively. Methanol and n-hexane extracts at 20 mg/ml showed 83.3% and 76.6% larval mortality respectively. Adult motility test with both extracts showed maximum immobilization of worms after 6 hours of treatment at 20 mg/ml concentration. It is concluded that peels of *Arachis hypogaea* possess significant anthelmintic potential against nematodes. It may be suggested that the plant can be used further to investigate the *in-vivo* activity.

Keywords: *Arachis hypogaea*, *Haemonchus contortus*, Anthelmintic, Egg inhibition, Larval mortality

Corresponding Author:
Raheela Akhtar, Department of Pathology, University of Veterinary and Animal Sciences, Lahore, 54000, Pakistan
E-mail address: raheela.akhtar@uvas.edu.pk

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INTRODUCTION

Livestock sector plays a vital role in Pakistan economy. It is the second most important sub-sector in agriculture of Pakistan (Amin *et al.,* 2010) and High prevalence of gastrointestinal parasities in small ruminants have been reported in Pakistan (Gadahi *et al*., 2009; Bilal *et al.,* 2009). Gastrointestinal parasites are the major problem worldwide that can lead to weight reduction, lowered milk and meat production (Githigia *et al.* 2005). The severe clinical signs include diarrhea, anorexia, oedema and anaemia that are further related with poor performance and mortality particularly in young, aged and in those animals that lack immunity (Eysker and Pleoger 2003).

Haemonchus contortus, commonly known as the *barber's pole worm* is a highly pathogenic, blood-feeding nematode of small ruminants, and is a major constraint on ruminant health and production worldwide (Sargison, 2012)**.** It has a remarkably high propensity to develop resistance to anthelmintic drugs, making control increasingly difficult. The expected increase in prevalence and severity of resistances in field isolates of this species to the available drugs will make it difficult for producers to control *H. contortus* on a worldwide scale (Kaplan, 2004).

Due to development of parasitic resistance against synthetic anthelmintics, veterinarians are now exploring anthelmintic potential of traditional medicinal plants. Peanut is one of the most important crops in the world, both as oil and as a protein source. It contains flavonoids, carbohydrate, mineral and vitamins. The former studies suggest that peanut can exhibit potential activity against microbes and parasites. It has also endocrine, relaxing effects and reduces inflammation (Al-Snafi 2014). Some studies have showed inhibitory activity of legumes against larvae and adult motility of *H. contortus (*Naumann *et al.,* 2014; Von Son-de Fernex *et al.,* 2012). Current study has been planned to test the anthelmintic efficacy of *Arachis hypogaea* (peanut) peels against the eggs, larvae and adult forms of *Haemonchus contortus.*

MATERIALS AND METHODS

Arachis hypogeae peels were purchased from local market of Lahore. It was identified from Botany Department in Govt. College University Lahore with the batch number GCU-HERB-BOT-4001A. The peels of the plant were subjected to grinding to form fine powder. Powder was kept in air tight jar at 4°C until use. *H. contortus* infected sheep was taken from Department of Parasitology, UVAS, Lahore for eggs and worm collection. This study was approved from the ethical committee of UVAS, Lahore and all efforts were taken to minimize pain and discomfort to the animal while conducting these experiments.

Preparation of extracts

Soxhlet apparatus (Iqbal *et al.* 2006) was used to prepare the methanol and n-hexane extracts of *Arachis hypogaea.* Briefly, 50 gm of plant powder, wrapped in Brazil filter paper was introduced in into the Soxhlet extracter for extraction against 800ml methanol solvent to get crude methanol extract or N-hexane solvent for crude N-hexane extract. The extraction was preceded until the thimble of plant powder became almost colorless. The semi-solid extracts were used to formulate different concentrations (10 mg/ml, 15 mg/ml and 20 mg/ml) using PBS. The extract yield (% w/w) from the plant material was recorded. The parasites were collected from the abomasum of sheep with the help of forceps. Three *in vitro* assays were performed to check the efficacy of methanolic and n-hexane extracts against adults, larvae and eggs of *H. contortus*. EHA and LDA were conducted in triplicate and % egg inhibition and % larval mortality were then calculated as mean±SD.

Egg Hatch Assay (EHA)

Microscopic examination of fecal samples of infected sheep was done through floatation method and observed under microscope for eggs identification of *Haemonchus contortus*. Egg hatch assay was carried out according to the typical method pronounced by Coles *et al.*, (1992) to check the efficacy of methanol and n-hexane extracts of *Arachis hypogaea* peels on *Haemonchus contortus* eggs. Methanol extract was applied on eggs (150 eggs/well) at different concentrations of 10, 15 and 20 mg/ml in a microtiter plate. The activity of levamisole drug at the concentration of 0.55 mg/ml as positive control and PBS as negative control was also determined. Same procedure was done for n-hexane extract. The observations were recorded after examination of samples under microscope.

Larval Developmental Assay (LDA)

Eggs were incubated to develop into larvae. Larvae were then separated by Bearmann technique (Mehlhorn, 2008). Methanol extract was applied on larvae at different concentrations of 10, 15 and 20 mg/ml in a microtiter plate. The activity of levamisole drug at the concentration of 0.55 mg/ml as positive control and PBS as negative control was also determined.

Same procedure was repeated for n-hexane extract. The observations were recorded after examination of samples under microscope.

Adult Motility Assay (AMA)

H. contortus worms were collected from abomasum of the infected sheep after slaughtering it. These parasites were washed and confirmed by microscopy, then kept in PBS. Moving worms were placed in Petri dishes (10 worms in each dish) with 10, 15 and 20 mg/ml of the Methanolic extract of *Arachis hypogaea* and in petri dishes with same concentrations of n-hexane extract. PBS was used as negative control. Levamisole diluted in PBS at the concentrations of 0.55 mg/ml was used as a positive control. After 24hrs, the extracts were washed away and the parasites were suspended in PBS for 30min for possible recovery of the parasitic motility. Under dissecting microscope, the number of alive and dead worms was calculated and recorded for each concentration. The motility of worms was checked at intervals of 0, 1, 2, 3 and 6 hours. A mortality index was calculated as the number of departed or paralyzed worms divided by the total number of worms per petri dish. Mortality percentage was then calculated from mortality index.

$$\text{Mortality Index} = \frac{\text{Dead parasites}}{\text{Total number of parasites}}$$

STATISTICAL ANALYSIS

Statistical Program SPSS VERSION 16 was used to apply the technique ANOVA for assessing the significance of the anthelmintic activity of extracts in comparison with negative control.

RESULTS

The physical features and percentage yield of plant extracts are given in the table 1. The plant powder yielded 12% methanol and 10% n-hexane extracts.

The maximum egg inhibition and larval mortality was seen at high concentration of 20mg/ml with both extracts. Methanol and n-hexane extract showed 87% and 80% egg inhibition respectively at 20mg/ml concentration. At 20 mg/ml, methanol and n-hexane extract showed 83.3% and 76.6% larval mortality respectively. Results revealed significant dose dependent inhibitory activity ($p<0.05$) for both extracts (methanol and n-hexane) as well as for levamisole (positive control) in both tests when compared with the negative control. The ovicidal and larvicidal activity of both extracts is given in table 2.

Efficacy of *Arachis hypogaea* peels methanol extract against adult worms of *H. contortus* is represented in figure 1 while that of n-hexane extract is represented in figure 2. In both cases, number of motile worms decreased significantly ($p<0.05$) during 6-hour period. The maximum mortality was seen at high concentration of 20mg/ml after 6 hours. Methanol and n-hexane extract showed 77% and 60% mortality rate at 20mg/ml concentration after 6 hours of treatment respectively. Positive control (Levamisole) and negative control showed 100% and 1% mortality after 6 hours of treatment. Result of adult motility assay showed dose and time dependent anthelmintic activity of extracts of *Arachis hypogaea* peels.

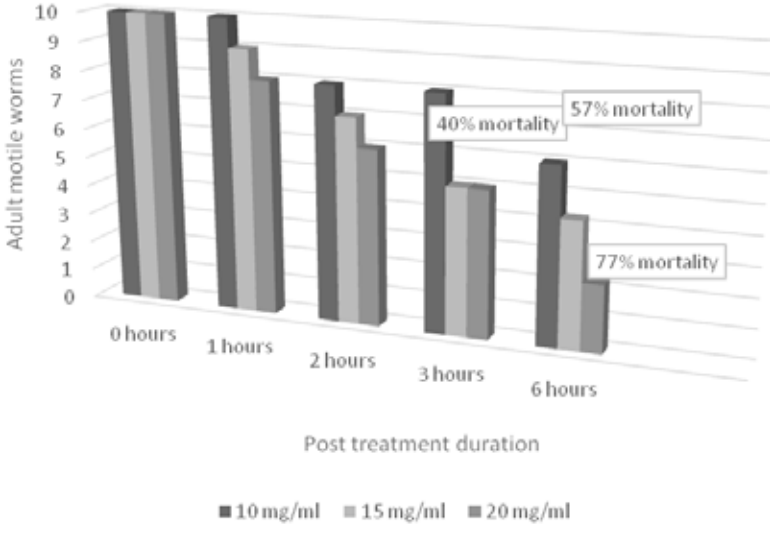

Figure 1. Efficacy of *Arachis hypogaea* methanol extract against adult worms of *H. contortus*

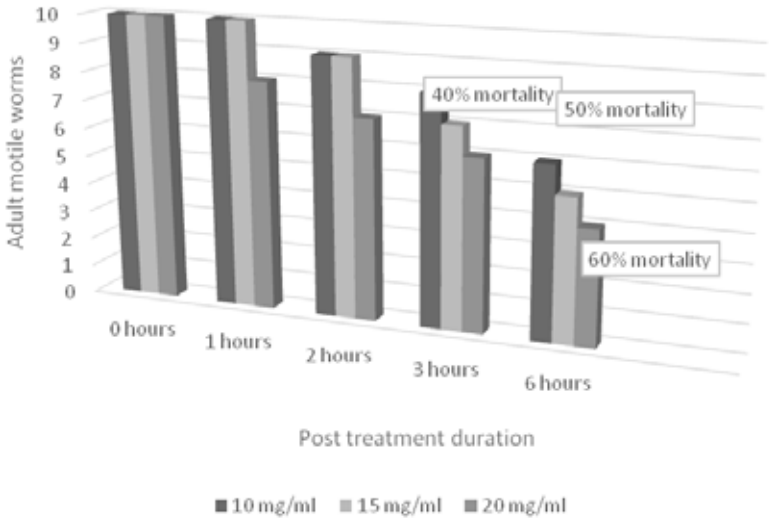

Figure 2. Efficacy of *Arachis hypogaea* n-hexane extract against adult worms of *H. contortus*

Table 1. Physical characteristic features of plant extracts

Wt. of dry powder (g)	Wt. of extract (g)	Yield (%)	Extract color	Extract consistency	Solvent used
50	12	24	Dark brown	Semisolid	Methanol
50	5	10	Yellow	Semiliquid	n-hexane

Table 2. Dose based ovicidal and larvicidal efficacy of methanol and n-hexane extracts

	Dose	% Egg inhibition (Mean±SD)	%larval mortality (Mean±SD)
Methanolic extract	10mg/ml	66.0±2.0	53.3±5.7
	15mg/ml	79.0±1.0	70±10.0
	20mg/ml	87.0±3.4	83.3±5.7
N-hexane extract	10mg/ml	62.0±1.0	53.3±5.7
	15mg/ml	71.0±1.0	66.6±5.7
	20mg/ml	80.0±1.0	76.6±5.7
Positive control	0.55mg/ml	97.3±0.5	96±5.7
Negative control	-	0.0±0.0	0.0±0.0

DISCUSSION

Traditional medicinal plants have been reported to have anthelmintic potential (Satyavati *et al.* 1976; Lewis, 1977). Scientists are exploring anthelmintic potential of traditional plants due to the increasing in-effectiveness of synthetic anthelmintics against parasites. As a part of this exploration, we aimed to assess the *in vitro* efficacy of *Arachis hypogaea* (Peanut) Peels extract on eggs, larvae and adults of *Haemonchus contortus*. The major benefit of using *in vitro* methods is to assess the antiparasitic properties present in plants and their extract including low expenditure as well as quick yield (Tiwari *et al.,* 2011). In our study, maximun egg inhibtion and larval mortality by methanol and n-hexane extracts of *Arachis hypogaea* was seen at higher dose of 20 mg/ml. Significant time-dependent inhibition in motility of adult worms was seen during 6 h of treatment with

different concentrations of extracts. Overall, all tests showed significant dose-dependent anthelmintic effect by both methanol and n-hexane extracts. These results suggest that *Arachis hypogaea* peels contain possible anthelmintic compounds which can be effective against other parasites in addition to *Haemonchus contortus*.

Hounzangbe-Adote *et al.* (2005) screened extracts of four tropical plants (*Zanthoxylum zanthoxyloides*, *Newbouldia laevis*, *Morinda lucida* and *Carica papaya*) in vitro for potential anthelmintic against eggs, larvae and adult *Haemonchus contortus.* Their results showed significant anthelmintic activity by extracts of all four plants. Egg hatching inhibition was dose dependant but larvaicidal effect and adult mortality was not found dose dependant in their results. In a similar kind of study, Ferreira *et al*. (2013), evaluated the *in-vitro* anthelmintic effect of *A. muricata* aqueous leaf extract against eggs, infective larvae and adult forms of parasitic nematode *H. contortus*. In their study, *A. muricata* extract at higher doses, showed 84.91% and 89.08% of efficacy in egg hatch test and larval motility test, respectively. In the adult worm motility test, worms were completely immobilized after 6–8 h of treatment with different concentrations of extract. On the base of phytochemical analysis, they suggested that phenolic compounds in the extract may be responsible for anthelmintic activity.

Kamaraj and Rahuman, (2012) tested ovicidal and larvicidal activities of methanol extracts of five medicinal plants (*Annona squamosa*, *Eclipta prostrata*, *Solanum torvum*, *Terminalia chebula*, and *Catharanthus roseus)* on *Haemonchus contortus.* They used extracts in different concentrations. Overall results of their study suggest that the tested plants contain anthelmintic compounds. Marie-Magdeleine *et al.* (2010) in their study also showed similar kind of results.

Results of our study are in concordance with the study conducted by Adama *et al*. (2009), who examined the anthelmintic effects *in-vitro* of *Anogeissus leiocarpus* leaf and *Daniellia oliveri* stem bark extracts on eggs, first stage larvae and adults of *Haemonchus contortus.* They used different concentrations of the extracts. PBS and levamisole (at 0.125 µg/ml in PBS) were used as negative and positive control groups, respectively. *Their results showed that* both plant extracts induced significant anthelmintic effects on the three life-cycle stages of *H. contortus. Moreover,* the effect was dose-dependent on egg hatching and first stage larvae but unlike the results of our study, the effect was not dose dependent on adult worms. This can be due to difference in the nature and properties of anthelmintic compounds of different plants

CONCLUSION

This study concludes that both extracts (methanol and n-hexane) of *Arachis hypogaea* peels show significant anthelmintic activity against eggs, larvae and adult motility of *Haemonchus contortus*. Both extracts displayed significant ($P<0.05$) dose and time dependent anthelmintic activity. The maximum activity was recorded at high dose concentration of 20mg/ml. At low concentrations, the extracts revealed no significant activity. These results suggest that *Arachis hypogaea* can be used against *Haemonchus contortus* as an alternative for synthetic anthelmintic drugs. Further research for evaluation of *in-vivo* efficacy of *Arachis hypogaea* against *Haemonchus contortus* in sheep is recommended.

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Research article
Ερευνητικό άρθρο

Effect of genotype on adipose tissue fatty acids profile of two autochthonous sheep breed

B. Miščević[1], Š. Rašković[1], D. Gajić[1], N. Puvača[2,*], T. Könyves[1], M. Stojšin[1], V. Bursić[3], D. Puhača[2], I. Gianennas[4], D. Ljubojević Pelić[5], G. Vuković[6]

[1]*University Megatrend Belgrade, Faculty of Biofarming, Department of Animal Science, Bačka Topola, Serbia*

[2]*University Business Academy in Novi Sad, Faculty of Economics and Engineering Management in Novi Sad, Department of Engineering Management in Biotechnology, Novi Sad, Serbia*

[3]*University of Novi Sad, Faculty of Agriculture, Novi Sad, Serbia*

[4]*Aristotle University of Thessaloniki, Faculty of Veterinary Medicine, Thessaloniki, Greece*

[5]*Scientific Veterinary Institute "Novi Sad", Novi Sad, Serbia*

[6]*Institute of Public Health of Belgrade, Belgrade, Serbia*

ABSTRACT: This research aimed to investigate the influence of different sheep genotype on the fatty acid profile of fatty tissue. Three months old lambs of Somborska cigaja (SC) breed and Čokanska cigaja (ČC) breed, the two different genotypes of autochthonous sheep, were used in the experiment. The fatty acid composition was determined in lambs adipose tissue samples. A total of sixty 24-week-old lambs were reared under standard productive conditions fed with compound concentrate feed and dried grass 5 weeks. Fatty acid profile analysis of lambs adipose tissue was performed on capillary gas chromatography with an FI detector. Total values of saturated fatty acids in the lambs adipose tissue ranged from 53.70% (SC) to 54.87% (ČC) with a statistically significant difference ($P < 0.05$). In our research C18:1 fatty acid with *cis*-Δ^9 configuration show significant differences ($P < 0.05$) between these two genotypes of lambs. Results of total PUFAs in our study indicate the significant influence of genotype ($P < 0.05$) adipose fatty acid profile of investigated two autochthonous sheep breeds. The recorded concentration of total PUFAs in SC amounted to 6.15%, while in ČC that amount was 4.69% with a significant difference, respectively. The obtained ratio of total n-6/n-3 fatty acids of 1.79 (SC), is highly lower compared to 21.33 obtained from ČC breed. According to obtain results, from the healthier aspect of consumer life, and decrease the incidence of possible inflammatory processes and disease, we would be recommended meat from Sobmorska cigaja as meat with better fatty acids profile.

Key words: Fatty acids, sheep, lambs, genotype, adipose tissue

Corresponding Author:
Nikola Puvača, University Business Academy in Novi Sad, Faculty of Economics and Engineering Management in Novi Sad, Department of Engineering Management in Biotechnology, Cvećarska 2, 21000 Novi Sad, Serbia.
E-mail address: nikola.puvaca@fimek.edu.rs; nikola.puvaca@gmail.com

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INTRODUCTION

The consumption of saturated fatty acids in human diets has been recommended to be reduced while the consumption of polyunsaturated fatty acids (PUFAs) to be increased (Simopoulos, 2002). Moreover, the regular consumption of n-6 PUFAs (C18:2n-6) should not be changed, while the consumption of n-3 fatty acids as an α-linoleic acid (C18:3n-3), eicosapentaenoic acid (C20:5n-3), and docosahexaenoic acid (C22:6n-3) should be increased roughly for twice of their existing level (Ebrahimi et al., 2018; Puvača et al., 2019). This is caused by the connection between long-chain n-3 nutritional fatty acids and a reduced thrombotic inclination to the blood and the risk of coronary heart disease (Edwards and O'Flaherty, 2008). Ruminant meat and meat products have made a significant contribution to providing total and saturated fatty acids (SFAs) in the diets of humans (Arsenos et al., 2006). The SFAs and PUFAs ratio of sheep meat is outlined as low, but the ratio C18:2n-6/C18:3n-3 and total n-6/n-3 ratio in lamb muscle is more favourable at 1.9 and 1.3, respectively. Nevertheless, a large issue presents the high proportion of SFAs in lamb meat which led to negative preference of customers for high concentration of saturated fats in meat (Cifuni et al., 1999). Having those facts in mind, methods for a fatty acid profile modification and its ratios balancing of lamb meat should be found. Although a great deal of work has been carried out with regard to nutritional methods to improve the PUFAs of lamb meat, there was little focus on the effect of the lamb genotype on fatty acid profiles (Arsenos et al., 2006). It has been confirmed that switching the lamb breed offers the ability to manipulate the composition of the meat fatty acids profile as well. Many scientists have noted that the levels of n-3 and n-6 PUFAs of Soay lambs breed in semimembranosus muscles were greater relative to the Suffolk breed of lambs (Laborde et al., 2001). This impact may be due to leaner lamb carcasses as ruminants were shown to have PUFAs preferentially deposited in phospholipids instead of in neutral storage lipids. Meat quality and the fatty acid structure of carcass fat in meat producing animals has been described among others as one of the most significant features of meat quality, besides the proximate, technological and sensory properties which play a very important role in the attractiveness of meat to customers (Schiavon et al., 2017). In the past, significant research has been carried out regarding the structure of fatty acids in lamb fat deposits (Enser et al., 1996). Designed research on the fatty acid structure of meat and carcass lipids in small ruminants has focused on several elements of feeding, breeding, and other management methods.

As already mentioned, there has been relatively little work done to assess the effect of lambs breed on the n-3 fatty acid composition of meat and adipose tissue (Hoffman et al., 2003). A lower ratio of n-6 to n-3 fatty acids is desirable for reducing the risk of many chronic diseases that have a high prevalence in developing countries (da Costa et al., 2013). For cardiovascular diseases, a ratio of 4/1 was associated with a 70% decrease in total mortality (Johnson, 2019). A reduced risk was connected to the reduced n-6/n-3 fatty acids ratio in females with breast cancer. A 2-3/1-ratio of rheumatoid arthritis suppressed inflammation, and a 5/1 ratio had a beneficial impact on asthma while a 10/1 ratio had negative effects. Studies of Simopoulos (2002) indicates that the optimal ratio of fatty acids may vary within the diseases. This is consistent with the fact that chronic diseases are multigenic and multifactorial. The possibility is that the therapeutic dose of n-3 fatty acids will depend on the degree of severity of disease resulting from the genetic predisposition. A lower ratio of n-6/n-3 fatty acids is more desirable in reducing the risk of many chronic diseases.

The study aimed to investigate the influence of sheep breed on the fatty acid profile of adipose tissue and to give possible suggestions for potential consumers of lamb meat in their daily nutrition.

MATERIALS AND METHODS

Animals, nutrition and experimental design

All experimental procedures with lambs have been approved by the competent Veterinary Authority according to the National legislation (Presidential Decree 56/2013 on harmonization of the Directive 2010/63/EU on the protection of animals used for scientific purposes).

A total of 60 lambs were introduced in the study at the age of 24-weeks. Lambs belonged to two autochthonous breeds of sheep, which were chosen as representatives of the most common breeds of sheep in Europe, north part of Serbia. Somborska Cigaja (SC) and Čokanska Cigaja (ČC) breed with an equal number of males and females were used, respectively. Lambs were reared under standard productive conditions fed with compound concentrate feed and dried grass 5 weeks. At the end of 29 weeks of age, six lambs of each breed as a statistically appropriate sample was

randomly selected and slaughtered, while their adipose tissue was stored for further analyses. Feedstuffs, the proximate and fatty acid composition of used compound feed in lamb nutrition is shown in Table 1.

Table 1. Feedstuffs, the proximate and fatty acid composition used in lambs nutrition

Feedstuffs, g/100g		Fatty acid, g/100g DM	
Dried grass	74.0	Lauric acid, C12:0	0.05
Wheat trop	10.5	Myristic acid, C14:0	0.05
Calcium soap of palm oil	4.4	Palmitic acid, C16:0	2.20
Soybean meal	5.6	Palmitoleic acid, C16:1	0.02
Molasses	2.5	Stearic acid, C18:0	0.30
Salt	0.5	Oleic acid, C18:1	1.33
Ammonium chloride	0.5	Linoleic acid, C18:2	1.14
Premix	2.0	α-linolenic acid, C18:3	0.70
Proximate composition, g/100g DM		Eicosapentaenoic acid, C20:5	0.002
DM, g/100g	89.3	Docosahexaenoic acid, C22:6	0.001
Organic matter	87.8		
Crude protein	13.4	Total	5.79
NDF	45.9		

Sample of adipose tissue collections

After 5 weeks on the adaptation diet, six lambs within each breed were transported to the slaughterhouse, according to EU regulations (Council Regulation, EEC No 1/2005). After 12 h of fasting, the animals were electrically stunned and slaughtered according to standard commercial procedures. Lambs carcasses subsequently were cooled at 4 °C for 24 h. Adipose tissue samples (full thickness, 50 × 50 mm) were dissected from the loin of the cold carcass, after which samples were vacuumed and stored at -18 °C, for the further fatty acids analysis.

Adipose tissue fatty acid profile analysis

Briefly, the fatty acids in the lambs loin were determined following the extraction of total lipids employing accelerated solvent extraction (ASE) on Dionex ASE 200. The mixture of n-hexane and isopropanol (60:40, v/v) was used for lipid extraction at 100 °C and a nitrogen pressure of 10.3 MPa in two static cycles lasting in total 10 minutes. Fatty acid methyl esters were separated on a polar cyanopropyl aril column HP-88 (column length 100 m, diameter 0.25 mm, film thickness 0.20 μm; Agilent, Santa Clara, USA), in a programmed temperature range, on capillary gas chromatography (Shimadzu 2010; Shimadzu, Kyoto, Japan), with an FID. The temperature of the injector was 250 °C and the detector temperature was 280 °C. The carrier gas was nitrogen, of flow rate 1.33 ml min and split ratio 1:50. The injected volume was 1 μl, and the duration of analysis 50 min 30 seconds. The identification of fatty acid methyl esters was based on their retention times compared with the standard, Supelco 37 Component FAME Mix (Supelco, Bellefonte, USA). The content of each fatty acid was expressed as the percentage of the total.

Statistical analyses

Before one-way ANOVA analysis, the data were checked for normal distribution and homogeneity of variances. The data expressed as percentages were transformed with the arcsine transformation. The mean values were compared by the Tukey HSD post hoc test. The results were presented as means ± SD, where the significance level of $P < 0.05$ was used. For statistical analysis statistical software Statistica 13 (TIBCO Software Inc., USA) was used.

RESULTS AND DISCUSSION

The obtained values of SFAs of both genotypes lambs adipose tissue are shown in Table 2. Total values of saturated fatty acids in the lambs tissue ranged from 53.70% (SC) to 54.87% (ČC) with a statistically significant difference ($P < 0.05$). The highest share of C16:0 (Palmitic acid) was recorded for both lambs breed (22.40 and 23.67%) with significant differences ($P < 0.05$). From the presented results it can be noticed that the higher share of SFAs is present in adipose tissue of Čokanska cigaja (ČC) breed of lambs. Significant differences between both lambs breed were not recorded ($P > 0.05$) regarding the only two SFAs C18:0 (Stearic acid) and C20:0 (Arachidic acid), while all other detected fatty acid showed significant differences between each other. Wachira et al. (2002) have shown similar results in their investigation which had the aim to investigate the influence of

nutrition with different fatty acids sources and breed. Their results show a significant influence of breed in C18:0 SFA which have ranged from 14.67% for Friesland breed to 15.07% for Soay breed, while nutrition didn't show any significant influence. The long-chain C20 fatty acids were present at very low levels in the subcutaneous adipose tissue of lambs in our research. This could be due to the small percentage of adipose tissue phospholipids as well as the small incorporation in ruminants of long-chain fatty acids into the triacylglycerol portion (Enser et al., 1996). As far as the fatty acid composition in lamb fat is concerned, our findings indicate that breed is very significant. The lambs breed affected most of the fatty acids profiles studied throughout the years in other experiments as well (Arsenos et al., 2006).

Table 2. Adipose tissue saturated fatty acids profile of lambs, %

Fatty acids	SC	ČC	*p*-value
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	
C8:0	4.68^{a} ± 0.632	3.48^{b} ± 0.705	0.004
C10:0	0.24^{b} ± 0.062	0.43^{a} ± 0.176	0.000
C14:0	3.61^{b} ± 1.393	4.94^{a} ± 1.380	0.001
C15:0	0.79^{b} ± 0.151	0.96^{a} ± 0.054	0.010
C16:0	22.40^{b} ± 0.801	23.67^{a} ± 0.404	0.001
C17:0	1.12^{a} ± 0.086	0.99^{b} ± 0.024	0.002
C18:0	20.60^{a} ± 1.780	19.87^{a} ± 1.53	0.332
C20:0	0.17^{a} ± 0.050	0.19^{a} ± 0.028	0.186
Total	53.70^{b} ± 0.610	54.87^{a} ± 0.530	0.009
Pooled SE	0.572	0.776	

Means in the same column with a common superscript letter are not significantly different ($P < 0.05$)
$\bar{x}$ - mean value of six replicates; SD – standard deviation; CV – coefficient of variation; SE – standard error

Besides our findings with two autochthonous breeds of lambs, the significant effect of breed on fatty acids composition in fat depots of suckled lambs from two dairy breeds was also reported (Cifuni et al., 1999; Hoffman et al., 2003). Also, it has been highlighted that sheep breed effects on fatty acid composition should be assessed at the same degree of maturity and fatness (da Costa et al., 2013; Hanuš et al., 2018; Laborde et al., 2001). Research of Arsenos et al. (2006) has shown that there are no significant differences between male and female lambs in the fatty acid composition of their carcass fat, except for C18:0 and C18:2 (Linoleic acid) fatty acids, what led to a conclusion that the effect of sex is generally very small and neglectable.

The results of monounsaturated fatty acids (MUFAs) obtain in our research have been shown in Table 3. Results of *cis* configuration (Δ^{9}, Δ^{13}, $\Delta^{5,8,11,14}$) of MUFAs with the two hydrogen atoms adjacent to the double bond stick out on the same side of the chain, show statistically significant ($P < 0.05$) differences in total MUFAs identified in our research. Significantly higher ($P < 0.05$) concentration of C16:1 (Palmitoleic acid) in SC lambs breed (1.68%) was recorded compared to ČC lambs breed (1.43%). The same significant tendency in C18:1 (Oleic acid) was maintained between investigated adipose tissue fatty acids in the experiment. On the other hand, a significant difference was not present ($P > 0.05$) considering the detected concentrations of C22:1 (Erucic acid) and C20:4 (Arachidonic acid) between investigated lambs (1.32 and 1.16%).

Table 3. Adipose tissue monounsaturated fatty acids profile of lambs, %

Fatty acid	SC	ČC	*p*-value
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	
C16:1n-7	1.68^{a} ± 0.294	1.43^{b} ± 0.060	0.037
C18:1n-9	33.10^{a} ± 2.472	29.46^{b} ± 1.4771	0.001
C22:1n-9 C20:4n-6	1.32^{a} ± 0.384	1.16^{a} ± 0.276	0.386
Total	36.15^{a} ± 0.788	32.05^{b} ± 0.600	0.004
Pooled SE	0.022	0.051	

Means in the same column with a common superscript letter are not significantly different ($P < 0.05$)
$\bar{x}$ - mean value of six replicates; SD – standard deviation; CV – coefficient of variation; SE – standard error

In research of Wachira et al. (2002) total muscle fatty acid concentration ranged from 3.66 to 2.90% with no significant effect of lambs breed, which is contrary to the results obtained from our research with adipose tissue of lambs. Further, in the dietary trial Wachira et al. (2002), have confirmed that lambs on the control diet, compared to lambs on the diet with flaxseed addition had higher concentrations of C18:1 fatty acid. Puvača et al. (2014) have shown that with the use of regression models is possible to predict deposition, e.g., concentrations of fatty acids in edible tissue of animals. In our research C18:1 fatty acid with *cis*-Δ^9 configuration show significant differences ($P < 0.05$) between two breeds, while in the research of Wachira et al. (2002), C18:1 fatty acid with *trans*-Δ^9 configuration (Elaidic acid) didn't show significant differences of this fatty acid in muscle tissue between different lamb breeds. Arsenos et al. (2006) showed no significant differences in fatty acids composition of different lambs breed fed with low and high concentrations of concentrate in the diet. Concentrations of C16:1 for low diet were in the range of 3.5 and 3.4% for Boutsko, Serres and Karagouniko indigenous Greeks breed, while the concentration of C18:1 in the high and low diet was higher and uniform for all mentioned breeds (41.9; 41.0 and 39.7%), respectively. Similarly to lambs, in other animal species such as fish, the same tendency was observed when fish fed with lower diets (Ljubojević et al., 2015). Gravador et al. (2018) have shown a significant difference in fatty acids profile in lambs of two different breeds after castration. In castrated lambs of Scottish Blackface and Texel × Scottish Blackface lambs breed significant increase in MUFAs and decrease in PUFAs was recorded.

Results given in Table 4 show a significant difference ($P < 0.05$) and the influence of sheep genotype on PUFAs profile of both investigated Somborska cigaja breed and Čokanska cigaja breed. The concentration of C18:2 (Linoleic acid) didn't show any significant differences ($P > 0.05$) between investigated breeds of lambs, with the equal presented amount of 3.83 and 3.84%, respectively. Opposite to C18:2 concentration in adipose tissue, the concentration of C18:3 PUFA (α-linolenic acid) showed significant ($P < 0.05$) influence of lambs breed with the recorded amount of 1.27% (SC) and 0.49% (ČC), respectively. The same tendency as for the C18:2 was observed for concentrations of C20:5 (Eicosapentaenoic acid) without the presence of significant differences ($P > 0.05$). The interesting results obtained in this study is a significant increase and difference ($P < 0.05$) in C22:5 (Docosapentaenoic acid). Adipose tissue of Somborska cigaja breed has recorded a significantly higher concentration of docosapentaenoic fatty acid (0.66%) compared to the concentration of the same fatty acid (0.17%) in adipose tissue of Čokanska cigaja breed ($P < 0.05$). Docosapentaenoic acid is an n-3 fatty acid that is structurally similar to eicosapentaenoic acid with the same number of double bonds, but two more carbon chain units (Yazdi, 2013). Docosapentaenoic acid designates any straight-chain C22:5 fatty acid, which is primarily used to designate two isomers, already known as n-6 and n-3. These designations describe the position of the double bond is 6 or 3 carbons closest to the carbon at the methyl end of the molecule and is based on the biologically important difference that n-6 and n-3 PUFAs are separate PUFAs classes, the n-6 fatty acids, and n-3 fatty acids, respectively (Edwards and O'Flaherty, 2008). Mammals, including humans, cannot interconvert these two classes and therefore must obtain dietary essential PUFAs from both classes to maintain normal health (Spector and Kim, 2015).

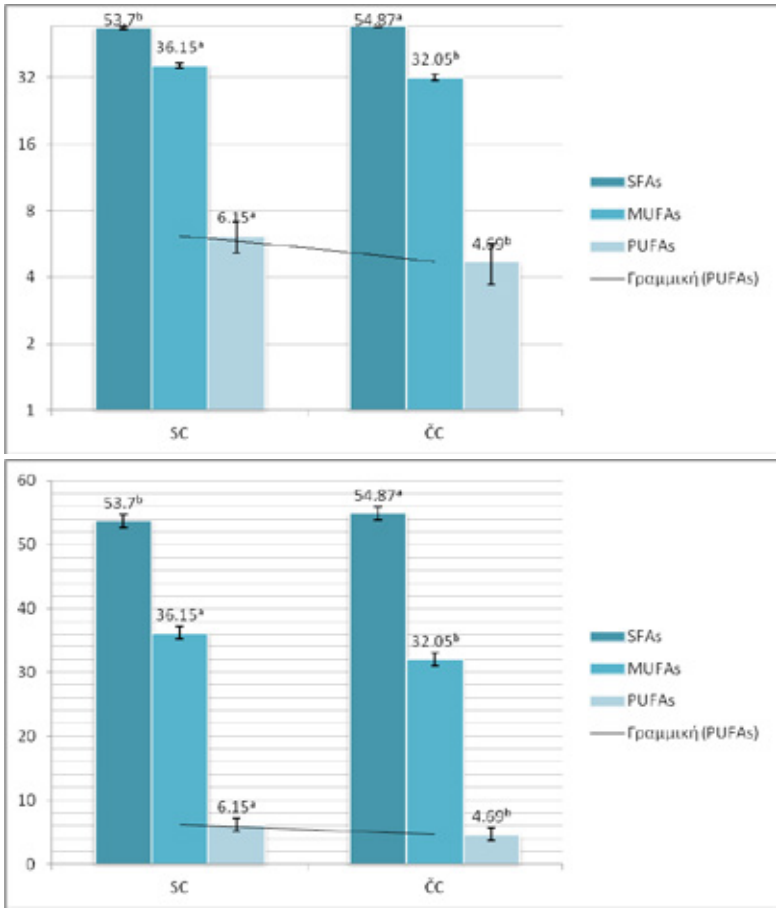
Table 4. Adipose tissue polyunsaturated fatty acids profile of lambs, %

Fatty acid	**SC**	**ČC**	*p*-value
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	
C18:2n-6	3.83^a ± 0.579	3.84^a ± 1.071	0.996
C18:3n-3	1.27^a ± 0.157	0.49^b ± 0.138	0.000
C20:5n-3	0.20^a ± 0.058	0.15^a ± 0.039	0.426
C22:5n-3	0.66^a ± 0.139	0.17^b ± 0.065	0.000
Total	6.15^a ± 0.150	4.69^b ± 0.328	0.040
Pooled SE	0.048	0.092	

Means in the same column with a common superscript letter are not significantly different ($P < 0.05$)
$\bar{x}$ - mean value of six replicates; SD – standard deviation; CV – coefficient of variation; SE – standard error

Results of total PUFAs in our study indicate the significant influence of genotype ($P < 0.05$) adipose fatty acid profile of investigated two autochthonous sheep breeds. The recorded concentration of total PUFAs in SC amounted to 6.15%, while in ČC that amount was 4.69% with a significant difference, respectively. Similar results were obtained in the research of Ebrahimi et al. (2018) with the different goats genotypes as well in the research of Obućinski et al. (2019) with different cows breeds. Schiavon et al. (2017) in their research with dietary rumen-protected conjugated linoleic acid didn't record any significant influence of PUFAs changes in adipose and liver tissues, but the influence of breed could not be investigated in these research having in mind that experiment was conducted on one lamb genotype. Contrary to our results for Somborska cigaja and Čokanska cigaja, the research of Wachira et al. (2002) showed a similar proportion of the longer-chain n-3 PUFAs in all three investigated breeds; Suffolk, Soay, and Friesland. These results could be explained as the genotype difference between all five investigated breeds. Besides the genotype influence on adipose fatty acid profiles of sheep, investigations were conducted in a way of investigation the sheep milk quality as well (Skoufos et al., 2018). Wachira et al. (2002) indicated the presence of significant interaction between lambs breed and feed for the total content of fatty acids in their research. The same research revealed that Friesland lambs breed were observed to have the greatest content of fatty acids in the adipose followed by Soay lambs breed, while the Suffolk lambs breed had the greatest content when fed regular daily diet.

From Figure 1 it can be easily seen the share of total SFAs, MUFAs, and PUFAs in adipose tissue of lambs obtain in our study. Highest share takes the SFAa, then MUFAs, and at the end PUFAs. The linear trendline shows that lambs of genotype Somborska cigaja have recorded significantly ($P < 0.05$) higher share of total PUFAs (6.15%), compared to total PUFAs obtained from adipose tissue of Čokanska cigaja genotype (4.69%).



Means in the same column with a common superscript letter are not significantly different ($P < 0.05$)

Figure 1. Share of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) in adipose tissue of lambs, %

The ratio of total n-6 to total n-3 is not very useful (Ljubojević et al., 2015). Based on the gain results in our study the ratio of total n-6/n-3 fatty acids in Somborska cigaja breed is 1.79, which is highly lower compared to a ratio of these fatty acids obtained from breed Čokanska cigaja which is 21.33 (Table 5). The ratio of linoleic acid to α-linolenic acid is of important value, since these compete for processing by delta 6 desaturase, and should be consumed in a balanced way. Again the ratio of arachidonic acid to eicosapentaenoic acid is important as too high a ratio could promote different inflammation processes in the body (Ljubojević et al., 2015; Puvača et al., 2014). Regarding the ratio of C18:2n-6 and C18:3n-3 fatty acids in our study, the tendency is similar to the previous ratio, but with the values of 3.07/7.84 (SC/ČC).

Table 5. The ratio of selected fatty acids from adipose tissue of lambs

Fatty acid/Ratio	**SC**	**ČC**
	$\bar{x}$	$\bar{x}$
Total n-6	3.83	3.84
Total n-3	2.13	0.18
n-6/n-3	1.79	21.33
C18:2n-6	3.83	3.84
C18:3n-3	1.27	0.49
C18:2/C18:3	3.07	7.84
C20:4n-6	1.32	1.16
C20:5n-3	0.20	0.15
C20:4/C20:5	6.60	7.73

The recorded ratio between PUFAs C20:4n-6 (Arachidonic acid) and C20:5n-3 (Eicosapentaenoic acid) in our study was 6.60/7.73 (SC/ČC). On the other hand, according to Simopoulos (2002), human beings evolved on a diet with a ratio of n-6 to n-3 essential fatty acids of approximately 1 whereas in Western diets the ratio is 15/1-16.7/1. Diets which are deficient in n-3 fatty acids, with increased amounts of n-6 fatty acids, e.g., a very high n-6/n-3 ratio, is found to promote the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases, whereas increased levels of n-3 PUFAs exert suppressive effects (Simopoulos, 2002).

CONCLUSIONS

Based on our findings, it can be concluded with certainty that the genotype of investigated sheep breeds Somborska cigaja and Čokanska cigaja have a significant influence on the fatty acid composition of adipose tissue. Obtained results indicate that Somborska cigaja compared to Čokanska cigaja have a higher share of MUFAs and PUFAs in adipose tissue, as well a much better ratio of total n-6/n-3; C18:2/C18:3 and C20:4/C20:5 fatty acids. According to obtain results, from the healthier aspect of consumer life, and decrease the incidence of possible inflammatory processes and disease, we would be recommended meat from Sobmorska cigaja as meat with better fatty acids profile. Nevertheless, the further investigation related to fatty acids profile of lambs influenced by breed, sex, and nutrition is more than necessary.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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Research article
Ερευνητικό άρθρο

Molecular characterization of Enterotoxigenic *Escherichia coli* isolates harboring genetic elements mediating multiple-drug resistance

M. Kohansal[1,2], A. Najafi[3], R. Vidal[4]

[1]*Department of Medical Genetics, School of Medicine, Fasa University of Medical Sciences, Fasa, Iran.*

[2]*Department of Biology, Payame Noor University, Tehran, Iran.*

[3] *Department of Medical Genetics, Tehran University of Medical Sciences, Tehran, Iran.*

[4]*Disciplinary Program of Microbiology, Institute of Biomedical Sciences, Faculty of Medicine, Universidad de Chile, Santiago, Chile*

ABSTRACT: Intensive antibiotics' use in the management of the disease in neonate calves, a major economic concern in bovine industry, is one of the contributors to high levels of antibiotic resistance of pathogenic bacteria. The objective of this study was to investigate the antibiotic resistance patterns and the frequency of integrons classes among Enterotoxigenic Escherichia coli (ETEC*)* strains isolated from neonatal calf diarrhea (NCD) in South of Iran. 412 recto-anal mucosal swabs from diarrheic calves were analyzed by biochemical fingerprinting and for virulence genes by polymerase chain reaction (PCR). The isolates were examined for their susceptibility to a panel of 8 antibacterial agents using the Kirby-Bauer disc diffusion method. Finally, the frequency of integron classes was detected in multi-drug resistant (MDR) strains by polymerase chain reaction restriction fragment length polymorphism (PCR- RFLP). 194 out of 412 (47.09%) diarrheic fecal samples harbored *E. coli* and 35 (18%) of them were identified as ETEC. The drug susceptibility test showed that all isolates were resistant to erythromycin, penicillin and trimethoprim/sulfamethoxazole and more than 80% were resistant to ampicillin and chloramphenicol. All isolates were MDR. 17 out of 35 (48.57%) isolates were identified possessed class 1 integron.
High prevalence of class 1 integron in ETEC isolates was mainly associated with multidrug resistance. Cefixime was the most effective antibiotic *in vitro*.

***Keywords*:** ETEC, Integron, MDR.

Corresponding Author:
M. Kohansal, Department of medical genetics, School of Medicine, Fasa University of Medical Sciences, Fasa, Iran.,Department of biology, Payame Noor University,Tehran, Iran.
E-mail address: k_kohansal@yahoo.com

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INTRODUCTION

Neonatal calf diarrhea (NCD) leads to high mortality and morbidity in young calves (de Verdier et al., 2012). It is a disease characterized by varying degrees of diarrhea and dehydration (Ahmed et al**.,** 2009).

Escherichia coli strains normally colonizes the mammalian intestines, however there are some clones with potential to produce diarrheic infections in human or animals (Souto et al. 2017). Six pathotype of diarrheagenic *E. coli*, have been described, namely: enterotoxigenic *E. coli (*ETEC*),* enteropathogenic *E. coli (*EPEC*),* enterohemorrhagic (Shiga toxin-producing*) E.coli (*EHEC/STEC*),* enteroaggregative *E. coli (*EAEC*),* and enteroinvasive *E. coli (*EIEC*) and diffusely adherent E.coli DAEC)* (Umpiérrez, et al., 2016).

Among these *E. coli* pathogroups, *ETEC* strains are the most common cause of NCD which is one of the most difficult diseases to treat responding to only a few antimicrobial drugs

(Abraham et al., 2014). Its pathogenicity mechanism involves the attachment to the bovine intestinal microvilli via specific fimbrial adhesion factors conferring to ETEC the ability to attach receptors on the enterocytes and production of heat-stable enterotoxins (*STa*). The *STa* proteins are responsible for fluid hyper-secretion and diarrhea that are common causes of NCD (Oliver et al. 2016). Major virulence factors associated to calf ETEC isolates are the heat-stable enterotoxin (*STa*),as well as F5 (K99) and F41 fimbrial adhesins. The K99 fimbriae is plasmid-encoded, whereas F41 is chromosome-encoded. The plasmid harboring the K99 fimbrial gene also contains the gene for *STa* (Shams et al, 2012).

Antimicrobial agents in food-producing animals have been used either for animal treatment or for animal growing and fattening (Harada and Asai, 2010). This practice leads to the inevitable selection of antimicrobial resistance among pathogenic and commensal bacteria in the intestinal tracts of these animals, which could serve as important reservoirs for colonization and infection in human beings. Antibiotics have long been considered as the first line of defense to prevent pathogenic *E. coli* infections (Umpiérrez et al., 2016). The use of antimicrobials in the treatment of ETEC diarrhea is problematic, due to the rapid emergence and dissemination of antibiotic-resistant strains. Recent studies have shown that the level of antibiotic resistance among ETEC has steadily increased (Moredo et al. 2015). The rapid emergence of antibiotic resistance among bacteria is mainly due to horizontal transmission of antibiotic resistance genes via different types of mobile genetic elements, such as transposons, plasmids and integrons with which multi-drug resistance in *Enterotoxigenic Escherichia coli* is associated (Abraham et al., 2014). Integrons are capable of integrating or mobilizing single or multiple gene cassettes encoding antibiotic resistance determinants (El-Sokkary and Abdelmegeed., 2015).

An integron is mainly composed of an integrase enzyme (*IntI)* that is responsible for gene cassette integration, a recombination site (*attI*) which is the target of the enzyme, and a promoter that is located upstream of the integration site (Díaz-Mejía et al., 2008).

Based on the sequences of integrase genes (Yu et al., 2004), there are at least eight classes of integrons (Nield et al., 2001) which could be distinguished by their respective integrase (*int*) genes (White et al. 2001). Class 1 and 2 of integrons are the most frequently detected in many bacterial species that carry different arrangements of gene determinants related to antibiotic resistance (El-Sokkary and Abdelmegeed., 2015). Significant association of class 1 integrons, most commonly found in clinical isolates of Gram-negative bacteria, with MDR has been shown (Yu et al., 2004). The distribution of integron in multi-drug resistant *Escherichia coli* strains has been previously studied in many different countries among them Germany (Friedrich et al., 2010), India (Mathai et al. 2004), USA (Diekema et al., 2004), Spain (Oteo et al., 2005) and Sudan (Ibrahim et al., 2013).

However, no studies on the prevalence of integron classes in MDR isolates of ETEC in diarrheic calves have been published in Iran to date. Consequently, the goal of this study was to investigate the genotypic screening of virulence genes in ETEC from diarrhoeic calves and to assess the occurrence of multi-drug resistant, dissemination of different classes of integrons in MDR isolates.

MATERIALS AND METHODS

Sampling and clinical signs of the studied population

A total of 412 fresh fecal samples were collected with rectal swabs from 412 untreated diarrheic neonatal calves, within the age of up to 30 days old. The calves were characterized as suffering from NCD by sudden onset of profuse yellow/white diarrhea, leading to rapid and severe dehydration. The fecal swabs were collected within a period of one year, from

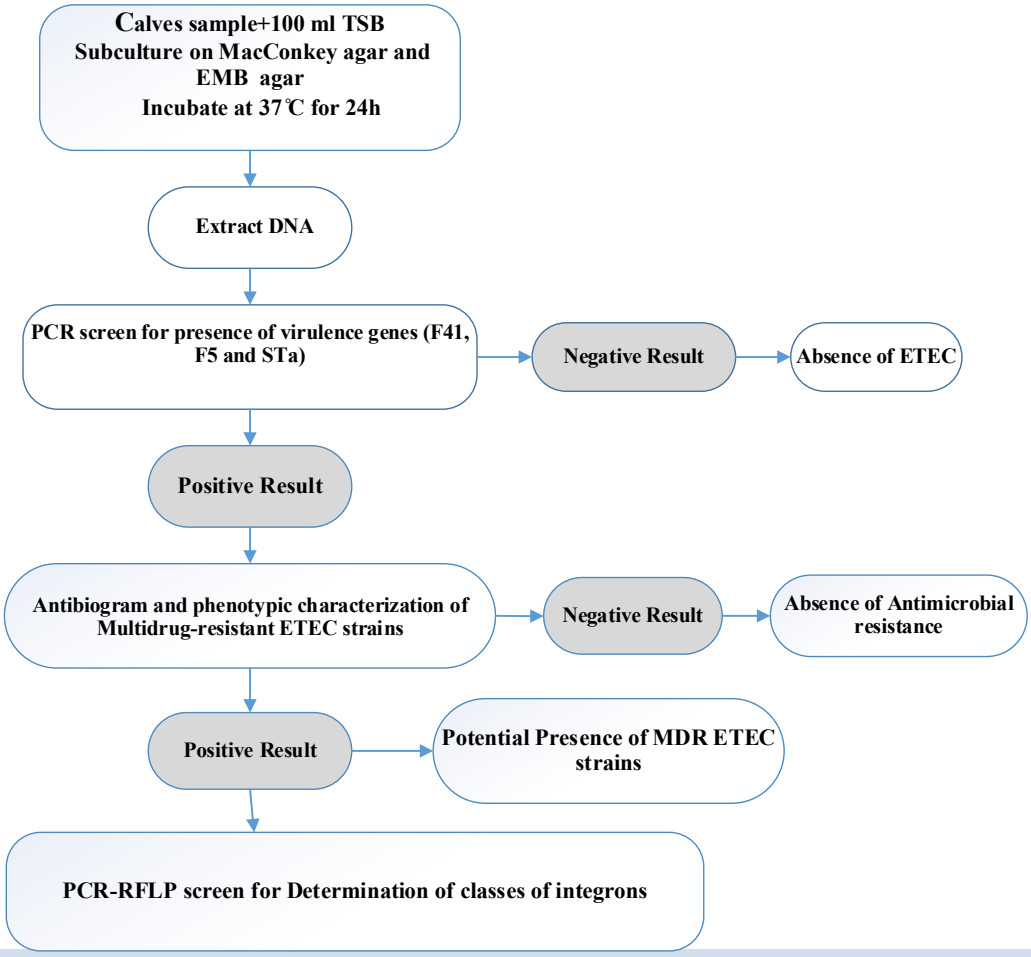

Figure 1. Preparation, screening and confirmation steps carried out for evaluating the multi drug resistant *ETEC* strains in samples

October 2016 to October 2017 from 25 farms belonging to six geographic areas of Fars province, Iran. The methods used in this study are summarized in Figure1.

Cultural and biochemical tests

A recto-anal mucosal swab sample from each diarrheic calf was collected and transferred to distinct tubes containing Tryptic Soy Broth (TSB) (Merk, Germany). The samples were transported within 6 hours to the main laboratory inside an ice box and subsequently incubated at 37°C for 18 hours. Then, the samples were subcultured on MacConkey agar and Eosin Methylene Blue (EMB) agar plates, at 37°C for 24–48 hours. The isolates were confirmed as *E. coli* using standard biochemical tests i.e. indole test (+ve) (Ehrlich's reagent) (Merk, Germany), oxidase test (−ve) (TMPD reagent) (Merk, Germany), urease (−ve) (Phenol red indicator) (Merk, Germany), Simon's citrate (−ve) (Bromothymol Blue, Reagent) and hydrogen sulfide (−ve) [(SO4 (NH4) Fe indicator] (Merk, Germany) (Atlas, 2010). The biochemically confirmed *E. coli* colonies were subjected to DNA analysis.

Bacterial DNA preparation for PCR

1ml of overnight mTSB culture from *E. coli* strains was employed to obtain template DNA for PCR. Bacterial cultures were pelleted at 3,000 rpm for 5 min (Hermle Z23o MA centrifuge) and then the SinaPure ™ DNA was used, as previously described by the provider (Sinaclon kit, Iran-Cat No.: EX6011). Extraction was performed according to the manufacturer's instructions. Briefly, bacterial pellet were lysed using lysis buffer then a precipitation solution was applied for 5 seconds, centrifuged, the supernatant recovered and precipitation solution was added. The sample mixture was then passed through a spin column, followed by two washes with wash buffer. The DNA was eluted in a volume of 200 µl of elution buffer, which was passed through the same column twice.

PRESENCE OF THE VIRULENCE GENES

Isolates were analyzed for 3 different genes encoding the virulence factors of *E. coli K99* (*F41*, *F5* and *STa*) by Multiplex PCR. Sizes of PCR products are shown in Table I. PCR was carried out in a 25-µl

reaction volume containing 10×PCR buffer (2.5 µl), MgCl2 (1.25 µl), dNTP (0.5 µl), primers (1 µl), DNA template (1 µl), Taq DNA polymerase (0.25 µl) with distilled water to reach 25 µL. The PCR protocol was as follows: 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec, and 1 cycle of 72°C for 7 min. DNA extracts from *E. coli* HB101 and RCCT 86 strains were used as negative and positive controls, respectively.

Antimicrobial susceptibility and multi drug resistance

Kirby–Bauer disk diffusion method was used to characterize the antibiotic sensitivity phenotypes of ETEC strains with standards and interpretive criteria of Clinical and Laboratory Standards Institute (CLSI) (Wayne 2012a). The following antibiotics (all obtained from Difco Laboratories, MI, U.S.A.) were used: ampicillin (AMP: 10 µg), tetracycline (TET: 30 µg), erythromycin (ERY: 25 µg), enrofloxacine (ENR: 10 µg), trimethoprim/sulfamethoxazole (SXT: 30µg), chloramphenicol (CHL: 30 µg), penicillin (PCN: 10 µ g), cefixime (CFM: 5µg).The inhibition zones were measured to the nearest millimeter and according to CLSI guidelines, where available (Wayne 2012b), the bacterial isolates were classified as: intermediate (I), resistant (R) or susceptible (S). Due to the small number of isolates with intermediate susceptibility, they were considered susceptible, for practical purposes. As multi-drug resistance phenotype was defined the simultaneous resistance to three or more categories of antibiotic agents (Magiorakos et al., 2012). *E. coli,* ATCC 25922 (beta-lactamase negative, sensitive to all these drugs), recommended by the Clinical and Laboratory Standards Institute (CLSI) was used for a quality control. Quality control result was within specified range as published in M100-S22 (Wayne 2012b).

Presence of integrons

To detect integron in confirmed ETEC isolates, a PCR protocol was performed. The base sequences and sizes of the amplified products for the specific oligonucleotide primers are shown in Table 1. The PCR reaction mixture included 1 µL of each primer, 0.25 µLTaq polymerase, 1.25 µL MgCl2, 1 µL dNTPs, 10×PCR buffer (2.5 µl), 1 µL of template DNA and nuclease free water to complete the reaction volume (25 µl). PCR amplification was performed in thermo-cycler (Eppendorf Mastercycler, Germany) under the following conditions: 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec, and 1 cycle of 72°C for 7 min. DNA extracts from *E. coli* 96K062 and ATCC 25922 strains were used as positive and negative controls, respectively.

Restriction Fragments Length Polymorphism (RFLP)

Determination of classes of integrons was done by RFLP analysis of integrase PCR products. The restriction reaction was performed using RsaI (Thermo Fisher) restriction enzyme (White et al., 2001). Based on manufacturer's recommendations, the PCR product (7 µl) was digested by the addition of 19 µl of double-distilled water, 3 µl of 10X buffer, and 1 µl of restriction enzyme, and incubated at 37°C for 1 hour. The digested PCR products were fractionated by gel electrophoresis (3 % agarose gel). Table 2 shows the size and number of generated fragments..

Table 1. Primers and PCR conditions used in this study.

Primers	Oligonucleotide sequence(5'-3')	Product size (bp)	References
F5(K99)	TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATTTC	314	(Shams et al, 2012)
F41	GCATCAGCGGCAGTATCT GTCCCTAGCTCAG TATTATCACCT	380	(Shams et al, 2012)
STa	GCTAATGTTGGCAATTTTTATTTCTGTA AGGATTACAACAAAGTTCACAGCAGTAA	190	(Shams et al, 2012)
Intg	TGCGGGTYAARGATBTKGATTT * CARCACATGCGTRTARAT	491	(White et al, 2001)

R=A or G , Y = C or T, B=C or G or T, K=G or T *

Table 2. RFLP classification of integrase PCR products.

product PCR	Enzyme	No. of fragments	Fragment size(s) (bp)	References
IntI1	RsaI	1	491	(White et al, 2001)
IntI2	RsaI	2	300,191	(White et al, 2001)
IntI3	RsaI	2	119,327	(White et al, 2001)

Data analysis

The frequencies of resistance to particular antimicrobials in integron-positive and -negative isolates were compared with Fisher's exact test. The level for statistical significance was <0.05.

Statistical calculations were made with GraphPad Prism5 for Windows Edition (GraphPad Software, SanDiego, CA, USA).

RESULTS

ETEC isolates

194 out of 412 isolates collected fecal samples from diarrheic calves (47%) were identified as *E. coli* . Multiplex PCR identified two fibrial genes (*F5* and *F41*) previously associated with colonization of the bovine intestinal epithelium and a *STa* toxin gene which lead to fluid secretion. 35 out of 194 *E. coli* isolates examined by PCR (18%) were tested positive for *f5, f41* and *sta* (*E. coli* K99 strains) (Table 3).

Table 3. Overview of the integron-positive *ETEC* strains.

Resistance phenotypes	MDR	Integron class	Virulence gene
CHL-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
CHL- ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
CHL-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-ERY- ENR -SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY- ENR -PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-CFM-ERY-SXT-PCN-TET	+	1	*F5*, *F41*, *STa*
ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-CFM-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY- ENR -PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY- ENR -PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY- ENR-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-ERY-PCN-SXT	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*
AMP-CHL-ERY-NFX-PCN-SXT-TET	+	1	*F5*, *F41*, *STa*

F5, F41, Sta: isolates carrying *F5, F41* and *STa* genes. MDR: multidrug-resistance isolates, S: Antibiotic susceptible isolates,R: Antibiotic resistant isolates.
AMP= ampicillin, TET = tetracycline, ERY= erythromycin, ENR = enrofeloxacin, SXT = trimethoprim/sulfamethoxazole, CHL =chloramphenicol, PCN = penicillin, CFM= cefixime.

Table 4. Association between integrons and antibiotics resistance in 35 multidrug-resistant isolates
Results are shown as the percentage resistant (% R), with the number of isolates (n) in parentheses

Antibiotic	Resistance integron PCR-RFLP positive		Resistance integron -PCR-RFLP negative		Total Resistance		Associatio with integron
	No.	%	No.	%	No.	%	
Ampicillin	13	37.1	18	51.4	31	88.5	0.312
cefixim	2	5.7	2	5.7	4	11.4	1.000
Chloramphenicol	13	37.1	16	45.7	29	82.8	0.401
trimethoprim/ sulfamethoxazole	17	48.5	18	51.5	35	100	1.000
enrofeloxacin	16	45.7	9	25.7	25	71.4	0.003*
erythromycin	17	48.5	18	51.5	35	100	1.000
Penicillin	17	48.5	18	51.5	35	100	1.000
tetracycline	16	45.7	10	28.5	26	74.2	0.017*

*, Correlation is significant at the 0.05 level.

Antibiotic resistance profiles

Table 4 shows the antimicrobial resistance profile among the 35 isolates of ETEC analyzed by the Kirby– Bauer disk diffusion method. Among the drugs under the study, all ETEC isolates were resistant to erythromycin, trimethoprim/sulfamethoxazole and penicillin. Susceptibility testing of ETEC revealed that 31 of 35 isolates (88.5%) were resistant to ampicillin, 29 (82.8%) to chloramphenicol, 26 (74.2%) to tetracycline, 25(71.4%) were resistant to enrofloxacine and 4 (11.4%) to cefixime .

PCR-RFLP analysis

17 out of 35 isolates (48.75%) were integron positive (Table 4) and RFLP analysis revealed that 100% of them contained class 1 integron (Figure2).

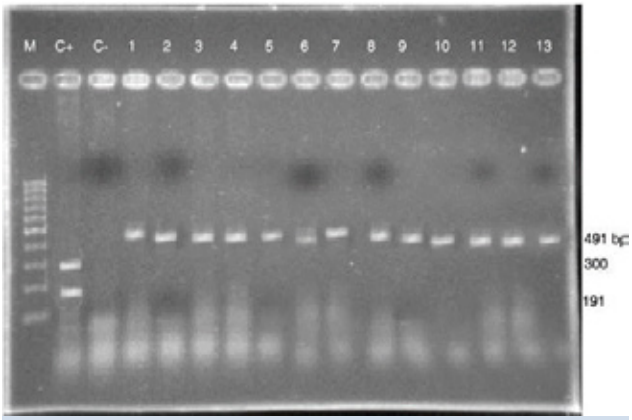

Figure 2. PCR-RFLP of integrase gene products. LanesC -,C+: negative and positive control (A 491 bp PCR product for *intI2* possessed a restriction site for *Ras1*, which was digested into 327 bp and 119 bp)
Lane1-13: PCR product of conserve of integrase; Lane2: RsaI treated of amplified products represent class1 integrons; M: molecular marker (100 base pair Ladder)

DISCUSSION

In pathogen detection systems, *E. coli* pathotypes, remarkably EHEC and ETEC isolates, had been identified by phenotypic methods for many years, including O- and H- serotyping and bacteriophage typing. These traditional techniques are generally time consuming and not always accurate. Therefore, they have been replaced by molecular-based techniques that have made it possible to more accurately assign the isolates to each pathotype based on their virulence-associated genes (Prapasawat et al. 2017). Accordingly, the K99, F41 fimbriae, and STa toxin genes were used in the present study for molecular identification of isolates. In the present study, among 194 *E. coli* strains from diarrheic calves, 35(18%) were positive for ETEC. The prevalence of ETEC coincided with the findings of another study in Tanzania (Lindblom et al. 1995); however, is higher than the 10.36% previously described by Younis *et al*., (2009) and the 5.8 % described by Yadegari *et al*. (2019). Conversely, it was lower than the 70%, 57.6% and 40% reported by El-Seedy *et al*.,(2016), Zhang et al. (2007) and Acha *et al*. (2004), respectively.

Most of the epidemiological studies in Iran have revealed that the prevalence of ETEC infection in diarrhoeic calves ranges between 5.3% and 28% (Shams et al., 2012; Pourtaghi-Shotorban et al., 2011; Shahrani et al., 2014). The differences of the prevalence rates of ETEC in diarrheic calves among different studies can be accounted to geographical locations and hygienic measures as well as management practice (El-Seedy et al., 2016).

In this study, high incidence of multi-drug resistant strains was detected among the ETEC isolates from diarrheic calves. Multidrug resistance (MDR) level among ETEC strains isolated from diarrhoeic calves varies in different countries. In previous studies conducted in Spain, 15% (Orden et al. 2002) and in another study from Australia, 87% of the isolates had MDR (Barigye et al., 2010). In addition, MDR was 10.4% in Egypt (Ahmed *et al*. 2009). Moreover, in our study MDR ETEC isolates were highly resistant to penicillin (100%) and high-level of resistance to penicillin among ETEC strains isolated from diarrhoeic calves has also been documented in Australia and Iran (Barigye et al., 2010; Shahrani et al., 2014).

In the present study, most of ETEC isolates were resistant to tetracycline (71.1%). High resistance of ETEC strains to this antibiotic has been reported in previous studies in Thailand (Prapasawat., 2017), Canada (Maynard *et al*., 2003, Boerlin *et al*., 2005), Iran (Shahrani *et al*., 2014)), where resistance to TET was 96%, 93%, 96%, and 100% respectively. A high percentage (88.5%) of ETEC isolates exhibited resistance to ampicillin nearly similar to the survey of Rusheeba *et al*., (86%) in India (Rusheeba *et al*., 2015), yet higher than other study in Sweden (31%) (de Verdier *et al*., 2012) whilst lower than that reported in Thailand (100%) by Prapasawat (2017).

These various levels of resistance to different classes of antibiotics among ETEC suggest a direct relationship between the percentages of resistance in different parts of the world and the prevalence of antibiotic consumption in each country (Kargar et al., 2014). Class 1 integrons are found embedded in transposons and conjugative plasmids, allowing their rapid dissemination via lateral gene transfer. As a con-

sequence, class 1 integrons have spread to nearly all species of Gram-negative pathogens.

Some reports indicate that the presence of class 1 integrons among intestinal bacteria such as *E.coli* is associated with MDR (Shahrani et al., 2014). Class 1 integrons that seems to be the most frequent of these genetic elements among commensals and pathogens isolated from livestock as well as among isolates cultured from clinical cases (Kohansal and Asad., 2018).

Previous studies have illustrated the wide distribution of class1 integrons in *E. coli* isolated from animals; 63% of isolates from chickens (Bass et al. 1999), 82% also from chickens (Keyes et al. 2000), 64% of swine diarrhea isolates (Kang et al. 2005), and 59% from calf diarrhea isolates (Du et al. 2005). In this study, the frequency of integrons was estimated as 48.75%. Only integron class 1 was detected. Other researches revealed the prevalence of class 1 integron in ETEC isolates as: 10.4% in Egypt (Ahmed et al., 2009), 60% in Canada (Maynard et al., 2003), 33% in Thailand (Prapasawat *et al*., 2017) and 68.6% in Australia (Abraham et al., 2014). These reports together with our findings emphasize the worldwide distribution of class 1 integrons among intestinal bacteria in food-producing animals. In these isolates class 1 integrons were associated with a variety of resistance gene cassettes, which encode resistance to different antibiotics.

We also detected a significant relationship between class 1 integrons and resistance to tetracycline and enrofloxacine (Table 4). Resistance to these antibiotics are probably attributable to embedded resistance gene cassettes within the integrons. Previous studies demonstrated that the presence of integrons is closely related to gene cassettes encoding_resistance to quinolones and β-lactam antibiotics (Prapasawat *et al*., 2017; Kargar *et al*., 2014).

CONCLUSION

Antibiotic resistance is common in pathogenic *E. coli* isolated from calves of Fars province experiencing problems with neonatal diarrhea. Our data demonstrate the presence of multiple drug resistance and class 1 integrons that can be easily dispersed among other bacteria, resulting in the rapid spread of antibiotic resistance genes. Clonal spread could not be the only reason for class 1 integron prevalence in different sources and the bigger player in this prevalence is probably the horizontal transfer with conjugative plasmids. Cefixime can serve as the drug of choice for treatment of multi resistant ETEC in calves with NCD. Therefore, it is advised to stop routine antimicrobial treatment, and test for antibiotic susceptibility as well as the sequential analysis of class 1 integrons in ETEC of fecal samples.

CONFLICTS OF INTEREST

The authors declare having no conflict of interests with this study.

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Research article
Ερευνητικό άρθρο

Heat killed *Dietzia maris* reduces lipopolysaccharide-induced inflammatory responses in murine adherent peritoneal cells

K. Nofouzi[1*], B. Ghahvechi-Dizaji[2], G.H. Zarrini[2], B. Baradaran[3], G.H. Hamidian[4], R. Safaralizadeh[2], G. McIntyre[5], P. Ripley[6], S. Mousavi[7]

[1]Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

[2]Department of Animal Biology, Faculty of Natural Science, University of Tabriz, Tabriz, Iran

[3] Immunology Research Center, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

[4]Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

[5] Centre for Infectious Diseases and International Health, Windeyer Institute of Medical Sciences, University College London, 46 Cleveland Street, London, United Kingdom

[6] BioEos Ltd., Mill House, Claygate, Marden, Near Tonbridge, Kent, UK

[7]Department of Food Hygiene and Aquatic Animals, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

ABSTRACT:

Dietzia maris (*D. maris*) is a gram-positive, aerobic, mycolic acid-containing actinomycete without mycelium. Actinomycetes such as *Tsukamurella inchonensis* reduce lipopolysaccharide- induced inflammatory responses in activated murine peritoneal macrophages. Here, the effects of *D. maris* on LPS-induced inflammatory responses were examined in mouse adherent peritoneal cells. *D. maris* was grown, harvested, and washed. Suspensions were standardized by wet weight, re-suspended in borate-buffered saline, and autoclaved. For *in vivo* study, each mouse was orally administered by bacterial suspension (5×10^7, 1×10^8 and 2×10^8 CFU/Mouse) consecutively for seven days. Control animals received the same amount of phosphate-buffered saline (PBS). Adherent peritoneal cells were harvested for *in vitro* experiments. Cells were lavaged and plated in RPMI 1640 medium, stimulated with LPS (100 ng/ml), and incubated for 2 h. Afterward, non-adherent cells were removed followed by adding freshly prepared medium. Supernatants (50 µl) were collected, centrifuged, mixed with Griess reagent, and the absorbance was measured at 560 nm. *D .maris* inhibited LPS-stimulated nitric oxide (NO) production in murine macrophages at concentrations of 5×10^7, 1×10^8 and 2×10^8 CFU/Mouse. Also, *D. maris* decreased LPS-induced production of pro-inflammatory cytokines of interleukin (IL)-6 at all doses. By contrast, tumor necrosis factor (TNF)-α was not effected by *D. maris* treatment of mice. Our results indicate that *D. maris* is a potent inhibitor of LPS-induced NO production. *D. maris* may be useful as a novel agent for the chemoprevention of inflammatory disease.

***Keywords*:** Adherent peritoneal cells; Anti-inflammation; *Dietzia maris*; IL-6; Lipopolysaccharide

Corresponding Author:
K. Nofouzi, Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
E-mail address: nofouzi@tabrizu.ac.ir

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INTRODUCTION

Inflammation is a process mediated by the host's microcirculatory system to protect against a wide range of injuries (Xu et al., 2012). Inflammation is the result of complex interactions between immune and inflammatory cells, their mediators, as well as regulators, and is part of the innate immune response. There are several differences between inflammation in health and disease. One of the most important differences is that in a disease state, inflammatory cells and pathways continue to perpetuate inflammatory cycles regardless of the tissue structure; on the contrary, during inflammation in a state of health, the response is highly self-limited to a specific stimulus working with a "pro-gramme" to minimize tissue damage (Gallo et al., 2017). Nitric oxide (NO) is a short-lived biomolecule that mediates many biological functions, including host defense, vasoregulation, platelet aggregation, and neurotransmission (Azadmehr et al., 2009). In addition to its physiological functions, NO is also implicated in the pathology of many inflammatory diseases, including arthritis, myocarditis, colitis, and nephritis, and a large number of pathological conditions such as amyotrophic lateral sclerosis (ALS), cancer, diabetes, and neurodegenerative disease (Davis et al., 2001). Therefore, inhibition of NO production has become a therapeutic target of treatment for inflammatory disease.

Current evidence indicates that macrophages play an essential role in the pathogenesis of the inflammatory responses by their ability to produce cytokines such as IL-6 and TNF-α. TNF-α is a monocyte-macrophage derived cytokine that acts as an essential mediator in the defense mechanism of the host in response to bacterial colonization or invasion and causes immunopathologic disorders when secreted in excess (Delgado et al., 1999). The toxicity caused by Gram-negative bacteria has been ascribed to LPS (Lipopolysaccharides), an outer membrane component of bacteria. LPS represents one of the most potent inducers of TNF-α and at high concentrations causes tissue injury, fever, disseminated vascular coagulation, and septic shock, often resulting in death (Tracey and Cerami, 1993).

Interleukin-6 (IL-6) is a cytokine with a broad range of biological activities (Remick et al., 2005). IL-6 functions as an essential and sensitive indicator of inflammation within the body. IL-6 is a key player in chronic inflammation, and IL-6 levels are elevated in inflammatory diseases in humans. Expression of IL-6 is increased at the site of inflammation and blockade of IL-6, and IL-6 signaling is effective in the prevention and treatment in models of inflammatory diseases.

Dietzia maris (*D. maris*) is a Gram- positive, aerobic, mycolic acid-containing actinomycete without aerial mycelium (Pidoux et al., 2001). In a previous report, it was found that *Tsukamurella inchonensis* (*T. inchonensis*), an aerobic species of Actinomycetales, could reduce inflammatory responses in murine peritoneal macrophages (Nofouzi et al. 2017). Therefore, our study aimed to assess the effects of another actinomycete, *D. maris* on mice adherent peritoneal cells.

MATERIALS AND METHODS

Animals

This experimental study was approved (FVM. REC. 1395.59) by the Ethics Committee of the School of Veterinary Medicine, Tabriz University. Male albino laboratory mice, *Mus musculus* Linn. (20-22 g; 7-8 weeks old) were obtained from the Pasteur Institute (Tehran, Iran). Mice (four groups, five mice per group) were randomized and housed in polyester cages. Because study did not aim to examine sex-associated differences, only male mice were used here to minimize the stress associated with intracage fighting. The animals were maintained under standard laboratory conditions at 25 ± 2 ºC and a photoperiod of L/D 12: 12 h, and received a standard mouse chow and water *ad libitum*. Animal care and handling throughout the experimental procedures was in accordance to Iran National Committee for Ethics in Biomedical Research legislations and Presidential Decree 56/2013, in compliance with the Directive 2010/63/EU on the protection of animal used for scientific purposes.

Strain preparation

D. maris was grown in Sauton's medium, harvested by centrifugation, and washed in borate-buffered saline (pH 8.0). Suspensions were standardized by wet weight, re-suspended in borate-buffered saline, and autoclaved at 121 ºC for sterilization.

In vivo exposure to *D. maris*

For the *in vivo* study, after successful completion of a two-week quarantine period, each mouse received orally 200 µl of administered bacterial suspension (5×10^7, 1×10^8 and 2×10^8 cells/mouse) by gavage consecutively for 7 days (Fig. 1). An expert experimenter in oral gavage of mice administered all treatments

using a straight, 20-gauge stainless steel bulb tipped gavage needle once daily between 09:00 and 10:00 am. The needle was never forced down the esophagus and all animals were monitored for at least 15 min after oral gavage. The control animals received the same amount of phosphate-buffered saline (PBS). After 7 days of treatment, the mice were anesthetized with isoflurane (1.5-2.5%) to harvest peritoneal macrophages.

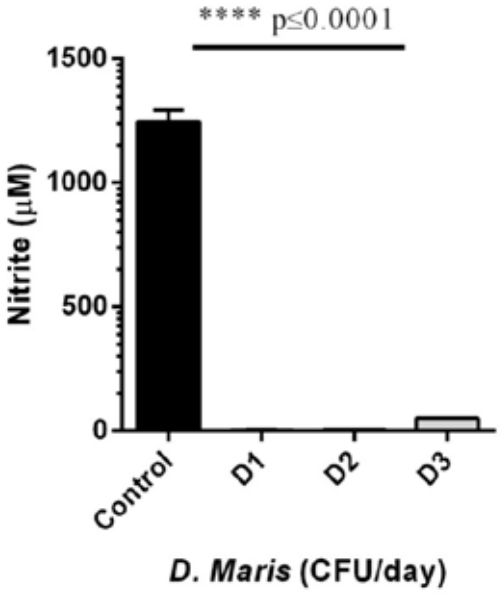

Figure 1. Inhibition of LPS-induced NO production by ***D. maris*** in murine macrophages. Murine macrophages were treated with LPS (100 ng/ml) in PBS (control), 5×10^7 of ***D. maris*** (D1), 1×10^8 of ***D. maris*** (D2) and 2×10^8 of ***D. maris*** (D3) for two h. The cell culture supernatant was collected and used to determine NO levels. Data are expressed as the mean ± SEM of triplicate experiments. Statistical analysis was performed using one-way ANOVA andTurkey's multiple-comparison post hoc tests.

Adherent peritoneal cells isolation, cell culture, and determination of NO production

For *in vitro* experiments, adherent peritoneal cells were harvested immediately by lavaging with ice-cold sterile PBS. Cells were lavaged twice and plated in RPMI 1640 medium (Sigma Chemical Co.) containing 100 U/mL penicillin/100 µg/mL streptomycin (Sigma Chemical Co.), 10% Fetal Bovine Serum (GIBCO), stimulated with LPS (100 ng/ml), and incubated in a 5% CO_2 humidified incubator at 37 ˚C for 2 h. Afterward, non-adherent cells were removed by gently washing with PBS, followed by adding a freshly prepared medium (Nofouzi et al., 2017). Supernatants (50 µl) were collected, centrifuged at 600 × g for 5 min, and mixed with 100 µl of Griess reagent [1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamide dihydrocholide in distilled water]. Absorbance was measured at 530 nm using a Wallac 1420 ARVO Sx (Perkin Elmer, Inc., Waltham, MA).

Cytokine protein array

Peritoneal cells were harvested by lavage of the corresponding site with ice-cold PBS. The lavage was performed in a single round with 60 ml of PBS. The cell extract was concentrated by centrifugation (600×g, 10 min, 4 °C), and the pellet was resuspended and incubated with ACK lysis buffer at room temperature for 3 min. The resulting cell extract was then pelleted by centrifugation, and resuspended in RPMI 1640 medium (Sigma Chemical Co.) containing 100 U/mL penicillin/100 µg/mL streptomycin (Sigma Chemical Co.), 10% Fetal Bovine Serum (GIBCO), stimulated with LPS (100 ng/ml), and incubated in a 5% CO_2 humidified incubator at 37 ˚C for 2 h. The cells were then plated at a density of 1×10^6 live cells (trypan blue exclusion) per well in 12-well, flat-bottom polystyrene plates, and cultured at 37 ˚C and 5% CO2. Non-adherent cells were discarded twice: the first time at 30 min after incubation with a medium containing 2% FBS, and the second time after overnight incubation with a medium containing 10% FBS. Subsequently, the adhered cells were examined in the medium containing 10% FBS. A 1-h resting period was allowed between the second medium change and the stimulation with LPS to generate supernatants. Macrophages (5×10^5 cells) on 24 well plates were preincubated for 24 h and then treated with LPS (100 ng/ml) for 2 h. IL-6 and TNF-α in the culture medium were screened using commercial ELISA kits (Ebioscience, Austria).

Statistical analysis

Data are shown as means ± SD (standard deviation) from five mice. Comparisons between groups were made using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison post hoc test (SPSS software for windows release 21.0; SPSS Inc., Chicago, USA). Differences were considered statistically significant at $p < 0.05$. In order to monitor the risks for decision error in statistical inference from a minimum of five mice per group, the statistical power of the study sample size was analyzed by G Power software (Version 3.1., Heirich Heine University, Düsseldorf, Germany) to detect an efficient group size at a 80% power (1-β err prob = 0.8), 95% confidence (α = 0.05), and an anticipated effect size

d>1. The actual power of statistical analysis of this study was 0.82.

RESULTS

Effects of *D. maris* on LPS-induced NO production in mouse macrophages

As shown in Figure 2, *D. maris* significantly inhibited LPS-induced NO production ($p < 0.001$) in all groups. *D. maris* was not observed to have any effect on the viability of peritoneal macrophages using the trypan blue exclusion assay (cell viability was greater than 90% for all groups).

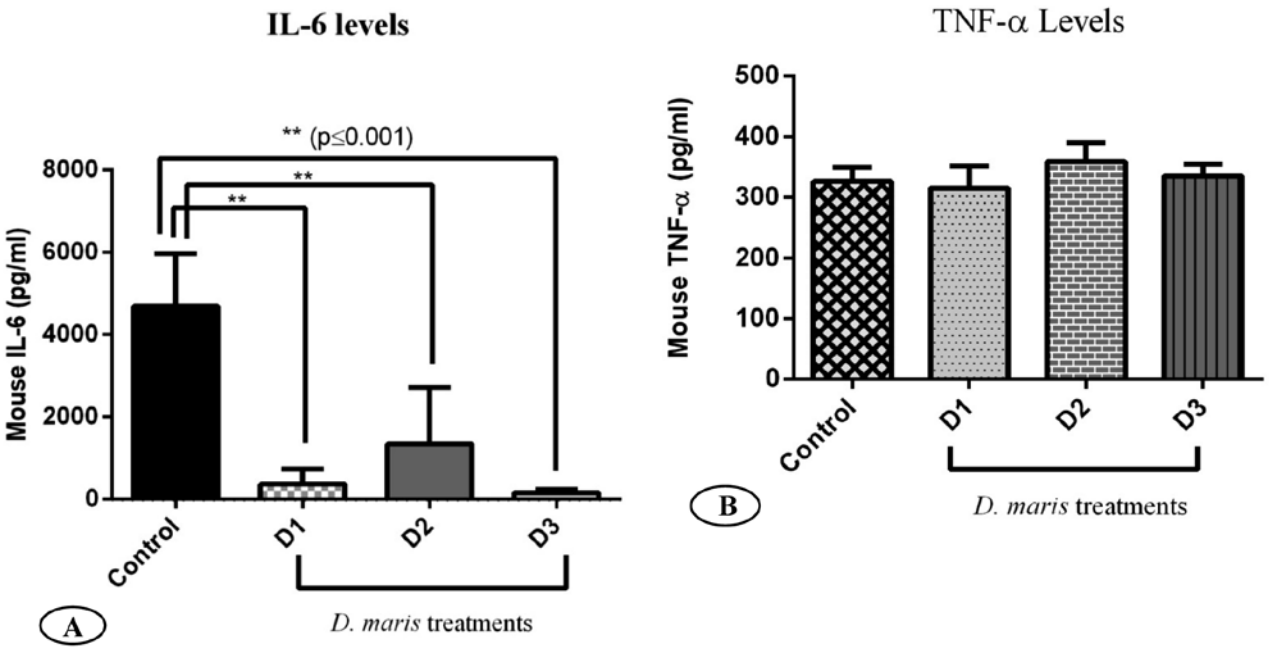

Figure 2. Levels of IL-6 and TNF-α produced by peritoneal macrophages from mice orally treated with *D. maris*. A) IL-6 level significantly different from PBS (control) group. B) *D. maris*did not alter TNF-α level. Murine macrophages were treated with LPS (100 ng/ml) in PBS (control), 5×10^7 of *D. maris* (D1), 1×10^8 of *D. maris* (D2) and 2×10^8 of *D. maris* (D3) for 2 h. Vertical bars represent the mean of measurements of five mice per group, with error bars denoting the standard error of mean.Statistical analysis was performed using one-way ANOVA and Turkey's multiple-comparison post hoc tests.

Effects of *D. maris* on IL-6 production

Since macrophage-derived cytokines are critical in a variety of inflammatory processes, the impact of *D. maris* was evaluated on the output of two cytokines by peritoneal macrophages using an ELISA technique (Fig. 3 and 4). The creation of IL-6 was suppressed by *D. maris* (by 5×10^7, 1×10^8 and 2×10^8 cells/mouse *vs*. LPS-treatment) as shown in Figure 3.

Effects of *D. maris* on TNF-α production

To investigate the suppression of TNF-α production by *D. maris*, a cell-based assay was performed using murine macrophages, which are known to respond to LPS and produce inflammatory cytokines such as TNF-α. *D. maris* could not suppress LPS-induced TNF-α production in murine peritoneal macrophages as measured by ELISA (Fig. 4).

DISCUSSION

D. maris has been isolated from soil, and the skin and intestinal tract of carp (Koerner et al., 2009). Several species of aerobic Actinomycetales, including *Gordonia bronchialis (G. bronchialis*), *Rhodococcus coprophilus (R. coprophilous*), and *T. inchonensis*, are capable of subtly different adjuvant or immunomodulatory activities when injected as suspensions of killed bacilli (Tarres et al., 2012). In animal models, it was found that preparation of this species was especially fortunate in preventing the inflammation of the intima of arteries damaged with a balloon catheter (Stanford and Stanford, 2012), and for the prevention and medical care of spontaneous type 2 diabetes mellitus (Tarres et al., 2012). Previously, we demonstrated the stimulatory effects of *T. inchonensis* on immune functions and enhancing immune barri-

er function in the intestines of mice (Nofouzi et al., 2016). A previous study reported even anti–inflammatory and immunomodulatory impacts of this species (Nofouzi et al., 2017). In the present study, therefore, the effects of *D. maris* at doses of 5×10^7 cell, 1×10^8 cell, and 2×10^8 cell *D. maris*/mouse were examined on NO production and some cytokines in mice. As our previous study, five mice were used per group because our ethics committee did not approve the use of extra mice in the present study. However, our work is confirmed by previous studies that used three to five mice per group (Azadmehr et al., 2009; Kalischuk et al., 2009; Gasting et al., 2010; Nofouzi et al., 2016). The main finding of our study is that prior exposure to certain actinomycete bacteria, alters IL-6, and nitric oxide production by peritoneal cells or not?

The spectra of products observed in our study with peritoneal cells suggest that macrophages are a crucial target for *D. maris* immunomodulatory activity. The bacteria capable of secreting IL-6 and NO could exert a wide scope of effects including stimulatory or inhibitory impacts. The potential to alter macrophage task is significant for several reasons. Macrophages constitute the other significant cell population of the immune system with phagocytosis being a primary task. They originate from bone marrow, and, after migration and maturation, settle in the tissue as mature macrophages (Tejada-Simon et al., 1999). These can be triggered by a variety of stimulants, and their main functions involve phagocytosis of foreign particles, antigen presentation, and making cytokines (IL-6, TNF-α, IL-1, IL-12) or reactive oxygen mediators, such as NO, which recruit other inflammatory cells. Thus, macrophages can participate in both humoral and cell-mediated immune responses.

Lipopolysaccharide has been exposed as the main initiating agent in Gram-negative sepsis due to the activation of inflammatory cells, particularly the mononuclear phagocyte, which releases a series of additional mediators including TNF-α, IL-1, and IL-6 (Revelli et al., 1999). Additionally, LPS stimulates iNOS gene expression and NO making. An earlier work demonstrated that *Helicobacter pylori* attenuated LPS-induced nitric oxide production by murine macrophages (Lu et al., 2011). The repressive effect was not due to cytotoxicity. As NO is an evaluative mediator in inflammation, increased NO production has been implicated in inflammatory and autoimmune diseases Although, NO has very high reactivity and a variety of physiological activities involved in the regulation of blood vessel dilation and immune response, and functions as a neurotransmitter (Azadmehr et al., 2009). If the inflammation becomes extreme or chronic, however, healthy host cells may also be damaged and killed by NO, resulting in inflammatory pathologies (Xu et al., 2012). The results of our study indicated that heat-killed *D. maris* enhanced the suppression of NO production in the two treatment groups. Similar results were observed with lactobacilli (Tejada-Simon et al., 1999) and *T. inchonensis* (Nofouzi et al., 2017).

In addition to the association of IL-6 in the generation of B and T cell-mediated responses, it should be recalled that this cytokine is primarily involved in the elicitation of the acute phase response to injury (Snick 1990). The IL-6 output by LPS-stimulated macrophages at all doses was significantly inhibited by *D. maris*. The same may be true for benzimidazole and *T. inchonensis* induced the suppression of IL-6 output (Revelli et al., 1999; Nofouzi et al, 2017). In contrast, Wang et al. demonstrated that *Ganoderma lucidum* enhanced LPS-induced IL-6 in mice macrophages (Wang et al., 1997).

There is plenty of confirmation that TNF-α is an essential mediator of shock and organ failure complicating Gram-negative sepsis (Delgado et al., 1999). TNF is combined and secreted very quickly by macrophages in response to endotoxin and may be detected in the circulation within one hour in experimental animals given endotoxin. Following synthesis, TNF is widely delivered in tissues and is then rapidly degraded (Morrison, 1987). TNF-α modulates the immune response by triggering the production of several other regulatory cytokines (Shin et al., 2006). The effect of *D. maris* on TNF-α release was inconclusive as clarifications should be made as to why NO is inhibited whereas TNF appears intact. Maybe the kinetics are different for testing both compound productions, for which more time point evaluations are necessary. Reis et al. (2008) found that proteasome inhibitors are potent inhibitors of NO production by LPS-stimulated macrophages, with TNF-α being inhibited to a lesser extent. *D. maris* likely causes depletions in inflammatory mediators by blocking proteasomal activity, rather than antagonizing one cytokine at a time. Our data are supported by those of Ahmadi-Renani and McCruden (1997) who found no change in TNF-α release with alpha dihydrotestestrone. Morrison and Silverstein (2000) reported that treatment of mice with killed *S. aureus* and *E. coli* did not affect TNF- α levels. Ishida-Fujii et al. (2007) described that peritoneal macrophages from rats fed

with *Lactobacillus casei* and intraperitoneally infected with *E.coli* could improve phagocytic activity and produced higher quantities of TNF- α. Neumann et al. (2009) showed that *L. delbrueckii* UFV-H2b20 stimulated the production of TNF-α in mouse *in vitro* . In our previous work on *T. inchonensis*, however, the bacteria decreased lipopolysaccharide-induced production of TNF- α level (Nofouzi et al., 2017).

CONCLUSION

According to our observations, *D. maris* acts as a NO inhibitor in adherent peritoneal cells. Also, this species attenuated LPS-induced IL-6 production. *D. maris*, therefore, demonstrates potential as a potent inhibitor of responses induced by inflammatory stimuli. Additional works will, however, be required before full potential therapeutic benefits of *D. maris* can be wholly understood and evaluated.

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CONFLICT OF INTEREST STATEMENT

None declared by the authors.

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Research article
Ερευνητικό άρθρο

A novel β-lactam-aminoglycoside combination in veterinary medicine: The co-use of ceftiofur and gentamicin to combat resistant *Escherichia coli*

M. Cengiz[a], P. Sahinturk[b], G. Hepbostanci[b], H. Akalin[c], S. Sonal[a]

[a]*Laboratory of Molecular Pharmacology, Department of Pharmacology and Toxicology,*

Faculty of Veterinary Medicine, Uludag University, Nilufer, Bursa, Turkey

[b]*Institute of Health Science, Uludag University, Nilufer, Bursa, Turkey*

[c]*Department of Infectious Diseases and Clinical Microbiology, Faculty of Medicine,*

Uludag University, Nilufer, Bursa, Turkey

ABSTRACT: The focus of this study was to evaluate the efficacy of ceftiofur+gentamicin combination to increase the success of antimicrobial inhibition against resistant *Escherichia coli (E.coli)* strains isolated from animals. Interaction between drugs was determined using checkerboard method and the fractional inhibitory concentration index was interpreted as synergism, antagonism and indifference. The combination was defined as bactericidal or bacteriostatic based on the minimum bactericidal test results. Mutant prevention concentration test was used to evaluate the resistance tendency suppression potential of the combination. The synergistic effect was detected for all *E. coli* strains by the checkerboard method; even the strains that were resistant to the individual compounds in the combination. Based on the results of minimum bactericidal concentration test, the combination exhibited bactericidal effect against all *E. coli* strains. In addition, the individual mutant prevention concentrations of ceftiofur and gentamicin decreased up to 125-fold by using the combination for the inhibition of resistant *E. coli* strains. The results indicated that killing potential of co-use of the compounds is much stronger than their individual use. The combination achieved to decrease the mutant prevention concentrations and this can reduce the risk of emergence of single mutations during treatment done with suggested doses.

***Keywords*:** Ceftiofur, gentamicin, resistant *Escherichia coli*, antimicrobial combination

Corresponding Authors:
Murat Cengiz, Laboratory of Molecular Pharmacology,
Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine,
Uludag University, 16059, Nilufer, Bursa, Turkey
E-mail address: cengizm@uludag.edu.tr

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INTRODUCTION

As a member of the intestinal microbiota, *Escherichia coli* (*E. coli*) is responsible for many intestinal or extraintestinal opportunistic infections in humans and animals (Hopkins et al., 2005; Ingerson Mahar and Reid, 2011). Due to the lack of effective therapeutic options in the veterinary field, the treatment of infections caused by resistant *E. coli* is a major concern. Resistance gene-based dose optimization is one pragmatic approach to combat resistant bacteria. However, resistance gene variability is a limiting factor affecting the success of treatment (Cengiz et al., 2013). Antimicrobial combinations should be considered for the treatment of infections caused by various resistance gene-containing strains. Combination therapy involves the co-use of two or more compounds with synergistic interactions and increases the treatment potential of the infection provided that the combination does not lead to increased toxicity (Sun et al., 2016; Tamma et al., 2012). The synergistic activity of β-lactam plus aminoglycoside has been widely investigated against many infectious agents (Tamma et al., 2012). β-lactams inhibit peptidoglycan biosynthesis, disrupting bacterial cell wall synthesis and increasing the influx of aminoglycoside to block ribosomal protein synthesis (Kohanski et al., 2010). Ceftiofur (CEF) is a semi-synthetic member of third generation cephalosporins and is resistant to several β-lactamases (Meegan, 2013). As an aminoglycoside, gentamicin (GEN) inhibits protein synthesis by binding to the bacterial 30S ribosomal subunit. Based on the results of recent studies, β-lactam+aminoglycoside remains an effective therapeutic option for the treatment of critical clinical cases (Theelen, 2019). The aim of this study was to determine the efficacy and resistance prevention potential of CEF+GEN as a novel β-lactam+aminoglycoside combination. Therefore, the interaction between CEF and GEN was determined, and the bactericidal characteristics of the combination and its resistance tendency toward *E. coli* strains under the combination pressure were evaluated.

MATERIAL AND METHODS

For isolation of *E. coli*, samples collected from cattle were directly spread onto Eosine Methylen Blue Agar-Levine and MacConkey Agar, and incubated under aerobic conditions. Candidate *E. coli* colonies were identified by API 20 E, and results were evaluated by API-Web system. Broth microdilution testing was performed to determine the minimum inhibitory concentrations (MICs) of the antimicrobials according to the guidelines of Clinical Laboratory Standards Institute (CLSI, 2016). Antimicrobials were obtained as an analytical standard powder (Fluka). MICs were defined as the minimum concentration of antibiotic that inhibited growth of the organism. PCR and qRT-PCR were used to characterize the presence and expression levels of molecular mechanisms of the resistance as described previously (Sahinturk et al., 2016). Briefly, *gyrA*, *parC* and *oqxB* genes were PCR amplified using specific primers and PCR products of *gyrA* and *parC* were sequenced. qRT-PCR was used to determine expression level of *marA*, *acrB*, *soxS* and *ompF* genes. *E. coli* AG100 was used as a control strain. Overexpression was defined as a 1.5-fold increase in the genes.

Six *E. coli* isolates with various resistance determinants and profiles were used in this study (Table 1). *E. coli* E245 was resistant to both CEF and GEN, and *E. coli* E246 was resistant to GEN only. The 4/6 of *E. coli* strains were susceptible to CEF and GEN. In this study, the efficacy of the combination was tested against *E. coli* strains resistant to compounds in the combination and the resistance preventive potential of the combination was evaluated against all *E. coli* strains. The interaction between drugs was determined by the checkerboard test. The fractional inhibitory concentration indexes (FICIs) provided from the checkerboard test were interpreted as follows: FICI≤ 0.5 = synergy; FICI > 4.0 = antagonism; and FICI > 0.5-4 = indifference (Table 1) (Elipoulos and Moellering, 1996). The minimum bactericidal concentration (MBC) and mutant prevention concentration (MPC) of the combination were determined as previously described (Blondeau et al., 2001; Hansen and Blondeau, 2005). The MBC was defined as the lowest concentration showing ≥ 99.9 % death compared with the initial inoculum. The combination was defined as bactericidal and bacteriostatic for MBC:minimum inhibitory concentration (MIC) ratios of 1-4 and ≥ 8, respectively (Table 2) (Maaland et al., 2015). The MPC was determined as the concentration that allowed no growth of bacteria at the end of the 72-h incubation period (Table 2). The inoculum densities used for MIC-MBC and MPC determination were 10^7 cfu/ml (equivalent to 0.5 MacFarland turbidity) and 10^{10} cfu/ml, respectively.

RESULTS

The FICI values of the combination therapy for *E. coli* strains are shown in Table 1. The FICIs of the combination ranged from 0.1 to 0.5. A synergistic ef-

fect was detected for all *E. coli* strains by the checkerboard method. Based on the MBC:MIC ratios, the combination exhibited bactericidal effect against all *E. coli* strains (Table 2). The MBC:MIC ratio was two for *oqxB* containing-*E. coli* E306 and one for the rest of *E. coli* strains. The individual MPCs ranged from 32 μg/ml to 512 μg/ml for CEF and from 16 μg/ml to 4096 μg/ml for GEN (Table 2). The MPCs of the combination ranged from 0.256 μg/ml to 64 μg/ml. The individual MPCs of CEF and GEN decreased by up to 128-fold using the combination for the inhibition of resistant *E. coli* strains. The MPC:MIC ratio of the combination ranged from 2 to 32 for *E. coli* strains. The highest MPC:MIC ratio was detected for the most susceptible isolate, *E. coli* E175.

Table 1. Resistance profiles and mechanisms of *E. coli* and checkerboard data with the interpretations

		Resistance mechanism						
		QRDR[a]		PMQR[b]	MDR[c]			
Isolate ID	Resistance profile[d]	gyrA	parC	oqxB	marA	acrB	soxS	ompF
E175	SMX				↓↓	↓	↓↓	↑
E222	NAL, CIP, SMX, TMP, TET, OTC, CHL	Ser83Leu	Ser80Ile		↓↓	↓↓	↑↑	↑
E245	NAL, CIP, ORB, GAT, AMP, CEF, GEN, TET, OTC, ERY, CHL	Ser83Leu Asp87Glu			↑	↑↑	↑↑	↑
E246	NAL, GAT, AMP, TMP, GEN, TET, OTC, CHL, CST	Ser83Leu			↑↑	↑↑	↑↑↑	↓↓↓
E269	NAL, SMX, TMP, TET, OTC, CST				↓↓	↓	↑↑	↑
E306	NAL, CIP, ORB, AMP, TMP, TET, OTC, ERY, CHL	Ser83Thr		+	↓↓	↓↓	↑	↑

[a]: quinolone resistance determining region
[b]: plasmid-mediated quinolone resistance
[c]: compared to AG100; ↑: 1–5 fold increased; ↑ ↑: 5–10 fold increased; ↓: 1–5 fold decreased; ↓↓: 5–10 fold decreased; ↓↓↓: ≥ 10 fold decreased.
[d]: SMX: sulfamethoxazole, NAL: nalidixic acid, CIP: ciprofloxacin, SMX: sulfamethoxazole, TMP: trimethoprim, TET: tetracycline, OTC: oxytetracycline, CHL: chloramphenicol, ORB: orbifloxacin, GAT: gatifloxacin, AMP: ampicillin, CEF: ceftiofur, ERY: erythromycin, CST: colistin

Table 2. Pharmacodynamic profile of ceftiofur+gentamicin combination

Isolate ID	Pharmacodynamic parameters															
	MICs (μg/ml)		FICI		MBCs (μg/ml)			MBC:MIC			MPCs (μg/ml)			MPC:MIC		
	CEF S≤8, R>8	GEN S≤2, R>4	Conc. (μg/ml)	Intp.[a]	CEF	GEN	CEF +GEN	CEF	GEN	CEF +GEN	CEF	GEN	CEF +GEN	CEF	GEN	CEF +GEN
E175	1	4	0,128/1	0,3	8	4	0.128/1	8	1	1	128	128	4/32	128	32	32
E222	2	4	0,128/1	0,3	8	4	0.128/1	4	1	1	128	128	2/16	64	32	16
E245	16	128	1/16	0,1	64	256	1/16	4	2	1	256	4096	4/64	16	32	4
E246	2	64	0,512/8	0.3	2	64	0.512/8	1	1	1	128	512	1/16	64	8	2
E269	1	4	0,256/1	0,5	1	4	0.256/1	1	1	1	32	32	2/8	32	8	8
E306	4	2	0,512/0,064	0,1	4	2	1/0.128	1	1	2	512	16	2/0.256	128	8	4

[a]: Synergistic interaction

DISCUSSION

β-lactam-aminoglycoside combinations are primarily preferred to expand the spectrum of action and have synergistic effects. These combinations have been used as an initial therapeutic option since 1984 (Rafei, 2018). All aminoglycosides can cause varying degrees of ototoxicity and nephrotoxicity. Nephrotoxicity is the most common adverse effect of aminoglycoside treatment (Prescott et al., 2013). β-lactam-aminoglycoside combinations are also considered a less toxic therapeutic option in addition to having potential synergistic and expanded spectrum activities. In veterinary medicine, aminoglycosides are most often combined with penicillin (EMA, 2017). Cephalosporins are also synergistic with aminoglycosides for the treatment of neutropenic human patients with infections caused by resistant strains (Prescott et al., 2013; Rafei, 2018). Therefore, the focus of this study was to compare the efficacy of the individual use of the antimicrobials with synergistically acting CEF+GEN combination against *E. coli* strains with resistance to many antimicrobials. The results of this study showed that CEF+GEN effectively inhibited *E. coli* strains with varying susceptibility profiles and different resistance determinants. Multidrug resistance (MDR) is a potential limiting factor in the treatment of infectious bacteria. FICI data showed that the CEF+-GEN combination could more effectively inhibit *E. coli* strains resistant to compounds in the combination and those resistant to many other antimicrobials from different groups. The use of a second antimicrobial can reduce the risks for patients infected with MDR organisms and will provide adequate coverage for potential pathogens causing an infection (Tamma et al., 2012). In clinical trials, the efficacy of β-lactam-aminoglycoside combinations has also been shown. For example, the combination of amikacin and ampicillin was found to be suitable for the treatment of foals with sepsis (Theelen, 2019). Similarly, the CEF+GEN combination can also be used to increase the clinical success of treatments for infections caused by *E. coli* strain even when it is resistant to CEF and/or GEN. Noel et al. (2018) showed that addition of amikacin to ceftalazone/tazobactam bacterial clearance were increased and emergence of resistance to ceftalazone/tazobactam was prevented. Tschudin-Sutter et al. (2018) indicated that combination therapy with β-lactam+aminoglycoside might improve mortality of *Pseudomonas aeruginosa* causing blood stream infection. Based on the results of previous studies, CEF+GEN combination can be preferred without risk of emergence of resistance to inhibit various resistance determinant-carrying *E. coli* strains instead of monotherapy with CEF or GEN alone. The results of this study showed that the MICs of the CEF+GEN combination were equal to their MBCs. Based on the MBC:MIC ratio, the combination was defined as bactericidal against all *E. coli* strains. The co-use of CEF and GEN caused a decrease in their individual MBCs by up to 64-fold. The MBCs of CEF and GEN were below the clinical breakpoint of CEF (S≤8 μg/ml, R>8 μg/ml) for all *E. coli* strains and below that of GEN (S≤2 μg/ml, R>4 μg/ml) for four *E. coli* strains. The genetic mechanism of resistance can be determinative for the bactericidal activity of antimicrobials (Cengiz et al., 2013). Therefore, sustaining of bactericidal activity of each compound in the combination is crucial by decreasing their individual MBCs. The other benefit of the CEF+GEN combination was a change in the individual MPCs of CEF and GEN. The MPCs decreased for all *E. coli* strains, and the MPC:MIC ratio decreased for four strains. The distance between

MPC and MIC is determinative for the emergence of resistant sub-populations during antimicrobial therapy (Hansen and Blondeau, 2005). The lowering MPC:MIC ratio may reduce the risk of emergence of resistance or evolving of resistant strains to highly resistant strains. As a clinical perspective, this improvement may increase the success of antimicrobial therapy applied by CEF+GEN combination.

CONCLUSION

In conclusion, the combination decreased the MPCs and narrowed the range between the MIC and MPC. This improvement can reduce the risk of the emergence of single mutations during treatment with currently approved doses. In addition, the bactericidal effects of the compounds sustained at concentrations below the clinical breakpoints of the individual compounds by their use in a combination. This result indicates that the killing potential of the co-use of the compounds is much stronger than their individual use.

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CONFLICT OF INTEREST STATEMENT

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Research article
Ερευνητικό άρθρο

Risk factors associated with reproductive disorders in dairy cows in Algeria

S. Boudelal[1], A. Niar[2]

Laboratoire de reproduction des animaux de la ferme, institut des sciences vétérinaires, université Ibn Khaldoun, Tiaret, Algerie.

Laboratory of Farm Animals Reproduction, Institute of Veterinary Science, Ibn Khaldoun University, Tiaret, Algeria.

ABSTRACT: In Algeria, there is a lack of information about the prevalence of reproductive health disorders in dairy farms. In this light, the aim of the present study was to determine the effect of parity, body condition score (BCS), calving season, calves' status at delivery and gender of the calf on the prevalence of dystocia, retained fetal membranes (RFM) and metritis under Algerian field conditions. The study took place from October 2016 to April 2019. It was conducted on 449 dairy cows from 22 commercial dairy farms located in Algerian highland regions (Medea, Tiaret and Tissemsilt areas).The prevalence of dystocia, RFM and metritis was 12%, 16.9%, and 11.8%, respectively. Cows with BCS ≤2.75 were the most affected by RMF (Odds ratio [OR] = 3.66). The main risk factors for RMF were calving abnormalities (OR=3.03), calving in warm season (May to October) (OR= 1.85) and the birth of male calves (OR= 1.75) (P<0.05). Stillbirth, dystocia and RFM were identified as potential risk factors for metritis (P<0.05). These findings confirm again the importance of herd management in prevention of these reproductive disorders.

***Keywords*:** dystocia; retained fetal membranes; metritis, prevalence; risk factors

Corresponding Authors:
Salah Boudelal, Kerbal Lakhdar city, N:03. Ksar El Boukhari, Medea, 26300, Algerie
E-mail address: salah.boudelal@univ-tiaret.dz

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INTRODUCTION

Dystocia, retained fetal membranes (RFM), and metritis are the major reproductive disorders that have a direct impact on farm profitability which lead to severe economic losses. Economic losses are related to the costs of treatment, perinatal mortality, maternal death, decline in milk production, and decrease in reproductive performances (De Amicis et al., 2017; Kim and Jeong, 2019). Dystocia is a welfare problem of cows and calves (Kaya et al., 2015). It is associated with a higher incidence of calf losses (25%) (De Amicis et al., 2017), which increases as the degree of calving difficulty increases (Lomabard et al., 2007; Kaya et al., 2015). Dystocia occurs in 10 to 30% of calvings (Lomabard et al., 2007). Feto-pelvic disproportion (FPD), fetal malposition, multiple fetuses, uterine inertia, and uterine torsion are the main causes of dystocia (Mee, 2008). There are several predisposing factors for dystocia such as gestation length, parity, gender, sire, breed, nutrition, climate, age and weight at service (Mee, 2008). One of the sequels of dystocia is RFM (Giuliodori et al., 2013). Normal expulsion of fetal membranes occurs within 8h after calf delivery (Qu et al., 2014). The breakdown collagen that links the interface together at several sites is likely to be a key factor in placental separation (Opsomer, 2015); nevertheless, the precise reason of failure of detachment of fetal membranes is still not fully understood (Szenci, 2016). The prevalence of RFM ranges from 5.8 % to 19.1% (Vergara et al., 2014), but it can be increased in cows with twins, and after dystocic calving (Sheldon et al., 2008), and even more in herds with infectious diseases such as bovine viral diarrhea (BVD) and brucellosis (Opsomer, 2015; Szenci, 2016). Reported risk factors for RFM include breed, parity, stillbirth, abortion, cesarean section, fetotomy, induction of parturition, shortened gestation, and management during the dry period (Tucho and Ahmed, 2017; Daros et al., 2017). Metritis is the inflammation of all the layers of the uterus (Deori and Phookan, 2015). As clinical condition, metritis is observed during the first 21st days of postpartum and characterized by abnormally enlarged uterus and the presence of putrid uterine discharges detectable in the vagina, with or without fever (Sheldon et al., 2006). *Arcanobacterium pyogenes*, *Escherichia coli*, coliforms and the Gram-positive cocci, *Fusobacterium necrophorum*, *Trueperella pyogenes* and *Clostridium spp* are the most isolated microorganisms in cases of metritis (Deori and Phookan, 2015; Ordell et al., 2016). The prevalence of metritis varies from 1 to 29.5% (Bruun et al., 2002, Vergara et al., 2014). Breed, herd, parity, climatic conditions, RFM, dystocia, fetal maceration, shorter gestation length, twins and stillbirth calves are the relevant risk factors of metritis (Piccardi et al., 2015; Kumari et al., 2015).

To the authors' knowledge, the prevalence and the risk factors of reproductive disorders in dairy farms of Algeria has been scarcely studied. The purpose of the present study was to report the prevalence and the main risk factors associated with dystocia, RFM and metritis under Algerian field conditions.

MATERIAL AND METHODS

Herds

The current study was conducted in 22 farms located in the Algerian highland regions (three provinces: Medea, Tiaret, and Tissemsilt), during the period from October 2016 to April 2019. The study area is characterized by two types of climates prevailing, namely the semi-arid climate and the Mediterranean climate, with two distinct seasons. The summer is hot and dry with temperatures that reach up to 38°C, while the winter is cold and windy with a minimum temperature below freezing. Four hundred and forty-nine (n=449) dairy cows (108 crossbred and 341 purebred: Holstein, Red Holstein, Flechvieh and Montbeliard cows), free of tuberculosis and brucellosis, were included in the study. The cows were kept in a loose housing system, and milked two times daily. The mean milk yields were 13.7 ± 0.6 kg per cow per day. The cows were dried 60 days before the expected date of calving. Seasonably, the cows received green fodder or meadow fodder with vetch oats hay, and supplemented with commercial concentrate (17% at 20% crude protein: crushed maize grains, soybean meal, barley and vitamin-mineral mixture), and were given ad libitum access to water. All herds had 10 or more cows.

Definition of traits

The trait descriptions were as follow**:** dystocia is defined as calving that requires assistance (Mee, 2008). Retained fetal membranes is defined as the failure to expel fetal membranes more than 24h (Kim and Jeong, 2019). Metritis is defined as a cow with an abnormal distention of uterus and the presence of putrid uterine discharge with or without fever over the 21st days postpartum (Sheldon et al., 2008). Stillbirth is defined as the death of a calf that occurs at birth or within the first 48h after parturition (Bicalho et al., 2007).

Data collection

Data were collected from 449 calvings. The enrolment of the cows, diagnoses of disorders, evaluation and data collection were made by the first author. Health data and calving conditions were documented by farmers using a questionnaire. Each herd was visited at least bimonthly. Information recorded included date of calving, cows' parity, calving ease score (CES), number of newborns, calf status at delivery (alive or dead), gender of the calf, body condition score (BCS), calving season, occurrence of RFM, and occurrence of metritis. The data of parity of cows was grouped into three classes (1st, 2nd and ≥3rd lactation). Calving ease score was scored on a scale ranging from 1 to 5. A scale in which 1 means no assistance is required, 2 means minor assistance is required, 3 stands for considerable assistance, 4 signifies extreme difficulty with veterinary assistance and mechanical traction, and 5 represents caesarean section or fetotomy. Cows with a CES of ≥3 are classified to have dystocia (Bicalho et al., 2007; Kim and Jeong, 2019). The calving season was classified into two major periods (1= cold season [November-April] or 2: warm season [May-October])(Johanson and Berger, 2003). The BCS was evaluated at calving (±5days) according to Fergusson et al. (1994) and grouped into three main classes (1= BCS≤ 2.75, 2= 2.75<BCS≤ 3.25 and 3 = BCS >3.25). Gender of the calf was classified into two categories (1= birth of single female, 2= birth of single male). There were a total of 18 cows (4%) in the 22 farms that had twins. Cows having twins were excluded since the sample size for affected cows was too small to make reliable estimates (only in the study of the effect of gender of the calf). Abnormal calving included cows having dystocia or stillbirth. A value of 2 for calving abnormality was assigned to cows with one or more of the following: dystocia or stillbirth. Otherwise, cows were assigned a value of 1 (normal calving: the birth of viable calves with CES ≤2).

Statistical analyses

The test data was processed using the IBM SPSS, version 24.0. First, we determined the effect of variables (parity, age, calving season, BCS around calving, gender of the calf, and calving abnormalities) on the occurrence of each disease using the chi-square test. Thereafter, the suspected risk factors for diseases were analyzed by binary logistic regression. Results were presented as percentages and Odds Ratios (ORs) with their respective 95% confidence intervals (CIs). The level of significance was considered at P<0.05.

Table1. Estimated odds ratios and 95% confidence intervals (CI) for factors affecting dystocia in Algerian dairy cows (n=449)

variables	Num cows	Dystocia		OR[Π]	IC[£]	P
		Num. cases	%			
Total	449	54	12	-	-	-
Parity *						0.523
1	143	19	13.3	1.332	0.653-2.157	
2	83	12	14.5	1.470	0.695-3.107	
3	223	23	10.3	referent	-	
Calving season †						0.762
1	291	34	11.7	referent	-	
2	158	20	12.7	1.095	0.607-1.976	
BCS ὐ :						0.277
1	69	12	17.4	2.105	0.778-5.697	
2	303	35	11.6	1.306	0.556-3.065	
3	77	7	9.1	referent	-	
Gender of the calf §						0.870
1	199	23	11.6	referent	-	
2	232	28	12.1	1.050	0.584-1.890	

* Parity: 1= first lactation; 2 = second lactation, 3= third or more lactation, † Calving season: 1= cold season [November to April]; 2= warm season [Mai to October], ὐ BCS: 1 = BCS≤ 2.75; 2 = 2.75<BCS≤ 3.25; 3= BCS >3.25, § Gender of the calf: 1= birth of single female; 2= birth of single male; the 18 cows that gave twins were excluded, Π Odds ratio, £ 95% confidence intervals.

Table2. Estimated odds ratios and 95% confidence intervals (CI) for factors affecting RFM in Algerian dairy cows (n=449)

variables	Num cows	RFM		OR£	IC**	P
		Num.cases	%			
Total	449	76	16.9	-	-	-
Parity *						0.203
1	143	25	17.5	1.265	0.714-2.239	
2	83	19	22.9	1.772	0.940-3.341	
3	223	32	14.3	referent		
Calving season †						0.015
1	291	40	13.7	referent	-	
2	158	36	22.8	1.852	1.124-3.051	
BCS ὺ						0.026
1	69	14	20.3	3.665	1.245-10.791	
2	303	57	18.8	3.337	1.289-8.637	
3	77	5	6.5	referent	-	
Gender of the calf§						0.038
1	199	24	12.1	referent	-	
2	232	45	19.4	1.755	1.026-3.001	
Calving abnormalityΠ						<0.001
1	353	46	13	referent	-	
2	96	30	31.3	3.034	1.783-5.161	

*: Parity: 1= first lactation; 2 = second lactation, 3= third or more lactation, † Calving season: 1= cold season [November to April]; 2= warm season [Mai to October], ὺ BCS: 1 = BCS≤ 2.75; 2 = 2.75<BCS≤ 3.25; 3= BCS >3.25, § Gender of the calf: 1= birth of single female; 2= birth of single male; the 18 cows that gave twins were excluded, Π Calving abnormality: 1= birth of viable calves without dystocia; 2= cows having dystocia or stillbirth, £Odds ratio, **:95% confidence intervals.

Table3: Estimated odds ratios and 95% confidence intervals (CI) for factors affecting metritis in Algerian dairy cows (n=449)

variables	Num cows	Metritis		OR**	IC††	P
		Num. cases	%			
Total	449	53	11.8	-	-	-
Parity *						0.417
1	143	14	9.8	referent	-	
2	83	13	15.7	1.711	0.762-3.843	
3	223	26	11.7	1.216	0.612-2.416	
Calving season †						0.305
1	291	31	10.7	referent	-	
2	158	22	13.9	1.357	0.756-2.434	
BCS ὺ						0.941
1	69	9	13.0	1.133	0.422-3.041	
2	303	35	11.6	0.987	0.453-2.151	
3	77	9	11.7	referent		
Gender of the calf§						0.424
1	199	20	10.1	referent	-	
2	232	29	12.5	1.279	0.669-2.339	
Calving abnormalityΠ						0.002
1	353	33	9.3	referent	-	
2	96	20	20.8	2.552	1.388-4.692	
Retained placenta£						<0.001
1	373	27	7.2	referent	-	
2	76	26	34.2	6.664	3.603-12.323	

* Parity: 1= first lactation; 2 = second lactation, 3= third or more lactation, † Calving season: 1= cold season [November to April]; 2= warm season [Mai to October], ὺ BCS: 1 = BCS≤ 2.75; 2 = 2.75<BCS≤ 3.25; 3= BCS >3.25, § Gender of the calf: 1= birth of single female; 2= birth of single male; the 18 cows that gave twins were excluded, Π Calving abnormality: 1= birth of viable calves without dystocia; 2= cows having dystocia or stillbirth, £Retained placenta: 1= disease free cows; 2= cows having retained placenta, ** Odds ratio, †† 95% confidence intervals

RESULTS

The overall prevalence of dystocia, RFM and metritis in the current study were 12%, 16.9% and 11.8%, respectively. Tables 1, 2 and 3 demonstrate detailed statistics describing risk factors associated with each disease. There was a significant difference between warm (May to October) and cold (November to April) calving seasons on the risk of experiencing RFM (OR= 1.85, P = 0.015). Also, the BCS at calving (±5days) had a significant association with RFM prevalence (P<0.05). The birth of male calves increased the prevalence of RFM to 19.4% (OR=1.75, P=0.038). Cows with abnormal calving had higher risk of having RFM compared to cows with normal calving (OR=3.03; P<0.001). The risk of diagnosing metritis was higher in cows with abnormal calving (OR= 2.5, P=0.002). Also, RMF was identified as risk factors for metritis (OR=6.6, P<0.001) (Table3).

DISCUSSION

The prevalence of dystocia (12%) and RFM (16.9%) in the present study were in agreement with the reports of other researchers (Johanson and Berger, 2003; Nguyen-Kien and Hanzen, 2016; Daros et al., 2017), who state that the prevalence of dystocia and RFM ranges from 11% to 23.7%, and 13.9% to 19.3%, respectively. The prevalence of metritis (11.8%) coincided closely with the prevalence of 11.2% found by Daros et al. (2017). Furthermore, this prevalence was lower than others reported in recent studies, where the prevalence of metritis ranges from 22.4% to 29.7% (Cui et al., 2015; Piccardi et al., 2015; Giuliodori et al., 2013). The variation in the prevalence of metritis may be attributed to the difference of disease definitions, study area, variation of sample size and the year of study.

The season of calving was associated with the occurrence of RFM, as cows calved in warm season had more (OR= 1.85, P=0.015) risk to develop RFM than in cold season. Similarly, the higher prevalence of RFM is reported in summer season in cows (Bahri Binabaj et al., 2014) and in buffaloes (Verma et al., 2018). Furthermore, heat stress during the warm periods may contribute to impair immune system functions (Dawod and Byeng, 2014) and indirectly to increase the prevalence of reproductive diseases in dry season (Verma et al., 2018). This is in agreement with Gilbert's report (2016) in which he declares that heat stress is one of factors associated with RFM, and to preserve optimum reproductive performances, Szenci (2016) suggests reducing summer heat stress.

We found that BCS proved to be a risk factor of RFM. In the cow that calved with a BCS ≤2.75, a RFM prevalence of 20.3% was registered, which is significantly higher than in the cows calved within the other BCS classes (P= 0.026). This finding was in accordance with the observation of Islam et al. (2012). Vieira-Neto et al. (2016) state that the occurrence of RFM was negatively correlated with BCS. Low BCS is an indicator of low energy statut which is often associated with impaired neutrophil functions (Giuliodori et al., 2013). Also, Cows with lower prepartum body condition (Qu et al., 2014), or those with greater BCS loss (Roche et al., 2009) are expected to be less healthy and more susceptible to infection compared to cows with a good BCS, losing less BCS, or to those gaining BCS (Roche et al., 2009).

The birth of male calves increased significantly the prevalence of RFM compared to the birth of female calves. Our finding agrees with those of Bahri Binabaj et al. (2014), in which, they showed that the birth of male calf is a significant risk factors for RFM. Male calves, due to their higher birth weight than heifer calves, are direct causes of dystocia (Johanson and Berger, 2003), and consequently to RFM. The prevalence of RFM was 13% in normal calving, and reached to 31.3% in abnormal calving (in cases of dystocia or stillbirth)(OR= 3.03, P <0.001). This result is in agreement with other studies (Vieira-Neto et al., 2016; Buso et al., 2018). Conversely, neither dystocia (Kaya et al., 2015; Nguyen-Kien and Hanzen, 2016), nor stillbirth (Kumari et al., 2015) affect the prevalence of RFM.

The risk of developing metritis was much higher in dystocic cows, or those that deliver stillborn calves (OR=2.5, P=0.002), and RFM was present in 34.2% of cases of metritis (OR= 2.55, P<0.001), which is supported in the literature (Deori and Phookan, 2015; Piccardi et al., 2015; Daros et al., 2017). According to Giuliodori et al. (2013), the risk for metritis increased with dystocia, RFM, and dead calf [Adjusted Odds Ratio (AOR) = 2.58, 95% CI: 1.189–5.559]. The prevalence of stillbirth is higher in dystocic calvings than in eutocic ones (Johanson and Berger, 2003; Kaya et al., 2015). Prolonged manipulation in cases of dystocia increased the introduction of bacteria, and hard pulls are traumatic for the uterus. The tissue trauma facilitates adhesion and invasion of the germs (Opsomer, 2015), which may disturb intrauterine cellular defense, and lead to the loss of the ability to control the uterine infections naturally (Deori and phookan, 2015). Retained fetal membranes pose a perfect media for bacterial growth (Bruun et al., 2002). It is

responsible of slowed uterine involution, impaired neutrophils functions and altered immune responses, which in turn, results in increasing the risk of metritis.

CONCLUSIONS

The current study aimed to identify the relevant risk factors for dystocia, RFM and metritis in dairy cows in Algeria. Cows at first and second lactations, and those that with low body condition, are more likely to experience dystocia and RFM. The important risk factors for RFM included dystocia, stillbirth, birth of male calves and calving in the warm season. Dystocia, stillbirth and RFM were the most implicated risk factors for metritis. The determination of relationships among disorders and the role of cows, calf and environment are important for developing an effective prevention strategy by better management of these risk factors that lead to decrease their prevalence in the dairy farms.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
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The Effects of Plant Fibers on Improving the Properties of Meat Emulsion

Ş. Kurt, H. G. Ceylan

Department of Food Engineering, Faculty of Engineering, University of Adıyaman, Adıyaman, Turkey

ABSTRACT: The effects of plant fibers (1%; pea, wheat, apple, carrot, lemon, oat, inulin, cellulose) on the emulsion stability (ES), capacity (EC), density (ED), activity (EA), viscosity (EV) and apparent yield stress of emulsion (AYSe) and emulsion gel (AYSg) of beef were studied using a model system. The fibers had significant (p<0.05) effect on the emulsion properties. Apple and lemon fibers increased EA values and addition of carrot and wheat fibers increased ED values. Carrot, lemon and oat fibers increased ES values while lemon fiber increased emulsion storage stability the most. Inulin and apple fibers decreased AYSe values while other fibers increased. AYSg values increased with the addition of wheat fiber, but decreased with the addition of other fibers. However, the fibers did not significantly (p>0.05) improve emulsion capacity. On the other hand, the added fibers improved the emulsion viscosity.

Keywords: beef, plant fiber, meat emulsion, apple, lemon, car1rot

Corresponding Authors:
Şükrü Kurt, Department of Food Engineering, Faculty of Engineering, University of Adıyaman, Adıyaman, Turkey
E-mail address: sukrukurt@hotmail.com

Huriye Gözde Ceylan, Department of Food Engineering, Faculty of Engineering, University of Adıyaman, Adıyaman, Turkey
E-mail address: hgyildiz@adiyaman.edu.tr

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INTRODUCTION

Plant-derived products can be used in various meat products (Hughes et al., 1997; Steenblock et al., 2005; Aleson-Carbonell et al., 2005). One of them is fiber. Fibers can be used to improve the technological properties and quality of meat products (Thebaudin et al., 1997; Rodriguez et al., 2006; Kilincceker and Yilmaz, 2019). Fibers are naturally occurring compounds present in different kinds of plants. They affect the water-binding capacity, oil adsorption capacity, viscosity, gel forming properties of meat products (Biswas et al., 2011). Dietary fiber plays an important role in the prevention of some diseases. They have a positive effect on health since its consumption has been related to a decreased incidence of several types of cancer (Fernández-Ginés et al., 2005; Rodriguez et al., 2006). Plant fibers have been studied in meat products to improve texture and maintain juiciness in the products due to the water retention ability (Rodriguez et al., 2006; Moller et al., 2011). Different types of fibers have different effects, depending on their chemical and physico-chemical properties that affect the behaviour of proteins in food systems during preparation, processing, storage and consumption, and contribute to the quality and organoleptic attributes of food systems (Thebaudin et al., 1997). They have been used as fat replacer, fat reducing agent, volume enhancer, binder, bulking agent and stabilizer (Ang and Miller, 1991; Hughes et al., 1997; Aleson-Carbonell et al., 2005). In particular, their hydration and fat binding properties are important in terms of functionality related to chemical and physico-chemical factors such as solubility. Hydration properties of fibers are described by water-holding capacity, water-binding capacity, swelling and solubility (Thebaudin et al., 1997). Meat processors generally use purified soluble fibers up to the 1 % for their functional properties (Thebaudin et al., 1997).

The technological properties of meat proteins and non-meat additives such as plant fibers affect emulsion stability, viscosity, gel strength and oil and water holding properties (Rodriguez et al. 2006; Moller et al., 2011). These properties can be predicted by the model system. Some plant fibers influence on emulsified meat products have been studied so far by only a few researchers, so there is some information available. The objective of this study was to determine the effects of eight different plant fibers on the emulsion properties of beef.

MATERIALS AND METHODS

Materials

The meat source used in this study was beef (*M. semimembranosus* muscle). Meat and refined sunflower oil were obtained from local markets. Apple (*M. domestica*), lemon (*C. limon)*, pea (*P. sativum*) and oat (*A. sativa*) fibers were obtained from Arosel Gida (Istanbul Turkey). Cellulose and carrot fibers were obtained from Kimbiotek Kimyevi Maddeler (Istanbul Turkey). Inulin and wheat fibers were obtained from Smart Kimya (Izmir, Turkey). Analytical grade chemicals were used.

Methods

Meat sample and homogenate preparation

The meat was ground using a grinder (Tefal, Le Hachoir 1500, France) with a 3 mm diameter hole plate. Each meat sample was divided into equal lots and packaged by using three layers of medium polyethylene and this was stored as –20 ˚C until it was used.

Homogenate was prepared as; 0.45 M NaCl solution was prepared and standardised to a pH of 6.5. 100 ml of NaCl solution (4ºC), 25 g ground meat and fiber were placed into a blender (Waring 80011S, USA) jar and comminuted for 1 min at 18 000 rpm. The fiber rate was adjusted to be 1% in the emulsion.

Emulsion preparation

One hundred and thirty ml of homogenate solution and 100 ml of oil (as the amount of starting oil) were placed in the blender (Kenwood KM010, UK). During emulsification at 6 500 rpm, 100 ml of oil was added at a rate of 0.5-0.6 ml/s. After all the oil was added, the emulsion was mixed for an additional 5 s.

pH determination

The pH of the prepared NaCl solution, homogenates and emulsions were measured by a pH meter (Hanna 2215, USA) equipped with a temperature probe.

Emulsion capacity (EC)

EC is the maximum amount of oil emulsified by a unit of protein. It was determined by using a model system described by Ockerman (1985). The end point was determined as described by Webb et al. (1970). 12.5 ml homogenate, 37.5 ml of 0.45 M NaCl and 50 ml of oil were placed into the blender (Kenwood KM010, UK) jar at first. During emulsification at 6500 rpm, oil was added at a rate of 0.5-0.6 ml/sec

until the emulsion broke. Water was circulated around the burette as to maintain the oil at a constant temperature of 20°C. The electrical conductivity of the emulsion was monitored with a microprocessor. At the breaking point of the emulsion, conductivity rapidly dropped and the addition of oil was stopped. The amount of oil added, including the first 50 ml, was recorded as the EC.

Emulsion stability and storage stability

Meat emulsions are not stable forever (Gökalp et al., 1999). Heat treated emulsions can be separated over time into the oil and water phase. ES was determined using model systems, as described by Ockerman (1985). Raw emulsion (10 g) was weighed into a centrifuge tube, which was capped and immediately heated at 80°C in a water bath for 30 min. The tubes were transferred in a cold water bath immediately and cooled to approximately 25°C. Then, the emulsion was held for temperature standardisation for 1h at ambient conditions and then centrifuged at 350 x g for 20 min. The water and oil separations were measured, and emulsion stability (ES) was calculated using the following equations:

ES (%) = 100 - (SW + SO)

SW (%) = ml of water separated x 10

SO (%) = ml of oil separated x d x 10 (d: specific gravity)

Storage stability of emulsion (ESs) was determined as described above with storage process. After the emulsion cooled to 25°C, they were stored at 4°C during 45 days.

Emulsion density

ED measurement is one of the simplest methods of determining emulsion properties. It can be required inexpensive equipment that is available in many laboratories (Mcclements, 1999). Emulsion of 20 ml was pipetted with enlarged mouth side of the pipette and weighed. ED was determined as the weight of 20 ml of emulsion.

Apparent yield stress of emulsion

Apparent yield stress was determined by using a cone penetrometer (Yüksel Kaya Makina, YKM-S216, Turkey). In rheological studies, it is recommended that the penetration depths are converted to "apparent yield stress" (AYS) values for a sharp-ended cone. A special conical head (with a 22° cone angle weighing 18.75 g) was placed just above the surface of the emulsions and released. Penetration depth was read after 5 s of penetration. Three replicates were performed for each sample. Then, apparent yield stress (AYS) was calculated as:

$$AYS\ (N/m^2) = \frac{g.w}{\pi.d^2.\tan^2(\varepsilon)}$$

Where; g is acceleration due to gravity, w is the weight of the cone assembly, ε refers to the cone angle and d is the penetration depth (Wright et al., 2001).

Preparation of emulsion gel and measurement of apparent yield stress

The emulsion was immediately transferred into the jars. After heat treatment at 80°C in a water bath for 30 min, the emulsion was transferred immediately to a cold water bath and cooled to approximately 25°C. Then, the emulsion gel (cooked emulsion) was held for temperature standardisation for 1h at ambient conditions and apparent yield stress was determined as described above for emulsion testing.

Emulsifying activity

Emulsifying activity was determined using the method of Neto et al (2001). The raw emulsion was immediately transferred into the tubes. After the centrifugation at 350 g for 20 min, the height of emulsified layer and that of the total contents in the tube was measured. The emulsifying activity (EA) was calculated as:

$$EA = \frac{\text{(Height of emulsified layer in the tube) x 100}}{\text{Height of the total contents in the tube}}$$

Emulsion viscosity

The viscosity of emulsion was measured with a rheometer (Brookfield DV3T). A spindle (type RV-3, viscosity range= 100-200 000 mPa.s) was used to measure the viscosities. The spindle was set to rotate at 15 rpm. The rheometer was checked with a calibration fluid (Brookfield, 4 700 cp at 25 ºC). Each sample was equilibrated for at least 3 hours to allow reaching the required temperature (25 ºC).

Statistical analysis

Analyzes were performed four times for each parameter. The study was repeated twice. The data were subjected to analysis of variance (ANOVA), and the results were expressed as mean ± standard deviation (SD). When there were differences among the sam-

ples, the differences were compared by using Duncan's multiple-range tests; a probability value of $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

The mean values of the effects of the fibers on the emulsion properties are given in Table 1. The effects of fibers on the pH values of the homogenate (pH-h) and emulsion (pH-e) were found to be significant ($p<0.05$). The pH-h and pH-e values varied between 4.69-5.54 and 4.65-5.60, respectively (Table 1, Figure 1). Oat fiber increased pH-h value the most compared to control. However, fibers were not increased significantly pH-e values. Apple, carrot and lemon fibers decreased pH-e values. In particular, lemon fibers reduced pH-h and pH-e to the lowest levels. Aleson-Carbonell et al. (2005) reported that pH values of raw, dehydrated raw and dehydrated cooked lemon albedo were 4.53, 3.73 and 4.64 respectively. As shown in Figure 1, pH values of emulsion were found to be higher than homogenate. The increase in pH-e might have influenced by the emulsification process.

The effects of fibers on the EC values were found to be significant ($p<0.05$). Oat, cellulose, pea and wheat fibers increased EC values significantly (Table 1, Figure 2). Their oil binding capabilities were found to be higher than other fibers. However, carrot, lemon and apple fibers decreased EC values compared to the control. Thebaudin et al. (1997) reported that fat-absorption capacity of wheat-bran were higher than apple, pea and carrot fibers. Moreover, they stated that insoluble fibers can retain up to a few times their mass in oil. Lower pH values of emulsions with carrot, lemon and apple fibers might be resulted from lower pH values. Emulsion capacity is the maximum amount of oil which can be emulsified by proteins. It is strictly related to protein solubility which can be affected by pH values (Ockerman, 1985). However, hydrophilic and lipophilic characteristics and the balance between them played an important role on EC values. These characteristics of proteins reduced interfacial tension between oil and water allowing the formation of emulsion with a much reduced energy input (Elizalde et al., 1988). Also, Biswas et al. (2011) stated that oil binding properties of fiber related to its chemical composition, but is more largely a function of the porosity of fiber structure. However, hydrated fiber reduces oil-binding because water occupies pores.

The effects of fibers on the EA and ED values were found to be significant ($p<0.05$). EA values of the emulsions with lemon and apple fibers were found to be higher than control (Table 1, Figure 3). As shown in Figure 1, the pH-h values of these two fibers were found to be lower than others. As pH moves away from the isoelectric point of muscle proteins, the net electrical charge increases. This causes an increase in polarity (Gokalp et al., 1999). Neto et al. (2001) reported that the emulsion activity related to pH values. The hydration and swelling properties of the fibers might have affected the emulsion volume. The additions of fibers without lemon fiber were caused significant differences on the ED values compared to control (Table 1, Figure 4). While carrot and wheat fibers increased ED values, inulin, apple, cellulose, oat and pea fibers decreased ED values significantly. Emulsion density might be affected by the size of oil globules and air inclusion because of lower density of oil and air.

The effects of fibers on the ES and ESs (storage stability of the emulsion) values were found to be significant ($p<0.05$). The highest ES and ESs values of beef emulsions were found with lemon fiber (Table 1, Figure 5). Lemon fiber improved stability and storage stability of beef emulsion. Saricoban et al. (2008) reported that raw and dehydrated lemon albedo increased emulsion stability of mechanically deboned chicken meat. Moreover, Aleson-Carbonell et al. (2005) reported that dehydrated lemon albedo increased water retention of meat products. Carrot and oat fibers increased ES values and wheat fiber increased ESs values significantly (Table 1, Figure 5). Researchers (Hughes et al., 1997; Steenblock et al., 2001) stated that oat fiber increased water holding capacity and stability of emulsified meat products. Moller et al. (2011) reported that the addition of carrot fiber has a positive effect on water-binding capacity of processed meat products. Han and Bertram (2017) observed that the addition of dietary fibers improved water binding capacity of the meat batter. Thebaudin et al. (1997) reported that the thickening and gelling properties and the water-retention ability of the polysaccharides contribute to the stabilization of the structure of foods (dispersions, emulsions and foams) by modifying the rheological properties of the continuous phase. Moreover, the thermal process affects globular protein structure and protein-polysaccharide interactions at the surface of protein-coated droplets, with significant implications for stability and rheology (Dickinson, 2003). When ES compared to ESs, the stabilities of beef emulsions containing carrot, lemon or oat decreased during the storage period (Figure 5).

However, stability of beef emulsions containing other fibers increased during the storage period. Storage conditions and duration of the emulsion could affect the emulsion stability.

The effects of the fibers on the EV, AYSe and AYSg values were found to be significant ($p<0.05$). Each fiber without inulin increased viscosity of the beef emulsion significantly (Table 1, Figure 6). Highly soluble fibers such as inulin and oligosaccharides have low viscosity (Biswas et al., 2011). Of all the fibers, lemon fiber increased emulsion viscosity the most. Elleuch et al. (2011) reported that water soluble fibers are the major component that would influence the viscosity of a solution. However, Thebaudin et al. (1997) reported that owing to their water-retention ability and swelling properties, insoluble fibers can influence rheological properties of foods. The protein solution rheological properties, particularly viscosity, are a function of molecular size, shape, flexibility, degree of hydration and intermolecular interactions (Elizalde et al., 1988).

Apple fiber and inulin decreased and other fibers increased AYSe values significantly (Table 1, Figure 7). Of all the fibers, carrot fiber increased AYSe values the most. However, wheat fiber increased and other fibers decreased AYSg values significantly (Table 1, Figure 7). Solubility and chemical structure of fibers plays an important role on the rheological and textural properties of meat products (Biswas et al., 2011). Moreover, viscosity and apparent yield stress of emulsion are changed with coalescence of oil globules or the oil retention ability of protein matrix. Moreover, behaviour of non-protein additives might be influence the behaviour of proteins in meat emulsions. Partial unfolding of globular proteins may make them susceptible to complex formation with hydrocolloids (Dickinson, 2003). The addition of fibers into foods modifies the rheological properties as a function of the processing condition (Thebaudin et al., 1997; Aleson-Carbonell et al., 2005; Fernández-Ginés et al., 2005; Steenblock et al., 2005). The rheological properties of such foods are related to the solubility and hydration (swelling) properties of fibers (Thebaudin et al., 1997). When AYSe values compared to AYSg values, AYSg values were found to be higher than AYSe (Figure 7). An important functional characteristic of proteins is gel forming ability. Myofibrillar proteins play an important role in gel formation after heat treatment. Gel formation contributes to the desirable texture and oil-water stabilisation in emulsified meat products. Also, during gel formation, some components can be retained inside the protein matrix (Ziegler and Acton, 1984). Non-meat proteins, gums and fibers can control and improve the texture and stability of meat emulsions (Meullenet et al., 1994; Lin and Huang, 2003). Decrease AYSe and increase AYSg values might have some advantages in practice. Decreased AYSe values cause easier automation and increasing AYSg values give a strong gel structure.

Table 1. Effects of added fibers on the pH values of homogenate (pH-h) and emulsion properties of beef

Fiber	pH-h	pH-e	EC	EA	ED	ES	ESs	EV	AYS_e	AYS_g
Control	5.49±0.01 ^{abc}	5.59±0.01 ^{a}	80.50±2.12 ^{a}	55.78±1.93 ^{b}	0.908±0.000 ^{bc}	60.97±1.82 ^{bc}	64.50±1.77 ^{ab}	1739±51.62 ^{e}	265.17±4.38 ^{e}	649.06±1.76 ^{b}
Pea	5.35±0.01 ^{c}	5.53±0.03 ^{a}	79.85±1.77 ^{a}	55.77±1.34 ^{b}	0.902±0.002 ^{e}	59.86±0.81 ^{c}	65.00±3.18 ^{ab}	2109±60.10 ^{d}	285.05±3.31 ^{c}	622.98±5.40 ^{c}
Wheat	5.47±0.06 ^{abc}	5.56±0.02 ^{a}	79.95±1.06 ^{a}	50.34±2.16 ^{c}	0.912±0.002 ^{b}	59.99±2.84 ^{c}	63.94±2.21 ^{b}	2085±92.63 ^{d}	273.31±3.03 ^{d}	689.20±1.49 ^{a}
Apple	4.56±0.04 ^{d}	4.65±0.01 ^{c}	71.95±0.78 ^{b}	59.83±1.49 ^{a}	0.906±0.004 ^{bce}	60.14±1.75 ^{c}	63.63±1.59 ^{bc}	2110±65.05 ^{d}	255.74±1.45 ^{f}	379.32±1.74 ^{f}
Carrot	5.37±0.11 ^{bc}	5.43±0.04 ^{b}	67.90±1.84 ^{c}	49.85±0.22 ^{c}	0.921±0.002 ^{a}	72.36±2.32 ^{a}	62.63±0.18 ^{bc}	3112±42.43 ^{b}	315.24±4.69 ^{a}	634.16±6.97 ^{c}
Lemon	4.69±0.04 ^{d}	4.70±0.05 ^{c}	68.10±0.57 ^{c}	60.49±1.27 ^{a}	0.910±0.004 ^{bc}	75.88±1.41 ^{a}	68.38±1.24 ^{a}	3678±97.58 ^{a}	299.10±3.54 ^{b}	571.31±6.98 ^{e}
Oat	5.54±0.11 ^{a}	5.57±0.01 ^{a}	81.10±1.56 ^{a}	53.13±1.47 ^{bc}	0.904±0.003 ^{ce}	64.47±1.46 ^{b}	61.19±2.21 ^{bc}	2101±80.61 ^{d}	276.84±1.96 ^{d}	580.88±6.89 ^{de}
Inulin	5.51±0.05 ^{ab}	5.60±0.05 ^{a}	74.75±0.49 ^{b}	54.30±1.10 ^{b}	0.908±0.001 ^{bce}	58.63±0.18 ^{c}	59.25±1.06 ^{c}	1763±6.36 ^{e}	262.60±4.38 ^{ef}	619.64±8.80 ^{c}
Cellulose	5.48±0.02 ^{abc}	5.58±0.01 ^{a}	81.40±0.57 ^{a}	52.86±0.83 ^{bc}	0.906±0.002 ^{bce}	60.35±0.40 ^{c}	61.01±1.24 ^{bc}	2358±84.85 ^{c}	287.62±3.58 ^{c}	588.31±9.27 ^{d}

pH-h: pH of homogenate, pH-e: pH of emulsion, ES: Emulsion stability, EC: Emulsion capacity, ED: Emulsion density, EA: Emulsion activity, EV: Emulsion viscosity, AYSe: Apparent yield stress of emulsion, AYSg: Apparent yield stress of emulsion gel (AYSg).

$^{a-f}$: Different lowercase letters in a column show significant differences between the groups ($p<0.05$)

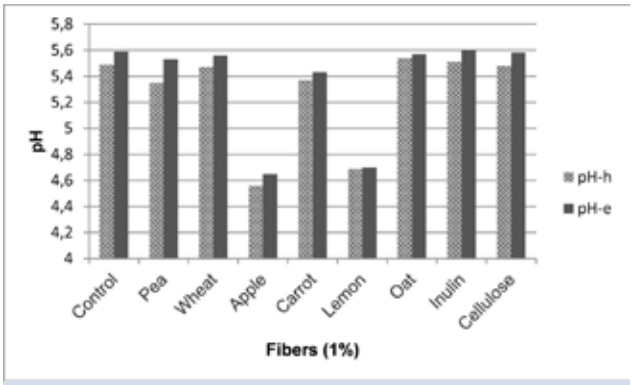

Figure 1. Effects of added fibers on the pH values of homogenate (pH-h) and emulsion (pH-e)

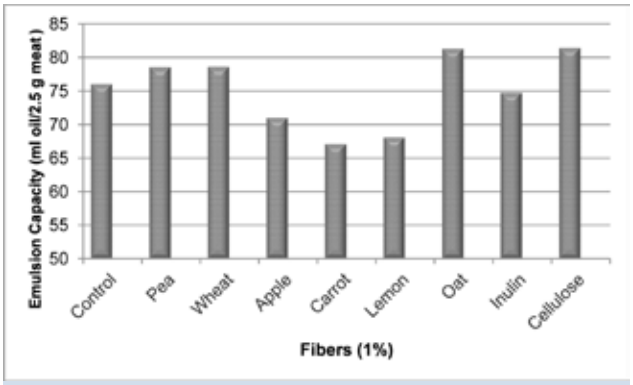

Figure 2. Effects of added fibers on the emulsion capacity

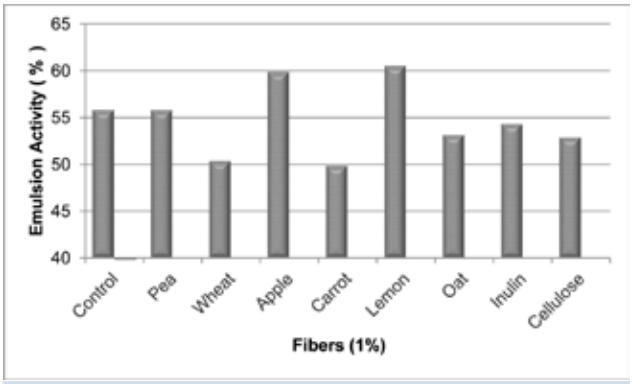

Figure 3. Effects of added fibers on the emulsion activity

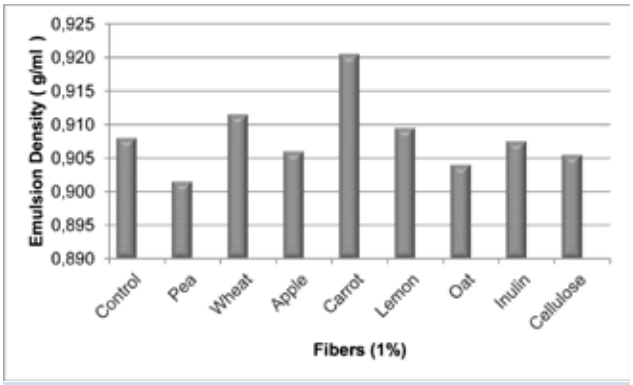

Figure 4. Effects of added fibers on the emulsion density

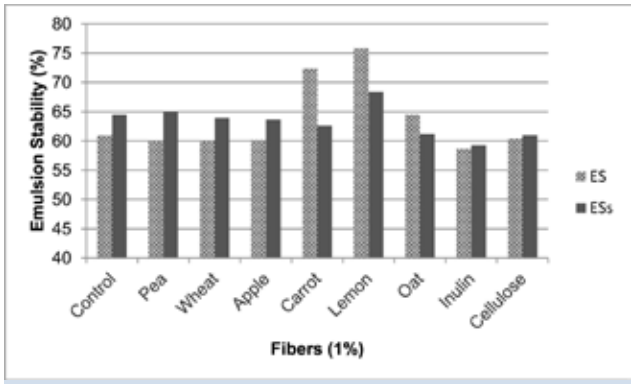

Figure 5. Effects of added fibers on the emulsion stability (ES) and emulsion storage stability (ESs)

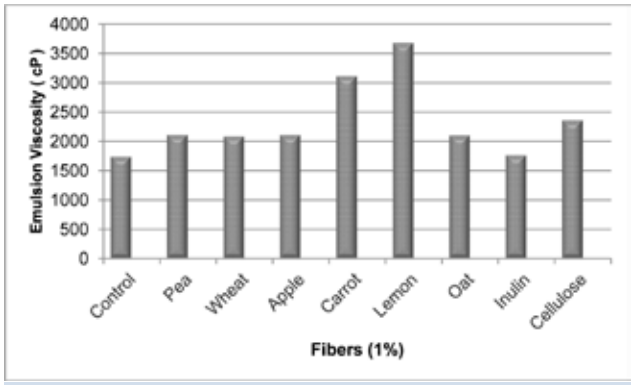

Figure 6. Effects of added fibers on the emulsion viscosity

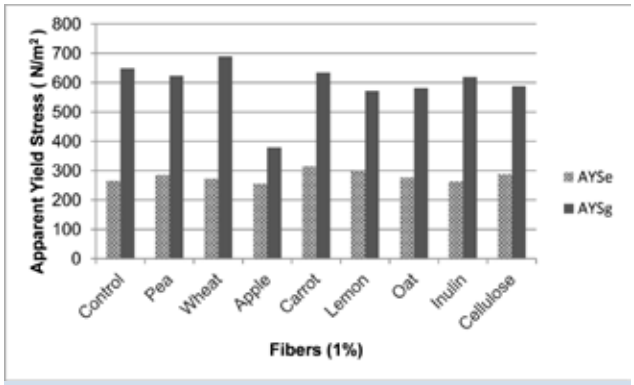

Figure 7. Effects of added fibers on the apparent yield stress values of emulsion (AYSe) and emulsion gel (AYSg)

CONCLUSION

Although oats and cellulose had a positive effect, it was found that the fibers did not improve the emulsion capacity. Emulsion activity was improved by the use of apple and lemon fibers. However, wheat and carrot fibers had a significant effect on the increase of ED. Although the lemon fiber decreased the pH values of the homogenate, it was the fiber that increased the EV and ES values the most. The highest increase in AYSE and AYSg values was caused by carrot and wheat fibers, respectively.

Plant fibers can be used as functional ingredients in emulsified meat products to improve beef emulsion activity, stability, rheology and texture properties. Each fiber has different effects on the different parameters of emulsion. They can be selected according to the emulsion type or desired properties of emulsified products.

CONFLICT OF INTEREST

None declared by the authors.

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Research article
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Harmful effects of dietary supplementation of boron on blood parameters of Rainbow Trout (*Oncorhynchus mykiss*)

M. Öz[1], T. Karaşahin[2], N. H. Aksoy[3], B. E. Inanan[4], S. Dikel[5]

[1]*Department of Fisheries and Diseases, Faculty of Veterinary Medicine, Aksaray University, Turkey*

[2]*Aksaray University, Faculty of Veterinary Medicine, Department of Physiology, Aksaray, Turkey*

[3]*Aksaray University, Faculty of Veterinary Medicine, Department of Biochemistry, Aksaray, Turkey*

[4]*Department of Veterinary Science, Eskil Vocational School, Aksaray University, Aksaray, Turkey*

[5]*Department of Aquaculture, Faculty of Fisheries, University of Cukurova, Adana, Turkey*

ABSTRACT: Even though boron (B), as a trace micronutrient, occurs in natural waters and organisms, its high concentration could cause harmful and even toxic effects for organisms. Lately, studies about positive and negative effects of B on aquatic organisms have been increased with the growing scientific awareness. The aim of the present study was to determine the effects of B-containing feed (0.01%, 0.05%, 0.10%, and 0.20% of B in feed) on hematological and serum biochemical parameters of Rainbow Trout (*Oncorhynchus mykiss*) compared to the control feed without B. Among the most remarkable results, red blood cell, hemoglobin, and hematocrit values were dramatically decreased in the 0.20 % of B group compared to the control ($P<0.05$). Also, activities of liver enzymes increased with the increasing B level in the feed. Consequently, B supplementation (> 0.01%) to feed induced negative changes in blood parameters of rainbow trout.

***Keywords*:** Boron, fish feed, hematological parameters, serum biochemical parameters, rainbow trout

Corresponding Author:
Mustafa Öz, Department of Fisheries and Diseases, Faculty of Veterinary Medicine, Aksaray University, Aksaray, Turkey
E-mail address: ozmustafa@aksaray.edu.tr

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INTRODUCTION

Blood is a major bio-material in assessing the health and disease status of all fish species, just as in all other animals (Coles, 1986; Bush, 1991). Hematological and biochemical parameters of blood in fish are general metabolic, physiological, biological, pathological and biochemical health-disease indicators. These parameters are also important indicators of the effects of environmental stress sources and water eco-system conditions. Fish, which are cold-blooded organisms, are very easily affected by various factors such as environmental and nutritional factors (Lusková, 1997; Gabriel et al., 2011). It is known that factors such as nutrition, stress, seasonal changes, disease, age, gender, species and race affect the physiological values of blood parameters (Fazio et al., 2013; Gabriel et al., 2004).

Rainbow trout (*Oncorhynchus mykiss*), a member of the Salmonidae family, is of high commercial importance and is widely produced and consumed in the world. This species is extensively cultured in various European countries, owing to its rapid growth and high nutrient content (Öz, 2018a; Öz, 2018b; Öz et al., 2017a; Öz, 2016).Boron (B) is considered an essential element for plant growth and development (Ahmad et al., 2009). It is not yet certain whether it is essential for humans and animals. However, after the 1980s it has been examined various studies on human and animal metabolism as a nourishing micro element (Devirian and Volpe, 2003; Yıldız et al., 2009; EFSA, 2013). Recently, it has been defined as a trace element that can affect the metabolism of macromolecules, triglycerides, glucose, amino acids, proteins and estrogenic compounds (Nielsen, 1997). It can have a role in minerals (Kurtoglu et al., 2001), lipids (Eren et al, 2006), energy metabolism (Hunt and Herbel, 1991) and in enzyme and steroid hormone activities (Hunt, 1994; Naghii and Mofid, 2008). Moreover, it is thought that the mechanism of boron mineral, which is thought to be essential for humans and animals in trace amounts, has important functions in lipid metabolism and energy metabolism, immune and endocrine system and brain, positively affects performance, and may be effective in preventing osteoporosis, osteoarthritis and arthritis (Nielsen, 1997). A part of dietary boron intake from nutrition is digested through the gastrointestinal system and accumulated in tissues and organs in various concentrations (Sayli, 2000). Boron concentrations in tissues are increased with the consumption of boron (Rossi et al., 1993).

In recent years, studies on the evaluation of the ecological and nutritional effects of water borne and food borne boron in different fish specieshave increased in number(Öz et al., 2017; Acar et al., 2018; Öz et al., 2018; Alak et al, 2018). These studies have reported that there were some positive effects of boron at certain levels, but higher concentrations of boron caused toxic effects in fish. For this reason, the aim of the current study was to investigate the effects of dietary boron on some hematological and serum parameters of Rainbow Trout (*Oncorhynchus mykiss*).

MATERIALS AND METHODS

Experimental Design and Diet composition

This study was approved by the Animal Experiments Local Committee (no: 4, 24.04.2017). The feeding experiment was carried outin Pozantı, Adana for 132 days. A total of 750 fish (of20.14 ± 1.21 g body weight) were randomly assigned to 15 cages (1 × 1 × 1 m; 10 mm mesh size, 50 individualsper cage) for 5 treatments including a control group.A commercial trout feed produced by Skretting (Stavanger, Norway) was used as the control diet and the base feed for the preparation of the other experimental diets. In a previous study, 0.05% boron in feed has been reported to increase the immune response of Nile tilapia (*Oreochromis niloticus*) (Ardó et al. 2008). Another study in rainbow trout reported that boron supplemented to fish feed enhanced growth (Oz et al. 2018). The dose recommended in these two studies was taken into consideration while determining the boron rates used in our study.Thus, four different diets containing 0.01%, 0.05%, 0.10%, and 0.20% of boron were prepared by boric acid (Sigma–Aldrich, Steinheim, Germany). As described in our previous study (Oz et al., 2018), the powdery boric acid was diluted with 500 mL of water, impregnated by spraying to the feed and the oiled. 5 kg feed batches were dried in the shade and stored in buckets with covers. The fish were fed with the experimental and the control diets two times a day at 08:30 and 16:30, according to visual satiation determined as the fish did not approach the surface when the feeds was offered.

Determination of blood parameters

The fish were anaesthetized by 0.30 ml/L 2-phenoxyethanol (Velísek and Svobodová, 2004), and the blood samples were collected from the caudal vein by vacuum tubes with anticoagulant for hematological parameters and without anticoagulant for biochemical analyses of serums. Serums were separated with cen-

trifugation at 3000 rpm for 10 min (Coles, 1986). The serums were stored in a -20 ºC freezer until biochemical analyzes were performed. The hematological parameters were carried out with the commercial test kits (Mindray V-28 Reagent Kit, China) of Mindray BC-2800-Vet (China) Auto-hematology analyzer. White blood cells (WBC, 10^9 / L), hemoglobin (Hgb, g / dL), Hemotocrit (Hct , %), red blood cells(RBC, 10^{12} / L), mean red blood volume (MCV, fL), mean red blood cell hemoglobin (MCH, pg) and the mean red blood cell hemoglobin concentration (MCHC, g / dL) values were measured. The serum biochemical analyses were determined by colorimetric estimation using semi auto-analyzer (Humalyzer 3000 Semianalizer, Germany) with commercial test kits (Assel, Italy). The measured biochemical parameters were total protein (TP, g/dL), glucose (Glu, mg/dL), albumin (Alb, g/dL), globulin (Glu, g/dL), urea (Ure, mg/dL), aspartate amino transferase (AST, U/L), alanine aminotransferase (ALT, U/L), alkaline phosphatase (ALP, U/L). Also, albumin and globulin ratios (A/G) were calculated for the samples.

Statistical analysis

The data are expressed as means±standard deviation (SD). Significance differences in the treatments were determined by one-way analysis of variance (ANOVA), followed by a Tukey's pair- wise multiple comparison test using SPSS 15.0 (SPSS, Inc., Chicago, USA) software. Statistical significance was established at $P<0.05$.

RESULTS

The effects of dietary boron on the hematological and serum biochemical parameters are presented in Table 1 and 2, respectively. Significant differences were found in all treatments regarding all blood parameters. WBC values were found similar in all treatments. RBC, Hgb, and Hct values were dramatically decreased ($P<0.05$) while the boron concentrations were increased in the feed. The highest values were found in the control group, while the lowest values were observed in the 20% boron treated group. MCV, MCH, and MCHC values were increased in the 0.20 % of boron group. Decrease in the serum TP, Alb, Glu, and Ure values were determined compared to the control ($P<0.05$). Also, A/G values were found significantly lower in the 0.20 % of boron group when compared to others ($p<0.05$). Glu values and the activities of liver enzymes the 0.20 % of boron group were higher than the control and the other boron groups.

Table 1. Hematological parameters of rainbow trout (*Oncorhynchus mykiss*) fed diets containing boron for 132 days.

Parameters	Control	0.01% B	0.05% B	0.10% B	0.20% B
WBC (10^9/L)	*26.40±0.07^a*	*25.26±0.07^d*	*24.99±0.06^e*	*25.75±0.03^c*	*26.00±0.04^b*
RBC (10^{12}/L)	*1.64±0.01^a*	*1.09±0.02^b*	*0.94±0.00^c*	*0.92±0.02^d*	*0.82±0.00^e*
Hgb (g/dL)	*11.03±0.13^a*	*9.05±0.01^b*	*8.86±0.02^c*	*8.52±0.01^d*	*8.37±0.00^e*
Hct (%)	*30.44±0.01^a*	*28.32±0.01^b*	*27.48±0.18^c*	*25.11±0.02^d*	*22.64±0.03^e*
MCV (fL)	*186.12±0.81^d*	*260.19±4.10^c*	*291.86±1.83^a*	*273.52±6.42^b*	*276.18±0.90^b*
MCH (pg)	*67.40±0.84^e*	*83.18±1.30^d*	*94.12±0.44^b*	*92.81±2.20^c*	*102.18±0.25^a*
MCHC (g/dL)	36.21±0.43^b	31.97±0.01^e	32.25±0.23^d	33.93±0.04^c	36.99±0.01^a

WBC (white blood cell), RBC (red blood cell), Hgb (hemoglobin), Hct; (hematocrit), MCV (mean red blood cell volume), MCH (mean red blood cell hemoglobin), MCHC (mean red blood cell hemoglobin concentration)

Table 2. Serum biochemical parameters of rainbow trout (*Oncorhynchus mykiss*) fed diets containing boron for 132 days.

Parameters	Control	0.01% B	0.05% B	0.10% B	0.20% B
TP (g/dL)	3.56±0.15^a	3.19±0,05^b	2.93±0.08^c	2.68±0.04^d	2.08±0.11^e
Alb (g/dL)	1.58±0.02^a	1.41±0.01^b	1.28±0.02^c	1.17±0.02^d	1.04±0.07^e
Glo (g/dL)	1.98±0.15^a	1.78±0.06^b	1.65±0.09^c	1.51±0.04^d	1.40±0.07^e
A/G	0.80±0.05^a	0.79±0.03^a	0.78±0.04^a	0.77±0.03^a	0.74±0.07^a
Glu (mg/dL)	70.63±1.26^e	82.36±1.55^d	92.04±1.55^c	100.56±1.54^b	108.09±2.17^a
Ure (mg/dL)	4.51±0.08^a	4.16±0.07^b	3.84±0.05^c	3.20±0.06^d	2.97±0.11^e
ALP (U/L)	81.84±84.67^a	102.16±0.83^b	123.95±5.13^c	149.21±3.89^d	193.87±11.19^e
ALT (U/L)	12.71±0.68^e	16.59±0.53^d	18.33±0.40^c	21.23±0.33^b	27.87±0.85^a
AST (U/L)	325.57±4.94^a	355.61±3.53^b	375.88±11.82^c	421.87±8.52^d	474.82±12.15^e

Total protein (TP), glucose (Glu), albumin (Alb), globulin (Glo,), urea (Ure), aspartate amino transferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP). Also, albumin and globulin ratios (A/G)

DISCUSSION

The increase in WBC indicates that fish increase their defense capacity against microbial or bacterial infection. In the present study, WBC values were found higher than those in previous studies in rainbow trout (Atamanalp et al., 2008; Talas and Gülhan 2009; Zorriehzahra et al., 2010; Bianchi et al., 2014; Khodadadi et al., 2018). Similar results were found regarding WBC values obtained from fish exposure to cobalt chlorite (Atamanalp et al., 2011). RBC values in the current study were found higher than in some studies (Atamanalp et al., 2008, Atamanalp et al., 2011) and lower than in some studies (Vazquez and Guerrero, 2007; Bianchi et al., 2014; Qadir et al., 2014). RBC values were found to have similar values in some studies(Talas and Gülhan, 2009, Zorriehzahra et al., 2010; Kankaya and Kaptaner, 2016). The number of erythrocytes decreases as the amount of boron in feed increases. It should be taken into consideration that the addition of boron minerals to the feed in small amounts and in short intervals stimulates the body defense system in fish while minimizing the decrease in erythrocyte count. The Hgb values obtained in the study showed a statistically significant decrease as the amount of boron mineral. Despite this decrease, the Hgb value obtained from all groups was higher than some studies (Vazquez and Guerrero, 2007; Atamanalp et al., 2008; Talas and Gülhan, 2009; Atamanalp et al., 2011; Kankaya and Kaptaner, 2016; Bianchi et al., 2014). The values found in the control group were higher than the values reported by Zorriehzahra et al., (2010), Fazio et al., (2013) and Cakici and Aydin (2006).

The reduction in MCV suggests that boron mineral may interfere with normal physiology of RBC (Latif et al., 2015). The main constant values of erythrocytes, MCV, MCH and MCHC are important parameters for the assessment of adaptation of respiratory function to practical oxygenation conditions in water (Radu et al., 2009). In terms of MCV, MCH and MCHC, there are values given in literature (Çelik et al., 2006). The increase of MCV, MCH and MCHC indicate that there is a relevant reaction. MCV, MCH and MCHC values were obtained by investigating and while their values were found to be high (Latif et al., 2015, Bianchi et al., 2014, Charoo et al., 2014), the MCV and MCH values are found lower by researching (Altun and Diler, 1999).Some researchers reported thatin *Channa marulius*MCV and MCH values were different in different seasons (Latif et al. 2015). In the literature, very different reports cause difficulties in the evaluation of hematologic values in fish physiology. It is necessary to prevent this confusion with studies conducted with a large number of animals.

In a review published in 2007, the mean values of some biochemical parameters commonly used in different healthy fish species were provided (Celik and Bilgin, 2007). According to this review, the published values were: total protein 3,49 ± 1,007 (0,10-7,50) g / dL, albumin 1,23 ± 0,639 (0,10-3,20) g / dL, globulin, 2,38 ± 0,664 (0,40 -4,37 g / dL, urea 5,33 ± 4,056 (0,00-18,00) mg / dL. Based on this information, the amount of serum TP 3.56 g / dL measured in the control group is within normal physiological limits; the amount of Alb (1.58 g / Dl) is higher than normal and globulin (1.98 g / Dl) (Table 2) is observed as lower than normal values. When the literature was reviewed, it was observed that *O. mykiss* TP values differed even in the same type:

The lowest serum TP amount mentioned in literature was 0.88 g / dL (Handy et al., 1999), while it was reported as 4.14 g / dL. in the study of Shimma et al. (1984). The results of TP (Giles, 1984) reported as 3.60 g / dL in the control group in literature are consistent with the results in the control group of our study. Furthermore, low serum protein may result from increased proteolytic activity in order to compensate for increased energy demand for coping with molecular stress which may be caused by high doses of boron.

Albumin, while increasing in the rate of dehydration, is quite reduced in cases of liver, kidney and circulatory system diseases or in cases of malnutrition and intoxication. Some of globulinsare connected to hemoglobin, and globulins are responsible for the transport of metals such as iron in blood and help with the anti-inflammatory system (Kaneko et al., 2008). The albumin / globulin ratio is used to identify the causes of changes in total serum protein. However, it is not a specific indicator in the diagnosis due to not showing which particular proteins are changed. The specific A / G ratio in mammals is between 0.8-2.0. In all living organisms, this ratio increases in cases where albumin increases, while the ratio decreases in the table where globulin increases (Kaneko et al., 2008).

In parallel to the total protein decrease, Alb and Glo values decreased in 4 experimental groups in comparison to the control group (table 2). This is quite natural if the total protein content is considered to be a

total of Alb + Glo + fibrinogen. Protein catabolism for the homeostatic balance of protein metabolism and energy cycle may be reflected in blood parameters in the forms of intense enzymatic activities, protein degradation in response to stress, possibly liver damage due to chemical materials and the decrease in serum protein.

Average reported urea values in rainbow trout grown in three different environments were: 4-10 mg / dL in *Oncorhynchus mykiss* raised in natural environment, 5-11 mg / dL for ones in cages and 5-8 mg / dL for ones raised in pools (Ural et al, 2013). The urea value reported here in the control group is in accordance with the reference values. The amount of urea measured in the control group of our study was 4.51 mg / dL, which was slightly less than the value 5.33 mg / dL which was reported in a review on the mean biochemical parameters in all fish species by Çelik and Bilgin (2007).

From this point of view, it can easily be said that the low urea level found in our study primarily indicates gill and kidney dysfunction, especially if we keep in mind that blood urea nitrogen and urea levels are important metabolic products of protein catabolism in fish.

Enzymes are proteins that convert substrates into products. It is clinically important to know the serum levels of various enzymes. The presence of these enzymes in serum (low and high amounts) indicates that damage to cells occurs and causes intracellular components to be released into the blood. It is important to measure the enzyme levels in serum or plasma to detect, diagnose and monitor diseases, monitor status and control of treatment, and to detect tissue response to toxic substances exposed. In the bone, alkaline phosphatase (ALP) allows the progression of mineralization by destroying the pyrophosphate, a potent inhibitor of mineralization in bone tissue. It is also involved in the detoxification with the *invivo* dephosphorylation of bacterial endotoxin, especially in the liver and intestines. They are varying values according to species, subspecies, regional changes, age and gender differences. They are reliable indicators for liver damage (Kaneko et al., 2008). Transminases (AST and ALT) are critical and important enzymes in biological processes. ALT and AST enzymes are frequently used in the diagnosis of damages caused by pollutants (liver, kidney, intestine, placenta) in different tissues of fish (Sastry and Subhadra, 1985; Kaneko et al., 2008).

In the present study, aminotransferases (AST and ALT) and alkali phosphatase (ALP) values have also been researched as liver enzymes. In the control group ALP values have been found as 81.84 U/ L, AST values as 325.57 U/L and ALT values as 12.71 U/L. A increase has been observed in the values of ALP, ALT and AST due to increasing boron concentrations in the experimental groups. In the study, published in 2016, which researched the effects of permethrin insecticide on fish it has been reported that *O. mykiss* control group had AST values of 125,6 and ALT values of 14.5 U/L (Mozhdeganloo et al, 2016).

As is known, metals can alter the enzyme activity by binding enzymes to functional groups such as sulfhydryl, carboxyl, or inhibit the activity of the enzyme by replacing the metal in the active site of the enzyme (in metal-containing enzymes such as ALP). Increased levels of heavy metals in living organisms cause the formation of ROS (Reactive Oxygen Species) which causes lipid peroxidation, inhibition of enzymes and DNA damage. Especially serum levels of liver enzymes are also used in clinical biochemistry as a stress indicator (Kaneko et al., 2008). Enzymes used as biomarkers of aquatic contamination are frequently used to determine the effects of environmental pollutants on the organism due to their sensitivity to the effects of very low pollutants and their immediate response (Sastry and Subhadra, 1985). Significant changes in serum activities of these enzymes express the target tissue damage caused by stress.

The concentration-physiological response curve of boron mineral on living things can vary for many animal species. While negative effects can be observed in very high and very low concentrations, there is no negative effect at the intermediate concentrations and it has been determined that there are positive effects in physiological doses. The most sensitive tests report that the acute effects on the fish are in the range of 10-20 mg-B / L. Toxic effects of boron compounds on aquatic organisms such as fish, insects, molluscs etc. have also been reported (Loewengart, 2001). Although these studies provide toxicological assessments of the boron, there is still no sufficient information about the metabolic systematic and toxic effects of boron. Our study demonstrated that boron mineral can have a nutritional compound effect until certain dosages while it can also show toxic effects above a certain dose.

This type of descriptive and determinant biochemical research in animals in different countries, or even

in different regions of the same country, provides useful information for academic perspectives as well as for clinical studies (Kaneko et al., 2008).

CONCLUSION

When all parameters were taken into consideration, it was observed that differences in region, race, sex, age, season and nutrition sources affect hematological and biochemical values and cause changes. The detection and monitoring of hemo-biochemical parameter values reflecting the metabolic profile may indicate whether homeostatic mechanisms can maintain blood composition under different conditions (different races, different regions & areas, different feed-nutrition regimes, different age and gender characteristics) in physiological limits.

It is very important to include the physiological, biochemical and hematological values among the investigations that complement and support the clinical findings in a way to shed light on academic, clinical and economic scientific studies. At this point, it will be the most useful approach to determine, calculate and use hemo-biochemical reference ranges of species with racial and regional differences.

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CONFLICT OF INTEREST

None declared.

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Research article
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Effect of embryo quality on pregnancy outcome in recipient cows and heifers

U. Kara[1], S. Çoban[4], E. Say[1], H. Hızlı[1], H. Mutlu[1], Y. Nak[2], H. Sağırkaya[3], T. Ayaşan[5], M. A. Çoşkun[1], A. Asarkaya[1], H. E. Erten[1], İ. Yilmaz[1], E. Yazgan[1], İ. E. Erdoğan[1], Z.M.Erten[4], Ş. Ergül[1], F. Sert[1]

[1]Eastern Mediterranean Agricultural Research Institute, Adana, Turkey

[2]Uludağ University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology, Bursa, Turkey

[3]Uludağ University, Faculty of Veterinary Medicine, Department of Reproduction and Insemination, Bursa, Turkey

[4]Food Control Laboratory Directorate, Adana, Turkey

[5]Osmaniye Korkut Ata University, Kadirli Academy of Applied Sciences, Osmaniye, Turkey

ABSTRACT: This study was performed to compare the effects of embryo quality on pregnancy outcome in recipient cows and heifers. Embryos obtained from 83 *Holstein* donors were used in the study. In order to synchronize the recipients, 500 µg of cloprostenol were administered intramuscularly (i.m.), twice, at an interval of 11 days. In all recipients, one embryo was transferred to the upper 1/3 of the uterine horn ipsilateral to the ovary with the corpus luteum on the 7th day after oestrus onset (day 0). Each recipient received an i.m. injection of 5 µg of buserelin at the time ofembryo transfer. Pregnancy examinations were performed by ultrasonography on the day 30th post-transfer. A total of 345 (262 grade 1, 64 grade 2 and 19 grade 3) fresh embryos were transferred to 171 recipient cows and 174 recipient heifers. The pregnancy rates of the recipient cows and heifers were 41.52% and 52.29%, respectively ($p<0.05$).The pregnancy rates achieved with first, second and third quality grade embryos were 45.16%, 25.0% and 12.5% in the cows ($p<0.05$), and 55.11%, 41.66% and 54.54% in the heifers ($p>0.05$), respectively. In conclusion, the pregnancy rates was significantly higher in heifers than in cows ($p<0.05$) and, the embryo quality had a significant impact on recipient pregnancy outcome ($p<0.05$). The effect of embryo quality on pregnancy was significant in cows ($p<0.05$), but not significant in heifers ($p>0.05$).

Keywords: Embryo transfer, embryo quality, pregnancy rate, cows, heifers

Corresponding Author:
Uğur Kara, Doğu Akdeniz Tarımsal Araştırma Enstitüsü, Karataş Yolu 17. Km. Doğankent Mah. Yüreğir-Adana, Turkey
E-mail address: ugurvetkara@hotmail.com

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INTRODUCTION

Embryo transfer (ET) is a prominent technique used to shorten the generation interval of genetically valuable individuals, and thereby, to increase herd potential through the transfer of targeted traits to the offspring (Hasler, 2014). While artificial insemination enables genetic improvement with the use of valuable male breeders, ET renders it possible to make use of the genetic potential of high-producing female breeders (Tekin, 2007). Today, ET is a well-established method, which has found common use. Research on the use of multiple ovulation and embryo transfer (MOET) in the bovine has increased the pace of genetic selection and shortened the generation interval via the progeny of high-producing females and genetically superior bulls (Mapletoft and Hasler, 2005). The establishment of an industry specialized in commercial embryo production and transfer has brought about major advances in the technology and practical use of ET (Roper et al., 2018). Today, each year approximately 1.25 million transferable embryos are produced worldwide from high-producing females in vivo and in vitro. Of these embryos, nearly a million are transferred per year. According to data published by the International Embryo Transfer Society (IETS), as of 2016, the mean number of transferable embryos recovered in vivo per donor per flushing ranges from 6 to 7 (Perry, 2017).

Two important factors, affecting the success of ET in cattle, are the selection and the management of recipients. Although the improved management and nutrition of recipients increase the cost of ET, they also increase the profitability of ET by increasing the resulting pregnancy rate (Siqueira et al., 2009). The variability in the pregnancy outcome of embryo transfer is considered to be associated with embryonic, maternal and environmental factors and variances in the combination of these factors. The most significant among these factors are the skill and experience of the practitioner performing ET, the quality of the transferred embryo, and the fittingness of the recipient (Hasler, 2001). Multiple factors, including among others, poor embryo quality, temporal incompatibility between the recipient female, the embryo and uterine enviroment, and inadequate uterine-embryonic interaction, may cause embryonic death (Hasler, 2001). Furthermore, it is reported that the particular site, where the transferred embryo is deposited in the uterus, as well as the complication score of the transfer procedure and the length of time required for embryo transfer also influence the pregnancy outcome (Ferraz et al., 2016). Pregnancy rates achieved with ET in beef and dairy heifers are similar (Hasler, 2001). Nonetheless, pregnancy rates achieved with the transfer of fresh and frozen-thawed embryos are lower in dairy cows, compared to dairy heifers and beef cows and heifers (Putney et al., 1988).

This study was carried out at the premises of the Eastern Mediterranean Agricultural Research Institute under the “Project for the Improvement of the Anatolian Multi-coloured Cattle” with the aim to assess the superovulatory response of dairy donors and compare the pregnancy rates, achieved after the transfer of different-quality-embryos to recipient cows and heifers.

MATERIAL AND METHODS

Animals

Donor animals used in this study were regularly cycling 3 to 7-year-old, healthy, 3-3.5 body condition scored, 500-550 kg body weight, <8500 L first lactation milk yield. Holstein cowswhich were raised at the Research Farm of the Eastern Mediterranean Agricultural Research Institute; they were reproductively sound, regularly cycling, had given birth at least once once and were at <100 days of postpartum stage. The recipients were selected among healthy and reproductively sound Holstein cows (2 to 5 year-old, at <80 days of postpartum stage and in their 1st-3rd lactation) and heifers (over 370 kg body weight). All animals were fed a total mixed ration (TMR) that consisted of wheat straw, alfalfa hay and corn silage as roughage supplemented with concentrate. Donor and lactating recipient cows were fed with 63.21% roughage and 36.79% concentrate (containing 2700 kcal / kg ME and 19% crude protein). Recipient heifers were fed with 74.60% roughage and 25.40% concentrate (containing 2600 kcal/kg ME and 14% crude protein). Prior to the study, each animal underwent uterine and ovarian examination by ultrasonography (5 MHz, Honda HS-101V, Japan). The experiments on animals were conducted in accordance with local Ethical Committee laws and regulations as regards care and use of laboratory animals.

Superovulation

In total, 83 Holstein cows were used as donors. Some of the donors underwent superovulation treatment more than once (55 donors once, 18 donors twice, eight donors three times and two donors four times) a total of 120 uterine flushings were performed. For superovulatory treatment, between days 8-12 of

the sexual cycle, the donors received a total amount of 400 mg FSH (Follitropin V, Bioniche Animal Health Canada Inc, Canada), which was administered by i.m., twice daily for 4 days, at decreasing doses (Day 1 - 80/80 mg, Day 2 - 60/60 mg, Day 3 - 40/30 mg, and Day 4 - 30/20 mg). Two i.m. doses of 500 μg of cloprostenol (Esrumate, Schering Plough/Essex Animal Health, Sedelsberger Strasse 2, 26169 Friesoythe-Germany; Lutelen, Topkapı Pharmaceuticals and Premixes Industry and Trade Inc., Turkey) were co-administered with the 5th and 6th FSH injections. Oestrus monitoring and control of the donors were performed. Oestrus onset was observed 12 h after last FSH injection. Starting from the 12th hour after the last FSH injection, each donor was inseminated (bulls were selected from Holstein top 100 list and each dose contained at least 7 million motile spermatozoa) three times at an interval of 12 h. At the time of the second insemination, all cows were given 10 μg of buserelin (Receptal, Veterinary Pharmaceuticals Marketing and Trade Inc., Turkey) by i.m. route.

Collection and evaluation of embryos

Uterine flushings were performed seven days after the second insemination. Prior to this procedure, each donor cow underwent ultrasonographic examination (5 MHz, Honda HS-101V, Japan and 5 MHz, Honda HS- 2000VET, Japan) to determine the total numbers of corpora lutea (CL) and follicles in the ovaries.

Flushings were performed using 1000 ml of lactated Ringer's solution (Ringer-Fleks, Eczacıbaşı-Baxter Hospital Products Industry and Trade Inc., Ayazağa/Istanbul) containing 1% calf serum (Foetal Bovine Serum Sigma F 9665, Germany) and 0.1% kanamycin (Kanovet, Vetaş Veterinary Pharmaceuticals and Pesticides Joint Stock Company, Küçükçekmece/Istanbul) (13). Prior to the flushings, epidural anaesthesia was established with 4 ml of a local anaesthetic (L-Anestin, Alke Health Products Industry and Trade Inc., Turkey). After the flushings were completed, each donor received 500 μg of cloprostenol i.m. and 500 mg cephapirin benzathine (19 g, Metricure, MSD Animal Health, Turkey) intrauterinely.

Embryo quality was assessed based on morphological integrity, in line with the guidelines of the International Embryo Transfer Society (IETS) (Robertson and Nelson, 2010). The recovered flushing medium was examined under a stereomicroscope to grade the embryos for their quality and morphology. Embryos of first, second and third grade quality were considered to be transferable. The transferable embryos were washed three times in a solution containing TCM-199 (Sigma-M7528, Germany), L-glutamine (Sigma-G5763, Germany), Gentamicin (Sigma-G1264, Germany) and 20% Fetal Calf Serum (38.5 °C, 5% CO_2) and transferred to recipients within 6 hours.

Recipient synchronization

The recipient heifers and cows received two IM injections of 500 μg of cloprostenol, at an interval of 11 days, for oestrus synchronization. The animals were monitored twice daily for signs of oestrus. The oestrus starting time difference between the recipients and the donors was at maximum ±24 hours. The second cloprostenol injection to recipient cows was performed synchronous to the first cloprostenol injection to donors (5th FSH injection). In the case of recipient heifers, the second cloprostenol injection was performed synchronous to the second cloprostenol injection to the donors (6th FSH injection). Epidural anaesthesia was established prior to embryo transfer.

Embryo transfer

The embryos were deposited in the uterine horn ipsilateral to the ovary with the corpus luteum (CL) on the 7th day after oestrus (Day 0). Following the embryo transfer procedure, the recipients were administered with 5 μg of buserelin acetate by intramuscular route. Pregnancy examinations were performed by ultrasonography on the 30th day after embryo transfer.

Statistical analyses

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, Version 20.0. The pregnancy rates of the recipient cows and heifers were compared with the t test. The Kruskall-Wallis test was used to determine the impact of embryo quality on recipient pregnancy outcome and to make a comparison of this impact between cows and heifers. The findings are presented as means and percentages, and mean values are expressed with their standard error or standard deviation.

RESULTS

In this study, 83 Holstein cows were used as donors. Some of the donors underwent superovulation treatment more than once (55 donors once, 18 donors twice, eight donors three times and two donors four times), and in total 120 uterine flushings were performed. Four of the donors did not respond to superovulation treatment, as was confirmed by the absence

of two or more corpora lutea in their ovaries on the day of flushing. On the other hand, from eight of the donors that responded to superovulation treatment, as was confirmed by the presence of two or more corpora lutea in their ovaries on the day of uterine flushing, yet no embryo was recovered from their flushing medium. Pre-flushing ovarian findings, post-flushing embryo recovery rates (Total Ova and Embryos / Total Number of CL x 100) and the numbers of embryos of the different quality grades are presented in Table 1.

Table 1. Ovarian findings, embryo recovery rate and numbers of the different quality grade embryos (Mean±SD), after superovulation of Holstein cows.

Parameter	Value
Number of Donors	83
Number of Uterine Flushings Performed	124
Total Number of CL	1264
Total Number of Follicles	334
Number of First Quality Embryos	471
Number of Second Quality Embryos	90
Number of Third Quality Embryos	21
Number of Unfertilized Oocytes	280
Number of Degenerate Embryos	94
Embryo Recovery Rate (%)	75.63
Mean Number of CL in Ovaries Per Uterine Flushing	10.24 ± 6.41
Mean Number of Follicles in Ovaries Per Uterine Flushing	2.70 ± 3.01
Mean Number of Total Ova/Embryos Achieved Per Uterine Flushing	7.72 ± 7.23
Mean Number of Transferable Embryos Achieved Per Uterine Flushing	4.71 ± 5.86
Mean Number of Dejenere Embryos Achieved Per Uterine Flushing	0.75 ± 1.49
Mean Number of Unfertilized Oocytes Achieved Per Uterine Flushing	2.25 ± 4.60

The pre-flushing total numbers of CL and follicles, and the mean numbers of transferable embryos, degenerate embryos and oocytes are presented in Table 2.

Table 2. Corpora Lutea and follicles recorded, and total ova/embryos, transferable embryos, degenerate embryos and oocytes collected (mean ± SE), after superovulation of Holstein cows.

Donor data	Total Number of Uterine Flushings (n=124) X± SE
Total Number of CL	10.24 ± 0.57
Total Number of Follicles	2.71 ± 0.27
Total Number of Ova/Embryos	7.72 ± 0.64
Transferable Embryos	4.71 ± 0.52
Degenerate Embryos	0.75 ± 0.13
Unfertilized Oocytes	2.25 ± 0.41

X: Mean, SE: Standard Error

In this study, a total number of 582 transferable embryos (of 1st, 2nd and 3rd grade quality) were recovered. Of these embryos, 174 were transferred to recipient heifers and 171 were transferred to recipient cows. The onset of oestrus in all recipients was detected ± 24 hours to that of the donors. The pregnancy number and rate achieved in the heifers were higher than those achieved in the cows (91 - 52.29% and 71 - 41.52%, respectively). The remaining embryos, which were not transferred, were frozen for future use. As can be seen in Table 3, the pregnancy numbers and rates of the recipient cows and heifers differed significantly ($P<0.05$).

Table 3. Single embryo transfers performed and pregnancy rates achieved in the recipient cows and heifers.

Recipient	Number of Transfers	Number of Pregnancies	Pregnancy Rate (%)	P Value
Heifer	174	91	52.29	0.045
Cow	171	71	41.52	0.045
Total	345	162	46.95	

The distribution of the pregnancy rates achieved with respect to the recipient group and embryo quality grade is presented in Table 4. Overall, it was determined that the impact of embryo quality on recipient pregnancy outcome was statistically significant ($p<0.05$). While embryo quality was observed to have a statistically significant effect on pregnancy in recipient cows ($p<0.05$), it was ascertained that the effect of embryo quality on pregnancy was statistically in significant in recipient heifers ($p>0.05$).

Table 4. Pregnancy rate distribution for the different recipient groups after single transfer of grade 1, 2 and 3 embryos.

Recipient	First Grade Embryos			Second Grade Embryos			Third Grade Embryos			P Value
	Number of Transfers	Number of Pregnancies	Pregnancy Rate (%)	Number of Transfers	Number of Pregnancies	Pregnancy Rate (%)	Number of Transfers	Number of Pregnancies	Pregnancy Rate (%)	
Heifer	127	70	55.11	36	15	41.66	11	6	54.54	0.359 P>0.05
Cow	135	63	45.16	28	7	25.0	8	1	12.5	0.025 p<0.05
Total	262	133	50.76	64	22	34.37	19	7	36.84	0.042 p<0.05

DISCUSSION

The success of embryo transfer (ET) procedures depends on the recovery of multiple high-quality embryos from donors and the achievement of the targeted pregnancy and calving rates with the transfer of these embryos to favourable recipients. Several studies have been carried out with the aim to increase both the number of embryos recovered from superovulated animals and the rate of pregnancy achieved after ET in recipients. In order to increase the response to superovulation, protocols have been developed after the synchronization of follicular wave emergence in the donors. In addition, fixed-time embryo transfer programs are implemented in which recipients can be synchronized (ovysynch or ovysynch + P4 etc.) without the need for oestrus detection (Bó et al., 2012).

Several researchers have reported embryo recovery rates of 60% to 70% (Barati et al., 2006; Bülbül et al., 2010). In our study, uterine flushing was performed by fixing the catheter 5 cm from the bifurcatio uteri, as indicated by Bülbül et al. (2010). These previously reported rates being lower than the mean embryo recovery rate in the present study (75.63%) could be attributed to possible differences in several factors, such as the day of embryo recovery, the type of catheter used for embryo recovery, the position of the catheter during uterine flushing, and the skill/experience level of the practitioner performing the flushing procedure, similar to the influential factors suggested in literature (Bülbül et al., 2010).

The success of embryo production in cattle depends on many factors. Donor age, breed, lactation status and milk yield are important. The hormones used for the induction of superovulation affect the success of the application. Another important factor is the difference between starting time of FSH administration (usually 8-12th days of the estrous cycle) and the time of follicular wave emergence (Kaymaz M, 2015). Inaddition, the number, method, dose and duration of FSH administration (Kaymaz M, 2015; Lovie et al., 1994), initiation of FSH administration during the emergence of different follicular waves (Lovie et al., 1994), the effectiveness of hormones and methods used to synchronize wave emergence are factors that affect embryo production (Bó et al., 1995; Wiley et al., 2019).

According to data published by the IETS (1998-2016), the mean number of transferable bovine embryos recovered per flushing ranged from 5.50 to 6.90 at a global level (Perry, 2013; Perry, 2014 ; Perry, 2015; Perry, 2016; Perry, 2017). The reasons for low mean numbers of transferable embryos recovered in the present study (4.71) compared to above studies of other researchers could be related to several widespread factors such as selected breed, yield, and superovulation method, as well as to animals non-responsive to superovulation and the donor animals from which embryos were not recovered. In addition, the IETS data report the average results of beef and dairy donors worldwide while only dairy cattle were used in our study. Kim et al. (2001) induced superovulation to Holstein donors after the aspiration of dominant follicle in the first group and on the 8th day of the estrous cycle in the second group. Their recorded 9.6±1.1;6.1±0.9 CL, 7.7±1.3;3.9±1.0 oocytes and embryos and 4.6±0.9;2.3±0.8 transferable embryos for each flushing, in the two study groups, respectively. The results obtained in the first group are consistent with our results. The inferior results obtained in the second group may be due to the differences in starting time of FSH administration in relation to the follicular wave emergence.

Another major factor, which affects the success of ET, is the recipient animal. In bovine ET practice, heifers and cows are used as recipients (Schmidt, 2010). It has been reported that pregnancy rates achieved in recipient heifers are 10% to 23% higher than those achieved in recipient cows (Hasler, 2014). On the other hand, the use of heifers as recipients presents with disadvantages such as dystocia and difficulties

in calving management and calf care. Although pregnancy rates achieved in cows are lower, 3 to 8-year-old cows are recommended to be used as recipients in view of the advantages they offer, including higher milk yield, known reproductive history, and colostrum production (Schmidt, 2010). It has been reported that the pregnancy rates achieved with ET in cattle range from 50% to 70% (Gordon, 2005). Assumed that the practitioners performing the procedure have the required technical skills, the main factors that affect the pregnancy rate achieved with ET are embryo quality and recipient suitability (Hasler, 2004). In addition, the synchronization between donor and recipient estrus is very important for pregnancy success in embryo transfer (Hasler, 2014). In cases where the difference in the time of estrus onset between the donor and recipient does not exceed ± 24 hours, the success of pregnancy is not affected highly (Kanagawa, 1995; Hasler, 2014).

In the present study, the mean pregnancy rate achieved in the recipient heifers (91/174, 52.29%) was significantly higher ($P<0.05$) than in the recipient cows (71/171, 41.52%). It has been reported that higher pregnancy rates are achieved with the transfer of either fresh or frozen-thawed embryos in heifers than in cows (Hasler, 2001). Lower pregnancy rates in cows have been attributed to lactation-related management requirements and metabolic factors (Hasler, 2005). High-producing dairy cows suffer embryonic death mostly during the first two weeks after fertilization due to physiological and metabolic changes associated with negative energy balance. This high early embryonic mortality has been attributed to the impact of the poor follicular microenvironment on oocyte and embryo quality as well as to the suboptimal uterine environment provided to the embryo and inadequate maternal-embryonic interaction (Lonergan et al., 2016). Köse et al. (2006) have reported pregnancy rates of 27.2% and 56.5% in Brown Swiss-cows and heifers, respectively, following the transfer of in vivo-produced fresh embryos. High pregnancy rates achived in the present study compared to previous studies, after the transfer of fresh embryos, could be related to embryo quality and supportive hormone treatment (GnRH) to increase progesterone levels of recipients at time of ET. Furthermore higher results previously obtained in recipient heifers compared to the present study could be related to different types of cattle breeds (meat or mixed-type) invovled. Julón et al. 2012 reported pregnancy rates of 54.1% and 34.6% in Brown Swiss recipient cows following the transfer of in vivo-produced fresh and frozen-thawed embryos, respectively. These better results could be attributed to differences in the type of cattle used (meat or mixed-type) and physiological and metabolical disturbances of high milk yielding cows included in the group. Greater success is achieved with ET in meat-type breeds than in dairy breeds (Putnry et. al., 1988). Wallacea et al. (2011) achieved a pregnancy rate of 66.3% in meat-type recipient cows with the transfer of in vivo-produced fresh embryos. The lower rate observed in the present study may be related, for the reasons explained above, to the high-producing dairy cows used in the present study.

Embryo quality has a significant role in pregnancy rates after ET. Low pregnancy rates are recorded when low quality embryos are transferred (Hasler, 2001). Nevertheless, in some studies, pregnancy rates achieved with the transfer of first and second quality embryos were not significantly different (Spell et al., 2001). In the present study, the mean pregnancy rates achieved in heifers and cows respectively after the transfer of embryos of different quality grades were 55.11% and 45.16%, for first quality embryos; 41.66% and 25.0%, for second quality embryos and 54.54% and 12.5%, for third quality embryos ($P<0.05$). While the effect of embryo quality on recipient pregnancy outcome was found to be statistically significant in cows ($P<0.05$), it was not significant in heifers ($p>0.05$). Freitas et al. (2004) reported pregnancy rates of 64.7% and 57.4% after the transfer of first and second grade quality in vivo-produced fresh embryos, respectively ($P>0.05$).

Today ET practice involves hormone administration to synchronized recipients, [human chorionic gonadotropin (hCG), gonadotropin-releasing hormone (GnRH) or luteotropic hormone (LH)] (Marques, 2003), concurrent with the transfer procedure, in order to generate an accessory CL, and thereby to increase the serum progesterone level and pregnancy rate. Wallacea et al. (2011) observed a higher pregnancy rate in recipients, which were treated with hCG at the time of ET (61.85%), compared to recipients that did not receive any supplementary hormone treatment (53.9%). Based on this result, they concluded that pregnancy rates achieved with ET could be increased by elevating serum progesterone levels through the generation of an accessory CL with hCG administration. On the other hand, Niles et al. (2019) reported that hCG administered to Holstein heifers during ET increased serum progesterone levels, but did not have

any effect on the resulting pregnancy rate. Torres et al. (2013) reported that treatment with hCG at ET significantly increased the survival rate of demi-embryos and the pregnancy rate of high-yielding lactating dairy cows (untreated 26% and hCG treated 53%, on day 42 pregnancy rate). In our study, pregnancy results in recipient cows were lower than the results in above studies, but higher than those obtained by Köse et al (2006) did not treat the recipients with GnRH, hCG or LH.

CONCLUSION

In conclusion, embryo production and transfer technology in cattle has shown great improvement over the last 20 years. Today, practical applications have become routine. With fresh embryo transfer applications in cattle breeding programs, successful breeding is possible in a much shorter time thus accelerating genetic progression on the female side. In this study, the results obtained with superovulation and ET meet MOET study requirements. In our study, the average number of transferable embryos obtained for each flush, which is an important criterion for the success of superovulation applications, was optimal for high yield dairy donors. This study demonstrated that the pregnancy rates achieved with ET inrecipient cows and heifers differed significantly. The overall impact of embryo quality on recipient pregnancy outcome was statistically significant. However, while the effect of embryo quality on pregnancy was significant in recipient cows, no such effect was observed in recipient heifers. In view of the results obtained in the present study, we suggest that if cows are used as recipients for embryo transfer, first quality embryos should be used to achieve the targeted pregnancy rate. Furthermore, in order to increase the success of embryo transfer, heifers should be preferred to be used as recipients. Moreover, if second and third quality embryos are to be transferred, heifers should be preferred as recipients in the first place.

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CONFLICT OF INTEREST

None declared.

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