Journal of the Hellenic Veterinary Medical Society

Vol 71, No 4 (2020)

Whole issue

Ελληνική Κτηνιατρική Εταιρεία

doi: 10.12681/jhvms.25912

To cite this article:

Εταιρεία Ε. Κ. (2021). Whole issue. *Journal of the Hellenic Veterinary Medical Society*, *71*(4), 2419–2582. https://doi.org/10.12681/jhvms.25912

ISSN 2585-3724

JOURNAL OF THE HELLENIC VETERINARY MEDICAL SOCIETY

ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ

Quarterly Edition
Volume 71, No 4
OCTOBER - DECEMBER 2020

Τριμηνιαία Έκδοση
Τόμος 71, No 4
ΟΚΤΩΒΡΙΟΣ - ΔΕΚΕΜΒΡΙΟΣ 2020

Ε.Κ.Ε. Πατησίων 158, 112 57 Αθήνα
H.V.M.S. 158, Patission str., 112 57 Athens, Greece
ΕΝΤΥΠΟ ΚΛΕΙΣΤΟ Α.Ρ. ΑΔΕΙΑΣ 1

ΠΛΗΡΩΜΕΝΟ ΤΕΛΟΣ
Ταχ. Γραφείο ΑΓ. ΔΗΜΗΤΡΙΟΥ
Αριθμός Άδειας 1

ΚΩΔΙΚΟΣ: 2782

JOURNAL OF THE HELLENIC VETERINARY MEDICAL SOCIETY
ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ

CODE: 2782

Quarterly Scientific Journal
6th Period, Volume 71, Issue no.4
OCTOBER - DECEMBER 2020

Τριμηνιαίο Επιστημονικό Περιοδικό
Περίοδος ΣΤ', Τόμος 71, Νο.4
ΟΚΤΩΒΡΙΟΣ - ΔΕΚΕΜΒΡΙΟΣ 2020

ΚΩΔΙΚΟΣ: 2782

J Hellenic Vet Med Soc is covered by THOMSON REUTERS
Science Citation Index Expanded & Journal Citation Reports/Science Edition
ISSN 2585-3724

EBVS EUROPEAN BOARD OF VETERINARY SPECIALISATION

Update: July 2019
Ενημέρωση: Ιούλιος 2019

EUROPEAN COLLEGES OF VETERINARY SPECIALISTS
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)

REPRESENTATIONS / ΕΚΠΡΟΣΩΠΗΣΕΙΣ

Organisation / Οργανισμός	Representative / Εκπρόσωπος	Alternative representatives (alphabetically) / Αναπληρωτές εκπρόσωποι (με αλφαβητική σειρά)
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International Sheep Veterinary Association (ISVA)	G.C. Fthenakis	G. Arsenos, V.S. Mavrogianni
World Association for Buiatrics (WAB)	G.C. Fthenakis	G.S. Amiridis, N. Panousis
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Περιεχόμενα

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
Member:	Apostolos Rantsios

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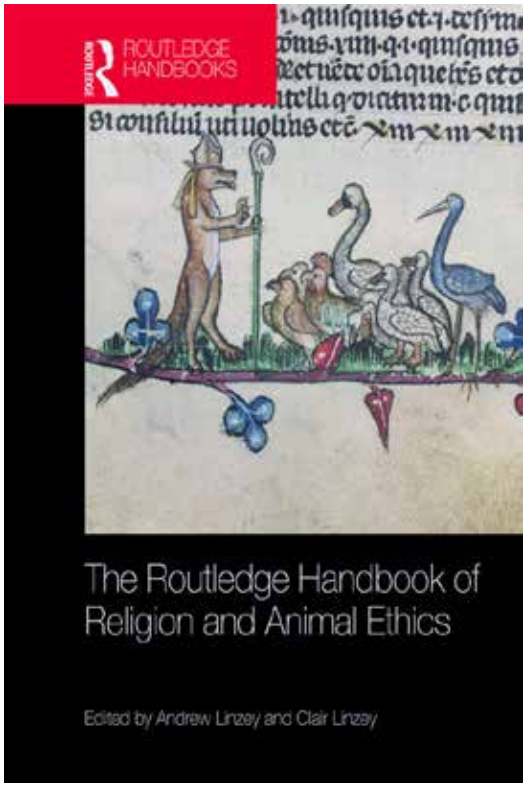
The Routledge Handbook of Religion and Animal Ethics

Edited by Andrew Linzey and Clair Linzey

Routledge Handbooks in Religion.

London: Routledge, 2019. 390pp ISBN 978-1-138-59272-8



The ethical consideration and treatment of non-human animals (a term used by the book's authors) is, especially nowadays, an important issue that is directly related to the way people treats natural environment. The religious beliefs of each person influence and very often determine his attitude towards life itself, other living beings and nature. What is the role that the Creator gave to human? Is the sovereign, the administrator or his is equal to every living creature? The answers to these questions also determine the way humans share the planet with other creatures. These are exactly the issues that this book deals with, examining in particular the role that religion plays in shaping animal morality.

The book consists of thirty-five chapters, written by reputable contributors, and is divided into two parts. The first part gives a general overview to the position of the major religions on issues related to animal ethics and animal protection. Through a colorful mosaic of fifteen different religions, and even more variations of them, from Africa, America, Asia and Europe, the reader has the opportunity to "travel" to the different approaches, from the anthropocentric approach of Western religions to the Hindu approach and the theory of yoga.

The second part consists of five sections dealing with topics/questions of particular interest such as human interaction with animals, the killing and exploitation of animals, religious and secular law, evil and theodicy, as well as souls and afterlife. For these issues, comparative presentations and analyzes are made between the different religions, describing at the same time the modern trends that are being formed. Particular interest is given to issues related to the use of animals for food as well as to the philosophy of vegetarianism.

Ultimately, this book demonstrates that religious traditions, despite their usually anthropocentric character, have much to offer those seeking a framework for a more enlightened relationship between humans and non-human animals. That is why this book is considered important for students and researchers dealing with animal theology and ethics, as well as those who study the philosophy of religion and ethics in general.

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Biomedical Research Foundation of the Academy of Athens

J HELLENIC VET MED SOC 2020, 71(4): 2419-2424
ΠΕΚΕ 2020, 71(4): 2419-2424

Review article
Ανασκόπηση

Peritoneal dialysis in dogs and cats

B.A. Viţălaru

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ABSTRACT: Dialysis represents the separation process of a colloidal dispersion substance from molecular dispersion particles, based on the property of certain membranes to retain only colloidal particles. In veterinary medicine, the most common use for peritoneal dialysis is the therapy of acute kidney injury, although it can be employed for removing dialyzable toxins and treating pancreatitis, electrolyte disorders and acid-base imbalances, refractory congestive heart failure and metabolic congenital disorders. Peritoneal dialysis is contraindicated in patients with peritoneal adhesions, fibrosis or abdominal malignant tumours. The ideal catheter for dialysis allows for an adequate administration and evacuation of the dialysate, it determines minimum subcutaneous losses, it minimizes infection both in the peritoneal cavity and in the subcutaneous tissue. The placement method for peritoneal dialysis catheters depends on the catheter itself, on the patient and on the approximate duration of the dialysis. In cases of extreme emergency, when the peritoneal dialysis should not be used more than 72 hours, the placement of a short-term catheter is justified. The peritoneal dialysis system is placed immediately after the peritoneal dialysis catheter is inserted and attached to a closed collection system, being carefully prepped in place with dry sterile dressings. The ideal peritoneal dialysis solution should achieve the low absorption clearance of osmotic agents, provide missing or insufficient electrolytes and nutrients, correct acid-base imbalances, inhibit growth of microorganisms, and be inert with regard to the peritoneum. When initiating peritoneal dialysis in acute kidney injury, the main goal is not to immediately normalize uraemia. The initial objectives should be to normalize the hemodynamic status of the patient and the acid-base and electrolyte imbalances, as well as to reduce uraemia to a BUN of 60-100 mg/dl and to reach a creatinine of 4.0-6.0 mg/dl in 24-48 hours. Complications of peritoneal dialysis are common, but they can be easily managed if discovered or addressed in due time. The most common complications are: peritoneal catheter obstruction, electrolyte imbalances, hypoalbuminemia and bacterial peritonitis.

***Keywords*:** peritoneum, AKI, CKD, peritoneal dialysis

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Invited Review Article

INTRODUCTION

Dialysis represents the separation process of a colloidal dispersion substance from molecular dispersion particles, based on the property of certain membranes to retain only colloidal particles.

In veterinary medicine, the most common use for peritoneal dialysis is the therapy of acute kidney injury, although it can be employed for removing dialyzable toxins and treating pancreatitis, electrolyte disorders and acid-base imbalances, refractory congestive heart failure and metabolic congenital disorders.

The principle of peritoneal dialysis is represented by the transfer of solutions through a semipermeable membrane based on the principle of diffusion. These membranes are the visceral and parietal peritoneum.

Peritoneal dialysis is an excellent substitute for hemodialysis in cases of patients who, out of various reasons, cannot withstand hemodialysis. (Acierno, M.J., Labato, Mary, 2011)

INDICATIONS

The most important indication for peritoneal dialysis in dogs and cats is anuria generated by an acute kidney injury (AKI) that is unresponsive to fluid therapy. (Barges, J., Polzin, D. J., 2011)

Dialysis is also indicated in patients showing acute non-anuric uraemia, when blood urea nitrogen (BUN) reaches levels over 100 mg/dl or when creatinine is higher than 10 mg/dl.

Peritoneal dialysis can also be employed to stabilize patients with uroabdomen or other urinary tract obstructions before surgery. (Mazzaferro, Elisa, 2012)

Peritoneal dialysis is indicated in various cases of intoxication and metabolic abnormalities. It can also be employed to remove dialyzable toxins, such as ethylene glycol, ethanol, barbiturates, propoxyphene and hydantoin, as well as in cases of electrolyte imbalances, such as hyperkalemia. (Holowaychuk, Marie, Marks, S. L., 2011)

Peritoneal dialysis is indicated when hemodialysis or charcoal hemoperfusion is not available, when vascular access is difficult to gain or when patient suffers from refractory hypotension, thus making hemodialysis too risky a procedure. (Viţălaru, B. Al., Micşa, C., 2015)

Peritoneal dialysis is also used on a wide scale in new born humans showing urea cycle disorders. It is used as an emergency tool when correcting hyperammonemia along with supplementary drug management in order to stabilize the condition until a liver transplant. (Viţălaru, B. Al., Ştefănescu Alina, 2015)

Congestive heart failure refractory to medical management is another indication for peritoneal dialysis in human medicine. (Langston, Cathy, 2011)

CONTRAINDICATIONS

Peritoneal dialysis is contraindicated in patients with peritoneal adhesions, fibrosis or abdominal malignant tumours. (Munteanu, Raluca et. all, 2017)

Peritoneal dialysis is also contraindicated in patients with pleuroperitoneal accumulations, because of their predisposition to develop pleural accumulations during dialysis. (Michell, A. R., 2010)

Relative contraindications are required in patients that have recently undergone abdominal surgery or in those that have inguinal or abdominal hernia, because of the increased risk of high intraperitoneal pressure. (Ross, Linda, 2011)

Patients in a severe hypercatabolic state, such as burn victims or patients in extreme malnutrition states, have a relative contraindication because of their tendency to lose proteins across the peritoneum. (Donohoe, Charlotte, 2012)

Patients who have recently undergone surgery, especially gastrointestinal surgery, present a risk for dehiscence and infection during peritoneal dialysis because of the higher intraperitoneal pressure and because of the extravasation potential of the dialysis fluid across the incision. (Ross, Linda, 2011)

Peritoneal dialysis presents as well as a high risk of complications in patients presenting polycystic kidney disease, extreme obesity, peripheral vascular disease and hyperlipidemia. (Elliott, D. A., 2011)

TYPES OF PERITONEAL CATHETERS

The ideal catheter for dialysis allows for an adequate administration and evacuation of the dialysate, it determines minimum subcutaneous losses, it minimizes infection both in the peritoneal cavity and in the subcutaneous tissue. (Thornhill, J. A., 1981)

Catheters for acute dialysis are placed percutaneously, under local anaesthesia, with the help of a stiletto and they require immediate heparinization.

These catheters are usually straight with orifices at the distal end. Acute catheters generally do not have Dacron cuffs in order to protect the patient against bacterial infection and catheter migration, that may lead to a high peritonitis rate in case of extensive use. There is also a high risk of bowel perforation during catheter placement. (Viţălaru, B. Al., 2014)

Catheters for chronic peritoneal dialysis have specific models, both intraperitoneal and extraperitoneal, in order to reduce secondary effects and to minimize blockage. These catheters are made of silicon, rubber or polyurethane. (Viţălaru, B. Al., Petrescu, V. F., 2016)

Catheters designed for chronic peritoneal dialysis have Dacron cuffs meant to protect the patient against bacterial infection and catheter migration that may lead to a high peritonitis rate in case of extensive use. (Thornhill, J. A., 1981)

The intraperitoneal segment of catheters has numerous lateral orifices at the distal end that allow the free flow of dialysate. The distal end of the peritoneal dialysis catheter may be straight or coiled. The coiled end may help with avoiding blockage. The segment of the catheter that comes out of the abdomen usually presents one or two Dacron cuffs. Usually, the middle segment is incorporated in the right abdominal muscle. The Dacron cuffs generate a local inflammatory response that triggers the emergence of granulation or fibrous tissue. This tissue fixes in place the catheter and it prevents the migration of bacteria from skin to the peritoneal cavity. In peritoneal dialysis, studies show that, in catheters with a single Dacron cuff, peritonitis shows up in a much shorter amount of time and that the infection rate for the placement area is higher, although other studies revealed no difference between catheters with different numbers of Dacron cuffs. (Viţălaru, B. Al., 2014)

The extraperitoneal segment of the catheter may be straight or with a curve between the two cuffs. The swan-neck catheters are meant to have a subcutaneous tunnel oriented straight down, in order to reduce the risk of infection at catheter level. There are no studies performed in veterinary medicine meant to assess the practicality of a certain type of catheter for peritoneal dialysis. (Viţălaru, B. Al., et. all, 2014)

In human medicine, the Tenckhoff catheter is the one most widely used in chronic peritoneal dialysis. This silicon catheter boasts a straight extraperitoneal segment and either a straight intraperitoneal segment, or a curved one with various orifices on the distal end. The Tenckhoff catheter may have one or two Dacron cuffs.

Among other catheter types used in veterinary medicine, there are Blake 15 Fr surgical drain, straight or curved Swan Neck, Missouri catheter, 10 cm PD catheter, Quinton pediatric catheter for peritoneal dialysis, and Dawson-Mueller drain. All these catheters require surgical placement. (Holowaychuk, Marie, Marks, S. L., 2011)

Simple tubular catheters with trocar can be placed on conscious animals, using local anaesthetics in case of major emergencies. The catheter for percutaneous cystostomy can also be used successfully in veterinary medicine instead of a peritoneal catheter in renal patients. (Viţălaru, B. Al., 2015)

PERITONEAL CATHETER INSERTIONS

The placement method for peritoneal dialysis catheters depends on the catheter itself, on the patient and on the approximate duration of the dialysis. In cases of extreme emergency, when the peritoneal dialysis should not be used more than 72 hours, the placement of a short-term catheter is justified. (Viţălaru, B. Al., 2015)

In order to place a percutaneous peritoneal dialysis catheter for short-term use, the animal is placed in dorsal decubitus and the abdomen is trimmed and prepared in sterile conditions using proper asepsis and antisepsis techniques. It is very important that each aspect is taken into account in order to preserve the asepsis and the antisepsis and in order to prevent catheter-related infections. A 1-2 mm incision, 3-5 cm from the umbilicus, in the direction of the pelvis, will be made. The trocar is tunnelled subcutaneously a few centimetres before inserting it into the abdomen. The catheter is then passed over the trocar until it fully penetrates the abdomen.

After catheter insertion, a tobacco pouch suture will be performed to secure the catheter. The suturing material is extremely important in the prevention of catheter related infections. Non-absorbable monofilament sutures (Nylon - polyamide, polypropylene) are to be preferred. Therefore, it is indicated to perform long-term peritoneal dialysis via peritoneal catheters with Dacron cuffs. (Viţălaru, B. Al., Ştefănescu Alina, 2018)

When placing a peritoneal dialysis catheter, which is meant to be used for more than three days, it is recommended to use a permanent catheter. The long-term peritoneal dialysis catheter should be surgically placed. The surgical placement of the catheter allows the visualization of the abdominal cavity and it is useful in omentectomy. Surgical omentectomy is also recommended due to the high risk of catheter blockage by the omentum.

Omentectomy is a surgical procedure which should be performed under maximum safety with regard to asepsis and antisepsis conditions and under general anaesthesia. (Costea, Ruxandra, Vitalaru, B. Al., 2015)

Peritoneal dialysis catheters can be trimmed (cut), resizing them at the desired level for entering the peritoneal cavity, in order to reach the bottom of the Douglas pouch. The catheters are pre-measured and, after being cut, they are inserted into the cavity. The abdominal muscles are sutured using an absorbable monofilament thread and the last suture, or a separate suture point, is anchored in the distal Dacron cuff of the peritoneal catheter. Subsequently, the anterior end of the catheter is passed through a subcutaneous lateral tunnel and it is exteriorized through the skin at 3-5 cm from the main incision line, so that the second Dacron cuff is located at subcutaneous level. (Viţălaru, B. Al., el. all, 2019)

After the placement, the catheter should be sutured at the skin level using Chinese finger trap or Roman sandals suture. (Viţălaru, B. Al., el. all, 2020)

In the first 24-48 hours after catheter placement, it is not recommended to use large volumes of dialysate in order to reduce intraperitoneal pressure, since this may cause leakage at the catheter placement site. (Viţălaru, B. Al., 2020)

PERITONEAL DIALYSIS SYSTEM

The peritoneal dialysis system is placed immediately after the peritoneal dialysis catheter is inserted and attached to a closed collection system, being carefully prepped in place with dry sterile dressings.

The peritoneal dialysis catheter is connected to the dialysis bag through a plastic tubing called a transfer set.

The Y transfer set consists of a Y-shaped tubular piece connected to both a dialysis bag and a drainage bag.

During the exchange, the dialysate is allowed to flow into the effluent bag. Prior to introducing fresh dialysis fluid into the peritoneum, a low volume of fresh dialysate solution is drained from the dialysis bag directly into the effluent bag, bypassing the patient. This step is supposed to eliminate the bacteria that were introduced into the system at the time of connection. Upon completion of the lavage, the dialysate may be instilled into the peritoneum.

It is absolutely necessary to use strictly sterile techniques at any time when manipulating the peritoneal dialysis catheter and the drainage system. (Viţălaru, B. Al., 2020)

PERITONEAL DIALYSIS SOLUTIONS

The specific composition of the dialysate is an important factor to consider when performing peritoneal dialysis.

The ideal peritoneal dialysis solution should achieve the low absorption clearance of osmotic agents, provide missing or insufficient electrolytes and nutrients, correct acid-base imbalances, inhibit growth of microorganisms, and be inert with regard to the peritoneum. (Thornhill, J. A., 1981)

The standard dialysis solution contains glucose as an osmotic agent. The glucose-based dialysate is present in three different concentrations: 1.5%, 2.5% and 4.25%. The dialysis performed in order to remove uremic toxins is generally done using a 1.5% solution.

The use of a hypertonic glucose solution (4.25%) is reserved for overhydrated patients, in which case the strong osmotic dialysate is used for the removal of water from the body through osmosis.

Peritoneal dialysis can be performed using commercial dextrose dialysate solutions or a mixture formulated by adding dextrose to Ringer's lactate solution. (Holowaychuk, Marie, Marks, S. L., 2011)

Recipes for "Homemade" peritoneal dialysis fluids can also be used. "Homemade" solutions contain 0.45% or 0.9% sodium chloride or lactated Ringer solution, depending on the patient's needs and the dextrose added.

Dextrose at a concentration of 1.5% is suitable for a dialysate solution and it can be obtained by adding 30 ml of 50% dextrose to one litter of lactated Ringer solution or 50 ml in order to obtain a 2.5% solution. (Viţălaru, B. Al., 2020)

PERITONEAL DIALYSIS PROTOCOL

When initiating peritoneal dialysis in acute kidney injury, the main goal is not to immediately normalize uraemia. The initial objectives should be to normalize the hemodynamic status of the patient and the acid-base and electrolyte imbalances, as well as to reduce uraemia to a BUN of 60-100 mg/dl and to reach a creatinine of 4.0-6.0 mg/dl in 24-48 hours. (Viţălaru, B. Al., Ştefănescu Alina, 2018)

The volume of the introduced dialysate varies depending on the concentration, composition and individual needs of the patient with an average of 40-60 ml/kg/exchange in a complete peritoneal dialysis cycle. In practice, a much more convenient formula can be used, essentially the amount of dialysate introduced being of 1 l/1sqm/exchange.

The dialysate should be heated to 38°C or even 39°C to improve peritoneal permeability and patient comfort prior to instillation into the peritoneal cavity.

Most patients with acute kidney injury will experience hyperkalemia, and most dialysis solutions do not have added potassium. In the initial cycles of peritoneal dialysis this is an ideal situation. However, hypokalemia may occur over time and, in order to prevent it, 2-4 mEq/l of potassium may be added to the dialysate after several cycles. (Viţălaru, B. Al., 2015)

To initiate a peritoneal dialysis session, the new dialysis bag is placed above the patient while the effluent bag is placed under the patient. A small amount of dialysate is washed from the dialysis bag directly into the effluent bag. At the first instillation, 10 ml/kg of dialysate solution is instilled by gravity into the peritoneum for 10 minutes and the solution is allowed to remain inside for 30-40 minutes.

The peritoneal cavity is allowed to drain by gravity into the sterile effluent bag for 10 to 20 minutes. The system is closed and the line washing procedure is repeated.

After the initial shifts in the first 24 to 48 hours, the patient can be transferred onto a chronic peritoneal dialysis protocol. Cycles of 3 to 6 hours will be performed and then these may be transformed into 3 to 4 daily shifts as renal function is restored.

It is recommended to gradually reduce the number of shifts and to prolong the duration of dialysis until reaching intermittent peritoneal dialysis which is performed at 3 to 4 days, with frequent patient re-evaluation before deciding on discontinuation of dialysis. (Viţălaru, B. Al., 2020)

PERITONEAL DIALYSIS COMPLICATIONS

Complications of peritoneal dialysis are common, but they can be easily managed if discovered or addressed in due time. The most common complications are: peritoneal catheter obstruction, electrolyte imbalances, hypoalbuminemia and bacterial peritonitis. (Holowaychuk, Marie, Marks, S. L., 2011)

Dialysis solution retention is defined as the recovery of less than 90% of the dialysate solution, which represents approximately 45% (on average) in animals undergoing peritoneal dialysis. (Acierno, M.J., Labato, Mary, 2011)

The most common causes of dialysate solution retention are the occlusion created by entrapping the omentum in the catheter pores and the accumulation of fibrin on the catheter. Performing partial omentectomy prior to placing the peritoneal catheter considerably decreases the frequency of this complication. (Viţălaru, B. Al., Ştefănescu Alina, 2018)

Accumulation of the dialysate solution at the subcutaneous level is another common problem in about 35% (on average) of patients. Regardless of the peritoneal, percutaneous or surgical catheter placing method, in veterinary medicine, extravasation of the dialysate solution at subcutaneous level is a frequent complication because, most of the time, the catheter is used immediately after placing it. (Viţălaru, B. Al., 2020)

The presence of hypoalbuminemia in animals undergoing peritoneal dialysis has been reported to be in the range of 41-90%. (Viţălaru, B. Al., 2015)

Peritonitis is diagnosed when two of the three following criteria are identified: turbid dialysate fluid when extracted, extracted dialysate fluid with more than 100 inflammatory cells/μL, or a positive bacterial culture test in the extracted dialysate solution, and clinical symptomatology characteristic of peritonitis. Peritonitis is the most common complication in peritoneal dialysis.

Pleural effusion and dyspnea are rare complications in patients undergoing peritoneal dialysis. Pleural effusions may be caused by hyperhydration or peluro-peritoneal leaks. Careful monitoring of the patient's hydration status, central venous pressure, urinary output and body weight may reduce the inci-

dence of hyperhydration.

Respiratory insufficiency due to intra-abdominal pressure and the respiratory rate in accordance with the introduced dialysis fluid flow should be carefully monitored. The volume of the dialysate solution should be decreased if intra-abdominal pressure causes ventilatory dysfunctions. (Viţălaru, B. Al., 2020)

Dialysis disequilibrium syndrome is a very rare complication of peritoneal dialysis and it is characterized by convulsive seizures, coma and/or exitus. This is secondary to the sudden drop in blood osmolarity. (Polzin, D., 2006)

Peritoneal membrane exchanges and ultrafiltration loss associated with peritonitis are the most serious complications of peritoneal dialysis. (Viţălaru, B. Al., 2020).

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J HELLENIC VET MED SOC 2020, 71(4): 2425-2436
ΠΕΚΕ 2020, 71(4): 2425-2436

Review article
Ανασκόπηση

Colibacillosis in poultry: A disease overview and the new perspectives for its control and prevention

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ABSTRACT: *Escherichia coli* (*E.coli*) is a common bacterium that can be naturally found in the intestinal tract of birds and as a result in their environment. However, it can cause clinical disease called colibacillosis which is regarded as one of the most common and important diseases in poultry. Strains of *E.coli* that have the ability to cause clinical disease are described as Avian Pathogenic *Escherichia Coli* (APEC). Colibacillosis can affect birds of all ages and different types of poultry production including broiler and commercial layers and breeders. The ability of *E.coli* to cause colibacillosis is not always the same; that is why its role as primary or secondary pathogen triggered by various predisposing factors is contradictory and differs from case to case. Antibiotics have been used as the main tool against colibacillosis for many decades. However, the emergence of increased antibiotic resistance has posed the need of alternative treatment to colibacillosis as well as emphasizing on preventive measures to avoid disease. The scope of this article is to assess recent scientific literature data on avian colibacillosis emphasizing on disease characteristics and recent data on prevention and control of the disease.

Keywords: antibiotic resistance, APEC, colibacillosis, epidemiology, incidence, poultry, vaccination

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Date of initial submission: 09/01/2020
Date of revised submission: 28/02/2020
Date of acceptance: 21/03/2020

INTRODUCTION

Escherichia coli (E.coli) is a bacterium that causes disease in different animal species, mainly localized in the intestine of mammals, but also they can cause septicemic or special type disease like 'Oedema disease' in pigs and 'watery mouth in calves' (Quinn et al., 2011; Tseng et al., 2014; Bashahun and Amina, 2017; Luise et al., 2019). Poultry is also affected by *E.coli* which causes serious clinical disease named colibacillosis. Those *E.coli* strains are called avian pathogenic *Escherichia coli* (APEC) and more often they can cause disease outside of the intestinal tract in poultry (extraintestinal APEC) (Dho-Moulin and Fairbrother, 1999; Ewers et al., 2009).

Escherichia coli is described as the most frequent bacterial agent that causes general health and production problems in poultry (Barnes et al., 2008; Kabir, 2010; Zhuang et al., 2014; Landman and van Eck, 2015).

Avian pathogenic *E.coli* strains seem to have restricted role in causing clinical disease in humans. However, some human uropathogenic *E.coli* strains are found to share similar genetic factors with APEC indicating a relationship between them (Skyberg et al., 2006; Ewers et al., 2007; Zhu Ge et al., 2014; Stromberg et al., 2017; Jorgensen et al., 2019). Furthermore, APEC antibioresistance can be a source of resistance for human pathogens (Singer and Hofacre, 2006; Johnson et al., 2007; Mellata, 2013; Singer, 2015).

The aim of this article is to provide important recent information concerning epidemiology, incidence, prevention and control of colibacillosis to anyone concerned in research or poultry production.

MICROBIOLOGY

Escherichia coli is a bacterium that belongs to the family of *Enterobacteriacae*. The family consists of many different important pathogenic genera like *Salmonella* and *Yersinia* and other members that can act like opportunistic pathogens (*Enterobacter*, *Citrobacte*r, *Proteus* etc) (Quinn et al., 2011). Bacteria of the *Escherichia* genus are Gram negative rods, with a flagella or not, that can grow under aerobic or anaerobic conditions. They have the ability to ferment different sugars like glucose and lactose; however, some strains do not ferment lactose, they are oxidase negative and indole positive (Barnes et al., 2008; Quinn et al., 2011).

Their colony morphology varies according to the medium that is used for culturing and the biochemical properties of the strain. For example, in MacConkey agar they have a pink appearance because of lactose fermentation and the production of acid, with the exception of a few non-lactose fermenting strains that are orange-pale strains (Filho et al., 2013).

The classification of various strains in different serotypes is based on 3 different antigens found in the bacterial cell of *Escherichia coli*: the O antigen (described as somatic antigen from the Greek word 'soma' that means 'body') that consists of polysaccharides (Stenutz et al., 2006), the H antigen that is cited on the flagella (flagellar antigen) and the K antigen (capsular antigen) (Brugere-Picoux et al., 2015). Up to now, about 180 somatic antigens, 60 flagellar and 80 capsular antigens have been reported.

The ability of certain *E.coli* bacteria to cause disease has been attributed to certain serogroups.

DISTRIBUTION

A big variety of serogroups/serotypes have been isolated from birds that were suffering from clinical colibacillosis with different patterns according to country/region using mainly the agglutination method and pulsed-field gel electrophoresis (PFGE). The agglutination method is a classical and simple method for serotyping *E.coli* strains (Orskov et al., 1977; Bettelheim and Thompson, 1987; Fratamico et al., 2016). The technique of PFGE is the most commonly used one to study the molecular epidemiology of infectious pathogens such as *Escherichia coli*, *Streptococcus spp., Staphylococcus spp., Neisseria meningitides, Vibrio cholerae, Bordetella pertussis* and *Campylobacter jejuni* (Ahmed et al., 2012; Natsos et al., 2019).

A big number of different serogroups were isolated from septicemic birds in Spain (Blanco et al., 1998). In a study conducted in Canada, 14 different serotypes were reported while most of the strains were untypeable (Allan et al., 1993). The O111 serogroup has been involved with polyserositis in layers in Italy (Zanella et al., 2000); furthermore, the O111 along with O166 and O64 were predominant isolates from egg peritonitis in layers in a study conducted in India (Srinivasan et al., 2013) while O111 with O78 were isolated from layer flocks in USA and Greece (Trampel et al., 2007; Koutsianos et al., 2017). A huge diversity of serotypes was revealed in a European study investigating the characteristics of APEC isolates from UK,

Italy and Germany (Cordoni et al., 2016). Heydel et al. (2019a) investigated the characteristics of *E.coli* strains isolated from broiler and layer chickens from Germany, Hungary, Belgium and the Netherlands. They reported that the majority of isolates (42.2%) belonged to various serogroups followed by O78 (27.5%), O2 (25%) and O1 (5.9%). A wide diversity of serotypes was also reported by Ali et al. (2019) for APEC strains of different geographical areas in Egypt. Different serotypes (O78, O1, O2, O86, O63, O125, O27, O169) were also found to be predominant in different country regions. In Brazil, the O6 serogroup was reported as the most prevalent serogroup in APEC isolates from broiler farms (Knobl et al., 2012) while the O2, O53 and O78 were found to be the predominant *E.coli* serogroups in a recent research project in Korea (Kim et al., 2020).

In general, the most described serogroups that are reported to cause clinical disease in poultry are O1, O2, O35, O36 and O78 (Barnes et al., 2008). The prevalence of 3 serogroups (O78, O2, O1) in birds suffering from colibacillosis has been reported in many studies (Ngeleka et al., 1996; Ewers et al., 2004; Mc Peake et al., 2005; Zhao et al., 2005; D'Incau et al., 2006; Camarda et al., 2008; Wang et al., 2010; Wun Jeong et al., 2012; Dou et al., 2016).

Two different research projects performed in broilers with coliseptisemia in the USA, have referred the O78 as the most common serogroup among others isolated from samples (Ngeleka et al., 1996; Zhao et al., 2005).

A German study, using coliseptisemic birds (layers and broilers), found that 49.6% of the isolated *E.coli* strains belonged to 3 serotypes (O2:28.7%, O78:14.7%, O1:6%) (Ewers et al., 2004). A similar research project conducted in coliseptisemic broilers and layers in the UK, revealed that the majority of *E.coli* isolates belonged to the O78 serogroup (45.6%) while 14.9% belonged to the O2 serogroup (Mc Peake et al., 2005).

An Italian study that focused on colibacillosis in layers reported that the 3 most common serogroups of the isolates were O78 (49%), O88 (15%) and O2 (9%) (D'Incau et al., 2006). A second Italian survey in layers revealed a variety of 15 serotypes, with the O78, O2 and O129 being the most dominant while a 45.4% remained untypeable (Camarda et al., 2008).

A south Korean study reported O78 as the most common group (19.4%) for *E.coli* isolates coming from diseased poultry with colibacillosis (Wun Jeong et al., 2012) while Chinese researchers revealed that the two most common groups for *Escherichia coli* isolated from birds suffering from colibacillosis were the O78 (35.8%) and the O2:14.4% groups (Dou et al., 2016). Another survey that was conducted in Southern China in broilers suffering from colibacillosis identified *E.coli* isolates that belonged to 21 different serogroups. The most prevalent serogroups among strains were the O65 (27%), O78 (10%) and the O8 (9%) groups (Wang et al., 2010).

Finally, in a French survey, the most important *E.coli* serogroups isolated from colibacillosis cases in poultry were O78:17.6%, O2:17.3%, O18:9%, O1:6%, O5:4.5% and O8:2% (Schouler et al., 2012).

ROUTES OF INFECTION

Escherichia coli is a natural inhabitant of the poultry intestinal tract. It returns in the bird environment through droppings and can insert to other birds by the oral-feacal route (Dho-Moulin and Fairbrother, 1999). It seems that bird's intestine and environment are the most important reservoirs for *Escherichia coli* strains capable of causing infection (Ewers et al., 2009). This study reported that *E.coli* strains isolated from the intestine of healthy birds and from their environment are phylogenetically similar to APEC strains that have been isolated from coliseptisemic birds. Therefore, they have the zoonotic potential to cause colibacillosis in poultry (Ewers et al., 2009; Kabir, 2010). Vectors like darkling beetle (Goodwin and Waltman, 1996), flies, insects, mites (Wales et al., 2010), rats and wild birds can play a role in the spreading of *Escherichia coli* (Barnes et al., 2008). *Escherichia coli* can be transmitted either horizontally, directly or indirectly, or vertically from breeders carrying the organism in their reproductive tract to their progeny (Giovanardi et al., 2005).

The respiratory route seems to be also very important for the appearance of clinical disease, while the oviduct can also be another route of infection for *Escherichia coli* concerning layers and breeders (Antao et al., 2008; Ozaki and Murase, 2009; Landman et al., 2013). Finally, penetration of *E.coli* through the skin can produce an inflammation in the form of cellulitis (Norton et al., 2000).

VIRULENCE FACTORS

The ability of APEC to cause disease has been

identified in specific genes which are responsible for coding the production of specific components of the bacterial cell, the virulence factors. These factors can either increase the ability of *E.coli* to attach to the host cells, increase the bacterium ability to multiply and attack the bird cell or can protect *E.coli* against the immune response of the host (Dho-Moulin and Fairbrother, 1999).

The most important virulence factors described in *E.coli* bacteria cells are adhesins like the type 1-F adhesin (La Ragione and Woodward, 2002) and the P-adhesin , iron related factors, like aerobactin system consisting of iuc, iut factors (Dziva and Stevens, 2008), protectins like the K1 and the iss factor (Mellata, 2013), invasins like ibeA (Cortes et al., 2008; Flechard et al., 2012), toxins/bacteriocins like stx1,2, hlyD and hlyF and miscellaneous factors (Barnes et al., 2008).

However, despite the fact that many specific virulence factors and their genes have been described, there is not a certain pattern attributed to APEC strains (La Ragione and Woodward, 2002). Different factors and combinations can be present in different avian pathogenic *Escherichia coli* strains. Ewers et al. (2004) reported a wide diversity of virulence genes combinations for isolates that belonged to O78, O1 and O2 serogroups, while strains that belonged to other serogroups had a higher variability. Schouler et al. (2012) reported that 13 virulence genes are more often present in APEC strains and 4 specific virulence gene combinations can be linked with the identification of APEC strains. However, it was found that 30% of the strains could not be confirmed as pathogenic because of the presence of different virulence factors that contributed in the strain pathogenicity. Guabiraba and Schouler (2015) also reported that although some virulence gene patterns can help us determine the pathogenicity of APEC strains, certain virulence gene combinations cannot be used in general to describe APEC strains. Furthermore, the presence of virulence factors patterns that are observed in APEC strains can also be present in nonpathogenic *E.coli* strains (Dziva and Stevens, 2008).

CLINICAL DISEASE

E.*coli* can infect and damage different bird systems causing various syndromes and localised or systemic disease.

In day old chicks, a very common type of *E.coli* infection is the yolk sack infection (omphalitis) which is characterized by inflammation of the yolk sack and high mortality (Dinev, 2007).

In birds of different ages, *E.coli* can cause severe infection of the upper or lower respiratory system through the nasal route or the trachea. In cases of localised infection of the upper respiratory system, facial oedema and sinuses swelling can be presented and described as swollen head syndrome. However, in case of lower respiratory infection, colibacillosis is characterized by the presence of fibrinous whitish yellow exudates in the air sacks and fibrinous pneumonia (Barnes et al., 2008). Another type of localized colibacillosis is cellulitis that is characterized by inflammation of the skin and presence of subcutaneous fibrinous exudates especially in broilers. Cellulitis is a very common reason for carcass condemnations in the slaughterhouse (Norton, 1997).

In layers and breeders, the most common type of colibacillosis is the infection of reproductive system; which is known as salpingitis, oophoritis and peritonitis (Jordan et al., 2005; Barnes et al., 2008; Brugere-Picoux et al., 2015). Regarding the macroscopic findings in necropsy, fibrin in different quantities can be present inside the peritoneum, the oviduct or around the ovaries (Dinev, 2007). Egg peritonitis syndrome caused by APEC is of great economic significance in layer production because of its high incidence, the induced flock mortality and drop in egg production (Landman et al., 2013). Furthermore, there is an added cost of flock antibiotic treatment. In males, *E.coli* infection can cause reduced fertility due to testicles inflammation (Barnes et al., 2008)

The septicemic or systemic form of colibacillosis is mainly found in broilers but can also be present in layers. It is characterized by polyserositis and presence of fibrin in various organs (pericarditis, perihepatitis, air-sacculitis, peritonitis) (Randall, 1985; Dinev, 2007). If birds survive the acute phase of coliseptisemia, colibacillosis can revert into a chronic localized type of disease. In such cases, coli infection can be present in unusual sites like brain, eyes, joints and bones (Barnes et al., 2008).

Another type of systemic colibacillosis is reported as Hjarre disease or coligranuloma. This type of colibacillosis is caused by specific *E.coli* strains and appears as multiple granuloma like lesions in various organs like liver, proventriculous and intestine. Coligranuloma is rarely seen but mortality rates in these

cases can be high (Brugere-Picoux et al., 2015). However, there is a new claim regarding a different aetiological agent responsible for granuloma disease in poultry. Landman and Van Eck (2017a) reported that in many case that granuloma disease was present, it was not possible to isolate E.coli or reproduce experimentally the granuloma lesions. They revealed that the role of a parasite, Tetratrichomonas gallinarum that has the ability to cause granulomatosis should be taken into consideration. This parasite has been isolated in cases of granuloma disease in poultry (Landman et al., 2019).

INTERACTION OF *E.COLI* WITH OTHER INFECTIOUS AND NON INFECTIOUS AGENTS

As it was mentioned before, *E.coli* is a bacterium can be related to other diseases. However, its role can differ from case to case, acting individually as a primary causative agent or act as a secondary pathogen complicating other viral, bacterial or parasitic disease as well as bad environmental management (Barnes et al., 2008).

Respiratory viruses such as paramyxovirus (Newcastle disease) (El Tayeb and Hanson, 2002), coronavirus (Infectious bronchitis) (Matthijs et al., 2003; Dwars et al., 2009), metapneumonivirus (Avian rhinotracheitis-swollen head syndrome) (Nakamura et al., 1997; Al-Ankari et al, 2001), herpesvirus (Infectious laryngotracheitis) (Nakamura et al., 1996), orthomyxovirus (Avian influenza) (Mosleh et al., 2017; Samy and Naguib, 2018) can trigger avian colibacillosis. Bacterial causes like *Mycoplasma gallisepticum* (Bradbury, 2005; Barnes et al., 2008) or *Mycoplasma synoviae* (Raviv et al., 2007) can also be complicated with *E.coli* creating serious respiratory syndromes with severe adverse effects on the birds. Those infectious agents can either damage the upper respiratory system cilia which is a significant defensive mechanism against *E.coli* penetration or act as immunosuppressive agents (Guabiraba and Schouler, 2015).

Immunosuppressive viruses as Birnavirus (Infectious bursal disease) and circovirus (chicken infectious anaemia) can predispose a secondary infection with *E.coli* (Gowthaman et al., 2012; Umar et al., 2017).

Apart from the aforementioned infectious agents, the environmental factors are well established to play a crucial role as a risk factor for horizontal disease transmission (Natsos et al., 2016). Some important non infectious factors that are predisposing to colibacillosis are shown in Table (1).

Table 1. Non infectious predisposing risk factors related to colibacillosis in poultry

RISK FACTORS	REFERENCE
Air quality/ammonia level/ventilation	Patterson and Adrizal, 2005
Water quality	Amaral, 2004; Dhillon & Jack,1996
Vaccination stress	Friedman et al., 1992; Nakamura et al., 1994; Matthijs et al., 2009
Bird density	Vandekerchove et al., 2004
Distance between poultry farms	Vandekerchove et al, 2004
Temperature/heat stress	Omer et al., 2010
Productive period/peak of lay	Zanella et al., 2000
Hatchery hygiene	Hill, 1994; Samberg and Meroz, 1995

TREATMENT USING ANTIMICROBIALS-ANTIBIOTIC RESISTANCE

Antimicrobials have been used for many decades as a tool against colibacillosis. However, *E.coli* like many other bacteria have the ability to create antibiotic resistance under the pressure of antibiotic usage (Aarestrup and Wegener, 1999). This resistance is created when antibiotics are used extensively on a prophylactic schedule or when antibiotic treatment is applied incorrectly using an underdosage (OIE, 2016).

The creation of antibiotic resistance is based on the ability of bacteria to change the composition of their outer membrane, to produce enzymes that damage antimicrobials or alter their metabolism (Quinn et al., 2011). Bacteria also have the ability to transfer genetic material that is regulating antibiotic resistance genes to other bacteria that infect animals or human, transforming susceptible bacteria to resistant and posing great concern for their health (Quinn et al., 2011; OIE, 2016). The transformation of a sensitive *E.coli* bacterium to a resistant one can occur either by genetic mutations, or transformation of genetic material like plasmids (Gyles, 2008; Fricke et al., 2009; Dheilly et al., 2013) between two bacterial cells horizontally.

The trends antibioresistance for *E.coli* strains vary from country to another country according to worldwide use of antibiotics and has become a field of research for many projects.

In a survey performed in Morroco, *E.coli* isolates from coliseptisemic birds revealed high resistance for sulphonamides, oxytetracycline, trimethoprim & sulphamethoxazole and chloramphenicol (Amara et al., 1995).

American scientists tried to investigate the antimicrobial patterns of avian *E.coli* strains isolated from broiler chickens suffering with colibacillosis (Zhao et al., 2005). It came out that the highest resistance levels were traced for sulfamethoxazole (93%), tetracycline (87%), streptomycin (86%) and gentamicin (69%). Furthermore, a high percentage of the isolates (92%) was proved to be multi-resistant (Zhao et al., 2005).

In a similar Chinese research project, the tested *E.coli* strains showed high levels of resistance to tetracycline (97.5%), nalidixic acid (82.3%), ampicillin (81.1%), sulphafurazole (80.7%), streptomycin (79.0%), trimethoprim (78.2%) and cotrimoxazole (78.2%). Most of these strains (80.2%) were resistant to more than 3 antimicrobial classes (Dou et al., 2016).

A survey that was conducted in India, revealed high *E.coli* resistance to chlortetracycline (88.58%), streptomycin (85.72%), penicillin-G (82.86%), amikacin (82.86%), furazolidone (77.14%), ampicillin (74.29%) and tetracycline (74.29%) (Sahoo et al., 2012) while an Iranian survey revealed high resistance of broiler *E.coli* strains(>80%) for many different antimicrobials (Salehi and Bonab, 2006).

Antibioresistance was traced even also for antimicrobials that were not supposed to be used. Resistance against cephalosporines in broilers which are not allowed to be used in Belgium since 2000 was recorded in broilers (Smet et al., 2008).

EU has also set a monitoring program for controlling of *E. coli* infection in poultry, as all participating member countries must report their data especially for broiler chickens and turkeys. In Table (2), the antimicrobial resistance EU data for 2014 in broilers are shown in comparison to Greek data (Valkanou, 2016; EFSA, 2016).

Table 2. Antimicrobial resistance to different antimicrobials in broilers (EFSA journal, 2016)

TYPE OF ANTIMICROBIAL	%RESISTANCE EU	%RESISTANCE GREECE
Ciprofloxacin	65.7	89
Nalidixic acid	62.6	86
Ampicillin	58.6	69.8
Sulfamethoxazole	53.1	70.3
Tetracycline	50.1	68
Trimethoprime	40.6	61.6
Chlorapmhenicol	21.6	35.5
Gentamicin	11.6	12.8
Azithromycin	6.7	9.3
Cefotaxime	5.1	2.9
Ceftazidime	5.0	2.9
Colistin	0.9	0
Tigecycline	0	0
Multi drug resistance	54.6	49.1

* The European Union reported information includes data from 27 countries belonging to European Union plus Norway.

The table reports a big variation of resistance among different classes where high resistance is observed to some antimicrobials (ciprofloxacin, nalixidic acid, ampicillin, sulfamethoxasole, tetracycline and very low resistance to others (tigecycline, colistin). Resistance to cephalosporins was observed to be low in general, even though there were reports of higher levels of resistance in some countries (Cyprus, Latvia, Lithuania, Malta, Spain) (EFSA, 2016). *E.coli* strains with multidrug resistance reached the overall level 54.6% following a trend of increasing resistance. However, antibiotic resistance of indicator *E.coli* varied among different countries. For example, Scandinavian countries (Norway, Sweden, Denmark and Finland) revealed low resistance levels for most antimicrobial classes (EFSA, 2016).

Resistance to colistin, was reported to be restricted

(0.9%) in total while no resistance was observed in Greece. The discovery of the *mcr-1* gene, that is responsible for bacterial resistance to colistin, is located on a plasmid and can transfer to other bacteria has increased concern for colistin resistance spreading (Liu et al., 2015).

Since colistin is used as a last resort treatment for serious human diseases caused by Gram negative bacteria like (Enterobacteriacae, Acinetobacter, Pseudomonas) when other antimicrobial treatments prove to be ineffective, its sensitivity is crucial for human Medicine (Falagas and Kasiakou, 2005). Since 2014, EU has decided to apply a monitoring schedule for colistin resistance which was updated with a surveillance scheme for the *mcr-1* presence as well for bacteria like *Escherichia coli* and *Salmonella* (EFSA, 2016).

ALTERNATIVE CONTROL STRATEGIES

Since antibioresistance is increasing, it is necessary to adopt alternative measures and implement new tools and methodologies in order to control bacterial diseases. As many strains of *E.coli* are harboring in the bird's intestinal tract, compounds that can control the gut overpopulation with *E.coli* can be useful against colibacillosis. The term 'competitive exclusion' has also been introduced to describe their mechanism.

Probiotics and prebiotics proved their efficacy against colibacillosis (Patterson and Burkholder, 2003).

Probiotics are microorganisms that belong mainly to the Bacillus family (*Lactobacillus, Bacillus, Bifidus* and *Enterococcus*) and the yeasts/moulds family (*Aspergillus/Candida*). Probiotics proved their positive effect in improving the bird's health by minimizing pathogenic bacterial intestinal colonization, producing antagonist pathogen metabolites and stimulating the immune response (Kabir, 2009; Papatsiros et al, 2013; Wang et al., 2017). Jaiswal et al. (2019) showed that the use of *Lactobacillus reuteri* can reduce the pathogenic bacterial population including *E.coli* in broiler intestine. Similar findings of decreased caecal *E.coli* counts in broilers after the use of probiotics eas revealed by Salim et al.(2013). Two similar projects by Dong et al. (2019) and Cao et al. (2013) revealed the beneficial effect of a probiotic (*Enterococcus feacalis*) supplementation in broilers diet. The use of probiotic contributed in the reduction of *E.coli* numbers in broilers intestine, after challenge with *E.coli*-K88.

Prebiotics are nutritional substances that are not digested and are necessary for the survival of specific intestinal bacteria which help the normal functioning of intestinal microflora (Hazati and Rezaei, 2010). The two main categories of prebiotics are fructo-oligosaccharides and Mannan-oligosaccharides (Hazati and Rezaei, 2010). Prebiotics are adjusting the type and number of poultry beneficial microflora in comparison to pathogenic bacteria and also stimulate the bird immune system (Pourabedim and Zhao, 2015). Xu et al. (2003) managed to reduce the *E.coli* population in broilers intestine after the use of fructo-oligosaccharide in their diet. Kim et al. (2011) showed that the use of fructo-oligosaccharide (FOS) and mannan-oligosaccharide (MOS) in broilers' diet managed to decrease significantly the number of *E.coli* in the gut.

Some essential oils are also found to to reduce the number of *E.coli* in the gut (Hammer et al., 1999). Ebani et al. (2018) tested the antimicrobial activity of 16 essential oils against and reported good activity for at least 5 oils against *E.coli* isolates deriving from poultry. Another study from Iran tried to test the efficacy of different essential oils coming from plants extracts. Some of the tested essential oils were proved to have antimicrobial activity against *E.coli* (Habibi et al., 2018)

Acidifiers or organic acids can decrease the number of *E.coli* by changing the intestinal PH and consequently reducing the intestinal pathogens metabolism, as well as by adjusting the population of beneficial bacteria (Khan and Iqbal, 2016). Furthermore, it has been found that organic acids can trigger the birds' immunity (Khan and Iqbal, 2016). Emami et al. (2017) investigated the antimicrobial activity of organic acids after *E.coli* K88 challenge in broilers and showed that the use of 3 commercial organic acids managed to reduce the *E.coli* counts in ceca. Finally, another study revealed that the use of a product consisting of formic acid, acetic acid and propionic acid contributed in the reduction of *E.coli* that was resistant to ampicillin, tetracycline, ciprofloxacin and sulfamethoxazole (Roth et al., 2017).

Another tool for controlling E. coli infection is the use of viruses which destruct the bacterial cells or bacteriophages. Bacteriophages can be naturally found in the environment and found to have an efficacy in colibacillosis control (Barrow et al., 1998; Huff et al, 2002; Huff et al., 2003 and 2004; Brussow, 2005; Huff et al., 2005 and 2006).

Good hygiene practice in the hatchery reduces significantly the risk of new hatcher birds with omphalitis. Rejection of dirty eggs containing large number of coliforms on their outer shell is a first step for minimizing hatchery infections. Spraying hatching eggs with disinfectants reduces contamination of egg surface with *E. coli* (Barnes et al., 2008) and also using of ultraviolet light was found to limit the possibility of *E. coli* infection in chicks (Coufal et al., 2003).

Maintaining optimum environmental conditions inside poultry house (air quality, temperature or stocking density) maintaining a good health status as well as the application of a novel vaccination scheme minimize the risk of predisposing factors that are usually trigger colibacillosis outbreaks (Barnes et al., 2008).

Apart from all the above, vaccination against colibacillosis is the most interesting tool as an alternative mean of control of colibacillosis. Live, subunit and inactivated vaccines have been tested to prevent E. coli infection in poultry (Ghunaim et al., 2014). Live vaccines are used in commercial poultry and they are administrated by mass applying route like spraying and seem to offer a wider protection against different serotypes (Ghunaim et al., 2014). Subunit vaccines are applied by injection as they contain proteins (*E.coli* virulence factors) and seem to produce better heterologous immunity than inactivated vaccines. Inactivated vaccines also taken by injection and have strict homologous protection as they contain more than one strain of *E. coli* (Ghunaim et al., 2014). Vaccines mainly trigger a cell mediated immunity against *E.coli* challenge as this type of immunity plays the most important role in bird's protection when compared with humoral immunity and antibodies (Filho et al., 2013; Sadeyen et al., 2014 and 2015).

Live vaccines to control *E. coli* infection in poultry have been tested in Germany (Heydel et al., 2019b), in Italy and USA (Alberti et al., 2019), in Taiwan (Lee Guo-Wei et al., 2019), in Japan (Nagano et al., 2012), in USA (Cookson et al., 2008), in the UK (La Ragione et al., 2013), in Morocco (Mombarg et al., 2014), in Iran (Sadeghi et al., 2018) and in Israel (Frommer et al., 1994) and the results revealed that this type of vaccine reduced mortality, macroscopic lesions of colibacillosis and in some cases diminished bacterial recovery after challenge with *E.coli* strains. Live *E.coli* vaccines have also been reported to reduce mortality and increase egg laying rates in layer breeders in Japan (Asano et al., 2019; Uotani et al., 2017) and in Thailand (Awaiwanont and Chotinum, 2019) where the *E.coli* vaccination also reduced the applied antibiotic treatment.

However, another study showed that live *E. coli* vaccines could not control colibacillosis after homologous or heterologous challenge (Kariyawasam et al., 2004).

Inactivated *E.coli* vaccines have already been reported to be beneficial in experimental trials. A Canadian study tested the efficacy of inactivated vaccines with different adjuvants and found reduced recovery of the challenging *E.coli* in comparison to unvaccinated birds (Gomis et al., 2007). Yaguchi et al. (2009) reported that the use of an inactivated liposomal E.coli vaccine managed to reduce clinical symptoms and bacterial numbers in chicken blood after *E.coli* challenge of specific pathogen free chickens.

Another survey tried to evaluate the benefits conferred by a commercial inactivated vaccine to broiler breeders and their progeny. A broiler breeder flock was divided in 2 groups of birds. Half of the birds were vaccinated with a commercial inactivated *E.coli* vaccine while the rest birds received no vaccination against *E.coli.* Even though, overall mortality between the 2 groups was similar, the vaccinated group was reported to have reduced losses due to colibacillosis in comparison to the unvaccinated group of birds (Gregersen et al., 2010).

Shehata et al. (2019) investigated the efficacy of a formalin inactivated *E.coli* vaccine that consisted of 7 different APEC serotypes, after challenge of SPF chicks. The efficacy was assessed by means of mortality, clinical signs and seroconversion. It was observed that the vaccinated birds were 100% protected when challenged with an O157:H7 and partially protected after an O125 challenge.

Autogenous vaccines are another type of vaccines that are used with increasing interest against *E. coli* infections. The term autogenous vaccine is used to describe vaccines that are produced specifically to protect a certain flock. Their production procedure uses bacterial isolates from a specific flock suffering from colibacillosis and produces a vaccine containing the homologous *E.coli* strains of the flock. Those vaccines may delay the clinical onset of colibacillosis and reduce mortality.

Landman and Eck (2017b) demonstrated complete protective effect of autogenous vaccines after aerosol homologous challenge with pathogenic *E.coli* in layer

hens. However, protection was not proved after heterologous challenge. The efficacy of an autogenous vaccine has also been reported after homologous intratracheal challenge in layer pullets in a Greek study (Koutsianos et al., 2019). Another Egyptian study showed reduced mortalities in *E. coli* challenged birds after vaccination with an autogenous vaccine (El Jakee et al., 2016). On the other hand, there are reports of incomplete protective efficacy of using autogenous vaccines against *E.coli* challenges (Li et al., 2016). The authors reported that the reasons for this may be the high infective dose that was used or the insufficiency of the stimulating humoral immunity to confer protection against *E.coli* challenge.

So, vaccines seem to be an interesting tool against colibacillosis and also contributing in the limitation of treatment with antimicrobials. However, there are still some cases with weak vaccine protection against colibacillosis. Further investigations related to avian *E. coli* vaccine production, and efficacy are in need.

CONCLUSIONS

Colibacillosis is the most common bacterial disease in poultry having great economic significance for the producers. Disease controlling using antimicrobials has led to the emergence of antibioresistance creating a big concern for animal and human health. It is crucial that judicious use of antimicrobials will be combined with a different approach for controlling colibacillosis including various preventing measures along with alternative treatments. Vaccination can have an important role in the control of the disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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J HELLENIC VET MED SOC 2020, 71(4): 2437-2444
ΠΕΚΕ 2020, 71(4): 2437-2444

Research article
Ερευνητικό άρθρο

Influence of rumen bypass fat fed with total mixed ration on growth performance in Nili-Ravi buffalo calves

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ABSTRACT: This study was conducted to know the influence of rumen bypass fat supplement on growth performance of Nili-Ravi mal buffalo calves. Nili Ravi buffalo male calves (n=12) were randlomly selected and divided into two groups i.e., A and B based on two different levels of age. These groups (A and B) were further divided into two respective subgroups i.e., A1, A2 and B1, B2. Sub groups A1 and BI served as control (without supplement) whilst subgroups A2 and B2 were fed RBF supplement (at the rate of 2.35% of dry matter intake) with basel diet with total mixed ration. Results showed no statistical difference (P>0.05) in dry matter intake, body weight gain and body condition score on rumen bypass fat supplementation. In blood metabolites, rumen bypass fat supplementation increased (P<0.05) blood triglyceride and cholesterol levels, however, it reduced (P<0.05) blood glucose level in Nili-Ravi buffalo male calves. This study suggests that supplementation of rumen bypass fat at the rate of 2.35% per day in TMR possess no impact on growth performance parameters in Nili Ravi buffalo male calves.

Keywords: buffalo calves, rumen bypass fat, growth performance, body condition score, blood metabolites

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Date of initial submission: 06-04-2019
Date of revised submission: 09-06-2020
Date of acceptance: 17-06-2020

INTRODUCTION

Nutrition plays key role in the maintenance and body development of animals (İmran *et al*., 2018; Nawab *et al*., 2019; Aydogdu *et al*., 2019). The available information on the nutrients requirements of buffalo male calves for growth is limited. Of all nutrients, energy is an important constituent of a ration for beef animals (Basra *et al*., 2003). Traditionally, cereal grains are energy sources which are used for feeding of ruminants, possessing good production potential however, high levels of grains adversely affect feed intake, fermentation characteristics of rumen and consequently digestion (Palmquist and Jenkins, 1982). The growth potential of calves can be fully exploited by incorporating fats in the ration. Rumen bypass fat is vegetable oil which contains 85% palmitic acid and has higher melting point, it bypasses rumen degradation and does not melt in rumen. This undegraded bypass fat degraded by lipase enzyme in small intestine (Singh *et al.,* 2014). Rumen bypass fat remain partly unaffected by process of bio-hydrogenation of rumen microorganisms and hence it supports in leaasening the looming danger of metabolic diseases (Naik *et al.,* 2009). Zinn and Plascencia (1996) stated that consumption of energy by feedlot steers augmented linearly with cumulative nutritional energy density. Moreover, a direct reduction in feed consumption and unfilled body gain is reported due to addition of fat in the diets of steers (Zinn, 1989; Jenkins and Jenny, 1989). In dissimilarity, Palmquist and Jenkins (1982) stated that calcium could get better fiber digestibility in additional fat diets by creating unsolvable soaps, which eliminate fatty acids (FA) from rumen fluid, therefore they are not available for a long time for rumen bacteria. Though, Sutton *et al*. (1983) stated that protected linseed oil caused a large depression in neutral detergent fiber breakdown in the rumen. It was designated that the seeming discrepancy might be due to the extent of defence, and the result fatty acid slowly released in the rumen which permissible hydrogenation of fatty acid to happen, therefore decreased microbial activity**.** Considering varying nature of reported literature due to different species, physiological status of experimental animals, the protection level of rumen bypass fat (RBF), basal diet and many other reasons, this study was designed to know the influence of RBF supplemented with total mixed ration (TMR) on growth perofrmance in Nili-Ravi buffalo male calves.

MATERIALS AND METHODS

This study was carried out in University of Veterinary and Animal Sciences, Ravi campus, Pakistan. For this purpose, Nili Ravi buffalo male calves (n=12) were randomly selected and divided into two groups i.e., A and B based on age. The age of buffalo calves in group A were 21-24 months (average body weight 308±8.8kg) whilst age of buffalo calves in group B were 08-11 months (average body weight 130±7.6kg). These groups (A and B) were further divided into two respective subgroups i.e., A1, A2 and B1, B2. Sub groups A1 and BI served as control (without supplement whilst subgroups A2 and B2 were fed RBF supplement (@ 2.35% of dry matter intake). RBF (in fine flanks, white to pale in color) is commercially available product with different trade names and are prepared by 100% vegetable oil. This product was made by palm oil fatty acids and fractionated by physical method (where in fatty acids of wanted configuration were achieved). It contained 99 % total fat, 85% plametic acid, 20% stearic acid with 29 (MJ/kg) NEL and 56-58^{0}C melting point. This procudt is developed to provide maximum energy from a protected fat with great small intestine digestibility and low rumen solubility.

As basal diet, total mixed ration (Table 1) containing 2.5 Mcal/kg mtabolizabe energy and 12% crude protein fed to all groups at the rate of 3% body weight on dry matter basis. The duration of experiment lasted for 70 days with 10 days adjustment period of calves to their respective diets. Clean and freshwater was given *ad libitum* daily for 24 hours. The refusals were collected and recorded (if any). Nili Ravi Buffalo calves were de-wormed for ecto and endo-parasites during the adjustment period.

Data on daily feed intake was recorded on daily basis. Body weight of individual Nili-Ravi buffalo were taken before the commencement of feeding experiment, subsequent weight of each Nili Ravi calf was recorded after every 14 days using a digital weighing balance. Body weight gain (kg) of each calf was determined by deducting initial body weight from final body weight. Body condition score (BCS) was recorded by using 1 to 9 measurement scale in which different scores were allotted according to the body conditions of animals with 1 being very thin and 9 being extremely obese (Eversole *et al*., 2009). BCS in terms of brisket, ribs, pins, hooks, back and tail head of every male calf was noted before the start of experiment and at the end of feeding trial.

Table 1. Ingredient and chemical composition of TMR fed to experimental calves

Ingredients %	Control group	Supplemented group
Maize grains	25.00	20.00
Soybean meal	05.00	05.00
Canola meal	06.00	06.00
Molasses	06.00	06.00
Oat silage	45.00	47.65
Wheat straw	11.00	11.00
Mineral mixture	02.00	02.00
RBF	0.00	2.35
Total	100	100
Nutrients %		
DM	56.90	56.90
CP	12.00	12.00
ME (Mcal/kg)	2.50	2.50
NDF	28.70	28.70
NFC	38.34	38.34
Calcium	0.98	0.98
Phosphorus	0.40	0.40
Fat	2.08	4.07

TMR Total mixed ration; RBF= Rumen bypass fat; DM= Dry matter CP= Crude protein;
M.E= Metabolizable energy; NDF= Neutral detergent fiber;
NFC= Non fiber carbohydrates

Blood samples of Nili-Ravi buffalo male calves were taken at day zero of experiment and then at the completion of experiment by puncturing the jugular vein in 20 ml sterilized disposable syringes and were transferred to EDTA coated tubes for biochemistry analysis. Plasma was collected from the samples of blood by the process of centrifugation at 3000 rpm for five minutes in a centriguge. All samples were labeled carefully and plasma was stored at the temperature of -20 ^{0}C until further analysis (Ashmawy**,** 2015)**.** The blood glucose, cholesterol and triglyceride levels were measured by standard procedure using Human cat # 10260, 10017 and 12851, respectively.

Collected data on all studied parameters were stayistically analysed (Steel *et al.*, 1997) under 2x2 factorial design using Statistical Analysis System software (SAS, 2004).

RESULTS AND DISCUSSION

Daily average and total dry matter intake (Mean ± S.E) of Nili-Ravi buffalo male calves on experimental diets are given in Table 2. In our findings, feeding additional RBF did not improve average daily matter intake (DMI) and total dry matter intake. Similar response with and without RBF supplementation in DMI may be due to nature of basal diet fulfilling basic nutrient requirement of calves. In our study, TMR was fed as basal diet and it is well established fact that TMR provides all ingredients containg different nutrients in one bolus. Therefore, it is deduced that it may have attributed to the non-interference of RBF with digestibility of nutrients and its relatively stable nature and minimum dissociation in the rumen (Reddy *et al*., 2003). Findings of this study are inagreement with the earlier findings (Garg *et al*., 1997) where feed intake was not different on RBF feeding at the rate of 500 gm per day per head in Holstein Friesian cows. Similarly, Raval *et al*. (2017) reported no difference in DMI on RBF feeding in Surti buffaloes. Tyagi *et al*. (2010) also reported statistically same DMI on RBF feeding at the rate of 2.5% in multiparous crossbred cows. However, some studies have shown increased DM intake on RBF feeding. Gajera *et al*. (2013) performed an experiment on growing Jaffrabadi heifers and reported increased (P<0.05) DMI. Likewise, Sirohi (2010) reported higher DMI with additional bypass fat supplementation in crossbred cows. The increased DMI in these studies may be due to different physiological stage of animals and nature of basal diet.

Table 2. Daily DMI* and total DMI (Mean± SE) intake in Nili-Ravi male buffalo calves fed experimental diets

Diet	Average DMI* (kg /animal/)	Total DMI (kg)
Control	6.80±0.90	407.86±54.10
RBF**	6.80±1.06	407.82±63.74
Age (months)		
21-24	8.88±0.20^a	532.90±12.09^a
8-11	4.71±0.40^b	282.78±24.23^b
P-value		
RBF	0.9990	0.9990
Age	<.0001	<.0001

$^{a-b}$ different superscript in a column shows significant differences;
*Dry matter intake ;
**RBF Rumen bypass fat

In the present study, as expected, younger age calves (8-11months age) consumed less (average dialy DMI, Total DMI) as compared to older age calves (21-24 months age). These findings explains higher requirement of DMI in comparatively older age and weight calves. These findings are similar with the Kumar *et al*. (2007) who fed RBF to Murrah buffalo calves and found low DM intake in calves of younger age (less than one year) buffalo calves. The lower DMI in younger age calves may be due to the fact that

unsaturated long chain fatty acids reaching the small intestine may reduce the gastrointestinal motility and thus DMI (Reidelberger 1994). However, Meshram *et al*. (2017) conducted study on growing crossbred calves having less than one year of age and compared RBF fed group with non RBF fed group.They found significantly higher dry matter intake with feeding RBF diets.

Influence of RBF supplementation with TMR on body weight gain (Mean ± S.E) in Nili-Ravi buffalo male calves is given in Table 3 and Table 4. In our study, RBF supplementation did not show significant body weight gain as compared to claves fed same diet without RBF supplementation. However, age and weight variation influenced body weight gain. Eearlier studies (Wadhwa *et al*., 2012; Long *et al*., 2014; Len *et al*., 2016; Mangrum *et al*., 2016) are in agreement to our findings where weight gain in experimental animals was increased with supplementation of RBF. However, in his studies Vahora *et al*. (2012) found increased (P<0.05) body weight gain in growing buffalo calves on RBF feeding. Higher gain on RBF supplementation in some studies may be correlated with nature of basal diet and consequent nutrient digestibility, nitrogen balance and utilization, better rumen environment and higher microbial-nitrogen synthesis. (Bhatti *et al*., 2016).

Table 3. Fortnight body weight (Mean ±SE) in Nili-Ravi buffalo calves fed experimental diets

Diet	0 day (Kg)	14 day (Kg)	28 day (Kg)	42 day (Kg)	60 day (Kg)
Control	255.67±42.77	261.17±43.06	268.00±43.67	279.33±45.13	295.00±45.77
RBF*	266.33±40.00	277.33±42.95	286.83±43.75	298.67±45.10	311.67±46.21
Age (months)					
8-11	173.50±13.08[b]	177.67±13.13[b]	184.00±13.37[b]	192.67±13.99[b]	205.50±14.31[b]
21-24	348.50±14.40[a]	360.83±14.06[a]	370.83±13.66[a]	385.33±14.16[a]	401.17±14.99[a]
P-value					
RBF	0.8591	0.7958	0.7668	0.7681	0.8030
Age	<.0001	<.0001	<.0001	<.0001	<.0001

[a-b] different superscript in a column shows significant differences; *RBF= Rumen bypass fat

Table 4. Weight gain (Mean ±SE) in Nili-Ravi buffalo calves fed experimental diets

Diet	14 day (Kg)	28 day (Kg)	42 day (Kg)	60 day (Kg)	ADG/anim (kg)	AGF (Kg)	TWG (Kg)
Control	5.50±1.23	6.83±1.74	11.33±1.58	15.67±1.23	0.66±0.07	9.83±1.05	39.33±4.22
RBF	11.00±3.46	9.50±2.39	11.83±1.74	13.00±1.21	0.76±0.12	11.33±1.73	45.33±6.92
Age (months)							
8-11	4.17±0.79[b]	6.33±2.01	8.67±1.09[b]	12.83±1.30	0.53±0.05[b]	8.00±0.76[b]	32.00±3.03[b]
21-24	12.33±3.04[a]	10.00±2.02	14.50±0.99[a]	15.83±1.05	0.88±0.07[a]	13.17±1.04[a]	52.67±4.17[a]
P-value							
RBF	0.0427	0.4048	0.7673	0.1133	0.4761	0.2575	0.2575
Age	0.0073	0.2611	0.0073	0.0805	0.0025	0.0030	0.0030

[a-b] different superscript in a column shows significant differences
ADG = Average daily gain; AGF = Average weight gain on fortnight basis; TWG = Total weight gain

In our study, average daily and total weight gain increased (P<0.05) in older age calves (21-24 months) as compared to younger age (8-11 months) calves which may be due to more weight and age of calves that consequently resulted in increased DMI. In other words, average daily gain was not significantly increased in lower age and weight calves due to less and decreased feed intake (Crystal 2015). Similar to our findings, Gajera *et al*. (2013) reported significant differences (P<0.05) with RBF supplementation on body weight gain in Jaffrabadi buffalo heifers in more than one year age).

Initial and final BCS in terms of brisket, ribs, pins, hooks, back and tail head of Nili-Ravi buffalo calves is given Table 5. RBF supplementation and age difference showed no influence on BCS of buffalo calves. These results explain that iso-caloric and iso-nitrogenous nature of basal diet (TMR) may have fulfilled nutritional requirement of calves according to their respective age and weight, hence additional suuplenetation of RBF may have not further improved BCS. Findings of this study are in accordance with Raval *et al*. (2017) who did not report any change in BCS of Surti buffaloes fed RBF. Likewise, Long *et al*. (2014) reported no difference in BCS of beef heifers fed with different dietary treatments of RBF. On the other hand, an improved BCS in animals with RBF supplementation is reported in litearature. Naik *et al*. (2009) recorded significant differences (P<0.05) in BCS of cows fed RBF as compared to control diet. However, lactating animals may give repsose to RBF supplementation due to high demand of rich energy source in small intestine particularly at early stage of lactation.

Table 5. Body condition score (Mean ±SE) in Nili Ravi male buffalo calves fed experimental diets

Diet	**Day zero (BCS)***	**Day Final** (Day 60) (BCS)
Cnotrol	3.00±0.26	4.33±0.42
RBF**	3.00±0.26	5.00±0.26
Age (months)		
8-11	2.67±0.21	4.33±0.33
21-24	3.33±0.21	5.00±0.37
***P*-value**		
RBF	1.0000	0.2191
Age	0.0805	0.2191

*BCS=Body condition score; 1 emaciated; 2 very thin; 3 thin; 4 moderate thin; 5 moderate ideal; 6 moderate fleshy; 7 fleshy; 8 very fleshy; 9 obese.
** RBF= Bypass fat

Effect of diet and age on blood glucose, cholesterol and triglycerides level (Mean±S.E, mg/dl) of Nili-Ravi buffalo male calves are given in Table 6. RBF feeding to buffalo calves significantly reduced blood glucose level whilst blood cholesterol and triglycerides level were increased. Findings of this study are in line with Hammon *et al.* (2008) who found significantly reduced glucose concentration in RBF fed animals as compared to control group. The decrease in plasma glucose concentration was not associated with decreased post absorptive availability of glucose, because rate of appearance of glucose was not affected by the diet. Therefore, the reduced glucose concentration was likely due to an increased utilization of glucose (Hammon, 2008). On a similar pattern, in our study the reduced glucose concentration was likely due to increased utilization of glucose. This may be due to elevated fat oxidation in the liver, changes in gastrointestinal hormones, or impact on rumen digestion (Benson et al., 2001). Barley and Baghel (2009) fed RBF at the rate of 100gm per day/buffalo and found significantly increased serum triglyceride levels in buffaloes fed RBF as compared to control group. Our findings are also similar with Ranjan *et al*. (2012) who found significantly increased serum cholesterol level with feeding RBF in Murrah buffaloes. Raval *et al.* (2017) also found that supplementation of RBF significantly (P<0.01) increased serum cholesterol and triglyceride levels in Surti buffaloes calves of more than one year age. Increased serum triglycerides and cholesterols levels with the advance in feeding of supplemental RBF might be due to enhanced uptake of dietary fatty acid (Grewal *et al*. 2014). However, contradictory finding were also reported by Tyagi *et al*. (2010) who fed ration 2.5% RBF supplement (on DMI basis) and did not found any effect on cholesterol concentration between groups in cows. In this case, the probable reason might be level of RBF feeding (2.5% of DMI) which was not sufficient to cause increase in serum cholesterol level.

Table 6. Blood level (Mean ±SE, mg/dl) in Nili-Ravi male buffalo calves fed experimental diets

Diet	Glucose		Cholesterol		Triglyceride	
	Initial	Final	Initial	Final	Initial	Final
Control	99.86±8.01	138.42±10.86[a]	214.34±10.95	228.25±15.36[b]	231.94±9.13	264.62±10.19[b]
RBF*	92.01±3.52	109.51±5.52[b]	221.09±15.94	365.07±53.46[a]	205.60±6.18	351.76±44.71[a]
Age (months)						
8-11	95.86±6.36	113.65±9.48	227.89±14.45	251.74±20.38[b]	214.97±10.17	262.85± 11.00[b]
21-24	96.00±6.49	134.29±9.97	207.54±11.33	341.58±61.19[a]	222.57±9.05	353.53±43.81[a]
***P*-value**						
RBF	0.4450	0.0361	0.7371	0.0072	0.0578	0.0074
Age	0.9891	0.1102	0.3248	0.0467	0.5413	0.0060

[a-b] different superscript in a column shows significant differences; *RBF=Rumen bypass fat

Influence of different age groups on blood metabolites in calves, Kumar and Thakur (2007) also reported no influence on blood glucose level in different age groups of Murrah buffalo calves. The researon may be due to more utilization of glucose (high metabolic rate) and other factors (homeostatic mechanism) in ruminants and other animals that limits glucose level (Tyagi and Thakur, 2007). Further, similar to our findings, Sharma *et al.* (2016) reported significantly increased cholesterol level in more than one year old buffalo calves. The higher plasma cholesterol in buffaloes may be due to the positive energy balance of animals associated with prilled fat feeding (Ranjan *et al*., 2012).

CONCLUSION

It is concluded that supplementation of RBF at the rate of 2.35% per kg in total mixed ration did not improve dry matter intake, body weight gain and body condition score. In blood metabolites, RBF supplementation significantely reduced blood glucose level however, it increased blood triglyceride and cholesterol levels in Nili-Ravi buffalo male calves.

CONFLICT OF INTEREST

In this research trial, the authors declare that there is no conflict of interests regarding the publication of this article

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J HELLENIC VET MED SOC 2020, 71(4): 2445-2454
ΠΕΚΕ 2020, 71(4): 2445-2454

Research article
Ερευνητικό άρθρο

Comparison of hematological and biochemical profile between Podolian grey steppe and Holstein cows

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ABSTRACT. This research aimed to investigate the influence of different age of Podolian grey steppe and Holstein Friesian cows, as well as the effects of different breeds on their hematological and biochemical blood profile.A total of 128 adult cows aged from 3 to 12 years were used in the study. Gained results show statistically significant differences ($P < 0.05$) regarding all hematological parameters. The concentration of NE was recorded in the optimal range for Podolian grey steppe cows, while for Holstein Friesian breed was elevated.Values for LY was in the optimal range, while the values for MO, EO, and BA were slightly higher for both breeds of cow in all groups, with recorded significant differences ($P < 0.05$).Count of RBC in the Podolian grey steppe breed ranged between 5.83 and 11.21×10^{12}/L, with significant influence ($P < 0.05$) of cows age in obtained values. In contrast,Holstein Friesian cows breed recorded RBC values lover then optimal, without any significant difference ($P > 0.05$) related to the age of cows. Total protein has ranged between 7.10 and 8.50 g/dl (Podolian grey steppe), and 8.13 to 8.48 g/dl (Holstein Friesian), without recorded statistically significant differences ($P > 0.05$) between the age, breed as well as the interaction of age and breed. Asimilar tendency can be seen regarding the otherbiochemical values ($P > 0.05$), respectively.Gain results have mainly shown a significant influence of breed on the parameters above, while the significant influence of age of cows was not present as well the interaction between age and breed.

Keywords: cows, hematological profile, blood, enzymes, WBC, RBC

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Date of initial submission: 22-08-2019
Date of revised submission: 10-06-2020
Date of acceptance: 03-08-2020

INTRODUCTION

To evaluate animal health and welfare, regular monitoring of the health status of dairy herds is essential(Roland et al., 2014).Cows' health can be assessed and depends on the blood profile of hematology and biochemistry(Obućinski et al., 2019). Hematology, in addition to clinical examination or other diagnostic procedures, is exceptionally Informatique as a diagnostic instrument in bovine medicine(Roland et al., 2014). Dairy cow's nutrition could be the primary technological factor that can lead to significant modifications in the metabolic profile of animals.The mineral, protein,and vitamin deficiency in cows nutrition is a source of profound metabolism alterations(Coroian et al., 2017). Hematological parameters indicate the adaptability of cows and other stressors to adverse environmental circumstances. On the other hand, besides hematological parameters, biochemical blood status in a range of normal physiological limits reflects a good health status and is highly correlated with milk production(Obućinski, Soleša, et al., 2019). Over the years biochemical and hematological blood profile of cowshasinvestigated the age-related changes and comparison with blood composition in adults(Mohri et al., 2007), location-related changes (Hagawane et al., 2009), effects of parity, stage of lactation, and season of production(Cozzi et al., 2011), mineral dietary supplementation (Sobhanirad and Naserian, 2012), influence dependent on the management system (Obućinski, Soleša, et al., 2019; Radkowska & Herbut, 2014), changes during drying-off and the beginning of lactation (Bertulat et al., 2015), the prevalence of uterine infection(Sarkar et al, 2016), improvement of the biochemical and metabolic biomarkers in response to the therapeutic management in ketotic dairy cows(Biswal et al., 2017)milk and blood of ketotic dairy cows in and around Bhubaneswar, Odisha, India, before and after treatment. Thirty of 100 ketotic cows identified from a population of 1014 cows were equally divided into three groups of 10 animals each while group IV selected from the population under investigation was treated as control. Following treatment in group III, the ALT, AST, ALP and LDH levels observed in ketotic animals at pre-treatment were decreased maximum at post-treatment. It can be concluded that the treatment package comprising of Dextrose (25%, lactation stage on milk composition (El-Tarabany et al., 2018), even the prevalence of ticks in cattle (Ullah et al., 2019), respectively.

Könyves et al. (2017)feed intake and feed efficiency of Holstein-Frisian cows in different seasons of the year. Five hundred and sixty three cows were monitored in spring, 557 cows in summer, 594 cows in autumn and 567 cows in winter, for a total period of two years. In contrast to the spring, autumn and winter periods, the summer period was characterized by heat stress conditions. Average T and THI exceeded the 25°C and 72 critical points, respectively, on all, 90% and 93% of test days for this period, indicating that the cows were exposed to heat stress during the summer trial. The heat stress reduced daily milk yield by 1.32 kg or 9.46%, by 0.92 kg or 9.62% and by 1.27 kg or 9.48% as the THI values went from 64 in the spring, from 66 in the autumn and from 42.34 in the winter periods to 79 in the summer period. Forage intake was decreased by 1.63 kg, by 1.42 kg and by 1.25 kg compared to those in spring, autumn and winter, respectively, and the efficiency of conversion of feed to milk was increased (from 1.6 to 1.59 kg milk/kg milkinvestigated the relationship of the temperature-humidity index with milk production and feed intake of Holstein-Frisian cows in different year seasons, while it has been confirmed that lactation phase the, as well as the duration of the production cycle,also influencethe biochemical profile of cows blood according to the research ofCoroian et al.(2017).Some investigations have shown that blood profile differs between different breeds of cows(Vordermeier et al., 2012; Widayati etal., 2018) indicating that breed as well the age of cows (Kumar et al., 2017) reflects on hematological and biochemical blood profile of cows.

Metabolic diseases are much more prevalent in cows with elevated milk yield than in indigenous breeds of the same species. In Podolian grey steppe cows, the diseases occur sporadically, while in the cows with high milk production, metabolic diseases are much more common(Di Lorenzo et al., 2018; Keros et al., 2013)BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, BM1824. Early detection of nutrient deficiency or metabolic disease can contribute to the successful diagnosis, faster recovery of animal and heart health approach to prevent deviations before they become significant. A significant number of factors such as dairy herd management, different cattle breed, various technological phases and productive life of cows, as many others, have a particular influence on cows blood parameters, each in their way(Coroian et al., 2017; Cozzi et al., 2011; Obućinski et al., 2019).

This study aimed to investigate differences in the

hematological and biochemical profile between Podolian grey steppe and Holstein Friesian cows in different age groups.

MATERIALS AND METHODS

Animals and experimental design

All animals in this study were treated in compliancewith 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 128 adult cows aged from 3 to 12 years were used in the study. Cows were divided into equal numbers of 64 Podolian grey steppe, and 64 Holstein Friesian cows breeds, respectively. Further, each breed was divided into three groups according to age. The first group included cows aged from 3 to 6 years, the second from 7 to 10 years, and the third group cow over ten years old.The cows both used breed were clinically healthy. All cows were in the early stage of lactationbetween 65 and 85 days.The cows were kept in stables with temporary access to pastures.

During the experiment, cows were fed with hay (94.7% dry matter, 9.7% crude protein and 1.4% crude fat, respectively) which was provided *ad libitum*, and with compound feedsupplementation (91.3% dry matter, 12.6% crude protein and 1.6% crude fat, respectively) of an average 6 kg/day/cow.Freshwater was available to cows all the time.

Sample collections

At the time of blood sample collection, all cows were clinically healthy.Ten cows from each breed group were randomly selected for blood samples collection (Total of 60 blood samples were collected; 10 blood samples from each group (10 samples × 3 groups of each breed × 2 breeds = 60 blood samples). Blood wasobtained from the jugular vein (*V. jugularis*) of each selected cowin the morning before feeding. Blood was collected in test tubes with and without EDTA as an anticoagulant and refrigerated at a temperature of 4 °C, and immediately delivered to the laboratory for further analyses. Haematological and biochemical blood parameters were analyzed with the devices ADVIA 120 (Bayer Diagnostics Siemens, Germany) and ANALYSER A15 (Biosystems S.A., Barcelona, Spain).

Hematological and biochemical blood analyses

All blood samples analyses were performed at accredited laboratory VetLab (Belgrade, Serbia).Analyzed hematological parameters include white blood cells (WBC), neutrophils (NE), lymphocytes (LY), monocytes (MO), eosinophils (EO), basophils (BA), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelets (PLT), and mean platelet volume (MPV).All parameterswere determined on the hematological apparatus ADVIA 120 (Bayer Diagnostics Siemens, Germany).

Analyzed biochemical parameters included total protein (TP), albumin (AL), creatinine (Cr), urea, calcium (Ca), phosphorus (P), alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol (TC), and triglycerides (TG). All mentioned parameters were determined on the biochemical auto ANALYSER A15 (Biosystems S.A., Barcelona, Spain).

Statistical analyses

For statistical analysis,statistical software Statistica 13 (TIBCO Software Inc., USA)was used. First, the data were checked for normal distribution and homogeneity of variances. Obtained values within each group used for comparisons were not normally distributed. Values transformations (x^2) were necessary to acquire normal distributionafter which one-way ANOVA analysis was performed. The mean values were compared by the Tukey HSD post hoc test. With the usage of factorial analysis of variance,the significance level of the factors has been estimated, as well as their interactions (age; breed; and age × breed) within the observed values.The results were presented as means ± SD, wherea significance level of $P<0.05$ was used.

RESULTS AND DISCUSSION

The novelty of this research is reflected in that way that in the field and available scientific literature exists luck in data on the specific parameters of the health status of this autochthonous cattle breed. Asimilar investigation has been conducted in other research(Bedenicki et al., 2014) on the Istrian cattle breed, which pointed constant genetic similarity to Podolian grey steppe cattle breed. Muchresearch has shown different values of hematological and biochemical factors that

are understandable because of the many differences of genetic factors between different cattle breeds and many existing paragenetic factors(Keros et al., 2013) BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, BM1824.Results in table 1 show hematological parameters related to white blood cells of both breeds Podolian grey steppe and Holstein Friesian for different age groups. Results of WBC for Podolian grey steppe ranged between 6.75 and 11.94×10^9/L without a statistically significant difference between the same breed and between different ages ($P > 0.05$). Statistically, a significant difference was observed within two investigated breed ($P < 0.05$) where WBC was significantly higher for Holstein Friesian breed compared to Podolian grey steppe, ranged from 11.68 to 14.90×10^9/L, but without present statically significant difference in age between same breed ($P > 0.05$). The overall effect of interaction between ages and breed shows a significant difference ($P < 0.05$) regarding the WBC parameters of cows.According to Merck's veterinary manual, values for Podolian grey steppe cows in our research wereoptimal, while WBC of Holstein Friesian breed was slightly elevated. Results of Coroian et al.(2017) study showed higher WBC values in the six lactations compared to those results obtained in the research of-Radkowska and Herbut(2014), who observed hematological changes which were related to the management system. Obtained results in our study and related studies are in agreement with results obtained in the research of Obućinski et al.(2019).

Table 1. Hematological white cell parameters (Mean ± SD) of two breed for different age groups

Breed	Age	White cell parameters, ×10^9/L					
		WBC	NE	LY	MO	EO	BA
Podolian grey steppe	3 - 6	8.96^b±1.44	2.22^c±0.99	4.70^b±1.21	0.50^b±0.15	1.33^a±0.30	0.12^b±0.12
	7 - 10	6.75^b±0.21	0.81^d±1.17	2.60^c±0.75	0.29^c±0.11	0.96^b±0.05	0.04^c±0.01
	> 10	11.94^b±1.01	3.86^c±0.94	7.13^a±0.51	0.92^a±0.07	1.84^a±0.98	0.51^a±0.23
Pooled SE		0.021	0.457	0.033	0.018	0.029	0.034
Holstein Friesian	3 - 6	11.68^a±0.57	5.84^b±0.03	5.02^b±0.28	0.06^e±0.09	0.76^b±0.07	0.00^c±0.00
	7 - 10	13.80^a±0.31	5.30^b±0.27	7.30^a±0.03	0.14^d±0.05	0.99^b±0.07	0.00^c±0.00
	> 10	14.90^a±1.02	9.59^a±1.45	4.62^b±0.99	0.28^c±0.08	0.21^c±0.04	0.50^a±0.13
Pooled SE		0.057	0.011	0.032	0.196	0.025	0.132
Statistical significance of effect	Age	NS	*	*	*	*	*
	Breed	*	*	*	*	*	*
	Age × Breed	*	*	*	*	*	*

The value (Mean ± SD) with a different superscript in each column differ significantly among the same age group of different cows breed ($P < 0.05$); * - in each column differ significantly ($P < 0.05$); NS – not significant;SD – standard deviation; SE – standard error

Results presented in Table 1 showed statistically significant differences ($P < 0.05$) regarding all other hematological parameters. High influence in our study was recorded regarding age, breed as the well significant influence of age and breed interaction ($P < 0.05$). The concentration of NE was recorded in the optimal range for Podolian grey steppe cows, while for Holstein Friesian breed was elevated.Differences tendency in NE concentration in our study is similarto thefindings of Aarif et al.(2011) with3 groups of Sahiwal cows in different physiological stages, while the difference in research ofKumar et al.(2017) was not present.According to Merck's veterinary manual values for LY werein the optimal range.

In contrast, the values for MO, EO, and BA were slightly elevated for both breeds of cow in all groups, with recorded significant differences ($P < 0.05$).Higher increase of MO number in the blood occurs in response to chronic infections, in autoimmune disorders, in blood disorders, and in certain cancers, while elevated levels of EO in the blood can be an indicator of an illness or infection, what was not the situation in our study, as well as the elevated levels of BA which cannot be related to basophilia.In the study of Coroian et al.(2017), lymphocytes number was under the influence of lactation within extensive limits, which was not following the results in our study, as well as the other studies (Obućinski et al., 2019).The results obtained byKumar et al.(2017) shown that the breed did not have a significant influence on cow's lymphocyte profile.

Table 2. Hematological red cell parameters (Mean ± SD) of two breed for different age groups

Breed	Age	Red cell parameters				
		RBC ($\times10^{12}$/L)	HGB (g/L)	HCT (%)	MCV (fL)	MCH (10^{-12})
Podolian grey steppe	3 - 6	7.49^{b}±1.56	128.6^{a}±0.89	36.99^{a}±2.04	50.85^{a}±7.28	17.84^{a}±2.40
	7 - 10	5.83^{c}±0.25	112.0^{b}±0.93	32.50^{a}±1.92	33.60^{b}±3.48	12.30^{b}±1.27
	> 10	11.21^{a}±1.11	139.0^{a}±0.58	39.30^{a}±1.51	60.20^{a}±6.46	20.50^{a}±2.55
Pooled SE		0.058	0.096	1.027	0.983	1.203
Holstein Friesian	3 - 6	6.62^{c}±0.21	102.2^{b}±1.98	26.10^{b}±0.02	39.40^{b}±1.61	15.60^{b}±0.37
	7 - 10	6.69^{c}±0.69	105.8^{b}±0.96	27.60^{b}±0.97	39.90^{b}±1.12	15.80^{b}±0.88
	> 10	6.93^{c}±1.23	105.5^{b}±0.32	27.90^{b}±0.50	39.50^{b}±1.00	15.60^{b}±0.24
Pooled SE		0.262	0.011	0.021	0.046	0.033
Statistical significance of effect	Age	*	NS	NS	NS	NS
	Breed	*	*	*	*	*
	Age × Breed	*	NS	NS	NS	NS

The value (Mean ± SD) with a different superscript in each column differ significantly among the same age group of different cows breed ($P < 0.05$); * - in each column differ significantly ($P < 0.05$); NS – not significant; SD – standard deviation; SE – standard error

Table 3. Hematological red cell parameters (Mean ± SD) of two breed for different age groups

Breed	Age	Red cell parameters			
		MCHC (g/L)	RDW (%)	PLT ($\times10^{9}$)	MVP (fL)
Podolian grey steppe	3 - 6	351.1^{a}±0.90	20.09^{b}±0.93	231.50^{b}±65.67	10.51^{b}±1.30
	7 - 10	331.0^{a}±0.72	18.20^{b}±0.18	154.00^{b}±35.14	7.80^{b}±0.97
	> 10	370.0^{a}±0.66	21.80^{b}±0.56	386.00^{a}±69.22	12.00^{a}±1.28
Pooled SE		0.299	0.029	3.470	0.150
Holstein Friesian	3 - 6	394.0^{a}±0.31	24.11^{a}±0.27	401.00^{a}±82.11	13.48^{a}±0.15
	7 - 10	397.0^{a}±0.22	24.96^{a}±0.13	397.21^{a}±53.18	13.11^{a}±0.55
	> 10	394.0^{a}±0.54	23.18^{a}±0.85	342.39^{a}±51.10	13.79^{a}±0.63
Pooled SE		0.874	0.013	2.997	0.183
Statistical significance of effect	Age	NS	NS	NS	NS
	Breed	NS	*	*	*
	Age × Breed	NS	NS	NS	NS

The value (Mean ± SD) with a different superscript in each column differ significantly among the same age group of different cows breed ($P < 0.05$); * - in each column differ significantly ($P < 0.05$); NS – not significant; SD – standard deviation; SE – standard error

Results in Tables 2 and 3 show the obtained values of red blood cell parameters between two different breed of cows used in our study. Statistically significant ($P < 0.05$) differences in RBC can be seen between these two breeds. Count of red blood cells in the Podolian grey steppe breed ranged between 5.83 and 11.21×10^{12}/L,whichisthe following values provided in Merck's veterinary manual, with significant influence ($P < 0.05$) of cows age in obtained values. On the other hand, Holstein Friesian cows breed recorded RBC values lover then optimal, without any significant difference ($P > 0.05$) related to the age of cows.Results obtained in our study regarding the RBC of Holstein Friesian cows agree with the results ofRadkowska and Herbut(2014). These differences in RBC cannot be attributed to acute hemorrhage or abnormal destruction of red blood cells, which wasn't the case in our study so that it could be related to cows breed. All other red blood cell parameters in our study are consist, and the overall statistical significance test has shown significant influence ($P < 0.05$) of breed on red blood cell values, while age and interaction of age and cows breed didn't show statistically significant differences ($P > 0.05$).

Some research has shown that red blood cell parameters and hemoglobin concentration do not show seasonal differences, while according toKoubková et al. (2002), seasonand oscillations in temperature could influence increase values of red blood cell count and hematocrit. Besides changes in blood parameters in dairy cows, similar changes have been recorded in blood parameters of meat cows(Miglior et al., 2017), respectively. The parameters of red blood cells are interrelated tightly, and rely, among other things, on hemoglobin levels, hematocrit count, and value.

Results of mean corpuscular hemoglobin concentration, red cell distribution width, platelets, and mean platelet volume (Table 3), gained in our study shown no statistically significant differences ($P > 0.05$) related to the age of cows, while significant differences ($P < 0.05$) was recorded between the breeds of cows, except for the MCHC values, which was not affected by the breed influence.Overall statistical significance of age × breed in our study has shown no significant influence ($P > 0.05$).Our results of MCHC values are in agreement with the results ofCoroian et al.(2017). The hematological profile of dairy cattle suffering from the reproductive problem was examined byRuginosu et al.(2011) compared with healthy cows with changes in hematocrit, hemoglobin and erythrocytes were reported. Some hematological parameters were influenced by the age of the cows(Botezatu et al., 2014).

Results of biochemical blood parameters of Podolian grey steppe and Holstein Friesian cows breeds gained in our study are shown in Tables 4, 5, and 6.

Total protein has ranged between 7.10 and 8.50 g/dl (Podolian grey steppe), and 8.13 to 8.48 g/dl (Holstein Friesian), without recorded statistically significant differences ($P > 0.05$) between the age, breed as well as the interaction of age and breed. The same tendency can be seen regarding the albumin values obtained in our study ($P > 0.05$). The indicators of protein metabolism are urea, total protein, and albumins, while creatinine is the primary parameter reflecting kidney function(Stojević et al., 2005). Having in mind that the optimal values for creatinine are between 53.0 to 159.0 (µmol/L), we can see that Podolian grey steppe cows breed over ten years old shows increased levels, which can be an indicator of renal problems in that age.

Statistically significant differences ($P < 0.05$) are present between thesetwo investigated breeds regarding the values of Cr in blood.

Table 4. Biochemical blood parameters (Mean ± SD) of two breed for different age groups

Breed	Age	TP (g/dl)	AL (g/dl)	Cr (µmol/L)	Urea (mmol/L)	Ca (mmol/L)	P (mmol/L)
Podolian grey steppe	3 - 6	7.65^{a}±0.35	3.05^{a}±0.30	170.80^{b}±28.64	2.99^{b}±0.72	1.93^{b}±0.34	0.83^{b}±0.13
	7 - 10	7.10^{a}±0.18	2.20^{b}±0.94	145.10^{b}±29.14	1.70^{b}±0.66	1.30^{b}±0.47	0.70^{b}±0.22
	> 10	8.50^{a}±0.24	3.40^{a}±0.63	251.30^{a}±32.97	2.40^{b}±0.67	3.60^{a}±0.99	2.10^{a}±0.61
Pooled SE		0.015	0.147	1.998	0.024	0.019	0.009
Holstein Friesian	3 - 6	8.26^{a}±0.32	3.52^{a}±0.12	79.56^{c}±15.41	8.57^{a}±1.24	2.89^{a}±0.21	1.90^{a}±0.14
	7 - 10	8.13^{a}±0.41	3.14^{a}±0.19	83.98^{c}±19.20	7.99^{a}±0.86	3.00^{a}±0.55	1.96^{a}±0.05
	> 10	8.48^{a}±0.28	3.51^{a}±0.43	75.14^{c}±26.11	8.97^{a}±0.73	3.09^{a}±0.48	2.12^{a}±0.16
Pooled SE		0.053	0.091	1.014	0.029	0.087	0.017
Statistical significance of effect	Age	NS	NS	NS	NS	NS	NS
	Breed	NS	NS	*	*	NS	NS
	Age × Breed	NS	NS	NS	NS	NS	NS

The value (Mean ± SD) with a different superscript in each column differ significantly among the same age group of different cows breed ($P < 0.05$); * - in each column differ significantly ($P < 0.05$); NS – not significant; SD – standard deviation; SE – standard error

Urea concentration in blood and milk is one of the indicators of nutritional status.The higher blood urea concentration in the Holstein Friesian cows breed compared to the Podolian grey steppe, maybe since Holstein Friesian breed had high rumen degradable protein content of the diet, which mainly are indicative of dietary nitrogen wastage, while thelow urea levels are characteristic of low dietary crude protein consumption.The urea content indicates that feed protein is efficiently used by rumen microflora(Calsamiglia et al., 2010)and while major improvements in our understanding of N requirements and metabolism have been achieved, the overall efficiency remains low. In general, maximal efficiency of N utilization will only occur at the expense of some losses in production performance. However, optimal production and N utilization may be achieved through the understanding of the key mechanisms involved in the control of N metabolism. Key factors in the rumen include the efficiency of N capture in the rumen (grams of bacterial N per grams of rumen available N.

Values of Ca and P obtained in our research was optimal with slightly lower and higher values, without any statistically significant differences ($P > 0.05$) between breeds, age, and their interaction. Our results show no significant differences in Ca levels between

two breeds, while Mamun et al.(2013)founded a slight increase of calcium in females compared to the male, and alkaline phosphatase was higher in growing cattle. In research ofCoroian et al.(2017) Ca levels in the first three days of the postpartum period presented the lowest values, while the highest values were shown between 4 and 7 days, while phosphorus gave the most moderate average values on day one postpartum and the highest on day 7.

The alkaline phosphatase test is used to help detect liver disease or bone disorders. In conditions affecting the liver, damaged liver cells release increased amounts of ALP into the blood(Sato et al., 2005). The optimal range for ALP levels in cow's blood is 17.5 to 153.0 (U/L). The results of our study show no significant ($P > 0.05$) irregularities in ALP levels in Holstein Friesian cows breed (149.71 to 154.22 U/L). Significantly high ($P < 0.05$) levels of ALP in the blood of Podolian grey steppe cows breed over ten years old (314.40 U/L) indicates possible problems with liver disease or bone disorders.

Table 5. Biochemical blood parameters (Mean ± SD) of two breed for different age groups

Breed	Age	ALP (U/L)	ALT (U/L)	AST (U/L)
Podolian grey steppe	3 - 6	94.57^{c}±73.24	52.39^{a}±7.18	125.84^{a}±16.12
	7 - 10	14.50^{d}±2.84	38.40^{b}±4.12	93.70^{b}±17.31
	> 10	314.40^{a}±87.11	63.00^{a}±3.18	166.30^{a}±8.22
Pooled SE		2.144	0.991	1.034
Holstein Friesian	3 - 6	154.22^{b}±1.13	22.90^{b}±1.88	62.50^{b}±5.37
	7 - 10	149.71^{b}±2.36	23.30^{b}±2.67	63.80^{b}±8.22
	> 10	153.00^{b}±3.18	23.50^{b}±1.89	61.10^{b}±10.25
Pooled SE		1.065	0.084	1.001
Statistical significance of effect	Age	NS	NS	NS
	Breed	*	*	*
	Age × Breed	NS	NS	NS

The value (Mean ± SD) with a different superscript in each column differ significantly among the same age group of different cows breed ($P < 0.05$); * - in each column differ significantly ($P < 0.05$); NS – not significant; SD – standard deviation; SE – standard error

Table 6. Bloodlipid profile (Mean ± SD) of two breeds for different age groups

Breed	Age	HDL (g/L)	LDL (g/L)	TC (g/L)	TG (g/L)
Podolian grey steppe	3 - 6	0.84^{b}±0.02	0.53^{b}±0.27	2.10^{a}±0.08	0.08^{a}±0.04
	7 - 10	0.55^{b}±0.01	1.11^{a}±0.16	1.60^{b}±0.01	0.08^{a}±0.02
	> 10	1.17^{a}±0.03	0.19^{c}±0.02	1.30^{b}±0.06	0.09^{a}±0.01
Pooled SE		0.143	0.074	0.033	1.225
Holstein Friesian	3 - 6	0.96^{a}±0.01	0.28^{b}±0.02	1.84^{b}±0.46	0.09^{a}±0.01
	7 - 10	0.94^{a}±0.07	0.31^{b}±0.02	1.89^{b}±0.34	0.09^{a}±0.09
	> 10	0.91^{a}±0.09	0.33^{b}±0.01	1.82^{b}±0.51	0.09^{a}±0.01
Pooled SE		0.022	0.554	0.021	0.086
Statistical significance of effect	Age	NS	NS	NS	NS
	Breed	*	*	NS	NS
	Age × Breed	NS	NS	NS	NS

The value (Mean ± SD) with a different superscript in each column differ significantly among the same age group of different cows breed ($P < 0.05$); * - in each column differ significantly ($P < 0.05$); NS – not significant; SD – standard deviation; SE – standard error

Determining AST activities in dairy cows is most often connected with fatty liver syndromelow appetite and the appearance of ketosis in dairy cows during early lactation. Increased AST activity in the serum is a sensitive marker of liver damage, even if the damage is subclinical. Ruminant liver cells do not show high ALT activity, and the increased activity of that enzyme in the serum during liver damage, even in necrosis, is insignificant(Stojević et al., 2005). Results gained in our research (Table 5) show that the age of cows didn't influence significantly ($P > 0.05$) on ALT and AST enzyme activity. Statistically significant influence ($P > 0.05$) was recorded regarding the breed influence. Holstein Friesian cows have significantly lower levels of both ALT and AST enzyme activity compared to Podolian grey steppe cows breed, while the interaction of age and breed effects didn't show significant influence ($P > 0.05$). Our results have

been in agreement with the results of other researchers(Cozzi et al., 2011; González et al., 2011; Krsmanovic et al., 2016; Sun et al., 2015), respectively.

Cholesterol, triglycerides, and different density lipoproteins are important constituents of the lipid fraction of the body. Cholesterol is unsaturated alcohol of the steroid family of compounds, and it is essential for the normal function of all animal cells and is a fundamental element of their cell membranes(Puvača et al., 2016; Puvača et al., 2015). Results presented in Table 6, show significant influence ($P < 0.05$) of breed on HDL and LDL levels in the blood of cows. Our results are in agreement with the investigation ofTajik and Tahvili(2011). In dairy cows, an important lipid-related metabolic disorder is the fatty liver, which has also been associated with the changes in TC and TG levels. Fatty liver develops when TG synthesis exceeds the export of TG as very low-density lipoproteins(Kessler et al., 2014).Levels of TC in Podolian grey steppe cows have ranged between 1.30 and 2.10 g/L, while in Holstein Friesian breed levels of TC ranged between 1.82 and 1.89 g/L, respectively. The significant difference in this parameter was recorded between thesetwo breeds ($P < 0.05$) only in cows 3 to 6 years old, while the interaction between age and breed did not show any statistical significance ($P > 0.05$). A similar tendency without statistically significant differences ($P > 0.05$) was recorded for levels of TG in the blood of both cows breed.

CONCLUSIONS

The results of our study have shown the influence of different age and breed of cows on hematological and biochemical parameters. Gain results have mainly demonstrated a significant influence of breed on the parameters mentioned above, while the significant influence of cows age was not present as well the interaction between age and breed.

It has been shown that the values of hematological and biochemical parameters were generally situated within the reference intervals. Due to the shortage of studies on the hematology and clinical biochemistry of the Podolian grey steppe ordinarily, the obtained results represent a novelty and contribution to achieving a better understanding of the metabolic profile and hematological indicators for estimating the physiological status of both Podolian grey steppe and Holstein Friesian breed, and for future diagnostic purposes, but more investigation in this field is certainly more than necessary.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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J HELLENIC VET MED SOC 2020, 71(4): 2455-2462
22ΠΕΚΕ 2020, 71(4): 2455-2462

Research article
Ερευνητικό άρθρο

Antibiotic resistance pattern and frequency of some beta lactamase genes in *Klebsiella pneumoniae* isolated from raw milk samples in Iran

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ABSTRACT: *Klebsiella pneumoniae* have become an important cause of mastitis in dairy cows. Resistance to beta lactam antibiotics resulted from beta lactamases enzyme production. The aim of this study was to investigate the antibiotic resistance pattern and frequency of some beta lactamase genes in *Klebsiella pneumoniae* isolated from raw milk samples in Iran. 200 raw cow milk samples were collected from different villages of north west of Iran. The samples were cultured and biochemical tests were performed for phenotypic diagnosis. Then, antibiotic resistance pattern was determined by antibiogram test. Finally, the presence of *CTX*, *SHV* and *TEM* genes in *Klebsiella pneumoniae* isolates was found by PCR method. Of total 200 raw cow milk samples, 80 samples (40%) contained *Klebsiella pneumoniae*. The frequency of *CTX*, *SHV* and *TEM* genes in *Klebsiella pneumoniae* isolates was 50 (62.5%), 34 (42.5%) and 70 (87.5%), respectively. 14 *Klebsiella pneumoniae* isolates (17.5%) possessed all three intended genes simultaneously. All strains of *Klebsiella pneumoniae* (100%) were resistant to ampicillin. The most strains were resistant to ceftriaxone (75%), gentamicin (70%) and nitrofurantoin (70%). 4 *Klebsiella pneumoniae* strains (5%) were resistant to all of tested antibiotics. The results showed high frequency of ESBLs and antibiotic resistance in *Klebsiella pneumonia* samples isolated from raw milk. It may occur exchange of resistance genes within and across species and with commensal bacteria of the human and animals.

Keywords: *Klebsiella pneumoniae*; Raw milk; Antibiotic resisrance; Beta lactamase genes

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Date of initial submission: 29-08-2019
Date of revised submission: 31-08-2020
Date of acceptance: 01-10-2020

INTRODUCTION

Klebsiella pneumoniae is a gram-negative intestinal bacterium that forms part of the natural microflora of the human body. This bacterium is responsible for a wide range of diseases including bacteremia, pneumonia and urinary tract infection in human (Hashemi et al., 2014). Studies worldwide have revealed that Klebsiella can contaminate meat (Messaoudi et al., 2009) and dairy products (Yilma et al., 2007) and contribute to disease and spoilage (Clegg and Sebghati, 2002). Raw milk can be contaminated with ESBLs (Extended Spectrum Beta Lactamases) producing enterobacteriaceae in several entities such as mastitis, directly by animal feces or indirectly during milking (Dahmen et al., 2013). Clinical mastitis resulting from *Klebsiella pneumoniae* infection causes high milk losses and mortality of the affected cows (Grohn et al., 2004). The sources of *Klebsiella* spp. in dairy operations include organic bedding material such as wood by-products (Hogan et al., 1989). In addition, fecal shedding by cows contributes to the presence of a large variety of *K. pneumoniae* strains in dairy herds (Munoz et al., 2006; Munoz and Zadoks, 2007). Humans may become colonized or infected by ESBL producing *K. pneumoniae* upon contact with blood, saliva, feces and urine of ESBL carrier animals or consumption of contaminated water or food products (Founou et al., 2016). *Klebsiella pneumoniae* are more capable than most strains of *E. coli* to overcome the inhibitory effects of lactoferrin and infect involuted mammary glands (Todhunter et al., 1990). According to different studies, 1- 5% of food borne intoxications are associated with milk consumption and dairy products (Mansuri Najand and Ghanbarpour, 2006). Unfortunately, the majority of the population in Iran, especially in rural families, still consume raw dairy products without pasteurization including traditional Lighvan cheese (most popular soft white cheese). The general assumption is that raw milk is generally safer and has more beneficial health effects, and that pasteurization would drastically affect the milk quality (Zeinhoma and Abdel-Latefb, 2014). The entry of ESBL-producing enterobacteriaceae in the food chain and environment could be considered a possible interface for the exchange of resistance genes between humans and animals (Walsh and Fanning, 2008). ESBL-producing *Klebsiella* spp. and *E. coli* are now listed among the six drug-resistant microbes for which new therapies are urgently needed (Shah et al., 2004). Approximately 20% of *K. pneumoniae* infections in intensive care units in the United States are now caused by strains not susceptible to third-generation cephalosporins (Paterson 2006). The most frequent and clinically relevant ESBL genes belong to *CTX-M*, *TEM*, and *SHV* families, with CTX-M enzymes emerging as the predominant type. *K. pneumoniae* commonly produces all three groups of enzymes (Perovic et al., 2016). These resistance genes are generally carried on mobile genetic elements (MGEs) facilitating their dissemination within and between bacterial species (Founou et al., 2016). ESBL can hydrolyse penicillins first, second and third-generation cephalosporins and aztreonam (but not cephamycins or carbapenems). Resistance to beta lactam antibiotics is most commonly found in *E. coli* and *K. pneumonia*, and today this resistance mechanism is recognized globally. During the past few years, there has been an increase in the detection of ESBL-producing strains in the general community (Mesa et al., 2006). The aim of current study was to investigate the antibiotic resistance pattern and frequency of some beta lactamase genes in *Klebsiella pneumoniae* isolated from raw milk samples in Iran.

MATERIALS AND METHODS

Sample collection

This cross-sectional study, was performed from April to October 2018. A Total of 200 raw cow milk samples from 35 dairy farms (which had not received antibiotics for at least five days) were collected randomly from different villages in north west of Iran. The breasts of the studied cows were apparently healthy. First, each teat was disinfected with ethanol 70̊, then the first few showers of milk were thrown away and 30-50 ml of milk was taken from the animal separately and transported to the laboratory under chilled conditions and processed for microbiological analysis.

Isolation and identification of bacteria from raw milk samples

The samples were inoculated into Eosin Methylene Blue (EMB) agar and MacConkey agar (Merck, Germany) and the plates were incubated at 37$^{\circ C}$ for 24 h. Then, separation of pure colonies were performed by streaking onto sterile nutrient agar slants as pure culture and subjected for standard morphological (Gram staining) and biochemical tests such as oxidase, catalase, methyl red, voges proskauer, citrate, indole and urease.

Antimicrobial susceptibility testing and ESBL detection

The antimicrobial susceptibility testing of all identified isolates were done according to the criteria of the Clinical and Laboratory Standards Institute method (CLSI 2017) (Padtan teb, Iran) (Kirby-Bauer method). In addition, all isolates were screened in terms of ESBL presence by using combined disk method. Cefotaxime + clavulanic acid (*CTX*/CLA) and Ceftazidime + clavulanic acid (CAZ/CLA) were used to confirm ESBL-producing isolates (Becton, USA). The strains in which the diameter of growth inhibition zone of cephalosporin + clavulanic acid disc were ≥ 5 mm compared to cephalosporin discs only were considered as ESBL-producing bacteria (Coudron et al., 2000).

DNA extraction

Genomic DNA was extracted using the boiling method (Chen et al., 2009). DNA extraction was performed on 80 cultured isolates of *Klebsiella pneumoniae* in brain heart infusion (BHI) agar (Merck, Germany) medium at 35℃ for 24 h. 3-5 colonies of each sample were poured in 1.5 ml eppendorf tube containing 200 µl of sterile TE buffer and were mixed thoroughly using a shaker. Then, the vials were boiled in boiling ban Marry (100 ℃) for 10 minutes, so that the boiling water level covered two-thirds of the vials. Finally, the vials were centrifuged at 9000 g for 5-10 minutes. The supernatant of vials containing DNA was transferred to sterile ependorf for PCR test. The quantity and quality of DNA extracted were investigated by nano-drop and electrophoresis apparatuses.

PCR test to detect intended genes

The polymerase chain reaction (PCR) method was done in 25 µl, including 11µl of Master mix PCR, 1 µl of each specific primers (25 nano moles) (Table 1), 1 µl (50 ng) of DNA template and 11 µl of double distilled water. Timetable and thermal schedule for each gene is presented in Table 2. The amplified products were run on 1% agarose gel and staining with ethidium bromide (0.5 mg/ml) in a dark room.

Table 1. Characteristics of specific primers related to the genes under investigation

Gene	Primer sequence	Amplicon size (bp)	Reference
CTX	5′-CGCTTTGCGATGTGCAG-3′ 5′-ACCGCGATATCGTTGGT-3′	550	Lin et al., 2012
SHV	5′-TACCATGAGCGATAACAGCG-3′ 5′-GATTTGCTGATTTCGCTCGG-3′	450	Doosti et al., 2015
TEM	5′-TCCGCTCATGAGACAATAACC-3′ 5′-ATAATACCGCACCACATAGCAG-3′	296	Doosti et al., 2015

Table 2. PCR test conditions for *Klebsiella pneumoniae* samples for replication of the tested genes

Stage	Number of cycles	Tested gene Time	Temperature (℃)
		CTX/*SHV*/*TEM*	
Primary denaturation	1	5′	95
Denaturation	32	60”	94
Annealing	32	40”	55/55/58
Extension	32	40”	72
Terminal extension	1	5′	72

RESULTS

Of total 200 raw cow milk samples, 80 samples (40%) contained *Klebsiella pneumoniae*. The results showed that 50 (62.5%) samples of *Klebsiella pneumoniae* isolates harbored *CTX* gene (Figure 1).

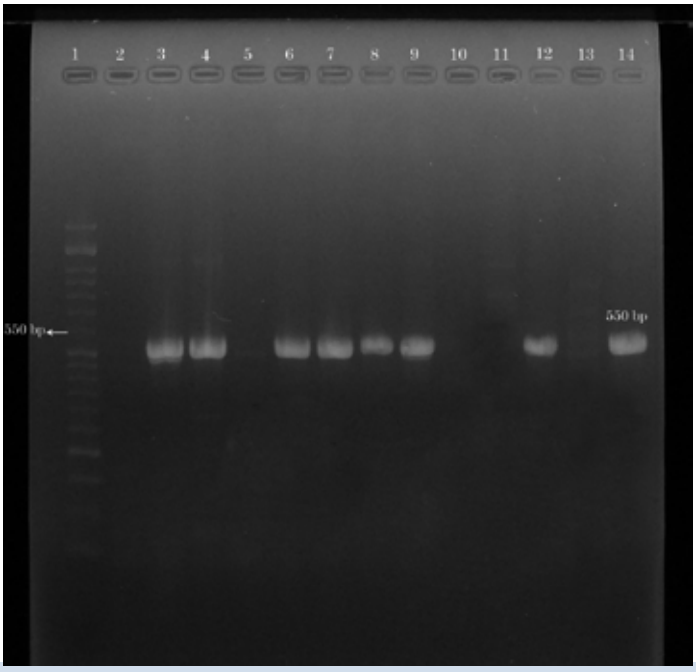

Figure 1. Lane 1 is marker (50 bp). Lane 2 is negative control (Double distilled water). Lane 3 indicates *Klebsiella pneumoniae* PTCC 1290 as positive control that bands within 550 bp and is associated to *CTX* gene. Lanes 4, 6-9, 12 and 14 are positive samples. Lanes 5, 10, 11 and 13 are negative samples

34 (42.5%) samples of *Klebsiella pneumoniae* isolates harbored *SHV* gene (Figure 2).

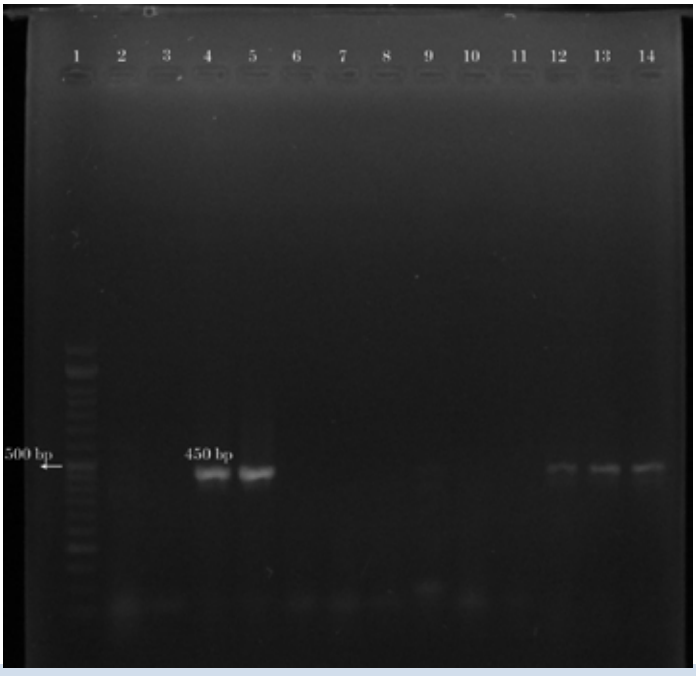

Figure 2. Lane 1 is marker (50 bp). Lane 2 is negative control (Double distilled water). Lane 4 indicates *Klebsiella pneumoniae* ATCC 700603 as positive control that bands within 450 bp and is associated to *SHV* gene. Lanes 5, 12-14 are positive samples. Lanes 3, 6-11 are negative samples

70 (87.5%) samples of *Klebsiella pneumoniae* isolates harbored *TEM* gene (Figure 3).

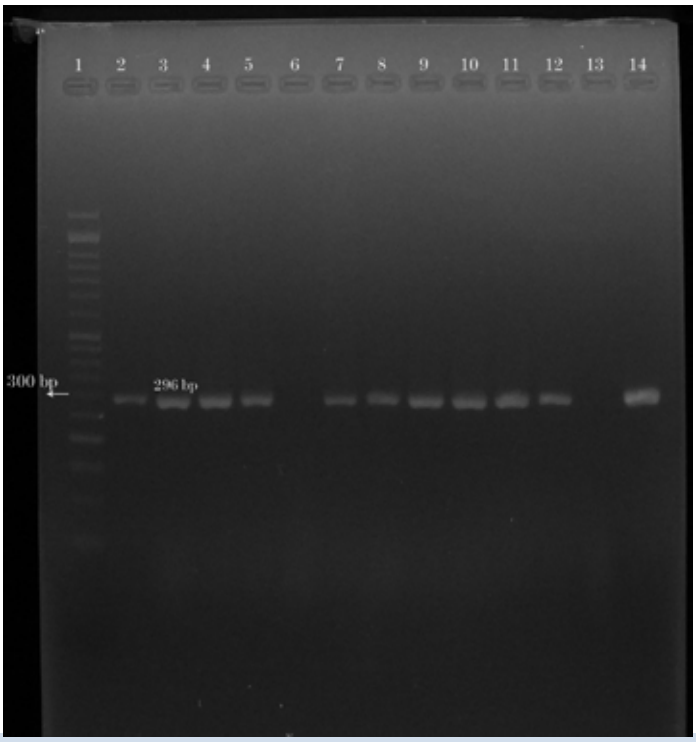

Figure 3. Lane 1 is marker (50 bp). Lane 6 is negative control (Double distilled water). Lane 2 indicates *Klebsiella pneumoniae* PTCC 1290 as positive control that bands within 296 bp and is associated to *TEM* gene. Lanes 3-5, 7-12 and 14 are positive samples. Lane 13 is negative samples

Furthermore, 14 (17.5%) *Klebsiella pneumoniae* isolates possessed all three intended genes simultaneously. 4 (5%) *Klebsiella pneumoniae* isolates lacked the tested genes. On the basis of Chi square test, the frequency of *CTX+TEM* genes was significantly higher than the other double combination genes ($P< 0.05$).

The results revealed that the frequency of studied genes within *TEM* and *SHV* genes showed statistical significance ($p< 0.05$). Moreover, the frequency of studied genes among gene groups showed statistical significance ($p< 0.05$) (Table 3).

Table 3. Frequency of intended genes in the studied bacterial samples

	CTX	TEM	SHV
Yes	50	70	34
No	30	10	46
Total	80	80	80
Chi square	0.8	45.0	7.2
P value	0.371	0.0001	0.007

The obtained results recorded in Table 4 releaved that, all strains of *Klebsiella pneumoniae* (100%) were resistant to ampicillin. Most of the strains were resistant to ceftriaxone (75%), gentamicin (70%) and nitrofurantoin (70%). Much of the sensitivity of *Klebsiella pneumoniae* strains was to amikacin (47.5%) and chloramphenicol (47.5%). Of total 80 *Klebsiella pneumoniae* isolates, only 56 samples (70%) were considered as ESBL producing strains in combined disk test. The percentages of resistance of *Klebsiella pneumoniae* isolates to cefotaxime and ceftazidime were 55% and 67.5%, respectively.

The antibiogram results showed that 4 *Klebsiella pneumoniae* strains (5%) were resistant to all of tested antibiotics (Table 5).

Table 4. Antibiogram results obtained from *Klebsiella pneumoniae* samples isolated from milk

Antibiotic	Abbreviation	Concentration (µg)	Susceptibility R(%)	S(%)	I(%)
Ampicillin	AM	10	0 (0)	0 (0)	80 (100)
Amikacin	AN	30	8 (10)	38 (47.5)	34 (42.5)
Ceftriaxone	CRO	30	14 (17.5)	6 (7.5)	60 (75)
Chloramphenicol	C	30	6 (7.5)	38 (47.5)	36 (45)
Ciprofloxacin	CP	5	12 (15)	32 (40)	36 (45)
Cotrimoxazole	SXT	23.75	4 (5)	26 (32.5)	50 (62.5)
Gentamicin	GM	10	6 (7.5)	18 (22.5)	56 (70)
Imipenem	IMP	10	16 (20)	12 (15)	52 (65)
Nitrofurantoin	FM	300	10 (12.5)	14 (17.5)	56 (70)
Tetracycline	TE	30	14 (17.5)	22 (27.5)	44 (55)
Cefepime	FEP	30	8 (10)	20 (25)	52 (65)

R: Resistant, S: Sensitive, I: Intermediate

Table 5. Frequency and percentage distribution of multidrug resistant (MDR) ***Klebsiella pneumoniae*** **isolates**

Resistant isolates (%)	1 fold	2 fold	3 fold	4 fold	5 fold	6 fold	7 fold	8 fold	9 fold	10 fold	11 fold
	80 (100)	78 (97.5)	76 (95)	70 (87.5)	66 (82.5)	56 (70)	50 (62.5)	34 (42.5)	26 (32.5)	16 (20)	4 (5)

DISCUSSION

Klebsiella pneumoniae is a causative agent of coliform mastitis. Mastitis is one of the major diseases affecting livestock breasts and is considered to be the most costly disease of dairy cattle worldwide, causing major damage to the livestock industry worldwide annually. In the US, milk production decline due to subclinical mastitis costs about 1 billion dollars (110 $ per cow) annually in the dairy industry, and 70% of cases of milk production decline in the herd is associated with subclinical mastitis (Seegers et al., 2003). The incidence of mastitis has been between 0.5-25 percent per month in Iran. It has been estimated that milk production decline from subclinical mastitis alone in 2006 had been approximately 150,000 tons in national level in Iran (Bolourchi et al., 2008). The results of current study revealed that the percentages of *CTX*, *SHV* and *TEM* genes in *Klebsiella pneumoniae* strains isolated from raw cow milk were 62.5%, 42.5% and 87.5%, respectively. In a study in Kenya, it was reported that the percentage of *SHV* and *CTX* genes in *Klebsiella pneumoniae* strains isolated from raw camel milk were 97.1% and 57.1% , respectively (Njage et al., 2012). In another study in Sudan, it was revealed that 61% of *Klebsiella pneumoniae* strains isolated from cow milk harbored *CTX* gene. They also showed that 23% and 16% of these strains possessed *SHV* and *TEM* genes, respectively (Badri et al., 2018). In a research in India, 1.5% of *K. pneumoniae* strains was identified as ESBL from raw milk (Koovapra et al., 2016). Significant differences in the frequency of *CTX*, *SHV* and *TEM* genes in different studies may be due to different sources of isolation in different regions, their methods of investigation and sensitivity, and number and types of samples. The results of the present study indicate the difference in dispersion of *CTX*, *SHV* and *TEM* genes in *Klebsiella pneumoniae* strains; this difference probably originates from geographical diversities and also differences in the ecological origin of the isolated strains (milk, human and different animals). In present study, the antibiogram results showed that, all strains of *Klebsiella pneumoniae* (100%) were resistant to ampicillin and 4 (5%) strains were resistant to all of tested antibiotics. In a study in Egypt, it was reported that all of *Klebsiella pneumoniae* strains (100%) isolated from buffalo and cow milk were resistant to ampicillin and most of the strains (82.6%) were resistant to chloramphenicol. Furthermore, much of the sensitivity of *Klebsiella pneumoniae* strains was to gentamicin (100%) and nitrofurantoin (91.3%) (Osman et al., 2014). In another study, the resistance of *Klebsiella pneumoniae* strains isolated from cow milk to ampicillin, ciprofloxacin, gentamycin, amikacin and cefepime was 94%, 89.2%, 46%, 82,5% and 92%, respectively (Badri et al., 2018). The cause of mismatch between the results of the antibiogram test in several studies is probably due to differences in geographical area, type of treat-

ment regimen and measure of antibiotic use in different regions. In present study, the results of combined disk test showed that 70% of *Klebsiella pneumoniae* isolates were considered as ESBL producing strains but PCR test revealed that 95% of *Klebsiella pneumoniae* isolates were ESBL producing strains. This inconformity in the results of these two methods may be due to insufficient sensitivity of the antibiogram discs or errors in performing test. Prolonged and wasteful use of antibiotics in the treatment of livestock diseases such as mastitis as well as the use of antibiotics as growth promoters has created antibiotic resistant strains. This raises concerns about the introduction of bacterial resistant strains into the food chain.

CONCLUSION

The results of current study showed high frequency of ESBLs and antibiotic resistance in *Klebsiella pneumonia* samples isolated from raw milk. It may occur due to the exchange of resistance genes within and across species and with commensal bacteria of the human and animals. Therefore, in order to control infection and prevent distribution of antibiotic resistance genes among clinical or foodborne isolates, correct management of treatment is necessary.

ACKNOWLEDGEMENT

This research project is funded by the personal authors of the article.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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J HELLENIC VET MED SOC 2020, 71(4): 2463-2470
ΠΕΚΕ 2020, 71(4): 2463-2470

Research article
Ερευνητικό άρθρο

Etomidate anesthesia in chicks: Effect of xylazine

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ABSTRACT: The current study aimed to evaluate the etomidate anesthetic action, its toxicity profile and safety alone and to determine the benefit of xylazine coadministration to enhance its anesthetic duration, efficacy and to reach a state of balanced anesthesia in chicks. By using the up-and-down technique, it was found that the hypnotic Median Effective Dose (ED_{50}) of the etomidate was 4.30 mg/kg, IM, whereas the acute Median Toxic Dose (TD_{50}) was 17.90 mg/kg, IM in the chicks. In response, the calculated Therapeutic Index (TI) and Standard Safety Margin (SSM) indicate that the etomidate has a wide safety margin. Etomidate injection at 4, 8 and 16 mg/kg, IM yields a significant dose-response and dependent hypnosis in the chicks by evaluating the onset of the righting reflex loss, its period and regaining from it. The combination composed of etomidate and xylazine at 5 mg/kg, IM for each, reduced the onset of hypnosis and significantly distended its period besides a significant rise of the recovery time when compared with the group receiving etomidate alone. At the same time, this coadministered drugs elicited a significant raise in analgesic efficacy. Concerning plasma glucose, Alanine Transaminase (ALT) and Aspartate Transaminase (AST) concentrations, neither etomidate nor etomidate plus xylazine differ significantly from the control group. The results of this study propose the likelihood of using etomidate as an anesthetic agent for short surgical trials in the chickens that can be more effective by using xylazine to yield balanced anesthesia without causing significant side effects.

Keywords: Analgesia, chicks, etomidate, hypnosis, xylazine

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Date of initial submission: 12-11-2019
Date of revised submission: 28-07-2020
Date of acceptance: 05-10-2020

INTRODUCTION

Etomidate is an imidazole derivative that belongs to non-barbiturate anesthetics acting as a short anesthetic agent used for rapid induction of general anesthesia. The mechanism by which etomidate induced anesthesia via its potentiation of the neurotransmitter Gamma Aminobutyric Acid (GABA) and tighten its binding to $GABA_A$ receptor, leading to depression of the central nervous system (Finkel et al., 2009; Forman, 2011). Etomidate has a good hypnotic effect but less analgesic and muscle relaxation efficacy with little to negligible effect on the cardiovascular system (unlike ketamine) so it is used for cardiac and vascular surgeries (Finkel et al., 2009; White and Trevor, 2009; Forman, 2011). Etomidate has a side effect by maintaining the sympathetic outflow (Ebert et al., 1992) besides the suppression of the adrenal gland cortex (Jabre et al., 2009) and causing cognitive deficits as found in a rat model (Dixon et al., 2003). Furthermore, xylazine considered to have analgesic and sedative effects as well as muscle relaxant effect which results from its action by stimulating α_2–adrenoceptor causing an inhibition of the release of noradrenaline neurotransmitter and leading to depression of the central nervous system. Xylazine is used commonly with ketamine to produce balanced anesthesia characterized by good hypnotic, analgesic and muscle relaxant effects (Pawson, 2008; Kleinz and Spence, 2008).

The main goal of this study was to evaluate the anesthetic profile of etomidate (as well as its toxicity and safety) in the chickens and to determine the benefit of xylazine coadministration to enhance its anesthetic duration, efficacy and to reach a state of balanced anesthesia which is characterized by good hypnosis, analgesia, muscle relaxation and hyporeflexia. The above goal is of a major outcome to be applied in the surgical operations and may be substituted the ordinary protocol of ketamine-xylazine combination in animals since the latter combination lack the complete criteria of balanced anesthesia with the presence of cardiovascular side effect of ketamine.

MATERIAL AND METHODS

Birds

7-14 days-old chicks (70 overall chicks were used in this study) of both sexes were used in all the experiments with mean body weights between 0.1–0.15 kg. They retained in 25 chicks per cage at 30–33°C of temperature (which is the optimal temperature at this age of chicks) besides incessant light and the litter made up of wood shreds. The birds' advent to water and food at will. The dose of etomidate (Hypnomidate 0.2%, Janssen-Cilag Ltd., UK) and xylazine (2%, alfasan, Holland) were diluted with a physiological normal saline solution in 10 ml used to make the desired concentration to be injected in the chicks for each kg of bird mass as intramuscularly (IM) route (10 ml/kg, IM).

For ethical respects, the professional scientific committee of the department of physiology, biochemistry and pharmacology / College of the Veterinary Medicine / University of Mosul agreed and permitted this research for the necessarly utilization of optimal experimental animals.

Determination of the hypnotic ED_{50} of etomidate in chicks

Hypnotic ED_{50} value of etomidate was estimated conferring to the up and down mode (Dixon, 1980). First etomidate's dosage of 5 mg/kg, IM depends on an introductory study. The chicks were superintended for two hours to the incidence of the etomidate's hypnosis (lack the righting reflex). The doses of etomidate then would be diminished or augmented 1 mg rely on the incidence or absence of the hypnotic effect in the chicks, respectively (Mousa and Al-Zubaidy, 2019). The ED_{50} value was calculated as follows:

ED_{50} value = xf + K d were:

xf = The last dose used

K = Table's value which extracted from Dixon, 1980 (depend on X and O symbols obtained)

d = ± in the dosage

The marks elicited by etomidate hypnosis in chicks (during 1-2 min.) were ataxia, recumbency, closed eyelids, loss of righting reflex and the hypnosis characterized by quiet sleep.

Evaluation of acute TD_{50} of etomidate in chicks

This experiment was conducted also conferring the up and down described before (Dixon, 1980) mentioned above. Rudimentary dosage of etomidate at 20 mg/kg, IM which depends on an initiative procedure. The chicks were watched twenty-four hours aimed at the appearance of etomidate's toxic marks thereafter,

the doses of etomidate should be reduced or amplified 3 mg according to the death presented by etomidate in the chicks. The TD_{50} was calculated from the equation in the same manner as previous experiment.

The toxicity signs observed in the chicks were recumbency, defecation, excitation, paralysis, increased sensitivity to external stimuli, an increase in the wings and leg movements and the outcome was death during 4-8 min.

Estimation of the drug safety indices of etomidate

The ED_{50} and TD_{50} values of etomidate mentioned above were used for the interpretation and extrapolation the drug safety of etomidate through its use in the following equations (Muller and Milton, 2012):

$TI = TD_{50} \setminus ED_{50}$ (The higher the number of TI resulted in means that the drug is safe and vice versa).

$SSM = (TD_1 \setminus ED_{99} - 1) \times 100$ (TD_1 is the minimum dose which produces an adverse effect in 1% of the experimental sample and ED_{99} is the minimum dose required to produce the therapeutic effect in 99% the experimental sample; large SSM value means the drug is safer).

The dose-responsive hypnotic effect of etomidate in chicks

Etomidate's hypnosis (lack the righting reflex) were watched in three groups of chicks (six chicks/dose group) that injected with different doses of etomidate at 4, 8 and 16 mg/kg, IM procured from the hypnotic ED_{50} value of etomidate from the previous experiment in chicks. The onset of hypnosis (indicated by the loss of the righting reflex) was calculated as the time from etomidate injection to the loss of righting reflex. The duration of hypnosis is the time from the loss of the righting reflex till the chick return and corrected their body to normal position while the recovery time was defined as the time between the onset of hypnosis until the chick begin to move and restore the normal activity (Roder et al., 1993; Mousa and Al-Zubaidy, 2019).

Hypnotic and analgesic efficacy of etomidate and the effect of xylazine combination in chicks

A. Effect of xylazine on etomidate hypnosis

Two groups of chicks (6 chicks/group) were used, the first one consisted of etomidate injection alone at 5 mg/kg, IM and the second one composed of injection of etomidate and xylazine at 5 mg/kg, IM for each drug. The dose of etomidate was selected from the previous two experiments while the dose of xylazine was selected from another study as it resembles the effective dose of xylazine in the chicks (Mousa and Mohammad, 2012a; Mousa et al., 2019). Onset, duration and recovering from hypnosis were written down in two groups for every chick individually.

B. Effect of xylazine on etomidate analgesia

Other chickens of the same groups mentioned above composed of etomidate alone or etomidate plus xylazine were used in this trial. The voltage of electro-stimulator device (Harvard apparatus, USA) that induce nociceptive effect (specified by distress call) was recorded pre- and post-5 minutes of therapy. The proportion of analgesic effect (the number of chicks that show analgesia to total), its volts including voltage (delta) for every group was also noted (Mousa and Mohammad, 2012b; Mousa, 2014; Mousa, 2019a).

Measurement of plasma glucose, ALT and AST concentrations in the chicks treated with etomidate alone or etomidate plus xylazine

After 2 hours of etomidate injection alone (5 mg/kg) or etomidate plus xylazine (5 mg/kg, IM for both drugs), the blood got from the jugular vein of the neck (6 chicks/group), experiencing centrifugation to gain the plasma which refrigerated pending analysis during 48 hours. The procedure for estimation of glucose concentration (Wotton, 1974), AST and ALT (Yang et al., 2018) concentrations in plasma were analyzed for all the groups mentioned earlier, in addition to the –ve saline control which all analyzed with the specified kit (Biolabo, France) for glucose and liver enzymes by mean of Chemistry Analyzer Smart-150 apparatus (GenoTEK, USA).

Statistical analysis

Parametric data (onset, duration and recovery from hypnosis as well as glucose, ALT and AST concentrations of three groups) were analyzed by analysis of variance (one-way) followed by the LSD, while student T-test implemented to analyze the two groups (onset, duration and recovery from the hypnosis of two groups besides the pre and post-voltage recorded) (Katz, 2011; Petrie and Watson, 2013). The non-parametric outcome (% antinociception) were resolved by the Fisher exact probability and Mann-Whitney-U-test (delta voltage)(Kvam and Vidakovic, 2007; Katz, 2011). The outcome reflected significantly differs once p was < 0.05.

RESULTS

Hypnotic ED_{50} value for etomidate in chicks

The ED_{50} value of etomidate that generates hypnosis (lack the righting reflex) in 50 % of the subjected chicks was at 4.30 mg/kg, IM as resolved by the up and down technique (Table 1).

Table 1. Etomidate's hypnotic ED_{50} in the chicks

Parameter	Result
ED_{50} value = xf + K d	4.30 mg/kg, IM
The extent of the doses applied	4-5 mg/kg
Starting dose	5 mg/kg
Ending dose (xf)	5 mg/kg
± in the dosage (d)	1 mg
Chicks used	5 (XOXOX)*
Starting of hypnosis	1-2 minutes

X: effect (hypnosis), O: no effect (no hypnosis)
*: Symbols (XOXOX) used to extract table value (K) from Dixon, 1980

Acute TD_{50} for etomidate in experimental chicks

Table 2 exhibits acute etomidate's TD_{50} value in chicks determined to became 17.90 mg/kg, IM.

Table 2. Determination of acute TD_{50} of etomidate in chicks

Parameter	Result
Acute TD_{50} value = xf + K d	17.90 mg/kg, IM
The extent of the doses applied	17-20 mg/kg
Starting dose	20 mg/kg
Ending dose (xf)	20 mg/kg
± in the dosage (d)	3 mg
Chicks used	5 (XOXOX)*
Occurence of death	4-8 min.

X: effect (death), O: no effect (alive)
*: Symbols (XOXOX) used to extract table value (K) from Dixon, 1980

Drug safety of etomidate as estimated by safety indices

By using the values of ED_{50} and TD_{50} of etomidate mentioned above, The TI of etomidate estimated will be 4.

$TI = TD_{50} \setminus ED_{50}$ = (17.90 mg/kg) \ (4.30 mg/kg) = 4.16 = approximate to 4

The TI number (4) means that the effective dose (ED_{50}) used of etomidate should be multiplied 4 times to induce the toxic effect of etomidate and kill 50% of the animals (TD_{50}).

In contrast, The SSM estimated from its equation is as follows:

$SSM = (TD_1 \setminus ED_{99} - 1) \times 100$ = (0.358 mg/kg \ 8.514 mg/kg – 1) × 100 = 100 % (The TD_1 and ED_{99} values were calculated in response to TD_{50} and ED_{50} values respectively mentioned above).

The SSM calculated (100%) means that the effective dose of etomidate (ED_{99}) that produces 99 % anesthesia in animals should be increased by 100% (i.e. from 8.514 to 17.028 mg/kg, IM) to kill 1% of the animals (TD_1) and produces its toxic and deleterious effects. Thus the TI and SSM values reflect that etomidate possesses a preferable and had a wide margin of safety.

The dose-response hypnotic effect of etomidate in chicks

Etomidate injection at 4, 8 and 16 mg/kg, IM produces significant narcosis as dosage hooked-on mode. Hypnotic onset (which is the lack of righting reflex) was fast during 1-5 min. while the period of hypnosis was short between 18-43 min. as well as the regaining from the hypnotic effect of etomidate lasts for 28-84 min. which depend on the doses of etomidate (Table 3).

Table 3. Hypnosis (dose-response) of multiple etomidate dosages in chicks

Etomidate (mg/kg, IM)	Hypnosis		
	Onset (min.)	Duration (min.)	Recovery (min.)
4	5.67 ± 1.15	18.00 ± 1.65	28.67 ± 2.00
8	1.33 ± 0.21 *	27.67 ± 2.25 *	43.83 ± 1.90 *
16	1.00 ± 0.00 *	43.83 ± 1.89 *,a	84.00 ± 6.04 *,a

The values typify Mean ± Std.E. for 6 chicks per dose group of chicks
*: significantly difference ($p < 0.05$) from etomidate dosage 4 mg/kg, IM
a: significantly difference ($p < 0.05$) from etomidate dosage 8 mg/kg, IM

Effect of xylazine on anesthetic efficacy of etomidate in chicks

A. The Effect of xylazine on hypnosis produced by etomidate

The combination composed of etomidate at 5 mg/kg, IM and xylazine at the same dose, shortened the onset of hypnosis and significantly prolong its period as well as there was a significant increase in the regaining time from the hypnotic effect when compared with the group that receives etomidate alone (Table 4-A).

Table 4-A. Etomidate's hypnotic effect alone or with xylazine in chicks

Groups	Hypnosis		
	Onset (min.)	Duration (min.)	Recovery (min.)
Etomidate alone (+ve control)	1.67 ± 0.33	32.67 ± 1.91	51.33 ± 2.93
Etomidate and xylazine	1.00 ± 0.00	59.00 ± 2.93 *	86.83 ± 4.82 *

The values typify Mean ± Std.E. for 6 chicks per group of chicks
Etomidate treatment at 5 mg/kg, IM with or without xylazine at 5 mg/kg, IM
*: significantly difference ($p < 0.05$) from etomidate alone

B. The Effect of xylazine on analgesia produced by etomidate

The 5 min. recording of the analgesic effect pre-injection of etomidate and xylazine combination denotes that there was a significant increase in the antinociceptive efficacy in comparison to the group that treated with etomidate alone. At a similar moment, a significant rise in the proportion of analgesic efficacy and a significant increase in the voltage (delta) that produces nociception in comparison to the group that injected with etomidate alone (Table 4-B).

Table 4-B. Etomidate's analgesia alone or with xylazine in the chicks

Variables	Antinociception %	Pre-treatment (Volts)	Post-treatment (Volts)	Voltage (delta)
Etomidate alone (+ve control)	50	6.00 ± 0.45	11.00 ± 2.65	5.00 ± 2.42
Etomidate and xylazine	100 *	7.00 ± 0.52	23.00 ± 0.82 *,+	16.00 ± 1.06 *

The values typify Mean ± S.E. for 6 chicks per group of chicks
Nociception induced by electro-stimulation (registered pre- and post 5 minutes) of etomidate therapt (5 mg/kg, IM) with or without xylazine (5 mg/kg, IM)
*: significantly difference ($p < 0.05$) from etomidate alone
+: significantly difference ($p < 0.05$) as of volts (pre-treatment) in the same group of chicks

Determination of plasma glucose, ALT and AST concentrations in the chicks treated with etomidate alone or etomidate plus xylazine

Table 5 revealed that there is no significant difference in plasma glucose, ALT and AST concentrations between groups that received normal saline (-ve control), etomidate alone (+ve control) and that treated with etomidate plus xylazine at 5 mg/kg, IM for each drug.

Table 5. Plasma glucose, ALT and AST concentrations in chicks treated with etomidate alone or etomidate plus xylazine

Groups	Glucose concentration (mg/dl)	ALT (U/L)	AST (U/L)
Normal saline (-ve control)	133.32 ± 11.93	15.35 ± 2.10	114.00 ± 5.51
Etomidate alone (+ve control)	135.67 ± 14.97	18.37 ± 2.19	104.10 ± 4.71
Etomidate and xylazine	142.67 ± 10.24	14.93 ± 1.12	111.73 ± 2.55

The values typify Mean ± Std.E. for 6 chicks per group of chicks
Etomidate and xylazine were injected at 5 mg/kg, IM for each drug

DISCUSSION

The main goal of this study was to evaluate the anesthetic profile of etomidate in the chickens and to determine the benefit of xylazine coadministration to enhance its anesthetic duration, efficacy and to reach a state of balanced anesthesia which is characterized by good hypnosis, analgesia, muscle relaxation and hyporeflexia. The mentioned goal is of a major and valuable outcome to be applied in the surgical operations and may be substituted the ketamine-xylazine combination in animals since the latter combination lack the complete criteria of balanced anesthesia with the presence of cardiovascular side effect of ketamine. Etomidate is considered a general anesthetic agent that produces rapid induction with a short duration of anesthesia by its potentiation of the GABA neurotransmitter and $GABA_A$ receptor effect, causing an inhibition of the nervous system. Etomidate has a good hypnotic effect but less analgesic and muscle relaxation efficacy with little to negligible effect on the heart and circulation so it is used for cardiovascular surgeries (Finkel et al., 2009; White and Trevor, 2009; Forman, 2011) though, etomidate is considered a safe drug of choice for using in anesthesia in human because it possesses many advantages including a well-known mechanism, protection from myocardial and cerebral ischemia, decreasing histamine release with a uniquely stable hemodynamic status (Bergen and Smith, 1997; Vinson and Bradbury, 2002; Falk and Zed, 2004; Forman, 2011), has a neuroprotective efficacy on the nervous system in contradiction of the diabetic oxidative injury (Ates et al., 2006) and was found to have efficient anesthesia than pentobarbital (Baxter et al., 2007). In response, the values of TI and SSM calculated in this study refers that the etomidate is safe and possesses a wide margin of safety (Janssen et al., 1975; Forman, 2011). Furthermore, xylazine preferred to use commonly with anesthetic agents like ketamine to produce balanced anesthesia characterized by worthy hypnosis, analgesic and muscle relaxant properties because it possesses analgesic, sedative, and muscle relaxant efficacy that are resultant from nits action on α_2–adrenoceptor (Pawson, 2008; Kleinz and Spence, 2008). The result of this study clarifies the anesthetic profile of etomidate in the chicks through determining the hypnotic ED_{50} and acute TD_{50} values as well as by evaluating the hypnotic dose-response fashion for etomidate which came close and in accordance to what's found in other laboratory animals (Janssen et al., 1975). As found in this study, etomidate when combined with xylazine, produce balanced anesthesia. That etomidate-xylazine combination enlarged the hypnotic effect and increased the analgesic efficacy as well as a decrease in the doses of both agents with minimizing the side effects of both drugs when compared with etomidate alone and it is typically of beneficial value for use in surgeries and may replace the classical remedy of ketamine-xylazine combination. The interaction between etomidate and xylazine may be regarded as their synergistic inhibition of the various areas of the brain with different mechanisms of action on their receptors. Xylazine alone was known to cause an increase in plasma glucose concentration, as a side effect, due to its mechanism of action (Roder et al., 1993). Neither etomidate alone, nor etomidate plus xylazine differ significantly from the negative control (saline group) concerning plasma glucose, ALT and AST concentrations as showed in this study that came close to the normal concentration ranges in chickens of another study (Cruz et al., 2018) and the reason may be attributed to their single and small doses of each drug used for a short period of time and the short time for plasma estimated suggesting another reason for using this combination for inducing prolonged anesthesia in the chicks.

CONCLUSIONS

The results of this study propose the likelihood of using etomidate as an anesthetic agent for short surgical trials in the chickens that can be more effective by using xylazine to yield balanced anesthesia (characterized by good hypnosis and analgesia) without causing significant side effects; supplementary studies are required in other animal species.

AKNOWLEDGEMENTS

The author grateful for the Veterinary Medicine College, University of Mosul for its facilities obtainable.

CONFLICT OF INTEREST

None declared by the author.

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J HELLENIC VET MED SOC 2020, 71(4): 2471-2482
ΠΕΚΕ 2020, 71(4): 2471-2482

Research article
Ερευνητικό άρθρο

Class1-3 integrons and antimicrobial resistance profile in *Salmonella* spp. isolated from broiler chicken in Western Iran

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ABSTRACT: *Salmonella* spp. are major etiologies of food-borne infections that are disseminated primarily through poultry to human. Nowadays, the high rate of antimicrobial resistance and the emergence of multi-drug resistant (MDR) strains, worse the threat imposed to the public health. Integrons are attributed as important contributors to MDR isolates. The present research aimed to identify the frequency of integrons 1-3 and the antimicrobial resistance patterns in *Salmonella* spp. isolated from broiler chicken in Western Iran. A total of 500 fecal samples were screened for *Salmonella* phenotypically. The isolates were confirmed genotypically and the frequency of integrons 1-3 was evaluated molecularly among the isolates. Besides, the antimicrobial resistance of the isolates was determined through the agar disk diffusion method. In general, 67 (13.4%) isolates of *Salmonella* spp. were recovered phenotypically, all of which were confirmed molecularly. The incidence of class 1, 2, and 3 integrons was 40.29% (27 isolates), 28.35% (19 isolates), and 11.94% (eight isolates), respectively. Coexistence of integrons was also detected in 26.86% of the isolates including class 1+2 (13 isolates, 40.62%), class 1+3 (2 isolates, 6.25%), and class 1+2+3 (3 isolates, 9.37%). No statistical association was detected between the frequencies of *Salmonella* spp. or *Salmonella*-bearing integron isolates with age, season, and location. The most frequent antimicrobial resistance was exhibited to ampicillin, nalidixic acid, trimethoprim-sulphamethoxazole, and tetracycline; while ciprofloxacin, gentamicin, and ceftazidime were the most effective drugs. 35.82% of the isolates were MDR, all of which harbored at least one class of integrons. Statistical assessment represented an association between the prevalence of integrons and tetracycline, chloramphenicol, streptomycin, and ceftazidime resistance rates. An alarming rate of integrons and MDR frequency among poultry-originated *Salmonella* spp. in the studied region demands the constant stewardship and prudent prescription and use of antibiotics to prevent human infections and preserve the effectiveness of those antibiotics in treating human salmonellosis.

Keywords: *Salmonella*, broiler, integrons 1-3, antimicrobial resistance, Western Iran

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Date of initial submission: 26-11-2019
Date of revised submission: 25-07-2020
Date of acceptance: 25-08-2020

INTRODUCTION

Poultry is incriminated as a leading reservoir of non-typhoid *Salmonella* spp. (Parry et al., 2008). This healthy carrier state may lead to food-borne infections following direct or indirect contact (egg and carcass contamination) (Hugas and Beloeil, 2014). Some previous studies have reported the prevalence of *Salmonella* spp. in broiler in different districts of Iran; like 22.5% in Shiraz (Ansari-Lari et al., 2014), 26% in Mazandaran province (Seifi et al., 2016), 1.8% in Sanandaj (Doulatyabi et al., 2016), 19% in Lorestan province (Haeri and Ahmadi, 2019), and 19.2% in Mashhad (Peighambari et al., 2019). Likewise, the frequency of *Salmonella* spp. isolated from pediatric diarrhea in Tehran, the capital of Iran, was reported as 7% (Farahani et al., 2018).

Although antibiotic therapy is crucial in controlling and treating infectious diseases, recent indiscriminative application of antibiotics has been linked as a major contributor to the emergence of substantial antibiotic resistance in bacterial agents (Gong et al., 2013). The antimicrobial agents which are frequently prescribed in Iranian poultry industry include fosfomycin, enrofloxacin, florfenicol, linco-spectin, colistin, trimethoprim-sulphamethoxazole, tetracycline, tilmicosin, tiamulin, streptomycin, doxycycline, gentamycin, amoxicillin, and bacitracin. In addition to the zoonotic importance of the infection, emergence and dispersion of multi-drug resistant (MDR) *Salmonella* spp. constitutes a pre-eminent health issue in both public and veterinary sectors worldwide (Shan and Huang, 2010). Integrons are attributed as ambulatory DNA elements to the dissemination of antimicrobial resistance genes in *Enterobacteriaceae* including *Salmonella* spp. Their capability of acquisition and distribution of resistance determinants facilitates the development and propagation of subsequent MDR strains (Correa et al., 2014). The presence of two conserved segments, separated by a variable region supports them to harbour one or several cassettes in the latter. These gene cassettes are mobile and mostly encode antimicrobial resistance proteins (Labbate et al., 2009). Insertion of these elements in plasmids enables the spread of resistance among bacteria (Su et al., 2004). Despite the retrieval of several classes of integrons from GenBank, only some of them are sustained in Gram-negative bacteria. Class 1, the most ubiquitous category, is associated with encoding over 130 resistance gene cassettes. However, six cassettes and more limited diversity are appraised in class 2 and 3, respectively (Cambray et al., 2010; Correa et al., 2014; Mazel et al., 2006; Rahmani et al., 2013).

Considering the elevating antimicrobial resistance frequency in *Salmonella* spp., it is of great magnitude to determine the route of resistance dissemination, vertical or horizontal, in the evolution of MDR strains. Surveillance programs for the presence of mobile genetic contributors of resistance genes in *Salmonella* spp. have been underscored regarding the mentioned issue (Goldstein et al., 2001; Mahero et al., 2013; Asgharpour et al., 2014; Correa et al., 2014; Lu et al., 2014; Trongjit et al., 2017; Doosti Irani et al., 2018). Hence, the aim of the present survey was to evaluate the frequency of class 1, 2, and 3 integrons and the distribution of phenotypic antimicrobial resistance among *Salmonella* spp. derived from a selection of broiler chicken in the west of Iran.

MATERIALS AND METHODS

Collection of samples

A total of 500 fecal samples were collected aseptically from apparently healthy broilers from March to September 2019 based on random cluster sampling method in Kurdistan province, in the west of Iran. Each poultry house of a farm was divided at least to four sections and an approximately 2-3 gram of fresh fecal sample from two birds were gathered from each part. The average number of houses in each farm and the density of chickens in each house were three and 5000, respectively. Besides, the biosecurity measures in all farms were implemented based on the principles of OIE biosecurity procedures in poultry production (OIE, 2019). Samples were then chilled until delivery to the laboratory within maximum three to five hours. Demographic information regarding the samples is presented in Table 1.

Isolation of *Salmonella* spp.

The initial step for *Salmonella* spp. isolation from each sample was undertaken by hemogenizing 10 gram stool in 90 mL Buffered peptone water (BPW, Merck, Germany) and incubation for 18 to 24 hours at 37 °C, followed by inoculation of 0.1 mL of the pre-enrichment medium into 10 mL Rappaport Vassiliadis enrichment (RV, Merck, Germany) broth and incubation at 41.5-42 °C for 15-18 hours. Next, a loopful of the previous medium was streaked onto xylose lysine deoxycholate (XLD, Merck, Germany) that were incubated for 24 hours at 37 °C. An individual red colony with black center on XLD, presumptive to *Salmonella* spp. was further subcultured on MacConkey (MAC, Merck, Germany) agar and identified based on Gram staining and biochemical reactions including Urea, Tryptic Sugar Iron (TSI), Indole, Methyl Red, Voges Proskauer, and Simmons Citrate (IMViC) reactions.

Table 1. Characterization of the samples in the present study

City	Farm code	Season of sampling	Age (days-old)	No. of sampling	No. of *Salmonella* isolates	No. of *Salmonella* isolates harboring integrons	Class of integron	No. of MDR* isolates	Antimicrobial resistance profile
Sanandaj	A	spring	20	25	2	1	Int1	1	AMP, NAL, CAZ, STR, TET, CHL
		summer	12	25	2	1	Int1 + Int2	1	AMP, NAL, CAZ, STR, TET, CHL
Sanandaj	B	spring	27	25	1	0	-	0	-
		summer	33	25	1	1	Int1	1	AMP, NAL, TET, CHL
Saggez	C	spring	45	25	7	3	Int2	2	AMP, NAL, STR, SXT, TET, CHL
							Int1 + Int2		AMP, NAL, STR, SXT, TET, CHL
							Int1 + Int2		AMP. STR
		summer	17	25	9	5	Int1	3	AMP, NAL
							Int1		AMP, NAL
							Int2		AMP, NAL, CAZ, STR, SXT, TET
							Int1 + Int2		AMP, NAL, SXT, TET, CHL
							Int1 + Int2 + Int3		AMP, NAL, CAZ, TET, CHL, GEN
Saggez	D	spring	52	25	0	0	-	0	-
		summer	43	25	0	0	-	0	-
Marivan	E	spring	49	25	4	2	Int1	2	AMP, NAL, STR, SXT, TET, CHL
							Int1 + Int2 + Int3		AMP, NAL, CAZ, STR, TET, CHL
		summer	25	25	6	4	Int 1	3	AMP, NAL, STR, SXT, TET, CHL
							Int2		AMP, CHL
							Int1 + Int2		AMP, NAL, STR, SXT, TET, CHL
							Int1 + Int3		AMP, CAZ, STR, TET, CHL
Marivan	F	spring	20	25	4	3	Int1	2	AMP,TET
							Int1 + Int2		AMP, NAL, STR, SXT, TET, CHL
							Int1 + Int2		AMP, NAL, STR, SXT, TET, CHL
		summer	24	25	5	2	Int1	1	AMP, TET
							Int1 + Int2		AMP, NAL, STR, SXT, TET, CHL

Bijar	G	spring	17	25	1	1	Int1 + Int2	1	AMP, NAL, CAZ, STR, SXT, TET, CHL
		summer	34	25	3	1	Int1 + Int2 + Int3	1	AMP, NAL, CAZ, STR, SXT, TET, CHL, GM
Bijar	H	spring	25	25	11	3	Int1 + Int2	3	AMP, NAL, STR, SXT, CHL
							Int1 + Int2		AMP, NAL, STR, SXT, TET, CHL
							Int1 + Int3		AMP, NAL, CZA, STR, SXT
		summer	36	25	3	2	Int3	1	AMP, STR, TET, CHL
							Int3		AMP, CAZ
Baneh	I	spring	14	25	7	4	Int1	2	AMP, NAL, SXT, TET, CHL
							Int3		AMP, CAZ
							Int1 + Int2		AMP, CAZ, SXT, TET, CHL
							Int1 + Int2		AMP, NAL, CAZ, STR, SXT, TET
		summer	36	25	1	0	-	0	-
Baneh	G	spring	32	25	0	0	-	0	-
		summer	50	25	0	0	-	0	-

Resistance to ≥3 classes of antibiotics

Table 2. Details of primer sequences and thermal conditions used in the present study.

Gene	Primer Sequence (5′→3′)	PCR thermal condition					Product Size (bp)	Reference
		Initial denaturation	Denaturation	Annealing	Extension	Final Extension		
invA	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGGAACC	94 °C for 5 min	94 °C for 1 min	53 °C for 2 min	72 °C for 3 min	72 °C for 7 min	284	Rahn et al., (1992)
			35 cycles					
IntI	GGTGTGGCGGGCTTCGTG GCATCCTCGGTTTTCTGG		94 °C for 30 S	50 °C for 30 S	72 °C for 30 S	72 °C for 5 min	164	Koeleman et al., (2001)
IntII	CTAGAATAGGCTGTATAGGCAGA GAGTGACGAAATGTATGACAAG			52 °C for 1 min			233	Goldstein et al., (2001)
IntIII	CAGTCTTTCCTCAAACAAGTG TACATCCTACAGACCGAGAAA			47 °C for 1 min			600	
			35 cycles					

Molecular characterization of *Salmonella* spp.

Total DNA of each isolate was extracted by boiling method of its overnight culture in Luria Bertani (LB, Merck, Germany) broth. The isolates were molecularly delineated as *Salmonella* spp. in an *invA*-based polymerase chain reaction (PCR) in accordance with the primers and thermal protocol introduced by Rahn et al. (1992) (Table 2). The master mix used in this study included 12.5 μL of 2X ready-to-use PCR master mix (CinnaGen, Iran), 50 ng (2 μL) of template DNA, and 0.7 μL of each primer in a final volume of 25 μL. The positive and negative controls used in the reaction were *Salmonella* Typhimurium ATCC 1730 and DNA-free master mix, respectively.

Molecular detection of integrons

All of the *Salmonella* spp. isolates were screened for class 1-3 integrons in three separate reactions in accordance with the method prescribed elsewhere (Goldstein et al., 2001; Koeleman et al., 2001). The primer sequences and PCR protocols are represented in Table 2. The final PCR master mix consisted of 12.5 μL of 2X ready-to-use PCR master mix (CinnaGen, Iran), 50 ng (2 μL) of template DNA, 0.4 μL of each primer in a total volume of 25 μL. Positive and negative controls were used in all reactions.

Antimicrobial susceptibility testing

The antimicrobial sensitivity profile of the isolates was designated using the agar disk diffusion method, following the Clinical and Laboratory Standards Institute (CLSI, 2012) guidelines. The antibiotic disks included ampicillin (AMP, 10 μg), nalidixic acid (NAL, 30 μg), streptomycin (STR, 10 μg), trimethoprim-sulphamethoxazole (SXT, 25 μg), tetracycline (TET, 30 μg), chloramphenicol (CHL, 30 μg), ceftazidime (CAZ, 10 μg), gentamicin (GEN, 5 μg), and ciprofloxacin (CPR, 5 μg). *E. coli* ATCC 25922 was used for quality control.

Statistical analysis

The statistical associations of the frequency of *Salmonella* spp. and integron determinants with the variables of age, season, city, and antimicrobial resistance patterns were analyzed in SPSS software (version 21.0, Chicago, IL) using Chi-square test. A P value of ≤ 0.05 was considered to be statistically significant.

RESULTS

In general, 67 (13.4%) *Salmonella* spp. isolates were detected phenotypically, all of which were confirmed molecularly, producing the expected 285 bp amplicon (Figure 1). The production of 164 bp (Figure 2), 233 bp (Figure 3), and 600 bp (Figure 4) amplicons were representative of the presence of classes 1, 2, and 3 integrons, respectively. The total frequency of integrons among the isolates was 49.25% (33 isolates). In details, the frequency of class 1, 2, and 3 were 40.29% (27 isolates), 28.35% (19 isolates), and 11.94% (eight isolates), respectively. The coexistence of the integrons among the isolates, from the most to the least, were related to class 1+2 (13 isolates, 40.62%), class 1+2+3 (3 isolates, 9.37%), and class 1+3 (2 isolates, 6.25%). While no isolate was detected with the coexistence of class 2+3 integrons. As shown in Table 1, no statistical relationship was observed between the frequency of *Salmonella* spp. with age (P = 0.225), season (P = 0.392), and city (P = 0.107). The same results were obtained for the frequency of integron-harboring *Salmonella* spp. with age (P = 0.666), season (P = 0.724), and city (P = 0.314).

Figure 1. Agarose gel electrophoresis of PCR products with *invA* primers (285 bp). M: 100 bp DNA ladder (CinnaGen, Iran), NC: negative control, PC: positive control, Lanes 1–8: field samples.

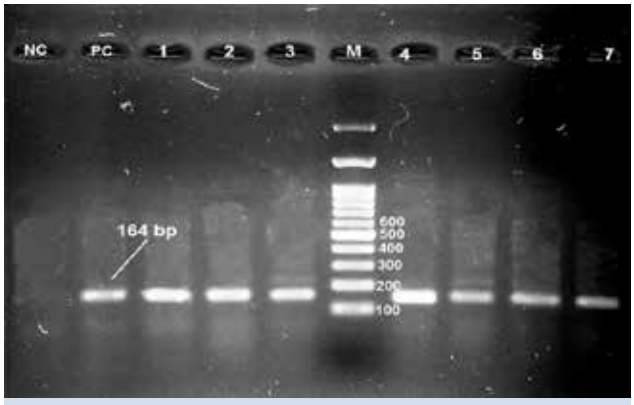

Figure 2. Agarose gel electrophoresis of PCR products with *Int1* primers (164 bp). M: 100 bp DNA ladder (CinnaGen, Iran), NC: negative control, PC: positive control, Lanes 1–7: field samples.

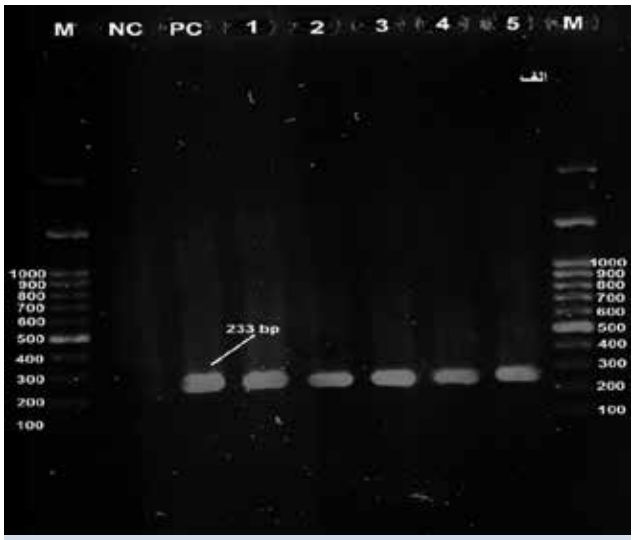

Figure 3. Agarose gel electrophoresis of PCR products with *IntII* primers (233 bp). M: 100 bp DNA ladder (CinnaGen, Iran), NC: negative control, PC: positive control, Lanes 1–5: field samples

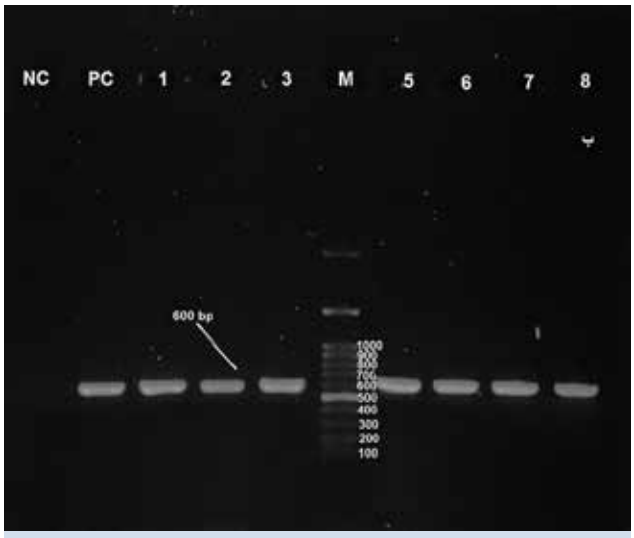

Figure 4. Agarose gel electrophoresis of PCR products with *IntIII* primers (600 bp). M: 100 bp DNA ladder (CinnaGen, Iran), NC: negative control, PC: positive control, Lanes 1–8: field samples

Phenotypic antimicrobial assessment represented the most resistance against AMP (100%), NAL (73.13%, 49 isolates), SXT (58.20%, 39 isolates), and TET (43.28%, 29 isolates). In comparison, CPR (100%), GEN (92.53%, 62 isolates), CAZ (73.13%, 49 isolates), and TET (55.22%, 37 isolates) represented the most antimicrobial susceptibility among the isolates (Table 3). None of the isolates was sensitive to all challenged antibiotics. Besides, 24 (35.82%) isolates were nominated as MDR due to their resistance to ≥ 3 classes of antibiotics. Moreover, all of the MDR isolates harbored at least one of the studied classes of integrons. The frequency of antimicrobial resistance profile of MDR isolates regarding the presence of integrons and the antimicrobial resistance in relation to integron classes is depicted in Tables 1 and 4, respectively. Additionally, statistical association was revealed between the frequency of integrons and resistance against TET (P = 0.001), CHL (P = 0.048), CAZ (P = 0.045), and STR (0.045). However, analyzing the statistical relationship between the frequency of individual integrons with resistance to the studied antibiotics depicted a merely association between integron 1 with TET (P = 0.035).

Table 3. The frequency of antibiotic resistance among *Salmonella* spp. isolated in the current study

Antibiotic code	Sensitive % (n)	Intermediate % (n)	Resistance % (n)
AMP	0	0	100% (67)
NAL	17.91% (12)	8.95% (6)	73.13 (49)
STR	52.23% (35)	0	47.76% (32)
SXT	34.32% (23)	7.46% (5)	58.2% (39)
TET	55.22% (37)	1.49% (1)	43.28% (29)
CHL	49.25% (33)	16.41% (11)	34.32% (23)
CAZ	73.13% (49)	2.98% (2)	23.88% (16)
GEN	92.53% (62)	0	23.88% (5)
CRP	100% (67)	0	0

Table 4. The frequency of antimicrobial resistance in relation to individual integrons

Antibiotic code	Class of integron	Sensitive % (n)	Intermediate % (n)	Resistance % (n)
AMP	1	0	0	100% (26)
	2	0	0	100% (19)
	3	0	0	100% (8)
NAL	1	19.23% (5)	7.69% (2)	73.07% (19)
	2	15.78% (3)	0	84.21% (16)
	3	50% (4)	0	50% (4)
STR	1	30.76% (8)	0	69.23% (18)
	2	21.05% (4)	0	78.94% (15)
	3	37.5% (3)	0	62.5% (5)
SXT	1	41.66% (10)	0	61.53% (16)
	2	26.31% (5)	5.26% (1)	68.42% (13)
	3	75% (6)	12.5% (1)	12.5% (1)
TET	1	19.23% (5)	3.84% (1)	76.92% (20)
	2	15.78% (3)	0	84.21% (16)
	3	37.5% (3)	0	62.5% (5)
CHL	1	26.92% (7)	15.38% (4)	57.69% (15)
	2	15.78% (3)	15.78% (3)	68.42% (13)
	3	37.5% (3)	0	62.5% (5)
CAZ	1	61.53% (16)	0	38.46% (10)
	2	57.89% (11)	0	42.10% (8)
	3	12.5% (1)	12.5% (1)	75% (6)
GEN	1	92.30% (24)	0	7.69% (2)
	2	89.47% (17)	0	10.52% (2)
	3	75% (6)	0	25% (2)
CPR	1	100% (26)	0	0
	2	100% (19)	0	0
	3	100% (8)	0	0

DISCUSSION

Salmonella spp. is a major etiological agent of food-borne infection and poultry is characterized as a main source of human contamination. Induction of subclinical carriers under stressful conditions leads to shedding bacteria in feces. This may contaminate carcasses following evisceration in poor sanitation practices (Mannion et al., 2012). It is claimed that the frequency rate of less than 5% *Salmonella* contamination in a farm may constitute 50-100% of carcass contamination in a retail outlet (Barrow, 2000). The 13.4% occurrence of *Salmonella* in the present research was lower than the frequency reported from Turkey (41.30%) (Carli et al., 2001), Basrah province of Iraq (19%) (Al–Abadi and Al-Mayah, 2012), Shiraz (southern Iran) (22.50%) (Ansari-Lari et al., 2014), Mazandaran province (North of Iran) (26%) (Seifi et al., 2016), Lorestan province of Iran (19%) (Haeri and Ahmadi, 2019), and Mashhad (Northeast of Iran) (19.2%) (Peighambari et al., 2019). In contrast, some studies corroborated lower recovery of *Salmonella* in broiler including 2.11% and 1.80% in Tehran and Sanandaj cities of Iran, respectively (Morshed et al., 2010; Doulatyabi et al., 2016). Plausible explanations for diverse frequencies of infection among different studies may be due to several causes such as geographical location, sampling season and methodology, culture media, diet, management measures particularly at early rearing stages, hygienic status, and biosecurity measures at farms (Zhao et al., 2017). For instance, inappropriate fencing of warehouses in farms may confer easier traffic of insidious animals and *Salmonella* vectors, which may increase the oral attainment of infection (Pui et al., 2011). Although cold seasons (fall and winter) were not included in the present study, the approximate recovery of *Salmonella* spp. in the two studied seasons were higher than the frequency reported from Spain (Lamas et al., 2016). Haeri and Ahmadi (2019) also found a similar distribution. Further, as the immunity status is extended at advanced ages, the most distribution of the bacteria in the present study was in lower ages, which was in line with the results from northeastern Algeria. The authors stated that the samples collected at

the age of 15–30 days were more contaminated with *Salmonella* spp. than those collected at 45–60 days (Djeffal et al., 2017). Moreover, Haeri and Ahmadi (2019) represented the statistical association between the frequency of *Salmonella* spp. with age in broiler; the lower the age, the more frequency of contamination was observed.

The frequent use/abuse of antimicrobial agents in animal husbandry is a serious menace threatening the effectiveness of chemotherapy through emergence and spread of antimicrobial resistance among both commensal and pathogenic bacteria. Exchange of genetic materials not only may lead to cross-resistance among the microbial community but also may confer genes prerequisite for bacterial survival in stressful conditions (Lamas et al., 2016). The massive consumption of different classes of antibiotics in poultry husbandry in Iran, including macrolides, fosfomycin, quinolones and fluoroquinolones, lincosamides, tiamulin, polymyxins, sulfonamides, penicillins, aminoglycosides, bacitracin, tetracyclines, and florfenicol, is worrying. Based on the empirical experience of the authors, antimicrobial agents are occasionally used without a veterinarians' prescription or antibiogram testing in the studied region. A recent increasing trend of resistance to common antimicrobial agents has been delineated in *Salmonella* spp. (Antunes et al., 2004). Discordant with the results obtained in the present study, 100% of *Salmonella* isolated from poultry in a former study in Babol, a city in the north of Iran, were susceptible to NAL, TET, and STR (Asgharpour et al., 2014). In agreement with the present study, high rates of resistance to NAL have been reported in *Salmonella* spp. in some studies from Iran and other countries (Yan et al., 2010; Rahmani et al., 2013). TET, a commonly used antibiotic in poultry husbandry in Iran, represented 43.28% resistance. An increasing trend of resistance against this antimicrobial agent has been recently reported in Iran (Mirzaie at al., 2010; Morshed et al., 2010; Asgharpour et al., 2018). High rates of resistance to TET and AMP are also consistent with the findings reported elsewhere (Wannaprasat et al., 2011; Trongjit et al., 2017, Zhao et al., 2017). CHL, a prohibited antibiotic in the poultry industry in Iran, represented 34.32% resistance, which may be related to co-selection and co-resistance (Trongjit et al., 2017). A relatively high rate of sensitivity was revealed to CAZ (73.13%), which was lower than the report published in Thailand (Trongjit et al., 2017). Third-generation cephalosporins are choice drugs for the treatment of human invasive salmonellosis (Wilke et al., 2005). Prolonged and widespread application of trimethoprim in veterinary practices leads to approximately high rates of resistance to it. The resistance rate against the latter in the present study was higher than the one reported by Okamoto et al., (2009). Susceptibility to CPR was represented in 100% of the isolates, which was in contrast with the 35.9% resistance reported from China (Zhao et al., 2017). The identified profound and regulated use of antibiotics, particularly not at their inhibitory concentrations, is the clue for lowering the resistance rates. This is related to omit the selection pressure for the maintenance of resistance genes (Lamas et al., 2016).

Meanwhile, the emergence of MDR *Salmonella* spp. is a matter of global concern. It is proved that MRD *Salmonella* is associated with particular serotypes including *S.* Indiana, *S.* Typhimurium, and *S.* Enteritidis (Clemente et al., 2014). Hence, it is highly recommended to determine the serotype of the isolates in upcoming studies. Approximately high rate of MDR *Salmonella* spp. in the current research concurs with that stated previously from Iran (Asgharpour et al., 2018), China (Zhao et al., 2017), and Egypt (Abdel Aziz et al., 2018). Integrons, particularly class I, have been recognized as important contributors of MDR isolates (Idrees et al., 2011). The mechanism of MDR in integron-harboring isolates is due to diminished sensitivity to antimicrobial agents, either their respective genes are incorporated or not in integron cassettes (Malek et al., 2015). Because of their ability to propagate resistance gene cassettes through acquisition and shearing modes and to integrate in mobile genetic elements, dispersion of antimicrobial resistance genes may be facilitated. They capture one or more mobile gene cassettes to form cassette arrays which can readily be lengthened by incorporation of new cassettes, shortened by excision of one or more cassettes, or reshuffled to create new orders. All these reactions are mediated by integrases which catalyze recombination between two primary or a primary and a secondary recombination sites and allows the rapid formation and expression of new combinations of genes in response to selection pressures. This acquired resistance can be transferred among bacteria via horizontal gene transfer by conjugation and transformation (Mazel 2006; van Hoek et al., 2011). The consequence is limited options in treating infectious diseases (Recchia and Hall, 1995). Resistance genes, related to various classes of antimicrobial genes, including beta-lactams, sulfonamides, macrolides, aminoglycosides, trimethoprim, chloramphenicols, and

rifampin may be transported by integrons (Peters et al., 2001). The most common gene cassettes and arrays and the corresponding antimicrobial resistances which have been frequently reported in class 1 integrons are *aadB* (resistance to dibekacin, gentamicin, kanamycin, sisomicin and tobramycin) (Shaw et al., 1993), *dfrA7* (resistance to trimethoprim) (Roberts et al., 2012), *aadA1a* and *aadA2* (resistance to spectinomycin, streptomycin, and kanamycin) (Ramirez and Tolmasky, 2010), $bla_{\text{CARB-2}}$ (resistance to penicillins including carbenicillin) (Matthew and Sykes, 1977), *dfrA1–gcuC* (resistance to trimethoprim) (Roberts et al., 2012), *dfrA1–aadA1a* (resistance to trimethoprim, spectinomycin and streptomycin) (Ramirez and Tolmasky, 2010; Roberts et al., 2012), *dfrA17–aadA5* (resistance to trimethoprim, spectinomycin and streptomycin) (Ramirez and Tolmasky, 2010; Roberts et al., 2012), *oxa10/aadA1* (resistance to ampicillin, cefaclor and spectinomycin) (Tennstedt et al., 2003), *gca-L/M/P* and *catB* (resistance to chloramphenicol) (Tennstedt et al., 2003), *dfrA12–gcuF–aadA2* (resistance to trimethoprim, spectinomycin and streptomycin) (Sandvang et al., 1997), $floR_{st}$ (resistance to florfenicol) (Ramirez and Tolmasky, 2010; Roberts et al., 2012), and *tetR* and *tetA* (resistance to tetracyclines) (Briggs et al., 1999; Sandvang et al., 1997). Some of the most prevalent gene cassettes and arrays integrated in class 2 integron are *aadA* and *sat-1* (resistance to aminoglycosides and sulfonamides) (Hansson et al., 2002), and *ere(A)* (resistance to erythromycin and rifampin) (Tribuddharat et al., 1999). Class 3 integron has been reported to harbor the bla_{IMP} gene cassette that confers resistance to broad-spectrum β-lactams including carbapenems (Carattoli 2001). The overall prevalence of integrons were 49.25%, with the highest frequency related to class 1 (40.29%), followed by class 2 (28.35%) and 3 (11.94%), herein. In a former study carried out in the southwest of Iran, the prevalence of class 1 to 3 integrons in *Salmonella* spp. isolated from broiler chicks were 50%, 28%, and 48%, respectively (Doosti Irani et al., 2018). Moreover, 82% of the *Salmonella* spp. isolated from poultry in Cairo, Egypt, harbored class 1 integron, with the total absence of class 2 among the isolates (Abdel-Maksoud et al., 2015). Most of the reports regarding the frequency of integrons in *Salmonella* spp. originated from poultry are related to class 1, ranging from 0 to 100% (Okamoto et al., 2009; Asgharpour et al., 2014; Lu et al., 2014; Halawa et al., 2016; Zhao et al., 2017; Abdel Aziz et al., 2018; Shabana et al., 2019). Our results were in contrast with those that claimed class 3 integron is not harbored in *Salmonella enterica* serovars Enteritidis, Typhimurium, and Infantis (Eshraghi et al., 2010; Ranjbar et al., 2011). Nevertheless, the presence of the aforementioned class in other *Enterobacteriaceae*, retrieved from random French hospital effluent samples, may reflect its role in antimicrobial resistance (Barraud et al., 2013). Higher frequency of integrons reported in some studies may imply the abundant use of antimicrobials in those study regions (Trongjit et al., 2017). The statistical association between the presence of integrons with resistance genes in *Salmonella* spp. in literature points toward their role in distribution of resistance genes (Abdel Aziz et al., 2018). However, amplification and sequencing of cassette regions should be performed in order to genetically confirm this association.

Besides, the discrepancy about selective and non-selective pressure of antibiotics in dispersion of integrons has been demonstrated, as environmental pollution with quaternary compounds may influence the dissemination of integron 1 (Gaze et al., 2005). Phenols, formaldehyde and aldehydes, chlorhexidine, sodium hydroxide, quaternary ammoniums, and iodophors are frequently used as disinfectants in Iranian poultry farms. Although the antimicrobial patterns of *Salmonella* isolates harbouring different classes or a combination of different classes of integrons revealed varieties, it was less than the diversities observed in other studies (Macedo-Viñas et al., 2009; Dessie et al., 2013; Rahmani et al., 2013; Asgharpour et al., 2018). This may be due to the fact that prescribing antibiotics in the studied region is relatively based on implementing empirical therapy rather than surveillance analysis. This may limit the usage of different antimicrobial classes in the region. Further, the relationship between the presence of integrons with MDR *Salmonella* was 100%, as a confirmation with previous investigations (Molla et al., 2007; Lu et al., 2014). This renders a scientific basis to guide the prudent clinical use of antibiotics (Lu et al., 2014). A high frequency of penta-resistance ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) phenotype was manifested among MDR *Salmonella* isolates, which is in agreement with other studies (Mahero et al., 2013; Lu et al., 2014). Besides, 15 other different antimicrobial resistance profiles were detected among the isolates. The patterns NAL-SXT-STR-TET-CHL and NAL-CAZ-STR-SXT-TET-CHL have also been reported in *Salmonella* Infantis isolated from chicken in Iran (Asgharpour et al., 2018). To the best of our knowledge, other patterns

were not found in similar studies; this does not mean their specificity to the isolates of this research, but rather relates to assessing the other antimicrobial agents' resistance in various studies.

In brief, a relatively high distribution of integrons was identified among *Salmonella* spp. isolated from broiler chicken in the west of Iran. In addition, antimicrobial and multi-drug resistance was detected in approximately high rates. This emphasizes the continuous surveillance measures and cautious application of antimicrobial agents in poultry husbandry in order to reduce the emergence of resistant strains and to prevent food-borne diseases caused by them.

CONFLICT OF INTEREST

None declared.

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J HELLENIC VET MED SOC 2020, 71(4): 2483-2490
ΠΕΚΕ 2020, 71(4): 2483-2490

Research article
Ερευνητικό άρθρο

A study on the use of henna plant (*Lawsonia inermis Linn*) for the treatment of fungal disease (*Trichophypton verrucosum*) in calves

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ABSTRACT. The aim of this study was to investigate the usability of henna plant (*Lawsonia inermis Linn*) in the treatment of dermatophytosis lesions (*Trichophypton verrucosum*) in cattle. The animal material of the trial consisted of 50 holstein calves between the ages of 4 and 6 months, who were found to have a dermatophytosis lesion on their face and neck in their clinical examination. The experiment was organized on a three-group repeated measurement trial plan. I. Group: Trichlorfon (Neguvon 75%, Bayer) ointment, II. Group: Henna applied, and III. Group: Control Group, no treatment, and 20 (10 females, 10 males), 20 (10 females, 10 males), and 10 (5 females, 5 males) totally 50 calves used, respectively. The research was continued for 14 days until the lesions were completely healed. I. and II. Groups were observed the best healing in the calves, respectively. In the III. Group without any treatment, there was no improvement and the lesions were enlarged. In addition, the effect of gender in the treatment process of dermatophytosis lesions was insignificant. As a result of this study, it is thought that henna plant can be used in the treatment of dermatophytosis.

***Keywords*:** Dermatophytosis; *Trichophypton verrucosum*; Henna (*Lawsonia inermis Linn)*; Repeated measurement; Ringworm

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Date of initial submission: 25-12-2019
Date of revised submission: 09-06-2020
Date of acceptance: 14-06-2020

INTRODUCTION

Henna (*Lawsonia inermis Linn)* is one of the oldest and most popular plants known among medicinal plants and is a perennial shrub widely grown in North Africa (Solecki, 1975; Bhattacharjee and De 2003; Borade et al. 2011). Henna contains phenol compounds, naphthoquinone derivatives (mainly lawson), terpenoids, sterols, xanthines, aliphatic compounds, coumarins, carbohydrates, flavanoids, essential oils and other chemical components (Makhija et al. 2011; Santosh et al. 2013). Henna leaves contain a dense pigment called hennotannic acid (2-hydroxy-1,4-naphthoquinone) lawsone. This pigment has orange-red colorant properties and in studies conducted in different countries, it has been observed that naftokinone-derived dyes such as lawsone have antibacterial and antifungal activities (Jain et al. 2010; Rayavarapu et al. 2011).

It is used as an antifungal antibacterial compound since it contains *Cassia obovata* anthraquinones in henna leaf powder (Trease and Evans, 1983; Vaidya, 2000; Wallis, 2001). It has been reported that henna has antibacterial effects, antifungal activity against dermatophytes, wound healing, antitumoral effects, hypotensive, astringent and sedative effects, Henna has been reported by many researchers to have different healing effects, antibacterial effects especially for gram positive bacteria, antifungal activity against dermatophytes and wound healing (Berenji et al., 2010; Elmanama et al., 2011; Jain et al., 2010; Muhammad and Muhammad. 2005).

Dermatophytosis (Ringworm / dermatomycosis) is an infectious and zoonotic disease characterized by various lesions in many animal species and different parts of the body (Arda, 2006; Barbieri et al., 2017; Cabañes et al., 1997; Chermette et al., 2008; Sahal, 1994). *Trichophyton*, *Microsporum* and *Epidermophyton* are the three genera that cause dermatophytosis of fungal agents and there are many species within them. Many species of *Trichophyton* and *Microsporum* cause clinical infections in farm animals. The species known as *Trichophyton verrucosum* causes clinical infection especially in cattle (Yılmazer and Aslan, 2010; Şennazlı, 2018; Or and Bakırel, 2002). The infection caused by dermatophytosis in humans and animals is called “ringworm” or fungal in society (Wabacha et al., 1998; Weber, 2000).

Wagini et al., (2014) repored that together with henna, the fungitoxic effects of 13 species known as dermatophytes on 30 plant species were tested and only the full toxic effect of henna was observed. In addition, Ponugoti, (2018) repored that aqueous extract of henna leaves were tested for antifungal potential against eight important *Aspergillus* species isolated from sorghum, corn and paddy seed samples, and petroleum ether, benzene, chloroform, methanol and ethanol extract of the plant showed significant antifungal activity. Ponugoti, (2018) reported this finding suggested that henna extract may be used as an alternative source of antifungal agent for the protection of fungal infection.

Başoğlu et al., (1998), Kırmızıgül et al. (2008a, 2009b, 2013c), Cam et al., (2009) repoted that systemic antifungal and topical treatment were used for the treatment of wounds caused by dermatophytes. In a study has done with henna by Muhammed H and Muhammed S, (2005) reported that the mixture of powder henna leaves with water prevents the wound from growing in burns. Polat, (2014) reported that a stray dog that has two pecuniary lossy wound on applied a mixture of henna, butter and povidin iodee in certain proportions to the injured area and reported that the wound healed quickly.

This study was carried out to investigate the possibility of using henna in the treatment of dermatophytosis lesions in cattle.

MATERIAL AND METHODS

Ethical scope

This study was conducted in accordance with the principles of the Local Ethics Committee in the framework of the ethics confirmed by the Çukurova University Directorate of Local Ethics Committee of Animal Experiments (29.01.2018).

Fifty head Holstein Friezian calves aged between 4-6 months and with a live weight of 80-90 kg were used at the Eastern Mediterranean Agricultural Research Institute. I. Group: Trichlorfon (Neguvon 75%, Bayer) ointment was applied, II. Group: Henna slurry that obtained from the mixture of henna leaf powder with 1/2 ratio of water was applied, and III. Group, Control Group: calves was not applied any treatment, and 20 (10 females, 10 males), 20 (10 females, 10 males), and 10 (5 females, 5 males) totally 50 calves used, respectively. Calves were randomly distributed according to the wound size, and care and feeding were applied homogeneously in all Groups. In all Groups the lesions were brushed with a medium hardness brush before any treatment. For the initial

measurements of the experiment, the wound size was drawn on the acetate paper placed on the wound and then the size of the wound was calculated in mm^2 with the help of millimetric paper. In the III th Group only the lesions were measured by brushing every day, and since there was no improvement the trial was terminated on the 14th day when complete recovery was achieved in the I th, II th Groups.

Since each of the observations were obtained from the same experimental unit (calf) and included a period of time in terms of healing time. One-way analysis of variance was performed in the General Linear Model approach by the Repeated Measurement procedure in IBM SPSS 22. Differences in means between applications were compared by Tukey multiple comparison test statistic. In the analysis of variance, Mauchly's Sphericity test was used to ensure the validity of the F test. Greenhouse-Geisser, Huynh-Feldt, or Lower-bound corrections, which correct the degrees of freedom, were used to determine whether the differences between sphericity test and all dependent group combinations were equal (Box, 1954; Greenhouse and Geisser, 1959; Huynh and Feldt, 1976).

RESULTS

Mauchly's Sphericity test was applied to the F-test to be valid in one-way analysis of variance of repeated measured data and it was found to be significant at (Table 1) ($P < 0.001$).

Thus, Greenhouse-Geisser, Huynh-Feldt or Lower-bound estimates, which corrected degrees of freedom, were used to interpret the ANOVA F test and The Analysis of Variance Table is divided into two sources as Test-Between-Subjects Effects, Test-Within-Subject Effects. As seen Table 2. Test-Between-Subjects effects of the variance analysis results the difference between the groups was statistically significant ($P < 0.0001$) and the difference between the genders was not statistically significant ($P > 0.05$). On the other hand, Test-Within-Subject Effects of The variance analysis results the days (recovery time of calves) and the interaction of the days and groups were statistically significant ($P < 0.0001$) and the results are shown in Graph 1 and Graph 2.

Table 1. Mauchly's Test of Sphericity[a]

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b]		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Day	0.000	1784.546	90	0.000	0.117	0.128	0.077

Table 2. Repeated Measures Variance Analysis

Tests of Between-Subjects Effects

Source		**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept		290907	1	290907	225,021	0.000
Groups		102167,6	2	51083,81	39,514	0.000
Gender		168,894	1	168,894	0,131	0,719
Error		59468,83	46	1292,801		
Tests of Within-Subjects Effects						
Day	Sphericity Assumed	12341,96	13	949,381	14,602	0.000
	Greenhouse-Geisser	12341,96	1,526	8089,462	14,602	0.000
	Huynh-Feldt	12341,96	1,67	7389,194	14,602	0.000
	Lower-bound	12341,96	1	12341,96	14,602	0.000
Day * Groups	Sphericity Assumed	119904,8	26	4611,723	70,93	0.000
	Greenhouse-Geisser	119904,8	3,051	39295,45	70,93	0.000
	Huynh-Feldt	119904,8	3,341	35893,82	70,93	0.000
	Lower-bound	119904,8	2	59952,41	70,93	0.000
Day * Gender	Sphericity Assumed	341,519	13	26,271	0,404	0,969
	Greenhouse-Geisser	341,519	1,526	223,847	0,404	0,614
	Huynh-Feldt	341,519	1,67	204,469	0,404	0,632
	Lower-bound	341,519	1	341,519	0,404	0,528
Error(Day)	Sphericity Assumed	38880,62	598	65,018		
	Greenhouse-Geisser	38880,62	70,181	554,002		
	Huynh-Feldt	38880,62	76,832	506,044		
	Lower-bound	38880,62	46	845,231		

Differences between the groups were found with Tukey test statistics and the results were given in Table 3 and the best healing was seen in I th Group where Trichlorfon ointment was applied. This was followed by henna application II th Group. The mean, standard deviations, minimum and maximum values of fungal lesions according to treatment Groups and genders were given in Table 4.

Table 3. The means of trial groups comparison by Tukey test statistics

Groups	N	Mean	Std. Dev.	Std. Error
I. Group	20	7.49a	7.73	2.15
II. Group	20	16.50b	7.71	2.15
III. Group	10	40.47c	5.52	3.04

P < 0.05; I. Group: Trichlorfon; II. Grup: Henna: III. Group: Control

The results of I., II., III th Groups were used in the treatment in calves were shown in Table 4.

Table 4. Descriptive statistics of experimental groups in daily recovery process

	Female					**Research Groups**								
	III. Group					II. Group					I. Group			
	N	**Mean**	**±Ss**	**Min.**	**Mak.**	**N**	**Mean**	**±Ss**	**Min.**	**Mak.**	**Mean**	**±Ss**	**Min.**	**Mak.**
1. day	5	20.1	7.34	12.8	30.2	10	33.68	21.71	12	80	34.14	21.2	11.25	70.3
2. day	5	20.76	7.69	13.5	32	10	33.68	21.71	12	80	30.4	21.2	9.3	66.5
3. day	5	21.68	7.5	15	33	10	32.71	21.65	11	78	22.3	17.8	2	57.4
4. day	5	22.86	8.08	16	35	10	30.67	21.73	9	75	14.79	15.4	**0**	48.2
5. day	5	24.54	8.39	17.5	37	10	27.43	20.96	6	70	8.38	11.5	0	35.3
6. day	5	26.34	8.79	19	40	10	23.38	19.57	4	61.5	3.85	7.02	0	22.5
7. day	5	31.2	8.26	23	43	10	18.53	16.93	2	50	1.24	3.02	0	9.4
8. day	5	37.78	8.58	28.9	47	10	14.5	15.11	**0**	43	0.32	1.01	0	3.2
9. day	5	43.2	10.38	33	58	10	10.92	12.26	0	33	**0**	**0**	**0**	**0**
10. day	5	48.3	12.33	38	66	10	6.75	8.37	0	20	0	0	0	0
11. day	5	55.5	15.26	44	79	10	3.3	4.52	0	12	0	0	0	0
12. day	5	63	18.06	47	91	10	1.6	2.41	0	7	0	0	0	0
13. day	5	72.32	26.47	47	115	10	0.3	0.67	0	2	0	0	0	0
14. day	5	79.5	32.94	48	133	10	**0**	**0**	**0**	**0**	0	0	0	0
	Male													
	N	**Mean**	±Ss	**Min.**	**Mak.**	**N**	**Mean**	±Ss	**Min.**	**Mak.**	**Mean**	±Ss	**Min.**	**Mak.**
1. day	5	17.38	7.36	10	26.3	10	33.65	18.18	11.3	65	32.05	16.2	15	62
2. day	5	18.05	7.83	10	28.3	10	33.61	18.23	11.3	65	25.85	17.5	8.5	58
3. day	5	19.27	8.97	10.5	32	10	32.95	18.07	11	65	18.4	16.6	2	50
4. day	5	20.8	8.91	13.2	34	10	30.6	17.86	9	63	11.37	13.2	**0**	37.7
5. day	5	22.84	10.12	14.6	38.6	10	27.1	17.27	6	59	5.1	6.89	0	20
6. day	5	25.86	12.3	15.8	45	10	22.1	16.38	3	52	1.3	2.36	0	7
7. day	5	28.3	13.45	17	49.5	10	16.9	14.69	1	44	0.3	0.95	0	3
8. day	5	33.05	13.6	21	53.3	10	12	12.33	**0**	36	0	0	0	0
9. day	5	39.96	10.05	29.8	54	10	8	9.45	0	27	0	0	0	0
10. day	5	47.2	10.16	35	61	10	4.6	6.1	0	18	0	0	0	0
11. day	5	56.2	11.69	43	69	10	2.2	3.52	0	10	0	0	0	0
12. day	5	66.2	11.52	51	79	10	0.7	1.64	0	5	0	0	0	0
13. day	5	77.6	14.36	58	97	10	0.2	0.63	0	2	0	0	0	0
14. day	5	93.4	20.53	65	123	10	0	0	0	0	0	0	0	0

(P < 0.0001); Grup I: Trichlorfon; Grup II: Henna: Grup III: Control

It was exemined results that although, the mean lesion areas in both male and female calves increased in the III th Group. The healing process in the I th Group started on 2th day, the mean lesion areas continued to shrink and females recovered completely on the 9th day and, in males on the 8th day. In the II th Group, recovery started in males on the 2nd day, females on the 3rd day and finished on the 14th day completely. Also, no improvement was observed in the Group III and lesions were enlarged. Lesion size increased during the 14-day period from 20.1 ± 7.38 mm to 79.5 ± 32.94 mm in females and from 17.38 ± 7.36 mm to 93.4 ± 20.53 mm in males. In experiment, triple interaction Groups x Gender x Recovery Time is shown in Graph 1. The effects (daily change) of Grup I, II, III applications on healing time throughout the trial are shown in Graph 2.

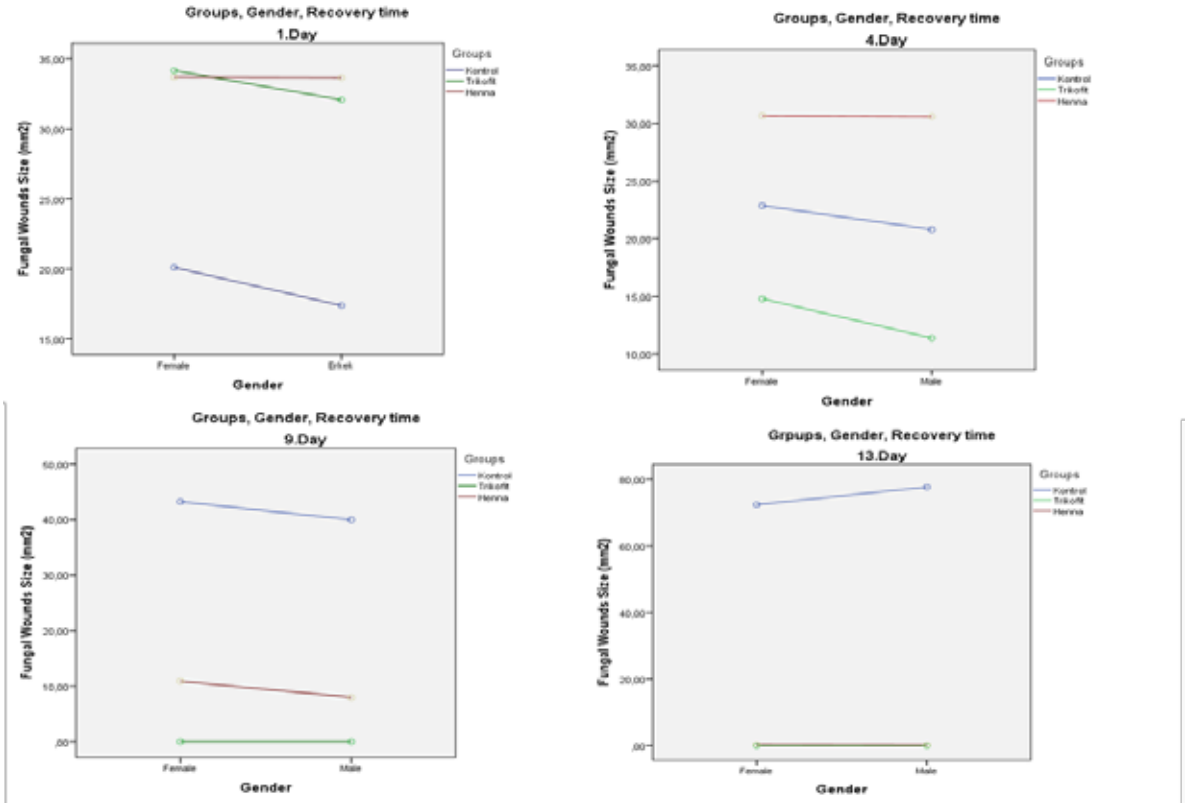

Figure 1. Change of interaction of group and gender

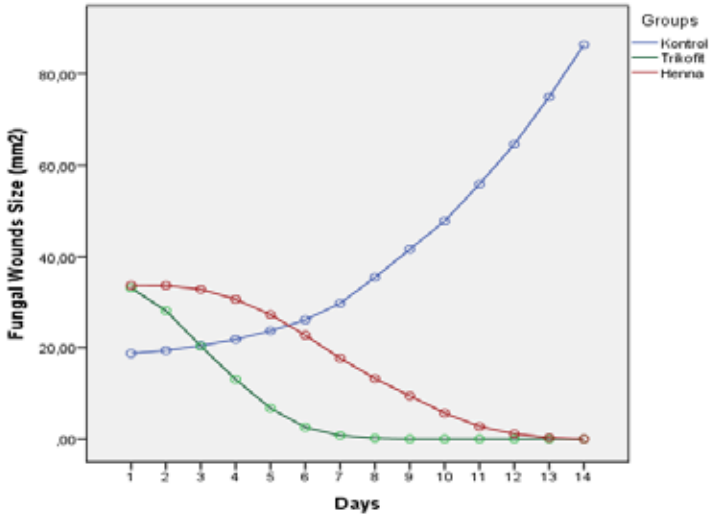

Figure 2. Distribution of the effects of groups on recovery time

DISCUSSION

The differences between the minimum and maximum values (mm^2) increase in the I. and II. Groups from the beginning of the recovery and cause the standard deviations to increase. In I th Group mean and standard deviation 8th day in females(14.5 ± 15.11), minimum and maximum values (0; 43) and, in males (12 ± 12.33) and (0; 36) as it has been realized. In II th Group mean and standard deviation 4th day in females (14.79 ± 15.4), minimum and maximum values (0; 48.2) and, in males (11.37 ± 13.2) and (0;37.7) realized. The standard deviation was never greater than the mean because the wounds in the III. Group were constantly growing. Also, Başoglu et al., (1998) reported that 57 calves with 1-6 cm ringworm in the neck area applied henna slurry four times a month and reported that all calves healing at the end of one month. This result is similar to the results of this study in that the henna completely heals the fungal wounds in calves.

The results, reflected by Yılmazer and Aslan (2010) that effectiveness of using of neguvon and whitfield's ointment in the treatment of fungal disease in cattle were in agreement with the results of the present study and the application of trichophyte ointment. Additionally, Kırmızıgül et al., (2008, 2009, 2013) reported that the healing of dermatophytosis lesions started on day 3 and ended on day 7 completely. These findings were similar to the results of the present study. In the current study, improvement starts on the 4th day and ends on the 8th day. It is thought that the longer duration of the healing is due to the different wound size and the different feeding program applied and the different environmental conditions such as climate and season. Abdulmoneim, (2007) reflected that leaf samples of *Lawsonia inermis* examined their antimicrobial potentials by phytochemical analyzes and determined the presence of anthraquinones as the main component in henna leaves. As a result, since this condition is known to have antimicrobial activity in general, it has been reported that henna leaves have antibacterial, antifungal activity and can be used in the treatment of bacterial infections. Collected henna samples from different regions of Oman have been shown to have the highest antibacterial activity against *P. aeruginosa* organisms. During screening of barks of 30 plant species against *Microsporum gypseum* and *Trichophyton mentagrophytes*, only *Lawsonia inermis Linn* extract exhibited absolute toxicity, the leaves of *L. inermis L.* were also found to exhibit strong fungi toxicity and non-phytotoxicity reported by Habbal et al. (2011). Lawsone isolated from the leaves of *L. inermis* has shown significant antifungal antibiotic effetcs reported by Santosh et al. (2013). All these stated results were similar to the results of this study Also, Rahmoun et al. (2013) stated that henna was widely used on the skin and was clearly safe to use. In addition, Yadav et al. (2013) reported that henna plant and extract against *Microsporum gypseum* and *Trichophyton mentagrophytesine fungi*, absolute toxicity, even in high temperature even if this toxicity stated that the activity continues. In addition, Kızılgül et al. (2008), Karpe et al. (2011) and Jamshidi-Kia et al. (2018) reported that dermatophytosis lesions started to heal on the 3rd day with different drugs and they completely healed on the 7th day. These findings were similar to the results of the present study.

In the present study, healing starts on the 2th day and ends on the 14th day and it is thought that the longer duration of the healing is due to different wound size and different feeding program, different environmental conditions such as climate and season.

CONCLUSION

Although the treatment of fungal disease in cattle (*Trichophypton verrucosum*) requires a slightly longer treatment period than synthetic medicine, it is thought that henna can be used instead of synthetic drugs, but further researches are needed on this subject.

ACKNOWLEDGMENTS

I would like to acknowledges to Professor Dr. Mikail BAYLAN, Aysun ÇAMLIDAĞ and Orhan Gazi DÜNDAR for their contributions during the conduct of the research.

CONFLICT OF INTEREST

None declared.

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J HELLENIC VET MED SOC 2020, 71(4): 2491-2498
ΠΕΚΕ 2020, 71(4): 2491-2498

Research article
Ερευνητικό άρθρο

Effects of *Alchemilla vulgaris* on growth performance, carcass characteristics and some biochemical parameters of heat stressed broilers

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ABSTRACT. This study aimed to investigate the effects of *Alchemilla vulgaris* (*A. vulgaris*) on growth performance, carcass characteristics, and biochemical parameters in broilers exposed to high environmental temperature conditions. A total of 45 broilers of 21 days of age (Ross 308) were used and grouped as the control group (C), and the groups with the addition of 1% (G1), or 3% (G2) *A. vulgaris* to chicken diet, respectively. In this study, the body weights of heat stressed broilers were significantly different on the 35th day and onwards. Feed intake was higher in the control group. Feed conversion ratio (FCR) was better in groups G1 and G2 compared to control on the 36th and 42nd day, the FCR was better on the 21st- 42nd days. The highest hot and cold carcass performance were observed in group G2 (79.72±0.93% and 78.02±0.99, respectively), and the lowest values were observed in group C (76.26±1.13% and 75.70±1.20%, respectively). *A. vulgaris,* reduced serum malondialdehyde (MDA) levels as numerically, and suppressed lipid peroxidation in a dose-dependent manner. It had significant effects on monocytes (MON %, MON count), platelets (PLT), and plateletcrit (PCT) parameters only. In conclusion, the deleterious effects of high environmental temperature in broilers could be partially reversed by *A. vulgaris* addition to the diets between days 21 and 42.

Keywords: Alchemilla vulgaris, Broiler, Carcass characteristics, High environmental temperature, Growth performance

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Date of initial submission: 30-12-2019
Date of revised submission: 13-07-2020
Date of acceptance: 17-07-2020

INTRODUCTION

Heat stress has become a serious problem in poultry production along with global warming. High environmental temperature has harmful effects on the physiology and immunology of poultry and reduces efficiency. The harmful effects include delayed growth rate, reduced feed intake, and impaired blood biochemistry and immunity. It was reported that feed intake of poultry decreased over 25 ℃, and that heat stress-related reduced capacity reached a serious level (Bollengier-Lee, 1999; Imik *et al*., 2013).

It was recommended to increase either feed intake or the nutrient ingredients within the diet to compensate for the reduced feed intake due to high environmental temperature (Ayhan *et al*., 2000). Studies have been conducted to investigate the effects of various herbal ingredients into the diets to increase heat stress-related reduced feed intake, especially in poultry (Karslı and Dönmez, 2007) and to reduce the other negative effects of heat stress. Thus, in the study of Akhavan-Salamat and Ghasemi (2016), the effects of betaine (Bet) and dried turmeric rhizome powder (TRP) on leukocyte profile, humoral immunity and antioxidant status alone or in combination in broilers exposed to heat stress were studied, and it was observed that the negative effects of heat stress on body weight, feed intake, FCR, and death rate were partially eliminated with dietary Bet and TRP. Likewise, it was observed in the study of Hassan *et al.* (2018) that addition of rutin (is a flavonol glycoside) into the rations resulted in increased body weight, protein efficiency rate, leukocyte and lymphocyte count, but did not affect the total blood protein, albumin, globulin and alanine transaminase levels in broilers. It was also determined in the study that high rutin concentration (0.5 and 1 g/kg) added into the rations significantly reduced the serum total cholesterol (TC), concentrations of triacylglycerol, low density lipoprotein (LDL) cholesterol ($p<0.001$) and malondialdehyde (MDA) ($p=0.001$), and increased the activities of superoxide dismutase, catalase, and glutathione peroxidase.

Alchemilla vulgaris is a biologically active herb as an immunomodulatory agent, which is rich in phyto components. It is widely referred to as Lady's Mantle, Bear's Foot or Lion foot, and used as a public medicine in European countries to a significant extent (Al-osaj, 2016). It is a member of the Rosaceae family and is rich primarily in flavonoids such as quercetin and kaempferol (Al-osaj, 2016), tannins, and chemical compounds such as gallic acid and ellagic acids (Neagu *et al*., 2015; Kostadinović *et al*., 2019). Dried lion's foot leaves have rich in total polyphenolic and flavonoids content (395.65 and 183.10 mg/100g, respectively) (El-Hadidy *et al*., 2018). The antioxidant, anti-inflammatory, anti-microbial and the anti-carcinogenic effects of the *A. vulgaris* have been attributed to its flavonoid components (9.8 mg/g) (Havsteen, 2002; Pietta, 1998).

In research, of all studied extracts, ethyl acetate extract from Lady's Mantle roots characterized by the highest content of catechins in comparison with other samples demonstrated the highest activity *in vitro* towards the studied viruses (neutralization index for vaccinia and ectromelia viruses were 4.0 and 3.5 lg, respectively) (Filippova, 2017). *A. vulgaris* (leaves and flowers) have the highest effect on lipase activity and α –amylase activity comparative with the extracts of Sophora Japonica and Crataegus Azarolus (leaves and fruits) which suggest that the chemical content of polar extracts of these plants might be of therapeutic interest concerning the treatment of obesity (Samah *et al*., 2018).

And it is determined that 3% *A. vulgaris* supplementation in the heat-stressed quail prevented decreased egg production and improved FCR (Akdemir *et al*., 2019). *A. vulgaris* L. can be proposed to protect the toxicity induced by Carbon tetrachloride (CCl_4) in rats, also to help inhibit the improvement of cardiovascular diseases and cystic fibrosis (El-Hadidy *et al*., 2019). Another research results demonstrate that the wound-healing properties of *A. vulgaris* associated with promitotic activity in epithelial cells and myofibroblasts (Shrivastava *et al*., 2007).

Efforts to eliminate stress-related productivity in broiler chickens are promising for the future of poultry production. Therefore, adding antioxidant plants to the diets of broilers is considered a simple, effective, and profitable application in the field due to its oxidative stress reduction effects. However, in the literature, there is no study on the effect of A. vulgaris use on the growth, feed intake, carcass characteristic parameters, and blood and serum stress parameters in the chickens raised under heat stress conditions. As reported in the above studies, A. vulgaris has the potential to be used as a natural remedy due to its antioxidant and anti-inflammatory properties. The present study aimed to determine the effects of dietary A. vulgaris supplementation on growth performance, carcass characteristics, and blood and serum stress parameters in 21-42 day heat stressed broilers.

MATERIALS AND METHODS

This study was conducted in Akçadağ Vocational School Division of Malatya Turgut Ozal University. The experiment was approved (approval document no 2018/A-34) by the Committee on Animal Research at Inonu University, Malatya, Turkey. Broilers (total of forty-five 21-days old, Ross 308), purchased from a commercial company (Seher Tavukçuluk, Malatya, Turkey), with an initial average body weight of 956.4 ± 23.4 g were used in this experiment. Furthermore, the birds were randomly assigned to 3 groups (15 birds in each group) defined as the control group (C) fed with basal diet (Table 1), and a basal diet supplemented with 1% (G1) (1) or 3% (G2) (2) of *A. vulgaris* (purchased from Altinterim Co., Elazig, Turkey as a powdered form). The chicks were selected from all groups in the study in terms of male and female gender-balanced. Broilers were housed 10-12 birds per $1m^2$ floor area/10-12 bird. The birds were also exposed to a 23L:1D illumination cycle. Diets and freshwater were offered for *ad libitum* consumption throughout the experiment. The 21-day-old broilers classified were kept at 34±2 °C until 42 days of age. The birds were fed with a finishing diet (Table 1) were obtained from a commercial feed factory (Seher Tavukçuluk, Malatya, Turkey).

Table 1. Ingredient and nutrient composition of the basal diet[a]

Ingredients, %	Finisher (21-42 d)
Corn	61.13
Soybean meal (48 %)	30.6
Soy oil	4.2
Limestone	1.51
Dicalcium phosphate	1.53
Sodium chloride	0.41
DL-Methionine	0.12
Vitamin-mineral premix[b]	0.5
Chemical analyses, dry matter basis, %	
Crude protein	20.50
Crude fat	6.13
Crude fiber	3.89
Calcium	0.97
Phosphorus	0.42
Calculated compositions[c]	
Metabolizable energy, Kcal/kg	3130
Lysine, %	1.12
Methionine + cysteine, %	0.85

[a] *A. vulgaris* was added into the basal diet at a dosage of 0, 1 and 3 %.
[b] Vitamin premix provides the following per kg: all-trans- retinyl acetate, 1.8 mg; cholecalciferol, 0.025 mg; all-a-tocopherol acetate, 1.25 mg; menadione, 1.1 mg; riboflavin, 4.4 mg; thiamine, 1.1 mg; pyridoxine, 2.2 mg; niacin, 35 mg; Ca-pantothenate, 10 mg; vitamin B_{12}, 0.02 mg; folic acid, 0.55 mg; d-biotin, 0.1 mg. Mineral premix provides the following per kg: Mn (from MnO), 40 mg; Fe (from FeSO4), 12.5 mg; Zn (from ZnO), 25 mg; Cu (from $CuSO_4$), 3.5 mg; I (from KI), 0.3 mg; Se (from NaSe), 0.15 mg; choline chloride, 175 mg.
[c]Calculated value according to tabular values listed for the feed ingredients (20).

The broilers in each group were weighed following 8-12 hours of fasting time on the 28th, 35th and 42nd days, and the bodyweight gains were calculated. Additionally, the feed intake per week was measured, and FCR was determined (consumed feed, g/gained weight, g).

The blood samples (5 ml) were collected from *vena cutanea ulnaris* of 6 chickens by random sampling from each group into both EDTA (anticoagulant tubes) and biochemistry tubes (additive-free vacutainer) on the 42nd day. Blood was rapidly transferred to the laboratory for serum separation and other analyses.

The blood samples were taken into the anticoagulant tubes and thereafter hematological parameters (WBC; white blood cell, LYM; lymphocytes, MON; monocytes, GRAN; granulocytes, RBC; red blood cell, HGB; hemoglobin, HCT; hematocrit, MCV;

mean corpuscular volume, MCH; mean corpuscular hemoglobin, MCHC; mean corpuscular hemoglobin concentration, PLT; platelets, MPV; mean platelet volume, PDW; platelet distribution width, PCT; plateletcrit and P-LCR; Platelet larger cell ratio) were measured with hematological analysis device. Also, the blood samples collected into the additive-free vacutainers were centrifuged at 5000 rpm/min for 5 minutes and serum separation was performed for measurement of some stress parameters. Reduced glutathione (GSH) and malondialdehyde (MDA) were determined according to Uchiyama and Mihara (1978). The serum TAS (Total Antioxidant Status) and TOS (Total Oxidant Status) levels were determined by previously reported methods (Erel, 2004; Erel, 2005) with a spectrophotometer. The OSI (Oxidative Stress Index) was defined as the ratio of the TOS level to the TAS level. Specifically, OSI (arbitrary unit) = TOS (μmol H_2O_2 Eq/L)/TAS (μmol Trolox Eq/L).

In the groups, the slaughter weight was detected for all chickens for the detection of carcass characteristics and then the chickens were sent for slaughter.

Before slaughter, chickens were subjected to a total feed withdrawal of 8 hours. The previously weighed chickens were cut using the cervical dislocation method and then were plucked and eviscerated. Post-slaughter hot and cold carcass weights were measured and the carcass characteristics were determined.

For these aims, the organ weights (breast, leg, wing, neck, and back) were recorded. The carcass weight was obtained by removing the head, neck, shanks, and abdominal fat from bled, plucked, and eviscerated chickens. Then, the hot carcass, breast, leg (thigh+drumstick), and abdominal fat weights were recorded. Carcass yields were determined according to the main commercial parts of the breast meat (including pectoralis major and pectoralis minor muscles) and the leg (including thigh and drumstick meat) (Cömert, et al. 2016). The cold carcass weights were determined by waiting at +4 °C for 24 hours. The weights of the pieces were determined using a 1 g scale. Heart, liver, spleen, and abdominal fat weight was determined proportionally with a precision scale of 0.01 g.

Statistical Analysis

Descriptive statistics were calculated for each parameter according to the data obtained. The Kruskall Wallis variance analysis was used for the intergroup comparisons to determine the effect of *A. vulgaris* addition into rations of the broilers on the investigated parameters. The Duncan Multiple Range Test (DMRT) as post hoc test was used for parameters that demonstrated significance (Akgül, 2005). Analyses were performed using the SPSS 22.0 version program package (SPSS, 2015).

RESULTS

The effects of *A. vulgaris* added at different amounts into the diets of broilers exposed to heat stress on body weights and body weight gains have been presented in Table 2 and that on feed intake and FCR have been presented in Table 3. Effects of *A. vulgaris* on the carcass weights (g) and ratios, and various carcass part weights of broilers exposed to heat stress are presented in Table 4.

Table 2. Effects of ***A. vulgaris*** added at different amounts into the rations of broilers exposed to high environmental temperature conditions on body weight and body weight gain

		Bodyweight, g			
Groups	**n**	**day 21** $\bar{X} \pm S_{\bar{X}}$	**day 28** $\bar{X} \pm S_{\bar{X}}$	**day 35** $\bar{X} \pm S_{\bar{X}}$	**day 42** $\bar{X} \pm S_{\bar{X}}$
Control	15	953.20±24.97	1486.67±37.58	2100.00±68.87^{B}	2688.00±97.45
G1	15	960.40±18.46	1434.67±24.18	2001.33±35.91^{AB}	2569.33±63.39
G2	15	955.20±26.58	1382.67±34.02	1892.00±56.33^{A}	2430.67±67.72
p		-	-	*	-
		Bodyweight gain, g			
Groups	**n**	**day 21-28**	**day 29-35**	**day 36-42**	**day 21-42** $\bar{X} \pm S_{\bar{X}}$
Control	15	76.20	87.60	73.50	79.10±4.32
G1	15	67.80	81.00	71.00	73.27±3.98
G2	15	61.10	72.80	67.30	67.07±3.38
P		-	-	-	-

-:p>0.05, *:p<0.05, $^{A, B}$: Differences between the values of the different letters in the same column are important (p <0.05).

Table 3. Effects of *A. vulgaris* added at different amounts into the rations of broilers exposed to high environmental temperature conditions on feed conversion ratio (FCR) and feed intake

Groups	n	Feed intake, g			
		day 21-28	day 29-35	day 36-42	day 21-42 $\bar{X} \pm S_{\bar{X}}$
Control	15	120.20	147.60	154.00	140.60±10.37
G1	15	114.10	137.30	145.80	132.40±9.47
G2	15	107.00	128.60	139.30	124.97±9.50
p		-	-	-	-
Groups	**n**	**FCR**			
		day 21-28	day 29-35	day 36-42	day 21-42 $\bar{X} \pm S_{\bar{X}}$
Control	15	1.58	1.68	2.10	1.78±0.16
G1	15	1.68	1.70	2.05	1.81±0.12
G2	15	1.75	1.77	2.07	1.86±0.10
p		-	-	-	-

-:p>0.05

Table 4. Effects of *A. vulgaris* on the carcass weights and yields, and various carcass part weights of broilers exposed to high environmental temperature conditions

Groups	n	Hot carcass weight, g $\bar{X} \pm S_{\bar{X}}$	Cold carcass weight, g $\bar{X} \pm S_{\bar{X}}$	Hot carcass yield, % $\bar{X} \pm S_{\bar{X}}$	Cold carcass yield, % $\bar{X} \pm S_{\bar{X}}$
Control	15	2076.73±96.56	2034.80±96.61	76.26±1.13	75.70±1.20
G1	15	1983.80±59.95	1945.27±59.75	77.21±0.69	75.71±0.70
G2	15	1937.80±67.14	1896.47±67.58	79.72±0.93	78.02±0.99
p		-	-	-	-
Groups	**n**	**Breast, g** $\bar{X} \pm S_{\bar{X}}$	**Leg, g** $\bar{X} \pm S_{\bar{X}}$	**Wing, g** $\bar{X} \pm S_{\bar{X}}$	**Neck+Back, g** $\bar{X} \pm S_{\bar{X}}$
Control	15	786.67 ± 39.66^{B}	817.78 ± 44.63^{B}	218.89 ± 9.72^{B}	260.44 ± 13.64^{B}
G1	15	771.14 ± 40.56^{B}	749.14 ± 30.21^{AB}	199.43 ± 5.99^{AB}	230.00 ± 11.55^{B}
G2	15	652.00 ± 24.23^{A}	664.44 ± 25.66^{A}	180.00 ± 5.93^{A}	195.11 ± 8.86^{A}
p		*	*	**	**

-:p>0.05, *:p<0.05, **:p<0.01. ^{A,B}: Differences between the values of the different letters in the same column are important (p <0.05).

Effects of *A. vulgaris* on serum antioxidant levels in the blood used as stress parameters (MDA, GSH, TAS, TOS and OSI) of broilers exposed to heat stress have been presented in Table 5. Although no significant difference was observed in the serum levels of MDA, GSH, TAS, TOS and OSI, the MDA and GSH levels showed a tendency to decrease following dietary *A. vulgaris* supplementation, and TOS and OSI levels showed a tendency to increase (Table 5).

Table 5. Effects of *A. vulgaris* on serum stress parameters of broilers exposed to high environmental temperature conditions

Groups	n	MDA[a] $\bar{X} \pm S_{\bar{X}}$	GSH[b] $\bar{X} \pm S_{\bar{X}}$	TAS[c] $\bar{X} \pm S_{\bar{X}}$	TOS[d] $\bar{X} \pm S_{\bar{X}}$	OSI[e] $\bar{X} \pm S_{\bar{X}}$
Control	6	4.34±0.52	39.87±6.19	1.59±0.14	27.71±7.12	18.25±4.38
G1	6	3.73±0.08	32.32±0.99	1.34±0.12	20.67±1.79	16.38±2.34
G2	6	3.63±0.15	36.25±2.04	1.60±0.12	31.95±5.13	20.03±2.56
p		-	-	-	-	-

-: p>0.05. [a]Malondialdehyde, µmol/L; [b]Reduced Glutathione, µmol/L; [c]Total Antioxidant Status, mmol Trolox Equiv./L; [d]Total Oxidant Status, µmol H_2O_2 Equiv/L; [e]Oxidative Stress Index, mmol Trolox/µmol H_2O_2.

Also, effects of *A. vulgaris* on hematological parameters (WBC, LYM (%), MON (%), GRAN (%), LYM (10^3/µL), MON (10^3/µL), GRAN (10^3/µL), RBC, HGB, HTC, MCV, MCH, MCHC, RDV-SD, RDV-CV, PLT, MPV, PDW, PCT, and P-LCR) have been presented in Table 6. As demonstrated in Table 6, the effect of *A. vulgaris* on some blood parameters was observed to be significant only for MON (%), MON (10^3/µL), PLT, and PCT ($p<0.05$, $p<0.05$, $p<0.01$, $p<0.05$, respectively). MON (%) and MON (10^3/µL) were highest in group G2 (5.55±0.24 % and 1.42±0.08 10^3/µL, respectively), and PLT and PCT were highest in group C (53.00±2.96 10^3/µL and 0.06±0.01%, respectively) (Table 6).

Table 6. Effects of *A. vulgaris* on various blood parameters of broilers exposed to high environmental temperature conditions

Groups	n	WBC, 10^3/µL	LYM, %	MON, %	GRAN, %	LYM, 10^3/µL
		$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$
Control	6	25.25±0.65	93.68±0.84	3.95±0.35^{A}	2.37±0.54	23.65±0.44
G1	6	23.62±0.69	93.67±0.91	3.97±0.42^{A}	2.37±0.50	21.38±1.05
G2	6	25.87±0.91	89.07±1.20	5.55±0.24^{B}	4.90±1.04	23.82±1.35
p		-	-	*	-	-
Groups	**n**	**MON, 10^3/µL**	**GRAN,10^3/µL**	**RBC,10^6/µL**	**HGB, g/dl**	**HCT, %**
		$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$
Control	6	1.02±0.12^{A}	0.58±0.15	3.40±0.09	9.72±0.24	19.08±0.36
G1	6	0.92±0.07^{A}	0.48±0.11	3.13±0.13	8.83±0.32	17.77±0.61
G2	6	1.42±0.08^{B}	0.62±0.05	3.42±0.14	9.57±0.47	18.92±0.78
p		*	-	-	-	-
Groups	**n**	**MCV, fL**	**MCH, Pg**	**MCHC,g/dl**	**RDW-SD, fL**	**RDW-CV, %**
		$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$
Control	6	56.42±1.23	28.53±0.33	50.87±0.61	27.57±0.57	9.62±0.27
G1	6	57.02±0.52	28.22±0.22	49.68±0.25	28.20±0.30	9.77±0.10
G2	6	55.40±0.49	27.85±0.30	50.43±0.45	26.95±0.42	9.52±0.18
p		-		-	-	-
Groups	**n**	**PLT, 10^3/µL**	**MPV, fL**	**PDW, %**	**PCT, %**	**P-LCR, %**
		$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$
Control	6	53.00±2.96^{B}	11.07±0.21	11.87±0.58	0.06±0.01^{B}	21.43±0.66
G1	6	36.00±2.33^{A}	11.40±0.22	12.53±0.81	0.04±0.01^{A}	22.22±1.70
G2	6	46.50±3.85^{B}	11.57±0.23	12.12±0.40	0.05±0.01^{AB}	23.20±1.73
p		**	-	-	*	-

-:$p>0.05$, *: $p<0.05$, **:$p<0.01$. ^{A,B}: Differences between the values of the different letters in the same column are important ($p<0.05$).

DISCUSSION

Effect of *A. vulgaris* on the growth performance of broilers

It was observed in this study that the body weights of broilers exposed to heat stress were significantly different on the 35th day and onwards ($p<0.05$), and body weights in the control group were higher compared to the remaining groups (G1 and G2) (Table 2). No chickens died during the study.. Increases in feed intake were observed to be higher in the control group as well (Table 3). Although it was observed that FCR was better in groups G1 and G2 compared to control on the 36th and 42nd day of the study, the FCR was better on the 21st – 42nd days (Table 3). No comparison could be performed with similar studies since no study could be detected on the effects of *A. vulgaris* on the growth performance of the broilers exposed to heat stress in the literature. However, in a study comparing body weights of chickens not exposed to heat stress and fed with a mixture containing 4 different herbs including *A. vulgaris* as well, the body weights were found to be lower compared to the control group; and it was emphasized that it regulated activities of digestive enzymes in rats, and increased the metabolic rate and fat burning (Said *et al*., 2011). *A. vulgaris* has been reported to be rich in flavonoids and shown action via this biomolecule (Havsteen, 2002). Similar to the outcomes of our study, Karslı and Dönmez (2007) reported that the flavonoid-rich herb mixtures added into the broiler diets to reduce heat stress has negative effects on feed intake. Likewise, no improvement was reported in feed intake, carcass

weight, and FCR parameters of broilers exposed to heat stress and fed with propolis, which is known to be rich in flavonoids, compared to the control group (Chegini *et al*., 2018). On the other hand, flavonoids added to the rations such as quercetin, genistein, hesperidin and rutin have been reported to have no effect on feed intake, carcass weight and FCR of broilers exposed to heat stress, whereas high dose flavonoids have been reported to have a partial effect on CW on 22nd - 42th days (Kamboh *et al*., 2013).

Effect of *A. vulgaris* on the carcass characteristics of the broilers

No significant difference was observed between the groups concerning hot and cold carcass weight or both carcass yield (p>0.05). However, although not statistically significant, the highest hot and cold carcass performance values were observed in group G2 (79.572±0.93% and 78.02±0.99, respectively), and the lowest values were observed in group C (76.26±1.13% and 75.70±1.20%, respectively) (Table 4). Similar to our study, Karslı and Dönmez (2007) reported no negative effect of flavonoid-rich herb mixture added into the diets of broilers exposed to heat stress on carcass weight and reported the highest carcass weight in the control group and the lowest in the group fed with herbal extract mixture (P<0.05). This indicates that *A. vulgaris* added into the diets had no effect on the carcass weight, but showed a tendency to improve the carcass performance.

Effect of *A. vulgaris* on some blood and serum stress parameters in broilers

Different outcomes have been observed in numerous studies investigating the effects of stress on serum antioxidant levels, enzyme activities, and blood parameters of broilers raised at high temperature conditions. Erköse and Akşit (2009) reported significantly reduced serum cholesterol, alkaline phosphatase, and uric acid and hematocrit levels in broilers exposed to acute heat stress. Indeed, acute heat stress has been related to many problems in broilers, such as myopathy, blood acid/base imbalance, and increase in cholesterol, uric acid, alkaline phosphatase, and plasma creatine kinase (CK) levels (Sandercock *et al*., 2001). Overproduction of reactive oxygen species (ROS) related to heat stress has been known to cause lipid peroxidation and increase the level of MDA (Altan *et al*., 2003). Furthermore, in a study conducted on 44-day-old broilers, exposure to 39±1 °C heat stress for 2 hours was shown to result in an increase in heterophile and basophil ratios, reduction in monocyte and lymphocyte ratios, and no effect in the eosinophil count and hematocrit values (Altan *et al*., 2000). In the study of Dönmez and Atalay (2007), an increase was reported in the heterophile and basophil counts of broilers, and a decrease in eosinophil and lymphocyte counts. On the other hand, some studies have reported that antioxidants added to the diets have partially reduced or not affected the negative outcomes of heat stress. It was observed in our study that, *A. vulgaris,* which is known to have anti-inflammatory, antioxidant and anti-microbial effects, did not affect serum MDA, GSH, TAS, TOS and OSI values (p>0.05) (Table 5), but reduced serum MDA levels and suppressed lipid peroxidation in a dose-dependent manner, although not statistically significant. We believe that its lipid suppressing effect would be stronger if the rate added to the rations could be increased and if the duration of the study could be delayed. *A. vulgaris* was demonstrated to significantly affect certain blood parameters (MON (%), MON (10^3/µL), PLT, and PCT) (p<0.05, p<0.01) (Table 6). Among those, it was notable that the monocyte levels were significantly increased with the change in the dose of *A. vulgaris* added into the diets, because monocytes are phagocytic cells of the blood and support the immune system of the body by killing pathological microorganisms. This suggests that the addition of *A. vulgaris* to the diets may contribute to the immune system and the resistance of the chickens to diseases. It was observed in the study of Akhavan-Salamat and Ghasemi (2016) investigating the effect of dried turmeric powder addition into diets of broilers exposed to heat stress alone or in combination, which included betaine and flavonoids, that the serum SOD and GPx activities were higher in the study groups compared to the control group (P <0.05).

No effect of rutin addition of *A. vulgaris* into the diets of broilers was reported on total protein, albumin, globulin, and alanine transaminase levels in the study of Hassan et al. (2018), whereas increased leukocyte and lymphocyte counts were observed. Furthermore, as an ingredient, the routine was demonstrated to significantly reduce the levels of total serum cholesterol, triglyceride, LDL cholesterol, and malondialdehyde concentrations, and to increase the CAT, SOD, and GPx activity. Hasheimi et al. (2013) investigated the effect of Zingiber addition on broilers exposed to heat stress and observed increased plasma corticosterone concentration and heterophil/lymphocyte ratio independent from the diet on the 42nd day.

CONCLUSION

Especially 3% A. vulgaris supplement to heat stress broiler diets, although not statistically significant, decreases feed intake while increasing body weight and hot and cold carcass performance. This is important for broiler-producing farms to provide more profit. A. vulgaris also has significant effects on MON (%), MON (count), PLT, and PCT parameters. Also, addition of A. vulgaris to ration reduced MDA levels as numerically and suppressed lipid peroxidation partially, too. This result is important for farms growing broilers in hot climates. Besides, there may be a solution that will contribute to an effective broiler breeding today, where there is a global warming problem. However, to better define the effects of A. vulgaris on poultry exposed to heat stress, studies with different doses and durations should be conducted.

ACKNOWLEDGEMENTS

The authors thank YS. ÖZDEMİR and O. ORDU.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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J HELLENIC VET MED SOC 2020, 71(4): 2499-2504
ΠΕΚΕ 2020, 71(4): 2499-2504

Research article
Ερευνητικό άρθρο

Paraoxonase activity assessment in dogs suffering from *Parvovirus* infection

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ABSTRACT: Canine *Parvovirus* enteritis is one of the most common causes of diarrhea and death in dogs younger than 6 months of age. Clinical gastroenteritis in parvo-positive dogs is associated with increased levels of lipid peroxides and alteration in antioxidant enzymes. Paraoxonase/arylesterase 1 is considered as an antioxidant enzyme and acute phase protein in laboratory animals and human. The present study evaluated paraoxonase activity in 27 dogs suffering from *Parvovirus* infection and compared with 9 normal dogs. Blood samples were taken from all of the dogs and were sent to the laboratory for complete cell blood count and also biochemical factors assessment (paraoxonase/arylesterase 1 activity, liver, kidney and metabolic profiles). Infected dogs significantly showed decrease in paraoxonase/arylesterase 1 enzyme activity, an increase number of neutrophils and lymphopenia compared with parvo-negative dogs. Significant decrease in the enzyme activity was also observed in dogs with neutropenia or leukocytosis when compared to control group. It seems that in dogs paraoxonase/arylesterase 1 enzyme activity is decreased as a part of acute phase response in *Parvovirus* infection.

***Keywords*:** Paraoxonase; *Parvovirus* enteritis; acute phase proteins

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Date of initial submission: 11-01-2020
Date of revised submission: 11-05-2020
Date of acceptance: 17-07-2020

INTRODUCTION

Canine *Parvovirus* infection (CPV) is one of the major causes of morbidity and mortality in puppies and young dogs for more than three decades (Appel et al., 1979; Decaro and Buonavoglia, 2012; de Oliveira et al., 2019). High mitotic rate tissues such as thymic cortex and germinal epithelium of the intestinal crypts are the excellent targets for *Parvovirus* to lead lymphocytolysis, lymphopaenia, and collapse in the absorptive capacity of enterocytes (Greene, 2012). Clinicopathological biomarkers in this disease such as haematology or serum biochemistry and endocrine changes guide clinicians making right decision and help predicting outcomes (Goddard et al., 2008; Dossin et al., 2011; McClure et al., 2013). Recently, new renal biomarkers such as urinary immunoglobulin G (uIgG), C-reactive protein (uCRP), neutrophil gelatinase associated lipocalin (uNGAL) and urinary protein/ creatinine ratio (UPC) have been detected in parvo infected dogs suffering from acute kidney injury (Van den Berg et al., 2018). **S**erum paraoxonase or (PON1) (aryldialkylphosphatase, EC 3.1.8.1) is an antioxidant enzyme belonging to hydrolase family which is synthesized by the liver and causes degradation of lipid oxidation products and lactones such as homocysteine thiolactone (Rosenblat et al., 2006; Weijun et al., 2008; Altenhöfer et al., 2010; Ciftci et al., 2015). Alteration in level of PON1 had been approved in various diseases involving inflammation and oxidative stress such as cardiovascular, renal and liver diseases or obesity (Camps et al., 2007; Ferretti et al., 2010; Zhao et al., 2012; Zhang et al., 2015; Dalal et al., 2018). For these properties, this enzyme attracted scientists' attention in human and veterinary medicine as a novel biomarker of oxidative stress or acute phase response (Rossi et al., 2013; 2014[a,b]; Tvarijonaviciute et al., 2015; Moya and Manez, 2018). The current study was conducted to evaluate the PON1 activity in dogs suffering from *Parvovirus* infection.

MATERIALS AND METHODS

Animals

This retrospective study performed on 27 *Parvovirus* infected dogs (test group) and 9 healthy dogs (control group) that were admitted to the Hospital of Veterinary School of Shahid Bahonar University of Kerman in Iran for treatment and routine check-ups respectively. Animals were client-owned dogs aged between 2- to14-month-old from both genders and mixed breed.

Animal ethics

The study was conducted in accordance to the guidelines for use of animals in research and approved by the State Committee on Animal Ethics, School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran.

Blood samples

Blood samples were taken from cephalic vein (2.5 ml) and were divided into two parts. 0.5 ml of the blood sample was anticoagulated with EDTA for complete cell blood count (CBC) analysis and 1.5 ml of the blood sample was centrifuged at 750 g for 15 min in order to separate sera for measuring PON1 activity, liver, kidney and metabolic profiles.

Complete blood count parameters

Immediately after blood collection, samples with EDTA were used to prepare blood smear for measuring white blood cell (WBC), red blood cell (RBC) count and hematocrit.

Serum biochemical analysis

The sera were analyzed for total protein (Biuret method), albumin (Bromocresol green), alkaline phosphatase enzyme activity (ALP), blood urea nitrogen or BUN (Urease method), creatinine (Jaffe method), cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) and triglyceride (TG) using commercial kits (Pars Azmoon Co., Tehran, Iran). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using Reitman and Frankel colorimetric method (Reitman and Frankel, 1957). Analysis procedure was carried out using automated analyzer (Ames, Rome, Italy). PON1 enzymatic activity was assessed using commercial kit (ZellBio GmbH, Germany) according to manufacturer instruction briefly by measuring in vitro hydrolysis of paraoxon to ρ-nitrophenol. Product absorbance measurement (412 nm) was carried out using microplate spectrophotometer (Epoc, BioTek Instruments Inc., USA). Based on the results of the study by Rossi and her colleagues, paraoxon- based methods were accurate and precise for evaluating the esterase activity of PON1 in canine serum (Rossi et al., 2013).

Statistical Analysis

Statistical analysis was performed using the SPSS software® (Version 17 for Windows; SPSS Inc., Chicago, IL, USA). Data was checked for normal dis-

tribution using the Kolmogorov-Smirnov test. Comparison of mean values within groups was performed using Independent Samples T-Test. *P* value less than 0.05 was considered statistically significant. Potential correlation between PON1 and other parameters was determined using the Pearson Correlation test.

RESULTS

In this study, blood samples selected from healthy dogs and dogs suffering from *Parvovirus* infection. A complete evaluation of the history, physical examination findings, hematology and biochemical analysis were performed on all animals. *Parvovirus* infection was admitted by both clinical examination and a point-of-care test (ICT test, Quicking Biothech Co., Ltd, China). In order to prevent misdiagnosing *Parvovirus* infection, we excluded dogs that had been vaccinated two weeks earlier. Anorexia, vomiting and diarrhea were reported in the history of the dogs in the test group. Healthy dogs (n=9) were presented to clinic for routine check-ups. They did not present any clinical evidence of disease and absence of CPV was confirmed by a point-of-care test. Blood samples were taken within 2 hours of admission before any treatment. Data were presented as means ± standard errors (X ± SE) in Tables (1, 2) and Figures (1-5). As shown in Table 1, significant differences were observed in lymphocyte, segmented and band neutrophil between healthy and infected dogs. There was significant difference in PON1 activity between healthy and parvo-positive dogs (Fig 1); however, there was no significant difference in PON1 activity between infected dogs without neutropenia in comparison with neutropenic dogs (Fig 2). In Fig 3, lower PON1 activity also was seen in neutropenic dogs in comparison with healthy ones (*P=0.03*). There was no significant difference in PON1 activity between infected dogs without cytosis in comparison with dogs which had cytosis; in contrast, there was significant decrease in PON1 activity in dogs that had cytosis compared with healthy dogs (*P=0.02*) (Fig 4, 5). According to the results in Table 2, significant differences were observed in creatinine, albumin, total protein concentrations and ALT activity between two groups. There were no significant differences between triacylglycerol (TG), high density lipoprotein (HDL), low density lipoprotein (LDL) and cholesterol between groups.

Table 1. The Mean ± SE of blood components in healthy and infected dogs

Parameters	Healthy dogs	Infected dogs
Hematocrit	40.62±9.11	61.38±98.57
RBC	6.03±1.34	6.63±1.37
WBC	10.00±1.96	13.15±14.39
Segmented neutrophil	4.78±1.47	10.37±13.50*
Band neutrophil	0.09±1.13	0.63±0.77*
Lymphocyte	4.83±1.28	1.88±1.71*
Monocyte	0.05±0.13	0.12±0.23
Eosinophil	0.18±0.11	0.09±0.19

*Asterisk showed significant differences between groups (*P<0.05*).

Table 2. The concentration of biochemical factors (Mean ± SE) in healthy and infected dogs

Parameters	Healthy dogs	Infected dogs
AST (U/ml)	40.33±21.27	72.44±86.61
ALT (U/ml)	29.33±13.72	82.70±110.26*
ALP (U/ml)	436.67±320.28	492.37±466.46
BUN (mmol/L)	21.84±17.50	28.31±18.02
Creatinine (µmol/L)	102.54±19.44	79.56±32.70*
TG (mmol/L)	0.90±0.26	1.02±0.40
Cholesterol (mmol/L)	5.91±0.37	3.61±1.56
HDL(mmol/L)	2.85±0.70	3.12±0.92
LDL (mmol/L)	1.55±0.84	2.20±1.45
Albumin (g/L)	36.2±3.7	30.1±6.9*
Total protein (g/L)	720±63.6	711±168.8*

*Asterisk showed significant differences between groups (*P<0.05*).

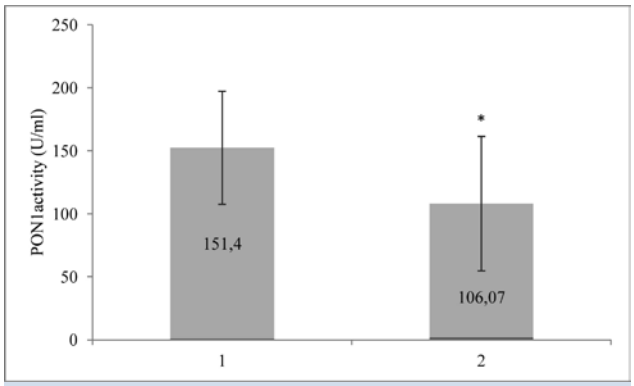

Fig 1. The activity of PON1 (U/ml) in healthy (1) and infected dogs (2)

*Asterisk showed significant differences between groups (*P<0.05*).

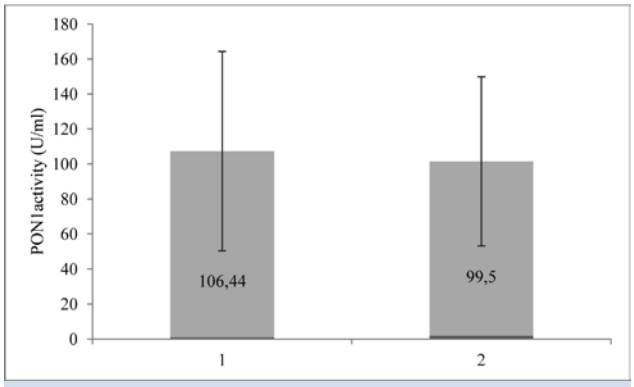

Fig 2. The activity of PON1 (U/ml) in dogs without neutropenia (1) in comparison with infected neutropenic dogs (2)

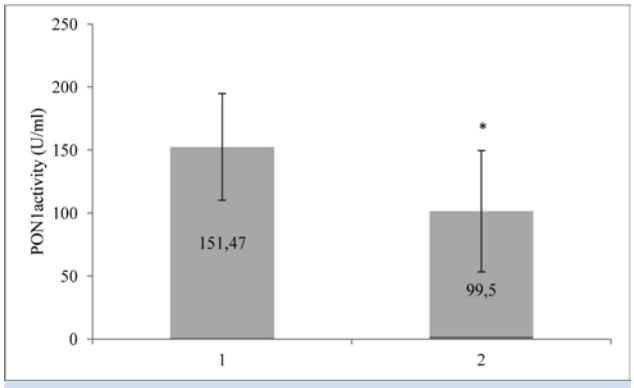

Fig 3. The activity of PON1 (U/ml) in healthy dogs (1) in comparison with neutropenic infected dogs (2)

*Asterisk showed significant differences between groups (*P=0.03*).

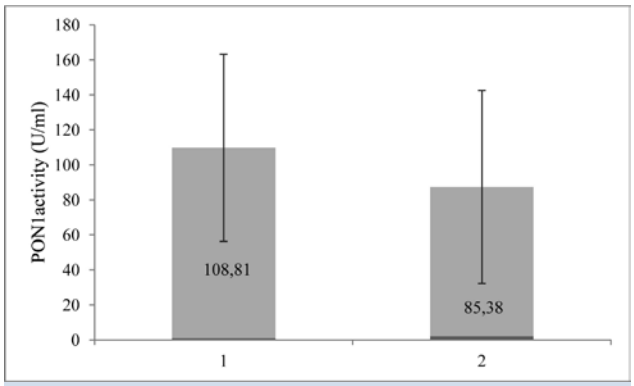

Fig 4. The activity of PON1 (U/ml) in infected dogs without cytosis (1) in comparison with infected dogs with cytosis (2)

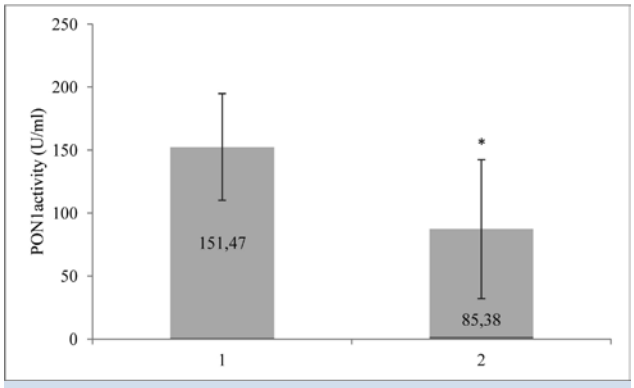

Fig 5. The activity of PON1 (U/ml) in healthy dogs (1) in comparison with infected dogs with cytosis (2)

*Asterisk showed significant differences between groups (*P=0.02*).

DISCUSSION

Findings of this study demonstrated that all of the *Parvovirus* infected dogs had lower PON1 activity compared with healthy ones. It seemed that PON1 enzyme was deactivated in the course of the *Parvovirus* infection as an acute phase response. Canine *Parvovirus* enteritis is an acute gastrointestinal illness that is seen mostly in puppies and young dogs. Since it's emerging in 1979, numerous scientific efforts were established in order to find reliable clinicopathological biomarkers for this contagious disease (Schoeman et al., 2013; Hoang et al., 2019). Some researches revealed that inflammation state and alteration in profile of serum acute-phase proteins occurred in the time of the canine *Parvovirus* infection; therefore, the anti-inflammatory body's reaction would be as reflex of disease severity (Kocaturk et al., 2010, McClure et al., 2013). PON1 is tightly associated with apolipoprotein A1 in HDL and exerts its protective function through hydrolyzing oxidized lipids (Aviram et al., 2000). During an acute phase response, HDL molecules lose

its associated enzymes, including PON1, which is replaced by serum amyloid A and ceruloplasmin. This phenomenon results in reduced antioxidative properties of HDL (Cabana et al., 2003; Novak et al., 2010). Alteration in serum PON1 had been correlated with inflammation and oxidative damage in many clinical conditions (Rosenblat et al., 2006; Zhao et al., 2012; Tecles et al., 2015; Arenas et al., 2018). The main goal of this experiment was attempting to find whether or not level of PON1 activity would have been changed in dogs that were infected with the *Parvovirus* enteritis. As noted in the results, the significant decrease in PON1 activity was observed in dogs suffering from *Parvovirus* enteritis when it was compared with healthy dogs (Fig 1). This phenomenon probably exhibited that low PON1 activity was linked to a part of acute phase response in the course of the *Parvovirus* infection. This was approved by Rossi and her coworkers (2014[a]) who revealed that PON1 activity was decreased in dogs that were affected by leishmaniasis. After medication of leishmaniasis, normalized PON1 activity was seen earlier than other inflammatory biomarkers in cases with systemic inflammation (Rossi et al., 2014[a]). These researchers concluded that low value of PON1 activity might indicate sever inflammation during leishmaniasis (Rossi et al., 2014[a]). Other investigation also suggested the relationship between reduction in serum PON1 activity and sternness of infection in dogs with acute pancreatitis (Tvarijonaviciute et al., 2015). Sensitivity of PON1 activity to inflammatory conditions and oxidative stress was approved in other small animals by Tecles and his colleagues in 2015. These researchers established that in cats with infectious peritonitis (FIP) not only PON1 level was considerably decreased but total antioxidant capacity was diminished (Tecles et al., 2015). Findings in Table 1 showed that, both segmented and band neutrophil were increased (probably because of secondary infections) and lymphocyte number was decreased noticeably in infected dogs compared with parvo-negative cases. Lower PON1 activity was seen in neutropenic infected dogs in comparison with healthy group ($P=0.03$) (Fig 3). There was significant decrease in PON1 activity in infected dogs that had cytosis compared with healthy dogs ($P=0.02$) (Fig 5). This result proposed the relationship between low PON1 activity and severity of the CPV in dogs. PON1 is an antioxidant enzyme so antioxidant capacity of infected dogs is decreased with lowered PON1 activity. This is in agreement with an investigation which was done by Panda and coworkers in (2009) which revealed that clinical gastroenteritis in parvo-positive dogs was associated with increased levels of lipid peroxides and alteration in antioxidant enzymes in the erythrocytes (Panda et al., 2009). PON1 is a glycoprotein that associated with HDL in circulating system so it was expected that level of HDL would have been declined if PON1 activity was reduced. Surprisingly as shown in Table 2, there were no significant differences in HDL and other metabolic parameters like TG, LDL and cholesterol between groups. This was in conflict with previous study which had shown that serum total cholesterol and HDL levels were decreased and TG level was increased in dogs with *Parvovirus* enteritis (Yilmaz and Senturk, 2007). This study performed on a limited number of samples, however; a large population of dogs with naturally occurring *Parvovirus* infection could lead to a higher generalization of our results.

CONCLUSIONS

It was found that all of the infected dogs and also cases with neutropenia or cytosis had lower PON1 activity compared with parvo-negative cases; therefore, it seemed that PON1 activity level in serum is sensitive to *Parvovirus* infection. This investigation emphasized possible contribution of PON1 level as an antioxidant enzyme and negative acute phase reactant in canine *Parvovirus* infection.

ACKNOWLEDGEMENT

The authors would like to thank the Research Council of Shahid Bahonar University and School of Veterinary Medicine, Shahid Bahonar University of Kerman for financial and technical support of this study (Grant No.96-GR-VT).

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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J HELLENIC VET MED SOC 2020, 71(4): 2505-2510
ΠΕΚΕ 2020, 71(4): 2505-2510

Research article
Ερευνητικό άρθρο

Effect of Cefazolin on endometrial cytology and reagent test strips parameters in bovine endometritis

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ABSTRACT: Endometritis is one of uterine disorders in dairy cows causing low fertility. This study explored effects of intrauterine (IU) infusion of Cefazolin on endometrial cytology and reagent test strips parameters (leukocyte esterase [LE] and pH) in bovine subclinical endometritis. A total of 90 cows at 49-56 days in milk (DIM) diagnosed for subclinical endometritis were divided randomlyinto three groups, group I (n = 35), treated with IU infusion of 2 grams Cefazolin (Zinol ®, Pharco) diluted with 50 ml saline; group II (n = 28), treated with IU infusion of 2 gramsCephalexin (Ceporex®, GlaxoSmithKline) diluted with 50 ml saline; group III (n = 27), cows kept as untreated control. Cytological examination and reagent test strips were performed in uterine material derived with the cotton swab technique before treatment program and repeated two times later, in 10 days interval. The cows were artificially inseminated at first oestrous after the end of treatment program and conception rates were evaluated. After the end of treatment program, polymorphonuclear cells (PMN) decreased significantly (P <0.05) in Cefazolin and Cephalexin groups (3.23% and 4.39% respectively) compared tocontrol (24.89%) group, also means of LE in these groups became significantly (P<0.05) lower. The pH value decreased after treatment, this reduction was significant after the first dose of Cefazolin, while in Cephalexin reduction of pH became significant after second dose. Cefazolin had conception rate (77.14%) significantly (P<0.05) higher than cephalexin and control groups (57.14% and 25.93% respectively). Cefazolin and Cephalexin decrease uterine PMN, LE concentration. The pH value after treatment by Cefazolin was lower than in Cephalexin. Moreover, treatment with Cefazolin resulted in a significantly higher conception rate. Thus, Cefazolin is recommended for the treatment of subclinical endometritis in dairy cows.

***Keywords*:** Polymorphonuclear cells; Leukocyte esterase; Conception rate; Cows

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Date of initial submission: 15-01-2020
Date of revised submission: 26-06-2020
Date of acceptance: 10-09-2020

INTRODUCTION

Endometritis is a common uterine disorder in dairy cows that is strongly associated with reproductive and economic losses (Giuliodori et al., 2013). Various diagnostic methods are needed in order to treat the condition quickly and effectively to reduce these losses. These methods include vaginal examination, ultrasound, cytology, uterine biopsy and reagent test strips (Cheong et al., 2012; Casarin et al., 2018). Cytological examination of the reproductive tract is one of these methods, often used to assess possible reproductive lesions in humans and animals (Kasimanickam et al., 2005). In this method of examination, it has been reported that the neutrophil ratio to endometrial cells becomes an indicator of the inflammatory process (Couto et al., 2013).

To properly evaluate this inflammatory process, it is necessary to know the threshold values for polymorphonuclear cells (PMN) through which the presence of inflammation is established. Several studies have discussed this point including a study reporting 5% PMN as a threshold value; another study reported that this value varies depending on the examination stage. It was ≥8%PMN at the period equal or less than 33 days postpartum and ≥6% PMN at 34 to 47 days postpartum.Moreover, it was ≥4% PMN at the period equal or more than 48 days postpartum (Madoz et al., 2014; Chaudhari et al., 2017). Reagent test strips results were strongly related with endometrial cytology for the diagnosis of endometritis(Cheong et al., 2012).Several studies have addressed the use of reagent test strips as a rapid test to diagnose the inflammatory process. These strips contain different parameters including leukocyte esterase (LE), protein concentration and pH value. It has been suggested that LE in vaginal discharge can be used as a method for screening subclinical endometritis in cattle (Hajibemani et al., 2016). Endometritis changes the physical and chemical properties of cervical mucus. Therefore, examination of this mucus for appearance, consistency and pH may be useful for its diagnosis (Kumar et al., 2017).After diagnosis of endometritis, the role of treatment is important, with a variety of intrauterine antibiotic, antiseptic chemicals, systemic antibiotics and hormones to have been used for this purpose (Jeremejeva et al., 2012; Singh et al., 2018). Cefazolin and Cephalexin are first generation cephalosporin antibacterialswhich have a wide range of activity against gram-positive and gram-negative bacteria (Moradi et al., 2020).Therefore, this study was designed to evaluate the effects of IU infusion of Cefazolin on endometrial cytology and reagent test strips parameters (LE and pH) in dairy cows affected withsubclinical endometritis.

MATERIALS AND METHODS

Animals

Ninety Holstein-Friesian cows aged from 3-5 years at 49–56 days in milk (DIM) affected by subclinical endometritis were used in the study during the period from March 2017 to February 2019. Cows were housed outdoors in open hygienic yards; they were fed grass silage, concentrate and hay. The cows had a good health status and they received ordinary vaccines against foot and mouth disease (FMD), Lumpy skin-disease (LSD) and rift valley fever (RVF).Cows with arthritis, hoof problems, retained placenta , cesarean section, mastitis and other clinical diseases were excluded from the study. A clinical examination of the reproductive tract was performed by vaginal examination and transrectal ultrasonography. Cytological examination and reagent test strips were performed initiallyand repeated twice in 10 days interval. The treatment program started after the first examination.

Vaginal examination

The perineum and vulva were cleaned with a paper towel and a lubricated vaginal speculum was inserted deepinto the vagina to enable visualization of the cervix with the help of a light source (Barlund et al., 2008).Cows with abnormal vaginal discharge were excluded from the study.

Ultrasonography

Ultrasonography was performed using a portable real-time B-mode transrectal ultrasound scanner (SonoScape, Model: M12, SonoScape Medical Corp, Guangdong, China). Ultrasonography was used to determine ovarian activity by the presence of a CL, a follicle, or both. Cows with ovarian abnormalities, i.e., ovarian cysts were excluded from the study (Pothmann et al., 2015).

Samples collection

A few drops of sterile normal saline were used to make the cotton swab wet before obtaining the uterine sample (Salah et al., 2017).Samples of uterine swabs were derived using sterilized transcervical guarded swabs. The swab was placed in the body of the uterus. The sterile swab was then removed from its protective tube and pressed against the uterine mucosa. Consequently, the swab was pulled into the protective tube and pulled out of the reproductive system.

Cytological examination

The cotton swab was rolled on a clean glass slide and was left to dry. The slide was stained by Rapi-Diff II Stain (Diff-Quick) according to the manufacturer's instructions (Constantin et al., 2017). Cytological evaluations were performed by counting a minimum of 100 cells at 400X and 1000X magnification of a microscope to determine the percentage of PMN cells. To evaluate the inflammatory process 5% PMN was considered as the cut-off value (Kumar et al., 2013). Polymorphonuclear cells(%) was calculated just before initiation of the treatment program and was repeated twice in 10 days intervals.

Evaluation of reagent test strips results

The cotton swab was immersed in a 3 ml glass tube containing 1 ml of 0.9% saline and was gently shaken for 30 seconds. Reagent test strip (Medi-Test Combi 10® SGL) was then immersed in the glass tube. The strip was removed from the tube and the result was recorded after 1-2 minutes(Couto, 2009). The pH results were recorded in five categories: 5, 6, 7, 8 and 9. Results of LE were recorded in five categories: negative, 25, 75 and 500 leukocytes / μL. Leukocyte esterase and pH results were evaluated in all stages of examination.

Treatment protocols

After first examination, subclinical endometritis cows were randomly divided into three groups: group I (n = 35), treated with IU infusion of 2grams Cefazolin (Zinol ®, Pharco) diluted with 50 ml saline;group II (n = 28), treated with IU infusion of 2grams Cephalexin (Ceporex®, GlaxoSmithKline) diluted with 50 ml saline.Both treatments were repeated after 10 days; group III (n = 27), cows kept as untreated control.

Evaluation of conception rate

The cows were artificially inseminated at first oestrous after theend of the treatment program (2nd dose of treatment). Conception rate was calculated as the total number of pregnant cows divided by the total number of inseminated cows multiplied by 100 (Schefers et al., 2010).

Statistical analysis

The results were statistically analyzed using the Statistical Package for Social Sciences version 22.0 (SPSS for Windows 22.0, Inc., Chicago, IL, USA). ANOVA test was performed for comparing mean values. Data are represented asmeans ± SE. Conception rates were analyzed by Chi-square test. Comparisons showing$P<0.05$ were considered to be significant.

RESULTS

We have assessed parity and milk yield. There were no significant differences ($P>0.05$) between all groups for these parameters.Parity ranged from 3.29 to 3.36 and milk yield from 35.60 to 36.70 kg/day (Table 1).

Table 1: Means (± SE) of parity and milk yield at first examination in the different treatment groups

Variable	Treatment groups			*P*-value
	Cefazolin	Cephalexin	Control	
Parity	3.29±0.15	3.36±0.16	3.33±0.18	0.77
Milk yield (kg/day)	35.60±0.36	36.25±0.51	36.70±0.48	0.10

After the end of the treatment, PMN%, LE concentration and pH value decreased significantly ($P<0.05$) compared to first examination for Cefazolin and Cephalexin groups, whereas this decrease was not significant for the control group. The PMN% at first examination ranged from 26.21% to 28.91%. At third examination, PMN% in Cefazolin and Cephalexin (3.23%and 4.39%,respectively) groups became significantly ($P<0.05$) lower than in the control (24.89%) group. Also, means of LE concentration ranged from 374.07 to 408.93Leukocytes /μL before treatment.At third examination, LE concentration in Cefazolin and Cephalexin groups became significantly ($P<0.05$) lower than in the control group. pH values before treatment ranged from 8.46 to 8.56.At second examination, the pH value in Cefazolin group (7.34) became significantly ($P<0.05$) lower than in Cephalexin and control groups (8.25 and 8.41, respectively). Moreover, at third examination the values in treated groups became significantly ($P<0.05$) lower than in the control group (Table 2).

Days Openin Cefazolin and Cephalexin (87.07and 97.75 days, respectively) groups were significantly ($P<0.05$) lower compared to the control(122.29 days) group. At first service, conception rate in Cefazolin group was significantly ($P<0.05$) higher than Cephalexingroup while there was no conception in con-

trol group. At second service, conception rates were 50.00%, 27.27% and 7.41% in Cefazolin, Cephalexin and control groups but this difference was not significant.Moreover, there was no significance difference between all groups at third service. Total conception rate in Cefazolin (77.14%) group was significantly higher than in Cephalexin and control groups (57.14 and 25.93%,P<0.05, respectively, Table 3).

Table 2: Means (± SE) of uterine PMNs %, LE concentration (Leukocytes /μL) and pH values in the different treatment groups

Variable	Stage of examination	Treatment groups		
		Cefazolin (N=35)	Cephalexin (N=28)	Control(N=27)
PMN %	Ex.1	28.91±2.37 ^{aA}	26.21±2.06^{aA}	27.74±2.73^{aA}
	Ex.2	12.57±2.37^{aB}	14.14±2.01^{aB}	27.30±3.09^{bA}
	Ex.3	3.23±0.38^{aC}	4.39±0.87^{aC}	24.89±3.14^{bA}
LE concentration (Leukocytes /μL± SE)	Ex.1	390.71±31.86^{aA}	408.93±33.56^{aA}	374.07±38.06^{aA}
	Ex.2	148.57±30.33^{aB}	166.07±33.56^{aB}	358.33±39.29^{bA}
	Ex.3	47.86±4.27^{aC}	68.75±16.65^{aC}	340.74±40.76^{bA}
pH values	Ex.1	8.51±0.51^{aA}	8.46±0.10^{aA}	8.56±0.10^{aA}
	Ex.2	7.34±0.48^{aB}	8.25±0.10^{bA}	8.41±0.10^{cA}
	Ex.3	7.29±0.08^{aB}	7.57±0.10^{bB}	8.37±0.09^{cA}

Ex.1:1st examination. Ex.2: 2nd examination, Ex.3:3rd examination
Different superscripts (small letters) indicate significant differences (P<0.05) between different groups within rows
Different superscript (capital letters)indicate significant differences (P<0.05) between different times of examination within each group and variable

Table 3: Days Open(Means ± SE) and conception rate (%) in the different treatment groups

Groups	N	Days Open	Conception rate			
			1st service*	2nd service	3rd service	Overall*
Cefazolin	35	87.07±2.84^{a}	48.57% (17/35)	50.00% (9/18)	11.11% (1/9)	77.14% (27/35)
Cephalexin	28	97.75±11.34^{a}	21.43% (6/28)	27.27% (6/22)	25.00% (4/16)	57.14% (16/28)
Control	27	122.29±7.71^{b}	0.00% (0/27)	7.41% (2/27)	20.00% (5/25)	25.93% (7/27)

Different superscripts(small letters) indicate significant differences(P<0.05) between different groups
*Denotes significant difference (P<0.05) within column for conception rate

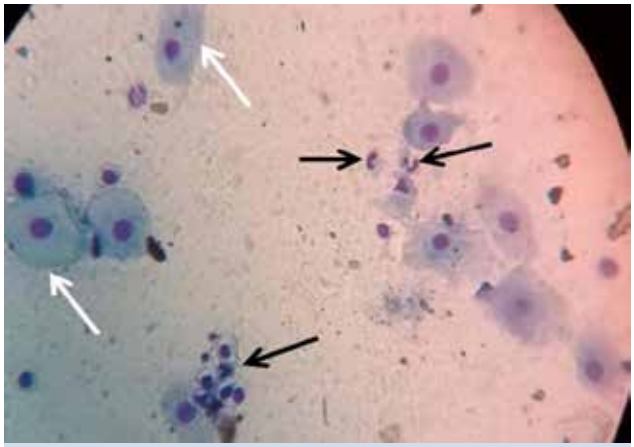

Figure 1: Cytology smear obtained from cow's uterine content at first examination, stained with Rapi-Diff II Stain. Endometrial epithelial cells (white arrow) and PMN (black arrow).

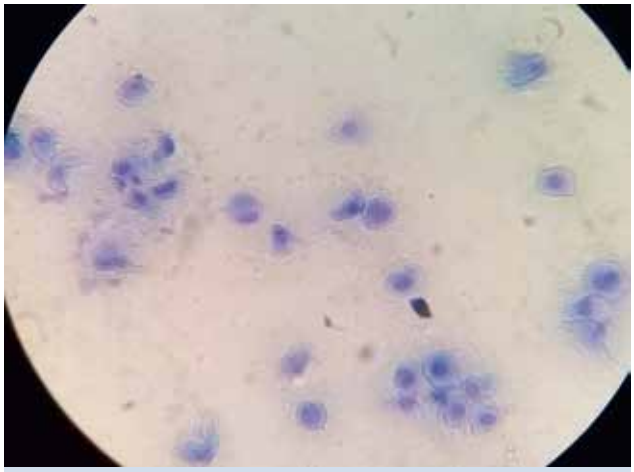

Figure 2: Cytology smear obtained from cow's uterine content at third examination, stained with Rapi-Diff II Stain. Endometrial epithelial cells.

DISCUSSION

The study design followed those of previous published studies that had collected similar information (Kumar et al., 2017; Tomar et al., 2017).The retrospective power analysis indicated that it is important to conduct further studies with a larger sample size. However, statistically significant differences between treatment groups with P-valuesof <0.05 have been detected.

Endometrial cytology has beenthe most reliable method for diagnoses of endometritis in cattle (Barlund et al., 2008). The presence of neutrophils within the uterine cavity indicates an inflammatory process (Brick, 2011). These neutrophils form the first line of defense against invasive pathogens, resulting in an increase in the number of PMN within the uterine cavity (Kasimanickam et al., 2004). Antibiotics reduce uterine bacterial load and inflammation of the endometrium (Lefebvre and Stock, 2012).Regarding our results, thefirst dose of treatment withCefazolin or Cephalexin significantly decreased PMNs. However,this decrease was not significant regarding the untreated group.At the third examination, which was10 days after the second dose of treatment, Cefazolin andCephalexin further reduced the percentage of PMNsfrom 28.91%and 26.21% to 3.23%and 4.39%, respectively. This reduction is in agreement with a previous study which reported a significance decrease in neutrophils after 14 days from an intrauterine infusion of antibiotic at 50-60 days after parturition. However, no significance difference was found at 20-30 after parturition(Ahmadi et al., 2005). An explanation may be that after antibiotic administration, the elimination of pathogenic organisms occurs and inflammation subsides; neutrophils become confined to the fluid in the uterine cavity and are expelled by uterine contractions (Couto et al., 2013).

Uterine LE activity was associated with neutrophil ratio as determined by endometrial cytology. Therefore, it is an alternative method for the evaluation of inflammatory cells in the uterine cavity (Couto et al., 2013). A previous study reported that LE activity in the genital discharge was significantly higher inendometritis than that in healthy cows anda significant difference in percentage of LE activity was observed after intrauterine infusion of penicillin and streptomycin (Hajibemani et al., 2016).In the present study LE concentration wassignificantly decreased from 390.71 and 408.93 Leukocytes /μLto 47.86 and 68.75Leukocytes /μL. after Cefazolin and Cephalexin treatment, respectively.

The pH value of genital discharge could be used for the diagnosis of endometritis in cattle (Palanisamy et al., 2014).In the present study pH value ranged from 8.53 to 8.57 before treatment. The change in pH of uterine fluid to the alkaline side is usually due to the presence of metabolites of bacteria and inflammatory exudates associated with uterine infection. This increase of pH is not suitable for the survival of sperm and fetus in the uterus (Palanisamy et al., 2014; Kumar et al., 2014). In this study, the pH value decreased after treatment, and this reduction was significant after the first dose of Cefazolin, while Cephalexin needed two doses to be effective. The reduction of the pH value in our results agrees with several studies reporting a decrease in cervical and uterine pH after antibiotic therapy, including those that reported a decrease in value from 7.83 to 7.26(Tomar et al., 2017). Another study reported a decrease in value from 8.10 to 7.05 after treatment with levofloxacin (Kumar et al., 2017).

Reproductive performance was monitored after treatment. Previous studies have discussed conception rate after Cephalexin treatment, including a study that achieved 40.00% and 33.33% conception rate respectively at first and second insemination and 60% total conception rate (Resum and Singh, 2016). Moreover, Parikh et al. (2017) achieved high conception rate (83%) after treatment by Cephalexin. In the present study, total conception rate in Cefazolin group was significantly (P<0.05) higher than in Cephalexin and control groups. This may be due to the decrease of high pH value after theend of treatment noticed in our study for theCefazolin group. This decrease of pH value can be critical for the survival of sperm and fetus in the uterus (Palanisamy et al., 2014; Kumar et al., 2014).

CONCLUSION

Intrauterine infusion of Cefazolin and Cephalexin leads to a decrease in uterine PMN and LE concentration. The pH value in Cefazolin group was significantly lower than in the Cephalexin group. Moreover treatment with Cefazolin resulted in a significantly higher conception rate. Thus, Cefazolin is recommended for the treatment of subclinical endometritis in dairy cows.

CONFLICT OF INTEREST

None declared.

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J HELLENIC VET MED SOC 2020, 71(4): 2511-2514
ΠΕΚΕ 2020, 71(4): 2511-2514

Research article
Ερευνητικό άρθρο

Seroprevalence of *Neospora caninum* infection in stray dogs in Chalkidiki, Northern Greece

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ABSTRACT: Despite the importance of *N. caninum* in veterinary medicine, knowledge on the prevalence of this parasitosis in dogs is limited in some countries. The aim of this study was to assess the prevalence of *N. caninum* infection in stray dogs in Chalkidiki, Northern Greece. This prospective study was conducted between January 2018 and December 2019 in stray dogs aged ≥6 months old. Blood samples were collected by venipuncture before the female and male dogs underwent the spay and castration procedures, respectively. The indirect enzyme-linked immunosorbent assay (ELISA) was used to screen dogs for the presence of IgG antibodies against *N. caninum*. Of 511 dogs included in this study, 39 (7.63%) were positive for IgG antibodies against *N. caninum*. Of all the dogs, 221 were males and 290 females, with positive results for IgG antibodies found in 16 (7.24%) males and 23 (7.93%) females. Preventive measures should be developed and implemented to break the domestic cycle between dogs and bovine. We want to highlight the importance of regional reporting of *N. caninum* infection prevalence in dogs and control measures by veterinarians and veterinary authorities to farmers and public, in order to avoid this disease's spread.

Keywords: neosporosis, prevalence, dog, Greece

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Date of initial submission: 16-01-2020
Date of revised submission: 23-01-2020
Date of acceptance: 25-01-2020

INTRODUCTION

Canine neosporosis is caused by *Neospora caninum*, an obligate intracellular parasite of the phylum Apicomplexa, and has a global distribution (Dubey et al., 2007a). Many morphological and biological features of this protozoan are similar to its relative *Toxoplasma gondii* (Dubey and Schares, 2011a) with key differences being observed in their natural host range, antigenicity, virulence factors and pathogenesis (Dubey et al., 2007a).

N. caninum has a complex facultative heteroxenous life cycle with canids such as domestic and wild dogs (*Canis familiaris*) (McAllister et al., 1998), grey wolves (*Canis lupus*) (Dubey et al., 2011b), coyotes (*Canis latrans*) (Gondim et al., 2004) and dingoes (*Canis lupus dingo*) (King et al., 2010) confirmed as definitive hosts in which sexual replication the parasite occurs (Donahoe et al., 2015; Dubey and Schares, 2011a). Ruminants, horses, rabbits and mice have been reported as intermediate hosts in which asexual replication takes place. Of these, cattle are the most frequently affected (Donahoe et al., 2015; Dubey et al., 2007b). Interestingly, dogs can also play the role of intermediate host in *N. caninum* life cycle (Dubey and Schares, 2011a; King et al., 2010).

Canids can acquire infection by ingestion of the infected tissues from the intermediate hosts or of the sporulated oocysts from the environment, and/or by vertical transmission (Dijkstra et al., 2001; Gondim et al., 2002; Schares et al., 2001). Dogs have been shown to shed oocysts into the environment after ingestion of infected offal or placental membranes from infected cows (Donahoe et al., 2015; King et al., 2010), maintaining *N. caninum* life cycle. Neosporosis has emerged as a serious disease in cattle and dogs worldwide (Dubey and Schares, 2011a; Dubey et al., 2007b).

Presence of the infected dogs in cattle farms is considered a great risk factor for bovines. Despite the importance of *N. caninum* in veterinary medicine, knowledge on the prevalence of this parasitosis in dogs is limited in some countries, including Greece.

The aim of this study was to assess the prevalence of canine *N. caninum* infection in dogs in Chalkidiki, Northern Greece. This is the first report on the seroprevalence of *N. caninum* in stray dogs in this region.

MATERIALS AND METHODS

Study design and size of the analyzed population

This was a prospective study conducted between January 2018 and December 2019 in Chalkidiki, Northern Greece including 511 stray dogs that were part of the municipal neutering program. All dogs were aged ≥6 months old, but the exact age was not assessed and recorded.

Blood samples were collected by venipuncture before the female and male dogs underwent the spay and castration procedures, respectively. The samples were separated, and serum was collected. The storage was performed in individual plastic tubes at -20 °C, pending testing examination for the presence of *N. caninum* antibodies.

N. caninum antibody detection

To determine canine's serological status for *N. caninum*, the blood samples were centrifugated at 2000 rpm for 15 minutes and serum samples were obtained for further examination. The indirect enzyme-linked immunosorbent assay (ELISA) was used to screen dogs for the presence of IgG antibodies against *N. caninum*.

The commercial kit ID Screen® Neospora caninum In Direct Multi-species ELISA (IDVet®, Montpellier, France) was used and manufacturer's instructions were followed. The same assay was used in other studies (Enăchescu et al., 2012; Sharma et al., 2015; Villagra-Blanco et al., 2018).

Statistical analysis

Statistical analyses were performed using the IBM SPSS statistics 26.0. Data were tabulated as categorical numbers and percentages. Results interpretation were descriptive. A statistical analysis was also performed using the chi-square (χ^2) test, with a statistical significance level of $p<0.05$.

RESULTS

Of 511 dogs included in this study, 39 (7.63%) were positive for IgG antibodies against *N. caninum*. Of all the dogs, 221 were males and 290 females, with positive results for IgG antibodies found in 16 (7.24%) males and 23 (7.93%) females (Table 1.) No differences of significant importance were recorded between the male and female groups of infected dogs ($p>0.05$).

DISCUSSION

Current information regarding to the prevalence of *N. caninum* infection in dogs suggests that neosporosis is spread in many areas worldwide. We recorded a prevalence of 7.22% in stray dogs from the region of Chalkidiki, Northern Greece. Compared to other stud-

ies, our findings suggest a lower prevalence in this part of Greece compared to North West Italy (36.4%) (Ferroglio et al., 2007), Romania (32.7%) (Gavrea et al., 2012), Czech Republic (19.2% in canine shelter dogs) (Vaclavek et al., 2007), Serbia (17.2%) (Kuruca et al., 2013), south-eastern Poland (16.4%) (Ploneczka and Mazurkiewicz, 2008), rural areas of central Poland (21.7%) (Gozdzik et al., 2011), Spain (12.2%) (Ortuno et al., 2002) and China (20%) (Gao and Wang, 2019).

Contrary to our findings, in some of the northern European countries, the prevalence of *N. caninum* infection were notably lower: Sweden with a prevalence of 0.5% (Bjorkman et al., 1994), Germany with 4% (Klein and Müller, 2001) and Austria with 3.6% (Wanha et al., 2005). In Korea (Nguyen et al., 2012) and Grenada, West Indies (Sharma et al., 2015) were also recorded lower prevalences (3.6% and 1.6%, respectively) compared to Chalkidiki (7.2%).

Even though the prevalence of *N. caninum* infection may be high in some areas, the clinical signs are rare in adult dogs. Bitches that have given birth to puppies congenitally infected with this parasite do not present any clinical signs (Dubey et al., 2007a; Villagra-Blanco et al., 2018). Nevertheless, naturally acquired *N. caninum* infection by transmission to offspring in succeeding generations can occur (Barber and Trees, 1998; Crookshanks et al., 2007).The study conducted by Barber and Trees (1998) has showed that the frequency of vertical transmission is variable, as long as 80% of puppies born to seropositive mothers were not infected (Barber and Trees, 1998).

Even if clinical canine neosporosis is rare, studies on the prevalence and epidemiology of this disease can contribute to a better organization of the preventive measures in individual areas in order to minimize both canine and intermediate hosts infection, especially in cattle.

In case of clinical neosporosis in dogs, neuromuscular signs including ataxia, ascending paralysis and other general nervous clinical symptoms are present (Lindsay and Dubey, 2000). Other manifestations include myocardial, pulmonary, dermatological and reproductive disorders (Barber and Trees, 1998; Dubey et al., 2011b; Dubey et al., 2007b).

As definitive hosts, *N. caninum*-infected dogs shed oocysts into the environment for long periods of time, contributing to the spread and maintenance of this parasite in the environment (Basso et al., 2001; Dubey et al., 2007b). Oocysts are the key factor in the epidemiology of neosporosis. Even if they are shed in an un-sporulated form, they can sporulate outside the host within 24 hours (Dubey et al., 2007b).

Regarding gender, in our study the percentages of *N. caninum* seropositive females and male dogs were similar with no statistically significant association (7.93% [N=23] versus 7.24% [N=16], respectively). This finding is in line with previous studies that have reported gender as not a risk factor for seropositivity (Cheadle et al., 1999; Collantes-Fernández et al., 2008; Ferroglio et al., 2007. On the contrast, Nazir et al. (2014) referred a significantly higher prevalence in male stray dogs. Regarding age, dogs of any age could be infected, with an increased prevalence reported in older dogs compared to younger ones (Basso et al., 2001; Capelli et al., 2004).

Reports on neosporosis prevalence in different areas worldwide could be of a great help for identification of regions at risk. The presence of stray dogs and their potential exposure to *N. caninum* should be considered a risk factor for neosporosis spread in canine and bovine populations.

N. caninum causes abortions from month 3 of gestation onwards in both dairy and beef cattle (Bartels et al., 1999; Dubey et al., 2007b; Reiterová et al., 2009). This parasite can also cause fetal viability disorders or neurological birth defects in newborn calves (Lassen et al., 2012; Malaguti et al., 2012) and those younger than 2 months of age (Dubey, 2003).

Because of their free ranging, stray adult dogs, in addition to ingestion of sporulated oocysts from the environment, they can have direct access to ingest tissue cysts originating from miscarriage products of cattle abortions or consumption of row meat of other intermediate hosts. Preventive measures should be developed and implemented to break the domestic cycle between dogs and bovine. This could lead to a better control of bovine neosporosis and reduction of the economic impact of this parasitosis.

To note, a worldwide general strategy to control neosporosis is not applicable due to the regional differences in the epidemiology of bovine neosporosis, reason why the regional epidemiology of neosporosis should assessed before elaborating on a control program (Dubey et al., 2007b).

CONCLUSIONS

N. caninum was found prevalent in adult stray dogs from Chalkidiki, Greece. 39 out of 511 dogs (7.63%)

were positive for IgG antibodies against *N. caninum*. This recommends an important prevalence that cannot be neglected. We would like to highlight the importance of regional reporting of *N. caninum* infection prevalence in stray dogs and control measures by veterinarians and veterinary authorities to farmers and public, in order to avoid this disease's spread.

CONFLICT OF INTEREST

None declared.

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J HELLENIC VET MED SOC 2020, 71(4): 2515-2524
ΠΕΚΕ 2020, 71(4): 2515-2524

Research article
Ερευνητικό άρθρο

The effects of water deprivation-induced dehydration on serum acute phase protein concentrations in sheep

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ABSTRACT: The effects of dehydration on serum acute phase proteins (APPs) concentrations are unknown in sheep. In this study, it was aimed to reveal the effect of dehydration on the blood concentrations of serum amyloid a (SAA), haptoglobin (Hp), ceruloplasmin (Cp) and fibrinogen (Fb) in Kivircik cross-breeds sheep. The animal materials of the study consisted of 20 healthy sheep. They were divided into 4 equal groups: systemic inflammation group (SIG), a single dose of 5 ml Freund's complete adjuvant (FCA) was administered intramuscularly, drinking water were provided as *ad libitum*; dehydration group (DEH), a single dose of 5 ml placebo 0.9% NaCl was administered intramuscularly and water was deprived for consecutive 5 days; systemic inflammation+dehydrationgroup (SIG+DEH), a single dose of 5 ml FCA was administered intramuscularly and water was deprived for consecutive 5 days; and the control group (CON), a single dose of 5 ml placebo 0.9% NaCl was administered intramuscularly and drinking water was provided as *ad libitum*. Also, feed was offered *ad libitum* throughout the experimental period in all study groups. Blood samples were collected on days 0 (baseline values), 1, 3, 5, and 7 while clinical examinations were performed daily during the study. Significant increases were found in serum Hp, SAA, Cp and plasma Fb concentrations in SIG and SIG+DEH groups. There was a significant increase only in serum Hp concentration over time in the DEH group. In conclusion, this study exhibited that Hp concentration increased as part of an acute phase reaction in water deprivation-induced dehydration in Kivircik cross-breeds sheep.

Keywords: acute phase protein, dehydration, Freund's complete adjuvant, sheep

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Date of initial submission: 06-02-2020
Date of revised submission: 17-04-2020
Date of acceptance: 30-04-2020

INTRODUCTION

Dehydration develops during the course of many diseases and mainly associated with decreased fluid intake and/or increased fluid loss (Cogan, 1992; Hartmann and Reder, 1995; Walz and Taylor, 2012). It is a vital symptom for both humans and animals due to its fatal consequences. In the first stage, interstitial and intracellular compartments of connective tissue, muscle tissue and skin are affected by negative fluid balance. This leads to loss of skin elasticity, drying of the skin and mucous membranes, and the collapse of the eyeball into the orbit with a decrease in the volume of postorbital fat deposits (Walz and Taylor, 2012; Constable et al., 2016). As a secondary response to the on-going negative fluid balance, a reduction in circulating blood volume and an increase in blood concentration (hemoconcentration) occur (Constable et al., 2016). Decreased circulation volume due to hemoconcentration leads to hypovolemia and arterial hypotension. The release of catecholamines by compensation mechanisms increases peripheral vasoconstriction and cardiac contraction (Kreimeier, 2000; Constable et al., 2016). Myocardial oxygen demand increases during tachycardia, but as a result of decreased perfusion, sufficient oxygen cannot be provided and thus myocardial insufficiency can be formed (Kreimeier, 2000; Constable et al., 2016). This myocardial insufficiency may also have consequences that may lead to multiple organ failure (Kreimeier, 2000). Furthermore, hypovolemia and hypotension caused by dehydration can also cause tissue damage in various organs due to circulatory failure. For example; in cases where gastrointestinal perfusion disrupts, ischemia occurs especially in the mucosal layers of the intestines. The disruption of the mucosal barrier leads to the passage of bacteria and endotoxins in the intestinal lumen into the blood. Thus systemic inflammatory response syndrome (SIRS) and shock can occur (Kirby and Ruddloff, 2000; Kreimeier, 2000; Constable et al., 2016).

The acute phase reaction (APR) is the earliest defence mechanism response to inflammation, trauma, infection and stress (Murata et al., 2004; Ceciliani et al., 2012). One of the most important metabolic changes in the APR is the production of acute phase proteins (APPs) by the liver and their release into the circulation following stimulation by pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (Cray et al., 2009; Dinler et al., 2017). APPs, which increase blood concentrations during the APR, are called positive APPs. Positive APPs are further categorized as major (100-1000 fold), moderate (2-10 fold) or minor (50-100%), depending on the degree of increase. Diagnostic significance of APPs is generally dependent on animal species. Haptoglobin (Hp) and serum amyloid-A (SAA) are major APPs, and ceruloplasmin (Cp) and fibrinogen (Fb) are minor APPs in sheep (Cecilianiet al., 2012; Dinler et al., 2017; Iliev and Georgieva, 2017). Possible factors affecting the blood concentrations of APPs after stimulation include the type of inflammation, the course of the disease, the etiology of the disease, the animal species and the type of APP evaluated.(Alsemgeest et al., 1994; Ceciliani et al., 2012; Iliev and Georgieva, 2017).

APPs concentrations were evaluated in many bacterial, parasitic and viral diseases causing diarrhea and dehydration and the pathogenicities of these diseases were put forth via the concentrations of APPs (Iliev and Georgieva, 2018; Dinler et al., 2017). Nevertheless, the extent to which dehydration stimulates the APR and the effects of dehydration on APP concentrations is unknown. We aimed in this experimentally study to the evaluate reflection of inflammatory processes formed during dehydration on the blood concentrations of major (SAA, Hp) and minor (Cp, Fb) APPs in sheep.

MATERIAL AND METHODS

All study procedures were reviewed and approved by the Animal Research Ethics Committee of the Aydin Adnan Menderes University, under protocol number 64583101/2014/107. The study was carried out between April-May 2016 at the Clinic for Large Animal Internal Medicine of the University.

Animals and environmental factors

This study was carried out on 20 Kivircik crossbreeds, 6-8 months of age sheep that were found to be healthy after clinical and laboratory examinations (haematological, biochemical, parasitological). The sheep were brought to Clinic for Large Animal Internal Medicine of the University and endoparasitic and ectoparasitic applications were performed. The animals were taken to a month of adaptation period in the same environment before the experiments were conducted and *ad libitum* water and feed were given. During the study period, each group of sheep was housed in a separate box and no vaccination and additional medication were administered to the animals. Ambient temperature and relative humidity were measured twice a day as a morning and evening. Dry matter and moisture analysis of feedstuffs to be given

to animals during the study were performed at two different times (just before the start of the study and on the 4th day of the study).

Research groups

The sheep were divided into 4 equal groups (n = 5) according to sex (3 males and 2 females in each group) and body weights and followed for 7 days.

Systemic inflammation group (SIG): A single dose of 5 ml FCA was administered intramuscularly into right *M. serratus cervicis*. Drinking water was provided as *ad libitum* during the study period (7 days).

Dehydration group (DEH): A single dose of 5 ml 0.9% NaCl for placebo was administered intramuscularly into right *M. serratus cervicis* and drinking water deprived for consecutive 5 days (Laden et al., 1987).

Systemic inflamation+dehydration group (SIG+-DEH): A single dose of 5 ml FCA was administered intramuscularly into right *M. serratus cervicis* and drinking water was deprived for consecutive 5 days.

Control group (CON): A single dose of 5 ml 0.9% NaCl for placebo was administered intramuscularly into right *M. serratus cervicis*. Drinking water was provided as *ad libitum*.

Drinking water was re-supplied on day 5 after taking blood in DEH and SIG+DEH groups. Feed was provided as *ad libitum* in all study groups through the study period.

Clinical examinations

The physical examinations including body temperature (T), heart rate (HR), respiratory rate (RR), mucous membranes status, appetite evaluations and body weight measurement in the sheep were performed daily. In addition, daily feed intake was recorded on a group basis. The degree of dehydration was determined by evaluating the change in body weight and according to the table of estimating the degree of dehydration from the physical examination findings reported by Walz and Taylor (2012). Sheep showing at least two findings of SIRS criteria (body temperature, heart rate, respiratory rate, total leukocyte count) were evaluated as SIRS positive (Badial et al., 2011; Chalmeh et al., 2013; Constable et al., 2016).

Laboratory analyses

Blood samples were collected from the jugular vein into both heparin tubes (Vacutainer®) and plain tubes (Vacutainer®) at days 0 (baseline values), 1, 3, 5, and 7 of the study. The leukocyte count (WBC) was performed on blood samples collected into tubes with heparin by use of an automatic cell counter (Abacus Junior vet5, Budapest, Hungary) within 2 h after sampling. Hematocrit (Hct) value was measured in % by the microhematocrit method (CLSI, 2000). Blood sodium (Na) concentration was measured from heparinized blood with a blood gas analyzer (Radiometer abl 80 flex). Blood samples in tubes with heparin were centrifuged at 1500*g* for 10 minutes and plasma osmolarity (pOsm) were measured with Osmomat 3000 device (Gonotec, Berlin, Germany) from obtained plasma samples. Plasma Fb concentrations were measured using a refractometer according to the heat precipitation method (Lepherd et al., 2009).

Sera were obtained by centrifugation at 1500*g* for 10 min of blood samples and stored at -20 ° C until analysis. Total protein (TP) and albumin (Alb) concentrations were measured on an autoanalyser (RaytoChemray 120, Hamburg, Germany) from serum sample. Both serum Hp and SAA were measured with commercially available ELISA kits (Phase Hp and Phase SAA kit, Tridelta Ltd., Ireland), according to the manufacturer's instructions. Serum Cp concentrations were determined with UV-spectrophotometer (Schimadzu, UV-1601, Japan) according to described by Sunderman and Nomoto (1970).

Statistical analyses

Statistical analyses were performed using the software Statistical Package for the Social Sciences (SPSS) version 19.0 (IBM Corporation, Armonk, USA). All analyses were considered statistically significant at $p<0.05$. Arithmetic means, standard error of the mean (SEM), medians, and interquartile ranges for each evaluated parameters and sample collection times were calculated using descriptive statistic. The Shapiro-Wilk test was used to assess the respective data distributions for the normality of each parameter. This test revealed that SAA was not normally distributed, whereas distributions of all other variables were normal. Although SAA was log transformed but still displayed a non-normal distribution. Therefore, this parameter was analysed using non-parametric tests.

For normally distributed parameters, repeated measure analysis of variance (ANOVA) was performed to evaluate the effects of the sampling time (day), group, and interaction between time and group. The Bonfer-

roni correction method was used to test time effects (the change from baseline) for parameters of interest within each group. When a significant group-by-time interaction was detected, between groups difference in the change in parameters at particular time points was tested using Student's t-test for independent samples. Serum SAA concentrations were evaluated by Friedman's rank sum test coupled with Wilcoxon test.

The sample size was estimated by analysis of variance (ANOVA) with a repeated mean using G*Power (ver. 3.1.9.4) analysis software. The parameters employed were α error = 0.05; power (1-α) = 0.8; and number of measurements = 5.

RESULTS

During the study, the mean ambient temperature was determined to be 21.9 ± 0.71 °C and the mean relative humidity was 55.28 ± 1.38 % in the boxes. Dry grass, hay and barley paste were given to sheep and the dry matter ratios of these feedstuffs were 88.31%, 91.62%, and 88.60%, respectively.

Clinical findings

The feed intake of the sheep in the DEH and SIG+DEH groups decreased from the 3rd day onwards and their feed intake completely stopped on the 4th and 5th days. On the 5th day of the study, the appetite returned to normal after providing drinking water again to sheep. There was no change in feed consumption in 3 of the sheep in the SIG group and in 2 of them there was a slight decrease in feed consumption on the 1st and 2nd days following FCA application. No change was observed in the appetite status of the sheep in the CON group during the study period.

The sheep in DEH and SIG+DEH groups showed no signs of dehydration until the 3rd day and they showed mild stagnation, loss of appetite, weakness together with 5% dehydration signs starting from the 3rd day. On the fifth day, 5-8 % (1 sheep), 8% (3 sheep) and 10% (1 sheep) clinically dehydration were detected in the DEH group. In addition, in the SIG+DEH group, dehydration was 8% in 3 sheep and 10% in 2 sheep on the same day. Also, sheep exhibiting 10% dehydration signs in both DEH and SIG+DEH groups on day 5 showed licking behaviour to tiles walls of the boxes. No dehydration was detected in other groups (SIG and CON) during the study period.

While no significant difference was found in the body weights of animals in SIG and CON groups during the study period, the significant decrease ($p<0.01$) was observed in DEH and SIG+DEH groups after 5 days of water deprivation. On the 5th day when the most severe clinical dehydration was observed, in DEH and SIG+DEH groups, the mean percentages decreases in body weight were 24.48% (DEH) and 26.63% (SIG+DEH), respectively.

The changes in T, HR, RR and WBC of sheep during the study period are presented in Table 1. In this context, 4 sheep in the SIG group and 5 sheep in the SIG+DEH group were evaluated as SIRS positive on day 1. No SIRS findings were observed in the SIG group on days 3, 5 and 7, while two sheep in the SIG+DEH group were evaluated as SIRS positive on days 3 and 5. The sheep in the DEH group did not show SIRS finding during the first 4 days of the study and the two sheep were evaluated as SIRS positive on day 5.

Laboratory findings

While there was no significant change in the WBC of the DEH and CON groups over time, time-dependent changes were found in the SIG ($p<0.05$) and SIG+DEH ($p<0.01$) groups. The mean WBC counts were higher on day 1 compared to day 0 in SIG and SIG+DEH groups (Table 1).

In the statistical evaluation of the Hct values; the significant ($p<0.01$) changes were determined in DEH and SIG+DEH groups. The Hct values of DEH ($p<0.05$) and SIG+DEH ($p<0.01$) groups were significantly high on days 3 and 5 compared to other days (Table 2).

The means and SEMs of pOsm, Na, TP and Alb levels and their statistical evaluations are presented in Table 2. In this context, these parameters in DEH and SIG+DEH groups were found significantly higher compared to SIG and CON groups. In addition, these parameters were significantly higher on days 3 and 5 compared to baseline values. When the Hct, pOsm, Na, TP and Alb levels are evaluated together with the clinical findings, it can be said that dehydration started to take occur on day 3 and exacerbated on day 5 of in the DEH and SIG+DEH groups. Moreover, the dehydration determined in the DEH and SIG+DEH groups was detected to be hypertonic dehydration in terms of serum Na concentration.

Table 1. Changes in temperature (T), heart rate (HR), respiratory rate (RR) and WBC during the experimental period in groups of the study. Results are represented as mean ± SEM

	Day	SIG	DEH	SIG+DEH	CON	Dif.[1]
T (°C)	0	39.22±0.34	38.86±0.40	38.82±0.31	38.88±0.46	-
	1	40.12±0.4	39.3±0.25	40.16±0.32	38.88±0.43	**
	3	40.18±0.47	39.02±0.38	39.56±0.87	39.06±0.40	**
	5	40.14±0.75	37.96±0.21	38.94±0.56	39.08±0.29	**
	7	39.58±0.50	39.02±0.08	39.9±0.43	39.12±0.19	-
	Dif.[2]	**	-	**	-	
HR (beat/min)	0	94±11.66	108.8±4.38	93.2±12.12	100.4±9.63	-
	1	119.6±25.93	111.6±17.68	147.8±33.51	105.4±12.75	-
	3	124±13.26	104±29.66	130.2±18.33	103.6±14.51	-
	5	119.2±26.29	129.2±9.95	125.6±20.36	105.8±9.54	-
	7	97.2±19.26	114±11.31	102.4±15.64	108±8.83	-
	Dif.[2]	**	-	**	-	
RR (breath/min)	0	36.2±3.34	38.0±3.80	40.4±2.96	37.6±4.56	-
	1	41.6±3.57	41.6±3.84	42.8±8.89	41.6±2.60	-
	3	41.6±3.79	36.8±5.21	45.2±8.67	41.2±1.78	-
	5	42±3.48	35.4±4.38	40.4±5.69	38.8±2.28	*
	7	39.2±5.21	40.4±2.60	34.4±2.9	40.4±1.67	-
	Dif.[2]	*	-	-	-	
WBC (x10⁹/L)	0	10.01±3.51	12.84±5.30	11.30±3.98	10.14±4.79	-
	1	14.97±5.03	10.12±3.38	16.99±2.58	9.58±3.83	*
	3	11.97±2.68	10.35±4.83	12.03±3.35	11.24±4.49	-
	5	10.13±2.89	9.77±3.92	10.16±3.46	10.09±3.81	-
	7	9.65±3.71	10.38±3.64	12.25±1.94	10.53±3.77	-
	Dif.[2]	*	-	**	-	

Dif. [1]: expresses the significance of the change in time within the same group. Dif.[2]: represents significance at the same time points between groups. *: p<0.05, **: p<0.001, NS: No significant difference (p>0.05)

Table 2. Changes in hemotocrit (Hct), plasmaosmolarity (pOsm), sodium (Na), total protein (TP) and albumin (Alb) values during the experimental period in groups of the study. Results are represented as mean ± SEM

	Day	SIG	DEH	SIG+DEH	CON	Dif.[1]
Hct (%)	0	26.60±4.15	29.8±1.78	27.00±1.58	30.00±1.87	NS
	1	28.40±4.15	30.20±1.92	28.00±1.41	30.40±1.34	NS
	3	26.20±3.56	32.40±2.96	29.20±1.64	30.00±1.87	*
	5	25.60±4.44	36.20±3.70	31.20±2.16	29.80±2.16	**
	7	26.20±4.32	28.20±6.18	24.80±1.30	30.40±2.30	NS
	Dif.[2]	NS	**	**	NS	
pOsm mosm/L	0	303.8±3.42	291.0±14.42	300.8±10.56	292.4±4.80	NS
	1	296.0±7.87	310.2±9.67	310.6±10.85	290.2±2.94	**
	3	279.8±5.11	338.2±15.41	325.2±7.46	291.2±4.08	**
	5	289.2±4.32	355.4±26.06	345.2± 3.42	292.8±6.76	**
	7	283.0±2.91	281.4±2.60	296.6±4.27	292.0±1.41	**
	Dif.[2]	NS	**	**	NS	
Na (mEq/L)	0	144.0±2.54	142.8±1.64	140.6±1.81	143.2±1.64	NS
	1	140.2±2.28	150.2±4.65	143.8±2.77	143.8±0.83	*
	3	138.4±2.96	155.4±7.16	158.2±3.56	143.8±0.83	**
	5	141.0±1.00	163.4±9.5	163.2±3.34	144.6±0.54	**
	7	141.0±0.7	141.6±1.94	143.8±3.70	144.0±1.58	NS
	Dif.[2]	NS	**	**	NS	
TP (g/dl)	0	4.67±0.45	4.53±0.24	4.77±0.21	4.77±0.18	NS
	1	4.75±0.37	4.66±0.29	4.78±0.22	4.75±0.30	NS
	3	4.73±0.28	5.01±0.33	5.10±0.54	4.78±0.34	NS
	5	4.77±0.31	5.10±0.21	5.59±0.50	4.49±0.38	**
	7	4.88±0.41	4.19±0.33	4.76±0.39	4.68±0.30	NS
	Dif.[2]	NS	**	**	NS	
Alb (g/dl)	0	2.98±0.31	3.23±0.10	2.98±0.17	3.21±0.07	NS
	1	2.93±0.42	3.44±0.16	3.09±0.15	3.18±0.10	*
	3	2.99±0.26	3.60±0.12	3.05±0.20	3.14±0.10	**
	5	2.90±0.28	3.78±0.19	3.22±0.13	3.08±0.12	**
	7	2.93±0.35	3.42±0.17	2.97 ±0.09	3.08±0.14	NS
		NS	**	*	NS	

Dif. [1]: expresses the significance of the change in time within the same group. Dif.[2]: represents significance at the same time points between groups. *: p<0.05, **: p<0.01, NS: No significant difference (p>0.05)

Haptoglobin, SAA, Cp and Fb concentrations were evaluated as the biomarkers of inflammation in the study groups and the results are presented in Fig. 1. All of the mentioned APPs concentrations in SIG and SIG+DEH groups which had systemic inflammation induced by FCA were detected significantly higher than those of DEH and CON groups. In addition, the changes of these APP concentrations over time were statistically significant in SIG and SIG+DEH group. Hp concentrations of the DEH group changed significantly over time (p<0.05). In this group, on day 5 when dehydration was most severely determined clinically, Hp concentration was significantly higher than the other days (Fig. 1A). Furthermore, it was noted as an important data that the Hp concentrations of the SIG+DEH group were higher compared to the Hp concentration of the SIG group on day 5 (Fig. 1A). Besides, in the DEH group, although SAA and Fb concentrations tended to increase on days 1 and 3, these changes did not reach statistical significance (Fig. 1B, D).

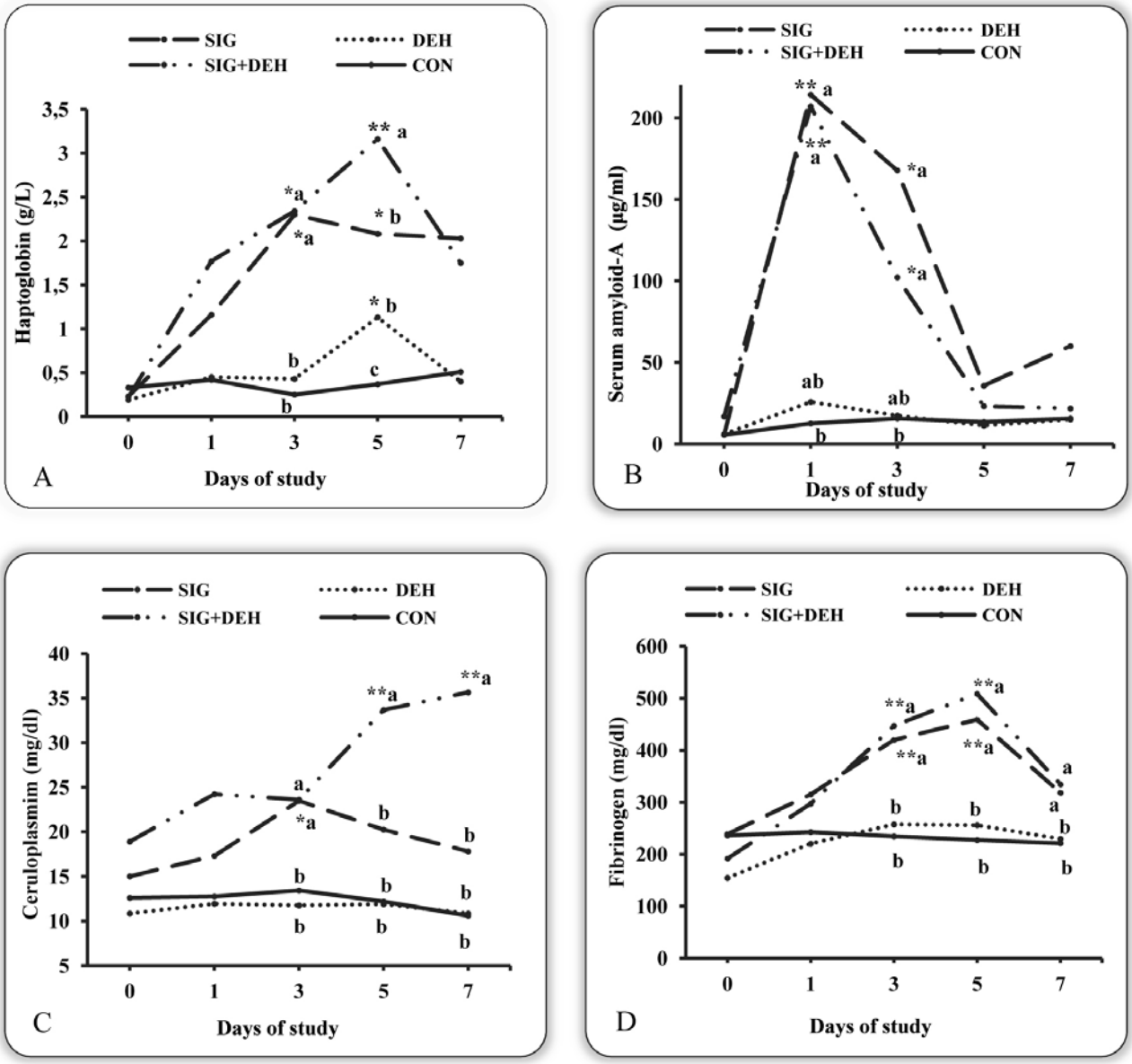

Fig. 1. Dynamics in changes of haptoglobin (Hp), serum amyloid-A (SAA), ceruluplasmin (Cp**)** and fibrinogen (Fb) in groups of this study. The dots represent the means for Hp (A), Cp (C) and Fb (D), and the median for SAA (B). *means are significantly different from day 0 (baseline) values. [a, b, c]express differences between groups at each time points (*: p<0.05, **: p<0.01).

DISCUSSION

The main objective of this study was to investigate the inflammatory changes associated with water deprivation-induced dehydration in sheep. To this purpose, we used an integrated approach based on different experimental methods. In this context; the effects of systemic inflammation, dehydration and their combination on serum APP concentrations were determined in Kivircik cross-breeds sheep.

In this study, FCA was used to induce systemic inflammation in SIG and SIG+DEH groups. The FCA achieves its stimulating effect by forming ligands with toll-like receptors (TLR), particularly TLR-2, TLR-4 and TLR-9, which are expressed by macrophages and dendritic cells. In this way, it stimulates cellular immunity and increases immunoglobulin production (Carroll, 2016). Due to this its feature, FCA has used frequently in animal studies for monitoring changes related to inflammation. In this context, an inflammatory reaction induced by FCA has been shown to occur in rabbits (Fishback et al., 2016), mice (Ferreira et al., 2001; Chillingworth and Donaldson, 2003), horses (Mills et al., 1998; Oliveira-Filho et al., 2014), and dogs (Botrel et al., 1994; Haak et al., 1996). It is reported that the inflammatory reaction may occur with FCA application in sheep as in other animal species (McClure et al., 1991; Badial et al., 2011). Intramuscular administration of FCA at a dose of 5 ml/sheep was preferred in this study as reported by Badial et al. (2011). It was determined SIRS was successfully established by evaluating SIRS criteria in sheep in SIG and SIG+DEH groups treated with FCA (Table 1). In addition to clinical signs, the APP concentrations of these groups were significantly higher than the DEH and CON groups (Fig. 1). Moreover, the APP concentrations reached peak concentrations at different times after the FCA administration. For example, after the application of FCA in the SIG group, Hp, SAA, Cp and Fb concentration reached a peak on days 3, 1, 3 and 5, respectively. The differences in the magnitude of the response and the timing of peak values between these APPs are related to the kinetics of these APPs (Murata et al., 2004; Tothova et al., 2014).

Dehydration is mainly related to increased fluid loss or decreased fluid intake (Cogan, 1991; Hartmann and Reder, 1995; Walz and Taylor, 2012). Experimental dehydration models are also based on these two foundations. Thus, in laboratory animals and domestic animals, dehydration can be achieved by reducing external fluid intake or increasing fluid loss by using diuretics, laxatives and enteropathogenic agents (Igbokwe, 1993; Silanikove, 1994; Walker et al., 1998; Constable et al., 2001; Khanvilkar, 2014). In this study, we preferred water deprivation for occur dehydration in DEH and SIG+DEH group as applied by many researchers (Alamer and Al-hozab, 2004; Hamadeh et al., 2006; Ghanem et al., 2008).

There are many factors affecting the severity of dehydration caused by total or partial water restriction. These have reported as animal-related factors (species, age, sex, sexual period etc.) and environmental factors (ambient temperature and humidity, the nature of the feed given) (Khanvilkar, 2014). It was reported that a decrease in body weight of 10.7% and 8-14% in horses (Carlson et al., 1979) and dogs (Cornelius et al., 1978) respectively, after 3 days of water restriction. In sheep, 13.3% and 15.3% decreases in body weight have been determined after 3 days of water deprivation under spring conditions (ambient temperature: 21.9^{o}C, humidity 45%) in Saudi Arabia (Alamer and Al-hozab 2004). In another study (Laden et al., 1987) conducted in sheep, a 33% reduction in body weight was observed following 5 days of water deprivation under 35-40^{o}C ambient temperature. Small ruminants adapt to extreme climatic conditions via behavioral, morphological, physiological, and largely genetic bases (Berihulay et al., 2019). They routinely experience dehydration and rapid rehydration cycles, and it was reported that most of these animals can withstand severe dehydration (18-40% of initial body weight), which exceeds considerably the capacity of most monogastric mammals (Silanikove, 1994). In this study, the sheep in DEH and SISY+-DEH groups showed a decrease in body weight by 24.48% and 26.63%, respectively, following 5 days of water deprivation as consistent with other studies. In addition, dehydration occurred 5% to 10% on the 5th day of total water deprivation in both groups. In this study, dehydration development time via water deprivation was longer than other animal species as consistent with Laden et al (1987). This situation may be related to the ability of sheep to use the large reservoirs in their rumen in case of long-term lack of water, as stated by Silanikove (1994).

It is known that the values of Hct and pOsm, TP and Alb are increased due to the decrease in plasma volume associated with dehydration (Cork and Halliwell, 2002; Hamadeh et al., 2006). In this study, the significant increases in Hct and pOsm values and serum TP and Alb concentrations were determined

following 5 days of water deprivation in DEH and SIG+DEH groups (Table 2). Both the clinical and laboratory findings showed that dehydration has successfully effectuated with 5-day water deprivation in sheep within these groups. In addition, when Na concentrations (Table 2) were considered, the dehydration formed was found to have hypertonic character in both groups.

Dehydration and its known consequences may lead to APR or aggravate the existing APR. Currently, the most common method to measure the presence and severity of APR is to determine the APPs concentrations (Cecilliani et al., 2012; Tothova et al., 2014). In this study, the most striking changes were determined in Hp concentrations of dehydration groups (Fig. 1A). However, the other measured APP concentrations did not change during water deprivation period in these groups (Fig. 1B-D). This can be explained by the fact that different types of stimuli can affect different types of APPs in different animal species (Alsemgeest et al.,1994; Ceciliani et al., 2012; Iliev and Georgieva, 2017). When the results are evaluated, it may be thought that water deprivation-induced dehydration significantly affects serum Hp concentration in sheep.These changes in Hp concentrations of DEH and SIG+DEH groups may be associated with the other mentioned consequences of dehydration together with starvation and/or stress resulting from dehydration.

Total or partial water restriction in ruminants decreases or completely stops feed consumption depending on the duration and severity of dehydration (Silanikove, 1994; Khanvilkar, 2014). This is explained by compensatory mechanisms like that prevent rumen enlargement after feeding, reduce the amount of fluid to be lost by salivary secretion and prevent the postprandial increase in rumen osmolality (Silanikove, 1994). In this study, feed intake completely stopped on days 4 and 5 of water deprivation in DEH and SIG+DEH groups. Therefore, it was observed that starvation accompanied to dehydration in these groups. Gonzalez et al. (2011) reported a significant increase in Hp concentration after 72 hours of fasting in goats, whereas no change in SAA and other APPs (alpha-1-acid glycoprotein, Fb). Similarly, Gurdogan et al. (2014) found a significant increase in serum Hp concentrations in both disease forms in sheep with clinical and subclinical pregnancy toxemia. Although studies in laboratory animals show that starvation has a negative effect on proinflammatory cytokine concentrations (Jain et al., 2011), negative energy balance and lipid metabolism were increased the Hp concentration in studies conducted in ruminants (Yoshino et al., 1992; Nakagawa et al., 1997; Gonzales et al., 2011). Stress may be another cause of increased serum Hp concentration in water deprivation. Water deprivation and starvation can cause stress. Cortisol, known as the stress hormone, is one of the most important glucocorticoids (Katsu and Iguchi, 2016). Glucocorticoids play a regulatory role in APR. Although glucocorticoids are reported to inhibit IL-1 and IL-6 synthesis from pro-inflammatory cytokines during APR (Ceciliani et al., 2012), less information is available on the regulation of ruminant cytokine expression, particularly of its suppression. Nevertheless, the authors have found that the administration to cows of the synthetic glucocorticoid dexamethasone results in the induction of Hp (Yoshino et al., 1993). This is explained by Hp induction that can be mediated by the direct action of this hormone on the liver in ruminants (Nakagawa and Yamamoto, 1997). Also, it is thought that the mode of induction of Hp is probably different in ruminants and non-ruminant animals such as rats or human beings (Yoshino et al., 1993; Nakagawa and Yamamoto, 1997).

Another remarkable point in the results of this study was that the SIG + DEH group had a higher Hp concentration than the SIG group. In light of these results; it can be made the interpretation that dehydration accompanying systemic inflammation exacerbates the present APR and this reflects on the concentrations of Hp.

One limitation of this study can be the results reflect the effect of the just water deprivation-induced dehydration on APPs concentrations in sheep. Maybe, they can differ by naturally occurred dehydration. Therefore, the one-to-one generalization of results for all types of dehydration and is not proper. Nevertheless, these results are valuable in that they provide a starting point for future studies and draw attention to Hp in relation to dehydration.

CONCLUSIONS

The study presented here exhibited that only Hp concentration among measured APPs increased in water deprivation-induced dehydration in Kivircik cross-breeds sheep. Further studies would be needed to ascertain whether the measurement of serum Hp concentration could be useful as a biomarker for the monitorization of dehydration degree.

ACKNOWLEDGEMENTS

This report is based on the corresponding author's PhD thesis. The financial support provided by the Teaching Staff Training Program Council of Aydin Adnan Menderes University (Turkey) was acknowledged.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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J HELLENIC VET MED SOC 2020, 71(4): 2525-2530
ΠΕΚΕ 2020, 71(4): 2525-2530

Research article
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Effects of Housing Locations on Feather Damages of Laying Hens in a Free-Range Housing System

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ABSTRACT: This study was made to evaluate the effects of different locations of a free range housing system on feather damages of laying hens. The experimental house consisted of three different locations as closed indoor plastic slats, closed indoor litter and outdoor range area. The birds were able to move freely between the locations of the experimental house and they had continuous access to outdoor range during the day. The feather damages of the birds was evaluated with a distance scoring system at 64 weeks of age. Five area in each location of the experimental house were determined at first and then feather damages of five body parts of ten birds in each location were scored to measure plumage quality. Total feather score was defined as the sum of the scores of five body parts of the birds. Best plumage quality was measured in neck in all housing locations ($P<0.01$, $P<0.05$ *and* $P<0.01$) and total feather score of the birds was significantly greatest (worst) in slats ($P<0.05$).

***Keywords*:** Free range, housing location, plumage quality

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Date of initial submission: 06-02-2020
Date of revised submission: 27-04-2020
Date of acceptance: 26-05-2020

INTRODUCTION

Feather pecking can represent an important welfare problem in all housing systems in commercial egg production, probably may be resulting in canibalism and mortality as well as production loss for the damaged birds (Petek and Mckinstry, 2010; Scherwin *et al*., 2010; 2013). Any outbreaks of feather pecking or injurious pecking is currently impossible to control, despite a number of proposed interventions (Lambton *et al.*, 2015). The causes of feather pecking and canibalism are very complicated and multi-factorial including drinker, genotype, insufficient or improperly placed feeder or drinking space, farm location, lighting program, housing system, lenght and type of perches (Lambton *et al*., 2010; Sherwin *et al*., 2010; Coton *et al*, 2019; Ellen *et al*.,2019; Kaukonen and Valros 2019). Therefore, any approach to prevent or reduce prevaleness of feather pecking in commercial flocks should use a multifactorial approach to reduce this problem (Bestman and Wagenaar, 2003; Petek *et al*., 2015; Petterson *et al*., 2017).

Currently, there has been increasing focus on understanding the risk factors and alternative methods to reduce feather pecking in laying hens (Ellen *et al*., 2019). The ability to predict feather damage and canibalism in advance would be a valuable research tool for identifying which management or environmental factors could be the most effective interventions in egg production. Some scientific findings suggests that feather pecking and canibalism could be largely prevented by the use of appropriate husbandry techniques without the use of beak trimming (Weeks *et al*., 2011; Lambton *et al*., 2013). Good housing design and layout of equipment are very important to reduce deletorius effects of injurious pecking in laying hens (Featherwell Booklet, 2013; Liebers *et al*., 2019). Pecking activity of a layers can be different in different location of a house depending on the condition as light intensity, feeding time, presence of something remarkable or competition (Petek *et al*. 2015; Temple *et al*. 2017) and this can be important to understand and solve the feather pecking problem of free range laying hens. Blokhuis and Arkes (1984) showed that birds housed on slatted floors showed more feather pecking and less ground pecking than birds housed on litter. When birds housed on litter were transferred to slatted floors, feather pecking increased in these birds (Lambton *et* al., 2010). Dust bathing and litter scratching in litter and range area are the key factors that decrease the risk of injurious pecking (Defra Booklet, 2005) Feather pecking is very rarely seen out on the outdoor grazing area (Ellen *et al.,* 2019) and it is clear that the risk of feather pecking in free-range layer chickens might be lower when an outdoor grazing area is provided as early as possible (Shimmura *et al*. 2008; Petek *et al.* 2015). Lambton *et al*.(2010) showed that risk of vent pecking may be reduced by delaying the onset of lay and subsequently encouraging range use. Whereas, it was reported earlier that there were no significant relationships between feather condition and use of outside run (Leenstra *et al*., 2012). Mahboup *et al*.(2004) reported that the percentage of time spent on grassland and feather damage were inversely correlated. The aim of this study was to determine the level of feather damage of layer chickens observed in different locations of a free range house such as slats, litter and range area.

MATERIALS AND METHODS

This study was carried out at Research and Experimental Farm of Bursa Uludag University, in Turkey. The experimental procedures employed in this study were in accordance with the principles and guidelines set out by the Committee of Bursa Uludag University on animal care. Data collected from a Lohman Brown layer hens housed in an experimental free-range house.

Management

The experimental house was consistent of three locations as indoor raised plastic slats, indoor ground litter (one third of total indoor floor space) and outdoor range area to the birds. White plastic slats (100x60 cm) and rice hull (about 10 cm deep) were used as a slatt and litter material. In this study, automatic nest boxes (1 m^2 of nest space in a group nest for every 100 hens), hanging feeders (30 cm in diameter with 10-15 kg capacity, each) and bell drinkers were provided for the birds in the experimental house. The birds were beak trimmed by hot blade method at first week of age and mean stocking density within the groups was 5 birds per 1 m^2 indoor and 4 birds per 10 m^2 outdoors. The standard layer diet was supplied (2700 kcal kg-1 metabolizable energy and 18 % crude protein) as ad libitum throughout the experiment (NRC, 1994). The birds were able to move freely between the indoor and foraging area of the experimental house and they had continuous access to outdoor range during the day. The daily photoperiod consisted of 16 h of light and 8 h of darkness and the lighting intensity was arranged as 3.0 lx m^2.

Data

In this study, a distance scoring system was used to assess feather damage in hens at 64 weeks of age (Bright *et a*l., 2006; Lambton *et al*., 2013). The birds' body divided into three regions and all of the body regions were scored for plumage damage using five point scale. Neck, back and rump regions of the birds' bodies were scored from 0 (no or very little feather damage) to 4 (severe damage to feathers or large naked areas on the body). Wing and tail of the birds were also scored from 0 (intac feathers) to 4 (all feathers missing or broken). At first, five area in each location (for the slat, litter and range area) of the experimental house were determined and then the feather cover of five different body regions of ten birds in each part in each location were scored to measure plumage quality (50 birds for slat, 50 birds for litter and 50 birds for range). Because it is considered that a sample of 50 hens will provide a good indication of the state of a flock about their plumage quality (Temple et al., 2017; Decina et al., 2019). Ensure that the hens are selected at random within the sampling point, every second bird sampled to avoid drawn towards specific birds with bad or good feather cover. Flock prevalence of feather pecking in all area was calculated as a percentage of birds with damaged feathers from the total birds scored. Total feather score was defined as the sum of the scores of five body parts in a part of experimental house or as average of the scores of three location of the experimental house for a body region.

During data collection, indoor temperature, relative humidity and ammonia concentrations, and outdoor temperature and relative humidity were continuously monitored at 10-minute intervals with a weather monitor (Kestrel Handhend Weather Monitor 3500) and an ammonia meter (GasBadge Pro: Single Gas Detector:Ammonia) and was recorded.

Statistical Analysis

ANOVA test procedure was used to analyze all the data investigated in SPSS version 13.00 (Spss, 2004). Duncan test was using for mean seperation (Snedecor and Cochran, 1989).

RESULTS

The average scores of plumage observed in different body region of the hens in relation to different location of the experimantal house were presented in table 1. There were a significant differences for plumage score/feather damage of different body region of the birds in every location of the experimental house including slats, litter and range area, respectively (*P<0.001, P<0.05 and P<0.001*). Best plumage quality in all housing location was measured in neck and it was significantly greatest in range area. Differences for the average feather scores of rump, tail and wing of the layers in slats, litter and range location were found to be significantly important in this study, respectively (*P<0.05, P<0.01 and P<0.01*).

Table 1. Observed plumage score in different body region of the birds in different house locations (Mean ± SEM)

House location/	Body Region					
	Neck	Back	Rump	Tail	Wing	*P*
Slatt	0.188 ± 0.23^{Bd}	0.531 ± 0.23^{Ac}	1.250 ± 0.22^{Ab}	1.742 ± 0.24^{Aa}	1.000 ± 0.22^{Ab}	*0.001*
Litter	0.182 ± 0.22^{Bc}	0.500 ± 0.23^{Ab}	0.875 ± 0.23^{Bb}	1.281 ± 0.23^{Ba}	0.818 ± 0.21^{Ab}	0.050
Range	0.265 ± 0.20^{Ab}	0.441 ± 0.21^{Ab}	0.500 ± 0.20^{Cb}	1.206 ± 0.23^{Ba}	0.529 ± 0.22^{Bb}	*0.001*
P	0.05	n.s	0.05	0.01	0.01	

a-d : within rows, values with different superscript letters differ significantly (P<0.05, P<0.001), n.s.;no significant.
A-B: within columns, values with different superscript letters differ significantly (P<0.05, P<0.01).
SEM; Standard error of means

Flock prevalence of feather damage, average and total feather scores of birds in different house locations and different body region were showed in table 2. Based on the study, plumage quality was the worst in birds in slatt location of the experimental house compare to the other two house locations. The mean proportion of birds affected by any feather damage in slatt, litter and range locations of the house were calculated as 60.00, 47.50 and 41.88, respectively. There were significant differences for total feather score between all body region (P<0.001). The flock prevaleness of feather damage was found to be significantly greatest in tail and 90.62% of the birds has a varied tail damages. The final total feather score in neck, back, rump, tail and wing of the birds were 0.211, 0.491, 0.875, 1.419 and 0.783, respectively and significantly important. The effects of house location and body region on average feather score were found to be significantly important, respectively (P<0.01, P<0.001).

Table 2: Flock prevalence of feather damage, average and total feather scores in different house location and body region of the birds (Mean ± SEM)

Factors	Flock Prevalance (%)	AFS[1]	TFS[2]
Housing location			
Slatt	60.00	0.942±0.067[a]	4.711±0.121[a]
Litter	47.50	0.731±0.066[b]	3.656±0.112[b]
Range	41.88	0.588±0.064[b]	2.941±0.098[b]
P		0.01	0.01
Body Region			
Neck	13.54	0.211±0.084[c]	0.635±0.080[c]
Back	34.37	0.491±0.085[c]	1.472±0.081[c]
Rump	59.37	0.875±0.083[b]	2.625±0.095[b]
Tail	90.62	1.410±0.086[a]	4.229±0.162[a]
Wing	51.04	0.783±0.085[b]	2.347±0.091[b]
P		0.001	0.001

a-c: within columns, values with different superscript letters differ significantly (P<0.01, P<0.001) , SEM; Standard error of means [1]; AFS: Average feather score was defined as average of the scores of each body parts in total of the experimental house and of the scores of each three locations of the experimental house.
[2]; TFS; Total feather score was defined as the sum of the scores of each five body parts in total of the experimental house and of the scores of each three locations of the experimental house.

The humidity level in slat, litter and range locations were found to be 60.63, 53.80 and 34.35%, respectively. Inside slat and litter locations temperature and outside temperature on range area at bird level were measured as 28.13, 28.03 and 30.50 C^o, respectively. Inside ammonia level on slat and litter location were 0.016 and 0.006 *p.p.m* during the data collection (table 3).

Table 3: Environmental measurements at hen head height in different house location

Location	Humidity (%)	Temperature (C^o)	Ammonia (p.p.m)
Slat	60.63	28.13	0.016
Litter	53.8	28.03	0.006
Range	34.35	30.50	*Not measured*

DISCUSSION

Free-range housing systems provide outdoor access for layers and they have a choice between indoor and outdoor areas. The behavioral demands of a free range bird may be higher than those experienced within enclosed indoor systems due to the large areas to navigate and variable environmental conditions (Campbell *et al*., 2018). In reality, birds may only use certain areas of the house throughout the flock cycle (Petterson *et al*., 2017), and fearful birds may be hesitant to go to outside (Campbell *et al.*, 2016, Hartcher *et al*., 2016) in a free range housing. In most flocks, many birds appear reluctant to leave the poultry house and only small proportions of the flocks are reported to be observed outside (Grigor, 2013). However, daily access to range and a greater proportion of range using during the laying period reduces the occurrence of feather pecking on a flock level (Bestman and Wagenaar, 2003; Jung and Knierim, 2018).

According to the results and in agreement with previous finding (Giesberg *et al.,* 2017), the best plumage quality within the all body region was measured in neck. Although, vent pecking is most prevalent in hens housed in a free range housing system (Sherwin *et al.,* 2010), we found that the tail was the most severely affected body part in all location of the experimental house. The second most commonly affected body region of the birds was rump in slats and litter locations, whereas it was wings in range location. Feather pecking is not uniformly directed to the whole body and the tail, back and rump receive most pecks (Gunnarson *et al*., 1995). Feather pecking is usually accepted the cause of plumage damage to the tail and rump (Petek and Mckinstry, 2010). Ramadan and Von Borel (2008) reported that wings, rump, tail and back were the main targets for feather pecking in laying hens and feather damage for these body region were found to be greater in slats in our study. While feather damage in back and rump is generally associated with injurious pecking, feather damage in the head and neck can be indicate aggression or equipment damage than to feather pecking behavior. Similar with findings of Ramadan and Von Borel (2008) feather damage in rump and tail were found to be significantly greater in slats compare to litter and range

location of the experimental house. In a study, Pickova *et al.(*2017) reported that the proportion of featherless areas in the rump region differed significantly between the housings from week 8 of the experiment and on the back and rump region from week 12.

In this study, the prevalence of feather damage were 60.00, 47.50 and 41.88% in slats, litter and range location of the experimental house, respectively. This means the birds located in slats had more feather damage or birds located in all area had more or less feather damage. Ramadan and Von Borel (2008) reported that the feather pecking occurred mainly on the floor (66%) and followed by feeding area (26%), perches (4%) and slats (4%). In this study, plumage quality as average and total feather score were found to be worst in slats probably due to feeder and drinker lines which is affecting bird distribution and pecking behaviour around. The result of this study clearly showed that litter or especially range using is very important to reduce pecking damage because less feather damage were observed in range or litter compared to slats. There is evidence flocks with many birds outside using all areas of the range have better feather cover (Chielo *et al*., 2016) and it would be beneficial to attract layers to litter or foraging area to reduce pecking behaviour. As reported previously, greatest range use can be achieved by letting the hens use the range as early as possible (Petek *et al*., 2015). Maintaining litter quality in litter area is the single most important enrichment you can provide to reduce the risk of feather pecking (Temple *et al*., 2017) and pecking stones or alfaalfa bales can be useful for attracting birds to the litter (Schreiter *et al*., 2019). Rearing environment can also play an important role in the later development of pecking problems (Janczak and Riber 2015).

In this study, the location of the hens (slats, litter or range area) did not affect air temperature or relative humidity. The ammonia concentration was 0.016 and 0.006 *ppm* in slat and litter area and no measurement outside range area. No significant differences for the relative humidity and ammonia levels were found between slat and litter location due to probably proper ventilation rate and optimum poultry stocking density with proper amount and type of litter. Because ammonia concentration and relative humidity are mainly affected by manure accumulation under the slats and higher moisture content of the litter (Oliveira et al., 2019). Moreover, free range access to fresh air helped to reduce the inside ammonia level.

When searching for an on-farm solution to reduce feather pecking behaviour, it is importance to identify the potential risk factors involved in the development of feather pecking activity on every flock. Free range housing systems should be designed so that birds can easily move throughout the house including slats, perches, feeder and drinker lines. This gives them ease of access to all facilities thus reducing the risk of feather pecking and it will make it easier for them to escape any pecking attempts. Slatted floors, some ramps and stairs may be used to facilitate an easy access to and movement through the system.

CONCLUSION

The study underlines the importance of housing locations, especially indoor housing design, in preventing pecking problems and indicates that increasing use of range area and spending less time on the slats location in a free range house would be very beneficial to reduce feather pecking of layer chickens. Further research covering a large number of flocks should be also very usefull to see the clear effects of house locations on pecking behavior and plumage quality in free range flocks.

CONFLICT OF INTEREST

None declared.

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J HELLENIC VET MED SOC 2020, 71(4): 2531-2538
ΠΕΚΕ 2020, 71(4): 2531-2538

Research article
Ερευνητικό άρθρο

Molecular Investigation and Genotyping of *Theileria equi* and *Babesia caballi* in Horses in Mus Province, Turkey

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ABSTRACT: Equine Piroplasmosis (EP) is a tick-borne disease caused by *Theileria equi* and *Babesia caballi* of the phylum Apicomplexa. In this study, 102 blood samples were randomly collected from the horses in Mus province of Turkey. PCR analysis, gene sequences, and phylogenetic analyses were carried out for detecting the presence and genotypic characteristics of species that cause piroplasmosis. Four (3.9%) of the 102 horses that were examined were found to be positive for *T. equi*, while *B. caballi* was not detected. *Theileria equi* isolates that were detected in the sequence analyses were found to be 100% identical to the isolates that were isolated from the horses in Turkey, the United States, and South Africa as well. In the phylogenetic analysis, all of the isolates were found to cluster with *T. equi* sequences in the genotype A. This study, in which we revealed intraspecies sequence heterogeneity of the parasite using the 18S rRNA gene region, provides important epidemiological data for equine piroplasmosis. However, we think that determining the characterization of genotypes that are common in different parts of our country is extremely important in terms of developing new diagnostic tools and vaccines.

Keywords: *Babesia caballi*, horse, genotyping, *Theileria equi*

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Date of initial submission: 19-02-2020
Date of revised submission: 19-04-2020
Date of acceptance: 21-04-2020

INTRODUCTION

Equine piroplasmosis is known as a protozoan disease transmitted through ticks and caused by *Babesia caballi* and *Theileria equi*. The disease is widespread throughout the world, especially in tropical and subtropical regions, and is treated as an important problem in transport processes at national and international levels (Taylor et al., 2007). It is known that *B. caballi* is transmitted transovarially and transstadially through ticks in the genera *Dermacentor*, *Hyalomma*, and *Rhipicehalus*, and *T. equi* is transmitted transstadially through ticks in the genera *Dermacentor*, *Hyalomma*, *Rhipicephalus*, and *Boophilus* (Inci et al., 2010; Rothschild, 2013). It is reported that the epidemiology of equine piroplasmosis is directly related to the spread of vector ticks (Altay and Aktas, 2013). Although they share the same vectors in certain regions, it was reported that *T. equi* infections are more common in the world when compared with *B. caballi* infections. The disease can occur in peracute, acute, and chronic forms, and characterized by fever, anemia, icterus, and hepatosplenomegaly (Rothschild, 2013).

The place of *T. equi* in the classification of the species that cause the disease has remained controversial for a long time. This parasite, described as *Piroplasma equi* at first, was named *B. equi* by Laveran in South Africa in 1901. In recent years, *B. equi* was found to be similar to Theileria species after it was revealed by experiments that *B. equi* developed in both vertebrates (lymphocytic schizogony) and vector ticks (no transovarial transmission), and on this basis, it was renamed *T. equi* in 1998 (Mehlhorn and Schein, 1998). *T. equi* has certainly been described as a new species with also genomic studies (Kappmeyer et al., 2012).

Thanks to major advances in molecular biology, it has now become easier to study the evolutions, ecologies, and epidemiology of parasites (Sant et al., 2019). In recent years, with conventional PCR (cPCR) method, Equimerozoite antigen, β-tubulin, and 18S rRNA genes are used to detect *T. equi* and *B. caballi* infections (Cacciò et al., 2000; Battsetseg et al., 2002; Alhassan et al., 2005). Because of its low rate of change, limited and preserved function, and the ability to create multiple copies, the 18S rRNA gene is considered to be superior to other genes (Qablan et al., 2013). Therefore, this gene region has been more widely used in defining *T. equi* and *B. caballi* species, phylogenetic, and genotypic studies (Bhooraet al., 2009; Seo et al., 2013; Hall et al., 2013; Veronesi et al., 2014; Liu et al., 2016; Bragaet al., 2017; Ketter-Ratzonet al., 2017; Peckleet al., 2018; Vieira et al., 2018; Wang et al., 2019). In consequence of the phylogenetic studies on the 18S rRNA gene, *T. equi* was reported to have five different genotypes as A, B, C, D, and E (Qablan et al., 2013;Liu et al., 2016; Ketter-Ratzonet al., 2017). There are fewer sequence variations in *Babesia caballi* when compared with *T. equi*. Three different genotypes, i.e., A, B, and C, were reported for *Babesia caballi* (Bhooraet al., 2009; Qablan et al., 2013; Manna et al., 2018).

The number of studies on the molecular epidemiology and genotypes of *T. equi* and *B. caballi* in Turkey is limited. To date, while three different genotypes (A, D, and E) have been detected for *T. equi*, only the genotype D has been reported for *B. caballi* (Kizilaslan et al., 2015; Ozubek et al., 2018). Therefore, this study aimed to identify these parasites in the horses raised in Mus province of Turkey using molecular methods and investigate their genotypic characteristics.

MATERIALS AND METHODS

Sample collection. The study materials were blood samples collected from 102 healthy horses ranging in age from 1 to 15 in 9 settlements in Mus province in June-August 2017 but was not found in any tick species (Akkoyun and Oguz, 2019). Mus is located in the Eastern Anatolian Region of Turkey and between the northern latitudes of 39° 29’ and 38° 29’ and the eastern longitudes of 41° 06’ and 41° 47’. Mus province has a harsh continental climate. The temperature is between -29°C and +37°C. The temperature is above +30°C on 120 days, and below 0°C on 120 days a year. It snows a lot in winter. The annual precipitation varies between 1000 mm and 350 mm. Winters are very cold and long, summers are short, hot, and dry. Whole blood samples were collected from the vena jugularis of the horses into EDTA tubes according to the technique, and the data on the animals were recorded with protocol numbers. The DNA samples were stored at -20°C until the PCR was performed. Ethics Committee approval for this research was obtained from the Local Ethics Committee for Animal Experiments of Yüzüncü Yıl University, Van (dated June 10, 2019, no. 42826).

DNA extraction and Multiplex-PCR amplification. Genomic DNA was obtained from the blood samples collected from the horses by using a commercial blood kit (EcoSpin Blood Genomic DNA Kit, Turkey). For the detection of *T. equi* and *B. caballi*, prim-

ers targeting the 18S rRNA gene were selectedfrom the literature (Alhassan et al., 2005). A multiplex PCR includes Bec-UF2 (5'-TCGAAGACGATCAGATACCGTCG-3') as a universal forward primer and Cab-R (5'-CTCGTTCATGATTTAGAATTGCT-3') and Equi-R (5'-TGCCTTAAACTTCCTTGCGAT-3') as reverse primers specific for *B. caballi* (540 bp) and *T. equi* (392 bp), respectively (Alhassan et al., 2005). The PCR reactions were carried out in a total volume of 50 µl containing 5 µl of genomic DNA for each sample amplification, 5 µl of $MgCl_2$, 1.25 mM of each dNTP, 5 µl 10 X PCR buffer, 0.5 IU Taq DNA polymerase and 20 pmol of each primer. The thermal profile used was 96 °C for 10 min; 40 cycles of 96 °C for 1 min, 60.5 °C for 1 min, 72 °C for 1 min, and final elongation step at 72 °C for 10 min. PCR products were electrophoresed in 1.5% agarose in Tris-acetate-EDTA (TAE) buffer and stained with ethidium bromide to visualize the amplified DNA fragments under ultraviolet light.

Sequence analysis. After all of the positive products obtained were purified using a commercial purification kit (High Pure PCR Cleanup Micro Kit, Roche, Germany) before the sequence analysis, they were subjected to capillary electrophoretic separation (Sentebiolab, Ankara, Turkey) and sequence analyses of the products were performed. The sequence chromatograms were checked and arranged using Bioedit software (Hall, 1999). Final consensus sequences of the isolates were subjected to "nucleotide BLAST" (National Center for Biotechnology Information, www.ncbi.nlmn.nih.gov/BLAST) analysis in the GenBank database and similarity rates were compared with the isolates reported from different countries. The 18S rDNA phylogenetic analysis data set was formed from the nucleotide sequences of 30 isolates in total. *Plasmodium falciparum* was used as the "out-group." A part of about 392 bp. was utilized for the phylogenetic analysis. The phylogenetic analyses and tree creation were carried out using the "maximum likelihood" method on MEGA 7.0 software with 1000 bootstrap replicates (Kumar et al., 2016). The nucleotide sequences obtained in the study were recorded in the GenBank with the accession numbers of MN586811, MN586812, MN586813, MN586814. Also, a list of accession numbers, which were obtained from GenBank and recorded in Turkey on this subject, was given in Table.

Table. List of the accession numbers for the *T. equi* and *B. caballi* from Turkey that is available the Genbank™ EMLB

Accession number	Name of piroplasm	Year Obtained	Genotype
JX826604	*Theileria equi*	2012	E
JX826603	*Theileria equi*	2012	E
KF840330	*Theileria equi*	2014	Unknown
KU921667	*Theileria equi*	2016	Unknown
KU921666	*Theileria equi*	2016	Unknown
KU921665	*Theileria equi*	2016	Unknown
KU921664	*Theileria equi*	2016	Unknown
KU921663	*Theileria equi*	2016	Unknown
KU921662	*Theileria equi*	2016	Unknown
KU921661	*Theileria equi*	2016	Unknown
MG569905	*Theileria equi*	2018	A
MG569904	*Theileria equi*	2018	A
MG569901	*Theileria equi*	2018	D
MG569900	*Theileria equi*	2018	D
MG569899	*Theileria equi*	2018	Unknown
MG569898	*Theileria equi*	2018	Unknown
MG569897	*Theileria equi*	2018	Unknown
MG569896	*Theileria equi*	2018	Unknown
MG569895	*Theileria equi*	2018	Unknown
MG569894	*Theileria equi*	2018	Unknown
MG569893	*Theileria equi*	2018	Unknown
MN481267	*Theileria equi*	2019	Unknown
MN481266	*Theileria equi*	2019	Unknown
MN481265	*Theileria equi*	2019	Unknown
MN481264	*Theileria equi*	2019	Unknown
KP792452	*Babesia caballi*	2015	A

RESULTS

While 4 (3,9%) of the 102 horses in total were found to be infected with *T. equi*, no *B. caballi* species were detected (Figure 1). *Theileria equi* isolates (MN586811, MN586812, MN586813, MN586814) were found to be 100% identical among themselves according to pairwise comparisons. Besides, they were also found to be 100% identical to the isolates that were isolated from the horses in Turkey (MG569905), the United States of America (JX177672), and South Africa (EU642508). Phylogenetic tree of the *T. equi* isolates detected in the horses in Mus province and some other *T. equi* isolates from the other regions on the world using the Maximum Likelihood Method (Kimura 2 Parameter Model) was given in Figure 2. As can be also seen on the phylogenetic tree, all of the detected isolates were found to be in the *T. equi* Genotype A.

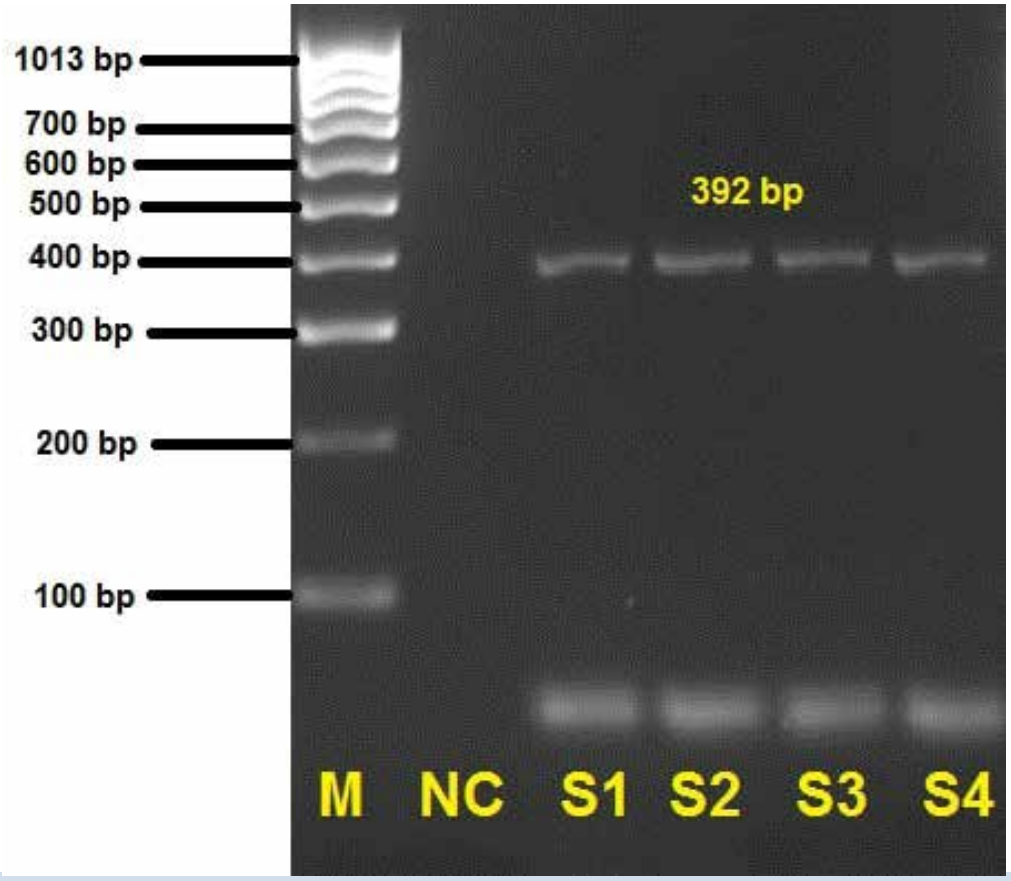

Figure 1. Bands resulting from amplification of the 18S rRNA with *Theileria equi*. M: 100 bp ladder, NC: Negative control, S1-4: Samples

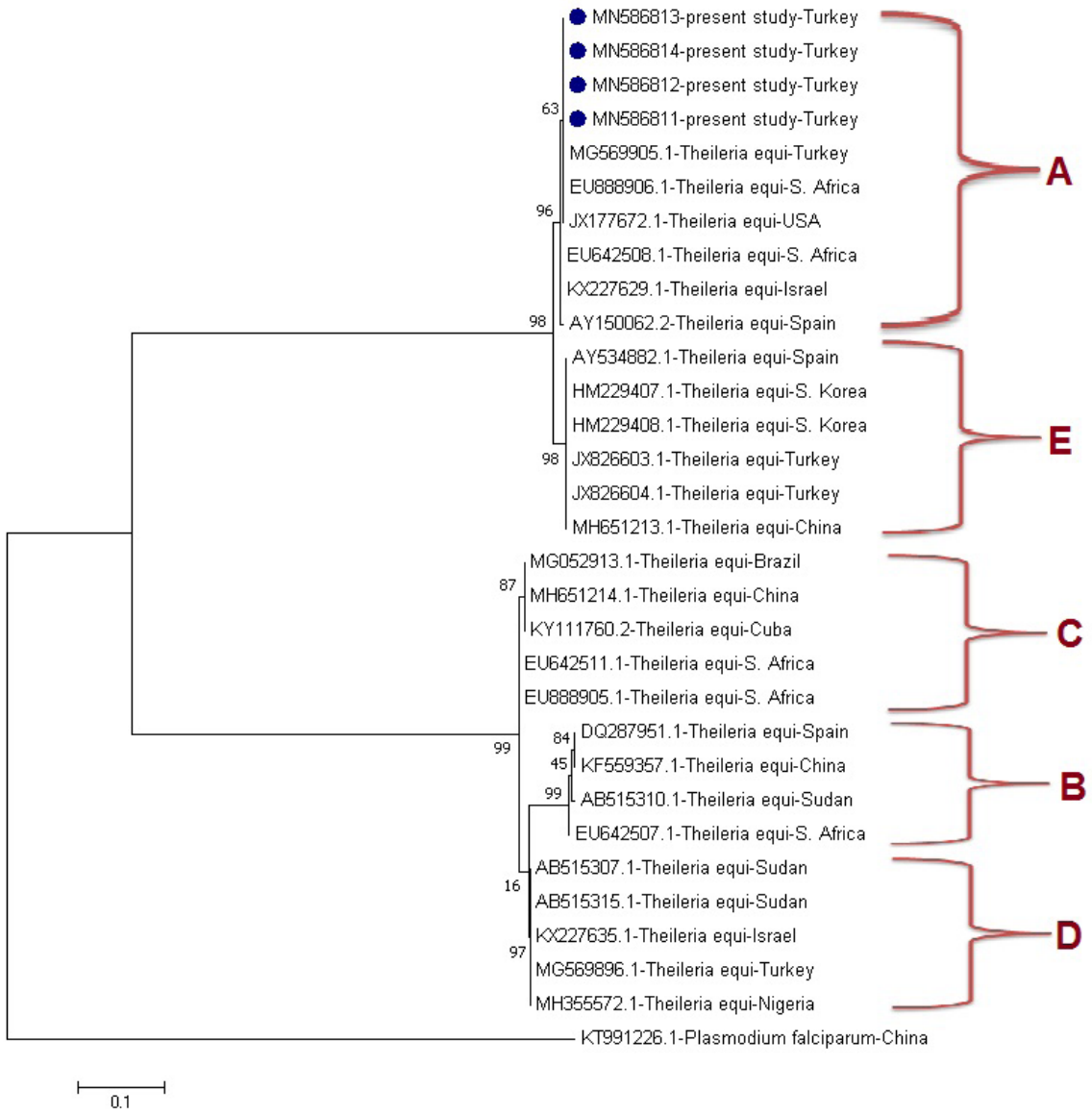

Figure 2. Maximum likelihood phylogenetic tree of *T. equi* 18S r RNA gene sequences with 1,000 bootstrap replicates. The evolutionary history was inferred by using the Kimura 2-parameter model. The tree with the highest log likelihood (-1416.91) is shown. Sequences were obtained from the GenBank database and GenBank accession numbers, animal host species and country of origin from which the sequences were derived are included for each sequence. Isolates from this study are indicated with a blue round

As can be seen in Figure 3, single-nucleotide polymorphisms (SNP) special to the genotypes were shown between the *T. equi* isolates detected according to the 18S rRNA gene region and the other isolates. For example, while transversion occurs upon conversion of base T into A at codon 101 between the genotypes A and C, transition mutation occurred in consequence of conversion of the base A into G at codon 155 between B and C. While transitions were observed at 403rd, 408th, 414th, 417th, and 421st codons between the genotype A and E, transversion occurred as a result of conversion of the base T into A at codon 420.

Figure 3. Multiple sequence alignments of *Theileria equi* genotypes. Identical bases are shown as dots and sites of some variation are shown in boxes

DISCUSSION

Theileria equi was compared with *Babesia caballi*, and *T. equi* infection was reported to be more common in endemic countries (Rothschild et al., 2013). To date, studies on piroplasmosis in horses were conducted with mainly conventional and serological diagnosis methods in Turkey, and the number of molecular prevalence and characterization studies is limited. In reviewing these studies, *T. equi* was found to be much more prevalent when compared with *B. caballi*, or no prevalence was found (Inci, 1997; Guclu and Karaer, 2007; Kizilaslan et al., 2015; Guven et al., 2017; Ozubek et al., 2018). Similarly, the prevalence of *T. equi* was found to be 3,9% (4/102) and *B. caballi* species was not found in the samples in our study. This can be explained by the fact that while parasites in the blood are eliminated about 4 years later in the infections with *B. caballi*, *T. equi* is resistant against antiprotozoal drugs and infected horses carry this agent for life even after treatment (Brüning, 1996; Bhoora et al., 2009).

Whether different *T. equi* genotypes form clinical differences in equine piroplasmosis cases is not fully known except for some unverified data. While Manna et al., (2018) found in a study that genotype B was more common in asymptomatic animals and genotype A was observed more in symptomatic ani-

mals. According to the same researchers, Hall et al., (2013)'s study supports their findings. Hall et al., (2013) found the dominant genotype to be A when they examined the isolates in *T. equi* epidemics that occurred in North America. Sant et al., (2019) detected the dominant genotype to be A in their study on the genotype of *T. equi* in mares and foals in Trinidad. All of the isolates obtained in Mus province in our current study were found to be genotype A, but we do not have any data on the course of the disease. Ketter-Ratzo et al., (2017) found the genotypes A, C, and D in their study on horses in Jordan, Palestine, and Israel. However, they claimed that the genotypes E and B were more (99%) similar when compared with others. On the other hand, it was reported that the genotype E is responsible for clinically fatal piroplasmosis cases in Greece and Spain (Nagore et al., 2004; Kouam et al., 2010). Similarly, Wang et al., (2019) reported that the dominant genotype was E in their study in the Gansu region of China. Whether there is a connection between the pathogenesis of the disease and genetic variations is still unknown. Further molecular epidemiological studies in which full genome sequencing and clinical findings can be assessed together are needed to clarify this situation.

The number of studies on genotypic variations is limited in Turkey. Kizilaslan et al., (2015) reported that they detected the genotype E for *T. equi* and genotype A for *B. caballi* in consequence of their phylogenetic analyses of the isolates they obtained in Bursa province. Ozubek and Aktaş (2018) reported that the genotype of the *T. equi* isolates in Şanlıurfa, Tunceli, and Iğdır provinces were A and D in consequence of their sequence and phylogenetic analyses, and they did not detect *B. caballi* species. In our current study, all of the isolates obtained in Mus province were found to be genotype A, and *B. caballi* could not be detected. Regarding the regions where the horses live, the distances from Mus to Bursa, Sanliurfa, Tunceli, and Igdır are 1351, 385, 256, 386 km, respectively. Geographical and climatic changes among the regions, the current condition of vector ticks species, and entries and exits of horses or interregional circulation of horses may cause both the infection and the genotypes to varying from one region to another.

CONCLUSION

To the best of our knowledge, our research is the first molecular epidemiological and genetic variation study on *T. equi* and *B. caballi* in Mus province of Turkey and only one genotype was identified for *T. equi*. Further molecular epidemiological studies are needed to discover the levels of pathogenicity in different genotypes of *Theileria equi* and *B. caballi*, block the emergence of new genotypes, and prevent the disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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J HELLENIC VET MED SOC 2020, 71(4): 2539-2548
ΠΕΚΕ 2020, 71(4): 2539-2548

Research article
Ερευνητικό άρθρο

Investigation of the relationship of apelin hormone response with some physiological parameters in Maedi-Visna infected sheep

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ABSTRACT: In this study, the objective was to assess the serological characteristics of sheep, during lactation and pregnancy periods, and rams belonging to the Red Karaman and White Karaman–Kangal race infected with maedi-visna infection and body condition score (BCS) of ≤2, 3- 3.5 and ≥4 (high) and to analyse the relationship between the level of apelin and its secretion. Apelin level in the blood serum samples obtained from the jugular veinof the sheeps was determined using ELISA method. As a result of the analyses, it was determined that the level of apelin was statistically different between the races; between lactating sheep and pregnant sheep; and sheep and rams. There was no difference between the BCS groups ($p > 0.05$). Race and sex interaction as well as race, sex and BCS triple interaction effects were found significant ($p < 0.05$). It was observed that the sheep belonging to the White Karaman–Kangal race were 18 times more likely to be infected with the virus. A decrease in apelin level was observed in the sheep with infection, and it was found that the risk of infection was 0.37 times higher in rams than in lactating sheep ($p < 0.05$).

Keywords: Maedi-Visna; ewe; Apelin; hormone; breed; gender; pregnancy; ELİSA

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Date of initial submission: 09-12-2019
Date of revised submission: 09-09-2020
Date of acceptance: 10-06-2020

INTRODUCTION

Maedi-visna (MV) is a viral infection that causes significant economic losses in the sheep breeding sector worldwide. Its causative agent, maedi-visna virus (MVV), belongs to the lentivirus class of Retroviridae family. It is in the small-ruminant lentivirus (SRLV) subgroup along with caprine arthritis encephalitis virus and classified under the same genus as the human lentivirus [human immunodeficiency virus (HIV)](Gayo et al. 2019;Thormar et al.2005).

MVV infection causes progressive weight loss as well as non-purulent chronic inflammation development in lungs, mammary glands, joints and central nervous system in adult sheep and goats(Russo et al., 1988; Phelps and Smith,1993). It is transmitted through ingestion of infected colostrum and milk and inhalation of respiratory secretions(Gomez-Lucia et al.2018;Peterhans et al.2004; Blacklaws et al. 2004). Primary targets of MVV are monocytes/macrophages and dendritic cells (Slingenbergh, 2019; Gendelman et al. 1986). These cells, which are affected by MVV, migrate to the regional lymph nodes through which the virus systemically spreads and dendritic cells (Slingenbergh, 2019) and persistently circulates throughout the body without getting affected by immune response. Thus, infection is classified as a slow virus infection owing to its long incubation period and the ability for continuous reservoir which is persistent without being affected by immune response and which can continue life-long (Gendelman et al. 1986;Stonos et al.2014;Singh et al., 2006).

Hormones are one of the important factors in the formation of suitable conditions for the reproduction and secretion of infected viruses. Cytokines such as interferon-gamma (IFN- γ) and granulocyte macrophage colony stimulating factor (GM-CSF) and hormones such as steroids provide the suitable cellular medium for virus reproduction (Zhang et al. 2002).

Hormones act as transcription factors by binding to regions called transcription binding sites in the lentivirus DNA (Puffer et al. 2000; Gomez-Lucia et al.2014;Gomez-Lucia et al.2018). Hypothalamic pituitary adrenal (HPA) axis plays an important immunomodulatory role in the development of viral infection (Bailey et al.2003).The apelin hormone which forms the basis of our study is excessively present in regions controlling the HPA axis activity such as the supraoptic nucleus, the paraventricular nucleus and the central nervous system (Newson et al.2009; O'Carroll et al. 2013).

Apelin is a hormone of the hormone–cytokine family; it is secreted from adipose tissue, has a 77 amino-acid precursor and has various isoforms such as apelin-12, 13, 17 and 36 (Tatemoto et al. 2001). Among its isoforms, apelin-13 has the highest biological activity owing to its pyroglutamate modification characteristics (Beltowski,2006; Kleinz and Davenport, 2005). Apelin–APJ (apelin receptor) is reported to have potential therapeutic effect in the prevention of HIV-1 infection (Fan et al. 2003; Kakizawa, 2016; Sing et al. 2006).

The primary clinical symptoms of MV infection are primary interstitial pneumonia, encephalitis, lymphadenopathy, arthritis, mastitis (Anker and Coats, 1999) and most distinctly, chronic weight loss (Russo et al. 1988; Phelps and Smith, 1993). The reproduction and secretion of virus in MVV infection are affected by the reproductive cycle (lactation, pregnancy, etc.) (Gomez-Lucia et al. 2014; Ouzrout and Lerondelle,1990). In this study, the objective was to comparatively analyse through serological and molecular tests the effect of race, gender and lactation on serum apelin levels in fat-tailed sheep belonging to the Red Karaman and White Karaman–Kangal race having different body condition scores (BCS of ≤2, 3–3.5 or ≥4) in terms of MVV infection.

MATERIALSANDMETHODS

Ethical Approval

Ethical approval for this study was obtained from the Bayburt University Local Ethics Committee (27/03/2019/01; Approval Number: 2019-2).

Animal Selection and Creationof Groups

In this study, 180 sheep were used analyse the relationship between apelin level and secretion in terms of MVV infection through serological and molecular tests. The animals used in the study were divided into three groups as Group I (lactation group), ewes in the early lactation period (n=60); Group II (pregnancy group), ewes in the first period of pregnancy (on the 100th day of first pregnancy) (n=60); Group III (rams) by randomization so that the average total live weight of the groups was equal.

The study was conducted in 6 ewe farms in Bayburt Province center and two districts (Demirozu and Aydıntepe) (40.16N,39.89-K;40.22N,40.26-K;40.3N,40.14-K) which ewe production is performed under intensive conditions and records are regularly followed up. BCS controls and scoring were

performed by four referees prior to the inclusion of rams. A scale of 5 points with 0.5 intervals was used in BCS scoring. In the trial plan, sheep and rams were analysed under three different groups according to their BCS of ≤2, 3 and ≥4 (Sarı et al. 2013). During the study period, the environment and feed factor were taken into account.The content of feeds used in this study was analyzed (Table-1) according to the standard AOAC methods (AOAC, 2005).

Table 1. Nutrient composition of diets used in the study (%)

Ration Composition	Red Karaman	White Karaman-Kangal
Barley	65.0	65.0
Wheatbran	10.5	7.0
Soybeanmeal	22.0	20.5
Dicalciumphosphate	1.0	1.0
Salt	0,5	0,5
Premix	0.5	0.5
Chemical composition %		
Dry Matter	90.39	90.60
Crudeprotein	17.37	16.99
Crudeash	5.59	5.78
ADF	8.84	11.26
NDF	34.44	32.72
MEKcal/kg	2642	2620

1 kg vit.-min. Premix contains vitamin A, 7,000,000 IU; vitamin D3, 1,000,000 IU; vitamin E, 30,000 IU; Mn, 50,000 mg; Zn, 50,000 mg; Fe, 50,000; Cu, 10,000 mg; I, 8,000 mg; Co, 200 mg; Se, 150 mg; and Mg, 100 mg

Collection of Blood Samples

Blood sample of 10 ml was collected from the jugular vein of the ewe into the tubes with EDTA and without anticoagulant (VACUETTE® TUBE 9 ml Serum Clot Activator). The blood samples without anticoagulant were centrifuged at 3000 rpm/min for 10 minutes on a refrigerated centrifuge (NF 1200, NUVE, Ankara, TURKEY) in the laboratory, then blood serums were separated. The separated serums were transferred into sterile tubes and stored in deep freezers (-80°C) until the laboratory analyses were carried out. The blood samples with EDTA were stored in deep freezers (-80°C) until the DNA extraction.

Measurement of apelin hormone levels in serum

The basic principle of the ELISA method is based on the use of enzyme to determine the antigen-antibody combination in the sample. The enzyme used converts the colorless layer (chromogen) into a colored product, indicating the presence of antigen-antibody, and the intensity of the resulting color is read by the elisa plate reader at the recommended wavelength so the relevant concentration is determined (Marai et al. 2007).The minimum detectable concentration of the apelin hormone kit used in the measurement of apelin levels in the blood serums obtained as a result of the study is reported as <18.75 pg/ml. The race-specific ewe APLN ELISA Kit (Apelin, FineTest, Product code: ESH0081, CHINA) was studied in accordance with the procedure described in the manufacturer's catalog using the determination of 31.25-2000 pg/ml, the intra assay coefficient of 8.0% and the inter-assay coefficient of 10.0% (Tsiodras et al., 2010).

Enzyme Linked Immunosorbent Assay (ELISA)

In the herd screening of MVV infection, the most sensitive and fastest screening test is performed by ELISA (Reina et al. 2009; Preziuso et al., 2010; Kurowska et al. 2018). Commercial kits (IDEXX MVV/CAEV p28 Ab screening, Lot:E891, IDEXX, USA) were used for the detection of Anti-MVV/CAEV antibodies (Arık et al. 2015; Çelik et al. 2018). The study was carried out with the ELISA method and in accordance with the suggestions of the producer company. The plates were measured at 450 nm to obtaining the optical density (OD) data, and the derived results were calculated in accordance with the procedures.

DNA extraction and PCR

Proviral DNA was extracted from whole blood (with EDTA) samples using High Pure Viral Nucleic Acid Kit (Roche, Germany) according to the manufacturers' instructions. Extracted MVV DNA was stored at -80°C until further use. LTR gene regions was amplified by PCR using specific primer pairs reported by

Extramania et al. (2002), producing fragments of approximately 300 nt. LTR-PCR was performed in a 20 uL reaction consisting of 10X reaction buffer, 2 mM MgCl2, 0.2 mM dNTP, 0.2 pmol each primer and 1.25 U Taq DNA polymerase (Fermentas, Lithuania) (Extramania et al. 2002; Yıldırım et al. 2011). The PCR cycling profile included a denaturation stage at 96 °C for 6 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. PCR products were analyzed after electrophoresis in 1,5 % agarose gel containing ethidium bromide and visualized on a UV transilluminator (Kodak, 2019).

Sequencing and Phylogenetic Analysis

PCR fragments were purified from gel using a purification kit (High Pure PCR Product Purification Kit, Roche, Germany) and sequenced using a sequencing kit (BigDye Terminator v3.1 Cycle Sequencing kit, Applied Biosystems, USA) on a genetic analyzer (ABI 3130xl, Applied Biosystems, USA). After sequence assembly and editing using Bioedit (Version 7.0.5.3) and Clustal W, the samples were then compared with the GenBank nucleotide sequence database for sequence similarities via the basic length alignment search tool (BLAST) software of the National Centre for Biotechnology Information (NCBI) (Extramania et al. 2002; Altschul et al. 1997; Hall and BioEdit, 1999). Phylogenetic tree for the 276 bp fragment of Maedi Visna Virus was constructed using the Maximum Likelihood (ML) method of the MEGA X v.10.0.4 software, based on the evolutionary distances between different sequences calculated by the Kimura two-parameter model (Tamura et al. 2011). The confidence level of the ML tree was assessed by bootstrapping, using 1,000 replicates.The nucleotide sequence of TR-Bayburt-2019 from this study have been submitted to GenBank and assigned the accession number MN197477. A phylogenetic tree of the TR-Bayburt-2019 sequence comparing it to other reference sequences in the GenBank database formed a separate branch in which only the Turkish, American and Greek sequences were included, suggesting a common origin.

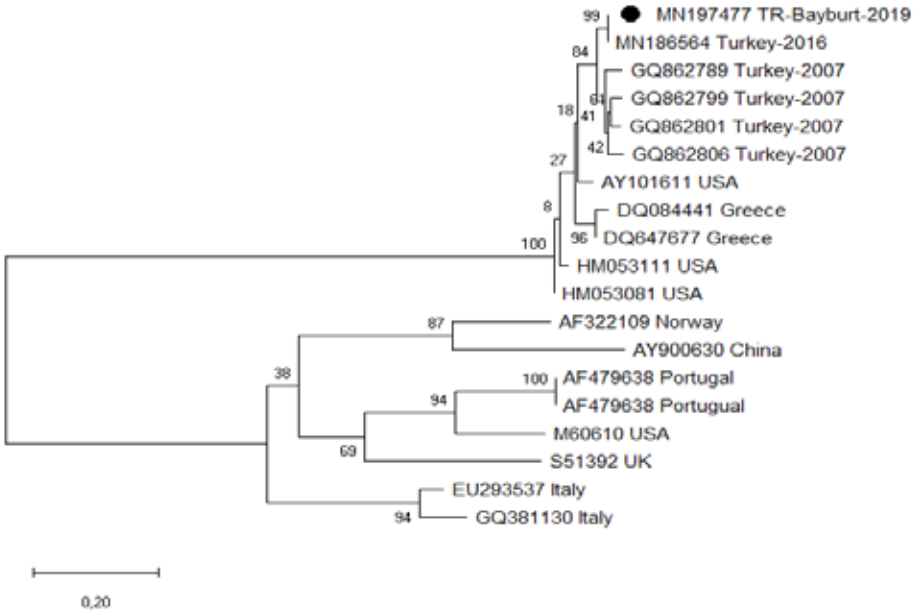

Figure 1. Molecular Phylogenetic analysis by Maximum Likelihood method

Maximum Likelihood tree constructed using the Kimura 2-parameter model, based on the LTR gene (276 bp). The numbers indicate bootstrap values (1,000 replicates) in the figure. Sequences characterized in this study are marked using black dots. The scale bar at the bottom represents genetic distances in nucleotide substitutions per site. Horizontal distances are proportional to sequence distances.

StatisticalAnalysis

In this study, the difference of serum apelin hormone levels in infected and non-infected animals was investigated by t-test. The effects of gender, body score and race factors on the serum apelin hormone levels for Red Karaman and White Karaman-Kangal ewe races in the lactation and pregnancy period were estimated. The gender, body score and race factors

were analyzed as fixed effects. The normality test was applied to the apelin measurements for the estimation of the effects of gender, body score and race on the serum apelin hormone levels of Red Karaman and White Karaman-Kangal ewe races at lactation and pregnancy period. Then, the univariate procedure was used in the generalized linear models (GLM) for the normally distributed apelin measurements. The analyse was performed in the full factorial setting with 3 factors (2x2x3) according to completely randomized design, and the interaction effects of the body score and race, body score and gender, race and gender, and body score, gender and race. The investigated effects and interactions with the statistical model as given below:

$$Y_{ijkl}=\mu+\alpha_i+\beta_j+\gamma_k+(\alpha\beta)_{ij}+(\alpha\gamma)_{ik}+(\beta\gamma)_{jk}+(\alpha\beta\gamma)_{ijk}+\varepsilon_{ijkl}$$

Where, Y is the apelin hormone, μ: Mean, α is the body score, β is the gender and γ is the race effect. Tukey's multiple comparison test was used to compare the differences for the means of apelin hormone. JMP 7 statistical package (JMP, 2019) was used in all analyses in this study. All significant differences were evaluated by testing at P<0.05 level.

Moreover, the gender, race and body score effects on Maedi Visna were investigated with binary logistic regression. Because the Maedi Visna was categorical variable and has just two categories (uninfected:0 and infected:1) the Wald test was used to verify that coefficient βi differs from 0. The applied binary logistic regression model was shown as follow: X_1, X_2, and X_3 are gender, race and body score independent effects and P(Y) probability of virus occurence or not.

$$P(Y)=\frac{1}{1+e^{-(\beta_o+\beta^1X_1+\beta_2X_2+\beta_3X_3)}}$$

RESULTS

Serum apelin values (pg/ml) measured in MVV-infected sheep and rams were presented in Table 2.It was determined that 47 of the 180 blood serums constituting the basis of the study were positive in terms of MVV antibody (Table 3). Accordingly, the apelin level was higher in non-infected animals than in infected animals, and this difference was statistically significant ($p < 0.01$). Moreover, serum apelin values (pg/ml) of the MVV-infected sheep measured during lactation and pregnancy, BCS periods and serum apelin values (pg/ml) of the rams were presented in Table 2.

Table 2: The Least square means (±SEM) and p values for simultaneous comparison of apelin in ewe and ramsinteraction of gender, races and body condition score (ng/ml)

Gender	N	Mean±SEM
Ewe in Lactation	60	2.806±0.160^{c}
Ewe in Pregnancy	60	5.820±0.160^{a}
Rams	60	4.497±0.160^{b}
Races	**N**	**Mean±SEM**
Red Karaman	90	4.69 ± 0.16^{a}
White Karaman-Kangal	90	4.17 ± 0.16^{b}
BCS	**N**	**Mean±SEM**
BCS ≤2	60	4.26±0.16
BCS = 3- 3.5	60	4.54±0.16
BCS≥4	60	4.32±0.16
Source of variation (P-values)		
Gender	0.00	
Races	0.04	
BCS	0.08	

Race*Gender (Mean±*SEM)*	**Ewe in Lactation**	**Ewe in Pregnancy**	**Rams**
Red Karaman	3.308±0.226^{c}	5.157±0.226^{b}	5.284±0.226^{b}
White Karaman-Kangal	2.304±0.226^{d}	6.482±0.226^{a}	3.709±0.226^{c}

Race*Gender*BCS	**Red Karaman(Mean±SEM*)***			**White Karaman-Kangal(Mean±SEM*)***		
BCS/Gender	**Ewe in Lactation**	**Ewe in Pregnancy**	**Rams**	**Ewe in Lactation**	**Ewe in Pregnancy**	**Rams**
BCS≤2	3.38±0.47^{defg}	5.29±0.47^{abcd}	5.05±0.47^{abcde}	2.25±0.47^{fg}	5.54±0.47^{abc}	4.05±0.47^{cdef}
BCS=3-3.5	3.38±0.47^{defg}	6.09±0.47^{abcde}	5.00±0.47$_{bcde}$	2.72±0.47^{fg}	6.90±0.47^{ab}	4.17±0.47^{cdef}
BCS≥4	3.17±0.47^{efg}	5.09±0.47^{abcde}	5.81±0.47^{abc}	1.95±0.47^{g}	7.01±0.47^{a}	2.90±0.47^{fg}

*Means within the same column showing different superscripts are significantly different (P < 0.05) * Significant at 0.05 level, ** Significant at 0.01 level, NS: Not significant (P > 0.05). SEM = standard error of the mean.* a, b, c, d, e, f, g: Means with different letters are statistically different (P<0.05).

As a result of the analyses of our study, it was determined that the apelin level showed a statistically significant difference between races ($p < 0.01$). Similarly, apelin levels showed statistically significant difference in rams and sheep in lactation and pregnancy ($p < 0.05$) (Table 2). It was detected that apelin levels did not change in BCS groups ($p > 0.05$). Moreover, it was detected that apelin levels in different sexes showed different trends according to races; in other words, it was found that the interaction effect of race and sex was statistically significant ($p < 0.01$). Conversely, race, BCS and race–BCS pairwise interaction effects were not significant, whereas triple interaction effect of race, sex and BCS was significant ($p < 0.05$).

Table 3. The Least square means standard errors of for serum apelin hormone level for virus infections

	The presence of the virus	N	Mean± Std. Error Mean	P
Apelin hormone level	0 (Negative)	133	5,22±0,121	0.00
	1 (Positive)	47	1,99± 0,073	

When the logistic regression analyses results are examined, it is obtained that the race effectis statistically significance on MVV enfection.The risk of MVV for White Karaman-Kangal race was18 times higher than Red Karaman race. In terms of gender, when the sheeps in lactation are accepted as indicators and pregnancy has no risk effect (P=0.996), but the risk of rams being infected is 0.37 times higher than sheeps in lactation. Moreover, when the BCS:3-3.5 was held as an indicator,the risk of MVV for BCS≤2 and BCS≥4 were 6.26 and 4.54 times higher risk than BCS:3-3.5, respectively (Table 4).

Table 4. Coefficient of factors, standard errors, Wald statistics and odds ratios

						Odds Ratio
	N	B	S.E.	Wald	P	Exp(B)
Race	180	2.89	0.54	29.12	.000	18.02
BCS:3-3.5	60			9.56	.008	
BCS≤2	60	1.84	0.62	8.64	.003	6.26
BCS≥4	60	1.51	0.61	6.13	.013	4.54
Ewe in Lactation	60			4.21	.122	
Ewe in Pregnancy	60	-21.81	4613.26	0.00	.996	0.00
Ram	60	-1.01	0.49	4.21	.040	0.37
Constant		-2.78	0.63	19.57	.000	0.06

Likelihoodratio(omnibüs,p<0.001)andHosmer-Lemeshowtest(p=0.099)

DISCUSSION AND CONCLUSION

MVV is an infection of adult sheep; it leads to symptoms associated with progressive interstitial pneumonia in its maedi form and symptoms associated with meningoencephalitis in its visna form. The development of infection usually takes 3–4 years for the maedi form and approximately 2 years for the visna form, and a large portion of the sheep can carry the virus through their lifetime or complete their economic lifetime without manifesting symptoms (Tan and Alkan, 2002;Lopez and Martinson, 2017).Seroprevalence studies are important because they affect the success of eradication and control programmes.

The first clinical symptom of MVV infection manifests as the disruption of body condition and with the progression of the disease, death occurs owing to respiratory problems(Karaoğlu et al. 2003). In our current study, although the apelin level was higher and statistically different in the non-infected animals compared with the infected ones ($p < 0.01$), the effect of BCS on apelin level was not different at a statistically significant level ($p \geq 0.05$). We believe that this may be caused by the duration for which the sheep were infected by the virus and the latent course of infection. It is consistent with the results of study where the relationship between MVV infection and various BCSs are analysed and the difference is shown in the results (Tefera et al. 2016; Özkan et al. 2014). Because our study is the first study analysing the change intervals and effects of the apelin level in various BCSs, literature data is limited in terms of discussion of the results. Conversely, although BCS levels are not significant in terms of apelin, according to the logistic regression analysis results, it was determined that the risk of infection by MVV was 6.26 and 4.54 times higher in the BCS ≤ 2 and BCS ≥ 4 groups compared with the BCS = 3–3.5 group. This is consistent

with the literature data stating that the first symptom of infection is the disruption of body condition.

Genetic factor is an important component in infection control. Breeding of races known to have genetic resistance (Wachendörfer et al.1995)is an important strategy in infection control. Several studies report that some races are extremely sensitive to MVV infection and that some show high resistance (Wachendörfer et al.1995; Simard and Briscoe, 1990; Houwers et al. 1984; Cutlip et al. 1992). The seropositivity in the sheep of White Karaman–Kangal race with MVV infection was lower than that in those without the infection. When the effect of race on apelin level was analysed, it was found statistically significant ($p < 0.05$). Indeed, according to the logistic regression analysis, the White Karaman–Kangal race sheep were 18 times more likely to be infected by the virus. Red Karaman race sheep were more resistant to infection compared with the White Karaman Kangal race sheep in the study sample, and this is consistent with results of previous study [42]and the studies reporting race resistance Simard and Briscoe, 1990; Burgu et al. 1990; Kandil et al.1997). We propose breeding of sheep belonging to the Red Karaman race in the regions with high infection seroprevalence owing to its resistance to MVV infection. However, there is a need for a larger number of studies to support our recommendation regarding the effect of the race.

Epidemiologic studies have reported that sex, particularly female sex, plays a primary role in infection transmission owing to transfer of MVV by infected sheep to their lambs through milk and colostrum (Slingenbergh, 2019; Simard and Briscoe, 1990; Burgu et al. 1990; Kandil et al. 1997; Muz et al. 2013). Sex difference and correlation of infection incidence have been reported in previous studies (Simard and Briscoe, 1990; Burgu et al.1990). According to the infection status of the rams in our study sample, the rate was 56% in the rams belonging to the White Karaman–Kangal race and 6% in those belonging to the Red Karaman race. It was 73% in the non-pregnant sheep belonging to the White Karaman–Kangal race and 20% in those belonging to the Red Karaman race. The results of our study are consistent with the studies evaluating the effect of gender(Simard and Briscoe, 1990; Burgu et al.1990). Burgu et al. (1990) found the effect of sex statistically insignificant (Burgu et al.1990). We believe that this may be caused by the increase in seropositivity due to close contact with infected female sheep during the mating period, although the rams are separately housed.

In pregnant animals, research and detection of infected pregnant sheep prevalence has a strategic importance in abortion events or the occurrence of smaller than normal and weak offspring births (Burgu et al.1990;Kandil et al. 1997; Oguma et al.2013) and the understanding of prevalence rate and ratio of the infection in the lambs to be born, development of disease prevention and eradication programmes. It should be emphasised that in our study sample, all sheep in the early pregnancy period had negative results for MVV infection because during pregnancy highly complicated physiological processes occur (Ouzrout and Lerondelle, 1990).

In early pregnancy, apelin levels increase to regulate normal placentation(Van Mieghem et al. 2010) thus, we concluded that the increased hormone concentration may have an inhibitory effect on virus expression. Due to current literature information being limited in comparison to our pregnancy period study results, when studies similar to our studies are examined, they show compatibility (Slingenbergh, 2019; Ouzrout and Lerondelle, 1990). Respiratory fluids, milk and colostrum containing infective monocytes and macrophages are one of the primary transmission paths. Thus, lactation is one of the important factors in the spread of infection(Gomez-Lucia et al.2018; Legastelois et al. 1998). It is reported that infection spreads at a rate of 28% at the end of 10 hours via milk and colostrum. Moreover, because of the lactation period providing the opportunity for the sheep to be isolated from milk within the 5 months (Simard and Briscoe, 1990) isolation during the lactation period may present a strategic importance in eradication programmes. Similarly, apelin levels are reported to show a significant increase in apelin expression during mammary gland, pregnancy and lactation periods (Habata et al. 1990).A decrease was observed in apelin levels in infected sheep; compared with lactating sheep with infection, rams were found to have 0.37 times higher risk of being infected ($p < 0.05$). There are no studies evaluating serum apelin levels in MVV-infected sheep during lactation; however, the results of our study are consistent with the results of the study reporting that there are severe increases in apelin expression during the lactation period (Habata et al. 1990).

MVV infection is also called ovine lentivirus-induced lymphoid interstitial pneumonia (Mingujion et al.2015). It is reported that sheep may acquire infec-

tion at any age by droplet inhalation via respiration. In pathogenesis, hyperplasia and fibrosis in muscle fibres develop as a result of infiltration and thickening in the intraalveolar regions in lungs (Luján et al.2019).It has been reported that apelin which forms the basis of our study is important in the prevention of fibrosis in lungs(Pchejetski et al2011; Wang et al. 2014) and regulation of pulmonary vascular homeostasis (Kim, 2014).

Hormones play an important role in viral infections owing to providing an appropriate medium for the reproduction and excretion of infectious viruses as a result of host cell reactivation (Ouzrout and Lerondelle, 1990). Thus, we hypothesise that hormonal treatments based on therapeutic molecules form an alternative and important strategic approach in viral therapies.

Apelin is a promising therapeutic agent in many pathologies such as inflammatory (Zhou et al.2016; Tsiodras et al. 2010) and cardiovascular diseases induced by oxidative stressZhou et al. 2016; Tsiodras et al. 2010),obesity (Wysocka,2018) and cancer (Rayalam et al.2011İ Uribesalgo et al. 2019). Moreover, apelin has the ability to prevent infection owing to its inhibitory effect on HIV and human lentivirus (Esposito et al. 2002;Gayo et al. 2019; Thormar, 2005), both of which have homolog similarity with MVV (Kleinz, 2005; Zou et al. 2000).Because there is no treatment or vaccination for MVV infection (Polledo et al. 2013). We hypothesise that apelin is a potential therapeutic agent owing to its physiological role. However, it is necessary to conduct studies on a larger number of animals and different sheep races regarding apelin treatment to determine the physiological mechanisms and to support with prospective studies which can be developed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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J HELLENIC VET MED SOC 2020, 71(4): 2549-2552
ΠΕΚΕ 2020, 71(4): 2549-2552

Research article
Ερευνητικό άρθρο

Chemical quality indices in local and imported beef meat

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ABSTRACT: Freshness of meat samples can be measured and evaluated by chemical tests based on their protein decomposition, lipid oxidation and/or pH values. A total of 100 random samples of local and imported beef meat (50 of each) were collected from different shops in Cairo governorate for evaluation of their chemical quality. Chemical examination of beef samples revealed that the mean values of pH, TVB/N (mg%) and TBA (mg/Kg) were 5.77 ± 0.32, 11.9±1.63 &0.58± 0.10 for local meat samples respectively , while they were 6.6 ± 0.12, 21.5 ± 1.95 & 0.95 ± 0.11 for imported samples respectively. Overall results obtained were considered important meat quality indicators. It is clear that there is a significant difference between fresh and frozen meat samples, which could be attributed to storage time and other conditions, particularly for frozen samples.

Keywords: beef, local, imported, chemical, evaluation.

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Date of initial submission: 17-02-2020
Date of revised submission: 05-04-2020
Date of acceptance: 27-04-2020

INTRODUCTION

Meat is one from the most nutrient-dense food that provides ideal conditions for microbes to grow and defines its perishable nature (Saucier, 2016). Freshness of any meat samples can be measured chemically based on protein decomposition and lipid oxidation. For example, thebreakdownofproteinleads to ammoniaproductiondue toamino acid deamination, which is reflected as liberation of volatile basic nitrogen (VBN) content (Min et al., 2007). Onthe other hand, lipid oxidation could be measured as a thiobarbituric acid value (Kruk et al., 2011). The increase in TVB/N value in the meat might be attributed to the destruction of proteinas a result of different microorganisms activity and their proteolytic enzymes (Hassan and Omama , 2011).It isimportantto mention that TBA values is a useful quality indicator for the assessment of rancidity during the storage of lipid rich food substance. Therefore, the present study was planned out to throw light on the chemicalexamination of meat samples .There is a significant effect of storage time. Beef samples are more susceptible than chicken and tuna to lipid oxidation changes, and by day 7 of storage, TBA values in beef reached above 1 mg /kg of meat samples (Khan, 2014). The TVB-N content of chilled beef increased rapidly with prolonged storage time, indicating that beef spoilage was accelerated. The average TVB-N contents increased from 9.42 mg/100g to 19.57 mg/100g at Days 1 and 13 of storage at4°C, respectively. This increase might be attributed to ammonia produced due to proteolysis of protein inthe chilled beef during storage (Li et al., 2019). pH values of meat under any kind of conditions must not exceed 6.4, otherwiseit should be considered as unfit for human consumption (Gracey and Collins, 1992) .The variation of pH values of the examined samples of meat could be attributed to the water holding capacity of the muscle proteins and storage life of the meat (Kadim et. al., 2006).

MATERIALS AND METHODS

A grand total of 100 random fresh and frozen meat samples (50 0f each) were collected from different shops in Cairo governorate. The imported meat samples were collected from raw frozen imported meat stored at a storage temperature -18°C to -20°C which were collected during its claimed shelf-life time, while the local fresh meat samples were collected from raw chilled meat stored at 4°C to 8°C, collected during the claimed shelf-life time then all samples were transferred directly to the laboratory, in an ice-box under complete aseptic conditions without any delay. Then they were subjected to following examinations to evaluate chemical quality.

Determination of pH value (ES 63/11, 2006)

Approximately 10 grams of the examined sample were homogenized with 25 ml of neutral distilled water in stomacher, and left to stand for 10 min at room temperature with continuous shaking and filtered. The pH was determined by using electrical pH meter (ACTWA-AD1200-1034678) calibrated by using two buffer solutions of exactly known pH (Alkaline pH 7.01, acidic pH 4.01) therefore, pH electrode was washed with neutralized water and then introduced into the homogenate.

Determination of Total Volatile Basic Nitrogen (TVB/N)"mg" % (ES 63/10, 2006)

A part of 10 grams of sample were minced in a stomacher for 1-2 minutes until homogenization. Then in a distillation flask add 2 grams of magnesium oxide and 300 ml distilled water to the minced sample. Make distillation and receive 100 ml distillate within 30 minute in a beaker contain 25 ml of 2% boric acid. Then titrate against H_2SO_4 0.1M until faint pink color.

$$\text{TVN mg/100g} = R \times 14$$

Where R is the volume of H_2SO_4 exhausted in titration.

Determination of Thiobarbituric Acid (TBA)"mg/Kg" (ES 63/9, 2006)

TBA number is expressed as milligrams of malondialdehyde equivalents per kilogram of sample.

A part of 10 grams of sample were blended with 48 ml of distilled water, to which 2 ml of 4% of ammonium chloride (to bring the pH to 1.5) were added in a stomacher for 2 minutes and left at room temperature for 10 minutes. The mixture was quantitatively transferred into Kjeldahl flasks by washing with additional 50 ml distilled water, followed by an anti-foaming preparation and few glass beads. The Kjeldahl distillation apparatus were assembled and the flask was heated to 50°C.

A part of 50 ml distillate was collected in 10 minutes from the time of boiling commences. The distillate was mixed, and then 5ml was pipette into a glass- Stoppard tube. 5ml of TBA reagent (0.2883g/100ml of 90% glacial acetic acid) were added. The tube was stoppered, shacked and immersed in boiling water bath for 35 minutes. A blank was similarly prepared using 5ml distilled water with 5ml TBA reagent and treated like

the sample.After heating, the tube was cooled under tape water for 10 minutes. A portion was transferred to a curette and the optical density (D) of the sample was read against the blank by means of spectrophotometer (Perkin Elmer, 2380, USA) at a wave length of 538 nm.

TBA value (mg malondialdehyde /kg of sample) = D× 7.8

D: the read of sample against blank.

RESULTS

Results show the chemical evaluation of fresh and frozen samples and revealed that PH values were varied from 4.22 to 6.80 and 6.36 to 7.32 with an average of 5.77±0.32 and average of 6.6± 0.12 for fresh and frozen meat samples respectively with a significance difference between the fresh and frozen samples, TVB/N values were varied from 6.81 to 20.95 and 14.22 to 28.4 with an average of 11.9 ± 1.63 and of 21.5 ± 1.95 for fresh and frozen meat samples respectively, moreover , and finally TBA values were in range of 0.12 to 1.02 and 0.47 to 1.55 with an average of 0.58 ± 1.63 and 0.95 ± 0.11 of fresh and frozen meat samples respectively and also there is a significance differences between the fresh and frozen samples .

DISCUSSION

Results of table (1) discuss the chemical evaluation of fresh and frozen samples and revealed that pH values were varied from 4.22 to 6.80 and 6.36 to 7.32 with an average of 5.77±0.32 and average of 6.6± 0.12 for fresh and frozen meat samples respectively with a significance difference between the fresh and frozen samples, TVB/N values were varied from 6.81 to 20.95 and 14.22 to 28.4 with an average of 11.9 ± 1.63 and of 21.5 ± 1.95 for fresh and frozen meat samples respectively, moreover, and finally TBA values were in range of 0.12 to 1.02 and 0.47 to 1.55 with an average of 0.58 ± 1.63 and 0.95 ± 0.11 of fresh and frozen meat samples respectively and also there is a significance differences between the fresh and frozen samples.

Results obtained in table (2) concluded the acceptance of samples accordingto Egyptian standard specification (ES 1522/ 2005) and revealed that 30% of fresh meat samples and 60 % of frozen meat were unfit for human consumption due to their unaccepted pH value according to ES (1522/ 2005). These findings of freshmeat samples were nearly similar to those reported by Edris et al. (2013**)** with mean values 5.69 ± 0.01, for Elbagour abattoir, 5.62 ± 0.01, for Menouf abattoir and 5.54 ± 0.01, for Shibin Elkom abattoir and Suleimenova (2016**)** when pH values ranged from 5.63±0.06 for Aberdeen Angus to 5.83±0.19 for Northern Finn cattle, low pH of meat is an indication of high quality and it is associated with a bright red colour, increased tenderness, increased flavor and increased shelf life and this may be attributed to the presence of lactic acid production during the postmortem phase as said by (Pethick et al.,1995**).** 10% of fresh meat samples and 40 % of frozen meat samples were unacceptable for human consumption due to high TVB/N content according to ES (1522/ 2005). Lower results have been reported by Edris et al. (2013) where mean values of TVB/N (mg%) were 7.63 ± 0.49 for Elbagour abattoir and 4.15 ± 0.32 for Shibin Elkom abattoir.

Table 1: Chemical evaluation of examined fresh and frozen meat samples (n = 50 each)

	Fresh meat			Frozen meat		
	Min.	Max.	Mean ±S.E.M*	Min.	Max.	Mean ±S.E.M*
pH	4.22	6.80	5.77 ± 0.32**	6.36	7.32	6.6 ± 0.12**
TVB/N	6.81	20.95	11.9±1.63	14.22	28.4	21.5 ± 1.95
TBA	0.12	1.02	0.58± 0.10**	0.47	1.55	0.95 ± 0.11**

*S. E.M = Standard error of mean

** significant difference (P<0.05).

N.B: No Significant difference in TVB/N (P>0.05)

Table 2: Chemical acceptability of examined fresh and frozen meat samples (n = 50 each)

	MPL[1]	Accepted fresh		Unaccepted fresh		Accepted frozen		Un accepted frozen	
		No.	%	No.	%	No.	%	No.	%
PH	5.6 – 6.5	35	70	15	30	20	40	30	60
TVN	< 20 mg%	45	90	5	10	30	60	20	40
TBA	< 0.9 mg/kg	40	80	10	20	25	50	25	50

MPL[1] = Maximum permissible limit according to ES (1522/ 2005).

Results obtained by Li et al. (2019) showed rapidly increase of content of TVB-N of chilled beef samples with prolonged storage time, indicating that beef spoilage was an accelerated process. The average TVB-N contents increased from 9.42 mg/100g to 19.57 mg/100g at Days1and 13 of storage at 4°C, respectively. This increase might be an indication to ammonia produced in the chilled beef during long storage and decomposition of meat.

Total volatile basic nitrogen (TVB-N) content in meat samples is a physicochemical indication in the judgment of meat freshness (Ma et al., 2013**).** According to Chinese National Standard GB2707-2016, TVB-N content is the vital direct measurement indicator form at freshness. TVB-N is an alkaline nitrogen-containing substance produced by meat protein decomposition under the action of enzymatic degradation and microbial action during the spoilage of animal-based foods (Leroi et al., 2001).

As the results of TBA, there was found that 20% of fresh meat samples 50 % of frozen meat samples were unacceptable for human consumption due to their increase of TBA value *according to ES (1522/ 2005).*, Khan (2014) recorded almost the same results of fresh samples with average (1.06 mg/kg) due to effect of storage time. While Edris et al. (2013**)** have got much lower results with mean values of TBA (mg/ Kg) were 0.18 ± 0.01 for Elbagour abattoir, 0.11± 0.01 for Menouf abattoir and 0.06 ± 0.01 for Shibin Elkom abattoir. Lipid oxidation primarily results in alkyl, alkoxyl and peroxy radicals which later result in secondary oxidation products such as aldehydes, ketones, alcohols, hydrocarbons, esters, furans, epoxides, and cyclic peroxides (Faustman et al., 2010).

The thiobarbituric acid (TBA) test is used for lipid oxidation measurements (Fernandez et. al., 1997**).** Overall, results of table (1) and Table (2) concluded that there is a significant difference between fresh and frozen meat samples.

CONCLUSIONS

Chemical examination of beef samples revealed that the mean values of pH, TVB/N(mg%) and TBA (mg/Kg) were 5.77 ± 0.32, 11.9±1.63 &0.58± 0.10 for local meat samples respectively , while they were 6.6 ± 0.12, 21.5 ± 1.95 & 0.95 ± 0.11 for imported samples respectively. Overall results which obtained were considered important meat quality indicators. It is clear that there is a significant difference between fresh local and imported frozen meat samples, which could be attributed to storage time and other handling and transportation conditions, particularly for frozen samples.

CONFLICT OF INTEREST

The authors declared no conflict of interest in this article.

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J HELLENIC VET MED SOC 2020, 71(4): 2553-2558
ΠΕΚΕ 2020, 71(4): 2553-2558

Research article
Ερευνητικό άρθρο

Effects of wild type lactic acid bacteria on histamine and tyramine formation in sucuk

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ABSTRACT: Biogenic amines (BAs) are formed by the decarboxylation of amino acids in fermented products and accumulate in these products due to the fermentation conditions, the natural microflora of the product, and the diversity of amino acids. Although they are inhibited by the human body, they are a hazard to public health. Starter cultures used in fermented sucuk should not have amino acid decarboxylase properties. The aim of the present study was to determine proteolytic activity, histidine and tyrosine decarboxylase enzyme activities of *Lactobacillus plantarum*, *Lactobacillus sake,* and *Lactobacillus curvatus* species and to evaluate the level of BA in sucuk groups containing these lactic acid bacteria (LAB). It was determined that none of the LAB generated these activities. While histamine values were not statistically significant in the sucuk groups (P> 0.05), tyramine values showed statistically significant differences (P< 0.05). The tyramine values of GI (= 1.43 ± 0.75) and GIII (= 2.73 ± 1.02) groups were lower than C (= 8.97 ± 5.29) and GII (= 7.58 ± 2.90) groups. According to the results of the study, *L. plantarum* or *L. curvatus* can provide more reliable fermented products with respect to tyramine formation. *L. plantarum*, *L. sake*, and *L. curvatus* could reduce histamine and tyramine formation in fermented sucuk.

***Keywords*:** histamine, lactic acid bacteria,sucuk, tyramine

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Date of initial submission: 21-02-2020
Date of revised submission: 17-05-2020
Date of acceptance: 09-06-2020

INTRODUCTION

Biogenic amines in foods are formed by the enzymatic decarboxylation of amino acids as a result of the activities of microorganisms (Halász et al., 1994).The accumulation of BA in foods is associated with the presence of amino acids, the presence of microorganisms containing amino acid decarboxylase enzyme, and the establishment of proper condition (Ten Brink et al.,1990). However, not only proper hygiene but also technological measures must be applied to avoid biogenic amine formation (Leroy and Vuyst, 2004; Vidal-Carou et al.,2007). Available data show that fermented products are susceptible to the accumulation of biogenic amines (Montel et al.,1999; Parente et al., 2001). Putrescine, histamine, cadaverine, tyramine, phenylalanine, spermidine, and spermine are the most common BAs in sucuk. Histamine and tyramine are the most studied biogenic amines due to their physiological activities (Silla-Santos, 1996).Histamine and tyramine have not effect on the sensory properties of the products.However, numerous cases of food poisoning have been reported related to histamine and tyramine (Lehane and Olley, 2000; Coton and Coton, 2005). The main reason for the different levels of BA determined in sucuk is associated with the amino acid amounts, decarboxylase activity of the natural microbiota, changes in the biosynthesis of BA, and the quality of meat (Shalaby, 1996). So, It has been suggested that *Micrococcus*, *Staphylococcus*, *Pediococcus,* and *Lactobacillus* spp. should be included in the quality standards during the production of fermented sausages. The microorganisms which have amine decarboxylase can contaminate foods during any stage of processing or be present due to the product's microbiota (Maijala and Eerola, 1993; Bover-Cid et al.,2000). Therefore, there is interest in taking technological measures to reduce BA formation during the producing of traditional fermented sausages, such as the use of an autochthonous starter culturewhich lacks amino acid decarboxylase activity and is well adapted to the ecology of traditional meat fermentation(Benito et al., 2007; Villani et al., 2007).*L. sake* and *L. curvatus* play an important role during fermentation under uncontrolled conditions with their competitive properties and adaptation capacity (Hammes and Knauf, 1994). During fermentation, it is necessary to consider the BA formation capacity of microorganisms (Bover-Cid andHolzaphfel, 1999). High level of biogenic amines cancause serious health problems like headache, respiratory distress, cardiac palpitation, hypertension or hypotension, facialflushing, itching, swelling, diarrhea, vomiting, migraine headache, and several allergy-related disorders, moreover anaphylactic shock syndrome and death (Silla-Santos, 1996; Sohrabvandi et al.,2012). Therefore, the amino acid decarboxylase activities of microorganisms must be taken into consideration in studies involving the addition of new starter culture collections.This study aimed to evaluate the proteolytic activity, histidine, and tyrosine decarboxylase enzyme activities of *Lactobacillus plantarum*, *Lactobacillus sake,* and *Lactobacillus curvatus* obtained from the natural microbiota of sucuk and verify them with molecular methods and to determine the level of BA in sucuk containing these microorganisms.

MATERIAL AND METHODS

Bacterial Strains

Twenty-two lactic acid bacteria that were isolated and identified from traditionally produced sucuk as *Lactobacillus plantarum*, *Lactobacillus sake*, and *Lactobacillus curvatus* were used in the study (Demirel and Gürler, 2016).*Lactobacillus* 30a ATCC 33222, *Lactobacillus brevis* ATCC 367 were used as positive control strains for histidine decarboxylase andtyrosine decarboxylase respectively. Proteolytic efficiency was determined according to the methods of Lee andSimard (1984) and Franciosi et al. (2009). The method of Mangia et al. (2013) was applied for the decarboxylase activities with the modified medium content of Maijala (1993) (Table 1).

Table 1. Decarboxylase Agar Content

Content	Quantity (L)
Tryptone	5 g
Yeast Extract	4 g
Meat Extract	8 g
Tween 80	0.5 g
$MgSO_4$	0.2 g
$MnSO_4$	0.05 g
$FeSO_4$	0.04 g
$CaCO_3$	0.1 g
Amino Acid	20 g
Brome Creosol Purple	0.06 g
Agar	20 g

Confirmation of Decarboxylase related DNA by Polymerase Chain Reaction

DNA extraction was carried out according to the manufacturer's instructions (Thermo Scientific, K0721).Polymerase Chain Reaction (PCR) amplification of DNA samples was performed according to the method of Marcobal et al. (2005). The PCR was

performed in a 25 µl amplification reaction mixture containing 20 mM Tris-HCl, pH8.0, 50 mM KCl; 2.5 mM $MgCl_2$; each 200 µM dNTP; 1 µM primers, 1U Taq polymerase and 12.5 ng target DNA (Marcobal et al., 2005).

Marcobal et al. (2005), Coton and Coton (2005) methods were modified and used for amplification. The reactions were carried out in a Thermal Cycler(Thermal Cycler, Biocyler, TC-S, Programmable thermostat)using the following cycling parameters: 5 min for the first denaturation at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 48 °C, 1min at 72 °C, and a final extension step of 7 min at 72 °C. The primer pairs used in the amplification process are shown in Table 2. Amplified products were examined on a 1.8% agarose gel with stained ethidium bromide.

Table 2. Oligonucleotide Primer pairs used in the PCR method for LAB

Target Gene[a]	Primer Sequence(5'-3')	Amplicon size (bp)	Reference
hdc	F: AGATGGTATTGTTTCTTATG R: AGACCATACACCATAACCTT	367 bp	Marcobal et al. (2005)
tyrdc	F: GCATACCAGAGTCCCTCAAG R: CGGATACGGACGCACAATTG	906 bp	Lucas et al. (2003)

[a]*hdc*, histidine decarboxylase; *tyrdc*, tyrosine decarboxylase

Inoculation of Lactic Acid Bacteria on Sucuk and Sucuk Production

L. plantarum, *L. sake,* and *L. curvatus* which did not show proteolytic, histidine, and tyrosine decarboxylase activities verified with molecular method were selected for sucuk production. Fresh cultures were prepared using physiological saline dilutions until the desired McFarland turbidity was achieved (Öztürk, 2013).After the degree of McFarland turbidity was determined to correspond to 10^7-10^8 cfu/g, microorganism suspensions were prepared and added to the sucukbatter.The LAB groups are given in Table 3.

Table 3. The lactic acid bacteria groups.

Groups	The combination of starter culture
Control (C)	Non starter culture
Group I (GI)	Sucuk Batter + *Lactobacillus plantarum*
Group II (GII)	Sucuk Batter + *Lactobacillus sake*
Group III (GIII)	Sucuk Batter + *Lactobacillus curvatus*

The formulation and method indicated by Gökalp et al. (2004) was modified and used for sucuk production. The additive ratios are shown in Table 4. Maturation conditions were applied according to the method of Kaban and Kaya (2007).

Table 4. The additives and their proportions

The additives	The ratios (%)
Salt	1.6
Sugar	0.4
Garlic	1
Red pepper	1.4
Black pepper	0.4
Cumin	0.9
Pimento	0.1

Measurement of Biogenic Amines

BAs were measured by the method of Köse et al. (2011). According to the method, 5 g of the sample was taken and 50 mL of 0.1 M hydrochloric acid was added. The homogenized mixture was centrifuged at 4000 rpm at 4ºC for 20 minutes. After the supernatant was removed, 100 µL of 2 N sodium hydroxide, 150 µL of saturated sodium bicarbonate, and 1 ml of dansyl chloride were added. The mixture was incubated at 40ºC for 45 minutes. After incubation, it was kept at room temperature for 10 minutes. 50 µl of 25% NH_3 was added and left to stand for 30 minutes at room temperature. Finally, 5 mL of ammonium acetate: acetonitrile was added and passed through a 0.45 µm filter and injected into the HPLC system(Shimadzu Prominence, ACE5 C-18 (250 x 4,6 mm, 5 µm)) (Köse et al., 2011). Histamine and tyramine analysis of the sucuk samples were carried out in Burdur Mehmet Akif Ersoy University Scientific and Technology Application and Research Center, Turkey. The analyses were repeated three times. The mean of three measurements was used in statistical analysis.

Statistical Analysis

The normality of data was verified using the Shapiro-Wilk test (P >0.05). One-Way ANOVA test was used to compare the variables according to the experimental groups. Moreover, the LSDpost-hoc test was used at a 5% significance level. The analyses were conducted with SPSS 22.0 program.

RESULTS

None of the LAB isolates (12, 6, and 4 of them were *L. plantarum*, *L. sake*, *L. curvatus*, respectively) displayed proteolytic, histidine, and tyrosine decarboxylase activities with culture methods. Subsequently, the negative activities of the isolates were verified by PCR. The amounts of histamine and tyramine obtained from the HPLC analysis are given in Table 5.When histamine and tyramine values were compared according to sucuk groups, it was determined that differences in the histamine values were not statistically significant (P>0.05).On the other hand, the differences in tyramine values among the sucuk groups were statistically significant (P<0.05).Accordingly, the tyramine values of GI (= 1.43 ± 0.75)and GIII (= 2.73 ± 1.02) groups were lower than C(= 8.97 ± 5.29) and GII (= 7.58 ± 2.90) groups.

Table 5. Comparison of histamine and tyramine values according to sucuk groups

BA		N	Mean ± SD	F	P
Histamine (mg/kg)	C	3	11.42±5.82	1.818	0.222
	GI	3	15.08±4.94		
	GII	3	7.17±1.51		
	GIII	3	9.41±3.71		
Tyramine (mg/kg)	C	3	8.97±5.29^{a}	4.227	**0.046***
	GI	3	1.43±0.75^{b}		
	GII	3	7.58±2.90^{a}		
	GIII	3	2.73±1.02^{b}		

Note: Values are the mean of triplicate measurements ± standard deviation; *P < 0.05; a,b: The different letters indicate the difference between the groups; N; Number of analysis.

DISCUSSION

The phenotypic properties of LAB may vary during fermentation, geographical conditions, and the origin of the isolates. It has been recommended to use molecular methods in LAB researches since false positive or false negative results can be obtained by culture methods. In this study, biogenic amine geneshave been confirmed by molecular methods. However, the same species of LAB may also be positive for amino acid decarboxylase. While Choudhury et al. (1990), De Llano, (1998), Leuschner and Hammes (1998), Bover-Cid and Holzaphfel (1999) submitted tyrosine decarboxylase of certain LAB; Dapkevicius et al. (2000), Maijala (1994), Bover-Cid and Holzaphfel (1999) reported histidine decarboxylase activity. Moreover, Straub et al. (1995) declared both histidine and tyrosine decarboxylase activity by culture methods. On the other hand, Maijala, (1993); Silla-Santos (1998); Montel et al. (1999); Bover-Cid et al. (2001) did not find histidine decarboxylase activity. AlthoughConstantini et al. (2006) reported that no histidine decarboxylase gene region in the LAB or tyrosine decarboxylase gene region in any of the others except for*L. brevis*,De las Rivas et al. (2008), Ruiz-Moyano (2009), Landeta et al. (2013) reported that some LAB contained tyrosine decarboxylase gene regions in molecular studies. It has been reported that BA levels can be reduced in fermented sucuk by using non-BA starter cultures and the ensuing competition with non-starter LAB (Maijala, 1994). BA formation is an important criterion for the selection of starter culture to be used in sucuk production. In this study, it has been determined with both culture and molecular methods that none of the evaluated strains affected BA formation.However, BA formation can be observed in fermented sucuk depending on the diversity of the microbiota, the competitive properties with other LAB, and the fermentation conditions.

According to the HPLC results, *L. sake*and control group weremore effective than *L. plantarum* and *L. curvatus*in terms of tyramine formation. Histamine and tyramine formation efficiency can be sorted from high to low as *L. plantarum*, *L. curvatus*,and*L. sake*; *L. sake*, *L. curvatus*, and *L. plantarum*, respectively. Various researchers (Ten Brink et al.,1990; Buncic et al., 1993; Şenöz et al., 2000; Ekici et al.,2004; Erkmen and Bozkurt, 2004) informed that histamine con-

centration varied between 1 mg/kg and 478.2 mg/kg in fermented sucuk.

The use of starter culture has decreased the histamine level in the study. However, the effect of *L. planturum* on histamine formation may change this situation compared to the control group.This difference may be due to the poor ability of *L. plantarum* to compete with microorganisms or non-starter LABas a result of initial contamination in the sucuk batter or during fermentation.

Erkmen and Bozkurt (2004) detected between 1.2 and316.3 mg/kg tyramine levels in 50 sucuk samples. Although using starter culture in Turkish sucuk reduces the formation of putrescine, spermine, and histamine,it does not affect the formation of tryptamine, 1.7-diamino heptane, serotonin, and spermidine(Bozkurt and Erkmen, 2004). After maturation at 15 ºC for 21 days, the level of tyramine was determined as 30 mg/kg in the sucuk samples which contained *L. curvatus* and *Staphylococcus xylosus.* Whereas, the tyramine level detected in the control group which did not contain starter culture was detected as 85 mg/kg.Şenöz et al. (2000) declared that the amount of tyramine in sucuk withoutstarter culture was 400-617 mg/kg. Furthermore, tyramine levels in the samples with starter culture have been reported as 125-1173 mg/kg. In summary, the amount of BA in sucuk with starter culture was significantly lower than sucuk samples with non-starter culture (Şenöz et al.,2000).It has been noted that *L. sake* is more effective in the formation of tyramine than *L. plantarum* and *L. curvatus* in the study. Taking into consideration the amount of tyramine in fermented sucuk,with *L. plantarum* or *L. curvatus* as mixing cultures it can be stated that such fermented products are more reliable than with other starter cultures.

CONCLUSION

Although fermented sucuk production has been carried outusing starter culture without the ability to form BA,a certain amount of BA formation which is harmful to human health is observed. The human body has a mechanism that decreases BA levels to a certain level. However, these mechanisms may not be effective under some circumstances like gastrointestinal disease, alcoholism. Therefore, control measures must be taken and monitoring systems of every production step until consumption to ensure hygienic quality, production, and storage conditions. When the relationship between the starter cultures has been examined, it has been found that *L. plantarum* and *L. curvatus*, having negative proteolytic and decarboxylase activity, significantly reduce tyramine levels. Similarly, histamine levels have been decreased by *L. sake* and *L. curvatus*in comparison to the control group. BAs are known to be food quality indicators and harmful to health. Therefore, it can be said that the use of starter culture reduces histamine and tyramine formation. Probiotic microorganisms in fermented products can be protected from environmental conditions by surrounding meat and fat. Also, they can produce bacteriocins or low molecular weight antibacterial compounds to combat pathogens and form the dominant flora.The LAB used in this study may have such an effect on biogenic amine forming microorganisms. However, further studies are needed to determine how they reduceBA formation. It has been determined that classical culture methods can give false positive results in BA decarboxylase determination.Therefore, molecular methods are recommended. The values determined in the study are below the toxic limits. However, this level does not mean that there is no risk for susceptible individuals.The activity of LAB on BA formation is particularly important for fermented foods. Biogenic amine formation factors should be identified and protective measures taken to during the product development to ensure public health.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENT

The authors declare that they have no conflict of interests. The research was supported by Afyon Kocatepe University, Scientific Projects Research Coordination Centre as 17.KARİYER.144 project number.

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J HELLENIC VET MED SOC 2020, 71(4): 2559-2568
ΠΕΚΕ 2020, 71(4): 2559-2568

Research article
Ερευνητικό άρθρο

Determinant and conditioning factors of feline asthma: a questionnaire-base study

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ABSTRACT: Feline asthma is a chronic inflammatory disease of the lower respiratory airways that has shown an increased incidence in the past decades, aside with human asthma. It is also important to acknowledge that human and feline asthma are very similar in their pathophysiology,being the housing conditions (pollutants, stress and obesity) major risk factors to its development. The present study aimed to investigate if these housing conditions could be determining and conditioning factors associated with the occurrence of feline asthma previously reported in literature.

A cross-sectional (self-completed) questionnaire-based study targeting Portuguese-speaking owners of cats was carried out, validated and applied (in both paper and digital form) between September 2018 and March 2019.

A total of 189 questionnaires were analysed, of which 18 corresponded to cats with respiratory disease. Most of studied cats were of mixed breed and neutered, living indoor exclusively, mainly in urbanareas and from the north mainland region. According to the owner's perception, the cats were mostly active and with the ideal weight. The clinical signs more often associated with asthma crisis were respiratory wheezes and cough, whereas the worsening of such clinical signs occurred mostly in spring. Stress symptoms were not common, but the correlation between stress-related diseases and asthma was close to significance *(P*=0.065). A mixed lifestyle was associated with less symptoms of stress *(P*=0.032). Although close to significance *(P*=0.07), the presence of pollutant industries was not associated to asthma in the enrolled cats. Finally, in most of the houses in which an asthmatic cat lived in, no owner or other co-inhabitant had asthma.

The paucity of similar epidemiological studies in cats demonstrates the importance of the current work and the need to conduct further studies on housing conditions associated with the disease. Eventually, further studies will clarify if cats could be used as sentinels for human asthma.

Keywords Allergens, conditioning factors, housing conditons, environment, feline asthma

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Date of initial submission: 27-02-2020
Date of revised submission: 03-05-2020
Date of acceptance: 13-08-2020

INTRODUCTION

Feline asthma is a chronic and recurrent inflammatory disease of the lower respiratory airways and it is becoming one of the most frequent disease affecting the respiratory tract of cats, causing significant morbidity and even mortality (Reinero, 2011; Taylor, 2017; Rosenberg and Druey, 2018).

Asthma is characterized by a reversible airflow limitation caused by an airway hyperactivity, increased mucus production and smooth muscle hypertrophy due to lower airway inflammation - type I hypersensitivity reaction (Taylor, 2017; Venema and Patterson, 2010).

According to recent literature, asthma affects approximately 1-5% of cats across all ages, breeds and genders. Nevertheless some studies described an increased incidence in the Siamese breed and age of occurrencebetween four and five years, although it can occur at any age (Venema and Patterson, 2010; Kettner, 2018; Rosenberg and Druey, 2018).

Feline asthma is the most frequently diagnosed lower airway disease.However,there are other non-infectious, inflammatory diseases affecting cats featuring the sampathophysiology and are often not properly diagnosed or misdiagnosed as asthma (Taylor, 2017).

Aswith feline asthma, an increase in human asthma was noticed during the latter part of the 20thcentury, in developed countries. In fact, the disease itself is very similar. The coexistence of cats and humans in the same environment raises the hypothesis that it can be triggered by common risk factors and that cats can be considered as sentinels for human asthma (Heller et al., 2014; Toskala and Kennedy, 2015; Mueller et al., 2016; Neo and Tan, 2017).

Bermuda grass, house dust mites and other grasses and weeds, cat litter, strong chemical smells, building dust, perfumes, hairspray, tobacco smoke, and other environmental pollutants, are associated with the development of the disease in cats, being most of them also common to human asthma (Reinero, 2009; Reinero, 2011; Fehrenbach et al., 2017; Taylor, 2017).

On the other hand, some of the common risk factors associated with human asthma, like obesity and stresshave not been studied in cats (Reinero, 2011; Toskala and Kennedy, 2015).These were positively correlated to an increase in childhood asthma in humans, and even though their prevalence is increasing in both species, such correlation has not yet been confirmed in cats (Toskala and Kennedy, 2015).

Indoor pollution, along with outdoor pollution, has also been recognized and became an important risk factor in human asthma and probably also in feline asthma, as reported in recent studies (Lin et al., 2018).

Furthermore, in human asthma according with the hygiene hypothesis an exposure to infection and allergens in infancy is correlated with a decrease of allergic diseases in adulthood (Toskala and Kennedy, 2015). Similarly previous studies showed a negative correlation between neonatal exposure to allergens and the development of feline asthma (Heller et al., 2014; Toskala and Kennedy, 2015).

Given the above, the present study aimed to investigate if some of the housing conditions previous reported could be determining and conditioning factors associated with the occurrence of feline asthma.

MATERIALS AND METHODS

Data collection

This was a cross-sectional (self-completed) questionnaire-based study targeting portuguese ownersof cats with or without asthma diagnosed by the refereeing veterinarian. The inclusion criteria included being a cat owner with at least 18 years old, living in Portugal or Portuguese speaker, and living with and taking care of the cat.Exclusion criteria were based on the extent of reply of the questionnaire, being excluded if one or more sections were not completely answered.

The study design complied with the recent General Data Protection Regulation (EU, 2016)as well as other relevant legislation. The University's Scientific Council approved the study (Minuten#44_CC_2018 of October 10th 2018).

The initial drafting version of the questionnaire was constructed after a literature review regarding feline and human asthma. To enable internal validation, a panel (comprisingone veterinary epidemiologist, one statistical professional and five small animal clinicians) was asked to answer the questionnaire and give their direct feedback to the authors, regarding the extension and structure of the questionnaire, and if responseswere clear and correctly evaluated the issue, allowing data analysis.In addition, in the validation phase, 10 cat owners were also asked to answer the questionnaire to assess the extension of the questionnaire and if responses were unambiguous and of sim-

ple understanding. The questionnaire was adjusted accordingly and the final version included 55 questions structured in five main sections, regarding information on [A] animal identification and characterization, with 9 questions, [B] respiratory disease, with 10 questions, [C] animal stress, with 18 questions, [D] animal physical condition, with 4 questions and [E] animal environmental pollutants, with 14 questions.

The first section [A], included questions regarding the breed, age, sex and reproductive stage, weight, district of residence, lifestyle and acquisition site.

In the second section [B],the questions addressed the presence or absence of respiratory disease. If present, the frequency and characteristics of the episodes and in which type of scenarios it worsened. In this same section, questions were included relating to the exposure and development of diseases in the paediatric period.

In the third section [C], to perceive the animal's stress level,a grading scheme (Mariti et al., 2017; Atkinson, 2018) with several circumstances related to stress was presented. Besides playing, hiding, appetite, vocalizing and sleep and hygiene habits, the existence of other animals and their behaviour with the cat was also questioned as well as the occurrence of urinary tract disease symptoms.

The fourth section [D] considered the cat´sphysical condition through the adaptation of a body condition score scheme system based in a five-point scale adapted from the WSAVA (World Small Animal Veterinary Association) scale (Laflamme, 2013), it was also assessed the cat´s activity and the amount of treats given.

The fifth and last section [E],was directed at the environment in which the animal lives. Absence or existence (with corresponding distance)of outdoor main pollution producers, as industries and highways, was questioned. As for indoor pollutants, questions regarded the use of chemicals, gas ovens, fireplaces and smoke. Lastly, it was asked if the owner or other co-inhabitant relatives were diagnosed with asthma.

Both paper form (hard copies) and digital form questionnaires were distributed. Paper questionnaires were distributed via personal contacts and via veterinary clinics. The digital questionnaire was developed in Google platform Google forms™ following the same structure as the paper questionnaire. Digital questionnaires were disseminated through email to different veterinary care centres, as well as in social media platforms, namely Facebook™ and Instagram™. Questionnaires were received between September 2018 and March 2019.

Data analysis

Before analysis, coding was applied to all the questions. A descriptive analysis of the responses to each question was performed. After this, a series of independence tests with Chi-Square, trough SAS (Statistical Analysis System) program PROC FREQ (SAS Institute Inc., 2019), was performed to evaluate associations between the different variables included in the questionnaire.

The probability of an animal having respiratory disease was analysed by logistic regression with the same SAS program, with a model that included the several risk factors individually. Subsequently, probability ratios were estimated between the different risk factors levels that significantly influenced the probability of respiratory disease development in inquired animals.

Other variables, as weight, were submittedto several variance analyses developed with SAS program PROC GLM, to understand which significantly influenced it individually.

RESULTS

From the210 questionnaires received, only 189 (90.0%) met the defined inclusion and exclusion criteria. Of the 189 included, 27 were responded in paper form, with only two cats with diagnosed asthma, whereas the remaining ones (n=162) were replied in digital form, of which 16 cats were diagnosed with asthma.

Individual identification and characterization

Among the study population (characterization summarized in table 1),the breed with the higher prevalence was the European or mixed breed (82.0%). The male/female ratio was practically equal (95:93), being most of the cats neutered (78.3%). The mean age was 5±3 years old, ranging from 1to 15 years old and the mean weight was 4±1kg, ranging from 0.5 to 8kg.

Geographical origin of participating owners was broad, with questionnaires been completed also by Portuguese speakers living in foreign countries. Considering only the Portuguese mainland residents (96.6%), the higher number of respondents lived in the north region (48.6%; Figure 1).

Table 1- Characterization of studied cat population by signalment, lifestyle and acquisition

Variable	Categories	Absolute frequency (Relative, %)
Breed	European or mixed breed	n = 155 (82.0%)
	Siamese	n = 16 (8.5%)
	Other	n = 18 (9.5%)
Gender	Male	n = 95 (50.2%)
	Female	n = 93 (49.2%)
	No response	n = 1 (0.5%)
Reproductive status	Neutered	n = 148 (78.3%)
	Non-neutered	n = 40 (21.2%)
	No response	n = 1 (0.5%)
Age		
	< 3 years	n = 91 (48.2%)
	4 - 7 years	n = 48 (25.4%)
	8 - 11 years	n = 31 (16.4%)
	12 - 15 years	n = 14 (7.4%)
	> 15 years	n = 2 (1.0%)
	No response	n = 3 (1.6%)
Weight		
	< 2kg	n = 18 (9.5%)
	3 - 5kg	n = 126 (66.7%)
	6 - 8kg	n = 30 (15.9%)
	No response	n = 15 (7.9%)
Residence area	North mainland region	n = 89 (47.1%)
	Centre mainland region	n = 70 (37.0%)
	South mainland region	n = 18 (9.5%)
	Extra-continental	n = 6 (3.2%)
	No response	n = 6 (3.2%)
Lifestyle	Outdoor only	n = 10 (5.3%)
	Outdoor & indoor	n = 62 (32.8%)
	Indoor only	n = 117 (61.9%)
Acquisition	Street	n = 108 (57.1%)
	Pet shop	n = 10 (5.3%)
	Breeder	n = 8 (4.2%)
	Acquaintance	n = 61 (32.3%)
	No response	n = 2 (1.1%)

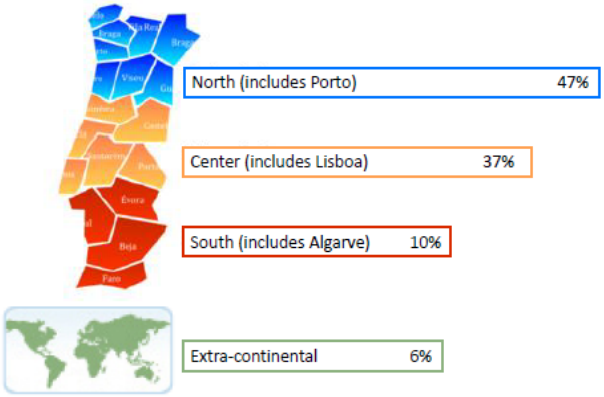

Figure 1. Geographical distribution of inquired cats

Regarding lifestyle, the vast majority of respondents identified their cats as "indoor only" (61.9%).In most cases (57.8%) cats were taken off the streets, and only 5.3% were commercially acquired in pet shops. None of the variables addressed in this sectionwas found to be statistically associated with asthma.

Feline asthma

Respiratory disease was only present in 18 (9.5%) of' cats, of which 16 were diagnosed with feline asthma. The mean age of the cats with respiratory disease (Table 2) was 6±3 years old, ranging from 1to 15 years. Their mean weight was 4±1kg, varying from 3up to 6kg.

Of those diagnosed with feline asthma, 43.8% (n=7/16) were diagnosed before the first year of age and 37.5% (n=6/16) between the second and the fifth year.

The signsthat owners more often associated with asthma crisis were respiratory wheezes and cough (Figure 2).

Table 2. Characterization of cats with respiratory disease studied by signalment, lifestyle and acquisition

Variable	Categories	Absolute frequency (Relative, %)
Breed	European	n=13 (72.2%)
	Siamese	n=3 (16.7%)
	Other	n=2 (11.1%)
Gender	Male	n = 9 (50.0%)
	Female	n = 9 (50.0%)
Reproductive status	Neutered	n = 17 (94.4%)
	Non-neutered	n = 0 (0%)
	No response	n = 1 (5.6%)
Age		
	< 3 years	n = 6 (33.3%)
	4 - 7 years	n = 6 (33.3%)
	8 - 11 years	n = 4 (22.2%)
	12 - 15 years	n = 2 (11.1%)
	> 15 years	n = 0 (0%)
Weight		
	< 2kg	n = 0 (0%)
	3 - 5kg	n = 15 (83.3%)
	6 - 8kg	n = 1 (5.6%)
	No response	n = 2 (11.1%)
Residence area	North mainland region	n = 10 (55.6%)
	Centre mainland region	n = 7 (38.9%)
	South mainland region	n = 1 (5.5%)
	Extra-continental	n = 0 (0%)
Lifestyle	Outdoor only	n = 0 (0%)
	Outdoor & indoor	n = 5 (27.8%)
	Indoor only	n = 13 (72.2%)
Acquisition	Street	n = 8 (44.4%)
	Pet shop	n = 3 (20%)
	Breeder	n = 1 (5.5%)
	Acquaintance	n = 6 (33.3%)

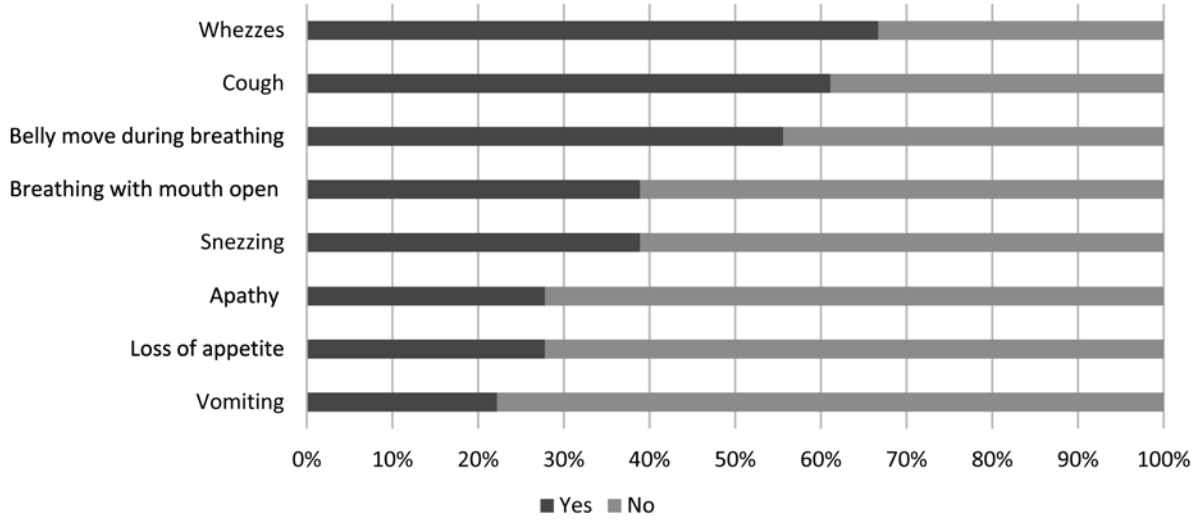

Figure 2. Signs associated with asthma crisis among diagnosed cats (n=16)

There are also situations in which the owners observed a worsening of clinical signsor that were associated with triggering an asthma crisis. As detailed in Figure 3, owners reported worsening of clinical signs mostly associated with season (specifically spring).

With the results gathered it was possible to identify some features of the disease in asthmatic cats, as displayed in Table 3. Crisis and medication intake were not frequent, with nearly half of the animals being under no medication.

Most cats had access to the outdoors in the paediatric age, and most did not develop any disease throughout that period.However, when they developed disease during the paediatric age, it was mainly asthma.

Stress

The frequency of the majorsituationsthat are commonlythe cat´s manifestations of stress as reported by respondent owners are displayed in Figure 4. To those that usually vocalize during traveling it was asked the frequency of the travelling, and most of them did it only about one to two times a year (83.3%).

Regarding other co-inhabitant animal as a potential source of stress, most of the enrolled cats lived with another cat(s) (68.3%), but co-inhabitants were also from other species, namely dogs, birds or small mammals. None of co-inhabitant cats were diagnosed with feline asthma, and the majority slept, ate and played together (Figure 5).

Table 3. Features of the disease in asthmatic cats

Variable	Categories	Absolute frequency (Relative, %)
Crisis frequency	1-2 times a year	n =7 (43.8%)
	Monthly	n =4 (25.0%)
	Weekly	n =1 (6.3%)
	Daily	n = 1 (6.3%)
	No response	n = 3 (18.6%)
Asthma medication	Yes	n = 9 (56.2%)
	No	n = 7 (43.8%)
Last medication	Daily	n = 2 (12.5%)
	Weekly	n = 1 (6.3%)
	Monthly	n = 1 (6.3%)
	In the last 6 months	n = 2 (12.5%)
	In the last year	n = 4 (25.0%)
	No response	n = 6 (37.4%)
Exterior access during pediatric age	Yes	n = 9 (56.2%)
	No	n = 3 (18.8%)
	No knowledge	n = 4 (25.5%)
Respiratory disease in pediatric age	Yes	n = 4 (25.0%)
	No	n = 6 (37.5%)
	No knowledge	n = 6 (37.5%)
Disease developed in pediatric age	Feline asthma	n = 4 (25.0%)
	No response	n = 12 (75.0%)

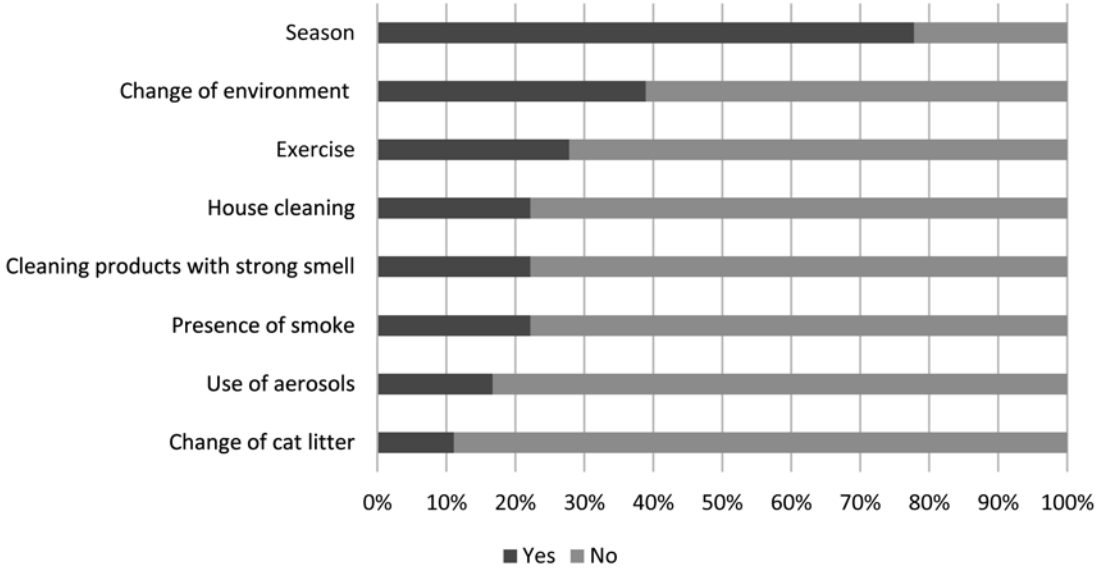

Figure 3. Circumstances associated with increased clinical signs or triggering crisis

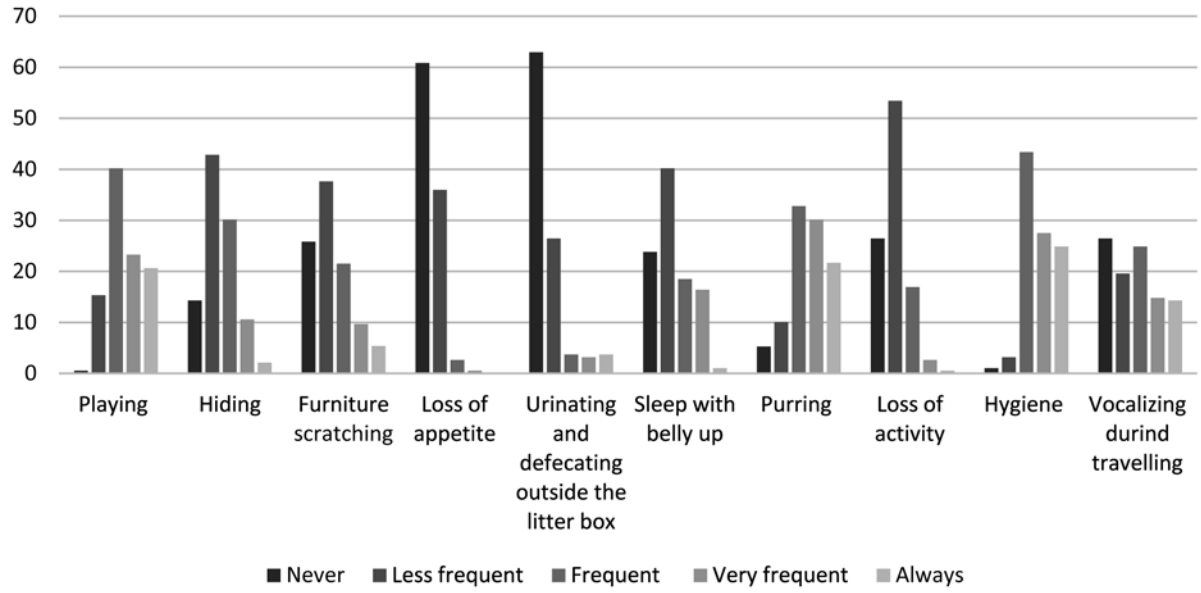

Figure 4. Relative frequency (%) of signs associated with cat stress, as reported by owners

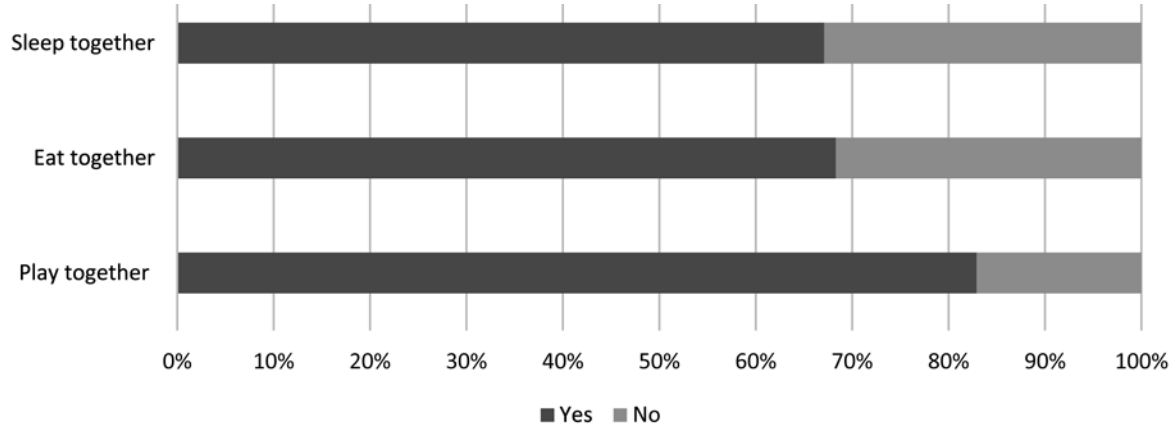

Figure 5. Stress related behaviors in the presence of co-inhabitating cats

Diseases correlated to stress had low prevalence (10.6%), with urinary tract disease symptomsas the most frequently reported one (65%). It was observed a significant association between lifestyle and stress *(P*=0.032). The smaller percentage of animals with disease (3%; n=2/62) were the ones that had access to both indoor and outdoor. Additionally, although not statistically significant, the correlation between stress-related diseases and asthma was close to significance *(P*=0.065).

Body condition

Most owners identified their cat as being in the ideal weight (54.4%) and active (84.4%). The majority did not give treats frequently to their cats (64. 2%) (Table 4).

It was possible to prove a significant relation between weight and age, Weight was significantly influenced by age*(P*< 0.01). There was no specific association found with asthma.

Environment

The majority of the respondents lived in an urban environment (55.6%). Among those living in urban areas, a public park or gardenexisted in 71.8% of cases, frequently (87.7%)less than 1 km away.

Regarding smoking habits, most respondents had no smokers in the house (66.1%), but of those who actually smoked, most did so in the areas to which the cat had access (48.4%).

Other indoor pollution sources included gas ovens or heaters (42.0%), fireplaces (36.5%) and aerosol-sources (28.0%).

Proximity with highways or freeways was observed in 32.3% of all enrolled cats, being in most cases at two to 5km away (61.4%) (Table 5).

Table 4. Characterization of body condition regarding all the studied population and cats with asthma/respiratory disease.

Variable	Categories	Absolute frequency (Relative, %)		P value
		Total cat population (n=189)	Cats with asthma/ respiratory disease (n=18)	
Body condition	Lean	n = 42 (22.2%)	n = 2 (11.1%)	*P*=0.38
	Ideal	n = 102 (54.0%)	n = 10 (55.5%)	
	Overweight	n = 44 (23.3%)	n = 6 (33.3%)	
	No response	n = 1 (0.5%)	n = 0 (0%)	
Activity	Active	n = 157 (83.1%)	n =13 (72.2%)	*P*=0.35
	Sedentary	n = 29 (15.3%)	n = 4 (22.2%)	
	No response	n = 3 (1.6%)	n = 1 (5.6%)	
Treat given	Frequent	n = 67 (35.4%)	n =4 (22.2%)	*P*=0.21
	Not frequent	n = 120 (63.5%)	n =14 (77.8%)	
	No response	n = 2 (1.1%)	n = 0 (0%)	

Table 5. Characterization of environment regarding all the studied population and cats with asthma/respiratory disease

Variable	Categories	Absolute frequency (Relative, %)		P value
		Total cat population (n=189)	Cats with asthma/ respiratory disease (n=18)	
Urban vs. Rural	Urban	n = 105 (55.6%)	n =8 (44.4%)	p=0.30
	Rural	n = 84 (44.4%)	n =10 (55.5%)	
Smoking	Yes	n = 52 (27.5%)	n = 5 (27.8%)	p=0.50
	No	n = 125 (66.1%)	n = 13 (72.2%)	
	No response	n = 12 (6.4%)	n = 0 (0%)	
Pollutant industries	Yes	n = 19 (10%)	n = 4 (22.2%)	p=0.07
	No	n = 170 (90%)	n = 14 (77.8%)	
Highways/ freeways	Yes	n = 61 (32.3%)	n = 6 (6.4%)	p=0.92
	No	n = 128 (67.7%)	n = 12 (66.7%)	

Presence of nearby pollutant industries only occurred for 10.1% of the participants and most of them were 2to 10km away (62.5%). Although close to significance *(P*=0.07), the presence of these industrieswas not correlated to asthma in enrolled cats.

None of the remaining environmental factors showed to be significantly associated with asthma.

Finally, in the last question that inquired if anyone living in the household had asthma, most answered negatively (78.8%). In addition, 93% of asthmatic individuals did not owned or lived with an asthmatic cat.

DISCUSSION

This study enrolled a considerable number of animals (n=189), nevertheless most of the parameters tested did not had a statistically significant association with feline asthma. This was probably because of the small number of asthmatic cats (n=16) and the fact that feline asthma is frequently underdiagnosed. In fact, asthma was present in approximately 8.5% of the animals analysed, which is slightly superior to the results found in literature: 1-5% (Venema, 2010; Kettner, 2018; Rosenberg and Druey, 2018). A possible justification may lie in the fact thatthe questionnaire was disseminated online as being a study regarding respiratory disease in cats. It is also important to mention that the diagnosis of feline asthma was not confirmed.

There is an increasing incidence of asthma, in both humans and cats, living in the same housing conditions (Neo and Tan, 2017). In addition, consideringthe studies conducted in humans, most are focused in treatment and not so much in prevention. It would be important to conduct further studiesto try to understand if exposure to the same risk factors can lead to the development of the disease both in humans and cats or even if cats could be used as sentinels for the disease in humans (Neo and Tan, 2017).

Although most of the associations proposed were not statistically proven,this study enabled us to better characterize cats with respiratory disease, namely asthma.Such as reported in previous studies (Venema and Patterson, 2010; Kettner, 2018; Rosenberg

and Druey, 2018) no correlation was found between asthma and the breed, age or sex of the cats, probably given the reduced number of enrolled asthmatic cats. However, other studies found a higher prevalence in the Korat (19%) and Siamese (7%) breeds (Vapalahti et al., 2016).

Disease prevalence in the different Portuguese areas, which was not studied up until now, was higher in the north mainland region (55.5%), although no significant association was found between asthma and a Portuguese region in particular.

It was observed that respiratory wheezes and coughingwere the most frequently reported signsassociated with asthma crisis. Season was the main reported leading cause ofworsening of clinical signs, probably related to aeroallergenslike pollens and fungal spores. These aeroallergens, usually associated with spring,are capable of inducing bronchial obstruction therefore increasing asthma symptoms in humans (D'Amato et al., 2005), and probably also in cats.

Other aspects of the cat's life have also been hypothesized as being associated with asthma, namelypaediatric exposure to pathogens. In both human and feline immunity, benefits were found with exposure in the paediatric period and the development of diseases (Heller et al., 2014; Toskala and Kennedy, 2015), although recurrent respiratory infections in infancy has been correlated with the development of asthma in humans later on (Arshad et al., 2005; Subbarao et al., 2009). A previous study (Heller et al., 2014) determined that exposure to allergens in early neonatal period of the cat, until the fourth to sixtieth month of age, prevented the development of airway eosinophilia when exposed to the same allergens later onespecially in children. Rodent and human studies suggest an opportunity to \"prevent\" asthma in the perinatal period. The aims of this study were to create a more \"natural\" model of feline asthma by exposing offspring of asthmatic queens to Bermuda grass allergen (BGA.In the cited study, no statistical association between early exposure to allergens and asthma was found, although most of the animals in the study evidenced paediatric exposure and did not develop any respiratory disease in early life nor in adult life.

Even though there are no studies analysing possible correlation between asthma and stress in cats, in humans evidence exist that suggests a casual association between both (Reinero, 2011; Toskala and Kennedy, 2015). For example, in children living in the north hemisphere there is a predictable annual exacerbation of symptoms every September, correlated to seasonal allergens and possibly to the stress of returning to school (Subbarao et al., 2009). Thus, it could be reasonable to assume that in cats other stress-related circumstances could lead to exacerbation of clinical signs (Atkinson, 2018; Mariti et al., 2017). It is worth mentioning that,in the present study, the association of stress related diseases with asthma was close to significance *(P*=0.065).

An association was also described with asthma and obesity in humans (Venema and Patterson, 2010; Toskala and Kennedy, 2015). However, Subbarao et al. (2009) suggested that such relation might be casual given their mutual increase. Associated with obesity, a sedentary lifestyle was also positively associated with asthma symptoms in humans (Beasley et al., 2015). Nevertheless such significant association was not found in the present study.

Although there are studies relating a rural environment to a reduced risk of development of asthma in childrensuch relation was not found in this study. On the other hand it was observed an increased prevalence of asthma in humans in urban areas for diverse factors like vehicle emissions and industrialization (D'Amato et al., 2005; Beasley et al., 2015). Although no positive association between asthma and urban area residence was determined, the association between proximity with pollutant industries and occurrence of feline asthma was close to significance *(P*= 0.07). Along with industries, tobacco smoke, chemicals, and highways are recognized as producers of different pollutants that can lead to the development of the disease and that are associated with increasing respiratory symptoms in children (D'Amato et al., 2005; Taylor et al., 2017; Lin et al., 2018;). Nevertheless, such associations were not found to be significant in the current study.

Lastly, it was asked if someone in the house was diagnosed with asthma, however such association was not found.

This study provided important information regarding overall housing and health conditions of Portuguese cats. Most of the cats are of European or mixed breed and sterilization is a common practice, among the cats enrolled in the study. In addition, most of the cats (61.9%) live exclusively indoor which is in line with the previously described (64%; GFKTrack. 2Pets, 2015). As for stress-related parameters most of

them were very low or even non-existent, suggesting that studied cats have a low level of stress, or at least they were not detected by the criteria used to evaluate this parameter.

Likewise, obesity was not frequent, with the majority of responses indicating an ideal body condition of the cats.Nonetheless, these results must consider that ownersanswered the questionnaire, which can influence body-conditioning scoring results. Owners most frequently consider their animals as having an ideal body condition even when a graphic chart is used (Eastland-jones, German, Holden, Biourge, and Pickavance, 2014)

CONCLUSIONS

This questionnaire-based study directed to Portuguese-speaking owners of cats focused on respiratory diseases, namely asthma. The study showed that most of studied cats were of mixed breed and neutered, living indoor exclusively, mainly in urbanareas and from the north mainland region. According to the owner's perception, the cats were mostly active and with the ideal weight. The clinical signs more often associated with asthma crisis were respiratory wheezes and cough, whereas the worsening of such clinical signs occurred mostly in spring. Stress symptoms were not common, but the correlation between stress-related diseases and asthma was close to significance. A mixed lifestyle was associated with less symptoms of stress. Although close to significance, the presence of pollutant industries was not associated to asthma in the enrolled cats. Finally, in most of the houses in which an asthmatic cat lived in, no owner or other co-inhabitant had asthma.

It would be important to do further research in this subject, with higher number of enrolled animals, to try to understand if such results are transversal to different study populations and eventually identify further predisposing and conditioning factors that may be involved in the disease both in cats and in humans and if the housing conditions may be determining for the development and exacerbation of other diseases.

ACKNOWLEDGEMENTS

The authors acknowledge all the tutors that participated in the study.

CONFLICT OF INTEREST

None declared.

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J HELLENIC VET MED SOC 2020, 71(4): 2569-2576
ΠΕΚΕ 2020, 71(4): 2569-2576

Research article
Ερευνητικό άρθρο

Effect of GnRH treatment following a short-term estrous induction protocol on estrus and ovulation in Saanen goats, during the transitional period

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ABSTRACT: The objective of this study was to compare the effect on the ovulation time and estrus parameters of a GnRH agonist (buserelin acetate) administered 24 or 36 h following a short-term estrus induction and synchronization treatment in non-lactating Saanen goats during the transitional period. Goats received 20 mg FGA sponges for 6 days plus 300 IU eCG and 125 μg d-cloprostenol 24 h prior to sponge removal. After removal of the sponges, goats were given either 1 ml physiological saline (0.9% NaCI) solution ($Group_1$; n = 9) after 12 h, 0.004 mg GnRH ($Group_2$; n = 10) after 24 h or 0.004 mg GnRH ($Group_3$; n = 10) after 36 h. The follicle development and ovulation in the ovaries were monitored by transrectal ultrasonography starting from the sponge application until the fifth day of the estrus cycle. Blood samples were collected on the same days to determine the plasma concentrations of progesterone (P_4) and estradiol (E_2). No statistical differences among groups were detected in any synchronization parameters, ultrasonic evaluations and plasma P_4 and E_2 concentrations. The evaluation of pooled data showed that the response of Saanen goats to treatments was comparable to the results of other published trials. Results of this study indicate that administration of GnRH at 24 h or 36 h after sponge removal, at the end of a short estrus induction and synchronization protocol, does not affect plasma P_4 and E_2 concentrations, estrus parameters and ovulation time in goats, during the transition period.

Keywords: buserelin acetate, estradiol, goat, progesterone, ovulation

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Date of initial submission: 24-02-2020
Date of revised submission: 27-03-2020
Date of acceptance: 26-05-2020

INTRODUCTION

Reproductive activity in goats is usually seasonal (Fonseca and Torres, 2005) regulated by the photoperiod and other environmental factors (Fatet et al., 2011). Thus, various protocols have been developed to control reproductive activity all-year-round, including exogenous hormone treatment, the male effect and photoperiod treatments (Whitley and Jackson, 2004; Lopez-Sebastián et al., 2014). Exogenous hormones such as progesterone or its synthetic analogues in combination with equine chorionic gonadotrophin (eCG) in goats are generally used more often in control of reproductive activity during the non-breeding season and transition period (Dogan et al., 2008a; Souza et al., 2011; Abecia et al., 2011; Pietroski et al., 2013). However, repeated use of eCG may lead to antibody formation (Baril et al., 1996), resulting in delays in estrus, LH surge and ovulation time and consequently to reduction in pregnancy rates (Baldassarre and Karatzas, 2004; Fatet et al., 2011). Nevertheless, conception rate in the non-breeding season is lower compared to the breeding season (Wildeus, 2000). The main factors which influence variation in the pregnancy rates in goats are genetics, type of estrus induction protocol, follicular status at the beginning of treatment, ovulation time, season and nutritional status (Gonzalez-Bulnes et al., 2003). These factors may affect the quality of oocytes or embryos and are directly or indirectly related to low pregnancy rates. As a result, the cost of sperm and hormones spent per goat per fertilization/pregnancy increases (Souza et al., 2011).

After estrus induction oocytes that have completed development are present in large follicles. On the other hand, significant variation is observed in the time of ovulations. Accurate control of peri-ovulatory events would provide more efficient estrus induction or synchronization programs in goats. Knowledge of the timing of ovulation would permit more precise timing of mating or artificial insemination (Freitas et al., 1997; Simões et al., 2008). More close synchronization of ovulation(s) could be achieved by supporting the preovulatory LH peak with exogenous hormones such as the gonadotropin releasing hormone (GnRH) or human chorionic gonadotropin (hCG), as in sheep (Balaro et al., 2016) and cows (Fonseca et al., 2001). Similarly, a single injection of GnRH 24 h after norgestomet implant removal has been used to induce ovulation of dominant follicle in Baladi goats during the non-breeding season (Medan et al., 2002). On the contrary Riaz et al. (2012) reported that the administration of GnRH at the time of breeding did not promote ovulation induction in the goats. However, the potential of GnRH in synchronizing ovulation and follicular emergence after the use of a short-term estrous synchronization protocol has not been assessed during the transitional period in goats. The current study was therefore designed to compare the effect on the ovulatory response, estrus parameters and plasma P_4 and E_2 concentrations of a GnRH agonist (buserelin acetate) administered 24 or 36 hours following a short-term estrous induction and synchronization protocol in non-lactating Saanen goats, during the transition period.

MATERIALS AND METHODS

Location and Animals

The study was carried out at the Research and Application Farm of the Veterinary Faculty at Uludag University, located in Bursa (latitude 40° 11`N, longitude 29° 04` E, altitude 155 m), Turkey. A total of 29 sexually mature Saanen does (20 nulliparous and 9 non-lactating multiparous) were used during the transition period from the non-breeding to breeding season in the region. The does were kept indoors in sand/hay-floored pens with access to outdoors in sheltered paddock under conditions of natural daylight and temperature and were fed dry oats hay supplemented with commercial pellets (18% crude protein; 1000 g/goat/day). No extra food was offered to the goats during the study. Clean water and mineral salt were provided *ad libitum* and no changes were made during the experiment. The does were separated physically and visually from the bucks. The experiment was approved by the Animal Ethics Committee of Uludag University (References No. B.30.2.ULU.0.8Z.00.00/09).

Synchronization of estrus and GnRH treatment

Estrus was synchronized in all goats by means of intravaginal sponges (Chronogest, Intervet Productions SA., France) impregnated with 20 mg fluorogestone acetate (FGA) inserted for 6 days, coupled with 125 μg of d-cloprostenol (PSG, Alke, Turkey) and 300 IU of equine chorionic gonadotropin (eCG, Chrono-Gest; MDS Animal Health, Netherlands) i.m. 24 h before sponge removal. The application day of intravaginal sponges was defined as day 0 of the experiment. After sponge removal, all does were divided into 3 different groups according to their age, body weight and body condition score (scale 0 to 5, according to model proposed by Morand-Fehr et al. (1989). Age, body weights and body condition score for the

1, 2 and 3 Groups averaged 21.67 ± 4.52, 22.30 ± 3.72 and 24.40 ± 4.33 months, 45.54 ± 4.22, 46.40 ± 3.41 and 46.70 ± 2.50 kg and 3.00 ± 0.14, 2.95 ± 0.12 and 3.00 ± 0.07, respectively, so there were not differences in these parameters between groups.

Goats in $Group_1$ (control; n=9) received 1 ml physiological saline solution (0.9% NaCI) i.m. 12 h after sponges removal. The rest of the goats received 0.004 mg buserelin acetate (GnRH, Buserin, Alke, Turkey), i.m., either 24 h ($Group_2$; n=10) or 36 h ($Group_3$; n=10) after sponge removal.

Estrous was monitored with the aid of 2 teaser bucks, every 12 h (8 am – 8 pm) from 12 to 72 h following sponge withdrawal. Estrous onset was defined as the time of the first accepted mount. Estrous duration was defined as the time between the first and last accepted mount.

Ultrasonographic examination and follicular assessment

The ultrasound examination of the ovaries in all does was performed using a real-time B-mode ultrasound scanner (RTU, Prosound 2, Hitachi Aloka Medical, Ltd., Tokyo, Japan), connected with a transrectal 7.5 MHz linear array probe (model UST-660-7.5) and designed for human prostatic examination. Transrectal ovarian ultrasonography was performed every 24 hours from sponge insertion until sponge removal and then every 12 h, from sponge removal until ovulation was confirmed. After ovulation, ultrasonography was performed again every 24 h until Day 5 of the estrus cycle. The day of ovulation was defined by the disappearance of follicles ≥5mm between two successive observations and was considered as the 0 day of the estrus cycle. The preovulatory follicle diameter was the last measurement obtained. The mean number of ovulations per goat was determined by the number of corpora lutea (CL) on the ovaries. After ovulation, the CL number was counted on the 5th day of the estrus cycle. During the examination, the goats were restrained in the standing position and the probe was gently inserted to the rectum, after appling hydrosoluble contact gel. In brief, after the probe was passed the urinary bladder and the uterine horns, it was rotated laterally 90° clockwise and then 180° counter-clockwise to observe both ovaries and their structures, as previously described by Simões et al. (2006). The ultrasonography evaluations were performed always by the same person.

All follicles ≥ 2 mm in diameter with spherical or oval conformation and, smooth walls as well as and the CL were measured. The measurement was carried out once daily throughout the test period and the ovary map was drawn on paper and compared with the previous day. The follicles were classified as small (2.0–3.4 mm), medium (3.5–4.9 mm), and large (≥5.0 mm). The follicles measured for the first time with a diameter of 4 mm were thought to have a diameter of 3 mm in previous day (Ginther and Kot, 1994) and counted as small follicle for the previous day.

Blood collection and hormonal analysis

Blood samples (10 ml) were collected from the jugular vein into heparinized tubes (BD Vacutainer®, Becton Dickinson, Plymouth, UK) once a day for 13 days, from sponge insertion to approximately 4 d after ovulation. Centrifugation was performed at 1500 x g for 15 min at 4 °C, and plasma was separated and stored frozen (-20°C) until assayed for P_4 and E_2. Concentrations of P_4 and E_2 in plasma were assessed with a goat P_4 (SRB-T-86624) and E_2 (SRB-T-87401) enzyme-linked immunosorbent assay (ELISA) kit and results were read by ELISA reader (ELISA, ELX-808IU Ultra Microplate Reader). The standard range of P_4 and E_2, kits used were 0.05-8.0 ng/ml and 1-300 pg/ml, respectively. The inter- and the intra-assay coefficients of variation (CV_s) for P_4 and E_2, were 2.7, 2.7, 4.6 and 4.6, respectively. The sensitivities of P_4 and E_2 assays were 0.048 ng/ml and 0.925 pg/ml, respectively. Plasma P_4 concentration (≥1 ng/ml) was considered as an indication of a functional corpus luteum (Thimonier, 2000).

Statistical analysis

SPSS for Windows, Version 20 was used to conduct the statistical analyses. ANOVA followed by Tukey was used to compare the onset of estrous and duration of induced estrous periods, the onset of ovulation from sponge removal and the onset of ovulation from the onset of induced estrus periods, ovulations and also the largest and second largest follicle diameter and number of small, medium and large follicles. Estrous response and the percentage of goats ovulating were analyzed using the chi-square test. Results are given as mean (± SEM) and the differences were considered significant when $P<0.05$.

Table 1. Clinical observations and ovarian findings (ultrasonography evaluation) of Saanen goats treated with FGA sponge for 6 days with eCG plus d-cloprostenol 24 h prior to sponge removal and administration of GnRH 36 (Group 3) or 24 (Group 2) h and saline 12 (Group 1) h after sponge removal (means ±SEM). No differences detected between groups (p>0.05, Tukey test, *chi square test).

Data	Treatment groups			Mean
	Group 1 (n = 9)	Group 2 (n = 10)	Group 3 (n =10)	
Clinical observations				
Estrus response (%)*	100.0 (9/9)	80.0 (8/10)	100.0 (10/10)	93.10 (27/29)
Duration of estrus (h)	34.67 ± 1.33	31.50 ± 3.89	34.80 ± 3.32	33.66 ± 2.85
Interval from sponge removal to onset of estrus (h)	29.33± 4.06	25.50 ± 1.50	27.60 ± 2.56	27.48 ± 2.71
Occurrence of ovulation from sponge removal (h)	48.25 ± 1.33	47.00 ± 1.39	48.89 ± 1.00	48.05 ± 1.24
Estrus onset to ovulation (h)	22.75 ± 1.92	20.88 ± 1.88	20.89 ± 3.30	21.51 ± 2.37
Ovarian findings (Ultrasonography evaluation)				
The percentage of goats ovulating (%)*	88.89	100.0	90.00	93.10 (27/29)
Mean number of ovulations per doe	2.33 ± 0.17	1.90 ± 0.23	1.80 ± 0.25	2.01 ± 0.22
Largest follicle diameter (mm)	6.64 ± 0.21	6.89 ± 0.30	7.02 ± 0.26	6.85 ± 0.26
Second largest follicle diameter (mm)	5.97 ± 0.16	6.25 ± 0.25	6.14 ± 0.30	6.12 ± 0.24

RESULTS

Estrous and ovarian findings

A summary of data regarding the estrous behavior and ultrasonography evaluation after the induced estrus in the does are set out in Table 1. All estrous behaviors and transrectal ovarian ultrasonography parameters were not significantly different among the 3 treatment groups. Thus, the data were pooled and analysed in relation to time only. The overall estrus response rate within 72 h was 93.10%.

Plasma P_4 and E_2 concentrations

Since the plasma concentrations of both hormones were not different between groups during the experiment period, data on hormone concentrations were combined. During the study period, both P_4 and E_2 plasma concentrations were affected by the day (P<0.05). According to plasma P_4 measurements, 37.93% (11/29) of goats had a functional corpus luteum at the beginning of the study (1> ng/ml). Although there was no statistical difference between plasma P_4 concentrations among the six days of sponges application in all goats, the highest mean plasma progesterone concentration was detected on the fifth day (2.46 ± 0.33 ng/ml). The lowest and highest mean P_4 concentrations were observed on days 2 and 5 after the withdrawal of the sponges (0.89 ± 0.23, 2.83 ± 0.40 ng/ml, respectively, P<0.05).

Among the six days of sponges application, the highest mean plasma E_2 concentration was detected on the fourth day (67.36 ± 7.75 pg/ml) and there was a statistical difference between the fourth day and the first three days (P<0.05). Plasma E_2 concentrations decreased from 53.90 ± 5.18 to 34.19 ± 3.44 pg/ml during the first four days after removal of the sponges, then an increase in E_2 concentration (42.27 ± 6.46 pg/ml) was observed again with the emergence of new follicle wave on the last day of the study period.

DISCUSSION

The use of eCG and $PGF_{2\alpha}$ in conjunction with a short-term intravaginal progestogen treatment (6 days), regardless of GnRH treatment, was found to be an efficient protocol for estrus induction and synchronization in the non-lactating Saanen goats during transitional period. The administration of 0.004 mg buserelin acetate (GnRH) 36 or 24 h after sponge withdrawal had no significant effect on estrus behavior and ultrasonography findings. In the present study, there were no significant differences among the three groups in term of estrous response rate, within 72 h after progestogen withdrawal. In small ruminants, estrus synchronization is normally defined as acceptable when 90% or more of treated goats come into estrus within observation period (Wildeus, 2000). In the present study, 93.1% of all goats exhibited estrous during the 72 h observation period following the cessation of treatment. This result was within the range of 89.5-100%, reported by other researchers (Fonseca et al., 2005; Dogan et al., 2008a, 2016) following treatment with FGA or MAP intravaginal sponges for 6 days in combination with eCG and $PGF_{2\alpha}$ 24 h prior to sponge removal, in various goat breeds and different seasons. Similar estrous response rate has been reported (Fonseca et al., 2005; Fonseca and Torres, 2005; Salvador et al., 2005; Dogan et al., 2008a, 2016) after long (9, 11, 13-day) progestagen protocols in goats,

without GnRH. Similar to the present study, Medan et al. (2002), reported no significant differences in estrus response in Baladi goats treated with norgestomet with or without GnRH (10.5 μg buserelin acetate) 24 h after ear implant removal during the non-breeding season. Kridli et al (2003) reported that 60% of ewes showed estrus after treatment with FGA sponges for 12 days either with or without GnRH administration 28 h after sponge removal. Considering our overall estrus rate, it can be concluded that short-term intravaginal progestogen treatment with eCG and $PGF_{2\alpha}$ is effective in the induction and synchronization of estrus in Saanen goats, during the transition period.

In the current study, the mean interval from progestagen withdrawal to estrus and the duration of estrus were 27.48 ± 2.71 h and 33.66 ± 2.85 h, respectively, with no significant difference among groups (Table 1). Similarly, Pierson et al. (2003) who used 50 μg GnRH 24 h after sponge withdrawal reported no significant effect on the onset of estrus in goats during the breeding and non-breeding season. These observations are similar to those reported by Fonseca et al. (2005), Fonseca and Torres (2005), Valentim et al. (2006) and Dogan et al. (2008a, 2016) after various synchronization protocols. In a previous study, Kridli et al. (2003), reported that GnRH administration, 28 h after the end of 12-day FGA sponge treatment, decreased (42.0 v.s 58.0 h; $P<0.05$) the interval from cessation of treatment to estrus. eCG stimulates follicular development enhancing the recruitment of small follicle in the ovary (Fatet et al., 2011). Therefore in goats, eCG given at the end of progestagen treatment reduces the interval to onset of estrus (Dogan et al., 2008b). Also, high eCG doses enhance estrogen concentration and induce LH surge; therefore, it reduces the time from progestagen removal to the onset of estrus (Dogan et al., 2004).

The duration of estrus was longer in the present study when compared with previous study that reported estrous duration of 14.7 and 17.3 h in Alpine and Saanen goats, respectively (Fonseca and Torres, 2005). In untreated goats, the duration of estrus is about 36 h but varies from 24-96 h (Fatet et al., 2011) depending on breed, age (Fonseca and Torres, 2005), season (Pierson et al., 2003), parity (Simões et al., 2008), the size of the dominant follicle, E_2 concentrations (Medan et al., 2003, 2005) and the presence of a male (Dogan et al., 2016).

In a previous study, Riaz et al. (2012) reported that the use of GnRH at the time of mating did not improve the reproductive performance of goats. Sponge removal and treatment with eCG indirectly initiated the endogenous GnRH peak which resulted in the LH surge, as suggested by Pierson et al. (2003). On the contrary, as observed in this study, administration of low dose eCG may not synchronize LH increase together with follicle development in goats coming to early and late estrus. This may also be related to the absence of large follicles at the time of administration of GnRH. In addition, ovulation occurring on average 48 hours after removal of the sponge may indicate that GnRH administration was performed too early.

In the current study, the mean overall intervals from sponge removal to the onset of estrus and to ovulation were 21.51 ± 2.37 h and 48.05 ± 1.24 h, respectively, with no significant difference among groups (Table 1). These results are supported by the earlier work in which compared with intravaginal progestogen plus eCG and $PG_{2\alpha}$. (Fonseca et al., 2005; Valentim et al., 2006) or double $PG_{2\alpha}$ alone (Simões et al., 2008). Nevertheless, these results are not in agreement with Riaz et al. (2012) who found interval from onset estrus to ovulation 31.2 and 36.0 h after Ovsynch and double $PGF_{2\alpha}$ treatment groups, respectively.

The GnRH agonist used in this trial had no effect on the timing of ovulation compared to $Group_1$ (no-GnRH). This is in agreement with the results of Cavalcanti et al. (2012), who applied a 6-day MAP-sponges treatment with 300 IU eCG and 37.5 μg d-cloprostenol 24 hours before sponge removal and 0.025 mg of a synthetic GnRH analogue (lecirelin) 24 h after sponge withdrawal and reported no significant difference in the interval from sponge removal to the onset of estrus in non-treated and GnRH-treated ewes (59.1 and 58.4 h, respectively; $P>0.05$).

In this study, no differences were observed in mean concentrations of plasma E_2 for Group1 compared with the other two Groups during the 13 days of the experiment. Additionally, GnRH injection 36 and 24 hours after sponge withdrawal did not change the concentration of P_4 and E_2 during and following ovulation. Similarly, Balaro et al. (2016) applied GnRH injection at 36 and 24 hours after sponge removal reported that, in ewes, plasma concentrations of P_4 and E_2 could not find any difference. Anyway, a direct comparison of ovulation time and hormone concentration in nulliparous and multiparous goats between studies is difficult due to differences in breed, hormonal milieu, age, nutrition status and synchronization protocols.

The percentage of goats ovulating of 93.10% (27/29), regardless of the application time of GnRH, showed the efficiency of the synchronization protocol used in this study (Table 1). Likewise, using the same protocols as in the present study Balaro et al. (2016) reported no significant difference in the percentage of ovulating (96.5%) between Santa Inês ewes treated with GnRH (0.025 mg, lecirelin) 36 and 24 hours after removal of the sponges. Similarly, using 20 mg FGA sponge for 6 days with 300 IU eCG and 30 μg of d-cloprostenol, Cavalcanti et al. (2012), reported 100 and 90 % the mean number of ovulations in ewes non-treated or treated GnRH (25 μg, licerelin) 24 h after sponge removal, respectively, during breeding season, and no significant difference between groups were also observed. Pierson et al. (2003) reported that the administration of GnRH results in an earlier and more regular formation of the LH peak, and shortens the time between the withdrawal of intravaginal sponges and the ovulation, and ultimately increases the mean number of ovulations per goat, but this was not observed in the present study.

The mean number of ovulations per doe, as observed by transrectal ovarian ultrasonography in the present trial, was not different between GnRH-treated and non-treated groups (Table 1); this finding was similar to that observed in other (Medan et al., 2003, 2005; Simões et al., 2006; Nogueira et al., 2015) or in the same goat breeds (de Castro et al., 1999) without exogenous GnRH administration. However, the mean number of ovulations per doe was higher to that reported by Riaz et al. (2012**),** (1.6 ± 0.2) in Beetal and Dwarf does, after using Ovsynch protocol. The differences in the results of these studies could be due to differences in dose of hormones, genetics, breed, age, nutrition and other environmental and management factors.

According to Cavalcanti et al. (2012), the ovulation response to GnRH injection might depend on the time of treatment and size of ovulatory follicle. In this study, though, the largest and second largest follicle diameter was similar in both the GnRH-treated goats and the untreated goats (Table 1).). According to previous studies, the largest follicle diameter is between 6.6-6.8 mm in acyclic (Cruz et al., 2005) and 5.9-8.0 in cyclic (Orita et al., 2000; Medan et al., 2005; Simões et al., 2006; Riaz et al., 2013) goats, which was similar to observed in Saanen in the our study. On the contrary, after using Ovsynch and double $PGF_{2\alpha}$ protocol in goats Riaz et al (2012) reported that ovulatory follicle diameter was 7.1 and 7.0 mm, respectively. Nogueira et al. (2015) reported that, in Boer goats, the largest follicle diameter was 6.7 mm in the non-breeding season and 7.8 mm in the breeding season.

As a consequence, six-day progesterone priming, in combination with eCG and $PGF_{2\alpha}$ can be used successfully for estrous induction and synchronization in Saanen goats during the transition period, but the administration 0.004 mg of GnRH 24 or 36 h after sponge removal had no effect on estrous behaviors, ovarian structures and plasma P_4 and E_2 concentrations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This research was supported by the Uludag University Scientific Research Projects Centre, Bursa, Turkey (Grant No. BUAP (V) - 2016/1). The authors would like to thank Prof. Dr. Veysi ASLAN and ALKE Co., Ldt for providing cloprostenol ($PGF_{2\alpha}$, PGS®) and buserelin acetate (GnRH, Buserin).

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J HELLENIC VET MED SOC 2020, 71(4): 2577-2582
ΠΕΚΕ 2020, 71(4): 2577-2582

Case report
Κλινικό περιστατικό

Treatment of Ventral Hip Luxation in a Puppy

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ABSTRAC: A Three months of age, 4.5 kg, female mixed (cross) breed presented to our clinic with complaint of the left hind leg lameness. In the clinical, radiographic and ultrasonographic examination, ventral hip luxation was diagnosed. Based on the examination findings which indicated the case was chronic, surgical treatment was decided. Hip joint was exposed firstly by craniodorsal approach. Flattened and full acetabulum, fractured greater trochanter, ruptured gluteal muscle attachment and irreparably broken joint capsule were determined. Excision arthroplasty was performed by ventral approach. Greater Trochanter was attached to its position by an L shape Steinman pin using craniodorsal approach. Then, a drill hole was created on the greater trochanter, and a screw was inserted to dorsal acetabular rim. A synthetic suture passed through the hole was tied to the screw in a figure of eight mode. Postoperative antibiotics and anti-inflammatory drugs were used. Physiotherapy such as swimming and massage treatments was proposed following skin sutures removal. In the 4th week control postoperatively, the dog began to use the operated leg effectively despite the moderate quadriceps' contracture. The contracture was mostly resolved 12th week by continuing physiotherapy.

Keywords: Hip, luxation, ventral, treatment, dog

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Date of initial submission: 01-03-2019
Date of revised submission: 07-07-2020
Date of acceptance: 13-08-2020

CASE PRESENTATION

An Approximately 3 months of age, 4.5 kg, female mixed breed dog with severe lameness of the left hind leg presented to our clinic. The dog was a stray and just found 4 days ago by its new owner. In the clinical examination of the affected leg, there were moderate muscle contracture manifested by constant abduction and hyperextension, and mild muscle atrophy. Also, a hard swelling palpated mass compatible with femoral head at the level of the obturator foramen was determined. (Figure 1a).

Ultrasonography of the swollen area on the inguinal region clearly showed the femoral head under the skin (Figure 1b). In ventro-dorsal extended radiograph, the superimposed to the acetabulum, femoral head was seen (Figure 1c). On 15° oblique medio-lateral radiograph of the coxofemoral joint, the luxation of femoral head was obvious, and the absence of the greater trochanter which indicated fracture can be seen (Figure 1d). Based on these findings, hip luxation in ventral direction and epiphysiolysis of the greater trochanter was diagnosed and surgical treatment was decided.

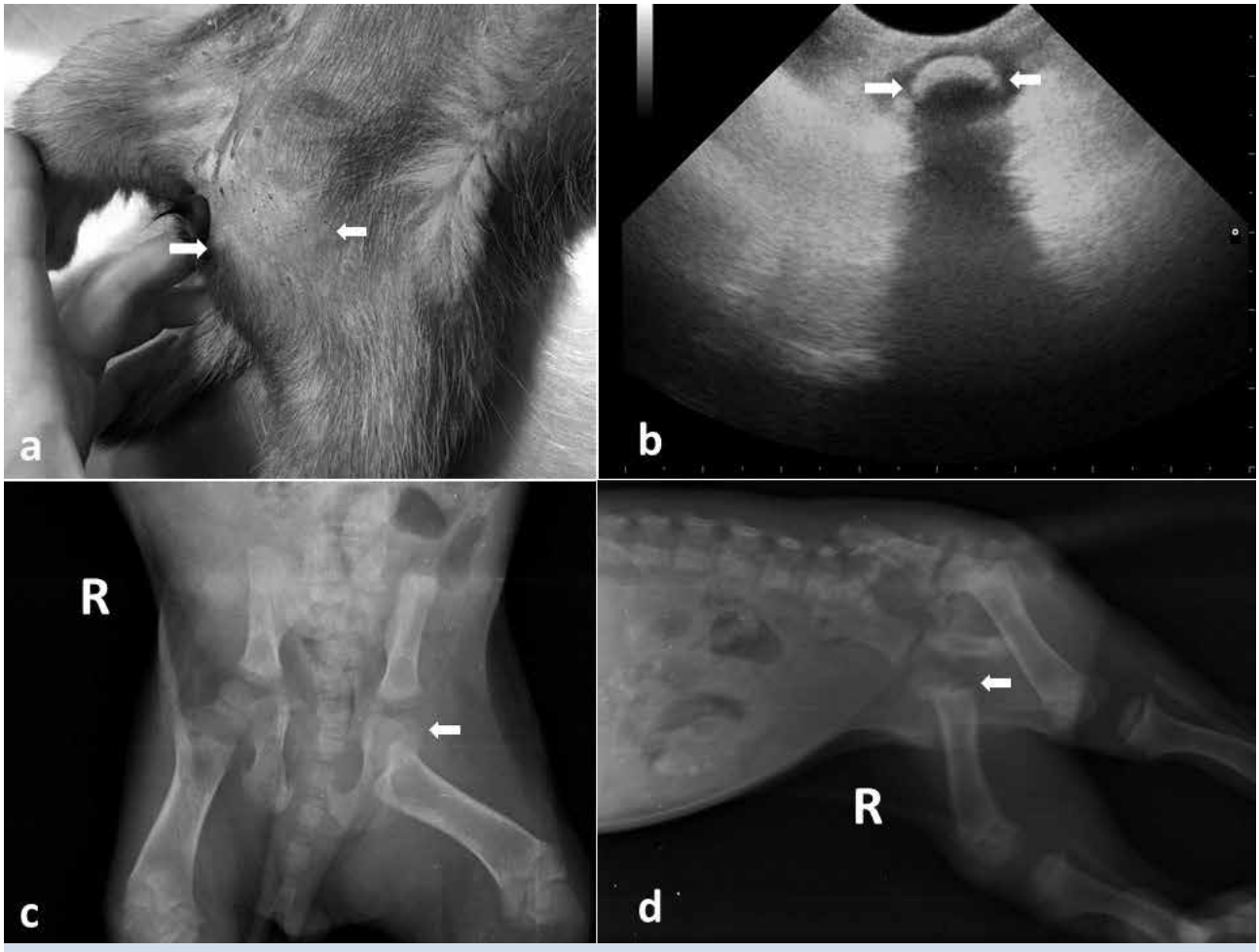

Figure 1. White arrows point out the clinical appearance of the luxated femoral head (between arrows) (a); White arrows indicates ultrasonographic imaging of the femoral head under the skin (b); The luxation pointed by arrow on the ventrodorsal radiograph of the dog (c); The arrow indicates the absence of the greater trochanter on 15° oblique medio-lateral radiograph of the dog (d).

Anesthesia was induced with combination of xylazine HCl (0.5 mg/kg, IM, Alfazyne®, Egevet, Turkey) and ketamine HCl (10 mg/kg, IM, Alfamine®, Egevet, Turkey) and maintained with inhalation of Isoflurane (Forane ®, Abbott, Latina, Italy) at a 2% flow rate.

Hip joint was exposed firstly by craniodorsal approach (Figure 2a). The joint capsule was broken irreparably, and the acetabulum was flattened completely, it was almost even slightly convex (Figure 2b). Also, the fractured greater trochanter diagnosed by the x-ray was seen, and gluteal muscle attachment rupture depending on avulsion fracture was detected during surgery. Because of the condition of the acetabulum and joint capsule, excision arthroplasty was performed. Since, the femoral head could not be reached by craniodorsal approach, excision was performed by ventral approach (Figure 2d, e, f). During this procedure, a small organized piece of the round ligament was seen on the femoral head (Fig 2e). Because of the muscle contraction probably depending on long time elapsed and, gluteal muscle rupture associated with avulsion fracture of the greater trochanter, the leg could not be re- positioned anatomically. Therefore, the greater trochanter was attached to its anatomical position by the use of an L shape Steinman pin. Then, a drill hole was created on the greater trochanter and a screw (2.7 mm Ø) was inserted to the dorsal rim of the acetabulum. A synthetic suture passed through the hole was tied to the screw in a figure of eight mode. (Figure 2c). By support of the figure of eight suture, the leg was pulled to more lateral and more dorsal direction to reach its anatomical position (Figure 2 g, h). Skin and subcutaneous tissues were routinely closed, postoperative antibiotics and anti-inflammatory drugs were applied. Physiotherapy such as swimming and massage treatments was recommended to the owner following removal of the skin stitches on around 10th day.

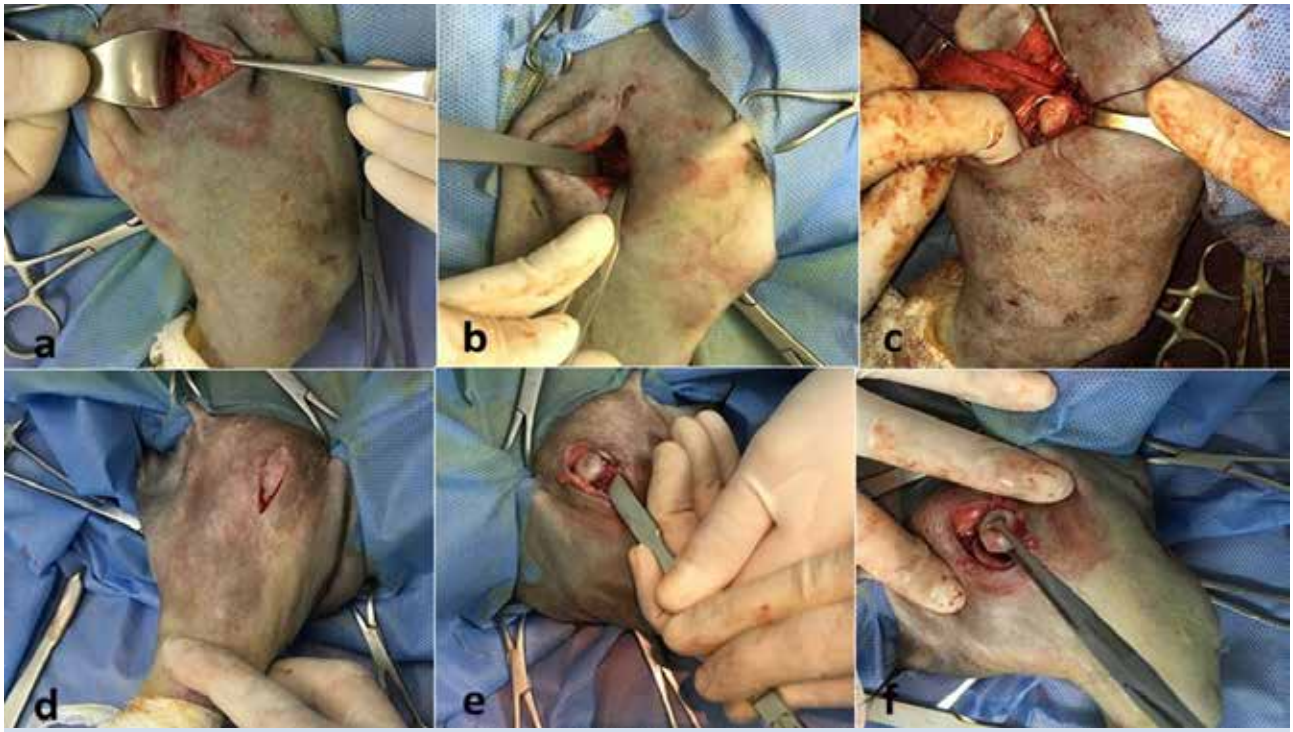

Figure 2. Intraoperative procedures. Dorsolateral approach to the articulatio coxae (a), Apperance of the flattened acetabulum (b); Attaching of the greater trochanter in its position by using L shape Steinman pin and, placing eight-shape synthetic suture material (c); Excision of the femoral head by ventral approach (d, e, f); Postoperative x-rays in ventrodorsal (g) and mediolateral (h) direction.

In the 4th week control postoperatively, despite the moderate quadriceps' contracture, it was seen that the dog began to use the operated leg effectively, and it was recommended to continue the physiotherapy (Figure 3). At week 12 postoperatively, the owner declared that the dog's leg condition is much better and she can use it as much as the opposite one.

Figure 3. Clinical appearance of the dog at postoperative 4th week

DISCUSSION

Luxation of the hip in small animals, with a rate of %39-90, is the most common one among all luxations (Johnson and Dunning, 2005; Piermattei et al., 2006). It is generally the result of trauma and various degree soft tissue damages (Piermattei, 1993; Piermattei et al., 2006). Hip luxations occur usually in the cranial direction due to pulling force of gluteal and iliopsoas muscles (Duff and Bennett, 1982; Fox, 1991). However, luxations of the femoral head in caudo-dorsal or ventral direction are rarely encountered (Piermattei et al., 2006; Fox, 1991; Harasen, 2005)

Following hip joint luxation, closed reduction is only possible within 48-72 hours because of inward folding of joint capsule, hypertrophy of the round ligament, inflammation and fibrosis within the acetabulum (Piermatte, 1993; Piermattei et al., 2006). There are different extracapsular methods such as capsulorrhaphy (Bone et al., 1984; Piermattei et al., 2006), synthetic capsule technique or its modifications (Denny and Butterworth, 2000; Piermattei et al., 2006), the greater trochanter transposition (Basher et al., 1986; Denny and Butterworth, 2000), and intracapsular techniques such as Toggle pin/rod fixation (Piermattei et al., 2006; Cetinkaya and Olcay, 2010), trans-acetabular pinning (Hunt and Henry, 1985; Denny and Butterworth, 2000) sacrotuberous ligament transposition (Kılıc et al., 2002; Ozaydın et al., 2003), for surgical treatments of hip luxation.

In our case, the exact time elapsed following the luxation was not known, because the owner found the limping dog in the street. Clinical findings implied that it was a chronic case, closed reduction was not an option. Also, intraoperatively it was seen the acetabulum was completely flattened and there was no trace of the acetabular cavity. In this condition, reposition of the femoral head was impossible. Therefore, femoral head and neck excision was performed.

The hip luxation of this case was complicated with an avulsion fracture of the greater trochanter and quadriceps contracture. Greater trochanter was attached to where it was separated from, by using an L shape Steinmann pin. A tension band wire would be the best option for uncomplicated avulsion fracture of the greater trochanter. But the hole created just below the fracture line of the greater trochanter was kept as small as possible considering the size of the suture material, to avoid another fracture. Also, an adequate support to keep it in its position was provided by using the L shape pin. However, the leg could not reach its anatomical position just by restoring by of the greater trochanter. For simple and new luxation cases, repairing of the joint capsule is usually recommended as a way of treatment and also highly recommended following femoral head and neck excision to support joint position (Basher et al., 1986; Piermattei et al., 2006; Off and Matis, 2010). Because of the excessive damage of the joint capsule, a figure of eight suture (inspired by synthetic capsule technique) between a drilled hole on the greater trochanter and a screw inserted to the dorsal acetabular rim was used to restore the anatomical position of the joint and support the capsule. Originally, synthetic capsule technique is performed by placing two screws on the dorsal acetabular rim, drilling femoral neck and placing a figure of eight suture between screws and drilled hole (Holsworth and De Camp, 2003; Johnson and Dunning, 2005). In this case, the hole was drilled on the greater trochanter as described by some studies which modified synthetic capsule technique (Belge et al, 2014), and one screw was placed on the dorsal acetabular wall because the dog was too small.

The flattening of the acetabular cavity in our case suggests that presence of the femoral head in the acetabulum contributes in the development of acetabular depth during growth of the dog, as also theoretically known for the patellar groove. (Piermattei et al., 2006). In this case report, physical consequences of prolonged duration of the hip luxation in very young aged (1-2 month-old) animals, such as complete flattening of the acetabulum, and successful results obtained by combining different treatment methods for a rare case of chronic and complicated ventral hip luxation was documented.

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JOURNAL OF THE HELLENIC VETERINARY MEDICAL SOCIETY

ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ

Quarterly Edition
Volume 71, No 4
OCTOBER - DECEMBER 2020

Τριμηνιαία Έκδοση
Τόμος 71, No 4
ΟΚΤΩΒΡΙΟΣ - ΔΕΚΕΜΒΡΙΟΣ 2020