Journal of the Hellenic Veterinary Medical Society

Vol 70, No 3 (2019)

Whole issue

Ελληνική Κτηνιατρική Εταιρεία

doi: [10.12681/jhvms.22351](https://doi.org/10.12681/jhvms.22351)

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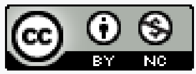

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To cite this article:

Εταιρεία Ε. Κ. (2019). Whole issue. *Journal of the Hellenic Veterinary Medical Society*, *70*(3), 1589–1748. https://doi.org/10.12681/jhvms.22351

ISSN 2585-3724

JOURNAL OF THE HELLENIC VETERINARY MEDICAL SOCIETY

ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ

ΚΩΔΙΚΟΣ: 2782
ΠΛΗΡΩΜΕΝΟ ΤΕΛΟΣ
Ταχ. Γραφείο Αγ. ΔΗΜΗΤΡΙΟΥ
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ΕΝΤΥΠΟ ΚΛΕΙΣΤΟ ΑΡ. ΑΔΕΙΑΣ 1

Quarterly Edition
Volume 70, No 3
JULY - SEPTEMBER 2019

Τριμηνιαία Έκδοση
Volume 70, No 3
ΙΟΥΛΙΟΣ - ΣΕΠΤΕΜΒΡΙΟΣ 2019

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CODE: 2782

Quarterly Scientific Journal
5th Period, Volume 70, Issue no.3
JULY - SEPTEMBER 2019

Τριμηνιαίο Επιστημονικό Περιοδικό
Περίοδος Ε', Τόμος 70, No.3
ΙΟΥΛΙΟΣ - ΣΕΠΤΕΜΒΡΙΟΣ 2019

ΚΩΔΙΚΟΣ: 2782

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

Publisher
Hellenic Veterinary Medical Society
Patission 158, 11257 Athens, Greece
Tel.: +.30.210.8642284
Fax: +.30.210.8645744
www.hvms.gr
E-mail: secretary@hvms.gr

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Redaction
Rotonda Publications
8 Kamvounion Str, 54621
Thessaloniki, Greece
Tel: +30210212212

Επιμέλεια έκδοσης
Εκδόσεις Ροτόντα
Καμβουνίων 8, 54621
Θεσσαλονίκη
Τηλ: 2310212212

Annual subscription
Europe: € 150
Other countries: € 180
Price per issue: € 50

Ετήσιες συνδρομές
Ελλάδα και Κύπρος
Μέλη ΕΚΕ: € 30
Μη μέλη ΕΚΕ: € 40
Φοιτητές: € 15
Νομικά πρόσωπα: € 100
Τιμή τεύχους: € 15

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D. Vourvidis
P.O. Box 3546, 10210 Athens, Greece

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Τ.Θ. 3546, 10210 Αθήνα

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J Hellenic Vet Med Soc (M. Zafiropoulou)
Hellenic Veterinary Medical Society
Patission 158, 11257 Athens, Greece

Ταχυδρομική διεύθυνση
ΠΕΚΕ (Μ. Ζαφειροπούλου)
Ελληνική Κτηνιατρική Εταιρεία
Πατησίων 158, 11257 Αθήνα

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	1
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)
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History of the Hellenic Veterinary Medical Society

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

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

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

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

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

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

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

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

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

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

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

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

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

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J HELLENIC VET MED SOC 2019, 70(3): 1589-1602
ΠΕΚΕ 2019, 70(3): 1589-1602

Review article
Ανασκόπηση

The laboratory diagnostic approach to thoracic and abdominal effusions in the dog, cat, and horse

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Η εργαστηριακή προσέγγιση των υπεζωκοτικών και περιτοναϊκών συλλογών στο σκύλο, τη γάτα και το άλογο

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ABSTRACT: Cases involving pleural and peritoneal effusions occur relatively frequently in clinical practice. Determining the underlying etiology in these cases relies mainly on fluid analysis. The technique used for obtaining the pleural or peritoneal fluid can impact greatly the results of the analysis. Most often used diagnostic tools include evaluation of gross appearance, Total Nucleated Cell Count / Total Protein (TNCC/TP) measurement, chemical/biochemical analysis (Lactate dehydrogenase and lactate, cholesterol, triglycerides, glucose, creatinine, pH, pO_2, pCO_2, and K measurements), cytology (identification of septic and non-septic inflammation and neoplasia), microbiology (Gram stain, culture, molecular techniques), and specific tests for certain clinical conditions and diseases. Classifying an effusion as transudate, modified transudate and exudate is traditionally based on the TNCC and TP values. New diagnostic methods encourage the clinician to approach the effusion etiologically instead of strictly following this traditional classification. Many of the diagnostic tests described in this review are simple and can be performed in-house, providing the clinician quickly with information about the cause of the effusion, essential for an effective treatment plan without wasting valuable time.

Keywords: effusion, pleural, peritoneal, laboratory, diagnostic approach

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Date of initial submission: 21-09-2018
Date of revised submission: 23-02-2019
Date of acceptance: 29-02-2019

ΠΕΡΙΛΗΨΗ: Περιστατικά στα οποία διαπιστώνονται υπεζωκοτικές και περιτοναϊκές συλλογές είναι αρκετά συχνά στην κλινική πράξη. Ο καθορισμός της υποκείμενης αιτιολογίας σε αυτές τις περιπτώσεις βασίζεται κυρίως στην ανάλυση του υγρού της συλλογής. Η τεχνική που χρησιμοποιείται για τη λήψη του υγρού της υπεζωκοτικής ή περιτοναϊκής συλλογής μπορεί να επηρεάσει σε μεγάλο βαθμό τα αποτελέσματα της ανάλυσης. Στις πιο συχνά χρησιμοποιούμενες διαγνωστικές εξετάσεις περιλαμβάνονται η αξιολόγηση των φυσικών ιδιοτήτων του υγρού, ο προσδιορισμός του συνολικού αριθμού εμπύρηνων κυττάρων / ολικών πρωτεϊνών (TNCC / TP), ο προσδιορισμός χημικών / βιοχημικών παραμέτρων (γαλακτική αφυδρογονάση και γαλακτικό οξύ, χολοστερόλη, τριγλυκερίδια, γλυκόζη, κρεατινίνη, pH, pO_2, pCO_2, K), κυτταρολογική εξέταση (διάγνωση σηπτικών και μη σηπτικών φλεγμονών και νεοπλασμάτων), μικροβιολογικές εξετάσεις (επιχρίσματα με χρώση Gram, καλλιέργεια, μοριακές τεχνικές) και ειδικές διαγνωστικές εξετάσεις για ορισμένες παθολογικές καταστάσεις και νοσήματα. Η ταξινόμηση μιας συλλογής ως διίδρωμα, τροποποιημένο διίδρωμα και εξίδρωμα βασίζεται παραδοσιακά στις τιμές των TNCC και TP. Νέες διαγνωστικές μέθοδοι συνεισφέρουν στην αιτιολογική διάγνωση χωρίς απαραίτητα να ακολουθηθεί αυστηρά η παραδοσιακή ταξινόμηση. Πολλές από τις διαγνωστικές εξετάσεις που περιγράφονται σε αυτήν την ανασκόπηση είναι απλές και μπορούν να πραγματοποιηθούν στο ιατρείο, παρέχοντας γρήγορα πληροφορίες στον κλινικό κτηνίατρο σχετικές με την αιτία της συλλογής. Η γνώση αυτή είναι απαραίτητη για την έγκαιρη και αποτελεσματική θεραπευτική αντιμετώπιση της υποκείμενης παθολογικής κατάστασης.

Λέξεις ευρετηρίασης: συλλογή, υπεζωκοτική, περιτοναϊκή, εργαστηριακές εξετάσεις, διαγνωστική προσέγγιση

INTRODUCTION

The pleura is a membrane lined by a single layer of mesothelial cells surrounded by elastic connective tissue that contains vascular and lymphatic channels. The pleural space exists as a cavity only when there is fluid or gas collected between the parietal and visceral pleura. It normally contains 2 to 3 ml of fluid in dogs and cats, with low cell numbers (<500 cells/μl) and protein content (<1.5 g/dl). Regarding horses, it has been reported that 2 to 8mL of thoracic fluid could be aspirated by 17 out of 18 clinically normal horses, however multiple attempts were made in order to obtain the fluid (Wagner and Bennett, 1982). Its production depends mainly on the colloid osmotic pressure and the hydrostatic pressure within the capillary and lymphatic beds which means that lymphatics gather the surplus left after fluid filtration by the capillaries. In dogs and cats increased fluid can be absorbed by the visceral pleura, while horses have to rely on their lymphatic system, a fact that may explain the quick development of parapneumonic effusions in horses (Mazan, 2018). Pleural fluid production is normally favored because systemic and pulmonary capillary hydrostatic pressures are greater than those of the pleural space (Radlinsky, 2012).

The peritoneal cavity is a potential space between the visceral and parietal peritoneum. It normally contains a very small amount of fluid produced by the mesothelial cells to act as a lubricant. The peritoneum is capable of both absorption and exudation or transudation. It allows exchange between peritoneal fluid and plasma. Normal peritoneal fluid is almost acellular, containing <300 cells/μl, with protein content <3 g/dl and does not clot, as it lacks fibrinogen. In an early study about peritoneal lymphatic drainage in dogs (Kirby, 2012), intraperitoneally injected particles cleared from the peritoneal cavity appeared quickly in the systemic circulation and in the lungs, which is why in bacterial peritonitis, bacteremia is an early and consistent finding. Contamination or injuries of the peritoneum activate a strong inflammatory response consisting of an inflow of protein-rich fluid along with macrophages and neutrophils. Mast cells and macrophages release histamine and prostaglandin, cause vasodilation and increase the permeability of peritoneal capillaries, leading to the massive entrance of fluid abundant in complement, immunoglobulins, clotting factors, and fibrin into the peritoneal cavity.

There are 4 main mechanisms by which a pleural or peritoneal effusion can occur; (1) it can be the result of an increase in hydrostatic pressure (e.g. right sided congestive heart failure), (2) reduction of osmotic pressure (hypoalbuminemia), (3) increased vascular permeability (e.g. inflammations in the pleural space), and (4) obstruction of lymphatics that can decrease fluid resorption (Kirby, 2012; Mazan, 2018).

Apart from the aortic, the caval and the esophageal hiatus, dorsally to the diaphragm and ventrally to the psoas muscles, the pleural and peritoneal cavities are separated only by a thin layer of fused endothoracic and transversalis fascia (Kirby, 2012). This explains how even when the diaphragm is intact, cases like

pneumothorax/pneumoperitoneum, pyothorax/septic peritonitis, or even uroperitoneum/urothorax can develop (Tsompanidou, 2015).

INDICATIONS AND COLLECTION TECHNIQUES

Fluid analysis can quickly narrow the differential diagnosis of an effusion. Thoracocentesis/abdominocentesis is not always strictly diagnostic as lessening the amount of fluid is essential for alleviating the symptoms caused by fluid accumulation. Ultrasound-guided paracentesis is the safest option to obtain a sample for analysis, especially in the presence of a small amount of fluid, or when the effusion is unilateral or compartmentalized. Furthermore, it has much higher yield than blind tapping (Valenciano et al., 2014). The procedure and the materials needed are described in Fig. 1 and Table 1, respectively.

Table 1. Materials used for sampling and collection

Sampling	Collection
Needle 19-21 G (cats, small dogs)	EDTA tube (TNCC, PCV, TP, cytology)
Needle 18-20 G (medium and large sized dogs)	Plastic or glass serum tube (chemical, biochemical analysis)
Needle 14 G (horse)	Sterile tube or culturette (PCR, culture)
Over-the-needle catheter	
Extension tube	
3-way stop-cock	
Tomcat catheter	
Peritoneal dialysis catheter (abdominocentesis)	
Teat cannula (horse)	

TNCC: Total nucleated cells, PCV: Packed Cell Volume, TP: Total protein

1. Preparation of the insertion site. Clipping and surgical scrubbing. Care should be given to the strict aseptic conditions of the chosen technique, especially in surgery patients. Preoperative thoracocentesis or intrathoracic biopsy were found to be risk factors that contributed to the development of pyothorax in animals following thoracic surgery (Meakin et al., 2013).

2. The animal is left standing (dog, cat, horse) or put in sternal recumbency (dog, cat). Restraining is crucial, especially if sedation or anesthesia is not used.

3. **Dog, cat**: insert a butterfly needle or an over-the-needle catheter approximately two thirds down the chest, near the costochondral junction between the 6th-8th intercostal space, close to the cranial surface of the rib. The caudal border should be avoided to minimize the possibility of lacerating the vessels. Use a 19-21 gauge needle for cats and small dogs and an 18-20 gauge for larger breeds. An over-the-needle catheter allows for the needle to be withdrawn before attaching the syringe, thus minimizing the risk of injuring the organs of the thoracic cavity. If the removal of a large amount of fluid is needed, the catheter should be attached to an extension tube and a three-way stopcock (Kirby, 2012). **Horse**: insert a 14-gauge catheter between the 7th-8th intercostal space, 4cm above the costochondral junction. Care needs to be taken not to lacerate the lateral thoracic vein. A stab incision needs to be made first, for a chest tube or a teat cannula placement. Due to the anatomical characteristics of the horse's mediastinum, to obtain a diagnostic sample for analysis it is important to sample the respected chest side (the condition of the two chest sides may differ) (Mazan, 2018).

Fig. 1. Synoptic presentation of thoracocentesis/abdominocentisis

Table 2. Traditional effusion classification

	Transudate (pure)	Modified transudate	Exudate
TP	<2.5 g/dL	2.5 – 7.5 g/dL	>3.0 g/dL
TNCC	<1500 cells/μL	1000 – 7000 cells/μL	>7000 cells/μL

TP: Total protein, TNCC: Total nucleated cells

Abdominocentesis is performed with similar materials as thoracocentesis. A tomcat catheter or a peritoneal dialysis catheter can also be used, especially if only a small amount of fluid is present. When a syringe is not attached the technique is considered more sensitive. Preparation also includes emptying the urinary bladder to avoid accidental cystocentesis. The animal is left standing or put in lateral recumbency and the puncture is made along the ventral midline to avoid the presence of falciform fat that can block the needle barrel, except in the case of a previous surgical incision. When a surgical scar is present, the insertion should be made at least 1.5 cm away to avoid any abdominal viscera that may have adhered in the area.

Method selection depends on factors such as time, cost, safety, and amount of fluid present. Using only a syringe is less expensive than an over-the-needle catheter but also less safe, while using a peritoneal dialysis catheter is the best method to collect a sample from an effusion with a very small amount of fluid -and it further allows for abdominal lavage but is also the most expensive. In dogs with septic peritonitis, the comparison among needle paracentesis, catheter paracentesis, and diagnostic peritoneal lavage showed an accuracy rate of 94.6% for the later, and less than 50% for the first (Culp and Holt, 2010). The needle and the cannula technique were compared in horses using repeated abdominocentesis and were found to be similar in the time needed and results yielded with no technique being superior over the other (Duesterdieck-Zellmer et al., 2014).

THORACIC/ABDOMINAL FLUID ANALYSIS

A. Gross appearance

Fluid appearance can sometimes be indicative of both the process that resulted in its accumulation and the Total Nucleated Cell Count (TNCC). An opaque sample is usually expected to have a higher nucleated cell number than a colorless clear sample. Transudates, which are low cellularity effusions usually produce more clear and colorless specimens. Modified transudates of moderate cellularity are often of amber color and clear to slightly opaque. However, fluids with more or less opaque appearance are most commonly exudates (high cellularity fluids). Whether the fluid is clear or turbid will also affect the laboratory process. More opaque fluid smears can be made directly with well-mixed, uncentrifuged samples, while non-turbid fluid specimens need to be centrifuged at 1000-1500 rpm for 5 minutes before making a smear from the sediment (or if centrifugation is not available, a line smear can be made instead of a traditional pull or squash smear). A milky appearance is often observed when chylous or, less often, pseudochylous effusions are present and the cause of this appearance is their consistence of chylomicron rich lymph fluid. Of course, the milky macroscopic appearance is only indicative, as there are 'white' appearing effusions that are not chylous, as well as chylous effusions that do not have a milky appearance (especially when the animal is fasted or anorectic). Determining the Total Protein (TP) concentration in clear fluids can be done without centrifugation but in opaque fluids is best estimated by using the supernatant after centrifugation to avoid falsely high TP readings. Comparing the appearance of the specimen before and after the centrifugation can give the clinician important clues. A turbid sample that yields clear supernatant indicates that the turbidity was a result of cell and debris accumulation (may be suggestive of empyema), whereas turbidity that persists despite the centrifugation may suggest high lipid concentration.

B. TNCC and TP measurement

Effusion classification as transudate, modified transudate, or exudate is traditionally based on these two tests (Table 2). TNCC measurement can be performed either by automated cell counters or manually with the use of a haemocytometer. It should be noted that TNCC measurement whether is performed manually or automatically, does not substitute cytological evaluation which is necessary to confirm cell counts (Brudvig and Swenson, 2015). When using an automated counter that also counts RBC numbers, the information obtained can be useful to determine blood contamination of the sample, haemorrhage, or increased capillary permeability. Counting errors can occur with either automated or manual methods by factors such as debris, cell fragmentation and clumping. Delaying of the analysis can also alter TNCC, as well as cell morphology (Valenciano et al., 2014; Hughes et al., 2016; Mazan, 2018). The TP measurement can be performed either with a biochemical analyser or refractometer. Refractometry has in fact shown acceptable results

for TP measurement in canine pleural and abdominal fluid samples, providing a quicker, convenient and relatively inexpensive method, compared to the biuret biochemical assay (Rose et al., 2016).

C. Chemical and biochemical analysis

- Lactate dehydrogenase (LDH) and Lactate: Difference between blood lactate and effusion lactate concentrations < -1.5 mmoL/dL in canine and feline effusions was found to be indicative of septic exudates (Dempsey and Ewing, 2011). Elevated lactate concentrations in fluid from effusions supports the diagnosis of septic exudates (lactate increase due to an anaerobic microenvironment, production of lactate by neutrophilic glycolysis, and from the presence of bacterial metabolites). It has been observed that LDH activity, LDH effusion/serum relationship, lactate concentration as well as lactate effusion/serum gradient present a high correlation with the classification of an effusion as exudate. On the other hand, an LDH effusion:serum ratio of < 0.5 has been associated with transudates. Lactate dehydrogenase activity seems to be significantly different depending on the method of measurement, therefore it is necessary that the method used is known and remains constant for comparison (Smuts et al., 2015; Smuts et al., 2016).

In horses with colic, higher levels of LDH activity may be present in the peritoneal fluid when the condition is caused by sepsis or neoplasia, rather than those caused by mechanical obstructions or non-septic inflammatory conditions, with the latter ones more probable to respond to treatment. Again, the use of a consistent method of determination seems to be crucial, as great variation exists among the results. A moderate to good correlation appears between LDH activity and lactate concentration (Smuts et al., 2015). Peritoneal fluid lactic acid concentration was found to be an indicator of intestinal ischemia, and higher concentrations correlate with a poor prognosis (not surprising, as there is likely a relationship between the amount of necrotic tissue and peritoneal fluid lactate concentration). Assessing survivability, serum lactate concentrations were shown to be significantly higher in non-surviving colic horses than those that eventually lived (Boom et al., 2010), and lower serum LDH levels were found in dogs with better survival time amongst animals suffering from lymphoma (independently of the clinical stage or cytological evaluation) (Zanatta et al., 2003).

- Cholesterol: This measurement has been proposed as a way to increase the diagnostic accuracy when classifying effusions as transudates or exudates. Although measuring cholesterol on its own is not suggested because of low sensitivity, it can be used in combination with other markers e.g. LDH (Zoia et al., 2009). The exact mechanism by which cholesterol levels are found higher in exudates remains poorly understood (serum leakage suggesting increased permeability and release due to cellular degeneration have been proposed but not yet proven).

- Triglycerides: Effusion triglyceride concentration on its own, and also in combination with effusion cholesterol and serum triglyceride concentration are methods used for cavitary fluid analysis. Effusion triglyceride concentration > serum triglyceride concentration, effusion cholesterol < triglyceride concentration, and effusion triglyceride concentrations >100 mg/dL suggest the presence of a chylous effusion. Triglyceride measurement in the pleural fluid has been compared to - and has shown good correlation to - the lipoprotein electrophoresis, the latter being used in humans for the differentiation between chylous and non-chylous effusions as it provides the detection of chylomicrons (Waddle and Giger, 1990).

- Glucose: As a bacterial infection develops in a cavity, the effusion glucose levels begin to decrease, either because of glucose usage by the bacteria and the inflammatory cells, possible glycolysis in the fluid, or low blood to fluid glucose transport. Measuring effusion glucose levels alone, despite the established concentration of 50mg/dL being specific for septic effusion, is not recommended due to low sensitivity. Comparing serum and fluid levels provides a rapid and more accurate screening test. A concentration difference between blood glucose and effusion glucose concentrations > 20 mg/dL is a quick and reliable diagnostic tool in differentiating septic from non-septic effusions (Dempsey and Ewing, 2011; Bonczynski et al., 2003). Cytology and additional diagnostics (e.g. culture) are however essential in the definitive diagnosis of a septic effusion. Veterinary point-of-care glucometers can be useful in the sense of a more rapid diagnosis of septic peritonitis, but diagnostic accuracy is limited, and cut-off points need to be different for maximizing sensitivity (Koenig and Verlander, 2015).

- Creatinine: In dogs, measuring creatinine levels in the peritoneal fluid can help diagnose uroabdomen. Effusion concentration 2 times above serum levels is considered indicative of the condition. In a case reported in 2015, the same criteria were also used to detect urothorax in a dog in which the accumulation of urine in the thoracic cavity was found to be secondary

to uroperitoneum (Tsompanidou et al., 2015). Fluid urea is not useful in the diagnosis of uroabdomen as it diffuses rapidly across membranes given its small size.

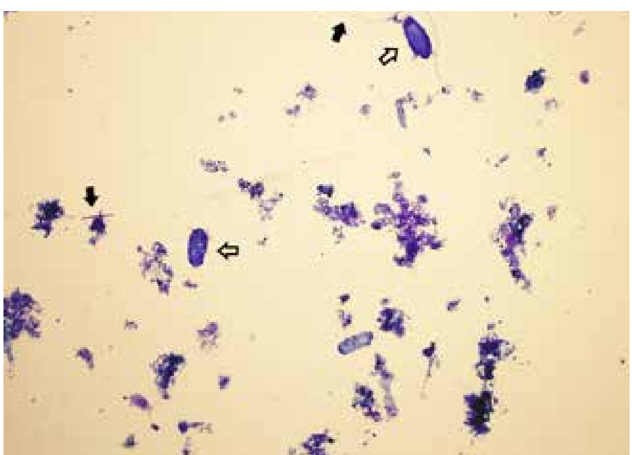
- pH, pO_2, and pCO_2: Attempts have been made in humans and animals to link these markers with bacterial and malignant effusions, often with ambiguous results. A low pH value in an effusion suggests high metabolic activity and indicates an inflammatory or an infiltrative process. In humans, low pH value in pleural fluid is used to facilitate the decision of fluid drainage in parapneumonic effusions (since clinical findings and radiographic imaging often provide poor diagnostic accuracy in determining which effusions need surgical treatment). In malignant effusions, low pH values were linked to shorter survival time and poorer response to chemical pleurodesis. A study analyzing the pH, pO_2 and pCO_2 values in the peritoneal fluid of dogs with ascites, showed statistically significant differences in the group of dogs with bacterial peritonitis (Glińska-Suchocka et al., 2016). As an inflammatory process presents within the peritoneal cavity, fibrin accumulation leads to decreased oxygen supply causing non-oxidative glucose metabolism that results in a decreased pH value, and also creates perfect conditions for the growth of anaerobic bacteria (e.g. preventing phagocytosis, activating proteolytic enzymes). The peculiarity of this measurement, which could be the cause of the differing results in many previous studies, seems to involve the sample collection and handling. Maximizing diagnostic accuracy requires recognizing factors that easily occur in a clinical practice and can have an impact in these results. In humans it has been demonstrated that residual air in the collection tube, topical anesthetics such as lidocaine (commonly used in hospitals as well as animal practices), and delay of analysis significantly alter the values measured (Rahman et al., 2008). In horses, exposure of peritoneal fluid to room air was shown to have significant effects on pH, PCO_2, PO_2, HCO_3 and ionized Ca (Romero et al., 2011). Not acknowledging the need for anaerobic collection and containment, the importance of rapid analysis or the possibility of clot formation in the blood gas analyzer leads to diagnostic inaccuracy that can influence case management.

Potassium: The effusion:serum potassium value has been used in the diagnosis of uroabdomen (Dempsey and Ewing, 2011; Fry, 2011; Tsompanidou et al., 2015, Ben Oz et al., 2016) but it was recently proposed that it could also be indicative of gastric perforation (Ben Oz et al., 2016).

More parameters such as leukocyte esterase reagent strips (Porcel, 2013; Thomovsky and Johnson, 2014), serum amino-terminal pro-C-type natriurettic peptide (Guieu et al., 2015), adenosine deaminase, growth factors as cancer-related biomarkers, the albumin gradient (ALBg) for distinguishing transudates from exudates (Zoia and Drigo, 2015) are currently being evaluated in human and veterinary medicine with promising results.

D. Cytology

Cavitary effusions are routinely examined cytologically to evaluate the presence of an inflammatory process and/or microorganisms and extracellular material (e.g. ingesta in peritoneal fluid resulting from gastrointestinal tract rupture as shown in Fig. 2a and Fig. 2b), to observe the cells included, or to determine if there is an underlying neoplastic cause for fluid accumulation.



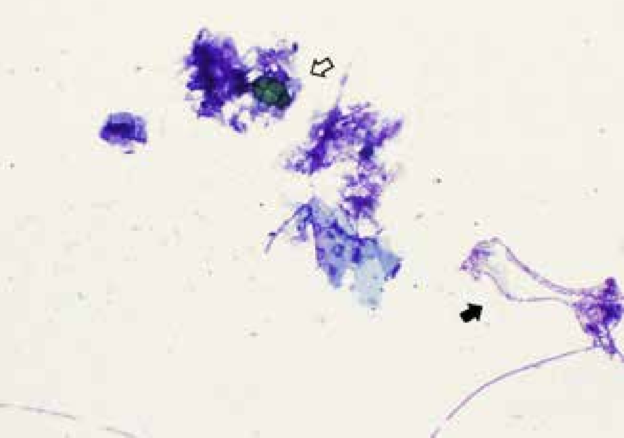


Fig. 2a and 2b. Accidental enterocentesis in a horse. Note the presence of aggregates of mixed, extracellular bacteria (black arrows) and amorphous plant material (intestinal contents). There are also large ciliated protozoa (white arrows) which are part of the normal gut microbiota in horses. An inflammatory response is not observed. Repeat abdominocentesis in this horse revealed a transudate with no obvious sign of gut rupture. Wright-Giemsa stain, 10x objective (2a) and 50x objective (2b)

A negative cytological result for malignancy cannot completely exclude the possibility from the differential, as cytology is a method that can be influenced by several factors. Paucity of representative cells (Fig. 3), cell overcrowding, cell changes and abundance of inflammatory cells can pose great difficulties. To overcome these difficulties many methods have been described in literature. From the more 'traditional' pull smears to the most recently described cell block, there are many methods to choose from, depending on various factors such as fluid quantity and character.

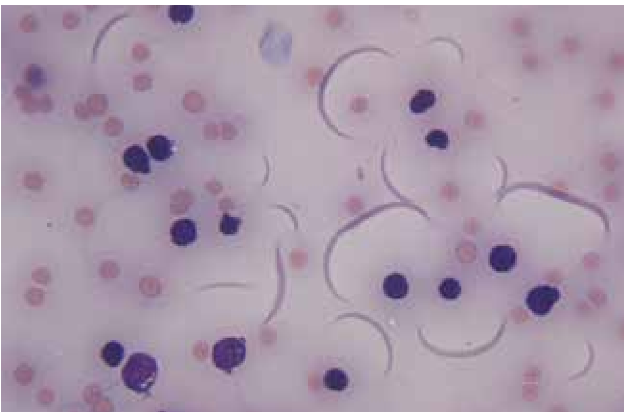


Fig. 3. Pleural fluid from a 6-year-old DSH cat with mediastinal lymphoma. Note the eosinophilic background and the crescent formations, as a result of the highly proteinaceous fluid. Cell morphology cannot be assessed due to the cells being too condensed – the definitive diagnosis in this case was reached post-mortem through histopathology (the cat was euthanized). Cytocentrifuged preparation, 50x objective

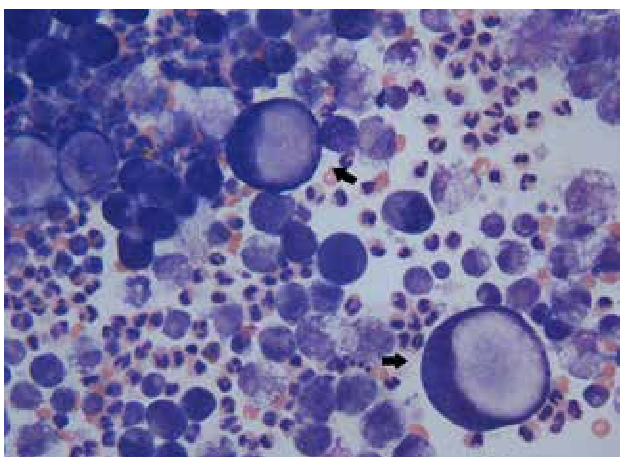


Fig. 4. Pleural fluid from a 14-year-old Collie dog. The cytological diagnosis was mesothelioma or carcinoma, however the possibility that these are reactive mesothelial cells could not be ruled out. There is also marked pyogranulomatous inflammation (probably secondary to the neoplastic process). Amongst high numbers of neutrophils and reactive macrophages there are cells of epithelial/mesothelial origin with marked pleomorphism, including two ''signet ring'' cells (arrows), each with a single large vacuole that displaces the nucleus to the margin of the cell. Cytocentrifuged preparation, 50x objective

Despite its limitations, cytology is extremely valuable in evaluating effusions for the presence of malignant cells (Fig. 4 and Fig.5). Diagnostic accuracy depends mainly on the observer's ability and experience (Brudvig and Swenson, 2015), and on whether the causative neoplasia is exfoliative. The most challenging cases to diagnose are those in which no primary neoplasm can be found anywhere else (e.g. in the lungs, or the abdomen), like in cases with mesothelioma. Most dogs with mesothelioma require immunological testing or histology, apart from cytological evaluation, and even computed tomography has begun to be evaluated as a non-invasive diagnostic tool for this purpose (Watton et al., 2017). A flow chart of a basic microscopic examination of an effusion is presented in Fig.6.

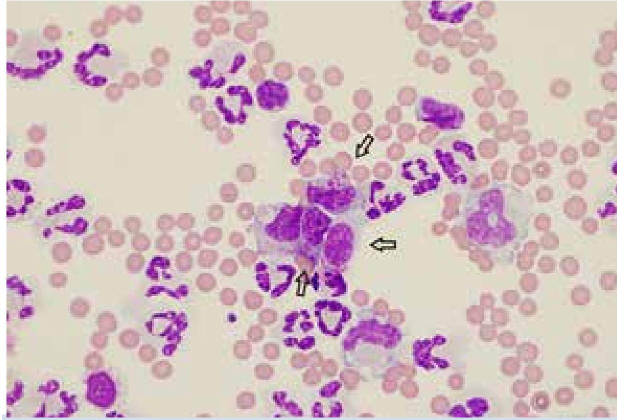


Fig. 5. Abdominal fluid (exudate) from a horse with intestinal lymphoma. Note the presence of intermediate-sized lymphocytes with large, magenta, cytoplasmic granules in the center of the image (arrows). Wright-Giemsa stain, 100x objective

Slide preparation: Opaque fluids usually just need a direct smear (pull or squash prep from well-mixed uncentrifuged sample) because of their high cellularity. Staining can be done using a variety of choices, as long as the cellular elements are depicted well and there is as little precipitate as possible to be confused with bacteria. It is important to prepare as many slides as possible, especially if they are going to be submitted in another laboratory. Generally, when submitting samples, the clinician should include an EDTA tube and at least one fixed stained slide. The slides should be prepared as soon as possible to avoid cell hypersegmentation and morphology alteration due to prolonged storage in the EDTA tube. However, unstained and/or unfixed slides are commonly requested by several laboratories.

The most difficult samples to prepare are those of low cellularity specimens. If centrifugation is not possible, a line smear can be made instead of the more traditional pull smear. This allows for the majority of

cells to appear more concentrated and thus more easily observed under the microscope. If centrifugation is possible, after removing the supernatant (which can be used for TP measurements as referred above) slide preparation can proceed with the known techniques.

The cell block method was first reported in human medicine more than 100 years ago but started to be used widely just in the mid-20th century. The technique was developed as a way for liquid samples to be converted in solid material, thus provide sections in a cytologic sample that can be used for histopathology, immunohistochemistry and molecular biology testing. In veterinary medicine, cell block began to be used only in the last few years. It is not recommended as a replacement, but when used in combination with conventional cytology it increases the diagnostic accuracy (Koksal et al., 2013; Wallace et al., 2015; Assawasaksakul et al., 2017).

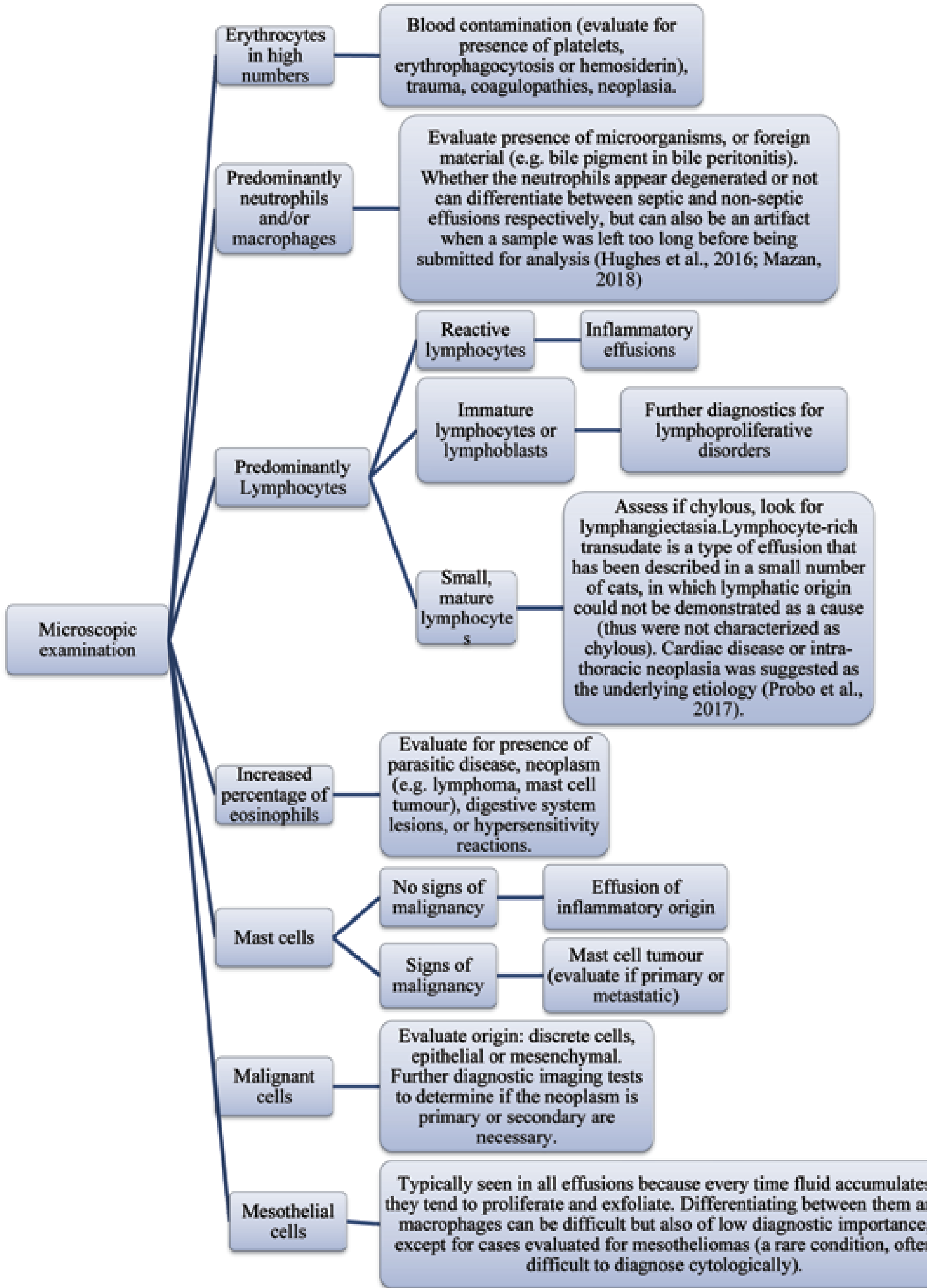


Fig. 6. Flow chart of the microscopic examination of an effusion

Table 3. Specific diagnostic tests for characteristic effusions

Condition	Specific diagnostic tests
Haemorrhagic effusions	Effusion PCV > 10%
Pancreatitis	Effusion lipase activity > 4 times the upper reference limit for serum lipase activity Effusion/serum lipase ratio > 2
Feline infectious peritonitis (FIP)	Rivalta's test (Fischer et al., 2012) Feline Coronavirus (FCoV) antibodies 1:1600 Effusion γ-globulin concentration >1.0 g/dL Effusion albumin/globulin ratio < 0.9 g/dL Immunofluorescent staining of FCoV antigens in effusion macrophages Nested reverse transcription PCR of effusions for FCoV
Chylous effusion	Effusion triglyceride concentration > serum triglyceride concentration Effusion cholesterol < triglyceride levels Effusion triglyceride concentrations >100 mg/dL
Uroabdomen	Effusion/serum creatinine ratio >2.0 Effusion/serum potassium ratio >1.4 Effusion creatinine >4 times greater than normal serum creatinine concentration
Bile peritonitis	Bile pigment crystals in effusion fluid (cytological evaluation) Effusion/serum bilirubin > 2.0
Lymphosarcoma	Flow cytometry PCR for Antigen Receptor Rearrangements (PARR)
Septic exudates	Effusion TNCC > 13,000 cells/ μL (Dempsey and Ewing, 2011) Blood glucose - effusion glucose concentrations difference >20 mg/dL Effusion lactate - blood lactate concentrations < 1.5 mmoL/dL Phagocytized bacteria on cytology

PCV: Packed Cell Volume, TNCC: Total nucleated cells

Cell blocks can be used for all effusion samples but they are more useful for the low cellularity fluid specimens. Advantages include separation of erythrocytes from nucleated cells (especially important in haemodiluted specimens, e.g. when only a few neoplastic cells are present among large concentrations of erythrocytes), maintenance of cellular architecture even when cell clusters are present, use of histochemistry and immunohistochemistry, storage of material and possibility to be re-examined later, cost-effectiveness, and simplicity, as it can be done using equipment present in most practice environments (e.g. microhematocrit centrifuge) (Marcos et al., 2017).

E. Effusion pathology and classification, traditional criteria and beyond

In 1972, the first study about the Light criteria was published. It involved a combination of 3 tests: TNCC, TP and LDH to help clinicians in human medicine distinguish between transudates and exudates. Since then, numerous studies have built upon these criteria in order to classify effusions. Forty years later, the Light criteria remain relevant; however, although they can correctly identify the vast majority of exudates, they misclassify almost 25% of transudates as exudates (Light, 2013). Many additional tests have been suggested to improve the misclassification ratio.

In veterinary medicine, effusions have traditionally been classified according to TNCC and TP measurements (Table 2), with cell types and a few other characteristics taken into account. However, the traditional classification algorithm is occasionally criticized (Bohn, 2017). Multiple cut-off points suggested (Dempsey and Ewing, 2011), confusion over how the published recommendations were established and a large number of cases that seem to defy the classic criteria often restrict the clinician rather than provide a coherent diagnostic tool. Furthermore, in horses, TNCC in the pleural space can be up to 5000 cells/μL (Piviani, 2014; Mazan, 2018).

Classification according to the underlying cause of the effusion instead of a numerical value seems to be a more useful tool for clinicians, enabling them to choose further diagnostic approach more easily (Dempsey and Ewing, 2011). Additional tests for etiological diagnosis are shown in Table 3. The traditional effusion classification helps narrowing down the differential list, as it provides an insight on the mechanism that has resulted in their formation. However, effusions are not static, can be influenced by various factors (e.g. multiple resampling attempts may increase the TNCC

and/or TP) and despite of their mechanism do not always "fit the box". A characteristic example of the latter is FIP. In the ''wet'' form, a cat can present with a pleural, abdominal or even pericardial effusion. These effusions are usually highly proteinaceous and could be classified as exudates according to the TP value, but are often moderately or even poorly cellular, which would bring them in the modified transudate category (Tasker, 2018). Moreover, effusion classification is not always diagnostic of a specific disease. For all these reasons, a holistic approach should be implemented, taking into account the history, clinical examination and other clinicopathological findings (such as imaging), alongside the laboratory analysis.

-Transudates: As there is no compromise in capillary permeability involved in the formation of transudates, cells are not leaked into the cavity. Thus, cell numbers are not increased but instead appear decreased due to dilution as fluid accumulates. The mechanism behind a transudative effusion involves passive fluid shifting due to increased hydrostatic or decreased oncotic pressure. The cause of this is most commonly severe hypoalbuminemia (serum TP <1.0 g/dL) resulting from conditions such as hepatic insufficiency, protein losing enteropathy (PLE), renal glomerular disease, malnutrition/malabsorption, or even iatrogenic. Other causes can include heart failure, portal hypertension or portosystemic shunt, and very early stages of uroabdomen.

-Modified transudates: Generally, modified transudates are formed due to leakage from blood vessels or lymphatics carrying protein-rich fluid. Conditions resulting in modified transudative effusions are numerous and include cardiovascular disease, neoplasms, feline infectious peritonitis (FIP), early stages of uroabdomen, hepatic disease, and, less commonly, glomerulonephritis, lung lobe torsion, and diaphragmatic hernias. Effusions containing blood elements are also usually classified as modified transudates. In these cases, care must be given in distinguishing blood contamination of the sample from intracavitary haemorrhage. Most noncoagulopathic, nontraumatic, spontaneous haemothorax/haemoabdomen, have neoplasms as the underlying cause (in dogs most commonly lymphoma, hemangiosarcoma, pulmonary carcinoma, mesothelioma, osteosarcoma, in cats most commonly hemangiosarcoma, hepatic carcinoma, mast cell tumor).

Exudates: They are associated with a variety of conditions, such as infectious diseases and noninfectious conditions, but they are generally inflammatory in nature and occur in response to a foreign material within a cavity (whether this material is endogenous e.g. urine, bile, pancreatic enzymes, or exogenous e.g. fungi, parasites, bacteria). Cytological evaluation will determine the cell type that predominates in the effusion (Fig. 7 and Fig. 8). Exudates are usually subcategorized in septic (Fig. 9) and non-septic (Fig. 10) depending on the presence or absence of an infectious cause. Although the gold standard method for the diagnosis of a septic effusion is bacterial isolation, when it comes to patient management there often is little time to wait for culture results before starting treatment. Diagnosis can be helped by analyzing certain biochemical markers and by cytology.

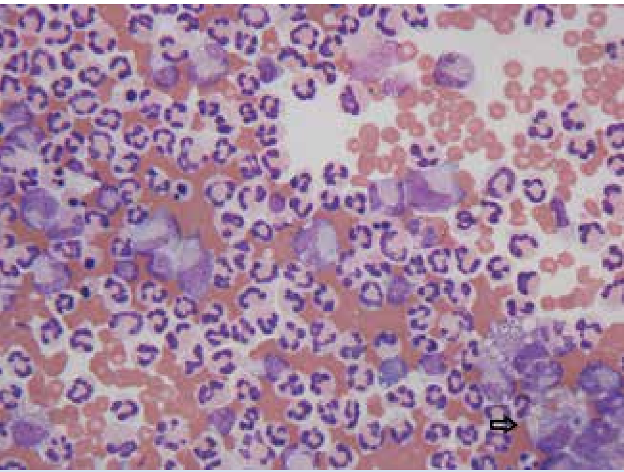


Fig. 7. Pleural fluid from a 12-year-old Golden Retriever dog with carcinoma. Mixed inflammation with non-degenerate neutrophils and reactive macrophages. Many pyknotic neutrophils are shown. Arrow showing a macrophage in the lower right corner exhibiting erythrophagia (evidence of recent or ongoing haemorrhage). Erythrophagia can also be present if the fluid is left to stand as macrophages can phagocytize erythrocytes in vitro. Cytocentrifuged preparation, 50x objective

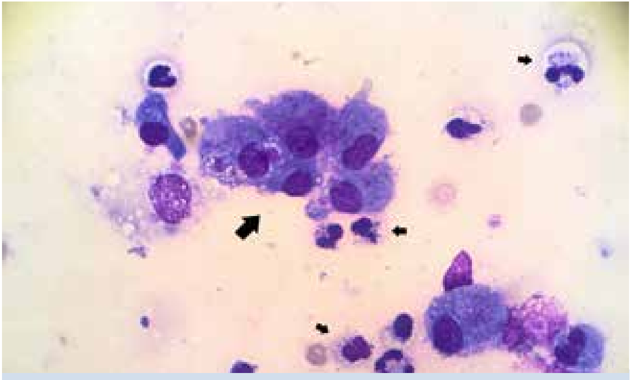


Fig. 8. Abdominal fluid from a dog with septic peritoneal and pleural effusion. Degenerate neutrophils with phagocytized rods (small arrows), and an aggregate of macrophages in the center (large arrow). Direct smear, 100x objective

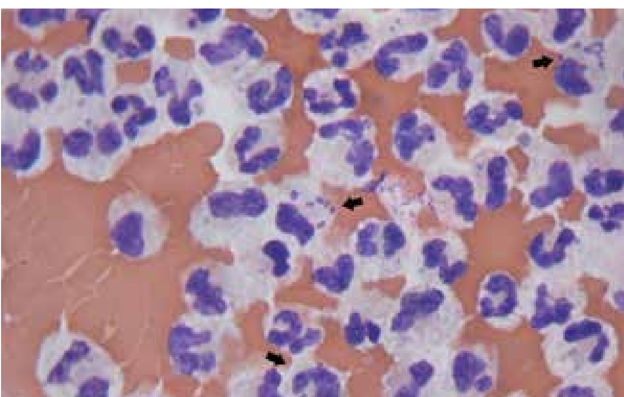


Fig. 9. Abdominal fluid from an 8-year-old Jack Russell with septic peritonitis. Neutrophils with signs of degeneration (nuclear swelling) and phagocytized cocci in a haemodiluted sample. Cytocentrifuged preparation, 100x objective

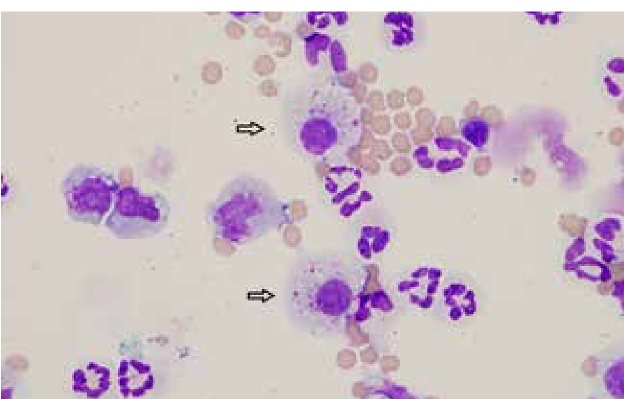


Fig. 10. Abdominal fluid from a horse with a history of colic surgery 3 days prior to abdominocentesis. Fluid analysis including cytology revealed a non-septic exudate with 12,000 leukocytes/μL, total protein 39 g/dL and predominantly non-degenerate neutrophils. Note the presence of vacuolated macrophages in the center of the image (arrows) which contain variably sized pink to purple cytoplasmic material, consistent with carboxymethyl cellulose (CMC) which was administered intraperitoneally during surgery to prevent adhesion. Wright-Giemsa stain, 100x objective

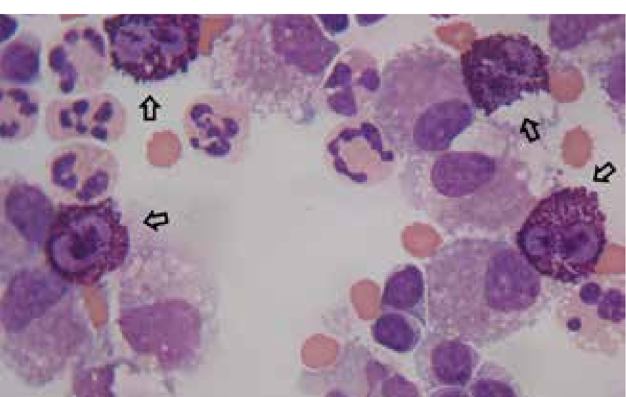


Fig. 11. Pleural fluid from a 4-year-old German Shorthaired Pointer with mast cell tumor. Four well-granulated, occasionally binucleated mast cells are noted. Mixed inflammation with neutrophils, macrophages and small lymphocytes is also evident. Cytocentrifuged preparation, 100x objective

Less commonly, an exudative effusion can form due to exfoliation of cells into a cavity, which can either be neoplastic (e.g. mesothelioma, mast cell tumors (Fig. 11), lymphomas) or reactive mesothelial (Fig. 12), or secondary to a chronic chylous effusion (Fig. 13).

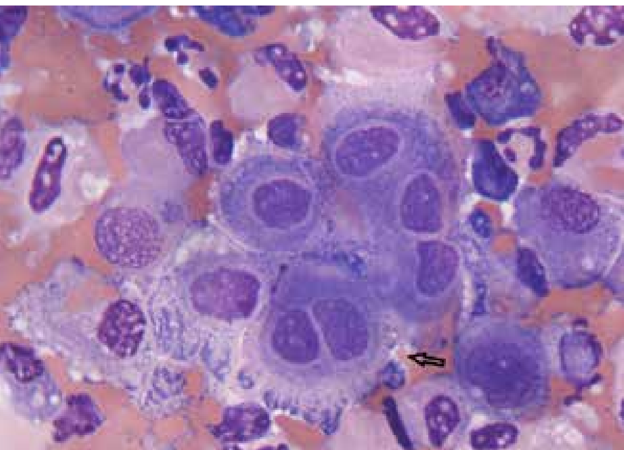


Fig. 12. Pleural fluid from a 6-year-old dog with a marked mixed inflammation secondary to a mast cell tumor. Poorly preserved neutrophils, and a cluster of reactive/dysplastic mesothelial cells with cytoplasmic basophilia are shown. Arrow showing a reactive binucleated mesothelial cell. Cytocentrifuged preparation, 100x objective

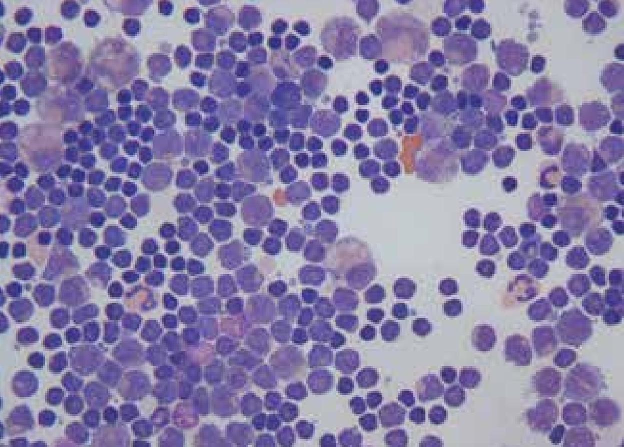


Fig. 13. Abdominal fluid from a 14-year-old DSH with chronic dyspnea, showing high numbers of lymphocytes (predominantly small) and occasional macrophages. The cat was diagnosed with cardiac disease. Cytocentrifuged preparation, 50x objective

F. Microbiology

Septic pleural effusions: The pathogenesis in the majority of pyothorax cases in companion animals, involves the aspiration of microorganisms that normally inhabit the oral cavity and upper respiratory tract. Most pleural effusions seem to be caused by polymicrobial infections. Most frequently involved microorganisms are presented in Table 4.

Table 4. Commonly isolated microorganisms in septic pleural and peritoneal effusions in dogs, cats and horses

Pleural effusions		
	Dogs, Cats	Horses
Bacteria	*Escherichia coli* *Streptococcus spp.* *Staphylococcus spp.* *Corynebacterium spp.* *Pasteurella spp.* *Bacteroides spp.* *Fusobacterium spp.* *Actinomyces spp.* *Nocardia spp.* *Porphyromonas spp.* *Peptostreptococcus spp.* *Prevotella spp.* *Mycoplasma spp. (kittens, immunosuppressed adults)*	*Streptococcus equi subspecies zooepidemicus* (Stillion and Letendre, 2015; Reuss and Giguère, 2015) *Staphylococcus spp.* *Escherichia coli* *Pasteurella spp.* *Klebsiella spp.* *Enterobacter spp* *Actinobacillus spp.* *Bordetella spp.* *Enterobacter spp.* *Salmonella enterica* *Clostridium spp.* *Bacteroides spp.* *Prevotella spp.* *Peptostreptococcus spp.* *Fusobacterium spp* *Rhodococcus equi (immunosuppressed individuals)* *Mycoplasma spp.* (controversial) (Stillion and Letendre, 2015
Fungi	*Candida albicans (Bradford et al., 2013)* *Cryptococcus spp.* *Blastomyces dermatitidis*	
Peritoneal effusions		
Bacteria	*Escherichia coli* *Klebsiella* spp. *Bacteroides* spp. *Streptococcus* spp. *Clostridium* spp. *Enterococcus* spp. *Staphylococcus* spp.	Same as for dogs and cats

Septic peritoneal effusions: In companion animals, most common etiology of septic peritonitis (Fig. 14 and Fig. 15) is perforation of an abdominal organ (usually the gastrointestinal tract) causing fluid containing bacteria to leak into the peritoneal space (Culp and Holt, 2010). Like in septic pleural effusions, in most septic peritonitis cases multiple microorganisms are often isolated, although Gram- bacteria are predominant (Table 4).

More recent case reports have indicated various other infectious microorganisms as potential causes for pleural effusions. Cytauxzoonosis is reported as an emerging tick-borne feline disease (Lloret et al., 2015; Alho et al., 2016). *Streptococcus equi* subsp. *zooepidemicus* (a commensal organism of horses) has been reported as an emerging pathogen in dogs (Priestnall and Erles, 2011), having caused several outbreaks of haemorrhagic pneumonia. The particular subspecies has also been isolated from purulent pleural effusions in humans and seems to be of animal origin (Held et al., 2014). In dogs, *Bartonella henselae* was found in high prevalence in haemorrhagic pleural effusions but whether it plays a primary role in the pathogenesis, or it causes an opportunistic infection requires further research (Cherry et al., 2009; Weeden et al., 2017).

The diagnostic value of the microbiological analysis (Gram stain, culture, enrichment culture before PCR) is controversial. In human medicine, the routine use of microbiological testing of patients with parapneumonic effusions, was shown to be of very limited

value, having no impact whatsoever in treatment decision or mortality rate, and found to add very little to the diagnosis, compared to other diagnostic tools such as biochemical analysis (Jimeneza et al., 2006).

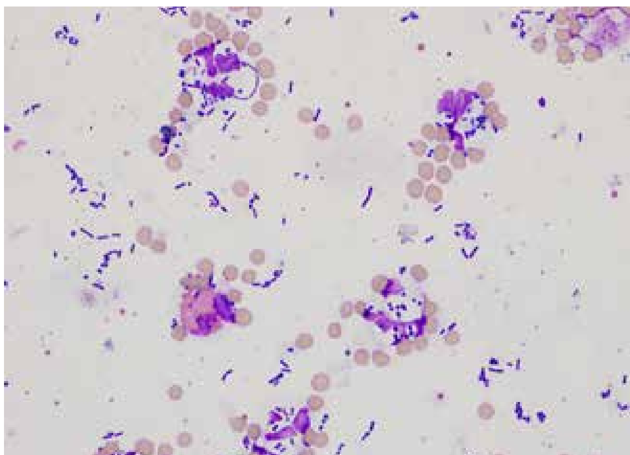


Fig. 14. Abdominal fluid from a horse, cytospin preparation. Septic exudate. Total nucleated cell count 89,000/μL, total protein 4.2 g/dL. Cytology revealed a predominance of frequently degenerated neutrophils with intracellular and extracellular bacteria (mixed population of cocci and rods). An eosinophil is also seen. The increased numbers of erythrocytes can be related to red cell diapedesis (due to serositis), secondary haemorrhage, and/or blood contamination during abdominocentesis. Wright-Giemsa stain, 100x objective

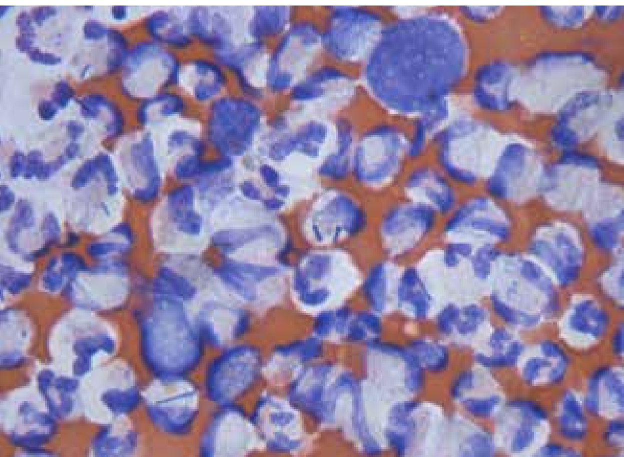


Fig. 15. Abdominal fluid from a 3-year-old Cocker Spaniel dog with septic peritonitis. Neutrophils with signs of degeneration and intracellular bacterial rods, against a bloody background (haemodiluted sample). Cytocentrifuged preparation, 100x objective

CONCLUDING REMARKS

Cases involving thoracic and/or abdominal effusions occur frequently in a clinical setting and are often life threatening. Using the established algorithm for TNCC/TP parameters, is a useful initial step. The diagnostic tools described in this review are an important part of forming a complete picture that will help the practitioner to reach a diagnosis, develop effective therapeutic plans, and improve in-hospital stay and mortality rates.

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J HELLENIC VET MED SOC 2019, 70(3): 1603-1610
ΠΕΚΕ 2019, 70(3): 1603-1610

Review article
Ανασκόπηση

Effects of diet consistency on mandibular growth. A review

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ABSTRACT. This article is a review that focuses on the diet consistency and how this affects mandibular morphology. Various published studies focused on the relationship between mastication and growth of the mandible because it is considered that mandibular growth is dependent on the loads exerted by the function of the masticatory muscles. Moreover it has been pointed out that the increase of orthodontic anomalies is due to the modern softer diet. Even in rats, soft diet is one of the factors causing malocclusions. All of the studies have been experimental, mainly in rodents, since this research is impossible to be applied on humans in a short period of time. Most experimental studies suggested that occlusal loading affects bone mass, bone amount, bone density, the length and the width of the bone, the degree of mineralization, the genetic expression, the collagen immunoreaction and the chondrocytes action on the cartilage. It is stated that bone volumes and thickness of the mandible of rats fed with soft diet were smaller when compared to animals fed with hard diet. Also the mandibles and condyles were smaller and less dense in the rats of soft diet as compared to controls. Furthermore the length and the width of the condyle in the soft diet group of animals were smaller as compared to the condyle of the hard diet group of animals. Soft diets affect also the degree of mineralization, and the action of the chondrocytes on the cartilage.

Keywords: soft diet, mandible, condyle

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Date of initial submission: 08-02-2017
Date of revised submission: 04-12-2017
Date of acceptance: 19-09-2018

ΠΕΡΙΛΗΨΗ. Το παρόν άρθρο είναι μια ανασκόπηση που διαπραγματεύεται την επίδραση της σύστασης της διατροφής στην μορφολογία της κάτω γνάθου. Πολλές δημοσιευμένες εργασίες έχουν επικεντρωθεί στη μελέτη της σχέσης της μασητικής λειτουργίας και της αύξησης της κάτω γνάθου, εξαιτίας της θεώρησης ότι η αύξηση της κάτω γνάθου εξαρτάται απο τις φορτίσεις που ασκούνται απο τους μασητήριους μύες. Επιπρόσθετα ο σύγχρονος τρόπος διατροφής με μαλακές τροφές έχει ενοχοποιηθεί για την αύξηση των ορθοδοντικών ανωμαλιών. Ακόμα και στους επίμυς η διατροφή με μαλακή τροφή θεωρείται ένας απο τους παράγοντες που συντελεί σε ανωμαλίες της σύγκλεισης. Όλες οι δημοσιευμένες εργασίες είναι πειραματικές, κυρίως σε τρωκτικά ζώα, επειδή είναι αδύνατον να εφαρμοσθούν ανάλογες μελέτες σε ανθρώπους σε σύντομο χρονικό διάστημα. Οι περισσότερες πειραματικές μελέτες συμπεραίνουν πως οι μασητικές φορτίσεις επηρεάζουν την οστική μάζα, τη ποσότητα του οστού, τη πυκνότητα του οστού, το μήκος και το πλάτος του οστού, το βαθμό της επιμετάλωσης, την γενετική έκφραση, και την ανοσοιστοχημική αντίδραση του κολλαγόνου, όπως και τη δράση των χονδροκυττάρων στο χόνδρο του κονδύλου της κάτω γνάθου. Έχει διατυπωθεί πως το βάρος και η πυκνότητα των γνάθων επίμυων που είχαν διατραφεί με μαλακή τροφή ήταν μικρότερα απο τα αντίστοιχα των ζώων που είχαν διατραφεί με σκληρή τροφή. Επίσης οι γνάθοι και οι κόνδυλοι επίμυων που έχουν διατραφεί με μαλακή τροφή ήταν μικρότεροι σε μέγεθος και παρουσιάζουν μικρότερη πυκνότητα σε σύγκριση με τις γνάθους και τους κονδύλους των ζώων ελέγχου. Επιπρόσθετα το μήκος και το πλάτος των κονδύλων των ζώων που είχαν διατραφεί με μαλακή τροφή ήταν μικρότερα απο τα αντίστοιχα των κονδύλων των ζώων που είχαν διατραφεί με σκληρή τροφή. Η μαλακή διατροφή διαπιστώθηκε ότι επηρεάζει τέλος το βαθμό επιμετάλλωσης όπως και τη δράση των χονδροκυττάρων στο χόνδρο.

Λέξεις ευρετηρίασης: μαλακή διατροφή, κάτω γνάθος, κόνδυλος

It is well accepted that the growth of the craniofacial system is determined both by hereditary and environmental factors (Watt and Williams 1951). Also bone growth and development is closely dependent on the loading forces exerted by muscular function. The mandibulofacial growth is closely associated to the movements of the jaws and loads of the orofacial region. Thus environmental factors including mastication seem to be responsible for a variety of developmental changes in the stomatognathic system. This may be the rationale for explaining the mean increase of malocclusions in industrial societies in the 20th Century (Beecher and Corruccini 1981). During the last century, the modern dietary habits have been pointed out as contributors to the increased frequency of malocclusion, due to their reduced demands on masticatory muscles (Waugh, 1937;Corruccini and Lee 1984). Also, Soft diet causes malocclusions in rats (Dontas et al 2010). Several experiments on the consistency of diet provided to animals and especially to rodents have been carried out in order to test the interelationship between function and mandibular growth.

INTERACTION BETWEEN MUSCULAR FUNCTION AND BONE GROWTH

The human masticatory system is a complex musculoskeletal system where activation of the masticatory muscles, movements of the jaw, and loads and deformations in both the temporomandibular joint and jaw are closely interrelated (Berger, 1992; Ciochon et al, 1997, Odman et. al. 2008). If one of these

factors changes by means of intrinsic or extrinsic cause, it will affect all the others. This evidently concerns the activation patterns of the masticatory muscles adapting to a new environment and it occurs as a consequence of, for instance, craniofacial growth and development (Ciochon et al, 1997). Studies in growing rodents have shown that reduced masticatory function causes morphological changes in the mandible. Recent studies reveal that myostatin deficient mice with increased muscle mass, physiological cross-section, and contractile muscle forces exhibit greater bone mineral density than normal mice in the spine and temporomandibular joint (Hamrick et al, 2003; Nicholson et al, 2006; Ravosa et al, 2007). Therefore, the craniofacial mandibular structure and its degree of mineralization can be considered to be closely associated with masticatory muscle behavior. A method used to alter the masticatory function is feeding young animals a soft diet. Altering the consistency of the diet in this way has been shown to cause overall size differences in the ramus region (Watt and Williams, 1951).

Bone is a dynamic tissue, which continuously undergoes adaptive remodelling, i.e. resorption and apposition, to meet the requirements of its functional environment. The remodelling rate is a major determinant of the degree of mineralization of bone (DMB) (Boivin andMeunier, 2002). A higher remodelling rate decreases the time available for secondary mineralization, which results in bone with a lower DMB (Boivin et al, 2009). The remodelling rate of bone is related to the magnitude of intermittent mechanical loading and the resulting dynamic strains in the tissue (Turner, 1998). In general, more heavily loaded bone has a higher remodelling rate and is therefore less mineralized and less stiff than lower loaded bone (Cullen et al, 2001). This regionally heterogeneous organization of bone mineral has been attributed to regional differences in the magnitude and mode of strain brought about by mechanical loading (Skedros et al,1994). Under physiological conditions, intermittent mechanical loading of bone is caused predominantly by muscular contractions.

The muscles thus provide an important mechanical stimulus for bone remodelling by inducing strains in the skeletal system (Turner, 2000). The significance of muscle-generated bone loading is illustrated by the effect on the skeleton under conditions of increased or decreased muscle activity. For example, the loss of normal physiologic loading after spinal cord injury causes rapid severe bone loss in the paralyzed extremities of affected individuals, which can be counteracted by long-term electrical stimulation of muscles (Dudley-Javoroskiand Shields, 2008).

In 1996, Kiliaridis et al. (1996) demonstrated that masticatory hypofunction caused the reduction of “radiographic” bone mass in the dental alveolar process, the condylar costa, the condylar process, and the lower anterior border of the ramus in the mandible of growing rats. However, it is not clear whether the reduction in radiographic bone mass was due to changes in the amount/thickness of bone and/or to changes in the density of the cortical and trabecular bone, and whether these changes would have a different regional pattern. Three years later Bresin et al. (1999) reported that the amount of bone amount and bone density may be two possible mechanisms used to adjust local mechanical properties within the mandibular functional units. They found that bone mass was larger in the hard diet group in all areas except lateral to incisors. Bone density was higher for the hard diet group only medial to cortical bone on the dentoalveolar process of the first molar and the pterygoid fossa. Thickness of the cortical bone was higher in the hard diet group in the cortical bone below the incisor, adjacent to the mental foramen and through the first molar, the lateral cortical bone on the dentoalveolar process, ramus region above condylar costa, pterygoid fossa and the lateral cortical plate of the pterygoid ridge in the ramus.

DIET AND MANDIBULAR GROWTH

Watt and Williams (1951) compared the mandibles of soft and hard diet rats and they concluded that the weight volume and thickness of the mandibles of the soft group was smaller than the hard diet group. This was explained by the change in density of bone structure. Beecher and Corruccini confirmed these findings in 1981. They also compared the morphology of the mandible in growing rats fed hard and soft diet. In conclusion, they found that the soft diet animals where slightly smaller in body mass, they had smaller mandibles and condyles and they were radiographically more dense. Furthermore, soft diet rats had less width of the maxillary dental arch and they had smaller masseter and temporal muscles. Finally, they found that the soft diet group had skulls consistently smaller in mass and in linear dimensions, although with no significant differences in shape. In 2008 Odman et al, found that a period of 7 months with low masticatory demands in the hypofunctional group during ado-

lescence and early adulthood had a significant effect on the lateral shape of the rat mandible as compared to controls. The area of the mandible was smaller in the hypofunctional compared to the normal group. Interestingly, the alveolar process was shorter in the normal group. Morphometric analysis revealed significant differences such as the area of the angular process and the inclination of the condylar process. The rehabilitation group was only marginally different compared to the hypofunctional group, although a general tendency to approach (catch-up) the normal group was observed, and one morphometric variable (condylar base inclination) was indeed significantly different. Morphometric analysis revealed only marginal changes of the adult rat mandibular morphology during a 6-week period of masticatory function rehabilitation. However, the observed catch-up tendency might suggest that a longer rehabilitation period may have significant effect on mandibular morphology. In 2007 Tanaka et al. studied the effect of food consistency on the degree of mineralization in the rat mandible. They found that the degree of mineralization was significantly lower in the trabecular than in the cortical bone and in the anterior area the mandibular body showed a significantly higher degree of mineralization than the posterior area. In both areas the soft diet group had a significantly higher degree of mineralization than the hard diet group. The trabecular bone in the condyle of the hard diet group showed a significantly higher degree of mineralization than in the soft diet group. Their results indicated the importance of proper masticatory muscle function for craniofacial growth and development. Four years after Grunheid et al. (2011) had the same hypothesis, using rabbits as experimental animals, but their result suggested that a moderate reduction in masticatory functional load does not significantly affect the remodelling rate and the degree of mineralization in areas of the mandible that are loaded during mastication but might induce a more heterogeneous mineral distribution. More specifically, the degree of mineralization of bone did not differ significantly between the experimental and control animals at any of the sites assessed. However, in the rabbits that had been fed soft pellets, both cortical bone at the attachment sites of the temporalis and digastric muscles and cortical bone in the alveolar process had a significantly higher degree of mineralization than cortical bone at the attachment site of the masseter muscle, while there were no significant differences among these sites in the control animals.

DIET AND TEMPOROMANDIBULAR GROWTH

Approximately 10% of the population over the age of 18 has pain in the temporomandibular joint (TMJ) regionand about 15% of the people who have TMJ pain have degenerative diseases of the TMJ (TMD). (LeResche, 1997; Grünheid et al, 2011). The exact etiology for TMD is unknown; however, most dentists and physicians have been inclined to believe that the single most important etiological factor is mechanical loading that surpasses the adaptive capacity of the joint.(Emshoff et al, 2003; Milam, 2005). In dentofacialorthopaedics an effort is made to influence mandibular growth. The main target in this effort is the condylar cartilage of the mandible. There has been a great deal of controversy over the years concerning the true effects of this treatment rationale. Animal experiments and condylar cartilage tissue examination revealed that the condylar cartilage responded favorably in functional mandibular advancement and extra growth of the cartilage tissue was evident (Zarb and Carlsson, 1999). For the aforementioned reasons recent studies focused more on the TMJ response to the mechanical forces. The TMJ is formed by the mandibular condyle and the mandibular fossa of the temporal bone. Separating these two bones from direct contact is the articular disc. Unlike other joints, which are composed of hyaline cartilage, the articular portion of the mandibular condyle and disc is comprised of fibrocartilage. The mandibular condylar cartilage can be organized into four zones. The most superficial layer is called the articular zone and cells in this zone are characterized by their expression of Proteoglycan 4 (Prg4) (McNamara and Carlson, 1979). The second zone is the polymorphic zone, which contains the precursor cells for the flattened and hypertrophic zones4. The third zone is the flattened zone. The cartilage cells in this layer are characterized by the expression of Collagen type II (Ohno et al, 2006). The fourth and deepest zone is the hypertrophic zone. In this zone, the chondrocytes are characterized by the expression of Collagen type X (Chen J. et al, 2009).

In 1999 Kiliaridis et al supported that low masticatory function leads to decreased growth of the condyle and changes in the thickness of the cartilage. They found that the rats fed a soft diet showed a thinner condylar cartilage in the anterior part of all portions. In contrast, the cartilage was thicker in the soft group in the posterior part of the condyle. The length and the width of the condyle in the group who fed soft diet

were significantly smaller. Chen J. et al (2009) examined the effects of altered functional loading on the expression of other genes found in the various zones of the mandibular condylar cartilage in female mice. They found that altered functional loading for 2-6 weeks caused significant reduction in the thickness of the condylar cartilage whereas, only at 4 weeks was there a significant decrease in the bone volume fraction and trabecular thickness of the subchondral bone. Gene expression analysis showed that altered functional loading for 4 weeks caused a significant reduction in the expression of SRY-box containing gene 9 (Sox9), Collagen type X (Col X), Indian hedgehog (Ihh), Collagen type II (Col II) and Vascular endothelial growth factor (Vegf) and altered loading for 6 weeks caused a significant decrease in the expression of Sox9, Col II, Vegf and Receptor activator of NF-kB ligand (Rankl) compared to the normal loading group. Altered functional TMJ loading in mice for 2-6 weeks leads to a loss of the condylar cartilage and a transient loss in the density of the mandibular condylar subchondral bone. In 2015 Uekita et al studied the effects of a soft diet on the collagens and chondrocytes in the growing TMJ cartilage. They examined the condylar and glenoid fossa cartilage of rats fed a liquid diet by histology, immunohistochemistry with anti-types I, II, and X collagen antibodies, and transmission electron-microscopy (TEM). The results of this research suggested that the condylar cartilage in the experimental rats showed weak immunoreactions for three types of collagens. The ultrastructure had fewer fine collagen fibrils, the glenoid fossa cartilage showed narrower Alcian blue-positive areas and the immunoreactions for three types of collagen were also weaker compared to those of the controls. The chondrocytes in the experimental rats had extended thin cytoplasmic processes, and had formed gap junctions, as assessed by transmission electron microscopy. Fewer fine collagen fibrils, but thick bands of collagen fibrils were observed in the glenoid fossa of the experimental cartilage. Their results proposed that liquid diet impairs the quality and quantity of collagens and chondrocytesin the TMJ cartilage of growing rats. The same year, another study from Polur et al (2014) looked at the role of Estrogen Receptor (ER) beta in mediating these effects. They used 21-day-old male and female mice. They were exposed to decreased occlusal loading (soft diet administration and incisor trimming) for 4 weeks. At 49 days of age the mice were sacrificed. Proliferation, gene expression, Col 2 immuno-histochemistry and micro-CT analysis were performed on the mandibular condyles. Their results suggested that decreased occlusal loading induced inhibition of early chondrocyte maturation markers in female mice was attenuated by ER beta deficiency. In 2006 Papachristou et al. examined the involvement of components of the AP-1 transcription factor family such as Fra-1, Fra-2, JunB and JunD in the signalling pathway of mechanical loading of the condylar cartilage, and subsequently the association of mechanical loading to cell differentiation and apoptosis through the involvement of these proteins. They concluded that the JunB, JunD, Fra-1, and Fra-2 members of the AP-1 transcription family in the response of condylar cartilage chondrocytes to functional loading alterations, suggesting that mechanical loading in chondrocytes triggers biochemical responses associated with AP-1 cellular functions such as maturation, differentiation and apoptosis. Downstream, these biological phenomena influence the overall growth of the condylar cartilage.

CONCLUSIONS

They have been various studies looking at the diet consistency and how this affects the mandibular and condylar morphology. The correlation of diet consistency and head growth has high clinical importance since it might give an answer to the evolution of human growth and development. We can understand the human growth and achieve a better treatment for orthodontic anomalies and condylar disorders. Through the aforementioned researches is suggested that occlusal loading affects the bone mass, bone amount and bone density of the mandible,as well as the mandibular length and the width. Also muscular loading affects the degree of mineralization, the genetic expression, the collagen immunoreaction and the chondrocytes action on the cartilage. Never less there are no works studying the long term effects of changes in the diet consistency of the animals and there is a need for new studies focusing on this.

CONFLICT OF INTEREST

None declared.

Author (year)	Aim	Experimental animals	Interventions	Study duration	Outcomes	Methods of outcome assessment	Results	Conclusions
Beecher and Corruccini (1981)	Dietary consistency on craniofacial and occlusal development in the rat.	90 Sprague-Dawley 21 days of age GroupI:15 F, 15 M. GroupII: 15 F, 15 M Group III: 15F, 15 M	GrI: Fed pelletted rat chow GrII: Fed a gruel-like porridge consisting of ground chow moistened with water GrIII: Fed soft diet six days with dry pellets provided every seventh day only	4 months	O_1: Body mass O_2: Mass of the entire masseter O_3: Maxillary arch length O_4: Maxillary arch breadth O_5: Mandibular length O_6: Anteroposterior length of condylar articular surface	O_1: measurement of Body mass O_2: excision of masseter O_3: Measurement from incisor to distal edge of last molar O_4: Measurement from buccal points of MI O_5: Measurement from incisor to MI O_6: From the anterior to posterior part of articular surface.	O_1: Significantly larger GrI O_2: Significantly larger in GrI O_3: Increased in GrI O_4: Increased in GrI O_5: Increased in GrI O_6: Increased in GrI	Soft diet animals were smaller in size of entire body, maxilla mandible and condyle.
Kiliaridis Et. al (1996)	The effect of altered masticatory muscle function on bone mass at different sites in the rat mandible	42 growing male rats GroupI:14 M. GroupII: 14 M Group III: 14 M	GrI: sacrificed at the beginning of the experiment. Used as base line. GrII: Fed hard diet (control) GrIII: Fed soft diet	28 days	O_1: Bone Mass	O_1: Lateral radiographs of mandibular halves together with aluminium stepwadge and then image analysis	O_1: Significant incresed in GrII in the alveolar bone of the molars and the incisor, condylar costa and condyle process area.	**Altered masticatory function influences the amount of bone mass in certain parts of the mandible.**
Kiliaridis et. al. (1999)	The influence of functional alterations on the size of the mandibular condyle.	40 male growing rats Group I: 20M Group II: 20M	GroupI: Fed hard diet GroupII: Fed soft diet.	28 days	O_1: Size of the mandibular condyle in histologic terms O_2: Size of mandibular condyle in morphometric terms	O_1: -GrI:10 rats for histologic analysis -GrII: 10 rats for hystologic analysis O_2:-GrI: 10 rats for morphometrics -GrII: 10 rats for morphometrics	O_1: Cartilage thinner in the anterior part and thicker in the posterior for GrII. O_2: Increase size of the condyle for GrI	**Increased condylar cartilage thickness is not necessarily evidence of increased condylar growth**
Papachristou et. Al. (2006)	To examine the involvement of the Fra-1, Fra-2, JunB and JunD proteins in the mandibular condylar cartilage.	30 female, 14-day-old Wistar rats Group I: 15F Group II: 15F	GroupI: Fed hard diet GroupII: Fed soft diet.	21 days	O_1: Expression of Fra-1, Fra-2, JunB and JunD proteins	O_1: The condylar cartilage of both groups was immunostained using specific antibodies against Fra-1, Fra-2, JunB and JunD.	O_1: Group II had higher expression of Fra-1, Fra-2, JunB and JunD proteins	**Mandibular condylar chondrocytes sense functional loading changes and ultimately influence the growth of the condylar cartilage.**
Tanaka et. al. (2007)	To analyze the degree of mineralization in the mandible of growing rats fed with a hard or soft diet.	15 Male Wistar Rats Group I: 6M Group II: 9M	Group I: Fed hard diet Group II: Fed soft diet	9 weeks	O_1: Degree of mineralization of cortical bone O_2: Degree of mineralization of trabecular bone	O_1, O_2: Cortical and trabecular bone of mandibles were obtained using a microCT system	O_1: Group II had a significantly higher degree of mineralization O_2: Group I showed a higher degree of mineralization	**Muscle function affects the craniofacial growth and development.**
Odman et. al. (2008)	The effect of masticatory functional changes on the lateral view morphology of the mandible in adult rats.	60 Male Sprague-Dawley rats (21 day old) Group I: 16M Group II: 44M, Group II_1: 12M Grouo II_2: 12M	Group I: Fed hard diet Group II: Fed soft diet for 21 weeks and then divided Group II_1: Fed soft diet Group II_2: Fed hard diet	27 weeks	O_1: morphometric differences between the groups.	O_1: cephalometric Xrays and morphometric analysis.	O_1,: Mandible was smaller in Group II, Group II_2 had only marginal differences to Group II_1, Group II_2 had a tendency to catch up the development as Group I.	**Marginal changes of the adult rat mandibular morphology during a 6-week period of masticatory function rehabilitation..**

Author (year)	Aim	Experimental animals	Interventions	Study duration	Outcomes	Methods of outcome assessment	Results	Conclusions
Chen et. al. (2009)	To develop a mouse TMJ altered functional loading model.	134 Female mice (21 day old)	Group I: Fed hard pellet diet Group II: Altered functional loading (incisor trimming every other day and soft dough diet)	12 weeks	O_1: mandibular condylar cartilage O_2: subchondral bone volume O_3: genetic expression	O_1: Histology O_2: Microcomputed tomography O_3: real time polymerase chain reaction (PCR) analysis.	O_1: There was a decrease in Safranin O staining in the mandibular condylar cartilage for group II. O_2: Group II had a significant decrease in the width of the condylar cartilage and in the thickness of the mandibular condylar cartilage. O_3: Group II caused a significant decrease in the expression of *Sox9, Col II, Vegf* and Receptor activator of NF-κB ligand (*Rankl*).	**Altered functional TMJ loading in mice for 2–6 weeks leads to a loss of the condylar cartilage and a transient loss in the density of the mandibular condylar subchondral bone.**
Dontas et. al. (2010)	The incidence of malocclusion in a longitudinal study of the normal growth and aging of Wistar rats carried out in our laboratory.	40 Wistar rats (20 male, 20 female,3 wk age)	All animals fed *ad libitum* a pelleted commercial balanced rat diet consisting of 21% protein, 6.2% fat, 4.5% fiber, 7.5% ash, 1.1% calcium, 0.9%phosphorus, 0.35% sodium	Rats life span	O_1: presence of malocclusion	O_1 : Clinical examination	O_1 : Malocclusion was found on 60th week of age in a male rat and the 76th week of age in a female rat. Sex did not influence malocclusion: 46% affected rats were female and 54% were male.	**Special attention should be given to the potential appearance of malocclusion during long-term rodent studies, because its incidence may adversely affect the animals' health and general wellbeing**
Grunheid et. al. (2011)	The effect of a reduction in masticatory functional load on the degree and distribution of mineralization of mandibular bone	16 male juvenile New Zealand White rabbits Group I : 8M Group II: 8M	Group I: Fed soft pellet diet Group II: Fed hard pellet diet	12 weeks	O_1:Degree of bone mineralization (DMB)	O_1: Excision of jaw muscles, condylar head and alveolar process.	O_1: In Group I, both cortical bone at the attachment sites of the temporalis and digastric muscles and cortical bone in the alveolar process had a significantly higher DMB than cortical bone at the attachment site of the masseter muscle, while there were no significant differences among these sites in the Group II.	**A moderate reduction in masticatory** functional load **does not significantly affect the remodelling rate and the DMB in areas of the mandible** that are loaded but might induce a more heterogeneous mineral distribution.
Polur et. al. (2015)	To examine the role of Estrogen Receptor (ER) beta in mediating these effects.	49 Mice (21-day-old) Group I:24 male Group II:25 female	Fed soft diet administration and incisor trimming was performed	4 weeks	O_1: Gene expression O_2: Condylar cartilage O_3: Bone volume	O_1: PCR O_2: Col 2 immunohistochemistry O_3: Micro-CT	O_1: Both groups had significant decreased Col 10 expression O_2: Decreased subchondral volume O_3: Decreased bone volume.	**Decreased occlusal loading induced inhibition of Sox9 and Col 2, did not occur in female ER beta deficient mice.**
Uetika et. al. (2015)	To determine the effect of a soft diet on the collagens and chondrocytes in the growing TMJ cartilage.	48 Male Wistar rats Group I: 24M Group II: 24M	Group I: Fed hard diet Group II: Fed soft diet	8 weeks	O_1: Changes on chondrocytes O_2: Changes on collagens	O_1: Electron microscopy O_2: Immunohistology	O_1:Gr II had extended thin cytoplasmic processes, and formed gap junctions O_2: Gr IIhad weak immuno-reactions for 3 types of collagens	Soft diet had deleterious effects on the quality and quantity of collagens and chondrocytes in the TMJ cartilage in growing rats.

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J HELLENIC VET MED SOC 2019, 70(3): 1611-1616
ΠΕΚΕ 2019, 70(3): 1611-1616

Review article
Ανασκόπηση

Nanotechnology and its Considerations in Poultry Field: An Overview

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ABSTRACT. Nanotechnology *is an emerging science field that in the years to come could have countless beneficial effects on every aspect of everyday life including animal farming.* Recently*, research in this sector has shown the potential for* many different applications in Veterinary *practice. In poultry farming in particular, various nanoparticles have been experimentally used for several purposes such as: alternative to antibiotics as growth promoters, as* feed additives to enhance and improve the growth rate, performance, immunity, resistance to pathogens and increase the quality of meat. In *laying hens*, *they can have a positive influence* to both quantity and quality of eggs. Moreover, nanomaterials applied in embryonated chicken eggs *can improve* embryos development. The aim of this overview *is* to provide a description of potential *nanotechnology applications* for poultry sector and discuss *any* challenges or obstacles *including the matters of safety of application of these nanomaterials in animals, in humans and of course in the environment*.

Keywords: Nanomaterials, Broilers, Layers, *In ova*, Types

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Date of initial submission: 30-01-2019
Date of revised submission: 29-03-2019
Date of acceptance: 03-06-2019

INTRODUCTION

Nanoscience and nanotechnology have been implicated in Veterinary Medicine as a tool for improving animal nutrition, breeding and reproduction (Sagadevan and Periasamy, 2014), for diseases diagnosis, prevention and therapy (Muktar et al., 2015) as well as for food safety (Lee et al., 2011). In poultry industry, certain antibiotics are used as feed additives growth promoters, but their use *now faces certain restrictions* due to development of antibiotics-resistant human pathogenic bacterial strains and *the presence of residues in bird's meat* (Verma et al., 2012). Social pressure *leads the industry to minimize their use* in animal feeding. European community banned use of antibiotics as growth promoters since 2006. Therefore, there is *a lot of research* for other safe alternatives to *substitute their use*. Nanotechnology is a very *promising* new approach that has the potential to substitute the antibiotics as feed additive. *Research in this area has* led to many innovations in animal production sector.

DEFINITION OF NANOTECHNOLOGY

Nanotechnology can be referred to as an area of science and technology focused on the *study* and manufacture of nano-sized materials (Lövenstam et al., 2010 and Gruère, 2012). *In U.S.A.* National Nanotechnology Initiative defined nanotechnology as the procedures focused on the production of materials and structures *at a scale of approximately 1-100 nanometres in at least one dimensio*. These materials possess unique and novel physical, chemical and biological properties that differ fundamentally from their bulk form (Buzea et al., 2007 and Feng et al., 2009). Nanomaterials can be produced through the utilization of two broad approaches known as top-down and bottom-up. The top-down approach is mostly used for processing inorganic materials through traditional methods such as milling, grinding, sieving, and chemical reactions (Cockburn et al., 2012). The bottom-up approach involves the assembly of smaller molecules through self-organization, resulting in the formation of supra-molecular structures which possess novel functionalities (Cockburn et al., 2012).

NANOMATERIALS TYPES

It is difficult to classify nanomaterials due to their complex structures and diverse properties. They present in different forms including; metal and metal oxide nanoparticles nanoemulsions, nanoporous materials, such as nanoclay, nanoshells, nanorods, dendrimers, carbon nanotubes etc. (Sekhon, 2012). Bai et al (2018) presented different types of nanomaterials including liposomes nanoparticles, micellar nanoparticles, polymeric nanoparticles, dendrimer nanoparticles, metallic nanoparticles, and carbon nanoparticles that have been used for disease diagnosis, treatment, drug delivery, animal nutrition, animal breeding and reproduction.

MECHANISM OF NANOPARTICLES ACTION

The mode of action of nanoparticles depends mainly many factor such as particles size where sizes lesser than 300 nm spread in the blood, but particles smaller than 100 nm reach tissues (Hett, 2004). Other factors also like particles solubility and charges are important. Nanoparticles could be administrated either by ingestion or inhalation and exert their actions through different ways. Chen et al. (2006) explained nanoparticles mode of action by increasing the surface area for better interaction with biological support, prolonging the residence time in gut, reducing the intestinal clearance mechanisms, increasing tissues penetration, crossing epithelial lining fenestration, efficient cells uptake and effective delivery to target sites.

APPLICATIONS OF NANOTECHNOLOGY IN POULTRY INDUSTRY

IN BROILERS SECTOR

Certain studies were conducted previously to investigate the impact of dietary inoculation of some nano elements on performance parameters as well as their antimicrobial activities in broilers.

Nano-silver ionic particles showed destructive influence on pathogenic intestinal microorganisms and consequently induced better nutrients absorption, improvement in feed intake, weight gain and feed efficiency of broilers chicks (Andi et al., 2011). On other study of Ahmadi (2012), nano-silver feed supplementation with 20, 40 and 60 ppm for 42 days showed dose dependent reduction in lymphoid organs weights which may be correlated with the antimicrobial property of silver nanoparticles that induced favorable proportion of non-pathogenic organisms than pathogenic ones in the gut. Dobrzanski et al. (2010) detected that nano-silver particles reduced the number of *Escherichia coli*, *Streptococcus*, harmful *Salmonella* and total number of mesophilic bacteria in the litter of broilers indicating bactericidal activities of these particles. Pineda et al. (2012) demonstrated the anti-

bacterial activity of silver nanoparticles in chickens. Basal broilers control diet supplemented with 4 ppm silver nanoparticles/kg for 5 weeks experimental period produced the best final body weight, body weight gain, feed conversion ratio, European production efficiency index and total serum antioxidant, while lowest levels of lipids and cholesterol as compared with other levels and control groups (El-Moustafa et al., 2015). In the same study, broilers fed different levels of silver nanoparticles (2, 4, 6, 8 and 10 ppm/kg) decreased the number of harmful bacteria as *E. coli* compared to control and had no effect on microflora as *Lactobacilli*. The harmful effect of using nano-particles in broiler was detected by Loghman et al. (2012) who concluded that higher levels of nano-silver (8 and 12 ppm) may induce severe pathological lesions in broilers liver.

Hu et al. (2012) studied the effect of dietary inoculation of 1.20 mg kg^{-1} nano-selenium (nano-Se) and found wider range between the optimal and toxic dietary levels with efficient body retention when compared with sodium selenite, increasing in the survival rate and improving both the average daily gain and feed to gain ratio.

Supplementation of broiler basal diet with 0.06 ppm organic zinc non-particles 0.06 ppm improved the bird's immunity and bioavailability as compared to inorganic zinc (Sahoo et al., 2014).

Some Egyptian studies were conducted to test the efficacy of using zinc oxide (ZnO) nano particles in poultry production. In the study of Hassan et al. (2013), it was found that feed treatments with different concentrations of ZnO nano particles inhibited the growth of mycotoxic fungi (A. *flavus, A. ochraceus and A. niger*) and consequently mycotoxicosis conditions. Ibrahim et al. (2017) suggested that replacing traditional inorganic ZnO source with nano-ZnO or combining nano-ZnO and Zn methionine at applied concentration, promoted the growth of broilers, enhanced Zn uptake and antioxidant status without negative influence on selected minerals distribution in broilers tissues. In addition, compared to inorganic-Zn form, supplementation and/or substitution with organic-Zn and/or nano-Zn form (20 ppm) had a positive influence on broilers body weight, body weight gain and feed conversion rate, Zn concentration in bird's serum and tissue, and increased return and net profit (Badawi et al., 2017). Feeding zinc nanoparticles to chickens encouraged chickens growth, immunity and reproduction (Swain et al., 2016).

A trial of Vijayakumar and Balakrishnan (2014) revealed that feeding of broilers with calcium phosphate non-particles to replace 50% of dicalcium phosphate requirement induced significant better feed conversion rate in comparison with control groups. Moreover, Hassan et al. (2017) concluded that using nano di-calcium phosphate (NDCP) in broiler diets allow successfully to reduce the dietary di-calcium phosphate (DCP) by 75%, diet formulated containing only 25% of the required non phytate P in form of NDCP could be used instead of 100% conventional DCP (CDCP) and using of dicalcium phosphate in nanoparticle size allow to reduce the excreted Ca and P by about 50% which reduce the impact of poultry on environmental pollution.

Using of copper nano particles in chickens was studied by Wang et al. (2011) who reported that copper nano particles loaded chitosan enhanced growth performance and immune status, enhanced protein synthesis and was beneficial to the caecal microbiota of broiler chickens. Copper silicate nanoparticles modified the intestinal microbiota of chicken, increasing counts of *Lactobacillus* species and decreasing *E. coli* (Minglei et al., 2013). Further, Miroshnikov et al. (2015) found improved growth, increased haemoglobin level, enriched copper and protein levels in serum and increased the arginine content in the chicken liver after intramuscular injection of copper nano particles. Nguyen et al. (2015) confirmed that addition of nano-crystalline metals of iron, copper, zinc oxide and selenium to chicken diet premix decreased inorganic minerals in diet premixes by at least four times, allowing chickens to absorb feed minerals more efficiently and consequently decreasing the risk of environmental pollution. Copper nano particles at level below 50mg/kg reduced intestinal coliform bacterial count in chickens than copper salts with concentration up to 200 mg/kg (EFSA, 2016).

IN LAYERS SECTOR

A study on nano-Se showed that supplementation with 0.3 mg kg^{-1} in dry diet induced better physiological effects of chicks (Mohapatra et al., 2014). In Egypt, Radwan et al. (2015) concluded that layer hen diet supplemental with nano-Se (0.25 ppm) showed improving in the productive performance and glutathione peroxides activity producing Se enriched egg which could supply 50% (35 µg) of the human Se recommended daily allowances.

Moreover, Ismail et al. (2016) observed that dietary

supplementation of 32-44 weeks old layer turkeys with nano forms of zinc and Se induced significant (P≤0.05) increase in serum total protein and antibody titres against Newcastle disease virus, high level of blood haemoglobin, increase in relative weight of spleen and oviduct length, increase hen-day egg production rate and egg weight, better albumen and egg shell quality and improve feed conversion ratio compared with their control groups. Just recently, it was shown that supplementation of some minerals in nano form into turkey hen's diet, especially of Cu and Zn in the dose covering 10% of the demand is relevant to maintain homeostasis in turkey muscles, as indicated by the activity of the aminopeptidase (Jó´zwik et al., 2018).

IN EMBRYONATED CHICKEN EGGS

In-ova inoculation of copper nano particles at concentrations of 4, 8, 12 and 16 µg/egg, no bad effect was observed on chicken embryos (Joshua et al., 2016). It was recorded that copper nano particles at concentration of 50 mg/kg revealed an increase in oxygen consumption and heat production that are important regulators in different developmental stages of chicken embryos (Scott et al., 2016). Mroczek-Sosnowska et al. (2014 and 2015) demonstrated that copper nano particles at concentration of 50 mg/kg exhibit pro-angiogenic properties at a systemic level, with the promotion of blood vessel development during embryogenesis, and consequently, increase the body weight, improve feed conversion ratio and increase breast and leg muscles of broiler chickens. A concentration of 50 mg/kg silver nanoparticles chelated with amino acids, threonine and cysteine improved immune competence in embryos and chickens (Bhanja et al., 2015). Moreover, proliferating cell nuclear antigen positive cells in the long bones of broiler chickens was observed after *in-ovo* inoculation of copper nano particles indicating a stimulatory effect during embryogenesis (Mroczek-Sosnowska et al., 2017). Sawosz et al. (2012) showed that silver nanoparticles potentially improve muscle morphology without affecting broiler performance at embryo growth.

CHALLENGES FACING NANOTECHNOLOGY

The potential of nanotechnology in poultry production cannot be fully appreciated yet because of insufficient knowledge. *It can lead to many* beneficial effects, *but may also* have many adverse effects or unclear hazards to humans, animals and the environment (Huang et al., 2015). Kannaki and Verma (2006) mentioned the future challenges of nanotechnology as; improving feed efficacy, controlling nutrient absorption, counteracting pathogens, *targeted* drug delivery, growth promoter, modifying the contents of the egg like full protein or cholesterol free eggs, decreasing energy and protein wastage, and reducing the cost of poultry meat. Nanoparticles materials could be included in poultry feed as a trial for prevention of diseases. Nevertheless, there are certain obstacles facing feed inoculation of these particles including; particles processing, their distribution and their condition in case of feed storage as well as their low bioavailability (Gallocchio et al., 2015). The meat of animals fed on these particles showed poor quality due to low resistance to micro-organisms growth, poor water retention and poor carcass traits (Fang et al., 2015). In addition, there is a lack of knowledge on the fate of ingested nanoparticles in human body and it is essential to investigate routes of exposure and also it is important to *accumulate* basic knowledge *on* their absorption, distribution, metabolism, and excretion (Bai et al., 2018). Potential toxicity of nanomaterials *is* not fully studied and risk assessments including hazard identification, hazard characterization and exposure assessment are essential (Oberdörster et al., 2005).

CONCLUSION

Encouraging results from recent studies proved that supplementation of poultry with nano materials in different forms seems promising on the performance, health and immunity as well as reducing pathogen gut load. These nanomaterials are still in their infancy, so much work is still required to support the safety of application of them in animal nutrition, avoiding any harm to livestock, the environment and human beings and to reduce the gaps between the knowledge and application.

CONFLICT OF INTEREST STATEMENT

The author declares no conflict of interest.

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J HELLENIC VET MED SOC 2019, 70(3): 1617-1624
ΠΕΚΕ 2019, 70(3): 1617-1624

Research article
Ερευνητικό άρθρο

Molecular analysis of Peste des Petits Ruminants Virus from outbreak in Turkey during 2010-2012

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ABSTRACT. The aim of the study is to determine the epizootiology of *Peste des petits ruminants* (PPR) in Turkey during 2010-2012, using molecular genotyping.

Samples of blood (n=193), swab (n=7) and tissue (n=374) were collected from sheep (n=473) and goats (n=101) suspected of having PPRV infection from an outbreak in 50 provinces of Turkey during 2010–2012. These samples (n=574) were tested using reverse transcription polymerase chain reaction (RT-PCR) and real-time reverse transcription polymerase chain reaction (RT-qPCR) targeting selected parts of the fusion (*F)* and the nucleocapsid (*N)* genes. Positivity ratios were 35.5%, 39.3%, and 44.4% with regards to RT-PCR targeting the *F* and the *N* genes, and RT-qPCR targeting the latter gene (*N*), respectively. The overall positivity rate was 45.8%.

For sequence analyses, *F*-gene (n=53) and *N*-gene (n=60) positive samples representing different provinces were selected. After phylogenetic analysis, the circulating PPRV was located in lineage IV according to two gene regions. The *F*-gene partial sequence analysis at the nucleotide level showed 98.2-100% resemblence among 53 for *F*-gene, and 97.9-98.9% and 91.3-92.4% to Turkey2000 and Nigeria75/1 sequences, respectively. The *N*-gene partial sequence analysis at the nucleotide level showed 94.2-100% resemblence among 60 for *N*-gene, and 94.2-98.3% and 89.3-90.9% to Turkey2000 and Nigeria75/1 sequences, respectively.

The result of this study indicates that PPRV infection is enzootic in Turkey, and belongs to the lineage IV, which is present in three haplogroup. The phylogenic analysis indicates the spread of the virus is associated with unauthorized movement of stock.

Keywords: Fusion gene, molecular epidemiology, Nucleocapsid gene, Peste des petits ruminants virus, Sequence analysis,

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Date of initial submission: 03-07-2018
Date of revised submission: 26-09-2018
Date of acceptance: 04-03-2019

INTRODUCTION

Peste des petits ruminants (PPR) is a highly contagious and economically important viral disease affecting small ruminants, caused by *Peste des petits ruminants virus* (PPRV) classified within the genus *Morbillivirus* in the family *Paramyxoviridae* (Banyard *et al.*, 2010). PPRV is a nonsegmented, negative sense, single-stranded RNA virus that encodes six structural proteins; nucleocapsid protein (*N*), phosphoprotein (*P*), matrix protein (*M*), fusion protein (*F*), hemagglutinin (*H*), RNA-dependent RNA polymerase (*L*), and two nonstructural proteins (*V* and *C*). According to phylogenetic analysis, PPRV can be classified into four genetic lineages based on the fusion (*F*) and/or nucleocapsid (*N*) gene (Couacy-Hymann *et al.*, 2002; Ozkul *et al.*, 2002; Kwiatek *et al.,* 2007; Munir *et al.*, 2012a; Munir, 2012b; Mahajan *et al.*, 2014). These lineages are generally correlated with the geographical distribution of the virus (Shaila *et al.*, 1996). Lineage IV is prevalent in Asian countries although all four lineages have been found in Africa (Libeau *et al.*, 2014).

The presence of PPR in Turkey was first reported in lambs in 1993 based on postmortem and immunohistochemical findings (Alcigir *et al.*, 1996). Then it was detected serologically and virologically in 1998 (Tatar and Alkan, 1999). The presence of the disease in Turkey was declared officially by the World Organisation for Animal Health (OIE) in 1999 (Food and Agriculture Organization, 2012). PPR has been a notifiable disease in Turkey since 1997. The complete genome of PPRV isolated from infected sheep in 2000 was sequenced in 2004 (isolate Turkey2000, GenBank acc. No. AJ849636). Phylogenetic analysis revealed that Turkey2000 is closely related to lineage IV, originating from the Middle East, the Arabian peninsula and Asia (Bailey *et al.*, 2005). Since 1993, PPR constitutes a significant health threat for sheep and goat farmers in Turkey despite to the unnual vaccination program. Together with PPRV, Rinderpest virus, the etiological agent of Rinderpest or cattle plague, is grouped in the same family *Paramyxoviridae* (Banyard *et al.*, 2010). Eradication status of Rinderpest in Turkey was declared by OIE (Food and Agriculture Organization, 2011), so experiences gained during in this eradication process had become very importand for designing of control and eradication program of PPR.

In the light of information mentioned above, the aim of the study was to determine the epizootiology of PPR in Turkey during 2010-2012, using molecular genotyping.

MATERIALS AND METHODS

Clinical Specimens

The samples (193 blood, 7 nasal swab, 162 lung, 116 spleen, 4 rectum, 3 small intestine and 89 lymph node) were taken from sheep (n=473) and goats (n=101) raised in 50 provinces in Turkey during

Figure 1. Sampled provinces (marked with yellow) and the positivity rates per provinces: **Province's number:** *The positivity rate per province*; **1:***59.25%*; **2:***57.14%*; **3:***50%*; **4:***66.66%*; **5:***28.57%*; **6:***100%*; **7:***50%*; **8:***100%*; **9:***38.46%*; **10:***33.33%*; **11:***100%*; **12:***0%*; **13:***100%*; **14:***44.44%*; **15:***0%*; **16:***30.95%*; **17:***33.33%*; **18:***100%*; **19:***16.66%*; **20:***25%*; **21:***100%*; **22:***50%*; **23:***50%*; **24:***36%*; **25:***100%*; **26:***40.42%*; **27:***53.33%*; **28:***100%*; **29:***0%*; **30:***52.63%*; **31:***60%*; **32:***36.84%*; **33:***55.55%*; **34:***50%*; **35:***75%*; **36:***33.33%*; **37:***47.61%*; **38:***50%*; **39:***100%*; **40:***66.66%*; **41:***46.66%*; **42:***100%*; **43:***25%*; **44:***100%*; **45:***100%*; **46:***100%*; **47:***61.53%*; **48:***80%*; **49:***100%*; **50:***0%.*

2010–2012 (Figure 1). Suspecion of PPRV infection was established based on clinical findings. Most of the animals had PPR symptoms such as fever, discharges from the eyes and nose, mouth lesions, respiratory distress, sometimes diarrhea, death and abortion. All the collected samples were kept at -80°C for the nucleic acid extraction. All procedures performed in this study involving animals were in accordance with the ethical standards of the Animal Experiments Local Ethics Committee of Ankara University (Approval date: 24/03/2010, Approval number: 2010-59-298).

Detection of RNA Using RT-qPCR Assay

Detection of RNA belonging to the *N* gene segment of PPRV using RT-qPCR was performed as previously described (Kwiatek *et al*. 2010). According to this, the RT-qPCR amplification conditions for *N* gene were as follows: an initial reverse transcription for 30 mins at 50ºC and RT-inactivation/Taq-activation for 15 mins at 95ºC, followed by 40 cycles of amplification (95ºC for 1 min, 60ºC for 1 min). All samples were tested further using *F* and *N* genes conventional RT-PCR.

Detection of RNA Using Conventional RT-PCR Assays

PPRV RNA was extracted from tissue samples using Qiagen RNeasy Mini Kit (Qiagen, Germany) and from blood samples using High Pure Viral Nucleic Acid Kit (Roche, Germany) according to the manufacturers' instructions. Extracted PPRV RNA was stored at -80°C until further use.

The One-step RT-PCR kit (Qiagen, Germany) was used for PPRV RNA detection. The assay was carried out in two seperate reaction mixtures for *F* and *N* genes. Each 20µl reaction mixture contained 10pmol primers, 4µl of 1x One-Step RT-PCR buffer, 0.8µl of 10mmol dNTPs, 0.8µl of One-Step RT-PCR enzyme mix and 3µl of extracted (Ozkul *et al*., 2002; Kerur *et al*., 2008). The RT-PCR amplification conditions for *F* and *N* genes were as follows: a reverse transcription step of 30 mins at 50ºC and 15 mins at 95ºC, followed by 40 cycles at 94ºC for 1 min, 50ºC for 1 min and 72ºC for 2 mins, with a final extension step at 72ºC for 10 mins. RT-PCR products then were electrophoresed in 1.5% agarose gel and visualized by ethidium bromide staining. A 448bp fragment for *PPRV-F* primers and a 463bp fragment for *PPRV-N* primers were amplified in positive reactions.

Lyophilized freeze-dried live PPR vaccine (Nigeria75/1 isolate) obtained from commercial PPR vaccine (Vetal Pestvac K, Vetal Inc., www.vetal.com.tr) was used as a reference virus for this study.

Sequencing and Phylogenetic Analysis

At least one positive sample from each province was selected for sequence analysis and purified from gel using a purification kit (High Pure PCR Product Purification Kit, Roche, Germany) and sequenced using the BigDye Terminator kit (v3.1, Applied Biosystems, USA) with an ABI 3130xl DNA Analyzer (Applied Biosystems, USA). Sequence reads were assembled and edited with DNAStar software package (DNAStar Inc., Madison WI; USA, https://www.dnastar.com/). Assembled sequences were also compared with reference publicly available sequences using the MegAlign tool available in the same software. Tables showing sequence similarity were produced (Data not show). Phylogenetic trees were constructed for the 290 and the 238 base pair (bp) fragments of *PPRV-F* and *PPRV-N*, respectively, using the same publicly availabe sequences used for sequence comparison. Neighbor-joining (NJ) trees were constructed using Molecular Evolutionary Genetics Analysis version 5 (MEGA5) software (https://www.megasoftware.net/), based on the evolutionary distances between different sequences calculated by the Kimura two-parameter model (Tamura *et al*., 2011). The confidence level of the NJ tree was assessed with bootstrapping, using 1,000 replicates.

A phylogenetic network was drawn for *PPRV-F* and *PPRV-N* to determine nucleotide substitutions and molecular evolution at *F* and *N* gene sites targeted RT-PCR. The data were processed using DnaSP v.5 software and the star contraction algorithm and median-joining (MJ) network algorithm (Bandelt *et al*., 1999; Forster *et al*., 2001; Librado and Rozas, 2009).

RESULTS

Detection of RNA in the field samples using RT-PCR assays

PPRV RNA was detected using the RT-qPCR assay targeting the *N* gene in 44.4% of the samples (255/574) and using RT-PCR assays targeting the *F* nad *N* genes in 204 (204/574, 35.5%) and 226 samples (226/574, 39.3%), respectively. RT-PCR results for specific test materials were shown in Table 1. Depending on year, positivity rates in the targeted population were 46.0-47.1% and 42.8-44.0% with regards to sheep and goats, respectively (Table 2).

Table 1. Positivity rates according to test materials

PCR	Organs	Blood	Swab	Total Positivity
N gene RT-qPCR	150/374 (40.1%)	100/193 (51.8%)	5/7 (71.4%)	255/574 (44.4%)
F gene RT-PCR	119/374 (31.8%)	81/193 (41.9%)	4/7 (57.1%)	204/574 (35.5%)
N gene RT-PCR	130/374 (34.7%)	92/193 (47.6%)	4/7 (57.1%)	226/574 (39.3%)

Table 2. Positivity rates according to year and species

Year	**Total test material**	**Sheep**	**Goat**	**Total positivity**
2012	74	25/53 (47.1%)	9/21 (42.8%)	34/74 (45.9%)
2011	389	156/339 (46.0%)	22/50 (44.0%)	178/389 (45.7%)
2010	111	38/81 (46.9%)	13/30 (43.3%)	51/111 (45.9%)
Total	574	219/473 (46.3%)	44/101 (43.5%)	263/574 (45.8%)

Table 3. Nucleotide and amino acid substitutions in *PPRV-N* sequences compared to TU00

Position in whole genome	Nucleotide substitutions	Amino acid substitutions	**Position in whole genome**	Nucleotide substitutions	Amino acid substitutions	**Position in whole genome**	Nucleotide substitutions	Amino acide substitutions
1415.nt	G→A	R→K	**1473.nt**	T→C		**1583.nt**	C→T	P→L
1452.nt	A→C	K→N	**1482.nt**	C→T		**1615.nt**	A→G	R→G
1460.nt	G→A	G→E	**1529.nt**	G→A	R→K			

Sequence and phylogenetic analysis

The *F* (n=53) and *N* genes (n=60) sequence corresponding to the strains of the PPRV that were detected, were deposited in GenBank under three groups with the following accession numbers: from JQ388615 to JQ388664; from JQ519907 to JQ519965, and from JX117877 to JX117880. Analysis of the *F* gene nucleotide sequences revealed that the nucleotide sequence identity among *PPRV-F* samples ranged from 98.2% to 100% whereas similarity with previously characterized Turkish isolates (Tu96 and Tu00) ranged from 97.9% to 99.3%. Amino acid sequence identity for *PPRV-F*, Tu96 and Tu00 was 97.8-100%. No amino acid substitutions were detected in *PPRV-F* compared to Tu96 and Tu00. Compared to the Nigeria75/1 vaccine strain, *PPRV-F* had 28 nucleotide substitutions with a similarity of 91.3-92.4%. A phylogenetic tree for *PPRV-F* gene was drawn using sequences analysed in this study, and sequences of strains from Iran, Iraq, Syria, Egypt, India and China (Tibet), and the reference sequences of the four lineages. As shown in figure 2, all *PPRV-F* studied here and other Asian sequences grouped together in lineage IV. The oligonucleotide sequences of strains from Egypt, India and Iraq were more similar than those of other countries (Iran, China (Tibet), Morocco, Kuwait, Pakistan) compared to those isolated in Turkey.

Analysis of the *N* gene nucleotide sequences revealed that the nucleotide sequence identity among *PPRV-N* samples ranged from 97.2% to 100% whereas similarity with previously characterized Turkish isolate (Tu00) was 97.5%-98.9%. Amino acid sequence identity was 94.2-100% among *PPRV-N* samples and 94.2-98.3% for *PPRV-N* and TU00. Nucleotide sequence identity for *PPRV-N* with the Nigeria75/1 vaccine strain were 89.3-90.3%. The phylogenetic tree of *PPRV-N* and other sequences from GenBank was shown in figure 3. All *PPRV-N* samples were clustered into lineage IV, which is exclusive to Asian and Middle East countries. All *PPRV-N* isolates sequenced here and reported earlier were similar to the PPRV isolates obtained from Iran and Iraq. But Turkey96 formed a separate branch within lineage IV with PPRV isolates from Iran, Israel, Nigeria, and

Morocco. Seven-12 nucleotide substitutions were detected between Turkey96 sequence and other *PPRV-N* sequences analysed here (Table 3).

To look for variation among the sequences, a phylogenetic network analysis was drawn for the *PPRV-F* and *PPRV-N* nucleotide sequences. According to this, all *PPRV-F* sequences were clustered under three haplogroups (H1, H2, H3) in lineage IV (Figure 4). As shown in the phylogram, sequences of strains from India (India03-FJ750562) and Iraq (Iraq2009-AY948429) were closest to the Turkish sequences, with one nucleotide substitution. Similarly, all *PPRV-N* sequences were mainly clustered under three haplogroups (H1, H2, H3) in lineage IV (Figure 5). In general, sequences of strains from Iraq (Iraq2011-JF969755) and Iran (JX898860 and JX898861) were closest to the *PPRV-N* sequences analysed here. Especially, sequences of strains from Iran were identical with H1.

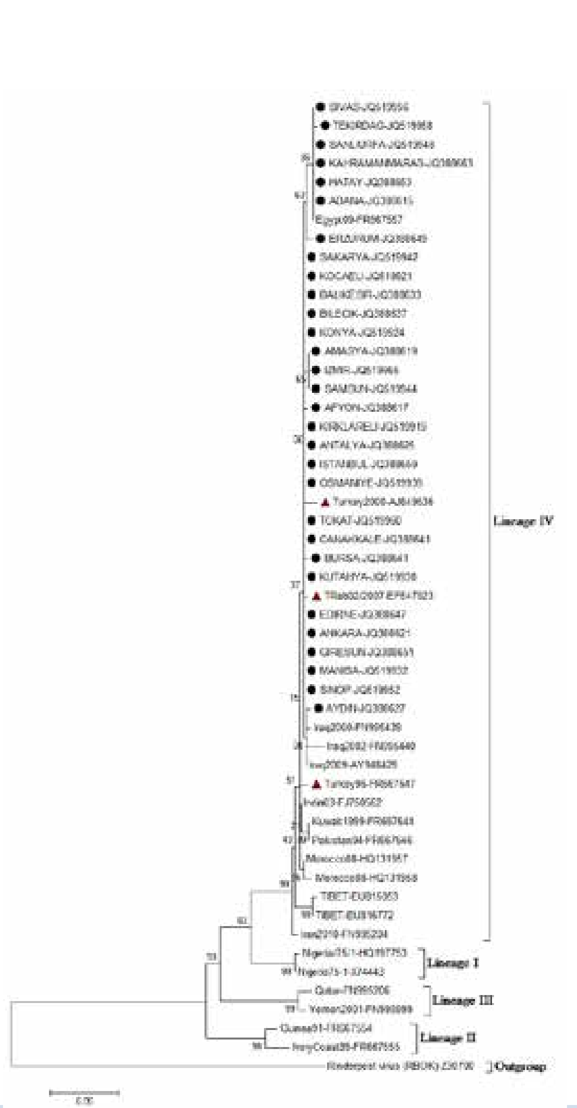

Figure 2. Phylogenetic tree of PPRV based on the PPRV-F gene (290 bp), constructed using the neighbour-joining method in the Kimura two-parameter model in Mega5 v.5. Numbers indicate bootstrap values (1,000 replicates). The scale bar at the bottom represents genetic distances in nucleotide substitutions per site. Horizontal distances are proportional to sequence distances. The phylogenic tree indicates clear division of PPRV strains into four lineages. ▲: Previously characterized sequences.

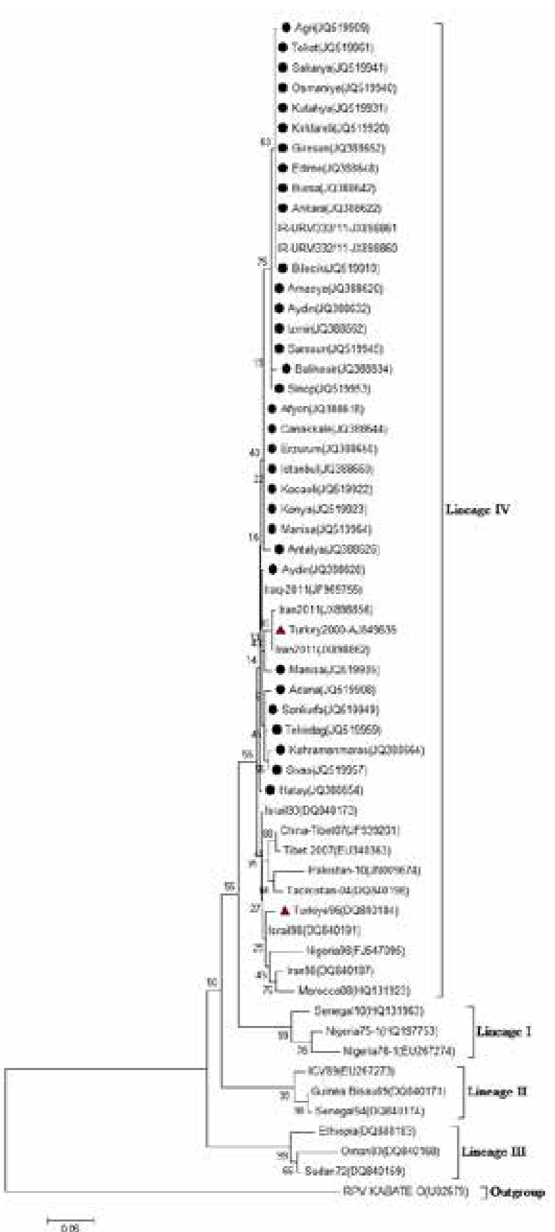

Figure 3. Phylogenetic tree of PPRV based on the *PPRV-N* gene (238 bp), constructed using the neighbour-joining method in the Kimura two-parameter model in Mega 5 v.5. Numbers indicate bootstrap values (1,000 replicates). The scale bar at the bottom represents genetic distances in nucleotide substitutions per site. Horizontal distances are proportional to sequence distances. The phylogenic tree indicates clear division of PPRV strains into four lineages. ▲: Previously characterized sequences.

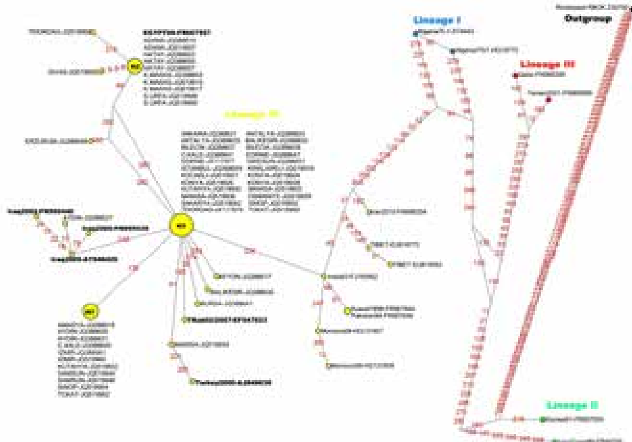

Figure 4. Phylogenetic network analysis based on the *PPRV-F* gene (290 bp). The numbers along the branches represent nucleotide changes. Sequences obtained from this study were usually clustered in three haplogroups (H1, H2 and H3) within lineage IV, marked in yellow.

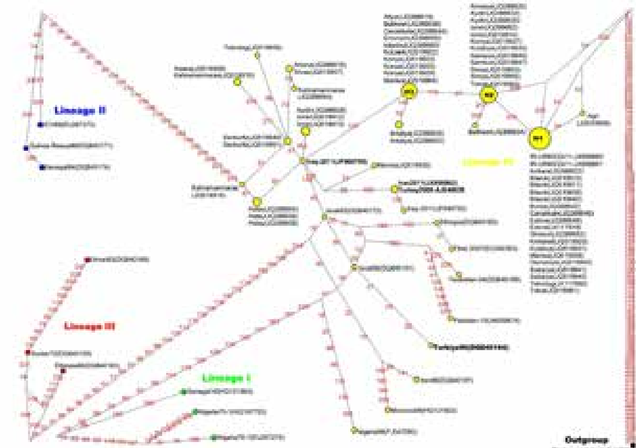

Figure 5. Phylogenetic network analysis based on the *PPRV-N* gene (238 bp). The numbers along the branches represent nucleotide changes. Sequences obtained from this study were clustered in lineage IV, marked in yellow.

DISCUSSION

The infection of small ruminants with PPRV causes significant economic losses across a wide geographical area, including Turkey (Ozkul *et al*., 2002; Kul *et al*., 2007; Banyard *et al*., 2010). After the successful Global Rinderpest Eradication Program in cattle, national and international organisations have undertaken initiatives to control and eradicate PPR. The determining of the seriousness and variability of PPRV infection in susceptible populations is impossible without any effective diagnostic method. Because of that, the accurate and reliable diagnosis constitute the first step of these initiatives (Banyard *et al.,* 2010). Especially in early stage of the disease, the differential diagnosis of PPRV infection is difficult from other diseases with similar symptoms. Thus, rapid alleviation programs, supported by rapid, specific and sensitive diagnostic methods are critical.

For the reasons mentioned above, the existing situation regarding disease prevalence was recorded for the first time, within the context of this study. The mean level of positivity for years 2010 to 2012 using RT-PCR was 45.8% (263/574), which corresponds to 45.9% for year 2010, 45.7% for 2011 and 45.9% for 2012. At same time, the level of positivity for sheep and goats was 46.7-47.1% and 42.8-44%, respectively. The Ministry of Agriculture and Forestry has an annual vaccination program since the first detection of the disease. According to this, all sheep and goats of all ages are vaccinated with PPR vaccine every year in the autumn. In 2010, PPR vaccination campaigns for individually identified lambs and kids were implemented in Turkey as part of a three-year European Union Project. For this, 30 million doses of PPRV vaccine were produced and consigned to the field. Between 2010-2012, in parallel with vaccination, approximately 27 million sheep and goat were ear-tagged and registered in all provinces. Despite all these vaccination campaigns, similar positivity

rates were determined between 2010-2012. In addition to problems mantioned previous studies (Ozkul *et al*., 2002; Banyard *et al*., 2010), the political situation on the south east borderline of Turkey may have hampered the efforts to control of disease and animal movement during this study.

In view of the results of detection limit and field samples, the *N* gene RT-qPCR was more valuable than *N* gene RT-PCR and *F* gene RT-PCR for detecting PPRV infection in this study. These results, which are compatible with those of Kwiatek *et al*. (2007) and Bao *et al.* (2008), indicate that the RT-qPCR assay used in this study was more sensitive than the *F* and *N* genes RT-PCR assays for the diagnosis of PPRV in field samples (Bao *et al.,* 2008; Kwiatek *et al*., 2010; Batten *et al*., 2011). Because the *N* gene is located at the 3' end of the PPRV genome, it is the most expressed gene due to a transcriptional gradient from the 3' to the 5' end of the genome (Ghosh *et al.,* 1995). Therefore, it is probably one of the best targets for maximising sensitivity with regards to RT-qPCR (Kwiatek *et al*., 2010).

In this study, a total of 473 sheep and 101 goats from various flocks were sampled. There was also a difference between sheep and goats in terms of positivity (Table 2). It was found that this finding is in accordance with the studies (Ozkul *et al*., 2002; Kul *et al*., 2007) that suggest that PPRV infection is more prevalent in sheep than goats in Turkey. However it is not compatible with the other reports (Anderson and McKay, 1994; Zhiliang *et a*l., 2009). Possible explanations for these results may be sample size relative the general population, the age of the animals and difference in species-specific animal trade. According to years, the rate of positivity was similar in 2010, 2011 and 2012 (Table 2). The absence of a reduction in rates despite vaccination can be attributed to uncontrolled animal movement on the east and southeast border of the country, which seems to be consistent with the outcome of the relevant network phylogenetic analyses that was conducted (provinces' numbers: 1, 24, 30,40), (Figure 1).

Although several RT-PCR methods have been developed since 1995 for rapid and specific detection of PPRV, genome sequencing has remained the gold standard for confirming the virus (Zhao *et al.,* 2009). This method has been also found useful for analyzing the genetic relationships between PPRV isolates and supported epidemiological investigations on the origin and spread of the virus. In light of this information, phylogenetic analyses of PPRV were conducted by using partial sequences of the *PPRV-F* and *N* genes (Munir et al., 2012a; Munir, 2012b). As in previous studies (Ozkul *et al.,* 2002; Bailey *et al.,* 2005; Yesilbag *et al.,* 2005; Kul *et al.,* 2007), this study showed that prevailing PPRV in Turkey belongs to lineage IV consisting of three main haplogroups. Detection of haplogroups indicates a number of introductions into Turkey. However, these haplogroups also show that PPRV strains in Turkey may have followed different evolutionary courses. It is plausible that multiple introductions from diverse sources were combined, resulting to mixed virus population in Turkey. The belief that border crossing of the virus (Ozkul *et al*., 2002; Banyard *et al*., 2010) is supported by the fact that sequences obtained in this study were clustered with sequences obtained in Iran, Iraq, Egypt and India. Specifically, the greater nucleotide sequence identity in the *N* gene network phylogenetic analysis suggests that there has been a close contact between sequences of strains from Iran and Turkey. To determine this possibility, sequencing should be added to regular virologic surveillance to characterise PPRV in the country.

Through *F* gene partial sequence analysis, it was found out that the PPRV strains showed a level of nucleotide sequence identity that was determined in Turkey to a minimum of 98.2%. Several studies (Kwiatek *et al*., 2007; Kerur *et al*., 2008; Banyard *et al*., 2010; Anees *et al*., 2013) indicated that substitution in the *N* gene is more probable than in the *F* gene, which was confirmed by our results indicating lower level of nucleotide sequence identity with regards to the *N* gene (94.2%). In general, the topology of the phylogenetic and network phylogenetic tree indicated that PPRV samples from different provinces differ mainly in their *PPRV-F* and *PPRV-N* gene sequences. While the PPRV strains in this study are more closely related strains from Egypt and Iraq in the *F* gene phylogenetic tree, they are more closely related to strains from Iran and Iraq in the *N* gene phylogenetic tree. When evaluated together with geographical proximity, the *N* gene-based phylogenetic tree becomes more meaningful. Because the probability of livestock transition from neighboring countries is higher than non-border countries. The possible relationship could be due to the fact that Turkish breeders share the same pastures with their Iraqi and Iranian counterparts. Another possible cause is uncontrolled animal movement, which especially increases before religious ceremonies, such as the festival of the sacrifice.. Like similar studies (Kwiatek *et al.,* 2007; Kerur *et al.,* 2008; Banyard *et al.,* 2010) our data indicate that a molecular genotyping survey targeting the *N* gene of PPRV would be more reliable than the *F* gene based.

CONCLUSIONS

It was concluded that PPR is enzootic in Turkey according to the PCR results of this study. By conducting molecular epidemiological analyses, it was understood that the main cause of this situation is animal movement from different sources according to three haplogroups. So PPR continues to be a major economic burden for sustainable animal production in Turkey. In this respect, an appropriate control and eradication campaign for PPRV infection should be considered similar to the successfully completed RPV eradication program.

ACKNOWLEDGEMENTS

We thank Dr. Aysel Unsal Baca for her kind assistance and support, and the Scientific Research Project Directorship of Ankara University (Project no. 10B3338012) and General Directorate of Agricultural Research and Policies of Ministry of Food, Agriculture and Livestock (Project no. TAGEM/HSYGAD/12/A07/P02/07) for financial support.

CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest. The funders had no role in study design, datacollection and analysis, decision to publish or preparation of the manuscript.

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J HELLENIC VET MED SOC 2019, 70(3): 1625-1630
ΠΕΚΕ 2019, 70(3): 1625-1630

Research article
Ερευνητικό άρθρο

Effect of *Ferula communis* L. on reproductive parameters in Awassi ewes

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ABSTRACT. Nutrient composition of the diet affects follicular activity, embryonic development and reproductive hormones in ruminants. The objective of the present study was to investigate the effects of *Ferula communis* L. on some reproductive parameters of Awassi sheep. The experiment was carried out on 29 (15-16 months old) Awassi ewes. All ewes were allocated to receive either a control (14% CP and 11.7 MJ ME/kg, n=9) or a diet supplemented with 5% (75g, n=10) or 10% (150g, n=10) powdered *F. communis* root, respectively for 21 days. Oestrus was synchronized using intravaginal sponges, while oestrus behaviour was observed 24, 36 and 48h after the sponge removal. Blood samples were collected for the assessment of oestradiol and LH. At the end of the 21-day period, animals were slaughtered and ovarian structures were recorded. Corpora lutea tissues were cultured *in vitro*, and progesterone production was measured. The results indicate that the treatment of animals with 5% of *F. communis* root increased the percentage of animals in heat (80%, 60% and 10% for 5C, 10C, and the control group, respectively). Furthermore, the number of small follicles (1-3 mm) in treated groups was significantly higher than those of the control group. Moreover, the number of large follicles (>4 mm) in the control group was higher than those of the treatment groups. Plasma concentration of oestrogen and LH peak were similar in the control and treatment groups. Progesterone production by luteal cells cultured *in vitro* was higher for both treatment groups compared to the control. Herewith, supplementation of the diet of Awassi Sheep with *F. communis* root during the breeding season may enhance ovulation rate and luteal activity.

Keywords: Awassi, *Ferula communis*, reproductive hormones, luteal tissue, ferutinin

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Date of initial submission: 0-07-2018
Date of revised submission: 27-03-2019
Date of acceptance: 11-04-2019

INTRODUCTION

The genus *Ferula* belongs to the family *Apiaceae* which consists of 170 species distributed to Mediterranean area and Central Asia (Canogullari et al., 2009; Rahali et al., 2018). *Ferula communis* L. subsp. *communis* (giant fennel) is a perennial phytoestrogenic plant with dense roots, found across the Mediterranean coast. Sub-varieties with poisonous and non-poisonous properties also exist (Appendino et al., 2001; Akaberi et al., 2015). Giant fennel has been used as a traditional medicine. *In vitro* and *in vivo* pharmacological properties have been reported recently (Akaberi et al., 2015). Phytoestrogens are oestrogen-like substances with similar molecular composition to plasma oestrogen. Non-poisonous chemotype of *F. communis* contains ferutunin, an aromatic ester of daucane alcohol, reported to contain phytoestrogen of the daucane type (Sacchetti et al., 2003; Akaberi et al., 2015, Rahai et al., 2018) which acts through stimulating the receptors in the hypothalamus to release luteinizing hormone (Canogullari et al., 2009). Ferutinin may interact with oestrogen receptors especially with oestrogen receptor (ER) α (Zanoli et al., 2009). Roots of *F. communis* especially rich in daucane esters.

Beside its pyhtoestrogenic effect, *F. communis* can prevent lipid peroxidation through its anti-oxidant properties. Both synthetic and natural anti-oxidants counteract detrimental effects of oxidative stress on reproduction. Synthetic anti-oxidants may have possible side effects in addition to their toxicity (Rahali et al., 2018). Local farmers in Mediterranean coast of Turkey traditionally feed livestock with *F. communis* root for its uterine stimulant activities to improve fertility rates. The present study was designed to further investigate the effects of *F. communis* on ovulation rates, ovarian structures and reproductive hormones of Awassi sheep.

MATERIALS AND METHODS

This experiment was carried out during the breeding season, on 29 sexually mature and cyclic Awassi ewes from the same farm, in Hatay province of Turkey (35° 52' - 37° 40' N and 35° 40' - 36° 35' E). Sheep were separated into groups of Control (C, n=9), 5% *Ferula communis* (5C, n=10) and 10% *F. communis* (10C, n=10) considering live weights (40.4, 40.3 and 40.2 kg, respectively) and body condition scores (2.40, 2.35 and 2.35, respectively). Feed consumptions were recorded daily to calculate subsequent feed. Animals were fed 1.5× maintenance ME requirement (Maintenance=0.42 MJ/kg0.75) for 21 days with a concentrated diet containing 18% CP and 13.5 MJ ME/kg. Animals in treatment groups received 75 g (5C) or 150 g (10C) *F. communis* (diluted in 0.5 L water) orally, with the aid of a cannula.

Oestrous cycles were synchronized using intravaginal progestogen sponges (an impregnated sponge containing progestogen; 0.3g cronolone, Intervet) for 14 days. Teaser ram were introduced 24h, 36h and 48h following progestogen withdrawal to find ewes in heat and hand mating was carried out.

Blood samplings were performed on 5 ewes in each group on days 12, 14, 16 and 17 after sponge insertion, once daily, for oestradiol assessment; on days 16 and 17 after sponge removal, in 2-hour intervals, for LH assessment. All samples were centrifuged at 2060×*g* for 10 min to separate the plasma and stored at -20ºC until the analyses.

At the end of feeding period of 21-days, all animals were slaughtered and reproductive tracts were taken immediately in a flask to the laboratory. The number of corpora lutea (CLs) and follicles were recorded. Mature CLs, identified on the basis of their gross appearance (Oldham and Lindsay, 1980) were isolated from ovarian tissues. Excisions were performed on a sterile warm stage (35ºC) and each CL was then bisected and sliced by scalpel to provide a pair of luteal tissue sections (approx. diameter = 0.3 mm). These sections were weighed and cultured individually in 3 mL of Medium 199 with Earle's salts (Sigma) containing 0.68 mM L-glutamine, 26.19 mM sodium bicarbonate, 50 IU/mL penicillin and 50 mg/mL streptomycin sulphate in 30 mm culture dishes for 4h at 38.5ºC in a humidified atmosphere of 5% CO_2 in the air. For each pair of sliced CL tissue sections, one section was incubated in the absence and one in the presence of 100 μIU equine LH (Sigma; 1 μIU ≡ 1 mg NIH-LH-S1). At the end of the culture period, culture medium was collected and stored at -20ºC for further analysis.

Plasma concentration of LH was measured using a sensitive competitive enzyme immunoassay method (Yildiz et al., 2002). Briefly, D-Biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (Biotin-X-NHS; Sigma-Aldrich, Taufkirchen, Germany) was used for labeling oLH [NIDDK-oLH-I-4 (AFP-8614B)]. Affinity purified goat IgG antirabbit IgG was attached to the solid phase and labelled and non-labeled (sample) oLH were competed against the

anti-oLH raised in rabbit [NIDDK-anti-oLH-1 (AFP-192279)]. Optimum dilutions of biotinyl-LH and oLH antiserum were found to be 1:5000 and 1:3200000, respectively. Standards used in the current study were between 0.39 and 50 ng oLH/mL. The minimum detection limit of the assay was 0.70 ng oLH/mL. Intra and interassay coefficients of variation were calculated at two levels of control samples and as quadruplicates in two different locations of the plate. At 12.2 ng/mL level, the intra- and inter-assay coefficients of variation were 10.8 and 11.5%, respectively.

The estradiol and progesterone concentrations were measured according to ELISA procedure using two commercially available assay kits (DRG EIA-2693 and DRG EIA-1561, DRG International Inc., Mountainside, New Jersey 07092, USA). The procedures were based on competition principle and microplates used as solid phase.

A sample containing unknown amount of progesterone and estradiol present in the sample and a fixed amount of estradiol or progesterone conjugated with horse-radish peroxidase compete for the binding sites of a polyclonal estradiol or progesterone antiserum coated onto the wells. After 120 min for estradiol and 60 min for progesterone incubation, the microtiter plates were washed three times in order to stop competition reaction. The substrate solution was added into each well for 15 min to stop competition reaction. The optical density was measured at 450 nm, and the concentrations of estradiol and progesterone are inversely proportional to the optical density measured.

It has been suggested that intra- and inter-assay coefficient of variation should be <20–25% (Findlay et al., 2000; Shah, 2007; Valentin et al., 2011; Birdwhistell et al., 2017). The values in the current study are much lower than that of the reported criteria and therefore tests are quite reliable (Table 1).

Table 1. Intra- and inter-assay coefficients of variations (CV) at different quality control (QC) levels

Analyte	Intra-assay variation		Inter-assay variation	
	QC	**CV (%)**	**QC**	**CV (%)**
Progesterone (ng/mL)	1.21	8.3	1.31	9.9
	2.62	4.6	2.71	4.8
	12.3	5.2	11.9	6.5
Estradiol (pg/mL)	478	6.3	91.89	2.0
	1167	5.0	276.1	3.8
			562.5	2.6

The IBM-SPSS (v23 for Windows) program was used to analyse all data. Data were analysed using ANOVA (GLM procedure) for evaluation of differences between treatments. Data were presented as mean±standard error (SE). The p value used to determine significance in all tests was 0.05.

RESULTS

The number of ewes in heat following sponge removal was increased ($P<0.05$) due to supplementation of *F. communis*, while no differences were observed 36h later. Feeding animals with *F. communis* root decreased the number of large follicles (≥ 4 mm) and an increase for 5C group was observed in terms of small to large follicles ($P<0.05$). *F. communis* root induced formation of CL ($P<0.05$) but the fact remains that CL weights remained similar for each group (Table 2).

Table 2. The effects of powdered F. *communis* root supplementation to diet on the manifestation of estrus and on ovarian structures

	Control (n=9)	5% (n=10)	10% (n=10)
1-3 mm follicles /ovary	4.5±0.45[a]	6.7±0.50[b]	5.7±0.55[ab]
≥ 4 mm follicles /ovary	0.89±0.20[a]	0.05±0.05[b]	0.06±0.05[b]
Number of corpus luteum / ovary	0.22±0.14[a]	0.70±0.2[b]	0.41±0.1[b]
Corpus luteum (weight; mg)	0.37±0.01	0.35±0.01	0.38±0.01
Ewes in oestrus % (24 h)	10[a]	80[b]	60[b]
Ewes in oestrus % (36 h)	40	90	70

[*]a,b Different superscript in the same row differ at P<0.05. Values presented as mean±SE

Plasma peak LH (Table 3) and estradiol concentrations (Figure 1) were similar in control and *F. communis* root supplemented groups. However, plasma peak LH concentration in 5C group was tended to be higher than in control and 10C group (P>0.05).

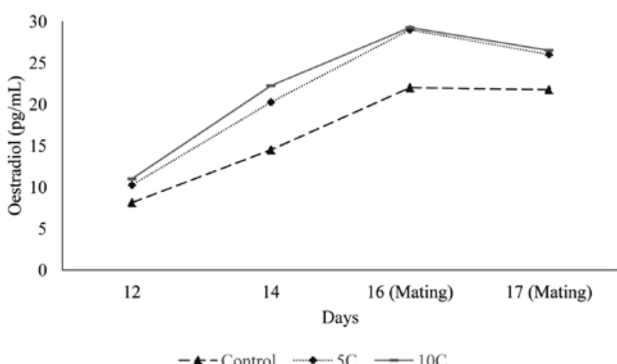

Figure 1. Plasma oestradiol concentrations of ewes fed with 0 g (control), 75 g (group 5C) and 150 g (group 10C) *F. communis* extract

Basal concentrations of LH (mean±sem; ng/mL) did not differ (*P*>0.05) between treatment groups and the control group. Similar to basal LH concentrations, neither plasma concentrations (C, 80.9±42; 5C, 123.3±13; 10C, 58.9±11) nor the time (C, 30.2±1.4; 5C, 35.2±1.8; 10C, 36.7±2.3) of LH peak were affected by supplementation of powdered *F. communis* root. Peak concentrations of LH (ng/ml) at the pre-ovulatory surge were higher (*P*>0.05) within 5% *F. communis* root supplemented group (Table 3).

Table 3. The effects of powdered *F. communis* root supplementation to diet on plasma peak LH concentration and on the time of LH peak

	Control (n=5)	5C (n=5)	10C (n=5)
Plasma peak LH (ng/ml)	80.9±42	123.3±13	58.9±11
Time of LH peak (h*)	32.0±1.4	35.2±1.8	36.7±2.3

* hours after sponge removal

Results showed that cultured luteal cells from the treatment groups produced more progesterone than those of the control (*P*<0.01). Also, progesterone production by luteal cells was stimulated by the supplementation of 100 µIU LH.

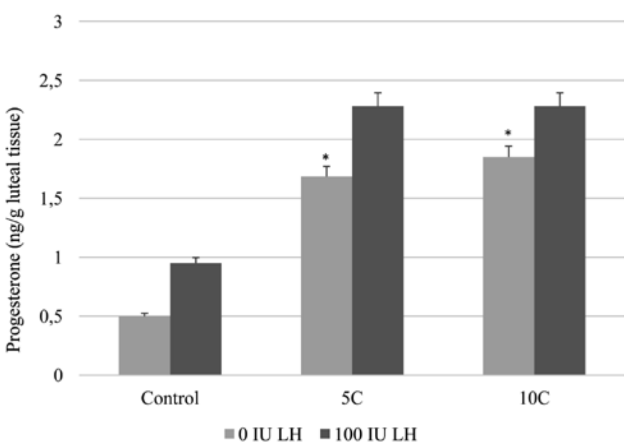

Figure 2. In vitro progesterone production by luteal cells originating from ewes fed with 0 g (control), 75 g (group 5C) and 150 g (group 10C) F. communis extract for 21 days. * Statistically significant at p<0.01

DISCUSSION

Many researchers have analysed the constituents of different organs of *F. communis*, previously (Manolakou et al., 2013; Rahali et al., 2018). Despite the fact that the chemical composition of *F. communis* root has not been analysed in the present study, mean live weights and body condition scores remained similar for each treatment group during the experimental period.

Formononetin and biochanin A are biotransferred to estrogenic isoflavone equol, a metabolite produced *in vivo*, by the action of ruminal bacteria and gut microflora. (Muthyala et al., 2004; Retana-Marquez et al., 2012). The main route of entry of phytosterols into body is via the diet. According to recent studies, consumption of some plants which contain phytoestrogens such as soybean may result in infertility problems, abortions and even stillbirth. Such plants exert these effects through agents like isoflavens and ferutinin (Appendino et al., 2001). Isoflavens, ferutinin and plasma oestrogen compete for binding to oestrogen receptors located in the anterior pituitary, since they have similar molecular structure (Adams, 1995; Ikeda et al., 2002). The estrogenic activity of pituitary decreases about 1/1000 to 1/10000 times once isoflavens bounded to the oestrogen receptors (Raymer et al., 1999). In line with this hypothesis, ovulation rates and the number of small follicles increased in the present study. This increase in the number of small to large sized follicles is in agreement with the assumption of Billig et al. (1993) who reported that phytoestrogens may prevent apoptosis. Increased oestrogen levels in follicular fluid is related to the number of decreased

atretic follicles. Oestrogen treatment was reported to prevent ovarian apoptotic DNA fragmentation (Billig et al., 1993). Recent studies reported that oestrogenic isoflavens resulted an increase in expression of anti-apoptotic factor while decrease in expression of pro-apoptotic factors (Kundu et al., 2018).

In the present study we demonstrated that ingestion of *F. communis* resulted enhanced earlier manifestation (24 h) of oestrus behaviour of Awassi ewes. These results seem to support the folkloric reputation of *F. communis* root as a sexual stimulant. Recent studies are available which reported both stimulating effects of the genus Ferula on reproductive behaviour in male rats. Hadidi et al. (2003) observed a significant increase in the copulatory behaviour of rats depending on the extract, when fed orally.

Overproduction of reactive oxygen species (ROS) in follicular microenvironment may cause meiotic arrest and impaired oocyte development (Rizzo et al., 2012). The present findings lead us to suggest that *F. communis* powder might exert its provocative action on female receptivity and proceptivity through acting like ROS scavenger and thus promoting the development of small to large sized follicles in this regard.

Plasma oestrogens and androgens have been reported to inhibit luteal function through different mechanisms. Wocławek-Potocka et al (2013) reported that high concentrations of active metabolites of phytoestrogens may disturb CL function by inhibiting progesterone secretion. However, our findings suggest that *in vitro* progesterone production by luteal tissues has been increased after the ingestion of *F. communis*.

Similarly, higher plasma LH concentration (Bindon et al., 1982) and/or enhanced sensitivity of LH secretion induced by GnRH-A (Arispe et al., 2013) for sheep consuming phytoestrogenic plants has been reported.

Finally, Guan et al. (2006) has shown that lipid activities of soybean and kudzu improved lipid profile, including total cholesterol and certain lipoprotein ratios. The authors also pointed that research on phytoestrogens other than genistein and daidzein is limited. Ferutinin in the *F. communis* has been reported to have effects on secretory gland activities by increasing Ca^{2+} channel activity (Zamaraeva et al., 1997).

CONCLUSION

In conclusion, *F. communis* root extract should exert pharmacological role on reproductive parameters of Awassi ewes dose-dependently and can be added in the diet at 5% with no adverse effects.

Concerning the potential therapeutic role of ferutinin, it could exert some beneficial endocrinological effects to the current hormonal replacement therapy in livestock, especially for ewes. In addition, the pharmacological profile of *F. communis* as a strong antioxidant and oestrus promotive could lead to further therapeutic benefits, which will be investigated in our future research.

CONFLICT OF INTEREST

None declared.

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J HELLENIC VET MED SOC 2019, 70(3): 1631-1638
ΠΕΚΕ 2019, 70(3): 1631-1638

Research article
Ερευνητικό άρθρο

Prevalence of *Trypanosoma evansi* in horses (*Equus caballus*) and donkeys (*Equus asinus*) in El-Bayadh district, southwestern Algeria

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ABSTRACT. *Trypanosoma evansi* is a parasite that causes surra in a variety of wild and domestic animals and is mainly transmitted by biting flies in Africa, Asia and Latin-America. Horses infected by *Trypanosoma evansi* present a chronic weight loss, icterus, oedema, anemia, abortions and neurological troubles. Due to this parasite, cases of human trypanosomiosis have been reported in different countries by contacting with infected animals. In this study, 206 healthy equines (177 horses and 29 donkeys) from El-Bayadh district, located in southwest Algeria, were tested for the presence of parasites in blood using Giemsa-stained blood films and for the presence of antibodies against *T. evansi* using CATT /*T. evansi*. While none of the equines showed detectable parasites in the blood, the individual seroprevalence of *T. evansi* was found to be 46.6% (CI 95%, 40.7-54.4%). Out of 98 positives samples, 56.1% (55/98) were shown at level 1 (+), 27.5% (27/98) at level 2 (++) and 16.3% (16/98) at level 3 (+++). The results show that out of 177 tested horses, 80 were seropositive to *T. evansi*, 45.2% (CI 95%, 37.8-52.5%) and out of 29 tested donkeys, 18 were seropositive to *T. evansi,* 62.1% (CI 95%, 44.4-79.7%). A questionnaire for the owners, targeted to associate risk factors for surra in horses, showed that environmental factors that are favorable for Tabanids, such as water and vegetation, but also promiscuity with dromedaries were positively associated with the seroprevalence rate in the horses. El-Bayadh district is a highly endemic region for surra in Algeria.

Keywords: *Trypanosoma evansi*, Prevalence, Horses, Donkeys, Algeria

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Date of initial submission: 08-10-2018
Date of revised submission: 15-01-2019
Date of acceptance: 08-02-2019

INTRODUCTION

Trypanosoma evansi was first identified in India where it caused an endemic disease known as "surra" in equines and camels (Evans, 1880-1881). Since then, this parasite has been found to infect many more domestic and wild animals, such as bovines, small ruminants and dogs (Fernandez et al., 2009, Desquesnes et al., 2013). Surra is widespread throughout Africa (Hilali et al., 2004, Birhanu et al., 2015, Fikru et al., 2015); Asia (Tuntasuvan et al., 2003, Hasan et al., 2006); and Latin-America (Hoare, 1965) and even shows occasional outbreaks in Europe (Tamarit et al., 2010). The disease is transmitted mechanically by insect vectors such as *Tabanus* and *Stomoxys* (Luckins, 1988, Brun et al., 1998) and also by vampire bats (*Desmondus rotundus)* in South America (Desquesnes et al., 2013).

Surra is a neglected tropical disease with devastating clinical impacts for the affected animals. Infected Horses with *T. evansi* have fever usually associated with parasitaemia, chronic weight loss, icterus, oedema, anemia, abortions and neurological deficits (Gardiner and Mahmoud, 1992). The parasite can cross the blood brain barrier and cause ataxia, hyperexitability and progressive paralysis of hind quarters (Rodrigues et al., 2009). In contrast, *T. evansi* infected donkeys present in general a subclinical infection (Mahmoud and Gray, 1980). In Brazil, losses due to mortality and treatment in equines infected by *Trypanosoma evansi* have been estimated to amount to 2.4 million dollars per year (Herrera et al., 2004). Human trypanosomiosis due to *T. evansi* has been reported in different countries (Joshi et al., 2005, Powar et al., 2006, haridy et al., 2011). A transmission by ingestion of meat from infected animals has been demonstrated in Vietnam (Van Vinh Chau et al., 2016).

In Algeria, there is little information about the prevalence of surra in most animals and districts. One previous article reported a 14% prevalence of the parasite in blood smears of camels in southwest Algeria (Bennoune et al., 2013).

The objectives of this study were twofold: first, to determine the prevalence of *T. evansi* parasites and antibodies in equines in El-Bayadh region and second, to associate physical and environmental risk factors for contracting surra in equines using a questionnaire.

MATERIALS AND METHODS

Study design and sample collection

EL-Bayadh district is located on the southwest of Algeria. It covers a total area of 71 697 km² (3 % of national territory) between 33°40′49″ N and 1°01′13″ E. The district consists of three distinct agro-climatic zones: high plains, near saharan and saharan atlas.

The population of horses and donkeys in El-Bayadh region is about 1250 and 1897 heads, respectively (MADR, 2016).

The study is achieved during March 2016. A number of 206 equines have been collected from the three agro-climatic zones.

A total of 177 horses and 29 donkeys were sampled, 139 males (121 horses and 18 donkeys) and 67 females (56 horses and 11 donkeys). These animals are divided into 3 different classes according to their age: < 5 years (104 horses and 17 donkeys); 6 to 11 years (58 horses and 12 donkeys) and 15 horses older than 11 years old. Different breeds of horses were sampled: Barb (n=155), Arab/barb (n=22). The chosen horses were used either for racing (n=10), or for pleasure riding (n=167). Donkeys were used for working.

Blood samples were obtained via a jugular vein by using 10 ml vacutainer tubes (which contained no anti-coagulants or preservatives), and then centrifuged at 3000 rpm for 10 min to get serum. The serum was collected in 1.5 ml Eppendorf tubes and kept at −20 °C until analysis by CATT/*T. evansi*.

Questionnaire

A case study has been done for each sampled animal. It contains information for the physical characteristics (gender, age, breed, purpose and herd structure), the environmental characteristics (promiscuity with dromedaries, contact with watering points and vegetation) and health care (vaccination, vermifugation). Each animal was examined for clinical signs of trypanosomiosis (anemia, icterus, oedema, weight loss and neurological signs).

Blood smears

Blood samples were collected into sterile vacuum tubes from the jugular vein of each animal. A blood film was prepared for each animal sampled. The smear was fixed by May-Grunwald stain for 3 minutes. It was washed with distilled water and then covered by

Giemsa stain for 30 minutes and again washed with distilled water to eliminate excess of stain. The slides were left to dry and examined with a light microscope by immersion oil at objective X100.

Serology

The CATT /*T. evansi*™ (Card agglutination test for trypanosomiosis due to *Trypanosoma evansi*) (Prince Leopold Institute of Tropical Medicine, Antwerp. Belgium) is a direct agglutination test used for detection of specific antibodies in blood, plasma and sera. It is included in the terrestrial manual of the word organization for animal health. The performance of the CATT /*T. evansi*™ was evaluated with two complement fixation tests in horses in Kazakhstan, the results determined the sensitivity of the kit at 80.2% and the specificity at 98.5% (Claes et al., 2005).

The test was used for serodiagnostic of trypanosomiosis due to *T. evansi* according to the manufacturer's instructions. Briefly, the agglutination reaction is done on card composed of 10 circles. In each circle, 25µl of diluted sera and one drop of Ag (about 45µl) are deposited and then mixed. Once the ten mixtures were made, the card was placed on an electric rotator at 70 rpm for 5 minutes. Each card had two circles for the positive and negative controls delivered by the manufacturer. Samples were considered positives when they have a blue granular agglutination. Agglutination levels were scored as: -, ± (negatives) or +, ++, +++ (positives).

Statistical analysis

The seroprevalence of *Trypanosoma evansi* in horses and donkeys were calculated as the number of seropositive animals divided by the total of animals sampled. For risk factors analysis, the independent variables were subjected to univariate analysis which was performed using the chi^2 test (n>5) or Fisher's exact test (n<5). The P value < 0.05 was considered statistically significant. Associations between individual animal serostatus and independent variables were assessed using logistic regression. A backwards stepwise approach was used to find the best fitting model to describe the dataset. Multivariate model selection was based on the Akaike information criterion (AIC) and the best model was selected using the lowest AIC. The P value, odds ratio with 95% CI for explanatory variables were also calculated. All analyses were carried out using RStudio (version 1.1.383, RStudio Inc., Boston, MA).

RESULTS

Blood smears

All the examined blood smears (177 horses and 29 donkeys) were negative for the presence of *Trypanosoma evansi*.

Serology

Out of 206 samples (177 horses and 29 donkeys) tested by CATT /*T. evansi*, 98 were found to be seropositive. Among the 98 seropositive samples, agglutination reactions were scored level 1 (+) in 56.1% (55/98), level 2 (++) in 27.5% (27/98) and level 3 (+++) in 16.3% (16/98). Out of 177 tested horses, 80 (45, 2% [CI 95%, 37.8-52.5%]) were seropositive for *T. evansi:* 27% (47/177) scored level 1 (+), 11% (20/177) level 2 (++) and 8% (14/177) level 3 (+++).

Out of 29 tested donkeys, 18 (62.1% [CI 95%, 44.4-79.7%]) were seropositive for *T. evansi:* 28% (8/29) scored level 1 (+), 24 % (7/29) at level 2 (++) and 7% (2/29) at level 3 (+++).

Risk factors

Horses

Results of the univariate analysis of physical and environmental variables compared to the prevalence of *Trypanosoma.evansi* infection in horses (*Equus caballus*) in El-Bayadh region are shown in table 1. Seropositivity for *T. evansi* in horses was detected in all three agroclimatic zones of EL-Bayadh district. The highest seroprevalence rate was founded in the high plains area: 71.8% (CI 95%, 65.6-78.09%), followed by the near-Saharan area: 41.67% (CI 95%, 34.8-48.5%), while the lowest rate was observed in the Saharan Atlas: 26.9% (CI 95%, 20.8-33.1%). The area's in the agro-climatic zones showed a significant association with seropositivity (P= 0.00001).Female horses had higher seropositive rates than male horses, with 57.1% (CI 95%, 44.2-70.1%) versus 39.66% (CI 95%, 30.9-48.4%) respectively. There was a significant association between gender and seropositivity (P = 0.029).

The horses that avoided promiscuity with dromedaries had a lower seropositivity rate 33.3% (CI 95%, 21.1 – 45.6%) than horses that had contact with dromedaries 50.8% (CI 95%, 41.9-58.9%) . The association between promiscuity with dromedaries and seropositivity was significant (P = 0.028).

Table 1. Univariate analysis of *Trypanosoma.evansi* infection in horses (Equus caballus) compared to physical and environmental variables

Risk factors	Category	No	Positive No	Seroprevalence % (95% CI)	P-value
Age	≤ 5	106	46	43.4 (34 – 52.87)	0.258
	6 - 11	58	29	50 (37.1 – 62.9)	
	> 11	15	4	26.7 (4.3 – 49)	
Gender	Male	121	48	39.7 (30.9 – 48.4)	
	Female	56	32	57.1 (44.2 – 70.1)	0.029
Breed	Barbe	155	69	44.5 (36.7 – 52.4)	0.628
	Arabe-Barbe	22	11	50 (29.1 – 70.9)	
Housing	Box	10	2	20 (23.6 – 28.1)	
	Stable	167	78	46.7 (16.1 – 26.2)	0.099
Purpose	Racis	10	2	20 (4.8 – 44.8)	
	Hobies	167	78	46.7 (39.1 – 54.3)	0.099
Promiscuity with Dromedaries	Yes	120	61	50.8 (41.9 – 59.8)	0.028
	No	57	19	33.3 (21.1 – 45.6)	
Watering points	Yes	133	66	49.6 (41.1 – 58.1)	
	No	44	14	31.8 (18 – 45.6)	0.039
Vegetation	Yes	157	76	48.4 (40.6 – 56.2)	
	No	20	4	20 (2.5 – 37.5)	0.000000
Zone	High Plains	64	46	71.8 (65,6-78,1)	0.00001
	Saharan Atlas	89	24	26,9 (20.8 – 33.1)	
	Near Sahara	24	10	41.7 (34.8 – 48.5)	

Univariate analyses (χ2 test for significance)

Table 2: Univariate analysis of *Trypanosoma.evansi* infection in donkeys (Equus asinus) compared to physical and environmental variables

Risk factors	Category	No	Positive No	Seroprevalence % (95% CI)	P-value
Age	< 5	17	12	70.6 (48,9-92,2)	0,438
	6 - 11	12	6	50 (21,7-78,3)	
Gender	Male	18	10	55.5 (32,6-78,5)	0,448
	Female	11	8	72.7 (32,9-93,9)	
Promiscuity with Dromedaries	Yes	20	12	60 (38,5-81,5)	1
	No	9	6	66.7 (35,9-97,5)	
Watering points	Yes	23	15	65.2 (45,7-84,7)	0,645
	No	6	3	50 (9,1-90)	
Zone	High Plains	15	11	73,3 (39,4-92,2)	0,371
	Saharan Atlas	8	4	50 (17,2-84,3)	
	Near Sahara	6	3	50 (13,9-88,2)	

Univariate analyses (χ2 test for significance)

Horses that did not live near water points had a lower seroprevalence rate 31.8% (CI 95%, 18 – 45.6%) than those who did 49.62% (CI 95%, 41.1 – 58.1%). Similarly, horses that are not surrounded by dense vegetation had a lower seroprevalence 20% (CI 95%, 2.5 – 37.5%) than those who did 49.03% (CI 95%, 40.6 – 56.2%). Both environmental characteristics: water points (P=0.039) and vegetation (P=0.000000) were associated with seropositivity (P< 0.005).

The age of animals, horse breed and housing conditions were not statistically significantly associated with seropositivity (p > 0.05).

Results of the multivariate logistic regression are summarized in table 3. Two risk factors were associated with *T.evansi* infection: sex and promiscuity with dromedaries (P<0.005). In this study, the risk of males for being seropostive was decreased by 67% than in femals (P=0.006, OR=0.37, 95% CI= 0.17-0.74%). Horses living in promiscuity with dromedaries were 2.49 times more likely to be infected by *T.evansi* (P=0.013, OR=2.49, 95% CI= 1.23-5.25%).

Table3: Factors influencing the risk of *Trypanosoma.evansi* infection in horses (Equus caballus)

Risk factors	Category	Odds ratio	95% confidence interval (OR)	P-value
Gender	Male	0.37	0.17-0.74	0.006
	Female	Ref		
Age	≤ 5	Ref		
	6 – 11	1.35	0.68-2.69	0.386
	> 11	0.28	0.07-0.95	0.054
Watering points	Yes	2.00	0.94-4.36	0.074
	No	Ref		
Promiscuity with Dromedaries	Yes	2.49	1.23-5.25	0.013
	No	Ref		

Donkeys

No significant differences were found in the study of the influence of physical and environmental characteristics on the seroprevalence of donkeys' samples (Table 2).

DISCUSSION

Trypanosoma evansi is the most widely distributed pathogenic salivarian trypanosome in animals. This parasite is cosmopolitan, affects a wide range of hosts (Desquesnes et al., 2013). In this paper, we have studied the prevalence of *T. evansi* parasites and antibodies in equines and the risk factors for surra in El-Bayadh district, southwestern Algeria.

A general weakness of this study is the fact that we were not able to microscopically observe the infecting parasite. The prevalence of parasites with the blood smear technique was 0%. This result is in agreement with a study on horses in Jordan (Abo-Shehada et al., 1999). Direct examination of the parasite in blood films usually fails in detecting the parasite if their concentration is less than 2.5 x 10^6 parasites per ml of blood (Chappuis et al., 2005). The sensitivity of detecting parasites in stained blood smears is thus very low. Concentration techniques such as the micro-haematocrite centrifugation technique (MHCT) or the mini-anion-exchange centrifugation technique (MAECT) are recommended to increase the sensitivity of direct observation of the parasite (Tehseen et al., 2015). Alternatively, PCR is the most sensitive diagnostic tool for the molecular confirmation of *T. evansi* infection (Ramírez-Iglesias et al., 2011). PCR has been demonstrated to be a method of choice to detect infection by *T. evansi* in horses (Clausen et al., 2003); this technique was not available in our study.

To our knowledge, this study is the first report of the detection of *T. evansi* antibodies in equines in Algeria. The total CATT/*T. evansi* seroprevalence in equines in El-Bayadh district was 47.6% (CI 95%, 40.7-54.4%). The majority of the tested animals (56.1%) scored the lowest agglutination score (+). This result confirms that trypanosomiosis due to *Trypanosoma evansi* is endemic in Algeria.

The seroprevalence obtained in horses alone was 45.2% (CI 95%, 37.8-52.5%), similar rates were found in Egypt and Jordan (Abo-Shehada et al., 1999; Zayed et al., 2010). Other publications reported higher seroprevalence rates, 73% were found in Brazil using IFAT (Herrera et al., 2004) and 92% were demonstrated in Philippine (Dargantes et al., 2009). Some studies report lower seroprevalence, as is the case in Malaysia (13%) (ELshafie et al., 2013) and India (27%) (Laha and Sasmal, 2008).

For donkeys, the seroprevalence was 62.1%. This rate is much higher than the seroprevalence reported in other countries; Egypt (44%) (Zayed et al., 21010) and India (11.53%) (Kumar et al., 2013).

The serological test used in this study, CATT/ *T. evansi* is a rapid and easy test to use but it cannot differentiate between past and present infection (Tehseen et al., 2015).

In this study, female horses are more exposed to the infection than males (P=0.006, OR=0.37, 95% CI= 0.17-0.74%). That result may be explained by the immunosuppression due to the gestation and to the difference of activities between females and males. This result agrees with some studies in horses, Elshafie et al (2013) were reported that female horses were 2.1 times more likely to be infected by *T.evansi*. In

contrast, a study in camels demonstrated that higher rates of male camels were infected with *T. evansi* than females (Njiru et al., 2004).

The seroprevalence of *T. evansi* varied in relation to the 3 agroclimatic zones. The highest rate was detected in the high plains zone (P=0.00001). Here, Tabanids encounter favorable conditions (water, vegetation and woodland) that allow proliferation of these vectors.

Several studies have showed that the Tabanids in North Africa are implicated as the main vectors of *T.evansi* transmission (Baldacchino et al., 2014). Also, Animals that live near watering points (P=0.039) and dense vegetation (P=0.000000) are the most infected. Watering points and vegetation constitute a favorable environment to the survival of vectors.

The dromedaries' population in EL-Bayadh district is about 1450 heads (MADR, 2016). In this study, the contact of horses with dromedaries was considered to be a real risk factor, horses in contact with dromedaries were 2.49 times more likely to be positive for serology. Camels are known to reach high parasitemia in their blood, and therefore there mere proximity to horses in an area with vectors already likely increase risk for transmission. A study realized in El-Oued province, southeast Algeria, reported parasites in 14% of the Giemsa-stained blood smears of dromedaries (Bennoune et al., 2013).

No significant association was found between seropositivity and age, breed, housing and purpose of horses. Age was not significantly associated with the infection by *T.evansi* but horses aged between 6 and 11 years had higher rates of seroprevalance. A previous study revealed an augmentation of seropositivity to *T. evansi* in camels aged 5-11 years (Dia et al., 1997).

Horses that are living in individual boxes are less exposed to the infection by *T. evansi* because the contact with vectors and other sick animals is decreased. In this study, the breed and the purpose of horses were not significantly associated with the seropositivity. In the contrary, a significant difference was been reported in Malaysia (Elshafie et al., 2013).

No physical or environmental characteristic was significantly associated in this study for donkeys. Donkeys are less susceptible to the infection by *Trypanosoma evansi* than horses (Desquesnes et al., 2013).

CONCLUSION

This is the first study of seroprevalence of *T. evansi* in horses and donkeys in Algeria. It has shown that the seroprevalence of *Trypanosoma evansi* in horses was 45.2% (CI 95%, 37.8-52.5%) and 62.1% (CI 95%, 44.4-79.7%) in donkeys. The results indicate that El-Bayadh district is a highly endemic area. Gender, high plains zone, promiscuity with dromedaries, presence of animals near than watering points and vegetation are risk factors to be infected by *Trypanosoma evansi*. Other characteristics like breed, purpose and housing are not revealed as risk factors for the infection by trypanosomiosis. However, physicals and environmental characteristics were not significantly associated with the infection by *Trypanosoma evansi* in donkeys.

Despite this first detection of *Trypanosoma evansi* in equines, in Algeria, and the real risks that may be caused by its spread in many areas of the country, it's essential to increase the epidemiological surveillance of animals in infected and unaffected areas. Therefore, the scientific community and health authorities must pay attention to this parasite, which can be opportunities for new developments in its geographical distribution, especially to the North.

CONFLICT OF INTEREST

None declared.

ACKNOWLEDGEMENTS

This study was supported by the ministry of Higher Education and Scientific Research in Algeria and carried out within the High National Veterinary School of Algiers.

We gratefully thank all those who contributed to the realization of this work.

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J HELLENIC VET MED SOC 2019, 70(3): 1639-1646
ΠΕΚΕ 2019, 70(3): 1639-1646

Research article
Ερευνητικό άρθρο

The *in vitro* toxicity of atrazine on kinematics and DNA fragmentation in common carp (*Cyprinus carpio*) sperm cells

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ABSTRACT. This study investigated the in vitro effects of different concentrations of Atrazine (0.001, 0.01, 0.1, 0.5 mg/L) added to motile and immotile solutions on kinematics quality of sperm cells of common carp, *Cyprinus carpio*, which is a fish of economic significance. The kinematics of the sperm cells was analyzed by a computer-assisted sperm analysis system (CASA). As a result of the study, while there was a significant difference ($P < 0.05$) between the groups in terms of the VSL (µm/s) and VCL (µm/s) values after the Atrazine-added immotile solution's (IMS) and incubation for 3 hours, there was a significant difference ($P < 0.05$) in only the VSL values directly activated by the Atrazine-added motile solution (MS). DNA fragmentation was evident but not in higher numbers in the 0.1 mg/L atrazine group. Finally, it was determined the effective concentration (EC50) values of the VSL value of the motile and immotile solution as 0.34 mg/L and 0.03 mg/L, respectively.

Keywords**:** *Cyprinus carpio, sperm cells kinematics, DNA fragmentation, atrazine, reproduction*

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Date of initial submission: 08-10-2018
Date of revised submission: 13-03-2019
Date of acceptance: 20-03-2019

INTRODUCTION

With increased pollution in the aquatic environment, in order to achieve environmental risk evaluation, it is needed to monitor the toxicity of pollutants, understand their mechanism and determine the areas they affect. Studies are limited on finding the ranges of concentration to determine the maximum levels of environmental pollutants that are not effective on reproduction. *In vitro* techniques allow application of important methods for comparing toxicity levels of pollutants, application of different concentrations and times of exposure, and determining the mechanisms and areas affected by pollutants (Hatef et al., 2013). As opposed to mammals, the sperm cells of teleost fish start to move right away with water and this motility lasts only for a few minutes. However, they may be preserved for 24 hours using some diluents and re-animated via water. For observing the sub-lethal effects of pollutants on reproductive systems, it is possible to test toxicity by both incubation in these diluents for 24 hours and adding pollutants during the last dilution (Kime et al., 1996).

In most teleost fish with ability of external fertilization, sperm activity is short and has a tendency to decrease during the motility period. While the duration of motility is very short (20-25 seconds in the rainbow trout, *Oncorhynchus mykiss* and 1-2 minutes in the common carp, *Cyprinus carpio*), it rapidly decreases after activation (Alavi and Cosson, 2005). Computer-assisted sperm analysis (CASA) is very popular for fast and practical analysis of sperm motility in different species. It has replaced traditional methods and estimation methods based on the analysts' personal considerations (Kime et al., 2001).

The weeds in asparagus, maize, sorghum, sugarcane and pineapple have controlled by the triazine herbicide atrazine in worldwide and USA for over 40 years (Xing et al. 2014). Atrazine and its metabolites have found in surface and subsurface waters (around levels of 0.002 mg/L, under 0.0001 mg/L in general, probably higher in agricultural areas with intensive usage). Atrazine leads to acute toxicity in rats, while this toxicity is LD_{50}: 1870-3090 mg/kg body weight orally and LD_{50}>2000 mg/kg body weight on the skin. Various *in vitro* and *in vivo* test system studies have reported that immune system modulation takes place after treatment of Atrazine, it affects the neuroendocrine function and reduces development. Many studies also stated that Atrazine leads to prostate and ovary cancer and/or increased risks of such cancers in humans (Graham, 1999). Similarly, according to the United States Environmental Protection Agency (US EPA), 0.01-0.02 mg/L of Atrazine poses a significant environmental risk for aquatic life forms and communities. It was reported that it is moderately toxic for fish, while it is severely toxic for aquatic invertebrates (USEPA, 2006). It is stated that Atrazine is a herbicide with a moderate-severe toxic effect on aquatic life forms and especially fish. Its LC_{50} value was found as 76 mg/L in carps, 4.4 mg/L in Guppy, *Poecilia reticulata*, 4.5-8.8 mg/L in trout, and 98-154 mg/L in tilapia. It was observed that a low Atrazine concentration of 1 **µg**/L led to endocrine function changes in male Atlantic salmons (*Salmo salar)*. Recent studies reported that Atrazine harms especially the reproductive system, leads to abnormalities in sperm cells, ovaries and reproductive organs and reductions in sperm cells motility and numbers of sperm cells and ovaries (Wang et al., 2011).

Several and diverse studies have been conducted on acute, sub-acute and chronic toxicity of metals, nanomaterials, herbicides, pesticides and other toxic substances in fish and other aquatic life forms. In last decades, there have studied some researches for toxicity of atrazine such as general health, disease susceptibility and gene expression (Shelley et al., 2012b), behavioral responses (Shinn et al., 2015) and immunotoxic and cytotoxic effects (Shelley et al., 2012) in rainbow trout (*Oncorhynchus mykiss*), cytochromes of liver (Xing et al., 2014), hepatic damage and inflammation (Toughan et al., 2018) of common carp (*Cyprinus carpio*). However, *in vitro* studies on sperm toxicity are rather recent, and there is a dearth of research on the effects of Atrazine on fish sperm cells motility. Various studies were recently conducted on *in vitro* estimation of sperm toxicity via CASA systems regarding some toxic materials (Dietrich et al., 2010; Li et al., 2010b; Fabbrocini et al., 2012). No studies were found to investigate particularly the effects of Atrazine on the sperm cells of the common carp, *Cyprinus carpio* with *in vitro* methods.

This study aimed to investigate the *in vitro* effects of Atrazine on the sperm cells of the common carp. For this purpose, the study examined motility parameters after different levels of Atrazine intervention via a computer aided sperm analysis system (CASA), and analyzed kinematics such as the velocities values of VSL: straight line velocity (µm/s), VCL: curvilinear velocity (µm/s), VAP: angular path velocity (µm/s) and as the movement style values of LIN, linearity

(%,VSL/VCL), the ratio of net distance moved to total path distance, BCF: beat cross frequency (cross/second), ALH: amplitude of lateral displacement of the spermatozoa head (μm) and MAD: mean angular displacement (°), average change in direction of the sperm head from frame to frame which are used to quality-control sperm cells in fish (Kime et al., 2001; Fauvel et al., 2010).

MATERIALS AND METHODS

Broodstocks and chemicals

The stocks of the species common carp, *Cyprinus carpio* were caught from the Karakaya reservoir located on the River Euphrates in end of March, 2018, and the fish were then transported to the fish breeding research unit of İnönü University, Sürgü Vocational School of Higher Education. After the period of adaptation there, the sperms were collected directly from 10 males (265±**1**5 g, Mean±SD) without hormonal injection as they were in the reproduction period. All experiment procedures were approved with protocol no: 2016/A-113 by a committee on the ethical use of animals at İnönü University.

Atrazine [2-chloro-4-ethylamine-6-isopropylamino-l,3,5-triazine] was obtained from the Department of Analytical Chemistry, Faculty of Pharmacy, İnönü University. (FLUKA Atrazine, PESTANAL®, analytical standard, 250 mg, CAS No.:1912-24-9). It was dissolved in distilled water and added into the motile and immotile solutions.

Experimental design

In most species that have external fertilization, fish sperm cells remain immotile in the testis and in the seminal plasma. Then sperm cells become motile at spawning when released into the surrounding water (Kime et al., 2001). Therefore, the experimental design was done two basic *in vitro* settings were created to investigate the effects of Atrazine on the sperm samples:

Setting 1: Analysis after adding nominal concentrations of Atrazine (0.001, 0.01, 0.1, 0.5 mg/L) into the immotile solution (IMS) as artificial seminal plasma and incubation for 3 hours.

Setting 2: Analysis after adding nominal concentrations of Atrazine (0.001, 0.01, 0.1, 0.5 mg/L) into the motile solution (MS) as fertilization media and directly activating sperm cells.

For sperm samples preparation and motility analysis, the stock solutions were prepared to obtain the immotile solution (IMS) containing 200 mM KCl and 30 mM Tris-HCl with pH 8.0, and the motile solution (MS) containing 45 mM NaCl, 5 mM KCl and 30 mM Tris-HCl with pH 8.0, (Poupard et al., 1998). The sperm samples were firstly diluted in Eppendorf tubes with the IMS of 100 times their quantity, and Atrazine was then added into the solution, stirred gently, left at 4 ^{0}C for the duration of incubation. All sperm samples were kept over ice during the procedures. The sperm samples were analyzed under a microscope by activating them with the MS in proportion of 1:20. The final rate of dilution was 2000 times. The dilution rates of the sperm samples were set based on the 2-set procedure (Billard and Cosson, 1992). The microscope used in computer aided sperm analysis system (CASA) was an Olympus BX 53 phase contrast microscope with 20x1.25 magnification coupled with a Sony CCD VB600B camera in the BASA-Sperm Aqua module produced by Merk Biotechnology Co. from Turkey.

DNA fragmentation

For DNA fragmentation, the Sperm Chromatin Dispersion (SCD) test method previously described by Fernandez et al. (2005) was modified and the DNA fragmentation of sperm cells was determined by the present halos. In this method, firstly, 5 μL of sperm samples from control group and 0.1 mg/L dose of atrazine group in Setting 1 medium was diluted with 995 μL immotile solution. So, the concentration of sperm cells were about 16 x10^{9} cells/mL. Then, the preparation method was modified with lysing solution (2 M NaCl, 0.5% SDS, 0.01 Triton X, 0,2 M Tris–HCl and 0.02 M EDTA, pH 7) for 5 minutes at room temperature. After dehydration, the sperm samples were stained with the Diff-Quick reagent for bright field microscopy. In staining, the samples immersed in Diff-Quick A solution for 10 seconds, Diff-Quick I solution for 5 seconds and Diff-Quick II solution for 5 seconds. Then slides was washed with water, dried air for 1 minute. The DNA fragmentation of sperm cells was determined to under 100x lens with immersion oil. The stained images were taken and converted to greyscale in Photoshop 8 version.

Statistics

The SPSS 17 software was used for statistical analysis. All results were expressed as mean ± standard deviation (Mean±SD) and the statistically significant

level was accepted as P < 0.05. The homogeneity of the data in all variables in the groups was tested using the Test of Homogeneity of Variances, and one-way ANOVA and Duncan Post-Hoc tests were used to make comparisons among the groups. Graph Pad Prism 5 was used to form the graphs.

RESULTS

In comparison to the control group, the Atrazine-added samples had gradual reductions in Setting 2 and decreased-increased velocities in Setting 1 at VSL (Straight line velocity) values. While the mean VSL value of the control group was found as 16.44±5.91 µm/s, it was seen that all sperm cells died in the samples with the 0.5 mg/L dose of Atrazine. The changes in the mean VSL values due to Atrazine addition were found to be statistically significant (P < 0.05) in Setting 1. Similar statistical results for VSL were observed in Setting 2. The highest reduction was seen in the group that was exposed to 0.1 mg/L of Atrazine in Setting 2 and the mean value was determined as 11.84±1.99 µm/s. This reduction in the mean VSL values due to mg/L of Atrazine in Setting 2 was statistically significant (P < 0.05) (Figure 1 a, b). The control group's mean VCL (Curvilinear velocity) value was found as 114.70±10.01 µm/s. While the velocity of the sperm cells suddenly increased with the minimum dosage of Atrazine (0.001 mg/L) but then it started to decrease in Setting 1. The highest decrease in velocity was seen in the sample with the dose of 0.1 mg/L in both Setting 1 and Setting 2. While it was found that the differences between VCL mean values were statistically significant (P < 0.05) between 0.001 mg/L and 0.1 mg/L of Atrazine doses in setting 1, they were insignificant (P > 0.05) for all groups in setting 2 (Figure 1 a, b).

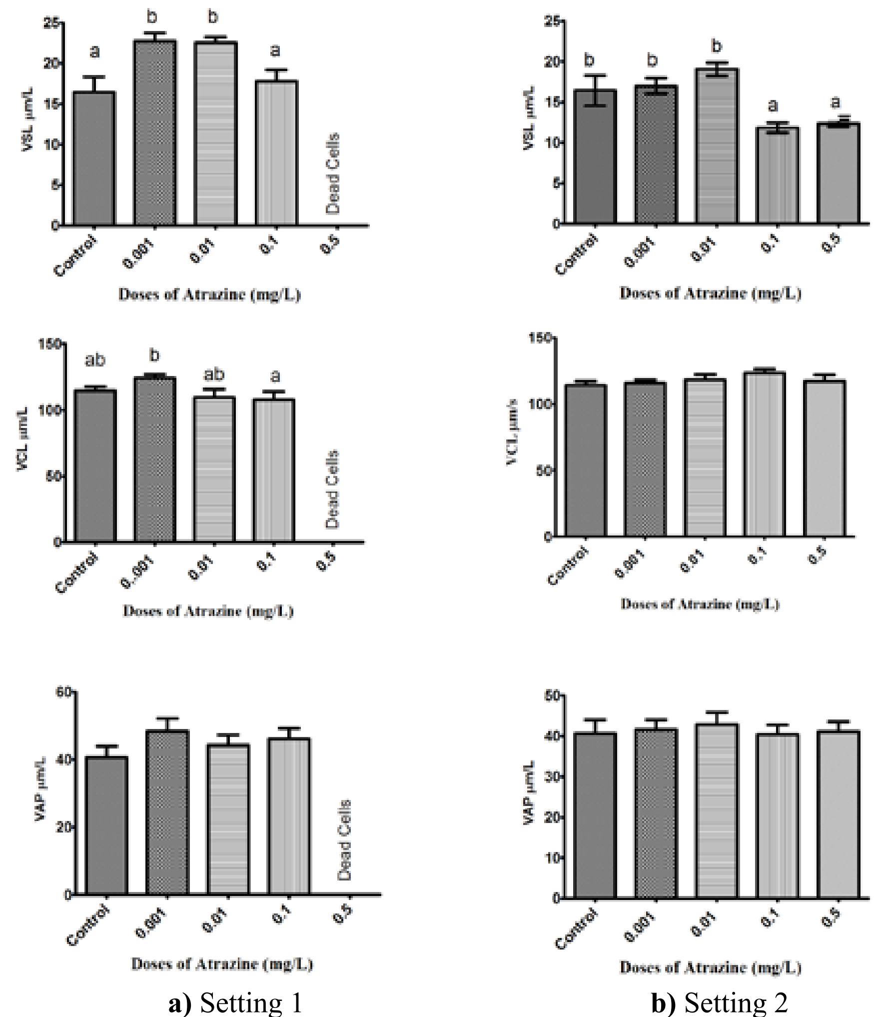


Figure 1. The velocities values as VSL, VCL and VAP in different Atrazine doses. [a,b] Different letters show differences between groups and plots show Mean±SD (P< 0.05)

The VAP (Angular part velocity) values of the sperm samples exposed to different rates of Atrazine increased as the dosage was increased, but these increased values were not significant (P > 0.05). While the mean VAP value of the control group was 40.65±10.73 μm/s after incubation, the highest increase was found in the group that was given the dosage of 0.001 mg/L by 48.49±11.50 μm/s in Setting 1. Although the similar statistical results were observed for VAP in Setting 2, but the VAP values increased slightly by increased doses. In the sperm samples in both Setting 1 and Setting 2 after exposure, it was found that the differences were insignificant (P > 0.05) regarding the control values of LIN (19.74±8.17), BCF (11.07±2.98), ALH (20.91±6.13) and MAD (0.03±0.01) (Figure 2).

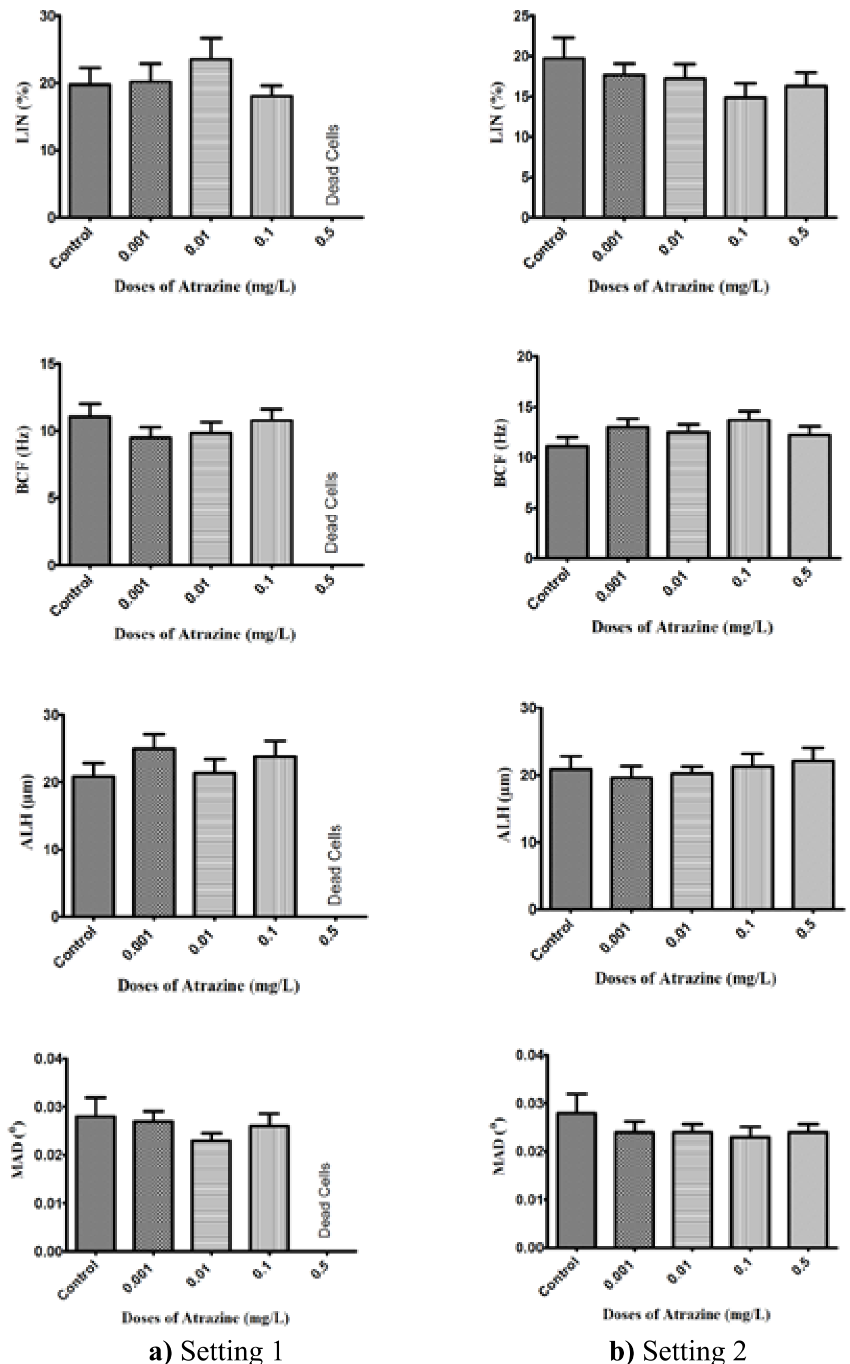
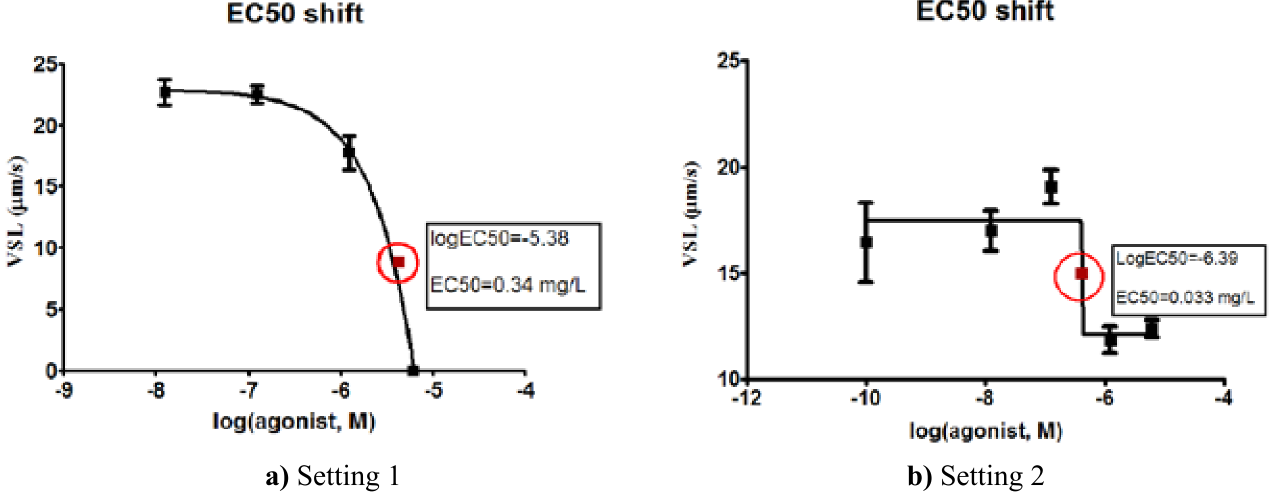


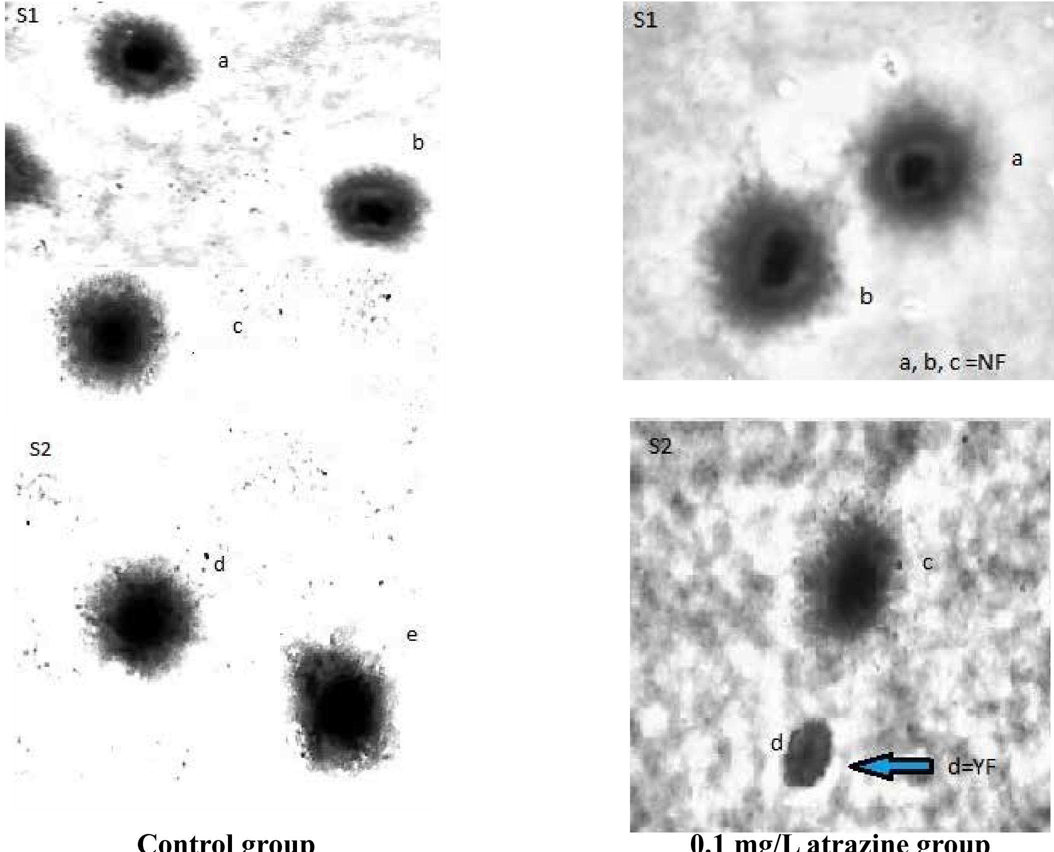
Figure 2. The movement style values as LIN, BCF, ALH and MAD in different Atrazine doses. [a,b,] Different letters show differences between groups and plots show Mean±SD (P < 0.05)

Additionally, on the basis of these experiments, the EC50 values of the VSL parameters after exposure to different doses of Atrazine were calculated as 0.34 mg/L in the immotile solution (Figure 3a) and as 0.033 mg/L in the motile solution (Figure 3b). In the SCD test, although we determined no fragmentation in DNA of sperm cell at control group, there was fragmentation in DNA of sperm cells at 0.1 mg/L Atrazine group but the DNA fragmentation was not increased after 3 hours incubation at +4 °C (Figure 4).



a) Setting 1 **b)** Setting 2

Figure 3. EC50 shift values for VSL in different Atrazine doses



Control group **0.1 mg/L atrazine group**

Figure 4. The Sperm Chromatin Dispersion (SCD) test processed sperm cells showing DNA dispersion halo in Control group (a, b, c, d) and 0.1 mg/L Atrazine group (a, b, c); no showing DNA dispersion halo in 0.1 mg/L Atrazine group (d) with arrow; YF= Yes fragmentation in DNA

DISCUSSION

While *in vitro* sperm toxicity studies are very recent, no studies that were particularly conducted with a herbicide like Atrazine were found. On the other hand, some studies were conducted to estimate *in vitro* sperm toxicity of some toxic substances using CASA systems.

A study investigated the effects of mercury on sperm cells of goldfish, *Carassius auratus,* and considered the sperm motility parameter VCL and sperm morphology (Van Look and Kime, 2003). In their study, they found that 1-10 mg/L concentrations of mercury chloride decreased VCL values dramatically, and 100 mg/L concentration of mercury chloride shortened the length of the flagellum in the sperm cells. In another study (Abascal et al., 2017), 0.01, 0.1, 1, 10 and 100 mg/L concentrations of lead chloride ($PbCl_2$), copper chloride ($CuCl_2·2H_2O$) and mercury chloride ($HgCl_2$) were added onto the activation solution on the sperm cells of sea bass, *Dicentrarchus labrax*, and their effects were studied. In the study, a reduction in all sperm motility parameters after 20 seconds was recorded in this fish species, whose sperm motility has a very short duration (<50s). Additionally, it was found that 100 mg/L of copper and lead added to the activation solution did not affect the sperm cells motility parameters. On the other hand, it was found that 0.4-1 mg/L (1:39 dilution rate) of mercury chloride changed the final swimming morphologies of the sperm cells, and dosages lower than 0.1 mg/L (1:2500 dilution rate) affected the motility parameters with a tendency for a complete halt (Abascal et al., 2007).

Dietrich et al. (2010) treated the sperm cells of the rainbow trout, *Oncorhynchus mykiss* with 1-10 mg/L of mercury and 10 mg/L cadmium, and observed changes in the motility of the sperm cells after 4 hours of incubation (Dietrich et al., 2010). Li et al. (2010a) conducted studies on the motility parameters of sterlet sturgeon (*Acipenser ruthenus*) sperms and their antioxidant responses against environmentally risky heavy metals. They studied the effects of heavy metals Cd, Cr and Cd+Cr after 2 hours of incubation, and found that the dosages of 5 mg/L of Cr, 0.05 mg/L of Cd and 5 mg/L of Cr + 0.05 mg/L of Cd affected sperm motility values negatively (Li et al., 2010a). Moreover, the ecotoxicological effects of cadmium on storing the sperm cells of the gilt-head sea bream, *Sparus aurata* were also investigated (Fabbrocini et al., 2012). The authors found that 50 mg/L of cadmium affected sperm motility values insignificantly in comparison to the control group. Nevertheless, in teleost fish, sperm cells generally move on a straight or slightly curved route right after activation. Under these conditions, Linearity (LIN, the ratio of net distance moved to total path distance (VSL/VCL)) may become a very important determining parameter in finding the curvature of the route. Fertilization may be based on both the number of motile sperm cells, and their velocity (Rurangwa et al., 2004; Bozkurt and Yavaş, 2017).

In our experimental design, the sperm cells incubated in Atrazine added artificial seminal plasma in Setting 1, while they did exposure directly with Atrazine in Setting 2. The value of VSL in Setting 1 did not more decrease than the values of VSL in Setting 2. Other hands, the values of VCL decreased at 0.1 mg/L dose of Atrazine in Setting 1. This results may be explained by protective role of artificial seminal plasma which inhibited to all acts of sperm cells in incubation time (Alavi and Cosson, 2006; Dietrich et al., 2010). Additionally, the decreasing of velocities in sperm cells may take place because of especially ATP consumption after activation (Dzyuba and Cosson 2014)spermatozoa must access, bind, and penetrate an egg, processes for which activation of spermatozoa motility is a prerequisite. Fish spermatozoa are stored in seminal plasma where they are immotile during transit through the genital tract of most externally fertilizing teleosts and chondrosteans. Under natural conditions, motility is induced immediately following release of spermatozoa from the male genital tract into the aqueous environment. The nature of an external trigger for the initiation of motility is highly dependent on the aquatic environment (fresh or salt water in Setting 2. However, our results on the VSL and VCL values were in parallel to those in studies conducted with other species (Van Look and Kime, 2003; Dietrich et al., 2010; Li et al., 2010a)**,** and are also in agreement with the results reported by Wang et al (2011).

Finally, it may be stated that Atrazine on the level of 0.5 mg/L showed lethal effects in all samples and 0.1 mg/L Atrazine level had negative effects on DNA fragmentations of sperm cells after *in vitro* incubation for 3 hours. However, it was shown that the motility parameters of sperm cells in *Cyprinus carpio* were affected negatively in direct exposures of the motile solution to Atrazine. Consequently, while the data obtained here showed that fish sperm cells are significant indicators in ecotoxicological studies, this study

may be seen as a contribution in determining the toxic dosages of Atrazine. Moreover, it is also expected that it will help understand ecotoxicology of herbicides in general.

CONFLICT OF INTERESTS

None of the authors has any conflict of interests to declare.

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J HELLENIC VET MED SOC 2019, 70(3): 1647-1654
ΠΕΚΕ 2019, 70(3): 1647-1654

Research article
Ερευνητικό άρθρο

Combined efficacy of silver nanoparticles and commercial antibiotics on different phylogenetic groups of *Escherichia coli*

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ABSTRACT. Silver nanoparticles (Ag-NPs) can attach to flexible polymeric chains of antibiotics, hence it can be used in combination with antibiotics against resistant bacteria. In this study, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and MBC/MIC ratio of Ag-NPs and antibiotics (gentamicin, tetracycline, erythromycin, ciprofloxacin, nalidixic acid, cefixime, cephalexin, amoxicillin, ampicillin, and penicillin) were quantified against 50 *Escherichia coli* isolates (25 human urinary tract infection and 25 avian colibacillosis). All isolates had been assigned as four phylogenetic groups A, B1, B2, and D. The results showed that the majority of the human and broiler isolates belonged to phylogenetic groups A and B2. MBC/MIC ratio of Ag-NPs in combination with antibiotics was assessed. It was found that the MIC of the majority of broiler isolates to Ag-NPs was equal to or greater than 50 µg/ml. To conclude, a combination of penicillin and ciprofloxacin with Ag-NPs exhibited profound impact against isolates, the combinations might be applicable for treating multidrug-resistant bacteria.

Keywords: *Escherichia coli*, MBC/MIC ratio, silver nanoparticles, phylotypes

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Date of initial submission: 30-09-2018
Date of revised submission: 30-05-2019
Date of acceptance: 19-08-2019

INTRODUCTION

Extra-intestinal pathogenic *Escherichia coli* (ExPEC) causes a variety of diseases outside the intestine, including urinary tract infections (UTIs) (Skjot-Rasmussen, et al. 2012), meningitis, sepsis, abdominal infections, osteomyelitis, cellulitis, and colibacillosis in poultry (Johnson, et al. 2007). *E. coli* has been characterized in four major phylogenetic groups including A, B1, B2, and D. Most commensal *E. coli* strains belong to phylogenetic groups A and B1 whereas the most virulent strains belong to groups B2 and D (Hussain, et al. 2012; Kazemnia, et al. 2014). Generally, human uropathogenic *E. coli* (UPEC) might be transferred from food chains (Singer 2015). A study indicates that retail meat contaminated with ExPEC strains, were associated with the antimicrobial-resistant *E. coli* causing urinary UTIs (Walk, et al. 2007). The application of antibiotics in the poultry industry is a risk factor for the emergence of antimicrobial resistant bacteria (Furtula, et al. 2010). Since multidrug resistant (MDR) bacteria is increasing, then, it requires to be considered as a major public health threat. The increase of MDR bacteria has led to find and develop a novel generation of safe antimicrobials to control those microorganisms. Because of their antimicrobial properties (Kim, et al. 2007), inorganic nanomaterials such as silver nanoparticles (Ag-NPs) are widely used in medical and consumer products, including antiseptic agents, medical devices, water purification systems, food packaging, and health care products (Park, et al. 2010). Mechanisms by which Ag-NPs exert their antibacterial effects to overcome bacteria are as follows: penetrating to bacterial cell which alters transportation of electrolytes and metabolites (Ansari, et al. 2014), and affects membrane-bound enzymes leading to disruption of ATP production; destroying the stability of LPS leading to an increase in the permeability of outer membrane; damaging vital enzymes, proteins, and DNA as a result of binding of Ag-NPs with them (Yang, et al. 2012; You, et al. 2012), owing to the fact that Ag-NPs have a great affinity to react with sulfur- or phosphorus-containing compounds (Silambarasan and Jayanthi 2013; Tamboli, et al. 2012); and generating reactive oxygen species which leads to death of bacteria (Xu, et al. 2012; You, et al. 2012). It has been reported that combination of antibiotics and Ag-NPs may act as an antibiotic carrier, facilitating the approaching of hydrophilic antibiotics to bacterial surface (Ghosh, et al. 2012), and then nano-silver drug carriers may help them to cross into bacteria, causing more damage to the cell. Additionally, Ag-NPs may share their mechanism of actions with antibiotics to cope with resistant bacteria. Moreover, regarding different modes of actions of antibiotics and Ag-NPs, it can be expected if a bacterium exhibits resistance to one agent, the other agent may overcome the bacterium with different mechanisms of action. Ag-NPs have a very lower natural tendency to induce microbial resistance than do antibiotics (Chudasama, et al. 2010). The aim of the present study was to investigate the effects of the simultaneous application of Ag-NPs and commonly used antibiotics on *E. coli* of different phylogenetic groups.

MATERIALS AND METHODS

Bacterial Strains

A total of 50 *E. coli* strains (25 from UTIs and 25 from broiler colibacillosis) were included in this study. All strains (tables 1 and 2) had previously been classified using triplex PCR as proposed by Clermont *et al.* (Clermont, et al. 2000) in our previous work (Kazemnia, et al. 2014). The results of phylogenetic typing of strains are in tables 1 and 2.

Susceptibility of *E. coli* isolates to Ag-NPs

Commercially manufactured 20 nm Ag-NPs (US Research Nanomaterials, Inc., Houston, USA) was used to treat the isolates. Firstly, all *E. coli* isolates were grown overnight and washed twice, then cells were re-suspended in Muller-Hinton broth until their concentration reached 10^8 CFU/mL. The concentration was evaluated by optical density at 600 nm (OD_{600}~ 0.1-0.13). Microdilution method was employed to examine the susceptibility of isolates to different concentrations of Ag-NPs. An amount of 100 µL of each overnight culture and 800 µL of fresh Mueller-Hinton broth were added to each well in a 48-well microplate. To treat each strain with a final concentration of Ag-NPs (2.5, 5, 10, 20, 30, 40, 50, 100 µg/mL), 100 µL of stock solutions of prepared Ag-NPs (25, 50, 100, 200, 300, 400, 500, 1000 µg/mL) was added to each well. The microplates were then incubated at 37 °C for 24 h. The MIC was determined by measuring the optical density of cultured bacteria using a spectrophotometer at 600 nm. (CLSI 2012). Five microliters of each well was plated on Luria–Bertani agar and incubated further at 37 °C in order to determine the minimum bactericidal concentration (MBC) (Ayala-Núñez, et al. 2009).

Table 1. MIC, MBC and MBC/MIC ratio of SNP and antibiotics alone and in combination against different urinary phylotypes of *E.coli.*

Samples	Phylotypes	SNP		Gentamicin			Penicillin		Ampicillin		Amoxicillin		Tetracycline	
		MIC MBC		MIC MBC		MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP
1	A	25	25	25	25	1	75 100	**	25 50	1	25 50	1	100 *	1.5
2	B2	25	25	*	*	**	* *	**	* *	**	* *	**	* *	1
3	A	50	75	*	*	**	* *	**	* *	**	* *	**	100 *	1
4	D	50	50	25	25	1	75 100	**	50 75	1	50 100	**	75 100	25
5	D	75	100	50	50	1	* *	**	* *	**	* *	**	* *	1
6	B2	100	100	25	25	1	* *	**	* *	**	* *	**	25 100	25
7	B2	50	50	25	25	1	* *	**	* *	**	* *	**	* *	**
8	D	50	75	*	*	1	* *	**	* *	**	* *	**	* *	**
9	D	75	100	50	50	1	* *	**	* *	**	* *	**	* *	**
10	A	25	25	25	25	1	* *	**	25 50	1	* *	1	25 *	75
11	D	75	75	100	*	1	* *	**	50 100	1	* *	1	* *	**
12	A	50	50	25	25	1	100 *	**	25 75	3	25 50	1	25 *	75
13	B2	100	100	*	*	2	* *	**	* *	**	* *	**	* *	1.33
14	A	25	25	75	*	2	* *	**	* *	**	* *	**	* *	1
15	D	100	100	25	25	1	* *	**	* *	**	* *	**	* *	**
16	A	100	100	*	*	1	* *	**	* *	**	* *	**	25 *	75
17	B2	*	*	*	*	**	* *	**	* *	**	* *	**	* *	**
18	B2	75	75	50	50	1	* *	**	* *	**	* *	**	* *	1
19	B2	75	75	25	25	1	100 *	**	50 75	1	25 50	1	50 *	75
20	B2	75	100	75	75	1	100 *	**	25 100	1	25 100	1	25 *	75
21	B2	100	100	50	50	1	100 *	**	50 100	1	25 100	1	25 *	75
22	B2	100	100	50	50	1	* *	**	* *	**	* *	**	* *	**
23	D	75	75	50	50	1	* *	**	* *	**	* *	**	* *	1.33
24	A	100	100	50	50	1	100 *	**	25 100	1	25 50	1	25 *	75
25	A	75	100	50	50	3	100 *	**	25 100	1	25 50	1	25 100	75

Table 1 (continued)

Samples	Phylotypes	SNP		Gentamicin			Penicillin		Ampicillin		Amoxicillin		Tetracycline	
		MIC MBC		MIC MBC		MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP
1	A	25	25	12.5	25	6	* *	1.33	100 *	1	25 50	1	* *	**
2	B2	25	25	12.5	25	1	* *	**	* *	**	100 *	2	* *	**
3	A	50	75	*	*	**	* *	**	* *	**	* *	**	* *	**
4	D	50	50	6	50	1	* *	8.33	50 100	8.33	25 50	25	50 *	**
5	D	75	100	12.5	50	1	* *	**	* *	4.17	* *	**	* *	**
6	B2	100	100	25	*	1	50 75	2	* *	**	50 50	8.33	* *	**
7	B2	50	50	75	100	25	* *	**	* *	**	50 50	2	* *	**
8	D	50	75	*	*	**	* *	**	* *	**	* *	**	* *	**
9	D	75	100	*	*	1	* *	**	* *	**	50 75	4.1	* *	**
10	A	25	25	100	*	1	25 50	8.3	50 100	4.17	25 50	3	75 *	**
11	D	75	75	*	*	**	* *	**	* *	**	50 50	8.33	* *	**
12	A	50	50	100	*	1	* *	8.3	* *	2	25 50	1	75 *	**
13	B2	100	100	*	*	**	* *	**	* *	**	* *	**	* *	**
14	A	25	25	*	*	**	* *	**	* *	**	* *	**	* *	**
15	D	100	100	12.5	*	25	75 100	4.17	* *	**	* *	**	* *	**
16	A	100	100	*	*	**	* *	**	* *	**	* *	**	* *	**
17	B2	*	*	*	*	**	* *	**	* *	**	* *	**	* *	**
18	B2	75	75	*	*	**	* *	**	* *	**	* *	**	* *	**
19	B2	75	75	25	50	1	50 75	25	50 100	**	25 50	1	* *	**
20	B2	75	100	12.5	25	1	50 75	25	50 100	**	50 50	6	* *	**
21	B2	100	100	12.5	25	1	50 75	25	50 100	**	25 50	12	100 *	**
22	B2	100	100	*	*	**	* *	**	* *	**	* *	**	* *	**
23	D	75	75	25	75	1	* *	8.33	50 100	**	25 25	2	* *	**
24	A	100	100	6	25	1	50 75	25	50 100	1	12.5 75	2	* *	**
25	A	75	100	6	25	1	50 100	25	100 *	**	25 75	2	* *	**

* refers to >100; ** refers to not determined; SNP refers to Ag-NPs.

Table 2. MIC, MBC and MBC/MIC ratio of SNP and antibiotics alone and in combination against different colibacillosis phylotypes of *E.coli.*

Samples	Phylotypes	SNP		Gentamicin			Penicillin		Ampicillin		Amoxicillin		Tetracycline	
		MIC	MBC	MIC	MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP
1	B2	50	75	25	50	3	* *	**	25 100	4.1	50 100	4.1	25 50	6
2	B2	100	100	25	50	1	* *	**	25 100	12	50 100	4.1	25 75	12
3	A	50	75	25	50	3	* *	**	50 100	25	50 *	4.1	25 *	8.3
4	B1	50	50	50	75	12	* *	**	* *	**	* *	**	* *	**
5	A	1	25	25	25	1	* *	**	25 100	12	50 100	4.1	25 75	4
6	B1	75	75	25	50	6	* *	**	* *	**	* *	**	* *	**
7	D	50	100	12	25	3	* *	**	50 *	12.5	75 *	4.1	25 50	12
8	B1	25	25	25	25	1	* *	**	* *	**	75 *	2.1	* *	**
9	B2	75	75	25	50	1	* *	**	50 *	8.3	50 *	4.1	25 100	8.3
10	B2	50	50	12	25	1	* *	**	50 *	8.3	50 100	2.1	100 *	4
11	B2	75	75	25	50	3	* *	**	75 *	8.3	100 *	4.1	25 50	6
12	A	50	50	25	25	1	* *	**	* *	**	* *	**	* *	**
13	A	3	25	12	25	1	100 *	**	50 *	8.3	50 100	4.1	* *	4
14	B2	1	25	12	25	1	100 *	2	75 *	8.3	100 *	4.1	25 75	12
15	A	50	100	25	50	3	* *	**	75 *	8.3	* *	4.1	* *	**
16	A	50	50	25	50	3	* *	**	* *	8.3	75 100	4.1	* *	**
17	A	50	50	12	25	1	* *	**	50 *	4.1	* *	4.1	* *	**
18	D	50	50	25	50	3	* *	**	50 *	8.3	25 100	4	* *	**
19	D	50	50	12	25	1	* *	**	50 100	8.3	100 *	4.1	* *	2
20	D	50	50	12	25	3	* *	**	75 *	8.3	* *	4.1	* *	**
21	B2	50	75	12	25	3	* *	**	* *	4.1	75 *	2.1	25 100	8.3
22	B1	25	25	25	50	3	* *	**	100 *	6.25	* *	3	* *	**
23	A	25	25	12	25	3	* *	**	* *	8.3	75 *	2.1	* *	**
24	A	12	25	12	25	1	* *	**	75 *	8.3	75 100	4.1	25 50	6
25	D	50	75	25	50	2	* *	**	75 100	8.3	100 *	**	* *	**

Table 2 (continued)

Samples	Phylotypes	SNP		Gentamicin			Penicillin		Ampicillin		Amoxicillin		Tetracycline	
		MIC	MBC	MIC	MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC MBC	MBC/MIC ratio in combination with 5µg/ml SNP
1	B2	50	75	75	*	100	* *	**	50 *	8.33	100 *	2	* *	**
2	B2	100	100	50	*	100	* *	**	100 *	8.33	25 100	16.7	50 100	3
3	A	50	75	*	*	**	* *	**	* *	**	25 100	12.5	* *	**
4	B1	50	50	*	*	4	* *	**	100 *	8.33	* *	**	* *	**
5	A	1	25	50	70	3	100 *	33.3	50 100	25	25 50	25	25 100	12.5
6	B1	75	75	100	*	8.3	* *	**	100 *	4	100 *	**	* *	**
7	D	50	100	50	*	50	* *	**	100 *	8.3	25 50	25	75 *	4
8	B1	25	25	50	*	16.7	* *	**	100 *	4	* *	**	* *	**
9	B2	75	75	50	*	75	* *	**	* *	2	75 *	8.3	50 100	2
10	B2	50	50	50	*	75	* *	**	100 *	**	100 *	8.3	* *	**
11	B2	75	75	75	*	75	* *	**	100 *	**	50 *	12.5	75 *	2
12	A	50	50	50	*	75	* *	**	100 *	6.25	* *	**	* *	**
13	A	3	25	*	*	6.25	* *	**	* *	**	25 50	12	75 *	**
14	B2	1	25	75	*	50	75 *	**	100 *	**	25 50	6	25 *	6.25
15	A	50	100	50	*	6.25	* *	**	* *	**	25 50	6	50 *	**
16	A	50	50	75	*	16.7	* *	**	* *	**	25 50	4	50 *	4
17	A	50	50	50	*	16.7	* *	**	* *	**	25 50	12	50 *	3
18	D	50	50	50	*	75	* *	**	* *	**	50 100	16.7	50 *	4
19	D	50	50	100	*	75	* *	**	* *	**	25 100	8.3	50 *	3
20	D	50	50	50	*	100	* *	**	* *	**	50 100	16.7	50 *	**
21	B2	50	75	50	*	33.3	* *	**	100 *	**	12 100	25	25 *	**
22	B1	25	25	25	50	12	100 *	**	100 *	**	50 *	12.5	50 *	**
23	A	25	25	50	*	33.3	* *	**	100 *	**	25 50	12	75 *	**
24	A	12	25	50	75	8.3	* *	**	* *	**	25 75	25	* *	**
25	D	50	75	*	*	**	* *	**	* *	**	* *	**	* *	**

* refers to >100; ** refers to not determined; SNP refers to Ag-NPs.

Susceptibility of *E. coli* isolates to antibiotics

E. coli strains were tested for antimicrobial susceptibility using the same procedure described for susceptibility testing of isolates for Ag-NPs. The antibiotics used in this essay belonging to six different classes of antibiotics: aminoglycosides (gentamicin); tetracyclines (tetracycline); macrolides (erythromycin); quinolones (ciprofloxacin and nalidixic acid); cephalosporins (cefixime and cephalexin); penicillins (amoxicillin, ampicillin, and penicillin) (Sigma-Aldrich, St. Louis, MO, USA). Stock solutions of each antibiotic were prepared with concentrations of 15.6, 31.25, 62.5, 125, 250, 500, 750, 1000 mg/mL. Each strain was then treated with a final concentration of 1.56, 3.125, 6.25, 12.5, 25, 50, 75, 100 mg/mL of each antibiotic in a 48-well microplate.

Susceptibility of *E. coli* isolates to Ag-NPs in combination with antibiotics

For determination of MIC and MBC of each isolate, the sub-inhibitory concentration of 5 µg/mL of Ag-NPs in combination with each of antibiotics was separately applied to each of the strains to examine MIC and MBC of combined agents. The interaction of antibiotics and Ag-NPs were evaluated using 5 µg/mL of Ag-NPs and 1.56, 3.125, 6.25, 12.5, 25, 50, 75, 100 mg/mL of each antibiotic to determine MIC and MBC. The procedure was performed as described previously (CLSI 2012).

Statistical analysis

All experiments were carried out at least in three replicates and obtained data were analyzed in the SPSS (Version 21, SPSS Inc., Chicago, IL).

RESULTS

MIC and MBC

The MIC and MBC patterns of 50 strains to 10 antibacterial agents and Ag-NPs are shown in tables 1 and 2. According to the MIC and MBC results, colibacillosis isolates exhibited the highest rates of resistance to penicillin and the lowest resistance to gentamycin. It was found that the MIC of the majority of isolates from broilers to Ag-NPs was equal to or greater than 50 µg/ml. Multi-drug resistance of *E. coli* isolates from human was higher than *E. coil* isolates from broilers. Statistical analysis demonstrated that, among colibacillosis isolates, bactericidal effect of 5 µg/ml in combination with penicillin, gentamycin, cephalexin, amoxicillin, erythromycin, tetracycline, cefixime, ciprofloxacin, nalidixic acid, and ampicillin was 100, 92, 80, 33.3, 33.3, 30.8, 10, 8.7, 0 and 0, respectively. The effect of simultaneous application of Ag-NPs and antibiotics in 34.8% of colibacillosis strains was bactericidal and in 65.2% was bacteriostatic. Maximum bactericidal effects of combined agents on the broiler strains belonged to genotypes B1, D, B2 and A calculated as 46.7, 35.7, 34 and 31.4 % respectively. The bactericidal effect of combination of 5µg/ml of Ag-NPs with each of antibiotics (ciprofloxacin, cefixime, erythromycin, tetracycline, gentamycin, nalidixic acid, ampicillin, and amoxicillin) in the case of human strains were 81.3, 60, 50, 44.8, 22, 16.7, 10, and 9%, respectively. The effect of the combination of 5 µg/ml of Ag-NPs with antibiotics in70.4% of strains from human was bactericidal, and in 29.6% of strains was bacteriostatic in total. The maximum bactericidal effect of combined agents against human strains belonged to genotypes of A, B2, and D calculated as 75, 70, and 63% respectively.

DISCUSSION

There is a need for a new generation of antibiotics to overcome drug-resistant bacteria because of the rapid development of antimicrobial resistance (Ansari, et al. 2013). According to previous studies, there is some evidence proving that Ag-NPs have potent antimicrobial effects individually (Ansari, et al. 2014; Radzig, et al. 2013) and show synergistic activity in combination with antibiotics (Dhas, et al. 2013; Lavanya, et al. 2013). As stated in some works, whether an agent by itself or in combination with the other agents is considered bacteriostatic when this ratio is equal to or greater than 16, and if this ratio is less than or equal to 4, the agent represents bactericidal manner (Das, et al. 2016; Prema, et al. 2017). In this study, an antibacterial performances using microdilution method against isolates elucidated that combination of a penicillin (in colibacillosis strains) and ciprofloxacin (in UTI strains) with Ag-NPs exhibited profound impact and could be used for coping with multidrug-resistant *E. coli*. According to the results, most of the combined agents used in this study had bactericidal effects. Gentamicin plus 5µg/ml offered itself to be a promising bactericidal compound against MDR *E. coli*. The results obtained from this study are consistent with that of other researches. Indeed, gentamycin plus Ag-NPs in several studies demonstrated enhanced or even synergistic antibacterial effect against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*,

Streptococcus agalactiae, *Streptococcus mutans*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Escherichia fergusonii*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Enterococcus faecalis*, *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, *Edwardsiella tarda* and *Pseudomonas aeruginosa* (Barapatre, et al. 2016; Chhibber, et al. 2017; Ebrahimi, et al. 2017; Jamaran and Zarif 2016; Katva, et al. 2017; Mohamed, et al. 2017; Panacek, et al. 2015; Satapathy, et al. 2017; Verma, et al. 2017; Wang, et al. 2016).Moreover, in a good agreement with reported data in the literature, penicillin (Ebrahimi, et al. 2017; Panacek, et al. 2015) in contrary to some studies (Deng, et al. 2016; Natan and Banin 2017; Wang, et al. 2016) and ciprofloxacin in the case of human strains (Mohamed, et al. 2017; Yallappa, et al. 2015) in accord with our data (Panacek, et al. 2015) showed significant increase in antibacterial activities when conjugated with Ag-NPs. Furthermore, tetracycline (Deng, et al. 2016; Ebrahimi, et al. 2017; Natan and Banin 2017), amoxicillin (Mohamed, et al. 2017), cephalosporin (Refat, et al. 2017), erythromycin (Yallappa, et al. 2015) showed partially increase in antibacterial activities in the presence of Ag-NPs. On the other hand, to the contrary to our study, ampicillin (Satapathy, et al. 2017), nalidixic acid (Tawfeeq, et al. 2017) have been mentioned to have a synergistic effect. This might be attributed to the joint mechanism of action between antibiotic and Ag-NPs (Wang, et al. 2016). Combination may trigger an enhanced membrane permeability and/or reactive oxygen species production. However, little is known about the interaction between Ag-NPs and antibiotic, and also the presence of resistant genes to Ag-NPs. The difference in the behavior of the combination of Ag-NPs with antibiotics discussed in the literature could be due to the presence of resistance genes to Ag^+, applied doses, the size, and shape of Ag-NPs, and the mechanisms of resistance to Ag-NPs in bacteria. The antibacterial capacity of Ag-NPs is dependent on the size, dose, and the shape of Ag-NPs (Majeed, et al. 2016; Prema, et al. 2017; Zheng, et al. 2018). The Ag-NPs in small size reveal higher antibacterial performance (Zheng, et al. 2018). The mechanisms by which bacterial cells exert resistance to Ag^+ are as follows: utilizing Ag^+ ATPase efflux pumps; multiple antibiotic resistance genes; *ybdE*, *ylcD*, *ylcC*, *ylcB*, *ylcA*, *ybcZ* genes (Nagy, et al. 2011; Silver, et al. 2006); some periplasmic Ag^+ binding proteins (Muhling, et al. 2009; Silver, et al. 2006); plasmids carrying metal resistance genes (Silver, et al. 2006). However, bacterial resistance to Ag-NPs has not been proven yet.

The MBC/MIC ratio has been used to consider whether an agent is a bactericide or bacteriostatic. Conversely, there were significant differences in bacteriostatic and bactericidal manners of combined agents among the phylotypes of human and broiler strains. The administration of 5μ/mL of Ag-NPs combined with antibiotics against the phylotypes A, B2, and D in the case of human strains exerted bactericidal action, whereas in the case of broiler strains it was bacteriostatic manner. The authors speculate that this effect is probably due to the tolerance (Das, et al. 2016) of broiler strains against Ag^+ and/or Ag-NPs. Since these differences among phylotypes of *E. coli* have not been studied yet, further studies are required.

CONCLUSION

The results of this study suggest that Ag-NPs combined with antibiotics exhibit excellent bactericidal effect towards drug resistant *E. coli*. Nevertheless, one of the profound concerns despite the benefits of Ag-NPs is now the toxicity of Ag-NPs on mammalian cells, which needs to be investigated further. To the best of our knowledge, it is the first report that of MBC/MIC ratio of simultaneous application of commercially prescribed antibiotic and Ag-NPs against clinical strains.

ACKNOWLEDGMENT

We express gratefulness to Urmia University for providing the financial support, facilities and instrumentations to execute this study.

CONFLICT OF INTEREST

There are no conflicts of interest.

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J HELLENIC VET MED SOC 2019, 70(3): 1655-1660
ΠΕΚΕ 2019, 70(3): 1655-1660

Research article
Ερευνητικό άρθρο

Investigation of the changes observed in scrotal circumference, and native and post-thaw semen characteristics in karayaka rams during the breeding and non-breeding seasons

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ABSTRACT. The aim of the present study was to investigate and compare the impact of the breeding (BS) and non-breeding (NBS) seasons on scrotal circumference, and native and post-thaw semen characteristics in Karayaka rams. Six (6) Karayaka rams were used as semen donors, and ejaculates were collected with the aid of an artificial vagina during the BS (October-November) and NBS (April-May). Interestingly, sperm freezability was found to be higher in the NBS, when compared to the BS, and the sperm motility rate (36.00±2.74%, 55.0±3.24%) and percentage of abnormal sperm (44.00±1.90%, 57.00±2.09%) were found to significantly differ with season (P<0.05). In conclusion, the post-freezing sperm motility was better during the non-breeding season. Post-thaw sperm abnormality was higher during non-breading season. However, if ram semen is used fresh for AI, it can be collected and used during both during the breeding and non-breeding season.

Keywords: freezability, karayaka rams, seasonal variation, semen characteristics

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Date of initial submission: 10-12-2018
Date of revised submission: 18-08-2019
Date of acceptance: 21-08-2019

INTRODUCTION

The Karayaka sheep is a native breed of Turkey, which is raised in the Black Sea Region. The Karayaka breed is renowned for its mutton production and high quality meat, while it is also well adapted to the warm, humid and irrigated semi-arid regions of northern Turkey (Kaymakcı et al., 2001). Sperm quality, which is an indicator of fertility in rams, is affected by several factors including season, temperature, humidity and day length. Furthermore, some other factors, such as breed and age of the animal and the production system used, have been reported to influence sperm production and quality (Tajangookeh et al., 2007). Among these factors, season is one of the most important factors that influences variations in semen quality and fertility. The season may show direct or indirect effects on variations in ram reproductive activity (D'Alessandro and Martemucci, 2005; Olah et al., 2013). Rams, that are raised in the northern hemisphere, are particularly affected by seasonal variations, and their testicular activity either increases or decreases, thereby altering sperm characteristics.

There is limited research on seasonal effects on cryopreservation of ram semen in the literature (D'Alessandro and Martemucci, 2005; Makawi et al., 2007). To our knowledge, there has not yet been any study about Karayaka rams concerning these issues and our study is the first to describe these parameters in this breed. Thus, this study aims at the investigation of the impact of seasonality on alterations in scrotal circumference, and native and post-thaw sperm quality in Karayaka rams. This study not only describes sperm characteristics of the Karayaka ram for the first time, but is also expected to help strengthen the preformance of AI in the Karayaka breed.

MATERIALS AND METHODS

Animals, feeding and management: In this study, the breeding season has been determined between October and November, while the non-breeding season has been defined as the months April and May (Olfaz et al., 2010). Six 2-3-year-old Karayaka rams, weighing 65-70 kg, were used in this study. The animals were raised under uniform feeding conditions, natural photoperiod and natural temperature and environmental humidity. All rams were housed in a covered shelter and were provided with 1 kg of hay per animal daily. A supplement containing 12% protein was also offered each morning (0,9 kg for each ram). Clean and safe water was available at all times. A general management schedule for de-worming, disease prevention, and hoof trimming was followed throughout the study.

Scrotal measurements, semen collection and evaluation: Scrotal circumference was assessed twice a month during the breeding and non-breeding season, with a flexible metric tape (Tape, Scrotal Metric, A Neogen Company) (Kulaksiz et al., 2010). Semen was collected in both the breeding season (BS, October-November) and the non-breeding season (NBS, April-May). During both seasons, the first five ejaculates of all rams were discarded to minimize the extragonadal sperm reserve. Semen collection was performed once a week on 8 occasions per each season for each ram. In total, ninety six (96) ejaculates were collected from six rams (sixteen ejaculates from each ram, eight collected in the BS and eight in the NBS) by means of an artificial vagina (Minitube model) after stimulating rams with ewes in estrous.

The ejaculates collected from each ram were evaluated for native sperm characteristics, including semen volume, sperm concentration, motility, and percentage of abnormal sperm. Ejaculate volume was measured immediately after collection using a graduated glass tube. Ejaculates were diluted 1:10 with a skim milk-based extender, and percentage of motile spermatozoa was estimated by subjective microscopic examination at x400 magnification using a phase-contrast microscope equipped with a heated stage at 37°C. Sperm concentration was determined using the haemocytometer method, after diluting the semen samples 1:400 with Hayem's solution. Sperm morphology was evaluated after fixation with Hancock's buffered formol saline solution (Schafer and Holzmann, 2000). Abnormal sperm morphology percentage was determined by counting a total of 200 spermatozoa under a phase-contrast microscope (×1000 magnification; oil immersion).

Semen cryopreservation: A skim milk-based egg yolk extender protocol was used for semen cryopreservation. The extender contained 10 g of skim milk powder and 0.9 g of glucose per 100 ml. Egg yolk and glycerol were added to the solution to final concentrations of 10% and 5% (v/v), respectively. The skim milk-egg yolk-glycerol (SEG) extender contained 500 IU of penicillin and 500 µg of streptomycin sulphate per mL, and was stored at 5°C. Diluted semen was loaded into 0.25 mL French straws were used, so that each straw contained a dose of 100 x 10^6 spermatozoa. Plastic straws were sealed with polyvinyl alcohol powder. Loaded straws were stored at

5°C and were allowed to equilibrate for 2 hours before being frozen. After equilibration, the straws were suspended on a styrofoam rack 4 cm above the liquid nitrogen (vapour) for 15 min. At this stage, the straws were rapidly transferred to LN2 containers at -196°C. Straws were stored in LN2 until evaluation. After being stored for a month, two straws belonging to each ram were thawed in a warm bath (37°C) for 1 min. Next, according to the method described above, the contents of the straw were examined for sperm motility and sperm abnormalities (Evans and Maxwell, 1987).

Statistical analysis

The data obtained in the present study was statistically analysed using the SPSS® (SPSS 18.0, Chicago, IL, USA) software package. Distribution of the data was assessed with the Shapiro-Wilk test. As the data had a normal distribution, the groups were compared with parametric tests. The comparison of all data for the breeding and non-breeding seasons was made with the t-test (independent). Results were expressed in mean±standard error of mean (SEM). Statistical significance was set at $P<0.05$.

RESULTS

Scrotal circumference and native and post-thaw sperm characteristics of the Karayaka rams included in this study are presented in Figures 1, 2, 3 and 4. Season had a statistically significant impact on scrotal circumference ($P<0.05$). Scrotal circumference was greater in the breeding season, when compared to the non-breeding season (Figure 1). While ejaculate volume was also significantly larger in the BS (1.45 ml, $P<0.05$), no statistical difference has been found in sperm concentration, sperm motility, or percentage of normal sperm with respect to season ($4.40x10^9$ ml, 85%, 95%, respectively) (Figures 2 and 3, $P>0.05$). Furthermore, post-thaw sperm motility rates and abnormal sperm percentages were significantly affected by season ($P<0.05$), whereas freezability of semen was better in the NBS, in comparison to the BS (Figure 4).

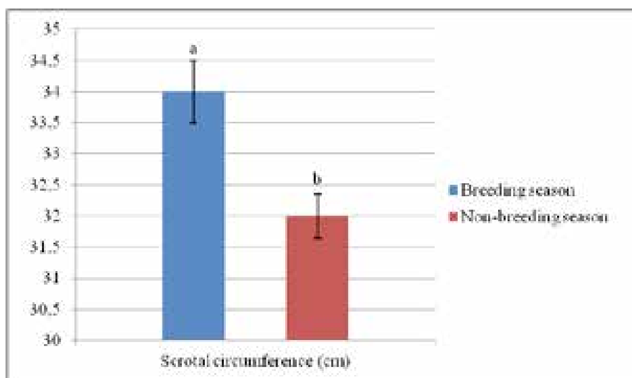

Figure 1. Scrotal circumference of Karayaka rams in the breeding and nonbreeding season
Different letters (a-b) indicate significanct difference (P<0.05)

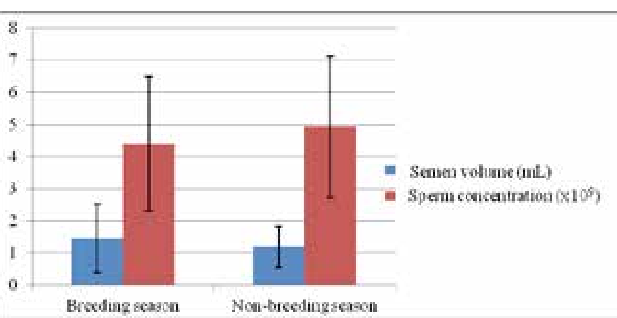

Figure 2. Semen volume and sperm concentration of Karayaka rams in the breeding and nonbreeding season. Different letters (a-b) indicate significanct difference (P<0.05)

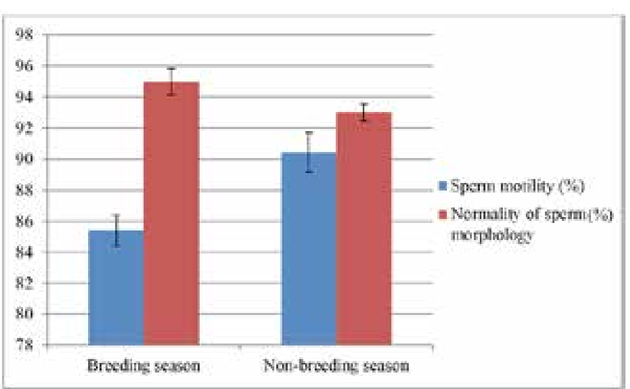

Figure 3. Sperm motility and normality of sperm morphology of Karayaka rams in the breeding and nonbreeding season

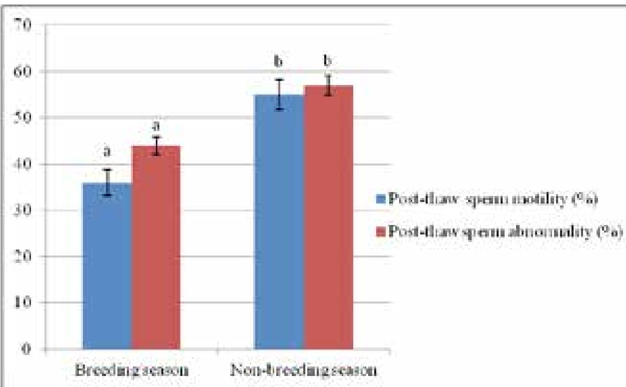

Figure 4. Post-thaw sperm motility and post-thaw sperm abnormality of Karayaka rams in the breeding and nonbreeding season. Different letters (a-b) indicate significanct difference (P<0.05)

DISCUSSION

This study is the first report on seasonal changes observed in reproductive indices of Karayaka rams reared in northern Turkey. In the present study, the highest values recorded for scrotal circumferences of the Karayaka rams, were measured in autumn, while the lowest values were recorded in spring. This is in line with the results reported by Zamiri et al. (2010). Similarly, in a study on Dorper rams, Budai (2014) reported the smallest scrotal circumference 30.1 cm in May, and the largest scrotal circumference 36.3 cm in October, and indicated the difference between these months to be statistically significant. Moreover, Oláh et al. (2013) reported the highest values for testicular circumference and scrotal width in winter in Merino rams. Kafi et al. (2004) have recorded the largest scrotal circumference (33.3 cm) in autumn and the smallest scrotal circumference (31.1 cm) in winter. On the other hand, other studies such as that of Jackson et al. (1990) suggest that season has no significant effect on scrotal circumference. Our findings showed that season affects scrotal circumference. These differences observed among the results of various similar studies probably occur due to several uncontrollable factors, including the absorption rate of nutrients.

Several factors including breed, body weight, age, management, weather, nutrition (feed quality and availability), method of semen collection, and degree of sexual stimulation affect the characteristics of ram semen (Folch 1984). In the current study, ejaculate volume of Karayaka rams significantly ($p<0.05$) increased during the BS, in agreement with the findings of Tajangookeh et al. (2007), Karagiannidis et al. (2000), Kafi et al. (2004) and Makawi et al. (2007). Surprisingly, our findings showed that season has no effect on fresh semen quality of Karayaka rams. In contrast to our expectations, the results of the present study showed that, in Karayaka rams, sperm concentration and motility measured in the BS (85.39%) were lower than the respective values measured in the NBS (90.43%). Mean sperm motility determined in the NBS was numerically higher than that determined in the BS, yet this difference was statistically insignificant. In contrast to the present study, Ali and Taha (2012) reported that sperm concentration of Awassi rams was highest during the natural breeding season (from late summer to early autumn), in comparison to the non-breeding season (winter). The increase detected in sperm concentration of Awassi rams was associated with a marked decrease of the photoperiod, which agrees with the findings of Zamiri and Khodaei (2005). Morever, Karagiannidis et al. (2000) reported that during the summer months, semen quality decreased in Chios rams. On the other hand, in a study carried out in crossbred Chios rams in the United Arab Emirates, Ibrahim (1997) found that semen quality did not decline during the hot months of summer. These findings suggest that semen quality of Karayaka rams is not affected by season in northern Turkey. The results of the present study showed that Karayaka rams had continuous and acceptable spermatogenic activity throughout the year. The differences among the results of the present study and previous research might be related to the length of the study period, as well as to age and breed differences of the rams used. These variations could also be attributed to management-related conditions and the laboratory method used to estimate sperm concentration.

Mandiki et al. (1998) recorded higher semen quality (lower percentage of abnormal sperm and higher percentage of live sperm) during the winter in both Suffolk and Ile-de-France rams. Karagiannidis et al. (2000) reported a higher abnormal sperm percentage during spring and summer, and indicated that winter is a transitional period (between the anestrous and breeding season) for the indigenous breeds of Greece. Furthermore, Aller et al. (2012) reported that seasonal changes significantly affected abnormal sperm percentage, that reached its highest level in winter and fall and its lowest in autumn. The low abnormal sperm percentage detected in these studies in autumn is in line with the results of the present study. On the other hand, in the present study the percentage of abnormal sperm was affected by seasonal changes only to a limited extent. Our findings showed that, excluding volume, individual motility, sperm concentration, and percentage of abnormal sperm remained stable throughout the study. Makawi et al. (2007) reported that the most significant ($p<0.05$) increase in seasonal changes occurred in semen volume, sperm concentration, mass motility, sperm individual motility and percentage of live sperm in Awassi rams in the period from late summer to early autumn; this finding is in agreement with that of Tajangookeh et al. (2007). Several factors such as age and breed of the animal, interspecies variations, number of specimens, sperm collection and evaluation methods, nutrition, management practice and environmental conditions may be responsible for the variations in the results of different studies.

One of the most striking findings obtained in the

present study was the fact that freezability of Karayaka ram sperm was significantly higher during the NBS, when compared to the BS, in contrast to the widely accepted view that freezability of ram semen is higher during the BS. Information available in literature reports regarding the freezability of ram sperm collected during the breeding and non-breeding seasons is contradictory. In contrast to the result, it has been reported that ram sperm freezability was higher during the breeding or non-breeding season (D'Alessandro and Martemucci, 2005; Makawi et al*.,* 2007). Olah et al. (2013) reported that freezability of ram sperm during the breeding season or outside may vary depending on the ram breeds.

In the present study, freezability of Karayaka ram semen was better in the non-breeding season. Endocrine status may also alter the resistance of sperm cells to freeze-thawing. It has been suggested that increased plasma testosterone level has a negative effect on the cryosurvival of Capra ibex spermatozoa (Coloma et al., 2010). Although testosterone levels were not measured in the present study, it is well known that, in rams, testosterone levels are much higher during the breeding season, in comparison to the non-breeding season (Ali and Taha 2012; Aller et al., 2012; Zamiri et al., 2010). Furthermore, seasonal alterations in seminal plasma composition may have also influenced sperm freezability *(*Rickard et al., 2014). In fact, in a study carried out in stallions, cryotolerance of sperm frozen in the breeding season was found to be lower than that of sperm frozen in the non-breeding season (Kumar et al., 2014).

CONCLUSION

The season in which semen is collected influences fresh semen quality and freezability of spermatozoa in Karayaka rams. The post-freezing motility of sperm was better during the non-breeding season. Overall, the most favourable fresh and post-thaw semen characteristics of spermatozoa, diluted with a skim milk-egg yolk extender, occur in spring, which corresponds to a sexually hypoactive season for this breed.

CONFLICT OF INTEREST STATEMENT

There is no conflict of interest.

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J HELLENIC VET MED SOC 2019, 70(3): 1661-1668
ΠΕΚΕ 2019, 70(3): 1661-1668

Research article
Ερευνητικό άρθρο

Isolation, genotyping and antimicrobial susceptibility of pathogenic *Escherichia coli* serotypes in ready to eat foods

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ABSTRACT. In this study, pathogenic *Escherichia coli* serotypes (*E. coli* O157:H7, O26, O111) and their molecular proximity and antimicrobial susceptibility were investigated in RTE foods. A total of 240 samples; consist of 105 stuffed mussel, 56 meatless cig kofte, 54 Russian salad, 25 cheese halva, were analyzed. The conventional culture and serotyping methods for determination of the organisms were performed and further confirmation by PCR was carried out. Confirmed *E. coli* O157 isolates were genotyped by the enterobacterial repetitive intergenic consensus (ERIC)-PCR. Antibacterial susceptibility testing of the isolates was performed by disc diffusion method. *E. coli* was detected in 7 (2.9 %) of 240 samples, including 3 (5.5%) Russian salad, 3 (2.8%) stuffed mussel, 1 (4 %) cheese halva. Two isolates from Russian salad, 1 from stuffed mussel and 1 from cheese halva were identified as *E. coli* O157 . In addition, stuffed mussel isolate was found to carry *stx*1 ve *hly*A genes whereas one Russian salad isolate carried the *stx*1 gene. *E. coli* isolates were found to be resistant to amoxycillin/clavulonic acid, gentamicin and ciprofloxacin, at the rate of 29%, 14% and 29 %, respectively. Only one (14 %) isolate from stuffed mussel was classified as multidrug resistant to three antimicrobials. Furthermore, the isolates, related to O157 and O157:H7, presented different ribotypes in this study. The results provide useful data for the development of public health policy concerning the potential presence of pathogenic antimicrobial resistant *E. coli* serotypes in RTE foods. Strict surveillance of RTE foods at retail points for emerging pathogens, their antimicrobial resistance patterns and the potential likelihood of cross-contamination is required.

Keywords: Antimicrobial susceptibility, cheese halva, ERIC-PCR, meatless cig kofte, Russian salad, EHEC, stuffed mussel.

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Date of initial submission: 13-11-2018
Date of revised submission: 19-05-2019
Date of acceptance: 15-06-2019

INTRODUCTION

In recent years, ready to eat (RTE) food consumption has increased because of rapid population growth and the modern lifestyle; longer working hours, increasing women's participation in the labour market and the change in cooking and eating habits (Tudoran et al., 2012; Oz et al., 2014). RTE foods do not generally require serious pretreatment process and are shelf-stable, delicious, inexpensive and easily accessible to consumers (Spencer, 2005; Jaroni et al., 2010). However, these types of foods present important microbiological risk since they have been implicated as vehicles of food borne microorganisms including *Escherichia coli* (Ateş et al., 2011; Kochakkhani et al, 2016).

E. coli, a member of *Enterobacteriaceae* family, is the main inhabitant of human and animal guts. They have been accepted as the indicator microorganisms of contamination with fecal and enteric pathogens (Montville et al., 2012). Although most *E. coli* strains are nonpathogenic, some are known to be responsible for serious human gastrointestinal diseases, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). Virulence factors such as shiga toxins (*stx*1 and *stx*2), enterohemolysin (*hly*A) and intimin (*eae*A) play an important role in the pathogenesis of these diseases (Bruyand et al., 2018). Three major surface antigens, O (somatic), H (flagellar) and K (capsule) antigens, are used to serologically to differentiate the *E. coli* isolates (Montville et al., 2012). Shiga toxin producing *E. coli* (STEC) strains are the non-O157 strains (O26, O45, O103, O104, O111, O121, O145) and contain O157: H7, the most important serotype (Paton and Paton, 1998; Durso et al., 2005). Although *E. coli* O157: H7 serogroup is responsible for most cases of STECs in humans, it is reported that non O157 STEC strains are increasingly causing diseases (Montville et al., 2012; Bruyand et al., 2018).

Antimicrobial resistance of *E. coli* has been traced world-wide in RTE foods (Musgrove et al., 2006; Zhao et al., 2012; Kochakkhani et al., 2016). Studies on antimicrobial resistant *E. coli* serotypes indicate that increasing antibiotic resistance has become a clinical and public health problem because of complicates treatment of infections caused by *E. coli* (Karlowsky et al, 2002).

Although there are studies focusing on the presence of *E. coli* and other pathogens in RTE foods (Bingol et al., 2008; Ateş et al., 2011; Cokal et al., 2012; Taban, 2012; Delikanli et al., 2014; Secim et al., 2017), to our knowledge, this study is the first report concerning the detection and genotyping of pathogenic *E. coli* serotypes in cheese halva, meatless cig kofte, Russian salad and stuffed mussel in Turkey. Studies on the pathogenic *E. coli* serotypes in RTE foods need to continue in order to complete food safety requires. For this reason, present study aimed to trace the current condition of toxin-producing *E.coli* contamination in RTE foods based on their prevalence, antimicrobial resistance and phylogenetic relationship.

MATERIALS AND METHODS

The samples of the study were purchased, weekly from January to March 2018, from supermarkets of Nigde and Kayseri cities of Central Anatolia /Turkey. A total of 240 RTE samples including 105 stuffed mussel, 56 meatless cig kofte, 54 Russian salad and 25 cheese halva from fishmongers, meatless cig kofte stores, grocery stores, restaurants and supermarkets (Table 1) were randomly collected. All samples were taken under aseptic conditions and transferred to the laboratory within 2 hours under the cold chain. Mix of stuffed mussels were removed from the shells before analysed.

Reference strains

E. coli O 157 NCTC 12900 (National Collection of Type Cultures 12900) reference strain was used as a positive control for isolation, identification and detection of virulence factors of *E. coli* O157: H7.

Table 1. RTE food samples

RTE food samples	N	Ingredients	Obtained from
Stuffed mussel	105	*Mytilus galloprovincialis* meat with mixed of spices, oil, salt and boiled rice in the cockleshells.	Fishmongers, street venders, cig kofte stores
Meatless cig kofte	56	Bulgur (pounded wheat) mixed with salt, tomato paste, onions, garlic and spices.	Cig kofte stores
Russian salad	54	Boiled peas, carrots and potatoes with cucumber pickles mixed in mayonnaise	Restaurants, supermarkets, grocery stores.
Cheese halva	25	Salt-free fresh cheese is melted and mixed with sugar, flour and semolina on the fire.	Restaurants, supermarkets, grocery stores.

Bacterial isolation

A 25 g of each sample was transferred aseptically to 225 mL Trypticase Soy Broth (mTSB, CM129 Oxoid, UK) containing novobiocin (20 g/ml, SR0181E' Oxoid, UK) and incubated at 37 °C for 18-24 h. Then, one loopful of enrichment cultures was inoculated onto Chromocult agar (CHROM agar O157, EE222, DRG International, Paris, France) and sorbitol MacConkey Agar (SMAC Agar-109202; Merck KGaA, Darmstadt, Germany) supplemented with 0.05 mg of cefixime and 2.5 mg of tellurite (CT Supplement 109202, Merck KGaA, Darmstadt, Germany). Plates were incubated at 37 °C for 24 h. After incubation, five suspected *E. coli* and *E. coli* O157 colonies were subcultured to blood agar (Oxoid, CM0271) for conducting confirmatory biochemical tests (indole, methyl red, Voges-Proskauer, citrate, urease, sorbitol fermentation and carbohydrate fermentation tests). Subsequently, they were further processed for serological identification (Chapman and Siddons, 1996; Dontorou et al., 2003).

Serological analysis

All suspected isolates were tested with *E. coli* O157, *E. coli* H7 antisera (221591, Difco), and *E. coli* O157 latex agglutination kit (DR0620M, Oxoid) according to the manufacturer's recommendations.

DNA exraction

Total genomic DNA extraction from the isolates was performed using a commercial DNA extraction kit (Axygen Bioscience, Union City, CA, USA) in accordance with the manufacturer's instructions.

Confirming *E. coli* isolates

The universal forward primer targeting the 3′ portion of *trp*B which, together with non-specific *trp*A reverse primer (trpA2.r, table 2), yields a 489 bp product from all *E. coli* strains was included in the reaction as an internal control as mentioned by Clermont et al. (2008).

PCR analysis for the detection of *fli*Ch7, *rfb*O111, *wzx-wzy*O26 and *rfb*O157 genes

The primer pairs for *fli*Ch7,*rbf*O157, *rfb*O111 and *wzx-wzy*O26 genes and the PCR assay conditions were performed in reference to Sarimehmetoglu et al. (2009), (Maurer et al. (1999), Paton and Paton (1998) and Durso et al. (2005), respectively.

Table 2. Primers and PCR amplification products used in this study

PCR Reaction	Target gene	Primer	Sequence (5′-to 3′)	Size of PCR ampl. (bp)	Reference
Internal control	*trpB*	trpBA.f	CGGCGATAAAGACATCTTCA	489	Clermont et al. (2008)
	trpA	trpA2.r	GCAACGCGGCCTGGCGGAAG		
H7	*fliCh7*	FLICH7-F	GCGCTGTCGAGTTCTATCGAGC	625	Sarimehmetoglu et al (2009)
		FLICH7-R	CAACGGTGACTTTATCGCCATTCC		
LPS O157	*rbf*O157	PF8	CGTGATGATGTTGAGTTG	420	Maurer et al. (1999)
		PR8	AGATTGGTTGGCATTACTG		
O26	*wzx-wzy* O26	wzx-wzyO26F	AAATTAGAAGCGCGTTCATC	596	Durso et al. (2005)
		wzx-wzy O26R	CCCAGCAAGCCAATTATGACT		
O111	*rfb*O111	O111F	TAGAGAAATTATCAAGTTAGTTCC	406	Paton and Paton (1998)
		O111R	ATAGTTATGAACATCTTGTTTAGC		
Shiga-like toxin 1	*stx1*	SLTI-F	TGTAACTGGAAAGGTGGAGTATACA	210	Fratamico et al. (2000)
		SLTI-R	GCTATTCTGAGTCAACGAAAAATAC		
Shiga-like toxin 2	*stx2*	SLTII-F	GTTTTTCTTCGGTATCCTATTCC	484	Fratamico et al. (2000)
		SLTII-R	GATGCATCTCTGGTCATTGTATTAC		
Intimin	*eaeA*	AE22	ATTACCATCCACACAGACGGT	397	Fratamico et al. (2000)
		AE20-2	ACAGCGTGGTTGGATCAACCT		
Enterohemolysin	*hlyA*	MFS1-F	ACGATGTGGTTTATTCTGGA	166	Fratamico et al. (2000)
		MFS1-R	CTTCACGTCACCATACATAT		

Detection of virulence genes (*stx*1, *stx*2, *eae*A and *hlyA*) by Multiplex PCR

Multiplex PCR (mPCR) targeting virulence genes of *E. coli* O157: H7, comprising *stx*1, *stx*2, *eae*A and *hlyA* (Table 2) was carried out in a study conducted by Fratamico et al. (2000).

Electrophoresis of all amplified products was carried out in 1.5% agarose gel containing 0.06% ethidium bromide for 50 minutes at 100 V (EC250-90, Thermo, Pittsburgh, Pa., USA) and visualized on a U.V transilluminator (Vilber Lourmat, Marne La Vallee, France).

ERIC-PCR

The ERIC-PCR was carried out on four isolates identified as EHEC. The total 50 µL of PCR mixture prepared including of 1xPCR buffer (Vivantis, Chino, CA, USA), 0.2 Mm dNTP mix (Vivantis), 4 mM $MgCl_2$(Vivantis), 5 U Taq polymerase (Vivantis), 25 pmol each primer and 1and 1µL target DNA. ERIC-PCR was performed under the following conditions: initial denaturation at 94 °C for 5 min, 94 °C for 1 min, 25 °C for 1 min, and 72 °C for 2 min (Techne TC-512, Keison Products, Chelmsford, Essex, UK).. The amplified product were subjected to electrophoresis at 100 V for 1h on 2 % agarose gel and was monitored by visual inspection under UV light for distinct DNA profiles (Houf et al., 2002). Banding patterns were photographed and analysed by scoring presence (1) or absence (0) of bands for prediction of similarity. Dendrogram was made by construction of a phylogenetic tree using the online software dendrogram construction utility, DendroUPGMA (http://genomes.urv.cat/UPGMA) (Garcia-Vallvé and Puigbo, 2002).

Antimicrobial susceptibility

Antimicrobial susceptibility of all *E. coli* isolates were tested using disk diffusion methods for Amoxicillin/Clavulanic acid (AMC) (30 µg), Ciprofloxacin (CIP) (5 µg), Gentamicin (GEN) (10 µg), Meropenem (MER) (10 µg) and Trimethoprim/ sulfamethoxazole (STX) (25 µg) according to EUCAST guidelines (European Committee on Antimicrobial Susceptibility Testing. Clinical breakpoint tables v. 8.1; http://www.eucast.org v.8.1, accessed: 12.08.2018).

Table 3. Results for pathogenic *E. coli* serotypes, their virulence genes from RTE foods

RTE food samples	N	n(%)	*rfb*O157, *fli*Ch7, *rfb*O111 and *wzx-wzy*O26 and virulence genes							
			*rfb*O157	*fli*Ch7	*rfb*O111	*wzx-wzy*O26	*stx*1	*stx*2	*hly*A	*eae*A
Stuffed mussel	105	3 (2.8%)	1(0.95%)	1(0.95%)	-	-	1(0.95%)		1 (0.95%)	-
Cig kofte	56	-	-	-	-	-	-	-	-	-
Russian salad	54	3 (5.5%)	2 (3.7%)	-	-	-	1 (1.85%)	-	-	-
Cheese halva	25	1 (4%)	1 (4%)	1 (4%)	-	-	-	-	-	-

n: Detected *E. coli* by *trp*A gene

Table 4. Antimicrobial susceptibility profiles of *E. coli* isolates

Antibiotics	Diameter of the inhibition zones *of E. coli* according to EUCAST, 2018 (mm)		Zone of inhibition (mm) in this study	
	S≥	R<	S/ (%)	R/(%)
Amoxicillin/clavulanic acid (AMC)	19	16	16±0.00 (71 %)	29 %
Ciprofloxacin (CIP)	26	24	26±0.05 (71 %)	29 %
Gentamicin (GEN)	17	14	18±0.00 (86 %)	14 %
Meropenem (MER)	22	16	28±0.00 (100 %)	-
Trimethoprim/ sulfamethoxazole (STX)	14	11	19±0.00 (100 %)	-

S:Susceptible, R: Resistant

RESULTS

Seven (2.9%) out of 240 RTE samples were found positive as a result of conventional culture methods and were confirmed by PCR. Furthermore, of the 7 *E. coli* isolates, 2 (3.7%) from Russian salad were identified as *E. coli* O157 based on PCR and serotyping and 1(1.85%) of them found to carry *stx*1 gene. *E. coli* O157:H7 was detected in 2 (0.83 %) out of 240 samples including 1 (0,95%) stuffed mussel and 1 (4%) cheese halva. One isolate from stuffed mussel were found to harbour the *stx*1 and *hly*A genes (As shown in Table 3). However, *E. coli* O111 and O26 were not detected in any sample.

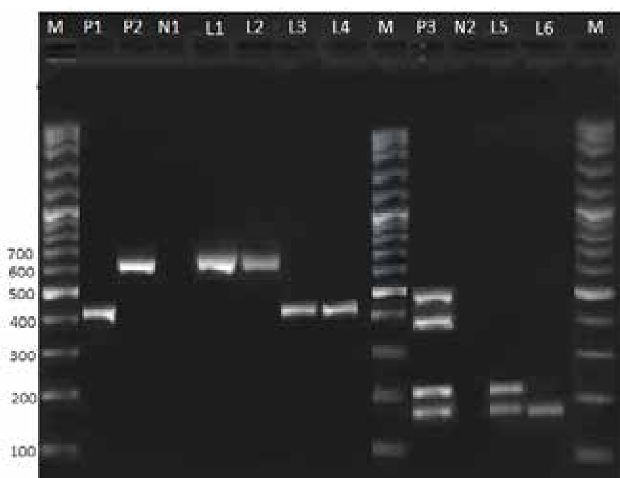

Figure 1. Agarose gel electrophoresis of PCR products of pathogenic *E. coli* isolates and their virulence genes. Lane M: molecular weight marker (Gene RulerTM 100 bp DNALadder Plus, Fermentas); lane P1: Positive control for *rfb*O157 (420 bp),P2 positive control for *fli*Ch7 gene (625 bp), P3: Positive control for toxin genes (for *stx*2 484 bp, for *eae*A 397 bp, for *stx*1 210 bp, for *hly*A 166 bp) N1-2: Negative Control (Sterile H_2O); Line 1-2: *E. coli* O157:H7 isolates; Line 3-4: *E. coli* O157 isolates; Line 5 *stx*1, *hly*A genes positive isolates. Line 6: *stx*1 gene positive isolate.

The results of antibiotic susceptibility test have been summarized in Table 4. All isolates of *E. coli* were highly sensitive to MER and STX. Resistance to AMC occured in 2 (29%) *E. coli* isolates from stuffed mussel, one of which was multidrug resistant to three antibiotics (AMC, CIP and GEN). Furthermore, *stx*1 gene carrying *E. coli* O157 isolate obtained from Russian salad was found to be resistant to CIP.

Figure 2 resumes the ERIC-PCR profiles of pathogenic *E. coli* serotypes. ERIC-PCR genotyping revealed 7-18 fragments resolved per isolate. All of 4 pathogenic *E. coli* isolates under analysis produced 3-7 amplicons ranging from 150 to 1500 bp. Phylogenetic tree (Fig. 2) showed that highly polymorphic DNA fragments among the 4 pathogenic *E. coli* isolates. The Jaccard similarity coefficient of the genotypes was ranging from 0.143 [(A (O157 serotype, carried *stx*1 gene, from Russian salad) and B (O157 serotype from Russian salad)] to 0.125 [B and C (O157 H7 serotype from stuffed mussel)].

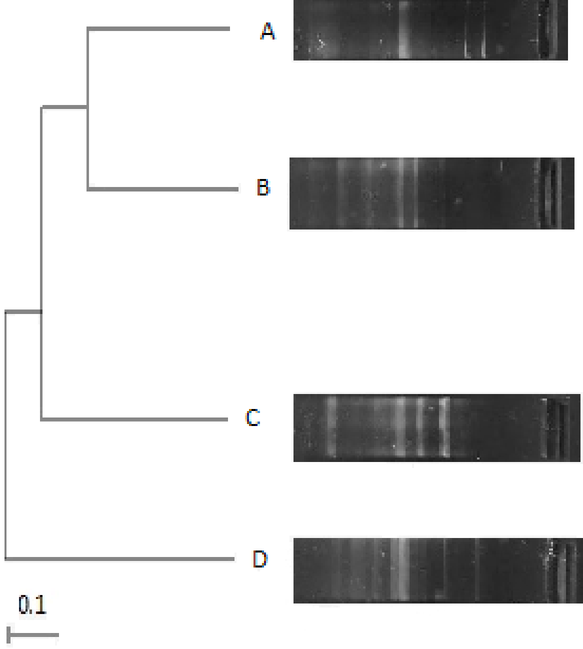

Figure 2. Phylogenetic tree of pathogenic *E. coli* isolates from Russian salad (A and B), stuffed mussel (C), cheese halva (D).

DISCUSSION

The RTE foods, frequently preferred by the consumers in recent years, are pre-cooked or prepared and packaged with a suitable material and often require minimal preparation (Spencer, 2005). Wide range of RTE foods, that can be bought from markets, street vendors, restaurants and stores, may contain a variety of microorganisms, while many of which are harmless, some are dangerous (Elobeid et al. 2014; Jaroni et al., 2008). In this study, pathogenic *E. coli* serotypes (*E. coli* O157:H7, O26, O111) was carried out from RTE foods in Central Anatolia region. The content of RTE foods examined in the study are raw and cooked materials, plants, cheese and shellfish with high protein, spices and sauces (Table 1).

Stuffed mussel is a highly consumed traditional shellfish in Turkey. Reported results demonstrated that 3 of 105 (2.85%) stuffed mussel were found to

be positive interms of *E. coli* and one of them was defined as O157 H7 (0.95%) containing *stx*1 and *hly*A genes. It was found that one *E. coli* strain was resistant to three antibiotics (AMC, CIP and CN); other *E. coli* strain was resistant to only AMC. Studies on the microbiological quality of stuffed mussels in Turkey demostrated that they may be contaminated with some foodborne pathogens including *E. coli* however no investigation is available on pathogenic *E. coli* serotypes in stuffed mussels samples (Bingol et al., 2008; Hampikyan et al., 2008; Ateş et al., 2011; Kocatepe et al., 2016). Similar to our results Surendraraj et al. (2010) in India also reported 8.3 % of shrimp samples were contamined with EHEC isolates which were positive for *eae*A, *stx* and *hly*A genes with low incidence of multiple antibiotic resistance. Prakasan et al. (2018) recently reported 33.33% of shellfish samples were contaminated with Shiga toxin-producing *E. coli*. *Mytilus galloprovincialis* is a filter feeder organism which collects pathogenic microorganisms and different harmfull residues including heavy metals and agricultural waste, as well as organic materials from the coastal and estuarine environments. In addition, high amino acid content, high pH (approximately 6.55) and high water activity (0.98) of mussels facilitate to colonization and transmission of *E. coli* and other pathogens (Sengor et al., 2004; Gourmelon et al. 2006). However preparation of the stuffed mussels includes cooking period that is high enough to kill most vegetative cells (Kisla ve Uzgun, 2008). According to Kisla ve Uzgun (2008), stuffed mussels were commonly exposed to unsuitable environmental conditions such as soil, dust, insects, flies etc and high ambient temperatures during retail sale for long times. We also collected stuffed mussel samples from fishmongers which was an outside sale under unsuitable environmental conditions. Furthermore, stuffed mussel mix (spices, oil, salt and boiled rice) is stuffed with hand in the cockleshells (Ates et al., 2011). *E. coli* is classified as faecal coliform and presence of this bacteria in the samples may indicate errors and omissions in handling, lack of sanitary practices by foodhandlers and possible cross-contaminations.

In this study, *E. coli* O157:H7 was isolated from only 1 of 25 (4 %) cheese halva samples. According to literature screening, there is no research related to *E. coli* O157:H7 in cheese halva in Turkey. Nevertheless Secim et al. (2017) investigated presence of *E. coli* in cheese halva samples and reported no contamination. The presence of *E.coli* has been investigated in cheese desserts in some studies; Cokal et al. (2012) and Secim et al (2017) reported that no *E. coli* contamination in Hosmerim desserts. The significance of *E. coli* O157:H7 contamination in milk and cheese samples has previously been reviewed (Zweifel et al., 2010; Lynch et al., 2012). As the cheese halva is a heat-treated dessert, the presence of *E. coli* O157:H7 in cheese halva might have originated from post heating contamination during packaging process or personel. Although *E. coli* is inactivated by some barrier factors like heat treatment in the processed foods, subsequent cross contamination could be of concern (Wahi et al., 2006).

In the present study, 3 Russian salad samples (5.5%) were found positive for *E. coli,* 2 of which (3.7 %) were determined as *E. coli* O157 with *stx*1 gene and CIP resistance was detected in one of them. Russian salad is a mayonnaise based salad. Althought mayonnaise is relatively resistant to microbial spoilage due to its low pH, it is known that *E. coli* and pathogenic *E. coli* serotypes have inducible acid resistance mechanisms. A study by Zhao and Doyle (1993) revealed that *E. coli* O157:H7 can survive at 5°C in mayonnaise for several weeks, in case of unsuitable manufacturing practices or any type of cross-contamination (contaminated vegetables in salad, dirty kitchen equipments, food handlers ect) of mayonnaise. In this study, Russian salad samples were bought from restaurants and grocery stores in which ready to eat foods were sold at retail without package. The contamination may be associated with unhygienic ingredients of salad, food handlers, utensils and contact surfaces.

In our study, no *E. coli* or pathogenic *E. coli* serotypes was detected in meatless cig kofte samples. Althought meatless cig köfte can serve as a vector for the transmission of some human pathogens (Taban, 2012; Delikanli et al. 2014), no reports are available about the examination of *E. coli* O157:H7 in meatless cig köfte samples. Several studies have demonstrated that garlic, spices and onion which are meatless cig kofte ingredients are able to inhibit pathogenic *E. coli* serotype growth, depending on the concentration, storage time and temperature (Koidis et al., 2000; Kim and Kim, 2007; Rounds et al., 2013).

In this study, one isolate found to carry *stx*1 and one isolate *hly*A gene. These results for detection rates of toxin genes were higher than the study conducted by Cho et al. (2010) which showed absence of the *stx* genes of street-vended foods in Korea. However, Gupta el al. (2012) reported from India the preva-

lence of *stx1* and *stx2* genes of RTE fish product were 5.55% and 7.4% respectively, higher than our results. The pathogenity of *E. coli* serotypes are related to their virulence factors, shiga toxins, enterohemolysin and intimin. Enterohemolysin (encoded by the *hly*A gene) causes the lysis of erythrocytes, which provide iron uptake in the intestinal environment (Dontorou 2003). Shiga toxins (*Stx* 1, 1c, 2, 2c, 2d, 2dact, 2e, 2f) are the primary virulence factor of pathogenic *E. coli s*erotypes which can be defined as the locus enterocyte effacement (LEE) of the adherence system (Obrig 2010). *Stx* lead to inflammatory and thrombogenic changes in the endothelial cells causing HUS and thrombotic microangiopathy (TMA), especially effects kidneys and other potential organs (Bruyand et al., 2018). *E. coli* O111 and O26 were not detected in any sample in our study. In contrast, the current results were reported by Hassanin et al. (2014), for RTE meat and chicken products, the rates of O111 and O26 serotypes were between 6.7-33.3%.

Results of this study demonstrated that MER and STX were the most effective agents against *E. coli* with susceptibility rate of 100%. Recent studies have also been describing STX and MER resistant *E. coli* isolates (Campos et al, 2013; Rasheed et al. 2014; Lima et al. 2017; Ye et al., 2018) in RTE foods. Of the 7 *E. coli* isolates examined, we found an overall prevalence of 42% (n=3) isolates showed resistance rate to AMC (29%), CIP (29%) and GEN (14%) (Table 4). AMC, CIP and GEN resistance has been reported in studies performed worldwide, concerning RTE foods (Rasheed et al. 2014; Kochakkhani et al. 2016; Baloch et al. 2017; Ye et al. 2018). Our results were lower than those found by Kochakkhani et al. (2016) (100% for AMC and CIP) and Baloch et al. (2017) (80.9% for AMC and 18% for GEN). Also, the low percentage of resistance to AMC, CIP and GEN was noted by Campos et al. (2013) (5% for CIP), Lima et al (2017) (none for CIP and GEN) and Rasheed et al. (2014) (8.6% for AMC). Moreover 1 (14.2%) multidrug resistant isolate also detected in the study (Table 4). This result is in accordance with those reported by Lima et al. (2017) and Baloch et al. (2017) as 13.3% and 17.6% resistance rate respectively. The existence of multidrug resistant strain could create serious threat to the patients because of transferring antimicrobial resistance genes to other pathogens and to humans through food.

The prevalence of pathogenic *E. coli* serotypes always should be carefully evaluated in RTE foods. To our knowledge, no study concerning the prevalence of pathogenic *E. coli* serotypes in RTE foods, including the detection of virulence genes, genotyping and antimicrobial susceptibility, has been conducted previously in Turkey. Results of the study would be useful for monitoring of pathogenic, antibiotic resistant *E. coli* serotypes and for providing information about possible role of RTE foods acting as a vehicle for this pathogen.

CONFLICT OF INTEREST

None declared by the authors.

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J HELLENIC VET MED SOC 2019, 70(3): 1669-1678
ΠΕΚΕ 2019, 70(3): 1669-1678

Research article
Ερευνητικό άρθρο

Biochemical characteristics of reduced-fat cheese made from high-heat treated goat's milk supplemented with *Penicillium candidum*

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Βιοχημικά χαρακτηριστικά τυριού με μειωμένη περιεκτικότητα σε λίπος που παρασκευάστηκε από κατσικίσιο γάλα υψηλής θερμικής επεξεργασίας με επιφανειακή ανάπτυξη του μύκητα Penicillium candidum

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ABSTRACT. Novel reduced-fat goat-cheese (R) was produced from high-pasteurized milk using *Penicillium candidum* as an adjunct. A full-fat goat-cheese (F) from pasteurized milk without mold addition was produced for comparison reasons. Physicochemical analyses of the two cheeses were performed through the 14-d period of ripening. The effect of *P.candidum* on proteolysis of goat-cheese caseins and the production of hydrophilic and hydrophobic peptides during cheese ripening were investigated. To our knowledge, similar results for reduced-fat, mold-ripened, goat-milk cheeses have not been previously reported before. R-cheese exhibited a higher organoleptic score and developed properties similar to Kopanisti, which is a Protected Designation of Origin Greek soft cheese with specific intense flavour manufactured from raw milk without the use of starters. Moreover, R-cheese had significantly higher moisture, protein in dry matter and water soluble nitrogen contents than F-cheese and was less adhesive. The high-pasteurization improved the texture and cheese yield, while the use of *P. candidum* as an adjunct improved the flavour, increased and accelerated proteolysis in R-cheese. According to the results, the technology for R-cheese employed in the present study can be easily adopted and could be used to produce a reduced-fat goat-cheese.

Keywords: Reduced-fat goat-cheese, physicochemical characteristics, proteolysis, texture, sensory attributes

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Date of initial submission: 11-07-2018
Date of revised submission: 17-10-2018
Date of acceptance: 11-11-2018

ΠΕΡΙΛΗΨΗ. Παρασκευάστηκε ένα νέο κατσικίσιο τυρί με μειωμένη λιποπεριεκτικότητα (R) από γάλα υψηλής θερμικής επεξεργασίας στο οποίο προστέθηκε ως συμπληρωματική καλλιέργεια ο μύκητας *Penicillium candidum*. Παράλληλα, για λόγους σύγκρισης, παρασκευάστηκε ένα κατσικίσιο τυρί από πλήρες παστεριωμένο γάλα (F) χωρίς την προσθήκη του μύκητα. Μελετήθηκαν οι φυσικοχημικές ιδιότητες των δύο τυριών κατά την διάρκεια της ωρίμανσής τους στους 20°C για 14 ημέρες. Επίσης, ερευνήθηκε η επίδραση του *P. candidum* στην πρωτεόλυση των καζεϊνών του κατσικίσιου τυριού και η παραγωγή υδρόφιλων και υδρόφοβων πεπτιδίων κατά την ωρίμανσή του. Στη βιβλιογραφία, δεν έχουν αναφερθεί μέχρι τώρα παρόμοια αποτελέσματα για κατσικίσιο τυρί με μειωμένη λιποπεριεκτικότητα που έχει παρασκευαστεί από γάλα υψηλής θερμικής επεξεργασίας και έχει ωριμάσει με επιταχυνόμενο ρυθμό από τον μύκητα *P. candidum*. Το τυρί R παρουσίασε υψηλότερη βαθμολογία στα οργανοληπτικά χαρακτηριστικά από το τυρί F και ανέπτυξε ιδιότητες παρόμοιες με το τυρί Κοπανιστή, το οποίο είναι ένα ελληνικό μαλακό τυρί Προστατευόμενης Ονομασίας Προέλευσης με ιδιαίτερη έντονη γεύση που παρασκευάζεται από νωπό γάλα χωρίς τη χρήση εκκινητών. Επιπλέον, το τυρί R είχε σημαντικά μεγαλύτερο ποσοστό υγρασίας, πρωτεΐνης υπολογισμένη σε ξηρή ουσία και υδατοδιαλυτού αζώτου από το τυρί F και λιγότερη συνάφεια. Η υψηλή παστερίωση βελτίωσε την απόδοση και την υφή του τυριού R, ενώ η χρήση του *P. candidum* ως πρόσθετη καλλιέργεια βελτίωσε τη γεύση, αύξησε και επιτάχυνε την πρωτεόλυσή του. Σύμφωνα με τα αποτελέσματα, η τεχνολογία που χρησιμοποιήθηκε για το τυρί R στην παρούσα μελέτη μπορεί να υιοθετηθεί εύκολα και να χρησιμοποιηθεί με επιτυχία στην παραγωγή κατσικίσιου τυριού με μειωμένη λιποπεριεκτικότητα.

Λέξεις κλειδιά: Κατσικίσιο τυρί με μειωμένη λιποπεριεκτικότητα, φυσικοχημικά χαρακτηριστικά, πρωτεόλυση, ρεολογικά χαρακτηριστικά , οργανοληπτικά χαρακτηριστικά.

INTRODUCTION

Greece is among the world's top ten producers of goat's milk and second within the European Union. According to FAO (2014) the goat population of Greece amounted to 4.255.000 for the year 2014. Many varieties of goat milk cheeses are produced around the world and especially in France. In Greece, goat's milk is mainly used to make cheese, but almost always mixed with sheep's milk, and only a small variety of home-made goat's cheese is produced. This is mainly due to a lack of data on the production methods and biochemical characteristics of goat cheeses, which precludes their production on an industrial scale. Goat's milk possesses unique nutritional and health properties (Jenness, 1980; Haenlein, 2004; Park *et al.*, 2007), such as high digestibility due to its fatty acid composition, the small size of the fat globules and its low levels of α_{s1} casein, as well as high hypoallergenicity due to structural differences in α-Lactalbumin (α-La) and β-Lactoglobulin (β-Lg), when compared with bovine milk. Goat's milk is richer than cow's milk in various elements, such as calcium, magnesium, phosphorus, potassium, and vitamins: vitamin A, niacin, thiamine and riboflavin (Kondyli *et al.*, 2007). Due to their desirable properties, goat's milk products have been increasingly developed over the past 15 years. Goat cheeses ripened with molds represent only a small proportion of the world cheese production. However, these cheeses are increasingly popular thanks to their distinct flavour and their soft texture, which is derived from the metabolism of lactose, fat, protein, citrate, and organic acids during ripening (Fox *et al.*, 1993).

Kopanisti cheese, according to Greek Codex Alimentarious (2014) is a soft traditional Greek cheese with Protected Designation of Origin (PDO) that is produced in the Cyclades islands from whole cow's, sheep's or goat's milk alone or mixed. The acidity of this cheese is produced by the flora of the raw milk without the use of lactic starter. It has a spreadable texture and a rich flavour similar to that of mold-ripened cheeses. Yeasts and molds are an important part of the essential microbial flora of Kopanisti cheese, with the species varying from production to production (Kaminarides and Anifantakis, 1989).

The last 30 years there is an increasing trend in favour of reduced-fat and reduced-energy products (Ritvanen *et al.*, 2005) to combat obesity, which contributes to serious health problems, such as arteriosclerosis, diabetes, cardiovascular damage, some forms cancer in the colon and breast (Hu *et al.*, 1997). Nevertheless, reduced-fat cheeses may be less acceptable to some consumers than full-fat cheeses due to defects in texture and flavour. Therefore, several attempts have been made to improve the flavour and texture of reduced-fat cheeses, for example by mod-

ification of conventional manufacturing process, the use of enzymes and additives (e.g., fat replacers), the introduction of adjunct cultures, or combinations of all these (Fenelon *et al.*, 2000; Mistry, 2001; Rodriguez, 1998).

In the present study, two soft goat-cheeses differing in fat content were produced, taking into consideration both the special dietary characteristics of goat's milk and consumers' preferences: a full-fat goat's cheese made from pasteurized milk using a mesophilic lactic starter and which is addressed to a wide consumer range. And a reduced-fat goat's cheese, made from high-pasteurized milk with the addition of *P. candidum*, which is aimed at customers who are on low-fat diets.

The first objective of this study was to encourage goat milk production in larger quantities for the production of high-quality, reduced-fat cheese. The second objective of this study was to determine whether *P. candidum* combined with high-pasteurization of milk could be used to improve appearance, flavour, yield, texture and to accelerate proteolysis and shorten the ripening of reduced-fat cheese.

MATERIALS AND METHODS

Milk, cultures and rennet

Raw goat's milk was obtained from the herd of the Agricultural University of Athens. The main constituents of 30 Kg whole raw milk, for the production of full-fat cheese (F), as determined by the Milkoscan apparatus (model 255 A/B, type 25700, Fosselectric, Denmark), were as follows (mean values ± the standard errors of the means): 3.01 ± 0.01% fat, 2.76 ± 0.03% total protein, 4.84 ±0.03% lactose, 0.76± 0.01% ash and 11.10 ± 0.03% dry matter. A further quantity of milk from the same source was subjected to separation for up to ~50% fat reduction. The mean values (±) the standard errors of the means of the standardized milk used for reduced-fat cheese (R) were: 1.51 ± 0.01% fat, 2.86 ± 0.03% total protein, 4.91 ± 0.03% lactose, 0.79 ± 0.01% ash and 9.68 ± 0.03% dry matter.

A 0.044% (w/w) freeze-dried lactic acid bacterial culture (DVS MO-10-Chr. Hansen Holding A/S, Hoershom, Denmark) of mixed mesophilic homofermentative species (*Lactococcus lactis subsp. lactis* and *Lactococcus lactis subsp. cremoris*) was used.

A spore suspension of the mold *P. candidum* (Chr. Hansen Swing FD PC A1) was made in sterile distilled water to an optical density of 0.4 at 525 nm (5.5 x 10^9 spores/ml). From this suspension was poured 0.2% to the surface of R-cheese drained curd.

Calf rennet powder HALA (0.3 gr per 100 l of milk; Chr. Hansen) was used in the cheese milk.

Cheese making and sampling

Two soft goat cheeses were produced at the Laboratory of Dairy Science and Technology on the basis of traditional Kopanisti cheese technology (Greek Codex Alimentarius, 2014), modified as shown in Figure 1. Full-fat cheese (F) was made as a control from full-fat milk pasteurized at 68°C for 10 min and using mesophilic lactic starter. Reduced-fat cheese (R) was made from 50% reduced-fat milk, pasteurized at 80°C for 10 min and using mesophilic lactic starter and spores of *P. candidum* as adjunct. Four replicates of each type of soft cheese were produced on four successive weeks. The cheeses from each treatment were weighed, sampled and analyzed. For the assessment of the physicochemical characteristics, cheeses were examined during ripening. Textural and sensory properties were examined in the ripened cheeses (14 days after manufacture).

Physicochemical analyses

The pH of the two cheeses was determined using a pH-meter (632 Metrohm, Herisau, Switzerland). Total solids were determined according to IDF (1982). Fat was analyzed according to the volumetric method of Gerber- Van Gulic (Schneider, 1954). Ash was determined by drying at 550°C to constant weight (IDF, 1964). Total nitrogen (TN), was determined by the Kjeldahl method according to IDF (1993). Water-soluble nitrogen (WSN) was obtained by homogenizing 25g cheese with 100 ml H_2O according to the method for WSN extraction, cited in Butikofer and Ruegg (1993) and determined by the Kjeldahl method (IDF, 1993). Chloride content was analyzed according to IDF (2006). Ca, Mg, K and Na contents were determined by Atomic Absorption Spectrometry (IDF, 2007). All analyses were performed in quadruplicate.

Electrophoretic analysis of cheese proteins (SDS-PAGE)

The electrophoresis of cheese proteins was performed using a Hoefer SE 600 Vertical Electrophoresis System according to the method of Kaminarides *et al.* (1995).

Analysis of waters extracts by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

Analysis of the water-soluble extracts of F- and R-cheese was carried out using the HPLC system of WATERS (WATERS, 34 Marple Street, Milford, MA 01757, USA) consisting of a pump capable of mixing four solvents (WATERS 600E), a photodiode array detector (WATERS 996), a helium degasser and an autosampler (WATERS 717). A Nucleosil 300-5-C18 column was used; the flow rate was 0.75 ml/min at room temperature. Solvent A was 0.1% trifluoroacetic acid (TFA, Serva Electrophoresis, Heidelberg, Germany) in ultra pure water (v/v) and solvent B was 0.1% TFA in 60% acetonitrile, (Lichrosolv grade, Merck, Darmstadt, Germany) (v/v).

The elution was as follows: 10 min, 100% A; 80 min 80% B, 101min, 100% A and 112 min, 100% B. Quantification was based on absorbance at 220 nm. 0.6 mL of WSN of each cheese sample (F- and R-cheese) was diluted with 0.6 mL solvent A. Diluted samples were kept for 10-15 min at room temperature. All samples were centrifuged at 8000 rcf for 5 min and filtered through 0.45μm membrane filters (Whatman, Rockwood, Germany) before injection; 100 μL of each sample were analyzed.

Textural evaluation

The textural properties of the cheese were measured with a Shimadzu testing instrument, (model AGS-500NG T, Shimadzu Corporation, Kyoto, Japan), as described by Kaminarides and Stachtiaris (2000).

Sensory evaluation

The cheese sensory characteristics were graded by a fourteen-member taste-panel of the Dairy Science and Technology Laboratory of the Agricultural University of Athens. Panel members, who were familiar with this type of cheese, evaluated each sample 14 days after its manufacture for appearance, texture and flavour on a 10-point scale, from one signifying the worst to ten signifying the best quality. More importance was given to flavour and to texture than appearance of the cheese as advised by the IDF Standard 99c (1997) by multiplying their scores by 5 and 4 respectively. The total score was obtained by adding the scores based on the three attributes. An excellent cheese would receive a total score of 100. The panel was asked to note any flavour defects.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using the statistical program Statgraphics Plus for Windows (v. 5.2, Manugistics, Inc., Rockville, MO, USA) to test the effect of different goat's cheese type on cheese parameters.

RESULTS AND DISCUSSION

Yield and physicochemical characteristics

Cheese yield, depends on the composition and quality of the milk used, the technology applied for cheese production and the conditions of cheese storage. Some reduction of cheese yield is inevitable during the production of cheese from milk with a low fat content, because the fat in the milk is one of the main components determining cheese yield (Romeih *et al.*, 2002). From the results (Table 1), it appears that the average yield of the full-fat cheese, one day after preparation, was 17.48%, compared with 17.91% for the reduced-fat cheese. Although the fat content of the R-cheese was half that of the F-cheese, the yield of the two cheeses did not differ significantly due mainly to the higher moisture content of the reduced-fat cheese (71.21%, day 1) compared to that of the full-fat cheese (65.01%, day 1) and also to the fact that in the case of R-cheese technology more proteins are transferred to the cheese (proteins in dry matter 49.06%) compared to that by F-cheese technology (proteins in dry matter 39.10%). The latter can be attributed to the incorporation of denatured serum proteins (mainly β-lactoglobulin) with κ- and α_{S2}-caseins in casein micelles via sulphur bridges (Walstra and Jenness, 1984) during high temperature pasteurization of the milk (80 °C/10 min) in R-cheese technology (Figure 1).

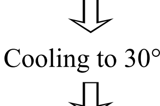
There were no significant differences ($P > 0.05$) in pH values between the two cheeses, although the pH values of both increased significantly ($P < 0.05$) from day 1 to day 14 during ripening (Table 1). This increase in pH may be attributed to the use of lactic acid by yeasts and molds, the numbers of which increased during Kopanisti cheese ripening according to Kaminarides and Anifantakis (1989).

The moisture content of the R-cheese was significantly higher ($P < 0.05$) than that of the F-cheese both on day 1 and day 14. The higher moisture content of ripe R-cheese (66.34%), compared with that of ripe F-cheese (55.99%), resulted from the use of high-pasteurization which is known to denature serum proteins (mainly α-La and β-Lg), resulting in enhanced water-retaining ability.

Full-fat cheese

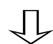
Receipt of raw full-fat goat's milk

Pasteurization at 68°C for 10 minutes



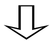
Cooling to 30°C

Addition of a mixture of mesophilic lactic acid bacteria (*Lactococcus lactis* subsp. *lactis* + *Lactococcus lactis* ssp. *cremoris*)



Biological ripening at 30°C for 2 hours

Addition of 15 g $CaCl_2$/100Kg of milk



Addition of 0.3 g rennet/100Kg of milk

Cutting the curd after 3h into slices 1cm

Left for 10 min.

Stirring very briefly

Transfer the curd into cloth bags

Press at 1 Kg / Kg curd at room temperature for 16h

Next day: chopping, grinding and salting (2%)

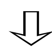
Ripening at 20°C for 14 days. During ripening milled the cheese mass 3 times

Reduced-fat cheese

Receipt of raw full-fat goat's milk

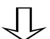
Standardization of milk fat to about half of the fat content of full-fat milk

High-pasteurization at 80°C for 10 minutes



Cooling to 30°C

Addition of a mixture of mesophilic lactic acid bacteria (*Lactococcus lactis* subsp. *lactis* + *Lactococcus lactis* ssp. *cremoris*)

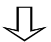


Biological ripening at 30°C for 2 hours

Addition of 15 g $CaCl_2$/100Kg of milk

Addition of 0.3 g rennet/100Kg of milk

Cutting the curd after 3h into slices 1cm



Left for 10 min.

Stirring very briefly

Transfer the curd into cloth bags

Press at 1 Kg / Kg curd at room temperature for 16h

Next day: chopping, grinding and salting (2%)

Spreading on the surface of the cheese curd spores of the mould *Penicillium candidum* ($5.5x10^9$spores/ml)

Ripening at 20°C for 14 days. During ripening milled the cheese mass 3 times

Figure 1. Flow chart for the production of soft goat-cheeses from full-fat, pasteurized milk and from reduced-fat, high-pasteurized milk supplemented with *Penicillium candidum*. Bold letters indicate the modifications between the two technologies

Moisture, expressed as % in non-fat ingredients (MNFS), did not differ significantly between the experimental cheeses on day 1 (Table 1), which proves that it was not affected by fat reduction. However, a statistical difference occurred after maturation (day 14) due to differences in moisture loss during ripening. R-cheese is characterized by fat ~10 %, moisture ~66% and MNFS~75%. According to the Greek Codex Alimentarious (2014), this cheese is classified as a soft cheese. The total solids of each type of cheese followed a trend that was the reverse of that relating to moisture.

Table 1. Yield and physicochemical characteristics of the two types of soft goat cheeses during their ripening. (Means of 4 trials ± standard error of mean)

Yield and physico-chemical characteristics	F		R	
	Day 1	Day 14	Day 1	Day 14
Cheese yield (%)	17.48^{a} ± 0.49	-	17.91^{a} ± 0.49	-
pH	4.09^{a} ± 0.11	4.73^{b} ± 0.13	4.15^{a} ± 0.11	4.63^{b} ± 0.13
Total solids (%)	34.99^{b} ± 1.21	44.01^{c} ± 1.33	28.43^{a} ± 1.21	33.67^{b} ± 1.33
Moisture (%)	65.01^{b} ± 1.20	55.99^{a} ± 1.31	71.21^{c} ± 1.20	66.34^{b} ± 1.31
MNFS (%)	77.48^{b} ± 1.96	71.59^{a} ± 1.96	78.66^{b} ± 1.96	74.75^{a} ± 1.96
Ash (%)	2.74^{a} ± 0.12	3.76^{b} ± 0.14	2.89^{a} ± 0.12	3.71^{b} ± 0.14
Fat (%)	16.66^{c} ± 0.49	19.47^{d} ± 0.57	8.81^{a} ± 0.49	10.02^{b} ± 0.57
Fat in dry matter (%)	46.75^{b} ± 0.62	46.65^{b} ± 0.72	31.03^{a} ± 0.62	30.96^{a} ± 0.72
Protein (%)	13.68^{a} ± 0.58	16.75^{b} ± 0.65	13.95^{a} ± 0.61	16.94^{b} ± 0.71
Protein in dry matter (%)	39.10^{a} ± 0.75	38.06^{a} ± 0.88	49.07^{b} ± 0.80	50.31^{b} ± 0.90
Water soluble nitrogen –WSN-(%)	0.105^{a} ± 0.012	0.196^{b} ± 0.015	0.105^{a} ± 0.014	0.289^{c} ± 0.013
Maturation index (WSN/TN%)	-	8.261^{a} ± 0.96	-	10.44^{b} ± 0.86
Salt (%)	2.14^{a} ± 0.16	2.89^{b} ± 0.15	2.10^{a} ± 0.16	2.69^{b} ± 0.15
Ca (mg/100g cheese)	-	97.76^{a} ± 2.72	-	98.77^{a} ± 2.72
Mg (mg/100g cheese)	-	11.49^{a} ± 0.50	-	12.75^{a} ± 0.50
Na (mg/100g cheese)	-	40.34^{a} ± 7.96	-	55.26^{a} ± 7.96
K (mg/100g cheese)	-	179.06^{a} ± 7.45	-	179.94^{a} ± 7.45

a,b,c,d: Means in the same row bearing a common superscript did not differ significantly (p>0.05).
F& R: as in Material and methods

The ripe R-cheese (14 days) had a fat content of 10.02%, compared with 19.47% in ripe F-cheese (14 days). Clearly, this statistically significant difference in fat between the two types of cheese was due to the 50% reduction of fat in the milk used for R- cheese production. According to the Commission Regulation (1924/2006) of the European Parliament and Council, R-cheese can be described as "reduced-fat cheese" as its fat content has been reduced by ~50% in relation to F-cheese.

A small, but statistically insignificant (*P > 0.05*) increase in protein content was found in R-cheese, which when expressed on a dry matter basis, was significantly higher in ripe R-cheese (50.31%) than in ripe F-cheese (38.06%) as shown in Table 1. This difference could be attributed to the denaturation of β-lactoglobulin during the high temperature pasteurization of milk for R-cheese production and its association with κ- and α_{S2-} casein in the milk through disulfide bond formation (Walstra and Jenness, 1984), thus coagulating with casein during cheese making.

There were no significant differences (*P > 0.05*) in ash and NaCl contents between the two types of cheese. Ash and NaCl contents were significantly higher at 14 days than at day 1 owing to moisture loss during ripening.

No significant difference *(P* > 0.05) was observed in the water soluble nitrogen (WSN) content of F- and R-cheese on day 1, but the levels of WSN increased during aging and differed significantly (*P < 0.01*) on day 14 (Table 1).

Water soluble nitrogen expressed as percentage of total nitrogen (WSN, % of TN), is used as a ripening index, and differed significantly *(P* > 0.05) between the two cheeses. Thus, R- cheese had a ripening index of 10.4%, which was significantly higher than that of F-cheese (8.3%), and was attributable to more intense proteolytic activity in R-cheese due to the presence of *P. candidum.* The ripening index is an indication of the extent of proteolysis and varies in cheeses from very limited (e.g. Mozzarella) to very extensive (Blue cheese, cheeses that mature with fungi) (Upadhyay *et al.*, 2004).

Electrophoretic profile analysis

Figure 2 demonstrates the electrophoretic patterns of proteins of the two types of cheese during ripening, together with that of pure isoelectric casein (ISO-CN) of caprine milk which was used as a standard (lane 5). The first strong bands correspond to β-caseins, and the other strong bands (following the β-caseins) correspond to α_s-caseins (Franco et al., 2003). In goat cheeses, β-caseins are predominant. On day 1, the first strong band of R-cheese (lane 2), appears to be diffusion (a tail), which probably corresponds to serum

protein denaturation by heat during high-pasteurization of milk and complexing of β-lactoglobulin with caseins (Walstra and Jenness, 1984; Considine *et al.*, 2007). The hydrolysis rate of α_s- and β-caseins differed significantly between the two cheeses. R-cheese (inoculated with *P. candidum*) had the higher hydrolysis rate of α_s-and β-caseins than those in F-cheese. The bands bellow a_s-caseins and above β-caseins correspond to peptides derived from their degradation by enzymes during cheese ripening (Franco *et al.*, 2003).

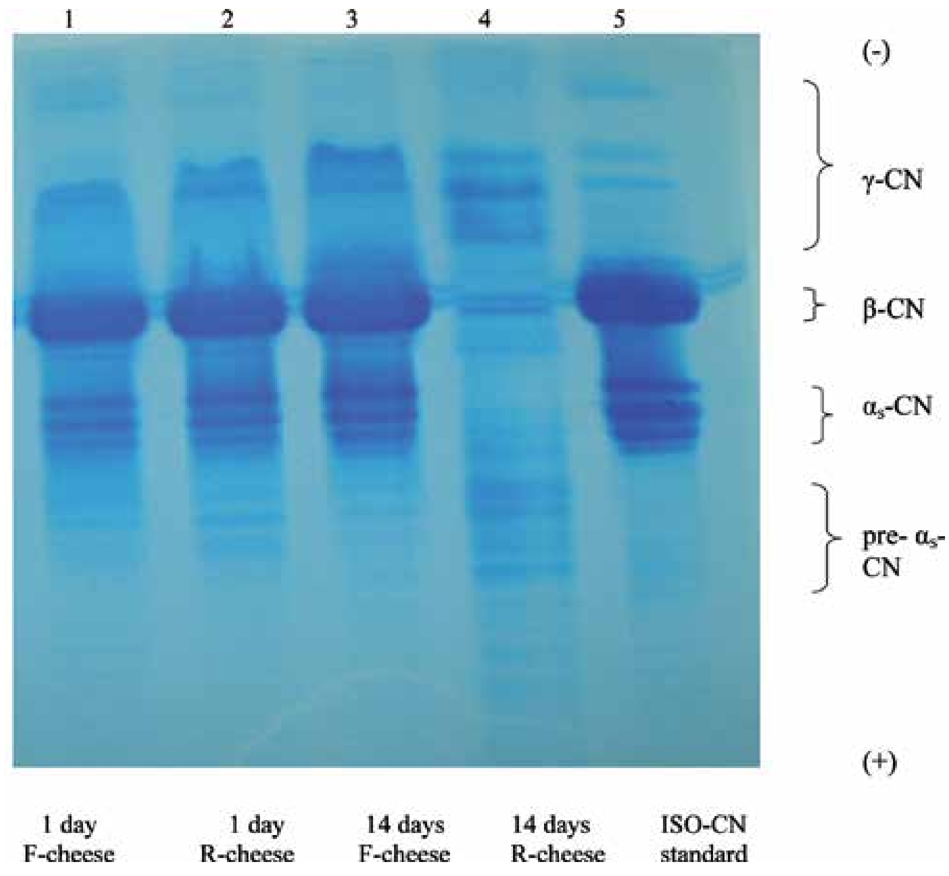


Figure 2. Polyacrylamide gel electrophoretic patterns of proteins of two goat cheeses during ripening. Lanes: 1 ; 2; 3; 4 and 5

RP-HPLC profiles of Water Soluble Extracts (WSN)

Figure 3 shows the peptide profiles of the water soluble fraction of experimental F-cheese and R-cheese on day 1 and after ripening for 14 days, obtained by means of RP-HPLC. Visual inspection of these chromatograms reveals that after 14 days of ripening new peaks appeared in both cheeses chromatograms, while the height of other peaks that existed on day 1 had changed. Michaelidou *et al.* (1998) reported that the first peaks eluted between 0 and 10 min in similar chromatographic analysis were free amino acids such as tyrosine and phenylalanine, the peaks eluted in the middle of the chromatogram corresponded to small and medium peptides mainly derived from the degradation of α_{s1}- and β-caseins, while the peaks eluted at the end of the chromatogram corresponded to α-La and β-Lg. Also, the peaks in the front region of RP-HPLC profiles (from 10-55 min) include hydrophilic peptides -HL-, while the peaks eluted between 55 and 80 min correspond mainly to the hydrophobic peptides -HB- (Nega and Moatsou, 2012). Differences were observed between the elution profiles of F- and R-cheese (Figure 3). It was evident that the cheese-making conditions strongly affected the major whey proteins β-Lg and α-La. On day 1, R-cheese contained less non-denatured (natural) whey proteins, apparently due to the high-heat treatment, β-Lg being more intensely affected (Sakkas *et al.*, 2014). Moreover, from day 1 to day 14 the area of α-La in R-cheese greatly decreased, indicating strong proteolytic activity of the mold. For quantitative analysis the chromatograms were divided into six parts according to the elution times given above, and the total area of the

peaks in each part was used for the assessment (Figure 4). The area of the part from 70-100 min was lower in R-cheese than in F-cheese due to denaturation. The area of peaks eluted between 0-55 min, which contains the hydrophilic peptides, was higher in R-cheese (with mold) than in F-cheese (no mold), indicating more intense proteolysis in the former. In contrast, the area of peaks eluted within 55-100 min, which contains the hydrophobic peptides and whey protein, was lower in the mold-ripened R-cheese than in F-cheese. For the assessment of proteolysis, the HB/HL peptide ratio of WSN profiles was calculated. After 14 days of ripening, HL peptides had increased in both cheeses, whereas the HB peptides decreased (Figures 3, 4). In particular, the ratio HB / HL of R-cheese decreased from 1.93 to 1.00 within 14 days. The respective values for F- cheese were 2.08 and 1.51. The decrease in the ratio HB/HL observed during ripening could be attributed to proteolytic activity, as reported for other cheese varieties (e.g. Gonzalez *et al.*, 1995; Katsiari *et al.*, 2000), again indicating higher proteolytic activity in R-cheese due to *P. candidum*.

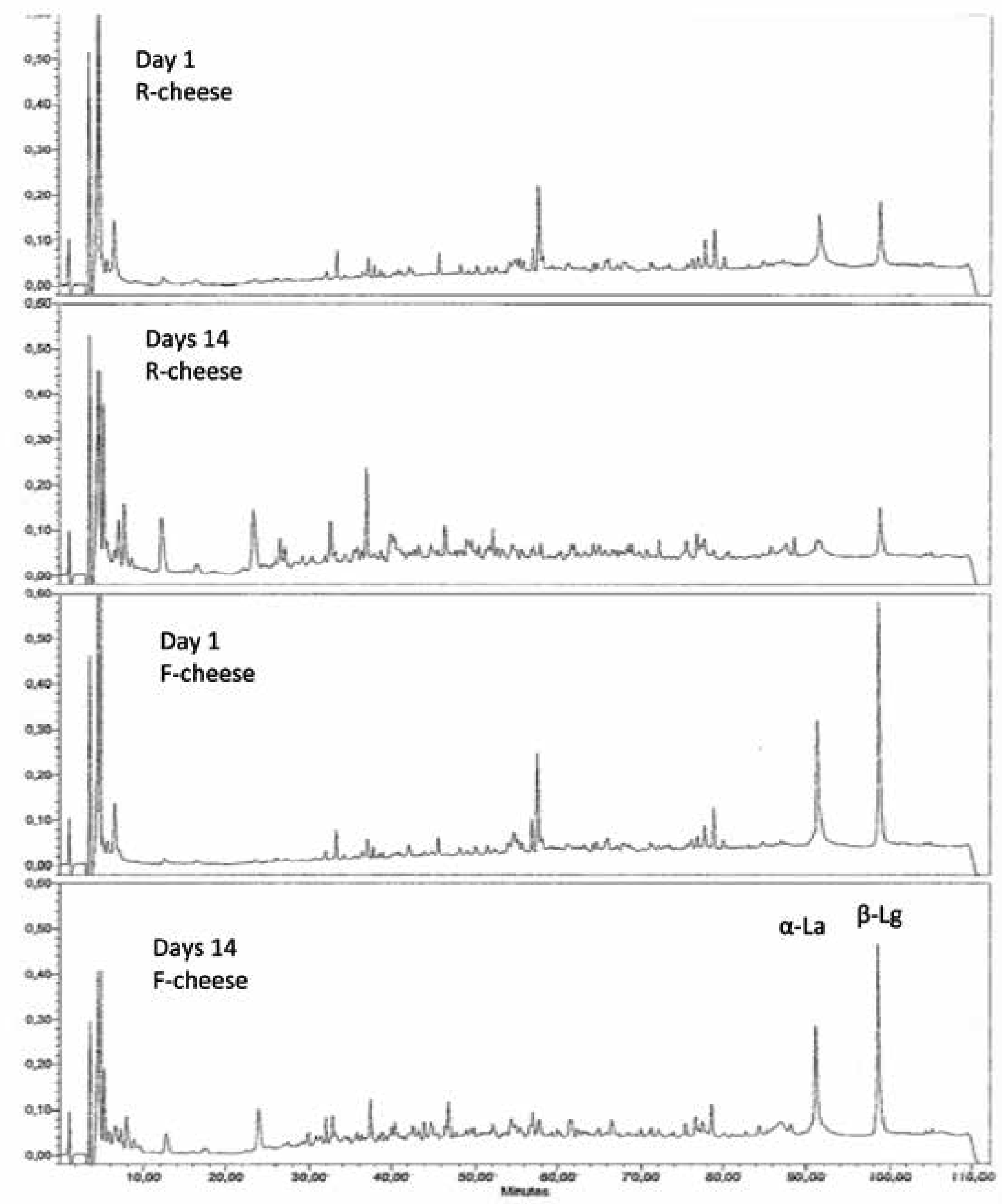


Figure 3. Reversed phase HPLC profiles of water-soluble extracts (WSN) of goat-milk cheeses during their ripening. F-cheese made from full-fat pasteurized milk without mold. R-cheese made from reduced-fat, high-pasteurized milk with the addition of *Penicillium candidum.*

Table 2. Textural and sensory properties of two types of soft goat cheeses after14 days' ripening. (Means of 4 trials ± standard error of mean)

Properties	Types of soft cheese from goat milk	
	F- cheese	R- cheese
Textural properties		
Hardness (N)	$2.58^a \pm 0.95$	$2.38^a \pm 1.24$
Cohesiveness (N.mm)	$0.37^a \pm 0.07$	$0.34^a \pm 0.07$
Adhesiveness (N.mm)	$7.89^b \pm 2.01$	$5.20^a \pm 1.47$
Sensory evaluation		
Taste and Flavour (0-50)	$39.9^a \pm 1.0$	$42.5^b \pm 0.7$
Texture (0-40)	$32.5^a \pm 0.6$	$33.3^a \pm 0.8$
Appearance (0-10)	$8.1^a \pm 0.3$	$8.9^b \pm 0.1$
Total (0-100)	$80.5^a \pm 1.2$	$84.7^a \pm 1.9$

a,b: Means in the same row bearing a common superscript did not differ significantly (p>0.05).
F& R: as in Material and methods

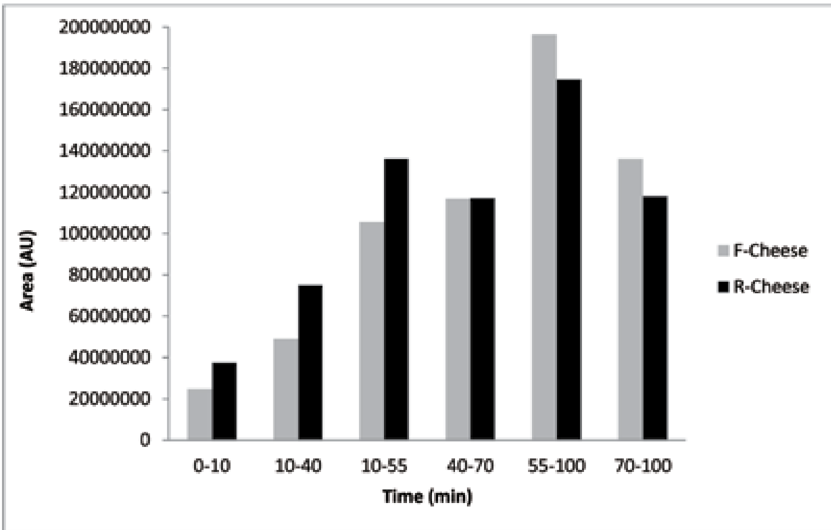


Figure 4. Area of isolated parts of the WSN profiles of goat-milk cheeses. F-cheese made from full-fat pasteurized milk without mold after 14 days of ripening. R-cheese made from reduced-fat, high-pasteurized milk with *Penicillium candidum* after 14 days of ripening.

Textural characteristics

No significant difference *(P > 0.05)* was observed in hardness and cohesiveness between F- and R-cheese. Although the reduction of fat content in R-cheese was half that of F-cheese, this did not increase its hardness, due to the high moisture content of the former (Table 1) and the incorporation of serum proteins into the curd, which loosened the casein matrix. Adhesiveness was found to differ significantly (*P < 0.05*) between F-cheese and R-cheese (Table 2). Similarly, a decrease in the adhesiveness of Cheddar cheese has been reported to result from a reduction in its fat content (Bryan *et al.*, 1995).

Sensory characteristics

Sensory evaluation results are given in Table 2. The mature cheeses obtained from both technologies were judged to be of good quality and characterized by soft texture, good spread ability and a rich flavor. No significant difference in texture was detected. These results were in accordance with the results of rheological tests, which showed that both cheeses had similar hardness and cohesiveness. R-cheese was whiter in color and received significantly (*P < 0.05*) higher scores for appearance, flavour and total sensory characteristics compared with F-cheese (Table 2) thanks to the mold, *P. candidum*, which improved the appearance and the flavour of R-cheese. No bitter taste was noticed by any member of the panel. In addition, R-cheese has less 'goaty' flavour due its lower level of milk fat.

CONCLUSION

The two new cheeses manufactured in this study possessed the special dietary nutritional properties of goat's milk. So, the use of goat's milk in the pro-

duction of these cheeses will help for an increased utilization of goat's milk. The technology applied to R-cheese resulted in a fast ripening period of two weeks and the cheese produced was of good quality, soft texture, rich flavour and good spread ability. So, R-cheese, which was produced from goat's milk with 10.02% fat content, had a whiter color and higher scores for appearance flavour and total sensory characteristics compared to F-cheese, which resulted from the inclusion of *P. candidum* in its manufacture. This mold in the R-cheese increased proteolytic activity during its ripening.

ACKNOWLEDGMENTS

The author thanks Theodore Paschos for his assistance in cheese-making.

CONFLICT OF INTEREST STATEMENT

We have no conflict of interest to declare.

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J HELLENIC VET MED SOC 2019, 70(3): 1679-1686
ΠΕΚΕ 2019, 70(3): 1679-1686

Research article
Ερευνητικό άρθρο

Risk assessment of mercury and methyl mercury intake via sardine and swordfish consumption in Algeria

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ABSTRACT. Total mercury (Hg) and methylmercury (MeHg) concentrations in the flesh of sardine *(Sardina pilchardus)* and swordfish *(Xiphias gladius)* fished in three Algerian coasts were determined by a direct mercury analyzer (DMA). We also assessed the risk to which the consumer was exposed to by calculating the estimated daily intakes (EDIs), target hazard quotient (THQ) and hazard index (HI). The average concentrations of Hg and MeHg in the flesh of sardine were similar (0.04 mg/kg wet weight) and in swordfish were 0.61 mg/kg wet weight; 0.57 mg/kg wet weight, respectively. These concentrations have not surpassed the thresholds set by the Algerian and European regulations. The estimated daily intakes for Hg and MeHg were similar in sardine (0.0064 µg/kg/day) and were 0.098 µg/kg/day and 0.092 µg/kg/day for Hg and MeHg, respectively, in swordfish. These values did not exceed the provisional tolerable weekly intake (PTWI) established by the European Food Safety Authority (EFSA) and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The target hazard quotient (THQ) and the hazard index (HI) calculated were < 1. Consequently, consumption of these fishes does not pose any risk for the adult groups of the Algerian population regarding mercury, and methylmercury studied.

Keywords: Mercury, Methylmercury, Sardine, Swordfish, Risk assessment.

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Date of initial submission: 05-12-2018
Date of revised submission: 23-06-2019
Date of acceptance: 25-06-2019

INTRODUCTION

Seafood is a source of energy and protein with high biological value, and contributes to the intake of essential nutrients, such as iodine, selenium, calcium, and vitamins A and D, with well-established health benefits. Seafood also provides n-3 (also called omega-3) long-chain polyunsaturated fatty acids (LC-PUFA) and is a component of dietary patterns associated with good health. Most European Food-Based Dietary Guidelines recommend a minimum of two servings of fish per week for older children, adolescents, and adults to ensure the provision of key nutrients (EFSA, 2014). However, there has been heightened concern about the presence of toxic metals such as mercury (Ricketts et al., 2016), which is released in the environment from both natural sources and human activities. It exists mainly in different forms of elemental mercury (HgO), inorganic mercury (Hg^{+}, Hg^{2+}) and organic mercury ($MeHg^{+}$, $EtHg^{+}$,$PhHg^{+}$, etc.) (Zhu et al., 2017). Mercury, an element also known as quicksilver, causes different toxic effects on the nervous, digestive and immune systems, as well as the lungs, kidneys, skin, and eyes. Exposure to it can result in severe illness and death (WHO, 2018). Consumption of fish is the main path for human exposure to mercury especially for coastal populations (Ricketts et al., 2016). Inorganic mercury is converted into the organic form (methylmercury) through methylation and the enzymatic process performed by bacteria and other aquatic microorganisms (Manavia et al., 2018). It is the predominant chemical form since the majority of total mercury that accumulates in the muscle tissue of fish is in this form methylmercury (95%-99%) (Carroll and Warwick, 2017) and has the ability to biomagnify through the aquatic food chain (Henry et al., 2017). It is the most toxic organic form of mercury which is considered by the International Agency for Research on Cancer (IARC) to be possibly carcinogenic to humans (Group 2B) (Torres-Escribano et al., 2010; Ricketts et al., 2016). Methylmercury is at the origin of neurologic damage such as mental retardation, seizures, vision and hearing loss, delayed development, language disorders, and memory loss or renal damage and has a long biological half-life. It can also cross the blood-brain and placental barriers (Torres-Escribano et al., 2010; Kral et al., 2017; Guérin et al., 2018). The first and most serious case illustrating the potential hazard of chemical contamination of food was encountered in Japan in the 1950s following industrial releases of mercury salts in a closed bay (Minamata Bay) producing fish for the consumer after concentration through the food chain and methylation, a mysterious neurological disease that reached alarming epidemic proportions (more than two thousand cases of poisoning and almost a hundred deaths were observed between 1953 and 1960, known as "Minamata Disease" (Roger and Guéry, 1991). Since then, the competent authorities have become aware of the hazard that these substances may represent and put in place standards to protect the health of consumers (JECFA, 1972). For this reason, it is necessary to monitor mercury and methylmercury levels in fishes and assess the risk they pose to the consumer. This study aimed to determine the levels of total mercury and methylmercury in the flesh of sardine *(Sardina pilchardus)* and swordfish *(Xiphias gladius)* collected in three Algerian coasts (Béjaia, Algiers, and Oran) and also assessed the risk related to consumption of these fishes for adult consumer.

MATERIALS AND METHODS

Sampling

A total of 87 samples of sardine (*Sardina pilchardus*) (n = 43) and swordfish (*Xiphias gladius*) (n = 44) were collected from April to September 2017 from three fishing major ports of Algeria, 15 samples of sardine and 20 samples of swordfish were collected from the coast of Algiers (North centre, NC), 12 and 13 from the coast of Bejaia (North East, NE), 15 and 11 from coast of Oran (North West, NW). The sampling procedure was carried out according to the EU (2016/582) regulation. The number of elemental samples that make up each aliquot varies according to the weight of the lot: elemental samples of 100 g in a number of 3, 5 and 10 were used if the weight of the lot was < 50 kg, 50 kg < weight < 500 kg or > 500 kg, respectively. The samples were placed immediately in blank polyethylene bags and transported to the laboratory in icebox for preparation.

Sample preparation

The preparation of the samples for the analysis was carried out, according to the requirements of the European standard EN 13804 (2013), in the laboratory of the National Center for Toxicology (CNT) in Algiers for the samples of the center (Algiers) and east (Bejaia) and at Federal Laboratory for the Safety of Food Chain Gentbrugge (FLSFC-G) of the Federal Agency for the Safety of the Food Chain (FASFC) in Gentbrugge (Belgium) for those of the west (Oran). The test samples were quickly prepared after the arrival of the fishes in the laboratory. The fishes were rinsed with potable wa-

ter before cutting to prevent leaching of the cut surfaces, then rinsed with distilled water. In the sardine samples, all inedible parts are removed. Swordfish samples were rinsed and peeled. Only the flesh was used for subsequent tests. From each sample, 100 g of matrix obtained was homogenized using a grinder (Retsch Grindomix 200), identified and placed in small closed black plastic bags and stored at -18°C.

Analysis of total mercury

The analysis was performed using a direct mercury analyzer (AMA-254), without any prior chemical treatment or digestion. A total of 50 to 100 mg of homogenate flesh was directly weighted in nickel boats. The nickel boats were rinsed with distilled water and cleaned by a thermal program by the apparatus to avoid contamination. Before the commencement of the analysis, a list was prepared in advance on the computer that was directly linked to the device where the date, the number of the samples, the weight and the position of nickel boats on the carousel are recorded. The nickel sample boats were automatically inserted into the combustion/ catalyst tube by the autoloader. The samples were firstly dried and then thermally degraded at 750°C. To determine the amount of mercury that was caught on the amalgamator, the amalgamator was briefly heated up to 900°C where by the mercury was released in the form of a cloud. The mercury cloud was transferred by the oxygen flow to the measurement cells. The amount of mercury was measured in each cell at 254 nm. Once finished, the detector was linked to a computer that gives the concentrations in ppm. The duration of the analysis for each sample was about 10min. The limit of detection and the limit of quantification of Hg and MeHg were 0.005 and 0.010mg/kg w.w.

Quality control of the analysis

Reference materials were used to control the quality of the analysis; canned fish (Fapas) with an internal reference number (12130869) and the tissue of lyophilized mussels ERM-CE278k with a known concentrations of Hg (0.359; 0.071mg/kg w.w, respectively). The results were in good agreement with the certified values located in this interval (0.404-0.674 mg/kg w.w; 0.053-0.089 mg/kg w.w) for both reference materials, respectively.

Methylmercury analysis

Analysis of MeHg was carried out according to the European document TC 275 WI0275321 (2017).

Extraction of the organic phase of mercury

Homogenate samples of 0.7 g to 0.8 g were weighed accurately (or 0.2 g in the case of lyophilised reference material) in a 50 ml centrifuge tube. 10 ml of hydrobromic acid was added and shaken manually for at least 2 min. Then, 20 ml of toluene was added and shaken vigorously for at least 20 min using an agitator (Stuart), centrifuged using a centrifuge (SIGMA^R) for 10 min at 2300 g according to the TC 275 WI0275321 (2017). 15 ml of the organic supernatant was transferred into a 50 ml centrifuge tube containing already 6 ml of L-cysteine solution. 15 ml of toluene was added to the initial centrifuge tube (containing the hydrobromic acid phase) and repeated the second extraction with the organic phase. After centrifugation, the remaining upper organic phase was transferred into the 50 ml centrifuge tube with the L-cysteine solution. It was then shaken vigorously using an agitator (Stuart) for at least 20 min and centrifuged in a centrifuge (SIGMA^R) for 10 min at 2300 g. An aliquot of 2 ml to 3 ml from the lower phase with the L-cysteine (and the extracted organic mercury) was taken. Ensured that the sample to be analysed is toluene free. Test samples were analysed as soon as possible to minimize instability issues.

Determination of concentration

The analysis was performed using direct mercury analyzer (AMA-254), where 500 µl to 200 µl of extract was put in a nickel boat cleaned by the apparatus and introduced in the DMA. Drying time, decomposition and waiting time were 350 s, 150s, and 55s respectively for samples and were150s, 150s and 55s respectively for reference materials, cleaning, and blanks. The results were given after a few minutes in µg/l Hg in the extract. The MeHg concentration was calculated using the formula:

$$\text{MeHg (mg/kg, expressed as Hg)} = \mathbf{C \times 6 \times D \div 1000 \times m}$$

where *6* is the volume of L-cysteine solution (6 ml); C is the concentration in the extract expressed in µg/l Hg; D is the dilution factor if needed; m is the mass of the test portion, in g.

Results of MeHg expressed in mg/kg.

The limits of detection and the limit of quantification of MeHg were 0.010 and 0.020mg/k w.w.

Quality control

To ensure the trueness of the method a (certified) reference material (RM) with a known content of

MeHg was used. Fish lyophilized TORT-2 with internal reference number (EU-RL-HM-15/IMP-115) and a known concentration (0.152 mg/kg w.w), the results were in agreement with the certified values located in this interval (0.0914-0.2126 mg/kg w.w).

Statistical analysis

Microsoft Excel ® (2007) software was used for calculating averages, standard deviation, minimum and maximum values. Statistical analyses were carried out using software R version (3.0.2). The Chapiro-Wallik normality test was used. The nonparametric Mann-Whitney test was used to compare the differences in the metal content studied in the two species (sardine and swordfish) (significant difference at a probability threshold of less than 5%). The Kruskal-Wallis test was conducted to compare the difference in the Hg and MeHg content of both species in the study areas.

Risk assessment for sardine and swordfish consumption

The risk assessment was performed using estimated dietary intakes (EDI/EWI), target hazard quotient (THQ) and hazard index (HI).

Determination of the estimated daily intake (EDI) for Hg and MeHg

The average concentrations of the metals analyzed were used to determinate the estimated daily intake (EDI; µg/kg/day) for an Algerian adult weighting an average of 60 kg and consuming 9.7 g per day (MFRR, 2018). The EDI was calculated using the following equation (Ju et al., 2017):

$$\mathbf{EDI = C \times DC \div BW}$$

Where C: the mean concentration of heavy metals in fish flesh (µg/g), DC: the daily fish consumption (g/day), BW: the mean body weight of population (kg).

Determination of the target hazard quotient (THQ)

The target hazard quotient (THQ) is a complex parameter introduced by the United States Environmental Protection Agency and is commonly used to assess the potential of non-carcinogenic risks associated with long-term exposure to contaminants, such as heavy metals from foods such as fish and water. THQ represents the ratio of chronic daily intake of metals studied (EDI) in mg/kg/day to the oral reference dose (RfD) also expressed in mg/kg/day. In addition, THQ parameter does not estimate the risk; it only indicates a level of risk associated with exposure to pollutants; if the value of THQ is < 1, it means that there are no adverse effects for the exposed population; when THQ > 1, there is a potential risk related to the metal studied in the exposed population (Al-Mahaqeri and Ahmad, 2015). The THQ can be calculated using the formula (Orosun et al., 2016):

$$\mathbf{THQ = (EFr \times EDtot \times FIR \times C) \div (RfDo \times BW \times ATn) \times (10\text{-}3)}$$

where EFris is the exposure frequency (365 days/year), EDtot the exposure duration (70 years, average lifetime), FIR the food-intake rate (g/day), C the mean of Hg and MeHg concentrations in sardine and swordfish muscular tissue (mg kg^{-1}), RfDo the oral reference dose of Hg and MeHg fixed by US EPA are 3×10^{-4} and 1×10^{-4} mg/kg/day, respectively (USEPA, 2017). BW is the average body weight (60 kg of body weight refers to adult people) and ATn the period of average exposure for non-carcinogens (365 days/year × number of exposure years, 70 years).

Determination of the hazard index (HI)

This is the sum of the hazard quotients for substances that affect the same organ or target organ systems. Ideally, hazard quotients should be combined for pollutants that cause adverse effects through the same toxic mechanism (USEPA, 2017). As with the hazard quotient, overall exposures below 1 calculated using hazard quotients are unlikely to result in any chronic systemic risk adverse health effects during a lifetime of exposure and would normally be considered as acceptable. The hazard index (HI) from THQs is expressed as the sum of the target hazard quotients (Núñez et al., 2018):

$$\mathbf{HI = THQ\ (Hg) + THQ\ (MeHg)}$$

RESULTS

Concentrations of Hg and MeHg

Concentrations of Hg and MeHg in the flesh of sardine (*Sardina pilchardus*) and swordfish (*Xiphias gladius*)

The results in (Table 1) showed that the concentrations of Hg and MeHg were higher in swordfish (0.61 ± 0.47; 0.57 ± 0.45 mg/kg w.w, respectively) than in the sardine (0.04 ± 0.03; 0.04 ± 0.028mg/kg w.w, respectively).

The nonparametric Mann-Whitney test showed a significant difference for both Hg and MeHg concen-

trations between the two species (sardine p-value = $9.99 \times 10^{-16} < 0.05$; swordfish p-value = $7.536 \times 10^{-10} < 0.05$).

Hg and MeHg concentrations in the three study areas for both species

The results in (Table2) showed that the concentrations of Hg and MeHg were higher in the swordfish flesh than in sardine in all study areas. The highest concentrations of Hg and MeHg were found in the swordfish of NC (0.77 ± 0.41 mg/kg w.w; 0.64±0.38 mg/kg w.w, respectively). The Kruskal-Wallis test showed no difference between the three study areas for MeHg concentrations, while a difference was recorded for the Hg concentrations between the NW area and the others.

In sardine, the statistical test showed a difference between NE and other areas for Hg and between NC and NE for MeHg.

The lower case letters showed the presence of difference or not according to the Kruskal-Wallis test.

The average concentrations of Hg and MeHg obtained in the flesh of sardine and swordfish in the three study areas were compared with the national and European regulatory thresholds (JORDPN°25/2011; EU N° 1881/2006). The results showed that the average concentrations of this metal and its organic form are lower than the set thresholds (0.5 mg/kg w.w in sardine and 1 mg/kg w.w in swordfish).The difference in threshold between these two species is due to the different concentrations of metals in fish flesh. Swordfish is a predatory fish found at the top of the marine food chain, which allows it to accumulate more mercury, particularly the organic form methylmercury than the sardine.

Risk assessment

Estimated dietary Intake (µg/kg/ body weight/day/week) to Hg and MeHg in sardine and swordfish

The estimated weekly intakes (EWI) of Hg and MeHg due to swordfish consumption (0.7; 0.64µg/kg/bw/week, respectively) were higher than the EWI's recorded for consumption of sardine (0.5/kg bw/week) for both Hg and MeHg. These values were lower than the provisional tolerable weekly intake (PTWI) established by the European Food Safety Authority (EFSA) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (Table 3).

Table 1. Concentrations of Hg and MeHg in the flesh of sardine *(Sardina pilchardus)* and swordfish *(Xiphias gladius)* (mg/kg w.w). n: number of samples; SE: standard deviation; Min: Minimum; Max: Maximum, ND: No detected concentration

	Sardine*(Sardina pilchardus)* (n= 43)		**Swordfish *(Xiphias gladius)* (n= 44)**	
	Mean ± SD	**Min.-Max. Value**	**Mean ± SD**	**Min.-Max. Value**
Hg	0.04±0.03	0.01-0.12	0.61±0.47	0.16-2.18
MeHg	0.04±0.02	ND-0.07	0.57±0.45	0.12-1.92

Table 2. Variations in concentrations of Hg and MeHg in sardine and swordfish flesh depending on the study areas

Species	**Study areas**	**N**	**Mean±SE (Max-Min)**	
			Hg	**MeHg**
Sardine (*Sardina pilchardus)*	**Algiers**	15	0.02^{d}±0.01(0.01-0.06)	0.02^{c}±- (ND-0.04)
	Bejaia	13	0.08^{c}±0.01(0.07-0.12)	0.06^{b} ± 0.006 (0.04-0.07)
	Oran	15	0.02^{d}±0.04(0.01-0.03)	ND
Swordfish *(Xiphias gladius)*	**Algiers**	20	0.77^{a}±0.41(0.25-1.25)	0.64^{a}±0.38 (0.12-1.92)
	Bejaia	13	0.69^{a}±0.59 (0.16-2.18)	0.59^{a}±0.52 (ND-0.01)
	Oran	11	0.23^{b}±0.02 (0.19-0.27	0.20^{a} ±0.02 (0.13-0.23)

Table 3. Estimated Dietary Intake (µg/kg/ body weight/ day/week) for the intake of Hg and MeHg in sardine and swordfish.

Heavy Metals		**EDI**	**EWI**	**Established PTWI by EFSA**	**Established PTWI by JECFA**
Hg	Sardine	0.0064	0.05	-	**5 (JECFA, 2010)**
	Swordfish	0.098	0.7		
MeHg	Sardine	0.0064	0.05	1.3 **(EFSA, 2012)**	1.6 **(JECFA, 2010)**
	Swordfish	0.092	0.64		

Table 4. Estimated Dietary Intake (µg/kg/ body weight/day/week) for the intake of Hg and MeHg in sardine and swordfish by region of study.

Heavy metals		Species	EDI	EWI	Established PTWI by EFSA	Established PTWI by JECFA
Hg	Algiers	Sardine	0.0032	0.0224	-	5 **(JECFA, 2010)**
		Swordfish	0.124	0.90		
	Bejaia	Sardine	0.013	0.91		
		Swordfish	0.111	0.78		
	Oran	Sardine	0.0032	0.0224		
		Swordfish	0.037	0.26		
MeHg	Algiers	Sardine	0.0032	0.0224	1.3 **(EFSA, 2012)**	1.6 **(JECFA, 2010)**
		Swordfish	0.103	0.721		
	Bejaia	Sardine	0.010	0.07		
		Swordfish	0.095	0.67		
	Oran	Sardine	-	-		
		Swordfish	0.032	0.224		

Estimated dietary Intake (µg/kg/ body weight/day/week) to Hg and MeHg by region of study

These results (Table 4) showed that the values of estimated daily/weekly intakes (EDI's/EWI's) were different among the studied regions for the same species. The estimated EDI's/EWI's of Hg and MeHg in the swordfish of NC gave the highest values (0.124; 0.103 µg/kg/bw/day; 0.90; 0.721 *µg/kg/bw/week*, respectively). All the estimated values in the three studied regions were lower than the fixed values (PTWI's) of EFSA and JECFA.

Target hazard quotient (THQ) and hazard index (HI)

We have estimated the THQ for Hg and MeHg and the HI due to the consumption of the two fish species. All the registered values have been < 1 (Table 5).

Table 5. Estimated of target hazard quotient (THQ) and hazard index (HI)

	THQ (Hg)	THQ (MeHg)	HI
Sardine	2.15×10^{-5}	6.46×10^{-5}	8.61×10^{-5}
Swordfish	3.28×10^{-4}	9.21×10^{-4}	1.24×10^{-3}

DISCUSSION

Hg and MeHg concentrations in the flesh of sardine and swordfish

The recorded average concentration of Hg in sardine flesh in the three studied regions (Table 1) was higher than that reported by Yabanli (2013) in Izmir (Turkey). However, it was much lower than reported in our previous study (2016) than that reported by Chahid (2016) in five areas (Agadir, Essaouira, Dakhla, Sidi Ifni and Laayoune) of Morocco.

The MeHg concentration recorded (Table 1) was higher than that reported by Cano-Sancho et al. (2015) and Carbonell et al. (2009) in Spain.

In swordfish, the Hg recorded average concentration (Table 1) was higher than that reported by Zaza et al. (2015) in the central Atlantic Ocean. While it was lower than that recorded in our previous study in Algiers (2016) and that reported by Liu et al. (2018) in the United States and Torres-Escribano et al. (2010) in Spain. It was observed that the values reported in our study in 2016 are different from those reported in the current study, which can be attributed to the changes of heavy metals concentrations with time, and also as a result of the different assay methods.

The average concentration of MeHg reported was higher than that reported by Cano-Sancho et al. (2015) in Spain.

Many studies on Hg and MeHg concentrations in sardine and swordfish have shown different results with a high or low concentration. This difference could be due to variations in the study area, the size, age, sex and the physiological status of the fish (Manavia and Mazumder, 2018). Other factors should be considered such as fishing seasons, microbial activity and mercury content in sediment, water chemistry characteristics (dissolved organic content, salinity, pH, and redox potential) (Ramos, 2012; Rajeshkumar and Li, 2018).

The concentrations of metals differ from one fish species to another; the highest concentrations of Hg

and MeHg were recorded in swordfish, which could be attributed to its high marine trophic position. This trophic position has variable effects on the bioaccumulation of metallic elements in pelagic fish depending on the element considered (Bodin et al., 2017). Hg levels in fish showed significant interspecific differences reflecting the trophic level and biomagnifications. Hg tends to bioaccumulate in organisms with higher trophic levels. Large predatory fish reaches high concentrations of Hg; usually larger individuals have higher concentrations than the smaller ones, due to age, diet and the time of exposure to pollutants. Casadevall et al. (2017) studied the contamination of swordfish muscle samples from Madrid markets and showed that approximatively 35% of the samples exceeded the maximum limit of Hg, some specimens reached levels up to 1900 ng/g, which agree with our results.

The lowest concentrations of total Hg and MeHg found in the sardine could be a result of their diet, which consists mainly of water plants and plankton (Vieira et al., 2011).

The literature on heavy metal concentrations in fish shows that the fishing areas are an important factor of variation of metal accumulations, which was observed in this study (Table 2).

The main results obtained in this study confirm what several researchers have reported previously. The majority of accumulated total mercury is in its organic form (methylmercury), due to its high lipophilicity and low solubility in water compared to other forms (Ramos, 2012; Carroll and Warwick, 2017).

Risk assessment

The risk assessment conducted for adult Algerian consuming sardine showed that the EWI for Hg in sardine (Table 3) was similar to that reported by Vieira et al. (2011) in Portugal but lower than that reported by Falcoä et al. (2006) in Spain in adults and Chahid (2016) for a Moroccan adult of 60 kg. The MeHg EWI (0.05 ug/kg/body weight/week) was higher than that reported by Cantoral et al. (2017) in Mexico.

In the swordfish, the Hg EDI and EWI (Table 3) were lower than that reported by Falcoä et al. (2006), Cano-Sancho et al. (2015) and Aranda et al. (2017) in Spain for adult men and women.

The values of estimated dietary intakes for both sardine and swordfish we recorded did not exceed the provisional tolerable weekly intake (PTWI) established by the European Food Safety Authority (EFSA) and Joint FAO/WHO Expert Committee on Food Additives (JECFA) (Table 3, 4).

From the results of this study, the consumption of sardine and swordfish, wherever they are caught, does not expose the consumer to toxic risk.

Since all calculated target hazard quotient and risk index are below 1 (Table 5), sardine and swordfish do not pose chronic systemic risk to the Algerian population.

CONCLUSIONS

The average concentrations of Hg and MeHg in swordfish were higher than those obtained in sardine fished in the three Algerian coasts; these values were lower than the national and European regulatory thresholds (OJPDRAN°25/2011; EU N°1881/2006).

The species and the fishing area are two important factors that influence the bioaccumulation of Hg and MeHg.

In the risk assessment performed, estimated dietary intake (EDI) was lower than the provisional tolerable weekly intake (PTWI) established by the European Food Safety Authority (EFSA) and Joint FAO/WHO Expert Committee on Food Additives (JECFA), the target hazard quotient (THQ) and the index hazard (IH) were below 1. As a result, the consumption of sardine and swordfish do not pose any risk to the adult Algerian population.

ACKNOWLEDGMENTS

The authors would like to extend their thanks to the Federal Laboratory for the Safety of the Food Chain Gentbrugge (FLSFC-G) of the Federal Agency for the Safety of the Food Chain (FASFC) (Belgium) and the laboratory of the National Center for Toxicology (CNT) in Algiers (Algeria) for their technical assistance and support. Special thanks go to Dr. Kamilou Ouro-sama and Dr. Michel Marengo for their support.

CONFLICT OF INTEREST

None declared by the authors.

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J HELLENIC VET MED SOC 2019, 70(3): 1687-1696
ΠΕΚΕ 2019, 70(3): 1687-1696

Research article
Ερευνητικό άρθρο

Effects of olive pulp addition to broiler diets on performance, selected biochemical parameters and antioxidant enzymes

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Επίδραση της προσθήκης πάστας ελαιόκαρπου στο σιτηρέσιο ορνιθίων κρεοπαραγωγής στην ανάπτυξη, επιλεγμένες βιοχημικές παραμέτρους και αντιοξειδωτικά ένζυμα

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Date of initial submission: 24-12-2018
Date of revised submission: 06-02-2019
Date of acceptance: 22-02-2019

ABSTRACT. Olive oil production generates various by-products that can be used in animal nutrition. These by-products contain several polyphenolic compounds that may exhibit antioxidant properties. The present study was designed to evaluate the effects of adding olive pulp to the feed on broiler performance, carcass yield and antioxidant enzymes. Two hundred (200), as hatched, day-old, Cobb 500 broilers were reared in total for 42 days. There were 4 dietary treatments. In T1 treatment, no olive pulp was added to starter, grower and finisher diet. In T2 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 2.5 and 5% respectively. In T3 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 5 and 5% respectively. In T4 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 5 and 8% respectively. Performance, carcass yield and a number of biochemical parameters were examined. Oleuropein and hydroxytyrosol were present in the olive pulp at 952 and 216 mg/kg respectively. Broilers performed well and no differences were observed between treatments on final body weight, carcass yield, total antioxidant activity and expression of selected antioxidant enzymes. Discriminant analysis was further applied and revealed that samples clustered according to added level of olive pulp. Samples from broilers fed T2 and T3 diet were located in the middle of the plot away from other treatments exhibiting high values of carcass, breast yield and most of the antioxidant enzyme activities. In conclusion, olive pulp can be used up to 5% in diets of broilers and future studies conducted on-farm conditions may pronounce its impact on growth performance and antioxidant potential.

Keywords: antioxidant, broilers, hydroxytyrosol, oleuropein, olive pulp

ΠΕΡΙΛΗΨΗ. Η παραγωγή ελαιόλαδου δημιουργεί ορισμένα υποπροϊόντα τα οποία μπορούν να χρησιμοποιηθούν στη διατροφή των ζώων. Αυτά τα υποπροϊόντα περιέχουν αρκετές πολυφαινολικές ενώσεις που ενδέχεται να παρουσιάζουν αντιοξειδωτικές ιδιότητες. Η παρούσα μελέτη σχεδιάστηκε για να αξιολογήσει την επίδραση της προσθήκης πάστας ελαιόκαρπου στο σιτηρέσιο ορνιθίων κρεοπαραγωγής στην ανάπτυξη, στην απόδοση σε σφάγιο και στην ενεργότητα ορισμένων αντιοξειδωτικών ενζύμων. Διακόσιοι (200), νεοσσοί κρεοπαραγωγής Cobb 500, ηλικίας μιας ημέρας, εκτράφηκαν για συνολικά 42 ημέρες. Υπήρξαν 4 διατροφικές επεμβάσεις. Στην επέμβαση Τ1 δεν προστέθηκε πάστα ελαιόκαρπου στο εναρκτήριο, ανάπτυξης και τελικό σιτηρέσιο. Στην επέμβαση Τ2, η πάστα ελαιόκαρπου προστέθηκε στο εναρκτήριο, ανάπτυξης και τελικό σε επίπεδο 0, 2,5 και 5% αντιστοίχως. Στην επέμβαση Τ3, η πάστα ελαιόκαρπου προστέθηκε στα τρία σιτηρέσια σε επίπεδο 0, 5 και 5% αντιστοίχως, ενώ στην επέμβαση Τ4 σε επίπεδο 0, 5 και 8% αντιστοίχως. Μελετήθηκε η ανάπτυξη, η απόδοση σε σφάγιο και ένας αριθμός βιοχημικών παραμέτρων. Η ολευρωπεΐνη και η υδροξυτυροσόλη ανιχνεύτηκαν στην πάστα ελαιόκαρπου σε επίπεδα 952 και 216 mg/kg αντιστοίχως. Τα ορνίθια αναπτύχθηκαν καλά και δεν παρατηρήθηκαν διαφορές μεταξύ των επεμβάσεων στο τελικό σωματικό βάρος, την απόδοση σε σφάγιο, την ολική αντιοξειδωτική ικανότητα και την ενεργότητα των αντιοξειδωτικών ενζύμων. Εφαρμογή της διακριτικής ανάλυσης έδειξε ότι τα δείγματα διακρίνονται βάση του επιπέδου προσθήκης πάστας ελαιόκαρπου. Τα δείγματα των επεμβάσεων Τ2 και Τ3 εντοπίστηκαν στο κέντρο του διαγράμματος απομακρυσμένα από τις άλλες επεμβάσεις παρουσιάζοντας υψηλές τιμές σε απόδοση σε σφάγιο, αναλογία βάρους στήθους προς σωματικό βάρος και ενεργότητα των περισσοτέρων αντιοξειδωτικών ενζύμων. Συμπερασματικά, η πάστα ελαιόκαρπου μπορεί να χρησιμοποιηθεί στα σιτηρέσια ορνιθίων κρεοπαραγωγής έως 5% και μελλοντικές μελέτες σε πραγματικές συνθήκες εκτροφής ίσως αναδείξουν περαιτέρω τις θετικές επιδράσεις αυτού στην απόδοση και αντιοξειδωτική προστασία των ορνιθίων.

Λέξεις Κλειδιά: αντιοξειδωτικά, ελιά, ολευρωπεΐνη, ορνίθια, πάστα ελαιόκαρπου, υδροξυτυροσόλη

INTRODUCTION

In the Mediterranean area, olive tree (*Olea europaea L.*) is cultivated for the production of table olives and edible olive oil. Long known to many generations that olive oil is an essential component of the healthy Mediterranean diet, it is now widely appreciated by consumers in Europe and many parts of the world for its unique aroma and nutritional properties (Frankel et al., 2013). The European Union is the leading producer of olive oil producing more than two thirds of world production. Four member states, namely Spain, Italy, Greece and Portugal produce 99% of the total EU olive oil production (European Commission, 2017).

Olive oil production generates various by-products that can be used in animal nutrition. By-products of olive oil extraction process include but not limited to crude olive cake, exhausted olive cake, partly destoned olive cake either crude or exhausted, olive pulp, vegetation waters and leaves (Sansoucy, 1985; Heuzé et al., 2015). The fruit of the olive tree (Kalogeropoulos and Tsimidou, 2014) as well as the by-products (Botsoglou et al., 2013; King et al., 2014; Gerasopoulos et al., 2015) contain several antioxidants that can potentially scavenge free radicals and provide antioxidant protection.

Table 1. Composition (g/kg) and calculated analysis of the experimental broiler diets

Ingredients (g/kg)	Starter 0%	Grower 0%	Grower 2.5%	Grower 5%	Finisher 0%	Finisher 5%	Finisher 8%
Maize	622.3	660.2	627.4	600.7	696.1	639.2	607.9
Soybean meal	268.9	234.6	235.1	241.3	184.8	193.1	197.5
Olive pulp	0	0	25.0	50.0	0	50.0	80.0
Gluten	50.0	36.5	43.9	39.9	55.0	55.1	55.4
Soybean oil	12.8	26.0	26.0	26.0	25.0	25.0	25.0
Monocalcium phosphate	13.5	12.4	12.5	12.7	10.6	10.9	11.0
Limestone	15.2	14.3	14.2	14.2	13.0	12.9	12.9
Lysine	5.8	5.5	5.4	5.3	5.1	4.9	2.0
Methionine	2.6	2.5	2.4	2.5	1.8	1.9	1.9
Threonine	0.7	0.6	0.6	0.6	0.3	0.3	0
NaCl	5.7	5.0	5.0	4.4	5.7	4.2	3.9
Premix[1]	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Calculated Analysis							
ME (MJ/kg)	12.7	13.2	13.1	13.0	13.5	13.3	13.2
CP (g/kg)	217.6	196.1	200.0	200.0	187.2	190.0	190.0
Sodium (g/kg)	2.3	2.0	2.0	1.8	2.3	1.7	1.6
Ca (g/kg)	9.0	8.4	8.4	8.4	7.6	7.6	7.6
Available P (g/kg)	4.5	4.2	4.2	4.2	3.8	3.8	3.8
Lysine (g/kg)	13.2	11.9	11.9	11.9	10.5	10.5	9.0
Methionine+ cysteine (g/kg)	9.8	8.9	8.9	8.9	8.2	8.2	8.2
Threonine (g/kg)	8.6	7.8	7.8	7.8	7.1	7.1	6.8
Arginine (g/kg)	13.8	12.5	12.5	12.5	11.3	11.3	11.3

[1]Premix supplied per kg of diet: 13,000 IU vitamin A (retinyl acetate), 5,000 IU vitamin D_3 (cholecalciferol), 100 mg vitamin E (DL-α-tocopheryl acetate), 4 mg vitamin K_3, 2.6 mg thiamin, 8 mg riboflavin, 3 mg pyridoxine, 0.015 mg vitamin B_{12}, 85 mg nicotinic acid, 22 mg pantothenic acid, 2 mg folic acid, 0.2 mg biotin, 10 mg ascorbic acid, 400 mg choline, 1 mg iodine, 40 mg iron, 120 mg manganese, 20 mg copper, 0.2 mg cobalt, 0.3 mg selenium, 100 mg zinc

In the latter years the contribution of the feed component of total costs for broiler production increased to approximately 70% (Donohue and Cunningham, 2009). Competition for plant sources between food, feed and biofuel producers may intensify the problem (Popp et al., 2014). Therefore, there is a need to successfully adopt a strategy to reduce feeding costs and find alternative, low-cost feedstuffs.

Three issues were taken into account during the design of the present study. Firstly, many of the olive oil's beneficial effects on human health are attributed to the polyphenolic compounds that may exhibit potent antioxidant properties (Kalogeropoulos and Tsimidou, 2014). Secondly, the availability of local olive oil by-products since Greece is a major olive oil producer (European Commission, 2017) and thirdly the need to use alternative low cost feedstuffs in order to reduce feeding costs (Donohue and Cunningham, 2009). The present study was designed to evaluate the effects of adding olive pulp to the feed on broiler performance and carcass yield.

MATERIALS AND METHODS

Animals, diets and experimental design

Two hundred (200), as hatched, day-old, Cobb 500 broilers were used in total. The broilers were obtained from a commercial hatchery. The duration of the experiment was 42 days with housing and care of broilers, conforming to the guidelines of the Ethical Committee of the Faculty of Animal Science and Aquaculture of the Agricultural University of Athens and complying with directive 2010/63/EC on the protection of animals used for scientific purposes. Pen was the experimental unit. There were five replicate pens of four (4) dietary treatments namely T1, T2, T3 and T4, randomly allocated in the house. There were 10 broilers per pen, 50 per treatment. Birds were assigned to a pen (2 m^2) with wheat straw shavings litter. The maximum stocking density in the pens did not

at any time exceed 33 kg/m^2 following EU directive 2007/43/EC. In house environmental conditions (light and ventilation) were controlled. Heat was provided with a heating lamp per pen.

Dried olive pulp was supplied by Sparta Life S.A, (Sparta, Greece). Chemical analysis revealed that the content of dry matter was 945 g/kg and the content of major nutrients (expressed in dry matter basis) was for crude protein (CP), crude fat, crude fibre (CF) and ash 85.7, 174.6, 276.0, and 61.4 g/kg respectively. Metabolisable energy was estimated at 11.2 MJ/kg (Van Der Klis and Fledderus, 2007).

Broilers were fed three different diets, namely starter (0 - 10 days), grower (11 - 22 d) and finisher (23 - 42 d). In T1 treatment, no olive pulp was added to starter, grower and finisher diet. In T2 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 2.5 and 5% respectively. In T3 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 5 and 5% respectively. In T4 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 5 and 8% respectively. Feed and water were provided *ad libitum*. Diets were isonitrogenous, isocaloric, with similar content of other oil sources (soybean oil). Composition (g/kg) and calculated analysis of the experimental broiler diets are shown in Table 1.

Sampling

On onset and at the end of each phase, broilers body weight (BW) was recorded and the mean body weight gain (MWG) was calculated. Furthermore, feed intake was measured (MFC) and feed to gain ratios (FCR) were calculated. Broilers were inspected daily and mortality was recorded on the appropriate data capture form. Total mortality was calculated as the number of broilers that died throughout the study compared to the initial number of broilers placed. At the end of the 6th week, 10 chickens per treatment were sacrificed to investigate treatment effects on carcass yield. The birds were weighed, anesthetized with a mild electric current, slaughtered, plucked, eviscerated and stored in the refrigerator for 24 h. The new weight was used for cold carcass yield calculation. Moreover, breast yield (boneless or with keel) was calculated as percentage of body weight.

At the end of the trial, the litter in each pen was scored to assess the degree of wetness based on method of Murakami et al. (2000) with minor modifications and representative samples were collected for dry matter determination. Furthermore, representative samples of freshly voided excreta were obtained for dry matter and wetness determination. Scoring was undertaken by a single operator using a scale based on shape and white cap definition of excreta, ranging from 0 to 3 with 0 referring to normal droppings with white caps in definition while 3 referred to completely liquid droppings. Dry matter determination was carried out according to standard procedures (AOAC International, 2005; method 930.15).

Haematology and Activity of Antioxidant Enzymes

At the end of the trial, blood samples were collected (n=5) for determination of haematocrit (%), aspartate aminotransferase (SGOT-AST) (IU/l), alanine aminotransferase (SGPT-ALT) (IU/l), blood urea nitrogen (BUN) (mg/dl), γ-glutamyltransferase (γ-GT) (IU/l), alkaline phosphatase (IU/l), cholesterol (mg/dl), total proteins (g/dl) and fractions of albumins (g/dl) and globulins (g/dl). Analysis was performed using an automated ABX Pentra 400 bench top analyser (Horiba-ABX, Montpellier, France).

Total antioxidant activity and selected antioxidant enzymes were determined in plasma. In detail, glutathione peroxidase (GPx), glutathione transferase (GST), superoxide dismutase (SOD), glutathione reductase (GR) and catalase (CAT) were determined according to Paglia and Valentine (1967), Habig et al. (1974), McCord and Fridovich (1969), Mavis and Stellwagen (1968) and commercial kit (CAT 100, Sigma-Aldrich, USA) respectively. Total antioxidant activity was determined with the ABTS (2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid)) and the FRAP (Ferric Reducing Ability of Plasma) assay according to Pellegrini et al. (1999) and Benzie and Strain (1996) respectively.

Oleuropein and hydroxytyrosol determination

All solvents were purchased from Baker as analytical (extraction) or HPLC (chromatographic analyses) grades. For the chromatographic analyses HPLC-grade water was prepared using a Milli-Q system, while all HPLC-solvents were filtered prior to use through cellulose acetate membranes of 0.45 μm pore size.

Calibration curves for oleuropein and 3-hydroxytyrosol standards (both obtained from Sigma–Aldrich) were constructed using the following concen-

tration levels (10, 50, 100, 150, 200, 250 and 300 µg/mL for oleuropein and 2, 20, 50, 100 and 150 µg/mL for 3-hydroxytyrosol).

The sample was prepared as follows: 10 g were poured into 60 mL Methanol (MeOH) and sonicated in an ultrasonic bath for 10 min. The solvent was separated by centrifugation (10 min, 7000 rpm) and the remaining solid was re-extracted for two additional times. The combined extracts were evaporated under vacuum and slurry obtained was purified with Solid Phase Extraction (SPE) using Methanol as eluent. The Methanolic solution was concentrated under vacuum to provide a semi-solid residue which was weighed, dissolved in Methanol/Water (1:1 v/v), membrane filtered (0.45 µm) and subjected to HPLC analysis. To avoid the degradation of bioactive molecules, all the aforementioned activities were performed in temperatures below 40 °C.

HPLC analysis was carried out using an Agilent 1100 system equipped with quaternary pump, degasser and diode array detector (DAD). The column used was a Kromasil C18 column (250 mm x 4.1 mm, particle size 5 µm) with a guard column of the same material (8 x 4 mm). Injection was by means of a Rheodyne injection valve (model 7725I) with a 20 µL fixed loop. Chromatographic data were acquired and processed using the Chemstation software.

The HPLC analyses was carried out at 40 °C (maintained by a column thermostat) and 120-140 bar pressure. The gradient eluted consisted of solvents A (aqueous solution with 0.2% v/v acetic acid) and B (Acetonitrile, ACN). Run time was 50 min, with a constant flow-rate of 1.0 mL/min in accordance with the following gradient time table: at zero-time, 98% A and 2% B; after 40 min, the pumps were adjusted to 70% A and 30% B. After the end of the run, a 30 min equilibration period was followed utilizing the zero-time mixture, prior to injection of the next sample. Peaks were identified by comparing their retention time and UV–Vis spectra with reference compounds and data were quantified in respect to the corresponding curves of the reference compounds which were used as standards. The peak area values (measured at 280 nm) constitute the average of three measurements. Results were expressed as mg/kg of olive pulp.

Statistical Analysis

Data were analysed using the Statgraphics Centurion statistical package (version 16.1) and are presented as least squares means ± pooled standard errors. Pen was the experimental unit. Dietary treatment effects on biochemical and haematological parameters, enzyme activities, carcass yield and all the other parameters were explored using one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. Kolmogorov–Smirnov test revealed that all variables, except score and the percentages of mortality, followed a normal distribution. Differences between these variables among the four treatments were examined using the Kruskal–Wallis non-parametric test, followed by Dunn's multiple range test. Moreover, principal components analysis was used to reduce the dimensionality of the data and to detect the relationships between the variables. Discriminant analysis was also applied to pooled data to establish those variables capable of distinguishing and classifying samples among the four treatments. Wilk's lambda (λ) criterion was used for selecting discriminant variables. For all tests, the significance was set at ≤0.05.

RESULTS

Broiler performance and carcass yield

Performance of broilers is presented in Table 2. Overall, broilers performed well with final body broiler weight at day 42 being about 2.4 kg. Statistically, no differences were observed between treatments. The FCR of broilers fed olive pulp up to 5% (treatments T2 and T3) did not differ with that of broilers fed the control diet. Broilers fed the diet with the highest inclusion level of oil pulp (T4) numerically consumed more feed and had lower weight gain and this was reflected in the FCR which was statistically higher compared to broilers fed the control or the olive pulp diets up to 5%. No difference between treatments were noted on mortality. It is worth noting that mortality rate of treatment T4 was nil.

Carcass yield is presented in Table 2. No differences were observed between treatments and average carcass yield (grand mean) was 74.5% of body weight. No differences were observed on breast yield between treatments.

Litter and excreta of broilers fed diets with increased levels of olive pulp revealed a tendency to be drier compared to those of broilers fed the control diet (data not shown) but this was not confirmed by data on dry matter since statistically no differences were observed between treatments (Table 2).

Table 2. Performance of broilers during the total experimental period (0-42 d), carcass yield (%) and dry matter content of excreta and litter (%) at the end of the trial

Parameter	Treatments[1]				SEM	*P-value*
	T1	T2	T3	T4		
BW[2] (g)	2411.9	2397.8	2369.2	2320.2	77.10	0.896
Mortality (%)	3.33	3.33	4.44	0	2.827	0.745
MFC[3] (g)	4419.2	4513.6	4454.2	4699.7	153.3	0.737
MBWG[4] (g)	2375.8	2361.9	2332.8	2283.7	77.08	0.894
FCR[5]	1.86^{a}	1.91^{ab}	1.91^{ab}	2.06^{b}	0.036	0.039
Carcass Yield (% live weight)	74.05	74.19	75.55	74.25	0.676	0.376
Breast Yield (% live weight)	28.27	28.95	29.55	27.76	0.671	0.269
Boneless Breast Yield (% live weight)	19.91	20.39	20.93	19.51	0.580	0.349
Excreta Dry Matter (%)	17.60	19.69	19.57	16.39	0.644	0.273
Litter Dry Matter (%)	44.64	47.25	43.11	43.85	1.890	0.811

Values are means of five replicate pens (n = 5). Means with different superscripts (a, b) in each row indicate significant differences (P≤0.05) between treatments.

[1]Broilers were fed three different diets, namely starter (0 - 10 days), grower (11 - 22 d) and finisher (23 - 42 d). In T1 treatment, no olive pulp was added to starter, grower and finisher diet. In T2 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 2.5 and 5% respectively. In T3 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 5 and 5% respectively. In T4 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 5 and 8% respectively.

[2]BW: body weight of 42 days old broilers.

[3]MFC: Mean feed intake of the total experimental period (0-42 days).

[4]MBWG: Mean body weight gain of the total experimental period (0-42 days).

[5]FCR: Feed conversion ratio of the total experimental period (0-42 days).

Biochemical parameters and antioxidant enzymes

Several biochemical and haematological parameters were examined in order to investigate potential effects on broiler's health. In Table 3, SGOT-AST, SGPT-ALT, BUN, γ-GT, alkaline phosphatase, cholesterol, total protein, albumins, globulins and haematocrit measurements are presented. No major differences were noticed in broilers fed olive pulp compared to broilers fed the control diet.

Oleuropein and hydroxytyrosol were present in the olive pulp at 952 and 216 mg/kg respectively. In Table 3, total antioxidant activity and expression of selected antioxidant enzymes is presented. No major differences were noticed among treatments.

Principal Components Analysis and Discriminant Analysis

Principal components analysis (PCA) was applied to pooled data in order to reduce the dimensionality of the data and detect the most important causes of variability, since a great correlation between the variables was noticed. PCA of the 26 variables (variables of broiler performance, carcass yield, dry matter, biochemical and haematological parameters and antioxidant enzymes) resulted in nine principal components with eigen-values greater than 1.0, a common statistical cut-off point. The nine selected components accounted for 83.23% of the total variability. In Figure 1 a plot of both first and second principal components is presented. The first principal component (PC) explained 19.05% of the total variability and was mainly defined by Total proteins, Globulins and Albumins. These haematological parameters were placed close together on the negative side of the horizontal axis, indicating that they were positively correlated with each other. They were away from the axis origin, suggesting that they were well represented from the first PC, which could be considered as representative of the haematological parameters. The second PC explained another 16.47% of the total variability and was mainly defined by the body weight (BW), the mean body weight gain (MBWG) and some enzyme activities (GR, GPx). BW and MBWG were located close together on the positive side of PC2, indicating a high positive correlation. GR and GPx were also close together and therefore they were positively correlated with each other. The second PC can be considered as a representative of the body weight and enzyme activities. The third PC explained another 10.67% of the total variability and it was mainly defined by FCR, MFC, Carcass yield and Breast yield. Carcass and Breast yield were placed close together indicating that they were positively correlated with each other. The third PC can be considered as a representative of feed intake and carcass and breast yield.

Table 3. Treatment effects on selected biochemical and haematological parameters and on total antioxidant activity and activity of enzymes

Parameter	Treatments[1]				SEM	*P-value*
	T1	T2	T3	T4		
Haematocrit (%)	33.33	33.17	32.83	33.50	1.127	0.978
SGOT-AST[2] (IU/l)	279.2	437.7	390.4	360.2	51.78	0.209
SGPT-ALT[3] (IU/l)	13.67	13.50	14.67	13.67	0.834	0.748
BUN[4] (mg/dl)	0.77	0.64	0.94	0.60	0.167	0.484
γ-GT[5] (IU/l)	16.33	16.67	20.33	21.00	1.902	0.215
Alkaline phosphatase (IU/l)	1106.5	2094.3	1319.5	1653.5	294.4	0.127
Cholesterol (mg/dl)	109.2	107.7	112.5	95.17	7.242	0.374
Total proteins (g/dl)	3.12	3.30	3.08	3.05	0.174	0.748
Albumins (g/dl)	1.78	1.82	1.75	1.65	0.072	0.416
Globulins (g/dl)	1.33	1.48	1.33	1.40	0.125	0.808
FRAP[6] (μmol ascorbic acid)	6.47	6.25	6.33	6.30	0.480	0.989
ABTS[7] (% inhibition)	30.21	32.08	32.41	32.15	1.935	0.835
GPx[8] (U/ml)	2.43	2.922	2.965	2.812	0.270	0.537
SOD[9] (U/ml)	11.90	12.00	11.74	12.20	0.269	0.697
GR[10] (U/ml)	0.020	0.023	0.023	0.023	0.002	0.341
CAT[11] (U/ml)	102.7	107.7	98.77	90.87	16.478	0.905
GST[12] (U/ml)	0.35	0.41	0.42	0.41	0.024	0.146

Values are means of five replicate pens (n = 5).
[1]Broilers were fed three different diets, namely starter (0 - 10 days), grower (11 - 22 d) and finisher (23 - 42 d). In T1 treatment, no olive pulp was added to starter, grower and finisher diet. In T2 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 2.5 and 5% respectively. In T3 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 5 and 5% respectively. In T4 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 5 and 8% respectively.
[2]SGOT-AST: aspartate aminotransferase.
[3]SGPT-ALT: alanine aminotransferase.
[4]BUN: Blood urea nitrogen.
[5]γ-GT: γ-glutamyltransferase.
[6]FRAP: Ferric Reducing Ability of Plasma
[7]ABTS: 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid)
[8]GPx: Glutathione Peroxidase
[9]SOD: Superoxide Dismutase
[10]GR: Glutathione Reductase
[11]CAT: Catalase
[12]GST: Glutathione Transferase

Since analysis of variance did not reveal remarkable differences on biochemical and haematological parameters, enzyme activities, carcass yield and the other parameters among the dietary treatments, discriminant analysis was further applied to the pooled data in order to investigate if the samples can be distinguished according to the type of diet that they were fed. Twenty six variables (performance, carcass yield, dry matter, biochemical and haematological parameters and antioxidant enzymes) were entered to develop a model to discriminate the samples. Even though one discriminant function was statistically significant (p=0.024), it can be seen in Figure 2, a discriminant plot of the first two discriminant functions, more readable than a one-dimensional plot. All the samples were correctly classified according to the treatment (100% of the total cases). On the left side of the plot, it can be seen a cluster of the samples that originated from treatment T1. These samples may be related to high body weight, body weight gain, percentages of mortality and cholesterol and on the contrary to low values of feed intake, FCR, SGOT-AST, γ-GT and phosphatase. Moreover, it must be mentioned that most of the enzyme activities had lower values in the samples treated with the control diet T1. On the other hand, samples from treatment T4 were clustered on the right side of the plot and they had the opposite characteristics. Samples originating from dietary treatments T2 and T3 were located in the middle of the plot separately from each other. These samples had high values of total proteins, albumins, globulins, carcass and breast yield and most of the antioxidant enzyme activities.

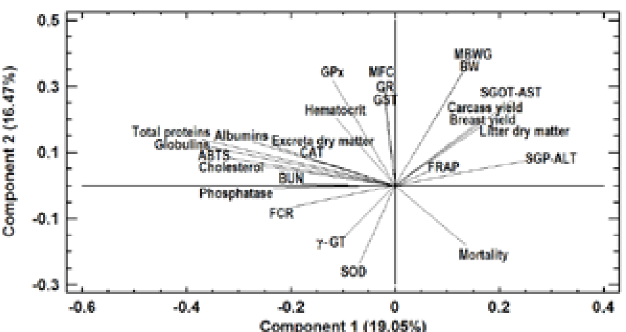

Figure 1. Plot of the first and second principal component

Principal components analysis of the 26 variables. 9 components have been extracted that account for 83.23% of the variability in the original data. First two are presented in the plot.

1. BW: body weight of 42 days old broilers. 2. Mortality: Average mortality of the total experimental period (0-42 days). 3. MFC: Mean feed intake of the total experimental period (0-42 days). 4. MBWG: Mean body weight gain of the total experimental period (0-42 days). 5. FCR: Feed conversion ratio of the total experimental period (0-42 days). 6. Carcass: carcass weight expressed as a percentage of live bird weight 7. Breast yield: Breast weight with keel expressed as a percentage of live bird weight. 8. Excreta dry matter: dry matter of excreta (%). 9. Litter dry matter: dry matter of litter (%). 10. SGOT-AST: aspartate aminotransferase. 11. SGPT-ALT: alanine aminotransferase. 12. BUN: Blood urea nitrogen. 13. γ GT: γ-glutamyltransferase. 14. Phosphatase: Alkaline phosphatase 15. Cholesterol. 16. Total proteins. 17. Albumins. 18. Globulins. 19. Hematocrit. 20. GPx: Glutathione peroxidase 21. SOD: Superoxide Dismutase. 22. GR: Glutathione Reductase. 23. CAT: Catalase. 24. GST: Glutathione Transferase. 25. FRAP: Ferric Reducing Ability of Plasma. 26. ABTS: 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid).

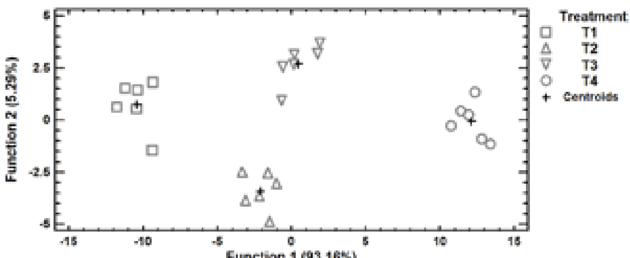

Figure 2. Discriminant plot separating samples according to the dietary treatment

Broilers were fed three different diets, namely starter (0 - 10 days), grower (11 - 22 d) and finisher (23 - 42 d). In T1 treatment, no olive pulp was added to starter, grower and finisher diet. In T2 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 2.5 and 5% respectively. In T3 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 5 and 5% respectively. In T4 treatment, olive pulp was added to starter, grower and finisher diet at a level of 0, 5 and 8% respectively.

DISCUSSION

The results of the present study indicate that olive pulp (8.6% CP; 27.6% CF) can be added to diets up to 5% in order to be utilised by broilers without impairment of feed efficiency. Previously, Taklimi et al. (1999) reported improved feed efficiency at 5% inclusion level of olive pulp (6.6% CP; 41.3% CF) in layer diets but declined at higher rates due to increased feed intake attributed to the increased crude fibre content. Similarly, in the present study, when broilers fed 8% olive pulp showed poorer FCR and numerically higher feed intake level. In ducks, Fathalla et al. (2015) examined the effects of an olive pulp (10.2% CP; 24% CF) on the growth performance and concluded that olive pulp added at 12% with or without enzyme complex resulted in improvement of weight gain and feed conversion ratio compared to that of ducks fed the control diet. In hens, inclusion of olive cake (5.2% CP) in diets at a ratio of up to 20% did not affect negatively performance and egg quality, but increased feed intake and impaired FCR compared with control group (Al-Harthi, 2015). Reported differences between studies may be related to differences in chemical composition and most notably the fibre content of olive by-products used. Recently it was reported that moderate amounts of fibre may improve the development of organs, enzyme production, and nutrient digestibility in poultry due to alterations in solubility, viscosity, and fermentation capability that in turn affects microbiota diversity and counts (Mateos et al., 2012).

Proper litter conditions are crucial for the survival of broilers and when not met bacterial growth and ammonia production may negatively affect health (Atapattu et al., 2008). Moisture of litter is of paramount importance for broiler growth since the decomposition of uric acid releases ammonia to the environment (Shah et al., 2007). It has been reported that dietary fibre may reduce the growth of pathogenic microorganisms and the occurrence of digestive disturbances, such as wet litter (Mateos et al., 2012), but in the present study, excreta and litter dry matter did not differ between treatments.

In the present study, carcass yield was similar between treatments and close to Cobb's yield for as hatched broilers (Cobb-Vantress, 2015). Similarly, increasing level of olive pulp in the diet of broilers up to 10% had no significant effects on dressing percentage and carcass composition of 35 day old broilers (Abo Omar, 2005). Previous study with olive cake added at 5 or 10% in broiler diets, with or without the presence of enzymes did not affect carcass yield and internal organs (Al-Harthi et al., 2017). Similarly, olive cake in broiler diets up to 10% did not adversely affect car-

cass traits and inner organs (Al-Harthi et al., 2016). In ducks, Fathalla et al. (2015) examined the effects of an olive pulp on the growth and carcass traits concluded that olive pulp added at 12% without enzyme complex did not affect dressing carcass weight but in the presence of enzyme increased dressing weight.

Olive tree fruits and olive by-products have gained considerable attention due to the interest on phenolic compounds as potential antioxidants (Silva et al., 2006). In detail, it has been shown that oleuropein, the main glycoside present in olive fruit, and hydroxytyrosol a major degradation product of oleuropein exhibit antioxidant and anti-inflammatory properties (Cardoso et al., 2006; Omar, 2010). In the present study, total antioxidant activity determined in blood was not altered by olive pulp addition to broiler diets. Contrary to our findings, Oke et al. (2017) reported that inclusion of olive leaf extract in the water of broilers, reared in a hot and humid tropical climate, improved performance and increased plasma SOD activity. Similarly to our results, Tarek et al. (2013) reported no significant difference in the performance of broiler chickens fed different doses of olive leaf extract in feed. Furthermore, Branciari et al. (2017) observed that dietary administration of a semi-solid olive cake improved the oxidative stability of broiler meat when added at a high dose but did not have any effect at a lower dose. Differences between studies regarding performance and response to olive tree extracts or by products may be attributed to experimental conditions, the presence of stress factors, inclusion level and duration of supplementation. Previous studies examined the polyphenol content and the antioxidant capacity of several by-products and reported that addition of by-products from olive mill wastewater processed using ceramic membrane microfiltration to chicken diet improved their redox status (Gerasopoulos et al., 2015). Furthermore, it was reported that olive leaves included on pig diets at 2.5% may improve the tocopherol content of meat without excessively compromising growth performance (Paiva-Martins et al., 2014). The noted differences may be attributed to different content of polyphenols between examined olive by-products. In detail, it was reported that total phenolic content of fresh olive leaves is 17 g/kg polyphenols while pulps may contain up to 30 g/kg (Silva et al., 2006). However, Paiva-Martins et al. (2014) reported that leaves and branches of olive tree may contain polyphenols up to 67 g/kg Under this context, it was reported that changes in phenolic composition may appear during olive processing, oil extraction and storage (Frankel et al., 2013).

In the present study, the determined values of several biochemical parameters examined were in line with normal values (Campbell, 2004) indicating that good health is maintained when olive pulp is added into broiler diets. Similarly, Sayehban et al. (2015) reported that processed olive pulp fed to broiler diets had no effect on hematological parameters. In contrast, Al-Harthi (2015) reported that plasma albumin was increased when olive cake was added to layer diets at 10 and 20% compared to control. However, in the present study total plasma protein concentration ranged within the normal range from 2.5 to 4.5 g/dl reported for birds by Carpenter (2004).

CONCLUSION

In conclusion, the present study showed that olive pulp added to broiler diets up to 5% can maintain good health and carcass yield without negatively affecting feed to gain ratio. This trial was a small scale one with low levels of stress however, under commercial conditions, any potential differences as those noted in the discriminant analysis of the present study could be more pronounced. Future studies may optimise the use of olive pulp in broiler nutrition in terms of both growth performance and antioxidant potential.

CONFLICT OF INTEREST

The authors declare no conflicts of interest

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J HELLENIC VET MED SOC 2019, 70(3): 1697-1706
ΠΕΚΕ 2019, 70(3): 1697-1706

Research article
Ερευνητικό άρθρο

Assessment of titanium dioxide nanoparticle as treatment of *Aeromonas hydrophila* infection in *Oreochromis niloticus*

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ABSTRACT. Nanoproducts became widely used materials all over the world. Antimicrobial properties of titanium dioxide (TiO_2) nanoparticle (NP) were examined against Aeromonas hydrophila (A. hydrophila) bacteria and the minimum inhibitory concentration (MIC) was found to be 20 µg/ml of TiO_2NP. In addition, the treatment efficacy of TiO_2NP was examined in Oreochromis niloticus (O. niloticus) infected with A. hydrophila. One hundred and eighty fish (54±2.4 g b.w.) were divided into six groups (G). O. niloticus in G1, G2 and G3 were fed for 30 days with 0, 20 and 100 µg/g b.w. TiO_2NP, respectively, while G4, G5 and G6 were i.p. injected with 0.2 ml distal water, 20 and 100 µg/g b.w. TiO_2NP, respectively, for three times with ten days of interval. The blood parameters as well as some of the biochemical parameters of O. niloticus that received high dosage of TiO_2NP were significantly affected regardless to the administration route. Elevation of the activities of glutathione peroxidase (GPx) and metallothionine (MT) were recorded with the high dosage. Furthermore, O. niloticus subjected to high dosage of TiO_2NP had the lower survival rate (SR%) especially with the injection route (50%). On the other hand, no significant changes were demonstrated with the perceived TiO_2NP MIC. The mortality rate (MR%) of challenged O. niloticus against A. hydrophila was decreased in case of TiO_2NP MIC exposure, as G2 and G5 revealed 20 and 30%, respectively. Therefore, the 20 µg/g b.w. of TiO_2NP could safely protect O. niloticus against A. hydrophila infection since no health hazards was observed. Meanwhile, health status of O. niloticus was adversely affected with high dosage of TiO_2NP irrespective to the route of administration.

Keywords: TiO_2, nanoparticles, *Oreochromis niloticus, Aeromonas hydrophila*, antioxidant.

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Date of initial submission: 27-12-2018
Date of revised submission: 30-03-2019
Date of acceptance: 12-04-2019

INTRODUCTION

Nanotechnology has provided the global market with novel nano-products with unique properties and functions that has emerged rapidly affecting economic sectors (Bour *et al.,* 2015). Nano-products have different physicochemical properties than their bulk forms; they are define as materials with a size between 1 and100 nm on at least one dimension, which provides surface area to volume ratio. Titanium nanoparticles (TiO_2NP) is one of the most manufactured NP worldwide; its production was expected to reach 201,000 tons during 2015 (Markets, 2015). TiO_2NP is widely used in the production of paints, coatings, plastics, papers, inks, foods, pharmaceuticals, cosmetics and toothpaste (Menard *et al.,* 2011 and Shi *et al.,* 2013).

The most frequently isolated bacterial pathogen in warm freshwater fishes is *A. hydrophila,* a Gram-negative motile rod bacterium that always associated with diseases outbreaks in the aquatic environment (Angka, 1990; Esteve *et al.,* 1993). *A. hydrophila* infection causes a systemic disease resulting in ulcerative dermatitis, tail or fin rot, ocular ulceration, which leads to hemorrhagic septicemia, the most common cause of mortality in the acute form is rapid septicemia (Cipriano, 2001).

Fish producers have used antibiotics and chemicals (malachite green, formalin, methyl blue, potassium permanganate and copper sulphate) as treatments for fish diseases, which unfortunately had severe impacts on fish consumers and environment. Therefore, a demand for new antibacterial agent that can avoid such hazards become essential (Sakr et al., 2014). Ravikumar *et al.* (2011) claimed that metal oxide nanoparticles antimicrobial property could be due to the reactive oxygen species mechanism. Nano-TiO_2 is a safe product (Rowe et al., 2003; Jacobs et al., 2010) and could be used as an additive in protocols for removal of arsenic from drinking water (EPA 2010). However, nano-TiO_2 had immune suppressive effect on fish health that enhance mortalities in fish exposed to infectious bacteria (Jovanovic *et al.,* 2015). Despite the high investments in nanotechnology the studies related with the antimicrobial property of metal oxide nanoparticle against bacterial fish diseases are too limited (Vale *et al.,* 2016). Therefore, this study was designed to investigate the potential antimicrobial role of TiO_2NP. Furthermore, the impacts of TiO_2NP on *O. niloticus* health were evaluated.

MATERIALS AND METHODS

Chemicals

Titanium dioxide nanoparticles (TiO_2NP) (Sigma Aldrich Corp, St. Louis, MO, USA) anatase, nano powder, < 25 nm, purity 99.7%, average zeta potential of 16.4 mV, conductivity of 16 mS/cm. The aggregate size had an average diameter of 86 nm, zeta potential of 8.87 mV and conductivity of 15.4 mS/cm.

Bacteria isolation and identification

A. hydrophila was isolated from diseased fish that were collected from a private freshwater fish farm. Bacterial swabs were obtained from fish hepatopancreas, spleen and kidneys according to Woo and Bruno (2014). Swabs were inoculated onto tryptic soya broth then the inoculum was streaked onto Rimler Schotts agar and incubated at 37^oC for 24 h. *A. hydrophila* was inoculated onto tryptic soy agar (Oxoid, Canada) then incubated in 28^oC for 24 h according to Austin and Austin (2012). Bacterial strain was confirmed by the polymerase chain reaction (PCR). DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^oC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged. Nucleic acid was eluted with 100 µl of elution buffer. Primers used were supplied from Metabion (Germany) and they are listed in Table 1. PCR amplification (35 cycles) was performed in a 25-µl reaction containing 12.5 µl of Emerald Amp Max PCR master mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler. Analysis of the PCR products was performed by a gel documentation system (Alpha Innotech, Biometra) and the data were analyzed through computer software Chip PCR (Rodiger and Burdukiewicz 2013).

Table 1. Primers sequences, target gene, amplicon size and cycling conditions

Target gene	Primers sequences 5'-3'	Amplified segment (bp)	Annealing temperature	Reference
***A. hydrophila* 16S rRNA**	GAAAGGTTGATGCCTAATACGTA CGTGCTGGCAACAAAGGACAG	625	50°C 40 sec.	Gordon et al. 2007

Determination of TiO_2NP minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was performed according to **Ravikumar *et al.* (2011)**. Briefly, 50 µl of 24h old *A. hydrophila* inoculum (corresponding to a concentration of 5 x 10^5 CFU) were exposed to a dilution series of TiO_2NP ranging from 500 to 10 µg/ml (500, 400, 300, 200, 100, 60, 50, 40, 30, 20 and 10 µg/ml). The culture was allowed to grow at 37°C for 48h and the whole setup was triplicated, while tryptic soya broth alone was considered as the negative control. The MIC of the nanoparticles was defined as the lowest concentration of the agent that restricted the growth of bacteria in the culture media.

Experimental design

Two hundred *O. niloticus* with an average 54±2.4 g b.w. was acclimated for two weeks at laboratory condition; water temperature 25.5±1.5°C, dissolved oxygen 5.2±0.5 mg/l, pH 7.5±0.4. Solid wastes of fish were removed daily with the exchange of one third of aquarium water. Following acclimation, one hundred and eighty *O. niloticus* were divided into six groups (G1-G6). Each group had three subdivisions (replicates) and fish were randomly distributed into 18 glass aquariums (50x50x40 cm), ten fish per aquarium. The Institutional Aquatic Animal Care and Use Committee, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University have approved the procedures.

Following the detection of the TiO_2NP MIC, the nanomaterial was dispersed in distal water by sonication to form a stock solution. Fish groups were either fed (for 30 days) or injected with TiO_2NP MIC. G1 (control) was fed a commercial fish diet with no additives, G2 was fed diet contained the revealed MIC of TiO_2NP per gram of fish body weight, while G3 was fed with a high dosage (100 µg/ g b.w.) of TiO_2NP per gram of fish body weight. On the other hand, fish under anesthesia with tricaine methanesulfonate (MS222; Sigma, St. Louis, MO, USA) were injected i.p. with 0.2 ml distal water (G4, control), with the obtained MIC of TiO_2NP suspended in 0.2 ml distal water (G5) or with high dosage (100 µg/g b.w.) of TiO_2NP suspended in 0.2 distal water (G6). Injections were repeated three successive times with ten days interval. By the due time, fish were euthanized by immersion in MS222 solution (250 mg/L; 25 to 30°C) that assumed to cause rapid unconsciousness, followed by decapitation and exsanguination (gill cut) according to Anonymous (2007).

By the end of the experimental period, the fish were counted to determine the survival rate percentage (SR %) according to the following formula:

SR %= Number of fish at the end/ Number of Fish at the beginning of the Experiment x 100

Hematological and biochemical analyses

For hematological analyses, blood samples were collected in vacutainers containing heparin (30 IU/l of blood) as an anticoagulant. For the serum biochemical analysis, blood samples were collected into vacutainers without anticoagulant, and serum was separated by centrifugation at 1000 g for 10 min and stored in a freezer at -20°C until use.

Red blood cells (RBCs) and white blood cells (WBCs) were counted by a haemocytometer according to Stoskopf (1993). Blood haemoglobin (Hb) was assessed by cyanomethemoglobin method (Drubkin, 1964). Packed cell volume (PCV) was determined by centrifuging heparinized blood in a capillary tube at 10,000 RPM for five minutes. In addition, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated according to the formulas mentioned by Dacie and Lewis (1975) as follows:

MCHC (g/dl) = (HB / PCV) ×100,

MCH (pg) = (HB / RBCs) × 10,

MCV (μm^3) = (PCV / RBCs) ×10.

Total protein (TP) was measured according to Weichselbaum (1946), albumin (Alb) was determined by colorimetric methods (Doumas *et al.,* 1971), while globulin concentrations (Glo) were determined by subtracting the Alb concentration from the concentration of TP according to Coles (1974). Serum creatinine was assessed according to Henry (1974). Liver enzymes aspartate amino transaminase (AST) and alanine amino transaminase (ALT) were determined according to Reitman and Frankel (1957).

At the end of the experimental period, the liver and spleen were carefully removed and weighed. Somatic indices including hepatosomatic index (HSI) and spleenosomatic index (SSI) were calculated (Htunhan, 1978) as follows:

HSI = weight of the liver/fish body weight,

SSI = weight of the spleen/fish body weight.

Liver tissues were examined for antioxidants activities. Glutathione peroxidase (GPx) activity was measured according to the method described by Mohandas *et al.* (1984). Briefly, the assay mixture was added to 0.2-0.3 mg protein of liver and 0.25 mmol/l hydrogen peroxide in a final volume of 1.0 ml NADPH. The activity of the enzyme was recorded at 340 nm at 25°C and was expressed as nmol of NADPH oxidized/min/mg protein by Enzyme-Linked Immunosorbent Assays (ELISA).

Standard ELISA protocol (Derango and Page 1996) was applied to measure the metallothionein content. Briefly, the primary antibody against metallothionein (Ab 36882) and the secondary antibody (Ab 6721) were purchased from Abcam, UK. An automatic micro titer plate ELISA reader (Wipro, India) was used to measure the absorbance at 650 nm. A second reading was recorded at 450 nm after addition of 2.0 M H2SO4 that stops the reaction. The high precision metallothionein value was achieved by plotting the ELISA values in the regression curve that obtained from a standard curve (Hornitzky and Searson 1986).

Infection trial

By the end of the experiment (after 30 days), 10 fish were randomly collected from each group and injected i.p. with 0.3 $x10^8$ cfu/ml of *A. hydrophila* according to Schaperclaus *et al.* (1992). Pure saline solution (0.65%) was parallel injected in a similar fashion, in three fish, for negative control injection (Boijink et al., 2001). *O. niloticus* were kept in the same experimental condition and under observation. Mortality rate (MR) % were estimated following 14 days as follows:

MR % = No. of death in specific period/ Total population during that period x 100.

Statistical analysis

Statistical analysis was performed by using the analysis of variance (ANOVA). All statistics were run on the computer using the SPSS program (SPSS, 2004).

RESULTS

Determination of TiO_2NP MIC

To determine MIC, a series of TiO_2NP dilutions ranging from 500 µg/ml to 10 µg/ml were mixed with adjusted *A. hydrophila* concentration while negative control contained only inoculated broth and the time and temperature of incubation being 48 h and 37ºC, respectively. MIC was determined to be 20 µg/ml, which was the minimum concentration that visually inhibited the growth of the microorganism.

The SR % in different tested groups (Table 2) revealed that it was significantly decreased in fish subjected to high dosage of TiO_2NP (100 µg/g b.w.), especially through the injection route (50% in G6). Meanwhile, the SR % was boosted in groups exposed to TiO_2NP MIC regardless to the administration route (G2 and, G5).

Table 2. Survival rate percentage of ***O. niloticus*** in different experimental groups

Items	Feed			Injection		
	G1 Control	**G2** 20 µg/g	**G3** 100 µg/g	**G4** Control	**G5** 20 µg/g	**G6** 100 µg/g
No	30	30	30	30	30	30
SR%	83.3^{ab}±3.3	86.7^{a}±3.3	66.7^{b}±8.8	73.3^{ab}±3.3	76.7^{ab}±3.3	50^{c}±5.8

No=Number of fish, SR= Survival rate. Different letters in the same row are significantly different at P≤0.05.

Hematological and biochemical analyses

As shown in Table 3, *O. niloticus* received high dosage of TiO_2NP (100 µg/g b.w.) displayed sever decrease in blood indices irrespective to the administration route. RBCs and Hb were severely decreased in G3 and G6, which received high TiO_2NP dosage, presenting 1.67 and 1.57 $X10^6$; 5.01 and 4.71 g/dl, respectively. In the same line WBCs were decreased to 65 and 61.2 $X10^6$ in G3 and G6, respectively. While no significant differences were observed in MCV, MCH, and MCHC.

Table 3. Blood analyses of *O. niloticus* received TiO_2NP. Mean±SE

Items	Feed			Injection		
	G1 Control	G2 20 µg/g	G3 100 µg/g	G4 Control	G5 20 µg/g	G6 100 µg/g
RBCs X10^6	2.4[a] ±0.25	2.35[a] ±0.2	1.67[b] ±0.1	2.16[a] ±0.3	2.3[a] ±0.24	1.57[b] ±0.15
WBCs X10^3	72[a] ±1.8	71[a] ±3.5	65[ab] ±3.3	73.4[a] ±1.5	74.5[a] ±2.7	61.2[b] ±2.1
Hb g/dl	7.44[a] ±0.55	7.28[a] ±0.35	5.01[b] ±0.5	6.48[a] ±0.4	6.9[a] ±0.8	4.71[b] ±0.6
PCV %	23.1[a] ±1.6	22.6[a] ±0.9	15.53[b] ±1.8	20.1[a] ±0.8	21.39[a] ±0.2	15.4[b] ±0.5
MCV Fl	96.25[a] ±2.1	96.17[a] ±1.9	92.9[b] ±1.5	93[b] ±0.5	93[b] ±0.4	92.7[b] ±0.8
MCH Pg	31[a] ±1.2	30.9[a] ±0.35	30[a] ±1.3	30[a] ±0.2	30[a] ±0.2	30[a] ±0.7
MCHC g/dl	32.2[a] ±2.1	32.13[a] ±0.5	32.3[a] ±2.4	32.26[a] ±0.3	32.26[a] ±0.1	32.36[a] ±0.1

Different letters in the same row are significantly different at P≤0.05.

For both groups subjected to 100 µg/g b.w. TiO_2NP (G3 and G6), a significant decrease in the values of TP (4.2 and 3.95) as well as Glo (1.8 and 1.52) was observed. Meanwhile, Alb revealed no significant difference among tested groups (Table 4). On the other hand, no significant differences were recorded in TP or Glo in fish exposed to the TiO_2NP MIC through either food or injection routes (G2 and G5).

Liver enzymes, AST and ALT, were significantly increased with the high TiO_2NP dosage in both G3 and G6, regardless to the administration route. Groups received a dosage that corresponds to the MIC were insignificantly different compared with control.

To evaluate the creatinine clearance, which reflects the glomerular filtration rate of fish kidneys, serum creatinine was measured. Values of serum creatinine had the same trend of liver enzymes since high dosage of TiO_2NP (G3 and G6) resulted in a remarkable elevation whatever the administration route was.

Table 4. Serum analyses of *O. niloticus* received TiO_2NP. Mean±SE

Items	Feed			Injection		
	G1 Control	G2 20 µg/g	G3 100 µg/g	G4 Control	G5 20 µg/g	G6 100 µg/g
TP g/dl	5.1[a] ±0.1	5[a] ±0.25	4.2[b] ±0.12	5.2[a] ±0.3	4.9[ab] ±0.2	3.95[b] ±0.38
Alb g/dl	2.65[a] ±0.24	2.62[a] ±0.28	2.4[a] ±0.1	2.79[a] ±0.25	2.51[a] ±0.2	2.23[a] ±0.3
Glo g/dl	2.45[a] ±0.15	2.38[a] ±0.18	1.8[b] ±0.2	2.41[a] ±0.2	2.39[a] ±0.1	1.52[b] ±0.3
AST U/L	23[d] ±2.3	29[c] ±2.4	75[b] ±3.2	25[d] ±1.3	36[c] ±3.9	112[a] ±5.8
ALT U/L	18[c] ±1.85	20[c] ±2.7	85[b] ±5.2	17[c] ±2.5	28[c] ±9.3	142[a] ±6.3
Creat mg/dl	0.2[c] ±0.02	0.71[b] ±0.05	0.8[b] ±0.06	0.18[c] ±0.01	0.81[b] ±0.1	1.3[a] ±0.02

Different letters in the same row are significantly different at P≤0.05. Creat denotes serum creatinine.

Both of the examined somatic indices (Table 5); HSI and SSI of *O. niloticus* in groups received the TiO_2NP MIC, were insignificantly different from control regardless to the administration route. Meanwhile, G3 and G6 revealed a significant increase as they recorded 1.76 and 2.4%, respectively. In addition, SSI showed similar trend as that for HSI, with a significant increase in G3 and G6 by 0.42 and 0.86%, respectively.

Table 5. HSI and SSI of *O. niloticus* received TiO_2NP. Mean±SE

Items	Feed			Injection		
	G1 Control	G2 20 μg/g	G3 100 μg/g	G4 Control	G5 20 μg/g	G6 100 μg/g
HSI %	1.6^c±0.02	1.62^c±0.03	1.76^b±0.01	1.59^c±0.01	1.59^c±0.1	2.4^a±0.12
SSI %	0.33^c±0.001	0.34^c±0.002	0.42^b±0.01	0.31^c±0.01	0.32^c±0.01	0.86^a±0.01

Different letters in the same row are significantly different at P≤0.05.

In hepatic tissues of ***O. niloticus***, a significant elevation of antioxidants activities for GPx and MT was remarkable in response to the high dosage of TiO_2NP in spite of the administration route. Groups received TiO_2NP MIC (G2 and G5) were insignificantly different from control groups (G1 and G4) (Table 6).

Infection trial

Ten *O. niloticus* from each group were challenged against *A. hydrophila*, and MR% was calculated and presented in Table 7. High TiO_2NP dosage, regardless to the administration route, in both G3 and G6 resulted in a higher MR% that recorded 70 and 80%, respectively followed by the control in both G1 and G4 (50%). On the other hand, the lower MR% was observed in G2 and G5 revealing 20 and 30%, respectively for those fish that received the TiO_2NP MIC (20 μg/g b.w.).

Table 6. Antioxidants activities in hepatic tissue of *O. niloticus* received TiO_2NP. Mean±SE

Items	Feed			Injection		
	G1 Control	G2 20 μg/g	G3 100 μg/g	G4 Control	G5 20 μg/g	G6 100 μg/g
GPx	190.8^c±6.5	190^c±4.8	253.7^b±3.8	195^c±2.8	198^c±3.9	287.5^a±5.2
MT	15.7^c±1.2	17.3^c±2.75	25.9^b±1.8	16.8^c±2.4	16.2^c±1.8	36.7^a±2.2

Different letters in the same row are significantly different at P≤0.05.

Table 7. MR% of *O. niloticus* received TiO_2NP and challenged with *A. hydrophila*

Items	Feed			Injection		
	G1 Control	G2 20 μg/g	G3 100 μg/g	G4 Control	G5 20 μg/g	G6 100 μg/g
NO.	10	10	10	10	10	10
MN	5	2	7	5	3	8
MR%	50	20	70	50	30	80

NO=Number of fish, MN= Mortality number and MR%= Mortality rate%.

DISCUSSION

Nile tilapia, *O. niloticus*, is a widespread teleost fish in tropical regions where it has a significant economic value in fishery and aquaculture industries. This study highlighted the antimicrobial properties of TiO_2NP and possible impacts on *O. niloticus* health. *A. hydrophila* bacteria is a common pathogen for fish. It is a Gram-negative motile rod and one of the highest isolated bacterial pathogens of freshwater fish in fish farms that occurred in warm climatic countries (Angka, 1990 and Esteve *et al.,* 1993).

Nanoparticles have a better and different quality compared to other forms of the same element. A small amount of them can have a great deal of antibacterial effect (Karimipour and Tanomand, 2016). The inhibitory effect of the nanoparticles may occur from their interference in the biological mechanisms of the bacteria as they penetrate the cell wall of the bacteria and change its properties. This increases the penetrability of cell membrane and interrupt the control of material intake and output from the cytoplasm. In the present study, the TiO_2NP MIC *for A. hydrophila* was determined to be 20 μg/ml. High SR % of ***O. niloticus*** was recorded with the determined MIC regardless to the administration route.

Concerning blood indices, high dosage of TiO_2NP (100 μg/g b.w.) displayed drastic impacts in both fed and injected fish although injected group (G6) revealed higher response. Meanwhile, no significant difference was observed in the tested blood indices

between the control groups and those received the TiO_2NP MIC. A number of studies have suggested that TiO_2NPs could pose toxicity to several aquatic organisms including microbes, algae, invertebrates and fish (Chen et al., 2012). In a species of ark clam known as the blood clam *Tegillarca granosa*, Shi *et al.* (2017) treated the clam with 10 and 100 mg/l TiO_2NP for 30 days. The authors reported that RBCs were significantly decreased from 79.76% to 70.98% in comparison to the negative control. In the same line, Barmo *et al.* (2013) and Balbi, *et al.* (2014) observed a reduction in different blood indices and phagocytic activity of the saltwater mussels *Mytilus galloprovincialis* in vivo acute toxicity (96 h) of TiO_2NP (size 15-60 nm) with a dosages 1-100 mg/l. The findings of the present study could be explained by the fact that high dosages of TiO_2NP perform a physical stress, which damaged blood cells. In agreement, Reeves *et al.* (2008) stated that the physical stress of TiO_2NP disrupted membranes of blood cell that was induced by the adhesion of TiO_2NP.

The examined liver enzymes of *O. niloticus* AST and ALT of fish exposed to high TiO_2NP were significantly increased by several times relative to control fish, while those subjected to the TiO_2NP MIC slightly increased. Along with the present results, Wang *et al.* (2007), Chen *et al.* (2009), Duan *et al.* (2009) and Liu *et al.* (2009) stated that the activity of a number of enzymes, including AST and ALT, were increased in mice treated with TiO_2NP. This was attributed to the increase in cellular membranes damages leading to liver enzymes leaking out. On the other hand, serum creatinine in fish exposed to the selected high TiO_2NP dosage was significantly increased by 4 to 7.2 times in G3 and G6, respectively with regard to the control. Meanwhile, fish groups subjected to TiO_2NP MIC also revealed a significant increase by 3.5 and 4.4 times in G2 and G5, respectively. In agreement, Banaee *et al.* (2016) found a significant increase ($P<0.05$) in creatinine levels 1.17 to 1.08 of *Cyprinus carpio* subjected to TiO_2NP compared to control group 0.14 to 0.17, respectively.

HSI is a widely known bioindicator of contaminant exposure (Sadekarpawar and Parikh, 2013). Because the liver is so important in detoxification, exposure to contaminants can lead to an increase in liver size from hypertrophy (an increase in size), hyperplasia (an increase in number) of hepatocytes (Sole et al., 2010), or both. On the other hand, fish spleen acts primarily as a blood filter, and plays important roles in regard to red blood cells and the immune system. Fish with larger spleens, may simply have a greater filtering capacity and thus increased immune function (Hadidi et al., 2008). *O. niloticus* which injected with a high dosage of TiO_2NP (G6) revealed high HSI as well as SSI, which may indicate the suffering of fish from hepatomegaly as well as splenomegaly, respectively. This could be attributed to TiO_2NP generation of free radicals, which in turn initiate an inflammatory response that leads to hepatocytes swelling together with dilatation of the central vein, increased permeability hepatocytes membrane and the endothelial lining of blood vessels (Johar *et al.,* 2004 and Alarifi *et al.,* 2013).

In the present study, significant differences were only demonstrated in TP and Glo levels in fish exposed to the high examined TiO_2NP level. Banaee et al. (2016) observed a decrease in total protein as well as globulin levels in *C. carpio* exposed to 125 μg/l of TiO_2NPs for 21 days that was attributed to reduced protein and globulin synthesis in hepatocytes. The authors claimed that the decrease in protein levels might be related to malnutrition, increased energy cost of homeostasis, tissue repair and the detoxification mechanism under stress conditions. Meanwhile, Griffitt et al. (2009) explained such decrease by the effect of TiO_2NPs exposure on the expression of genes involved in protein synthesis.

The increase activity of both AST and ALT besides the observed high HSI indicated that the fish hepatic tissue was adversely affected with the high TiO_2NP dosage. Therefore, antioxidants activity of GPx and MT were assessed to confirm this observation. Antioxidants activity were in the same line of the above mentioned results of liver enzymes and somatic indices since GPx and MT increased with the high TiO_2NP dosages while there was an insignificant difference between groups received MIC and control. A number of studies have painted a picture, which is in line with the currently predominant paradigm that nanomaterial toxicity is associated with the induction of oxidative stress (Lammel & Sturve, 2018). These authors have suggested that TiO_2NPs induce the formation of ROS leading to damage of biological macromolecules including lipids, proteins and DNA, and consequently to loss of vital cellular functions and cell death. Firat and Bozat (2018) reported that acute exposure of TiO2NPs caused decreases in activities of a number of enzymes including Gpx (37%), while its exposure for 14 days increased the activity of the

enzyme (32%). This was attributed to the fact that TiO_2NPs can potentially cause oxidative stress, which may lead to disturbance in the antioxidant enzymes systems by either stimulating or inhibiting their activities. Xiong *et al.* (2011) also observed that ROS in zebra fish exposed to 50 mg/l TiO_2NP, was elevated by 139.7% to 178.1% relative to the control group due to their protein carbonyl content. Similar changes were observed in zebrafish (Bar-Ilan *et al.,* 2013) and rainbow trout (Boyle *et al.,* 2013). In contrast, Federici *et al.* (2007) observed that rainbow trout subjected to high level of TiO_2NP (0.1-1 mg/l) for 14 days naturally still had the ability to scavenge the ROS. This conflict between the results of the present study and other studies could be explained by differences in animal's species as well as dosage, administration route, and duration of exposure to TiO_2NP.

O. niloticus challenged with *A. hydrophila* and either fed or injected with TiO_2NP MIC represented low MR%; 20 and 30% in fed and injected fish, respectively. Whereas fish treated with high TiO_2NP dosage showed a high MR% both in fed and injected groups revealing 70 and 80%, respectively. These results were anticipated due to the immunosuppression and compromised health status of the fish that occur, possibly because of high ROS activity, which was reflected by increased AST, ALT, creatinine, HSI, and SSI along with low WBCs, TP, and Glo; the decrease in globulin level may reduce the resistance of fish to pathogens (Griffitt *et al.,* 2009). Jovanovic *et al.* (2015) stated that Nano-TiO_2 is immunotoxic to fish and reduces the bactericidal function of fish neutrophils. The authors demonstrated that *Pimephales promelas* exposed to nano-TiO_2 (2 and 10 mg/g b.w.) and challenged with *A. hydrophila or Edwardsiella ictaluri* revealed a decrease in neutrophil phagocytosis rate, which resulted in increased fish mortality.

CONCLUSIONS

TiO_2NP had potential antibacterial properties and its MIC was determined to be 20 µg/g b.w. for *A. hydrophila* infection. This concentration proved no adverse impact on the health status of *O. niloticus*. The more appropriate route of administration was via fish feed. Meanwhile, irrespective to the route of administration, high dosage of TiO_2NP had immunosuppression as well as ROS generation effect therefore, not recommended to be used in fish treatment.

ACKNOWLEDGEMENT

The authors would like to thank the Agriculture Research Center, Animal Health Research Institute for financial support of this work.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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J HELLENIC VET MED SOC 2019, 70(3): 1707-1716
ΠΕΚΕ 2019, 70(3): 1707-1716

Research article
Ερευνητικό άρθρο

Seasonal variation of fatty acids composition of milk from grazing ewes in Thessaly, central Greece

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Εποχιακή διακύμανση στη σύνθεση των λιπαρών οξέων γάλακτος από προβατίνες ελευθέρας βοσκής στη Θεσσαλία της κεντρικής Ελλάδος

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Date of initial submission:
Date of revised submission:
Date of acceptance:

ABSTRACT. The aim of this work was to evaluate the changes in fatty acids (FAs) profile and conjugated linoleic acid (CLA) concentration of milk from grazing ewes in winter (December and January) and spring (April and May) in Thessaly, central Greece. No significant changes (P>0.05) in the physicochemical properties (pH and protein, lactose and total solids content) of winter and spring milk were observed. However, the fat content of spring milk was lower (P<0.05) than the winter milk. The saturated FAs content of milk was not significantly changed (P>0.05) during winter neither during spring, whereas the polyunsaturated FAs content was significantly changed (P<0.05) in each of the four months examined. Nevertheless, in the ovine milk of spring, the saturated FAs content was significantly decreased (P<0.05), but the monounsaturated and polyunsaturated FAs content was significantly increased (P<0.05) as compared to that of winter milk. In contrast to the saturated FAs decrease in spring milk, the saturated stearic acid (C18:0) content showed a significant increase (P<0.05) in the spring milk as compared to winter milk. In winter milk, the C18:2 *cis*-9, *trans*-11 CLA levels were 0.89±0.05 and 0.98±0.03g/100 g Fatty Acid Methyl Esters (FAMEs) in December and January, respectively, whereas, in spring milk, the CLA levels were significantly increased (P<0.05) to 1.36±0.04 and 1.27±0.03g/100 g FAMEs in April and May, respectively. The atherogenicity index (AI) associated with proatherogenic and antiatherogenic FAs was found significantly (P<0.05) lower in spring milk compared to winter milk.

Keywords: Fatty acids, conjugated linoleic acid**,** ovine milk, gas chromatographic analysis, grazed pasture.

ΠΕΡΙΛΗΨΗ. Σκοπός αυτής της εργασίας ήταν η αξιολόγηση των μεταβολών του προφίλ των λιπαρών οξέων και της συγκέντρωσης του συζευγμένου λινολεϊκού οξέος (CLA) του γάλακτος από προβατίνες ελευθέρας βοσκής το χειμώνα (Δεκέμβριος και Ιανουάριος) και την άνοιξη (Απρίλιος και Μάιος) στη Θεσσαλία της κεντρικής Ελλάδας. Δεν παρατηρήθηκαν σημαντικές μεταβολές (P>0,05) στις φυσικοχημικές ιδιότητες (pH και περιεκτικότητα σε πρωτεΐνη, λακτόζη και συνολικά στερεά) του γάλακτος χειμώνα και άνοιξης. Ωστόσο, η περιεκτικότητα σε λιπαρά του ανοιξιάτικου γάλακτος ήταν χαμηλότερη (P<0,05) εκείνης του χειμώνα. Η περιεκτικότητα σε κορεσμένα λιπαρά οξέα του γάλακτος δεν μεταβλήθηκε σημαντικά (P>0,05) τόσο κατά τη διάρκεια του χειμώνα όσο και κατά τη διάρκεια της άνοιξης, ενώ η περιεκτικότητα σε πολυακόρεστα λιπαρά οξέα μεταβλήθηκε σημαντικά (P <0,05) σε κάθε έναν από τους τέσσερις μήνες που εξετάστηκαν. Παρ ‘όλα αυτά, στο γάλα της άνοιξης, η περιεκτικότητα των κορεσμένων λιπαρών οξέων μειώθηκε σημαντικά (P<0,05), αλλά η συγκέντρωση των μονοακόρεστων και πολυακόρεστων λιπαρών οξέων αυξήθηκε σημαντικά (P<0,05) σε σύγκριση με το γάλα του χειμώνα. Σε αντίθεση με τη μείωση των κορεσμένων λιπαρών οξέων στο ανοιξιάτικο γάλα, η περιεκτικότητα του κορεσμένου στεατικού οξέος (C18:0) παρουσίασε σημαντική αύξηση (P<0,05) στο γάλα της άνοιξης σε σύγκριση με εκείνη του χειμώνα. Στο γάλα του χειμώνα, τα επίπεδα του C18:2 *cis*-9, *trans*-11 CLA ήταν 0.89±0.05 και 0.98±0.03 g/100g FAMEs τον Δεκέμβριο και τον Ιανουάριο, αντίστοιχα, ενώ στο γάλα της άνοιξης τα επίπεδα του CLA αυξήθηκαν σημαντικά P<0,05) σε 1.36±0.04 και 1.27±0.03 g/100g FAMEs τον Απρίλιο και τον Μάϊο, αντίστοιχα. Ο δείκτης αθηρογένεσης (AI), που σχετίζεται με λιπαρά οξέα που ευνοούν την πρόκληση αθηροσκλήρωσης αλλά και με λιπαρά οξέα που δεν ευνοούν την πρόκληση αθηροσκλήρωσης, βρέθηκε σημαντικά μικρότερος (P<0,05) στο γάλα της άνοιξης σε σχέση με εκείνο του χειμώνα.

INTRODUCTION

China is the largest producer of ovine milk in the world (12.2%) followed by Greece, which is the largest producer in Europe (8.7%) (Balthazar et al.,, 2017). According to the statistics of the Hellenic Agricultural Organization Elgo Dimitra, the production of the ovine milk amounted to 644,451,875 kg for the year 2017 (Elgo Dimitra, 2018). Also, according to the same data the regions with the highest production of ovine milk in 2017 were Thessaly (central Greece) followed by the Western Greece region. Karagouniko sheep breed is originated from Thessaly, but nowadays it is reared in several other regions in Greece. Among other local sheep breeds, Karagouniko sheep breed is characterized by a high milk production and lambing properties.

The nutritional value of ovine milk is considered quite high due to its protein, fat, minerals and vitamins content. Although the fat of the ovine milk is high and rich in saturated fatty acids (SFAs), which are incriminated for an increased risk of cardiovascular diseases, several (FAs) possess beneficial health properties, such as the monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) (Palmquist et al., 2005). Among MUFAs, the vaccenic acid C18:1 *trans*-11 VA exhibits a protective role against heart diseases and the metabolic syndrome (Wenjie et al., 2015). Among PUFAs, conjugated linoleic acid (CLA) was initially recognized for its anticancer activity (Pariza et al., 1979). CLA consists of several geometric and positional isomers of linoleic acid (C18:2) with conjugated double bonds, naturally found in ruminant dairy or meat products. In ovine milk, the predominant CLA isomer is C18:2 *cis*-9, *trans*-11 CLA (80 to 90% of total CLA) followed by C18:2 *trans*-10, *cis*-12 CLA but in lower quantities (Meluchova et al., 2008). The C18:2

cis-9, *trans*-11 CLA shows also, apart from its anticancer activity, anti-atherogenic, anti-diabetic, anti-inflammatory, and increased immune response properties, while C18:2 *trans*-10,*cis*-12 CLA exhibits anti-obesity properties and improves osteosynthesis (Fuke and Nornberg, 2017).

The FAs content of ovine milk may vary due to various reasons such as sheep breed, feeding conditions or season (De La Fuente et al., 2009). Grazing pasture is an effective feeding ways of improving the levels of MUFAs, PUFAs including CLA content in milk of lactating ewes (Soják et al., 2013). The FAs profiles of pasture varies in different seasons and thus, it affects also the profile of FAs of milk produced by lactated ewes under grazed pasture feeding conditions (Cabiddu et al., 2005; De Renobales et al., 2012). The FAs profile of milk produced by lactating Karagouniko sheep were mostly examined under conventional feeding conditions (Sinanoglou et al., 2015).

The aim of this study was to evaluate the changes in fatty acids (FAs) profile and conjugated linoleic acid (CLA) concentration of milk from grazing ewes in winter and spring in Thessaly, central Greece.

MATERIALS AND METHODS

Milk samples

Raw milk samples were taken from a Karagouniko sheep breed farm located in eastern Thessaly, central Greece. The lactating ewes were exclusively fed on grazing at the plain pastures of eastern Thessaly. Milk samples were collected in December 2016 and January 2017 as well as April 2017 and May 2017 from a bulk milk tank (a mixture of night and morning milk) for the winter and spring sampling seasons, respectively. The milk was obtained from two groups of 40 lactating ewes in each sampling season, approximately after 30-45 lamping days, according to the International Dairy Federation guidelines (IDF, 1995).

Physicochemical analysis of milk.

The fat, protein, lactose and total solids content of milk were measured using a Milko Scan 4000 (FOSS Electric, Integrated Milk Testing™, Hillerød, Denmark). The pH of the milk samples was estimated using a pH meter (WTW, type 525, Wissennchaftlich-Technische Werkstatten, GmbH, Weilheim, Germany)

Extraction of Fat

Milk fat was extracted using the method of Folch et al., (1975) with certain modifications as described by Castro-Gomez et al., (2014) and Fletouris et al., (2015). In brief, milk samples (1 ml) were mixed with 15 ml of chloroform – methanol solution (2:1, v/v). The resulting mixtures were initially homogenized for 20 min and subsequently centrifuged at 6500 rpm for 10 min at 4 °C. After 1 min pause, the mixtures were further shaken for 2 min and centrifuged at 6500 rpm for 5 min at 4 °C. The mixture was filtered through filter paper and the resulting filtrate was made to 15ml with chloroform – methanol solution. Then, 3ml of 0.74% KCl was mixed with the filtrate. After centrifugation at 6500 rpm for 5 min at 4 °C, the upper layer was removed and the bottom chloroform layer was mixed with 3g of anhydrous sodium sulfate and filtrated by using a Whatman 1-phase separator filter (Whatman, Maidstone, UK). Finally, the extract was concentrated by evaporating the chloroform in a rotary evaporator (Rotavapor RE111, BÜCHI, Switzerland) and subsequently dried over a gentle stream of nitrogen. The fat extract was kept in amber vials at -18 °C pending for (FAs) analysis.

Preparation of fatty acid methyl esters

Fatty acid methyl esters (FAMEs) were produced by using base-catalyzed methanolysis of the acyloglycerides with potassium methoxide following a standard IDF method (IDF, 2002). This IDF procedure was selected in order to restrict isomerization of FAs and CLA during methylation.

Gas chromatographic analysis of FAMEs

FAMEs analysis was accomplished using a GC-17A Shimadzu gas chromotograph (Shimadzu Scientific Instruments Inc., Kyoto Japan). The GC was equipped with an automatic injector, flame ionization detector (FID) and a split injection port. A fused capillary column of 60 m with internal diameter of 0.25 mm and film thickness 0.25 μm (model 122-2362 Agilent J&W, Agilent Technologies, Santa Clara, California, USA) was used for the FAMEs separation. After injection, the column temperature was held at 70 °C for 1min. Then, the column temperature was raised as follows, to 130 °C at a rate of 5 °C/min and kept for 14 min, to 170 °C at a rate of 5 °C/min and kept for 15 min, to 215 °C at a rate of 2 °C/min and kept for 2 min, and finally to 230 °C at a rate of 5 °C/min and kept for 6 min. The FID temperature was set at 270 °C, while the injector temperature was set at 250 °C. Helium was used as a carrier gas at a flow rate of 0.7 ml/min. The injection volume was 1 μl, while the split ratio was 1:50.

The FAMEs standards of a) Supelco FAME Mix 37 components (Sigma-Aldrich, Steinheim, Germany) and b) a mixture of *cis*-9 *trans*-11 CLA methyl esters and *trans*-10, *cis*-12 CLA methyl esters (Sigma-Aldrich, Steinheim, Germany) were used for the identification of the individual FAME by comparing their retention times. FAs percentages were measured by direct normalization of peak areas and using the Shimadzu GC solution data system program. Milk FAs were expressed as g/100 g FAMEs estimated by multiplying peak areas with correction factors according to AOAC 963.22 method (AOAC 2000). FAs analyses were carried out in triplicate.

Atherogenicity and Δ^9desaturase activity indexes

The atherogenicity index (AI) is associated with antiatherogenic and proatherogenic properties of FAs. The atherogenicity index (AI) was estimated as follows (Tsiplakou et al 2010):

AI = (C12:0 + 4xC14:0 + C16:0)/ (PUFA + MUFA).

The Δ^9-desaturase enzyme catalyzes the introduction of a *cis* double bond between carbons 9 and 10 of the saturated FA chain forming unsaturated FAs. The Δ^9desaturase activity indexes were estimated using two pairs of FAs that represent products and substrates for Δ^9desaturase action of the mammary gland. The fatty acids pairs were *cis*-9 C14:1/C14:0 and *cis*-9 trans-11 CLA/*trans*-11 C18:1. Thus, the desaturase activity index was calculated as: (product of Δ^9desaturase)/(product of Δ^9desaturase + substrate of Δ^9desaturase). The two Δ^9desaturase activity indexes C14DI and CLADI were estimated as follows (Lock and Garnsworthy, 2003):

1. C14DI: C14:1 *cis*-9 / (C14:1 *cis*-9 + C14:0)
2. CLADI: *cis*-9 *trans*-11 CLA/(*cis*-9 *trans*-11 CLA+ C18:1 *trans*-11).

Statistical analysis

All experimental data were subjected to statistical analysis of variance using the one-way ANOVA procedure of SPSS 10.05 statistical package (SPSS Ltd., Woking, Surrey, UK). The Tukey's test was applied in order to find the statistical differences between least-squares means. A probability level of P<0.05 was used in testing the statistical significance.

RESULTS AND DISCUSSION

The physicochemical properties of the ovine milk from lactating ewes of the Karagouniko sheep breed fed on grazing pasture at the Thessaly region, central Greece, in winter and spring, are shown in Table 1. No significant differences (P>0.05) as regards the pH and the protein, lactose and total solids content of ovine milk produced in winter (December or January) as well as that in spring (April or May) were observed. However, the fat content of the milk produced in spring was lower (P<0.05) than that produced in winter. The lower fat content in spring may be due to the diet inducing milk fat depression (MFD) syndrome (Bauman and Griinari 2001, Bauman et al., 2003). The MFD syndrome in lactating ruminants is caused by the high amounts of MUFA and PUFA found in the consumed feeds or pasture. It was previously reported that high amounts of MUFA and especially PUFA in ruminants' diet affects the biohydrogenation fat procedures in the rumen, forming certain fat components with antilipogenic properties which cause the fat content decrease of the milk (Bauman and Griinari, 2001). The pasture in spring was found to contain higher amounts of MUFA and PUFA than the one in winter in various Mediterranean countries (Cabiddu et al., 2005). Seasonal variations of the fat content have been reported in previous studies for ovine milk (Prandiniet al., 2004; Carloni et al., 2010), bovine (Lu et al., 2018) or caprine milk (Malissiova et al., 2015). The supplementation of feeds with fish oil, which is rich in MUFA and PUFA, affected the lipogenic genes of mammary gland and decreased the fat content of the ovine milk (Carreño et al., 2016). Previous studies on conventional feeding (hay and concentrates) of lactating Karagouniko ewes on the milk composition revealed that the fat content of the milk was dependent on the type of concentrates (Papadopoulos et al., 2002; Sinanoglou et al., 2015 ; Skoufos et al., 2017).

The changes of FAs profile of the milk produced by the Karagouniko sheep breed grazing in the pasture in winter and spring months are shown in Table 2. In all months, the predominant FA in the milk was the saturated palmitic (C16:0). Similarly, other major FAs were the myristic (C14:0) and stearic (C18:0) among SFA, *cis*-9 oleic (C18:1 *cis*-9) among MUFA, and *cis* linoleic (C18:2 *cis*) among PUFA. In line with the present study, previous studies on FA profile of ovine milk produced by sheep under grazing pasture (Atti et al., 2006; Carloni et al., 2010; Papaloukas et al., 2016) or conventional feeding conditions (Castro et al., 2009; De La Fuente et al., 2009), showed the same predominant FAs.

Table 1. Physicochemical properties of ovine milk produced by the Karagouniko sheep breed fed on grazing pasture in winter and spring

Month	Physicochemical properties of ovine milk*				
	pH	Fat (g/kg)	Protein (g/kg)	Lactose (g/kg)	Total Solids (g/kg)
December	6.69 ± 0.10	66.15 ±1.15[a]	54.24 ±1.24	46.07 ± 1.39	176.36 ± 2.52
January	6.70 ± 0.09	68.22 ± 1.27[a]	55.12 ± 1.32	45.09 ± 1.35	178.23 ± 2.23
April	6.70± 0.08	63.52 ±1.65[b]	53.62 ±1.45	± 1.27	172.14 ± 2.59
May	6.69 ± 0.09	62.75 ± 1.41[b]	52.93 ± 1.29	45.93 ± 1.35	171.51 ± 2.56

*Mean values ± standard deviation (n=9).
[a,b]Mean values followed by different letters in the same column are significantly different (P<0.05).

Comparing the sum of FA in the milk of all examined months, SFA were higher than MUFA, while PUFA were lower than MUFA (SFA>MUFA>PUFA). The same decreasing content order of the sum of the saturated and unsaturated FAs was also reported in previous studies in ovine (Ostrovsky et al., 2009; Tsiplakou et al., 2010), bovine (De Noni and Battelli, 2008) or caprine (Sampelayo et al., 2007) milk.

The FAs analysis of ovine milk produced in spring months showed that the SFA content was significantly decreased (P<0.05), but in contrast the MUFA and PUFA content was significantly increased (P<0.05) as compared to that of winter months. The FAs of ruminants' milk fat are derived from two sources: *de novo* synthesis in the mammary gland and the plasma lipids originating from the feed (Palmquist et al., 2005). MUFA and PUFA in blood plasma are also originated from the feed, by biohydrogenation of feed FA by rumen bacteria, and are excreted by the mammary gland in the milk (Bauman and Griinari 2001). The pasture plants are richer in unsaturated FAs in spring than in winter in countries with a moderate climate (Cabiddu et al., 2005). Feeding lactating ewes with pasture or lipid supplements rich in PUFA results in milk rich in MUFA and PUFA (Mierlita et al., 2011). In accordance with the present results, similar seasonal effect with a decrease in SFA, and an increase in MUFA and PUFA of the milk produced by lactating ewes (Atti et al., 2006; Meluchova et al., 2008; Abilleira et al., 2009; Carloni et al., 2010), cows (Sasanti et al., 2015) or goats (Sampelayo et al., 2007), under grazing pasture feeding conditions, were observed in previous studies.

The saturated stearic acid (C18:0) content showed a significant increase (P<0.05) in the spring milk as compared to that of winter, although total SFA showed a different behavior with a significant decrease (P<0.05) between the spring and winter months. This might be attributed to changes of the MUFA of the pasture by the rumen bacteria (Meluchova et al., 2008). It is known that stearic acid (C18:0) found in ruminants' milk is synthesized by biohydrogenation of linoleic or α-linolenic acids of the pasture to the end-product by the rumen bacteria (Palmquist et al., 2005). The levels of α-linolenic acid and linoleic acid of pasture flora are substantially increased by a percentage ranging between 20-55% in spring months as compared to those in winter months in the Mediterranean countries (Cabiddu et al., 2005). In line with our findings, an increase of stearic acid (C18:0) and a parallel decrease in SFA were also reported in previous studies for the milk produced in spring months as compared to that of winter months of lactating ewes (Abilleira et al., 2009), or cows (Chion et al., 2010) under grazing pastures feeding conditions.

In winter, the C18:2 *cis*-9, *trans*-11 CLA levels of the milk were 0.89±0.05 and 0.98±0.03 g/100 g FAMEs in December and January, respectively (Table 2). In spring, the C18:2 *cis*-9, *trans*-11 CLA levels of the milk were significantly increased (P<0.05) to 1.36±0.04 and 1.27±0.03 g/100 g FAMEs in April and May, respectively. The C18:1 *trans*-11 VA showed a similar increasing trend (Table 2). In winter, the C18:1 *trans*-11 VA levels were 2.16±0.07g/100 g FAMEs and 2.25±0.05 g/100 g FAMEs in December and January, respectively. In spring, the C18:1 *trans*-11 VA levels were significantly increased (P<0.05) to 2.52±0.09g/100 g FAMEs and 2.38±0.06 g/100 g FAMEs in April and May, respectively. The C18:2 *cis*-9, *trans*-11 CLA and C18:1 *trans*-11 VA are intermediate products of biohydrogenation of linoleic acid (C18:2) to stearic acid (C18:0) in the rumen. The C18:2 *cis*-9, *trans*-11 CLA is the initial intermediate product, while C18:1 *trans*-11 VA is rapidly formed by further biohydrogenation of the C18:2 *cis*-

9, *trans*-11 CLA. Before the complete transformation of C18:1 *trans*-11 VA to stearic acid (C18:0), parts of these two intermediate products of either C18:2 *cis*-9, *trans*-11 CLA or C18:1 *trans*-11 VA are absorbed in the small intestine of the ruminants and transferred by the blood plasma to milk. This biohydrogenation process of linoleic acid (C18:2) is accomplished by the action of *cis, trans* linoleate isomerase enzymes found in certain ruminant bacteria such as *Butyrivibrio fibrisolvens, Ruminococcus, Eubacterium*or, *Fusocillus* spp. (Palmquist et al., 2005). Other FAs such as α-linolenic acid and γ-linolenic acid, found in high amounts in the pasture, are also biohydrogenated to stearic acid in the rumen, with intermediate products of C18:2 *cis*-9, *trans*-11 CLA or C18:1 *trans*-11 VA (Bauman et al., 2003). In spring, the higher amounts of C18:1 *trans*-11 VA and C18:2 *cis*-9, *trans*-11 CLA found in ovine milk are derived from the higher PUFA amounts in grazing pasture (Abilleira et al., 2009).

In line with the present findings, seasonal variations of C18:2 *cis*-9, *trans*-11 CLA in ovine milk were observed in previous studies. Meluchova et al., (2008) reported that the bulk ovine milk produced by various Slovak sheep breeds under grazing pasture feeding conditions presented *cis*-9, *trans*-11 CLA levels of 0.71, 1.5 and 2.76 g/100 g FAME in April, June and September, respectively. De La Fuente et al., (2009) reported seasonal variations of *cis*-9, *trans*-11 CLA levels in ovine bulk milk produced by Churra sheep breeds under combined conventional and grazing pasture feeding conditions in Spain, which were 0.68, 1.02, 0.94 and 0.96 g/100 g FAMEs in winter, spring, summer and autumn, respectively. Carloni et al., (2010) examined ovine milk produced by various sheep breeds (Suffolk, Fabrianese, Sopravvissana, Sarda) in the plains and mountainous areas in Marche region (central Italy) under combined conventional or grazed pasture feeding conditions and reported seasonal variations of *cis*-9, *trans*-11 CLA values ranging from 0.01 to 0.07%, 0.08 to 1.96% and 0.50 to 2.22% FAME in December, May and July, respectively. Papaloukas et al., (2016) reported that bulk ovine milk produced by various sheep breeds fed on grazing pasture in mountainous areas in Northern Greece showed *cis*-9, *trans*-11 CLA values of 0.58, 0.98 and 1.12% FAME in winter, spring and summer, respectively. Seasonal variations of C18:2 *cis*-9, *trans*-11 CLA in milk from cows (Chion et al., 2010; Sasanti et al., 2015) or goats (Milewski et al., 2018) produced under grazing pasture feeding conditions were also reported.

Signorelli et al., (2008) examined the FAs composition of ovine milk produced by lactated ewes under the same combined pasture and concentrate feeding conditions and found *cis*-9, *trans*-11 CLA levels of 1.905%, 1.958% and 1.617% FAME for the Italian breeds, Altamurana, Gentile di Puglia and Sarda, respectively. Mierlita et al., (2011) examined the ovine milk produced by lactated ewes under the same conventional feeding conditions (hay, maize silage and concentrates) and reported high differences for the *cis*-9, *trans*-11 CLA reaching values of 1.01 and 2.67 g/100 g FAME for the Spanca and Turcana sheep breeds, respectively. However, Soják et al., (2013) did not observe any significant difference between the *cis*-9, *trans*-11 CLA levels (ca 1.54 g/100 g FAME) in the ovine milk produced under the same feeding conditions for the Tsigai, Valachian, Lacaune sheep breeds, respectively. Sinanoglou et al., (2015) examined the ovine milk produced by the Karagouniko sheep breed under conventional feeding conditions and found *cis*-9, *trans*-11 CLA levels of 0.52% and 0.66% FAME in December and June, respectively. Tsiplakou et al., (2010) reported *cis*-9, *trans*-11 CLA levels of 1.1% or 1.3% FAME for the ovine milk produced by the Karagouniko sheep breed under conventional and organic feeding conditions, respectively.

Previous studies also showed seasonal variations of C18:1 *trans*-11 VA in the ovine milk produced by lactating ewes under pasture grazing feeding conditions. The C18:1 *trans*-11 VA content in ovine milk was increased from ca 4 g/100 g FAME in March to 4.5 g/100 g FAME in April (Nudda et al., 2005). Ovine milk produced by lactating ewes fed on grazing pasture showed C18:1 *trans*-11 VA levels of 1.86%, 3.08% and 2.94% FAME in winter, spring and summer, respectively (Papaloukas et al., 2015). Various levels of C18:1 *trans*-11 VA were also reported for ovine milk produced from lactating ewes under grazing pasture feeding conditions such as 1.91-2.14 g/100 g FAME (Atti et al., 2006) or 1.05-3.95 g/100 g FAME (Meluchova et al., 2008). Sinanoglou et al., (2015) reported C18:1 *trans*-11 VA levels of 0.35 – 0.48% FAME in milk produced by the Karagouniko sheep breed under conventional feeding conditions. Seasonal variations of C18:1 *trans*-11 VA levels were also reported for milk from cows (Rego et al., 2008) or goats (Salari et al., 2016) fed grazed pasture.

Table 2. Fatty acid composition (g/100 g FAMEs) of ovine milk from lactating grazed pasture Karagouniko sheep breed in winter and spring months

Fatty acids		December		January		April		May	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
C4:0	Butyric	5.51	0.12[a]	5.36	0.12[a]	5.19	0.11[b]	5.31	0.13[b]
C6:0	Caproic	3.62	0.05	3.57	0.07	3.50	0.06	3.61	0.09
C8:0	Caprylic	2.77	0.07[a]	2.65	0.08[a]	2.49	0.06[b]	2.52	0.07[ab]
C10:0	Decanoiccapric	9.24	0.23	9.43	0.24	9.16	0.21	9.28	0.26
C11:0	Undecanoic	0.09	0.02	0.10	0.03	0.09	0.02	0.08	0.02
C12:0	Lauric	5.48	0.14[a]	5.39	0.13[a]	5.08	0.12[b]	5.14	0.14[b]
C13:0	Tridecanoic	0.73	0.08	0.75	0.09	0.79	0.07	0.77	0.08
C14:0	Myristic	11.51	0.19[a]	11.32	0.21[a]	10.67	0.24[b]	10.85	0.13[b]
C15:0	Pentadecanoic	0.91	0.08	0.90	0.09	0.83	0.07	0.76	0.09
C16:0	Palmitic	23.23	0.31[a]	22.97	0.34[a]	21.69	0.33[b]	21.94	0.27[b]
C17:0	Heptadecanoic	0.54	0.05	0.59	0.08	0.59	0.06	0.62	0.09
C18:0	Stearic	8.67	0.17[a]	8.58	0.19[a]	9.31	0.13[b]	9.36	0.14[b]
C20:0	Arachidic	0.27	0.05	0.28	0.04	0.25	0.05	0.24	0.06
C21:0	Heneicosanoic	0.06	0.01	0.06	0.02	0.08	0.02	0.07	0.02
C22:0	Behenicacid	0.06	0.02	0.06	0.02	0.08	0.01	0.08	0.02
C23:0	Tricosanoic	0.14	0.03	0.16	0.02	0.10	0.04	0.12	0.03
C14:1	Myristoleic	0.21	0.07	0.22	0.05	0.31	0.06	0.29	0.05
C15:1	*cis*-10- Pentadecanoic	0.25	0.04	0.24	0.03	0.28	0.06	0.29	0.04
C16:1	Palmitoleic	0.87	0.05[a]	0.85	0.06[a]	1.05	0.04[b]	1.01	0.05[b]
C17:1	*cis* -10- Heptadecanoic	0.25	0.02	0.26	0.04	0.23	0.02	0.24	0.02
C18:1 *trans* -n9	*trans* -9-Elaidic	0.41	0.02[a]	0.40	0.03[a]	0.50	0.04[b]	0.51	0.05[b]
C18:1 *trans* 11-n7	Vaccenic	2.16	0.07[a]	2.25	0.05[a]	2.52	0.09[b]	2.38	0.06[b]
C18:1 cis-9 n9	*cis* -9-Oleic	17.81	0.29[a]	18.13	0.17[a]	18.89	0.26[b]	18.52	0.18[b]
C22:1	Eruric	0.30	0.06	0.31	0.05	0.42	0.07	0.40	0.04
C24:1	Nervonic	0.24	0.02	0.26	0.02	0.26	0.03	0.27	0.02
C18:2	*cis* -9. *trans*-11 CLA	0.89	0.05[a]	0.98	0.03[b]	1.36	0.04[c]	1.27	0.03[b]
C18:2	*trans*-10.*cis* -12 CLA	0.08	0.01[a]	0.09	0.01[a]	0.14	0.02[b]	0.13	0.02[b]
C18:2 trans	*trans*Linolelaidic	0.37	0.05	0.39	0.06	0.44	0.04	0.41	0.05
C18:2 *cis*	*cis*Linoleic	2.35	0.08[a]	2.42	0.05[a]	2.59	0.10[b]	2.47	0.06[ab]
C20:4 n6	Arachidonic	0.17	0.03	0.18	0.02	0.19	0.02	0.17	0.04
C18:3 n6	γ-Linolenic	0.05	0.01	0.04	0.02	0.07	0.02	0.06	0.03
C18:3 n3	α-Linolenic	0.06	0.02	0.06	0.01	0.07	0.02	0.07	0.01
C20:2	*cis* -11-14- Eicosadienoic	0.08	0.02	0.09	0.02	0.08	0.02	0.09	0.02
C20:4 n3	*cis* 5.8.11.14 Eicostetranoic	0.29	0.05	0.31	0.07	0.31	0.04	0.29	0.05
C22:2	*cis* 13.16 Docosadienoic	0.10	0.02	0.11	0.02	0.13	0.03	0.12	0.02
C20:5	*cis* -5.8.11.14.17 Eicosapentanoic	0.12	0.03	0.12	0.02	0.14	0.02	0.13	0.03
C22:6	*cis* -4.7.10.13.16.19 Docosahexaenoic	0.11	0.02	0.12	0.03	0.12	0.02	0.13	0.02
	SFA	72.83	0.68[a]	72.17	0.58[a]	69.9	0.62[b]	70.75	0.54[b]
	MUFA	22.5	0.29[a]	22.92	0.23[a]	24.46	0.22[b]	23.91	0.24[c]
	PUFA	4.67	0.10[a]	4.91	0.11[b]	5.64	0.15[c]	5.34	0.12[d]
	Δ^9Desaturase index C14DI*	0.017	0.001[a]	0.019	0.002[a]	0.028	0.002[b]	0.026	0.001[b]
	Δ^9Desaturase index CLADI**	0.291	0.02[a]	0.303	0.02[a]	0.350	0.02[b]	0.347	0.01[b]
	AI***	2.868	0.07[a]	2.760	0.05[a]	2.307	0.07[b]	2.409	0.06[b]

[a,b,c,d]Mean values followed by different letters in the same row are significantly different ($P < 0.05$).
Abbreviations are: SFA=saturated fatty acids; MUFA= monounsaturated fatty acids; PUFA=polyunsaturated fatty acids
*Δ^9desaturase index C14DI = C14:1 *cis*-9 / (C14:1 *cis*-9 + C14:0)
**Δ^9desaturase index CLADI= *cis*-9 *trans*-11 CLA/ (*cis*-9 *trans*-11 CLA+ C18:1 *trans*-11)
***AI = (C12:0 + 4xC14:0 + C16:0)/ (PUFA + MUFA).

Table 3. CLA concentration (g/100 g lipids and g/100 g sample) in winter and spring months

Month	*cis*-9.*trans*-11 CLA		*trans*-10. *cis*-12 CLA	
	g/100 g lipids	g/100 g sample	g/100 g lipids	g/100 g sample
December	0.81 ± 0.08[a]	0.05 ± 0.01[a]	0.07± 0.01[a]	0.004 ± 0.001[a]
January	0.89 ± 0.07[a]	0.06 ± 0.01[a]	0.08 ± 0.01[a]	0.005± 0.001[a]
April	1.27 ± 0.09[b]	0.08 ± 0.01[b]	0.11 ± 0.01[b]	0.007 ± 0.001[b]
May	1.18 ± 0.07[b]	0.07 ± 0.01[ab]	0.10 ± 0.01[b]	0.006 ± 0.001[ab]

CLA analyses were carried out in triplicate.

[a,b]Mean values followed by different letters in the same column are significantly different (P < 0.05).

Since CLA possess several beneficial properties in human health and the large number of factors affecting its presence in dairy products, its concentration in milk as g/100 g lipids and g/100 g sample, were also estimated (Table 3). The recorded levels of *cis*-9, *trans*-11 CLA and C18:2 *trans*-10, *cis*-12 CLA in either winter or in spring milk, indicate that the ovine milk is an important source for the daily intake of CLA in the human diet. It is worthwhile to note that the recommended dietary daily intake of CLA is 0.8-3g per person (Benjamin et al., 2015).

The C18:2 *trans*-10, *cis*-12 CLA levels found in milk in winter (ca 0.08 g/100 g FAMEs) were also significantly increased (P<0.05) in spring (ca 0.13 g/100 g FAMEs). Compared to C18:2 *cis*-9, *trans*-11 CLA, the milk in both seasons presented lower levels of C18:2 *trans*-10, *cis*-12 CLA. Previous studies have also showed that the major CLA isomer is C18:2 *cis*-9, *trans*-11 CLA in ruminants' milk including ovine milk (Sampelayo et al., 2007). Under conventional feeding conditions of lactating ewes, Toral et al., (2010) and Buccioni et al., (2010) reported C18:2 *trans*-10,*cis*-12 CLA levels of 0.01-0.02 g/100 g FAME and 0.017 g/100 g FAME in the ovine milk, respectively. Under seasonal grazing feeding conditions of lactating ewes, the C18:2 *trans*-10,*cis*-12 CLA levels showed a significant increase (P<0.05) in spring (0.03-0.10% FAME) as compared to winter milk (0.01-0.02% FAMEs) (Carloni et al.,, 2010).

In the present study, the ovine milk produced in spring presented *Δ9*-desaturase activity indexes C14DI and CLADI higher (P<0.05) than those in winter. The use of Δ^9 -desaturase activity index C14DI represents better the Δ^9-desaturase activity, since C14:0 of the milk is totally formed via *de novo* synthesis in the mammary gland while *cis*-9 trans-11 CLA used in CLADI index is either formed in the rumen as an intermediate product of biohydrogenation of linoleic acid or endogenously in the mammary gland by the action of the *Δ9*-desaturase enzyme. In agreement to our work, similar seasonal variations of *Δ9*-desaturase activity indexes were reported for milk produced by lactating ewes (Soják et al., 2013) or cows (Lock and Garnsworthy, 2003) under grazed pasture feeding conditions.

The atherogenicity index (AI) is associated with proatherogenic and antiatherogenic FAs and may reflect also the atherogenicity status of FAs in produced milk. The AI index of the milk was found significantly (P<0.05) lower in spring than in winter. In agreement to the present study, seasonal variations of the atherogenicity index (AI) were reported for milk produced by lactated ewes (Gómez-Cortés et al., 2009) or cows (Nantapo et al., 2014) under grazed pasture feeding conditions.

In conclusion, the SFA were significantly higher (P<0.05) in the ovine milk of winter than that of spring, but the saturated stearic acid (C18:0) showed a different behavior with significantly lower (P<0.05) values in winter than in spring. The MUFA including C18:1 *trans*-11 VA, PUFA including C18:2 *cis*-9, *trans*-11 CLA and C18:2 *trans*-10,*cis*-12 CLA of the ovine milk were significantly higher (P<0.05) in spring than in winter months.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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J HELLENIC VET MED SOC 2019, 70(3): 1717-1726
ΠΕΚΕ 2019, 70(3): 1717-1726

Research article
Ερευνητικό άρθρο

The effect of progesterone on the anesthetic and analgesic requirements for ovariohysterectomy in the dog

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ABSTRACT. The objective of the current study was to investigate the effect of serum progesterone concentration, either endogenous, during the ovarian cycle and pregnancy, or exogenous, when administered during anestrus, and of its active metabolite allopregnanolone, on anesthetic and analgesic requirements, as well as post-operative pain intensity, for the performance of ovariohysterectomy in dogs. One hundred and fifty healthy female dogs, which were admitted to our clinic for elective ovariohysterectomy, were included in the present study. They were allocated to 6 groups according to the stage of the ovarian cycle and the corresponding serum progesterone concentration. The six groups consisted of dogs in anestrus (group A), in anestrus which received intramuscular progesterone injections prior to surgery (group Ap), dogs in diestrus (group D), in diestrus which received subcutaneous aglepristone injections prior to surgery (group Da), in diestrus which received oral trilostane prior to surgery (group Dt) and dogs in pregnancy of duration of 28-42 days (group P). Serum progesterone concentrations were measured in all dogs before and after any hormonal treatment and serum allopregnanolone concentrations were measured in selected dogs from all groups. The required dose of propofol for induction of anesthesia and the required isoflurane concentration for maintenance of anesthesia and the need for intraoperative fentanyl administration and extra postoperative pethidine analgesia were recorded. After statistical analysis, there were no significant differences between groups, regarding their anesthetic or analgesic requirements, that could be attributed to serum progesterone and/or allopregnanolone concentration. However, moderate correlations within certain groups were noted. Serum progesterone or allopregnanolone concentrations do not seem to have an effect on anesthetic and analgesic requirements for ovariohysterectomy in the dog or any potential effect is weak enough to be masked by the action of anesthetic premedication and/or analgesic and/or anaesthetic drugs used.

Keywords: Progesterone, allopregnanolone, dog, anesthesia, ovariohysterectomy.

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Date of initial submission: 21-01-2019
Date of revised submission: 19-08-2019
Date of acceptance: 25-08-2019

ΠΕΡΙΛΗΨΗ. Η παρούσα μελέτη διερεύνησε την επίδραση της συγκέντρωσης της προγεστερόνης, είτε ενδογενούς κατά τον ωοθηκικό κύκλο και την κυοφορία, είτε εξωγενούς, καθώς και του ενεργού μεταβολίτη της αλλοπρεγνανολόνη, στις απαιτήσεις σε αναισθητικά και αναλγητικά φάρμακα και στην ένταση του μετεγχειρητικού πόνου για τη διεξαγωγή ωοθηκυστερεκτομής στον σκύλο. Περιελήφθησαν 150 υγιείς, θηλυκοί σκύλοι, οι οποίο προσκομίστηκαν στην Κλινική Ζώων Συντροφιάς του ΑΠΘ για ωοθηκυστερεκτομή και οι οποίοι κατανεμήθηκαν σε 6 ομάδες με βάση το στάδιο του ωοθηκικού κύκλου και την ενδεχόμενη ορμονική αγωγή που έλαβαν. Συγκεκριμένα, σχηματίστηκαν 6 ομάδες από ζώα στο στάδιο του ανοίστρου (ομάδα Α), ζώα σε άνοιστρο που έλαβαν προγεστερόνη ενδομυϊκώς (Απ), ζώα στο στάδιο του διοίστρου (Δ), ζώα σε δίοιστρο που έλαβαν υποδορίως αγλεπριστόνη (Δα), ζώα σε δίοιστρο που έλαβαν κάψουλες τριλοστάνης (Δτ) και ζώα σε κυοφορία 28-42 ημερών (Ε). Σε κάθε ζώο καταγράφηκαν οι απαιτήσεις σε προποφόλη για εγκατάσταση της γενικής αναισθησίας, οι απαιτήσεις σε ισοφλουράνιο για διατήρηση, οι απαιτήσεις σε επιπλέον αναλγητικά φάρμακα είτε διεγχειρητικά είτε μετεγχειρητικά και έγιναν μετεγχειρητικές εκτιμήσεις πόνου. Επίσης έγιναν αιμοληψίες σε συγκεκριμένες χρονικές στιγμές για ορμονολογικές αναλύσεις. Μετά τη στατιστική επεξεργασία των αποτελεσμάτων δε βρέθηκε στατιστικώς σημαντική διαφορά στις απαιτήσεις σε αναισθητικά ή σε αναλγητικά φάρμακα μεταξύ των ομάδων, ούτε στατιστικώς σημαντική συσχέτιση μεταξύ των απαιτήσεων αυτών και των συγκεντρώσεων στο αίμα της προγεστερόνης ή της αλλοπρεγνανολόνης, εκτός από 2 συσχετίσεις εντός συγκεκριμένων ομάδων. Συμπεραίνεται ότι οι συγκεντρώσεις προγεστερόνης ή αλλοπρεγνανολόνης στο αίμα δεν επηρεάζουν τις απαιτήσεις σε αναισθητικά ή αναλγητικά φάρμακα για τη διεξαγωγή ωοθηκυστερεκτομής στο σκύλο ή ότι ενδεχόμενη τέτοια επιρροή επικαλύπτεται από τη χορήγηση προαναισθητικής αγωγής και αναλγητικών φαρμάκων.

Λέξεις ευρετηρίασης: Προγεστερόνη, αλλοπρεγνανολόνη, σκύλος, αναισθησία, ωοθηκυστερεκτομή.

INTRODUCTION

The anesthetic effect of steroid hormones was first reported by Hans Selye in 1941, who injected progesterone, desoxycorticosterone (DCA) and testosterone in rats (1941a). Selye documented deep sedation immediately after the first two intraperitoneal injections, and about an hour after the testosterone injection, with more pronounced effect on female rats and longer duration of anesthesia in rats that had been partially hepatectomised. The author also noted a synergistic action of progesterone with chloroform, increasing the potency and duration of its action (Selye 1941b). Tanifuji et al. (1986) injected male dogs with progesterone and reported a significant decrease in halothane's minimum alveolar concentration (MAC), the same way Datta et al. reported decreased halothane MAC in rabbits (1989). The most recent relevant study was published by Shimizu et al. (2010) regarding the significant sevoflurane sparing effect of a single subcutaneous injection of progesterone for the loss of righting reflex (LRR) in male mice.

Decreases in MAC of volatile anesthetics attributable to high concentrations of endogenous progesterone were first recorded in animal species during pregnancy, specifically in ewes (Palahniuk et al. 1974) and in rats (Strout and Nahrwold 1981). Chinese researchers studied groups of women receiving anesthesia for termination of early pregnancy, and found decreased MAC for isoflurane, halothane and enflurane compared with non-pregnant women (Chan et al. 1996; Gin and Chan 1994). Consequent studies found decreased thiopentone requirements for hypnosis and anesthesia and decreased propofol requirements for loss of consciousness in the same setting (Gin et al. 1997; Mongardon et al. 2009), and the first study to prove a correlation between the decreased sevoflurane MAC and high progesterone concentration was published in 2006 (Erden et al. 2005). Such correlations were shown again in women receiving anesthesia in 2014. In specific, significant negative correlations were found between progesterone serum concentration and propofol requirements for loss of consciousness in early pregnancy (Fu et al. 2014), as well as sevoflurane requirements in late pregnancy in women receiving anesthesia for caesarian section (Lee et al. 2014).

Atkinson et al. (1965) tested 142 progesterone metabolites on mice and discovered 67 to have anesthetic properties, depending on their chemical structure. Progesterone metabolites site of action was discovered to be the chloride ion channel of the $GABA_A$ receptor (Majewska et al. 1986), and the metabolite responsible for progesterone anesthetic effect was proven to be allopregnanolone (Korneyev and Costa 1996).

The serum concentration of progesterone during the ovarian cycle in the dog is not greatly affected by pregnancy, i.e. even in the case of non-pregnancy, after proestrus and estrus, there is a 2 month period,

called diestrus, during which the progesterone serum concentration is high (Concannon et al. 1975). This makes the dog an ideal candidate for the study of progesterone's effect on anesthetic requirements, excluding any effects of a simultaneous pregnancy, which is known to lower volatile anesthetic requirements through changes in respiratory function (Cugell et al. 1953). In theory, dogs undergoing surgery during a period of increased progesterone serum concentrations (diestrus) should have lower anesthetic requirements than dogs in anestrus, when the concentration of progesterone is baseline (Concannon et al. 1975). The aim of the present study was to investigate the effects of increased progesterone serum concentrations on the anesthetic and analgesic requirements and on the intensity of postoperative pain in dogs undergoing ovariohysterectomy (OHE).

MATERIALS AND METHODS

This double-blind, placebo-controlled, prospective clinical study was conducted at the Companion Animal Clinic, Veterinary School, Aristotle University of Thessaloniki, Greece and was approved by the university's Ethics Committee (number of approval 16/21-2-2012). All owners of animals included in the study were thoroughly informed and signed a consent form. The study included 150 female dogs that were admitted to the clinic for elective OHE. All dogs were classified as ASA status I (American Society of Anesthesiologists physical status I) after clinical examination, complete blood count and examination of the genital system. Exclusion criteria were bodyweight lower than 4kg, age less than 8 months or more than 8 years, abnormal findings in any examination performed, positive results for leishmaniosis, ehrlichiosis or heartworm disease, previous hormonal treatments for the manipulation of the ovarian cycle, blood serum progesterone concentration in the range 2-5 ng/ml, pregnancy of less than 28 days, considered insignificant, or more than 42, considered late pregnancy, as well as overt aggression, which complicates the hospitalization of the dog.

The stage of the ovarian cycle was determined based on the reproductive history of each animal, the results of the examination of the genital system, microscopic examination of vaginal smears and the serum progesterone concentration measurements. During the initial classification of the animals in groups, a commercial chromatographic progesterone kit was used (OVULATION® TEST, VIRBAC), and progesterone concentration was measured in blood serum later on. Animals with a progesterone concentration ≤2 ng/ml were considered to be in anestrus and those with a concentration ≥5 ng/ml in diestrus. Pregnancy was diagnosed by abdominal ultrasonography (micro convex head, 5-10 MHz) and pregnancy duration was calculated using suitable software (EsaoteMyLabOne Vet, Esaote Europe B.V.).

Groups, hormonal treatments, blood sampling and assays

All animals were hospitalized for a minimum of 5 days and OHE was performed on day 3. Dogs in diestrus were assigned to 3 groups according to the hormonal treatment they received prior to surgery, dogs in anestrus were assigned to 2 groups accordingly, and pregnant animals formed 1 group. Assignment of animals in the respective groups was random, based on a random order table. All animals received, on days 1 and 2, two injections plus po capsules per day, either of hormonal treatments or placebos. Placebos were normal saline in the injections and empty capsule shells. In specific, the animals were assigned to 6 groups, as follows:

- group A (anestrus): dogs in anestrus that received only placebos (n=40)
- group Ap (anestrus-progesterone): dogs in anestrus that received progesterone (GESTONE®, NORDIC PHARMA, 5mg/kg im SID) on days 1 and 2 and sc placebo injections and po placebo capsule cells (n=29).
- group D (diestrus): dogs in diestrus that received only placebos (n= 23)
- group Da (diestrus-aglepristone): dogs in diestrus that received aglepristone (ALIZIN®, VIRBAC, 10mg/kg sc SID) on days 1 and 2 and im placebo injections and po placebo capsule cells (n= 17).
- group Dt (diestrus-trilostane): dogs in diestrus that received im and sc placebo injections and trilostane (VETORYL®, ALTAVET, one 60 mg capsule in animals under 20 kg and two 60 mg capsules in animals over 20 kg po BID) from day 1 till the morning of day 3 prior to the surgery (n= 11).
- group P (pregnancy): pregnant dogs that received only placebos (n= 30)

Blood was collected 3 or 4 times for hormonal as-

says. In all dogs, blood sample 1 was collected on day 1, before administration of any hormonal treatment or placebo, sample 2 on day 3 just prior to surgery and after administration of all hormonal treatments or placebo, and sample 3 was collected 5 hours after the end of surgery. Sample 4 was collected only from animals of groups D and Dt on day 5. Progesterone was measured in all animals in samples 1-3, allopregnanolone was measured in sample 2 in randomly selected animals from all groups and cortisol was measured in samples 1-4 in the animals of groups D and Dt.

Progesterone and cortisol concentrations were measured from blood serum using commercial electrochemiluminescence kits (cobas® cortisol II, cobas® progesterone III, Elecsys 2010, Roche Diagnostics) and allopregnanolone was measured using a quantitative sandwich ELISA kit (www.mybiosource.com).

Anesthetic management

All animals received their last meal on the night of day 2 and had free access to water up to 1 hour prior to administration of pre-anesthetic medication. Anesthetic management was supervised in all cases by the same anesthesiologist in charge (CK), who was blinded to the group the animal had been assigned to. After standard pre-anesthetic clinical examination, anesthetic premedication was administered intramuscularly consisting of acepromazine (0.05 mg/kg, CALMIVET®, VETOQUINOL) and pethidine (3 mg/kg, FAMAR), followed immediately by a subcutaneous injection of carprofen (4 mg/kg, RIMADYL®, PFIZER). Thirty minutes later, the cephalic vein was cannulated and anesthesia was induced with propofol (PROPOFOL MCT/LCT/FRESENIUS 1%, FRESENIUS KABI) administered intravenously in consecutive doses of 1 mg/kg, approximately 1 minute apart, until intubation of the trachea was easily feasible. After a cuffed endotracheal tube of appropriate diameter was in place, anesthesia was maintained with isoflurane (Forrane, Baxter Healthcare Ltd) in 100% oxygen administered via a suitable anesthetic circuit. A circle rebreathing circuit was used for animals weighing 8 kg or more and a non-rebreathing circuit for smaller animals. Prophylactic cefuroxime (Zinacef, Glaxo Smith Kline) was then administered intravenously in all animals and an isotonic crystalloid solution (Lactated Ringer's injection, VIOSER) intravenous infusion commenced and was continued until the end of surgery. After clipping and surgical preparation of the abdomen, the dog was transferred to the operating room, where monitoring commenced, consisting of electrocardiography, pulse oximetry, oscillometric blood pressure measurement (PC Scout, SpaceLabs Medical Inc.) and capnography and measurement of inspired and expired isoflurane concentrations (CapnomacUltima, Datex-Engstrom). The monitors and gas analyzer were calibrated according to the manufacturers' instructions prior to the study. Heart rate (HR), respiratory rate (RR) and systolic, diastolic and mean blood pressures (BP), as well as end-tidal carbon dioxide and inspired and expired concentrations of isoflurane were constantly monitored and recorded every 5 minutes until the end of surgery.

The vaporizer dial was initially set to deliver 2.5% isoflurane, but within a few minutes it was adjusted so as to provide the appropriate depth of surgical anesthesia, based on clinical assessment and monitored parameters. The depth of anesthesia was constantly evaluated by the anesthesiologist in charge (CK). In case of a 20% or more increase in HR and/or RR and/or BP associated to noxious stimuli the depth of anesthesia was deemed inadequate and the isoflurane dial-setting was increased by 0.5% and the oxygen flow was temporarily increased to 4 L/min. If this manipulation proved ineffective in normalizing the value of the elevated parameter within 2-3 minutes, fentanyl (Fentanyl, Jannsen Pharmaceutica NV) was administered intravenously (0.002 mg/kg). When surgery was completed, the administration of isoflurane and Lactated Ringer's solution were discontinued and the dogs were allowed to recover. All operations were performed by the same team of surgeons.

Pain and sedation were evaluated 1, 3 and 5 hours after the end of surgery by the anesthesiologist in charge (CK) using a visual analogue scale (0%: no pain, 100%: worst pain imaginable) (0%: no sedation, 100%: very heavily sedated). The pain assessment was based on the animals' posture, movement and response to palpation of the abdomen as described previously by Savvas et al. (2008). Intramuscular pethidine was administered as rescue analgesia in case of a pain score higher than 50% in any assessment and repeated as needed.

Statistical analysis

The required number of animals in each group for an effect size of 0.3 and achieved power at least 0.8 was calculated to be 19 (total number of animals 114).

All data were copied to digital spreadsheets (Mic-

rosoft Excel 2007) and analyzed using the SPSS v.15 software. Propofol requirements were calculated as total mg/kg, fentanyl and rescue analgesia requirements were recorded as "required" or "not required" and isoflurane requirements were calculated using the serial end- tidal concentrations recorded during surgery. Area under the curve (AUC) was calculated from those measurements, as proposed by Matthews et al. (1990), and then divided by the total duration of surgery, which varied between cases, to calculate the time standardized AUC (AUC_std) in each case, as described by Lawrence and De Lange(1997).

The normality of distribution of the data was checked using the Shapiro-Wilk normality test. A generalized linear model was used to evaluate differences between serum cortisol concentrations between groups D and Dt, as well as post operation pain scores between groups. Differences in propofol or isoflurane requirements were evaluated using one way analysis of variance (one way ANOVA), when evaluating 3 or more groups, and Student's T-test when evaluating 2 groups. A chi-squared test was used to evaluate differences between groups regarding fentanyl or rescue analgesia requirements and possible correlations between various factors were checked using Pearson's test. Values of $p \leq 0.05$ were considered statistically significant. Descriptive statistics are presented as mean ± standard deviation.

RESULTS

The distributions of weight, age, duration of anesthesia, duration of surgery, propofol requirements for induction, isoflurane AUC_st, pain scores and sedation scores were found to be normal in all 6 groups (p = 0.103-0.983). There were no statistically significant differences between groups regarding age, weight, duration of anesthesia and duration of surgery (p=0.131-0.653).

The serum progesterone concentrations in group Ap were significantly higher compared to those of group A after the im administration of progesterone (p<0.0005). The serum progesterone concentrations of group Dt were significantly lower than those of group D after the administration of trilostane (p<0.0005), but those of group Da were not significantly affected, when compared to group D, after the administration of aglepristone (p=0.31).

Propofol requirements ranged from 1 to 10 mg/kg with a mean of 3.88 ± 1.11 mg/kg. There was no significant difference between main groups A, D and P (p = 0.713), diestrus groups D, Da and Dt (p = 0.378) or between anestrus groups A and Ap (p = 0.857). No correlation was found between propofol requirements and progesterone or allopregnanolone concentration, except a moderate inverse correlation between progesterone and propofol in group Da (r = -0.569, p = 0.017).

Isoflurane requirements (AUC_std) ranged from 0.7 to 2.68% with a mean of 1.65 ± 0.26%. No significant difference was discovered after comparison between groups A, D and P (p = 0.109), groups D, Da and Dt (p = 0.623) or groups A and Ap (p = 0.601). There was no significant correlation between isoflurane requirements and progesterone or allopregnanolone concentration, except a moderate inverse correlation between progesterone and AUC_std in group P (r = -0.39, p = 0.033).

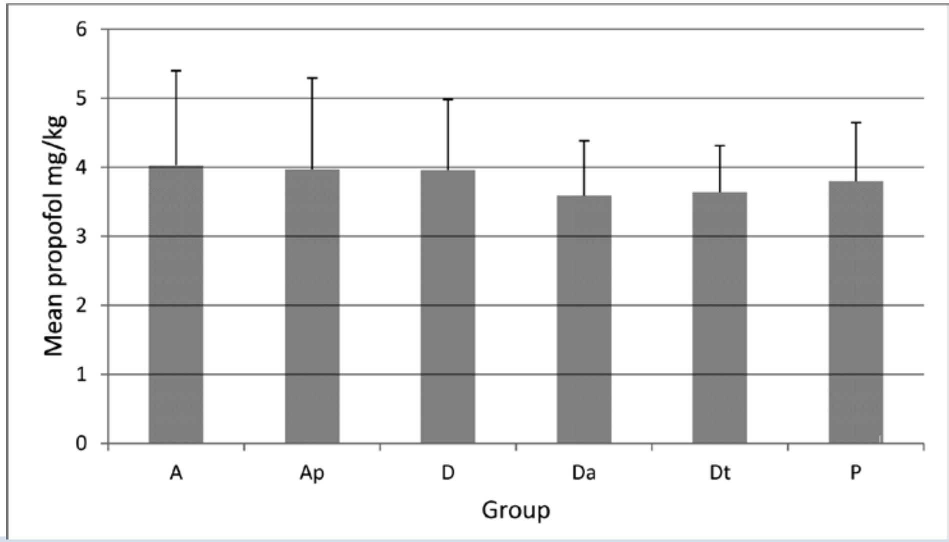

Figure 1.

There were no significant differences between the groups regarding the incidence of fentanyl intra-operative analgesia (p = 0.811) or the incidence of post-operative pethidine rescue analgesia (p = 0.858).

Concerning the post-operative pain scores, there were significant differences within each group over time (p<0.05). There were no significant differences between groups at the specific time points (p>0.05), with the exception of groups D and Dt, where significantly higher scores were found in group Dt at all 3 time points (1h p = 0.033, 3h p = 0.022, and 6h p = 0.01). There was no statistically significant correlation between pain scores (1h) and the progesterone (sample 2) (r=0.068, p=0.405) or allopregnanolone (r=0.064, p=0.565) concentrations.

Sedation scores did not differ significantly between groups at any time point (1h p=0.571, 3h p=0.198, 6h p=0.24).

The serum cortisol concentrations between groups D and Dt were significantly different in blood samples 2 and 3 (p = 0.02 and p = 0.09), the concentrations of group D being higher, as opposed to samples 1 and 4 (p = 0.566 and p = 0.075).

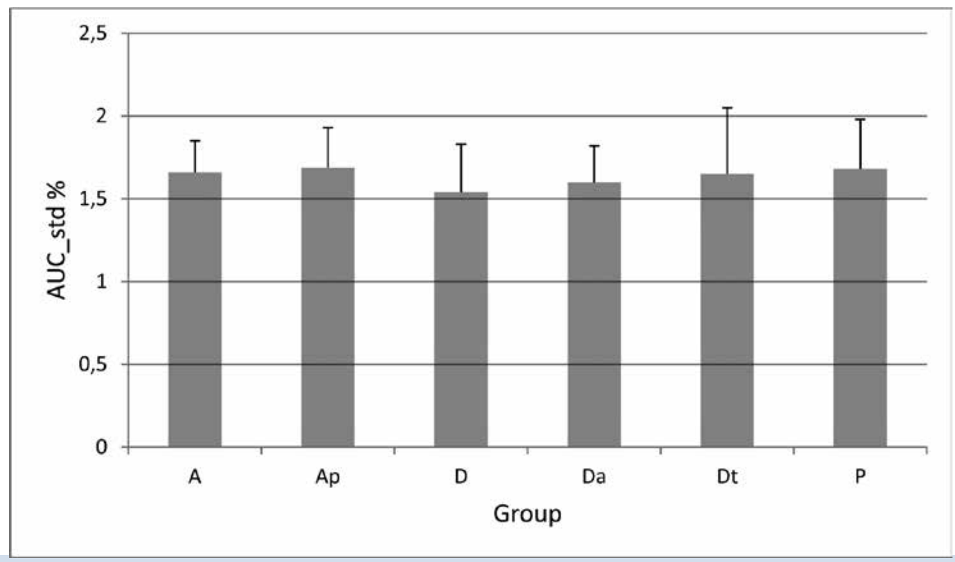

Figure 2.

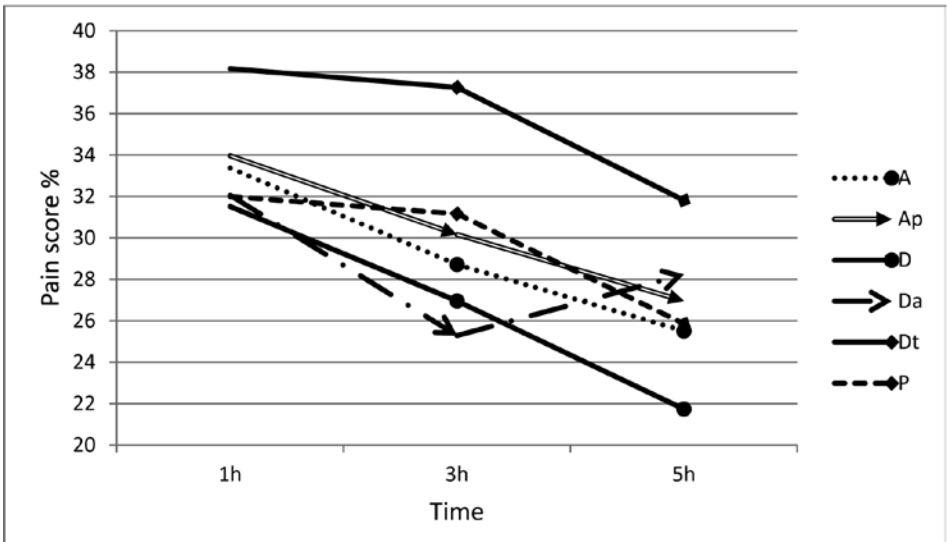

Figure 3.

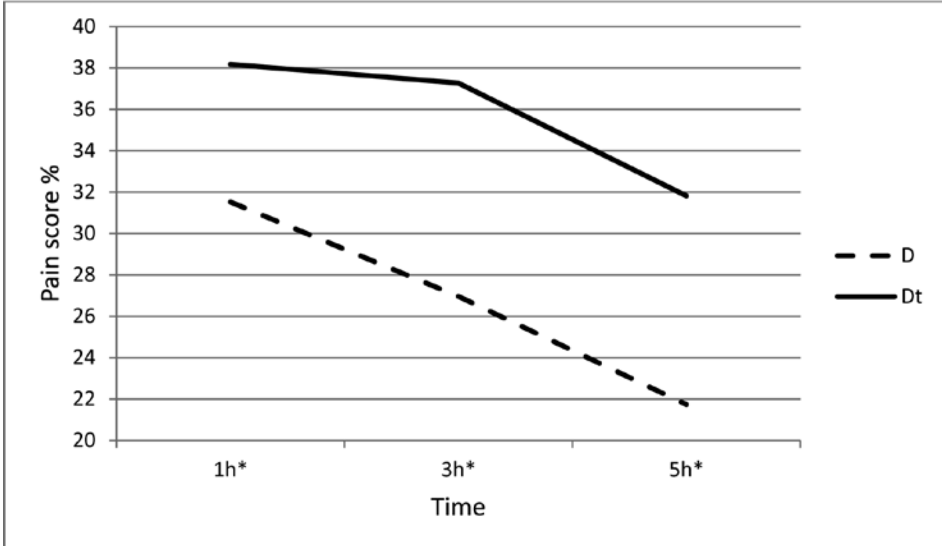

Figure 4.

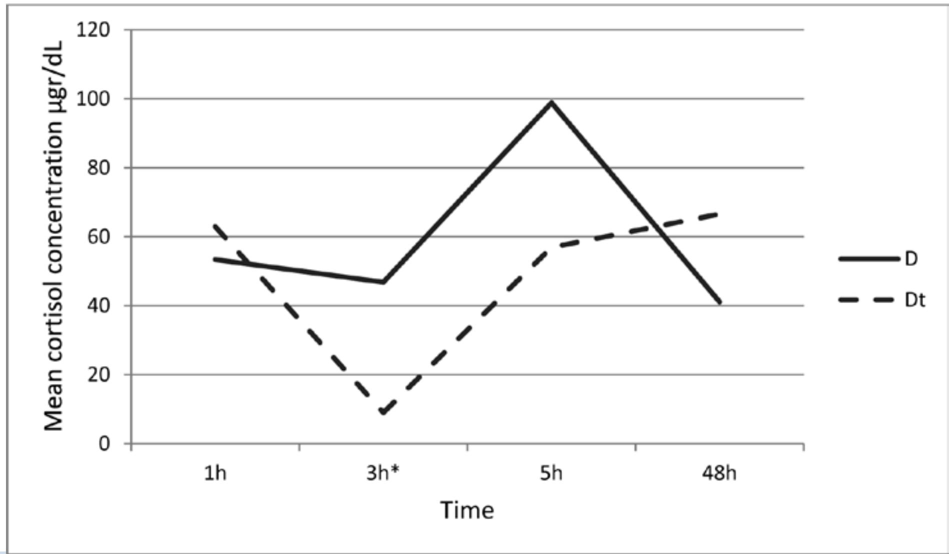

Figure 5.

DISCUSSION

The anesthetic effect of progesterone was discovered in 1941 by Hans Selye (1941a) and its volatile anesthetic sparing effect was documented in dogs, rabbits and mice (Datta et al. 1989; Shimizu et al. 2010; Tanifuji et al. 1986). High levels of endogenous progesterone have also been found to lower requirements for both volatile (Chan et al. 1996; Erden et al. 2005; Gin and Chan 1994; Lee et al. 2014; Palahniuk et al. 1974; Strout and Nahrwold 1981) and injectable anesthetics (Fu et al. 2014; Gin et al. 1997; Mongardon et al. 2009) in various species, including humans. The present study, on the contrary, did not detect any difference in anesthetic or analgesic requirements in dogs, regardless of their baseline or high serum progesterone concentration.

Selye's study was the first report of steroid anesthetic action and inspired all future studies, which eventually lead to the discovery and marketing of steroid anesthetics. However, very large doses of progesterone were used to achieve anesthesia and the blood progesterone concentrations were not reported, so one cannot compare to the endogenous levels normally occurring during the ovarian cycle or pregnancy. Selye injected 100gr rats with 35mg of progesterone, nearly 350 mg/kg. Merryman et al. (1954) tried to reproduce Selye's results in humans, by injecting 500mg of progesterone via infusion over 1 hour, which caused sleep, not anesthesia, similar to Arafat et al. who reported sleep after a 400mg per os

administration (1988). It seems, therefore, that progesterone's anesthetic effect requires large doses.

Progesterone's MAC-reducing effect is reported in many studies (Datta et al. 1989; Erden et al. 2005; Lee et al. 2014; Selye 1941a; Shimizu et al. 2010; Tanifuji et al. 1986). The determination of MAC for volatile anesthetics, although a universal standard for anesthetic potency, is based on a time consuming laboratory procedure and does not correspond to clinical surgical conditions (Eger et al. 1965). It also does not take into consideration preanesthetic or analgesic drugs, which were used in all cases in our study, and could have masked progesterone's anesthetic sparing effect. Other methodologies used in other studies, such as the modified sevoflurane pump used by Lee et al. to calculate the volume of liquid sevoflurane consumed (2014), Mongardon et al.'s automated propofol infusion pump which calculated a predicted effect site concentration of propofol based on a pharmacokinetic algorithm (2009), and even LRR, as used by Shimitzu et al. on mice (2010), are considered laboratory procedures, not common clinical practice, and most are not even applicable to dogs.

The main finding of the present study is that increased progesterone serum concentrations do not reduce the anesthetic and/or analgesic requirements or the intensity of post-operative pain in dogs undergoing OHE. Since no anesthetic-sparing effect of progesterone was shown in the present study, no speculations can be made about the potential role of progesterone receptors (blocked by aglepristone in group Da). Moreover, pregnancy did not influence the anesthetic and/or analgesic requirements or the intensity of post-operative pain. Pregnancy could have influenced via mechanisms unrelated to any potential action of progesterone (e.g. via changes in cardiovascular or respiratory function or via mechanical factors related to the size of the gravid uterus affecting the function of other systems), but no significant effect was shown. Exogenous administration of progesterone in our study did not induce any anesthetic-sparing effect, as one would expect since diestrus and pregnancy did not either induce such an effect.

The most comparable study to the present is the one by Tanifuji et al. (1986), as it was performed on dogs, and serum progesterone concentrations were measured. Tanifuji et al. injected 6 intact male dogs for 1 week with progesterone and calculated halothane MAC using the tail clamp technique. They used 2 dosages of progesterone, 2mg/kg and 5 mg/kg, and in both cases found halothane MAC to be significantly decreased with a strong negative correlation to progesterone concentration. The progesterone concentration increased from control concentration 1.46 ± 0.22 to 4.41 ± 0.7 and 8.91 ± 1.97 ng/ml. In our study, on the other hand, 29 female dogs were injected with 5 mg/kg of progesterone for 2 days and the serum progesterone concentration increased from 1.24 ± 2.97 to 36.44 ± 18.08 ng/ml, and still no statistically significant difference in isoflurane requirements was found when compared to the anestrus group. The difference in the results could be attributed to shorter duration of progesterone administration to female dogs in the present study (2 days) compared to a longer time of administration (1 week) to male dogs in the study by Tanifuji et al. However, it is the authors' opinion that a more likely explanation is that although progesterone shows MAC-reducing effects in a controlled setting, this effect is probably not intense enough to be detected during real-time surgery in the clinical setting.

The current study assessed the isoflurane requirements for performance of surgery in dogs, while in other relevant studies (Datta et al. 1989; Tanifuji et al. 1986) the MAC calculation was used as a simulation of surgical stimulation. It is likely that high serum progesterone levels indeed have an anesthetic effect that can be detected in a laboratory environment, but this effect is probably not so intense as to be shown as anesthetic-sparing effect in the clinical setting during surgery. When trying to answer the question whether the veterinary anesthetist should be prepared for the possibility that lower doses of anesthetics/analgesics might be required for performance of surgery in a dog in diestrus or pregnancy as opposed to a dog in anestrus, the methodology of the current study is more helpful in giving the correct answer than studies based on MAC determination.

The negative correlation that was detected in group P between serum progesterone concentrations and isoflurane AUC_std could be a reflection of this anesthetic effect that high serum progesterone exerts and which is depicted in studies based on MAC determination. Thus, there could be a real progesterone influence, at least in pregnant animals, but which, if present, seems to be too weak to be detected as reduced isoflurane requirements for maintenance of anesthesia, at least with the methodology used in the present study. Concerning the negative correlation detected between serum progesterone concentrations and propofol requirements in group Da, it could be

speculated that unavailability of cellular progesterone receptors occupied by aglepristone could result in more progesterone being available for metabolism to allopregnanolone and thus for action on GABA receptors, leading to central nervous system depression. As in group P for isoflurane, however, it seems that any effect of aglepristone is not intense enough to be detected as a reduction in anesthetic/analgesic requirements during surgery. The two correlations noted in the two groups P and Da taken together could be considered as indications of a potential central nervous system-depressive action of progesterone under specific circumstances that might be required for this action to be highlighted.

Trilostane was administered as it inhibits steroid synthesis from the adrenal cortex, including both cortisol and progesterone (de Gier et al. 2011), since more specific progesterone synthesis inhibitors, like epostane or azastane, were not available even for research purposes. Since trilostane inhibits cortisol synthesis, serum cortisol was monitored in groups D and Dt in blood samples 1 through 4. The serum cortisol concentration was significantly lower in group Dt compared to group D in blood samples 2 and 3 ($p = 0.002$ and $p = 0.009$ respectively) but was not lower in blood sample 4 ($p = 0.075$), indicating that cortisol synthesis returns to pre-administration levels within 48 hours after the last trilostane administration. This is the first report, to our knowledge, of cortisol's synthesis recovery after trilostane administration in healthy dogs, since all relevant publications study cortisol synthesis inhibition in dogs suffering from Cushing's disease.

Post-operative pain scores were significantly higher in group Dt compared to those of control group D, but the incidence of fentanyl intra-operative analgesia or pethidine post-operative analgesia was not higher in group Dt. Detection of higher pain scores in a group of dogs (Dt) that received a drug with a "pro-algesic action" (deprivation of synthesis of an endogenous hormone with anti-inflammatory effects) can be viewed as a confirmation that the pain-assessing tool used in the present study was effective in discriminating animals in more pain.

A limitation of the current study is the small number of animals in group Dt in comparison to the other groups, owing to the fact that trilostane, the only steroid synthesis inhibitor available, was commercially unavailable for a substantial period of the duration of the study. Regarding the methodology used in the current study for the detection of increased isoflurane requirements, instead of MAC calculation, it is the authors' opinion that it is more representative of clinical surgical conditions and would detect differences significant enough to suggest changes in the anesthetic management of such cases. A similar methodology was used by Lawrence and De Lange (1997) in their human study on surgeries of varying duration, and by Columbano et al. (2012) in dogs undergoing OHE.

CONCLUSIONS

In conclusion, progesterone, either endogenous during the ovarian cycle and pregnancy, or administered intramuscularly, does not affect anesthetic or analgesic requirements for the performance of ovariohysterectomy in dogs. In the author's opinion, any anesthetic effect of progesterone is not intense enough and/or is masked by the use of pre-anesthetic medications and analgesic drugs, such as non-steroidal anti-inflammatory drugs and opioids. Dogs receiving trilostane have significantly higher post-operative pain scores than dogs not receiving trilostane, so extra analgesic care is warranted in such animals undergoing surgery.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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J HELLENIC VET MED SOC 2019, 70(3): 1727-1732
ΠΕΚΕ 2019, 70(3): 1727-1732

Research article
Ερευνητικό άρθρο

Comparison of Lidocaine-Xylazine and Procaine-Xylazine for Lumbar Epidural Anesthesia in Cattle

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ABSTRACT. Lumbar epidural anesthesia is commonly utilized in veterinary medicine for diagnostic, obstetrical, and surgical intervention in the abdominal and perineal regions of large animals. The aim of this study was to directly compare the time to onset and duration of epidural anesthesia produced by lidocaine-xylazine and procaine-xylazine combinations to that produced by xylazine alone in cattle. A total of 24 healthy adult Holstein dairy cows were included in this study. The time to onset and duration of anesthesia were recorded. The heart rate, respiratory rate, and rectal temperature were recorded at 0 minute and at 10, 20, 30, 60, and 90 minutes after the epidural administration of each treatment. The time to onset of anesthesia did not significantly differ between the xylazine only group and the lidocaine-xylazine and procaine-xylazine combination groups. The duration of anesthesia in the xylazine only group was significantly shorter than that in the lidocaine-xylazine and procaine-xylazine combination groups ($p < 0.05$). Ataxia was not observed in any group. The heart rate, respiratory rate, and rectal temperature values in all the treatment groups throughout the study did not significantly differ from those at baseline.

We found that administration of procaine hydrochloride in combination with xylazine hydrochloride, an α2-adrenergic receptor agonist, offers the same time to onset and duration of anesthesia as does epidural anesthesia using a combination of lidocaine hydrochloride and xylazine hydrochloride. Furthermore, this combination of treatments did not cause adverse effects in the cardiovascular and respiratory systems. These findings indicate that combined administration of procaine and xylazine is an economic and useful approach for epidural anesthesia.

Keywords: Cow; epidural anesthesia; lidocaine; procaine; xylazine

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Date of initial submission: 17-01-2019
Date of revised submission: 04-02-2019
Date of acceptance: 27-02-2019

INTRODUCTION

Administration of general anesthesia to ruminants may involve aspiration due to regurgitation of the contents of the rumen and saliva (Trim, 1981; Hall et al., 2001), which creates a risk of bloating and muscle injury (Skarda and Tranquilli, 2007). In addition, the equipment used to deliver general anesthesia is expensive; it can be difficult to administer anesthesia to cattle in clinical fields, as it is necessary for another person to monitor the anesthesia during use. For this reason, local anesthesia is common when performing surgery or examination in ruminants with the animal in the standing position.

Local anesthesia procedures used in the ruminants include paravertebral nerve blocks, local administration, epidural anesthesia, and intravenous regional anesthesia of a distal limb (Skarda and Tranquilli, 2007). Among these approaches, the technique of epidural anesthesia includes the use of a dorsolumbar segment epidural block and a low-posterior or caudal epidural block. The former is commonly performed in conjunction with surgery of the gastrointestinal organs, such as abomasum reduction, rupture repair, intestinal surgery, and cesarean section with abdominal laparotomy assistance. The latter is widely used in conjunction with surgeries to repair anal and perineal cleft lacerations, or uterine or rectal prolapse, to alleviate difficulties occurring during delivery or ovum collection, and to restore control of urination and defecation (Elmore, 1980; Skarda, 1996). All the above-mentioned procedures can be performed with the animal maintaining the standing position.

Local anesthetics indiscriminately block motor, sensory, and sympathetic nerve fibers (Day and Skarda, 1991) that cause vasodilatation, which is attributed mainly to the inhibition of action potentials via sodium channel blocking in sympathetic nerves that cause vasoconstriction (Newton et al., 2007). They may also cause ataxia, hind limb weakness, and, occasionally, recumbency. Generally, lidocaine, mepivacaine, bupivacaine, and procaine are used for epidural anesthesia (Day and Skarda, 1991). However, these drugs are often administered in combination with α_2-adrenergic receptor agonists to extend the period of anesthesia (Grubb et al., 2002; McGrath and Richey, 2003) and minimize the risk of hind limb weakness and recumbency (Luttinger et al., 1985; Eisenach et al., 1996; Natalini and Robinson, 2000).

The purpose of this study was to compare anesthesia produced by lidocaine + xylazine, a combination of anesthetics commonly administered during epidural anesthesia of ruminants, and procaine hydrochloride + xylazine, a combination of ester-type local anesthetics, with respect to the time of onset and duration of anesthesia, heart rate (HR), respiratory rate (RR), and rectal temperature (RT).

MATERIALS AND METHODS

All animal experiments were performed in compliance with the Guide for the Animal Care and Use Committee at Azabu University, School of Veterinary Medicine (No. 160829-3).

The study included 24 healthy Holstein dairy cows (aged 28.2-98.9 months, weighing 406-698 kg, Body Condition Score 2.75-4.00) from a commercial dairy farm. These cows were randomized by age, body weight, and body condition score, and divided into five groups (Table 1).

Cows were restrained in stanchions. The epidural needle (16-gauge, 12 cm in length; Hakko Syoji., Tokyo, Japan) was inserted into the first to second lumbar (L1-L2) epidural space in standing cattle with the bevel pointed forward. Proper placement of the needle was determined by loss of resistance and by the hanging drop technique, which can be performed by placing a few drops of sterile water or lidocaine into the needle hub during insertion (Skarda and Tranquilli, 2007).

The dosage of anesthetics in each group was determined by the method explained in a previous report (Ismail, 2016). The xylazine only group (XY) was administered 0.05 mg/kg b.w. of xylazine hydrochloride (Selactar 0.2%; Bayer, Ltd., Tokyo, Japan). The xylazine hydrochloride and lidocaine hydrochloride combination groups (XL-1 and XL-2) were administered a combination of xylazine hydrochloride (0.05 mg/kg b.w. for both groups) and 2% lidocaine hydrochloride (0.10 and 0.20 mg/kg b.w., respectively; LIDOCAINE Hydrochloride Injection 2 %; Pfizer Japan Inc., Tokyo, Japan). The xylazine hydrochloride and procaine hydrochloride combination groups (XP-1 and XP-2) were administered a combination of xylazine hydrochloride (0.05 mg/kg b.w. for both groups) and 2% procaine hydrochloride (0.10 and 0.20 mg/kg b.w., respectively; Procaine Hydrochloride Injection KS; Kyoritsu Seiyaku Inc., Tokyo, Japan). All the drugs were diluted with 0.9% saline to a final volume of 5 ml.

Table 1. Characteristics of the experimental cows

No.	Group	xylazine (mg/kg)	Local analgesic (mg/kg)		Body weight (kg)	BCS[a)]
			lidocaine	procaine		
1					488.0	3.00
2	XY	0.05	–	–	582.0	3.25
3					593.5	3.50
4					560.0	3.50
					555.9 ± 47.3	3.3±0.2
5					575.0	3.25
6					478.0	2.75
7	XL-1	0.05	0.1	–	588.5	3.50
8					555.0	3.25
9					587.0	3.50
					556.7 ± 46.0	3.3±0.3
10					554.5	3.50
11					488.5	3.00
12	XL-2	0.05	0.2	–	579.3	3.50
13					623.0	4.00
14					530.0	2.75
					555.1 ± 50.7	3.4±0.5
15					578.5	3.00
16					481.0	2.75
17	XP-1	0.05	–	0.1	526.5	3.00
18					545.0	3.50
19					651.5	4.00
					556.5 ± 63.7	3.3±0.5
20					436.0	2.75
21					527.0	3.50
22	XP-2	0.05	–	0.2	595.5	3.50
23					645.0	4.00
24					559.0	3.00
					552.5 ± 78.5	3.4±0.5

a) Body condition score.

The administered fluids were warmed to rectal temperature and injected gradually for over approximately 30 s to prevent collapse.

The analgesic effect was evaluated in terms of the presence or absence of a reaction at the left or right abdominal skin surface as a result of a pinprick test, and application of pinch pressure owing to the hemostatic force applied using forceps (when set to the first ratchet). After drug administration, the time to loss of sensation was considered the time to onset of anesthesia. The time from loss of sensation to restoration of sensation was considered as the duration of anesthesia. Determination of the time to onset of anesthesia was achieved via monitoring until loss of sensation was confirmed and at 10, 20, 30, 60, and 90 minutes afterward.

The HR, RR, and RT were similarly measured prior to drug administration (at 0, baseline value), and at 10, 20, 30, 60, and 90 minutes after administration. The HR was measured via an electrocardiogram (BIO-SCOPE, AM 130; Fukuda ME Kogyo, Tokyo, Japan). The RR was measured based on a 1-minute auscultation, and the RT was measured using an electronic thermometer (ThermoVision J, TV-714J; Astec, Ibaragi, Japan).

Continuous data were reported as the mean ± standard deviation, if the distribution was normal. A one-way analysis of variance (ANOVA) was performed to determine differences in "time to onset of anesthesia" and "duration of anesthesia." A two-way repeated-measures ANOVA (treatment as a between-subject factor and time as the repeated measures variable)

was performed to determine differences in the HR, RR, and RT. Between-group comparisons for continuous variables were conducted using an unpaired t-test. Within-group comparisons for continuous variables were conducted using a paired t-test. A P-value of < 0.05 was accepted as statistically significant. Statistical analyses were performed with SPSS version 22.0 for Windows (IBM Japan, Tokyo, Japan).

RESULTS

The epidural injection was easy to perform and well tolerated by all the experimental animals.

The time to onset of anesthesia and duration of anesthesia are presented in Table 2. The time to onset of anesthesia did not significantly differ among the XY (14.5 ± 1.8 min), XL-1 (11.1 ± 3.3 min), XL-2 (12.3 ± 3.4 min), XP-1 (11.5 ± 3.5 min), and XP-2 (11.8 ± 3.2 min) groups.

The duration of anesthesia was significantly shorter in the XY group (68 ± 26.0 min) than in the XL-1 (215 ± 56.0 min), XL-2 (220 ± 55.1 min), XP-1 (205 ± 47.1 min), and XP-2 (214 ± 20.1 min) groups (P < 0.05 for all). Ataxia was not observed in any of the studies cows. The HR, RR, and RT are presented in Table 3. The values of these measures did not significantly differ from the baseline values throughout the study in any treatment group (Table 3).

Table 2. Anesthetic indices of epidural administration of xylazine (XY), xylazine-lidocaine (XL-1, XL-2), and xylazine-procaine (XP-1, XP-2) in cows (mean ± SD)

Indices	XY	XL-1	XL-2	XP-1	XP-2
Onset of analgesia (min)	14.5 ± 1.8	11.1 ± 3.3	12.3 ± 3.4	11.5 ± 3.5	11.8 ± 3.2
Duration of analgesia (min)	68 ± 26.0†	215 ± 56.0	220 ± 55.1	205 ± 47.1	214 ± 20.1

† Significant differences between the duration of XY with XL-1 and XL-2, XP-1, XP-2.

Table 3. Heart rate (HR), respiratory rate (RR), and rectal temperature (RT) in cows treated with epidural administration of xylazine (XY), xylazine-lidocaine (XL-1, XL-2), and xylazine-procaine (XP-1, XP-2) combination (mean ± SD)

Treatment	Time (min)					
	0	10	20	30	60	90
HR (beat/min)						
XY	68.3 ± 9.8	54.8 ± 4.3	57.2 ± 4.1	67.5 ±1.0	68.5 ± 3.4	66.5 ± 3.0
XL-1	68.8 ± 6.7	68.4 ± 1.7	70.6 ± 3.0	67.2 ± 2.3	70.4 ± 5.0	66.0 ± 2.8
XL-2	67.2 ± 9.8	69.2 ± 7.0	65.2 ± 4.4	66.4 ± 3.6	66.4 ± 4.8	67.2 ± 5.9
XP-1	69.6 ± 7.4	69.2 ± 5.2	66.4 ± 3.3	68.8 ± 2.3	69.6 ± 2.6	68.4 ± 2.2
XP-2	68.0 ± 5.3	68.4 ± 3.8	67.6 ± 5.4	67.6 ± 3.6	68.4 ± 1.7	68.0 ± 1.4
RR (breath/min)						
XY	23.5 ± 3.4	21.6 ± 2.5	24.4 ± 4.4	25.6 ± 1.9	30.8 ± 1.0	37.2 ± 1.6
XL-1	23.2 ± 2.3	23.2 ± 1.1	23.2 ± 1.1	21.6 ± 1.7	24.0 ± 2.0	23.6 ± 1.7
XL-2	23.6 ± 2.6	24.4 ± 5.0	24.4 ± 3.3	24.0 ± 1.4	25.6 ± 3.0	24.8 ± 3.0
XP-1	25.6 ± 3.3	24.4 ± 1.7	24.0 ± 2.0	25.6 ± 1.7	27.2 ± 1.8	25.6 ± 1.7
XP-2	24.4 ± 3.0	26.4 ± 2.6	26.4 ± 1.7	24.8 ± 3.3	25.6 ± 2.6	25.2 ± 1.1
RT (°C)						
XY	38.3 ± 0.1	38.4 ± 0.1	38.5 ± 0.1	38.5 ± 0.1	38.5 ± 0.2	38.5 ± 0.1
XL-1	38.3 ± 0.2	38.3 ± 0.2	38.4 ± 0.2	38.3 ± 0.2	38.4 ± 0.2	38.4 ± 0.2
XL-2	38.3 ± 0.1	38.3 ± 0.1	38.4 ± 0.3	38.4 ± 0.3	38.4 ± 0.3	38.4 ± 0.3
XP-1	38.2 ± 0.3	38.3 ± 0.2	38.4 ± 0.3	38.4 ± 0.3	38.4 ± 0.2	38.4 ± 0.3
XP-2	38.3 ± 0.2	38.3 ± 0.2	38.5 ± 0.1	38.4 ± 0.1	38.5 ± 0.1	38.3 ± 0.2

HR, Heart rate; RR, Respiratory rate; RT, Rectal temperature

DISCUSSION

Epidural anesthesia is a useful technique in ruminant animals, that is widely used in conjunction with not only abdominal surgeries, but also in perineal surgeries and procedures to implant fertilized eggs (Elmore, 1980; Skarda, 1996). In cattle and small ruminants, 2% lidocaine is used for epidural anesthesia (Mama, 2013). In addition, by administering an α_2-adrenergic receptor agonist in combination with lidocaine, the dosage of local anesthetic can be reduced while also extending the duration of the analgesic effect (Hall, 2000; Skarda, 1996; Grubb, 2002). Epidural xylazine also results in mild to moderate sedation and mild ataxia (Saifzadeh et al., 2007; Shekidef and Saleh, 2011).

Procaine was the first artificially-synthesized ester-type local anesthetic, with a mechanism characterized by prevention of neurotransmission via blockage of Na channels, which is similar to that of other local anesthetics. Procaine has a high ionization constant (pKa) at 8.9 and low lipid solubility at 0.6. Although procaine offers immediate anesthesia onset, it is classified as a short-acting local anesthetic, and has been used less frequently for non-invasive anesthesia in recent years. Thus, there have been few comparisons of procaine administration to lidocaine and bupivacaine administration as an epidural anesthetic.

Moreover, lidocaine hydrochloride offers stronger topical and visceral anesthetic effects for a longer duration, than does procaine. However, when converting the degree of neuropathic toxicity from the clinically applicable concentration, lidocaine hydrochloride has relatively greater toxicity. As per the reports, it is 2.5-fold more toxic than procaine hydrochloride and 13.2-fold more toxic than mepivacaine hydrochloride (Kasaba et al., 2003). Administration of lidocaine hydrochloride in high doses also increases the risk of ataxia and recumbency due to motor paralysis of the hind limbs (Ismail, 2016).

However, in this study, no subject developed ataxia following administration of either lidocaine or procaine. In addition, by administering procaine hydrochloride, a short-acting anesthetic that is rarely used for epidural anesthesia, in combination with xylazine hydrochloride, an α_2-adrenergic receptor agonist, it is possible to obtain the same duration of epidural anesthesia as that produced by lidocaine combined with xylazine, without the risk of adverse cardiovascular or respiratory effects. These results indicate that procaine+ xylazine is a useful combination of anesthetics for providing simple epidural anesthesia.

CONCLUSION

Lumbar epidural anesthesia is commonly used in veterinary medicine for diagnostic, obstetrical, and surgical interventions in the abdominal and perineal regions of large animals. This study was performed to directly compare the time to onset and duration of epidural anesthesia produced by lidocaine-xylazine and procaine-xylazine combinations with that produced by xylazine alone in cattle. The time to onset of anesthesia did not differ significantly among the groups, but the duration of anesthesia in the xylazine-only group was significantly shorter than that in the other groups.

We found that administration of procaine hydrochloride in combination with xylazine hydrochloride, an α2- adrenergic receptor agonist, offers the same time to onset and duration of anesthesia as does epidural anesthesia using a combination of lidocaine hydrochloride and xylazine hydrochloride. Furthermore, this combination of treatments did not cause adverse effects in the cardiovascular and respiratory systems. These findings indicate that combined administration of procaine and xylazine is an economic and useful approach for epidural anesthesia.

ACKNOWLEDGMENTS

The authors thank the staff and student assistants at Azabu University for taking care of the heifer during hospitalization. This work was partially supported by a research project grant awarded by the Azabu University Research Services Division.

CONFLICTS OF INTEREST STATEMENT

The authors declare no conflict of interest.

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J HELLENIC VET MED SOC 2019, 70(3): 1733-1736
ΠΕΚΕ 2019, 70(3): 1733-1736

Case report
Κλινικό περιστατικό

Caudal cruciate ligament avulsion at its origin in a dog

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Απόσπαση της έκφυσης του οπίσθιου χιαστού συνδέσμου σε σκύλο

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ABSTRACT. Caudal cruciate ligament (CaCL) rupture is uncommon in dogs and usually occurs with a concurrent rupture of the cranial cruciate ligament (CrCL). A 10-month-old cross-bred dog was presented with left hind limb lameness. Orthopaedic examination revealed positive craniocaudal drawer sign in the left stifle. Arthrotomy confirmed CrCL rupture, and showed CaCL avulsion fracture at its origin. The stifle was stabilized using extracapsular lateral fabellotibial suture. Eight months postoperatively the dog was free of obvious lameness and remained sound until the last re-evaluation (3 years). This case raises the possibility that restoration of the CaCL function is not always essential for animals' successful outcome.

***Keywords*: **Caudal cruciate ligament, cranial cruciate ligament, dog, lameness

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Date of initial submission: 06-10-2018
Date of revised submission: 8-11-2018
Date of acceptance: 03-12-2018

ΠΕΡΙΛΗΨΗ. Η ρήξη του οπίσθιου χιαστού συνδέσμου είναι σπάνια στον σκύλο και συνήθως συνυπάρχει με ταυτόχρονη ρήξη του πρόσθιου χιαστού συνδέσμου. Ένας ημίαιμος σκύλος ηλικίας 10 μηνών προσκομίστηκε με χωλότητα του οπίσθιου δεξιού άκρου. Η ορθοπαιδική εξέταση αποκάλυψε θετική προσθιοπίσθια συρταρωτή κίνηση στο δεξιό γόνατο. Η αρθροτομή επιβεβαίωσε τη ρήξη του πρόσθιου χιαστού συνδέσμου και έδειξε και απόσπαση του οπίσθιου χιαστού συνδέσμου στην πρόσφυσή του. Το γόνατο σταθεροποιήθηκε με τη χρήση εξωαρθρικής τεχνικής (νάιλον ράμμα μεταξύ έξω σησαμοειδούς και κνημιαίου κυρτώματος). Οκτώ μήνες μετεγχειρητικά ο σκύλος δεν παρουσίαζε εμφανή χωλότητα και παραμένει έτσι μέχρι και την τελευταία επανεξέταση (3 χρόνια). Το περιστατικό αυτό εγείρει την πιθανότητα ότι η αποκατάσταση της λειτουργίας του οπίσθιου χιαστού συνδέσμου δεν είναι πάντα απαραίτητη για την επιτυχή έκβαση των ζώων.

Λέξεις ευρετηρίασης: Οπίσθιος χιαστός σύνδεσμος, πρόσθιος χιαστός σύνδεσμος, σκύλος, χωλότητα

INTRODUCTION

The caudal cruciate ligament (CaCL) is the primary stabilizer against caudal tibial subluxation. In combination with the cranial cruciate ligament (CrCL), limits internal rotation of the tibia and hyperextension of the stifle (Arnoczky et al., 1977). Isolated rupture of the canine CaCL is very rare and in many cases coexists with rupture of the CrCL and/or medial collateral ligament of the stifle (Kowaleski et al., 2012). A rare case of CaCL avulsion fracture at its origin, which was treated successfully, is presented.

CASE HISTORY

A male, 10-month-old, 30 kg, cross-bred was referred due to sudden onset left hind limb lameness of 1 month duration. There was no history of trauma. Lameness was observed after the dog had run unattended in a grassland. Orthopaedic examination revealed mild to moderate lameness in the left hindlimb with severe pain, medial thickening and craniocaudal drawer movement in the left stifle. Tibial thrust was also present. Moderate joint effusion with reduced visibility of the infrapatellar fat pad was visible radiographically, while the popliteal sesamoid was caudodistally displaced. At least three bone opacities were visible superimposed on the lateral surface of the medial femoral condyle suggestive of possible avulsion fracture of the caudal cruciate ligament. Slight new bone formation was visible on the patellar apex and on the distal aspect of the medial gastrocnemius sesamoid bone is also visible (Figure 1a,b). Diagnosis of left stifle osteoarthritis compatible with CrCL rupture was established, but differential diagnosis included CaCL rupture, too.

Lateral arthrotomy of the affected stifle was performed and initial diagnosis of CrCL rupture was confirmed (Johnson, 2014). Also, CaCL avulsion fracture at its origin and an articular cartilage defect in the middle of the medial trochlear ridge were observed (Figure 2). The medial meniscus was not intact and partial meniscectomy was performed. An attempt was made to ostesynthesize the avulsed bone fragment, however, it was impossible to be reduced and it was removed. The stifle was stabilized using double extracapsular lateral fabellotibial suture 100 lb and both cranial and caudal drawer movements of the stifle were ceased.

Two months after surgery the lameness of the operated limb was very mild, while after 8 months the gait seemed to be normal on observation. Pain and crepitus were not clinically identified in the stifle. Osteoarthritic lesions were mainly restricted on the intercondyloid eminence and the lateral fabella. Two years postoperatively, the clinical view of the dog was unchanged, while signs of mildly worsened left stifle osteoarthritis were identified radiographically (Figure 3).

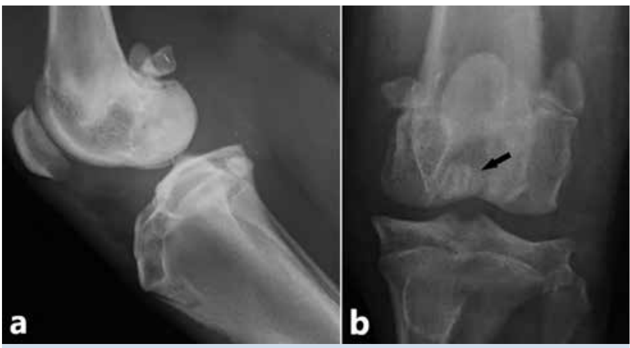

Figure 1. Mediolateral (a) and caudocranial (b) radiographs of the left stifle one month after both CLs rupture. Moderate joint effusion with reduced visibility of the infrapatellar fat pad. The popliteal sesamoid appears caudodistally displaced. At least three bone opacities are visible superimposed on the lateral surface of the medial femoral condyle (arrow) that likely reflect the multiple fragments of an avulsion fracture at the origin of the caudal cruciate ligament in the intercondylar fossa identified surgically. Slight new bone formation is visible on the patellar apex and on the distal aspect of the medial gastrocnemius sesamoid bone is also visible+

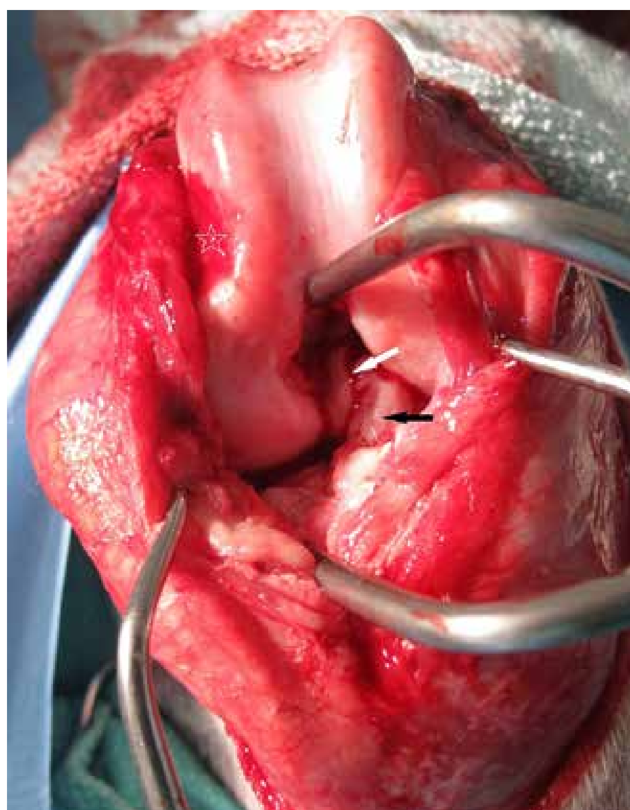

Figure 2. Lateral arthrotomy of the left stifle: Avulsion fracture of the femoral attachment of the CaCL (white arrow), CrCL rupture (black arrow) and articular cartilage defect in the middle of the medial trochlear ridge (white star)

Figure 3. Mediolateral (a) and caudocranial (b) radiographic views of the left stifle taken 2 years after extracapsular stabilization. There is mild increased new bone formation, most notably on the intercondyloid eminence and the lateral gastrocnemius sesamoid bone. Small osseous opacities are superimposed medially and caudally on the joint. The lucent area visible in the tibial tuberosity seen on the lateral view represents a hole drilled to pass the suture material in connection with the extracapsular stabilization procedure. Crimps are noted on the lateral aspect of the stifle. Moderate joint effusion is still visible, mildly less than preoperatively. Mild thickening of the patellar ligament is also visible

DISCUSSION

Avulsion fracture of the attachments of the CaCL is uncommon. Indeed, few cases of CaCL fracture at its insertion (Wong 1994; Soderstrom et al., 1998), and only one at its origin (Monotti et al., 2015) have been published. Our case was treated successfully using extracapsular lateral fabellotibial suture only for the management of CrCL instability.

The cause of CaCL rupture is usually traumatic (Fossum et al., 2013). Although, in this case, the owner was not aware of any accident, perhaps the defect in the femoral trochlear ridge is the evidence of a violent percussion.

Differentiation of caudal from cranial drawer movement of the stifle is difficult. In a retrospective study of 14 dogs of single CaCL rupture, half of the cases were misdiagnosed as having CrCL rupture, and also in 9/14 dogs with CaCL rupture, this was not included in the differential diagnoses list (Johnson and Olmstead 1987). Accurate diagnosis of CaCL avulsion fracture requires a high index of suspicion for this disorder; in addition, an understanding of the neutral position of the tibia and of the direction of displacement is necessary for correct diagnosis of caudal drawer (Kowaleski et al., 2012). In this animal, orthopaedic examination revealed only the CrCL rupture, while the CaCL one was suspected radiographically and confirmed with arthrotomy.

Radiographs are important in CaCL injuries because of their frequent association with other traumatic injuries and because of a higher percentage of avulsion injuries than with the CrCL. This probably results from the CaCL being larger and stronger than the CrCL and therefore resisting rupture but predisposing to avulsion (DeCamp et al., 2016). Computer tomography (CT) scan best demonstrated the CaCL avulsion fracture at its origin in a dog, while radiography and magnetic resonance imaging failed (Monotti et al., 2015). Unfortunately, in our case, CT was not performed due to financial constrains.

Stifles with CaCL injury could be treated with resection of the remnants of the ligament and stabilization by one of several extracapsular reconstruction techniques, e.g. suture stabilization, redirection of the medial collateral ligament, or popliteal tendon tenodesis (Fossum et al., 2013). Also, in case of avulsion fracture of the attachments of the CaCL various techniques have been proposed (DeCamp et al., 2016).

However, there must be some doubt about the necessity of stabilizing the stifle following CaCL injury, based on experimental transection in seven dogs. In a study, six months after transection and partial excision of the ligament, none of the dogs was lame al-

though there was still a positive caudal drawer movement present. It is interesting that none of the dogs developed osteoarthritis (Harari et al., 1987). Indeed, surgical stabilization of an isolated rupture of the CaCL is only suggested in working and sporting dogs or when it occurs with another ligamentous injury of the stifle (DeCamp et al., 2016). Monotti et al (2015) treated their case conservatively, due to minimally displaced avulsion, with strict rest and a non-steroid anti-inflammatory drug. Although their follow-up is short, the dog regained excellent use of the limb after 6 weeks (Monotti et al., 2015). In our case, the attempt to reduce the avulsed femoral attachment of the CaCL was failed due to the ligament contraction, which related to the chronicity. The use of extracapsular lateral fabellotibial suture to restore CrCL function stabilized the stifle enough to cease the craniocaudal drawer movement. So, additional stabilization for the CaCL rupture did not use. It is interesting that the dog seemed to be lameness free on re-examinations, even 3 years postoperatively, although osteoarthritis was worsened radiographically. This assessment was made during observation, which is a subjective method of estimating the movement of the animal, but if the equipment for gait analysis was available, mild disturbances could be detected. At any rate, it is well known that osteoarthritic clinical findings do not always correspond with radiographic ones.

In conclusion, rupture of the CaCL, with or without concurrent CrCL one, is difficult to diagnose clinically. Based on the clinical outcome from our study, restoration of the CaCL function is not always essential for animals' successful outcome.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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J HELLENIC VET MED SOC 2019, 70(3): 1737-1742
ΠΕΚΕ 2019, 70(3): 1737-1742

Case report
Κλινικό περιστατικό

Spinal nephroblastoma in a dog: a case report

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Νεφροβλάστωμα νωτιαίου μυελού σε σκύλο: αναφορά σε ένα κλινικό περιστατικό

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Date of initial submission: 06-12-2018
Date of revised submission: 10-02-2019
Date of acceptance: 19-02- 2019

ABSTRACT. The case report presents the clinical, clinicopathological, diagnostic imaging and histopathological findings of spinal cord nephroblastoma in a dog. It was admitted with a 2-week history of a "weight-bearing" lameness of the left posterior limb that evolved to paraplegia within hours. Neurological examination showed spastic paraplegia compatible with a T3-L3 spinal cord lesion. Differential diagnosis included ischemic myelopathy, myelitis and acute spinal cord compression. The spinal radiographs and cerebrospinal fluid analysis were unremarkable. Cisternal myelography indicated a focal intradural-extramedullary lesion at the level of T12 vertebra. Further diagnostic investigation was not performed because the owner decided to proceed to euthanasia. Histopathological examination of the spinal cord confirmed the diagnosis of intraspinal nephroblastoma.

Keywords: dog, lameness, myelopathy, neoplasm, nephroblastoma.

ΠΕΡΙΛΗΨΗ. Στην εργασία αυτή παρουσιάζεται περιστατικό νεφροβλαστώματος του νωτιαίου μυελού σε νεαρό σκύλο, που προσκομίστηκε με αιφνίδια παραπληγία. Συγκεκριμένα, στο ιστορικό αναφέρθηκε αρχικά χωλότητα στο οπίσθιο αριστερό άκρο που εμφανίστηκε δεκαπέντε ημέρες πριν την προσκόμιση του ζώου και εξελίχθηκε ραγδαία σε παραπληγία. Στη νευρολογική εξέταση διαπιστώθηκε σπαστική παραπληγία (θωρακοοσφυϊκό σύνδρομο, Θ3-Ο3) και η αρχική διαφορική διάγνωση περιλάμβανε την ισχαιμική μυελοπάθεια, τη μυελίτιδα και την οξεία συμπίεση του νωτιαίου μυελού. Στα ακτινογραφήματα της σπονδυλικής στήλης και στην ανάλυση του εγκεφαλονωτιαίου υγρού δεν υπήρξαν παθολογικά ευρήματα. Στη μυελογραφία διαπιστώθηκε εντοπισμένη υποσκληρίδια εξωμυελική χωροκατακτητική βλάβη στο ύψος του Θ12 σπονδύλου. Η διερεύνηση του περιστατικού δεν συνεχίστηκε ύστερα από απόφαση του ιδιοκτήτη να γίνει ευθανασία. Η οριστική διάγνωση τέθηκε με την ιστοπαθολογική εξέταση που έδειξε ότι επρόκειτο για υποσκληρίδιο νεφροβλάστωμα.

Λέξεις ευρετηρίασης: μυελοπάθεια, νεόπλασμα, νεφροβλάστωμα, νωτιαίος μυελός, σκύλος.

CASE HISTORY

A 2-year-old, client-owned, male, intact, mixed-breed dog, was presented with a history of acute paraplegia evolving within hours. According to case anamnesis the animal developed an acute, weight-bearing lameness of the left posterior limb 15 days prior to presentation that subsequently evolved to paraparesis and paraplegia within hours. The dog had been treated symptomatically with non-steroidal anti-inflammatory drugs (NSAID) without any noted improvement. Physical examination was unremarkable. The dog was paraplegic with preservation of spinal reflexes and deep pain sensation. Neurological signs were compatible with a grade IV T3-L3 spinal cord lesion. Differential diagnosis included ischemic myelopathy, myelitis and acute spinal cord compression due to traumatic intervertebral disk rupture, spinal arachnoid pseudocyst or congenital vertebral anomalies. Myelography showed a marked stop of contrast medium above the caudal end of T12 vertebra, with an evident "golf-tee" sign, suggesting a focal intradural-extramedullary lesion (Figure 1, 2). The owner declined further investigation and elected euthanasia, as the prognosis for recovery was poor.

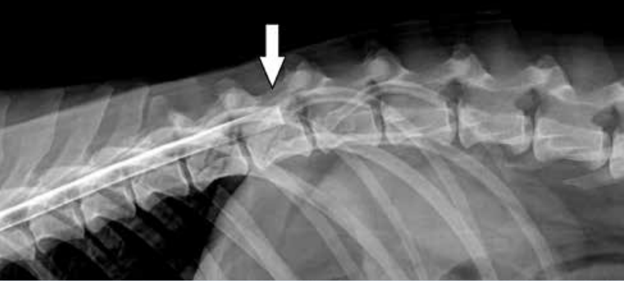

Figure 1. Cisternal myelogram of the thoracolumbar region of the spine indicating a complete obstruction (arrow) of the contrast medium at the caudal end of T12 vertebra in lateral view.

Gross macroscopic findings during necropsy included a lentiform, intradural-extramedullary multilobular, partially cystic proliferation with extensive lateral spinal cord compression, leading to a reduction for about 75% of the original diameter (Figure 3, 4). Samples from the mass were embedded in paraffin and were stained with Hematoxylin /Eosin and Giemsa staining. Histology revealed a predominantly extra-axial, lobulated and partially cystic mass which had grown in direct contact to the piamater, was attached to the nerve root as well as a focal, well circumscribed protrusion into the spinal cord. The mass was lined by a pseudo-capsule of flattened cells. The

lesion was mostly characterized by an epithelioid tubular pattern with delineated cystic and very cellular solid parts and very occasional glomeruloid features (Figure 5). Notably, there was a multifocal goblet cell differentiation and membrane specifications were seen throughout. The cysts varied in size, contained flocculent mucoid material and exfoliated cells. Between the surface structures, there were polymorphic spindeloid cells with some cellular exfoliation, high degree of basophilia, anisocytosis and anisokaryosis, round to elongated nuclei, highly hyperchromatic coarse chromatin and some areas with parachromatic vacuolation. Most of the cells contained 1-3 prominent paracentral and moderately anisometric nucleoli. Furthermore, there were small epitheloid nests scattered in between the tubular structures consisting of oval cells with an eosinophilic indistinct cytoplasm and hypochromatic round to oval nuclei and small central or paracentral nucleoli.The adjacent neuroparenchyma (spinal cord and nerve root) was deformed and presented with multiple enlarged myelin tubes, axonal spheroids and multiple gitter cells.

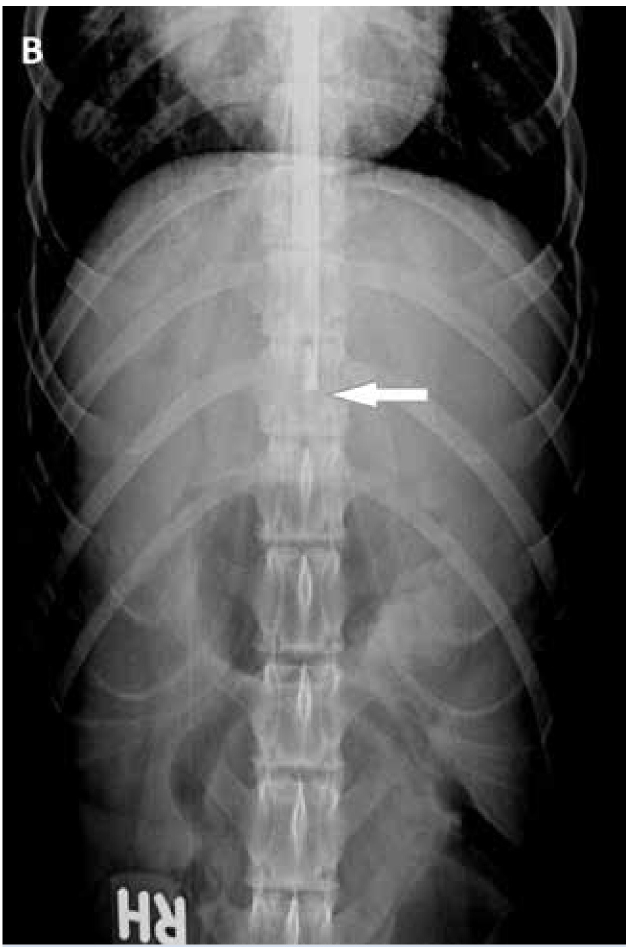

Figure 2. Cisternal myelogram of the thoracolumbar region of the spine indicating a “golf-tee” sign (arrow) in ventrolateral view (RH=right side).

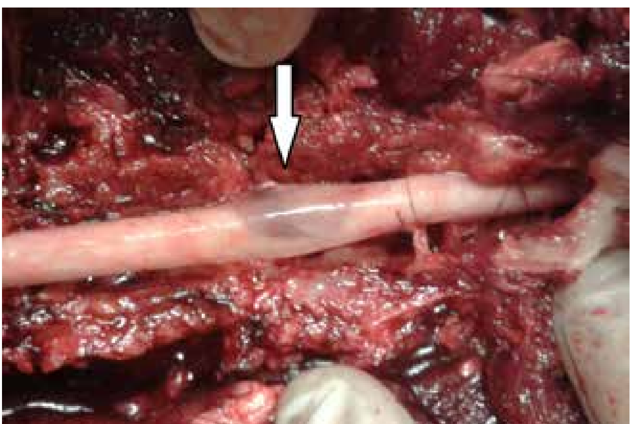

Figure 3, 4. Intradural- extramedullary mass with extensive lateral spinal cord compression.

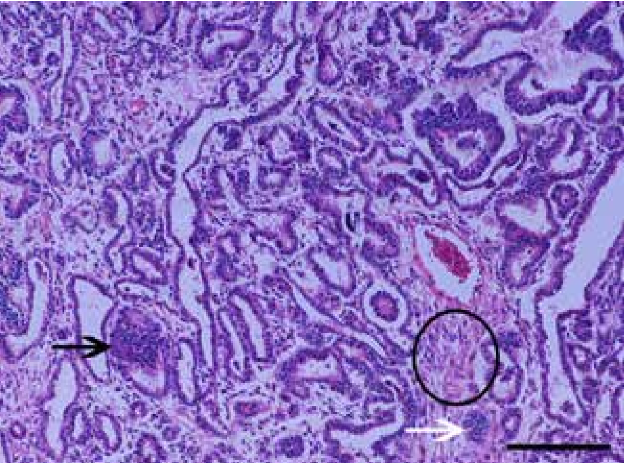

Figure 5. Primitive glomeruli (black arrow), embryonal mesenchyme (circle) and blastemal cells (white arrow), compose the ‘triphasic pattern’ of nephroblastoma. Hematoxylin-eosin staining. Bar: 500 μm.

Cranial to the lesion the histological examination revealed a mild diffuse lympho-plasma-cellular infiltration of the subarachnoid space and a severe bilaterally symmetric extracellular grey matter edema of the intermediate zone. This area and the ventral fascic-

ulus proprius contained numerous axonal spheroids. Caudal to the lesion the spinal cord presented with equal histopathological changes. In addition, the central canal in this segment was mildly enlarged. The histological picture was consistent with the pre-neuro-invasive stage of a spinal nephroblastoma of the young dog, due to embryonic misplacement of cells from the nephrogenic primordium (Figure 5).

DISCUSSION

Nephroblastoma is a rare neoplasm of the spinal cord occurring in young dogs aged from 6 months to 3 years, but it has also been reported in older animals (McConelli et al., 2003, Liebel et al., 2011). Nephroblastoma is also called Wilm's tumor and is the most common renal tumor in children, although extrarenal spinal localization is rare (Liebel et al., 2011). Large breed dogs seem to be predisposed, especially German shepherds and Retriever breeds (Liebel et al., 2011). The tumor is frequently located between the 10th thoracic and the 2nd lumbar spinal cord segments and may be intradural – extramedullary (ID – EM) or, less often intramedullary (IM) or extradural (ED) (McConelli et al., 2003, Liebel et al., 2011). Classification of these tumors in animals and humans is controversial, and they are invariably described as ependymomas, neuroepitheliomas, spinal cord blastomas, medulloepitheliomas, hamartomas, nephroblastomas, embryonal nephromas, embryonal adenosarcomas, renal adenocarcinomas, or Wilm's tumor (McConelli et al., 2003, Liebel et al., 2011).The recommended definition by the World Health Organization Histological Classification of Tumors of Domestic Animals is "thoraco-lumbar spinal cord tumor of young dogs" (Liebel et al., 2011).The histomorphologic and immunocytochemical evidence shows that nephroblastomas represent ectopic growths of undifferentiated metanephricblastema, entrapped within the dura or spinal cord parenchyma during fetal development (Liebel et al., 2011). This theory is supported by immunohistochemical staining using human Wilm's tumor (nephroblastoma) gene antibody, which was used to confirm the presence of primitive renal tissue (McConelli et al., 2003). Nephroblastomas are usually solitary but there are reports of potential spinal metastases (McConelli et al., 2003).

The clinical presentation of the current case is comparable to those of previously reported cases. The presenting signs of the tumor was sub-acute to chronic progressive, pelvic limb ataxia or paresis (T3 – L3 myelopathies) (Liebel et al., 2011).

A tentative diagnosis is based on advanced diagnostic imaging investigation such as myelography, magnetic resonance imaging (MRI) and ultrastructural or immunohistochemical examination of the mass (Ohta et al., 2009). In the majority of soft tissue spinal neoplasms, plain radiographs of the spine are normal, except of vertebral neoplasia cases where vertebral bone lysis with loss of cortical outlines is noted. Myelography, computed tomography (CT) or magnetic resonance imaging (MRI) are helpful for establishing the diagnosis of spinal tumors and for therapeutic planning. Evaluation of cerebrospinal fluid (CSF) rarely reveals neoplastic cells but may reveal increased protein levels with or without elevated cell counts. In the current case, CSF analysis did not reveal either increased protein concentration or elevated cell count. The myelographic appearance of central nervous system (CSN) tumors has been described in several reports as either an intramedullary or intradural-extramedullary space-occupying lesion (Dewey 2008). Nephroblastomas are intradural-extramedullary in origin, but may infiltrate the spinal cord giving rise to the appearance of an intramedullary mass. It can be difficult to differentiate intradural from intramedullary masses with myelography. Myelographic classification of a mass as intradural-extramedullary is based on the presence of a widened subarachnoid space and a filling defect within the contrast medium. If the lesion lies laterally, the spinal cord appears expanded on the lateral view and displaced on the ventrodorsal view. Focal widening of the subarachnoid space caused by a discrete mass in the region forms the "golf-tee" sign as was in the dog presented here (Kealy, McAllister, Gragam, 2005). In one study, it was found that conventional myelography was superior to CT myelography in differentiating intradural-extramedullary tumors from intramedullary tumors (Li et al., 1992).

In our case, based on cisternal myelography findings, the focal "golf-tee" lesion above the caudal end of T12 vertebra was considered compatible with intradural-extramedullary mass (Figure 1). However, the "golf-tee" sign was described in a case of extradural lesion, hence its identification should be interpreted with caution (Nderbisv2000).

A "triphasic pattern" may be observed in histopathologic examination of spinal neoplasms (De Lorenzi et al., 2007). The terminology refers to the presence of three different tumor cell populations, stromal/mesenchymal cells, epithelial cells and un-

differentiated small hyperchromatic blastemal cells (Liebel et al., 2011).

Therapy for dogs with this type of spinal tumor can be supportive (palliative) or definitive (Dewey, 2008). Supportive therapies are directed against secondary sequel of nephroblastoma (e.g., spinal cord edema, pain), whereas definitive therapies are aimed at elimination of neoplastic tissue (Dewey, 2008). Supportive therapies consist of administration of anti-inflammatory dose of glucocorticoids (e.g., prednisone), with or without additional pain-relieving drugs (e.g., narcotics)(Dewey, 2008, Liebel et al., 2011). Definitive therapy consist of cytoreductive surgery and radiotherapy (Dewey, 2008, Liebel et al., 2011). The cytoreductive surgical procedures include hemilaminectomy, dorsal laminectomy with unilateral facetectomy, durotomy, regional durectomy, myelotomy with or without radiotherapy (Liebel et al., 2011). In dogs, the goals of surgery for spinal cord tumors are decompression of the spinal cord, maximal neoplastic tissue resection and collection of samples for morphologic examination (Liebel et al., 2011). The outcome after cytoreductive surgery varies with survival times ranging from 2 months to more than 3 years (Liebel et al., 2011). The efficacy of radiotherapy after cytoreductive surgery or as a sole treatment modality for nephroblastoma in dogs is not well documented, but a few reports suggest that it may be beneficial (Liebel et al., 2011). Therapies can provide some temporary relief of clinical signs, but many patients will likely be euthanized because of poor general condition. The prognosis is considered poor, however, it may depend on the neuroinvansive and metastatic potential of the neoplasm, while distant extraneural and intraspinal-metastases appear to be rare (Liebel et al., 2011).

CONCLUDING REMARKS

Although rare, spinal cord tumors like nephroblastomas should be included in the differential diagnosis of canine cases with acute or sub-acute spinal cord disease.

ACKNOWLEDGEMENTS

The authors thank Dr. K. Matiasek and Dr. M. Rosati, Neuropathology Laboratory Institute of Veterinary Pathology, Ludwig-Maximilian University of Munich and Dr. D. Psalla, Laboratory of Pathology, School of Veterinary Medicine A.U.Th. for their support in the histopathological diagnosis of the case.

CONFLICT OF INTEREST

None declared by the authors.

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J HELLENIC VET MED SOC 2019, 70(3): 1743-1748
ΠΕΚΕ 2019, 70(3): 1743-1748

Research article
Ερευνητικό άρθρο

Clinical and molecular characterization of both methicillin-resistant and-sensitive *staphylococcus aureus* mastitis

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ABSTRACT. This study targeted bovine mastitis as a possible source of livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA), to identify clinical signs associated with MRSA- and non-MRSA-associated mastitis. Thirty-eight mastitis cases (68 infected quarters) were investigated. Gram-positive cocci-shaped isolates were selected based on Baird Parker agar growth as well as Gram-stained bacterial smears. Molecular screening for *Staphylococcus aureus* (*S. aureus)* yielded 17 isolates, of which five (29.41%) were methicillin resistant. The five isolates were *mecA* positive, but *mecC* negative. Multilocus sequence typing (MLST) indicated that sequence type 1 (ST1) was the identified type of all isolates of MRSA. *S. aureus*-associated cases showed different clinical forms of mastitis, including subclinical, acute, chronic, and gangrenous. Additionally, subclinical mastitis was the only detected condition associated with MRSA, which may represent a potential hidden risk for humans. Phenotypically, isolates of MRSA showed resistance to all of the tested β-lactam antimicrobials, with marked resistance to tetracycline and gentamycin. Based on our knowledge, this is the first report to identify MRSA ST1 in Egypt. Bovine mastitis could be a source for the dissemination of MRSA to humans and other animals. Additionally, while methicillin-resistance may have no effect on the clinical outcome of mastitis, it does affect therapeutic success, particularly when β-lactam antimicrobials are used.

***Keywords*:** *MRSA, Bovine, Mastitis*, *Staphylococcus aureus*

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Date of initial submission:
Date of revised submission:
Date of acceptance:

INTRODUCTION

Staphylococci, particularly *S. aureus*, are common pathogens of mastitis in bovines (Haveri et al., 2007), and the *Staphylococcus spp.* associated mastitis is responsible for considerable economic losses (Lammers et al., 2000). *S. aureus* accounts for 25–30% of all intra-mammary infections (IMI) in cows (Poutrel, 1985). Importantly, *S. aureus* IMI results in a 10–25% milk reduction in infected animals (Anderson, 1983). *S. aureus* can induce either clinical (CM) or subclinical (SCM) mastitis, but the subclinical form is more predominant (Anderson, 1983; Lammers et al., 2000; Akineden et al., 2001). While CM has detectable clinical symptoms, SCM has no detectable symptoms and necessitates screening using the California mastitis test (CMT) (Kasikci et al., 2012). The success in staphylococcal mastitis therapy is dependent on the individual animal, treatment, and pathogen factors (Barkema et al., 2006), and the resistance to antimicrobials is a major factor affecting the cure rates of staphylococcal mastitis (Barkema et al., 2006).

Staphylococci, notably *S. aureus*, have shown resistance to various antimicrobials (Wang et al., 2015), and MRSA strains have gained worldwide attention. MRSA is classified into three categories according to its origin: livestock-associated (LA-MRSA), healthcare-associated (HA-MRSA), and community-associated (CA-MRSA) (Stefani et al., 2012). There is an increasing global interest in LA-MRSA because of its animal and human associated health implications (Graveland et al., 2011). Many sequence types (ST1, ST9, ST97, ST130, ST398, and ST425) of MRSA had been recorded in both cattle and humans (García-Álvarez et al., 2011; Paterson et al., 2012; Spoor et al., 2013; Alba et al., 2015; Cuny et al., 2015). In humans, LA-MRSA strains can colonize tissues, resulting in pneumonia, endocarditis, and other life threatening conditions (Ekkelenkamp et al., 2006; Witte et al., 2007). Molecular epidemiology studies on MRSA in southern Mediterranean countries are limited (Borg et al., 2007). In Egypt, there are very few studies on MRSA strains originating from bovine mastitis (Elhaig &Selim, 2015). Additionally, studies describing the clinical aspects of MRSA- and non-MRSA-associated bovine mastitis are limited. Therefore, this study was intended to screen for MRSA and its sequence types associated with bovine mastitis in Egypt, and to describe the clinical aspects of MRSA- and non-MRSA-associated bovine mastitis.

MATERIALS AND METHODS

Animals and detection of mastitis

Study animals were reared in the Gharbia and Kafrelsheikh governorates, in the central and northern regions of the Egyptian Delta. In these areas, the dairy animals are reared in small groups rather than organized farms. Thirty-eight mastitis cases (37 Friesian-Balady crossbred cows and one Egyptian buffalo) were included in this study. CM cases were detected by clinical examination of the animals with special attention to the udder according to Houe et al. (2002). SCM cases were identified using the CMT (Kasikci et al., 2012).

Sampling

Clinically detected and CMT-positive quarters were aseptically sampled according to Quinn et Al. (1994). Milk samples were sent refrigerated to the laboratory for pathogen isolation.

Bacteriological examination

The samples were centrifuged (1000 *g*/5 min), the supernatant was discarded and sediment was streaked on Baird Parker agar. A 24 h incubation at 37 °C was done according to Silva et al. (2000). Gram-stained Smears of the colonies were examined. The putative *Staphylococcus species* isolates were preserved in glycerol stock at −20 °C until be used in molecular procedures.

In addition to clinical cases isolates, 33 *Staphylococcus species* isolates were obtained from the Animal Health Research Institute (AHRI), Tanta Branch, Egypt. These isolates originated from the study area (Gharbia Governorate) and were isolated from mastitis cases. Clinical data for these isolates was not recorded.

Molecular characterization and typing

For extraction of DNA, Luria-Bertani agar plates were streaked by the isolates and incubated for 24 h at 37 °C. The extraction of DNA was performed by InstaGene matrix (Bio-Rad Laboratories Inc.). The extracted DNA was preserved at −20 °C for use in PCR assays. Primer sequences of *S. aureus*, MRSA and MLST are shown in Table 1.

Table 1. Primers of *S. Aureus*, MRSA and MLST

Primer	Gene	Sequence (5'-3')	Size (bp)	References
au-F3	*Nuc*	TCGCTTGCTATGATT GTGG	359	Sasaki et al., 2010
au-nucR		GCCAATGTTCTACCA TAGC		
***MecA*147-F**	*MecA*	GTG AAG ATA TAC CAA GTG ATT	147	Zhang et al., 2005
***MecA*147-R**		ATG CGC TAT AGA TTG AAA GGA T		
$mecA_{LGA251}$MultiFP	$mecA_{LGA251}$	GAAAAAAAGGCTTAGAACGCCTC	138	Stegger et al., 2011
$mecA_{LGA251}$MultiRP		GAAGATCTTTTCCGTTTTCAGC		
***arcC*-Up**	*Arc*	TTGATTCACCAGCGCGTATTGTC	456	Enright et al., 2000
***arcC*-Dn**		AGGTATCTGCTTCAATCAGCG		
***aroE*-Up**	*aroE*	ATCGGAAATCCTATTTCACATTC	456	
***aroE*-Dn**		GGTGTTGTATTAATAACGATATC		
***glpF*-Up**	*GlpF*	CTAGGAACTGCAATCTTAATCC	465	
***glpF*-Dn**		TGGTAAAATCGCATGTCCAATTC		
***gmk*-Up**	*Gmk*	ATCGTTTTATCGGGACCATC	429	
***gmk*-Dn**		TCATTAACTACAACGTAATCGTA		
***pta*-Up**	*Pta*	GTTAAAATCGTATTACCTGAAGG	474	
***pta*-Dn**		GACCCTTTTGTTGAAAAGCTTAA		
***tpi*-Up**	*Tpi*	TCGTTCATTCTGAACGTCGTGAA	402	
***tpi*-Dn**		TTTGCACCTTCTAACAATTGTAC		
***yqiL*-Up**	*YqiL*	CAGCATACAGGACACCTATTGGC	516	
***yqiL*-Dn**		CGTTGAGGAATCGATACTGGAAC		

A PCR assay targeting a 359-bp region of the *S. aureus* thermonuclease (*nuc*) gene was used to detect *S. aureus* as described by Sasaki et al. (2010) with a few modifications. Briefly, a 25-µl reaction was prepared containing 5 µL of DNA, 0.2 mM dNTPs, 1× buffer, 0.5 U of AmpliTaq Gold (Applied Biosystems), and primers (each of 20 pmol). The thermal cycler conditions consisted of 95 °C /10 min, 35 cycles (95 °C/30 s, 56 °C/35 s, and 72 °C/1 min), followed by 72 °C/10 min.

MRSA was identified by PCR targeting 147- and 138-bp regions of *mecA* and *mecC* ($mecA_{LGA251}$) as described by Zhang et al. (2005) and Stegger et al. (2012), respectively, with a few modifications. The 25 µL reaction consisted of 5 µL of DNA, 0.2 mM dNTPs, 1× buffer, 0.5 U of AmpliTaq Gold (Applied Biosystems), and primers (20 pmol of each). Mixtures of *mecA* and *mecC* were initially heated at 94 °C for 4 min/15 min, followed by 35/30 cycles of 94 °C/30 s, 52 /59 °C for 30 s/1 min, and 72 °C for 45 s/1 min, respectively. A final extension was conducted at 72 °C/7 min. MLST was performed using seven housekeeping genes according to Enright et al. (2000) and allelic profiles were obtained from MLST web site (http://saureus.beta.mlst.net/).

Antimicrobial susceptibility testing

All *S. aureus* isolates were examined for their susceptibility to ampicillin, tetracycline, ciprofloxacin, gentamicin, sulfamethoxazole and trimethoprim, teicoplanin, ceftriaxone, amoxicillin and clavulanic acid, oxacillin, and cefoxitin. The antibiotic disc diffusion guidelines of The Clinical and Laboratory Standards Institute (2005) were followed up.

RESULTS

Clinical and descriptive aspects of mastitis cases

Most of the studied cases had mastitis in a single quarter (25/38 cases). However, two, three, and four quarters were found affected in four, one, and eight animals, respectively. Of the 68 individual infected quarters, 46 were diagnosed as SCM (67.6%) and 22 as CM (32.4%). Acute mastitis was observed in 19 (27.9%) quarters, with chronic and gangrenous mastitis detected in two (2.9%) and one (1.5%) quarters, respectively.

Identification of *S. aureus*

Gram-positive cocci-shaped bacteria were isolated from 36/38 mastitis cases (94.7%) and 49/68 infected quarters (72.1%). Based on Baird Parker agar growth and Gram-stained smears, 59 gram-positive cocci-shaped isolates were selected. Molecular screening for *S. aureus* yielded seven isolates from seven individual cases. Clinical findings of *S. aureus*- mastitis are shown in Table 2.

Table 2. Clinical findings of *S. Aureus* mastitis

Case number	Isolate name	Type of mastitis	Symptoms
1	Sa28	Chronic	Marked fibrosis of the quarter and yellow watery milk
2	Sa104	Acute	Animal depressed, fluctuation of body temperature between normal and subnormal, marked fibrosis of the quarter and intense bloody milk
3	Sa120	Gangrenous	Coldness and black discoloration of the teat and the quarter base in addition to intense bloody milk
4	Sa119	Subclinical	Normal
5	Sa101	Subclinical	Normal
6	Sa107	Subclinical	Normal
7	Sa69	Subclinical	Normal

Table 3. Phenotypic and genotypic profiles of *S. Aureus* isolates

Isolates	Phenotypic resistance		Genotypic resistance
	β-lactams	Other antimicrobials	
Sa28	AMP, FOX	TEC	
Sa69	AMP, AMC, FOX, CRO, OXA	TET, TEC	*mecA*
Sa70	AMP, AMC, FOX, CRO	TET, GEN, TEC	*mecA*
Sa101	AMP, AMC, FOX, CRO, OXA	TET, GEN, TEC	*mecA*
Sa104	FOX	TEC	
Sa107	AMP	TET, GEN,TEC	
Sa119	AMP	GEN, TEC	
Sa120	AMP	-	
Sa131	AMP, AMC, FOX, CRO, OXA	TET, GEN, TEC	*mecA*
Sa135	AMP, FOX	TET, TEC	
Sa136	-	TET, TEC	
Sa137	AMP, AMC, FOX, CRO, OXA	GEN, TEC	*mecA*
Sa140	AMP	-	
Sa144	AMP, AMC, FOX, CRO, OXA	TET, GEN, TEC	
Sa146	AMP, FOX	-	
Sa158	AMP, AMC	-	
Sa164	AMP	-	

TET, tetracycline; CIP, ciprofloxacin; SXT, sulfamethoxazole and trimethoprim; GEN, gentamicin; AMP, ampicillin; AMC, amoxicillin and clavulanic acid; FOX, cefoxitin; TEC, teicoplanin; CRO, ceftriaxone; OXA, oxacillin.

Molecular screening of the AHRI isolates resulted in an additional 10 isolates of *S. aureus*. Consequently, 17 *S. aureus* strains were used in further investigations.

Antimicrobial susceptibility of *S. aureus* isolated strains

A marked resistance of *S. aureus* to teicoplanin and ampicillin was observed. In addition, oxacillin-resistance was evident in five *S. aureus* isolates. *S. aureus* phenotypic and genotypic profiles are listed in Table 3.

Molecular screening/typing of MRSA

Out of the 17 *S. aureus* strains, five (two originated from the clinical cases and three AHRI isolates) strains contained *mecA* (29.41%) and were classified as MRSA ST1. However, *mecC* was absent in all of the *S. aureus* isolates.

Clinical nature of MRSA-associated bovine mastitis

The both cases which were infected by MRSA ST1 had showed SCM, which may indicate that MRSA ST1 may be unable to induce severe mastitis cases.

DISCUSSION

LA-MRSA strains are reported to induce endocarditis, pneumonia, soft tissue and skin conditions in humans (Ekkelenkamp et al., 2006; Witte et al.,

2007). Interestingly, the current study identified a high percentage of MRSA ST1 (29.41%) amongst the *S. aureus* strains isolated from mastitis in bovines. However, larger scale investigations are important to assess LA-MRSA of bovine mastitis origin and its potential risk for humans in Egypt. MRSA ST1 is a wide spread LA-MRSA lineage with a broad host range including humans (Alba et al., 2015). Although it is commonly isolated from pigs, recent studies had recorded MRSA ST1 associated with cattle and dairy farming in some countries such as Italy and Hungary (Juhász-Kaszanyitzky et al., 2007; Alba et al., 2015). Moreover, studies had showed high genetic similarity (90-100%) between human and cattle associated MRSA ST1 and confirmed complete ability of the later to colonize and infect humans (Juhász-Kaszanyitzky et al., 2007; Alba et al., 2015).

Because only a single quarter was infected in most cases, we hypothesize that the involved organisms are not highly contagious, particularly as Egyptian farmers often do not maintain ideal milking hygiene procedures. This was confirmed by the results of the molecular analysis that showed that most of the isolated strains were not *S. aureus*. SCM was also more prevalent than CM, which agrees with previous studies (Elhaig & Selim, 2015), and highlights the importance and widespread nature of SCM.

The clinical outcome of *S. aureus* associated mastitis is a multifactorial process. In addition to host immunity, factors such as exotoxins and other virulence determinants of *S. aureus* can influence the outcome (Haveri et al., 2007). According to the findings of the current study, there is no relationship between MRSA ST1 and the severity of mastitis symptoms. Previous research have shown that most LA-MRSA isolates lack many mastitis-associated virulence factors such as toxic shock syndrome toxin 1, hemolysins, and enterotoxins (Monecke et al., 2007; Walther et al., 2009). Despite this, the involvement of MRSA ST398 in CM cases has been reported previously (Vanderhaeghen et al., 2010). The previous study showed that 2/11 MRSA ST398 isolates were associated with CM; however, most of the isolates were related to SCM.

Antimicrobial susceptibility profiling of the MRSA and non-MRSA isolates revealed prominent differences. The MRSA isolates showed resistance to most of the tested antimicrobials (Table 3). The resistance of MRSA to oxacillin and other β-lactam antimicrobials can be attributed to the existence of *mecA*, which codes for a penicillin binding protein with low affinity for all β-lactams (Hartman & Tomasz, 1984). In conclusion, bovine mastitis is a source of LA-MRSA ST1 which is a health risk for humans and animals. Additionally, while methicillin resistance may not affect the severity of mastitis symptoms, it will affect therapeutic success, particularly when β-lactams are used for treatment.

ACKNOWLEDGMENTS

A. F. O. is supported by a postdoctoral fellowship from the Ministry of Higher Education, Egypt. H. O. K. is supported by a doctoral fellowship from the Ministry of Higher Education, Egypt. A. M. A. is supported by a postdoctoral fellowship (no. PU14012) from the Japan Society for the Promotion of Science. This work was supported in part by a Grant-in-Aid for Scientific Research (no. 25460532) to Tadashi S. from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Quarterly Edition
Volume 70, No 3
JULY - SEPTEMBER 2019

Τριμηνιαία Έκδοση
Volume 70, No 3
ΙΟΥΛΙΟΣ - ΣΕΠΤΕΜΒΡΙΟΣ 2019