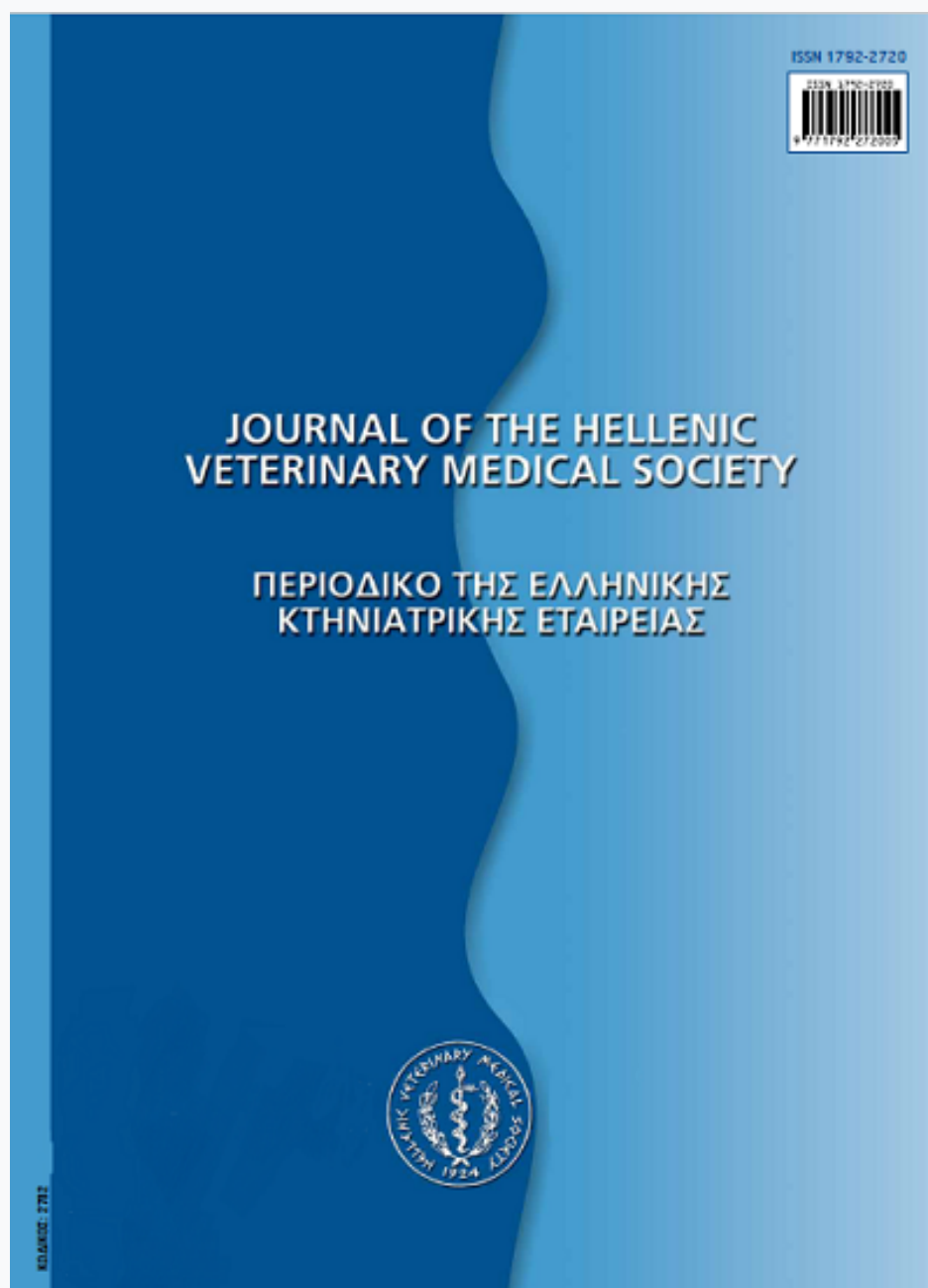


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EUROPEAN COLLEGES OF VETERINARY SPECIALISTS
ΕΥΡΩΠΑΪΚΑ ΚΟΛΕΓΙΑ ΕΙΔΙΚΕΥΜΕΝΩΝ ΚΤΗΝΙΑΤΡΩΝ

				Number of specialist veterinarians active in Greece Αριθμός ειδικευμένων κτηνιάτρων εργαζομένων στην Ελλάδα
1		ECAR	European College of Animal Reproduction	2
2		ECAWBM	European College of Animal Welfare and Behavioural Medicine	1
3		ECAAH	European College of Aquatic Animal Health	2
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	3
9		ECSRHM	European College of Small Ruminant Health Management	10
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	0
18		ECVO	European College of Veterinary Ophthalmology	0
19		ECVP	European College of Veterinary Pathology	0
20		ECVPH	European College of Veterinary Public Health	7
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	0
24		EVDC	European Veterinary Dentistry College	0
25		EVPC	European Veterinary Parasitology College	4
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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 Veterinary Microbiological Institute of Votanicos, from where all members 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, Institute 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 capabilities, 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 members. 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 execution of the veterinary profession.

Furthermore, the role of the HVMS has been determinative on the decision making of the Ministry of Agriculture on veterinary 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 Companion Animals (founding member)**
- **Veterinary Society of the Balkan and the Black Sea (founding member)**

The HVMS has a total of 1220 members many of which have been distinguished in the scientific field (University Professors, 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, Patisision 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. All the actions performed for possessing this new private office for the HVMS were performed during the presidency of Dr Theodoros Cl. Ananiadis and the following Governing Board:

President	: Theodoros Cl. Ananiadis†
Vice-President	: Veniamin Albalas
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The Board of Directors and the Editorial Board of the Journal of the Hellenic Veterinary Medical Society, warmly thank the reviewers that substantially contributed in the successful publication of the 68th volume 2017 of the J Hellenic Vet Med Soc, the names of which are cited below in alphabetical order:

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Kalogerakis N.	Καλογεράκης Ν.
Kantas D.	Καντάς Δ.
Katsoulos P.	Κατσούλος Π.
Kazakos G.	Καζάκος Γ.
Kiossis E.	Κιόσης Ε.
Kokozidou M.	Κοκοζίδου Μ.
Komnenou A.	Κομνηνού Α.
Koutinas Ch.	Κουτίνας Χ.
Koutoulis K.	Κουτουλής Κ.
Kritas S.	Κρήτας Σ.
Michailidis G.	Μιχαηλίδης Γ.
Milionis D.	Μηλιώνης Δ.
Panousis N.	Πανούσης Ν.
Papadopoulos E.	Παπαδόπουλος Ε.
Papadopoulos G.	Παπαδόπουλος Γ.
Papadopoulos I.	Παπαδόπουλος Η.
Papaioannou N.	Παπαϊωάννου Ν.
Papanna K.	Παπάννας Κ.
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Papazachariadou M.	Παπαζαχαριάδου Μ.
Pardali D.	Παρδάλη Δ.
Petridou E.	Πετρίδου Ε.
Pexara A.	Πεξάρα Α.
Polizopoulou Z.	Πολυζοπούλου Ζ.
Psalla D.	Ψάλλα Δ.
Samanidou V.	Σαμανίδου Β.
Samartzi F.	Σαμαρτζή Φ.
Samouris G.	Σαμούρης Γ.
Sergelidis D.	Σεργκελίδης Δ.
Sotiraki S.	Σωτηράκη Σ.
Symeonidou I.	Συμεωνίδου Η.
Tananaki Ch.	Τανανάκη Χ.
Tassis P.	Τάσσης Π.
Tsigotzidou A.	Τσιγκοτζίδου Α.
Tsiligianni Th.	Τσιλιγιάννη Θ.
Tsiouris V.	Τσιούρης Β.
Tsousis G.	Τσούσης Γ.
Tzivara A.	Τζιβάρα Α.
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Salmonella spp. in poultry: a constant challenge and new insights

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ABSTRACT. The knowledge about virulence mechanisms, resistance to antimicrobial agents and the biofilm formation ability of *Salmonella* spp. in poultry industry has been expanded over the years. However, in spite of the research efforts and significant investments to improve management systems in poultry industry, it has become evident that none of the methods applied in all stages of food production chain are 100% effective in eliminating *Salmonella* spp. Different serovars are manifesting different mechanisms of invasiveness which depend on their ability to invade lower zones of the lamina propria, their ability to gain accesses to parenchymatous organs and survive in macrophages. The ubiquitous nature of *Salmonella* spp. due to their adaptation to animal and plant hosts, as well as their survival in hostile environments and their enhanced capacity to produce biofilms, contribute to a long lasting contamination of the environment, feed and animals. The emergency and spread of antimicrobial resistances in *Salmonella* spp. raise additional concerns.

Keywords: poultry, *Salmonella*, pathogenesis, biofilm, resistance

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INTRODUCTION

Poultry farming presents one of the most important food manufacturing industries around the globe. Therefore, food safety standards are highly demanding and are generally better maintained in large scale production facilities than in small ones. In developing countries, rearing of backyard chicken flocks contributes to the continuous occurrence of some viral and bacterial diseases that are less likely present in well maintained farms. Except for a very few countries in the world, *Salmonella* spp. are detected in environmental specimens in practically all stages of the food production chain. Out of more than the 2600 serovars known today, only 10% are found in the commercial poultry and egg industry. Two of them, *S. Enteritidis* and *S. Typhimurium*, are of paramount importance to human health and can colonize the intestines of chickens (Velge et al., 2005). In most cases, the infected chickens either do not have clinical symptoms or the symptoms remain unnoticed.

With all this taken into account, it is evident that control programs for *Salmonella* spp. have to be implemented in all stages of the food production chain, starting from animal farms. According to European Directive 1003/2005, the occurrence of *S. Enteritidis* and *S. Typhimurium* in adult breeder flocks has to be $\leq 1\%$, in EU member states. However, this directive also targets serovars Hadar, Virchow and Infantis which are of public health significance in the EU (Carrique-Mas and Davies, 2008). It is very difficult to accomplish such a goal in developing countries, since implementing good management practice is expensive and requires the participation of educated staff. Even if biosecurity measures are well established on a farm, salmonellae can still be found in poultry and premises.

Other available measures to cope with *Salmonella* spp. in farms include the use of prebiotics and probiotics, antimicrobial therapy and vaccination of the birds. For serovars *S. Enteritidis* and *S. Typhimurium* commercial inactivated and attenuated vaccines have been developed and used widely. These vaccines target serogroups D and B respectively, but do not protect livestock against serovars from other serogroups. Therefore, vaccination against *S. Enteritidis* and *S. Typhimurium* could lead to the elimination of these two serovars on farms, opening

a vacant ecological niche, enhancing, thus, the emergence of new serovars, such as *S. Kentucky* or *S. Heidelberg* (Foley et al., 2011).

The framework of National control programs in European Union member states includes the vaccination of layer flocks during rearing which has to be mandatory in cases of 10% prevalence of *S. Enteritidis* (EC No 1168/2006 and EC No 1177/2006). Live vaccines could be used only in cases when the discrimination of vaccine versus wild type *Salmonella* is possible and the ban of antibiotic use in layers has been initiated (Carrique-Mas and Davies, 2008). Such high demands have motivated a number of research works aiming to find the best sampling strategy and the best monitoring systems for *Salmonella* spp. control all around the world.

The most convenient methods of taking samples for bacteriological analysis from poultry houses are using boot swabs or the “step on a drag swab” method (Buhr et al., 2007). Official sampling is carried out while birds are in the unit while own checks are carried out not only while livestock is in the unit but also after depopulation. Own check programs must be approved by the competent authorities. The sampling strategy aiming to detect and control *Salmonella* spp in adult breeding flocks of *Gallus gallus* is defined in Commission Regulation EU No 200/2010 and for laying hens in Commission Regulation (EU) No 517/2011. Reduction of the prevalence of the serovars *Enteritidis* and *Typhimurium* in flocks of turkeys is required and the sampling strategy is defined in Commission regulation (EC) No 584/2008. After cleaning and disinfection, swabs are collected from walls, floor, vents, drinking and feeding systems, changing rooms and other areas that may be exposed to external contamination. It is important to collect as many swabs as possible to determine the success of cleaning and disinfection. The same strategy applies for hatcheries which may become contaminated with the pathogen. In fact, *Salmonella* spp. can be effectively disseminated in the hatchery cabinet and chickens may become infected before removing from the hatchery (Bailey et al., 1998).

According to a longitudinal study of environmental *Salmonella* contamination in caged and free-range layer flocks carried out by Wales et al. (2007), the timing of taking samples has been shown to have a

significant influence on *Salmonella* spp. isolation. Flocks that remained longer on the premises yielded more isolates comparing to the new flocks. The temperature and the season also had an influence on *Salmonella* spp. populations, proving increased isolation rate during summer. The role of other animal reservoirs harboring *Salmonella* in and outside the farms is also significant (Guard-Petter, 2001). *Salmonella* spp. in wildlife vectors correlated well with the status of the flock and the same serovar and phage type could be found in wild predators caught around the farm and poultry. Cleaning and disinfection in cases when organic matter had been substantially removed and disinfectants were adequately applied and in proper concentration, had a positive influence on *Salmonella* control. However, the wildlife reservoirs, multiage farming and lack of “all in all out” strategy highlight the need for vaccination and the use of probiotics in flocks with high and low incidence of the pathogen’s load or even in cases that it is absent (Wales et al., 2007). Another study by Dewaele et al. (2012) which aimed to examine the *Salmonella enterica* serovar Enteritidis environmental contamination on persistently positive layer farms in Belgium during successive laying cycles showed that in contaminated poultry houses, neither vaccination nor cleaning and disinfection are considered as the only prerequisite for successful elimination of *Salmonella* spp. from the environment and that the chances for *Salmonella* spp. elimination were better in less contaminated poultry farms, comparing to those in highly contaminated environments. This is even more pronounced if rodents, flies and mites come into contact with poultry or equipment. In addition the authors concluded that there is a possibility that even if poultry houses are separately cleaned and disinfected, egg collection areas may still become a reservoir of *Salmonella* spp. In fact, the egg collection areas may become contaminated with a few serovars which are present on the entire farm.

THE PATHOGENESIS, TISSUE INVASION AND IMMUNE RESPONSES

Salmonella spp. possesses an arsenal of genetic determinants responsible for colonization, adhesion, invasion and proliferation in host cells, including

fimbriae, flagella, toxins, surface lipopolysaccharides (LPS), etc. Virulence genes are organized in clusters and spread throughout the chromosome, such as *Salmonella* Pathogenicity Islands 1 and 2 (SPI-1, SPI-2), or located on virulence plasmids, such as *spv* genes (associated with invasive strains). *Salmonella* pathogenicity genomic islands carry genes that are required for successful infection in poultry (Wisner et al., 2012). Noninvasive strains cause gastroenteritis, while invasive strains may cause systemic bacteremia in humans and animals. The outcome of infection depends on virulence factors, the pathogenesis of *Salmonella* spp. and their interaction with the host organism (Foley et al., 2013). Unlike noninvasive strains, invasive *Salmonella* strains penetrate through the epithelial lining to the lower parts of the lamina propria. Also, invasive strains are commonly isolated from parenchymatous organs (spleen, liver, ovaries) and a small number of bacteria become internalized by macrophages (Berndt et al., 2007). The survival in the acidic environment of the stomach is enabled by the activation of more than 50 acid tolerance response proteins (Bearson et al., 2006). The first phase of the infection has to provide a chance for the bacteria to invade intestinal epithelial cells. This process is accomplished by proteins encoded by *Salmonella* Pathogenicity Island (SPI-1) type III secretion system (T3SS). These organelles produce a special structure in the bacterial envelope called “the needle complex” which delivers toxins and other effector proteins and injects them into the host cells (Kubori et al., 2000). Bacterial effector proteins modulate the host actin cytoskeleton and initiate the signal transduction pathways required for the internalization of the bacteria. In addition, invasive strains recruit their own systems responsible for survival in macrophages. *Salmonella* spp. become internalized in a specific membrane bound compartment called “*Salmonella* containing vacuole” (SCV). The maturation of the SCVs and their migration to the basal membrane disable the destruction of the bacteria by phagolysosomes. Such intracellular trafficking and intracellular pathogenesis is also accomplished by the activation of the second T3SS encoded by the SPI-2. Hence, the type III secretory system encoded by SPI-1 and SPI-2 enables the attachment, invasion and survival of the pathogen within the

host cell, as well as the avoidance of antimicrobial compounds (Hensel, 2000; Foley et al., 2013). Most of *Salmonella* serovars contain SPI-1 to -5, while other pathogenicity islands are not so common. The colonization of the gastrointestinal tract and of the internal organs of poultry is enabled by the type VI secretion system encoded by the SPI-19 locus present in serovar Gallinarum (Blondel et al., 2010). In mice infected with serovar Typhimurium, the SPI-6 was necessary for the intracellular replication of the pathogen in macrophages and its systemic dissemination. The experimental work indicates that T6SS encoded by both SPI-6 and SPI-19 gene clusters are genetically involved in bacterial pathogenesis and that T6SS-SPI-6 play a role in gastrointestinal colonization and systemic spread of serovar Typhimurium in chickens (Pezoa et al., 2013).

Besides *Salmonella* pathogenicity islands-1 and 2, *Salmonella* strains involved in extraintestinal non-typhoid disease with bacteremia carry additional virulence genes in a *spv* locus, contained on virulence plasmids (Guiney and Fierer, 2011). Genes *spv* were found in serovars Typhimurium, Enteritidis, Choleraesuis, Abortusovis, Dublin, Gallinarum/Pullorum and in subspecies *arizonae*. The plasmid genes in the *spv* locus include *spvABCD* operon which is positively regulated by the upstream *spvR* gene. Only *spvR*, *spvB* and *spvC* are responsible for *spv* related virulence phenotype. In spite of having different biochemical pathways of action, SpvB and SpvC proteins are eventually involved in late apoptosis of macrophages, enabling the intracellular proliferation of *Salmonella* spp. Subsequent uptake of apoptotic macrophages by surrounding macrophages, facilitates cell to cell spread of *Salmonella* spp. (Guiney and Lesnick, 2005; Derakhshandeh et al., 2013). Consequently, it potentiates the systemic spread of the pathogen instead of causing a self limited gastroenteritis.

Salmonellae have different invading capacities in the poultry intestine and parenchymatous organs. They trigger systemic and local immune response which is in good correlation with their virulence. Experimental work was conducted by Berndt et al., (2007) to measure the immune response in cecum after the infection of White Leghorn day old chickens with serovars Enteritidis, Typhimurium,

Hadar and Infantis. At 2, 4 and 7 days post infection (pi) serovars Hadar and Infantis showed diminished invading capabilities for liver, compared to serovars Enteritidis and Typhimurium. *S. Enteritidis* was the best invader of the lower zones of the lamina propria, while *S. Infantis* was found in epithelial lining and subepithelial region. The increase of granulocytes, TCR1 gd and CD8 α in chicken cecum was most prominent for serovar Enteritidis, followed by serovars Typhimurium and Hadar, while Infantis provoked less significant immune cell influx. In the same study the reorganization of the extracellular matrix proteins, notably the increase of total fibronectin and tanascin-C, has been more pronounced after the infection of day old chickens with serovar Enteritidis comparing to the infection with the non invasive *Salmonella* Infantis. Furthermore, enhanced *Salmonella* spp. entry and the ability to disseminate in the gut epithelium support the concept that the most virulent strains utilize distinctive genetic mechanisms to invade the intestine and disseminate through the body, showing an important ability to provoke better immune responses in infected birds, as well (Berndt et al., 2009). It was experimentally shown that *S. Infantis* was found in higher numbers in avian macrophages in vitro comparing to *S. Typhimurium*, but the number of viable cells inside macrophages was higher for *S. Typhimurium* than for *S. Infantis* (Braukmann et al., 2015). Both serovars trigger active immune responses by activating genes involved in regulating immunological processes. The infection of avian macrophages with both serovars induced the increased expression of the immune mediators up to four hours post infection. The longer survival of serovar Typhimurium in macrophages was probably related to a higher and rapid SPI-2 genes activation, which explains the better invasiveness and the ability of causing systemic infection, something observed in serovar Typhimurium, but not in Infantis. The unfimbriated state of *Salmonella* spp. and *Escherichia coli* in chicken intestine are manifesting good colonizing ability in the intestine and oviducts of laying hens at 19 weeks of age as described by De Buck et al. (2004). However, the egg content, particularly the yolk and the egg shell, was contaminated by the wild type strain more efficiently.

Although the type 1 fimbriae deficient mutant caused prolonged bacteraemia in laying hens, the reduced egg shell contamination in mutant comparing to wild type strain, has shown that fimbriae are important for causing egg contamination in serovar Enteritidis (De Buck et al., 2004).

THE PREVALENCE OF *SALMONELLA* SEROVARS IN POULTRY FLOCKS

The rise of *S. Enteritidis* during the 1980s and 1990s coincided with the extensive measures undertaken to eradicate *S. Gallinarum*. It is suggested that *S. Enteritidis* has taken the ecologic niche previously occupied by *S. Gallinarum* in poultry flocks, via the mechanism of competitive exclusion, due to their antigenic similarity (Rabsch et al., 2000). Clearing the commercial flocks from *S. Gallinarum* enabled *S. Enteritidis* to colonize chickens without signs of disease (Andino and Hanning, 2015). In addition, serovar Enteritidis has a wider spectrum of natural reservoirs which makes it easier to persist on the farms. It has been isolated from insects, rodents, nematodes, wild birds and other animal hosts living in and around hen houses. Thus, after adequate disinfection of houses and stocking with culture-negative chicken, *S. Enteritidis* can be reintroduced from hen house pests, especially mice (Guard-Petter, 2001).

In the United States of America, *S. Enteritidis* which was dominant in the 1990s, was supplanted by serovar Heidelberg in the period 1997-2006, but since 2007 *S. Kentucky* has been the most prevalent serovar isolated from poultry (Foley et al., 2011). However, these serovars are less common in humans, with serovars Enteritidis and Typhimurium being the leading causes of alimentary toxoinfections in the USA. There are several possible reasons for the prevalence of serovars Kentucky and Heidelberg: flock immunity against *S. Enteritidis* gained due to vaccination or exposure might have opened the space for these two antigenically different serovars to which the flocks were susceptible (Foley et al., 2011). The ability of *S. Heidelberg* to colonize the reproductive tract in chickens and enter eggs, poses a threat to public health as another important egg transmitted pathogen, besides *S. Enteritidis* and *S. Typhimurium* (Gast et al., 2004). Although *S. Kentucky* is not so commonly involved in human infections, it is

very successful in colonizing chicken. One of the reasons might be the acquisition of the virulence plasmids ColBM and ColV from the avian pathogenic *Escherichia coli* (APEC) (Johnson et al., 2010).

In the past few years, the emergence of *S. Kentucky* strains resistant to multiple antimicrobial drugs has become a new threat to human and animal health. The international trade has facilitated the spread of those strains to the domestic poultry in the region of Mediterranean basin (Le Hello et al., 2013).

The experimental infection of two day old broiler chickens has revealed that serovar Kentucky persisted longer in the cecum comparing to Typhimurium and the peak was noted at 25 days pi (Cheng et al., 2015). Compared to *S. Typhimurium*, the expression of genes regulated by RNA polymerase sigma S factor (rpoS) was more pronounced in serovar Kentucky in the ceca content. The expression of genes from the metabolic pathway and the role of curli production seem to be in correlation with the ability of serovar Kentucky to colonize and persist in poultry.

Unlike other serovars, *S. Gallinarum* biovars Gallinarum and Pullorum are restricted to avian species and do not pose a risk to human health. However, among poultry, they cause septicemic fowl typhoid and pullorum disease (respectively) with high mortality and morbidity. Strict control programs using serological tests and elimination of positive birds has lead to the eradication of diseases from commercial poultry in the United States of America, Canada and most of Western Europe, although outbreaks occasionally occur (Barrow and Freitas Neto, 2011).

In the European Union, harmonized *Salmonella* control programs have lead to the overall decrease in the prevalence of five serovars (*S. Enteritidis*, *S. Typhimurium*, *S. Infantis*, *S. Hadar*, *S. Virchow*) of the public health relevance. However, in 2015 there was a slight increase in *S. Enteritidis* incidence comparing to 2014, but *S. Infantis* was the most prevalent serovar among domestic fowl (EFSA 2016a).

BIOFILM FORMING CAPACITY OF *SALMONELLA* SPP. IN POULTRY AND FEED INDUSTRY

Because of the profound ability to irreversibly bind to different types of biotic and abiotic surfaces,

the Most Prevalent Poultry-associated *Salmonella* serotypes (MPPSTs) usually have a capacity of biofilm (BF) formation on plant surfaces, in the host organism, as well as in a variety of materials commonly used in the poultry production and feed industry (Steenackers et al., 2012; White and Surette, 2006). Hence, BF formation is a common feature of bacteria and it is characterized as a complex surface associated community of microorganisms. Biofilm is defined as matrix-enclosed bacterial populations adherent to each other and/or on surfaces or interfaces (Donlan 2002; Donlan and Costerton 2002). Bacteria with the ability to form biofilms express different genes comparing to their planktonic counterparts, becoming increasingly resistant to antibiotics and disinfectants. Indeed, the resistance of bacteria in the BF may be 10 to 1000 times higher comparing to the bacteria in suspension, which is most often used for the examination of the effectiveness of disinfectants or other antimicrobial compounds, such as antibiotics (Mah and O'Toole, 2001). Hence, biofilm is the perfect microenvironment for the horizontal transfer of genetic material and the emergence of pathogens with new virulence factors and mechanisms of antibiotic resistance.

In a number of experimental studies, the ability of *Salmonella* to form BF on a variety of materials such as concrete, glass (Prouty and Gunn, 2003), cement (Joseph et al., 2001), stainless steel (Oliveira et al., 2007), plastic (Stepanović et al., 2004; Solomon et al., 2005), granite and rubber (Arnold and Yates, 2009) was confirmed (Solano et al., 2002; Steenackers et al., 2012). *Salmonella* spp. can rapidly colonize hydrophobic substrate, such as plastic, and they commonly produce a BF on them. Plastic materials are widely used on farms, in slaughterhouses and in food industry for the preparation of tanks, pipe-work, accessories and cutting surfaces (Díez-García et al., 2012). The microorganism easily forms a BF on galvanized steel, which is used for making transport containers for poultry (Ramesh et al., 2002). Various serovars of *Salmonella* spp. are characterized by a good ability to produce BF, which enables their persistence in poultry facilities, hatcheries, the water supply systems on farms, slaughterhouses, as well as in

processing and storage facilities of poultry products (Gradel et al., 2003; Gradel et al., 2004; McKee et al., 2008; Díez-García et al., 2012).

Biofilm is an important risk factor in feed contamination, and one of the critical points of controlling *Salmonella* spp. on poultry farms, having an increasing importance in the last decades (Cox and Pavic, 2010). The contamination of feed with *Salmonella* spp. may occur as a consequence of the use of contaminated raw materials or it may occur during the production process, by getting in contact with contaminated surfaces in production facilities. Biofilm formation is involved in both processes. The main components of the *Salmonella* BF-matrix, the protein surface aggregative fimbriae (curli) and the extracellular polysaccharide cellulose, are required for the colonization of plant surfaces and for the attachment to the surface of the feed factory environment. These biofilms allow the persistence of *Salmonella* spp. in feed and food factory environments for months, and even years (Vestby et al., 2009; Schonewille et al., 2012; Prunić et al., 2016).

In slaughterhouses and facilities for processing poultry carcasses, *Salmonella* spp. are found continuously, despite the regular use of strict measures for the control and reduction of pathogens (Rose et al., 2000; Joseph et al., 2001; Gradel et al., 2004; Marin et al., 2009). Research shows that conventional methods of disinfection are ineffective in eliminating *Salmonella* spp. from surfaces on which fresh meat processing is carried out (McKee et al., 2008). It is also experimentally evaluated that only two out of 13 commercially available disinfectants based on sodium hypochlorite, sodium chlorite and alkaline peroxide were effective against *Salmonella* biofilms formed on galvanized steel in the presence of organic matter (Ramesh et al., 2002). In field conditions, methods of cleansing and disinfection are often insufficient for *Salmonella* spp. elimination from poultry housing facilities (Marin et al., 2011; Davies and Breslin, 2003). The BF-matrix, particularly the extracellular polysaccharide cellulose, is considered to be an important factor for the protection against chemical agents.

The purpose of maintaining a dry environment in feed and food factories and low water activity in the finished product is to reduce pathogens, but these

measures are not effective in controlling *Salmonella* spp. In some *Salmonella* strains, including those of serovar Enteritidis, isolated from food products with low water activity, an increase in virulence and the reduction of the infective dose was found (Aviles et al., 2013; Andino et al., 2014). It is believed that the increasing virulence of *Salmonella* spp. in products with low water activity is the result of *rpoS* activation (the main stress response regulator), which directly affects the activation of virulence genes such as the *invA*, *hilA* and *sipC* (Aviles et al., 2013). However, experimental studies show that genes *invA* and *hilA* in *S. Enteritidis* are down regulated in low water activity, but the exact reason for the increased virulence of this serovar remains unknown (Andino et al., 2014).

Differences in the ability to produce the BF are established among different serovars, or strains of the same *Salmonella* serovar (Schonewille et al., 2012). However, *in vitro* conditions used in research on BF formation capacities, may not always reflect the conditions required for BF formation in the environment. Bacteria express important features that enable them to adapt under various challenges and the formation of the BF communities presents an important defense mechanism.

Biofilm is a risk that has been recognized recently as it causes long term contamination and persistency of some *Salmonella* serovars in all cycles of the poultry industry. It also presents actual research challenge in raising food producing animals and in safe food production. There are no effective measures to prevent or remove BF. Starting with the fact that multiple sources of contamination with *Salmonella* spp. are recognized, the only way to cope with *Salmonella* spp. in poultry production facilities is good management practice and high biological safety. Innovations in the field of BF control refer to the compounds that actually inhibit biosynthesis of signal molecules in BF, but they are not applicable in poultry and food industry at present.

RESISTANCE TO ANTIMICROBIAL AGENTS IN *SALMONELLA* SPP. FROM POULTRY SPECIMENS

Multiple drug resistance (MDR) of *Salmonella* spp. in poultry is developing because of the established

practice of using antibiotics in animal husbandry. There is evidence that some resistant *Salmonella* strains have increased virulence, which could be a result of the integration of virulence and resistance plasmids and their co-selection or up regulation of the virulence or the improved fitness of the bacteria (Mølbak, 2005).

It is widely considered that antibiotics used in human medicine should be avoided for the therapy of animals. Such practice is well established in developed countries except for rare cases, as for the treatment of infections caused by susceptible bacteria (Garcia-Migura et al., 2014). However, travelling and trade have a high impact on establishing MDR microorganisms in their communities. Besides the restrictive use of antibiotics in developed countries, growth promoter use was also banned in the year 2006 and the overall resistance rate in commensal and pathogenic bacteria from food producing animals has been decreasing. In developing countries resistance to fluoroquinolones and extended spectrum beta lactams is still worrisome. It has been recorded that multiple drug resistant *S. Kentucky*, *S. Typhimurium* and *S. Infantis* have a worldwide distribution and that poultry present permanent (*S. Infantis*, *S. Typhimurium*) or transient reservoirs (*S. Kentucky*). Emerging strains of *S. Kentucky* resistant to carbapenems and fluoroquinolones may cause life threatening disease in humans and they are among the most dangerous *Salmonella* serovars that have been diagnosed recently (LeHello et al., 2013). The first report of the occurrence of extended spectrum β -lactamase (ESBL) resistant *S. Kentucky* from poultry specimens (whole chicken, farm dust and chicken neck skin) in Ireland was attributed to *bla*_{SHV-12} and *bla*_{CMY-2} genes. Even though cephalosporins are not applicable for the therapy of chickens in Ireland, there is a possibility that the use of amoxicillin has favored the selection of β -lactamase producers over the time (Boyle et al., 2010). *Salmonella Kentucky* designated CVM29188 isolated from a chicken breast sample in the year 2003 has shown resistance to streptomycin, tetracycline, ampicillin and ceftiofur. All the genes determining resistances (*strAB* and *tetRA*, *bla*_{CMY-2'}, *sugE*) were found on two large transmissible plasmids. In addition, the pCVM29188_146 plasmid

is genetically similar to the virulence plasmids found in avian pathogenic *Escherichia coli* (APEC). These APEC-like plasmids were probably exchanged among the two bacteria species in the intestinal environment and they also possess virulence elements that have contributed to their establishment in predominant *Salmonella* Kentucky strains in chicken intestines and meat (Fricke et al., 2009).

Serovar Infantis is a typical poultry *Salmonella* serovar. It is well established on poultry farms with a tendency of clonal spread of the multidrug resistance phenotype. Clonal spread of *Salmonella* Infantis in poultry and poultry meat was reported in Japan (Shahada et al., 2006), Hungary (Nógrády et al., 2007), Israel (Gal-Mor et al., 2010), Italy (Dionisi et al., 2011), Germany (Hauser et al., 2012), Serbia (Rašeta et al., 2014; Velhner et al., 2014) but also in humans in Argentina (Merino et al., 2003) and Brazil (Fonseca et al., 2006). All these clonal strains were resistant to three or more antimicrobials except for Serbia, where the predominant resistance phenotype was nalidixic acid (NAL) / tetracycline (TET), while an approximate 30% of the isolates was showing resistance to ciprofloxacin (CIP), with the minimal inhibitory concentration (MIC) of $\geq 1\text{mg/L}$ (Velhner et al., 2014). The resistance to CIP was also found in some isolates of *Salmonella* Infantis from Hungary which belonged to the different pulsotype (Nógrády et al., 2007). The occurrence of novel multidrug resistant clones from human, food and poultry sources in Israel was established in 2007. These clones were resistant to NAL, TET, nitrofurantoin and trimethoprim/sulfamethoxazole (SXT). It was evident that the resistance to TET and SXT was encoded by a 280kb self-transmissible plasmid (pESI) and that new clones represented 33% of all *Salmonella* strains isolated in Israel (Aviv et al., 2014).

The most frequently detected serovars in poultry meat in the EU were *S. Infantis*, *S. Indiana* and *S. Enteritidis*. According to the epidemiological cut off breakpoints (ECOFFs), multi-drug resistance in *Salmonella* spp from broiler meat in the year 2014 fluctuated from high (Hungary) to low (France and Lithuania) or complete absence of resistance (Ireland). Resistance to colistin was 31.6% in *S. Enteritidis* while resistance to ciprofloxacin and nalidixic acid was 22.4%. No resistance toward

colistin was recorded in *S. Infantis*. High rate of multi-drug resistance was detected in some EU countries in *Salmonella* spp. isolates from turkey meat. In flocks of boilers, the most prevalent was *S. Infantis* with extremely high resistance rate to ciprofloxacin (except in Denmark and Spain). Second most frequently detected serovar in broilers was *S. Enteritidis* with overall resistance to ciprofloxacin and nalidixic acid of 23.3 and 24.6% respectively. Levels of resistance to ciprofloxacin were high in *Salmonella* spp. isolates from layer flocks in Cyprus, Hungary, Italy and Romania. However, trends in multi drug resistance were much lower in *Salmonella* spp. from layers comparing to broilers in the EU member states (EFSA 2016b). In the report of the National Antimicrobial Resistance Monitoring System (NARMS) USDA of 2011, it was documented that the most prevalent serovars from poultry in the USA were: Kentucky, Enteritidis, Heidelberg, Typhimurium var-5 and Infantis (NARMS-USDA, 2014). Resistance to beta lactam/inhibitor combination and cepheims was found in 17.9% of serovar Heidelberg isolates and in 0.7% of serovar Enteritidis isolates, regarding poultry. Resistance to (fluoro)quinolones was not found, while resistance to gentamicin was evident in 1.3% of the serovar Kentucky isolates, 14.3% of the serovar Heidelberg isolates and in 10.5% of the serovar Typhimurium var-5 isolates from poultry in 2011 (NARMS-USDA, 2014).

Poultry meat and products therefore present a significant reservoir of resistant *Salmonella* all around the world. However, the resistance patterns differ markedly from continent to continent and among countries. In this respect, the highest concern is the resistance of serovars Kentucky and Infantis which become well established in poultry flocks and frequently develop a multidrug resistant phenotype.

CONCLUDING REMARKS

Much effort has been put through to provide safe poultry meat and products worldwide. In spite of the fact that many biological, chemical products and vaccines have been invented and implemented in poultry production systems, it is still difficult to eliminate *Salmonella* spp. from the food chain. Different *Salmonella* serovars tend to take place

in commercially produced poultry, as soon as an ecological niche becomes vacant. In many developed countries, where measures, such as vaccination, were undertaken to eradicate certain *Salmonella* serovars, other less immunogenic serovars emerge and become dominant. *Salmonella* control programs in poultry industry has to cover all the segments of food production by implementing various procedures and strategies in integrated poultry production systems. It has to follow up new trends in raising free range chickens with respect to new challenges regarding food safety in upcoming years. In countries where comprehensive programs have been implemented to

eliminate *Salmonella* spp. from the food production chain, travelling and trade still pose and will continue to pose a substantial risk for infection of humans and efficient dissemination of *Salmonella* spp. globally.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests. ■

REFERENCES

- Andino A, Pendleton S, Zhang N, Chen W, Critzer F, Hanning I (2014) Survival of *Salmonella enterica* in poultry feed is strain dependent. *Poult Sci* 93:441–447.
- Andino A, Hanning I (2015) *Salmonella enterica*: survival, colonization and virulence differences among serovars. *Scientific World J* Article ID 520179. <http://dx.doi.org/10.1155/2015/520179>
- Arnold JW, Yates IE (2009) Interventions for control of *Salmonella*: Clearance of microbial growth from rubber picker fingers. *Poult Sci* 88:1292–1298.
- Aviles B, Klotz C, Smith T, Williams R, Ponder M (2013) Survival of *Salmonella enterica* serotype Tennessee during simulated gastric passage is improved by low water activity and high fat content. *J Food Prot* 76:333–337.
- Aviv G, Tsyba K, Steck N, Salmon-Divon M, Cornelius A, Rahav G, Grassi GA, Gal-Mor O (2014) A unique megaplasmid contributes to stress tolerance and pathogenicity of an emergent *Salmonella enterica* serovar Infantis strain. *Environ Microbiol* 16:977–994.
- Bailey JS, Cason JA, Cox NA (1998) Effect of *Salmonella* in young chicks on competitive exclusion treatment. *Poult Sci* 77:394–399.
- Barrow PA, Freitas Neto OC (2011) Pullorum disease and fowl typhoid – new thoughts on old diseases: a review. *Avian Pathol* 40:1–13.
- Bearson SM, Bearson BL, Rasmussen MA (2006) Identification of *Salmonella enterica* serovar Typhimurium genes important for survival in the swine gastric environment. *Appl Environ Microbiol* 72:2829–2836.
- Berndt A, Wilhelm A, Jugert C, Pieper J, Sachse K, Methner U (2007) Chicken cecum immune response to *Salmonella enterica* serovars of different levels of invasiveness. *Infect Immun* 75:5993–6007.
- Berndt A, Müller J, Borsi L, Kosmehl H, Methner U, Berndt A (2009) Reorganisation of the caecal extracellular matrix upon *Salmonella* infection-relation between bacterial invasiveness and expression of virulence genes. *Vet Microbiol* 133:123–137.
- Blondel CJ, Yang HJ, Castro B, Chiang S, Toro CS, Zaldivar M,

- Contreras I, Andrew-Polymeris HL, Santiviago CA (2010) Contribution of the type VI secretion system encoded in SPI-19 to chicken colonization by *Salmonella enterica* serotypes Gallinarum and Enteritidis. *PLoS One*. 5(7), e11724, doi:10.1371/journal.pone.0011724.
- Boyle F, Morris D, O'Connor J, DeLappe N, Ward J, Cormican M (2010) First report of extended-spectrum- β -lactamase-producing *Salmonella enterica* serovar Kentucky isolated from poultry in Ireland. *Antimicrob Agents Chemother* 54:551-553.
- Braukmann M, Methner U, and Brendt A (2015) Immune reaction and survivability of *Salmonella* Typhimurium and *Salmonella* Infantis after infection of primary avian macrophages. *PLoS One*. 10(3), e0122540, doi:10.1371/journal.pone.0122540.
- Buhr RJ, Richardson LJ, Cason JA, Cox NA, Fairchild BD (2007) Comparison of four sampling methods for the detection of *Salmonella* in broiler litter. *Poult Sci* 86:21-25.
- Carrique-Mas JJ, Davies RH (2008) *Salmonella* Enteritidis in commercial layer flocks in Europe: Legislative background, on farm sampling and main challenges. *Braz J Poultry Sci* 10:1-9.
- Cheng Y, Pedroso AA, Porwollik S, McClelland M, Lee MD, Kwan T, Zamperini K, Soni V, Sellers HS, Russell SM, Maurer JJ (2015) *rpoS*-Regulated core genes involved in the competitive fitness of *Salmonella enterica* serovar Kentucky in the intestines of chickens. *Appl Environ Microbiol* 81:502-514.
- Commission Regulation (EC) No 1177/2006: Implementing Regulation (EC) No 2160/2003 of the European Parliament and of the Council as regards requirements for the use of specific control methods in the framework of the national programmes for the control of salmonella in poultry.
- Commission Regulation (EC) No 1168/2006: Implementing Regulation (EC) No 2160/2003 as regards a Community target for the reduction of the prevalence of certain salmonella serotypes in laying hens of *Gallus gallus* and amending Regulation (EC) No 1003/2005.
- Commission Regulation (EC) No 584/2008: Implementing Regulation (EC) No 2160/2003 of the European Parliament and of the Council as regard a Community target for the reduction of the prevalence of *Salmonella enteritidis* and *Salmonella* Typhimurium in turkeys.
- Commission Regulation (EC) No 200/2010: Implementing regulation (EC) No 2160/2003 of the European Parliament and of the Council as regards a Union target for the reduction of the prevalence of *Salmonella* serotypes in adult breeding flocks *Gallus gallus*.
- Commission Regulation (EC) No 517/2011 implementing Regulation (EC) No 2160/2003 of the European Parliament and of the Council as regards a Union target for the reduction of the prevalence of certain *Salmonella* serotypes in laying hens of *Gallus gallus* and amending Regulation (EC) No 2160/2003 and Commission Regulation (EU) No 200/2010.
- Cox JM, Pavic A (2010) Advances in enteropathogen control in poultry production. *J Appl Microbiol* 108:745-755.
- Davies RH, Breslin M (2003) Observations on *Salmonella* contamination of commercial laying farms before and after cleaning and disinfection. *Vet Res* 152, 283-287.
- De Buck J, Van Immerseel F, Haesebrouck F, Ducatelle R (2004) Effect of type 1 fimbriae of *Salmonella enterica* serotype Enteritidis on bacteraemia and reproductive tract infection in laying hens. *Avian Pathol* 33:314-320.
- Derakhshandeh A, Firouzi R, and Khoshbakht R (2013) Association of three plasmid-encoded *spv* genes among different *Salmonella* serotypes isolated from different origins. *Indian J Microbiol* 53:106-110.
- Dewaele I, Van Meirhaeghe, H, Rasschaert G, Vanrobaeys M, De Graef E, Herman L, Ducatelle R, Heyndrickx M, De Reu K (2012) Persistent *Salmonella* Enteritidis environmental contamination on layer farms in the context of an implemented national control program with obligatory vaccination. *Poult Sci* 91:282-291.
- Diez-García M, Capita R, and Alonso-Calleja C (2012) Influence of serotype on the growth kinetics and the ability to form biofilms of *Salmonella* isolates from poultry. *Food Microbiol* 31:173-180.
- Dionisi AM, Lucarelli C, Benedetti I, Owczarek S, Luzzi I (2011) Molecular characterization of multidrug-resistant *Salmonella enterica* serotype Infantis from humans, animals and the environment in Italy. *Int J Antimicrob Agents* 38:384-389.
- Donlan RM (2002) Biofilms: microbial life on surfaces. *Emerg Infect Dis* 8:881-890.
- Donlan RM, Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 15:167-193.
- EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2016a. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. *EFSA Journal* 2016 14(12):4634, p231, doi:10.2903/j.efsa.2016.4634
- EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2016b. EU Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2014. *EFSA Journal* 2016 14(2):4380, p60, doi:10.2903/j.efsa.2016.4380.
- Foley SL, Nayak R, Hanning IB, Johnson TJ, Han J, Ricke SC (2011) Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. *Appl Environ Microbiol* 77:4273-4279.
- Foley SL, Johnson TJ, Ricke SC, Nayak R, Danzeisen J (2013) *Salmonella* pathogenicity and host adaptation in chicken-associated serovars. *Microbiol Mol Biol Rev* 77:582-607.
- Fonseca EL, Mykytczuk OL, Asensi MD, Reis EMF, Ferraz LR, Paula FL, Ng LK, Rodrigues DP (2006) Clonality and antimicrobial resistance gene profiles of multidrug-resistant *Salmonella enterica* serovar Infantis isolates from four public hospitals in Rio de Janeiro, Brazil. *J Clin Microbiol* 44:2767-2772.
- Fricke WF, McDermott PF, Mammel MK, Zhao S, Johnson TJ, Rasko DA, Fedorka-Cray PJ, Pedroso A, Whichard JM, Leclerc JE, White DG, Cebula TA, Ravel J (2009) Antimicrobial resistance-conferring plasmids with similarity to virulence plasmids from avian pathogenic *Escherichia coli* strains in *Salmonella enterica* serovar Kentucky isolates from poultry. *Appl Environ Microbiol* 75:5963-5971.
- Gal-Mor O, Valinsky L, Weinberger M, Guy S, Jaffe J, Schorr YI, Raisfeld A, Agmon V, Nissan I (2010) Multidrug-resistant *Salmonella enterica* serovar Infantis, Israel. *Emerg Infect Dis* 16:1754-1757.
- Garcia-Migura L, Hendriksen RS, Fraile L, Aarestrup FM (2014) Antimicrobial resistance of zoonotic and commensal bacteria in

- Europe: the missing link between consumption and resistance in veterinary medicine. *Vet Microbiol* 170:1-9.
- Gast RK, Guard-Bouldin J, and Holt PS (2004) Colonization of reproductive organs and internal contamination of eggs after experimental infection of laying hens with *Salmonella heidelberg* and *Salmonella enteritidis*. *Avian Dis* 48:863-869.
- Gradel KO, Jørgensen JC, Andersen JS, Corry JE (2003) Laboratory heating studies with *Salmonella* spp. and *Escherichia coli* in organic matter, with a view to decontamination of poultry houses. *J Appl Microbiol* 94:919-928.
- Gradel KO, Jørgensen JC, Andersen JS, Corry JE (2004) Monitoring the efficacy of steam and formaldehyde treatment of naturally *Salmonella* - infected layer houses. *J. Appl Microbiol* 96:613-622.
- Guiney DG, Lesnick M (2005) Targeting of the actin cytoskeleton during infection by *Salmonella* strains. *Clin Immunol* 114:248-255.
- Guiney DG, Fierer J (2011) The role of the *spv* genes in *Salmonella* pathogenesis. *Front Microbiol* 2:129. doi: 10.3389/fmicb.2011.00129
- Guard-Petter J (2001) The chicken, the egg and *Salmonella enteritidis*. *Environ Microbiol* 3:421-430.
- Hauser E, Tietze E, Helmuth R, Junker E, Prager R, Schroeter A, Rabsch W, Fruth A, Toboldt A, Malrony B (2012) Clonal dissemination of *Salmonella enterica* serovar *Infantis* in Germany. *Foodborne Pathog Dis* 9:352-360.
- Hensel M (2000) *Salmonella* pathogenicity island 2. *Mol Microbiol* 36:1015-1023.
- Joseph B, Otta SK, Karunasagar I, Karunasagar I (2001) Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. *Int J Food Microbiol* 64:367-372.
- Johnson TJ, Thorsness JL, Anderson CP, Lynne AM, Foley SL, Han J, Fricke WF, McDermott PF, White DG, Khatri M, Stell AL, Flores C, Singer RS (2010) Horizontal gene transfer of a ColV plasmid has resulted in a dominant avian clonal type of *Salmonella enterica* serovar *Kentucky*. *PLoS One* 2010; 5(12), e15524. doi:10.1371/journal.pone.0015524
- Kubori T, Sukhan A, Aizawa SI, Galán JE (2000) Molecular characterization and assembly of the needle complex of the *Salmonella typhimurium* type III protein secretion system. *Proc Natl Acad Sci USA* 97:10225-10230.
- Le Hello S, Harrois D, Bouchrif B, Sontag L, Elhani D, Guibert V, Zerouali K, Weill FX (2013) Highly drug-resistant *Salmonella enterica* serotype *Kentucky* ST198-X1: a microbiological study. *Lancet Infect Dis* 13:672-679.
- Mah TFC, O'Toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 9:34-39.
- Marin C, Hernandez A, Lainez M (2009) Biofilm development capacity of *Salmonella* strains isolated in poultry risk factors and their resistance against disinfectants. *Poult Sci* 88:424-431.
- Marin C, Balasch S, Vega S, Lainez M (2011) Sources of *Salmonella* contamination during broiler production in Eastern Spain. *Prev Vet Med* 98:39-45.
- McKee SR, Townsend JC, Bilgili SF (2008) Use of a scald additive to reduce levels of *Salmonella typhimurium* during poultry processing. *Poult Sci* 87:1672-1677.
- Merino LA, Ronconi MC, Navia MM, Ruiz J, Sierra JM, Cech NB, Lodeiro NS, Vila J (2003) Analysis of the clonal relationship among clinical isolates of *Salmonella enterica* serovar *Infantis* by different typing methods. *Rev Inst Med Trop S Paulo* 45: 119-123.
- Mølbak K (2005) Human health consequences of antimicrobial drug-resistant *Salmonella* and other foodborne pathogens. *Clin Infect Dis* 41:1613-1620.
- National Antimicrobial Resistance Monitoring System-NARMS, Annual Animal Report (2011) <https://www.ars.usda.gov/ARSTUserFiles/60401020/NARMS/NARMS2011/NARMS%20USDA%202011%20Report.pdf>
- Nógrády N, Tóth A, Kostyák A, Pászti J, Nagy B (2007) Emergence of multidrug-resistant of *Salmonella Infantis* in broiler chickens and humans in Hungary. *J Antimicrob Chemother* 60:645-648.
- Oliveira K, Oliveira T, Teixeira P, Azeredo J, Oliveira R (2007) Adhesion of *Salmonella Enteritidis* to stainless steel surfaces. *Braz J Microbiol* 38:318-323.
- Pezoa D, Yang HJ, Blondel CJ, Santiviago CA, Andrews-Polymenis HL, Contreras I (2013) The type VI secretion system encoded in SPI-6 plays a role in gastrointestinal colonization and systemic spread of *Salmonella enterica* serovar *Typhimurium* in the chicken. *PLoS One* 8(5), e63917. doi:10.1371/journal.pone.0063917
- Prouty AM, Gunn JS (2003) Comparative analysis of *Salmonella enterica* serovar *Typhimurium* biofilm formation in gallstones and on glass. *Infect Immun* 70:2640-2649.
- Prunić B, Milanov D, Velhner M, Pajić M, Pavlović Lj, Mišić D (2016) Clonal persistence of *Salmonella enterica* serovars *Montevideo*, *Tennessee* and *Infantis* in feed factories. *J Infect Dev Ctries* 10:662-666.
- Rabsch W, Hargis B.M, Tsois RM, Kingsley RA, Hinz KH, Tschäpe H, Bäuml AJ (2000) Competitive exclusion of *Salmonella Enteritidis* by *Salmonella Gallinarum* in poultry. *Emerg Infect Dis* 6:443-448.
- Ramesh N, Joseph SW, Carr LE, Douglass LW, Wheaton FW (2002) Evaluation of chemical disinfectants for the elimination of *Salmonella* biofilms from poultry transport containers. *Poult Sci* 81:904-910.
- Rašeta M, Teodorović V, Bunčić O, Katić V, Branković-Lazić I, Polaček V, Vidanović D (2014) Antibiotic resistance and molecular studies on *Salmonella enterica* subspecies *enterica* serovar *Infantis* isolated in human cases and broiler carcasses. *Acta Veterinaria* 64:257-268.
- Rose N, Beaudeau F, Drouin P, Toux J. Y, Rose V, Colin P (2000) Risk factors for *Salmonella* persistence after cleansing and disinfection in French broiler-chicken houses. *Prev Vet Med* 44:9-20.
- Schonewille E, Nesse LL, Hauck R, Windhorst D, Hafez MH, Vestby LK (2012) Biofilm building capacity of *Salmonella enterica* strains from the poultry farm environment. *FEMS Immunol. Med Microbiol* 65:360-365.
- Shahada F, Chuma T, Tobata T, Okamoto K, Sueyoshi M, Takase K (2006) Molecular epidemiology of antimicrobial resistance among *Salmonella enterica* serovar *Infantis* from poultry in Kagoshima, Japan. *Int J Antimicrob Agents* 28:302-307.
- Solano C, Garcia B, Valle J, Berasain C, Ghigo JM, Gamazo C, Lasa I (2002) Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. *Mol Microbiol* 43:793-808.
- Solomon EB, Niemira BA, Sapers GM, Annous BA (2005) Biofilm

- formation, cellulose production, and curli biosynthesis by *Salmonella* originating from produce, animal and clinical sources, *J Food Protect* 68:906-912.
- Steenackers H, Hermans K, Vanderleyden J, De Keersmaecker SCJ (2012) *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. *Food Res Int* 45:502-531.
- Stepanović S, Ćirković I, Ranin L, Svabić-Vlahović M (2004) Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Lett Appl Microbiol* 38:428-432.
- Velge P, Cloeckert A, Barrow P (2005) Emergence of *Salmonella* epidemics: The problems related to *Salmonella enterica* serotype Enteritidis and multiple antibiotic resistance in other major serotypes. *Vet Res* 36:267-288.
- Velhner M, Kozoderović G, Grego E, Galić N, Stojanov I, Jelesić Z, Kehrenberg C (2014) Clonal spread of *Salmonella enterica* serovar Infantis in Serbia: acquisition of mutations in the topoisomerase genes *gyrA* and *parC* leads to increased resistance to fluoroquinolones. *Zoonoses Public Health* 61:364-370.
- Vestby LK, Møretø T, Langsrud S, Heir E, Nesse LL (2009) Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal- and feed factories. *BMC Vet Res* 5, 20, doi:10.1186/1746-6148-5-20
- Wales A, Breslin M, Carter B, Sayers R, Davies R (2007) A longitudinal study of environmental *Salmonella* contamination in caged and free-range layer flocks. *Avian Pathol* 36:187-197.
- White AP, Surette MG (2006) Comparative Genetics of the *rdar* morphotype in *Salmonella*. *J Bacteriol* 188:8395-8406.
- Wisner A, Desin T, White A, Potter A, Köster W (2012) The *Salmonella* pathogenicity island-1 and -2 encoded type III secretion systems. In: *Salmonella - a diversified superbug*. InTech: pp 469-494. doi: 10.5772/2471

Current status and advances in ram semen cryopreservation

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Παρούσα κατάσταση και εξελίξεις στην κρυοσυντήρηση του σπέρματος του κριού

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ABSTRACT. Ram semen cryopreservation contributes to genetic improvement through artificial insemination, eliminates geographical barriers in artificial insemination application and supports the preservation of endangered breeds thus the conservation of biodiversity. Sperm freezing process induces ultrastructural, biochemical and functional changes of spermatozoa. Especially, spermatozoa's membranes and chromatin can be damaged, sperm membranes' permeability is increased, hyper oxidation and formation of reactive oxygen species takes place, affecting fertilizing ability and subsequent early embryonic development. Aiming to improve ram frozen-thawed semen's fertilizing capacity, many scientific investigations took place. Among them the composition of semen extenders, was a main point of interest. Semen preservation extenders regulate and support an environment of adequate pH and buffering capacity to protect spermatozoa from osmotic and cryogenic stress. Therefore, permeating (glycerol, dimethyl sulfoxide) and non-permeat-

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ing (egg yolk, skimmed milk) cryoprotectants, sugars (glucose, lactose, trehalose, raffinose), salts (sodium citrate, citric acid) and antioxidants (amino acids, vitamins, enzymes) have been added and tested. Moreover, semen dilution rate, storage temperature, cooling rate and thawing protocol, are also some key factors that have been studied. The research results of this scientific topic are encouraging, not only about the freezing and thawing procedures, but also about the improvement of the additives' properties. However, further research is needed to enhance the fertilizing ability of ram frozen-thawed semen, making its use practical in sheep reproductive management by the application of cervical artificial insemination.

Keywords: cryopreservation, ram semen, frozen-thawed, cryoprotectants

ΠΕΡΙΛΗΨΗ. Η κρυοσυντήρηση του σπέρματος του κριού αποσκοπεί στη γενετική βελτίωση μέσω της τεχνητής σπερματέγχυσης, στην άρση των γεωγραφικών περιορισμών, στην προστασία απειλούμενων με εξαφάνιση φυλών και στη διατήρηση της βιοποικιλότητας. Η διαδικασία κατάψυξης όμως, επιφέρει δομικές, βιοχημικές και λειτουργικές μεταβολές στα σπερματοζωάρια. Συγκεκριμένα, βλάπτονται οι κυτταρικές μεμβράνες και αυξάνεται η διαπερατότητά τους, μεταβάλλεται η δομή της χρωματίνης τους και προκαλείται υπεροξειδωση καθώς παράγονται δραστικές μορφές οξυγόνου. Οι αλλαγές αυτές, επηρεάζουν αρνητικά τη γονιμοποιητική ικανότητα των σπερματοζωαρίων του κριού και την πρώιμη εμβρυική ανάπτυξη. Στη βιβλιογραφία εντοπίζονται πολυάριθμες επιστημονικές προσπάθειες βελτίωσης της γονιμότητας του κρυοσυντηρημένου σπέρματος του κριού. Πρωταρχικό και κοινό σημείο διερεύνησης, υπήρξε η σύνθεση των αραιωτικών μέσων. Τα αραιωτικά του σπέρματος πρέπει να έχουν, μεταξύ άλλων, κατάλληλη ρυθμιστική ικανότητα και pH ώστε να προστατεύουν τα σπερματοζωάρια από την οσμωτική και τη θερμική καταπόνηση. Υπό αυτό το πρίσμα, έγινε προσθήκη και μελέτη πληθώρας ουσιών με ποικίλες ιδιότητες και αποτελέσματα. Μεταξύ αυτών, χρησιμοποιήθηκαν κρυοπροστατευτικά που διαπερνούν (γλυκερίνη, διμεθυλο-σουλφοξείδιο) ή όχι (κρόκος αυγού, αποβουτυρωμένο γάλα) την κυτταρική μεμβράνη, σάκχαρα (γλυκόζη, λακτόζη, τρεχαλόζη, ραφινόζη), άλατα (κιτρικό νάτριο, κιτρικό οξύ) και αντιοξειδωτικά (αμινοξέα, βιταμίνες, ένζυμα). Άλλοι παράγοντες που μελετώνται εκτενώς, αφορούν στη θερμοκρασία και στο χρόνο συντήρησης στα επιμέρους στάδια της κατάψυξης, στο ρυθμό πτώσης της θερμοκρασίας και στο πρωτόκολλο αναθέρμανσης. Τα αποτελέσματα των ερευνών είναι ενθαρρυντικά, όχι μόνο ως προς τις διαδικασίες κατάψυξης/αναθέρμανσης του σπέρματος, αλλά και ως προς τις ωφέλιμες ιδιότητες των προσθετικών. Παρά την πρόοδο που επιτεύχθηκε μέχρι σήμερα, εξακολουθούν να υφίστανται κενά, μέχρις ότου επιτευχθεί η διευρυμένη χρήση του κρυοσυντηρημένου σπέρματος του κριού στην αναπαραγωγική διαχείριση των ποιμνίων.

Λέξεις ευρετηρίασης: κρυο-συντήρηση, σπέρμα κριού, ποιοτικά χαρακτηριστικά σπέρματος

INTRODUCTION

Ram semen cryopreservation is of high interest, particularly in European countries, aiming to increase productive parameters by animal genetic improvement in selected flocks. The worldwide use of ram frozen-thawed semen eliminates the geographical barriers, supports the preservation of endangered breeds and conserves the biodiversity (Andrabi and Maxwell, 2007). Additionally, the need for widespread performance of sheep artificial insemination (AI) over extended periods or at different times of the year, stimulated more research on semen cryopreser-

vation scientific topic.

It has been comprehensively reported that sperm freezing process induces ultrastructural, biochemical and functional changes of spermatozoa (Salamon and Maxwell 1995b). Especially, spermatozoa's plasma and acrosome membranes are cryosensitive, so cells' membrane permeability is increased, sperm motility, morphology and chromatin integrity may be affected (Salamon and Maxwell, 2000; Gandini et al., 2006). Moreover, hyper oxidation and formation of reactive oxygen species takes place, damages of mitochondrial sheath and tail axoneme can be detected (Álvarez

and Storey, 1993).

These cryogenic changes have been associated with decreased sperm viability, membranes' functional integrity, and impaired transport and survival of spermatozoa into the female reproductive tract, affecting fertilizing ability and early embryonic development (Salamon and Maxwell 2000). Freezing - thawing processes can induce irreversible damage to ram spermatozoa. According to Medeiros et al. (2002), a relatively high proportion (40-60%) of ram spermatozoa preserve their motility after freeze-thawing, but only about 20-30% remain biologically functional.

Analysis of ram semen provides valuable information about the function of spermatozoa, with a reasonable degree of certainty about the outcome of AI (Tsakmakidis, 2010). However, interacting factors affect the results of AI with frozen-thawed ram semen; such are the technique of insemination, environmental elements and female-related parameters like estrous synchronization method, estrous detection and insemination time (Anel et al., 2005). Although, lower fertility rates are generally accepted owing to cryopreservation effect on ram spermatozoa (O'Meara et al., 2005), laparoscopic insemination of ram frozen-thawed semen is proposed as the selective technique to improve the outcome of AI in small ruminants (Milovanović et al., 2013). In spite of the fact that satisfying success rates are obtained by laparoscopic insemination, it is an expensive operation that has been criticized on welfare grounds (Fair et al., 2005). Additionally, it is not a practical method of fertilization, because it cannot be performed in routine.

Thus, attempts are being developed in cryopreservation methods and techniques with the goal to improve fertility after cervical insemination with frozen-thawed ram semen. This article summarizes and presents the current state of the topic.

BACKGROUND AND CURRENT STATUS

The scientific efforts to improve ram frozen-thawed semen quality and fertilizing ability are classified in two major categories: a) modifications and specificities of freezing methods, protocols and semen packaging, b) additives and modification of the extenders' contents.

α) "Modifications and specificities of freezing methods, protocols and semen packaging"

In summary, the common ram semen freezing process includes semen collection, two steps of dilution by supplemented extenders with egg yolk (first step) and glycerol (second step), cooling to a temperature close to 5° C and equilibration for some time to reduce cellular metabolic activity and increase life span of sperm cells. Semen is either packaged in straws of 0.25 and 0.5 ml for freezing and storage, or frozen as pellets on shallow depressions in dry ice (Ritar and Ball, 1993). Straws are placed above liquid nitrogen vapors or into a programmable bio-freezer machine. In the first traditional method, the freezing rate is regulated by the distance between the straws and the level of liquid nitrogen. Thus, it is advised to be placed 4-6 cm above the liquid nitrogen surface, as semen is being cooling according to a parabola-shaped curve (Salomon & Maxwell, 2000).

Many years ago, it was reported that the freezing rate must be slow enough to allow water to leave the cells by osmosis and fast enough to allow extracellular water freezing because intracellular ice formation causes irreversible damages to spermatozoa (Fisher et al., 1986). Additionally, a crucial temperature range (approximately from -5 to -50° C) has been confirmed as the range when ice crystal formation and spermatozoa dehydration take place (Kumar et al., 2003). Therefore, a lot of studies tried to improve ram frozen-thawed semen quality by freezing rate modifications, but controversial results can be found in the literature.

According to Lebouef et al. (2000) rapid cooling of extended semen from 30 to 15° C may not affect sperm survival. However, Watson (2000) reported that the fast cooling from 30 to 10, 5 or 0° C, causes injuries in some sperm cells, called "cold shock". This phenomenon is more pronounced in boar, but it is also occurs in ruminants' spermatozoa. During cooling process, the temperature range of 5-15° C is the most critical for cells' damage, because it is more related to changes in plasma membranes' fatty acid composition and lipid class ratios, whilst the uptake of calcium contributes to similar to acrosome reaction, capacitation changes (Drobnis et al., 1993). Additionally, the equilibration of ram semen at 5° C is necessary to secure freezing efficiency. Purdy et

al. (2006; 2010) reported that ram semen should not be cryopreserved immediately after collection, while when it is held at 5° C for either 48 h or 24 h prior to freezing, no injurious effects on post thaw motility, membrane integrity and fertilizing potential, are induced. These results were similar to those obtained by Câmara et al. (2011) and O'Hara et al. (2010), who demonstrated that the integrity of sperm membrane remained relatively stable for up to 12 or 72 h of equilibration at 5° C, respectively.

In practice, freezing rates from 50 to 100° C/ min are usually selected for ram semen. According to Anel et al. (2003), used rates from -10 to -60° C, should be faster than 50° C/min, but then slower rates (20–30° C/ min) may be applied up to the completion of freezing. In agreement with this indication, Vichas et al. (2017) also found that a slow freezing rate (25° C/ min from -8° C to -130° C) of Chios ram breed semen, provides higher motility and plasma membrane integrity compared to a fast freezing rate (50° C/ min from -8° C to -130° C) after thawing, as well as, after 3 h of incubation at 37° C.

Not only cooling/freezing, but also thawing conditions affect spermatozoa's survival. Thawing rate depends on whether cooling rate was sufficiently high to induce intracellular freezing or sufficiently low to produce cell dehydration. Thawing involves a reversal of these effects, so in the first situation, fast thawing is required to prevent re-crystallization of intracellular ice. During the semen freezing process, intercellular water is the first part of the sample which will be frozen, whilst ice formation under uncontrolled velocity and morphology may damage spermatozoa (Arav et al., 2002). Some efforts were made to avoid that issue. Arav et al. (2002) developed a device (Multi-Thermal-Gradient'-MTG®) to control temperature seeding and morphology of created ice crystals, achieving high rates of post thaw semen motility. Recently, Murawski et al. (2015) used an innovative technology preparing the commercial semen extender Triladyl® (Minitube, Germany) with water declusterized in the low-temperature plasma reactor, called "nanowater". This process led not only to lower crystallization rate of water but also to a substantial improvement in the fertilizing ability of frozen-thawed ram semen and higher conception rates after laparoscopic AI of ewes.

Many researchers studied the effect of thawing in relation to the types of semen packaging, taking different results. Paulenz et al. (2004) indicated that cervical AI using mini tubes resulted in the highest lambing rate, superior to mini straws. Regarding the thawing temperatures, there was a significantly higher lambing rate for 70° C/ 5 sec compared to 35° C/ 12 sec. However, another field study of the same research team didn't reveal significant differences on fertility after vaginal insemination of 719 Norwegian crossbred ewes with different packaging types (mini straws or mini tubes) and thawing protocols (mini tubes were thawed at 70° C for 8 sec and mini straws either at 50° C for 9 sec or at 35° C for 12 sec) (Paulenz et al., 2007). Nordstoga et al. (2009) also reported no significant differences between straw types (medium or mini) and thawing protocols (medium thawed at 35° C for 20 sec or at 70° C for 8 sec, mini straws thawed at 35° C for 15 sec) and neither a significant difference in non-return-to estrus or lambing rate, after vaginal insemination. A dissimilar approach was selected by Awada and Graham (2004), who demonstrated that cryopreservation of ram spermatozoa is more efficient in pellets which were frozen on the cold surface of cattle fat than in straws or pellets frozen on paraffin wax. (*Table 1*)

b) "Additives and modification of the extenders' contents"

Semen cryopreservation requires extension in specific diluents. The extenders must have adequate pH, buffering capacity and suitable osmolality to protect spermatozoa from cryogenic injury, by reducing the physical and chemical stress that induce both freezing and thawing processes (Purdy, 2006). Sperm cryopreservation extenders contain a penetrating cryoprotectant (glycerol, ethylene or propylene glycol, dimethyl sulfoxide), a buffer (Tris or TES titrated with Tris), one or more saccharides (glucose, lactose, raffinose, saccharose, or trehalose), salts (sodium citrate, citric acid) and antibiotics (penicillin, streptomycin). Penetrating cryoprotectants cause membrane lipid and protein rearrangement, resulting in increased membrane fluidity, greater dehydration at lower temperatures, reduced intracellular ice formation, and increased cryosurvival (Holt, 2000). On the other hand, a non-permeating cryoprotectant acts

Table 1: Modifications and specificities of freezing methods, protocols and semen packaging

Methods	Results	Publication
Rapid cooling from 30 to 15° C	Sperm survival may not be affected	Lebouef et al., 2000
Semen held at 5° C for either 48 h or 24 h prior to freezing	No effects on motility, membrane integrity, fertilizing potential	Purdy et al., 2006; 2010
12 or 72 h of equilibration at 5° C	Stable membrane integrity	-Cámara et al., 2011 -O'Hara et al., 2010
-From -10 to -60° C: faster rates than 50° C/ min -From -60 to -196° C: slower rates (20–30° C/ min) -Two-step glycerol addition	Kinetic parameters Conception and lambing rate	Anel et al., 2003
Control of temperature seeding and morphology of created ice crystals	Motility	Arav et al., 2002
Preparation of the extender Triladyl® with “nanowater”	Water crystallization rate Fertilizing ability Conception rates	Murawski et al., 2015
-Types of semen packaging -Thawing temperatures	-Mini tubes: the highest lambing rate -70° C/ 5 s: the higher lambing rate	Paulenz et al., 2004
-Semen frozen in pellets on the cold surface of cattle fat or on paraffin wax -Frozen in straws on paraffin wax	More efficient cryopreservation: pellets on the cold surface of cattle fat	Awada and Graham, 2004

extracellularly without crossing of plasma membrane (Aisen et al., 2000). Therefore, this may act as a solute, reducing the freezing temperature of the medium and decreasing the extracellular ice formation.

1. Research concerning “egg yolk”, properties and alternatives

Egg yolk is the most common component of Tris-based used diluents for semen cryopreservation. It is of animal origin and protects spermatozoa from cold shock (Holt et al., 1992). The constituents that protect the sperm membranes' phospholipids integrity during cryopreservation are the low density lipoproteins (LDL) of egg yolk (Moussa et al., 2002). The LDL adhere to the surface of sperm plasma membrane, reinstate the phospholipids and prevent the damage of

the membrane (Bispo et al., 2011). Many researchers studied the role of different species and concentrations of egg yolk, aiming to improve its benefits to ram semen cryopreservation.

A recent alternative investigation (Alcay et al., 2015) indicated that a modified extender containing lyophilized egg yolk provided similar cryoprotective effects with the fresh egg yolk extender. Additionally, Moustakas et al. (2011), who evaluated the suitability of using natural or lyophilized low density lipoproteins, concluded that in terms of post-thaw kinetics, total and progressive sperm motility, natural but not lyophilized LDL were appropriate for cryopreservation of ram semen. A different approach was followed by Del Valle et al. (2013), who used biologically safer components (casein, palm or coconut oil) as alternatives to egg yolk in ram freez-

ing process, but none of them was superior to egg yolk. Interesting information were provided from the study of Kulaksiz et al. (2010), who aimed to determine the effect of different egg yolk species, namely the domestic chicken (*Gallus gallus domesticus*), goose (*Anatidae anser*), turkey (*Meleagris gallopavo*), duck (*Anatidae anas platyrhynchos*), Japanese quail (*Coturnix japonica*) and chucker (*Alectoris chukar*), on ram semen cryopreserved by a Tris-citric acid-glucose extender containing 15% avian egg yolk and 5% glycerol. The most important finding was that ram frozen semen with 15% chucker egg yolk recorded higher sperm quality compared to the other tested egg yolks. Moreover, Gil et al. (2003) recommended that egg yolk concentration not higher than 5-10% supports frozen-thawed ram semen characteristics when milk-based extenders are used, whereas Bioexcell®, a free of animal origin additives extender (IMV, L' Aigle, France) which contains 6.4% glycerol, may be alternative to the conventional milk extender that contains 5% egg yolk. Taking into account that soybean lecithin is safer than egg yolk as the risk of microbial contamination is significantly lower (Bousseau et al., 1998) and not any cytotoxic effect of it has been reported (Fiume, 2001), it is widely used as an alternative of egg yolk in ram semen diluents. An improvement of sperm motility, viability and membrane integrity has been referred in soybean concentrations between 1-1.5% (Emamverdi et al., 2013; Forouzanfar et al., 2010). Supporting this theory, Masoudi's et al. (2016) showed that 1% soybean lecithin extenders have similar effects on post-thawed sperm quality with 20% egg yolk extenders. Notwithstanding the abovementioned positive properties of soybean lecithin, other surveys have noticed some detrimental effects of higher soy lecithin concentrations on post thaw ram semen quality (Mata-Campuzano et al., 2015; Valle et al., 2012). Although it is effective in protection of basic sperm quality characteristics against freezing-induced damage, high concentration of it (3.5%) induces loss of mitochondrial membrane potential without any significant reflection in motility (Valle et al., 2012). Mitochondrial membrane potential is a parameter that has been considered as a good indicator of sperm functionality which affects fertilizing capacity. In agreement with the aforemen-

tioned study, Mata-Campuzano et al. (2015) found a decrease of mitochondrial activity by soy lecithin extender at concentration of 3.5%. However, this study stated a protective efficiency of soy lecithin on sperm chromatin integrity, but it was attributed to its greater antioxidant, not cryo-protective, effect compared to egg yolk. Independently of the controversial results, the beneficial effects of soybean lecithin have been utilized in semen extenders' industry. Two commercial soybean lecithin-based extenders, AndroMed (Minitub, Tiefenbach, Germany) and BioXcell (IMV Technologies, L' Aigle, France), were evaluated in a field trial by Khalifa et al. (2013). Two hundred six ewes were laparoscopically inseminated with frozen-thawed ram semen and the authors reported that BioXcell is superior to AndroMed in preserving the fertilizing potential. Nonetheless, other studies did not demonstrate any significant variation among pregnancy rates of ewes inseminated with semen stored in AndroMed, milk, and egg yolk extenders (Fukui et al., 2008). Similarly, there was no difference in pregnancy rates after intracervical AI of ewes with frozen-thawed semen in milk-egg yolk and BioXcell extenders (Gil et al., 2003).

1) Research concerning "glycerol" concentration and addition methodology

Glycerol is one of the most widely used cryoprotectants. It is able to permeate the sperm cell, induce osmosis and prevent ice crystals formation. It can be added to semen during a two-step dilution in the second phase, or in one step as an essential component of the appropriate extender (Salamon and Maxwell, 1995a).

An acceptable concentration of glycerol in ram frozen semen aliquots has been established. Alvarez et al. (2012) stated that glycerol concentrations higher than 8% have great toxic effect on spermatozoa cryosurvival, while higher values of motility and viability were obtained at glycerol concentrations 4 or 6%. These results are in agreement with the study of Sönmez and Demirci (2004) who noted that the highest percentage of motile spermatozoa was determined in ram semen diluted with Tris-glucose-egg yolk based extender, containing 5% glycerol (testing levels between 3% - 7%). On the other hand, Forouzanfar et al. (2010) evaluated quality and *in*

vitro fertility of frozen-thawed ram sperm diluted in a Tris-based extender consisted of egg yolk or soybean lecithin and 5% or 7% glycerol. Sperm viability, motility and cleavage rate were higher for 7% glycerol group.

Moreover, many studies dealt with the time and temperature of glycerol addition, as well as, with the one or two steps of semen dilution process. Pelufo et al. (2015) demonstrated that the best procedure for ram semen cryopreservation in hypertonic disaccharide-containing diluents is the addition of glycerol after the cooling process, at 5° C, than before it at 30° C. Differences between 30° C and 5° C for spermatozoa quality were noticed just for HOST (hypo-osmotic swelling test), as plasma membrane showed higher sensitivity to glycerol addition at 30° C. Moreover, Anel et al. (2003) suggested that freezing of ram semen in a test-fructose-egg yolk medium containing 4% glycerol in a two-step dilution (to 2% at 35° C and to 4% at 5° C) along with a programmable biofreezer (-20° C/min), results in good field fertility rate. Furthermore, according to Gil et al. (2003) the addition of glycerol in a traditional milk extender supplemented with 5% egg yolk at 15° C does not present any improvement in thawed semen quality compared to 5° C. In contrast with the two steps dilution protocol studies, Morrier et al. (2002) reported that the addition of glycerol either immediately after collection (one-step) or at 5° C (two-steps) does not affect motility and viability of frozen- thawed ram sperm.

Finally, taking into account the osmolality of a Tris-based extender containing 6% glycerol in a two-step dilution procedure, Soyulu et al. (2007) concluded that glycerol based extenders with a high osmotic pressure (400 mOsm) was a better choice for ram semen cryopreservation, after testing post-thawed motility, acrosomal, morphological, and membrane integrity.

2) Research concerning “sugars” effects and properties

Sugars are non-permeating cryoprotectants that raise sperm cells dehydration by increasing the tonicity of the extender, interacting with phospholipid bilayers and contributing to plasma membrane stabilization against intracellular ice crystallization

(Molinia et al., 1994). Among the most commonly supplemented sugars, trehalose (disaccharide) is supposed to be the most effective, whereas raffinose (trisaccharide) and sucrose (disaccharide) are also widely used.

It has been strongly supported that the addition of trehalose to semen extenders before freezing, improves ram semen cryopreservation. Najafi et al. (2013) demonstrated that in a soybean lecithin-based extender, the combination of 5% glycerol and 100 mM trehalose resulted in higher post-thawing sperm quality (motility, viability, morphology, acrosome and plasma membrane integrity) than other combinations, or using glycerol alone, indicating a synergistic effect. These findings agree with Jafaroghli et al. (2011), who obtained the highest post-thawing semen quality by the combination of 5% glycerol with 100 mM trehalose in a Tris-citric acid-fructose-yolk extender. Similar findings were provided by Tonieto et al. (2010) by the supplementation of Tris-egg yolk-glycerol extender with 100 mM trehalose and 8% LDL, while Matsuoka et al. (2006) noticed that the addition of trehalose to an egg-yolk based ram semen diluent containing fructose, improves post-thaw sperm motility and viability. Although trehalose is the most investigated sugar additive in semen extenders, other sugars such as sucrose, raffinose and hypotaurine also resulted in higher ram semen cryoprotection (Bucak et al., 2013; Jafaroghli et al., 2011). Furthermore, a beneficial role of cyclodextrins, a family of cyclic oligosaccharides with a hydrophilic outer surface and a lipophilic central cavity, on semen has been found by Mocé et al. (2010). Specifically, they demonstrated that treating ram semen with cyclodextrins pre-loaded with cholesterol prior to cryopreservation, promotes sperm cryosurvival, since post thawed spermatozoa remain longer motile, showing increased binding capacity to zona pellucida.

3) Research concerning “seminal plasma” concentration and addition methodology

The role of seminal plasma in spermatozoa fertilization capacity is important and well known. It is produced by prostatic, epididymal and vesicular glands and provides sperm maturation, survival and fertilizing ability. It contains high concentration of

certain proteins that maintain the stability of the spermatozoa plasma membrane until capacitation begins in the female reproductive tract and also prevent cold-shock damage (Pérez-Pé et al., 2001). Considering the properties of seminal plasma, some researchers involved it in semen freezing or thawing process, with questionable results. Despite that some studies have shown an improvement of semen quality and fertility rate of ewes after cervical insemination with frozen-thawed sperm supplemented with seminal plasma (El-Rajj Ghaoui et al., 2007; Maxwell et al., 1999; Rebollo et al., 2007; Leahy et al., 2010), some other indicated that post-thaw ram semen incubated in seminal plasma had no identifiable beneficial effect on sperm quality (de Graaf et al., 2007; Dominguez et al., 2008; Rovegno et al., 2013). Controversial results may be explained by seasonality effects on seminal plasma patterns, in which the complex of protein content and composition changes among seasons (Dominguez et al., 2008). (Table 2)

3) Research concerning “antioxidants” addition

Spermatozoa contain a high ratio of polyunsaturated fatty acid (PUFA) and low cholesterol to phospholipids ratio which makes them sensitive to excessive production of reactive oxygen species (ROS) with subsequent lipid peroxidation (Holt and North, 1985). Spermatozoa regulate and resist to this phenomenon by an antioxidant protective system with the cooperation of seminal plasma, membranes, and cytoplasm, including superoxide dismutase, glutathione peroxidase and catalase. However, this system is partly removed and severely altered during cryopreservation procedures (Marti et al., 2008; Forouzanfar et al., 2013). A variety of biological and chemical antioxidants are presently under investigation, aiming to avoid the detrimental effects of oxidative stress on semen quality.

i) Enzymatic Antioxidants.

Enzymatic antioxidants are also known as natural antioxidants. They prevent cellular damage by the neutralization of ROS excessive production. Enzymatic antioxidants are composed of superoxide dismutase (SOD), catalase, glutathione peroxi-

dase (GPx), and glutathione reductase (GR) (Bansal and Bilaspuri, 2010). Two antioxidant agents that mimic superoxide dismutase (Tempo and Tempol) displayed protective effect on sperm DNA fragmentation, after their addition on ram semen extender during the cooling process (Mata-Campuzano et al., 2012; Santiani et al., 2014). Additionally, it has been shown that 150 μ M of a cell-permeable superoxide dismutase mimetic and peroxynitrite scavenger (MnTBAP), reduced the oxidative stress provoked by cryopreservation, leading to higher sperm motility and improved acrosomal membrane integrity (Forouzanfar et al., 2013). Moreover, Maia et al. (2006; 2009) reported a valuable impact of catalase on frozen-thawed ram spermatozoa's motility and stability of both acrosome and plasma membranes. On the other hand, some researchers demonstrated that the supplementation of a Tris-egg yolk extender with catalase does not influence the total antioxidant capacity of semen, nor it enhances post thaw ram semen quality (Camara et al., 2011; Sicherle et al., 2011).

ii) Non-enzymatic Antioxidants

Non-enzymatic antioxidants are also known as synthetic antioxidants or dietary supplements. Vitamins, minerals, fatty and amino acids are included in non-enzymatic dietary antioxidants.

Previous studies investigated the effect of supplementation of rams' diet with fatty acids. Taking into account their results, fish oil significantly improves frozen-thawed ram sperm motility, viability, membrane integrity and fertilizing ability, while palm and sunflower oil don't enhance or negatively affect semen characteristics (Esmaeili et al., 2014; Masoudi et al., 2016). Low-molecular weight amino acids were widely used as additives in ram semen extenders. Among them methionine, cysteamine and cysteine were extensively studied and were indicated as supplements that can reduce malondialdehyde's levels and improve post-thaw ram semen parameters (Bucak et al., 2007; 2008; Najafi et al., 2014; Sharafi et al., 2015; Toker et al., 2016). However, there are relative to the aforementioned amino acids studies, which report negative effects of cysteamine on semen motility, mitochondrial activity and DNA integrity (Cirit et al., 2013; Mata-Campuzano et al.,

Table 2: Additives and modification of the extenders' contents

Methods	Results	Publications
Lyophilized or fresh egg yolk	Similar cryoprotective effects	Alcay et al., 2015
Natural or lyophilized low density lipoproteins	Total and progressive motility	Moustacas et al., 2011
Casein, palm oil, coconut oil or egg yolk	Egg yolk is superior	Del Valle et al., 2013
Different species' egg yolk	Sperm quality by 15% chucker egg yolk	Kulaksiz et al., 2010
-Egg yolk concentration	-Optimal concentration: 5-10%	Gil et al., 2003
-Bioexcell [®] or egg yolk	-Bioexcell [®] may be an alternative	
Soybean concentrations between 1-1.5%	Motility, viability, membrane integrity	-Emamverdi et al., 2013 -Forouzanfar et al., 2010
Soybean lecithin or egg yolk	1% soybean lecithin similar with 20% egg yolk extenders	Masoudi et al., 2016
Soybean lecithin concentration	-Soy lecithin > 1% : semen quality -Soy lecithin > 3.5% : no significant effect on motility -Soy lecithin 3.5% : mitochondrial activity	-Valle et al., 2012 -Mata-Campuzano et al., 2015
BioXcell or AndroMed	BioXcell > AndroMed	Khalifa et al., 2013
-Fertility of semen stored in AndroMed, milk, or egg yolk extenders		-Fukui et al., 2008
-Fertility of semen stored in BioXcell or milk-egg yolk extenders	No significant difference	-Gil et al., 2003
Glycerol concentration	-8%: toxic effect on spermatozoa cryosurvival -4 or 6%: motility and viability -5%: motility -7%: viability, motility, cleavage rate	-Sönmez and Demirci, 2004 -Forouzanfar et al., 2010 -Alvarez et al., 2012
Temperature of glycerol addition	-Best results: at 5° C -At 30° C: plasma membrane sensitivity	Pelugo et al., 2015
Temperature of glycerol addition	No improvement in semen quality at 15° C, compared to 5° C	Gil et al., 2003
One-step or at 5° C two-steps glycerol addition	No effect on motility and viability	Morrier et al., 2002
4% glycerol in a two-step dilution (to 2% at 35° C and to 4% at 5° C)	Good field fertility rate	Anel et al., 2003
Osmolality of glycerol based extenders in a two-step dilution	High osmotic pressure (400 mOsm)	Soylu et al., 2007
Glycerol alone or in combination with trehalose	5% glycerol and 100 mM trehalose: post-thawing sperm quality	Najafi et al., 2013
-100 mM trehalose with 5% glycerol		-Jafaroghli et al., 2011
-100 mM trehalose and 8% LDL	High post-thawing semen quality	-Tonieto et al., 2010
Trehalose in combination with fructose	Motility and viability	Matsuoka et al., 2006
Trehalose, sucrose, raffinose, hypotaurine	High ram semen cryo-protection	-Bucak et al., 2013 -Jafaroghli et al., 2011
Cyclodextrins pre-loaded with cholesterol	Binding capacity to zona pellucida	Mocé et al., 2010
Seminal plasma	-Semen quality and fertility rate -No beneficial effect on sperm quality	-El-Rajj Ghaoui et al., 2007 -Maxwell et al., 1999 -Rebolledo et al., 2007 -Leahy et al., 2010 -de Graaf et al., 2007 -Dominguez et al., 2008 -Rovegno et al., 2013

2015), or no beneficial effect of cysteine to semen cryopreservation (Coyan et al., 2011). Furthermore, Sangeeta et al. (2015) demonstrated that the addition of 20 mM l-glutamine and 25 mM l-proline in a Tris-egg yolk-glycerol diluent, reduced lipid peroxidation and significantly improved sperm motility, viability and acrosome integrity. In contrast, supplementation of l-alanine in freezing extender resulted in lower cryoprotection, observed by a decrease of sperm motility and an increase of the immotile and non-progressive spermatozoa percentage. Moreover, Najafi et al. (2014) showed that when the freezing soybean lecithin extender was supplemented by 6 mM ergothioneine, motility, viability and membrane functionality were increased, while lipid peroxidation was decreased. However, Çoyan et al. (2011) found that ergothioneine, despite the satisfying motility results, reduces membrane integrity and mitochondrial activity. Concerning hypotaurine and taurine, their addition in Tris-based extenders enhances post thawed ram semen quality parameters (Bucak et al., 2007; 2013).

Regarding vitamins' antioxidant effects on ram frozen-thawed semen, it has been proved that when Tris extender supplemented with vitamin B₁₂, viability and motility are advanced, while the morphological defects are decreased (Asadpour et al., 2012; Hamedani et al., 2013). Likewise, Mata-Campuzano et al. (2015) indicated that the supplementation of Trolox, a water-soluble vitamin E analog, in a TES-Tris-fructose extender reduces lipid peroxidation after thawing of frozen ram semen. Silva et al. (2013) reinforced the previous finding, reporting that Trolox addition to Tris-egg yolk extender at 60 and 120 µM provides higher plasma membrane and mitochondria integrity, as well as, better kinematics for ram cryopreserved spermatozoa. These results confirm also Maia's et al. (2007; 2009) observations that Trolox addition at 50-100 µM to freezing extender resulted in increased semen motility. In contrast, Sicherle et al. (2011) noted no effect on any ram sperm kinematics and no sperm protection from spontaneous production of ROS after post thaw addition of 100 µM Trolox.

Furthermore, a big group of substances with antioxidant capacity are based on plant origin. Mata-Campuzano et al. (2015) indicated that crocin, a

carotenoid extracted from saffron (*Crocus sativus*), could be beneficial for ram semen freezing, because it protects sperm DNA. Moreover, Uysal and Bucak (2007) observed that the addition of lycopene, a natural carotenoid which occurs in certain fruits and vegetables, in a Tris-based extender exhibited high protection of all tested ram semen parameters after thawing. Similar results were obtained by the supplementation of soybean lecithin extender with 4 or 6% rosemary aqueous extract (Motlagh et al., 2014).

5) Substances with different main purposes of use, as antioxidant agents

Cirit et al. (2013) examined the cryosurvival of electro-ejaculated ram spermatozoa in the presence of iodixanol, a drug which contains iodine a substance that absorbs x-rays. Supplementation of 5% iodixanol was optimal to increase total and progressive motility and decrease acrosomal damage and total morphological abnormalities of frozen-thawed ram semen. Moreover, many studies investigated the role of insulin-like growth factor-I (IGF-I) in a variety of reproductive purposes. It is a polypeptide hormone, which is involved in follicular development, fetal growth and spermatogenesis in ruminants (Kumar and Laxmi, 2015). Padilha et al. (2012) demonstrated that IGF-I improves sperm motility and membranes' structural integrity after its addition in Tris-extender, but this positive effect didn't reflect on the pregnancy rate after laparoscopic insemination of ewes with frozen-thawed semen. Finally, it has been reported that bovine serum albumin (BSA), usually used as a protein source in media, eliminates free radicals and protects sperm acrosome and plasma membranes' integrity from heat shock during freezing-thawing procedures (Uysal and Bucak 2007).

(Table 3)

CONCLUSIONS

The majority of researches regarding frozen-thawed ram semen aim to improve spermatozoa's quality characteristics. On the other hand, these ones that have practically evaluated their results through artificial insemination are fewer. Nonetheless, there are seasonal, management, hygienic and individual factors, such as rams' and ewes' breed, age and inseminator's expertise, that

affect fertility results. As a consequence, although a progress on ram frozen-thawed semen has been achieved, there is a need for further research to enhance fertility results through cervical insemination of cryopreserved ram semen.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. ■

Table 3: Addition of enzymatic and non-enzymatic antioxidants

Antioxidants	Results	Publications
Tempo and Tempol	Protective effect on sperm DNA fragmentation	-Mata-Campuzano et al., 2012 -Santiani et al., 2014
MnTBAP	Motility Acrosomal membrane integrity	Forouzanfar et al., 2013
Catalase	Motility Acrosomal membrane integrity No effect on semen quality	-Maia et al., 2006, 2009 -Camara et al., 2011 -Sicherle et al., 2011
Fish oil, palm oil, sunflower oil	Motility, viability, membrane integrity, fertilizing ability No effect on semen characteristics	-Esmacili et al., 2014 -Masoudi et al., 2016
Methionine	Improvement of semen parameters	Toker et al., 2016
Cysteamine	Improvement of semen parameters Motility, mitochondrial activity, DNA integrity	-Bucak et al., 2007 -Najafi et al., 2014 -Toker et al., 2016 -Cirit et al., 2013 -Mata-Campuzano et al., 2015
Cysteine	Improvement of semen parameters or no beneficial effect	-Bucak et al., 2008 -Sharafi et al., 2015 -Toker et al., 2016 -Coyan et al., 2011
-l-Glutamine -l-Proline -l-Alanine	Motility, viability, acrosome integrity Immotile and non-progressive spermatozoa	Sangeeta et al., 2015
Ergothioneine	Motility, viability, membrane functionality Lipid peroxidation, membrane integrity and mitochondrial activity	-Najafi et al., 2014 -Çoyan et al., 2011
Hypotaurine and taurine	Semen quality parameters	Bucak et al., 2007; 2013
Vitamin B ₁₂	Viability, motility, morphological integrity	-Asadpour et al., 2012 -Hamedani et al., 2013
Trolox	Lipid peroxidation Kinematics, plasma membrane and mitochondria integrity motility	-Mata-Campuzano et al., 2015 -Silva et al., 2013 -Maia's et al., 2007, 2009
Trolox (post-thaw addition)	-No effect on sperm kinematics -No protection from ROS production	Sicherle et al., 2011
Crocin	Sperm DNA protection	Mata-Campuzano et al., 2015
-Rosemary aqueous extract -Lycopene	High protection of all tested semen parameters	-Uysal and Bucak, 2007 -Motlagh et al., 2014

REFERENCES

- Aisen EG, Alvarez HL, Venturino A, Garde JJ (2000) Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. *Theriogenology* 53: 1053-1061.
- Alcay S, Tokar MB, Gokce E, Ustuner B, Onder NT, Sagirkaya H, Nur Z, Soylu MK (2015) Successful ram semen cryopreservation with lyophilized egg yolk-based extender. *Cryobiology* 71: 329-333.
- Álvarez JG, Storey BT (1993) Evidence that membrane stress contributes more than lipid peroxidation to sublethal cryodamage in cryopreserved human sperm: glycerol and other polyols as sole cryoprotectant. *J Androl* 14: 199-209.
- Álvarez M, Tamayo-Canula J, Martínez-Rodríguez C, López-Uruena E, Gomes-Alves S, Anel L, Martínez-Pastora F, de Paz P (2012) Specificity of the extender used for freezing ram sperm depends of the spermatozoa source (ejaculate, electroejaculate or epididymis). *Anim Reprod Sci* 132: 145-154.
- Andrabi SMH & Maxwell WMC (2007) A review on reproductive biotechnologies for conservation of endangered mammalian species. *Anim Reprod Sci*, 99: 223-243.
- Anel L, de Paz P, Alvarez M, Chamorro CA, Boixo JC, Manso A, Gonzalez M, Kaabi M, Anel E (2003) Field and in vitro assay of three methods for freezing ram semen. *Theriogenology* 60: 1293-1308.
- Anel L, Kaabi M, Abroug B, Alvarez M, Anel E, Boixo JC, de la Fuente LF, de Paz P (2005) Factors influencing the success of vaginal and laparoscopic artificial insemination in churra ewes: a field assay. *Theriogenology* 63: 1235-1247.
- Arav A, Yavin S, Zeron Y, Natan D, Dekel I, Gacitua H (2002) New trends in gamete's cryopreservation. *Mol Cell Endocrin* 187: 77-81.
- Asadpour RM, Pourseif M, Moghadam G, Jafari R, Tayefi H, Mahmodi H (2012) Effect of vitamin B12 addition to extenders on some physicochemical parameters of semen in crossbred rams. *Afr J Biotechnol* 11: 11741-11745.
- Awada MM, Graham JK (2004) A new pellet technique for cryopreserving ram and bull spermatozoa using the cold surface of cattle fat. *Anim Reprod Sci* 84: 83-92.
- Bansal AK & Bilaspuri GS (2010) Impacts of oxidative stress and antioxidants on semen functions. *Vet Med Int* 2011.
- Baspınar N, Çoyan K, Bucak MN, Tuncer PB (2011) Effects of dithioerythritol on ram semen after the freeze-thawing process. *Cryobiology* 63: 152-156.
- Bispo CAS, Pugliesi G, Galvão P, Rodrigues MT, Ker PG, Filgueiras B, Carvalho GR (2011) Effect of low and high egg yolk concentrations in the semen extender for goat semen cryopreservation. *Small Rumin Res*, 100: 54-58.
- Bousseau S, Brillard JP, Marguant-Le Guienne B, Guerin B, Camus A, Lechat M (1998) Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology* 50: 699-706.
- Bucak MN, Atessahin A, Varışlı O, Yuce A, Tekin N, Akcay A (2007) The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen microscopic and oxidative stress parameters after freeze-thawing process. *Theriogenology* 67: 1060-1067.
- Bucak MN, Keskin N, Taspınar M, Çoyan K, Baspınar N, Cenariu MC, Bilgili A, Öztürk C, Kursunlu AN (2013) Raffinose and hypotaurine improve the post-thawed Merino ram sperm parameters. *Cryobiology* 67: 34-39.
- Câmara DR, Mello-Pinto MMC, Pinto LC, Brasil OO, Nunes JF, Guerra MMP (2011) Effects of reduced glutathione and catalase on the kinematics and membrane functionality of sperm during liquid storage of ram semen. *Small Rumin Res* 100: 44-49.
- Câmara DR, Silva SV, Almeida FC, Nunes JF, Guerra MMP (2011) Effects of antioxidants and duration of pre-freezing equilibration on frozen-thawed ram semen. *Theriogenology* 76: 342-350.
- Cirit U, Bagis H, Demir K, Agca C, Pabuccuoglu S, Varışlı O, Clifford-Rathert C, Agca Y (2013) Comparison of cryoprotective effects of iodoxanol, trehalose and cysteamine on ram semen. *Anim Reprod Sci* 139: 38-44.
- Çoyan K, Baspınar N, Bucak MN, Akalin PP (2011) Effects of cysteine and ergothioneine on post-thawed Merino ram sperm and biochemical parameters. *Cryobiology* 63: 1-6.
- Del Valle I, Souter A, Maxwell WMC, Muñoz-Blanco T, Cebrián-Pérez JA (2013). Function of ram spermatozoa frozen in diluents supplemented with casein and vegetable oils. *Anim Reprod Sci*, 138: 213-219.
- Dominguez MP, Falcinelli A, Hozbor F, Sanchez E, Cesari A, Alberio RH (2008) Seasonal variations in the composition of ram seminal plasma and its effect on frozen-thawed ram sperm. *Theriogenology* 69: 564-573.
- Drobnis EZ, Crowe LM, Berger T, Anchordoguy T, Overstreet JW, Crowe JH (1993) Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. *J Exp Zool* 265: 432-437.
- El-Rajji Ghaoui R, Thomson PC, Leahy T, Evans G, Maxwell WMC (2007) Autologous whole ram seminal plasma and its vesicle-free fraction improve motility characteristics and membrane status but not in vivo fertility of frozen-thawed spermatozoa. *Reprod Dom Anim* 42: 541-549.
- Emamverdi M, Zhandi M, Shahneh AZ, Sharafi M, Akbari-Sharif A (2013) Optimization of Ram Semen Cryopreservation Using a Chemically Defined Soybean Lecithin-Based Extender. *Reprod Dom Anim* 48: 899-904.
- Esmaili V, Shahverdi AH, Alizadeh AR, Alipour H, Chehrizi M (2014) Saturated, omega-6 and omega-3 dietary fatty acid effects on the characteristics of fresh, frozen-thawed semen and blood parameters in rams. *Andrologia* 46: 42-49.
- Fair S, Hanrahan J, O'Meara C, Duffi P, Rizos D, Wade M, Donovan A, Boland M, Lonergan P, Evans A (2005) Differences between Belclare and Suffolk ewes in fertilization rate, embryo quality and accessory sperm number after cervical or laparoscopic artificial insemination. *Theriogenology* 63: 1995-2005.
- Fiser PS, Fairfull RW, Marcus GJ (1986) The effect of thawing velocity on survival and acrosomal integrity of ram spermatozoa frozen at optimal and suboptimal rates in straws. *Cryobiology* 23: 141-149.
- Fiume Z (2001) Final report on the safety assessment of lecithin and hydrogenated lecithin. *Int J Toxicol* 20: 21-45.
- Forouzanfar M, Sharafi M, Hosseini SM, Ostadhosseini S, Hajian M, Hosseini L, Abedi P, Nili N, Rahmani HR, Nasr-Esfahani MH (2010) In vitro comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. *Theriogenology* 73: 480-487.
- Forouzanfar M, Ershad SF, Hosseini SM, Hajian M, Ostad-Hosseini S, Abid A, Tavalae M, Shahverdi A, Dizaji AV, Esfahani MH (2013) Can permeable super oxide dismutase mimetic agents improve the quality of frozen-thawed ram semen. *Cryobiology* 66: 126-130.
- Fukui Y, Kohno H, Togari T, Hiwasa M, Okabe K (2008) Fertility after Artificial Insemination Using a Soybean-Based Semen Extender in Sheep. *J Reprod Dev* 54: 286-289.
- Gandini L, Lombardo F, Lenzi A, Spano M, Dondero F (2006) Cryopreservation and sperm DNA integrity. *Cell Tissue Bank* 7: 91-98.
- Gil J, Lundeheim N, Soderquist L, Rodríguez-Martínez H (2003) Influence of extender, temperature and addition of glycerol on post-thaw sperm parameters in ram semen. *Theriogenology* 59: 1241-1255.

- Graaf SP, Evans G, Gillan L, Guerra MMP, Maxwell WMC, O'Brien JK (2007) The influence of antioxidant, cholesterol and seminal plasma on the in vitro quality of sorted and non-sorted ram spermatozoa. *Theriogenology* 67: 217-227.
- Hamedani MA, Tahmasbi AM, Ahangari YJ (2013) Effects of vitamin B12 supplementation on the quality of ovine spermatozoa. *Open Vet Sci J* 3: 140-144.
- Holt WV (2000) Basic aspects of frozen storage semen. *Anim Reprod Sci* 62: 3-22.
- Holt WV, Head MF, North RD (1992) Freeze-induced membrane damage in ram spermatozoa is manifested after thawing: observations with experimental cryomicroscopy. *Biol Reprod* 46: 1086-1094.
- Jafaroghli M, Khalili B, Farshad A, Zamiri MJ (2011) The effect of supplementation of cryopreservation diluents with sugars on the post-thawing fertility of ram semen. *Small Rumin Res* 96: 58-63.
- Khalifa T, Lymberopoulos A, Theodosiadou E (2013) Association of soybean-based extenders with field fertility of stored ram (Ovisaries) semen: A randomized double-blind parallel group design. *Theriogenology* 79: 517-527.
- Kulaksız R, Cigdem C, Akcay E, Daskin A (2010) The protective effect of egg yolk from different avian species during the cryopreservation of Karayaka ram semen. *Small Rumin Res* 88:12-15.
- Kumar A & Laxmi NA (2015) Role of IGF 1 in male and female reproduction in bovines: A Review. *Asia Pacific Journal of Research Vol: I. Issue XXIV*.
- Kumar S, Millar JD, Watson PF (2003) The effect of cooling rate on the survival of cryopreserved bull, ram, and boar spermatozoa: a comparison of two controlled-rate cooling machines. *Cryobiology* 46: 246-253.
- Leahy T, Marti JJ, Evans G, Maxwell WMC (2010) Seasonal variation in the protective effect of seminal plasma on frozen-thawed ram spermatozoa. *Anim Reprod Sci* 119: 147-153.
- Leboeuf B, Restall B, Salamon S (2000) Production and storage of goat semen for artificial insemination. *Anim Reprod Sci* 62:113-141.
- Maia MS (2006) Sperm viability and reactive oxygen species (ROS) generation in RAM semen cryopreserved in extenders with sodium lauryl sulfate (OEP), Trolox-c and catalase. Thesis. Faculty of Veterinary Medicine and Animal Science, UNESP, Botucatu.
- Maia MS, Sicherle CC, Biculo SD, Sousa SD, Azevedo HC (2007) Effect of Trolox addition on the motility and membrane integrity of ram spermatozoa with high and low freezability. *Reprod Domest Rumin VI. SRF* 64, 467-467
- Maia MS, Biculo SD, Azevedo HC, Sicherle CC, Sousa DB, Rodello L (2009) Motility and viability of ram sperm cryopreserved in a Tris-egg yolk extender supplemented with anti-oxidants. *Small Rumin Res* 85: 85-90.
- Marti E, Marti JJ, Muiño-Blanco T, Cebrián-Pérez JA (2008) Effect of the cryopreservation process on the activity and immunolocalization of antioxidant enzymes in ram spermatozoa. *J Androl* 29: 459-67.
- Masoudi R, Sharafi M, Shahneh AZ, Towhidi A, Kohram H, Zhandi M, Esmaeili V, Shahverdi A (2016) Effect of dietary fish oil supplementation on Ram semen freezability and fertility using soybean lecithin and egg yolk-based extenders. *Theriogenology*, <http://dx.doi.org/10.1016/j.theriogenology.2016.05.018> in press
- Mata-Campuzano M, Alvarez-Rodriguez M, Alvarez M, Anel L, de Paz P, Garde JJ, Martínez Pastor F (2012) Effect of several antioxidants on thawed ram spermatozoa submitted to 37°C up to four hours. *Reprod Dom Anim* 47: 907-914.
- Mata-Campuzano M, Álvarez-Rodríguez M, Álvarez M, Tamayo-Canul J, Anel L, de Paz P, Martínez-Pastor F (2015) Post-thawing quality and incubation resilience of cryopreserved ram spermatozoa are affected by antioxidant supplementation and choice of extender. *Theriogenology* 83: 520-528.
- Matsuoka T, Imai H, Kohno H, Fukui (2006) Effects of Bovine Serum Albumin and Trehalose in Semen Diluents for Improvement of Frozen-Thawed Ram Spermatozoa. *J Reprod Dev* 52: 675-683.
- Maxwell WMC, Evans G, Mortimer ST, Gillan L, Gellatly ES, Mcphie CA (1999) Normal fertility in ewes after cervical insemination with frozen-thawed spermatozoa supplemented with seminal plasma. *Reprod Fertil Dev* 11: 123-126.
- Medeiros CM, Forell F, Oliveira AT, Rodrigues JL (2002) Current status of sperm cryopreservation: why isn't better. *Theriogenology* 57: 327-344.
- Milovanović A, Maksimović N, Barna T, Lazarević M, Delić N (2013) Laparoscopic insemination of sheep in Republic of Serbia. *Biotechn Anim Husbandry* 29: 449-456.
- Mocé E, Purdy PH, Graham JK (2010) Treating ram sperm with cholesterol-loaded cyclodextrins improves cryosurvival. *Anim Reprod Sci* 118: 236-247.
- Molinia FC, Evans G, Casares PI, Maxwell WMC (1994) Effect of monosaccharide and disaccharides in Tris-based diluents on motility, acrosome integrity and fertility of pellet frozen ram spermatozoa. *Anim Reprod Sci* 36: 113-122.
- Morrier A, Castonguay F, Bailey JL (2002) Glycerol addition and conservation of fresh and cryopreserved ram spermatozoa. *Can J Anim Sci* 418: 347-356.
- Motlagh MK, Sharafi M, Zhandi M, Mohammadi-Sangcheshmeh A, Shakeri M, Soleimani M, Zeinoaldini S (2014) Antioxidant effect of rosemary (*Rosmarinus officinalis* L) extract in soybean lecithin-based semen extender following freeze-thawing process of ram sperm. *Cryobiology* 69: 217-222.
- Moussa M, Matinet V, Trimeche A, Tainturier D, Anton M (2002) Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. *Theriogenology* 57: 1695-1706.
- Moustakas VS, Zaffalon FG, Lagares MA, Loaiza-Echeverri AM, Varago FC, Neves MM, Heneine LGD, Arruda RP, Henry M (2011) Natural, but not lyophilized, low density lipoproteins were an acceptable alternative to egg yolk for cryopreservation of ram semen. *Theriogenology* 75: 300-307.
- Murawski M, Schwarz T, Grygier J, Patkowski K, Oszczeda Z, Jelkin I, Kosiek A, Gruszecki TM, Szymanowska A, Skrzypek T, Zieba DA, Bartlewski PM (2015) The utility of nanowater for ram semen cryopreservation. *Exp Biol Med* 240: 611-617.
- Najafi A, Zhandi M, Towhidi A, Sharafi M, Sharif AA, Motlagh MK, Martinez-Pastor F (2013) Trehalose and glycerol have a dose-dependent synergistic effect on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. *Cryobiology* 66: 275-282.
- Najafi A, Kia HD, Mohammadi H, Najafi MH, Zanganeh Z, Sharafi M, Martinez-Pastor F, Adeldust H. (2014) Different concentrations of cysteamine and ergothioneine improve microscopic and oxidative parameters in ram semen frozen with a soybean lecithin extender. *Cryobiology* 69: 68-73.
- Nordstoga AB, Soderquist L, Adnøy T, Paulenz H (2009) Effect of Different Packages and Freezing/Thawing Protocols on Fertility of Ram Semen. *Reprod Dom Anim* 44: 527-531.
- O'Hara L, Hanrahan JP, Richardson L, Donovan A, Fair S, Evans ACO, Lonergan P (2010) Effect of storage duration, storage temperature, and diluent on the viability and fertility of fresh ram sperm. *Theriogenology* 73: 541-9.
- O'Meara C, Hanrahan J, Donovan A, Fair S, Rizos D, Wade M, Boland M, Evans A, Lonergan P (2005) Relationship between in vitro fertilization of ewe oocytes and the fertility of ewes following cervical artificial insemination with frozen thawed semen. *Theriogenology* 64: 1797-1808.
- Padilha RT, Magalhães-Padilha DM, Cavalcante MM, Almeida AP, Haag KT, Gastal MO, Nunes JF, Rodrigues APR, Figueiredo JR, Oliveira MAL (2012) Effect of insulin-like growth factor-I on some quality traits and fertility of cryopreserved ovine semen. *Theriogenology* 78: 907-913.
- Paulenz H, Söderquist L, Ådnøy T, Nordstoga AB, Gulbrandsen B,

- Andersen BK (2004) Fertility results after different thawing procedures for ram semen frozen in mini tubes and mini straws. *Theriogenology* 61:1719-1727.
- Paulenz H, Adnøy T, Söderquist L (2007) Comparison of fertility results after vaginal insemination using different thawing procedures and packages for frozen ram semen. *Acta Vet Scand* 49: 26.
- Pelugo V, LópezArmengol MF, Malcotti V, Venturino A, Aisen EG (2015) Effects of glycerol and sugar mixing temperature on the morphologic and functional integrity of cryopreserved ram sperm. *Theriogenology* 83: 144-151.
- Pérez-Pé R, Cebrián-Pérez J, Muio-Blanco T (2001) Semen plasma proteins prevent cold-shock membrane damage to ram spermatozoa. *Theriogenology* 56: 425-434.
- Purdy PH (2006) The post-thaw quality of ram sperm held for 0 to 48 h at 5°C prior to cryopreservation. *Anim Reprod Sci* 93: 114-123.
- Purdy PH, Mocé E, Stobart R, Murdoch WJ, Moss GE, Larson B, Ramsey S, Graham JK, Blackburn HD (2010) The fertility of ram sperm held for 24h at 5°C prior to cryopreservation. *Anim Reprod Sci* 118: 231-235.
- Rebolledo AD, Sierra LN, Tamayo AC, Loria AA, Denis SE, Osés RB, Parra EG, Monsreal LP, Ugalde JR (2007) Fertility in hair sheep inseminated with freeze spermatozoa rediluted with seminal plasma. *Rev Cient* 17:73-76.
- Ritar AJ and Ball PD (1993) The effect of freeze-thawing of goat and sheep semen at a high density of spermatozoa on cell viability and fertility after insemination. *Anim Reprod Sci* 31: 249-262.
- Rovegno M, Feitosa WB, Rocha AM, Mendes CM, Visintin JA, MEO D'AvilaAssumpco (2013) Assessment of post-thawed ram sperm viability after incubation with seminal plasma. *Cell Tissue Bank* 14: 333-339.
- Salamon S & Maxwell WMC (1995a) Frozen storage of ram semen: I. Processing, freezing, thawing and fertility after cervical insemination. *Anim Reprod Sci* 37: 185-249.
- Salamon S & Maxwell WMC (1995b) Frozen storage of ram semen: II. Causes of low fertility after cervical insemination and methods of improvement. *Anim Reprod Sci* 38: 1-36.
- Salamon S & Maxwell WMC (2000) Storage of ram semen. *Anim Reprod Sci* 62: 77-111.
- Sangeeta S, Arangasamy A, Kulkarni S, Selvaraju S (2015) Role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen. *Anim Reprod Sci* 161: 82-88.
- Santiani A, Evangelista S, Sepúlveda N, Risopatrón J, Villegas J, Sánchez R (2014) Addition of superoxide dismutase mimics during cooling process prevents oxidative stress and improves semen quality parameters in frozen/thawed ram spermatozoa. *Theriogenology* 82: 884-889.
- Sharafi M, Zhandi M, Sharif AA (2015) Supplementation of soybean lecithin-based semen extender by antioxidants: complementary flow-cytometric study on post-thawed ram spermatozoa. *Cell Tissue Bank* 16: 261-269.
- Sicherle CC, Maia MS, Bicudo SD, Rodello L, Azevedo HC (2011) Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen supplemented with catalase or Trolox. *Small Rumin Res* 95: 144-149.
- Silva SV, Soares AT, Batista AM, Almeida FC, Nunes JF, Peixoto CA, Guerra MMP (2013) Vitamin E (Trolox) addition to Tris-egg yolk extender preserves ram spermatozoon structure and kinematics after cryopreservation. *Anim Reprod Sci* 137: 37-44.
- Sönmez M and Demirci E (2004) The Effect of Ascorbic Acid on the Freezability of Ram Semen Diluted with Extenders Containing Different Proportions of Glycerol. *Turk J Vet Anim Sci* 28: 893-899.
- Soylu MK, Nur Z, Ustuner B, Dogan I, Sagirkaya H, Gunay U, Ak K (2007) Effects of various cryoprotective agents and extender osmolality on post-thawed ram semen. *B Vet J Pulawy* 51: 241-246.
- Toker MB, Alcay S, Gokce E, Ustuner B (2016) Cryopreservation of ram semen with antioxidant supplemented soybean lecithin-based extenders and impacts on incubation resilience. *Cryobiology* 72: 205-209.
- Tonieto RA, Goularte KL, Gastal GDA, Schiavon RS, Deschamps LC, Lucia T (2010) Cryoprotectant effect of trehalose and low-density lipoprotein in extenders for frozen ram semen. *Small Ruminant Res* 93: 206-209.
- Tsakmakidis IA (2010) Ram semen evaluation: development and efficiency of modern techniques. *Small Rumin Res* 92: 126-130.
- Uysal O, Bucak MN (2007) Effects of Oxidized Glutathione, Bovine Serum Albumin, Cysteine and Lycopene on the Quality of Frozen-Thawed Ram Semen. *Acta Vet Brno* 76: 383-390.
- Valle I, Gomez-Duran A, Holt WV, Muino-Blanco T, Cebrian-Perez JA (2012) Soy lecithin interferes with mitochondrial function in frozen-thawed ram spermatozoa. *J Androl* 33: 717-725.
- Valle I, Souterb A, Maxwell WMC, Muino-Blanco T, Cebrián-Pérez JA (2013) Function of ram spermatozoa frozen in diluents supplemented with casein and vegetable oils. *Anim Reprod Sci* 138: 213-219.
- Vichas L, Tsakmakidis IA, Vafiadis D, Tsousis G, Malama E, Boscos CM (2017) The effect of antioxidant agents' addition and freezing method on quality parameters of frozen thawed ram semen. *Cell Tissue Bank* (DOI 10.1007/s10561-017-9633-6).
- Watson PF (2000) The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci* 60-61: 481-492.

Regional Acceleratory Phenomenon after Orthodontic Force Exertion in Ovariectomized Rats

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ABSTRACT. The application of orthodontic forces may be one of the factors that produce a regional acceleratory phenomenon (RAP) in mandibular and maxillary bones. The effect of exerted forces on bone tissue ahead of their point of application has not been extensively studied. Moreover, limited information exists regarding this phenomenon on osteoporotic bone. The study aim was to examine the role of orthodontic forces on the expression of RAP in normal and osteoporotic mature rats. Thirty-six eight-month-old skeletally mature female Wistar rats, half of which had been previously ovariectomized (OVX) at the age of 6 months, were subjected to orthodontic movement of the upper right first molar. An orthodontic force of 60 gr* was generated through a closed coil spring for 14 days. The maxillae were then removed and the area ahead of the first molar was examined histologically. On the side of orthodontic force application, distortion of bone structure and woven bone formation were observed in non-OVX rats, whereas in the OVX rats, extensive remodeling was apparent. In conclusion, the application of orthodontic forces on both normal and osteoporotic mature rats in the present study created a RAP ahead of the loaded teeth demonstrated histologically, indicating increased bone resorption and formation in the OVX rats.

Keywords: regional acceleratory phenomenon (RAP), orthodontic forces, osteoporosis, ovariectomy, rat

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INTRODUCTION

Orthodontic forces have been applied on growing and adult humans, in order to treat dentofacial abnormalities during the last decades. Research has focused on the tissues and the cellular changes surrounding the loaded teeth and their corresponding periodontal ligaments (Spyropoulos, 2008). Additionally, experimental studies have been conducted on normal and ovariectomized rats regarding the effects of orthodontic forces on tooth movement, root resorption, and both cortical and alveolar bone changes around the instrumented tooth (Tsolakis et al., 2008; Sirisoontorn et al., 2011; Sirisoontorn et al., 2012; Ru et al., 2013). However, there is limited information concerning the bone areas ahead to the application of forces.

A regional acceleratory phenomenon (RAP) may be produced by orthodontic forces, traumatic and stress fractures, lacerations, as well as by infectious and non-infectious inflammations (Frost, 1983; Frost, 2004). During a RAP, all ongoing regional processes are accelerated and it seems to represent an “SOS” mechanism during serious noxious stimuli (Jee and Yao, 2001). Moreover, RAPs normally improve the body's ability to resist and manage established infections, as well as the resulting healing in all hard and soft tissues (Frost, 2004).

Recent concepts interrelating bone biology and skeletal bio-mechanics consider microdamages of bone tissue as events that initiate bone modeling and remodeling (Parfitt, 2002; Martin, 2003). Moreover, it seems that the initial periodontal ligament response to orthodontic loading is related to hypertrophic and fatigue mechanisms (Katona et al., 1995; Roberts, 2000.). Furthermore, Verna et al suggest a role for microcracks in the initiation of bone remodeling following the application of orthodontic forces (Verna, 2004).

We therefore hypothesize that if orthodontic forces exerted on bone are perceived as traumatic or non-infectious stimuli by the local tissues, followed by microdamage, RAP phenomena may potentially appear in the surrounding bone tissue. The ovariectomized rat model of postmenopausal osteoporosis is an established model of skeletal osteopenia and is chosen for the present experimental study (Lelovas et al., 2008). Thus, the purpose of our study is to determine any RAP phenomena ahead of the application

of heavy orthodontic forces on the molars of mature rats, both on osteoporotic and intact animals.

MATERIALS AND METHODS

Laboratory animals

Thirty-six eight-month-old skeletally mature female Wistar rats were used in this study, with a mean body weight of 310 ± 20 g (mean \pm SD). The study protocol was approved by the Veterinary Directorate, according to the national legislation (PD 160/1991) which was in force at the time of the study and which was conformed to the European Directive 86/609 (license no. K/3816/26.10.05). The animals were housed in the Laboratory of Experimental Surgery and Surgical Research, School of Medicine, National & Kapodistrian University of Athens, Greece. They were housed two to a cage in conventional open-top cages, in environmentally controlled light conditions (12/12 hours light/dark), temperature range 22 ± 2 °C, relative humidity 55 ± 5 % and 12 air changes/hour, with unlimited access to standard pelleted rodent diet (Mucedola 4RF18) and tap water. The animals were divided into two different groups consisting of eighteen animals each, as follows: Group A included 18 rats that were subjected to orthodontic movement of the upper right first molars without any surgery. Group B included 18 rats that were subjected to orthodontic movement of the upper right first molars after bilateral ovariectomy which was conducted 2 months previously.

Ovariectomy

Bilateral ovariectomies were performed in the Group B female rats from the ventral approach at the age of 6 months, according to the model of postmenopausal osteoporosis. Briefly, the animals were weighed and anesthetized by i.m. injection of ketamine hydrochloride 100 mg/kg b.w. (Ketaset, Pfizer) and xylazine 5 mg/kg b.w. (Rompun, Bayer) for surgery. Following loss of consciousness, chemoprophylaxis with enrofloxacin 10 mg/kg b.w. (Baytril 5%, Bayer) and pre-emptive analgesia with carprofen 0.08 mg/kg b.w. (Rimadyl, Pfizer) were administered subcutaneously. Using aseptic procedures, a midline ventral incision through the linea alba was performed, the ovaries dissected, ligated and removed bilaterally, and the peritoneum and skin were closed in layers

using single interrupted sutures. The operation was performed on a heating pad to prevent hypothermia. Recovery from anesthesia was monitored in a heated recovery cage until the rats were mobile, following which they were returned to their respective cages. Chemoprophylaxis and analgesia were repeated for three post-operative days and the wound closure monitored daily for 10 days, after which the sutures were removed.

Application of orthodontic forces

Orthodontic rat molar movement was achieved by the application of a closed coil spring (0.010 X 0.045 inches) extending from the upper right first molar to the upper right central incisor. The nickel-titanium coil spring was 1 cm in length, and its activation for 0.25 cm produced a force of 60 gr*, that was measured with the Haldex precision force gauge. The orthodontic force was applied for 14 days. At the end of the experimental period, following euthanasia of the animals, the maxillary bone was dissected from the skull and the spring was removed carefully.

Histology

The specimens were cleaned from the surrounding soft tissues, fixed in 10% buffered formalin for 18 hours and decalcified in EDTA buffer for 6-8 weeks. Slices of 5 mm thickness were cut sagittally, including the cortical maxillary bone region from the central incisor to the first molar. The specimens were then dehydrated with ethanol and embedded in paraffin. Histological sections, 4 to 5 µm-thick, were obtained, stained with hematoxylin and eosin and were observed under transmitted light microscopy.

Quantitative estimation of the quality of the cortex was achieved using a scoring system as following: 0=no changes, 1=mild changes, 2=moderate changes and 3=severe changes. Mean values were determined for the final score. The cortex thickness was measured using a micrometer attached to the eyepiece of the microscope.

RESULTS

Laboratory animals

All animals survived the operations of ovariectomy and orthodontic force application uneventfully.

A non-significant transient body weight loss, ranging between 2 and 7 g per rat (less than 3% b.w.), in spite of the administration of powdered food (grinded pellets), was noticed in most animals during the first week of force application. This was attributed to the initial discomfort of the application and was followed by recovery of previous body weight in the next week.

Histological findings of non-ovariectomized rats

The cortical bone of the non-OVX rats, on the right side and ahead of the right first molar where the orthodontic forces were applied, showed distortion of

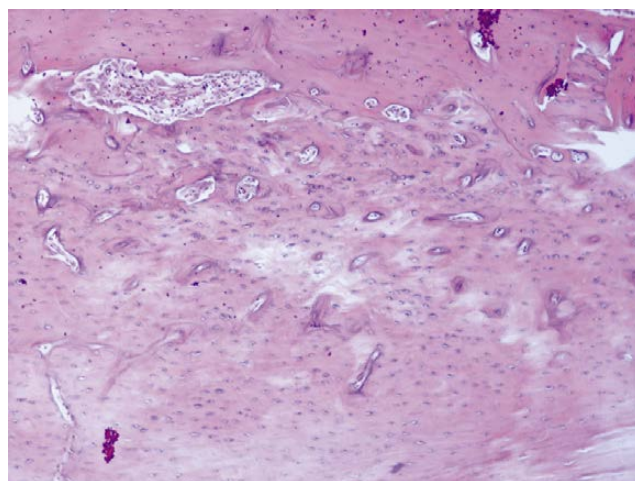


Fig 1. Cortical bone of non-ovariectomized rats, on the right side ahead of the 1st upper molar with force application, showing marked distortion of bone structure and woven bone (arrow) formation (Obj. X10 H-E).

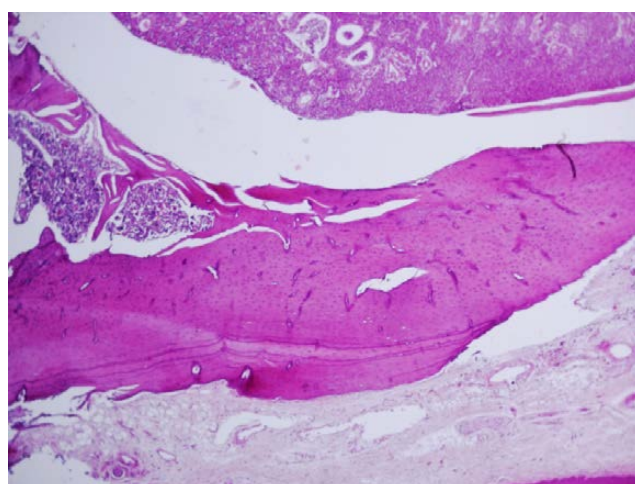


Fig 2. Cortical bone of non-ovariectomized rats, on the left side ahead of the 1st upper molar without any force application, where the bone is lamellar (arrow) and well oriented (Obj. X4 H-E).

bone structure and woven bone formation (Figure 1). The final score showed moderate changes=2.

In contrast, the left side, where no orthodontic forces were applied, the bone ahead of the left first molar was lamellar and well oriented (Figure 2) with a final score of mild changes=1.

Histological findings of ovariectomized rats

The cortical bone of the OVX rats, on the right side and ahead of the right first molar where the orthodontic forces were applied, showed distinct cement lines.

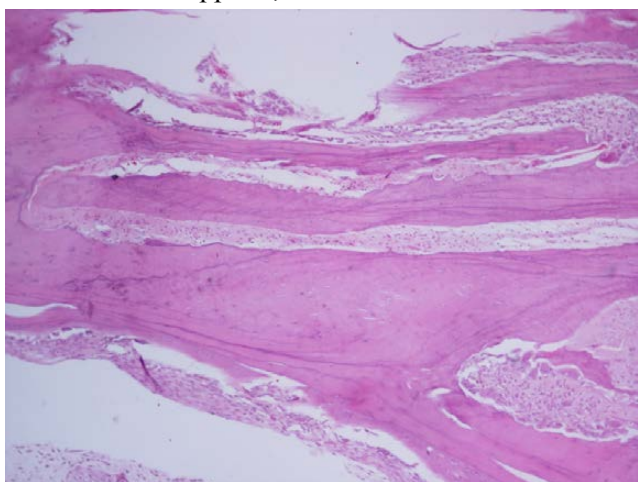


Fig 3. Cortical bone of ovariectomized rats, on the right side ahead of the 1st upper molar with force application, showing distinct cement lines (arrow), with increased bone resorption (arrowhead) and formation (white arrow) (Obj. X10 H-E).

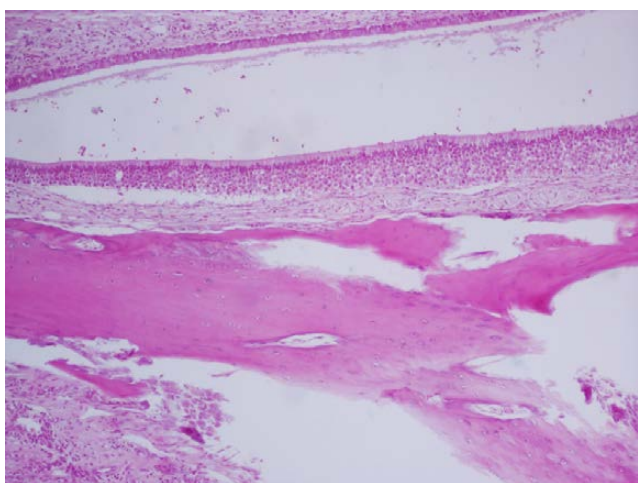


Fig 4. Cortical bone of ovariectomized rats, on the left side ahead of the 1st upper molar without any force application, showing a more compact structure (arrow) compared to the right side (Obj. X10 H-E).

researchers in the field of tooth movement concentrated their attention on the biologic changes mainly on the alveolus surrounding the displaced teeth (Reitan, 1951; Rygh, 1972; Davidovitch, 1991; Brudvik and Rygh, 1994; Masella and Meister, 2006; Meikle, 2006). Recent experimental work with orthodontic tooth movement in dogs has documented RAP in alveolar bone with histomorphometry (Deguchi et al., 2008).

The RAP is considered to increase regional or local bone remodeling, in response to an irritating intervention, such as, in the present case, force application (Jee and Yao, 2001; Verna, 2016). The RAP's severity depends on the intensity of the stimulus that has turned it on (Frost, 2004). In the present study, the magnitude of the applied orthodontic force was high (60 g*). The RAP was microscopically apparent at the cortical bone region between the central incisors and the 1st molar, two weeks following the application of the orthodontic loading.

Bone is a complex, living tissue that possesses the ability of constant adaptation to structural and metabolic demands. An interconnection and interrelationship of the osteons exists through the tubular canaliculae and therefore any stimulus on the external bone surface is distributed to the inner bone. Tubular canaliculae help osteocytes monitor and detect local bone stresses, strains and microdamages (Marotti, 2000). Furthermore, osteocytes communicate with each other and with bone lining cells via gap junctions (Jiang et al., 2007). In this sense, any change in tooth loading is distributed by osteocytes not only to the alveolar bone, but also to the entire surrounding bone tissue.

The effect of tooth loading in adjacent tissues in normal rats has been studied extensively, as mentioned above. However, there has not been systematic investigation of bone tissue changes ahead of the loaded tooth. Melsen (2001) observed woven bone formation ahead of the alveolus in the direction of the displacement in normal rats, and interpreted it as a RAP. In our study, the application of forces evoked accelerated changes, as shown by histological sections in both normal and osteoporotic mature animals. It has also been shown in dogs that there is accelerated tooth movement due to a regional acceleratory phenomenon following corticotomy-facilitat-

	Force application	No force application
OVX rats	3	1
Non-OVX rats	2	1

Table legend. Table indicating the cortical bone histological changes expressed with mean values of each rat group as calculated by the following scoring system:

1= Mild changes

2= Moderate changes

3= Severe changes

ed technique (Mostafa et al., 2009). Given the fact that the appropriate age of the female rat model for post-menopausal osteoporosis should be no less than 6 months, the rats of the present study were ovariectomized at that age. Additionally, as the earliest time of bone loss in different skeletal rat sites is noted between 14 and 60 days (Jee and Yao, 2001), we applied the orthodontic forces at the end of that period.

It seems that the strains exerted on the surrounding bone tissues ahead of the loaded teeth affected their modeling and remodeling processes. The effect of OVX on rat cortical bone ahead of the first molar was expressed by an increase of bone remodeling, whereas the effect of force application appeared to affect the structure of the bone.

The combination of OVX and retraction caused catabolic (resorption) and anabolic (increase in thickness) modeling of bone additionally to the structural distortion (woven bone). Our findings at the site ahead of the loaded tooth are similar to the tissue changes that Roberts et al. demonstrated adjacent to the loaded tooth, as initial responses to tooth movement (2004). According to their study, catabolic and

anabolic modeling occurs at the periodontal ligament of the loaded tooth. The findings of this experimental study may have significant clinical value in cases where orthodontic treatment of mature adults is warranted, in particular of postmenopausal women who suffer from systemic osteoporosis. The potentially increased mandibular and/or maxillary bone resorption and formation in these patients, in response to orthodontic forces applied, may necessitate modification of the normal adult treatment period and magnitude of applied forces.

CONCLUSION

The application of orthodontic tooth forces on both normal and osteoporotic mature rats, in the present study, created a regional acceleratory phenomenon ahead of the loaded teeth, which was demonstrated histologically. In the normal rats the RAP was expressed with distortion of bone structure and the presence of woven bone, whereas in the osteoporotic rats the RAP was expressed with increased catabolic, as well as anabolic remodeling. These experimental findings should be taken into account in clinical practice when orthodontic forces are applied to mature patients with osteoporosis.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest

REFERENCES

- Brudvik P, Rygh P. Root resorption beneath the main hyalinized zone. *Eur J Orthod* 1994;16:249-63.
- Davidovitch Z. Tooth movement. *Crit Rev Oral Biol Med* 1991;2:411-50.
- Deguchi T, Takano-Yamamoto T, Yabuuchi T, Ando R, Roberts WE, Garetto LP. Histomorphometric evaluation of alveolar bone turnover between the maxilla and the mandible during experimental tooth movement in dogs. *Am J Orthod Dentofacial Orthop* 2008;133:889-97.
- Frost HM. The mechanical bone signals; the RAP. In: *The Utah paradigm of skeletal physiology. International Society of Musculoskeletal and Neuronal Interactions*, ed. Athens: Hylonome, 2004;136-140.
- Frost HM. The regional acceleratory phenomenon. A review. *Henry Ford Hosp Med J* 1983;31:3-9.
- Jee WSS, Yao W. Overview: animal models of osteopenia and osteoporosis. *J Musculoskelet Neuronal Interact* 2001;1:193-207.
- Jiang JX, Siller-Jackson AJ, Burra S. Roles of gap junctions and hemichannels in bone cell functions and in signal transmission of mechanical stress. *Front Biosci* 2007;12:1450-62.
- Katona TR, Paydar NH, Akay HU, Roberts WE. Stress analysis of bone modeling response to rat molar orthodontics. *J Biomech* 1995;28:27-38.
- Lelovas PP, Xanthos TT, Thoma S, Lyritis GP, Dontas IA. The laboratory rat as a model of osteoporosis. *Comp Med* 2008;58:424-30.
- Marotti G. The osteocyte as a wiring transmission system. *J Musculoskelet Neuronal Interact* 2000;1:133-6.
- Martin RB. Fatigue microdamage as an essential element of bone mechanics and biology. *Calcif Tissue Int* 2003;73:101-7.
- Masella RS, Meister M. Current concepts in the biology of orthodontic tooth movement. *Am J Orthod Dentofacial Orthop* 2006;129:458-68.
- Meikle MC. The tissue, cellular, and molecular regulation of orthodontic tooth movement: 100 years after Carl Sandstedt. *Eur J Orthod* 2006;28:221-40.
- Melsen B. Tissue reaction to orthodontic tooth movement – a new paradigm. *Eur J Orthod* 2001;23:671-81.
- Mostafa YA, Mohamed Salah Fayed M, Mehanni S, ElBokle NN, Heider AM. Comparison of corticotomy-facilitated vs standard tooth-movement techniques in dogs with miniscrews as anchor units. *Am J Orthod Dentofacial Orthop* 2009;136:570-7.
- Parfitt M. Targeted and nontargeted bone remodeling: relationship to basic multicellular unit origination and progression. *Bone* 2002;30:5-7.
- Reitan K. The initial tissue reaction incident to orthodontic tooth movement as related to the influence of function. *Acta Odontol Scand* 1951;9:231-9.
- Roberts WE, Huja S, Roberts JA. Bone modeling: biomechanics, molecular mechanisms, and clinical perspectives. *Semin Orthod* 2004;10:123-61.
- Roberts WE. Physiology of bone remodeling during tooth movement. *Semin Orthod* 2000;6:173-82.
- Ru N, Liu SS-Y, Zhuang L, Li S, Bai Y. In vivo microcomputed tomography evaluation of rat alveolar bone and root resorption during orthodontic tooth movement. *Angle Orthod* 2013;83:402-9.
- Rygh P. Ultrastructural cellular reactions in pressure zones of rat molar periodontium incident to orthodontic tooth movement. *Acta Odontol Scand* 1972;30:575-93.
- Sirisoontorn I, Hotokezaka H, Hashimoto M, Gonzales C, Luppapanornlarp S, Darendeliler MA, et al. Tooth movement and root resorption; the effect of ovariectomy on orthodontic force application in rats. *Angle Orthod* 2011;81:570-7.
- Sirisoontorn I, Hotokezaka H, Hashimoto M, Gonzales C, Luppapanornlarp S, Darendeliler MA, et al. Orthodontic tooth movement and root resorption in ovariectomized rats treated by systemic administration of zoledronic acid. *Am J Orthod Dentofacial Orthop* 2012;141:563-73.
- Spyropoulos MN, Tsolakis AI. Mechanobiological perspectives of tooth movement related to bone biology. In: Enlow DH, Hans MG, eds. *Essentials of Facial Growth*. Second Edition. Ann Arbor: Needham Press, 2008;389-401.
- Tsolakis AI, Khaldi L, Makou M, Lyritis GP, Spyropoulos MN, Dontas IA. Cortical bone response adjacent to applied light orthodontic forces in ovariectomized rats. *J Musculoskelet Neuronal Interact* 2008;8:375-8.
- Verna C, Dalstra M, Lee TC, Cattaneo PM, Melsen B. Microcracks in the alveolar bone following orthodontic tooth movement: a morphological and morphometric study. *Eur J Orthod* 2004;26:459-67.

Prion protein gene polymorphisms in classical scrapie-affected flocks of sheep in Central Macedonia

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Πολυμορφισμοί του γονιδίου της πρωτεΐνης prion σε θετικές, ως προς την κλασσική τρομώδη νόσο, εκτροφές προβάτων στην Κεντρική Μακεδονία

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ABSTRACT. The allele and genotype frequency distributions of the prion protein gene polymorphisms at codons 136, 154 and 171 were determined by real-time PCR for 1,456 sheep from 7 classical scrapie-affected flocks of Thessaloniki and Imathia, Central Macedonia, Greece. The blood samples were collected by official veterinarians and were examined by the National Reference Laboratory (NRL) for TSEs, Veterinary Laboratory of Larisa, Greece, in the framework of the National Program for Scrapie Surveillance and Control between 2009 and 2013. Among the 1,456 sheep, 340 were of Chios breed, 633 Chios crossbred and 483 crossbred. The examined sheep showed high genotype variability, as a total of 7 haplotypes and 23 different genotypes were found. The predominant allele and the predominant genotype were ARQ and ARQ/ARQ respectively, in all breeds studied, followed by the ARR allele and the ARR/ARQ genotype. The TRQ allele was frequent in Chios and Chios crossbred, while the VRQ allele was rare for all the breeds. Interestingly, 3 genotypes (ARH/TRQ, ARR/ARK and ARK/VRQ) were detected for the first time in Greece and two of them (ARH/TRQ and ARK/VRQ) have, to our knowledge, never been previously reported. Furthermore, it is emphasized that our country outnumbers all European countries in classical scrapie cases of sheep every year. Therefore, there is an urgent need to reduce the incidence of classical scrapie through the implementation of selective breeding programs. This is supported by the fact that the prevalence of classical scrapie in the Greek sheep population is highly associated with the predominant genotype ARQ/ARQ. Therefore, the elimination of the ARQ/ARQ and the other susceptible genotypes (belonging to Risk Groups 3 and 5, according to the National Scrapie Plan of Great Britain) would reduce dramatically the incidence of classical scrapie in Greece.

Keywords: PrP gene polymorphisms, scrapie, Real-time PCR

ΠΕΡΙΛΗΨΗ. Για την έρευνα αυτή πραγματοποιήθηκε αιμοληψία σε 1.456 πρόβατα, ηλικίας 1-7 ετών, από 7 θετικές εκτροφές της Ημαθίας και της Θεσσαλονίκης, ως προς την κλασσική τρομώδη νόσο, στα πλαίσια της εφαρμογής του Εθνικού Προγράμματος Επιτήρησης, Ελέγχου και Εξάλειψης των Μεταδοτικών Σπογγωδών Εγκεφαλοπαθειών (ΜΣΕ) στα μικρά μηρυκαστικά του Υπουργείου Αγροτικής Ανάπτυξης & Τροφίμων, για τα έτη 2009-2013. Τα αιμοδείγματα συλλέχθηκαν από κρατικούς κτηνιάτρους των κτηνιατρικών υπηρεσιών της Ημαθίας και Θεσσαλονίκης και οι γονοτυπικές εξετάσεις πραγματοποιήθηκαν στο Εθνικό Εργαστήριο Αναφοράς για τις ΜΣΕ, Κτηνιατρικό Εργαστήριο Λάρισας με τη μέθοδο της αλυσιδωτής αντίδρασης της πολυμεράσης σε πραγματικό χρόνο (Real-time PCR). Από τα 1.456 πρόβατα που εξετάστηκαν, τα 340 ήταν φυλής Χίου, τα 633 ημίαιμα Χίου και τα 483 ημίαιμα. Τα εξεταζόμενα ζώα παρουσίασαν μεγάλη γενετική ποικιλομορφία, καθώς βρέθηκαν 7 διαφορετικά αλληλόμορφα και 23 διαφορετικοί γονότυποι. Ο κυρίαρχος γονότυπος σε όλες τις φυλές ήταν ο ARQ/ARQ, ακολουθούμενος από τον ARR/ARQ. Ο απλότυπος TRQ ήταν συχνός στα Χιώτικα και στα ημίαιμα Χίου πρόβατα, ενώ ο απλότυπος VRQ ήταν σπάνιος σε όλες τις φυλές. Είναι αξιοσημείωτο ότι 3 γονότυποι (ARH/TRQ, ARR/ARK και ARK/VRQ) ανιχνεύθηκαν για πρώτη φορά στην Ελλάδα. Επιπλέον, 2 από αυτούς τους γονότυπους (ARH/TRQ, και ARK/VRQ) δεν έχουν, καθ' όσον γνωρίζουμε, αναφερθεί ποτέ μέχρι σήμερα. Επίσης, δίνεται έμφαση στο γεγονός ότι στη χώρα μας παρατηρούνται τα περισσότερα κρούσματα κλασσικής τρομώδους νόσου των προβάτων κάθε χρόνο σε όλη την Ευρώπη. Συνεπώς είναι επιτακτική ανάγκη να μειωθεί η επίπτωση της νόσου στην Ελλάδα, μέσω της εφαρμογής προγραμμάτων επιλογής/αναπαραγωγής ανθεκτικών προβάτων (selective breeding programs) ως προς την κλασσική τρομώδη νόσο. Σε αυτό συνηγείται το γεγονός ότι ο επιπολασμός της νόσου στην Ελλάδα συνδέεται με τον γονότυπο ARQ/ARQ, ο οποίος είναι ο πιο συχνός γονότυπος. Άρα η δραστηκή μείωση του ARQ/ARQ και άλλων ευαίσθητων γονότυπων θα προκαλέσει δραματική μείωση της επίπτωσης της νόσου, προς όφελος της προβατοτροφίας και της εθνικής μας οικονομίας. .

Λέξεις-Κλειδιά: τρομώδης νόσος, γονοτυπική ανάλυση, πρωτεΐνη prion, αλυσιδωτή αντίδραση πολυμεράσης

INTRODUCTION

Scrapie is a fatal, progressive, neurodegenerative disease affecting sheep and goats. It has been known since 1732, when it was first described in the UK (McGowan 1922) and belongs to the family of Prion diseases or Transmissible Spongiform Encephalopathies (TSEs). The term “prion” was proposed by Prusiner in 1982, in order to denote a small proteinaceous infectious particle, which is resistant to inactivation by most procedures. According to the Prion Hypothesis, infectious prion particles are composed largely, if not entirely, of an abnormal isoform (PrP^{Sc}) of the prion protein, which is encoded by a chromosomal gene. (Prusiner 1991). The *PRNP* gene encodes the prion protein (PrP), which plays a major role in the disease process (Goldmann 2008, Houston et al., 2015). In the PrP genes of humans, mice and sheep, amino acid polymorphisms associated with disease susceptibility and pathogenesis have been found (Belt et al., 1995). In sheep, amino acid polymorphisms at many positions (89, 94, 101, 112, 127, 128, 132, 134, 135, 136, 137, 138, 141, 143, 145, 146, 149, 151, 152, 154, 157, 159, 160, 163, 164, 167, 168, 169, 171, 172, 174, 175, 176, 180, 183, 184, 185, 189, 193, 195, 196, 199, 211, 213, 220, 224, 241) have been described (Goldmann et al., 1990, 1991, 1994, 2005, Laplanche et al., 1993, Hunter et al., 1993, 1994, 1996, 1997, Ikeda et al., 1995, Belt et al., 1995, De Silva et al., 2003, Acutis et al., 2004, F. Guan et al., 2011, Alvarez et al., 2011, Oner et al., 2011, Zhao et al., 2012, Hautaniemi et al., 2012, Meydan et al., 2013, Curcio et al., 2015), but the polymorphisms at codons 136, 154 and 171 have been demonstrated to be of major importance, as they modulate the susceptibility/resistance of sheep for scrapie (Cloucard et al., 1995, Hunter et al., 1996, Smits et al., 1997). These polymorphisms are Alanine (A), Valine (V) or Threonine (T) at codon 136, Arginine (R) or Histidine (H) at codon 154 and Glutamine (Q), R, H or Lysine (K) at codon 171.

The five most common haplotypes are ARR, ARQ, AHQ, ARH and VRQ (Baylis et al., 2004, Dobly et al., 2013). Additional haplotypes (TRQ, ARK, VRR, AHR, VHQ and TRR) have been reported so far (Kutzer et al., 2002, De Silva et al., 2003, Acutis et al., 2004, Billinis et al., 2004, F. Guan et al., 2011, Psifidi et al., 2011, Oner et al., 2011, Alvarez et al., 2011,

Zhao et al., 2012, Meydan et al., 2012, Granato et al., 2013). According to the National Scrapie Plan for Great Britain, the fifteen most common genotypes have been classified into five risk groups (R1-R5), with R1 (ARR/ARR genotype sheep) indicating the lowest risk of scrapie and R5 (VRQ/VRQ, VRQ/ARQ and other VRQ-encoding genotypes) associated with the highest susceptibility (DEFRA, 2001). The disease is mainly associated with the ARQ/ARQ genotype (R3) in the sheep breeds (the so called “non-valine” or “alanine” breeds) where the VRQ allele is rare or absent, such as in many Mediterranean sheep breeds (Greek, Italian, Spanish), in Suffolk breed, in Canadian sheep etc (Hunter et al., 1997, Billinis et al., 2004, Ekateriniadou et al., 2007, Koutsoukou-Hartona et al., 2009, Harrington et al., 2010, Curcio et al., 2015).

A new form of scrapie was discovered in Norway in 1998 (named atypical scrapie or Nor98) (Benestad et al., 2003). It has been reported in several European countries (Fediaevsky et al., 2008), and the majority of sheep affected with atypical scrapie is between five and ten years of age or more, in contrast to the younger age (between three and five years) of the sheep affected with classical scrapie. It's noteworthy that only a single scrapie-positive per affected flock was identified in most cases (Luhken et al., 2007, Benestad et al., 2008). Recent evidence linking new variant Creutzfeldt-Jakob disease (nvCJD) in humans to BSE in cattle has increased attention for all TSEs, including scrapie (www.cabi.org). Scrapie (classical and atypical) is not considered a public health risk, but BSE has been linked to new variant Creutzfeldt-Jakob disease (Eloit et al., 2005, Spiropoulos et al., 2011).

Classical scrapie is a relatively common problem for Greek sheep farmers. Our country has the highest number of sheep scrapie cases in Europe every year. For example, in 2015 Greece reported 188 cases of classical scrapie in sheep, followed by Italy with 141, Romania with 98 and Spain with 69 cases, while all the other European countries presented only 50 cases in total (Boelaert et al., EFSA Journal 2016). Scrapie was firstly diagnosed in Greece in 1986 (Leontides et al., 2000), while nine positive flocks were found till 2001, as a result of the Passive Surveillance Program. From 2002, when the Active Surveillance Program

began, the positive flocks were increased year by year coming up to 223 (217 flocks with classical scrapie and 6 flocks with atypical scrapie) in 2008 (Koutsoukou et al., 2009). The total number of sheep classical scrapie cases in Greece during the period 2002-2015 was 5,448 (more than in any other European country), while only 30 atypical scrapie cases were also reported (Boelaert et al., EFSA Journal

The blood samples were collected by official veterinarians.

Among the 1,456 sheep, 340 were of Chios breed, 633 Chios crossbred and 483 crossbred (Table 1).

DNA Extraction, Amplification and Genotyping Analysis

All analyses were performed at the National Ref-

Table 1: Number of sheep sampled from each breed

Breed	Number of flocks	Number of sheep		Totals
		Rams	Ewes	
Chios	1	14	326	340
Chios Crossbred	4	30	603	633
Crossbred	2	17	466	483
Totals	7	61	1,395	1,456

2016). That's why it is imperative to combat classical scrapie in Greece, which can be achieved with proper implementation of selective breeding programs. The predominant genotype of the scrapie-affected sheep in Greece is ARQ/ARQ, at an approximately 70% percentage, followed by ARQ/AHQ, VRQ/VRQ and VRQ/ARQ (Billinis et al., 2004, Ekateriniadou et al., 2007a, Koutsoukou et al., 2009). Therefore, the elimination of these genotypes (belonging to Risk Groups 3 and 5, according to the National Scrapie Plan of Great Britain) with the successful implementation of selective breeding programs could reduce dramatically the incidence of classical scrapie in Greece.

The aim of this study was to present the allelic and genotype frequencies of 1,456 sheep from 7 scrapie-affected flocks in Central Macedonia (Thessaloniki and Imathia), sorted by their breed (Chios, Chios crossbred and Crossbred) from 2009 to 2013.

MATERIALS AND METHODS

Sheep

Blood samples were obtained from a total of 1,456 sheep (61 rams and 1,395 ewes), aged 1-7 years old, originating from 7 scrapie-affected flocks of Thessaloniki and Imathia (Central Macedonia, Greece), in the framework of the National Program for Scrapie Surveillance and Control, between 2009 and 2013.

erence Laboratory (NRL) for TSEs, Veterinary Laboratory of Larisa, Greece. The method used for scrapie genotype determination is based on multiplex real-time PCR technology.

DNA extraction

Sheep blood sample was collected using absorbing paper cards (Whatman FTA ELUTE Micro Cards). Blood spots (each of equal diameter per sample) were cut from the cards and the genomic DNA was extracted from circular spots using deionized water and direct heating using a thermoblock at 97 °C for 30min.

Real-time PCR melting curve genotyping

Genotyping was performed using the LightMix 480HT Scrapie Susceptibility Mutation Detection Kit (TIB MolBiol, Germany) and LightCycler® 480 Genotyping Master hot start reaction mix and probe melting-curve based genotyping kit (Roche Applied Science). The PCR reactions were conducted with the LightCycler® 480 II Real Time PCR thermal cycler (Roche Applied Science), according to the manufacturer's instructions. LightMix 480HT Scrapie Susceptibility Mutation Detection Kit uses 3 combinations of probes, each of them specific to codons 136, 154 and 171 respectively. In each real-time PCR reaction,

mutations within amplicons were detected during melting curve analysis, based on fluorescence resonance energy transfer (FRET) signal measurement. Melting peaks of PCR product–probe hybrids were obtained. Subsequently, melting curves peak analysis was performed to determine genotype at codons 136 [GCG (Ala-A), GTC (Val-V)], 154 [(CGT (Arg-R) and CAT (His-H)] and 171 [CAG (Gln-Q), CAT (His-H), CGT (Arg-R) and AAG (Lys-K)]. The melting peaks which were obtained by testing of samples were compared with the respective peaks of control samples, i.e. DNA extracts from the blood of sheep with known genotypes. These controls were purchased from VLA laboratories (UK) and were analyzed using the same protocol.

RESULTS

The PrP allelic frequencies of the 1,456 sampled sheep are summarized in Table 2. Seven alleles, of codons 136, 154 and 171 (ARR, ARQ, AHQ, ARH, VRQ, TRQ, ARK) were observed.

The most frequent allele was ARQ, with a mean frequency 62.74%. The second most frequent allele was ARR with 17.38%, followed by AHQ (8.41%), TRQ (6.05%) and ARH (3.57%). The ARK allele was found at an average frequency of 0.48%, while the VRQ allele was detected at low frequencies (1.37%) (Table 2).

The frequencies of PrP genotypes are presented in

Table 3. It is noteworthy that twenty three (23) different genotypes were identified in the sampled sheep. The fifteen (15) commonly reported PrP genotypes (included in the NSP) presented a total frequency of 87.91 %. Apart from them, eight (8) different genotypes were observed with a total frequency of 12.09%.

The ARQ/ARQ genotype, which is classified in the Risk Group R3, was the most frequent genotype (40.52%). The second most frequent genotype was ARR/ARQ (Risk Group R2) with 20.60 %, followed by ARQ/AHQ (Risk Group R3) with 10.10 % and ARQ/TRQ (which is not classified in any risk group by NSP) with 7.14 %. The genotype ARQ/TRQ was much more frequent in Chios breed and Chios crossbred (7.65 % and 10.58 % respectively) than in crossbred (2.28 %). The genotype ARH/ARQ (Risk Group R3) was found at frequency 4.33%, followed by the most resistant genotype ARR/ARR (Risk Group R1) with 3.85%, ARR/AHQ with 3.16 %, ARQ/VRQ (Risk Group R5) with 1.58%, ARR/TRQ with 1.51% and ARR/ARH with 1.17 %

NSP type III (Risk Group R3) was the most frequent (56.66 % of all animals), followed by type II (Risk Group R2) with 24.93%, type I (Risk Group R1) with 3.85%, type V (Risk Group R5) with 1.99% and type IV (Risk Group R4) with 0.48% in decreasing order of frequency. The remaining genotypes (12.09 %) were not classified in any NSP risk group (Table 3).

Table 2. Allelic frequencies of PrP polymorphisms at codons 136, 154 and 171

ALLELES	BREED							
	CHIOS		CHIOS CROSSBRED		CROSSBRED		TOTAL	
	n	%	n	%	n	%	n	%
ARR	68	10.00	190	15.01	248	25.67	506	17.38
ARQ	486	71.47	802	63.35	539	55.80	1,827	62.74
AHQ	39	5.74	97	7.66	109	11.28	245	8.41
ARH	37	5.44	31	2.45	36	3.73	104	3.57
VRQ	5	0.73	21	1.66	14	1.45	40	1.37
TRQ	42	6.18	115	9.08	19	1.97	176	6.05
ARK	3	0.44	10	0.79	1	0.10	14	0.48
TOTAL	680	100.00	1,266	100.00	966	100.00	2,912	100.00

Table 3. Genotype frequencies of PrP polymorphisms at codons 136, 154 and 171

BREED									
NSPa	PrP GENOTYPE	CHIOS						TOTAL	
		CHIOS		CROSSBRED		CROSSBRED			
		n	%	n	%	n	%	n	%
Type I	ARR/ARR	4	1.18	17	2.69	35	7.25	56	3.85
Type II	ARR/ARQ	45	13.23	119	18.80	136	28.16	300	20.60
Type II	ARR/AHQ	7	2.06	12	1.90	27	5.59	46	3.16
Type II	ARR/ARH	4	1.18	4	0.63	9	1.86	17	1.17
									24.93
Type III	ARQ/ARQ	180	52.94	258	40.76	152	31.47	590	40.52
Type III	ARQ/AHQ	24	7.06	62	9.80	61	12.63	147	10.10
Type III	ARH/ARQ	25	7.35	21	3.32	17	3.52	63	4.33
Type III	AHQ/AHQ	2	0.59	5	0.79	5	1.04	12	0.82
Type III	AHQ/ARH	1	0.29	2	0.31	6	1.24	9	0.62
Type III	ARH/ARH	2	0.59	0	0.00	2	0.41	4	0.27
									56.66
Type IV	ARR/VRQ	1	0.29	4	0.63	2	0.41	7	0.48
Type V	ARQ/VRQ	4	1.18	10	1.58	9	1.86	23	1.58
Type V	VRQ/AHQ	0	0.00	1	0.16	3	0.62	4	0.27
Type V	VRQ/VRQ	0	0.00	2	0.31	0	0.00	2	0.14
Type V	ARH/VRQ	0	0.00	0	0.00	0	0.00	0	0.00
									1.99
n.c	ARQ/TRQ	26	7.65	67	10.58	11	2.28	104	7.14
n.c	ARR/TRQ	2	0.59	16	2.53	4	0.83	22	1.51
n.c	ARQ/ARK	2	0.59	7	1.11	1	0.21	10	0.69
n.c	AHQ/TRQ	3	0.88	10	1.58	2	0.41	15	1.03
n.c	TRQ/TRQ	4	1.18	9	1.42	1	0.21	14	0.96
n.c	ARH/TRQ **	3	0.88	4	0.63	0	0.00	7	0.48
n.c	ARR/ARK *	1	0.29	1	0.16	0	0.00	2	0.14
n.c	ARK/VRQ **	0	0.00	2	0.31	0	0.00	2	0.14
									12.09
	TOTAL	340	100.00	633	100.00	483	100.00	1,456	100.00

^a Risk group classification, according to the National Scrapie Plan (NSP) of Great Britain

n.c Not classified in any risk group by NSP

* Genotype detected for the first time in Greece

** The 2 genotypes have, to our knowledge, never been previously reported

DISCUSSION

The haplotype with the highest frequency was ARQ, that was found at an average frequency of 62.74 % for all breeds. The frequency of the allele ARQ in Chios breed and in Chios crossbred was 71.47% and 63.35% respectively. These high percentages are in agreement with the previously reported results for Chios breed (Ekateriniadou et al., 2007b, Psifidi et al., 2011, Ekateriniadou et al., 2007a). Higher frequencies of the ARQ allele have been reported in scrapie-affected sheep (Ekateriniadou et al., 2007a). Similar frequencies of the ARQ allele were reported in Italy (Granato et al., 2013, Martemucci et al., 2015), in Brazil (Ianella et al., 2012) and in Turkey (Meydan et al., 2012), whereas higher frequencies (74-86%) were observed in Awassi and Assaf sheep populations (Gootwine et al., 2008).

The ARQ/ARQ genotype was the predominant genotype with a mean of 40.52%. The frequency in Chios breed was 52.94% and that of Chios crossbred was 40.76%. Compared with the results of our study, same frequencies were reported in Canada which concerned 1990 healthy sheep from 3 scrapie-affected flocks (ARQ allele 62.2%, ARQ/ARQ 38.4%, Harrington et al., 2010). It is noteworthy that the frequency of the ARQ allele has significantly decreased since then, as a result of selection for resistant PrP genotypes in Ontario sheep (frequency of the ARQ haplotype 28% in 2012) and in sheep from other provinces of Canada from 2005 to 2012 (Cameron et al., 2014).

Furthermore, the ARQ allele and the ARQ/ARQ genotype were found to be the predominant allele and genotype respectively, in Finland (Hautaniemi et al., 2012), in China (Zhao et al., 2012), in Brazil (Ianella et al., 2012), in Turkey (Alvarez et al., 2011, Meydan et al., 2012), in East Asian sheep (Tsunoda et al., 2010) and in Pakistan (Babar et al., 2009) among other countries. Sheep with the genotype ARQ/ARQ are highly susceptible to scrapie, while this is the most common genotype among scrapie-affected sheep of Greek positive flocks with 68.35% in Chios crossbred, 70.39% in other crossbred (Ekateriniadou et al., 2007a), 72.73% (88 ARQ/ARQ from 121 scrapie-affected sheep) in crossbred (Billinis et al., 2004) and 75.58% (Koutsoukou-Hartona et al.,

2009). Therefore, if the frequency of the ARQ/ARQ genotype is reduced through the selective breeding program, the incidence of classical scrapie in Greece will be dramatically reduced.

The second most frequent genotype was ARQ/ARR with a mean frequency of 20.60% for all breeds and 13.23% in Chios breed, which is similar to the 11.35% (Psifidi et al., 2011) and 14.50% (Billinis et al., 2004).

The most resistant genotype ARR/ARR was found with a low mean proportion 3.85% in the study population and 1.18% in Chios breed, which is in agreement with previous studies of Greek sheep breeds (Koutsoukou-Hartona et al., 2009, Ekateriniadou et al., 2007a, Ekateriniadou et al., 2007b, Psifidi et al., 2011 and Billinis et al., 2004). High frequencies of the ARR/ARR genotype were reported in the UK, France, the Netherlands, Belgium, Cyprus, Germany, Hungary, Poland, Canada and other countries where selective breeding programs have been implemented. For example, 77.14% was reported in Berrichon du Cher in Poland (Grochowska et al., 2014), 82.4% in Cyprus (Boelaert et al., EFSA Journal 2016), whereas the frequencies of the ARR allele in rams of French breeds ranged from 68% to 100% (Palhiere et al., 2008).

The genotype ARQ/AHQ was found with a mean proportion of 10.10%, while Billinis et al. found 17.6% in crossbred sheep from scrapie-affected flocks and 12.40% in crossbred scrapie-affected sheep and Ekateriniadou et al., found 24.51% in Chios crossbred scrapie-affected sheep. Therefore, the genotype ARQ/AHQ is often observed in sheep positive for classical scrapie and belongs to the susceptible genotypes. As for the VRQ allele, the frequency in the study holdings was 1.37% (0.73% in Chios breed), supporting previous studies in Chios breed, in which low (0.40%, Psifidi et al., 2011), or null (Ekateriniadou et al., 2007a, b, Billinis et al., 2004) frequencies of the VRQ allele were observed. Among classical scrapie-affected sheep in Greece, the total frequency of the VRQ-related genotypes (belonging to Risk Groups NSP-4 + NSP-5) was 7.63% for all Greek sheep breeds (Koutsoukou-Hartona et al., 2009), 0.63% for Chios crossbred and 17.32% for other crossbred sheep, especially Karagouniko breed (Ekateriniadou et al., 2007a). So the

VRQ-related genotypes, mainly ARQ/VRQ and less the VRQ/VRQ and the others, are sometimes observed in scrapie-affected sheep in Greece. The VRQ-related genotypes were responsible for the 202/257 (78.6%) of the positive cases for classical scrapie in the Netherlands (Hagenaars et al., 2010). In addition, in the UK the greatest scrapie risk by far was for the VRQ-encoding genotypes ARQ/VRQ, ARH/VRQ and VRQ/VRQ (Baylis et al., 2004). On the other hand, the VRQ allele was absent in 16 Chinese local sheep breeds, as all sheep were homozygous for A at codon 136 (AA₁₃₆) (Lan et al., 2014).

The five haplotypes described above and the fifteen PrP genotypes, which are pairings of these five alleles defined by the polymorphisms at codons 136, 154 and 171, were included in the National Scrapie Plan for Great Britain (DEFRA, 2001). Apart from them, two additional alleles and eight additional PrP genotypes were found in our study. The sheep included in the present study showed a high degree of genetic variability, which can be possibly attributed to the absence of scrapie genotype-based selection of animals. In all countries where selective breeding programs have been implemented, genetic variability was decreased. For example, in Canada 12 genotypes of sheep were observed in 2005 in Ontario sheep, whereas only 6 genotypes were observed in 2012 after 7 years of selection for resistant genotypes (Cameron et al., 2014). Also, in Belgium many genotypes (from Risk Groups R3 and R5) disappeared, as a result of the selection program from 2006 to 2011 (Dobly et al., 2013).

The mean frequency of the genotype ARQ/TRQ was 7.14% (7.65% in Chios breed and 10.58% in Chios crossbred), which is consistent with previous reports in Chios breed (10.00%, Billinis et al., 2004, 11.94%, Psifidi et al., 2011). This genotype has also been observed in Chinese Hu sheep, (9.44%, F. Guan et al., 2011), in Northwestern China (0.53%, Zhao et al., 2012) and in Turkey (4.03%, Oner et al., 2011 & 6.0%, Alvarez et al., 2011). The rare genotype TRQ/ARH was found only in Chios crossbred and in Chios breed and the frequencies were 0.63% and 0.88% respectively, at a mean of 0.48%. To our knowledge, it's the first time that this genotype is detected. The mean frequency of the rare genotype ARR/ARK was 0.14% (0.29% in Chios breed, 0.16%

in Chios crossbred). This genotype is detected for the first time in Greece. Till now, it has been reported in Chinese Hu sheep (0.56%, F. Guan et al., 2011), in North Western China (0.40%, Zhao et al., 2012), in Turkey (0.1%, Meydan et al., 2012) and in Italy (1.7% in Foza breed, Granato et al., 2013). The very rare genotype ARK/VRQ was found only in Chios crossbred (0.31%) and the average frequency in the study population was 0.14%. To our knowledge, it's the first time that it is detected.

To sum up, the dominant genotype (ARQ/ARQ with 40.52%) in the study sheep population is at the same time, by far, the most common genotype of the scrapie-affected sheep in Greece. Furthermore, the ARQ/AHQ (10.10%, third genotype in decreasing order of frequency) is also often observed in scrapie-affected sheep (Billinis et al., 2004, Ekateriniadou et al., 2007a). These 2 genotypes account for more than 50% of the study sheep population. The frequency of these genotypes, as well as of all the genotypes belonging to Risk Groups 3,4 and 5, could be significantly decreased as a result of the implementation of selective breeding programs, resulting in lower incidence of classical scrapie in the future. Experience with the successful implementation of selective breeding programs in the European countries (UK, France, Cyprus, the Netherlands, Belgium) has shown an impressive reduction in the incidence of the disease (Dawson et al., 2008, Palhiere et al., 2008, Hagenaars et al., 2010, Nodelijk et al., 2011, Ortiz-Pelaez and Bianchini, 2011, Dobly et al., 2013). For example, 3,063 classical scrapie cases in sheep were reported in Cyprus in the period 2002-2015. The 96.3% of them was reported until 2009 and only 3.7% in the period 2010-2015. The same happened in the UK and France (Boelaert et al., EFSA Journal 2016). This reduction in the incidence of classical scrapie in Cyprus, the UK and France is due to the beneficial effects of the selective breeding programs implemented in the 2000s.

CONCLUSIONS

The examined sheep from 7 scrapie-affected flocks of Central Macedonia showed high genotype variability, as a total of 7 haplotypes and 23 different genotypes were detected at codons 136, 154 and 171. Interestingly, three genotypes were detected for the first time in Greece, while two of them (ARH/TRQ

and ARK/VRQ) have, to our knowledge, never been previously reported.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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REFERENCES

- Acutis PL, Sbaiz L, Verburg F, Riina MV, Ru G, Moda G, Caramelli M, Bossers A.** (2004). Low frequency of the scrapie resistance-associated allele and presence of lysine-171 allele of the prion protein gene in Italian Biellese ovine breed. *J Gen Virol* 2004; **85**: 3165-3172.
- Alvarez L., Beatriz Gutierrez-Gil, Metehan Uzun, Fermin San Primitivo,**
- Juan Jose Arranz** (2011) Genetic variability in the prion protein gene in five indigenous Turkish sheep breeds *Small Ruminant Research* **99** (2011) 93–98
- Babar ME, Farid A, Benkel BF, Ahmad J, Nadeem A, Imran M.** (2009) Frequencies of PrP genotypes and their implication for breeding against scrapie susceptibility in nine Pakistani sheep breeds. *Mol Biol Rep* 2009 ;**36**(3):561-565.
- Baylis M., Chihota C., Stevenson E., Goldmann W., Smith A., Sivam K., Tongue S., Gravenor MB.** (2004) Risk of scrapie in British sheep of different prion protein genotype *J. Gen. Virol.* (2004) **85**:2735–2740.
- Belt, P. B., I. H. Muileman, B. E. Schreuder, J. Bos-de Ruijter, A. L. Gielkens and M. A. Smits** (1995). "Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie." *J Gen Virol* **76**: 509-517
- Benestad, S. L., P. Sarradin, B. Thu, J. Schonheit, M. A. Tranulis and B. Bratberg** (2003). "Cases of scrapie with unusual features in Norway and designation of a new type, Nor98." *Vet Rec* **153**: 202-208.
- Benestad SL, Arsac JN, Goldmann W and Noremark M,** (2008). Atypical/Nor98 scrapie: properties of the agent, genetics, and epidemiology. *Veterinary Research*, **39**, 39:19.
- Billinis, C., V. Psychas, L. Leontides, V. Spyrou, S. Argyroudis, I. Vlemmas, S. Leontides, T. Sklaviadis and O. Papadopoulos** (2004). "Prion protein gene polymorphisms in healthy and scrapie-affected sheep in Greece." *J Gen Virol* **85**: 547-554.
- Boelaert F, Hugas M., Ortiz Pelaez A., Rizzi V., Stella P. and Yves Van Der Stede** (2016) *EFSA Journal* **2016** "The European Union summary report on data of the surveillance of ruminants for the presence of transmissible spongiform encephalopathies (TSEs) in 2015" doi: 10.2903/j.efsa.2016.4643
- Cameron C., Bell-Rogers P., McDowall R., Ana R. Rebelo, Hugh Y. Cai** (2014) Prion protein genotypes of sheep as determined from 3343 samples submitted from Ontario and other provinces of Canada from 2005 to 2012
- The Canadian Journal of Veterinary Research* 2014; **78**:260–266
- Centre for Agriculture and Biosciences International (CABI)**
<http://www.cabi.org/isc/datasheet/64847> [accessed August 2nd, 2016]
- Clouscard, C., P. Beaudry, J. M. Elsen, D. Milan, M. Dussaury, C. Bounneau, F. Schelcher, J. Chatelain, J. M. Launay and J. L. Laplanche** (1995).
- "Different allelic effects of the codons 136 and 171 of the prion protein gene in sheep with natural scrapie." *J Gen Virol* **76**: 2097-2101.
- Curcio L., Sebastiani C., Ceccobelli S., Vaccari G., Pezzotti G., Lasagna E., Biagetti M.**(2015) PRNP polymorphisms in four Italian sheep breeds *Livestock Science* **181**(2015) 38–42
- Dawson M., Moore R., Bishop S.** (2008) Progress and limits of PrP gene selection policy *Veterinary Research* **39**: 25
- DEFRA** (2001). In National Scrapie Plan for Great Britain. Schemes Brochure, pp. 1–28. London: Department for Environment, Food and Rural Affairs.
- DeSilva, U., X. Guo, D. M. Kupfer, S. C. Fernando, A. T. Pillai, F. Z. Najar, S. So, G. Q. Fitch and B. A. Roe** (2003). "Allelic variants of ovine prion protein gene (PRNP) in Oklahoma sheep." *Cytogenet Genome Res* **102**: 89-94.
- Dobly A., Sara Van der Heyden, Stefan Roels** (2013)
- Trends in genotype frequency resulting from breeding for resistance to classical scrapie in Belgium (2006-2011) *J. Vet. Sci.* (2013), **14**(1), 45-51
- Ekateriniadou L.V., Kanata E., Panagiotidis C., Nikolaou A., Koutsoukou E., Lymberopoulos A.G., Sklaviadis T.** (2007a) PrP genotypes in scrapie-affected sheep in Greece—The contribution of the AHQ-I polymorphism *Small Ruminant Research* **73** (2007) 142–149
- Ekateriniadou LV, Panagiotidis CH, Terzis A, Ploumi K, Triantafyllidis A, Deligiannidis P, Triantaphyllidis C, Sklaviadis T.** (2007b) Genotyping for PrP gene polymorphisms in rare Greek breeds of sheep. *Vet Rec* 2007; **160**(6):194-195.
- Eloit M, Adjou K, Couplier M, Fontaine JJ, Hamel R, Lilin T, Messiaen S, Andreoletti O, Baron T, Bencsik A, Biacabe AG, Beringue V, Laude H, Le Dur A, Vilotte JL, Comoy E, Deslys JP, Grassi J, Simon S, Lantier F and Sarradin P,** (2005) BSE agent signatures in a goat. *Veterinary Record* (2005), **156**, 523-524.
- Fediaevsky A, Tongue SC, Noremark M, Calavas D, Ru G and Hopp P,** 2008.
- A descriptive study of the prevalence of atypical and classical scrapie in sheep in 20 European countries. *BMC Veterinary Research*, **4**.
- Goldmann, W., N. Hunter, J. D. Foster, J. M. Salbaum, K. Beyreuther and J. Hope** (1990). "Two alleles of a neural protein gene linked to scrapie in sheep."
- Proc Natl Acad Sci U S A* **87**: 2476-2480.
- Goldmann W, Hunter N, Benson G, Foster J & Hope J** (1991)
- Different scrapie-associated fibril proteins (PrP) are encoded by lines of sheep selected for different alleles of the Sip gene.
- J Gen Virol* **72**, 2411-2417
- Goldmann, W., N. Hunter, G. Smith, J. Foster and J. Hope** (1994). "PrP genotype and agent effects in scrapie: change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie." *J Gen Virol* **75** : 989-95
- Goldmann, W., M. Baylis, C. Chihota, E. Stevenson and N. Hunter** (2005). "Frequencies of PrP gene haplotypes in British sheep flocks

- and the implications for breeding programmes." *J Appl Microbiol* **98**: 1294-1302.
- Goldmann W. (2008).** "PrP genetics in ruminant transmissible spongiform encephalopathies" *Vet. Res.* (2008) **39**:30
- Gootwine E., Abdulkhalig A., Jawasreh K.I.Z., A. Valle Zárate (2008)** Screening for polymorphism at the prion protein (PrP) locus (*PRNP*) in Awassi and Assaf populations in Israel, the Palestinian Authority and Jordan
- Small Ruminant Research* **2008**; **77**: 80-83
- Granato A., Chiara Dalvit,, Mauro Caldon, Rosa Colamonico, Antonio Barberio, Franco Mutinelli (2013)** PRNP gene polymorphism in native Italian sheep breeds undergoing in situ conservation
- Small Ruminant Research* **113** (2013) 323-328
- Grochowska E., Piwczynski D., Portolano B., Mroczkowski S. (2014)** Analysis of the influence of the PrP genotype on the litter size in Polish sheep using classification trees and logistic regression. *Livestock Science* **2014**; **159**:11-17
- Guan F, Pan L, Li J, Tang H, Zhu C, Shi G. (2011)** Polymorphisms of the prion protein gene and their effects on litter size and risk evaluation for scrapie in Chinese Hu sheep. *Virus Genes* **2011**; **43**:147-152.
- Hagenaars TJ, Melchior MB, Bossers A, Davidse A, Engel B and van Zijderveld FG, 2010.** Scrapie prevalence in sheep of susceptible genotype is declining in a population subject to breeding for resistance. *BMC Veterinary Research*, **6**, 25.
- Harrington NP, O'Rourke KI, Feng Y, Rendulich J, Difruscio C, Balachandran A. (2010)** Prion genotypes of scrapie-infected Canadian sheep 1998-2008. *Can J Vet Res* **2010**; **74**:228-232.
- Hautaniemi M, Tapiovaara H, Korpenfelt SL, Sihvonen L. (2012)** Genotyping and surveillance for scrapie in Finnish sheep. *BMC Vet Res* **2012**; **8**:122.
- Houston F, Goldmann W, Foster J, Gonzalez L, Jeffrey M, Hunter N. (2015)** Comparative Susceptibility of Sheep of Different Origins, Breeds and PRNP Genotypes to Challenge with Bovine Spongiform Encephalopathy and Scrapie.
- PLoS One* **10**(11):e0143251. doi: 10.1371/journal.pone.0143251. eCollection 2015
- Hunter, N., Goldmann, W., Benson, G., Foster, J. & Hope, J. (1993)** Swaledale sheep affected by natural scrapie differ significantly in PrP genotype frequencies from healthy sheep and those selected for reduced incidence of scrapie.
- Journal of General Virology* **74**, 1025-1031.
- Hunter, N., Goldmann, W., Smith, G. & Hope, J. (1994).** The association of a codon 136 PrP gene variant with the occurrence of natural scrapie. *Arch Virol* **137**, 171-177.
- Hunter, N., Foster, J. D., Goldmann, W., Stear, M. J., Hope, J. & Bostock, C. (1996).** Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes. *Arch Virol* **141**, 809-824.
- Hunter, N., W. Goldmann, J. D. Foster, D. Cairns and G. Smith (1997a).** "Natural scrapie and PrP genotype: case-control studies in British sheep." *Vet Rec* **141**: 137-140.
- Hunter, N. (1997b).** Molecular biology and genetics of scrapie in sheep. In *The Genetics of Sheep*, pp. 225-240. Edited by L. Piper & A. Ruvinsky. Wallingford, UK: CAB International.
- Ianella P, McManus CM, Cactano AR, Paiva SR. (2012)** PRNP haplotype and genotype frequencies in Brazilian sheep: Issues for conservation and breeding programs. *Res Vet Sci* **2012**; **93**:219-225.
- Ikeda, T., Horiuchi, M., Ishiguro, N., Muramatsu, Y., Kai-Uwe, G. D. & Shinagawa, M. (1995).** Amino acid polymorphisms of PrP with reference to onset of scrapie in Suffolk and Corriedale sheep in Japan. *J Gen Virol* **76**, 2577-2581.
- Kioutsoukios C. (2011)** Genotype Analysis of sheep in the prefectures of Imathia and Thessaloniki for their predisposition to Scrapie disease. Thesis, Postgraduate Diploma in Public Health, National School of Public Health, Supervisor Dr. Papadogiannakis E., Professor Dr. Kontos V.
- Koutsoukou-Hartona H., Palaska V., Tsimpanoudi S., Papakostaki D., Tsimogianni P., Rempoutzakou E., Hondrokouki E., Ekateriniadou L., Ntountounakis S., (2009)** Greek National Scrapie Surveillance programme from 2002 up 2008-A review *NeuroPrion Newsletter* – Prion2009 special edition p.16-18
- Kutzer, T., I. Pfeiffer, and B. Brenig. 2002.** Identification of new allelic variants in the ovine prion protein (PrP) gene. *J. Anim. Breed. Genet.* **119**:201-208.
- Lan Z., Li J., Sun C., Liu Y., Zhao Y., Chi T., Yu X., Song F., Wang Z. (2014)** Allelic variants of PRNP in 16 Chinese local sheep breeds.
- Arch Virol.* **2014** ;**159**(8):2141-2144.
- Laplanche, J. L., Chatelain, J., Westaway, D., Thomas, S., Dussaucy, M., Brugerepicoux, J. & Launay, J. M. (1993).** PrP polymorphisms associated with natural scrapie discovered by denaturing gradient gel electrophoresis.
- Genomics* **15**, 30-37.
- Leontides, S., Psychas, V., Argyroudou, S., Giannati-Stefanou, A., Paschaleri-Papadopoulou, E., Manousis, T. & Sklaviadis, T. (2000).** A survey of more than 11 years of neurologic diseases of ruminants with special reference to transmissible spongiform encephalopathies (TSEs) in Greece. *J Vet Med Ser B* **2000**, **47**, 303-309.
- Lühken G., Buschmann A., Brandt H., Eiden M., Groschup M.H., Erhardt G., (2007)** Epidemiological and genetical differences between classical and atypical scrapie cases, *Vet. Res.* (2007) **38**:65-80.
- Martemucci G., Iamartino D., Blasi M., Angela Gabriella D'Alessandro (2015)** PrP genotype frequencies and risk evaluation for scrapie in dairy sheep breeds from southern Italy
- Preventive Veterinary Medicine* **122** (2015) 318-324
- McGowan J (1922)** Scrapie in sheep. *Scott J Agric* **5**: 365-375.
- Meydan H, Yüceer B, Degirmenci R, Özkan MM, Yildiz MA (2012)** Prion protein gene polymorphism and genetic risk evaluation for scrapie in all Turkish native sheep breeds. *Virus Genes*, **2012**; **45**(1):169-75.
- Meydan H, Özkan MM, Yildiz MA, Goldmann W. (2013)** Novel polymorphisms in ovine prion protein gene. *Anim Genet.* **2013**; **44**(5):588-591.
- Nodelijk G, H. JW van Roermund, L. JM van Keulen, Bas Engel, P. Vellemma, Hagenaars T (2011)** Breeding with resistant rams leads to rapid control of classical scrapie in affected sheep flocks. *Veterinary Research* **2011**; **42**: 5
- Oner Y, Yesilbag K, Tuncel E, Elmaci C. (2011)** Prion protein gene (PrP) polymorphisms in healthy sheep in Turkey. *Animal.* **2011**; **5**(11):1728-33.
- Ortiz-Pelaez and Bianchini (2011)** The impact of the genotype on the prevalence of classical scrapie at population level. *Veterinary Research* **2011**; **42**: 31
- Palhiere I., Brochard M., K. Moazami-Goudarzi, D. Laloe, Y. Amigues, B. Bed'Hom, E. Neuts, C. Leymarie, T. Pantano, E.P. Crihiu, B. Bibé, E. Verrier (2008)** Impact of strong selection for the PrP major gene on genetic variability of four French sheep breeds *Genet. Sel. Evol.* (2008) **40**: 663-680
- Prusiner SB. (1982)** Novel proteinaceous infectious particles cause scrapie. *Science.* **1982**; **216**:136-144.
- Prusiner, S. B. (1991)** Molecular biology of prion diseases. *Science* **252**, 1515-1522
- Psifidi A, Basdagianni Z, Dovas CI, Arsenos G., Sinapis E., Papanastassopoulou M., Banos G.(2011)** Characterization of the PRNP gene locus in Chios dairy sheep and its association with milk production and reproduction traits. *Anim Genet* **2011**; **42**:406-414.
- Smits MA, Bossers A, Schreuder BE (1997)** Prion protein and scrapie susceptibility
- Vet Q.* (1997); **19**(3):101-105.
- Spiropoulos J, Lockey R, Sallis RE, Terry LA, Thorne L, Holder TM, Beck KE, Simmons MM.** Isolation of prion with BSE properties from farmed goat. *Emerg Infect Dis* **2011**, **17**, 2253-2261.
- Tsunoda K, Namikawa T, Sato K, Hasnath MA, Nyunt MM, Rajbansary HB, Loc CB, Zanchiv Ts, Chang H, Sun W, Dorji T. (2010)** Prion protein polymorphisms and estimation of risk of scrapie in East Asian sheep. *Biochem Genet.* **2010** ;**48**(1-2):13-25.
- Zhao C-L, Wu R, Liu L, F-D. Li, X.-L.Zhang, C. Wang, F. Wang, X.-L. Diao , H.-W. Guan, X. Wang and L. Zhou (2012)** Ovine prion protein genotype frequencies in northwestern China. *Genet Mol Res* **2012**; **11**:1671- 1681.

■ Genomic identification of Toxic shock syndrome producing and methicillin resistant *Staphylococcus aureus* strains in human and sheep isolates

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ABSTRACT. Disease-associated *Staphylococcus aureus* strains often promote infections by producing potent protein toxins such as toxic shock syndrome toxin (TSST). The *mecA* gene allows a bacterium to be resistant to antibiotics such as methicillin, penicillin and other penicillin-like antibiotics. The aim of this study was to determine the prevalence of *Staphylococcus aureus* strains producing these two genes. In this study, within 110 cases isolated in Chaharmahal and Bakhtiari province, *Staphylococcus aureus* was isolated by microbiological methods. Then PCR was done for 66 samples to identify the *mecA* and TSST-1 genes. The results showed within 30 samples of human skin infection 18 cases (60%) were MRSA and 5 samples (16.66%) were positive for TSST-1 gene. Within 36 samples of ewe subacute mastitis 10 samples (27.77%) and 5 (13.88%) had *mecA* and TSST-1 genes respectively. Therefore the prevalence of methicillin resistance and toxic shock syndrome producing *Staphylococcus aureus* isolates was significant in Chaharmahal and Bakhtiari. Due to the presence of these isolates in Iran and their threatening role in public health, more attention for their monitoring and treatment is essential.

Keywords: *Staphylococcus aureus*, Toxic shock syndrome, methicillin resistance, human, sheep.

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INTRODUCTION

Staphylococcus aureus is the most common species of *Staphylococcus* to cause *Staph* infections. *S. aureus* can cause some illnesses, from minor skin infections, such as pimples (Tuncer et al., 2009), impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis. *S. aureus* can successfully cause such different illnesses due to a combination of nasal carriage and bacterial immunoevasive strategies (Kluytmans et al., 1997; Cole et al., 2001). It can infect skin, soft tissue, respiratory, bone, joint, endovascular or wound. It is one of the most common causes of hospital-acquired infections and is one of the cause of postsurgical wound infections (AL-Ruaily et al., 2011)

Strains of *Staphylococcus aureus* can produce some extracellular protein toxins and enzymes, including enterotoxins, toxic shock syndrome toxin 1 (TSST-1), exfoliative toxin (ET), hemolysins, and coagulase (Iandolo, 1989).

Toxic shock syndrome toxin (TSST) is a super antigen with a size of 22KDa produced by 5 to 25% of *Staphylococcus aureus* isolates. It causes toxic shock syndrome (TSS) by stimulating the release of interleukin-1, interleukin-2 and tumor necrosis factor. Mainly, the toxin is not produced by bacteria growing in the blood; rather, it is produced at the local site of an infection, and then enters the blood stream. *S. aureus* isolates producing TSST-1 cause the toxic shock syndrome of humans and animals (Schlievert, 1993).

The increase and emergence of *S. aureus* strains resistant to the antibiotic methicillin (MRSA strains), particularly in nosocomial settings has been reported (Haley et al., 1982). The intrinsic resistance to these antibiotics is attributed to the presence of *mecA*, that encodes for a protein with the size of 78-kDa called penicillin binding protein 2a. The *mecA* gene is a gene found in bacterial cells. *mecA* allows a bacterium to be resistant to antibiotics such as methicillin, penicillin and other penicillin-like antibiotics (Hartman and Tomasz, 1984; Utsui and Yokota, 1985).

The methods most commonly are used for the detection of staphylococcal toxins include immunodiffusion, agglutination, radioimmunoassay, and enzyme-

linked immunosorbent assay (Johnson et al., 1991). Among the techniques used to identify toxin genotypes, DNA-DNA hybridization and PCR have been established to be very successful and reliable (Johnson et al., 1991). There are several reports describing the use of multiplex PCR for detection of *Staphylococcus aureus* strains (Zambardi et al., 1994; Vannuffel et al., 1995; Salisbury et al., 1997; Schmitz et al., 1997). In this report, we detected the presence of two staphylococcal genes using 66 isolates of *S. aureus* which were first characterized with microbiological tests by using individual primers. We conclude that the prevalence of *S. aureus* strains with methicillin resistance and toxic shock syndrome genes in this area was significant but further studies are needed to determine the exact prevalence of *S. aureus* strains that are positive in phenotype and genotype for these toxins.

MATERIALS AND METHODS

Sampling

110 cases from two different groups (55 samples of human skin infections and 55 ewe subacute mastitis cases) were collected in Chaharmahal and Bakhtiari province.

Screening for subclinical cases was performed immediately before the collection of milk samples for the microbiological diagnosis of mastitis by the California Mastitis Test (CMT) according to the technique of Schalm and Noorlander (1957). Samples were also collected for somatic cell count (SCC) into flasks containing bronopol for counting in an electronic Somacount 300 (Bentley Instruments®). Mammary glands with a positive reaction in the CMT or SCC > 3.0 x 10⁵ cells/mL milk (McDougall et al., 2001) and that were bacteriologically positive were classified as subclinical mastitis.

The samples were transported to laboratory of microbiology and were stored at -20 until testing.

A complete history of recurrences due to failure of previous treatments, severity of skin lesions and the number of involved quarters was obtained and recorded.

Microbiological methods

Microbiological tests which include Re-cultivation, bio-chemical tests and coagulase were performed. The human skin lesions and sheep milk and skin lesions from each case were streaked for isolation

onto mannitol salt agar plates (PML Microbiologicals, Mississauga, Ontario, Canada). The plates were incubated at 36°C for 48 h in 5% CO₂. After incubation, the plates were examined for the characteristic morphology of *S. aureus*, and suspicious colonies were subcultured onto tryptic soy agar plates with 5% sheep blood (PML Microbiologicals) and incubated for 24 h. Gram stain, catalase, and coagulase tests were performed to confirm the identification of *S. aureus*.

DNA isolation

Total DNA was isolated from 5 ml of brain heart infusion broth culture grown overnight for all the bacterial strains used in the study. The DNA isolation method was a modification of the protocol by Doyle and Doyle (1990) (Chapaval et al., 2008). A total of 2.5ml from a 5ml overnight culture in BHI were centrifuged at 33000 x g for 30 sec. The supernatant was discarded and the pellet was re-suspended in 700 µl extraction buffer (1.4M NaCl; 100mM Tris-HCl [pH 8.0]; 200mM EDTA [pH 8.0], 40%PVP (polyvinylpyrrolidone); 2% CTAB (cetyltrimethylammonium bromide), 20mg/ml Proteinase K; 0.2% β-Mercaptoethanol). The tube was incubated at 65°C for 30min with occasional mixing at every 10min. Then, 650µl chloroform-isoamyl alcohol (24:1) was added and the solution was centrifuged at 33,000 x g for 7min. The upper aqueous phase was transferred to a 1.5-ml tube and 200 µl extraction buffer without proteinase K was added. The solution was gently mixed and 650µl chloroform-isoamyl alcohol (24:1) was added. The tube was centrifuged at 33,000 x g for 7min after which the upper aqueous phase was transferred for a fresh tube. Chloroform-isoamyl alcohol (24:1) extractions were performed twice using 650µl of the chemicals. The DNA was precipitated by adding an equal volume of isopropanol at room temperature. The solution was mixed and centrifuged at 33,000 x g for 7min. The isopropanol was removed and the pellet was washed twice with 70µl 70% ethanol. The DNA pellet was air-dried and re-suspended in 40µl TE buffer (10mM Tris-HCl [pH 8.0]; 1mM EDTA [pH 8.0] + 10µg/ml RNase) and incubated at 37 °C for 30 min. D concentration was determined as micrograms per milliliter according to A260 values. Template DNA in amounts ranging from 10 to 1,000 ng was used in the study.

PCR

PCR test was carried out using the primers designed for TSST-1 and *mecA* genes. The sequences of primers were as follows: TSST-1 F: ACCCCTGTTCCCTTATCATC-3', TSST-1 R: 5'TTTTCAGTATTTGTAACGCC -3' and *mecA* F: ACTGCTATCCACCCTCAAAC -3', *mecA* R: 5'-CTGGTGAAGTTGTAATCTGG -3'. The PCR thermal cycle programs were consisted of denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 45 s, 45 °C (TSST-1) or 47 °C (*mecA*) for 45 s and 72 °C for 45 s, followed by a final extension at 72 °C for 5 minutes. The negative and positive controls (ATCC: 25923) were used in each test. The PCR products were visualized after electrophoresis in 1.3% agarose by staining with ethidium bromide and compared to DNA markers (100 base pair ladder, Fermentas). Then the PCR products were sequenced in an automated fluorescent dideoxy sequencing system (Bioneer, Korea).

Sequencing

Two PCR positive samples in a volume of 50 ml were sent to Bioneer Company for sequencing.

RESULTS

In this study, 110 cases from two different groups (55 samples of human skin infections and 55 ewe

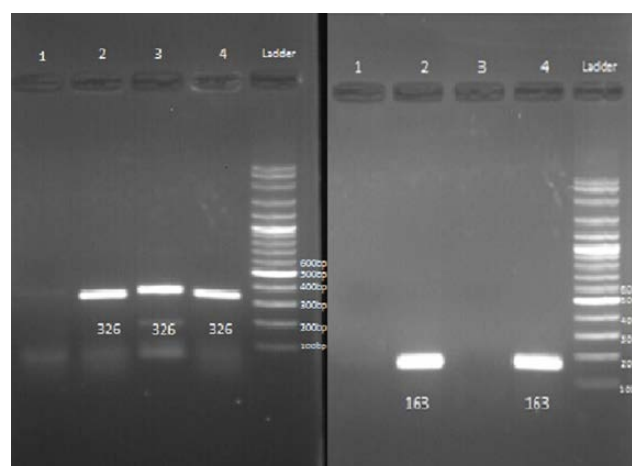


Figure 1: Amplification TSST-1 gene (Right), Amplification of *mecA* gene (Left)

Right to left: Gene ruler (100bp), 4: positive control for TSST-1, 2&3: TSST-1 band (326bp), 1: negative control for TSST-1, Gene ruler (100bp), 4: positive control for *mecA*, 2: *mecA* band (163bp), 1: negative control for *mecA*.

acute mastitis cases) were isolated in Chaharmahal and Bakhtiari province. *Staphylococcus aureus* was isolated from the samples by microbiological methods. Then PCR was done for 66 samples to identify the existence of TSST-1 and *mecA* genes. PCR reaction was performed to amplify a 326 bp fragment of TSST-1 gene and a 163 bp fragment of the *mecA* gene (Figure 1).

The results showed that within 30 cases of human skin lesions 5 cases had TSST-1 gene (16.66%) and 18 cases had *mecA* gene (60%). Within 36 samples of ewe subacute mastitis 5 samples had TSST-1 gene (13.88%) and 10 samples had *mecA* gene (27.77%). After sequencing, the BLAST of the read sequences, confirmed the presence of *S.aureus*- TSST-1 and *mecA* gene fragments in the positive samples (Figures 2&3).

The relationship between some variables and having TSST-1 and *mecA* genes was evaluated with statistical tests. Using Chi-Square test, there weren't any statistical relationships between having these two genes and recurrences due to failure of previous treatments, severity of skin lesions and the number of involved quarters ($P>0.05$). The results of gene expression within groups studied are presented in the tables.1-3.

DISCUSSION

The control of antibiotic resistant and toxigenic *Staphylococcus* infections is very difficult. The phenotypic and genotypic classification of these *Staphylococcus aureus* strains will be very beneficial in the diagnosis and better control of them. For any health care system in any society is very necessary and is essential that important nosocomial pathogens correctly be identified and the exact pattern of antibiotic resistance being determined. With such efforts effective prevention and treatment strategies against these pathogens can be used. Regional studies aimed at obtaining information about the strains of *Staphylococcus*, as well as their antibiotic resistance could help physicians to select suitable treatment guidelines.

Staphylococcus aureus is an opportunistic pathogen that under favorable conditions causes infection in humans and animals (Balaban and Rasooly, 2000). Different strains of this organism may produce toxins and virulence factors (Ertas et al., 2001).

We used a PCR-based diagnostic protocol to detect TSST-1 and the *mecA* genes in DNA extracted from human and sheep isolates of *S. aureus*. Individual primers were used to identify these *Staphylococcus* genes. The PCR primers were shown to be very specific, reliable, and very efficient for detection of all these two genes. There have been few well-documented cases of TSS in Iran (Varmazyar-najafi et al., 2016). It is unclear whether the small number of reported TSS cases is due to the failure to recognize the disease, underreporting of the disease, or it is because of microbiologic or immunologic reasons.

In recent decades antibiotic resistance is considered as a major public health problem and because of improper and abundant use of antibiotics, the prevalence of antibiotic-resistant strains is increasing rapidly (Najera-sanchez et al., 2003; Orwin et al., 2003; Hososaka et al., 2007). Because animals are one of the human's food sources, their infection with dangerous organisms may cause disease in human through consumption of contaminated food or contact with infected animals. The uncontrolled use of some medications, especially antibiotics has led to development of antibiotic resistance bacterial strains and if such bacteria spread to human beings, cause serious and dangerous diseases those common antibiotics may fail for their treatment (Khoei et al., 2014).

Nowadays methicillin is one of the most commonly used antibiotics for the treatment of nosocomial infections caused by *Staphylococcus aureus* strains. Unfortunately, the rapid emergence of strains resistant to methicillin causes this antibiotic lost their effectiveness for their treatment. So epidemiological studies to understanding the prevalence of this *Staphylococcus aureus* strains seems necessary (Dinges et al., 2000; Deurenberg et al., 2005; Zamani et al., 2007; Sajith Khan et al., 2012). Methicillin-resistant strains of *Staphylococcus aureus* have *mecA* gene or MIC of 4 µg / ml or more (Hososaka et al., 2007).

Increasing antibiotic resistance is a concern and should always be monitored. Methicillin resistance is independent of beta-lactamase production and is due to the presence of the *mecA* gene with the length of 1.2 kb. The prevalence of this type of resistance varies in different areas and different times (Gilbert and Humphrey, 1998; Enright et al., 2002; Japoni et al., 2004).

Query ID	Id Query_244509	Database Name	nr
Description	tst_PCR_tst_F	Description	Nucleotide collection (nt)
Molecule type	nucleic acid	Program	BLASTN 2.6.1+
Query Length	289		
Staphylococcus aureus gene for toxic shock syndrome toxin, complete cds, strain: TSST	274	274	56%
			9e-70
			97%
			LC075482.1
Staphylococcus aureus strain MRSA NN 353 Toxic Shock Syndrome toxin (TssT) gene, partial cds	237	237	44%
			1e-58
			100%
			KT124627.1
Staphylococcus aureus strain MRSA NN 226 Toxic Shock Syndrome toxin (TssT) gene, partial cds	231	231	43%
			5e-57
			100%
			KT124628.1

Staphylococcus aureus gene for toxic shock syndrome toxin, complete cds, strain: TSST

Sequence ID: **LC075482.1** Length: 1600 Number of Matches: 1

Range 1: 625 to 786

Score	Expect	Identities	Gaps	Strand	Frame
274 bits(148)	9e-70()	159/164(97%)	2/164(1%)	Plus/Plus	
Features:					
Query 126	GATGGCAGCATCAGCCTTATAATTTTCCGAGTCCTTATTATAGCCCTGCTTTTACaaaa	185			
Sbjct 625	GAT-GCAGCAT-AGCTTGATAATTTTCCGAGTCATTATTATAGCCCTGCTTTTACAAAA	682			
Query 186	ggggaaaaagtgtgacttaaacacaaaaagaactaaaaaaGCCAACATACTAGCGAAGGA	245			
Sbjct 683	GGGGAAGAGTTGACTTAAACACAAAAAGAACTAAAAAAGCCAACATACTAGCGAAGGA	742			
Query 246	ACTTATATCCATTTCCAAATAAGTGGCGTTACAAATACTGAAAA	289			
Sbjct 743	ACTTATATCCATTTCCAAATAAGTGGCGTTACAAATACTGAAAA	786			

Staphylococcus aureus strain MRSA NN 353 Toxic Shock Syndrome toxin (TssT) gene, partial cds

Sequence ID: **KT124627.1** Length: 316 Number of Matches: 1

Range 1: 1 to 128

Score	Expect	Identities	Gaps	Strand	Frame
237 bits(128)	1e-58()	128/128(100%)	0/128(0%)	Plus/Plus	
Features:					
Query 162	TATTATAGCCCTGCTTTTACaaaaggggaaaaagtgtgacttaaacacaaaaagaactaaa	221			
Sbjct 1	TATTATAGCCCTGCTTTTACAAAAGGGGAAAAAGTTGACTTAAACACAAAAAGAACTAAA	60			
Query 222	aaaaGCCAACATACTAGCGAAGGAACCTTATATCCATTTCCAAATAAGTGGCGTTACAAAT	281			
Sbjct 61	AAAAGCCAACATACTAGCGAAGGAACCTTATATCCATTTCCAAATAAGTGGCGTTACAAAT	120			
Query 282	ACTGAAAA	289			
Sbjct 121	ACTGAAAA	128			

Staphylococcus aureus strain MRSA NN 226 Toxic Shock Syndrome toxin (TssT) gene, partial cds

Sequence ID: **KT124628.1** Length: 314 Number of Matches: 1

Range 1: 1 to 125

Score	Expect	Identities	Gaps	Strand	Frame
231 bits(125)	5e-57()	125/125(100%)	0/125(0%)	Plus/Plus	
Features:					
Query 165	TATAGCCCTGCTTTTACaaaaggggaaaaagtgtgacttaaacacaaaaagaactaaaaaa	224			
Sbjct 1	TATAGCCCTGCTTTTACAAAAGGGGAAAAAGTTGACTTAAACACAAAAAGAACTAAAAA	60			
Query 225	agCCAACATACTAGCGAAGGAACCTTATATCCATTTCCAAATAAGTGGCGTTACAAATACT	284			
Sbjct 61	AGCCAACATACTAGCGAAGGAACCTTATATCCATTTCCAAATAAGTGGCGTTACAAATACT	120			
Query 285	GAAAA	289			
Sbjct 121	GAAAA	125			

Figure 2: Sequencing of a *S. aureus*-tst-1 PCR product: There is 97- 100% identity between PCR product sequence and *S.aureus* tst-1 sequences published in Genbank.

BLAST Results

Job title: mecA_PCR_mecA_F

RID [B936YGM901R](#) (Expires on 03-01 03:32 am)

Query ID lcl|Query_142319
Description mecA_PCR_mecA_F
Molecule type nucleic acid
Query Length 145

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.6.1+

Staphylococcus aureus strain MRSA19 penicillin binding protein 2a-like (mecA) gene, partial sequence	235	235	87%	2e-58	100%	GQ146438.1
Staphylococcus aureus strain MRSA17 penicillin binding protein 2a-like (mecA) gene, partial sequence	235	235	87%	2e-58	100%	GQ146439.1

Staphylococcus aureus strain MRSA19 penicillin binding protein 2a-like (mecA) gene, partial sequence
 Sequence ID: **GQ146438.1** Length: 138 Number of Matches: 1

See 1 more title(s)
 Range 1: 12 to 138

Score	Expect	Identities	Gaps	Strand	Frame
235 bits(127)	2e-58()	127/127(100%)	0/127(0%)	Plus/Plus	
Features:					
Query 9	CCTTGTAGCACACCTTCATATGACGTCTATCCATTTATGTATGGCATGAGTAACGAAGAA	68			
Sbjct 12	CCTTGTAGCACACCTTCATATGACGTCTATCCATTTATGTATGGCATGAGTAACGAAGAA	71			
Query 69	TATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCAACAAGTTCAGATTACAAC	128			
Sbjct 72	TATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCAACAAGTTCAGATTACAAC	131			
Query 129	TCACCAG	135			
Sbjct 132	TCACCAG	138			

Staphylococcus aureus strain MRSA17 penicillin binding protein 2a-like (mecA) gene, partial sequence
 Sequence ID: **GQ146439.1** Length: 138 Number of Matches: 1
 Range 1: 12 to 138

Score	Expect	Identities	Gaps	Strand	Frame
235 bits(127)	2e-58()	127/127(100%)	0/127(0%)	Plus/Plus	
Features:					
Query 9	CCTTGTAGCACACCTTCATATGACGTCTATCCATTTATGTATGGCATGAGTAACGAAGAA	68			
Sbjct 12	CCTTGTAGCACACCTTCATATGACGTCTATCCATTTATGTATGGCATGAGTAACGAAGAA	71			
Query 69	TATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCAACAAGTTCAGATTACAAC	128			
Sbjct 72	TATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCAACAAGTTCAGATTACAAC	131			
Query 129	TCACCAG	135			
Sbjct 132	TCACCAG	138			

Figure 3: Sequencing of a *S. aureus*-mecA PCR product: There is 97-100% identity between PCR product sequence and *S. aureus*-mecA sequences published in Genbank.

Table 1: The TST-1 and MecA positivity situation based on the number of involved quarters

the number of involved quarters	1	2	3	4
TST-1 positive	1	1	1	2
TST-1 negative	8	8	8	7
mecA positive	1	2	2	5
mecA negative	6	6	6	8

Table 2: The TST-1 and MecA positivity situation based on the number of recurrences

the number of recurrences	1	2	3	4
TST-1 positive	0	2	2	1
TST-1 negative	4	14	7	6
mecA positive	1	4	3	2
mecA negative	4	11	5	6

Table 3: The TST-1 and MecA positivity situation based on the severity of skin lesions

Severity of skin lesions	Mild	Moderate	Severe
TST-1 positive	1	2	2
TST-1 negative	9	12	10
mecA positive	3	3	4
mecA negative	6	7	7

In this study the findings about MRSA obtained by PCR. Of 30 isolates of *S. aureus* from human skin infection, 18 samples (60%) and of 36 *S. aureus* isolates from ewes subacute mastitis 10 samples (27.77%) were positive for mecA gene. Totally of 66 isolates of *S. aureus*, 28 samples (41.8%) carried mecA gene. In the study performed by Becker et al. in Germany (2003) of 219 isolates of *Staphylococcus aureus*, 40 samples (18.2%) carried tst gene (Becker et al., 2003). In a similar study in Canada, Mehrotra et al. detected tst gene in 15% of *Staphylococcus aureus* isolates (Mehrotra et al., 1996). Deurenberg et al. in the Netherlands (2005) investigated the presence of tst gene in 51 isolates of methicillin-resistant

Staphylococcus aureus strains. 24% of apparently healthy and 14% of hospitalized people was reported that were positive for this gene (Deurenberg et al., 2005).

Najjar pirayeh et al. in 2010 showed the prevalence of MRSA isolates was 48% (Varmazyar-najafi et al., 2016). As well as in the study which was performed in Shiraz (2005) the prevalence of MRSA was reported to be 38% (Hososaka et al., 2007). These results are consistent with the findings obtained in the present study. In this study, 41.8% of samples was mecA positive, which is in accordance with frequencies reported from Iran (Zeinali et al., 2011), India (Sajith Khan et al., 2012) and Turkey (Tuncer et al., 2009). In the study performed by AL-Ruaily and his colleagues in 2002 only 13% of strains were positive for mecA gene (AL-Ruaily et al., 2011), as well as the research was performed by Goud et al. in 2011 that isolated *S. aureus* from 22.5% of healthy individuals and 16.6% of these isolates were positive for mecA gene (Goud et al., 2011).

Identification of methicillin-resistant *Staphylococcus aureus* is sometimes complex due to heterogeneous expression of resistance gene and the influence of other variables such as PH, temperature and salt concentration (de Carvalho et al., 2009). The ability of *Staphylococcus aureus* strains to cause different diseases depends on producing several different types of extracellular toxins. The majority of *S. aureus* strains isolated from patients with symptoms of toxic shock syndrome produce a toxin called toxic shock syndrome toxin -1 (Mehrotra et al., 1996) which is a super antigen (Orwin et al., 2003).

In many countries, scientists have studied TSS as a health problem. TSS has been documented to occur worldwide. Colonization with *S. aureus* is generally highest (20 to 30%) in the nose or oropharynx while vaginal colonization with *S. aureus* has been determined to be lower (10% to 20%) in the United States, Europe, and Asia. However there is variation in incidence reports of TSST-1 producing strains of *S. aureus* in different countries (Parsonnet et al., 2008).

Risk factors for the staphylococcal type include the use of very absorbent tampons and skin lesions (Low, 2013). In other hand, *S. aureus* which caused

mastitis can potentially produce staphylococcal toxic shock syndrome toxin-1 (Dastmalchi Saei et al., 2013). Therefore in this study the sampling was performed using skin lesions absorbent tampons were used for them and mastitis cases.

Given the importance of the detection of *tst* gene, Johnson et al. (1991) showed that the use of PCR to identify *tst* genes is better than other methods, such as immunological assays. It is more sensitive, faster and less expensive (Mehrotra et al., 1996). Therefore, this method was used to identify *S. aureus* genes in this study. In the present study, of 36 *S. aureus* isolates from samples of human skin infection 5 cases (13.5%) and of 30 *S. aureus* isolates from samples of ewe subacute mastitis 5 cases (16.66%) were positive for *tst* gene. Totally, of 66 *S. aureus* isolates, 10 samples (15%) had *tst* gene.

In the study performed by Parsonnet et al. (2008), 159 *S. aureus* isolates were studied. 14 strains (9%) were *tst* positive and of 12 toxigenic strains, 2 strains were methicillin-resistant (Parsonnet et al., 2008). In this study of 10 *tst* positive isolates, 3 strains (30%) were methicillin-resistant. Of 28 MRSA isolates, 3 strains (10.7 %) had *tst* gene. Hoseini Alfatemani et al. (2014) performed a study in Shiraz to identify the profile of some virulence genes including: *sea*, *seb*, *sed*, *tst*, *eta*, *etb*, *LuKS/F-PV*, *hla* and *hld* in methicillin-resistant *S. aureus* strains by PCR technique. The frequency of the *tst* gene was 10.95% (Hoseini Alfatemani et al., 2014).

Nemati et al. (2015) in Ilam analyzed *S. aureus* isolates that were collected from different resources. Samples were screened for the *mecA*, *tst*-1, *eta* and *etb* genes by PCR. 50 isolates were selected from human *Staphylococcus* isolates and 100 from animal *Staphylococcus* isolates. Ten out of the 50 human *S. aureus* isolates and 5 out of 50 *S. aureus* isolates from cow milk were just positive for *mecA*. None of the poultry *S. aureus* isolates were positive for *mecA*. All of the isolates were negative for the *eta*, *etb* and *tst*-1 (Nemati et al., 2015).

Arfatahery et al. (2016) performed a research with the aim of characterization of toxin genes and antimicrobial susceptibility of *Staphylococcus aureus* isolates in fishery products in Iran. The results indicated that 34% of fish and shrimp samples were contaminated with *S. aureus*, and 23.8% of these

isolates were *mecA* positive. 3.9% were positive for *tst*-1 gene (Arfatahery et al., 2016). Varmazyar najafi et al. (2016) detected methicillin- resistance gene in *Staphylococcus aureus* isolated from traditional white cheese in Iran. 19 samples (31.67%) were contaminated with *S. aureus*. Three out of 19 *S. aureus* isolates (15.7%) were phenotypically resistant to methicillin (disk diffusion), while 4 (21.05%) of them were genotypically confirmed as MRSA strains (Varmazyar najafi et al., 2016). Khoei et al. (2015) studied antibiotic resistance pattern and frequency of *mecA* gene in *Staphylococcus aureus* isolated from Tabriz. 44.5% isolates were confirmed as MRSA by cefoxitin disc screening test and 53.3% isolates by showing the presence of *mecA* gene (Khoei et al., 2014).

The difference between cities in Iran and between different countries is most likely a reflection of differences in lifestyle and health practices. People in Chaharmahal and Bakhtiari province who described themselves as living in traditional conditions were more likely to have positive results than those who had assumed a better and more hygienic lifestyle. These results, taken together, suggest that the development of TSST-1 producing *S. aureus* strains in Chaharmahal and Bakhtiari province is large and probably a function of environmental and genetic factors.

Staphylococcus aureus infections are among the most important hospital- acquired infections. Enterotoxins and toxic shock syndrome toxin -1 secreted by *S. aureus* are some of important virulence factors and PTSAgs which have profound effects on their hosts.

S. aureus is a versatile microorganism that causes infection in different hosts. Moreover, this bacterium is one of the most important pathogens in the etiology of infectious mastitis in cows, goats, and sheep, causing chronic infection of the mammary tissue that is difficult to treat (Ai-res-de-Souza et al., 2007).

The infections caused by *Staphylococcus aureus* strains that are resistant to antibiotics (mainly hospital-acquired infections) and TSST-1-producing strains are growing in many countries. The results of this study showed there was a high prevalence of *tst* and *mecA* genes in the studied samples which may indicate that these strains are circulating in the

community. These strains may be as reservoirs for transmission of antibiotic resistance genes to the other strains and this could endanger public health. We characterized the *Staphylococcus* isolates for the production of TSST-1 and methicillin resistance.

The virulence profile of *S. aureus* strains, suggest that virulence factors spread among flocks and successfully establish an infection in the mammary gland of sheep, causing mastitis. The *tst* gene is located on a pathogenicity island, and can be transferred from one bacterium to another. The presence of this gene in one strain suggests that it probably acquired this virulence gene from other staphylococci, rendering it more virulent (McDougall et al., 2001; Schalm et al., 1957).

Staphylococcus aureus is a commensal and pathogen of several mammalian species, particularly humans and cattle. Animal lineages are closely related to human lineages and only a handful of genes or gene combinations may be responsible for host specificity. That's the point that people who work in veterinary farms or laboratories' or every work that deals with the animal and people that use unhealthy milk and milk products are at more risk (Sung et al., 2008).

The use of PCR assay help to provide valuable information required for appropriate treatment and control during outbreaks of *S. aureus* infections and diseases. It is important to recognize that this technique only will identify strains harboring the toxin genes and is independent of the expression and secretion of the toxin. To verify toxin production by any given isolate, time- and labor-intensive immunolog-

ical methods may be used to detect the excreted toxins. Considering the low cost and much shorter time required to detect the genes of *S. aureus* by PCR, we believe this to be a powerful tool for studying the genotypes of staphylococcus isolates. This procedure was specially developed to fit into the daily work pattern of a routine clinical laboratory, since genotypic detection of drug resistance and the presence of toxin genes is becoming an important component of the diagnostic inventory of such laboratories.

CONCLUSION

The results of this study showed that more likely abundant use of antibiotics causes a high prevalence of MRSA strains in Chaharmahal and Bakhtiari province. Furthermore, these results, suggest that the development of TSST-1 producing *S. aureus* strains in Chaharmahal and Bakhtiari province is large and probably a function of environmental and genetic factors. Therefore presentation of programs and rules for the controlled use of antibiotics and the application of new methods in identifying new strains of *S. aureus* seems necessary.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

- Aires-de-Sousa M, Parente CESR, Vieira-da-Mota OV et al. (2007) Characterization of *Staphylococcus aureus* isolates from buffalo, bovine, ovine, and caprine milk samples collected in Rio de Janeiro State, Brazil. *Appl Environ Microbiol* 73:3845-3849.
- AL-Ruaily MA, Khalil OM (2011) Detection of *mecA* gene in methicillin-resistant *Staphylococcus aureus* (MRSA) at prince A/Rhman, Sidery Hospital, AL-Jouf, Saudi Arabia. *J Med Genet Genomics* 3(3):41-45.
- Arfatahery N, Davoodabadi A, Abedimohtasab T (2016) Characterization of toxin genes and antimicrobial susceptibility of *Staphylococcus aureus* isolates in fishery products in Iran. *Sci Rep* 6: 34216.
- Balaban N, Rasooly A (2000) *Staphylococcal enterotoxin*. *Int J Food Microbiol* 61:1-10.
- Becker K, Friedrich AW, Lubritz G, Weilert M, Peters G, Von Eiff C (2003) Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. *J Clin Microbiol* 4:139-143.
- Chapaval L, Moon DH, Gomes JE, Duarte FR, Tsai SM (2008) An alternative method for *Staphylococcus aureus* DNA isolation. *Arq Bras Med Vet Zootec* 60(2):299-306.
- Cole AM, Tahk S, Oren A, Yoshioka D, Kim YH, Park A, Ganz T (2001) Determinants of *Staphylococcus aureus* nasal carriage. *Clin Diagn Lab Immunol* 8(6):1064-1069.
- de Carvalho MJ, Pimenta FC, Hayashida M, Gir E, da Silva AM,

- Barbosa CP, Canini SR, Santiago S (2009) Prevalence of methicillin-resistant and methicillin-susceptible *S. aureus* in the saliva of health professionals. *Clinics (Sao Paulo)* 64(4):295-302.
- Dastmalchi Saei h, Ahmadi M, Farahmand-Azar S, Anassori E (2013) Identification of Toxic Shock Syndrome Toxin-1 (TSST-1) gene in *Staphylococcus aureus* isolated from bovine mastitis milk. *Arch Razi Inst* 68(1):17-22.
- Dinges MM, Paul MO, Patrick M (2000) Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev* 13(1):16-34.
- Deurenberg RH, Nieuwenhuis RF, Driessen C, London N, Stassen FR, van Tiel FH, Stobberingh EE, Vink C (2005) The prevalence of the *Staphylococcus aureus* *tst* gene among community- and hospital-acquired strains and isolates from Wegener's Granulomatosis patients. *FEMS Microbiol Lett* 245:185-189.
- Enright MC, Robinson DA, Randle G, Feil EJ, Grundmann H, Spratt BG (2002) The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc Natl Acad Sci* 99:7687-7692.
- Ertas N, Gonulalan Z, Yildirim Y, Kum E (2001) Detection of *Staphylococcus aureus* Enterotoxins in sheep cheese and Dairy Desserts by Multiplex PCR Technique. *Int J Food Microbiol* 142(1-2):74-70.
- Gilbert RJ, Humphrey TJ (1998) Food-borne bacterial gastroenteritis. In: Topley and Wilson's microbiology and microbial infections. 3rd ed, A Hodder Arnold Publication, London: pp 577-600.
- Goud R, Gupta S, Neogi U, Agarwal D, Naidu K, Chalannavar R, Subhaschandra G (2011) Community prevalence of methicillin and vancomycin resistant *Staphylococcus aureus* in and around Bangalore, southern India. *Rev Soc Bras Med Trop* 44:309-312.
- Haley RW, Hightower AW, Khabbaz RF, Thorsberry C, Martone WJ, Allen JR, Hughes JM (1982) The emergence of methicillin-resistant *Staphylococcus aureus* infections in United States hospitals. *Ann Intern Med* 97:297-308.
- Hartman BJ, Tomasz A (1984) Low-affinity binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. *J Bacteriol* 158:513-516.
- Hoseini Alfatehi SM, Motamedifar M, Hadi N, Sedigh Ebrahim Saraie H (2014) Analysis of Virulence Genes Among Methicillin Resistant *Staphylococcus aureus* (MRSA) Strains. *Jundishapur J Microbiol* 7(6): e10741.
- Hosokawa Y, Hanaki H, Endo H, Suzuki Y, Nagasawa Z (2007) Characterization of Oxacillin-susceptible *mecA*-positive *Staphylococcus aureus*: A new type of MRSA. *J Infect Chemother* 13:79-86.
- Iandolo JJ (1989) Genetic analysis of extracellular toxins of *Staphylococcus aureus*. *Annu Rev Microbiol* 43:375-402.
- Japoni A, Alborzi A, Orafi F, Rasouli M, Farshad S (2004) Distribution Patterns of Methicillin Resistance Gene (*mecA*) in *Staphylococcus aureus* isolated from clinical Specimens. *Iran Biomed J* 8(4):173-178.
- Johnson WM, Tyler SD, Ewan EP, Ashton FE, Pollard DR, Rozee KR (1991) Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. *J Clin Microbiol* 29:426-430.
- Khoei F, Mobaiyen H, Nahaei MR, Sadeghi Mohammadi S (2014) Antibiotic resistance pattern and frequency of *mecA* gene in *Staphylococcus aureus* isolated from Shohada hospital, Tabriz. *J Med Microbiol Infect Dis* 2(3):105-108.
- Kluytmans J, van Belkum A, Verbrugh H, Van Belkum V (1997) Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* 10(3):505-520.
- Low DE (2013) Toxic shock syndrome: major advances in pathogenesis, but not treatment. *Crit Care Clin* 29 (3): 651-75.
- McDougall S, Murdough P, Pankey W et al. (2001) Relationship among somatic cell count, California mastitis test, impedance and bacteriological status of milk in goats and sheep in early lactation. *Small Ruminant Res* 40: 245-254.
- Mehrotra M, Wang G, Johnson WM (1996) Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxin, exfoliative toxin, toxic shock syndrome: Role of the environment, the host and the microorganism. *J Biomed Sci* 53(4):284-289.
- Nemati M, Shamsi M (2015) Detection of *mecA*, *eta*, *etb*, and *tst-1* genes from staphylococcal isolates. *J Bas Res Med Sci* 2(2):13-17.
- Najera-sanchez G, Maldonado-Rodriguez R, Ruiz Olvera P, de la Garza LM (2003) Development of two multiplex polymerase chain reactions for the detection of enterotoxigenic strains of *Staphylococcus aureus* isolated from foods. *J Food Prot* 66:1055-1062.
- Orwin PM, Fitzgerald JR, Leung DYM, Gutierrez JA, Bohach GA, Schlievert PM (2003) Characterization of *Staphylococcus aureus* enterotoxin L. *Infect Immun* 71:2916-2919.
- Parsonnet J, Goering RV, Hansmann MA, Jones MB, Ohtagaki K, Davis CC, Totsuka K (2008) Prevalence of Toxic Shock Syndrome Toxin 1 (TSST-1)-Producing Strains of *Staphylococcus aureus* and Antibody to TSST-1 among Healthy Japanese Women. *J Clin Microbiol* 46(8):2731-2738.
- Pesavento G, Ducci B, Comodo N, Lonostro A (2007) Antimicrobial resistance profile of *Staphylococcus aureus* isolated from raw milk: A research for methicillin resistant *Staphylococcus aureus* (MRSA). *Food control* 18:196-200.
- Sajith Khan AK, Shetty PJ, Sarayu Y L, Chidambaram A, Ranganaathan R (2012) Detection of *mecA* genes of Methicillin-Resistant *Staphylococcus aureus* by Polymerase Chain Reaction. *Int J Health Rehabil Sci* 1(2):64-68.
- Salisbury SM, Sabatini LM, Spiegel CA (1997) Identification of methicillin-resistant staphylococci by multiplex polymerase chain reaction assay. *Am J Clin Pathol* 107:368-373.
- Schalm OW, Noorlander DO (1957) Experiments and observations leading to development of the California Mastitis Test. *J Am Vet Med Assoc* 30:199-207.
- Schmitz FJ, Mackenzie CR, Hofmann B, Verhoef J, Finken-Eigen M, Heinz HP, Kohrer K (1997) Specific information concerning taxonomy, pathogenicity, and methicillin resistance of staphylococci obtained by a multiplex PCR. *J Med Microbiol* 46:773-778.
- Schlievert PM (1993) Role of superantigens in human diseases. *J Infect Dis* 167:997-1002.
- Sung JM, Lloyd DH, Lindsay JA (2008) *Staphylococcus aureus* host specificity: comparative genomics of human versus animal isolates by multi-strain microarray. *Microbiology* 154(7):1949-59.
- Tuncer I, Kalem F, Cosar M, Arsalan U (2009) Antibiotic susceptibility of *Staphylococcus aureus* strains isolated from blood stream infections. *Turk Mikrobiyol Cemiy Derg* 39(1-2):20-22.
- Utsui Y, Yokota T (1985) Role of an altered penicillin-binding protein in methicillin- and cephem-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 28:397-403.
- Vannuffel P, Gigi J, Ezzedine H, Vandercam B, Delmee M, Wauters G, Gala J (1995) Specific detection of methicillin-resistant *Staphylococcus* species by multiplex PCR. *J Clin Microbiol* 33:2864-2867.
- Varmazyar-najafi M, Pajohi -alamoti MR, Mohammadzadeh A, Mahmoodi P (2016) Detection of methicillin-resistance gene in *Staphylococcus aureus* isolated from traditional white cheese in Iran. *Arch hyg Sci* 5(4): 302-309.
- Zamani A, Sadeghian S, Ghaderkhani J, Alikhani MY, Najafimosleh M, Taghi Goodarzi M, Shahrabi Farahani H, Yousefi-Mashouf R (2007) Detection of methicillin-resistance (*mecA*) gene in *Staphylococcus aureus* strains by PCR and determination of antibiotic susceptibility. *Annu Rev Microbiol* 57(2):273-276.
- Zambardi G, Reverdy ME, Bland S, Bes M, Freney J, Fleurette J (1994) Laboratory diagnosis of oxacillin resistance in *Staphylococcus aureus* by a multiplex-polymerase chain reaction assay. *Diagn Microbiol Infect Dis* 19:25-31.
- Zeinali E, Moniri R, Safari M, Mousavi GA (2011) Molecular characterization and SCC *mec* typing in methicillin-resistant *Staphylococcus aureus* isolated from clinical samples. *Feyz J KAMUS* 14(4): 439-44.

Impact of different light colors in behavior, welfare parameters and growth performance of Fayoumi broiler chickens strain

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ABSTRACT. Light is considered one of the most managerial factors affecting poultry well-being. Therefore, the current study was conducted to investigate the effects of different light colors in behaviour, welfare and growth performance of Fayoumi chickens. A total of 300 one-day old chicks of Fayoumi broiler breed were weighed and randomly divided into 4 environmentally controlled chambers with different artificial light color (yellow, red, green and blue) until the end of the experimental period (12 weeks); each was divided into five replicate brooders (15 birds for each replicate). A scanning technique was used to report the chicken's behaviors. Moreover, the plumage condition, foot and toe hyperkeratosis, foot and toe lesions (foot pad dermatitis) and growth performance were evaluated. Light colors had significant ($P < 0.05$) effects of the impose of different light colors in all kinds of behaviour of Fayoumi chickens. It was found that eating frequency was the highest in blue light. Preening, dust bathing and drinking frequencies were the highest in green light. Birds reared in red light were more active, as expressed by greater walking, flying, head movement, litter scratching, body shaking, wing flapping, wing/leg stretching, feather pecking and aggression. While, birds in blue light were calmest, evidenced by more intense sleeping, sitting and idling behaviors. In spite of the fact that the light colors had no significant effect on plumage condition, health status of the foot and toe and growth performance, those parameters were better in birds kept in blue light than other light colors. We conclude that the blue light colour may improve the birds' welfare.

Key words: Fayoumi chickens, light color, welfare, performance.

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INTRODUCTION

Increasing competition and energy cost in the poultry industry are the mainsprings for chicken producers to minimize the cost of growth. From an economic standpoint, management strategies should focus on what is optimal for chicken welfare and growth performance simultaneously. Recently, producers and consumers are more concerned about the product quality and in which condition poultry is reared (Harper and Henson, 2001).

A high genetic diversity among commercial lines may be considered a key for selecting well adapted lines to tough environments, but these lines if exposed to novel environments, their welfare status may be affected (EFSA, 2010). Recent genomic studies confirmed that Fayoumi chickens were adapted and raised in hot and very dry areas of tropical and sub-tropical conditions (Hossary and Galal, 1994). It needs high capacity to tolerate harsh environmental Egyptian conditions with limited nutritional requirements when compared with breeds in the rest of the world (Ghamry et al., 2011).

Light is necessary for bird's vision influencing the visual acuity and color discrimination (Calvet et al., 2009). Moreover, the lighting system in chicken house must be designed and maintained in order to give a minimum illumination of 20 lux, that enables thorough inspection and vision without difficulty (RSPCA, 2013).

Domestic fowl differs from human in spectral sensitivity which illustrates the importance to identify the optimal light environment for health, behavior, welfare and production of broiler chickens. They are sensitive to ultraviolet, blue, green and red on the light spectrum (Prescott and Wathes, 1999) through retinal and deep brain photoreceptors (Kuenzel et al., 2015). Moreover, they also have 3 advanced light receptors within the brain that play a foremost role in the biological and physiological functions (Bertolucci and Foa, 2004; Wyse and Hazlerigg, 2009).

To increase their income, producers are capable of handling and modulating management factors such as temperature, humidity, ventilation, gases and light intensity, duration and color. From the aforementioned factors, light is the most critical one as it controls many physiological and behavioral functions (Olanrewaju et al., 2006). Light consists of 3 different

aspects; intensity, photoperiod (duration) and spectral content color (Olanrewaju et al., 2006). The light color is estimated by various outputs from different wave lengths in the visible spectrum (Anja, 2015). The efficacy of lighting is to achieve a maximum production performance of broilers with simultaneous conservation of the welfare (Škrbić et al., 2012).

The source of light color can affects poultry performance (Jin et al., 2011). The green light activated growth rate, while the reddish orange light stimulated reproduction (Rozenboim et al., 2004). The green light can increase the growth of young birds, while the blue light stimulates the older ones (Classen et al., 2004). Egypt is a member of the OIE (World Organisation for Animal Health), therefore it should align its national legislation with the OIE recommendations on the welfare of broiler chickens (FAO, 2014), but the majority of areas in Egypt still use outdated lighting technology (El-Sheikh, 2016). The objectives of this experiment was to assess the impact of different light colors in behavior, growth performance and welfare parameters of Fayoumi chickens under subtropical condition.

MATERIAL AND METHODS

The present study was carried out at Faculty of Veterinary Medicine, Zagazig University, Egypt. The experimental procedures were conducted in accordance with the Zagazig University Animal Ethics Committee guidelines (Licence No. ANWD-206).

Experimental animals and management: A total 300 one-day old chicks of Fayoumi breed purchased from a commercial hatchery were weighed upon arrival and randomly distributed into 4 environmentally controlled chambers with different artificial light color (yellow, red, green and blue) with 24 hrs of photoperiod program until the end of the experimental period (12 weeks); each was divided into five replicate brooders (15 birds for each replicate). A scanning technique was used to report the chicken's behaviors. Moreover, the plumage condition, foot and toe hyperkeratosis, foot and toe lesions (foot pad dermatitis) and growth performance were evaluated. Light intensity for all treatments was kept at the same lux level (25 lux), measured with lux meter along a horizontal plane 20 cm above the litter. Chickens were vaccinated against the most common chicken

diseases of the area, namely Infectious bronchitis (at 5 days of life), Newcastle disease (at 5 and 10 days of life), Gumboro disease (at 7 and 14 days of life) and fowl pox (at 30 days of life). The pen floors were covered with approximately 10-cm of wood shavings. Food and water were available *ad libitum* throughout the study. The basal diet was calculated to meet the nutrient requirements of broilers (AOAC, 2002) which fed two types of rations; The first ration during the starter period (0-4wks; crude protein "CP" 23.20% and metabolized energy "ME" 3218 kcal/kg diet) and the second ration during the growing-finishing period (4-12wks; CP 19.55% and ME 3218 kcal/kg diet).

Behavioral observation: Direct observations of chicken's behaviors were carried out using a scanning technique (Fraser and Broom, 1990). Behavioral observations were performed as follows: each group was observed three times a day (20 min/each time) for four days weekly at a circularly fixed time from 6 A.M to 5 P.M in order to record the different behavioral patterns. The behavioral patterns in all experimental groups were recorded by the same person through standing directly in front of each group and waiting for ten minutes before recording data to avoid any disturbance in behaviors (Mohammed et al., 2010). After scanning, the numbers of Fayoumi chickens were counted in the observed chambers to calculate the frequencies of various behaviours/one hour. These numbers were used to record the activities of chicken according to Senaratna et al., (2011) in all experimental groups.

Growth performance: Growth performance was recorded according to Taha and Abd El-Ghany, (2013), where broilers were weighed on 1st day of age as one day-old live weight (Mohammed et al., 2017) and then live body weights were subsequently estimated weekly until 12th week of age. Daily body weight gain (DBWG) was estimated by the difference between the recorded initial and final body weight (BW) divided by the number of days in the whole period of experiment. Also, the average feed intake (FI) was measured daily after calculating the feed residues. Furthermore, relative growth rate (RGR) and feed conversion ratio (FCR): (feed intake/weight gain) over a period of experiment were estimated.

Plumage scores: It was measured for all birds

from each group at the end of experimental period, where the plumage condition of the head, neck, breast, belly, back, wings, vent and tail was assessed by using 4- point scale from 1 to 4 for each part, where 4 was the best condition and 1 was the worst condition (Bright et al., 2006; Welfare Quality 2009).

Foot condition:-

At the end of experimental period, both feet of all chicken were examined, where the areas of possible foot pad dermatitis and hock burns should be distinguished and assigned using Tauson scale (Tauson et al., 1984) i.e. the scoring system assigned a value of 1 to 4 for each part, where 4 was the best condition and 1 was the worst condition.

Statistical analysis:- All statistical procedures were performed using the SAS statistical system Package V9.2 (SAS, 2002). Data were analyzed using one-way ANOVA with the general linear models

(GLM) procedure. The comparison of means was carried out using Duncan's multiple range tests.

RESULTS

There were significant ($P < 0.05$) effects of light color in all observed behaviour of Fayoumi chicken (Table 1). Eating and drinking frequencies were highest in chambers lit with blue and green light, respectively. Birds reared in chambers lit with red light were more active, as expressed by more intense walking, flying, head movement, litter scratching, body shaking, wing flapping, wing/leg stretching, feather pecking and aggression. In the meantime, birds kept in blue color chambers were calmer and this was evident by observing greater periods of sleeping, sitting and idling. An increase in preening and dust bathing was recorded in the green color chamber. In spite of the fact that there were no significant differences observed in plumage scores (Table 2), foot condition (Table 3) and growth performance (Table 4) among the experimental groups, all plumage scores, the health status of the foot and toe (hyperkeratosis and lesion appearance) and the final body weight, growth rate and daily weight gain of birds kept in chambers lit with blue light were relatively better than others light color, but the differences did not reach the significance (Table 2, 3 and 4). Feed intake and FCR were highest in chambers lit with red color.

Table 1. Frequencies of behaviour/hour in Fayoumi chicken under different light colors (mean±SE).

Behavior	Yellow	Red	Green	Blue
Eating	78.67±2.70 ^d	97.89±7.11 ^c	116.67±1.83 ^b	156.89±3.13 ^a
Drinking	64.89±3.97 ^b	40.89±1.37 ^c	78.89±1.49 ^a	67.33±3.25 ^b
Walking	217.33±1.83 ^b	279.67±1.94 ^a	220.00±6.65 ^b	222.22±4.55 ^b
Flying	17.33±1.13 ^b	28.00±0.94 ^a	9.77±0.52 ^c	11.56±0.69 ^c
Head movement	15.33±0.65 ^{ab}	17.00±0.62 ^a	14.00±0.53 ^b	10.11±0.63 ^c
Litter scratching	80.67±0.97 ^c	109.33±0.88 ^b	71.22±1.38 ^d	128.00±1.41 ^a
Sleeping	120.89±3.19 ^b	92.22±1.48 ^c	132.89±3.18 ^a	129.11±1.85 ^a
Siting	297.33±3.37 ^b	279.56±4.38 ^c	307.56±3.46 ^b	363.78±3.42 ^a
Idling	28.44±1.13 ^c	32.89±0.95 ^b	33.33±1.80 ^b	39.11±0.96 ^a
Preening	156.44±1.69 ^d	199.33±2.24 ^b	205.78±1.59 ^a	170.44±2.33 ^c
Dust bathing	2.22±0.36 ^d	9.78±0.55 ^c	16.89±0.54 ^a	15.11±0.56 ^b
Body shaking	15.22±0.72 ^c	24.67±0.78 ^a	23.11±0.77 ^a	18.67±0.97 ^b
Wing flapping	20.89±3.45 ^b	42.67±9.30 ^a	28.89±4.98 ^{ab}	36.44±0.80 ^{ab}
Wing/leg stretching	14.11±0.81 ^c	23.12±1.11 ^b	27.09±0.86 ^a	27.89±0.68 ^a
Feather pecking	9.22±0.46 ^b	14.22±0.64 ^a	8.11±0.51 ^b	9.33±0.53 ^b
Aggression	11.00±0.58 ^b	17.11±0.63 ^a	8.33±0.53 ^c	12.11±0.75 ^b

^{abcd} Means in the same row with different superscripts were significantly different at ($P \leq 0.05$). All observations were recorded with frequencies abases per one hour (scan sampling technique) in all birds of each group.

Table 2. Effect of different light colors in the plumage condition in all body parts of Fayoumi chickens.

Plumage condition	Yellow	Red	Green	Blue
Head	3.10±0.31	3.00±0.26	3.10±0.23	3.40±0.22
Nick	2.80±0.33	2.70±0.26	2.80±0.25	3.20±0.29
Back	2.70±0.30	3.10±0.22	2.70±0.21	3.20±0.24
Wing	2.80±0.36	2.30±0.34	2.70±0.37	2.80±0.32
Tail	2.70±0.36	2.40±0.34	2.60±0.37	2.80±0.33
Breast	2.50±0.37	2.40±0.40	2.50±0.31	2.50±0.30
Belly	2.30±0.36	2.50±0.34	2.60±0.40	2.70±0.37
Cloaca	2.50±0.31	2.30±0.30	2.70±0.33	2.50±0.34

The plumage condition in all body parts were recorded in all experimental birds, where 4 was the best condition and 1 was the worst condition.

Table 3. Effect of different light colors in the feet of Fayoumi chickens.

Feet condition	Yellow	Red	Green	Blue
Foot hyperkeratosis	2.30±0.33	2.70±0.34	2.70±0.21	3.30±0.30
Foot lesions	2.60±0.37	2.60±0.37	2.80±0.25	3.10±0.35
Toe hyperkeratosis	2.10±0.31	2.50±0.40	2.70±0.39	2.90±0.34
Toe lesions	2.40±0.33	2.40±0.34	2.90±0.35	3.10±0.35

Both feet of all chickens were examined with discriminating any problems like hyperkeratosis and lesions. The scoring system assigned a value of 1 to 4 for each part, where 4 was the best condition and 1 was the worst condition.

Table 4. Effect of different light colors in (means \pm SE) the growth performance of Fayoumi chickens.

Performance parameters	Yellow	Red	Green	Blue
Initial body weight (g)	34.15 \pm 0.41	34.35 \pm 0.66	35.09 \pm 0.80	34.48 \pm 0.45
Final body weight (g)	1048.6 \pm 8.52	1039.8 \pm 12.99	1053.1 \pm 8.69	1066.8 \pm 7.51
Feed intake (g/daily)	59.27 \pm 0.43	58.93 \pm 0.49	58.77 \pm 0.85	58.27 \pm 0.60
Growth rate	187.43 \pm 0.16	187.28 \pm 0.37	187.17 \pm 0.18	187.54 \pm 0.21
Daily weight gain (g/daily)	11.94 \pm 0.04	11.84 \pm 0.04	11.98 \pm 0.01	12.15 \pm 0.05
Feed conversion ratio	4.96 \pm 0.02	4.98 \pm 0.01	4.91 \pm 0.03	4.81 \pm 0.04

g= gram

DISCUSSION

Poultry vision is more affected by light through influencing color discrimination in the eye retina, which can differentiate colors with different sensitivity levels (Lewis and Morris, 2000), therefore we aimed to evaluate the effects of light colors on the behavior, growth performance, plumage and foot conditions in Fayoumi chickens that is considered one of the most famous Egyptian breeds. Broilers should be reared at light levels that let them see clearly and stimulate their activity (RSPCA, 2013).

Recently, Sejian et al., (2011) defined animal welfare as “the ability of an animal to cope physiologically, behaviorally, cognitively and emotionally with its physiochemical and social life environment, including the animal’s subjective experience of its condition”. Behavioural studies are of great importance for improving the understanding and appreciation of animals. Our results indicated that all studied behaviors were significantly affected by light colors (Senaratna et al., 2012).

In this study, high eating and drinking behavior frequencies were recorded in the blue and red light color chambers, respectively which supported by the findings of Prayitno et al., 1997^b who reported that bright red light considerably increased feeding and drinking behaviors, particularly when applied early in the growth period. In contrast, Senaratna et al., (2011) stated that light colors had no effect on eating behavior. Kinetic behaviors (walking, flying and head movement) were significantly affected by light colors, where chicken was more active under red light color than other colors (Senaratna et al., 2012; Son

and Velmurugu 2009). These results may be due to the different wavelengths of light, where the blue and green light color had a shorter wavelength (480 nm, 520 nm, respectively) than red (700 nm) and yellow colors (580 nm) (Shabiha et al., 2013).

Litter scratching was highest in blue light color chambers in the current study, which was supported by the results obtained by Senaratna et al., (2011) who stated that the litter scratching was usually associated with eating behavior (Senaratna et al., 2011) unlike chicken reared under blue light color were more comfortable and calmer than others due to the significant increase in sleeping, sitting and idling behaviors. This study supported the theory that blue color created a calming effect on birds, where the frequencies of physical activities was lower in chambers lit with blue light. Moreover, in the present experiment, sleeping, sitting and idling activities were much higher under blue light color, that may be attributed to the fact that blue light color alleviated the stress response in broilers through the reduction in the level of serum interleukin-1, as described by Xie et al., (2008).

Comfort behavior (feather preening and dust bathing) was significantly higher in green light color. But, body shaking was observed in significantly higher frequencies in red and green light colors. Chicken in chambers lit with red color were higher in wing flapping that may be due to more activity than those raised under other colors (Rusty, 2011). In the current study wing/leg stretching was highest in chambers lit with blue and green color, which may be due to the fact that chicken were more calm than others

light colours. Abnormal behavior (aggression and feather pecking) were significantly higher in chickens reared under red light color than other colors due to the increase of activities of birds under red light color. This result may be due to light stimulation and may have an impact on the coping ability to the stressors (Campo et al., 2007). Moreover, the light color have an effect on brain organization that impact the behavioral responses, including fear behaviour (Dharmaretnam and Rogers, 2005). These results and interpretation were suggested by previous studies (Prayitno et al., 1997^{a,b}; Danang, 2014), while another study stated that feather pecking and aggressive behaviors were higher with blue light, but the observed differences did not reach significance (Mohammed et al., 2010).

Light may be the most critical for chickens as it controls many behavioral patterns (Olanrewaju et al., 2006). Recently poultry producers and consumers are concerned with raising poultry in improved and comfortable conditions (Harper and Henson, 2001). Consumers equate good animal welfare standards with healthy feather and feet. Therefore, selecting optimal lighting colors allow birds to live a normal life. Previous studies mentioned that lighting treatments had insignificant effects on each of plumage and foot conditions (Farghly and Mahrose, 2012; Senaratna et al., 2011) which was comparable with our results.

The results on plumage condition was in disagreement with the observations of Van emous et al. (2003), who stated that different light sources might affect plumage condition as reflected by the incidence of feather pecking. Chicken reared under blue light color possessed the higher final body weight

with higher daily weight gain while those reared under red light color were the lowest. The lighting is very important factor in regulating behavioral processes in chickens and different wavelengths of light have found to affect growth rate in broiler chickens (Bradley, 2015). This was also reported by Olanrewaju et al., (2011), who found that light was one of the major environmental factors for poultry production that influences growth development and physiological functioning.

The light wavelength affected broiler performance, including of those reared in open houses with natural (solar) lighting, which are frequent in tropical climates (Guevara et al., 2015).

CONCLUSION

In conclusion, Fayoumi chickens raised under different light colors had significant differences in all behaviors, but with no significant differences observed in plumage scores, foot condition or growth performance among different light colors. This study helps to support the theory that blue light creates a calming effect on birds.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. ■

REFERENCES

- Anja BR (2015) Effects of color of light on preferences, performance, and welfare in broilers. *Poult Sci* 94: 1767-1775.
- AOAC (2002) Association official analytical chemists. Official Methods of Analysis. Gaithersburg, MD, U.S.A. Chapt. 4, pp 20-27.
- Bertolucci C, Foa A (2004) Extraocular photoreception and circadian entrainment in nonmammalian vertebrates. *Chronobiol Int* 21: 501-519.
- Bright A, Jones TA, Dawkins MS (2006) A non-intrusive method of assessing plumage condition in commercial flocks of laying hens. *Animal Welfare* 15, 113-118.
- Bradley TH (2015) The effects of light color on performance and immune response of broiler chickens. Undergraduate research thesis, Department of Animal Sciences, The Ohio State University, USA.
- Calvet S, Van den Weghe H, Kosch R, Estellés F (2009) The influence of the lighting program on broiler activity and dust production. *Poult Sci* 88: 2504-2511.
- Campo JL, Gil MG, Davila SG, Munoz I (2007) Effect of lighting stress on fluctuating asymmetry, heterophil-to-lymphocyte ratio, and tonic immobility duration in eleven breeds of chickens. *Poult Sci* 43:355-363.
- Classen HL, Annet CB, Schwan-lardner KV, Gonda R, Derow D (2004) The effects of lighting programmes with twelve hours of darkness per day provided in one, six or twelve hour intervals on the productivity and health of broiler chickens. *British Poult Sci* 45:S31-S32.
- Danang B (2014) Effects the combinations of light color and intensity of light to age at first laying and production egg of Alabio laying ducks. *IJB* 5:80-85.
- Dharmaretnam M, Rogers LJ (2005) Hemispheric specialization and dual processing in strongly versus weakly lateralized chicks. *Behav Brain Res* 162:62-70.
- EFSA (European Food Safety Authority) (2010) Scientific Opinion on the influence of genetic parameters on the welfare and the resistance to stress of commercial broilers. EFSA Panel on Animal Health and Welfare. *EFSA Journal* 2010 8 (7):1666.
- El-Sheikh (2016) Using led light in poultry production. 36th Annual meeting of Egyptian Poultry Science Association (EPSA), Faculty of Agriculture, Alexandria University, Egypt
- Farghly MF, Mahrose KM (2012) Effects of light during storage and incubation periods on pre and post hatch performance of Japanese quail. *Egyptian Poult Sci* 32: 947-958.
- FAO (2014) Review of animal welfare legislation in the beef, pork, and poultry industries, page 21.
- Fraser AF, Broom DM (1990) Farm animal behavior and welfare. Baillière Tindall Publ., London, UK.
- Ghamry AE, Allaway E, Hewida M, Yassein SA, Mallah GM (2011) Evaluation of Dietary calcium Requirements in Fayoumi laying hens. *Iranian Appl Anim Sci* 1:81-86.
- Guevara BD, Pech PS, Zamora BR, Navarrete SL, Magana SH (2015) Performance of broilers reared under monochromatic light emitting diode supplemental lighting. *Brazilian Poult Sci* 17:553-558.
- Harper G, Henson S (2001) Consumer concerns about animal welfare and the impact on food choice. Department of Agricultural and Food Economics. The University of Reading, United Kingdom.
- Hossary MA, Galal ES (1994) Improvement and Adaptation of the Fayoumi chickens. *Anim Genet Resour Inform* 14:33-39.
- Jin E, Jia I, Li J, Yang G, Wang Z, Cao J, Chen Y (2011) Effect of monochromatic light on melatonin secretion and arylalkylamine N-acetyltransferase mRNA expression in the retina and pineal gland of broilers. *The anatomical record* 294:1233-1241.
- Kuenzel WJ, Kang SW, Zhou ZJ (2015) Exploring avian deep-brain photoreceptors and their role in activating the neuroendocrine regulation of gonadal development. *Poult Sci* 94:786-798.
- Lewis PD, Morris TR (2000) Poultry colored effects of color and intensity of light on behavioral and light. *World Poult Sci* 56:189-207.
- Mohammed HH, Enas NS, Shereen EA (2017) Impact of different litter materials on behaviour, growth performance, feet health and plumage score of Japanese quail (*Coturnix japonica*). *Europ Poult Sci* 81.
- Mohammed HH, Grashorn MA, Bessei W (2010) The effects of lighting conditions on the behavior of laying hens. *Arch Geflügelk* 74:197-202.
- Olanrewaju HA, Thaxton JP, Dozier WA, Purswell J, Roush WB, Branton SL (2006) A Review of Lighting Programs for Broiler Production. *International Poult Sci* 5:301-308.
- Olanrewaju HA, Purswell JL, Collier SD, Branton SL (2011) Effect of varying light intensity on growth performance and carcass characteristics of broiler chickens grown to heavy weights. *Int. J. Poult. Sci* 10:921-926.
- Prayitno DS, Phillips CJ, Omed H (1997^a) The effects of color of lighting on the behavior and production of meat chickens. *Poult Sci* 76:452-457.
- Prayitno DS, Phillips CJ, Stokes DK (1997^b) The effects of color and intensity of light on behavior and leg disorders in broiler chickens. *Poult Sci* 76:1674-1681
- Prescott NB, Wathes CM (1999) Spectral sensitivity of the domestic fowl (*Gallus g. Domesticus*). *British Poult Sci* 40:332-339.
- Rozenboim I, Biran I, Chaiseha Y, Yahav S, Rosenstrauch A, Sklan D, Halevy O (2004) The effect of a green and blue monochromatic light combination on broiler growth and development. *Poult Sci* 83:842-845.
- RSPCA (2013) RSPCA welfare standards for chickens, page 10, 11.
- Rusty DR (2011) Broiler preference for light color and feed form, and the effect of light on growth and performance of broiler chicks. Master thesis in Dept. of Animal Sciences and Industry College of Agriculture, Kansas state university
- SAS (2009) SAS statistical system Package-Jmp 8 User's Guide. 2nd Cary, NC, SAS Institute Inc. USA. ISBN 978-1-60764-301-2.
- Seijan V, Lakritz J, Ezeji T, Lal R (2011) Assessment methods and indicators of animal welfare. *Journal of Animal and Veterinary Advances* 6 (4): 301-315.
- Senaratna D, Samarakone TS, Madusanka AP, Gunawardane WD (2011) Performance, behavior and welfare aspects of broilers as affected by different colors of artificial light. *Tropical Agricultural Res Extens* 14:38-44.
- Senaratna D, Samarakone TS, Madusanka AP, Gunawardane WD (2012) Preference of broiler chicken for different light colors in relation to age, session of the day and behavior. *Tropical Agricultural Res* 23:193-203
- Shabiha S, Hassan R, Choe HS, Ryu KS (2013) Impact of different monochromatic LED light color and bird age on the behavioral output and fear response in ducks. *Italian Journal Animal Sci* 12:573-579.
- Škrbić Z, Pavlovski Z, Lukić M, Petričević V, Milić D (2012) The effect of lighting program and type of litter on production and carcass performance of two broiler genotypes. *Biotechnology in Animal Husband* 28:807-816.
- Son JH, Velmurugu R (2009) The effects of light colors on the

- behavior and performance of broiler chickens. *Korean Journal of Poultry Science* 36 (4):329-335
- Taha AE, Abdel-Ghany FA (2013) Improving production traits for el-salam and mandarah chicken strains by crossing. *Alexandria Vet Sci* 39:18-30.
- Tauson R, Ambrosen T, Elwinger K (1984) Evaluation of procedures for scoring the integument of laying hens— independent scoring of plumage condition. *Acta Agriculturae Scandinavica* 34:400-408.
- Van Emous RM, Fiks-van niekerk TG, Reuvekamp BF (2003) Verrijkte kooien. *Praktijk Rapport Pluimvee* 9. Postbus 2176, 8203 AD, Lelystad, The Netherlands.
- Welfare Quality (2009) Welfare Quality®assessment protocol for poultry (broilers, laying hens). In: Welfare Quality®Consortium, Lelystad, Netherlands. pp. 1-14
- Wyse C, Hazlerigg D (2009) Seasonal biology: Avian photoreception goes deep. *Curr Biol* 19:R685-R687.
- Xie D, Wang ZX, Dong YL, Cao J, Wang JF, Chen JL, Chen YX (2008) Effects of monochromatic light on immune response of broilers. *Poult Sci* 87:1535-1539.

Evaluation of Intraocular Pressure (IOP) Regarding Circadian Rhythm, Age, Sex and Eye Side in Awassi Sheep

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SUMMARY. Measurement of intraocular pressure (IOP) in domestic animals has become a part of routine eye examination with advent of applanation tonometer. Delayed control of high IOP may lead to permanent blindness due to retinal ganglion cells dysfunction and optic nerve degeneration. This study aimed at evaluating IOP of Awassi sheep with respect to circadian rhythm, age, sex and eye sides and finally to establish a reference (baseline, normal) IOP value for this particular species. A total of 24 healthy sheep with different ages and sexes were used. The animals were divided into 2 equal groups, <1 (6 male, 6 female, n = 12) and ≥1 (6 male, 6 female, n = 12) years old. IOP measurements were performed twice, in the morning (6:00 a.m.) and in the evening (8:00 p.m.) with Tono-pen Vet[®] applanation tonometer.

Mean IOP in the animals decreased from 16.21 mmHg in the morning to 12.65 mmHg in the evening with an approximately rate of 22% ($P < 0.0001$). Comparison of mean IOP values of right eyes (n=12) to the left (n=12), male (n=48) to female (n=48), and ages < 1 (n=48) to ≥ 1 (n=48) showed no difference ($P > 0.05$). The reference IOP for this animal was calculated as 14.43 ± 2.72 mmHg notwithstanding any variable.

It was concluded that in this breed IOP values can vary significantly as far as circadian rhythm is concerned and Tono-pen Vet[®] can be used for sheep IOP measurement as an alternative to other applanation tonometry.

Keywords: Age, Awassi Sheep, Circadian Rhythm, IOP, Tono-pen Vet[®]

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INTRODUCTION

Intraocular pressure (IOP) represents the balance between aqueous humor production and drainage (Park et al., 2011; Pigatto et al., 2011). Routine IOP measurement is very important for the early diagnosis and effective treatment of glaucoma and other ocular diseases associated with ocular hypertension, such as uveitis, local or generalized corneal edema, orbital trauma and lens luxation (Andrade et al., 2012; Park et al., 2011; Rusanen et al., 2010). Delayed control of high IOP may lead to permanent blindness due to retinal ganglion cells dysfunction and optic nerve degeneration (Andrade et al., 2012).

IOP values may vary according to animal breed (Barsotti et al., 2013; Ghaffari et al., 2012; Pereira et al., 2011; Pigatto et al., 2011), age (Pereira et al., 2011; Verboven et al., 2014) and sex (Ofri et al., 1998), the measurement techniques applied (Pereira et al., 2011; Pigatto et al., 2011), the examiner's practice (Pereira et al., 2011; Pigatto et al., 2011), circadian rhythms (Giannetto et al., 2009; Pereira et al., 2011; Pigatto et al., 2011), stress (Pigatto et al., 2011) and anesthetic applications (Pigatto et al., 2011).

IOP measurement is performed via two basic techniques, manometry and tonometry (Andrade et al., 2012). Manometry is an invasive technique that requires anterior camera cannulation/ paracentesis (Von Spiessen et al., 2015) and general anesthesia and thus it is not practical for clinical use (Park et al., 2011; Von Spiessen et al., 2015). Tonometry, a noninvasive and indirect measurement of IOP, works on indentation, applanation or rebound principle and today it is a method of choice for clinical practice (Jeong et al., 2007; Park et al., 2011; Von Spiessen et al., 2015).

In recent years, several noninvasive applanation tonometers such as Tonopen-XL[®], Tonopen Avia[®] and Tono-pen Vet[®] have managed to find a place in veterinary clinical practice due to be portable, easy and practical to use and less influenced by the sizes and postures of animals (Andrade et al., 2012).

Ophthalmic diseases in farm animals are important because they may cause significant economic losses. Similarly, ocular studies on these animals have a research value in comparative ophthalmology (Ribeiro et al., 2014). Among farm animals the sheep has become a popular animal model in a range of diseases including steroid-induced hypertension; therefore, it is

important to know the mean intraocular pressure (Gerometta et al., 2010; Pigatto et al., 2011).

Awassi breed, a fat-tailed and combined trait sheep, is well adapted to tropical environments and widespread through the Mediterranean region and the Arab peninsula (Al-Atiyat and Aljumaah, 2014). This breed is found mainly in Eastern Mediterranean and South-east Anatolia of Turkey and constitutes 4-5% of its total sheep population (Internet).

According to our literature research no study has so far tried to measure IOP in Awassi sheep. The aim of this study was to measure this parameter with applanation tonometry, to evaluate that with respect to circadian rhythm, age, sex and eye side and finally to establish a reference (baseline, normal) IOP value for this particular species.

MATERIALS AND METHODS

The study was carried out at Agriculture and Livestock Research Farm of Firat University after official approval from the university ethic committee. Following thorough ophthalmologic examination including direct and indirect ophthalmoscopy and slit-lamp biomicroscopy (XL-1[®], Shin-Nippon, Japan), and assessments of the pupillary light reflex, Schirmer tear test (Tear Flo[®], Rose Stone Enterprises, India) and fluorescein staining, a total of 24 healthy Awassi sheep aging from 6 months to 4 years were selected as the study material.

The animals were divided into 2 equal groups, <1 (6 male, 6 female, n = 12) and ≥1 (6 male, 6 female, n = 12) years old. IOP measurements were performed twice, in the morning (6:00 a.m.) and in the evening (8:00 p.m.) with an applanation tonometer (Tono-pen Vet[®], Reichert, U.S.A.).

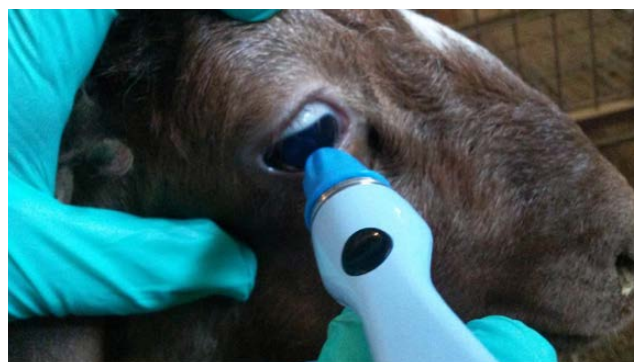


Figure 1. IOP measurement with Tono-Pen Vet[®].

Table 1. Distribution of IOP data of Awassi sheep according to the various variables: measurement time points, age, sex and eye sides.

Measurement Time Points (Circadian Rhythm)	Age	Sex	Eye Sides	IOP (mmHg)
Morning (08.00 a.m.)	<1 years	Male	Right	17.17 ± 1.94
			Left	16.33 ± 2.58
			Mean	16.75 ± 2.22
		Female	Right	16.67 ± 1.75
			Left	16.33 ± 3.61
			Mean	16.50 ± 2.71
	MALE AND FEMALE MEAN			16.63 ± 2.42
	≥1 years	Male	Right	14.50 ± 1.37
			Left	15.50 ± 1.51
			Mean	15.00 ± 1.47
		Female	Right	16.33 ± 1.86
			Left	16.83 ± 1.16
			Mean	16.58 ± 1.50
MALE AND FEMALE MEAN			15.79 ± 1.66	
TOTAL MEAN			16.21 ± 2.10	
Evening (08.00 p.m.)	<1 years	Male	Right	11.83 ± 0.75
			Left	11.67 ± 0.51
			Mean	11.75 ± 0.62
		Female	Right	11.83 ± 0.75
			Left	11.17 ± 1.16
			Mean	11.50 ± 1.00
	MALE AND FEMALE MEAN			11.63 ± 0.82
	≥1 years	Male	Right	12.83 ± 1.72
			Left	13.33 ± 2.33
			Mean	13.08 ± 1.97
		Female	Right	14.17 ± 3.06
			Left	14.33 ± 2.33
			Mean	14.25 ± 2.59
MALE AND FEMALE MEAN			13.67 ± 2.33	
TOTAL MEAN			12.65 ± 2.01	
ALL TOTAL MEAN			14.43 ± 2.72	
SEM			0.73	
ANOVA*			---	
			Time	0.0001
			Age	0.135
			Sex	0.164
			Eye	0.959
			Time x Age	0.001
			Age x Sex	0.046

*There is no significant interactions between others at $P > 0.05$

All ocular examination and measurements were carried out by the same investigator (KK). Great attention was paid to avoid the animals from unnecessary stress, abnormal pressure on the head and neck, disproportional physical constraints and abnormal body posture during the measurement. Prior to the measure-

ment the tonometer was calibrated and the data were recorded using the ear tag numbers.

Thirty seconds after instilling topical anesthesia the eyelids were gently opened while the animals held in sitting position head right in midline (Figure 1). Three consecutive IOP measurements were obtained from

each side of the eyes and their average was recorded. The latex Tono-pen Vet® tip coating was changed from animal to animal and the measurements were repeated if the average rate of device measurement error was higher than 5%. All the measurements performed in the morning, were repeated in the evening.

Data are given as mean \pm standard error of the mean (SEM). The data were analyzed by general linear models (GLM) procedure of SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Sample size was calculated based on a power of 85% and a P value of 0.05.

RESULTS

In the morning measurements, mean IOP data in male and female animals <1 years old were found to be 16.75 ± 2.22 and 16.50 ± 2.71 mmHg ($P > 0.05$), those of the right and left eyes to be 16.92 ± 1.78 and 16.33 ± 2.99 mmHg ($P > 0.05$), respectively. In animals ≥ 1 years old IOP data were 15.00 ± 1.47 in males and 16.58 ± 1.50 mmHg in females. Mean right and left eye data were 15.42 ± 1.83 and 16.17 ± 1.46 , respectively ($P > 0.05$). At this point, cumulative means of IOP data were 16.63 ± 2.42 mmHg in animals <1 years old and 13.67 ± 2.33 in animals ≥ 1 years old and that was 16.21 ± 2.10 mmHg regardless of age variability (Table 1).

For evening measurements, in male and female animals <1 year old mean IOP data were recorded as 11.75 ± 0.62 and 11.50 ± 1.00 mmHg and mean IOP data of their right and left eyes as 11.83 ± 0.71 and 11.42 ± 0.90 mmHg ($P > 0.05$), respectively. These data were determined to be 13.08 ± 1.97 in males and 14.25 ± 2.59 mmHg in females; 13.50 ± 2.46 in the right eyes and 13.83 ± 2.29 mmHg ($P > 0.05$) in the left eyes of the animals ≥ 1 year old. At this time point regardless of sex and eye variability, overall mean IOP data were 11.63 ± 0.82 mmHg in animals <1 and 11.63 ± 0.82 mmHg in animals ≥ 1 years old and also overall mean of 12.65 ± 2.01 mmHg was recorded when sex, age and age variability were neglected (Table 1).

According to these parameters, mean IOP ratio in morning measurements (16.21 ± 2.10 mmHg) was about 22% higher than that (12.65 ± 2.01 mmHg) of the evening and the difference between these time points was found to be statistically significant ($P < 0.0001$). The reference IOP for this animal was calculated as

14.43 ± 2.72 mmHg notwithstanding any variable. An interaction ($P > 0.05$) was found between IOP data of the variables such as age, sex, eye sides and measurement time point. This was determined to be between age \times measurement time points ($P < 0.001$) and age \times sex ($P < 0.05$) according to the statistical test (Table 1).

DISCUSSION

The aim of this study was to determine reference IOP mean for Awassi sheep, an indigenous breed in Southeast Anatolia and Mediterranean with an applanation tonometer and to reveal an interaction of this value with the variables such as age, sex, circadian rhythm and eye side. In recent years, many studies have been performed on reference IOP values of various animal species in a variety types of applanation tonometers, i.e. of eurasian eagle owls with TonoPen XL® (Jeong et al., 2007), dogs and cats with TonoPen XL® and Perkins® (Andrade et al., 2012) and TonoPen-XL® (Park et al., 2011), rabbits with TonoPen Avia® (Pereira et al., 2011), calves and dairy cows with Mackay-Marg® and TonoPen-XL® (Gum et al., 1998), long-eared hedgehogs with Tono-Pen Vet® (Ghaffari et al., 2012), eurasian tawny and little bred owls, common buzzards, european kestrels with TonoPen-XL® (Barsotti et al., 2013), ferrets (Montiani-Ferreira et al., 2006) and koala (Grundon et al., 2011) with TonoPen-Vet®, Kapacin monkey with TonoPen-XL® (Montiani-Ferreira et al., 2008), horses with Tono-Pen Avia (Marzok et al., 2014). Some studies have measured IOP values in Texel and Santa Ines bred sheep with Tono-Pen XL® (Pigatto et al., 2011; Ribeiro et al., 2014), Sanjabi bred male sheep with Tono-Pen Vet® (Ghaffari et al., 2011) and Corriedal bred sheep by Gerometta *et al.* (2009) utilizing Perkins® tonometer. In the present study, we measured IOP in Awassi bred sheep using Tono-Pen Vet® with a mean value of 14.43 ± 2.72 mmHg (Table 1). This value is higher than that (9.37 ± 2.45 mmHg) of Sanjabi sheep reported by Ghaffari *et al.* (2011), and that (10.6 ± 1.4 mmHg) of Corriedal bred sheep by Gerometta *et al.* (2009) and is near to that (14.56 ± 1.14 mmHg) of Santa Ines bred sheep by Riberio *et al.* (2014), however, it is lower than that (16.36 ± 2.19 mmHg) of Texel bred sheep reported by Pigatto *et al.* (2011). Despite the usage of the same type tonometer, these studies results show great variations, which

indicates that IOP value may vary according to different breeds.

In the present study mean IOP values of measured as 14.42 ± 2.61 mmHg in the right eyes and 14.44 ± 2.85 mmHg in the left eyes with a resultant of no significant difference between them ($P > 0.05$) were similar to those reported previously (Ghaffari et al., 2012; Ghaffari et al., 2011; Gum et al., 1998; İşler et al., 2014; Pigatto et al., 2011). It has been reported that IOP values can be influenced markedly by stress factors including abnormal pressure on the head and neck, disproportional physical constraints and abnormal body posture during the measurement (Broadwater et al., 2007; Komaromy et al., 2006; Rusanen et al., 2010). In this study an ultimate care was paid to these remarks during the measurement. To avoid individual diversity (Gelatt and MacKay, 1998; Pigatto et al., 2011), all measurement was performed by the same investigator.

The effects of sex on IOP is arguable, while many researchers (Ghaffari et al., 2012; Grundon et al., 2011; İşler et al., 2014; Montiani-Ferreira et al., 2006; Montiani-Ferreira et al., 2008; Nuhsbaum et al., 2000; Pereira et al., 2011) deny the presence of such an effect on IOP, Ofri et al. (1998) study on lions and Wu et al. (2006) study on human have reported higher IOP value in males as compared to females. The present study results in males (14.15 ± 2.51 mmHg) and females (14.71 ± 2.91 mmHg) shows no statistical difference ($P > 0.05$) in consistency with the majority opinion. The studies of Pamuk et al. (2011) in Anatolian Buffalo using TonoPen XL® and Gelatt and Mackay (1998) in dogs applying MacKay-Marg® and TonoPen XL® reported that average IOP decreased with age contrary to that of Montiani-Ferreira et al. (2006) in mountain ferrets and Montiani-Ferreira et al. (2008) in capuchin monkeys. Present study measured IOP values of Awassi sheep ≥ 1 (14.73 ± 2.27 mmHg) and < 1 (14.13 ± 3.09 mmHg) year old with a resultant of no significant difference ($P > 0.05$) between these two age groups were similar to the findings of the last two studies.

IOP is not a constant value and may vary according

to different times of a day, termed circadian rhythm (Gelatt et al., 1981; Giannetto et al., 2009; Jaen-Diaz et al., 2007; Pereira et al., 2011). In veterinary ophthalmology there is a limited number of studies investigating the relation of IOP with circadian rhythm. Studies on rabbit IOP utilizing rebound tonometer (Tonovet®) and applanation tonometer (Tono-Pen Avia®) determined a significantly higher IOP value in the morning compared to that later in the day, which has been reported to be associated with the transition from the dark phase to the light phase (Pereira et al., 2011). Schuster et al. (2015) in a study on IOP measurements of 56 dragons with rebound tonometry have set a higher value in the morning compared to that taken later in the day. Similarly to this, as well as to that of Giannetto et al. (2009) on healthy dogs and Gelatt et al. (1981) on healthy and glaucomatous beagles the present study determined IOP value in the morning (16.21 ± 2.10 mmHg) reduced about 22% when ration to that (12.65 ± 2.01 mmHg) late in the day. As a result, these findings appear to confirm the claim that IOP measurement tends to reduce during the light phase of a day.

CONCLUSION

So far no study has investigated the effect of age, sex and circadian rhythm on IOP in sheep, the reference IOP data in Awassi sheep has also not been determined. Thus, the present study may contribute to relevant literature deficiency. Studies performed on the same species with even the use of the same type of tonometer, may produce different IOP values indicating breed variability in that species. The presence of circadian rhythm in IOP of the sheep as in people suggests that this species may be a proper animal model for experimental ocular studies.

CONFLICT OF INTEREST STATEMENT

None of the authors of the present paper has a financial and personal relationship with other people or organization that could inappropriately influence their work. ■

REFERENCES

- Al-Atiyat RM and Aljumaah RS (2014) Genetic distances and phylogenetic trees of different Awassi sheep populations based on DNA sequencing. *Genetics and Molecular Research*. 13: 6557-68.
- Andrade SF, Palozzi RJ, Giuffrida R, De campos RJ, Santos GC, Fukui RM (2012) Comparison of intraocular pressure measurements between the Tono-Pen XL® and Perkins® applanation tonometers in dogs and cats. *Veterinary Ophthalmology*. 15: 14-20.
- Barsotti G, Briganti A, Spratte JR, Ceccherelli R, Breggi G (2013) Schirmer tear test type I readings and intraocular pressure values assessed by applanation tonometry (Tonopen XL®) in normal eyes of four European species of birds of prey. *Veterinary Ophthalmology*. 16: 365-369.
- Broadwater JJ, Schorling JJ, Herring IP, Pickett JP (2007) Ophthalmic examination findings in adult pygmy goats (*Capra hircus*). *Veterinary Ophthalmology*. 10: 269-73.
- Gelatt KN, Gum GG, Barrie KP, Williams WW (1981) Diurnal variations in intraocular pressure in normotensive and glaucomatous Beagles. *Glaucoma*. 3: 21-24.
- Gelatt KN and Mackay EO (1998) Distribution of intraocular pressure in dogs. *Veterinary Ophthalmology*. 1: 109-114.
- Gerometta R, Alvarez LJ, Candia OA (2010) Effects of sildenafil and tadalafil on intraocular pressure in sheep: implications for aqueous humor dynamics. *Investigative Ophthalmology & Visual Science*. 51: 3139-3144.
- Gerometta R, Podos SM, Danias J, Candia OA (2009) Steroid-induced ocular hypertension in normal sheep. *Investigative Ophthalmology & Visual Science*. 50: 669-73.
- Ghaffari MS, Hajikhani R, Sahebjam F, Akbarein H, Golezard Y H (2012) Intraocular pressure and Schirmer tear test results in clinically normal Long-Eared Hedgehogs (*Hemiechinus auritus*): reference values. *Veterinary Ophthalmology*. 15: 206-209.
- Ghaffari MS, Shojaei M, Sabzevari A, Khorami N (2011) Reference values for intraocular pressure and Schirmer tear test in clinically normal Sanjabi sheep. *Small Ruminant Research*. 97: 101-103.
- Giannetto C, Piccione G, Giudice E (2009) Daytime profile of the intraocular pressure and tear production in normal dog. *Veterinary Ophthalmology*. 12: 302-305.
- Grundon RA, Anderson GA, Lynch M, Hardman C, O'reilly A, Stanley RG (2011) Schirmer tear tests and intraocular pressures in conscious and anesthetized koalas (*Phascolarctus cinereus*). *Veterinary Ophthalmology*. 14: 292-295.
- Gum GG, Gelatt KN, Miller DN, Mackay EO (1998) Intraocular pressure in normal dairy cattle. *Veterinary Ophthalmology*. 1: 159-161.
- Işler CT, Altuğ ME, Cellat M (2014) Evaluation of tear secretion and intraocular pressure in healthy disorders with amaurosis on holstein calves. *Pensee Journal*. 76: 426-430.
- Jaén-Díaz JI, Cordero-García B, López-De-Castro F, De-Castro-Mesa C, Castilla-López-Madrdejos F, Berciano-Martínez F (2007) Diurnal variability of intraocular pressure. *Archivos de la Sociedad Española de Oftalmología*. 82: 675-679.
- Jeong MB, Kim YJ, Yi NY, Park SA, Kim WT, Kim SE, Chae JM, Kim JT, Lee H, Seo KM (2007) Comparison of the rebound tonometer (TonoVet®) with the applanation tonometer (TonoPen XL®) in normal Eurasian Eagle owls (*Bubo bubo*). *Veterinary Ophthalmology*. 10: 376-379.
- Komáromy AM, Garg CD, Ying GS, Liu C (2006) Effect of head position on intraocular pressure in horses. *American Journal of Veterinary Research*. 67: 232-235.
- Internet. KoyunIrklarımız: http://www.tarimkutuphanesi.com/KOYUN_IRKLARIMIZ_00164.html (accessed 10.10.2016).
- Marzok MA, El-Khodery SA, Oheida AH (2014) Effect of intravenous administration of romifidine on intraocular pressure in clinically normal horses. *Veterinary Ophthalmology*. 17: 149-53.
- Montiani-Ferreira F, Mattos BC, Russ HH (2006) Reference values for selected ophthalmic diagnostic tests of the ferret (*Mustela putorius furo*). *Veterinary Ophthalmology*. 9: 209-213.
- Montiani-Ferreira F, Shaw G, Mattos BC, Russ HH, Vilani RG (2008) Reference values for selected ophthalmic diagnostic tests of the capuchin monkey (*Cebus apella*). *Veterinary Ophthalmology*. 11: 197-201.
- Nuhsbaum MT, Gionfriddo JR, Powell CC, Aubin ML (2000) Intraocular pressure in normal llamas (*Lama glama*) and alpacas (*Lama pacos*). *Veterinary Ophthalmology*. 3: 31-34.
- Ofri R, Horowitz IH, Kass PH (1998) Tonometry in three herbivorous wildlife species. *Veterinary Ophthalmology*. 1: 21-24.
- Pamuk K, Saritas ZK, Demirkan I, Acar A, Korkmaz M, Acar DB (2011) Animal welfare related to evaluate intraocular pressure in Anatolian Buffaloes: Preliminary Report. *Journal of Animal and Veterinary Advances*. 10: 987-990.
- Park YW, Jeong MB, Kim TH, Ahn JS, Ahn JT, Park SA, Kim SE, Seo K (2011) Effect of central corneal thickness on intraocular pressure with the rebound tonometer and the applanation tonometer in normal dogs. *Veterinary Ophthalmology*. 14: 169-73.
- Pereira FQ, Bercht BS, Soares MG, Da Mota MG, Pigatto JA (2011) Comparison of a rebound and an applanation tonometer for measuring intraocular pressure in normal rabbits. *Veterinary Ophthalmology*. 14: 321-326.
- Pigatto JAT, Pereira FQ, Albuquerque I, Corrêa LFD, Bercht BS, Hünning PS, Silva AAR, Pacicco De Freitas LVR (2011) Intraocular pressure measurement in sheep using an applanation tonometer. *Revista Ceres*. 58: 685-689.
- Ribeiro AP, Crivelaro RM, Teixeira PP, Trujillo DY, Guimarães PJ, Vicente WR, Martins BDAC, Laus JL (2014) Effects of different mydriatics on intraocular pressure, pupil diameter, and ruminal and intestinal motility in healthy sheep. *Veterinary Ophthalmology*. 17: 397-402.
- Rusanen E, Florin M, Hässig M, Spiess BM (2010) Evaluation of a rebound tonometer (Tonovet®) in clinically normal cat eyes. *Veterinary Ophthalmology*. 13: 31-36.
- Schuster EJ, Struve J, Fehr MJ, Mathes KA (2015) Measurement of intraocular pressure in healthy unanesthetized inland bearded dragons (*Pogona vitticeps*). *American Journal of Veterinary Research*. 76: 494-499.
- Verboven CA, Djajadiningrat-Laanen SC, Teske E, Boevé MH (2014) Development of tear production and intraocular pressure in healthy canine neonates. *Veterinary Ophthalmology*. 17: 426-431.
- Von Spiessen I, Karck J, Rohn K, Meyer-Lindenberg A (2015) Clinical comparison of the TonoVet® rebound tonometer and the Tono-Pen Vet® applanation tonometer in dogs and cats with ocular disease: glaucoma or corneal pathology. *Veterinary Ophthalmology*. 18: 20-27.
- Wu SY, Nemesure B, Hennis A, Leske MC (2006) Barbados Eye Group, Nine-year changes in intraocular pressure: the Barbados Eye Studies. *Archives of Ophthalmology*. 124: 1631-1636.

Consumer Protection and Food Safety in Greece: Sanctions imposed by Hellenic Food Authority, in the years 2005-2013

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ABSTRACT. The enforcement of food safety legislation consists of a number of procedures, that may lead in certain cases in imposing administrative penalties and fines, in an effort to alter the nonconformity status of certain food establishments, according to the predefined legislative standards. The aim of this study is to evaluate data upon nonconformity of food establishments in Greece, in order to define trends and frequencies in the general framework of food safety and consumer protection. Hellenic Food Authority (EFET), the competent authority for food safety in Greece, during the period 2005-2013, imposed fines to food establishments that mount to 17,513,900€ for food safety violations. Most of the fines were imposed at mass catering establishments (21.6%) followed by supermarkets (16.2%), food industry (15.1%) and food manufacture establishments (10.7%). Moreover Attica Prefecture is the region with the highest, in number, imposed fines (32.4%), followed in descending order by the Prefecture of Central Makedonia (31.5%) and of Crete (9.6%). Significant difference, in imposing fines (χ^2 test, $p \leq 0.05$), was observed between mass catering establishments and violations concerning: i. Good Hygiene Practice (GHP) ii. infrastructure, iii. consumer misleading, iv. sale of unsuitable foods, v. preservation temperatures, vi. lack of food handlers training in food safety, vii. lack of food handlers booklet and viii. traceability systems. Moreover significant differences were observed between the level of the imposed fine and the type of violations (t-test, $p \leq 0.05$) concerning: i. only or and GHP, ii. only or and the sale of unsafe foods and iii. only or and issues of consumer misleading. According to Pearson coefficient there is a weak negative although significant ($p \leq 0.001$) correlation between years and the level of the imposed fines ($r = -0.079$). In addition violations related to HACCP system, that resulted in imposing fines to food establishments by EFET in 2012, corresponded to 31.8% of the total delinquency concerning HACCP system ascertained by the Prefectural Directorates, that are in charge of official control in the field of food hygiene.

Keywords: food safety, hygiene, violations, sanctions

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INTRODUCTION

High profile food threats in the industrialized world, amplified by the media, have served to fuel consumer concerns and erode confidence in prevailing mechanisms of food safety controls (Henson & Jaffe, 2008; World Bank, 2005; Henson & Caswell, 1999). As a result consumer confidence in the efficacy of the enforcement of food safety legislation has been undermined (Berg, 2004; de Jonge *et al.*, 2007; Eiser *et al.*, 2002; Frewer *et al.*, 1996; Houghton *et al.*, 2008), with conspicuous instances of food safety failure perceived as “signals” of problems in the wide system of control. This public concern has placed increasing pressure on government agencies to be more proactive.

In this context, in Greece, in 1999, the Hellenic Food Authority (EFET) was enacted by Law 2741/1999. Its mission is the consumer protection by ensuring the import, production and distribution of safe food, and the prevention of consumer deception in relation to hygiene, composition, labeling, presentation and advertisement of foods. EFET, within its responsibilities, through its departments of Food Control in Prefectural level, conducts inspections to food establishments, in preventive and repressive level.

The fines to food establishments, until December 2013, had been imposed under Ministerial Decisions 15523/2006 and 10755/2006, for violations of food safety legislation. In January 2014 the Law 4235/2014 was adopted and introduced a new common ratification system in the field of food safety. Penalties with the new legislative framework were imposed from September 2014, due to administrative procedures for issuing circulars for the application of the new legislative framework. Though since February 2014 no official reports concerning fines at food establishments have been published in regular basis.

MATERIAL AND METHODS

The data of the study were derived from reports (press releases) issued by EFET, public available at its official website at: www.efet.gr, as well as from the Annual Reports of the Multi-Annual National Control Plans (MANCP) in the field of food safety. The data refers to 8 calendar years, from January 2005 until December 2013. The reports were coded per year and penalty case and the contexts of the press releases

were studied meticulously, in order to extract information concerning i. the type of the food establishment to which fine was imposed (mass catering, supermarkets, food industry establishments, food manufacture establishments, bakeries, butcher shops, groceries, pastries, hotels' food services units, storage and food marketing firms, food production and trade establishments, bakeries & pastry shops, dairy plants, food import and trade establishments, confectionery outlets, fish stores, bottling companies, flea markets, hospitals' food services units, olive oil mill plants, street outlets, various other food businesses), ii. the type of infringements for which fine was imposed, iii. the number of infringements per case for which fines were imposed, iv. the amount (in number) of the fines imposed, v. the range of the imposed fines, vi. the Prefecture where the food establishment was active and vii. whether the infringements were observed after re-inspection. Following data entry, the data file was subject to a number of data validation procedures, as well as inter and intra variable checks. The data were registered in spreadsheets that turned out to have 2654 cases (rows) and 22 variables (columns).

The registered infringements were classified in thematic categories as follows, infringements associated to: i. infrastructure of the food establishment, ii. equipment of the establishment, iii. Good Hygiene Practice (GHP) iv. keeping of records, v. sale of unsafe food, vi. lack or inefficient application of Hazard Analysis Critical Control Point (HACCP) system, vii. lack of establishment's license, viii. modification of operation conditions of the food establishment without the appropriate licence (alteration of licence terms), ix. sale of irregular food, x. food preservation conditions, xi. lack of staff training in food safety, xii. lack of staff health booklets, xiii. Good Manufacture Practice (GMP), xiv. traceability systems and xiv. inhibition of official control. All these categories were grouped by the year that the fine was imposed, the amount of the fine, the range of the fine, the Prefecture where the food company was active and whether the infringement was found in re-inspection.

Moreover, the infringements concerning HACCP, GHP, the sale of unsafe food and the total number of violations, for which fines were imposed to food establishments from 2007 to 2013 from EFET, were compared to the overall delinquency data, as they are

presented at the public available official reports of the MANCP, published annually since 2007. The data presented at the above mentioned reports originated from reports from the Prefectural Directorates responsible in the field of food safety in Greece and more specifically the Prefectural Directorates of i. Public Health, ii. Rural Economy and Veterinary Services, iii. Trade and iii. EFET Prefectural Directorates.

The statistical analysis was performed with IBM SPSS 20 and StatSoft Statistika 8.0 and for data corrections, transformations and graphical displays Open Office Calc 3.0 was used.

Descriptive statistics were performed, as well as chi-square test ($p \leq 0.05$), Pareto analysis and contingency coefficient test (for qualitative variables), Pearson correlation coefficient, ANOVA and t-tests ($p \leq 0.05$) (for quantitative variables, as the asymptotic normality assumption can be safely assumed because of the sample size) and eventually data mining analysis, with the “a priori” algorithm, among non parametric data in order to identify frequent item sets and association rules.

RESULTS

During the period 2005 - 2013 EFET imposed 2,654 fines to food establishments. The overall amount of the imposed fines was 17,513,900€, the average fine was 6,600.94€ (± 275.17) while the maximum was 500,000€ and the minimum 500€. The highest percentage of fines was imposed in 2012 (19.9%), followed by the years 2013, 2011 and 2010 as it is shown in **Figure 1**.

The 64.2% of fines were below 5,000€, 28% of

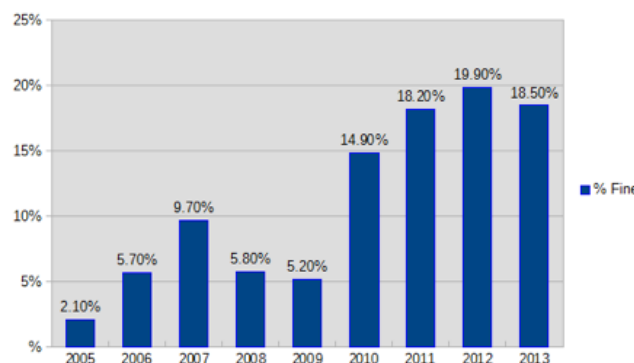


Figure 1: Per year percentage of the imposed fines

fine ranged from 5,000€ to 20,000€, while fines over 50,000€ were less than 1%.

According to the Pareto analysis the 80% of the fines were imposed in descending order at mass catering (21.6%), supermarkets (16.2%), food industry establishments (15.1%), food manufacture establishments (10.7%), bakeries (6.1%), butcher shops (4.1%), groceries (3.8%), various other food businesses (3.6%), storage and food marketing firms (3.4%) and pastries (2.2%)

Moreover the 80% of the fines imposed for violations pertained, in descending order: to sale of unsafe foods (20.84%), GHP (20.65%), inefficient or lack of implementation of HACCP system (11.55%), sale of unsuitable foods (10.31%), lack of operation licenses (8.43%) and infrastructure issues (5.91%). It should be mentioned that in a number of cases, fines were imposed because of more than one type of violations in a food establishment.

At **Table 1** we notice that in 58.5% of cases, fine

Table 1: Number of violations for which fines were imposed per food establishment

<i>N of violations/food establishments</i>	<i>%</i>
<i>1 violation</i>	<i>58,5</i>
<i>2 violations</i>	<i>28,6</i>
<i>3 violations</i>	<i>9,8</i>
<i>4 violations</i>	<i>2,2</i>
<i>5 violations</i>	<i>0,6</i>
<i>7 violations</i>	<i>0,2</i>
<i>6 violations</i>	<i>0,1</i>

was imposed for one (1) violation, in 28.6% for two (2) and lower rates for the rest of cases.

In 2007 the number of violations, for which fines were imposed by EFET, accounted to 6.1% of the total figures of delinquency reported by the Prefectural Directorates in charge of official control in the field of food safety in a wide range of food establishments (manufacturers & packers, distributors & transporters, retailers, food service business, manufacturers selling primarily on a retail basis), which fall under the provision of Regulation 852/2004. The relevant rates for the years 2007-2013 are shown in **Figure 2**.

The violations upon GHP regulations, that resulted in imposing fines in 2010 by EFET, accounted to 9.4% of the total figure delinquency concerning GHP, reported by the Prefectural Directorates, in the above

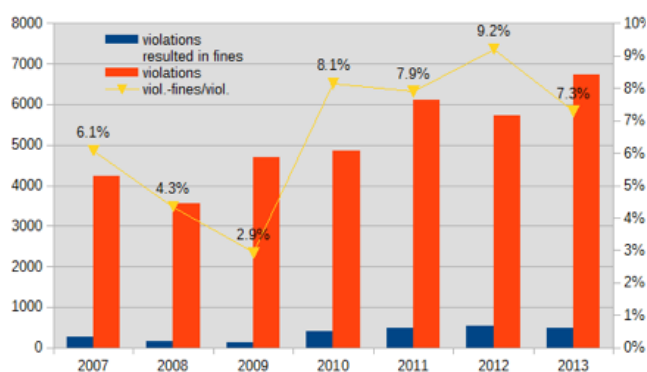


Figure 2: Rate of violations, where fines were imposed, over the total figures of delinquency per year (2007-2013).

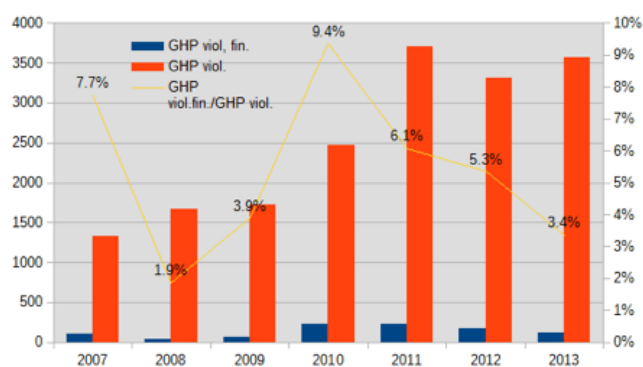


Figure 3: Rate of violations concerning GHP, where fines were imposed, over the total figures of delinquency concerning GHP, per year (2007-2013).

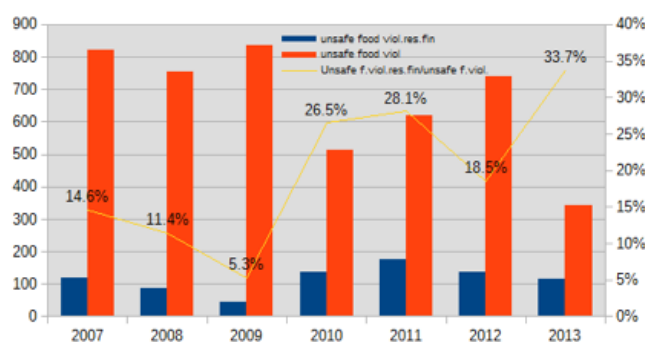


Figure 4: Rate of violations concerning sale of unsafe food, where fines were imposed, over the total figures of delinquency concerning unsafe food per year, (2007-2013).

mentioned food establishments. The relevant rates for the years 2007-2013 are shown in **Figure 3**.

The violations pertained to sale of unsafe foods, that resulted in imposing fines to food establishments by EFET in 2013, accounted to 33.7% of the total figures of delinquency concerning unsafe foods reported by the Prefectural Directorates, that were in charge of official control in the field of food safety in the above mentioned food establishments. The relevant rates for the years 2007-2013 are shown in **Figure 4**.

The violations pertained to the inefficient or lack of implementation of HACCP system, that resulted in imposing of fines to food establishments by EFET in 2012, accounted to 31.8% of the total delinquency concerning HACCP system reported by the Prefectural Directorates, that were in charge of official control in the field of food safety in the above mentioned food establishments. The relevant rates for the period 2007-2013 are shown in **Figure 5**.

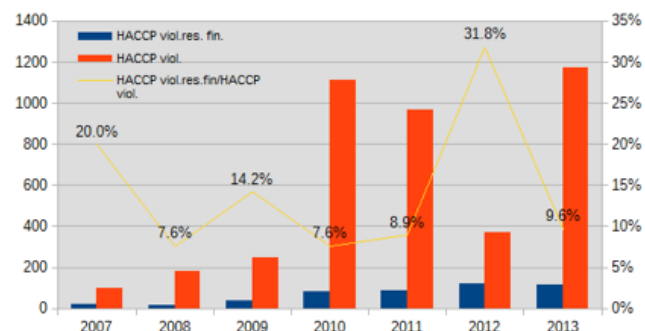


Figure 5: Rate of violations concerning HACCP system, where fines were imposed, over the total figures of delinquency concerning HACCP per year (2007-2013).

Significant differences were observed between the type of food establishments and the type of violations, in imposing fines (χ^2 test, $p \leq 0.05$), in the following cases:

- mass catering and violations concerning: i. GHP, ii. infrastructure, iii. consumer misleading, iv. alteration of license's terms, v. sale of unsuitable foods, vi. preservation temperature of foods, vii. lack of food handlers training in food safety, ix. lack of food handlers booklet and x. traceability system.
- supermarkets and violation concerning: i. sale of unsafe foods, ii. inefficient or lack of implementation of HACCP system, iii. consumer misleading, iv. alteration of terms of license, v. sale of unsuitable foods, vi. lack of operation license, vii. lack of food handlers booklet and ix. traceability system.
- food industry and violation concerning: i. GHP ii. sale of unsafe foods, iii. inefficient or lack of implementation of HACCP system, iv. consumer misleading, v. alteration of terms of license, vi. sale of unsuitable foods, vii. lack of operation license, viii. preservation temperature of foods, ix. lack of food handlers training in food safety, x. lack of food handlers booklet and xi. traceability systems

According to Contingency Coefficient test the relationship between food establishments and violations, for which fines were imposed, is low (o -0,30),

The 80% of fines, according to the Pareto analysis, imposed, in descending order, to Prefecture of Attica (32.4%), of Central Makedonia (31.5%) and of Crete (9.6%) (**Table 2**).

Significant differences were observed between the following types of violations and Prefectures, in imposing fines (χ^2 test, $p < 0.05$):

- violations concerning the sale of unsafe foods and Prefecture of: i. Attica, ii. Central Makedonia, iii. Thessaly, iv. Crete, v. Ionian Islands and vii. East Makedonia & Thrace.
- violations concerning GHP and Prefecture of: i. Attica, ii. Central Makedonia, iii. Thessaly and iv. Crete.
- violations concerning the inefficient or lack of implementation of HACCP system and Prefecture of: i. Attica, ii. Central Makedonia, iii. Epeirous and iv. West Greece.
- violations concerning the sale of unsuitable foods and Prefecture of: i. Thessaly, ii. Crete, and iii. Epeirous.
- violations concerning the lack of food handlers training in food safety and Prefecture of: i. Attica, ii. Central Makedonia, iii. Thessaly and iv. Crete
- According to Contingency Coefficient test the relationship between food establishments, to which fines were imposed and Prefectures where these companies were active, is medium (0,677).

Table 2: Pareto Analysis of the frequency of fines' per Prefecture

Prefecture of	Count	%
Attica	860	32.4%
Central Makedonia	837	31.5%
Crete	255	9.6%
Thessaly	230	8.7%
West Greece	158	6.0%
Ipeirous	136	5.1%
East Makedonia & Thrace	105	4.0%
Ionian Islands	30	1.1%
Peloponnese	17	0.6%
North Aigaïou	11	0.4%
Stereia Greece	9	0.3%
West Makedonia	5	0.2%

In addition significant differences were observed between the level of the imposed fine and the type of violations (t-test, $p \leq 0.05$) concerning: i. only or and GHP (mean=8,162.13€, ± 737.61 €, minimum=1,000€), ii. only or and sale of unsafe foods (mean =7,632.71€, ± 672.63 €, minimum=1,000€), iii. only or and consumer misleading (mean=9,842.79€, $\pm 1,044.90$ € minimum=500€). According to Pearson correlation, as the years go by, the level of the imposed fines is reduced ($r = -0.079$) ($p \leq 0.001$).

Moreover significant differences were observed between the level of the fines imposed to food establishments and Prefectures of Central Makedonia (mean=7,801.55€, ± 397.56 €), Thessaly (mean=5,126.09€, ± 509.32 €), Crete (mean=3,970.59€ ± 266.85 €) and West Greece (4,481.01€ ± 334.97 €) (t-test, $p < 0.05$).

Table 3: Association rules resulted from the use of “a priori” algorithm

Body	==>	Head	Support(%)	Confidence(%)	Correlation(%)
2013	==>	<5,000	14.43	78.00	41.88
Mass catering	==>	<5,000	15.97	73.86	42.88
2012	==>	<5,000	14.50	73.05	40.63
2011	==>	<5,000	12.05	66.25	35.28
Unsuitable foods	==>	<5,000	10.17	63.98	31.84
Attica Prefecture	==>	<5,000	20.68	63.83	45.36
Unsafe food	==>	<5,000	18.19	56.62	40.07
GHP	==>	<5,000	17.93	56.33	39.67
Central Makedonia Prefecture	==>	<5,000	17.25	54.71	38.36
5,000 to 20,000	==>	Unsafe food	11.34	40.51	37.80
Central Makedonia Prefecture	==>	GHP	12.32	39.06	38.88
5,000 to 20,000	==>	Central Makedonia Prefecture	10.88	38.89	36.64
GHP	==>	Central Makedonia Prefecture	12.32	38.69	38.88
Central Makedonia Prefecture	==>	Unsafe food	12.17	38.59	38.22
Unsafe food	==>	Central Makedonia Prefecture	12.17	37.86	38.22
5,000 to 20,000	==>	GHP	10.58	37.81	35.46
Unsafe food	==>	5,000 to 20,000	11.34	35.28	37.80
Central Makedonia Prefecture	==>	5,000 to 20,000	10.88	34.52	36.64
GHP	==>	5,000 to 20,000	10.58	33.25	35.46
<5000	==>	Attica Prefecture	20.68	32.23	45.36
<5000	==>	Unsafe food	18.19	28.36	40.07
<5000	==>	GHP	17.93	27.95	39.67

The association rules that resulted from the “a priori” algorithm are presented below in Table 3. These rules have got a certain rate of support, confidence and correlation and can be used for the composition of logical assumptions, where the “**body**” is the “**IF**” and the “**head**” is the “**THEN**” and “**,**” is the “**AND**”.

So:

If the fine was imposed in 2013, **then** its level would be <5000€ (with a rate of confidence 78%).

If the fine was imposed to mass catering establishment, **then** its level would be <5.000€, with a rate of confidence 73,86%.

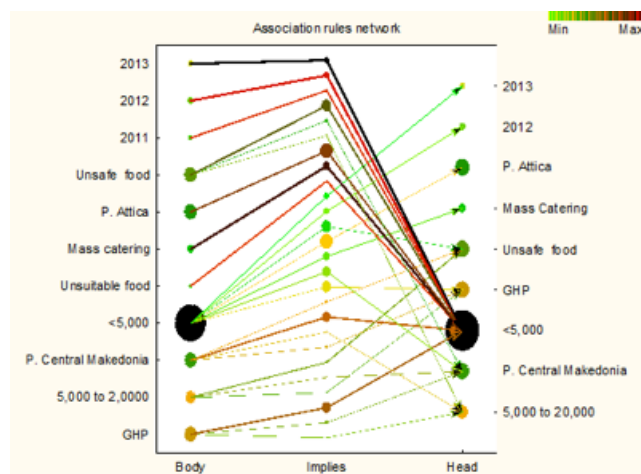
If the fine was imposed for the sale of unsuitable food, **then** its level would be <5.000€, with a rate of confidence 63,98%.

The following Graph (Graph 1) illustrates the most important association rules.

DISCUSSION

The changes in food systems and in consumer expectations have placed additional stress on the need for better control of food safety risks (Marian Garcia Martinez, *et. al.*, 2007; Lupien JR, 2007) and for imposing adequate sanctions.

Of particular interest is the implementation of



Graph 1: Net of association rules with min support of 10% occurrence (at this graph the thickness of each line is proportional to the percentage of confidence, the size of each dot is proportional to the rate of support, the central dots implies the rate of support of the rule, while the lateral dots implies the support rate of each case separately).

HACCP system, that is obligatory according to Regulation 852/2004, since 2006, at all types of food establishments. During the period 2005-2013, the implementation of HACCP was not the case for several types of food establishments, like mass catering, food industry, food manufacturing establishments, supermarkets, hotel catering and bakeries. In 2010 the rate of delinquency concerning HACCP was high, though the rate of violations concerning HACCP that resulted in imposing fines from EFET to the total delinquency concerning HACCP that year was only 7.6%. In 2012 the delinquency concerning HACCP was lower than 2010, although the rate of violations concerning HACCP that resulted in imposing fines from EFET to the total delinquency concerning HACCP this year was very high.

The number of fines concerning violations of GHP predominate in the case of mass catering establishments in comparison with all the other types of food establishments. The delinquency concerning GHP was the highest in 2011 (during the period 2007-2013), according to MANCP, though the rate of violations concerning GHP, that resulted in imposing fine from EFET in relation to the total delinquency concerning GHP this year, was at 6.1%.

In addition, in 2009 we notice the lowest rate of violations that resulted in imposing fines from EFET in relation to the total delinquency according to the MANCP, while the highest rate was observed in 2012, during the period 2007-2013.

Moreover the rate of violations that resulted in imposing fines during the period 2007-2013 to the overall delinquency according to the MANCP was higher for the violation concerning unsafe food, than to violations concerning GHP or HACCP.

As the years go by, the level of the imposed fines is reduced that proves that the food law enforcement practices gradually have an effect on reducing delinquency. On the other hand the increase of the number of fines imposed to food establishments, during the period 2005-2013, from EFET, is due to the increase of the establishment of EFET's Prefectural Directorates and the increase of the number of its staff.

The high rates of fines imposed to mass catering, super markets and food industry, is due to the policy design of food control plan, by EFET administration,

derived from the analysis and evaluation of food control results of each year and from the application of a risk based food inspection program.

Most of the fines are imposed to food establishments, that have their premises in Prefecture of Attica (32.4%) and Central Makedonia (31.5%). This is due to the fact that more than half of Greek population is gathered to these two (2) Prefectures and it's a parameter that EFET administration takes into consideration in the design of food control plans. Though it's remarkable that in Cyclades, EFET has imposed no fine during the period 2005-2013 and at Dodekanisa Prefecture only one (1). This is due to the fact that EFET organizational development has not been completed, because of the lack of financial resources and political will.

CONCLUSION

According to article 11 of Regulation (EU) 2017/625

on official controls the competent authorities in the field of food safety shall ensure the regular and timely publication on information on the type, number and outcome of official controls, the type and number of cases of non-compliance detected and establish procedures to ensure that any inaccuracies in the information made available to the public are appropriately rectified.

The development of a European platform, where all the Member States of the European Union would be able to publish enforcement measures and penalties imposed to food establishments could contribute to the increase of transparency in the sector of food safety, the protection of European consumer health and interests and the comparison of sanctions in this field between the competent authorities of the Member-States (M-S)

CONFLICT OF INTEREST

The authors declare no conflict of interest..

REFERENCES

- Berg, L. (2004) Trust in food in the age of mad cow disease: a comparative study of consumers' evaluation of food safety in Belgium, Britain and Norway. *Appetite* 42 (1), 21–32.
- De Jonge, J, van Trijp, JCM, Renes, RJ, Frewer, LJ, (2007) Understanding consumer confidence in the safety of food; it is two dimensional structure and determinants. *Risk Anal* 27 (3), 729–740.
- Eiser, JR, Miles, S, Frewer, LJ, (2002) Trust, perceived risk, and attitudes toward food technologies. *J. Appl. Soc. Psychol* 32 (11), 2423–2433.
- Frewer, LJ, Howard, C, Hedderley, D, & Shepherd, R (1996) What determines trust in information about food-related risks? Underlying psychological constructs. *Risk Anal* 16, 473–486.
- Henson, SJ and Caswell, JA (1999) Food Safety Regulation: An Overview of Contemporary Issues. *Food Policy* 24: 589–603
- Henson, SJ and Jaffee, S (2008) Understanding Developing Country Strategic Responses to the Enhancement of Food Safety Standards *The World Economy*, 31(1): 1–15.
- Houghton, JR, Rowe, G, Frewer, LJ, van Kleef, E, Chrysoschoidis, G, Kehagia, O, Korzen-Bohr, S, Lassen, J, Pfenning, U, Strada, A, (2008) The quality of food risk management in Europe: perspectives and priorities. *Food Policy* 33 (1), 13–26.
- Joint Ministerial Decision 15523/2006 “Additional measures to implement Regulations (EC) no 178/2002, 852/2004, 853/2004, 854/2004 and 882/2004 of the European Parliament and of the Council and harmonization of Directive 2004/41/EC of the European Parliament and Council (OJ 1187/B/31.08.2006).
- Joint Ministerial Decision 10755/2006 “Authorization to the Management Board of EFET to impose fines and procedure for imposing fines of par. 8, artic. 5 of Law 2741/99, as amended by par. 19, artic. 37 of Law 3066/2002, par. 5, artic. 11 of Law 3438/2006 and in accordance with par. 6, artic. 11 of Law 3428/2006” (OJ 727/15.06.2006).
- Law 2741/1999 “Hellenic Food Authority and other provisions of the Ministry of Development” (OJ199/A/28.09.1999).
- Law 4235/20145 “Administrative measures, procedures and sanctions in the implementation of European and National legislation in the field of food, feed, animal health and welfare and other provisions of the Ministry of Rural Development and Food” (OJ 32/A/11.02.2014).
- Lupien JR, (2007) “Prevention and control of food safety risks: the role of governments, food producers, marketers, and academia”, *Acia Pav J Clin Nutr*, 16 Suppl 1:74–9.
- Martinez, MG, Fiarne, A, Caswell, JA, Henson, S (2007) “Co-regulations as a possible model for food safety governance: Opportunities for public-private partnerships”. *Food Policy* 323 (3): 299–314, doi:10.1016/j.foodpol.2006.07.005
- Regulation (EC) 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs (EE L 139/30.04.2004).
- Regulation (EC) 2015/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, amending Regulations (EC) No 999/2001, (EC) No 396/2005, (EC) No 1069/2009, (EC) No 1107/2009, (EU) No 1151/2012, (EU) No 652/2014, (EU) 2016/429 and (EU) 2016/2031 of the European Parliament and of the Council, Council Regulations (EC) No 1/2005 and (EC) No 1099/2009 and Council Directives 98/58/EC, 1999/74/EC, 2007/43/EC, 2008/119/EC and 2008/120/EC, and repealing Regulations (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council, Council Directives 89/608/EEC, 89/662/EEC, 90/425/EEC, 91/496/EEC, 96/23/EC, 96/93/EC and 97/78/EC and Council Decision 92/438/EEC (Official Controls Regulation)
- World Bank, 2005 Food Safety and Agricultural Health Standards: Challenges and Opportunities for Developing Country Exports. Washington DC, World Bank.

Effects of iron nano-particle's on expression of tetracycline resistance encoding genes in *Staphylococcus aureus* by Real Time-PCR

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ABSTRACT. Increasing bacterial resistance towards traditional/conventional antibiotics is a major global health concern worldwide. Iron oxide nanoparticles (Fe nanoparticles, with average size of 20 nm) have considerable potential as antimicrobial agents in food safety applications due to their structure, surface properties, and stability. The aim of this work was to investigate the antibacterial effects and mechanism of action of iron nanoparticles against the expression of the tetA gene in Tetracycline Resistant *Staphylococcus aureus* strains by real time PCR. In the cross-sectional study, a total of 60 *S. aureus* were collected. Antibiotic susceptibility test was performed on the muller hinton agar according to the Clinical and Laboratory Standards Institute (CLSI). Then all strains were evaluated for tetA, tetB, tetC and tetD genes by multiplex-PCR method. In-vitro activity of iron oxide nanoparticles was evaluated against all resistant strains by microbroth dilution method. Therefore, the expression of tetA gene was measured in treated with iron oxide nanoparticles and untreated resistant *S. aureus* strain by Real time PCR. Our results indicated 25 (41.66%) strains resistant to Tetracycline. The prevalence of tetA, tetB, tetC and tetD genes were 5 (8.33%), 2 (2.33%), 20 (33.33%) and 10 (10.67%), respectively. The expression of tetA genes in resistant *S. aureus* strains treated with Iron oxide nanoparticles was lower than the untreated isolates. Iron oxide nanoparticles have strong antibacterial activity against resistant to Tetracycline *S. aureus* strains. In addition to, these nanoparticles reduce the expression of antibiotic resistance gene.

Key words: Iron oxide nanoparticles, *Staphylococcus aureus*, tet genes, Real time PCR.

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INTRODUCTION

Staphylococcus aureus is a gram-positive, round-shaped bacterium that is considered as a natural bacterial flora in different tissues such as the nose, respiratory tract, and on the skin. The biochemical analysis showed that it is often positive for catalase and nitrate reduction and is a facultative anaerobe that can grow without the need for oxygen (Masalha et al, 2001). Pathogenic strains often promote infections by producing virulence factors such as potent protein toxins, and the expression of cell-surface proteins that bind and inactivate antibodies. The emergence of antibiotic-resistant strains of *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) is a worldwide problem in clinical medicine. Despite much research and development there is no approved vaccine for *S. aureus* (Tsiodras et al, 2001). Tetracycline is a broad spectrum antibiotic that its general usefulness is reduced because of onset of antibiotic resistance, but still remains the treatment choice for specific indications in different bacterial infections (Tiwari et al, 2006). The Tetracycline family antibiotics are generally used in the treatment of infections of the urinary tract, respiratory tract, and the intestines and are also used in the treatment of chlamydia, especially in patients allergic to β -lactams and macrolides. However, their use for these indications is less popular than it was due to widespread development of resistance in the causative organisms (Chow et al, 1975).

Mechanisms in which the bacteria became resistant to Tetracycline are cytoplasmic exocytosis channels, ribosomal conservation and deactivation of enzymatic system. TetB gene encodes exocytosis pumps that makes the bacterium resistant to Tetracycline and Minocycline. Most of the exocytosis pumps in bacteria are encoded by TetA, TetB, TetC, TetD and TetG genes (Noble et al, 1992). Metallic element's nanoparticles can disrupt the transcription and translation in bacteria (Soenen et al, 2010), these elements can also effect gene expression by inducing breaks in DNA molecules (Panáček et al, 2006). Recent studies showed that some of chemical element oxides of Calcium, Magnesium and nanoparticles of Zinc and Copper have a noticeable antibacterial activity (Hadi et al 2011, Ohira et al, 2008). The ion of these elements by attaching to -SH groups of enzymes, will react with proteins and finally deacti-

vate them (Tawale et al, 2010). Iron oxide nanoparticles (Fe nanoparticles, with average size of 20 nm) have considerable potential as antimicrobial agents in food safety applications due to their structure, surface properties, and stability. The aim of this work was to investigate the antibacterial effects and mechanism of action of iron nanoparticles against the expression of the tetA gene in Tetracycline resistant *Staphylococcus aureus* strains by real time PCR.

MATERIAL AND METHODS

Samples were taken from different tissue sources in patients referred to the department of infectious diseases in 3 major hospitals in Tehran-Iran. 60 *Staphylococcus aureus* positive samples were detected by biochemical and microbiological analysis on cultured colonies on Blood Agar medium. The disc diffusion test based on CLSI (Clinical and Laboratory Standards Institute, 2014) was performed to identify resistant strains of *S. aureus* against Tetracycline according to the Kirby and Bauer protocol. Minimum inhibitory concentration (MIC) on the iron nanoparticles was done according to broth dilution method. *S. aureus* reference strain (accession number: ATCC25923) was used as positive control. All the 60 isolated strains were used for culture on blood-agar and incubated for 24 hours at 37°C. 0.5 McFarland bacterial suspension made in PBS and the bacterial MIC in Fe nanoparticle suspension were recorded according to the CLSI standard method.

DNA Extraction and primer design

DNA was extracted from selected samples using DNA Extraction Kit (MBST-Iran) according to the manufacturer protocol. The quantitative evaluation of Extracted DNA samples was done by OD measuring with spectrophotometry. The quality of DNA samples was evaluated by electrophoresis on agarose gel at 100 V (data not shown). Specific primer pairs were designed for amplifying Tetracycline resistance inducing encoding genes showed in Table 1.

PCR for detection Tetracycline resistance inducing encoding genes

For amplification of the target genes 10 ng of total DNA was subjected to Multiplex-PCR micro tubes in

Table 1: Primer sequences for amplification of *Target encoding genes*.

Target Gene	Primer sequences no	PCR fragment size (bp)
<i>tet(A)</i>	5'-GCT ACA TCC TGC TTG CCT TC-3'	210
	5'-CAT AGA TCG CCG TGA AGA GG-3'	
<i>tet(B)</i>	5'-TTG GTT AGG GGC AAG TTT TG-3'	659
	5'-GGTA ATG GGC CAA TAA CAC CG-3'	
<i>tet(C)</i>	5'-CTT GAG AGC CTT CAA CCC AG-3'	418
	5'-ATG GTC GTC ATC TAC CTG CC-3'	
<i>tet(D)</i>	5'-AAA CCA TTA CGG CAT TCT GC-3'	787
	5'-GAC CGG ATA CAC CAT CCA TC-3'	

100 microliter total volume including 10X PCR buffer, 2.5 U Taq polymerase enzyme (Cinnagen, Iran), 2 µl of each primers (20µM, Cinnagen, Iran), 2 µl of each dATP, dTTP, dGTP and dCTP (200µM Fermentase), 1.5 mM MgCl₂ in automated Thermo cycler (MWG, Biotech Primus, Germany) under the following program: Denaturation step for 10 min at 95°C, followed by 35 cycles of 30 S in 94°C, annealing step at 55°C for 35S and the elongation for 45S at 72°C.

10 µl of all PCR products were subjected to electrophoresis on 1.5% agarose gel in TBE buffer at 100 V and were visualized under UV light by Ethidium Bromide staining.

RNA extraction and cDNA synthesis

0.5 McFarland tetA positive *S. aureus* suspension were added to 0.1 mg/ml nanoparticles in 10 ml BHI broth and incubated for 15 h at 37°C. Fifteen hours after incubation RNA extraction was performed using RNA Extraction Kit (Cinnagen, Iran) and Trizol buffer (Life Technology, Belgium) according to the manufacturer protocol. One microgram of total RNA was subjected to cDNA synthesis using the AccuPower RT PreMix (Bioneer, South Korea) according to the manufacturer protocol. The synthesized Single strand DNA was then analyzed with agarose gel electrophoresis.

Real Time PCR

For quantification expression of the resistance against Tetracycline encoding genes, Real Time PCR was done using 1.5 microliter of single strand cDNA subjected in 12.5 µl SYBR Green I PCR Master Mix (Thermo, Denmark), 2 µg of each specific miRNA-

212 designed primers (sequences not shown) and 4 µl double distilled water under the following conditions; Denaturation 30 seconds at 94 ° C followed by 45 amplification cycles of 5 S at 94 ° C (Denaturation step), 30 S at 59 ° C (Annealing step) and 45 S at 72 ° C (Extension step). As housekeeping gene/s the Gyrase or/and 16S gene/s was used. The DDCT results were analyzed determining the ratio of each studied case. The graphs were drawn using Graphpad Design version 5 program.

RESULTS

60 isolated from all taken samples were detected as *Staphylococcus aureus* by biochemical and microbiological analysis on cultured colonies on Blood Agar medium. Twenty five out of 60 (41.66%) *S. aureus* samples detected as anti-Tetracycline resistant by disc diffusion test based on CLSI Standards.

Multiplex PCR results for detection *S. aureus* Tetracycline resistant strains

Multiplex PCR using four primer pairs (Table. 1) was done and the frequency of TetA, TetB, TetC and TetD were 8.33%, 2.33%, 33.33% and 16.67%, respectively. In one of the total 60 studied strains (1.6%) all the 4 target genes were detected (Figure 1).

Results of MIC broth dilution test of Fe nanoparticles on *S. aureus* Tetracycline resistant strains

MIC broth dilution test of Fe nanoparticles on incubated *S. aureus* Tetracycline resistant strains was recorded as 32µg/ml and the minimum bactericidal concentration was 64 µg/ml (data not shown).

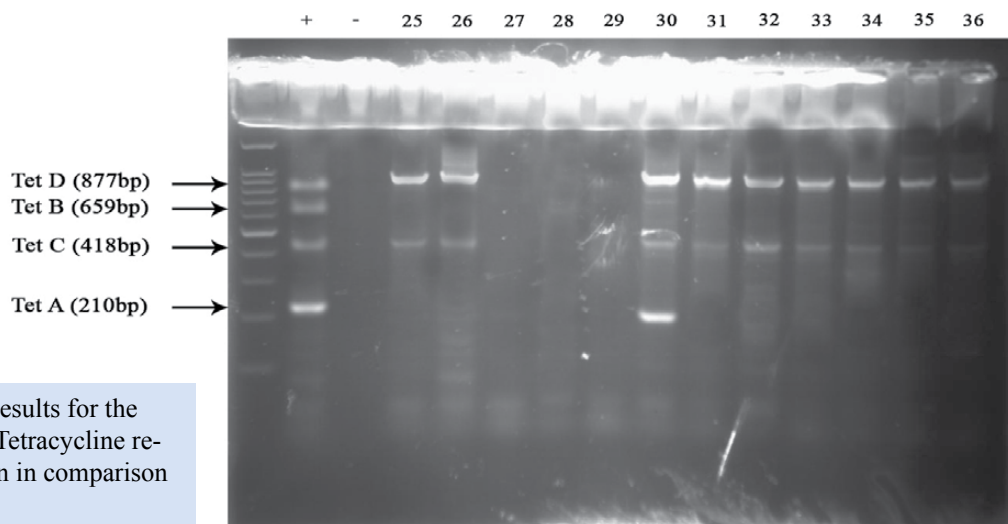


Fig 1: Multiplex PCR results for the detection of *S. aureus* Tetracycline resistant strains are shown in comparison to 100 bp DNA ladder.

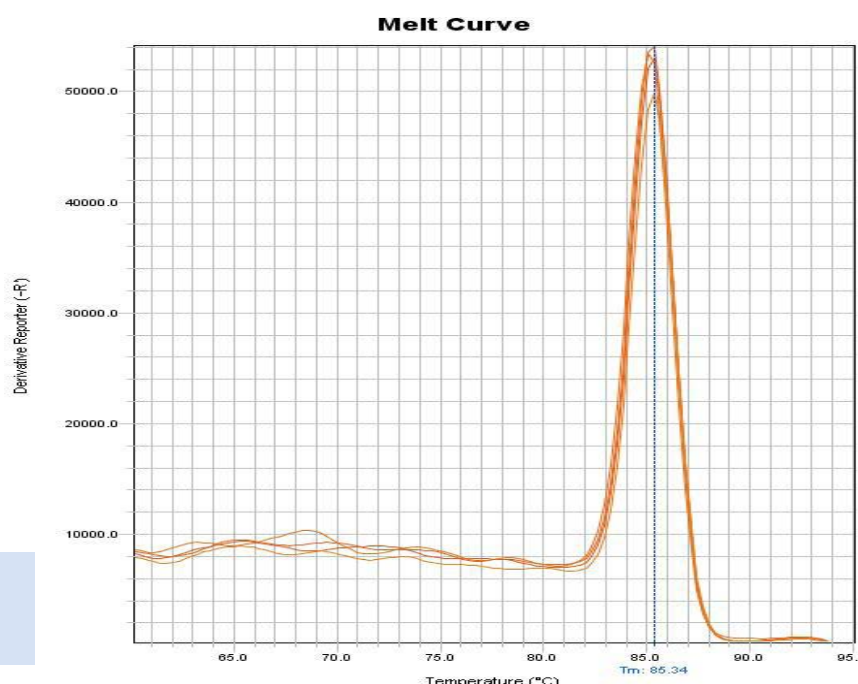


Fig 2: Melting curve recorded in Real Time-PCR on *S. aureus* strains treated with Fe Nano-particle

Real Time PCR results of quantification expression of the resistance against Tetracycline encoding genes in *S. aureus* strains treated by Fe Nano-particles

34.85 °C recorded as the best melting curve for TetA gene amplification by Real Time-PCR. It seems that the rate of TetA expression decreases in *S. aureus* treated with Fe nano-particles in comparison to untreated strains in our study (Figure 2).

TetA expression level in *S. aureus* treated with Fe

Nano-particles was two times lesser than the TetA expression level in untreated strains. The decrease of the expression level of TetA genes in treated strains is shown in Fig. 3.

Gene expression decreased in Fe nano-particle treated strains (green and blue curves) in comparison to non-treated ones (purple and red curves).

$\Delta\Delta C_t$ Analysis Method showed that the gene expression in Fe nano-particles treated strains was decreased 2 times than the non-treated strains.

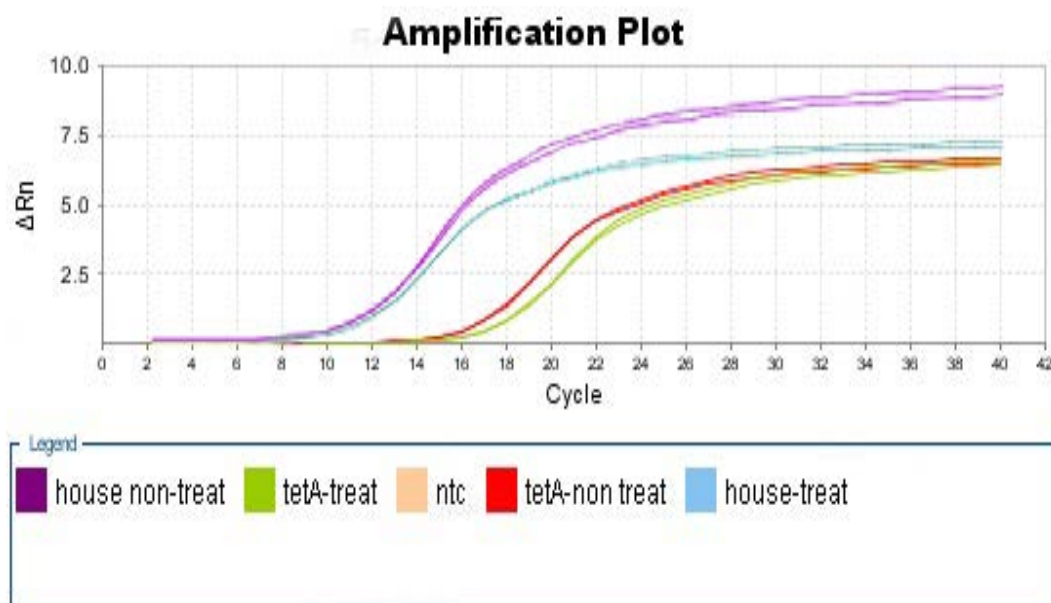


Fig 3: Comparison of the TetA expression level in *S. aureus* treated with Fe Nano-particles and non-treated strains.

DISCUSSION

The Tetracycline family antibiotics are generally used in the treatment of infections of the urinary tract, respiratory tract, and the intestines and are also used in the treatment of chlamydia, especially in patients allergic to β -lactams and macrolides however, their use for these indications is less popular than it once was due to widespread development of resistance in the causative organisms (Chow et al, 1975). Bondarenko in 2012 showed that Cu Oxide nano particles react with Amin and Carboxyl groups on the surface of microbial cells and release Cu in Oxidation reactions which leads to antibacterial effects by releasing Hydroxyl radicals (Bondarenko et al, 2012). Sondi in 2004 showed that nano-particles have a reductive effect during DNA replication process (Sondi et al, 2004). Warsa in 1996 showed that all of the 215 studied strains of *S. aureus* isolated from Asian countries carried both of the TetK and TetM (Tetracycline and Minocycline resistant inducing genes, respectively), on the contrary, the tetK gene was not detected in isolated from Japan and Korea (Warsa et al, 1996) This study was carried out in order to evaluate the expression level of TetA encoding gene in *S. aureus* strains treated with Fe nanoparticles. Antibacterial agents are widely used in different

levels of social hygiene, medicine and industrials (Aruoja et al, 2009). Growth of studied strains were suppressed after 2 hours treatment with 32 μ g/ml Fe nanoparticles which led to the conclusion that Fe nanoparticles at the least concentration play an effective role in decreasing the bacterial growth without time consuming side effects. Our results indicated 25 (41.66%) strains resistant to Tetracycline. The prevalence of tetA, tetB, tetC and tetD genes were 5 (8.33%), 2 (2.33%), 20 (33.33%) and 10 (10.67%), respectively. The expression of tetA genes in *S. aureus* resistant strain treated with Iron oxide nanoparticles was lower than the untreated isolates. Iron oxide nanoparticles have strong antibacterial activity against resistant to Tetracycline *S. aureus* strains. In addition to, these nanoparticles reduce the expression of antibiotic resistance gene. Azam in 2012 showed that the antibacterial activity of CuO nanoparticles was found to be size-dependent and the highly stable minimum-sized monodispersed copper oxide nanoparticles demonstrated a significant increase in antibacterial activities against both Gram-positive and -negative bacterial strains (Azam et al, 2012). So, further studies needed in order to evaluate the anti-bacterial effect of Fe nano-particles in different size and treatment conditions.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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REFERENCES

- Aruoja V, Dubourguier H-C, Kasemets K, Kahru A (2009) Toxicity of nanoparticles of CuO, ZnO and TiO₂ to microalgae *Pseudokirchneriella subcapitata*. *Science of the total environment* 407(4):1461-8.
- Azam A, Ahmed A, Oves M, Khan MS, Memic A (1989) Size-dependent antimicrobial properties of CuO nanoparticles against Gram-positive and -negative bacterial strains. *Int J Nanomedicine* 7: 3527-3535.
- Bondarenko O, Ivask A, Käkinen A, Kahru A (2012) Sub-toxic effects of CuO nanoparticles on bacteria: kinetics, role of Cu ions and possible mechanisms of action. *Environmental pollution*.169:81-90.
- Chow, Anthony W.; Patten, Valerie; Guze, Lucien B. (1975) Comparative Susceptibility of Anaerobic Bacteria to Minocycline, Doxycycline, and Tetracycline". *Antimicrobial Agents and Chemotherapy* 7 (1): 46–49.
- Hadi M, Shokoohi R, Ebrahimzadeh Namvar A, Karimi M, Solaimany Aminabad M (2011) Antibiotic resistance of isolated bacteria from urban and hospital wastewaters in Hamadan City. *Iranian Journal of Health and Environment* 4(1):105-14.
- Masalha M; et al. (2001) Analysis of Transcription of the *Staphylococcus Aureus* Aerobic Class Ib and Anaerobic Class III Ribonucleotide Reductase Genes in Response to Oxygen. *Journal of Bacteriology*. 183 (24): 7260–7272.
- Noble W, Virani Z, Cree RG (1992) Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. *FEMS microbiology letters* 93(2):195-8.
- Ohira T, Yamamoto O, Iida Y, Nakagawa Z-e (2008) Antibacterial activity of ZnO powder with crystallographic orientation. *Journal of Materials Science: Materials in Medicine* 19(3):1407-12.
- Panáček A, Kvítek L, Prucek R, Kolář M, Večeřová R, Pizúrová N, et al (2006) Silver colloid nanoparticles: synthesis, characterization, and their antibacterial activity. *The Journal of Physical Chemistry B* 110(33):248-253.
- Soenen SJ, Himmelreich U, Nuytten N, Pisanic TR, Ferrari A, De Cuyper M (2010) Intracellular nanoparticle coating stability determines nanoparticle diagnostics efficacy and cell functionality. *Small* 6(19):2136-45.
- Sondi I, Salopek-Sondi B (2004) Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. *Journal of colloid and interface science* 275(1):177-82.
- Tiwari HK, Sen MR (2006) Emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) from a tertiary care hospital from northern part of India. *BMC Infectious diseases* 6(1):156.
- Tawale J, Dey K, Pasricha R, Sood K, Srivastava A (2010) Synthesis and characterization of ZnO tetrapods for optical and antibacterial applications. *Thin Solid Films* 519(3):1244-7.
- Tsiodras S, Gold H, Sakoulas G, Eliopoulos GM, Wennersten MD, Venkataraman L, Moellering Jr (2001) Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. *The Lancet* 358: 207–208.

Seroprevalence of *Neospora caninum* in dairy cows in Belgrade city area, Serbia

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ABSTRACT. The protozoan parasite *Neospora caninum* is one of the most important abortifacient pathogen in cattle. Serological investigations are often used in order to estimate seroprevalence in herds. Aims of our study were to determine the seroprevalence among aborting and non-aborting dairy cattle in Belgrade city area as well as epidemiological factors that are important for *N. caninum* infection. Using commercial ELISA kit, we examined 188 sera. Out of 188, 142 samples originated from pregnant (non-aborting) cows from five farms, while 46 were from cows who had aborted. Overall seroprevalence was 25% (48/188). Seroprevalence was significantly higher ($p \leq 0.05$) in aborting than in non-aborting group of cows (37% and 21.1% respectively). At least one positive sample was detected on four (80%) out of five examined farms while seroprevalence among farms varied from 0 to 43.5%. On all examined farms crucial epizootiological factors (presence of dogs and low biosecurity measures) that favor the maintenance and spreading of the infection were identified. Our study revealed the presence of *N. caninum* antibodies in population of dairy cows in Belgrade city area. Infection is established in enzootic pattern on examined farms and high seroprevalence among aborting cows suggests that *N. caninum* could be important abortifacient pathogen.

Keywords: *Neospora caninum*, seroprevalence, dairy cow, Serbia

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INTRODUCTION

The protozoan parasite *Neospora caninum* is considered as a major cause of abortion in cattle (Dubey and Schares, 2006). *N. caninum* is a coccidian parasite with a wide host range (Dubey and Schares, 2011). In general, neosporosis is primarily disease of cattle which are intermediate host while dogs and related canids are definitive hosts of *N. caninum* (Dubey et al., 2007). The life cycle of *N. caninum* is typified by 3 infectious stages: tachyzoites, tissue cysts, and oocysts. Tachyzoites and tissue cysts are found in the intermediate hosts and appear intracellularly. Dogs, as definitive host, got infected after ingestion of placentas and/or aborted fetuses containing tissue cysts. Infected dogs excrete unsporulated oocysts in feces (Dubey, 2003). Cattle can be infected through vertical or horizontal transmission. Vertical or trans-placental transmission from infected dams to their offsprings appears to be the major natural route of infection. Prenatally infected, but healthy calves remain persistently infected and can pass the infection to their own offsprings. Post-natal infection (horizontal transmission) occurs through ingestion of oocyst-contaminated fodder or drinking water (Innes et al., 2005; Conraths and Gottstein, 2007). Main feature of *N. caninum* infection in cattle is abortion although fetuses can be reabsorbed, mummified, stillborn, born alive with clinical signs or born clinically normal but persistently infected (Dubey and Schares, 2006). Cows of any age may abort starting from 3rd month of gestation to the term. Majority of neosporosis-induced abortions occur in 5-6 month of gestation (Dubey, 2003).

Infected cattle are three to seven times more likely to abort compared to uninfected cattle. Infected heifers are at the highest risk during the first pregnancy (Innes et al., 2005). Cows with *N. caninum* antibodies are more likely to abort than seronegative cows while up to 95% of live born calves from seropositive dams will be congenitally infected and clinically normal (Dubey, 2003; Dubey and Schares, 2006).

Many countries, such as Turkey (Kurtede and Ural, 2009; Kacar et al., 2012) Romania (Mitrea et al., 2012), China (Wang et al., 2010), Poland (Wisniewski et al., 2002), Netherland (Wouda et al., 1999), France (Ould-Amrouche et al., 1999), Brasil (Corbellini et al., 2002) and Hungary (Hornok et al., 2006) reported about *N. caninum*-associated abortions and sero-

prevalence in aborting as well as non-aborting cows. There are several reports regarding seroprevalence (Gavrilović et al., 2006; Klun et al., 2008; Vidić et al., 2011; Pavičić et al., 2011; Savović et al., 2012; Kuruca et al., 2013; Klun, 2014) and detection of *N. caninum* genome by molecular methods (Cvetojević et al., 2013, 2015; Klun, 2014) in Serbia.

The aims of our study were to determine the seroprevalence of *N. caninum* in dairy cattle as well as to identify epizootiological factors important for introduction, maintenance and spreading of *N. caninum* infection in examined farms in Belgrade city area, Serbia.

MATERIAL AND METHODS

Samples

We examined 188 serum samples from cows. This group consisted of 46 cows who had aborted and 142 pregnant (non-aborting) dairy cows. Serum samples from cows who had aborted were selected randomly from archive of samples that were submitted for diagnostic investigations of abortions during 2015 and 2016 from the Belgrade city area. For each of those cows, month of gestation in which abortion was detected was available and used for further evaluation of results. Pregnant cows included in this study originated from 5 farms (designated as A, B, C, D and E) from Belgrade city area. All pregnant cows were between fourth and seventh month of gestation in the moment of blood sampling. Farms included in our study were medium to large, breeding between 200 and 2500 black and white Holstein Friesian cows. A questionnaire form was completed in each farm with following data: number of cows on farm, abortion rate in previous year (sporadic: <3%; low: 3-5%), data regarding previously confirmed *N. caninum*-associated abortions (confirmed or not confirmed), presence of dogs on farm (present or absent), availability of fodder to dogs (available or not available), procedure for removing placentas after delivery and/or aborted fetuses in case of abortion (collecting and disposal to special container or leaving in the maternity barn and dispose with manure).

Serological analyses

Blood samples were collected by tail vein venipuncture. Blood was left at room temperature until the separation of serum. The presence of *N. caninum* antibodies

were determined using commercial enzyme-linked immunosorbent assay (ELISA) kit (Neospora caninum Antibody Test Kit, Idexx, Switzerland) according to manufacturer's instructions.

Statistical analyses

An odds ratio (OR) was used to measure the association between an exposure of cows to *N. caninum* and abortion as an outcome.

RESULTS

The overall seroprevalence of *N. caninum* was 25% (48/188). Antibodies were detected in 18 (37%) out of 46 cows who had aborted and in 30 (21.1%) out of 142 pregnant cows. These results showed significant difference ($p=0.016$) between cows that have been infected and healthy ones meaning that seropositive cows were 2.4 times more likely to abort than seronegative (OR = 2.4; 95% CL: 1.17-4.91). In positive aborting cows, abortions were detected from the third to the seventh month of gestation (mean 4.7 months). At least one positive sample was detected on four (80%) out of five examined farms while seroprevalence among farms varied from 0 to 43.5% (Table 1.). Abortion rate was low on two farms (farm A and C) and sporadic on three farms (farms B, D and E). Previous *N. caninum*-associated abortions were confirmed on three (B, D and E) out of five farms. To dogs, kept on all farms, fodder was easily accessible. Only on one farm (farm C), placentas and/or aborted fetuses were collected to special container while on other farms afterbirth material were left in maternity barn and later were disposed with manure.

Table I.

DISCUSSION

Researches from different countries report about various seroprevalences of *N. caninum* depending on category (aborting or non-aborting cows). Kacar et al. (2012) detected 7.4% (11/148) seropositivity among cows that had had abortion in region of Kars while Kurtede and Ural (2009) reported that *N. caninum* seroprevalence in Turkey varied from 2 to 13.96% depending on the region of the country. Mitrea et al. (2012) detected high seroprevalence (41.7%) among randomly sampled dairy cows on farms in southern Romania. Results of seroprevalence in non-aborting cows in our study was similar to prevalence in Poland (20.7%) (Wisniewski et al., 2002). Seroprevalence in our group of aborting cows was lower comparing to results from the Netherland where Wouda et al. (1999) found seroprevalence from 17 to 87% in herds with history of *N. caninum* abortion. Ould-Amrouche et al. (1999) reported on seroprevalence of 5.6% (107/1924) in Normandy in France. Much lower seroprevalence (2.5%) comparing to our study was reported from northeast Hungary (Hornok et al., 2006).

Seroprevalence of *N. caninum* obtained from our study is higher than previously reported from other researches in Serbia. Savović et al. (2012) detected overall seroprevalence of 17.3% (18.8% in aborting and 16.7% in non-aborting cows) in Vojvodina province. In south Banat region, Gavrilović et al. (2013) detected *N. caninum* antibodies in 4.6% (23/500) cows while among 27 aborting cows they found 7 (26%) positive. Kuruca et al. (2013) obtained overall seroprevalence of 15.4% (55/356) and among them 12.2% (9/74) in cows with reproductive disorders and 16.3% (46/356) in reproductively healthy cows. Vidić et al. (2011) found that 3.7% (5/132) of cows in

Farm	Number of cows on the farm	Abortion rate	Previously confirmed <i>N. caninum</i> -associated abortions	Presence of dogs	Availability of fodder to the dogs	Removing of placentas and/or aborted fetuses	Examined/positive samples
A	250	low	not confirmed	present	available	leave in barn	20/4 (20%)
B	1100	sporadic	confirmed	present	available	leave in barn	39/17 (43.5%)
C	200	low	not confirmed	present	available	special container	20/0 (0%)
D	1000	sporadic	confirmed	present	available	leave in barn	25/6 (24%)
E	2500	sporadic	confirmed	present	available	leave in barn	38/8 (21%)

Vojvodina province had *N. caninum* specific antibodies. In two different investigations, Klun et al. (2008, 2014) performed serological examinations from samples from all regions of Serbia. In pilot study, Klun et al. (2008) detected seroprevalence of 8.6% from 350 cows. In more detailed study, Klun (2014) found that 108 (7.6%) out of 1496 cows were seropositive to *N. caninum*. Despite global and regional differences in seroprevalence, *N. caninum* is globally widespread. However, results from different investigations should be interpreted with caution because of many variables that can influence on the obtained results (number of examined animals, animals included in study – aborting or non-aborting, diagnostic test used, different husbandry systems, regional specificity etc).

Specific antibodies may persist longlife but fluctuate, sometimes even below detection limit of serological tests (Dubey and Schares, 2006). As a confirmation of its significant fluctuation, Sager et al. (2001) reported, in repetitive serological investigations (at 3–12 months interval), decrease of the overall *N. caninum*-seroprevalence from 17 to 12%. According to Innes et al. (2005), serum antibody response to *N. caninum* in pregnant cattle fluctuates throughout gestation. Therefore, in order to increase the possibility to detect seropositive cows, we sampled cows between the fourth and the seventh month of gestation when rise of antibody levels is expected. Despite the fact that seropositive cows are three to seven times more likely to abort (Innes et al., 2005), an important question in disease understanding why not all infected cows abort has raised? To better understand mechanism of parasite-associated abortions and control of the disease, it would be of uttermost importance to define the markers related to the diagnosis and more importantly to the risk of abortion in the infected cow (Almeira and Lopez-Gatius, 2015).

Possible reasons for high seroprevalence obtained in our study could be low biosecurity which was common characteristic for all farms included in our study. Dogs on farms and accessibility to fodder along with lack of infrastructure and procedures for disposal of

afterbirth and/or abortion material (placentas, aborted fetuses) contribute to maintaining and spreading *N. caninum* throughout the farm. Interestingly, only one farm (farm C) in our study did not have any positive cow. Although, dogs were also present on this farm as well as had access to fodder, on that farm special attention was given to disposal of afterbirth and/or abortion material. It could be assumed that good management of disposal of afterbirth and/or abortion material could prevent and interrupt parasite life cycle on the farm. Nevertheless, the most important preventive measure is avoiding dogs, which are definitive hosts and source of this infection, to come into either direct or indirect contact with cows. Neosporosis-associated abortions in bovine herds may have an epizootic or enzootic pattern (Dubey and Schares, 2006). Annual abortion rates on examined farms are rather stable. Therefore, we can conclude that *N. caninum*-associated abortions on those farms would be expressed through enzootic pattern.

CONCLUSIONS

In conclusion, our study revealed the high seroprevalence of *N. caninum* antibodies in population of dairy cows in Belgrade city area. Based on epizootiological data from examined farms, infection is established in enzootic pattern. High seroprevalence among aborting cows suggests that *N. caninum* could be important abortifacient pathogen in examined region. Improving biosecurity on farms is the first precondition for implementation of possible programs for control or eradication of the disease.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. ■

REFERENCES

- Almeira S, Lopez-Gatius F (2015) Markers related to the diagnosis and to the risk of abortion in bovine neosporosis. *Res Vet Sci* 100:169-175.
- Conraths FJ, Gottstein B (2007) Neosporosis. In: *Protozoal abortion in farm ruminants: Guidelines for diagnosis and control*, Athenaeum Press, Gateshead: pp 42-122.
- Corbellini LG, Driemeier D, Cruz CFE, Gondim LFP, Wald V (2002) Neosporosis as a cause of abortion in dairy cattle in Rio Grande do Sul, Southern Brazil. *Vet Parasitol* 103:195-202.
- Cvetojević Đ, Kureljušić B, Jezdimirović N, Radanović O, Jakić-Dimić D, Veljović Lj, Ivetić V (2013) Usage of PCR in diagnosis of *Neospora caninum* induced abortions in cattle. In: *Proceedings of the Scientific symposium "Reproduction of domestic animals"*, Divčibare, Serbia: pp 179-180.
- Cvetojević Đ, Savić B, Stanojević S, Kureljušić B, Jezdimirović N, Bojković-Kovačević S, Pavlović M, Katić M, Jakić-Dimić D (2014) Cattle abortions caused by contagious infectious agents on PKB corporation's farms during 2014. In: *Proceedings of XXIX Conference of Agronomists, Veterinarians, Technologists and Agricultural Economists*, Belgrade, Serbia: pp 1-6.
- Dubey JP (2003) Review of *Neospora caninum* and neosporosis in animals. *Korean J Parasitol* 41:1-16.
- Dubey JP, Schares G (2006) Diagnosis of bovine neosporosis. *Vet Parasitol* 140:1-34.
- Dubey JP, Schares G, Ortega-Mora LM (2007) Epidemiology and Control of Neosporosis and *Neospora caninum*. *Clin Microbiol Rev* 20:323-367.
- Dubey JP, Schares G (2011) Neosporosis in animals—The last five years. *Vet Parasitol* 180:90-108.
- Gavrilović P, Živulj A, Todorović I, Jovanović M, Parunović J (2013) Investigation of importance of *Neospora caninum* in aetiology of abortion in dairy cows in Serbia. *Revue Méd Vét* 164:100-104.
- Hornok S, Edelhofer R, Hajtos I (2006) Seroprevalence of neosporosis in beef and dairy cattle breeds in northeast Hungary. *Acta Vet Hung* 54:485-491.
- Innes EA, Wright S, Bartley P, Maley S, Macaldowie C, Esteban-Redondo I, Buxton D (2005) The host-parasite relationship in bovine neosporosis. *Vet Immunol Immunopathol* 108:29-36.
- Kacar C, Gokce HI, Akca A, Gunsor O, Kaya S (2012) Seroprevalence of *Neospora caninum* in cows with abortion history and in dogs sharing the same area in the Kars Region, Turkey. *Revue Méd Vét* 163:343-347.
- Klun I, Nikolić A, Vujanić M, Ivović V, Bobić B, Djurković-Djaković O (2008) Pilot study of *Neospora caninum* seroprevalence in cattle in Serbia. In: *Program & Abstract Book of 10th European Multicolloquium of Parasitology*, Paris, France: pp 192.
- Klun I (2014) Seroprevalence and molecular diagnosis of *Neospora caninum* infection in cattle in Serbia, PhD Thesis, Faculty of veterinary medicine, University of Belgrade, Serbia.
- Kurtede A, Ural K (2009) Serological status, epidemiology and prevalence of bovine *Neospora caninum* infection in Turkey: a review of published studies. *Revue Méd Vét* 160:244-251.
- Kuruca Lj, Spasojević-Kosić Lj, Simin S, Savović M, Lauš S, Lalošević V (2013) *Neospora caninum* antibodies in dairy cows and domestic dogs from Vojvodina, Serbia. *Parasite* 20:40.
- Mitreă IL, Enăchescu V, Radulescu R, Ionita M (2012) Seroprevalence of *Neospora caninum* infection on dairy cattle in farms from southern Romania. *J Parasitol* 98:69-72.
- Ould-Amrouche A, Klein F, Osdoit C, Mohammed HO, Touratier A, Sanaa M, Mialot J (1999) Estimation of *Neospora caninum* seroprevalence in dairy cattle from Normandy, France. *BMC Vet Res* 30:531-538.
- Pavičić Lj, Lalošević V, Spasojević-Kosić Lj, Lauš S, Simin S (2011) Seroprevalence of *Neospora caninum* in dogs. *Contemporary agriculture* 60:453-457.
- Sager H, Fischer I, Furrer K, Strasser M, Waldvogel A, Boerlin P, Audige L, Gottstein B (2001) A Swiss case-control study to assess *Neospora caninum*-associated bovine abortions by PCR, histopathology and serology. *Vet Parasitol* 102:1-15.
- Savović M, Lalošević V, Simin S, Pavičić Lj, Boboš S (2012) Seroprevalence of *Neospora caninum* in dairy cows with reproductive disorders in Vojvodina province, Serbia. *Lucrari Științifice Medicină Veterinară* 65:161-166.
- Vidić B, Grgić Ž, Savić S, Bugarski D (2011) *Neospora caninum* causes abortions in cows. In: *Proceedings of the Scientific Symposium "Reproduction of Domestic Animals"*, Divčibare, Serbia: pp 133-134.
- Wang C, Wang Y, Zou X, Zhait Y, Gao J, Hou M, Zhui X (2010) Seroprevalence of *Neospora caninum* Infection in Dairy Cattle in Northeastern China. *J Parasitol* 96:451-452.
- Wisniewski M, Cabaj W, Moskwa B, Wedrychowicz H (2002) The first detection of *Neospora caninum* DNA in brains of calves in Poland. *Acta Vet-Beograd* 52:393-400.
- Wouda W, Bartels CJM, Moen AR (1999) Characteristic of *Neospora caninum*- associated abortion storms in dairy herds in the Netherlands. *Theriogenology* 52:233-245.

Effect of natural neosporosis on levels of testosterone and thyroid hormones in bulls

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ABSTRACT. Present study was aimed to investigate the effect of *Neospora caninum* seropositivity on testosterone and thyroid hormones levels of Iranian bulls. Two groups containing bulls with neosporosis and bulls without neosporosis were considered. To achieve bulls with neosporosis, *N. caninum* agglutination test (NAT) and ELISA was performed. Testosterone, thyroxin (T4) and tri-iodothyronine (T3) levels were measured and compared between infected and non-infected bulls. Testosterone of bulls with neosporosis was decreased but the difference was not significant. Serum T4 of bulls with neosporosis was decreased significantly but changes of serum T3 was not significant. The hypothalamic–pituitary–adrenal axis (HPA axis) impairment, direct effect of *N. caninum* and its antigens on testis function, interrelationships between thyroid dysfunction and hypogonadism, adaptive response of infected animals to *N. caninum* and occurrence of oxidative stress in testis due to the presence of *Neospora* may be the reasons of hormonal changes. The effect of *N. caninum* on hormonal changes remains incomplete at present and is an area for future research so as to better characterize the effects and mechanisms.

Keywords: *Neospora caninum*; Testosterone; Thyroxin; Tri-iodothyronine, Bull

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INTRODUCTION

The coccidian parasite *Neospora caninum* is close to *Toxoplasma gondii* (Dubey and Lindsay, 1996) and it causes abortions and economic losses in cattle (Dubey, 1999). Dogs have been proved to be both intermediate and definitive hosts and cattle and other animals to be its natural intermediate hosts (Dubey, 1999). Routes of *Neospora* transmission include transplacental infection through tachyzoites, ingestion of tissues harbouring cysts and oral uptake of sporozoite-containing oocysts. Transplacental transmission seems to be very efficient for *N. caninum* in naturally infected cattle and plays a major role in the maintenance and spread of the disease (Davison et al. 1999). Other sources of vertical transmission, such as cow to calf transmission via pooled colostrum or milk could also be possible (Uggla et al. 1998). Previous reports showed that the seroprevalence in cattle is high in many countries, and that 12–42% of aborted fetuses from dairy cattle is infected with *N. caninum* (Dubey, 2003). Due to its high prevalence and association with abortion in cattle worldwide, neosporosis has emerged as an economically important disease for the livestock industry (Dubey et al. 2003). More recently, it has been shown that *N. caninum* is detectable in semen from infected bulls but there is no evidence to suggest that this is an important route of transmission (Ortega-Mora et al. 2003). Despite the existence of *N. caninum* in genitalia its effect on testosterone levels has not been studied. Testosterone and their derivatives (dihydrotestosterone and dehydroepiandrosterone) are androgens produced mainly in male gonads, adrenal glands and the brain. Testosterone can act directly as a ligand of androgen receptors (AR) found in several target tissues. Androgens stimulate the development of the secondary sexual characters in males; participate in reproduction and maturation of fetal testes (O'Shaughnessy and Fowler, 2014).

Thyroid gland is an essential gland in the body of cows that produces essential hormones regulated by the hypothalamic-pituitary-thyroid axis (Laposata, 2010). The main function of thyroid gland is to secrete thyroxin to regulate basal metabolic rate mostly this hormone acts through nuclear receptors that are transcribed by

numerous genes and these genes regulate a number of critical physiological functions in development and metabolism (Boelaert and Franklyn, 2005). Male reproduction is adversely affected by both thyrotoxicosis and hypothyroidism. Thyrotoxicosis induces abnormalities in sperm motility, whereas hypothyroidism is associated with abnormalities in sperm morphology; the latter normalize when euthyroidism is reached (Poppi et al. 2007). Based on the absence of documented data about testosterone and thyroid hormones levels of bulls with neosporosis the present study aimed to compare testosterone, thyroxin (T4) and triiodothyronine (T3) levels in non-infected and *N. caninum* infected bulls.

MATERIALS AND METHODS

Study design and samples preparation

In the present study samples were collected from the Ahvaz slaughterhouse (center of Khuzestan province, southwest of Iran) from January 2016 to April 2016. To reduce the impact of temperature and season sampling was restricted to winter. Khuzestan province has a border of about 64236 km², between 47 degree and 41 minutes to 50 degree and 39 min of eastern longitude from prime meridian and 29 degree and 58 min to 33 degree and 4 min of northern latitude from equator (Statistical book of Khuzestan province, 2006). The province has hot and wet summers, mild spring and cold winters. The bull's population mainly comprises local domestic species, which are well adapted to the climate of the area. The slaughterhouse was visited twice a week and blood were collected from mature bulls aged 24–48 months; estimated based on the dental combination (Cockrill, 1974). Since, the plasma levels of thyroid hormones may be altered also by other nutrition- and metabolism-related factors, such as selenium and/or iodine deficiency/supplementation (Wichtel et al. 1996; Awadeh et al. 1998), therefore it was tried to take samples from known farms in slaughterhouse (farms with acceptable management, fed with proper rations, with frequent veterinary care and etc.). The blood samples were collected from the jugular vein into sterile vacuum tubes. Sera were kept at –20 °C pending analysis.

Diagnosis of neosporosis by agglutination test and Elisa

In the present study two groups containing bulls with neosporosis and bulls without neosporosis were considered. To achieve bulls with neosporosis, *N. caninum* agglutination test (NAT) was performed in 96 round-bottom-well microplates according to the method previously described for toxoplasmosis (Desmonts and Remington, 1980). In brief, 50 µl of 0.2 M 2-mercaptoethanol in PBS was distributed in each well and sera were diluted two-fold up to 128, starting at 1:2. Tachyzoites of *N. caninum* NC-1 isolate were resuspended in alkaline buffer (7.02 g NaCl, 3.09 g H₃BO₃, 24 ml of 1 N NaOH, 4 g bovine plasma albumin (fraction V), and enough distilled water to bring the volume to 1 l; pH 8.7) and their concentration was adjusted at $2 \times 10^4/\mu\text{l}$. After the sera had been diluted, 50 µl *N. caninum* antigen suspensions were distributed in each well. Plates were gently agitated to allow for complete mixing and were then incubated overnight at 30 °C. A clear-cut button-shaped deposit of parasite suspension at the bottom of the well was interpreted as a negative reaction, and a complete carpet of agglutinated organisms was considered positive. Each assay included two negative controls and one positive control. A serum sample obtained from a rabbit with an experimental *N. caninum* infection was selected as the positive control. Those samples with doubtful results were re-tested.

For definitive diagnosis of bulls infected with *N. caninum* and non-infected ones, positive and negative samples from NAT test were re-examined by an indirect non comparative ELISA. The sera were analyzed for detecting antibody to *N. caninum* by using the commercially ELISA kit (IDEXX, USA) according to the manufacture's instruction. OD of 0.15 was considered as cut-off based on the instruction of manufacture and, the ratio of sample for positive control was $\geq 0.2\text{OD}$. Overall, 30 bulls infected with neosporosis and 15 non-infected bulls were selected for the present study.

Screening bulls for brucellosis

Since, brucellosis can affect the bulls reproduction therefore, all serums were tested for *Brucella* genus using slide agglutination by rose bengal test at cell

concentrations and tube agglutination test (TAT) by 2-mercaptoethanol, using whole cell antigen (Razi Vaccine and Serum Research Institute) used for the presence of antibodies against *B. abortus* strain. The positive animals were deleted from the study.

Hormone analysis

For each sample, T3 serum was measured by competitive enzyme immunoassay and using T3 kit of Auto bio Diagnostic Co. with sensitivity of 0.4 (µg/dl) and T4 serum was gauged by competitive enzyme immunoassay utilizing T4 kit of Auto bio Diagnostic Co. with sensitivity of 0.2 (µgr/dl).

The testosterone level was assayed using the testosterone test kits (Monobind Inc., Lake Forest, USA) based on the enzyme-linked immunosorbent assay (ELISA) technique (Ekins, 1998).

Statistical analysis

Data generated from the study were subjected to one-way analysis of variance (ANOVA). Variant means were separated using the least significant difference (LSD) method. Significance was accepted at a probability level of less than 0.05.

RESULTS

The levels of testosterone and thyroid hormones in bulls with or without neosporosis are presented in Table1. Testosterone of bulls with neosporosis was decreased but the difference was not significant ($p=0.06$). Changes of serum T3 was not significant in bulls with or without neosporosis ($p=0.12$) but serum T4 of bulls with neosporosis was decreased significantly ($p=0.04$).

DISCUSSION

Tyroxine (T4) has been known as the predominant product of the thyroid gland for many years. Its production and liberation is governed by the hypothalamus/anterior pituitary axis. First, thyrotropin-releasing hormone (TRH), a neuropeptide produced in the paraventricular nucleus (PVN) of the hypothalamus, controls the release of thyroid-stimulating hormone (TSH) from the anterior pituitary. TSH acts on receptors on the thyroid to

promote synthesis and release of the thyroid hormones, mainly of T4, but also in a small quantity of 3,3',5-triiodothyronine (T3). T4 is changed to the active T3 and T3 is more potent than T4 (Yen, 2001). Thyroid hormones mainly influence the thermoregulation and homeostasis of energy and protein metabolism. Also, they involve in the metabolic response of animals to certain nutritional, environmental and/or disease-related challenges, as well as in regulation of certain ovarian functions. Furthermore, normal thyroid hormone levels play an important role in testicular development and its function (Achermann et al. 1999). Alteration in thyroid hormones (particular hypothyroidism) negatively affects gonadotropin secretion (like testosterone) and semen quality (Choksi et al. 2003; Wagner et al. 2008). Testosterone is the primary male sex hormone and an anabolic steroid. testosterone plays a key role in the development of male reproductive tissues such as the testis and prostate, as well as promoting secondary sexual characteristics such as increased muscle and bone mass, and the growth of body hair (Mooradian et al. 1987). In addition, testosterone is essential for health and well-being (Bassil et al. 2009), and for the prevention of osteoporosis. Insufficient levels of testosterone may lead to abnormalities including frailty and bone loss (Tuck and Francis, 2009). In the present study, serum T4 of bulls with neosporosis was decreased significantly but changes of serum T3 was not significant. Furthermore, testosterone of bulls with neosporosis was decreased but the difference was not significant. In association with *N. caninum* effect on thyroid and testosterone hormones five hypotheses can be raised.

In the first hypothesis central nervous system involvement is concerned. The hypothalamic–pituitary–adrenal axis (HPA axis) is a complex set of direct influences and feedback interactions among three endocrine glands: the hypothalamus, the pituitary gland and the adrenal glands (Otmishi et al. 2008). The mentioned axis has the key role on reproduction. The hypothalamus senses low circulating levels of thyroid hormone (T3 and Thyroxine T4) and responds by releasing thyrotropin-releasing hormone (TRH). The TRH stimulates the pituitary to produce thyroid-stimulating hormone

(TSH). The TSH, in turn, stimulates the thyroid to produce thyroid hormones. On the other hand, gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus by GnRH-expressing neurons. The anterior portion of the pituitary gland produces luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and the gonads produce estrogen and testosterone. Due to the importance of hypothalamic–pituitary–adrenal axis in controlling hormones, any disorders in the mentioned axis can disturb the hormones levels. Nishimura et al. (2013) suggest that the cerebrum, especially amygdala, hippocampus, and hypothalamus, are the main areas that can be infected with *N. caninum*. Focal necrosis, glial activation and perivascular cuffing was the histopathological changes of cerebrum in their study. Based on their results and due to the injuries in cerebrum (hypothalamus) hypothalamic–pituitary–adrenal axis disorder can be proposed. Hypothalamic–pituitary axis dysfunction affects thyroid and sex steroid hormones which influence on male reproduction (Achermann et al. 1999, Choksi et al. 2003). Thyroid dysfunction (hypothyroxinaemia) as a result of thyrotropin-releasing hormone impairment (TRH), thyroid-stimulating hormone (TSH) and decreasing serum thyroxine (T4) levels reported in *T. gondii* infected mice by Stahl et al. (Stahl and Kaneda, 1998a; Stahl and Kaneda, 1998b).

Studies in Nylar female mice infected with *T. gondii*, exhibited hypogonadotrophic hypogonadism secondary to hypothalamic dysfunction. These mice infected with *T. gondii* Cornell strain, present atrophy in the thymus, ovaries, and uterus, cessation of cycling, anovulation, and decline of serum thyroxine (T4) levels (Stahl et al. 1985).

The second hypothesis about reduction of testosterone in bulls with neosporosis can be due to the direct effect of *N. caninum* and its antigens on testis function. Recently, the presence of *N. caninum* in the semen of naturally and experimental infected bulls have been described and it appears that certain cell types that are present in the cellular fraction of semen could harbor *N. caninum*. It seems that immune cells, such as mononuclear phagocytic cells, are responsible for protozoa transport in blood and semen, as circulating antibodies and complement could kill extra cellular parasites during reactivation

(Serrano- Martinez et al. 2007). Trafficking of leukocytes to disseminate intracellular parasites via a Trojan horse- type mechanism has been approved for *T. gondii* and *N. caninum* (Barragan et al. 2003). Abnormality of testis structure or function can lead to testosterone level changes.

The next hypothesis about testosterone and thyroid hormones imbalance can be related to the interrelationships between thyroid dysfunction and hypogonadism in bulls with neosporosis. Several studies have confirmed that thyroid hormone deficiency affects all tissues of the body, including multiple endocrine changes that alter growth hormone, corticotrophin, glucocorticoids, and gonadal function. Primary hypothyroidism is associated with hypogonadotropic hypogonadism, which is reversible with thyroid hormone replacement therapy (Meikle, 2004). It seems that neosporosis may affect this relationship.

High concentrations of testosterone are known to have immunosuppressive effects (Roberts et al. 2009). Therefore, the results of the present study, namely the decreased concentration of testosterone in *Neospora* infected bulls, make the immunosuppression based explanation of the association between *Neospora* infection and testosterone concentration unlikely. Therefore, the forth hypothesis is raised about the adaptive response of infected animals to *N. caninum*. In fact, it could be speculated that the decrease of testosterone concentration could be an adaptive response of infected animals to *Neospora*- induced immunosuppression. By decreasing the concentration of testosterone, the infected bulls could partly compensate the chronic neosporosis associated down regulated cellular immunity. Such compensation might increase the probability of the survival of infected bulls after contact with various pathogens in their natural environment.

Despite the low oxygen tensions that characterize the testicular micro-environment, this tissue remains vulnerable to oxidative stress due to the abundance of highly unsaturated fatty acids (particularly 20:4 and 22:6) and the presence of potential reactive oxygen species (ROS)-generating systems. ROS generation can be from the mitochondria and a variety of enzymes including the xanthine- and

NADPH-oxidases (Banfi et al. 2001; Kumagai et al. 2002), and the cytochrome P450s (Zangar et al. 2004). These enzymes specialize in the professional generation of ROS or produce these toxic metabolites as an inadvertent consequence of their biochemical activity. In order to address this risk, the testes have developed a sophisticated array of antioxidant systems comprising both enzymatic and non-enzymatic constituents. Concerning the enzymatic constituents of this defense system, the induction of oxidative stress in the testes precipitates a response characterized by the NF κ B mediated induction of mRNA species for superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities (Kaur et al., 2006). Recent investigations indicate that parasitic infections with high tolerance of the host are the result of defense mechanisms which include enhanced generation of reactive oxygen species (ROS) (Boczon et al., 1996; Sanchez-Campos et al., 1999). Therefore, occurrence of oxidative stress in testis of bulls with neosporosis can be the fifth hypothesis. Presence of *N. caninum* in testis and its antigens may trigger oxidative stress. In our previous study there was no significant differences in SOD activity and MDA levels in testis of bulls with neosporosis but GPX activity was significantly elevated in infected bulls and this finding indicated oxidant/antioxidant imbalances in testis of bulls with neosporosis (Bahrami et al., unpublished).

In conclusion, it seems that during the neosporosis alterations of thyroid hormones and testosterone can occur that can affect several behavioral, physiologic and immunological parameters for a long time. Albeit several influencing factors including parasite strain, doses, and routes of parasite inocula, as well as host variation in susceptibility to infection may directly affect the course of infection and hormones alterations. It's our future plan to investigate experimental neosporosis effects on male and female reproductive parameters and hormonal alterations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1: Mean \pm standard deviation of Testosterone, T3 and T4 levels (ng/ml) in non-infected and *N. caninum* infected bulls.

Groups	Hormones (ng/ml)		
	Testosterone	T3	T4
Infected	3.1 \pm 0.8 ^a	3.31 \pm 0.1 ^a	7.42 \pm 1.9 ^a
Non- infected	4.6 \pm 1.4 ^a	3.42 \pm 0.2 ^a	9.61 \pm 1.7 ^b

Values in columns with different lowercase superscripts are significantly different ($p < 0.05$).

REFERENCES

- Achermann JC, Jameson JL (1999) Fertility and infertility: genetic contributions from the hypothalamic-pituitary-gonadal axis. *Mol Endocrinol* 13(6): 812-818.
- Awadeh FT, Kincaid RL, Johnson KA (1998) Effect of level and source of dietary selenium on concentrations of thyroid hormones and immunoglobulins in beef cows and calves. *J Anim Sci* 76: 1204-1215.
- Bánfi B, Molnár G, Maturana A, Steger K, Hegedűs B, Demareux N, Krause KH (2001) A Ca(2+)-activated NADPH oxidase in testis, spleen, and lymph nodes. *J Biol Chem* 276:37594-37601.
- Barragan A, Sibley LD (2003) Migration of *Toxoplasma gondii* across biological barriers. *Trends Microbiol* 11: 426-430.
- Bassil N, Alkaade S, Morley JE (2009) The benefits and risks of testosterone replacement therapy: a review. *Ther Clin Risk Manag* 5 (3): 427-448.
- Boczón K, Hada's E, Wandurska-Nowak E, Derda M (1996) A stimulation of antioxidants in muscles of *Trichinella spiralis* infected rats. *Acta Parasit* 41: 136-138.
- Boelaert K, Franklyn JA (2005) Thyroid hormone in health and disease. *J Endocrinol* 187: 1-15.
- Choksi NY, Jahnke GD, St Hilaire C, Shelby M (2003) Role of thyroid hormones in human and laboratory animal reproductive health. *Birth Defects Res B Dev Reprod Toxicol* 68(6): 479-491.
- Cockrill WR (1974) Management, conservation and use. In: *The Husbandry and Health of the Domestic Buffalo*. Cockrill WR (ed). Food and Agricultural Organization, Rome, Italy, pp. 276-312.
- Davison HC, Otter A, Trees AJ (1999) Estimation of vertical and horizontal transmission parameters of *Neospora caninum* infections in dairy cattle. *Int J Parasitol* 29: 1683-1689.
- Desmonts G, Remington JS (1980) Direct agglutination test for diagnosis of *Toxoplasma* infection: method for increasing sensitivity and specificity. *J Clin Microbiol* 11: 562-568.
- Dubey JP (2003) Review of *Neospora caninum* and neosporosis in animals. *Korean J Parasitol* 41(1): 1-16.
- Dubey JP, Lindsay DS (1996) A review of *Neospora caninum*. *Vet Parasitol* 67: 1-59.
- Dubey JP (1999) Recent advances in *Neospora* and neosporosis. *Vet Parasitol* 1589: 1-19.
- Ekins RP (1998) Ligand assays: from electrophoresis to miniaturized microarrays. *Clin Chem* 44: 2015-2030.
- Kaur P, Kaur G, Bansal MP (2006) Tertiary-butyl hydroperoxide induced oxidative stress and male reproductive activity in mice: Role of transcription factor NF-kappa B and testicular antioxidant enzymes. *Reprod Toxicol* 22: 479-484.
- Kumagai A, Kodama H, Kumagai J, Fukuda J, Kawamura K, Tanikawa H, Sato N, Tanaka T (2002) Xanthine oxidase inhibitors suppress testicular germ cell apoptosis induced by experimental cryptorchidism. *Mol Hum Reprod* 8: 118-123.
- Laposata M (2010) Laboratory medicine, the diagnosis of disease in clinical laboratory. 1st Edn. McGraw Hill Company, USA. pp.394-400.
- Meikle AW (2004) The interrelationships between thyroid dysfunction and hypogonadism in men and boys. *Thyroid* 14 (1): 17-25.
- Mooradian AD, Morley JE, Korenman SG (1987) Biological actions of androgens. *Endocr Rev* 8 (1): 1-28.
- Nishimura M, Kohara J, Hiasa J, Muroi Y, Yokoyama N, Kida K, Xuan X, Furuoka H, Nishikawa Y (2013) Tissue distribution of *Neospora caninum* in experimentally infected cattle. *Clin Vaccine Immunol* 20: 309-312.

- Ortega-Mora LM, Ferre I, del-Pozo I, Caetano-da-Silva A, Collantes-Fernandez E, Regidor- Cerrillo J, Ugarte-Garagalza C, Aduriz G (2003) Detection of *Neospora caninum* in semen of bulls. *Vet Parasitol* 117: 301-308.
- O'Shaughnessy PJ, Fowler PA (2014) Development of the human fetal testis. *Ann endocrinol* 75: 48-53.
- Otmishi P, Gordon J, El Oshar S, Li H, Guardiola J, Saad M, Proctor M, Yu J (2008) Neuroimmune interaction in inflammatory diseases. *Clin Med Circ Respirat Pulm Med* 2: 35-44.
- Poppe K, Velkeniers B, Glinioer D (2007) Thyroid disease and female reproduction. *Clin Endocrinol* 66(3): 309-321.
- Roberts Mark L, Buchanan Katherine L, Evans Matthew R, Marin Raul H, Satterlee DG (2009) The effects of testosterone on immune function in quail selected for divergent plasma corticosterone response. *J Experimental Biol* 212: 3125-3131.
- Sanchez-Campos S, Tunon MJ, Gonzales P, Gonzales-Gallego J (1999) Oxidative stress and changes in liver antioxidant enzymes induced by experimental dicroceliosis in hamsters. *Parasitol Res* 85: 468-474.
- Serrano- Martinez E, Ferre I, Martinez A, Osoro K, Mateos- Sanz A, Del- Pozo I, Aduriz G, Tamargo C, Hidalgo CO, Ortega-Mora LM (2007) Experimental neosporosis in bulls: parasite detection in semen and blood and specific antibody and interferon-gamma responses. *Theriogenology* 67(6): 1175-1184.
- Stahl W, Dias JA, Turek G (1985) Hypothalamic-adenohypophyseal origin of reproductive failure in mice following chronic infection with *Toxoplasma gondii*. *Proc Soc Exp Biol Med* 178: 246-249.
- Stahl W, Kaneda Y (1998a) Impaired thyroid function in murine toxoplasmosis. *Parasitol* 117(Pt 3): 217-222.
- Stahl W, Kaneda Y (1998b) Aetiology of thyroidal dysfunction in murine toxoplasmosis. *Parasitol* 117(Pt 3): 223-227.
- Tuck SP, Francis RM (2009) Testosterone, bone and osteoporosis. *Front Horm Res* 37: 123-132.
- Uggla A, Stenlund S, Holmdahl OJM, Jakubek EB, Thebo P, Kindahl H, Björkman C (1998) Oral *Neospora caninum* inoculation of neonatal calves. *Int J Parasitol* 28: 1467-1472.
- Wagner MS, Wajner SM, Maia AL (2008) The role of thyroid hormone in testicular development and function. *J Endocrinol* 199(3): 351-365.
- Wichtel JJ, Craigie AL, Freeman DA, Varela-Alvarez H, Williamson NB (1996) Effect of selenium and iodine supplementation on growth rate and on thyroid and somatotrophic function in dairy calves at pasture. *J Dairy Sci* 79: 1865-1872.
- Yen PM (2001) Physiological and molecular basis of thyroid hormone action. *Physiol Rev* 81: 1097-1142.
- Zangar RC, Davydov DR, Verma S (2004) Mechanisms that regulate production of reactive oxy- gen species by cytochrome P450. *Toxicol Appl Pharmacol* 199: 316-331.

Effects of testosterone administration and feeding level on reproductive activity in sexually inactive goat bucks

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ABSTRACT. The aim of this study was to investigate the effect of the administration of testosterone to a well-fed or underfed sexually inactive goat bucks on their sexual behavior and semen characteristics. Twenty sexually experienced mixed-breed goat bucks (1.5 years of age) were randomly assigned to one of four treatments for a period of 71 days. Treatments consisted of diets with amount of nutrients to meet 1.0 times the nutrient maintenance requirements with the application of 25 mg testosterone every third day during 21 d (treatment 1T) or saline (treatment 1C). A third and fourth treatments received a diet 1/2 times the nutrient maintenance requirements with testosterone (1/2T) or saline (1/2C). Bucks on 1T and 1C had higher body weight, body condition score and scrotal circumference compared to 1/2T and 1/2C ($P<0.05$). Also, bucks on 1T and 1/2T had higher sexual odor compared to 1C and 1/2C ($P<0.05$). Mean serum testosterone concentrations were highest in 1T (4.78 ± 6.78 ng/ml) and lowest in 1/2C (0.56 ± 0.96 ng/ml; $P<0.01$). Semen volume, sperm concentration, and mass motility were not affected by treatments, but sperm progressive motility was lower ($P<0.05$) in 1/2C (46%) than the other treatments (52 to 57%). Courtship traits and mounts were more frequent ($P<0.05$) in 1T compared with the other treatments. Likewise, bucks in the 1T group had the shortest latency to first mount (82 seconds) compared to other treatments (110 to 164 seconds). These results indicate that testosterone administration to well-fed sexually inactive bucks provokes clearly defined sexual activity during the non-breeding season. However, these benefits are overridden by underfeeding goat bucks.

Keywords: Semen characteristics; Flehmen; Sexual odor; Sperm motility; Scrotal circumference

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INTRODUCTION

Most goats in developing countries are exploited on pastures, managed under traditional extensive systems. Grazing goat bucks need an even and continuous supply of feed for their maintenance, and other productive processes, such as reproduction. Goat bucks on arid and semi-arid rangelands subsist solely on native vegetation throughout the year, which implies successive periods of shortages and surfeits of forage. Under these circumstances nutrient supply and body energy reserves vary widely during the year, affecting the reproductive activity of goat bucks (Zarazaga et al., 2009). Another consequence of underfeeding goat bucks is a significant detrimental effect on the testicular development and semen quality in these animals (Almeida et al., 2007; Zarazaga et al., 2009).

Another important factor affecting buck sexual drive is the season of the year. In temperate zones, libido, fertility, and semen quality and volume are better in late summer and fall (Roca et al., 1992; Karagiannidis et al., 2000; Barkawi et al., 2006). As the daylength gets longer, sperm concentration is lower, whereas it is noticed that the percentage of morphologically abnormal spermatozoa may be increased. (Barkawi et al., 2006; Ramadan et al., 2009). Recent reports indicate that the administration of testosterone to bucks during the non-breeding period (Spring) elicits a strong sexual behavior in goat bucks (Luna-Orozco et al., 2012; Ángel-García et al., 2015a), which in turn induces ovulation in anestrus goats. However, it is unknown if the effect of exogenous testosterone is modified by the nutritional status of bucks. Therefore, the objective of this study was to determine whether nutritional status combined with the administration of testosterone influence the sexual behavior and semen quality of goat bucks during the non-breeding season.

MATERIAL AND METHODS

Study area and animal housing and management

The Autonomous Agrarian University Antonio Narro ethics committee approved all the experimental work. This study was carried out in northern Mexico (25° N, mean annual temperature 23.5°C). The climate is semi-arid, with hot spring and summer and

mild winters. Most of the precipitation occurs during the summer and fall.

A total of 20 mature mixed-breed (native x dairy breeds) goat bucks, initially averaging 41 ± 3.7 kg body weight (BW) and 2.9 ± 0.3 body condition score (5-point scale), with similar testis size at the start of the study were used. Prior to the experiment, the goat bucks were kept under rangeland conditions. Then the goat bucks were housed in a 4 x 10 m outdoor roofed pens with dirt floors, under ambient temperature and lighting. The trial was carried out over a period of 71 days (February 3 to April 15, 2014).

The bucks were randomly allotted to 4 experimental groups: 1.0 x the maintenance nutrient requirements with (1T, n= 5) or without (1C, n=5) administration of testosterone, and 0.5 x the maintenance nutrient requirements with (1/2T, n= 5) or without (1/2C, n=5) testosterone. The goat bucks on the 1.0 x the maintenance nutrient requirements diet were offered 500 g day⁻¹ of alfalfa hay, 1100 g of oat hay, 200 g of molasses and 300 g day⁻¹ of concentrate (14% crude protein). Those on the 0.5 diet were offered half of the feed previously described. The different nutritional planes were based on NRC (2007) and the goat bucks were provided with these diets for 71 days. Water and salt-mixed trace minerals were offered ad libitum. From March 24 to April 14, bucks on the 1T and 1/2T received 25 mg testosterone (Testosterona 50®, Brovel Laboratory, Mexico D.F., Mexico) i.m. every third day. Bucks on the 1C and 1/2C groups received saline injections.

Collection of data

The change in body weight and body condition score on each treatment was recorded weekly. Body condition score (BCS) was assessed by palpating the muscle mass at the lumbar region of goat bucks, and values were assigned based on a 1–5 points scale (1= very thin, 5= very fat; Detweiler et al., 2008). Scrotal circumference measurements were recorded weekly on all goat bucks throughout the experimental period. Measurements were done by using a flexible measuring tape placed in the widest part of both testicles.

The intensity of the odor was recorded weekly for each buck, by smelling the dorsum of the neck

approximately 15 cm behind the caudomedial border of each horn base (Walkden-Brown et al., 1999). The score scale used was from 0 (neutral odor, not different from a female) to 3 (strong male odor).

Ejaculates were successfully collected on all bucks during 5 consecutive weeks, at the middle of the supplementation period (5 weeks after the beginning of the feed supplementation), by means of an artificial vagina. The total volume of the ejaculate was measured using a calibrated test tube. Sperm mass motility of undiluted semen was subjectively estimated by a light microscope (x 400) at 37° C. Three different microscopic fields for each semen sample were observed and the mean of these estimations gave the motility score using a scale from 0 (no activity) to 5 (rapid swirling motion). Sperm concentration was evaluated by a spectrophotometer calibrated for goat bucks (Minitube, Tiefenbach, Germany) following the protocol described by Prathalingam et al. (2006). Furthermore, progressive motility was assessed by a light microscope (x400) at 37°C.

On 14 and 15 of April of the experimental period, a sexual behavior test was conducted. Goat bucks from all experimental groups were individually exposed to one unrestrained estrogenized female for 20 min in an isolated pen. The sexual behaviors were recorded uninterruptedly during the test period by an observer positioned outside the pen at a distance of approximately 2.5 m. Each buck was used in only one test per day. Two females were estrogenized by administering 2 mg estradiol cypionate i.m. every third day (ECP®, Lab. Pharmacia & Upjohn, Mexico) during 3 consecutive weeks. The behavioral components recorded were: latency to first mount, number of vocalizations, anogenital sniffing, flehmen response, approximations to females, mount attempts, mounts without ejaculation, mounts with exposed penis, mounts with ejaculation (buck thrusting forward and leaping off the ground, propelling the doe forwards). Total serum testosterone was assessed using an ELISA assay according to the kit protocol (Neogen Corporation #402510; Lexington, KY, USA). Ten blood samples (5 ml) were collected weekly during the study period into Vacutainers (Corvac Kendall Health Care, St. Louis, USA) from the jugular vein of each goat buck, and allowed to clot at room temperature during 30 min. Serum was separated by

centrifugation at 5000 g for 10 min, immediately transferred to polypropylene micro tubes (Axygen Scientific, Union City, USA) and stored at -20°C until further analysis.

Statistical analyses

The Shapiro Wilk W-test was used to analyze data for normality. Heterogeneity of variance was found for courtship behaviors, mount attempts, and mounts with ejaculations using Bartlett's Box F-test specifying this option in the MEANS Statement in SAS (SAS Inst. Inc., Cary, NC). The data for courtship behaviors, mounts, and mounts with ejaculations were transformed to the reciprocal of square root of $X + 1$, log of $X + 1$, and square root of $X + 0.1$, respectively, which stabilized the variances among goat bucks for the fixed effects. Data of sperm motility were arcsine transferred prior to analysis. The effect of feeding level and testosterone administration on goat buck body weights and BCS was determined using mixed-model methodology using PROC MIXED in SAS. Within these analyzes, goat buck was treated as a random effect and feeding level and administration of testosterone were included as fixed effects in the model.

Odor intensity data were analyzed using the Wilcoxon non-parametric tests (NPAR1WAY procedure; SAS) to test for differences between groups of goat bucks. Plasma testosterone data were analyzed using mixed model procedures of SAS (SAS Inst. Inc., Cary, NC) for specific repeated measures, considering 10 blood collection times. Data for testosterone showed heterogeneous variance using Bartlett's Box F-Test in SAS. To normalize variances among goat bucks for fixed effects, testosterone values were transformed to the inverse square root. Testosterone means were changed back to their original units after analysis.

A total of 5 ejaculates, each from the 20 goat bucks, were examined by repeated measures ANOVA using a general linear model (PROC GLM of SAS) procedure. The statistical model included the effects of feed level, testosterone administration and their first-order interactions on sperm characteristics. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

The mean values of body weight, body condition score, sexual odor intensity and scrotal circumference throughout the study are presented in Table 1. Body weight was lower ($P<0.05$) for underfed groups than for well-fed goat bucks. Likewise, BCS increased when animals were adequately fed compared with the underfed goat bucks. The intensity of sexual odor was greater ($P<0.05$) in goat bucks treated with testosterone, regardless of the feeding level, compared to bucks non-receiving testosterone. Bucks fed adequate levels of nutrients had larger mean scrotal circumference ($P<0.05$) after 70 d of the trial (Table 1). No

to all other groups. The main effect of feeding level and the feeding level \times testosterone treatment interaction were found to be significant at statistical level ($P<0.05$; Table 2).

The total numbers of sexual behaviors of each buck on the tests with estrogenized does are given in Table 3. There was a greater frequency ($P<0.01$) in the act of the male sniffing the anogenital region and urine of does in well-fed testosterone-treated bucks, compared with bucks in other groups. Moreover, during the season of sexual inactivity, well-fed and testosterone treated bucks emitted a greater ($P<0.05$) number of vocalizations than bucks in the other groups.

Table 1: Body traits, sexual odor intensity and scrotal circumference of well-fed or underfed mixed-breed goat bucks treated with exogenous testosterone or saline during the non-breeding season. Values are means \pm standard deviation.

Item*	1C	1T	1/2C	1/2T
Body weight, kg	42.8 \pm 9.9 ^a	45.8 \pm 3.2 ^b	39.1 \pm 5.6 ^c	39.7 \pm 8.9 ^c
Body condition score*	2.4 \pm 0.4 ^a	2.5 \pm 0.3 ^a	2.1 \pm 0.4 ^b	2.2 \pm 0.6 ^b
Sexual odor intensity**	0.15 \pm 0.23 ^a	0.29 \pm 0.38 ^b	0.12 \pm 0.21 ^a	0.26 \pm 0.34 ^b
Scrotal circumference, cm	26.8 \pm 2.0 ^a	26.9 \pm 1.2 ^a	25.1 \pm 1.3 ^b	1.9 \pm 2.7 ^b

1C=100% nutrient requirements for maintenance; 1T= 100% nutrient requirements for maintenance plus testosterone; 1/2C= 50% nutrient requirements for maintenance; 1/2T= 50% nutrient requirements for maintenance plus testosterone.

*5-point scale; **0 to 3-point scale.

For all variables, no feeding level \times testosterone treatment interactions ($P>0.05$) were found.

^{a,b,c}Means in the same row with different superscript differ ($P<0.05$).

feed level \times testosterone treatment interactions were detected for the aforementioned variables.

A beneficial effect of improved feeding and exogenous testosterone administration was observed on serum testosterone levels. Serum testosterone levels of group 1T were 5.7 times higher ($P<0.05$) than 1C bucks (Table 2). Likewise, serum testosterone levels in 1T bucks were about twice that observed in 1/2T bucks ($P<0.05$). The feed level \times testosterone treatment interaction was important ($P<0.05$), with better response of well-fed bucks to the testosterone treatment compared to underfed bucks.

Data revealed that no significant differences existed between the four groups for semen volume, sperm concentration and mass motility. Underfed goat bucks treated with testosterone had a significant ($P<0.05$) decline in sperm progressive motility as compared

However, higher plane of nutrition and exogenous testosterone did not increase the flehmen reaction of bucks. Likewise, the flehmen reaction was expressed at similar frequencies in well-fed and underfed bucks.

Approximations to does were performed more often ($P<0.05$) by the well-fed testosterone-treated bucks compared with all other groups. There was no difference in approximations neither between well-fed and underfed bucks nor between bucks treated with or without testosterone. Regarding the act of mounts attempts, there was a greater frequency in bucks treated with testosterone compared to bucks not treated with testosterone and no effect of nutritional status was observed. However, neither feeding level nor testosterone treatment influenced number of complete mounts. Both mounts with the penis exposed and mounts with ejaculation were greater ($P<0.05$)

in group 1T than in the other groups. Latency to first mount of 1T goat bucks was half the time recorded from the 1C goat bucks.

DISCUSSION

At the beginning of the study (spring; increasing daylength), the goat bucks were sexually quiescent with low serum testosterone, no male sexual odor, and few signs of peripheral androgen stimulation. Underfed animals under these conditions presented retardation in body development and scrotal circumference shared this retardation. This study confirms earlier reports that have indicated a significant association between plane of nutrition, body weight and scrotal circumference (Mellado et al., 2012; Ghorbankhani et al., 2015). The fact that testosterone administration did not affect scrotal circumference indicates that changes in testicular size could be induced solely by diet. The lack of nutrients was apparently associated with changes in gonadotrophin secretion, suggesting that there is an effect of nutritional status on the hypothalamic-pituitary axis regarding the testicular development of mixed-breed goat bucks (Martin et al., 1994; Blache et al., 2000). Scrotal circumference was enhanced with the higher feeding level, which is contrary to findings of Tufarelli et al. (2011) and Bielli et al. (1999), who found no significant effect of improved diets on scrotal dimensions. However, findings of the present study are consistent with data of Hötzel et al. (2003) and Fernández et al. (2004). These differences can mainly be attributed to greater amounts of sub-

cutaneous fat in the scrotal skin in goat bucks fed the 100% maintenance diet, as it has been observed by Fourie et al. (2004).

Sexual odor intensity was strongly affected by the administration of testosterone, regardless of the nutritional status of goat bucks. This finding can be ascribed to the fact that the primer pheromone responsible for the male effect is produced in the sebaceous glands (primarily in the cornual gland; Van Lancker et al., 2005) under the control of testosterone (Iwata et al., 2000). Thus, apparently, the sebaceous glands developed during the course of the testosterone treatment inducing the pheromone (odor) production.

The serum testosterone levels were clearly much higher in the well-fed testosterone-treated bucks, confirming the findings of previous studies showing that serum testosterone levels are three times higher in testosterone-treated bucks compared to control during the non-breeding season (Ángel-García et al., 2015b). The fact that testosterone levels were lower in underfed bucks compared to the well-fed bucks indicates that diets significantly affected the blood testosterone patterns. The interaction between feeding regime and testosterone treatment further shows that the energy body reserves alters the serum testosterone levels of bucks during the non-breeding season. Other studies have demonstrated that testosterone secretion in goat bucks is influenced by feed (Al-Sobayil et al., 2008). Therefore, it could be hypothesized that these differences in serum testosterone could be related to the alteration of body mass between the experimental groups of bucks.

Table 2: Serum testosterone levels and semen quantitative and qualitative characteristics of mixed-breed goat bucks during the non-breeding season, regarding nutritional status and testosterone treatment. Values are means \pm standard deviation

Item	1C	1T	1/2C	1/2T
Testosterone, ng/mL ^A	0.84 \pm 1.58 ^a	4.78 \pm 6.78 ^b	0.56 \pm 0.96 ^a	2.76 \pm 4.71 ^c
Semen volume (mL)	0.14 \pm 0.06	0.33 \pm 0.22	0.22 \pm 0.19	0.30 \pm 0.28
Sperm concentration ($\times 10^6$ mL ⁻¹)	826 \pm 276	1037 \pm 380	916 \pm 389	1026 \pm 307
Mass motility (1–5 scale)	1.3 \pm 1.0	2.3 \pm 1.4	1.6 \pm 1.1	1.8 \pm 1.5
Sperm progressive motility (% ^A)	57 \pm 11 ^a	58 \pm 14 ^a	52 \pm 16 ^a	46 \pm 15 ^b

1C=100% nutrient requirements for maintenance; 1T= 100% nutrient requirements for maintenance plus testosterone; 1/2C= 50% nutrient requirements for maintenance; 1/2T= 50% nutrient requirements for maintenance plus testosterone.

^AFeeding level \times testosterone treatment interaction ($P < 0.05$).

^{a,b}Means in the same raw with different superscript differ ($P < 0.05$).

Table 3: Sexual behaviors exhibited by well-fed or underfed goat bucks with or without testosterone administration during the non-breeding season by exposure to estrogenized does. Values are means \pm standard deviation

Item	1C	1T	1/2C	1/2T
Anogenital sniffing	10.0 \pm 7.6 ^a	59.8 \pm 38.1 ^b	11.2 \pm 7.2 ^a	26.0 \pm 20.1 ^a
Flehmen responses	1.0 \pm 0.7	5.2 \pm 6.3	0.6 \pm 1.3	3.6 \pm 4.6
Vocalizations	10.6 \pm 12.7 ^a	78.2 \pm 30.3 ^b	20.8 \pm 33.0 ^a	27.2 \pm 18.2 ^a
Approximations to does	12.6 \pm 11.3 ^a	82.6 \pm 41.5 ^c	33.0 \pm 34.5 ^{ab}	54.0 \pm 34.2 ^{bc}
Mount attempts	0.4 \pm 0.5 ^a	12.8 \pm 11.6 ^b	1.8 \pm 1.3 ^a	7.8 \pm 5.6 ^b
Complete mounts	0.0 \pm 0.0	0.8 \pm 1.3	1.2 \pm 1.6	1.8 \pm 1.9
Mounts with penis exposed	0.8 \pm 1.7 ^a	4.8 \pm 3.4 ^b	0.6 \pm 0.5 ^a	2.4 \pm 1.5 ^a
Mounts with ejaculation	0.4 \pm 0.9 ^a	4.0 \pm 1.2 ^b	0.6 \pm 0.5 ^a	2.8 \pm 1.6 ^a
Latency to first mount, sec ^A	164 \pm 43 ^a	82 \pm 63 ^c	145 \pm 51 ^a	110 \pm 44 ^b

1C=100% nutrient requirements for maintenance; 1T= 100% nutrient requirements for maintenance plus testosterone; 1/2C= 50% nutrient requirements for maintenance; 1/2T= 50% nutrient requirements for maintenance plus testosterone. Values are expressed as total behavioral events recorded (two days; 20 min per day).

^AFeeding level \times testosterone treatment interaction ($P < 0.05$).

^{a,b}Means in the same row with different superscript differ ($P < 0.05$).

Semen volume, sperm concentration and mass motility were not affected by feeding level or exogenous testosterone. This is in contrast to Tufarelli et al. (2011), who reported that semen volume and concentration were positively influenced by concentrate supplementation in rams. Many other studies have found improved semen characteristics when the nutritional condition of rams (Ghorbankhani et al., 2015) and goat bucks (Almeida et al., 2007) was improved. Although Kheradmand et al. (2006) found that neither semen volume nor sperm progressive motility were improved with an enhanced diet in Bakhtiary rams. Many other studies have found improved semen characteristics when the nutritional condition of rams (Ghorbankhani et al., 2015) and goat bucks (Almeida et al., 2007) was improved, although Kheradmand et al. (2006) found no improvement in semen volume with an enhanced diet in Bakhtiary rams. It was expected that semen volume and sperm concentration would be lower in the underfed goat bucks, which had slightly lower scrotal circumference. Kheradmand et al. (2006) argued that in rams, sperm cell concentration was higher after 7 weeks of improved diet administration. However, in the present study, no significant differences among buck groups were recorded for sperm concentrations. An explanation could be found in the fact that this trial only

lasted for 29 days, insufficient for the completion of a spermatogenesis cycle in goat bucks of about 7 weeks (Franca et al., 1999). Sperm concentration, in this case, is, therefore, a consequence of the management these animals were subjected prior to the onset of the experiment. Significant differences were observed for sperm cell progressive motility (lowest in the 1/2T group), indicating that the administration of testosterone was not helpful to maintain adequate sperm progressive motility in underfed goat bucks.

In group 1T, goat bucks exhibited more investigatory anogenital sniffing behaviors toward the female stimulus animals compared to the other groups. Likewise, these bucks showed an increased frequency of vocalizations and greater mounts with penis exposed and mounts with ejaculation in response to estrous females. In group 1T, bucks exhibited a notable enhancement of almost all sexual behavior characteristics as it has been demonstrated previously (Ángel-García et al., 2015a,b) following exposure to stimulus females. So, it could be considered that the greater investigatory behaviors exhibited by these goat bucks derived from an enhanced neuroendocrine response due to greater blood levels of testosterone in these animals, in response to testosterone administration.

The ejaculation is the culmination of the recog-

dition and preparatory behaviors in the courting of does, therefore, it is an important indicator of the libido and motivational state of bucks. In the present study, there was a positive relationship between the precopulatory behaviors and the mounts accompanied with ejaculation, which supports the view that the frequency of precopulatory behaviors in goat bucks reflects their underlying sexual motivation.

The precopulatory response toward estrous does by the underfed and saline-treated bucks could be due, at least in part, to their low body energy reserves and above all, to their low serum testosterone levels. This situation apparently limited their investigatory interest toward the estrogenized does. In low-performing and male-oriented rams, the sensory signals exuded by the estrous females are neither detected nor sufficiently provocative to elicit further investigation by the ram (Alexander et al., 1999).

Latency to the first mount, a good index of libido, was much shorter in the group1T compared to the other groups. The adequate feeding level combined with exogenous testosterone had an enhancing effect on time to first mount. As it has been noted by Zarazaga et al. (2009), in goat bucks, when the level of nutrition increased, sexual activity increased, too. Additionally, testosterone concentrations are directly linked to sexual and aggressive behavior in rams (Ungerfeld and Lacuesta, 2015).

The flehmen reaction was expressed more frequently in the testosterone-treated goat bucks. In this species, flehmen response was displayed when a determination of estrus occurred (Ungerfeld et al., 2006), while goat bucks receive information from females through the primary olfactory mode. Thus, well-fed bucks with higher blood testosterone levels were more competent to detect odorants derived from receptive goats.

CONCLUSIONS

This study provides evidence that high levels of exogenous testosterone administered to sexually inactive mature mixed-breed bucks at 25° N provoke a perceptible sexual activity, which suggests that exogenous testosterone in the non-breeding season stimulates cerebral structures mediating male sexual drive. However, this response is mainly attained in well-fed goat bucks. Therefore, supplementary feed should be supplied in conjunction with testosterone to goat bucks during the nonbreeding season in order to potentiate their sex drive.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. ■

REFERENCES

- Al-Sobayil KA, Zeitoun MM, Khalil MH Abdel-Salam M (2008) Effect of oral administration of a functional symbiotic syrup on libido, semen characteristics, serum testosterone and liver and kidney function of goat's bucks. *Asian J Biol Sci* 1: 11-18.
- Alexander BM, Stellflug JN, Rose JA, Fitzgerald JD, Moss GE (1999) Behavior and endocrine changes in high-performing, low-performing, and male-oriented domestic rams following exposure to rams and ewes in estrus when copulation is precluded. *J Anim Sci* 77: 1869-1874.
- Almeida AM, Schwalbach LMJ, Cardoso LA, Greyling JPC (2007) Scrotal, testicular and semen characteristics of young Boer bucks fed winter veld hay: The effect of nutritional supplementation. *Small Rumin Res* 73: 216-220.
- Ángel-García O, Meza-Herrera CA, Contreras-Villarreal V, Guillen-Muñoz JM, Leyva C, Robles-Trillo PA, Rivas-Muñoz R, Rodríguez-Martínez R, Mellado M, Véliz FG (2015a) Effect of different male-to-female ratios and testosterone administration upon the male sexual behavior and the out-of-season reproductive response of anestrus goats. *Small Rumin Res* 123: 21-29.
- Ángel-García O, Meza-Herrera CA, Guillen-Muñoz JM, Carrillo-Castellanos E, Luna-Orozco JR, Mellado M, Véliz-Deras FG (2015b) Seminal characteristics, libido and serum testosterone concentrations in mixed-breed goat bucks receiving testosterone during the non-breeding period. *J Appl Anim Res* 4: 457-461.
- Barkawi AH, Elsayed EH, Ashour G, Shehata E (2006) Seasonal changes in semen characteristics, hormonal profiles and testicular activity in Zaraibi goats. *Small Rumin Res* 66: 209-213.
- Bielli A, Pedrana G, Gastel MT, Castrillejo A, Moraña A, Lundeheim N, Forsberg M Rodríguez-Martínez H (1999) Influence of grazing management on the seasonal changes in testicular morphology in Corriedale rams. *Anim Reprod Sci* 56: 93-105.
- Blache D, Chagas LM, Blackberry MA, Vercoe PE, Martin GM (2000) Review Metabolic factors affecting the reproductive axis in male sheep. *J Reprod Fert* 120: 1-11.
- Detweiler G, Gipson T, Merkel R, Goetsch A, Sahl T (2008) Body condition scores in goats In: *Proceedings 23rd Annual Goat Field Day*, Langston University, Langston, OK. pp 127-133.
- Fernández M, Girálde FJ, Frutos P, Lavín P, Mantecón AR (2004) Effect of undegradable protein supply on testicular size, spermogram parameters and sexual behavior of mature Assaf rams. *Theriogenology* 62: 299-310.
- Fourie PJ, Schwalbach LM, Neser FWC, Van der Westhuizen C (2004) Scrotal, testicular and semen characteristics of young Dorper rams managed under intensive and extensive conditions. *Small Rumin Res* 54: 53-59.
- Franca LR, Becker-Silva SC, Chiarini-Garcia H (1999) The length of the cycle of seminiferous epithelium in goats (*Capra hircus*). *Tissue Cell* 3: 274-80.
- Ghorbankhani F, Souri M, Moeini MM, Mirmahmoudi R (2015) Effect of nutritional state on semen characteristics, testicular size and serum testosterone concentration in Sanjabi ram lambs during the natural breeding season. *Anim Reprod Sci* 153: 22-28.
- Hötzel MJ, Walkden-Brown SW, Fisher JS, Martin GB (2003) Determinants of the annual pattern of reproduction in mature male Merino and Suffolk sheep, responses to a nutritional stimulus in the breeding and non-breeding seasons. *Reprod Fert Dev* 15: 1-9.
- Iwata E, Wakabayashi Y, Kakuma Y, Kikusui T, Takeuchi Y, Mori Y (2000) Testosterone-dependent primer pheromone production in the sebaceous gland of male goat. *Biol Reprod* 62: 806-810.
- Karagiannidis A, Varsakeli S, Karatzas G (2000) Characteristics and seasonal variations in the semen of Alpine, Saanen and Damascus goat bucks born and raised in Greece. *Theriogenology* 53: 1285-1293.
- Kheradmand A, Babaei H, Batavani RA (2006) Effect of improved diet on semen quality and scrotal circumference in the ram. *Vet Arch* 76: 333-341.
- Luna-Orozco JR, Guillen-Muñoz JM, De Santiago-Miramontes MA, García JE, Rodríguez-Martínez R, Meza-Herrera CA, Mellado M, Véliz FG (2012) Influence of sexually inactive bucks subjected to long photoperiod or testosterone on the induction of estrus in anovulatory goats. *Trop Anim Health Prod* 44: 71-75.
- Martin GB, Tjondronegoro S, Blackberry MA (1994) Effects of nutrition on testicular size and the concentrations of gonadotrophins, testosterone and inhibin in plasma of mature male sheep. *J Reprod Fert* 101: 121-128.
- Mekasha Y, Tegegne A, Rodríguez-Martínez H (2007) Effect of supplementation with agro-industrial by-products and khat (*Catha edulis*) leftovers on testicular growth and sperm production in Ogaden bucks. *J Vet Med Series A: Physiol Pathol Clin Med* 54: 147-155.
- Mellado M, Herrera CAM, Arévalo JR, García JE, Véliz FG (2012) Effect of dietary energy intake and somatotropin administration after weaning on growth rate and semen characteristics of Granadina goat bucks. *Turk J Vet Anim Sci* 36: 338-345.
- NRC-National Research Council (2007) Nutrient requirements of ruminants: sheep, goats, cervids, and new world camelids. Washington, DC National Academy Press.
- Prathalingam NS, Holt WW, Revell SG, Jones S, Watson PF (2006) The precision and accuracy of six different methods to determine sperm concentration. *J Androl* 27: 257-262.
- Ramadan TA, Taha TA, Samak MA, Hassan A (2009) Effectiveness of exposure to long day followed by melatonin treatment on semen characteristics of Damascus male goats during breeding and non-breeding seasons. *Theriogenology* 71: 458-468.
- Roca J, Martínez E, Vázquez JM, Coy P (1992) Characteristics and seasonal variations in the semen of Murciano-Granadina goats in the Mediterranean area. *Anim Reprod Sci* 29: 255-262.
- Tufarelli V, Lacalandra GM, Aiudi G, Binetti F, Laudadio V (2011) Influence of feeding level on live body weight and semen characteristics of Sardinian rams reared under intensive conditions. *Trop Anim Health Prod* 43: 339-345.
- Ungerfeld R, Lacuesta L (2015) Competition between different social ranked rams has similar effects on testosterone and sexual behaviour throughout the year. *Reprod Dom Anim* 50: 1022-1027.
- Ungerfeld R, Ramos MA, Möller R (2006) Role of the vomeronasal organ on ram's courtship and mating behaviour, and on mate choice among oestrous ewes. *Appl Anim Behav Sci* 99: 248-252.
- Van Lancker S, Van Den Broeck W, Simoons P (2005) Morphology of caprine skin glands involved in buck odour production. *Vet J* 170: 351-358.
- Walkden-Brown SW, Restall BJ, Norton BW, Scaramuzzi RJ, Martin GB (1994) Effect of nutrition on seasonal patterns of LH, FSH and testosterone concentration, testicular mass, sebaceous gland volume and odour in Australian cashmere goats. *J Reprod Fert* 102: 351-360.
- Zarazaga LA, Guzmán JL, Domínguez C, Pérez MC, Prieto R (2009) Effects of season and feeding level on reproductive activity and semen quality in Payoya buck goats. *Theriogenology* 71: 1316-1325.

■ Presence of endoparasites in the Greek buffalo (*Bubalus bubalis*) from Northern Greece

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■ Ενδοπαράσιτα του Ελληνικού βουβάλου (*Bubalus bubalis*) στην περιοχή της Βορείου Ελλάδας

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ABSTRACT. This study was conducted in order to determine the presence of parasitic infections of the Greek buffalo (*Bubalus bubalis*) in the Prefecture of Serres, Northern Greece. During the period from February to October 2014, faecal samples from 110 buffaloes of the Greek buffalo breed (*Bubalus bubalis*), from 9 farms located in proximity to Lake Kerkini, in the Prefecture of Serres, Northern Greece, were examined, in order to find reproductive elements of parasites. Out of 110 faecal samples examined, 102 (92.73%) were found infected with reproductive elements (eggs, larvae, cysts and oocysts) of parasites. Specifically, the parasites found were: *Eimeria* spp. (40%), *Entamoeba bovis* (16.36%), *Paramphistomum cervi* (10%), *Fasciola hepatica* (16.36%), *Dicrocoelium dendriticum* (28.18%), *Moniezia*

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benedeni (27.27%), *Toxocara vitulorum* (11.82%), Strongylida (gastrointestinal nematodes) (31.82%) and lungworms (28.18%). The present study appears to be the first report of the detection of lungworms in buffaloes, in Greece.

Keywords: Greek buffalo, parasites, Northern Greece

ΠΕΡΙΛΗΨΗ. Σκοπός της παρούσας έρευνας ήταν η διερεύνηση της παρουσίας παρασίτων σε δείγματα κοπράνων βουβάλων, της φυλής του Ελληνικού βουβάλου (*Bubalus bubalis*), που εκτρέφονται σε περιοχές της Περιφερειακής Ενότητας Σερρών στη Βόρεια Ελλάδα. Κατά το χρονικό διάστημα Φεβρουάριος – Οκτώβριος 2014, εξετάστηκαν δείγματα κοπράνων από 110 βουβάλους της φυλής του Ελληνικού βουβάλου (*Bubalus bubalis*), από 9 εκτροφές που βρίσκονται σε αγροτικούς οικισμούς περιμετρικά της λίμνης Κερκίνης, με σκοπό την ανεύρεση αναπαραγωγικών στοιχείων παρασίτων (αυγών, προνυμφών, κύστεων και ωοκύστεων). Κατά την εξέταση των 110 δειγμάτων κοπράνων, τα 102 (92,73%) βρέθηκαν μολυσμένα με αναπαραγωγικά στοιχεία παρασίτων. Συγκεκριμένα, τα παράσιτα που βρέθηκαν ήταν: *Eimeria* spp. (40%), *Entamoeba bovis* (16,36%), *Paramphistomum cervi* (10%), *Fasciola hepatica* (16,36%), *Dicrocoelium dendriticum* (28,18%), *Moniezia benedeni* (27,27%), *Toxocara vitulorum* (11,82%), Στρογγυλοειδή (γαστρεντερικά νηματώδη) (31,82%) και πνευμονικά παράσιτα (28,18%). Στην παρούσα έρευνα γίνεται η πρώτη αναφορά της ανεύρεσης πνευμονικών παρασίτων στους βουβάλους, στην Ελλάδα.

Λέξεις ευρετηρίασης: Ελληνικός βούβαλος, παράσιτα, Βόρειος Ελλάδα

INTRODUCTION

Bubalus bubalis is one of the important species of domestic livestock as a source of good quality meat and milk (Hinrichs, 2004; Infascelli et al., 2004; Zicarelli, 2004; Zotos and Bampidis, 2014). The largest population of these animals in Europe is in Italy, Bulgaria and Romania (Galiero et al., 2005; Kobak and Pilarczyk, 2012).

The Food and Agriculture Organization of the United Nations refers to the Greek buffalo (*Bubalus bubalis*) population as a separate breed named “Ellinikos vouvalos (Greek buffalo)”, and characterizes this population as endangered-maintained species (FAO, 2007). The Greek buffalo is part of biodiversity of many Greek wetlands (mainly Lake Kerkini, Prefecture of Serres, Northern Greece), thereby enriching ecosystems with its aesthetic value. Moreover, the Greek buffalo, as a food producing animal, provides valuable products (milk, meat; Zotos and Bampidis, 2014), thus increasing the interest in breeding this particular species in Greece; current population is approximately 4,000 head (GBBC, 2018).

The parasitic diseases in buffaloes cause economic losses in a variety of ways: lowered fertility, reduced rates of weight gain, lower milk production, treatment costs etc. The survey of parasitic infection is an

important aid to combat infections more effectively and in controlling economic losses by adopting effective control measures (Sreedevi and Hafeez, 2014). Thus, the aim of the present study was to determine the current presence of the endoparasites in the population of the Greek buffalo breed, in the Prefecture of Serres, Northern Greece.

MATERIALS AND METHODS

During the period from February to October 2014, faecal samples from 110 buffaloes of the Greek buffalo breed (*Bubalus bubalis*), from 9 farms located in proximity to Lake Kerkini, in the Prefecture of Serres, Northern Greece (41°14' N, 23°06' E), were examined in order to find reproductive elements of parasites. The number of buffaloes per farm (adult females/males and heifers) and the number of examined buffaloes (faecal samples) per farm are given in Table 1. All faecal samples examined were obtained from adult buffaloes, randomly selected and apparently healthy, 105 samples from females and 5 samples from males, and were collected from the pasture directly after defecation of the animals. Each faecal sample was collected with hand glove and was kept in separate plastic bag to avoid contamination, tied carefully, labeled and sent to the Laboratory of Para-

sitology and Parasitic Diseases, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, for examination.

Faecal samples were examined using the sedimentation method Teleman (Pierkazski, 1954), which is a qualitative method that detects all parasitic elements in faeces, in order to find reproductive elements of parasites (eggs or larvae from worms and cysts or oocysts from protozoa) and to identify them (Soulsby, 1982; Thienpont et al., 1986) using microscope ($\times 10$, $\times 40$). Descriptive statistics were performed with the Statistical Package for the Social Sciences (2008).

RESULTS

Out of 110 faecal samples examined, 102 (92.73%) were found infected with reproductive elements of parasites. Detailed results are presented in Table 2. Single infection was observed in 30 (27.27%) faecal samples and mixed infection in 69 (62.73%) faecal samples with two, three and four species of parasites. In addition, mixed infection was revealed in one sample (0.91%) with five species of parasites and in two samples (1.82%) with six species of parasites.

DISCUSSION

The results of the present study revealed two species of protozoa, *Eimeria* spp. and *E. bovis*, both of them were found in a previous research, in our country and indicated that the prevalence of infection was 100% for both of them (Himonas et al., 1998). In another investigation, Founta et al. (2007) observed that the infection rate of *Eimeria* spp. and *E. bovis* were 0.16% and 11%, respectively. They also found the presence of *Buxtonella sulcata* (55%) and *Blastocystis* spp. (8%), which was not found in this survey. In the present study, the higher presence of *Eimeria* spp. (40%) infection, possible due to the intensive breeding in buffalo farms in the last years, implies a high density of animals thus leading to the spread of protozoa (Rinaldi et al., 2007). Moreover, buffaloes are exposed to ingestion of *Eimeria* spp. infective oocysts with drinking water contaminated with faeces and the presence of older buffaloes, acting as asymptomatic carriers, in the same place where newborns live (Bastianetto et al., 2007). Similar findings were reported by Karanikola et al. (2012) in Greece, where

the mean prevalence of *Eimeria* spp. and *E. bovis* were 40% and 14.8%, respectively. Different results were obtained by Nalbantoglou et al. (2008) in the Province of Afyon, Turkey, where the prevalence of *Eimeria* spp. in buffaloes was 3.8%-55.1%, whereas Singh et al. (2012) in India (Ludhiana District, Punjab) found prevalence of the infection which amounted to 0.95% and Mamun et al. (2011) in Bangladesh (Kurigram district) found 3.39%.

In the current study, the presence of helminths in buffaloes is much higher than the previous report of Founta et al. (2007), who noticed lower infection rate related to *P. cervi* 1.15%, *D. dendriticum* 0.49%, *M. benedeni* 0.16% and Strongylida 12.6%. Furthermore, the present study appears to be the first report of the detection of lungworms in buffaloes, in Greece. The high presence of infection is caused by the lack of appropriate parasite control programme, which is necessary for these animals. Buffaloes are raised on marshy pasture with high humidity which favors the growth and multiplication of parasites as well as their vectors (Kobak and Pilarczyk, 2012). Karanikola et al. (2012) also found high prevalence of helminthes infection in Greece (*F. hepatica* 11.1%, *P. cervi* 5.9%, *M. benedeni* 13.3%, *T. vitulorum* 8.9% and Strongylida 3.7%), but lower when compared to our results. Lower prevalence of helminths infection, using the FLOTAC technique, was observed in buffaloes by Cringoli et al. (2009) in the Lazio region in Italy and by Condoleo et al. (2007) in central Italy (*F. hepatica* 1.3%, *P. cervi* 2.1%, *D. dendriticum* 0.2%, *Moniezia* spp. 0.2%, Strongylida 5.4% and *Strongyloides* spp. 0.4%). Kobak and Pilarczyk (2012) found *F. hepatica* in 32% of buffaloes in the Notecka Forest region in Poland, which is almost twice the rate of infection with the present study. Racioppi et al. (2007) also found high prevalence of *F. hepatica* infection (28.5%) in the province of Corrientes, Argentina. Buffaloes are usually exposed to a higher risk of infection with snail-borne helminths due to their tendency to seek rivers, pools or swamps for wallowing (Cockrill, 1974). This research work indicated that the buffaloes were very much susceptible to parasitic infection with possible effects on their productivity. It is imperative that integrated strategies and measures be taken to control parasitic infections in buffaloes in Kerkini district and elsewhere in Greece.

CONCLUSIONS

The present study appears to be the first report of the detection of lungworms in buffaloes, in Greece. The high presence of parasites infection, in this study, should be an indication for devising an appropriate parasite control programme, it also highlights the need to carry out a larger study, including more samples and estimating the parasitic burdens, in order to estimate economic losses associated with parasitic

invasions. To prevent contamination of buffaloes by parasites, preserve their population and to improve their yields, early detection and treatment of infected animals is recommended, in combination with improving their breeding conditions.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest. ■

Table 1: Number of buffaloes (total and examined) per farm.

Farm number	Total number of buffaloes/farm	Number of examined buffaloes (faecal samples)/farm
1	451 (307 ♀, 126 ♂, 18 heifers)	10 ♀
2	159 (88 ♀, 51 ♂, 20 heifers)	15 ♀
3	72 (63 ♀, 9 ♂)	10 ♀
4	362 (241 ♀, 100 ♂, 21 heifers)	10 ♀
5	183 (115 ♀, 40 ♂, 28 heifers)	12 ♀
6	30 (30 ♂)	5 ♂
7	151 (85 ♀, 41 ♂, 25 heifers)	12 ♀
8	49 (33 ♀, 15 ♂, 1 heifer)	12 ♀
9	603 (423 ♀, 125 ♂, 55 heifers)	24 ♀
Total	2060	110

Table 2. Presence of parasitic infections in the faeces of examined buffaloes (n=110).

Parasites	Parasitic elements	Number of infected samples (%)	95% Confidence interval (%)
Protozoa			
<i>Eimeria</i> spp.	oocyst	44 (40.00%)	30.8 – 49.8 %
<i>Entamoeba bovis</i>	cyst	18 (16.36%)	10.0 – 24.6 %
Helminths			
Strongylida	egg	35 (31.82%)	23.3 – 41.4 %
<i>Dicrocoelium dendriticum</i>	egg	31 (28.18%)	20.0 – 37.6 %
Lungworms	larva	31 (28.18%)	20.0 – 37.6 %
<i>Moniezia benedeni</i>	egg	30 (27.27%)	19.2 – 36.6 %
<i>Fasciola hepatica</i>	egg	18 (16.36%)	10.0 – 24.6 %
<i>Toxocara vitulorum</i>	egg	13 (11.82%)	6.4 – 19.4 %
<i>Paramphistomum cervi</i>	egg	11 (10.00%)	5.1 – 17.2 %

REFERENCES

- Bastianetto E, Filho EJF, Lana AMQ, Cunha AP, Teixeira LV, Bello ACP, Teixeira C, Leite RC (2007). Epidemiology of *Eimeria* sp. infection in buffaloes (*Bubalus bubalis*) bred in Minas Gerais, Brazil. *Ital J Anim Sci* 6:911–914.
- Cockrill WR (1974). The working buffalo. In: Cockrill, W.R. (Ed.), *The Husbandry and Health of the Domestic Buffalo*, Food and Agriculture Organization of the United Nations, Rome, Italy.
- Condoleo RU, Veneziano V, Bruni G, Santaniello M, Carbone S, Pennacchio S, Rinaldi L, Cringoli G (2007). Distribution of helminths in buffalo farms from central Italy. *Ital J Anim Sci* 6:920–922.
- Cringoli G, Musella V, Maurelli MP, Morgoglione ME, Santaniello A, Condoleo R, Guariglia I, Rinaldi L (2009). Helminths and arthropoda in buffalo farms from the Lazio region (Italy). *Vet Res Commun* 33:129–131.
- Food and Agriculture Organization (FAO) (2007). Breeds currently recorded in the Global Databank for Animal Genetic Resources. Rome, Italy, pgs. 155.
- Founta A, Papazahariadou M, Papadopoulos E, Chliounakis S, Antoniadou-Sotiriadou K, Karamitros A, Efremidis K (2007). Fauna of gastrointestinal parasites of buffaloes in the area of Macedonia, Greece. *Anim Sci Rev* 36:15–21.
- Galiero G, Conedera G, Alfano D, Caprioli A (2005). Isolation of verocytotoxin-producing *Escherichia coli* 0157 from water buffaloes (*Bubalus bubalis*) in Southern Italy. *Vet Rec* 156:382–383.
- Greek Buffalo Breeders Cooperation (GBBC) (2018). Data concerning the Greek buffalo population. Vironia, Serres, Greece. Personal communication.
- Himonas C, Antoniadou-Sotiriadou K, Sotiraki S, Papazahariadou M (1998). Intestinal protozoa of animals in areas of Macedonia. *Bull Hellenic Vet Med Soc* 49:300–306.
- Hinrichs J (2004). Mediterranean milk and milk products. *Eur J Nutr* 1:112–117.
- Infascelli F, Gigli S, Campanile G (2004). Buffalo meat production: Performance *infra vitam* and quality of meat. *Vet Res Commun* 28:143–148.
- Karanikola S, Ptochos S, Diakou A, Karamitros A, Papadopoulos E (2012). Gastrointestinal parasitism of water buffaloes (*Bubalus bubalis*) in Northern Greece. In *Proceedings of the 12th Hellenic Veterinary Congress* (5-8 April 2012, Athens, Greece).
- Kobak P, Pilarczyk B (2012). Prevalence of gastrointestinal parasites of water buffaloes raised in the Notecka Forest region (Poland). *Bull Vet Inst Pulawy* 56:33–36.
- Mamun MAA, Begum N, Mondal MMH (2011). A coprological survey of gastro-intestinal parasites of water buffaloes (*Bubalus bubalis*) in Kurigram district of Bangladesh. *J Bangladesh Agric Univ* 9:103–109.
- Nalbantoglou S, Sari B, Cicek H, Karaer Z (2008). Prevalence of coccidian species in the water buffalo (*Bubalus bubalis*) in the Province of Afyon, Turkey. *Acta Vet Brno* 77:111–116.
- Piekarski G (1954). *Lehrbuch der parasitologie unter besonderer berucksichtigung der parasiten des menschen*. Springer-Verlag, Berlin-Gottingen-Heidelberg.
- Racioppi O, Moriena RA, Alvarez JD, Crudeli GA (2007). Fasciola hepatica: New buffalo (*Bubalus bubalis*) parasitosis in the province of Corrientes, Argentina. *Ital J Anim Sci* 6:948–950.
- Rinaldi L, Musella V, Condoleo R, Saralli G, Veneziano V, Bruni G, Condoleo RU, Cringoli G (2007). Giardia and cryptosporidium in water buffaloes (*Bubalus bubalis*). *Parasitol Res* 100:1113–1118.
- Singh NK, Singh H, Jyoti Haque M, Rath SS (2012). Prevalence of parasitic infections in buffaloes in and around Ludhiana district, Punjab, India: A preliminary study. *J Buffalo Sci* 1:1–3.
- Soulsby E.J.L. (1982). *Helminths, Arthropods and Protozoa of Domesticated Animals*, 7th Ed. Bailliere, Tindall and Cassell, London, UK.
- Sreedevi C, Hafeez Md (2014). Prevalence of gastrointestinal parasites in buffaloes (*Bubalus bubalis*) in and around Tirupati, India. *Buffalo Bull* 33:251–255.
- Statistical Package for the Social Sciences (2008). Release 17.0. SPSS Inc., Chicago, IL, USA.
- Thienpont D, Rochette F, Vanparijs OFJ (1986). *Diagnosing helminthiasis by coprological examination*, 2nd edition, Janssen Research Foundation, Beerse, Belgium.
- Zicarelli L (2004). Buffalo milk: Its properties, dairy yield and mozzarella production. *Vet Res Commun* 1:127–135.
- Zotos A, Bampidis VA (2014). Milk fat quality of Greek buffalo (*Bubalus bubalis*). *J Food Comp Anal* 33:181–186.

A case of pulmonary aspergillosis in white storks

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ABSTRACT. Aspergillosis is a fungal infection affecting respiratory system both in mammals and avian species. It is more commonly encountered in birds, in comparison with its mammalian counterpart. Mostly isolated strains are *Aspergillus fumigatus* (95%) and *Aspergillus flavus* (5%). Affected lungs and air sacs reveal miliary to gross lesions like gray-yellowish or white-grayish granulomatous foci surrounded by white halos indicative of inflammatory infiltration. Five storks found dead in the rural areas near Istanbul were submitted to our faculty between years 2008 and 2014. Two of them were thought to be younger than 1-year-old and the other three were older than one year of age. Necropsies were performed right after their submissions. Aspergillosis lesions were observed in the lungs and thoracic air sacs of the first four storks. In addition to these changes the lesions were detected at the aortic bifurcation and on the testicular and renal capsule of the fifth stork. Histopathology revealed encapsulated granulomas with foci of caseous necrosis at the center surrounded by numerous macrophages, heterophil leukocytes, lymphocytes and foreign body giant cells in all the storks. Following the gross, histopathological and mycological examinations the agents were detected as *Aspergillus fumigatus*.

Although, the number of reported deaths due to Aspergillosis is not high in storks, we believe that these birds are quite susceptible to the disease and stress factors such as migration increases the risk of pathogenicity. This report was designed as a contribution to literature since there is only one reported case available with respect to aspergillosis associated death in storks and stress factors such as migration may also predispose storks to the disease.

Keywords: *Aspergillus fumigatus*, avian aspergillosis, stork, histopathology, necropsy

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INTRODUCTION

Aspergillosis is a fungal infection affecting respiratory system both in mammals and avian species. Most common clinical manifestations occur in trachea, bronchioles, lungs and air sacs. Furthermore, eye, brain, skin, joints and visceral organs are involved, as well (Atasever and Gumussoy, 2004, Beyaz et al., 2008, Cacciuttolo et al., 2009). Aspergillosis is more commonly encountered in birds, in comparison with its mammalian counterparts. Avian aspergillosis is frequently seen in turkeys and chickens followed by ducks, geese, quails, ostriches, parrots, canaries, pigeons, flamingos and penguins, respectively (Atasever and Gumussoy, 2004, Tell, 2005). Acute and chronic diseases develop in birds. Acute form of the infection affects mostly young animals with high morbidity and mortality rates. Chronic disease develops in adults and in turkey chicks (Cacciuttolo et al., 2009, Tell, 2005).

Aspergillus spores are found in large numbers within the soil, decomposing meat, forage, hay and any kind of food (Arda, 1980). Mostly isolated strains are *Aspergillus fumigatus* (95%) and *Aspergillus flavus* (5%) (Tell, 2005). Affected lungs and air sacs reveal miliary to gross lesions like gray-yellowish or white-grayish granulomatous foci surrounded by a layer of darker zone, which is an evidence of inflammatory infiltrations. Histopathology reveals encapsulated granulomas with foci of caseous necrosis at the center, surrounded by numerous macrophages, heterophil leukocytes, lymphocytes and foreign body giant cells. Fungal elements like septate or aseptate hyphae and spores are visualized by special stains scattered around within these lesions (Cacciuttolo et al., 2009, Tell, 2005).

Despite the frequency of the cases of aspergillosis in poultry and waterfowl (Atasever and Gumussoy, 2004, Beernaert et al., 2008, Beyaz et al., 2008, Cacciuttolo et al., 2009, Carrasco et al., 2009, Tell, 2005, Yokota et al., 2001), a very small number of cases were reported in white storks (Garcia et al., 2007, Olias et al., 2010). There are only two articles available with the former pointing out aspergillosis associated death in white storks (Olias et al., 2010), and the latter describing the occurrence of *aspergillus* strains obtained by tracheal swabs in live animals (Garcia et al., 2007). In our country, although aspergillosis has

been reported in poultry and waterfowl (Akan et al., 2002, Akkoc et al., 2009, Atasever and Gumussoy, 2004, Beyaz et al., 2008), it has not yet been reported in storks. Turkey is located both on migration routes of the storks through from North Europe to North Africa and it is one of their breeding grounds. Istanbul Bosphorus is also seated upon this important migration route. Thus, we aimed to report the detection of aspergillosis in five storks.

MATERIALS AND METHODS

Five storks were found dead in the rural areas near Istanbul and were submitted to our faculty between the years 2008-2014. The common submission period for all the birds is during the end of October and the beginning of November. Two of them were thought to be younger than 1-year-old and the other three were older than one year of age. Necropsies were performed just after submissions of the birds. Different portions of lungs, air sacs, spleen, kidneys, heart and liver were collected from all of the birds and all the samples were fixed in 10% buffered formalin and then submitted for histopathology. For this purpose, the samples were embedded in paraffin and cut at 3-5 μ m thickness and then the sections were placed onto slides and stained with Hematoxylin-Eosin (H&E), periodic acid Schiff (PAS), Grocott and Ziehl-Neelsen. Spleen samples were stained also with Kongo-Red. Portions of lesioned lungs were stained with Gram stain and lactophenol in addition to the other stains. Fresh specimens of the mentioned organs were submitted for mycological culture examination, as well. Lung samples were cultured on a 7% defibrinated sheep blood agar plate (Oxoid), on a MacConkey agar plate and on two Sabouraud Dextrose Agar plates (SDA). Blood agar plate, MacConkey Agar plate and one of the SDA plates were incubated at 37 °C and the other SDA plate was incubated at 25°C. The blood agar plate and MacConkey agar incubated for 5 days while the two SDA plates incubated for 7 days (De Hoog et al. 2000).

RESULTS

Postmortem findings were similar in the first four birds examined: Numerous white nodules measuring from 0.5 cm to 5 cm in diameter were observed

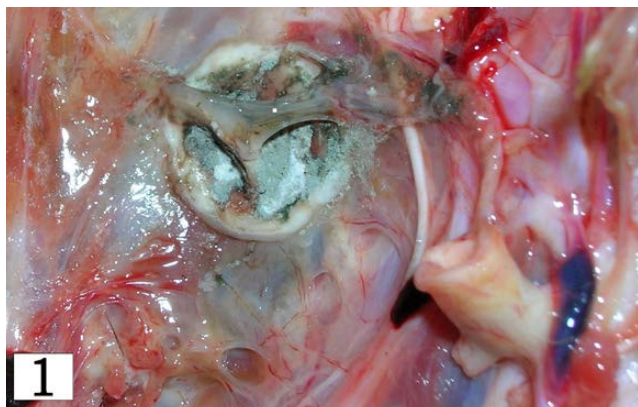


Figure 1. Moldy, greyish white depositions that resemble craters showing typical appearance of Aspergillosis in the lungs.



Figure 2. White round pyogranulomatous nodules filled with pus.

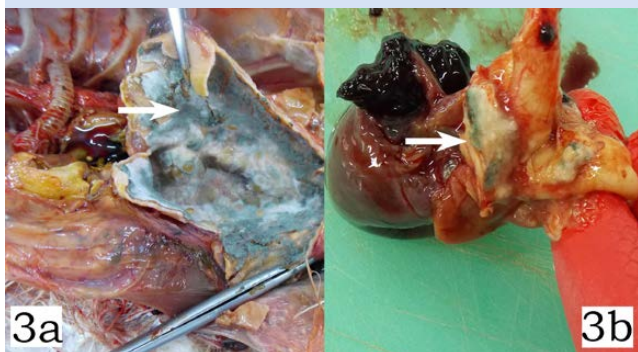


Figure 3
a. Aspergillosis. Greenish- gray moldy depositions in the abdominal air sac in the fifth stork.
b. Aspergillosis. Greyish dust like material at the aortic bifurcation in the fifth stork.

both in the lungs and thoracic air sacs. At the center of some of the nodules, there were craters covered with grayish dust like material that were surrounded

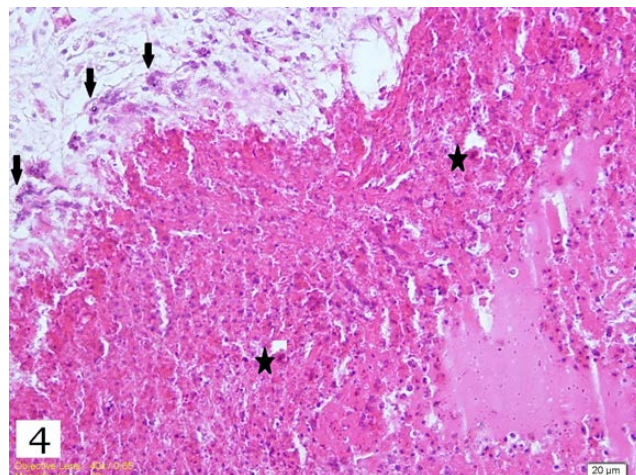


Figure 4. Focus of caseification necrosis (star) surrounded by a demarcation zone that consists of numerous macrophages, heterophil leukocytes, lymphocytes, foreign body giant cells (arrows) and fibrocytes and fibroblasts in the lung (H&E).

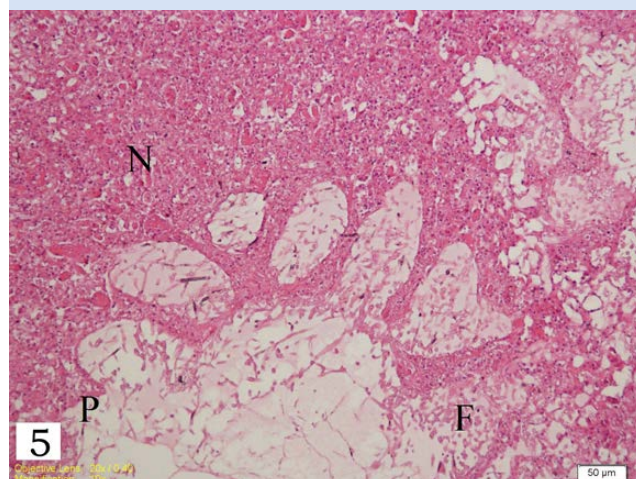
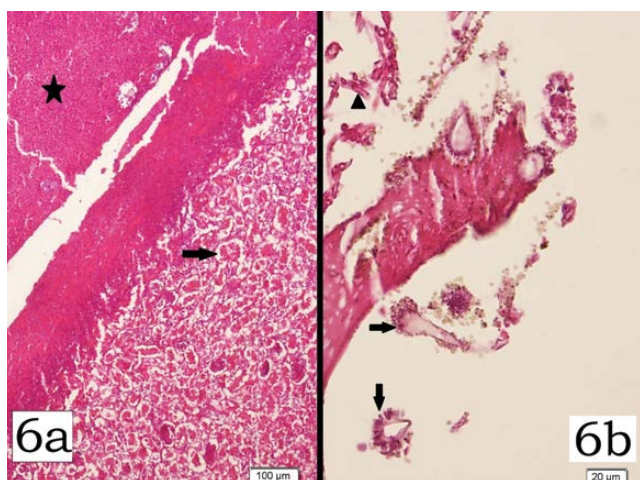


Figure 5. Necrotic pyogranulomatous areas (N) and numerous septate and non-septate hyphae (F) were present in the lumens of parabronchioles (P) (H&E).

by white halos (Fig.1). There were many nodules also on the cut surfaces of the lungs (Fig.2). The kidneys were congested. In the fifth stork, grayish dust like material identical to that seen in the lungs were observed also in the abdominal air sacs (Fig. 3a) and at the aortic bifurcation (Fig.3b).

Gram staining of the lungs revealed no evidence of acid resistant bacilli. However, lactophenol staining demonstrated numerous fungal hyphae.

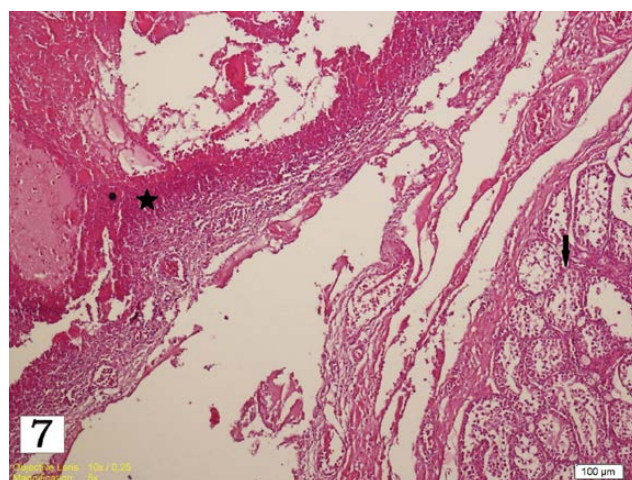
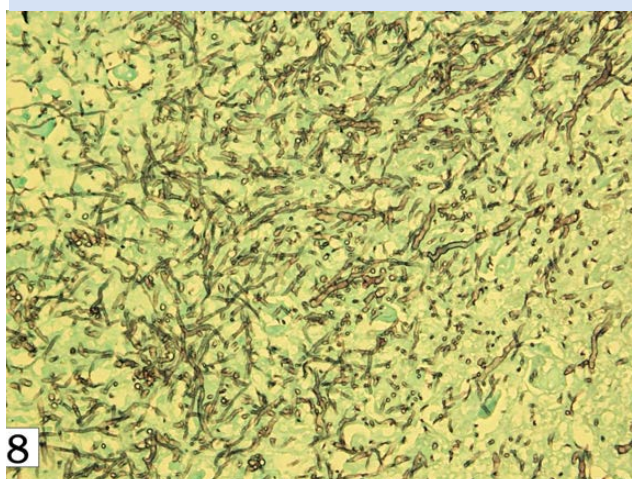
Histopathological examination of the organs revealed an exudative cellular inflammation composed of heterophil leukocytes, macrophages and foreign body giant cells in the lungs (Fig. 4). There

**Figure 6.**

- a.** Caseification necrosis (star) in air sacs adjacent to the kidney (arrow),
b. Conidiophores of *Aspergillus fumigatus* (arrows) and hyphae (arrowhead) (H&E).

were necrotic pyogranulomatous areas containing several septated and non-septated hyphae and conidia. Numerous septated and non-septated hyphae were also present in the lumina of parabronchioles (Fig 5). Furthermore, necrotic areas with numerous septated and non-septated hyphae and conidia and foreign body giant cells were observed in the abdominal air sacs, kidneys (Fig. 6a, 6b) and testis (Fig.7) of the fifth stork. In the slides that were stained with Grocott stain; hyphae and conidia of the aspergillosis were detected clearly (Fig. 8). Slides that were stained with Ziehl-Neelsen revealed no acid-fast bacilli. There were granulomatous lesions and foreign body cells in the air sacs, as well. In two young birds, there was urate crystal deposition in the kidneys. Hyalinization of trabeculae and amyloid like material deposition in the follicular areas were observed in the spleens. However, the Congo red stain was negative. Mononuclear inflammatory cells were seen around the portal veins and in the mid-zonal areas in the liver in both young birds and one of the adults.

There was no bacterial growth after a 7-day incubation period. Growth of fungal colonies was observed in the SDA plates that were incubated at 37 °C and 25 °C for 7 days. The microscopic examination revealed fungal colonies that were green at the center and white at the periphery. Lactophenol cotton blue staining revealed typical septated hyphae of *aspergillus*. Round vesicles, sterigmata and spores

**Figure 7.** Caseification necrosis (star) in air sacs adjacent to the seminiferous tubules (arrow) (H&E).**Figure 8.** Several fungal hyphae in the parabronchi (Grocot Stain-GMS)

were also seen. Single lined sterigma and conidia that were seated upon them were clearly observed. According to the gross and microscopic findings the agent was detected as *Aspergillus fumigatus*.

DISCUSSION

Pulmonary aspergillosis is among the most frequently seen mycotic disease in captive and free-ranging birds all around the world (Cacciuttolo et al., 2001, Charlto et al., 2008, Garcia et al., 2007). It is more common in birds than mammals due to the differences in their anatomic and immunocellular mechanisms (Garcia et al., 2007, Olias et al., 2010). Anatomic characteristics that might predispose birds to this disease include the lack of an epiglottis

that prevents particulate matter from entering the lower respiratory tract, the lack of a diaphragm which results in inability to produce a strong cough reflex and a limited distribution of pseudostratified ciliated columnar cells throughout the respiratory tract (Tell, 2005). Cellular characteristics that might predispose birds to respiratory aspergillosis include the lack of surface macrophages for phagocytizing *Aspergillus* spp. conidia and dependence on heterophils that use cationic proteins, hydrolases, and lysozymes rather than myeloperoxidase and oxidative mechanisms for killing fungal hyphae (Harmon, 1998). The most characteristic lesions in *Aspergillus* infections occur mainly in lungs and in air sacs. However, they can also be seen in the liver, spleen, myocardium, bones, brain, glandular stomach, bursa fabricius and eyes (Atasever and Gumussoy, 2004, Caciuttolo et al., 2009, Carrasco et al., 2009). In the present report, we detected numerous conidia and hyphae in both lungs and air sacs of the five white storks. And the mycological examination revealed *A. fumigatus* only in those organs. In two of the young birds, mononuclear cells were present in the liver and uric acid crystals were present in the kidneys and there was hyalinization in the white pulp of the spleen but there was no indication of Aspergillosis. Olias et al. (2010) reported mycotic pneumonia due to *A. fumigatus* in the necropsy of 22 storks and they also detected fungi only in the lungs and air sacs. They indicated two different types of pneumonia in the lungs: pneumonia of grade I was characterized by multifocal poorly circumscribed aggregates of epitheloid macrophages and multinucleated giant cells surrounding filamentous fungal structures while pneumonia of grade II was characterized by heterophilic granulomas with central necrosis and

degenerate heterophils surrounded by intact heterophils and a layer of epitheloid macrophages and multinucleated giant cells. In our cases, we detected the histopathological lesions of both grade I and grade II.

Garcia et al. (2007) reported that they took tracheal swab samples from 10 storks in a wildlife rehabilitation center and they detected *Aspergillus* spp. in one stork and *Candida* spp. in another one. Both of the storks survived despite the fungal infection. Nordani et al. (2006) reported that many birds are able to host the spores of the *Aspergillus* spp in the lungs and air sacs, leading to a dormant or chronic infection, with no clinical symptoms or apparent anatomopathological lesions. All these reports prove that *Aspergillus* spp. are facultative pathogens. They become pathogenic with the existence of predisposing factors such as stress, migration conditions and captivity of the wild birds, toxic substances and immunodeficiency. Two of the storks in our case were younger than 1 year-old and the other three were older than one year of age. They all died at the end of October and in November, which is the migration season, and thus we believe that Aspergillosis infection in our cases was related with stress, poor weather and environmental conditions during migration as it was reported in the literature (Caciuttolo et al., 2009, Tell, 2005).

In conclusion, although the number of reported deaths due to Aspergillosis is not high in storks, we believe that these birds are quite susceptible to the disease and stress factors such as migration, increases the risk of pathogenicity.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. ■

REFERENCES

- Akan M, Haziroglu R, Ilhan Z, et al. (2002) A case of aspergillosis in a broiler breeder flock. *Avian Dis* 49: 497-501.
- Akkoc A, Yilmaz R, Cangul I.T, et al. (2009) Pulmonary aspergillosis and amyloid accumulation in an ostrich (*Struthio camelus*). *Turk J Vet Anim Sci* 33: 157-16.
- Arda M (1980): Mikoloji. 185-192. In: Mikoloji Ankara Üniversitesi Basımevi, Ankara: pp 185-192
- Atasever A, Gumussoy K (2004) Pathological, clinical and mycological findings in experimental aspergillosis infections of starlings. *J Vet Med A Physiol Pathol Clin Med* 51(1): 19-22.
- Beernaert LA, Pasmans F, Haesebrouck F, et al. (2008) Modelling *Aspergillus fumigatus* infections in racing pigeons (*Columba livia domestica*). *Avian Pathol* 37: 545-549.
- Beyaz L, Gümüşsoy KS, Çam Y, et al. (2008) Kayseri Hayvanat Bahçesi'nde bulunan bazı yabani kanatlı türlerinde rastlanan sistemik Aspergillozis. *Ankara Üniv. Vet. Fak. Derg* 55: 31-35.
- Cacciottolo E, Rossi G, Nardoni S, et al. (2009) Anatomopathological aspect of avian aspergillosis. *Vet Res Commun* 33: 521-527.
- Carrasco L, Lima JS, Halfen DC, et al. (2001) Systemic Aspergillosis in an Oiled Magallanic Penguin (*Spheniscus magellanicus*). *J Vet Med B* 48: 551-554.
- Charlton BR, Chin RP, Barnes HJ (2008) Fungal Infections. In: *Diseases of Poultry*. 12th ed. Blackwell Publishing, Iowa, USA: pp 989-1001
- De Hoog GS, Guarro J, Gene J, et al. (2000) *Aspergillus* spp. In: *Atlas of Clinical Fungi*. 2nd edn. Utrecht: Centraalbureau voor Schimmelcultures: pp 442-519.
- Garcia ME, Lanzas P, Rodas VL, et al. (2007) Fungal flora in the trachea of birds from a wildlife rehabilitation centre in Spain. *Vet Med Czech* 52: 464-470.
- Harmon B (1998) Avian heterophils in inflammation and disease resistance. *Poultry Sci* 77: 972-977.
- Nardoni S, Ceccherelli R, Rossi G, et al. (2006) Aspergillosis in *Larus cachinnans* *miccaellis*: survey of eight cases. *Mycopathologia* 161: 317-321.
- Olias P, Gruber AD, Winfried B, et al. (2010) Fungal pneumonia as a major causes of mortality in white stork (*Ciconia ciconia*) chicks. *Avian Dis* 54: 94-98.
- Tell LA (2005) Aspergillosis in mammals and birds: impact on veterinary medicine. *Med Mycol* 43: 71-73.
- Yokota T, Shibahara T, Wada Y, et al. (2001) *Aspergillus fumigatus* infection in an ostrich (*Struthio camelus*). *J Vet Med Sci* 66: 201-204.

Granulosa Cell Tumor In A Spayed Young Queen

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ABSTRACT. A four years old cat was presented to Clinic of Obstetrics and Gynaecology with complaints of weakness, inappetency, vomiting and estrus signs although it was spayed. Blood tests, radiography and ultrasonography revealed abdominal mass and uterine stump which were then removed surgically. Multilobular mass was defined as solid granulosa cell tumor (GCT). Increase of estrogen (E2) and insulin-like growth factor-1 (IGF-1) values were detected on the 10th postoperative day. On the 40th postoperative day, the cat was brought to Internal Medicine Clinic with the complaints of weakness, inappetency and cachexia. Anemia, leucocytosis, uremia, hyperglycemia, sensitiveness and pain in the right abdomen were determined. A tumor was detected in the liver by radiography and ultrasonography and was suspected to be GCT metastasis. Despite medical therapy, the cat died after four months.

In conclusion; retained ovarian tissue after erroneous ovariectomy may cause, regular estrus signs and GCT development. Even if GCTs are removed by surgical approach, they have metastatic potential that deteriorates the prognosis. Evaluating IGF-1 and E2 in the short postoperative term are beneficial for determining the metastatic potential of GCTs.

Keywords: Granulosa cell tumor, IGF-1, estrogen, ovary, queen.

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CASE HISTORY

A four-years-old female cat, that was spayed when was one year old at a private clinic, was brought to Obstetrics and Gynaecology Clinic with regular estrus signs for three years. The cat had received a treatment because of vomiting, anorexia and weakness for 15 days beforehand in a proprietary clinic.

Complete blood cell count (CBC), ALT (Alanine aminotransferase), AST (Aspartate aminotransferase), ALP (Alkaline phosphatase), GGT (Gamma glutamyl transpeptidase), urea, creatinine, glucose, cholesterol, IGF-1, E2, progesterone (P4) analysis, thoracoabdominal radiography and abdominal ultrasonography were performed. Blood parameter results on operation day are notified in Table 1.

On ultrasonographic examination, a mass was viewed behind the left kidney (Figure 1a.,1b.) and uterine stump was determined. On radiography there was no visible metastasis in thorax; however the abdominal mass was clearly seen (Figure 2). A surgical approach was decided.

The anesthesia protocol consisted of sc atropine (Atropin, Vetaş, Turkey) as premedication (0.005 mg/kg) followed by the induction of the anesthesia 15 min. later with iv propofol (Pofol 1%, Dongkook Pharm, Korea) (6 mg/kg), and the intubation and maintenance with 3% isoflurane (Forane likid, Abbott Laboratories, England). Uterine stump (Figure 3) and a mass sized 12x10x5 cm which was located behind left kidney, around the left ovary, were removed by

Table 1. Blood parameter results on operation day and postoperative 10th, 40th and 45th day.

Blood Parameters	Reference Ranges In Cats	Operation Day	Postoperative 10th Day	Postoperative 40th Day	Postoperative 45th Day
RBC	5.8-11 uL*	4.5	6.84	4.5	3.6
HGB	8.6-16 g/dl*	6.5	9.6	6.3	4.6
HCT	28-47%*	20	31	18	14
WBC	(3.7-20.5) x1000/uL*	51.5	14.7	55.8	51.6
PLT	(160-660) x1000/uL*	494	386	95	157
MCV	37.7-50 fL*	45	46	39	40
MCH	12.3-17.2 pg*	15	14	14	13
MCHC	(31.1-36) x10 g/L*	32	31	36	32
GLU.	56-153 mg/dL*	224	164	170	190
UREA	18-36 mg/dL*	71	57	56	61
CREA.	0.6-2 mg/dL*	0.7	0.6	0.6	0.4
AST	14-54 U/L*	17	34	22	19
ALT	26-128 U/L*	26	51	28	20
ALP	14-102 U/L*	90	41		
GGT	0-5 U/L*	1	1		
Cholesterol	71-218 mg/dL*	134	165		
IGF-1	5-70 nmol/L**	88	118		
E2 Base level	5-14 pg/ml***	56.2	67.1		
In estrus	50-70pg/ml***				
P4 Luteal phase	1-4 ng/ml***	0.48	0.37		
In estrus	< 1ng/ml***				

RBC= Red Blood Cell Count; HCT= Hematocrit; HGB= Hemoglobin; MCV= Mean Corpuscular Volume; MCH= Mean Corpuscular Hemoglobin; MCHC= Mean Corpuscular Hemoglobin Concentration; WBC= White Blood Cell Count; PLT = Platelet Count; CREA= Creatinine; GLU= Glucose; AST= Aspartate Aminotransferase; GGT= Gamma Glutamyltransferase; ALP= Alkaline Phosphatase; ALT= Alanine aminotransferase; IGF-1= Insulin-like growth factor-1; E2= Estrogen; P4= Progesterone. Reference values: *Plumb (2008), **Maden and Çuhadar (2013), ***Shille et al., (1979).



Figure 1a. The mass behind left kidney on cranioabdominal regio, **1b** Tumoral mass on ultrasonographic examination.



Figure 2. Tumoral area on thoraco-abdominal radiography of the cat.



Figure 3. Uterine stump

median laparotomy. Before laparotomy incision was closed, we were sure that there was no residual ovarian tissue in the abdomen. Monofilament and absorbable suture material (Monocryl No: 2/0, Medeks, Turkey) was used for ligations, muscular, subdermal and dermal sutures. The mass and stump uterus were delivered to Pathology Department for histopathologic examination. Twenty ml/kg iv 0.9 % isotonic sodium chloride twice a day (b.i.d), 20 ml/kg iv Lactated Ringer (Laktatlı Ringer, Polifarma, Turkey) b.i.d., 40 µg/kg intramuscular (im) vitamin B12 (Dodex, Deva, Turkey) once daily (s.i.d.), B and C complex vitamin (Hepargriseovim, Deva, Turkey), 20 mg/kg im ampicillin sodium (Ampisina, Mustafa Nevzat, Turkey) b.i.d. and 15 mg/kg im clindamycin (Klindan, Bilim, Turkey) b.i.d. were administrated to the cat for ten days postoperatively. Also inactivated paravoxvirus ovis (Zylexis, Pfizer, USA) was injected once a week for four weeks by subcutaneous way.

The first control was made on the 10th postoperative day. On anamnesis; having appetite, absence of vomiting and vitality were informed. Blood parameter results on the 10th postoperative day are notified in Table1. After isotonic serum infusion and low protein diet for seven days, blood profile was as follows; hematocrit 28%, hemoglobin 8.6 g/dl, glucose 166 mg/dl, urea 45 mg/dl and creatinine 0.7 mg/dl. According to this blood profile, 0.9% isotonic sodium chloride (Izotonik, Eczacıbaşı, Turkey), Lactated Ringer (Laktatlı Ringer, Polifarma, Turkey), B and C vitamin complex (Hepargriseovim, Deva, Turkey) and vitamin B12 (Dodex, Deva, Turkey) were administrated. Three weeks after the prescrip-

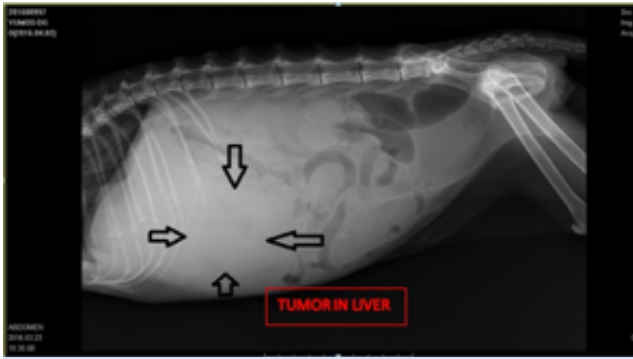


Figure 4. Abdominal radiography of the tumor in liver.

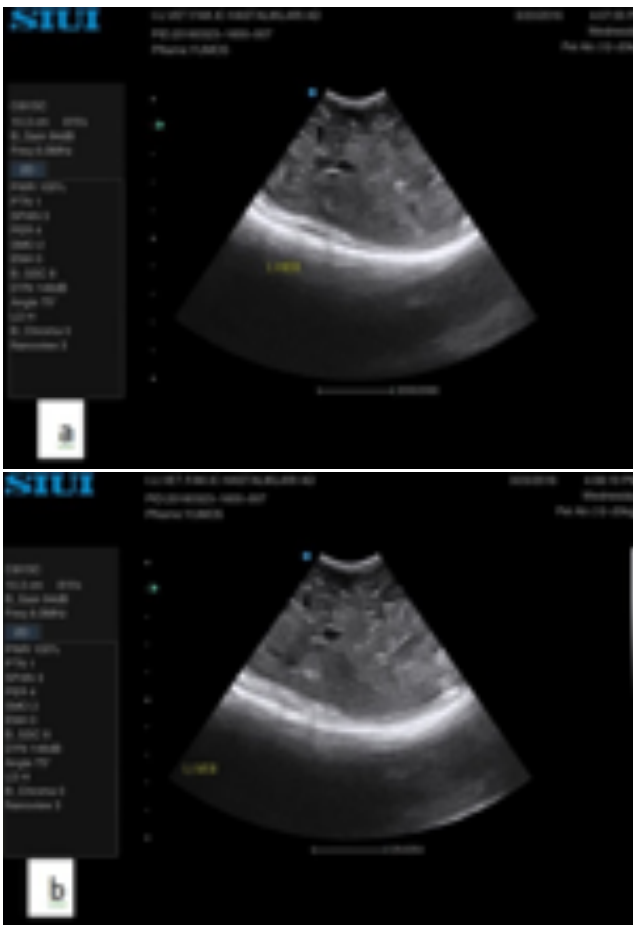


Figure 5a,b Hyperechoic areas within liver on ultrasonography.

tion applied, the cat was brought again to Internal Medicine Clinic with the complaint of weakness, anorexia and cachexia. Anemia, leucocytosis, uremia and hyperglycemia were detected on the 40th postoperative day (Table 1). As a result, 20 mg/kg ampicillin sodium and sulbactam combination (Sulcid, İ.E. Ulagay, Turkey), 15 mg/kg clindamycin (Klindan,

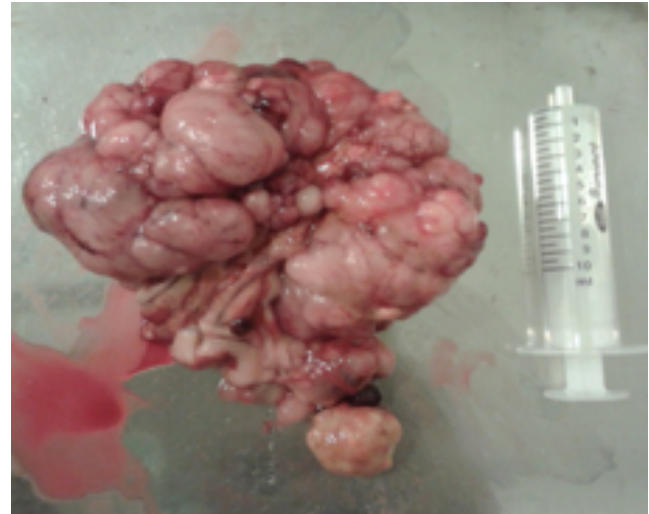


Figure 6. Tumoral mass in macroscopic examination.

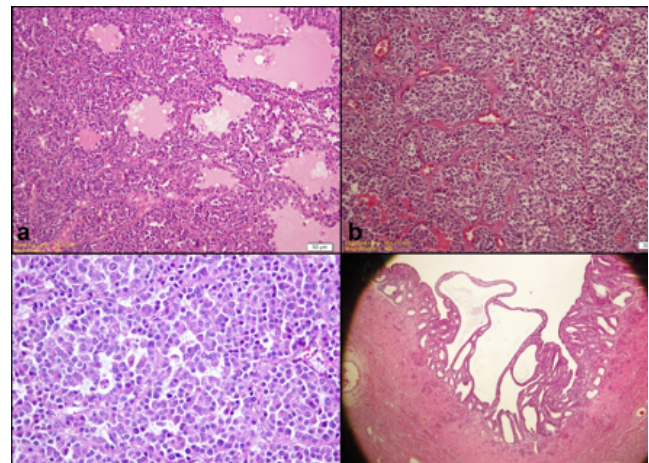


Figure 7a Cystic structures with diffuse, atypical granulosa cells. **7b, 7c** Density of distinctive tubular pattern in stroma. **7d** Cystic glandular metritis.

Bilim, Turkey) and vitamin B12 (Dodex, Deva, Turkey) were prescribed for 5 days. At the end of this therapy, blood tests (Table 1) were repeated. Because signs of pain were detected on palpation of the right abdominal region, abdominal radiography (Figure 4) and ultrasonography (Figure 5a,b) were performed. According to ultrasonographic examination, a tumor on the liver was determined. Because of the poor prognosis, surgery was not performed. Medical therapy with 25 mg/kg im Seftriaxon (Novosef, Zentiva, Turkey), 15 mg/kg im clindamycin (Klindan, Bilim, Turkey), 0.9% iv isotonic sodium chloride (Izotonik, Eczacıbaşı, Turkey) and 5% iv dextrose (Dekstroz, Eczacıbaşı, Turkey) serum infusion, im vitamin B12 (Dodex, Deva, Turkey) and 16 mg im cortisone

(Prednol, Mustafa Nevzat, Turkey) was applied for 7 days. After that last therapy, survival time was only four months.

Histopathologic examination was performed at Department of Pathology. In the macroscopic examination, the tumoral mass of the ovary was measured 12x10x5 cm in size and had multinodular structure (Figure 6). Tumor samples were fixed in 10% neutral buffered formalin for at least 24 h and 5 µm thick serial sections were cut, deparaffinized, rehydrated with water in descending concentrations of ethanol and used for hematoxylin and eosin (HE) staining. Tissues were observed under a light microscope. The histological evaluation revealed diffuse, atypical, granulosa-like cells with spherical to oval, hyperchromatic nuclei, distinct nucleoli, and scant eosinophilic cytoplasm. In the diffuse areas, there were cystic structures surrounded by granulosa cells (Fig 7a). Also, a distinctive tubular pattern in which a dense stroma was present (Fig 7b, c). In some slides, areas of luteinisation and hemorrhage were seen. Cystic glandular metritis was also observed in the same patient (Fig 7d).

DISCUSSION

GCTs are the most common sex-cord stromal tumor in all animal species (Nielsen and Kennedy, 1990). Holzworth (1987) reported that mean age of GCT occurrence in cats is 11 years. Contrarily, reported cat which affected with GCT was four years old.

In our case, GCT was detected in a spayed cat as Spoor et al. (2014) detected GCT in spayed Bulldog. In accordance with Gelberg and McEntee (1985), the cat had also estrus signs although spayed in early age. In line with Ball et al. (2010), it is thought to be as a result of incomplete excised ovarian or ectopic ovarian tissues.

Gelberg and McEntee (1985) reported that GCTs are the most common ovarian tumor type in cats which have malignant character. The appearance of the tumor in liver 45 days after the ovarian tumor resection supports GCTs' malignancy as Gelberg and McEntee (1985) reported.

In accordance with Gündüz et al. (2010) ultrasonographic imaging of the ovaries is used for diagnosis of GCT. In line with Hayes and Harvey (1979),

we performed surgery for treatment and also after surgery immunotherapeutic agent was used for four weeks.

Noakes (2009) reported that ovariectomy cause elimination of E2, P4, inhibin, activin, and follistatin in ovaries. Shille et al. (1979) notified that E2 in plasma elevate from a base level of 5-14 pg/ml to peaks of 50-70 pg/ml in estrus. Contradicting to Noakes (2009), in this report, we expected base level in plasma E2 after the GCT resection but E2, which was measured 56 pg/ml in the operation day, increased to 62 pg/ml on the 10th postoperative day.

Fontbonne et al. (2007) reported that ovarian remnant syndrome (ORS) leads to elevated levels of ovarian hormones. Shanbhogue et al. (2010) notified that hyperandrogenism and hyperestrogenism are the most common causes of ovarian tumors. Although we had no data about the E2 levels of the erroneous ovariohysterectomized cat before the GCT diagnosis, in line with the researchers (Fontbonne et al. 2007, Shanbhogue et al. 2010) this ORS case was followed by GCT formation.

Buijters et al. (2010) reported that GCTs produce E2 and small amount of P4 which causes endometrial growth and glandular secretion. In accordance with Buijters et al (2010), the cat with GCT had high E2 level and also cystic glandular metritis in the uterine stump.

Boormann (2002) notified that increase of E2 levels after surgery happen if a residual hormone producing tumor may exist. Increased E2 levels detected on the 10th postoperative day and presence of tumor on liver 45 days after surgery are in accordance with Boormann (2002).

In accordance with Spicer and Echternkamp (1995), that reported a positive correlation between follicular growth and IGF-1 concentration, IGF-1 concentration was measured high on the operation day in this case.

Despite the resection of GCT and uterine stump, the increased IGF-1 concentration on the 10th postoperative day may be related to IGF-1 synthesis by the metastatic liver as reported (Maden and Çuhadar, 2013).

The histopathological findings of this granulosa tumour did not differ from the literature and they

are in accordance with the aggressive behaviour described for this tumour in cats (Giacóia et al., 1999).

A second operation for tumor resection in liver was not performed due to the poor body condition. It is in accordance with Boormann's (2002) report which defends that second-look operations are unnecessary unless significant masses and complications such as obstruction of the bowel occur.

In conclusion; retained ovarian tissue after erro-

neous ovariectomy may cause regular estrus signs and GCT development. Even if GCTs are removed by surgical approach, they have metastatic potential that deteriorates the prognosis. Evaluating IGF-1 and E2 in the short postoperative term are beneficial for determining the GCT metastasis.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- Ball RL, Birchard SJ, May JR, Threlfall WR, Young GS (2010) Ovarian remnant syndrome in dogs and cats: 21 cases (2000-2007). *J Am Vet Med Assoc* 236:548-553.
- Boormann EV (2002) Granulosa cell tumors. In: *Proceedings of UCLA Health Care* 6:1-6. <http://www.med.ucla.edu/modules/xfsection/article.php?articleid=177>
- Buijts JJCWM, De Gier J, Kooistra HS, Veldhuis Kroeze EJB, Okkens AC (2010) Alterations of the pituitary-ovarian axis in dogs with a functional granulosa cell tumor. *Theriogenology* 73:11-19.
- Fontbonne A, Levy X, Fontaine E, Gilson C (2007) Guide pratique de reproduction clinique canine et feline. Med'com.
- Gelberg HB, Mc Entee K (1985) Feline ovarian neoplasms. *Vet Pathol* 22:572-576.
- Giacóia MR, Maiorka PC, Oliveira CM, Sinhorini IL, Daglı MLZ (1999) Granulosa cell tumour with metastasis in a cat. *Braz J Vet Res Anim Sci* 36(5).
- Gündüz MC, Kaşıkçı G, Kılıçarslan MR, Uçmak M, Düzgün O, Tek Ç (2010) Reproductive performance following unilateral ovariectomy for treatment of ovarian tumors in 7 mares. *Turk J Vet Anim Sci* 34(3):283-287.
- Hayes A, Harvey HJ (1979) Treatment of metastatic granulosa cell tumor in a dog. *JAVMA* 174(12):1304-1306.
- Holzworth J (1987) Tumours of the female reproductive system In: *Diseases of the cat: medicine and surgery*. 1st. ed., W.B. Saunders, Philadelphia: pp 520-521.
- Maden M, Çuhadar F (2013) Kedi ve köpeklerde endokrin hastalıkların laboratuvar tanısı. *Türkiye Klinikleri J Vet Sci* 4(3): 35-60.
- Nielsen SW, Kennedy PC (1990) Tumours of the genital systems In: *Tumours in domestic animals*. 3rd. ed., Berkeley University of California Press, California: pp 458-475.
- Noakes D. (2009). Endogenous and exogenous control of ovarian cyclicity In: *Veterinary reproduction and obstetrics*. 9th ed., Pa: Saunders, Philadelphia: pp 6-9.
- Shanbhogue AKP, Shanbhogue DKP, Prasad SR, Surabhi VR, Fasih N, Menias CO (2010) Clinical Syndromes Associated with Ovarian Neoplasms: A Comprehensive Review. *RadioGraphics* 30(4): 903-919
- Shille VM, Lundström KE, Stabenfeldt GH (1979) Follicular function in domestic cat as determined by estradiol-17 β concentrations in plasma: relation to estrous behaviour and cornification of exfoliated vaginal epithelium. *Biology of Reproduction* 23: 953-963.
- Spicer LJ, Echternkamp SE (1995) The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. *Domestic Animal Endocrinology* 12: 223-245.
- Spoor MS, Flesner BK, Trzil JE, Whitney MS, Shaw DP, Selting KA (2014) What is your diagnosis? Intra-abdominal mass in female spayed dog. *Vet Clin Pathol* 43(1): 109-110.

The zoonotic protozoan of sheep carcasses in the north of Algeria: A case of ovine toxoplasmosis

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ABSTRACT. *Toxoplasma gondii* is a zoonotic protozoan parasite of great importance in veterinary and public health. The aim of the present study was to determine the seroprevalence for *T. gondii* in 580 sheep sera slaughtered for human consumption in the slaughterhouses of El Harrach by a commercial kit ELISA (enzyme-linked immunosorbent assay), and to evaluate the presence of *T.gondii* in 335 sheep from 580 (335 oesophagi and 335 diaphragms) by the histopathological analysis. Antibodies to *T. gondii* were found in 8.28% (48 /580) of sheep. All positive sheep were male. Seropositivity for *T. gondii* increased with age, but the difference was not statistically significant.. While the seroprevalence was significantly higher in summer and in the North /Center of Algeria. Thus, season and origine of animals were considered as risk factors associated with *T. gondii* infection. Histopathological analysis showed that only 2 sheep presented dubious cysts of *T. gondii*. However, tissues cysts compatible with *Sarcocystis spp.* were visible in the histological sections of 94.03% (315/335) of sheep. These results suggest that infection with *T. gondii* in sheep is present in the north of Algeria and as sheep with antibodies usually carry tissue cysts, this indicates that undercooked lamb and mutton may indeed be a sources of human toxoplasmosis.

Keywords: Sheep, Toxoplasmosis, *T.gondii*, ELISA, Tissue cyst.

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1. INTRODUCTION

Sheep are commonly infected with a cosmopolitan zoonotic infection, Toxoplasmosis, caused by the coccidian protozoan parasite, *T. gondii* (Dubey, 2009), which naturally infects human beings, wild and domestic animals, as well as birds. It is a geographically wide spread infection (Germani Fialho and al., 2009). Also, it has substantial medical and veterinary significance (Tenter and al., 2000). Infection with *T. gondii* during pregnancy in sheep has a major economic impact and represents a serious risk for congenital disease including embryonic or fetal death and mummification, abortion, stillbirth, and neonatal death (Dubey, 2009). In humans, infection results from the ingestion of oocysts released into the environment with the faeces of cats, from the consumption of raw or lightly cooked meat containing tissue cysts, or through congenital transmission (Dixon, 1992). The ingestion of undercooked infected lamb is considered as an important source of infection for humans (Dubey, 2009). Approximately one-third of the world population is likely to be exposed to this parasite (Tenter and al., 2000). While toxoplasmosis is often mild or asymptomatic, it can be a devastating illness in immunocompromized patients and in congenitally infected infants (Dixon, 1992). Seroprevalence of *T. gondii* in sheep has been reported extensively in different countries and the positive rates ranged from 3% to 95% (Dubey, 2009). In Algeria, the incidence of toxoplasmosis in man or its prevalence in sheep is not well known. As a result, its impact on sheep production still remains unknown and abortions are attributed to other diseases such as brucellosis. Also, the possible sources of infection to humans through lamb consumption are not well established. Considering the importance of toxoplasmosis and the lack of epidemiological information of ovine toxoplasmosis in Algeria, the presented study aimed to obtain data on the prevalence of *T. gondii* infection in 580 sheep in the slaughterhouses in the north of Algeria, particularly those of El Harrach, and the probable role of ovine meat consumption in human toxoplasmosis. To this end, ELISA test and histological technique were established for the examination of sheep sera and the tissue fragments.

2. MATERIALS AND METHODS

2.1. Sample collection and storage

Samples from randomly selected sheep were collected. The animals came from different regions of

Algeria and were intended for human consumption. Most of them males and few females were sampled because females are normally kept for breeding purpose, age was estimated and animals were divided into three age groups: ≤ 1 year, 1.5-3 years, and 3-5 years. A total of 580 blood samples were collected directly from the jugular vein during bleeding in sterile assay tubes. Each animal was then tagged on the right anterior limb for oesophagus and diaphragm collection. The samples were tagged, refrigerated, and transported immediately to the laboratory of parasitology and mycology of the Superior National Veterinary School - Algiers -. In the laboratory, sera were separated after centrifugation at 3000 rpm for 10 minutes and stored at -20°C in microtubes until assayed by ELISA technique and the tissue fragments (oesophagi and diaphragms) were stored in 10% buffered formalin and were submitted to the laboratory of anatomy pathological ENSV- Algiers - for histopathological evaluation and identification of the parasite.

2.2. Serological analysis

Serological analysis of 580 sera of sheep was realised at the laboratory of parasitology and mycology of the University Hospital of Mustapha Bacha - Algiers -. Toxoplasma-specific antibodies were measured by a commercial kit ELISA (ID Screen® Toxoplasmosis Indirect Multi-species, ID vet, Grabels - France). According to the manufacturer's instructions, 90 microliter (ul) of the dilution buffer 2 were distributed into the 96 polystyrene wells of the ELISA microplate coated with Toxoplasmosis P30 Antigen. Ten ul of the positive and negative control were included in A1 and B1 cupules for the negative control, and C1 and D1 cupules for the positive one, the sera were deposited at 10 μl /well (into the other cupules) and incubated for $45 \text{ min} \pm 4 \text{ min}$ at $21^{\circ}\text{C} (\pm 5^{\circ}\text{C})$. Next, the plates were washed 3 times with washing buffer, 100 ul of Anti-multi-species IgG-HRP peroxidase-labeled conjugate previously diluted 1:10 in dilution buffer 3 was added and incubated for $30 \text{ min} \pm 3 \text{ min}$ at $21^{\circ}\text{C} (\pm 5^{\circ}\text{C})$. Then, the wells were washed, 100 ul of substrate solution was distributed and incubated for $15 \text{ min} \pm 2 \text{ min}$ at $21^{\circ}\text{C} (\pm 5^{\circ}\text{C})$ in the darkness, the reaction was stopped by adding 100 ul of stop solution. The optical density (OD) of each well was measured at 450 nm using a absorbance microplate reader (BIO-RAD PR

4100). The test results were interpreted according to the manufacturer's instructions. The test is validated if the average optical density value of the positive controls $OD_{pc} > 0.350$ and the ratio: OD of the positive controls / OD of the negative controls > 3.5 . For each sample, the percentage (S/P%) was calculated according to the schema provided by the manufacturer: $S/P\% = \text{Optical density of sample} \times 100 / \text{Optical density of positive control}$. Samples with $S/P\% \leq 40\%$ were considered to be negative, samples with $S/P\%$ between 40% and 50% were considered to be dubious, samples with $S/P\%$ between 50% and 200% were considered positive, whereas values $\geq 200\%$ indicated acute toxoplasmosis.

2.3. Histological examination

670 samples were studied from 335 sheep (335 oesophagi and 335 diaphragms). The fixed samples were cut into 0.5 cm-thick sections, dehydrated through serial dilutions of ethanol and xylene, before being embedded in paraffin wax using routine procedures. From each block, four to six sections 4 to 5 micron thick were cut, deparaffinised, rehydrated and stained with haematoxylin and eosin (H&E). All the slide was analyzed by a light microscopy (Leica DMLS®, objective (40×, 100×) for presence of tissue cysts of *T. gondii* or histopathological lesions.

2.4. Statistical analysis

Statistical analyses of *T. gondii* prevalence from different regions, age groups, gender and season were performed by Chi Square test using the software program Microsoft Excel 2010. The differences were considered statistically significant if $P < 0.05$.

3. RESULTS

3.1. Research of antibodies against *T. gondii* by ELISA

3.1.1. The overall seroprevalence of *T. gondii*

The overall *T. gondii* seroprevalence was 8.28% (48/580) with S/P% ranging from 79% to 336%. While 2/580 (0.34%) samples gave a dubious results and 530/580 (91.38%) were negative. The chi-square test was very significant between the positive and negative results. From 48 seropositive animals 13 sera had $S/P\% \geq 200$, while 35 sera had $S/P\%$ between 50% and 200% (Figure 1). The chi-square test was very sig-

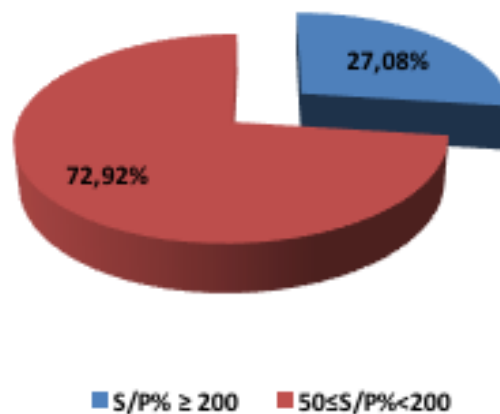


Figure 1: Repartition of the 48 seropositive sheep according to S/P% value.

nificant between the two levels of seropositive cases ($S/P\%$ between 50% and 200%, $S/P\% \geq 200$).

3.1.2. Seroprevalence of *T. gondii* according to the risk factors

Results of the seroprevalence of *T. gondii*, according to the risk factors are shown in the following Table.

3.2. Research of *T. gondii* tissue cysts by histological technique

It was difficult to identify cysts of *T. gondii* in histological sections stained with HE by a light microscopy, because the parasite can be confused with other protozoan parasites (*Sarcocystis*,

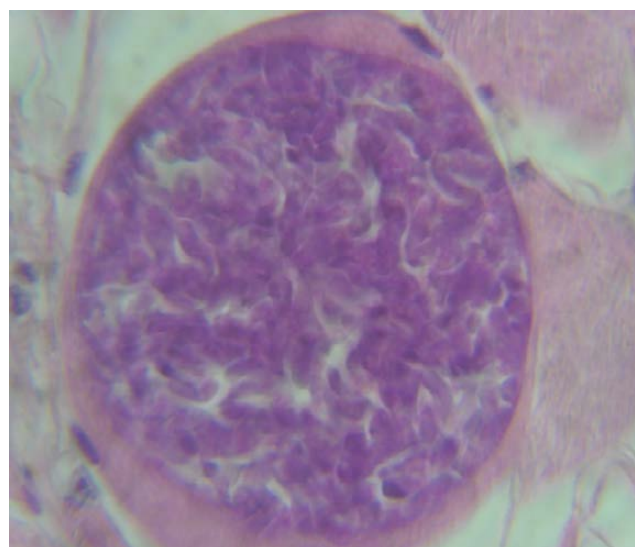
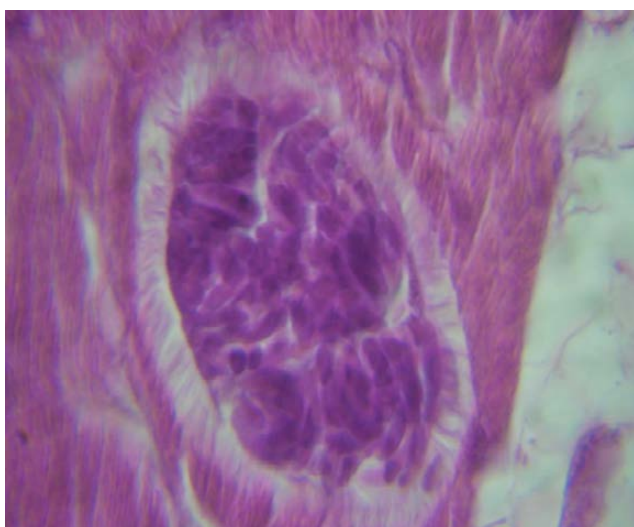
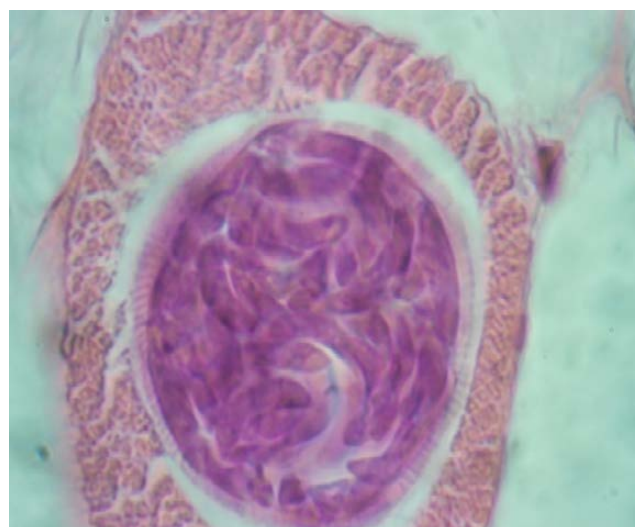


Figure 2: Dubious tissue cyst of *T. gondii* in diaphragm (H&E, 1000×)

Table: Seroprevalence of *T.gondii* in sheep according to the risk factors

Factors	Category	Sheep tested		Sheep with <i>T.gondii</i> anti-bodies		95%CI	Degree of significance and P value
		N	%	N	%		
Gender	Male	574	98.96	48	8.36	6 -10.5	Not significant p = 0.375
	Female	6	1.03	0	0		
Age Year (s)	≤ 1	87	15	5	5.75	0.9- 10.6	
	[1.5-3]	288	49.65	22	7.64	4.6-10.7	
	[3-5]	205	35.34	21	10.24	6.1-14.4	
Season	Winter	144	24.82	0	0		Very significant (p < 0.0001)
	Spring	144	24.82	7	4.86	1.3-8.4	
	Summer	144	24.82	32	22.22	15.4-29	
	Autumn	148	25.51	9	6.08	2.2-9.9	
Region (in Algeria)	Center	335	57.75	37	11.04	7.7-14.4	Very significant (p < 0.01) Without south
	Western	169	29.13	5	2.96	0.4-5.5	
	Eastern	65	11.20	6	9.23	2.2-16.3	
	South	11	1.89	0	0		

**Figure 3:** *Sarcocystis* spp cyst in oesophagus, thin wall with hair like projections (H&E, 1000×).**Figure 4:** *Sarcocystis* spp cyst in oesophagus, thick wall with radial striations (H&E, 1000×).

Hammondia, *Neospora*). Dubious tissue cysts of *T.gondii* were found in 2 sheep corresponding to a prevalence of 0.59% from 335 sheep examined and 4.16% from 48 seropositive sheep. One cyst was found in the diaphragm and the other in the oesophagi

Observed tissue cysts were spherical, had diameters of 40 to 50 μ m and surrounded by a thin wall cyst (Figure 2). However, tissue cysts compatible with *Sarcocystis* spp. were visible from both muscles tissues of 94.03% (315/335) of the sheep. These cysts

variable in size and in shape were widely dispersed throughout the tissues and according to the morphology of their walls cyst, two types of microcysts were differentiated. *Sarcocystis* with thin wall cyst with hair like projections (Figure 3) and *Sarcocystis* with thick wall cyst with radial striations (Figure 4). No significant histopathological changes were found in the evaluated organs.

4. DISCUSSION

4.1. Overall seroprevalence

The choice of the ELISA technique for this study is justified by the fact that it is considered to be one of the most sensitive immunologic techniques. A number of studies have been conducted on the seroprevalence of *T. gondii* infection in sheep from various geographical regions in the world. The present results agree almost with those obtained in Algeria by Dechicha and al. (2015) who studied sheep from different herds in the north of Algeria and found 11.59% positivity for 276 serum samples using the indirect fluorescent antibody test (IFAT). Also, similar results were observed in Chile where 12% from 408 sheep were positive to *T. gondii* antibodies by the indirect haemagglutination test (IHAT) (Gormana and al., 1999). However, our results were higher than the 7.7% obtained by Da Silva and Langoni (2001) in Brazil through IFAT and the 3.0% reported by Wang and al. (2011) in the northeastern China by IHAT. In addition, low prevalence was observed in the present study compared to those obtained in some localities from : China (20.3%) by using modified agglutination test (MAT) (Yin and al., 2015), Morocco (27.6%) by ELISA (Sawadogo and al., 2005), Ghana (33.2%) by ELISA (Van Der Puije and al., 2000), Portugal (33.6%) by means of the modified agglutination (Lopes and al., 2013), Iran (35%) by IFAT (Sharif and al., 2007), Mexico (37.9%) by IFAT (Cruz-Vazquez and al., 1992), Italy (49.9%) by ELISA (Vesco and al., 2007), Caribbean islands (65.25%) using an in house ELISA (Hamilton and al., 2014), Brazil (75%) of samples with ELISA and (80%) by using IFAT (Rossi and al., 2011). Serbia (85%) by the modified agglutination test (Klun and al., 2006).

The differences observed could be due to the sampling techniques, husbandry methods used in the different regions, frequency of cats on the farms and the

climatic variations from one region to another, which are essential elements in epidemiological studies. In addition, hygiene also presented a greater risk of *T. gondii* infections (Liu and al., 2015). Another probable explanation may be related to strain variation, since *T. gondii* strains from South America present significant genetic differences from Eurasia, Africa and North America populations (Lehmann et al., 2006). A positive association was observed between seroprevalence of *T. gondii* and the presence of cats in the herds, indicating that the presence of, and intimate contact with, feline species is important in the epidemiology of toxoplasmosis (Lopes and al., 2010). Cats are, however, likely to be found in almost all areas where sheep are kept, and the probability that a young cat may shed oocysts on a farm will always be present and any fecal material from infected cats will represent a hazard (Skjerve and al., 1998).

In Algeria, sheep raised in an extensive system and fed on fresh bulk feed or pasture presented a greater risk of toxoplasmic infection. These results support the scenario of the presence of sporulated *T.gondii* oocysts in the local environment.

Lopes and al. (2010) noted that, the lack of mineral supplementation, also had an influence in toxoplasmosis infection, this could be related to a decrease in immune defenses. Sheep that received supplementation were shown to be more immunocompetent than those that did not receive mineral supplements.

In the present study, 13 sera had a high titre of antibodies (≥ 200). This may be an indication of frequent exposure to the parasite on farms (Lopes and al., 2013). The seroprevalence demonstrated in this study indicates levels of environmental contamination with oocysts, which could potentially offer an alternative route of transmission for humans through contaminated fruit or vegetables or water (Hamilton and al., 2014).

4.2. Seroprevalence according to the risk factors

This study used samples from abattoir where the majority of the slaughtered animals were male. The difference in the occurrence of *T. gondii*-specific antibodies between genders was not considered. However gender-related tendency of prevalence had been reported previously and some data had suggested that the sex was a significant factor in determining

previous exposure to *T. gondii* infection in sheep (Clementino and al., 2007). Thus, some reports indicated that female animals were more susceptible than males to infection with *T. gondii* (Wang and al., 2011, Van Der Puije and al., 2000, Clementino and al., 2007) which was probably due to the lower levels in immune response or antibody persistence of females in some periods of their lives (Yin and al., 2015). Furthermore, according to some authors, the prevalence in males was higher than females (Alvarado-Esquivela and al., 2013, Holec Gąsior and al., 2015). However, according to the last, these results may differ because the male and female population consisted of a different number of animals, thus, the group of males represented only 5.6% of the tested population of animals (Holec Gąsior and al., 2015).

Results from our study showed an increase in *T. gondii* seroprevalence with age confirming that a major source of infection for sheep is likely to be through the consumption of sporulated oocysts from the environment, and suggesting that most sheep acquired the infection post-natally (Dubey, 2009). These results are similar to those of some previous investigations (Wang and al., 2011, Van Der Puije and al., 2000, Rossi and al., 2011, Clementino and al., 2007, Holec Gąsior and al., 2015, Gebremedhin and al., 2014, Katser and al., 2011) indicating that age was an important factor for being seropositive as a measure of the cumulated life-time risk (Katser and al., 2001). In contrast, Rahman and al. (2014) noted that seroprevalence for young and adult sheep was similar. The infection may have occurred because of poor hygiene conditions at the farm and ingestion of food or water contaminated with oocysts (Lopes and al., 2013).

On the contrary, other researchers found that there was no correlation between the seroprevalence and age and that age was not a crucial risk factor for *T. gondii* infection (Yin and al., 2015, Alvarado-Esquivela and al., 2013). Also, a study conducted in Italy; noted that the seroprevalence was already 39.6% in young animals less than 1 month of age, and suggested that these animals received *Toxoplasma*-specific IgG-antibodies from their mothers through the colostrums and milk or congenitally during the later part of gestation

(Vesco and al., 2007).

Yin and al. (2015) showed in their study that the season was considered as a risk factor associated with *T. gondii* infection and found that the seroprevalence was higher in summer and in spring, compared to winter (Yin and al., 2015). According to them, in spring and summer, the climate is warm and damp, conditions which are favorable for the survival of *T. gondii* oocysts. In addition, cats are more active at warm temperature and expand their range which lead to oocysts widely distribution (Yin and al., 2015). Also, in Ethiopia, a study revealed that the risk of *T. gondii* infection was significantly higher in sheep sampled during the dry season where the climate was more suitable for survival of the oocysts than those sampled during wet season (Gebremedhin and al., 2014). In Algeria, the climate in summer, spring and autumn is more suitable for survival of the oocysts compared to winter where we note a high mean rainfall and low mean temperature. This might be a reflection of fluctuations or differences in rate of transmission between seasons. In Mexico, the observations of a study performed on ovine toxoplasmosis showed that sheep in the driest (600 mm of mean annual rainfall) municipality had the highest seroprevalence of *T. gondii* infection and *T. gondii* oocysts remained longer in an environment with little rainfall because there is little wash or removal by the rain (Alvarado-Esquivela and al., 2013). These results suggested that environment climatic variables including mean annual temperature and mean annual rainfall are important factors correlating with the seroprevalence of *T. gondii* infection in sheep and have epidemiological significance and point toward a limitation in reporting an overall seroprevalence of *T. gondii* infection in sheep (Alvarado-Esquivela and al., 2013).

Differences in *T. gondii* prevalence across geographic locations were also reported in Scotland (Katser and al., 2011), Ghana (Van Der Puije and al., 2000), China (Yin and al., 2015), Mexico (Alvarado-Esquivela and al., 2013), Ethiopia (Gebremedhin and al., 2014) and Morocco (Sawadogo and al., 2005). According to (Katser and al., 2011) this distribution disequilibrium may be due to the spread and survival of oocysts on

pasture and lambing areas (Katser and al., 2011). While Gebremedhin and al. (2014) recorded that this might be due to climatic differences between the area which influence the tenacity and infectivity of oocysts (Gebremedhin and al., 2014). In our study, the high prevalence of toxoplasmosis in Northern Algeria (Center, Eastern and Ouestern) may be due to the high relative humidity which is favourable to the viability of *T. gondii* oocysts compared to the south with dry environments. While, significant differences in prevalence were found among the three regions in the north of Algeria, these differences may be attributed to the variable levels of contamination with *T. gondii* oocysts in different regions where sheep were exposed.

4.3. Detection of tissue cysts of *T. gondii* by histological technique

From Brazil, Da Silva and Langoni (2001) found that the examination of both brains and diaphragms by histopathological technique was negative in all examined sheep, while, forty of the sheep (7.7%) were IFAT positive. Also, Cremers et al. (1991) did not find *Toxoplasma* in smears of swine and ovine tissues. Esteban Redondon and al. (1999) reported the difficulty in detecting the parasite in tissue sections from large animals due to the low density of microorganisms and the limitation of sample size, as the parasite may be present in the unexamined tissues (Esteban Redondon and al., 1999). Also, a negative result from any sample does not necessarily mean that the whole tissue is free of the parasite (Barreto Tenório Nunes and al., 2015). The location and number of tissue cysts in animals differed with hosts and the strain of *T. gondii* (Dubey, 1998). In higher mammals (cattle, cats, sheep, goats) more tissue cysts were present in muscular tissues than in the brain (Dubey, 1998). However, the results from (Esteban Redondon and al., 1999) showed that *T. gondii* was more frequently and consistently detected within brain and heart tissues of sheep given the higher dose of *T. gondii* suggesting that the brain and heart are the favoured site for detection of *T. gondii* in experimentally infected adult sheep (Esteban Redondon and al., 1999). No significant histopathological changes were found in the evaluated organs. According to Barreto Tenório Nunes

and al. (2015), the histological alterations may differ between studies, especially with regard to the intensity of the mononuclear infiltrate observed in tissues targeted by the parasite. According to the last, the predominant finding histopathologically was the presence of mononuclear cell infiltrate in the heart and a perivascular cuff in the cerebrum and the cerebellum from sheep tissue. Dubey (1998) noted that intact tissue cysts probably do not cause any damage to the tissue and can persist without causing any inflammatory response by the host and only in cases where cysts have ruptured releasing parasites does a severe inflammation occur along with local necrosis (Dubey, 1998). As observed in the present study, this technique may have low sensitivity, because most of the samples that were positive in serological evaluation were negative by histology. We therefore consider that the results were insufficient for this technique to be adopted in routine diagnostic laboratories and we recognize that cysts are difficult to identify in histological sections, and that it is therefore more appropriate to use an association with another techniques in order to increase the chances of identifying the parasite.

5. CONCLUSION

T. gondii infection in sheep used for human consumption is prevalent in the present study, suggesting a dispersion of oocysts and parasite reservoir hosts in the environment. Season, region are risk factors of seropositivity in sheep. Considering the presence of *T. gondii* in slaughtered sheep from the study area in the north of Algeria, meat should be considered as a source of infection in the human population when consumed raw or undercooked. In Algeria, this risk is further reduced due to the customary prolonged cooking of meat. Also, the infection by oocysts eliminated by cats is another source of contamination which needs to be considered. Prevention of the spread of the disease is essential. The present serological survey does not represent a national population prevalence, other studies must be carried out to determine the true prevalence of the sheep toxoplasmosis in different regions in Algeria.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- Alvarado Esquivela C, Aguilarb DS, Villenac I, Dubey JP (2013) Seroprevalence and correlates of *Toxoplasma gondii* infection in domestic sheep in Michoacán State, Mexico. *Prev Vet Med* 112:433-437.
- Barreto Tenório Nunes AC, Da Silva Vieira EM, De Oliveira JA, Yamasaki Elise M, De Cássia Peixoto KP, De Almeida Jonatas C, Barros Nunes K, Aparecido MR (2015) Application of different techniques to detect *Toxoplasma gondii* in slaughtered sheep for human consumption. *Braz J Vet Parasitol* 24(4):416-421.
- Clementino MM, Souza MF, Andrade Net VF (2007) Seroprevalence and *Toxoplasma gondii*-IgG avidity in sheep from Lajes, Brazil. *Vet Parasitol* 146:199-203.
- Cremers, HJWM, Van Knapen F, Panggabean SO, Den Hartog JMP (1991) Problems in detecting *Toxoplasma gondii* in the muscular tissues of sheep. *Tijdschr. Diergensekd* 116:3-6.
- Cruz-Vazquez C, Garcia-Vazquez Z, Rosario-Cruz R, Solorzano- Salgado M (1992) Ovine toxoplasmosis in Huitzilac, Morelos, Mexico. *Pre Vet Med* 12:27-33.
- Da Silva AV, Langoni H (2001) The detection of *Toxoplasma gondii* by comparing cytology, histopathology, bioassay in mice, and the polymerase chain reaction (PCR). *Vet Parasitol* 97:191-198.
- Dechicha AS, Bachi F, Gharbi I, Gourbdji E, Baazize D, Brahim-Er-rahmani M, Guetarni D (2015) Sero-epidemiological survey on toxoplasmosis in cattle, sheep and goats in Algeria. *Afr J Agric Res* 10(20):2113-2119.
- Dixon BR (1992) Prevalence and control of toxoplasmosis - a Canadian perspective. *Food Control* 3:68-75.
- Dubey JP (1998) Advances in the life cycle of *Toxoplasma gondii*. *Int J Parasitol* 28:1019-1024.
- Dubey JP (2009) Toxoplasmosis in sheep—the last 20 years. *Vet Parasitol* 163:1–14.
- Esteban-Redondo I, Maley SW, Thomson K, Nicoll S, Wright S, Buxton D, Innes EA (1999) Detection of *Toxoplasmosis gondii* in tissues of sheep and cattle following oral infection. *Vet Parasitol* 86:155–171.
- Gebremedhin EZ, Abdurahman M, Hadush T, Tesfaye Sisay T (2014) Seroprevalence and risk factors of *Toxoplasma gondii* infection in sheep and goats slaughtered for human consumption in Central Ethiopia. *BMC Research Notes* 7:696.
- Germani Fialho C, Caetano Teixeira M, Pacheco de Araujo FA (2009) Animal Toxoplasmosis in Brazil. *Acta Sci Vet* 37(1):1-23.
- Gormana T, Arancibia JP, Lorcab M, Hirdc D, Alcaino H (1999) Seroprevalence of *Toxoplasma gondii* infection in sheep and alpacas (Llama pacos) in Chile. *Prev Vet Med* 40:143-149.
- Hamilton CM, Katzer F, Innes EA, Kelly PJ (2014) Seroprevalence of *T. gondii* in small ruminants from four Caribbean islands. *Parasite Vector*; 7:449.
- Holec- Gąsior L, Dominiak -Górski B, Kur J (2015). First report of seroprevalence of *Toxoplasma gondii* infection in sheep in Pomerania, northern Poland. *Ann Agr Env Med* 22(4): 604-607.
- Katzer F, Brülisauer F, Collantes-Fernández E, Bartley PM, Burrells A, Gunn G, Maley SW, Cousens C, Innes EA (2011) Increased *Toxoplasma gondii* positivity relative to age in 125 Scottish sheep flocks; evidence of frequent acquired infection. *Vet Res* 42:121.
- Klun I, Djurkovic'-Djakovic' O, Radivojevic' SK, Nikolic A (2006) Cross-sectional survey on *Toxoplasma gondii* infection in cattle, sheep and pigs in Serbia: Seroprevalence and risk factors. *Vet Parasitol* 135:121-131.
- Lehmann T, Marcet PL, Graham DH, Dahl ER, Dubey JP (2006) Globalization and the population structure of *Toxoplasma gondii*. *Proc Natl Acad Sci U.S.A* 103:11423-11428.
- Liu Z-K, Li Jian-Y, Pan H (2015) Seroprevalence and risk factors of *Toxoplasma gondii* and *Neospora caninum* infections in small ruminants in China. *Prev Vet Med* 118:488-492.
- Lopes AP, Dubey JP, Neto F, Rodrigues A, Martins T, Rodrigues M, Cardoso L (2013) Seroprevalence of *Toxoplasma gondii* infection in cattle, sheep, goats and pigs from the North of Portugal for human consumption. *Vet Parasitol* 193:266-269.
- Lopes WDZ, dos Santos TR, dos Santos da Silva R, Rossanese Walter M, de Souza FA, de Faria J D'A R, de Mendonça RP, Soares VE, da Costa AJ (2010) Seroprevalence of and risk factors for *Toxoplasma gondii* in sheep raised in the Jaboticabal microregion, São Paulo State, Brazil. *Res Vet Sci* 88:104-106.
- Rahman M, Azad Md, Thoufic A, Nahar L, Rouf S Md A, Ohya K, Chiou S-P, Baba M, Kito K, Takashima Y (2014) Age-Specificity of *Toxoplasma gondii* Seroprevalence in Sheep, Goats and Cattle on Subsistence Farms in Bangladesh. *J Vet Med Sci* 76(9):1257-1259.
- Rossi GF, Cabral DD, Ribeiro DP, Pajuaba ACAM, Corrêab RR, Moreira RQ, Mineoa TWP, Mineoa JR, Silva DAO (2011) Evaluation of *Toxoplasma gondii* and *Neospora caninum* infections in sheep from Uberlândia, Minas Gerais State, Brazil, by different serological methods. *Vet Parasitol* 175:252-259.
- Sawadogo P, Hafid J, Bellele B, Tran Manh Sung R, Chakdi M, Flori P, Raberin H, Bent Hamouni I, Chait A, Dalal A (2005) Seroprevalence of *T. gondii* in sheep from Marrakech, Morocco. *Vet Parasitol* 130:89-92.
- Sharif M, Gholami Sh, Ziaei H, Daryani A, Laktarashi B, Ziapour SP,

- Rafiei A, Vahedi M (2007) Seroprevalence of *Toxoplasma gondii* in cattle, sheep and goats slaughtered for food in Mazandaran province, Iran, during 2005. *Vet J* 174:422-424.
- Skjerve E, Waldeland H, Nesbakken T, Kapperud G (1998) Risk factors for the presence of antibodies to *Toxoplasma gondii* in Norwegian slaughter lambs. *Pre Vet Med* 35: 219-227.
- Tenter AM, Heckeroth AR, Weiss LM (2000) *Toxoplasma gondii*: from animals to humans. *Int J Parasitol* 30:1217-1258.
- Van der Puije WNA, Bosompem KM, Canacoo EA, Wastling JM, Akanmori BD (2000) The prevalence of anti-*Toxoplasma gondii* antibodies in Ghanaian sheep and goats. *Acta Trop* 76:21-26.
- Vesco G, Buffolano W, La Chiusa S, Mancuso G, Caracappa S, Chianca A, Villari S, Curro V, Liga F, Petersen E (2007) *Toxoplasma gondii* infections in sheep in Sicily, southern Italy. *Vet Parasitol* 146:3-8.
- Wang CR, Qiu JH, Gao JF, Liu LM, Wang C, Liu Q, Yan C, Zhu XQ (2011). Seroprevalence of *Toxoplasma gondii* infection in sheep and goats in northeastern China. *Small Ruminant Res* 97:130-133.
- Yin MY, Wang JL, Huang SY, Qin SY, Zhou DH, Liu GX, Tan QD and Zhu Q (2015) Seroprevalence and risk factors of *Toxoplasma gondii* in Tibetan Sheep in Gansu province, Northwestern China. *BMC Vete Res* 11:41.