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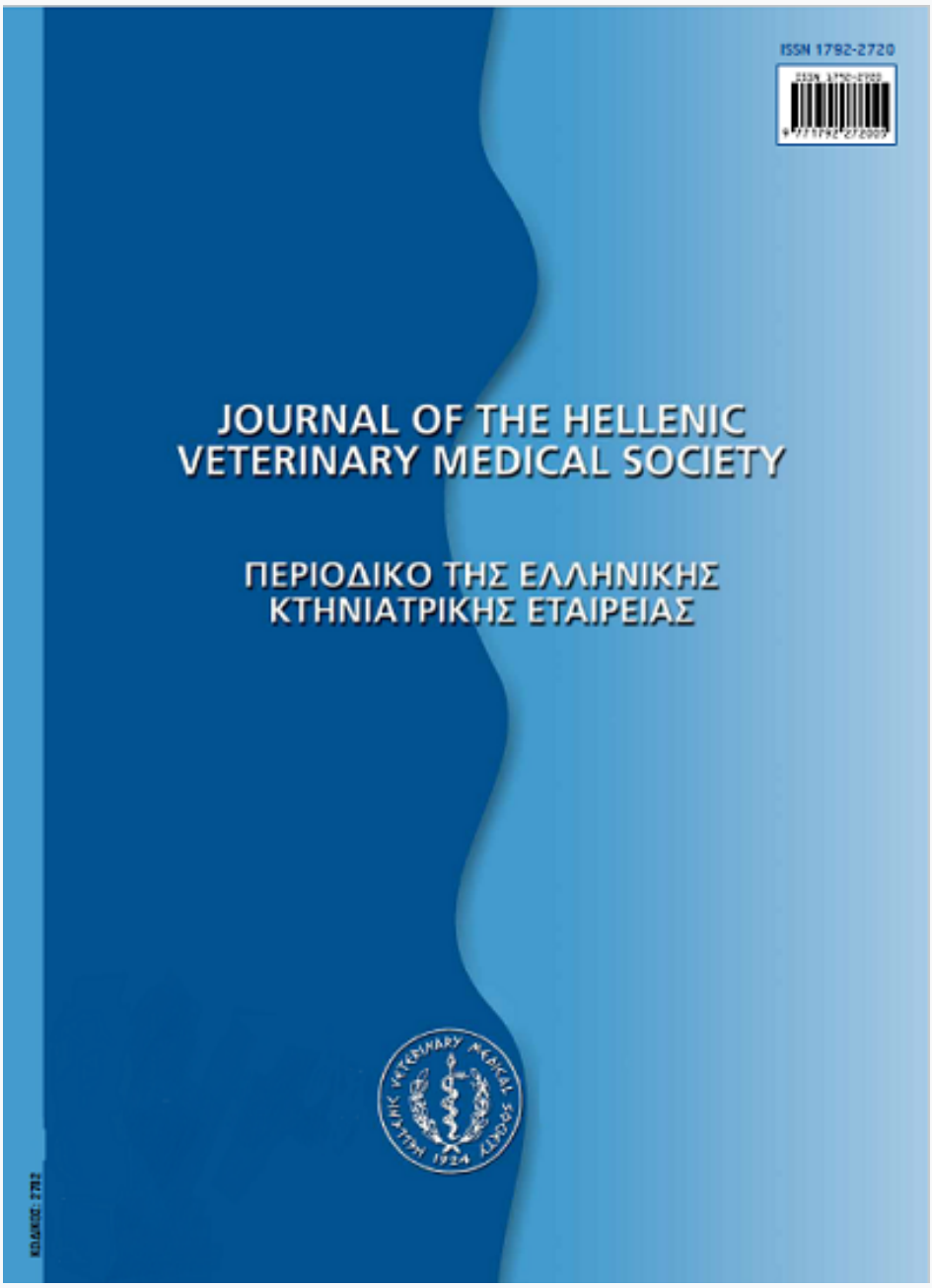

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1	ECAR	European College of Animal Reproduction	2
2	ECAWBM	European College of Animal Welfare and Behavioural Medicine	1
3	ECAAH	European College of Aquatic Animal Health	2
4	ECBHM	European College of Bovine Health Management	3
5	ECEIM	European College 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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ΕΛΛΗΝΙΚΗ ΚΤΗΝΙΑΤΡΙΚΗ ΕΤΑΙΡΕΙΑ (ΕΚΕ)
HELLENIC VETERINARY MEDICAL SOCIATY (HVMS)

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Περιεχόμενα

History of the Hellenic Veterinary Medical Society

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

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

The first post-war assembly of the HVMS took place in the private medical office of Petros Kiappe, on Peta Street in Athens. With its post-war first president Konstantinos Melanidis, the HVMS has been working by implementing its old memorandum of association and has been located in the premises of the 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, Patission street, of 265m2 area, including main lobby (14m^2), secretary (13m^2), lecture room (91m^2), the President's office (22m^2), the Governing Board meeting room & library (44m^2), the kitchen (18m^2), two big baths, a storage room and a large veranda. Al the actions performed for possessing this new private office for the HVMS were performed during the presidency of Dr Theodoros Cl. Ananiadis and the following Governing Board:

President	: Theodoros Cl. Ananiadis†
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Spec. Secretary	: Konstantinos Chandras
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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 sited below in alphabetical order:

Το Διοικητικό Συμβούλιο και η Συντακτική Επιτροπή του Περιοδικού της Ελληνικής Κτηνιατρικής Εταιρείας, ευχαριστούν θερμά τους κριτές που συνέβαλαν ουσιαστικά στην επιτυχή έκδοση του 68ου τόμου 2017 του ΠΕΚΕ, τα ονόματα των οποίων παραθέτουμε στον παρακάτω πίνακα:

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Review article
Ανασκόπηση

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 ≤ 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 parenhymatous 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 CD8α+ 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 *rpo*S activation (the main stress response regulator), which directly affects the activation of virulence genes such as the *inv*A, *hil*A and *sip*C (Aviles et al., 2013). However, experimental studies show that genes *inv*A and *hil*A 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 lactames 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 $bla_{\text{SHV-12}}$ and $bla_{\text{CMY-2}}$ 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,* $bla_{\text{CMY-2}}$, *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 ≥ 1mg/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/sulfametoxazole (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 colisitin was 31.6% in *S*. Enteritidis while resistance to ciprofloxacin and nalidixic acid was 22.4%. No resistance toward colisitin 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, Typhimurum var-5 and Infantis (NARMS-USDA, 2014). Resistance to beta lactam/inhibitor combination and cephems 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.

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Review article
Ανασκόπηση

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 υπεροξείδωση 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 cryopreservation 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.

BACKROUND 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	-Câmara 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, Moustacas 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 aforementioned 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, Soylu 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 with100 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; Rebolledo 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 peroxidase (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 μM 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 -Bioexcell ® or egg yolk	-Optimal concentration: 5-10% -Bioexcell ® may be an alternative	Gil et al., 2003
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 -Fertility of semen stored in BioXcell or milk-egg yolk extenders	No significant difference	-Fukui et al., 2008 -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	Pelufo 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 -100 mM trehalose and 8% LDL	High post-thawing semen quality	-Jafaroghli et al., 2011 -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 $B_{12,}$ 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 120μM 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	-Esmaeili 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

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Research article
Ερευνητικό άρθρο

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±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

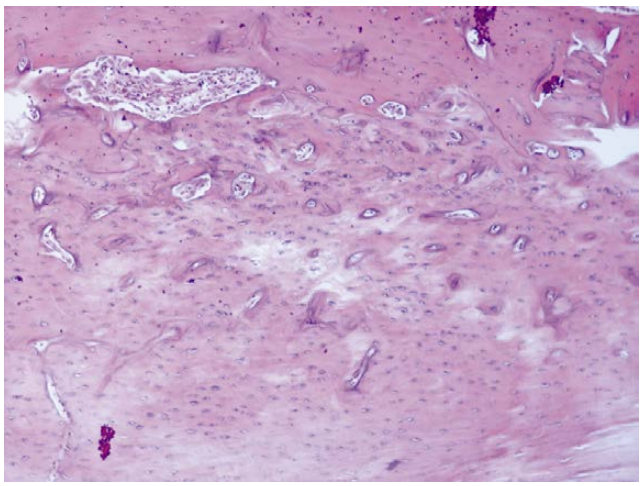

Fig 1. Cortical bone of non-ovariectomized rats, on the right side ahead of the 1st upper molar with force application, showing marked distortion of bone structure and woven bone (arrow) formation (Obj. X10 H-E).

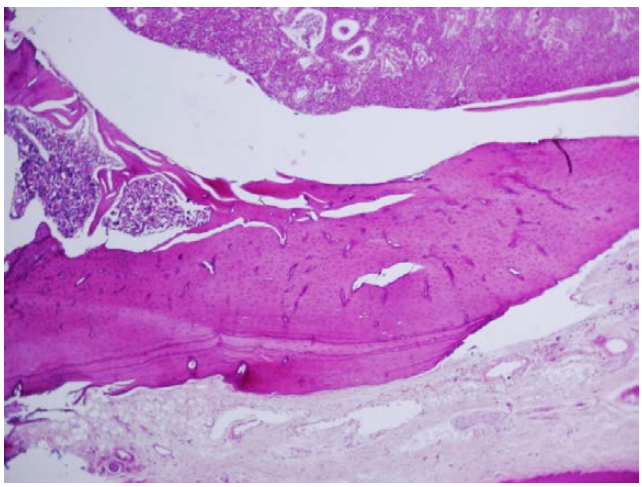

Fig 2. Cortical bone of non-ovariectomized rats, on the left side ahead of the 1st upper molar without any force application, where the bone is lamellar (arrow) and well oriented (Obj. X4 H-E).

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.

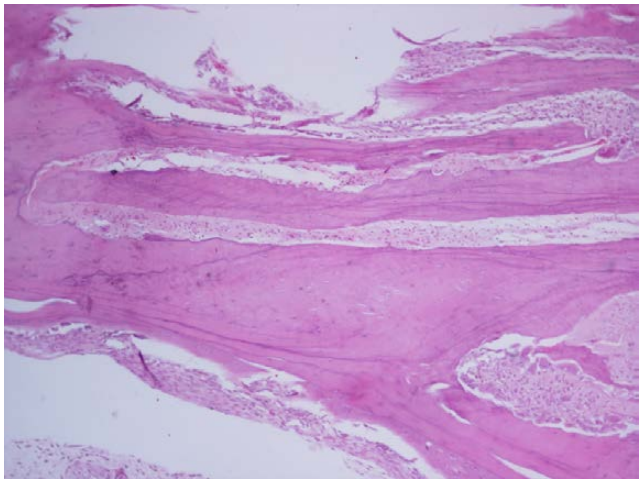

Fig 3. Cortical bone of ovariectomized rats, on the right side ahead of the 1st upper molar with force application, showing distinct cement lines (arrow), with increased bone resorption (arrowhead) and formation (white arrow) (Obj. X10 H-E).

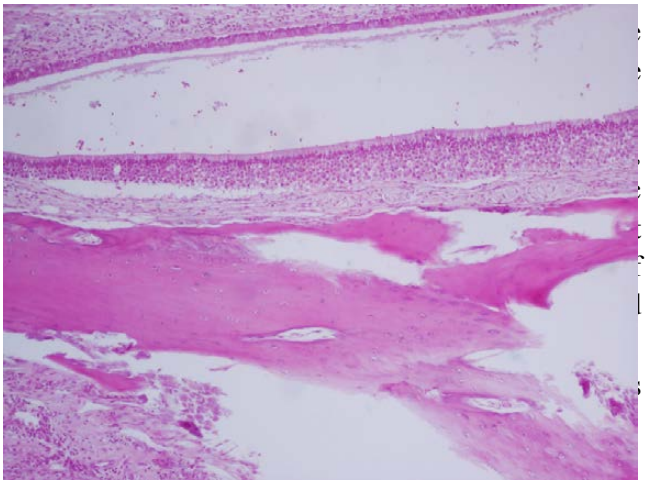

Fig 4. Cortical bone of ovariectomized rats, on the left side ahead of the 1st upper molar without any force application, showing a more compact structure (arrow) compared to the right side (Obj. X10 H-E).

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

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Research article
Ερευνητικό άρθρο

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 συνηγορεί το γεγονός ότι ο επιπολασμός της νόσου στην Ελλάδα συνδέεται με τον γονότυπο ARQ/ARQ, ο οποίος είναι ο πιο συχνός γονότυπος. Άρα η δραστική 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, πρωτεϊνη 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 (Clouscard 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 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. 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).

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

DNA Extraction, Amplification and Genotyping Analysis

All analyses were performed at the National Reference 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 ^{o}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		CHIOS CROSSBRED		CROSSBRED		TOTAL	
		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 (AA_{136}) (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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Research article
Ερευνητικό άρθρο

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 10^5 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] + 10µgml 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 º C for 5 min followed by 35 cycles at 94 º C for 45 s, 45 º C (TSST-1) or 47 º C (mecA) for 45 s and 72 º C for 45 s, followed by a final extension at 72 º 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

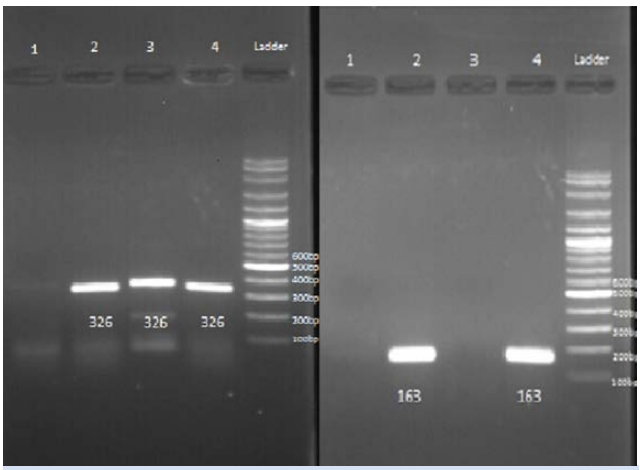

Figure 1: Amplification TSST-1 gene (Right), Amplification of mecA gene (Left)

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 TST-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*- TST-1 and mecA gene fragments in the positive samples (Figures 2&3).

The relationship between some variables and having TST-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 (Varmazyarnajafi 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 lcl|Query_244509
Description tst_PCR_tst_F
Molecule type nucleic acid
Query Length 289

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

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  GATGGCAGCATCAGCCTTATAATTTTTCCGAGTCCTTATTATAGCCCTGCTTTTACaaaa  185
            ||| |||||||| ||| | ||||||||||||||| ||||||||||||||||||||||||
Sbjct  625  GAT-GCAGCAT-AGCTTGATAATTTTTCCGAGTCATTATTATAGCCCTGCTTTTACAAAA  682

Query  186  gggaaaaaagttgacttaaacacaaaaagaactaaaaaaaGCCAACATACTAGCGAAGGA  245
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  683  GGGGAAAAAGTTGACTTAAACACAAAAAGAACTAAAAAAAGCCAACATACTAGCGAAGGA  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  TATTATAGCCCTGCTTTTACaaaaggggaaaaagttgacttaaacacaaaaagaactaaa  221
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TATTATAGCCCTGCTTTTACAAAAGGGGAAAAAGTTGACTTAAACACAAAAAGAACTAAA  60

Query  222  aaaaGCCAACATACTAGCGAAGGAACTTATATCCATTTCCAAATAAGTGGCGTTACAAAT  281
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AAAAGCCAACATACTAGCGAAGGAACTTATATCCATTTCCAAATAAGTGGCGTTACAAAT  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  TATAGCCCTGCTTTTACaaaaggggaaaaagttgacttaaacacaaaaagaactaaaaaa  224
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TATAGCCCTGCTTTTACAAAAGGGGAAAAAGTTGACTTAAACACAAAAAGAACTAAAAAA  60

Query  225  aGCCAACATACTAGCGAAGGAACTTATATCCATTTCCAAATAAGTGGCGTTACAAATACT  284
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AGCCAACATACTAGCGAAGGAACTTATATCCATTTCCAAATAAGTGGCGTTACAAATACT  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 Genebank.

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   TATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCAACAAGTTCCAGATTACAACT  128
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  72   TATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCAACAAGTTCCAGATTACAACT  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   TATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCAACAAGTTCCAGATTACAACT  128
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  72   TATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCAACAAGTTCCAGATTACAACT  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 Genebank.

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% (Varmazyarnajafi 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 shocksyndrome 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 Alfatemi 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 Alfatemi 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 mec-A 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 immunological 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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Research article
Ερευνητικό άρθρο

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 days of life) and fowl pox (at 30 days 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≤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±SE) the growth performance of Fayoumi chickens.

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

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Research article
Ερευνητικό άρθρο

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 Southeast 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*				---*P*---
			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 measurement 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 ± 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 x measurement time points (P <0.001) and age x 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; Işler 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; Işler 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 litterateur 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.

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Research article
Ερευνητικό άρθρο

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 ($\chi 2$ 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, flee 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 Prefectrural 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 overal 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

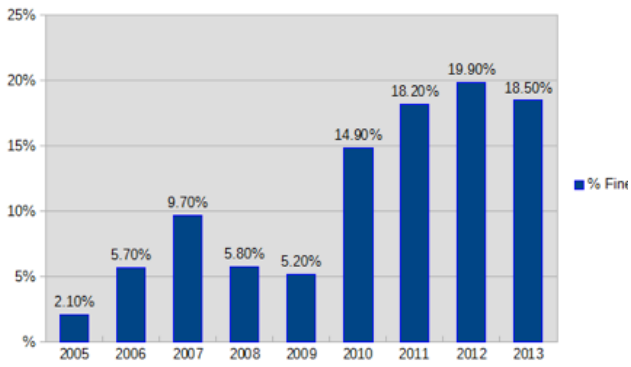


Figure 1: Per year percentage of the imposed fines

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

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

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

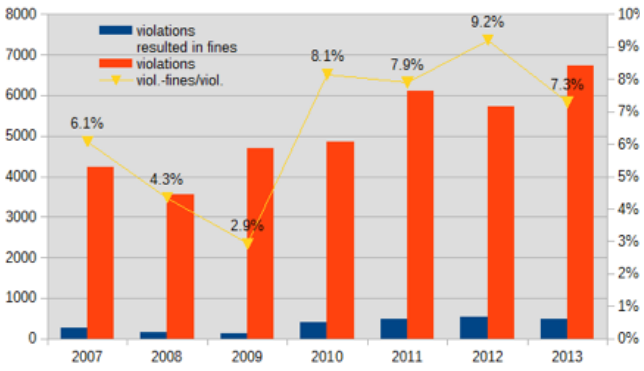


Figure 2: Rate of violations, where fines were imposed, over the total figures of delinquency per year (2007-2013).

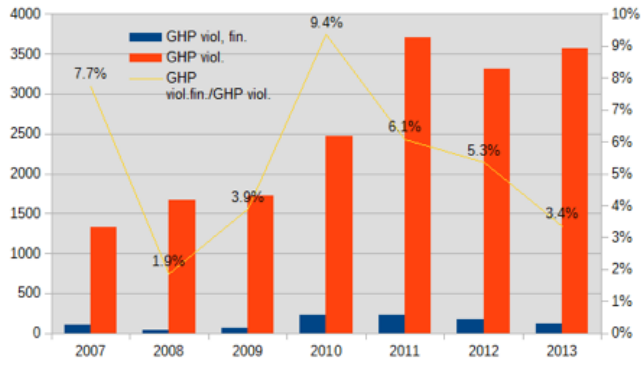


Figure 3: Rate of violations concerning GHP, where fines were imposed, over the total figures of delinquency concerning GHP, per year (2007-2013).

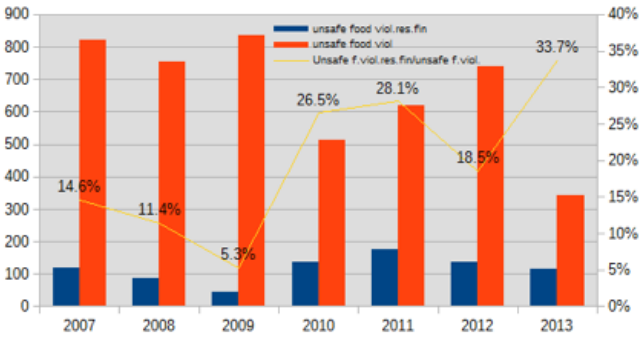


Figure 4: Rate of violations concerning sale of unsafe food, where fines were imposed, over the total figures of delinquency concerning unsafe food per year, (2007-2013).

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**.

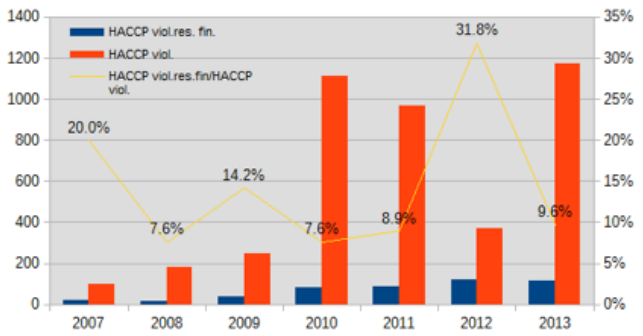


Figure 5: Rate of violations concerning HACCP system, where fines were imposed, over the total figures of delinquency concerning HACCP per year (2007-2013).

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),

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

Perfecture 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 Aigaiou	11	0.4%
Sterea Greece	9	0.3%
West Makedonia	5	0.2%

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).

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€, ±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€, ±509325€), 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%.

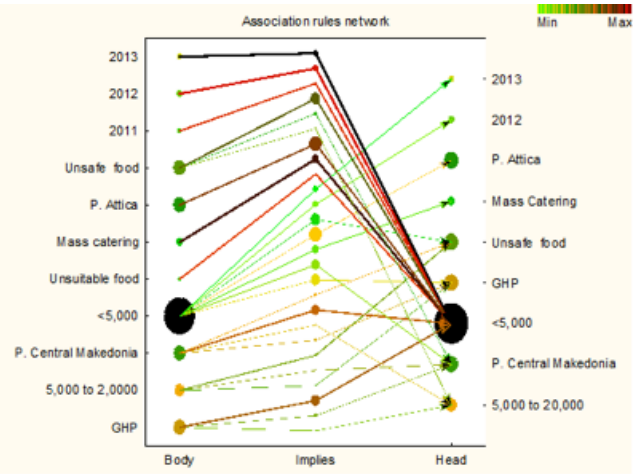
If the fine was imposed for the sale of unsuitable food, **then** its level would be <5.000€, with a rate of confidence 63,98%.

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 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.



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).*

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..

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Research article
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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 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 encodes 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 deactivate 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)
tet(A)	*5'-GCT ACA TCC TGC TTG CCT TC-3'* *5'-CAT AGA TCG CCG TGA AGA GG-3'*	210
tet(B)	*5'-TTG GTT AGG GGC AAG TTT TG-3'* *5'-GGTA ATG GGC CAA TAA CAC CG-3'*	659
tet(C)	*5'*-CTT GAG AGC CTT CAA CCC AG-*3'* *5'*-ATG GTC GTC ATC TAC CTG CC-*3'*	418
tet(D)	*5'*-AAA CCA TTA CGG CAT TCT GC-*3'* *5'*-GAC CGG ATA CAC CAT CCA TC-*3'*	787

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 $MgCl_2$, 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).

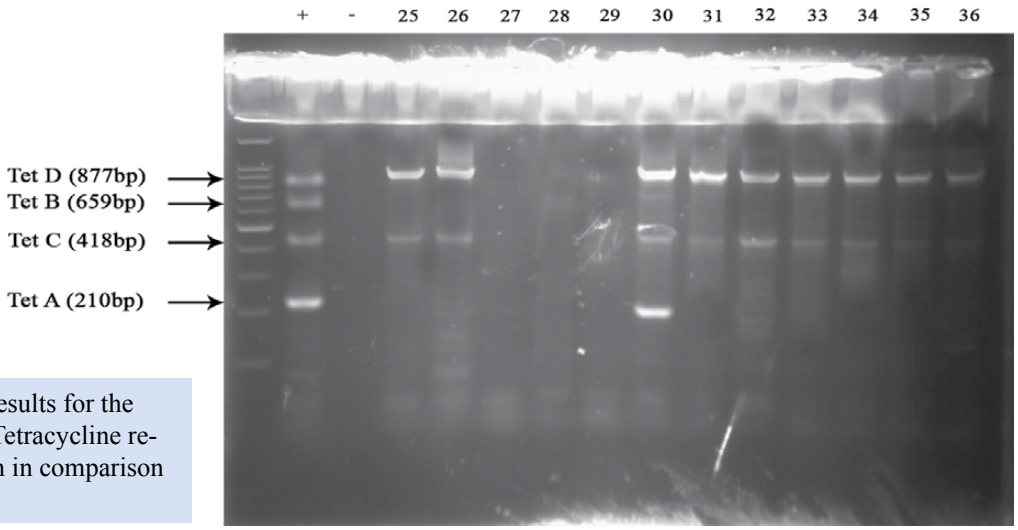

Fig 1: Multiplex PCR results for the detection of *S. aureus* Tetracycline resistant strains are shown in comparison to 100 bp DNA ladder.

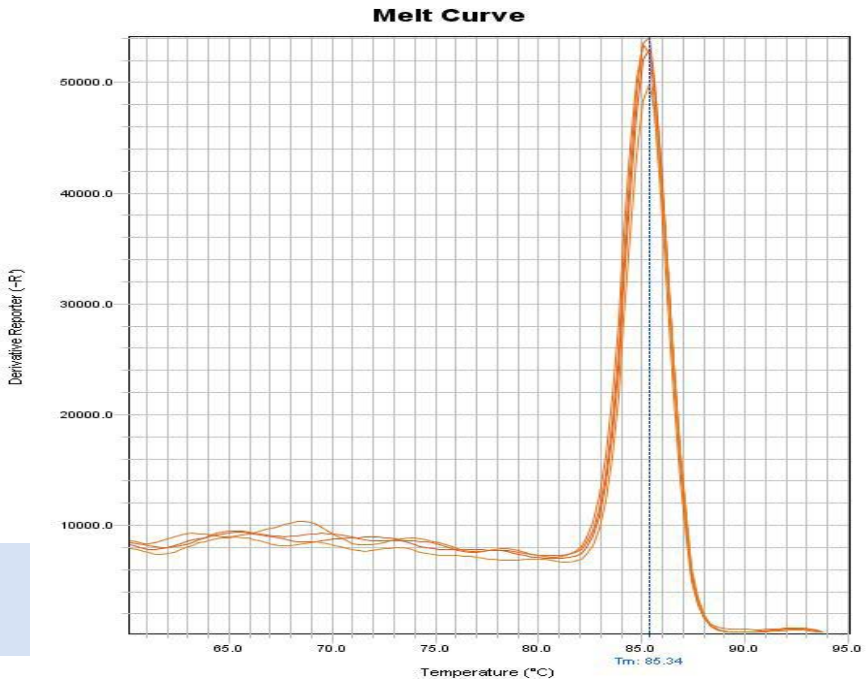

Fig 2: Melting curve recorded in Real Time-PCR on *S. aureus* strains treated with Fe Nano-particle

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).

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

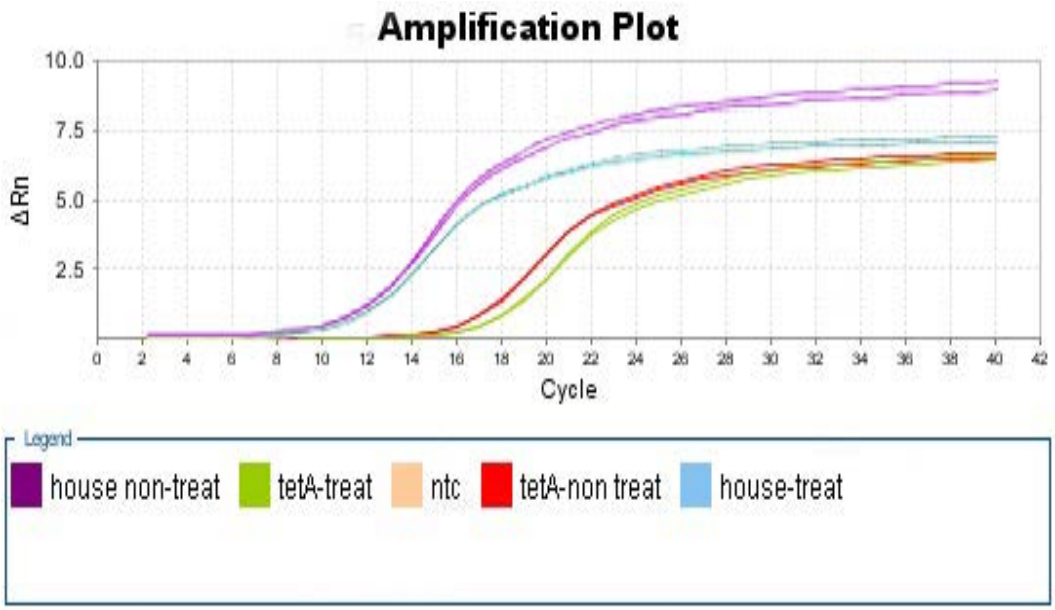

Fig 3: Comparison of the TetA expression level in *S. aureus* treated with Fe Nano-particles and non-treated strains.

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 (Bodarenko 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 32µg/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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Research article
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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 seroprevalence 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 seroprevelence 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 *N. caninum*-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.

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Research article
Ερευνητικό άρθρο

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 dehy droepiandrosterone) 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 transcripted 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 tri-iodothyronine (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, staring 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 2×10^4/µl. 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 ≥0.2OD. 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 neosporesis (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 NFκB 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 ± 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±0.8[a]	3.31±0.1[a]	7.42±1.9[a]
Non- infected	4.6±1.4[a]	3.42±0.2[a]	9.61±1.7[b]

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

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Research article
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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 anestrous 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 feed level x 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 x 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 to all other groups. The main effect of feeding level and the feeding level x 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. 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)

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 ± standard deviation.

Item*	1C	1T	1/2C	1/2T
Body weight, kg	42.8 ± 9.9[a]	45.8 ± 3.2[b]	39.1 ± 5.6[c]	39.7 ± 8.9[c]
Body condition score*	2.4 ± 0.4[a]	2.5 ± 0.3[a]	2.1 ± 0.4[b]	2.2 ± 0.6[b]
Sexual odor intensity**	0.15 ± 0.23[a]	0.29 ± 0.38[b]	0.12 ± 0.21[a]	0.26 ± 0.34[b]
Scrotal circumference, cm	26.8 ± 2.0[a]	26.9± 1.2[a]	25.1 ± 1.3[b]	1.9 ± 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 x testosterone treatment interactions (P>0.05) were found.
[a,b,c]Means in the same raw with different superscript differ (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 subcutaneous 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 ± standard deviation

Item	1C	1T	1/2C	1/2T
Testosterone, ng/mL[A]	0.84 ± 1.58[a]	4.78 ± 6.78[b]	0.56 ± 0.96[a]	2.76 ± 4.71[c]
Semen volume (mL)	0.14 ± 0.06	0.33 ± 0.22	0.22 ± 0.19	0.30 ± 0.28
Sperm concentration (x10^6 mL^{-1})	826 ± 276	1037 ± 380	916 ± 389	1026 ± 307
Mass motility (1–5 scale)	1.3 ± 1.0	2.3 ± 1.4	1.6 ± 1.1	1.8 ± 1.5
Sperm progressive motility (%)A	57 ± 11[a]	58 ± 14[a]	52 ± 16[a]	46 ± 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.
[A]Feeding level x 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 ± standard deviation

Item	1C	1T	1/2C	1/2T
Anogenital sniffing	10.0 ± 7.6[a]	59.8 ± 38.1[b]	11.2 ± 7.2[a]	26.0 ± 20.1[a]
Flehmen responses	1.0 ± 0.7	5.2 ± 6.3	0.6 ± 1.3	3.6 ± 4.6
Vocalizations	10.6 ± 12.7[a]	78.2 ± 30.3[b]	20.8 ± 33.0[a]	27.2 ± 18.2[a]
Approximations to does	12.6 ± 11.3[a]	82.6 ± 41.5[c]	33.0 ± 34.5[ab]	54.0 ± 34.2[bc]
Mount attempts	0.4 ± 0.5[a]	12.8 ± 11.6[b]	1.8 ± 1.3[a]	7.8 ± 5.6[b]
Complete mounts	0.0 ± 0.0	0.8 ± 1.3	1.2 ± 1.6	1.8 ± 1.9
Mounts with penis exposed	0.8 ± 1.7[a]	4.8 ± 3.4[b]	0.6 ± 0.5[a]	2.4 ± 1.5[a]
Mounts with ejaculation	0.4 ± 0.9[a]	4.0 ± 1.2[b]	0.6 ± 0.5[a]	2.8 ±1.[6]a
Latency to first mount, sec[A]	164 ± 43[a]	82 ± 63[c]	145 ± 51[a]	110 ± 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).
[A]Feeding level x testosterone treatment interaction (P<0.05).
[a,b]Means in the same raw 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-

nition 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.

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Research article
Ερευνητικό άρθρο

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, εξετάσθηκαν 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 (×10, ×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			
Eimeria spp.	oocyst	44 (40.00%)	30.8 – 49.8 %
Entamoeba bovis	cyst	18 (16.36%)	10.0 – 24.6 %
Helminths			
Strongylida	egg	35 (31.82%)	23.3 – 41.4 %
Dicrocoelium dendriticum	egg	31 (28.18%)	20.0 – 37.6 %
Lungworms	larva	31 (28.18%)	20.0 – 37.6 %
Moniezia benedeni	egg	30 (27.27%)	19.2 – 36.6 %
Fasciola hepatica	egg	18 (16.36%)	10.0 – 24.6 %
Toxocara vitulorum	egg	13 (11.82%)	6.4 – 19.4 %
Paramphistomum cervi	egg	11 (10.00%)	5.1 – 17.2 %

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Case Report
Κλινικό Περιστατικό

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, gooses, 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 at 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 μm 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

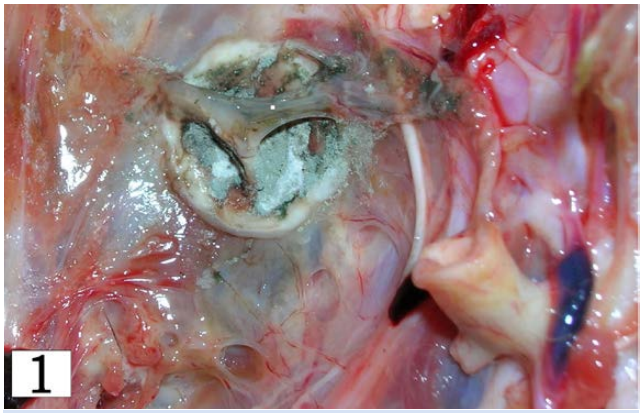

Figure 1. Moldy, greyish white depositions that resemble craters showing typical appearance of Aspergillosis in the lungs.

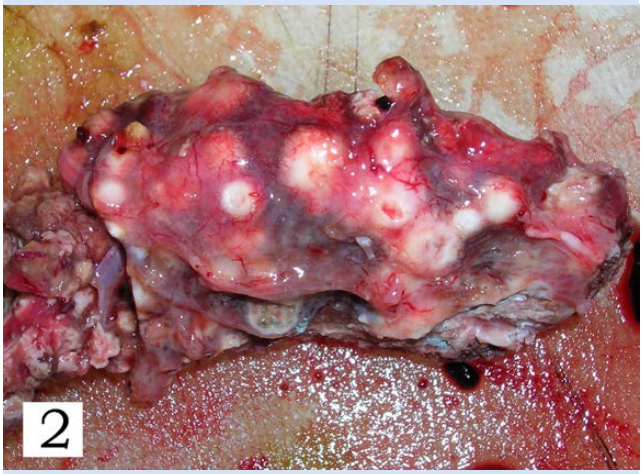

Figure 2. White round pyogranulomatous nodules filled with pus.

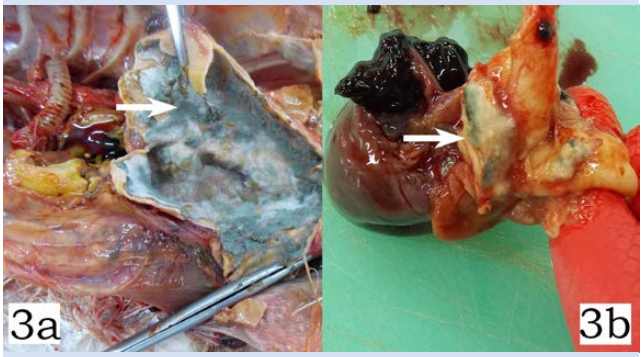

Figure 3

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

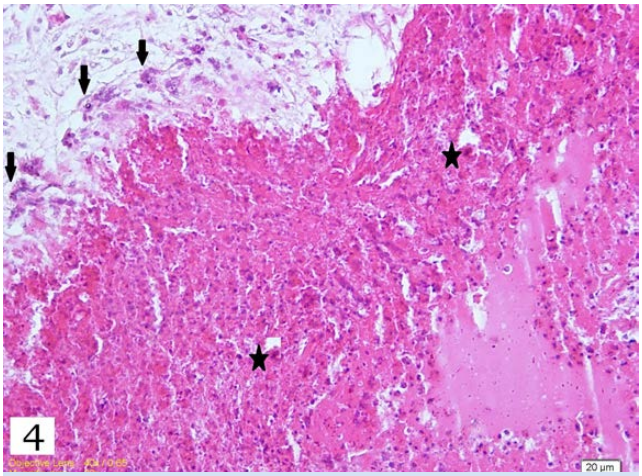

Figure 4. Focus of caseification necrosis (star) surrounded by a demarcation zone that consists of numerous macrophages, heterophil leukocytes, lymphocytes, foreign body giant cells (arrows) and fibrocytes and fibroblasts in the lung (H&E).

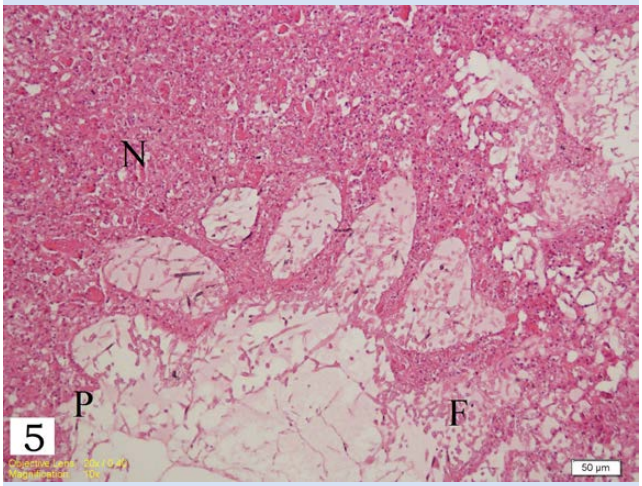

Figure 5. Necrotic pyogranulomatous areas (N) and numerous septate and non-septate hyphae (F) were present in the lumens of parabronchioles (P) (H&E).

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

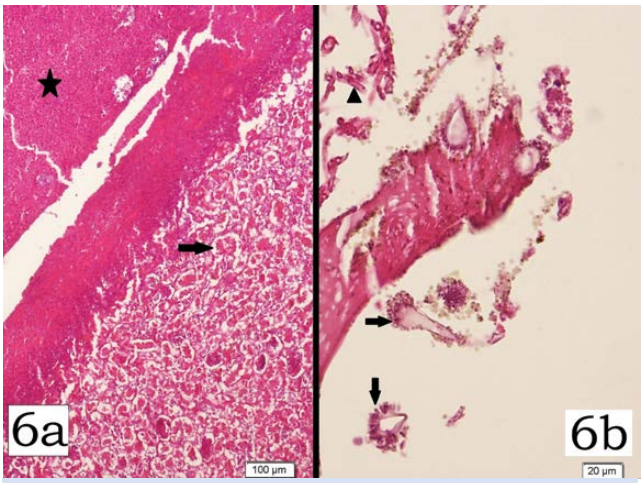

Figure 6.
a. Caseification necrosis (star) in air sacs adjacent to the kidney (arrow),
b. Conidiophores of *Aspergillus fumigatus* (arrows) and hyphae (arrowhead) (H&E).

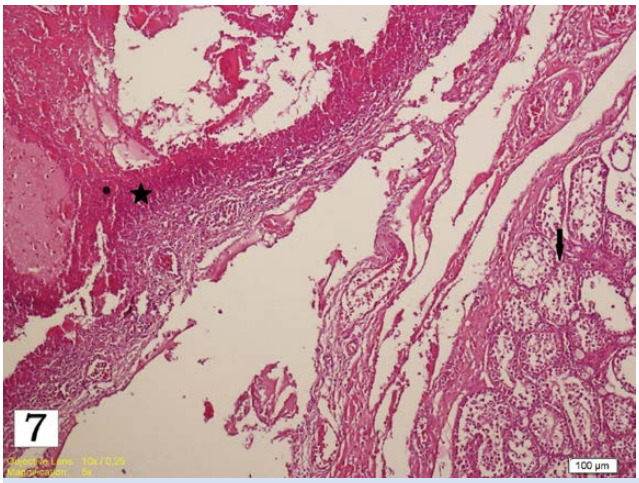

Figure 7. Caseification necrosis (star) in air sacs adjacent to the seminiferous tubules(arrow) (H&E).

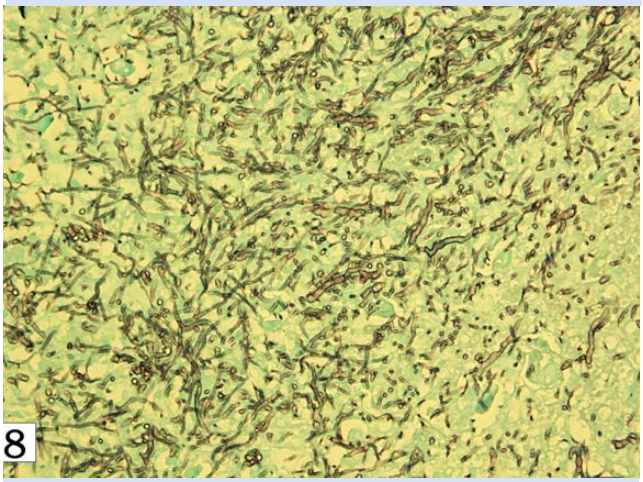

Figure 8. Several fungal hyphae in the parabronchi (Grocot Stain-GMS)

were 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 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 wereolder 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.

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Case Report
Κλινικό Περιστατικό

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 ovariohysterectomy 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 (Gama glutamyl transpeptidase), urea, creatinine, glucose, cholosterol, 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		Referance 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).

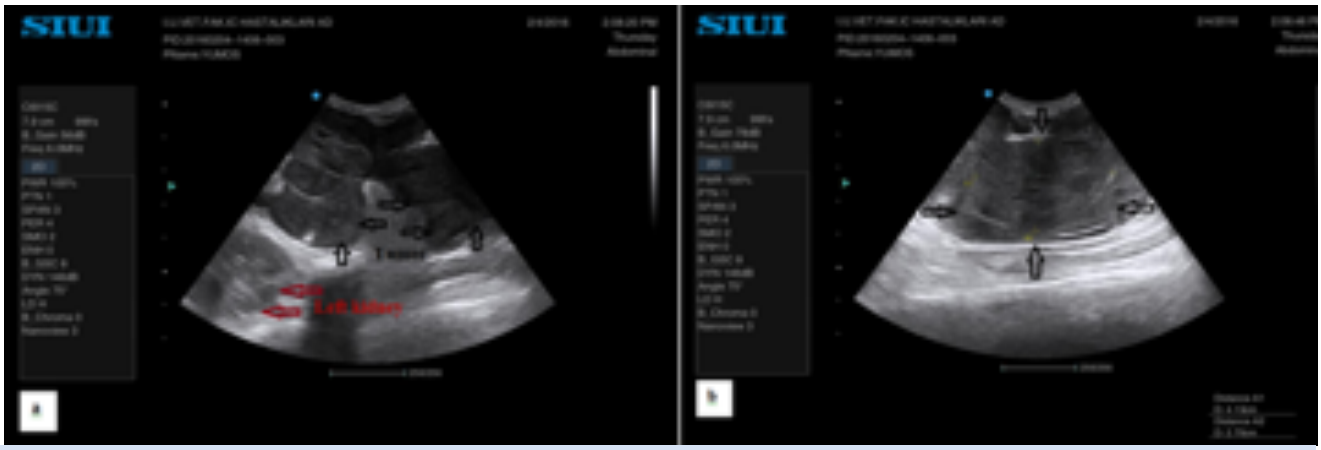

Figure 1a. The mass behind left kidney on cranioabdominal regio, **1b** Tumoral mass on ultrasonographic examination.

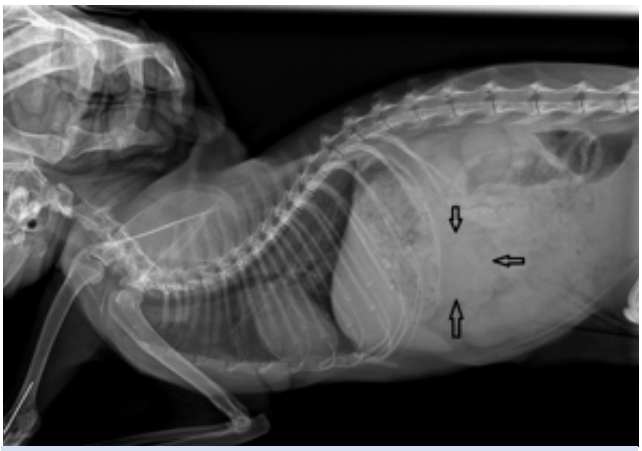

Figure 2. Tumoral area on thoraco-abdominal radiography of the cat.

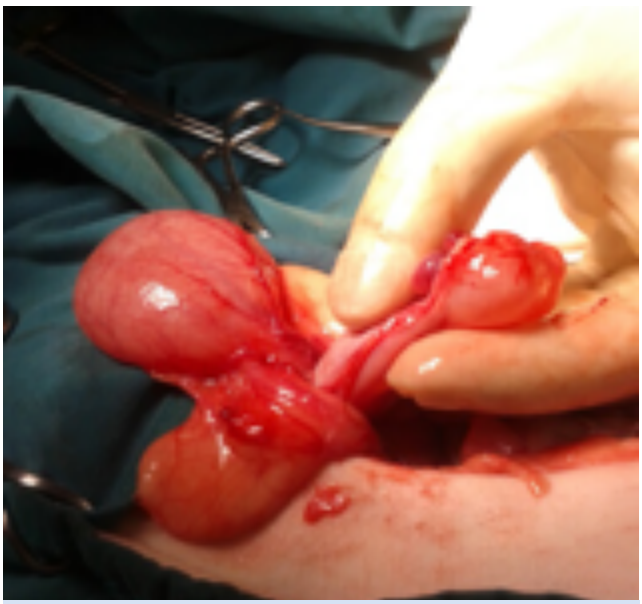

Figure 3. Uterine stump

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 parapoxvirus 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-

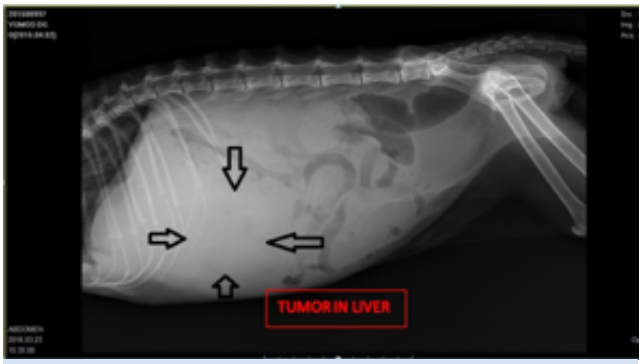

Figure 4. Abdominal radiography of the tumor in liver.

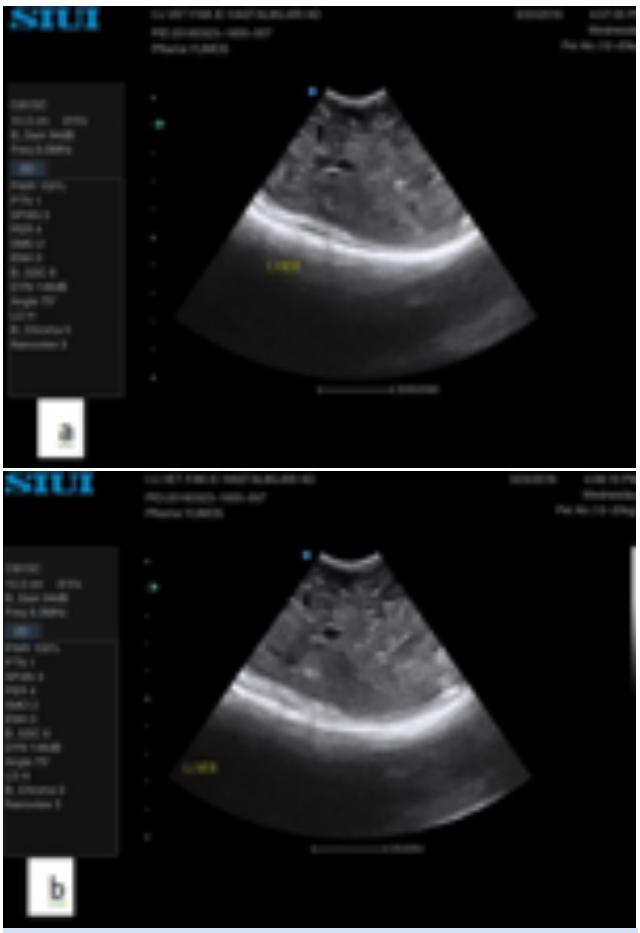

Figure 5a,b Hyperechoic areas within liver on ultrasonography.

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,

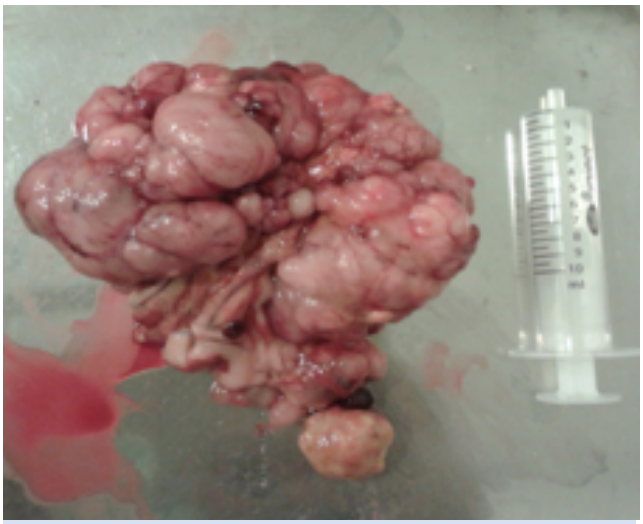

Figure 6. Tumoral mass in macroscopic examination.

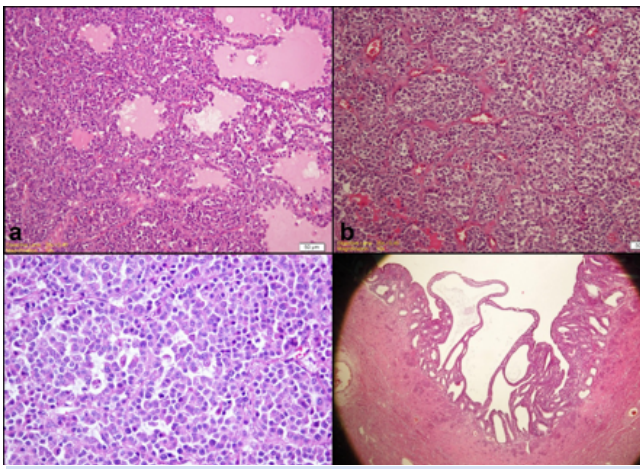

Figure 7a Cystic structures with diffuse, atypical granulosa cells. **7b, 7c** Density of distinctive tubuler pattern in stroma. **7d** Cystic glandular metritis.

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.

Buijtels et al. (2010) reported that GCTs produce E2 and small amount of P4 which causes endometrial growth and glandular secretion. In accordance with Buijtels 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 erroneous ovariohysterectomy 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.

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Case Report
Κλινικό Περιστατικό

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, particulary 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 Universitary Hospital of Mustapha Bacha - Algeirs -. 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 min ± 4 min at 21 °C (± 5 °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 min ± 3 min at 21°C (± 5 °C). Then, the wells were washed, 100 ul of substrate solution was distributed and incubated for 15 min ± 2 min at 21°C (± 5°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 ODpc > 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% = Optical density of sample × 100/ Optical density of positive control. Samples with S/P% ≤ 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 ≥ 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% ≥ 200, while 35 sera had S/P% between 50% and 200% (Figure 1). The chi-square test was very significant between the two levels of seropositive cases (S/P% between 50% and 200%, S/P% ≥ 200).

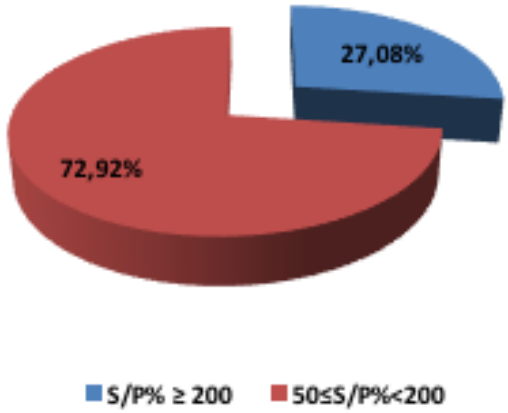

Figure 1: Repartition of the 48 seropositive sheep according to S/P% value.

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, beacause the parasite can be confused with other protozoan parasites (*Sarcocystis*,

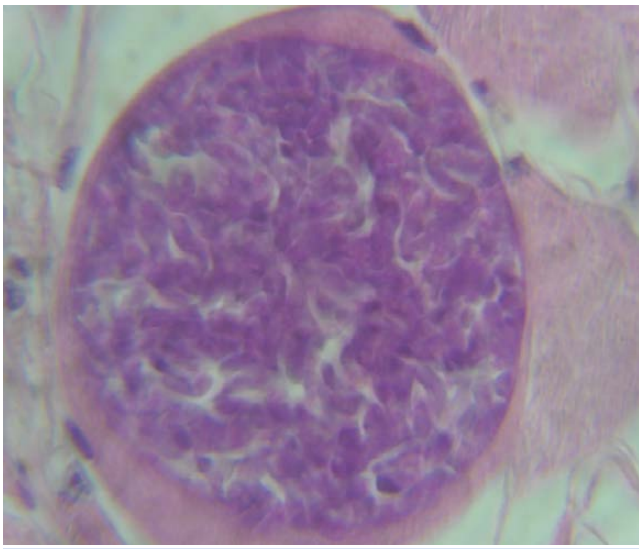

Figure 2: Dubious tissue cyst of *T.gondii* in diaphragm (H&E, 1000×)

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

Factors	Category	Sheep tested		Sheep with *T.gondii* antibodies		95%CI	Degree of significance and P value
		N	%	N	%		
Gender	Male	574	98.96	48	8.36	6 -10.5	
	Female	6	1.03	0	0		
Age Year (s)	≤ 1	87	15	5	5.75	0.9- 10.6	Not significant p = 0.375
	[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		

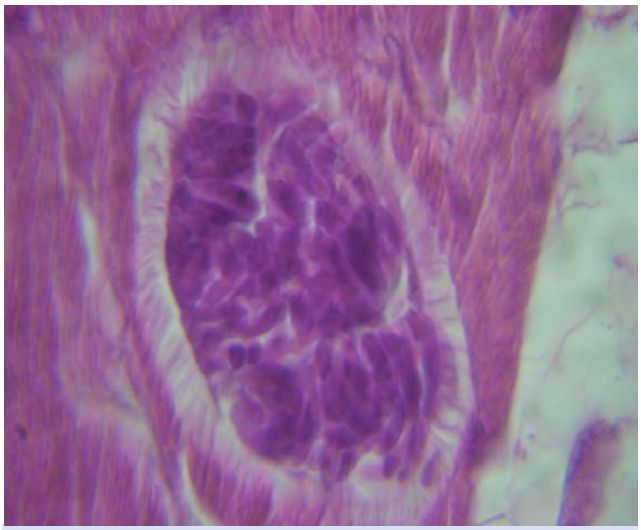

Figure 3: *Sarcocystis* spp cyst in oesophagus, thin wall with hair like projections (H&E, 1000×).

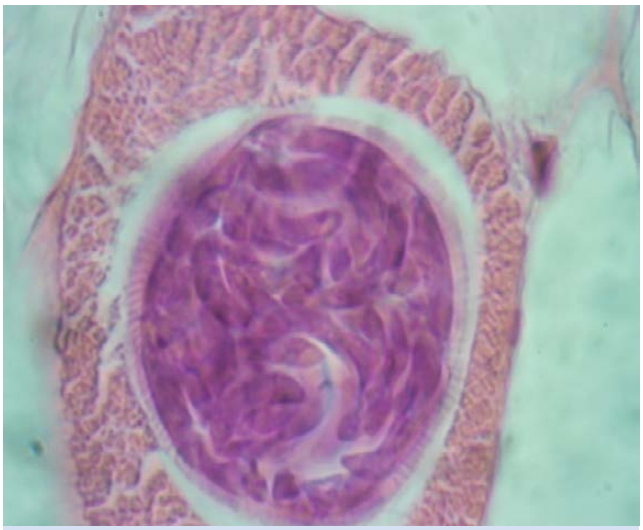

Figure 4: *Sarcocystis* spp cyst in oesophagus, thick wall with radial striations (H&E, 1000×).

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 um and surrouded 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., 20011, 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 attribuated 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 difered 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.

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