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ΕΥΡΩΠΑΪΚΑ ΚΟΛΕΓΙΑ ΕΙΔΙΚΕΥΜΕΝΩΝ ΚΤΗΝΙΑΤΡΩΝ

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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	ECBHM	European College of Bovine Health Management	3
5	ECEIM	ECEIM	European College of Equine Internal Medicine	0
6	eclam	ECLAM	European College of Laboratory Animal Medicine	0
7	ECPHM	ECPHM	European College of Porcine Health Management	3
8	ECPVS	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	ECVCP	European College of Veterinary Clinical Pathology	1
13	ECVD	ECVD	European College of Veterinary Dermatology	3
14	ECVDI	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	ECVN	European College of Veterinary Neurology	0
18	ECVO	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	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†
Vice-President	: Veniamin Albalas
General Secretary	: Athanassios E. Tyrpenou
Spec. Secretary	: Konstantinos Chandras
Treasurer	: Olga Sabatakou
Member	: Emmanuel Archontakis
Member	: Apostolos Rantsios

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Review article
Ανασκόπηση

The Potential Role of Probiotics (nutraceuticals) in Gut Health of Domestic Animals; an Alternative to Antibiotic Growth Promoters

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ABSTRACT. The term gut health is currently becoming more important for domestic animals including poultry. Gut health refers to the fundamental organ system which covers multiple positive functions like effective digestion, stabilizing intestinal microbiota, gut pH and modulation of effective immune response. Gut health depends on proper balance of microbial population. A wide range of feed and pathogen associated factors influence this balance, and adversely affect the animal health status and production performance. Antibiotic stimulators have been used in farm animals to achieve maximum production. But drug resistance and residual effects of antibiotics in animal products (milk, meat and egg etc.) have raised serious issues in human life. Therefore, The European Union (EU) has strictly banned the application of antibiotic stimulators in livestock nutrition in several others countries including China. As a result, an alternative to antibiotic growth promoters are required to support the profitable and sustainable animal production system. Probiotics as nutraceuticals has been categorized as an alternative natural feed supplement for commercial utilization. Such products have been recognized as safe feed additives in animal industry. Very few studies have comparatively described the effect of probiotics on gut health of domestic animals. Therefore, the aim of this review is not only to explore the beneficial effects of probiotics in improving gut health of domestic animals as an alternative to antibiotic growth promoters, but also to evaluate the probiotics associated health and risk factors, and to provide comprehensive scientific information for researchers, scientists and commercial producers.

Keywords: Animal production, Antibiotic growth promoters, Domestic animals, Gut health, Immune response, Probiotics

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INTRODUCTION

Optimum production level and best feed efficiency ratio are two main objectives in animal production system, which can be achieved by using of probiotics. A number of factors affect the production of animals including genetic potential, quality of feed, environmental stress and disease incidence. Excluding these factors, intestinal health has become the topic of great interest in animal production (Rinttila and Apajalahti, 2013). However, the term is specified to the gastrointestinal tract only and does not comprise other organs (Lalles et al., 2007). Gut mucosa acts as selectively permeable barrier between the lumen environment and the internal body tissues (Yegani and Korver, 2008; Markowiak and Śliżewska, 2018).

The gut is the major site for different processes such as digestion and fermentation of feed, nutrient absorption and metabolism, along with intestinal integrity and immune system development (Sommer and Kiel, 2013; Roselli et al., 2017). An animals' gut mucosa acts as an effective barrier between the tissues of the animal and its luminal content (Yegani and Korver, 2008). The gut is also a main site of extensive exposure to environmental benefits as well as harmful pathogens (Servin, 2006). Therefore, intestinal mucosa is a good determinant of gut health and optimal performances of the animals (Markowiak and Śliżewska, 2018). A lot of factors including feed, environment and infectious agents appear to affect the gut health and function which may consequently affect the animals health and production performance (Yegani and Korver, 2008).

Overuse and misuse of antibiotics cause antibiotic resistance in farm animals (Kabir, 2009) resulting in high residual effects in animal products such as meat, milk and egg which can develop drug-resistant microorganisms in human life and exerting deleterious effects on the environment (Olatoye and Ehinwomo, 2010; Markowiak and Śliżewska, 2018). Hence, in 2006, the European Union (EU) banned the usage of AGPs, which has now been followed by many other countries including China (Markowiak and Śliżewska, 2018). Therefore, in China, the poultry and livestock industry is now struggling to maintain animal production due to high feed costs and the restriction of AGPs in animal feeds. On the other hand, both consumers and manufacturers are now seeking alternatives to AGPs to confirm the safety of animal products (Smith et al. 2002). Experts have continuously worked to formulate natural, safe and effective growth promoters referred to as probiotics which might play a significant role for improving gut microbiota and gut health of domestic animals (Azzaz et al., 2012). Biotechnology has significant impact on animal nutrition and has given permission to produce large amounts of large amounts of probiotic supplements and their metabolites (Chauhan and Ak, 2014). Probiotics enhance feed intake, milk production, immune system and gut health (El-din, 2015; Roselli et al., 2017; Markowiak and Śliżewska, 2018) but it has reported that probiotic have different consequences on gut health of domestic animals (cattle, buffalo, pig and poultry) depending upon its composition, animals age and utilization in animal feeding (Markowiak and Śliżewska, 2018). Very few studies have thoroughly explored the effect of probiotic as nutraceuticals on gut health in domestic animals. Therefore, this review aims is to evaluate the potential role of probiotics (nutraceuticals) on intestinal health of domestic animals and their possible outcomes on animal physiology. The safety and hazards associated with probiotics have also been briefly summarized.

PROBIOTIC AS NUTRACEUTICALS: WHAT ARE THEY?

The term "nutraceutical" can be defined as any food or food particles that play an essential role in maintaining normal body function that provides health benefits, including the prevention and treatment of a disease (Das et al., 2012). Nutraceuticals are obtained from dietary supplements (probiotics, prebiotics, synbiotics, organic acids, clay minerals, exogenous enzymes, recombinant enzymes, nucleotides and polyunsaturated fatty acids), isolated nutrients (vitamin, mineral, amino acids, fatty acids) and herbal products (herbs or botanical products) (Das et al., 2012). Very specifically, they have been tested for their potential to replace AGPs in livestock and poultry nutrition (Khan et al., 2012; Sethiya, 2016). Probiotics as nutraceuticals are primarily used to improve animal health towards different infectious agents rather than normal nutrition. The potentials

of these probiotics in improving gut morphology, gut health and nutrient absorption may also encourage animal owners to utilize this feed supplementation to support intestinal health and production performance of farm animals.

SELECTION CRITERIA OF PROBIOTICS

Whereas selecting the probiotics, certain points must be taken into consideration: production, administration, application, colonial survival in the host and their physiological benefits. Probiotics should have the following properties in order to be effective: they must be able to produce antimicrobial property towards pathogens (Kullen and Klaenhammer 2000), they must have ability to adhere with intestinal epithelium and colonize the lumen of the gastrointestinal tract, they must have a positive effect on animals (non-pathogenic, non-reactive and non-toxic) (Roselli et al., 2017), they must be able to withstand the gastric acidity, bile salts and digestive enzymes (Parvez et al., 2006), they must have ability to reduce the incidence and severity of pathogen adhesion, they must have ability to stabilize normal gut microflora and be associated with health benefits (Markowiak and Śliżewska, 2018).

MODE OF ACTION OF PROBIOTICS

The significant property of probiotics is the ability to reduce incidence and severity of diseases due to development of colonization resistance or inhibitory effects towards pathogens. However Probiotics hinder pathogenic bacteria both in vitro and in vivo by different mechanisms of action, the exact method in which they exert their positive effects has not been fully determined (Kechagia et al., 2013). However, Seo et al. (2010) enlisted several possible modes of action of probiotics in domestic animals (ruminant, pig and poultry) (Table 1) such as: maintain the normal gut microbial growth by competitive exclusion and antagonism (Oliveira et al., 2000; Kabir 2009; Binek, 2016), alter the pattern of ruminal fermentation, improve feed intake and nutrient digestibility (Ghareeb and Zentek, 2006), and the supply of nutrients to the small intestine, higher nutrient retention rate and decreased stress by immunostimulation. Other mechanisms have been suggested specifically by several authors to illuminate positive effects of probiotics (Markowiak and Śliżewska, 2018) which can be explained as following; production of antimicrobial substances (acids, bacteriocins, antibiotics) (Vandenbergh, 1993), competition with detrimental organisms for adhesion sites (Retta, 2016), modulate immune response through increasing phagocytic activity of macrophages and natural killer cells (Erika et al., 2001), reduction of bacterial toxin metabolism metabolism (Markowiak and Śliżewska, 2018) and variation of enzymes secretion (Azzaz et al., 2015). These mechanisms may benefits ruminant by increasing nutrient absorption through reducing the

Table 1. Mode of action of probiotics

Item	Description
Competitive exclusion	Compete for nutrients in the gastro-intestinal Exclusion property towards pathogens
Antimicrobial effects	Produce antimicrobial substances which have bacteriostatic or bactericidal properties Reduce luminal pH and Inhibits the growth of bacteria (G-negative) by producing hydrogen peroxide
Immune booster	Stabilize intestinal integrity and improve the gut innate immune response Improve gut innate immune response through chloride secretion or increasing mucus production
Antitoxin effects	Inhibits toxin expression in pathogenic bacteria Neutralize pathogens by producing enterotoxins
Effect on nutrient digestibility	Increase digestive enzyme activity in the gastrointestinal tract Increase the digestion and absorption of nutrients
Ant-oxidative activity	Stress mitigation ability

thickness of an inflamed intestinal epithelium. If the thickness of the intestinal wall is decreased, bacterial feed supplementation could improve the efficiency of energy utilization by reducing the energy used for tissue turnover in the gastrointestinal tract (Peterson et al., 2007).

COMPETITIVE EXCLUSION

Competitive exclusion (CE) can be defined as the response of healthy gut microbiota to protect the intestine towards the establishment of pathogens and to reduce infection of the gastrointestinal tract in animals (Markowiak and Śliżewska, 2018). Probiotics have exclusion property towards pathogens both in case of preventive and therapeutic management. Gut epithelia have receptors for microorganism adhesion; both beneficial and pathogenic microorganisms for the same intestinal sites. Probiotic adhesion quality blocks the association between gut epithelia and infectious agents (Yang et al., 2015). Thus, probiotics based bacteria eliminate microorganism and prevent the gut infection of farm animal including cattle, buffalo, sheep, goat, pig and poultry (Liao and Nyachoti, 2017).

The mechanism of CE also specifies that probiotics and pathogenic bacteria compete for nutrient absorption (Yang et al., 2015). This competition between good and harmful bacteria can cause a reduction in pathogens. In addition, energy utilization may decrease bacterial growth and prevent pathogens from resisting the effects of gut peristalsis (Cho et al., 2011; Yirga, 2015). Hence, probiotics have been widely used in animal and poultry farming due to their ability to inhibit the harmful effects of pathogens like *Clostridium perferinges, Salmonella, Campylobacter jejuni and Escherichia coli* (Bermudez-Brito et al., 2012; Goudarzi et al., 2014; Syngai et al., 2016). Another study reported that with the administration of *L. rhamnosus* GG in rats, the first day pups reduce the adhesion and colonization of enteroinvasive *E. coli* (Sherman and Bennett, 2004). It has been observed that probiotic strains (*L. johnsonii* NCC 533, *L. casei* Shirota and *L. acidophilus* LB) control the infection of *H. pylori* and gastritis in mice models (Sgouras et al., 2005; Isobe et al., 2012).

ANTIMICROBIAL SUBSTANCES

Probiotic containing beneficial bacteria, once established in the intestine, may produce antimicrobial substances that may hinder the growth of pathogens in the gut of cattle, pig and poultry (Yirga, 2015; Bajagai et al., 2016). Many probiotic bacteria, comprising lactic acid bacteria (LAB) (Flynn et al., 2002), *bacillus* (Hyronimus et al., 2000) and *bifidobacteria* (Cheikhyoussef et al., 2008), can produce various types of heat resistant bacteriocins (Cotter et al., 2005) which have antimicrobial property towards pathogenic microorganism of animals including *Staphylococcus*, *Bacillus, Listeria, Enterococcus*, and *Salmonella* species (Flynn et al., 2002; Corr et al., 2007).

Probiotics such as *Lactobacillus* ferment lactose to lactic acid, reducing the pH of gut to a level that pathogenic bacteria cannot tolerate (Bajagai et al., 2016). Some strains also produce hydrogen peroxide, which hinders the growth of gram-negative bacteria (Yirga, 2015; Bajagai et al., 2016). These substances have detrimental effects on pathogens, which is mainly due to reducing pH of gut. A decline in pH may partially unbalance the secretion of hydrochloric acid in the stomach of young piglets. It can reduce the stomach ability to digest and absorb feed and kill off pathogens (Kenny et al., 2011). Furthermore, yeasts have also been reported to stabilize the ruminal pH and reduce the risk of acidosis by competing with lactic acid producing bacteria (Yirga, 2015). The digestion and feed intake can be improved by modifications of ruminal microbiota. Probiotics produce antioxidants, organic acids, reuterin, microcin and bacteriocins (Yirga, 2015). These substances may decrease not only the number of potential pathogens but may also hinder bacterial metabolism and toxin production (Eswara et al., 2010; Hou et al., 2015). LAB produce bacteriocins to deactivate the gram negative bacteria in combination with other environmental elements such as organic acids, low temperatures, and detergents (Alakomi et al., 2003). Furthermore, they can inhibit amine synthesis. Coliform bacteria decarboxylate amino acids to produce amines (toxic to epithelium) which can affect gut mucosa and cause diarrhea in young calf. If coliforms bacterial growth can be prevented, then amine production can also be hindered (Yirga, 2015), which may be advantageous in preventing neonatal diarrhea and calf mortality.

EXCLUSION OF NUTRIENTS

Probiotics have been designated to enhance the digestion and absorption of nutrients. The improved production of animals due to probiotics can be associated with an increase in digestion and absorption of nutrients (Markowiak and Śliżewska, 2018). The response of L. bulgaricus in broiler chickens diets was different depend on supplementation of various level of probiotic. There was no significant effect on digestibility of crude protein (CP) or fat at a rate of 2 ×106 cfu/g, but there was an increase in CP, fat and weight gain (WG) 7 to 11%, 6.5 to 13.4%, 7.9 to 11.7% respectively, at a rate of 6 ×106 cfu/g and 8 ×106 cfu/g (Apata, 2008). Another study observed that probiotic (AgiPro A100) offered to broiler chickens had increased dry matter (DM) digestibility by 12.4% at 42 day trial (Li et al., 2008) and no effects were reported on weight gain (WG), average daily gain (ADG), feed intake (FI) and feed conversion ratio (FCR). A similar study revealed that probiotics improved the ileal digestibility of essential amino acids (EAA), increased 5% WG (Zhang and Kim, 2014) and enhanced the bioavailability of calcium in broiler chickens (Chawla et al., 2013).

Probiotics increase the absorption of nutrients in the diet which may be due to the increase enzyme activity in the gastrointestinal tract. Probiotics containing *Lactobacillus* altered the enzyme activity in the gastrointestinal tract of domestic animals. L. acidophilus given at a rate of 2 $\times 10^6$ cfu/g of feed had increased the amylase activity in the small intestines of chickens (Jin et al., 2000). But, there was no change in proteolytic and lipolytic activity. The result indicated that a 4.6% increase in WG and a 5% increase in feed efficiency were due to the enhanced activity of amylase in the small intestine. A similar study has been reported that commercial probiotics (Probios) containing L. acidophilus, L. plantarum, L. casei and E. faecium increased the sucrose, lactase and amylase activity but no effects were observed on peptidase activity in the small intestine of young piglets (Collington et al., 1990). Bacillus amyloliquefaciens (spore forming bacteria) produce extracellular enzymes including α-amylase, cellulase, proteases and metalloproteases (Gangadharan et al., 2008) which may increase nutrient digestion. Probiotics improved the gut enzyme activity due to modification in the gut micro ecosystem and reduced the incidence of ruminal acidosis by stablizing the ruminal volatile fatty acids (VFAs) (Arcos-Garcıia et al., 2000).

Feed containing probiotics yeast culture (YC) exposed to lambs at concentration 0, 3, and 6 g/day, increased digestibility of dry matter (DM), organic matter (OM), crude protein (CP) at a concentration 3 g/day compared to the control group (Haddad and Goussous, 2005). Mukhtar et al (Mukhtar et al., 2010) reported that lambs given a concentrated probiotic diet had higher DM and CP digestibility than lambs without probiotics. In addition, it was reported that probiotics fed to growing lambs had enhanced digestibility of DM, OM, CP, CF, ether extract (EE), and nitrogen free extract (NFE) compared to the control group. No significant differences were observed in nutrients digestibility except for CP (Hillal et al., 2011). In contrast, another study indicated that probiotic mixed feed of weaned goats (Whitley et al., 2009) or lambs (Ding et al., 2008) did not affect the DM, OM, and CP digestibility compared to control group. Inconsistencies in the results of these studies may be due to variations in the animal models, environment, administration, composition and quality of probiotic, or supplementation times duration (Whitley et al., 2009). Probiotics improved the intestinal villi and villus height: crypt ratio in poultry (Biloni et al., 2013; Jayaraman et al., 2013; Afsharmanesh and Sadaghi, 2014), by increasing the surface area for nutrient absorption. Yeast also has the potential to change the metabolic process and to reduces the methane gas production in rumen (Chung et al., 2011). Hence, nutritionists have determined that probiotics have significant effects on nutrient digestibility.

REDUCING AMMONIA PRODUCTION

In poultry housing, ammonia is excreted due to rich protein diets. Ammonia has detrimental effects on the eyes and nasal cavity of affected chickens due to the gas alkalinity and corrosiveness. NH3 in respiratory tract reacts with the moisture and forms a corrosive alkaline solution (ammonium solution). The ammonium solution paralyze the respiratory cilia and reduce immunity in the respiratory system which increase the disease susceptibility especially E.coli (Maliselo and Nkonde, 2015). Ammonia emission causes keratoconjunctivitis in poultry birds including photopho-

bia, excessive lacrimation, respiratory distress, and/or closure of the eyelids. Regarding this concern, probiotics acts as antagonists of ammonifying bacteria that harbors the gut of poultry and prevents keratoconjunctivitis from developing (Patterson and Burkholder, 2003; Sarangi et al., 2016). They reduce nutrient deterioration and reduce ammonia production in the gut lumen. Probiotics *(Lactobacillus casei*) reduces the activity of urease in the gut of chickens and ultimately decrease uric acid, ammonia, urea and non-protein nitrogen sources (Fuller, 2001; Patterson and Burkholder, 2003). A diet con-

Table 2. Probiotics commonly used in animal nutrition

Genus	Species
Lactobacillus	*L. acidophilus*
	L. lactis
	L. amylovorus
	L. cellobiosus
	L. casei
	L. brevis
	L. plantarum
	L. fermentum
	L. crispatus
	L. curvatus
	L. farmicinis
	L. gasseri
	L. johnsonii
	L. paracasei
	L. reuteri
	L. rhamnosus
	L. sobrius
	L. bulgaricus
	L. delbrueckii subsp. bulgaricus
	L. salivarius
Bifidobacterium	*B. lactis*
	B. bifidum
	B. bifidus
	B. longum
	B. thermophilum
	B. breve
	B. pseudolongum
	B. adolescentis
	B. animalis
	B. infantis
Bacillus	*B. cereus*
	B. coagulans
	B. megaterium
	B. subtilis
	B. mesentricus
	B. amyloliquefaciens
	B. licheniformi
	B. polymyxa
	B. toyonensis
Leuconosto	*L. mesenteroides*
	L. citreum
	L. lactis
Enterococcus	*E. faecium*
	E. faecalis
Pediococcus	*P. acidilactici*
	P. parvulus
	P. pentosaceus subsp. Pentosaceous
Lactococcus	*L. lactis*
Streptococcus	*S. bovis*
	S. diacetylactis
	S. thermophilus
	S. gallolyticus
	S. salivarius
	S. faecalis
	S. infantarius
	S. faecium
	S. cremoris
	S. intermedius;
Aspergyllus	*A. oriza*
	A. niger
Escherichia	*E. coli strain nissle*
Propionibacterium	*P. jensenii*
	P. freudenreichii
	P. acidipropionici
	P. shermanii
Saccharomyces	*S. boulardii*
	S. cerevisiae
	S. carlsbergensis
	S. pastorianus
	S. servisia
Prevotella	*P. bryantii*
Clostridium	*C. butyricum*
Candida	*C. utilis*
	C. pintolepesii;
Brevibacillus	*B. laterosporus*
Megasphaera	*M. elsdenii*

References: (Pollmann et al., 1980; Azizpour et al., 2009; Le Bon et al., 2010; Meng et al., 2010; Daudelin et al., 2011different litters of pigs were randomly assigned to one of the following treatments: 1; Pan et al., 2011; Rastogi et al., 2011; Ibrahim et al., 2012; Kechagia et al., 2013; Yirga, 2015; Lv et al., 2015; Bajagai et al., 2016)

taining probiotics such as *Streptococcus faecium* and *Bacillus subtilis* also decreases the ammonia concentration in the excreta of poultry birds.

PROBIOTICS AND ITS SIGNIFICANCE

According to FAO/WHO, Probiotics are referred as "living microorganism which, when administrated in excessive amounts confer a healthy benefits to the host" via improving the host gut microbial population, improving the colonization resistance towards pathogens and stimulating the immune responses (Das et al., 2012; Bajagai et al., 2016; Jaiswal et al., 2017). Various microorganism strains are being used in probiotic preparations are vary in composition, such as LAB (*Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus lactis, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus salivarius, Streptococcus thermophilus, Enterococcus faecium, Enterococcus faecalis and Bifidobacterium spp*) are the most common type of probiotic bacteria (Kabir, 2009; Markowiak and Śliżewska, 2018) (Table 2).

EFFECTS OF PROBIOTICS ON ANIMAL PRODUCTION

The significant effects of probiotics in human and domestic animal health have been well documented. Probiotics have favorable effects on FCR, WG, milk yield, gastrointestinal microbiota, pH and intestinal immunity as well as animal health status (Table 3) (Kritas et al. 2006; Bhandari et al., 2010; Kenny et al., 2011; Upadhaya et al., 2015; Markowiak and Śliżewska, 2018). A study reported that probiotics given to sheep had increased feed intake and growth performance (Khalid et al., 2011). A small ruminant study determined that increased number of cellulolytic bacteria may improve growth rate, nutrient digestibility and fermentation process (Soren et al., 2013). Probiotics containing S. cerevisiae and E. faecium fed to cattle had increased milk fat concentration due to increased production of volatile fatty acids (VFAs) (Oetzel et al., 2007).

Effects of Probiotics on Cattle

LAB is a well-practiced probiotic strain in ruminant nutrition. Diarrhea is the main cause of death in young calves leading to major economic losses (Cowles et al., 2006; Markowiak and Śliżewska, 2018); thus, its prevention is important to economically support the calf producers (Servin, 2004; Timmerman et al., 2005). Numerous published data shows that probiotics can improve the balance of gut microbiota (Aattour et al., 2002), reduce the gut pH and infectious agents by enhancing immunological response (Musa et al., 2009; Sanchez et al., 2017).

For several years, AGPs have been used to prevent the economic losses in the animal industry. But antibiotic resistance in food animal and antibiotic residues in animal products has generated public health concerns (Martinez-Vaz et al., 2014) .In this circumstance, probiotics have been categorized as one of the alternatives in animal feed (Gyles, 2007), preventing the production of E. coliin the intestine, and to reduce the incidence of diarrhoea in ruminants (Reid and Friendship, 2002; Bahari, 2017; Markowiak and Śliżewska, 2018). However, the phenomenon of probiotics in gut microbiota currently remains unclear. The most common probiotics species, *lactobacillus*, *bifidobacterium* and yeast strains have been well documented in rumen development and gestrointestinal health status (Uyeno et al., 2010; Bahari, 2017). LAB strains modulate rumen microbiota (Weinberg et al., 2004; Han et al., 2014; Goto et al., 2016), increased the DMI, WG and improved animal health. Published data has reported that probiotics containing *Lactobacillus* or *Enterococcus* strains have reduced the incidence of acidosis in lactating cattle (Goto et al., 2016). The principle concept is that probiotics may reduce pH by decreasing lactic acid formation and enhancing the consumption of lactic acid by ruminal bacteria (Goto et al., 2016; Roselli et al., 2017).

Moreover, LAB strains may inhibit the adhesion of pathogens to gut mucosa during the initial days of colonization (Isolauri et al., 2001; Bahari, 2017). It has been investigated that feed containing *Lactobacillus* species had increased WG and immunocompetence in young calves (Al-Saiady, 2012). In contrast, previous findings related to probiotics have remained ambiguous in calf studies. The efficiency of probiotics may be different depending on the health conditions of calves, because in previous findings, the consequences of probiotics were signif-

Table 3. Effect of probiotics on animal production

Probiotic strains	Species	Consequences	References
Bacillus licheniformis *Bacillus subtilis*	Holstein calves	Higher ADG and live weight.	(Kowalski et al., 2009)
Bacillus licheniformis *Bacillus subtilis*	Holstein cows	Increases milk production, protein, ruminal digestibility and total VFA contents.	(Qiao et al., 2010)
Enterococcus faecium *Saccharomyces cerevisiae*	Cow	Improves milk fat % in first lactating cow	(Oetzel et al., 2007)
Propionibacterium strain P169	Cow	Improves energetic efficiency, propionate concentration, lower acetate contents	(Weiss et al., 2008)
Prevotella bryantii	Cow	Increases milk fat %, acetate and butyrate concentration, and decrease lactate concentration	(Chiquette et al., 2008)
Propionibacterium strain P169 *Yeast culture*	Cow	Increases propionate concentration, ruminal digestibility, microbial N synthesis, or passage rates did not report any difference	(Lehloenya et al., 2008)
Multi-species probiotic	Young cattle	Improves WG	(Bayatkouhsar et al., 2013)
S. cerevisiae	Lactating cattle	Increases milk fat production	(Alugongo et al., 2017)
S. cerevisiae	Camel	Improved weight gain and feed intake	(Mohamed et al., 2009)
S. cerevisiae	Buffalo calf	Improves cellulose digestibility	(Kumar et al., 1994)Saccharomyces cerevisiae plus growth medium
L. plantarum	Pig	Improved growth and pork quality	(Yang et al., 2015)
Enterococcus faecium	Weaned piglets	Improves FCR and growth rate	(Wang et al., 2016)the third and the fifth day after birth, while the control group received 2 ml of 10% sterilised skimmed milk without probiotics at the same time. Results showed that oral administration of E. faecium EF1 was associated with a remarkable increase on the body weight of piglets for both suckling and weaning periods, by 30.73% (P<0.01
E. faecium, *L. acidophilus, Pediococcus pentosaceus,* *L plantarum*	Weaned piglets	Improves FCR, feed intake and WG	(Giang et al., 2010)
Pediococcus acidilactici, *Lactococcus lactis,* *L. casei,* *Enterococcus faecium*	Weaned piglets	Improves growth rate, decreases coliform counts by facilitating antimicrobial substances	(Guerra et al., 2017)
Bacillus licheneformis	Broiler chicken	Improves FCR and growth performance	(Liu et al., 2012)
Lactococcus lactis CECT 539, *Lactobacillus casei* CECT 4043	Broiler chicken	Improves health and growth performance	(Fajardo et al., 2012)

icant in less healthy control calves (Timmerman et al., 2005; Bayatkouhsar et al., 2013). Under stressed conditions, probiotic bacteria can be used to decrease the severity of scours caused by imbalance of intestinal microbiota (Markowiak and Śliżewska, 2018). Amazingly, the particular lactobacilli and bifdobacteria strains reduce the pathogenicity by decreasing the effects of pathogens, while modulating the immune system to infections is still unclear (Servin, 2004; Al-Saiady 2012).

Effects of Probiotics on Pigs

Probiotics given to humans and livestock have improved gut microbiota, gut immunity, and shown good resistance to pathogens. It has also decreased harmful infectious agents and improved overall animal health (Bhandari et al., 2010; Kenny et al., 2011; Yirga, 2015; Roselli et al., 2017). The pathogenic bacteria like Salmonella enterica and Streptococcus suis caused diarrhea and a reduction in growth in young pigs (Kenny et al., 2011), during the first days of life. Probiotics utilization protects the neonatal piglets from intestinal infections during their initial age (Roselli et al., 2017). Post weaning, the piglets are highly exposed to enteric diseases due to the imbalance of beneficial and pathogenic gut bacteria. It has been reported that probiotics decreased 21% post weaning diarrhea out of 38% and 16.2% pre-weaning mortality out of 22.3%. (Taras et al., 2006; Lalles et al., 2007; Liao and Nyachoti, 2017). Supplementation of LAB species (L. acidophilus C3, E. faecium 6H2, L. fermentum NC1 and Pediococcuspentosaceus D7), B. subtilis H4 or cumulative with S.boulardii had found positive consequences in diarrhea reduction (Giang et al., 2012).

A study of piglets by Liu et al. (2014) stated that L. reuteri I5007 plays a beneficial role in the gut health of young pigs by modulating microbial population and intestinal development. Denaturing gradient gel electrophoresis (DGGE) examined that L. reuteri I5007 reduced the numbers of Clostridium spp by affecting the colonic microbial environment on day 14. Application of L. reuteri BSA131 reduced the population of enterobacteria in feces of weaning pigs (Chang et al., 2001). Significantly, *Lactobacillus* species comprising L. gasseri, L. reuteri, L. acidophilus and L. fermentum reduced *E. coli* and aerobic counts, and increased Lactobacilli and anaerobic counts in the digesta compared with a control group (Huang et al., 2004). Furthermore, a report suggested that LAB strains especially L. reuteri I5007 given through oral administration not only enhanced the butyrate and branched chain fatty acids concentration but also reduced the Clostridium spp by decreasing luminal pH to a level where pathogen bacteria cannot cause infection (Liu et al., 2014; Bajagai et al., 2016). It is compulsory to mention that different factors such as differences in doses, microbial strains, age, health status and pig husbandry management may help to explain the different consequences of same probiotic application in domestic animal trials (Bajagai et al., 2016). In addition, probiotic strains may not only decrease the pathogens but also reduce their metabolism and toxin production (Ng et al., 2009; Hou et al., 2015; Roselli et al., 2017). The probiotic strain E.coli produced microcin which may reduce intestinal pathogen, commensal E. coli, adhesion of *E. coli*, and Salmonella enterica associated pathogen (Setia et al., 2009; Bhandari et al., 2010; Krause et al., 2010; Sassone-Corsi et al., 2016). Therefore, available evidence has suggested that *E. coli* and L. reuteri strains have an essential role to improve gut health and immunity (Roselli et al., 2017).

Effects of Probiotics on Poultry

The probiotics application has become popular due to its favorable effects on gut health and production performance of farm animals including chickens (Khaksefidi and Ghoorchi, 2006; Zulkifli et al., 2000; Mookiah et al., 2014; Sarangi et al., 2016). Currently, antibiotic resistance in poultry products has forced scientific authorities to ban the application of AGPs (Park et al., 2016; Wang et al., 2017). Probiotic based bacterial diet given to day old chicks have ability to establish in the gut ecosystem (Jaiswal et al., 2017), hence they are well recognized as normal intestinal microbiota of chicken (Kizerwetter-Swida and Binek, 2005; Qin et al., 2018).

LAB, especially *Lactobacillus* strains, is commonly used as probiotics. Probiotics bacterial strains should be isolated from the natural gastrointestinal microbiota of the same animals in order to get more specific results (Kizerwetter-Swida and Binek, 2005). However, potential probiotic strains may improve the gastrointestinal health and microbiota by affecting the gut microbiota ecosystem (Khaksefidi and Ghoorchi, 2006; Nayebpor et al., 2007; Sugiharto, 2016; Markowiak and Śliżewska, 2018) Specifically, the literature findings indicated that the *Lactobacillus* strain has inhibitory action towards enteric pathogens like Salmonella, *E. coli* and Clostridium perfringens (Kizerwetter-Swida and Binek, 2005, Cao et al., 2013; Wang et al., 2017). This phenomenon is due to

production of antimicrobial substances by probiotics as well as nutrient competition between beneficial and pathogenic bacteria for adherence sites on the intestinal epithelium (Hayek et al., 2013; Song et al., 2012).

EFFECTS OF PROBIOTICS ON NUTRIENT DIGESTIBILITY

Probiotics products in market have an excellent ability to avoid digestive disorder (Nagaraja and Titgemeyer, 2007; Sanchez et al., 2017). Acidosis is common digestive disorder that not only affects the rumen ecosystem, but also decreases the production of animals (Enemark, 2008). In vitro scientific studies have found that yeasts (Saccharomyces cerevisiae) might affect the stability of lactate forming bacteria by reducing lactate production (Streptococcus bovis) and enhance lactate consumption by Selenomonas ruminantium or Megasphaera elsdenii (Rossi et al., 2004).

Significantly, it has reported that S. cerevisiae (yeast strain) plays a vital role in improving the cellulolytic bacterial activity (Arcos-Garcıia et al., 2000; Mosoni et al., 2007; Chung et al., 2011), which cause starch degradation and effectively competed with amylolytic lactate forming bacteria (Yutaka et al., 2015; Thrune et al., 2009). A trial in male goat (buck) has investigated that S. cerevisiae supplemented diet had improved nutrient digestibility then roughage feeding (El-Ghani, 2004). The potential effect of S. cerevisiae supplementation is generally considered a result of variations in the rumen fermentation process, which may improve nutrient digestibility and decrease the methane gas emission (Chung et al., 2011).

EFFECTS OF PROBIOTICS ON GUT MICROBIOTA

The gut of animals is inhabited by a complex and dense community of bacteria, archaea, fungi, protozoa and viruses (Markowiak and Śliżewska, 2018). In farm animals, the total number of gut microbial cells exceeds the host cells by at least one order of magnitude (Kim and Isaacson, 2015). The gut microbiota shows an increase in numbers, concentration and diversity from the proximal to the distal gastrointestinal tract. For example, in pigs, the stomach and proximal small intestine comprise moderately small numbers of bacteria (103–105 bacteria/g or ml of contents); but with increased *Lactobacillus* spp. and Streptococcus spp. (Roca et al., 2014). In contrast, the distal small intestine inhabits a greater number of bacteria (108 bacteria/g or ml of contents) (Gaskins, 2000). Numerous studies have found that the microbes are radially distributed within the gut tract (Gaskins, 2000; Wang et al., 2017). The gut micro ecosystem comprises of four points: i) the intestinal lumen, ii) the unruffled mucus layer (cover mucosa), iii) the deep mucus layer establish in the crypts, iv) surface of the intestinal epithelial cells. The variety of microbial populations within gut micro ecosystems is influenced by certain factors such as gut peristalsis, pH, anoxic conditions, dietary composition, inhibitory agents (bacteriocins), SCFA, and competitive exclusion (Gaskins, 2000; Pluske et al., 2003; Wang et al., 2017). Taking these factors into consideration, researchers have concluded that probiotics and their related health effects may perform a significant role in stabilizing the gut microflora and definitely gut health.

EFFECTS OF PROBIOTICS ON GUT HEALTH

In literature, the term ‘gut health’ lacks clear definition, however, it has been used constantly in human medicine (Tuohy et al., 2003; Jacobs et al., 2009) as well as in animal health (Lalles et al., 2007; Choct,

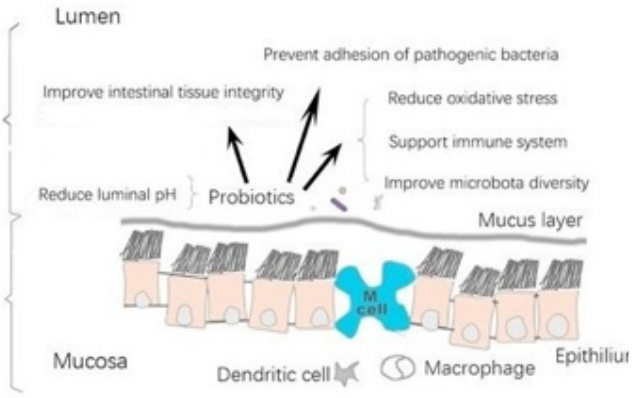

Fig 1. Effects of probiotics on gut health of domestic animals

Table 4. Effect of probiotics on gut health of domestic animals

Probiotic strains	Species	Consequences	References
Bacillus species or LAB species	Young calf	Balance the gut microbial ecosystem and reduce the adhesion of pathogen	(Yutaka et al., 2015)
LAB species (*Lactobacilli* and *Enterococci*)	Cattle	stabilize the rumen pH	(Jeyanathan et al., 2014)
M. elsdenii and *Selenomonas ruminantium* sub spp	Cattle	Stabilize the rumen pH, rumen microbiota, improve the immune action and enhance plant cell walls degradation in the rumen	(Johanne, 2009; El-Tawab et al., 2016)
Saccharomyces cerevisiae	Young calf	Improve the intestinal health, rumen microbiota and reduce the adhesion of pathogen	(Chaucheyras-Durand and Durand, 2010)
Bacillus cereus var. Toyoi *Saccharomyces boulardii*	Sheep	Improves humoral immunity	(Retta, 2016)
Enterococcus faecium	Pig	Reduce the intestinal *E. coli*, *Clostridium,* and *Enterobacterium* species	(Bajagai et al., 2016)
Lactobacillus species	Pig	Immunomodulators, improve antibody status, killer cells, macrophage response, and interferon production	(Cho et al., 2011)
Saccharomyces cerevisiae	Pig	Reduce risk of pathogens and diarrhea	(Liao and Nyachoti, 2017)
Bacillus subtilis	Chicken	Reduce 58% of the number *of S. heidelberg* colonization	(Knap et al., 2011)
L. acidophilus *L. salivarius*	Chicken	Improves T helper cells (Th), anti-inflammatory cytokines (IL-10) and transforming growth factor (TGFβ) in caecal tonsil cells	(Brisbin et al., 2010; Sugiharto, 2016)
Bacillus mesentericus, *E. faecalis* and *Clostridium butyricum*	Chicken	Reduce the diarrhea incidence	(Rodriguez-Fragoso et al., 2012)
Aspergillus, *Bacillus, Bifidobacterium,* *Candida Enterococcus, Lactobacillus, Saccharomyces Streptococcus,*	Chicken	Reduce risk of *E. coli*, *Clostridium perfringens or Salmonella*	(Kral et al., 2012; Syngai et al., 2016)
L. reuteri C1, C10, C16; *L. gallinarum* I16, I26; *L. brevis* I12, I23, I25, I218, I211, *L. salivarius* I24	Chicken	Increases the *lactobacilli, bifidobacteria* and decreases the *E.coli* caecal populations	(Mookiah et al., 2014)

2009). Gut health refers to the health status of the upper and lower gastrointestinal tract; with possibly more emphasis on the lower GI tract. The main function of gut is to stabilize nutrients, water and electrolyte proportions, mucus secretion, cytokine expression and immune system development (Corthesy et al., 2007; Niba et al., 2009; Yitbarek et al., 2015; Wang et al., 2017),

In addition, it acts as a barrier to eliminate toxins and infectious agents (Fig. 1) (Roselli et al., 2017). Even with these functions, certain types of bacterial

pathogens inhabit the gut and disturb the gut ecosystem (Markowiak and Śliżewska, 2018). For example, the numbers of pathogenic bacteria i.e. E.coli proliferates and exceeds other bacteria in post-weaning pigs, causing gastrointestinal disease (Fairbrother et al., 2005). The gut microbiota ecosystem is influenced by many factors such as feed composition (carbohydrates: protein), feed additives (probiotic, prebiotic, organic acids, feed enzyme), feeding practices, antibiotics agents, disease status, weaning age, seasonal stress, genetics and animal housing environment (Gaskins, 2000; Pluske et al., 2003; Zoetendal et al., 2004; Torok et al., 2011; Wang et al., 2017). These factors all have potential role in the health of gut microbiota.

The term 'optimal' and 'normal' gut microbiota has created confusion among nutritionist. Metzler et al. (2005) suggested that the term 'optimal' gut microbiota should be used rather than 'normal' microbiota, because it is very difficult to define what is 'normal' concerning the condition of growing pigs and chickens. Animal producers are trying to keep animals healthy and free of pathogens to achieve maximum healthy production (Roselli et al., 2017). Sometimes clinical illness and rarely death cause economic losses to the pig and poultry industry due to variation of gut microbiota (Lange et al., 2010). Therefore, probiotics have significant effects to improve the gut stability of domestic animals (Table 4). For example the outbreak of necrotic enteritis is a key problem in poultry caused by the intake of a concentrated diet (viscous grain) (Jia et al., 2009; Palliyeguru et al., 2010). The decrease in gut motility has been linked with high digesta viscosity which provides a favorable environment to Clostridium perfringens in the upper gestrointestinal tract (Timbermont et al., 2011). Swine dysentery and collibacillosis have been associated with consumption of a viscous fibrous diet (McDonald et al., 2001; Hopwood et al., 2004; Montagne et al., 2004; Wilberts et al., 2014). This has been related to an increase in digesta viscosity with a reduction in endogenous secretion and nutrient digestibility in the gastrointestinal tract. Therefore, probiotics have been given full consideration as alternatives to feed additives to stabilize the gut microbiota of domestic animals.

EFFECT OF PROBIOTICS ON GUT ASSOCIATED IMMUNE RESPONSES

The basic purposes of immunomodulation in domestic animals include: to initiate powerful and persistent immune system responses towards infectious agents, to modulate the maturation of acquired and innate immunity during the neonatal period and in young disease sensitive animals. Also to augment local defensive immune responses at susceptible sites such as in dairy cattle (mammary gland) or in young animals (gut), to overcome the immunosuppressive effects of stress and environmental pollution (Roselli et al., 2017).

Probiotics play a fundamental role in the development of immune system neonates (Balevi et al., 2001). Recently, it has become the topic of interest for researchers to explore the beneficial effects of probiotics in the gut and those associated with maintaining a healthy immune system in domestic animals. Regular utilization of probiotics stimulate both humoral and cell mediated immunity through increased production of natural cytokines, macrophage, lymphocyte, killer cell and immunoglobulin (IgG, IgM and IgA) (Balevi et al. 2001; Koenen et al. 2004; Yurong et al. 2005; Farnell et al., 2006; Cho et al., 2011; Roselli et al., 2017).

Several authors have revealed that microbial populations can support the animals defense mechanism towards pathogens by stimulating the gut immune response (Markowiak and Śliżewska, 2018). This may strengthen the immune systems reaction by enhancing phagocytic activity and the production of antibodies (Yirga, 2015). Probiotic bacteria are important to the immune system because when pathogens are recognized by antigen presenting cells (APC), they are eliminated by leukocytes (Butaye et al., 2003). Some strains of probiotics such as *Lactobacillus* have the capability to modulate the immune system. Yirga has explained two reasons of immunomodulation: i) They can either move through the intestinal wall as viable cells or multiply ii) the antigens released by the dead organisms definitely stimulate the immune system. Therefore, this factor induces the immune response (Yirga 2015).

Probiotics based L. reuteri may augment or reduce the innate immune action through stimulation of

pro-inflammatory cytokines in pigs. L. reuteri strains can be divided into two subgroups, immunosuppressive (ATCC PTA 6475 and ATCC PTA 5289) and immunostimulatory strains (ATCC 55730 and CF48-3A), and each subgroup has potential therapeutic value (Jones and Versalovic, 2009). Oral consumption of L. reuteri I5007 could improve T-cell differentiation and induce ileal cytokine expression, which proposes that this probiotic strain might modulate immune function in young piglets (Wang et al., 2009). Another study by Yu et al. (2008) reported that L. reuteri I5007 diets fed to young piglets had increased serum specific anti-OVA IgG level. In a recent study on neonatal piglets, it has been reported that L. reuteri decreases the mRNA expression of IL-1β in the ileum (Dowarah et al., 2017). A similar study reported that L. reuteri with L. acidophilus might help to maintain immunological homeostasis in young gnotobiotic pigs infected with rotavirus by regulating TGF-β production (Azevedo et al., 2012).

However, it is still unclear how a host body recognizes the pathogens and beneficial bacteria that ultimately cause immune activation or deactivation (Vinderola et al., 2005; Hardy et al., 2013), literature findings have revealed that Pathogen-associated molecular patterns (PAMP) or recent correct term microbe-associated molecular patterns (MAMPs) are pathogen associated molecules, that stimulate the innate immune system. They are recognized by pattern recognition receptors (PRRs) of the gastrointestinal mucosa (Lebeer et al., 2010). The gut epithelia and dendritic cells (DC) initially recognize the MAMPs (LPS and bacterial DNA etc.) and then interact with PRRs to stimulate innate as well as adaptive immunity (Rachmilewitz et al., 2004; Lebeer et al., 2010).

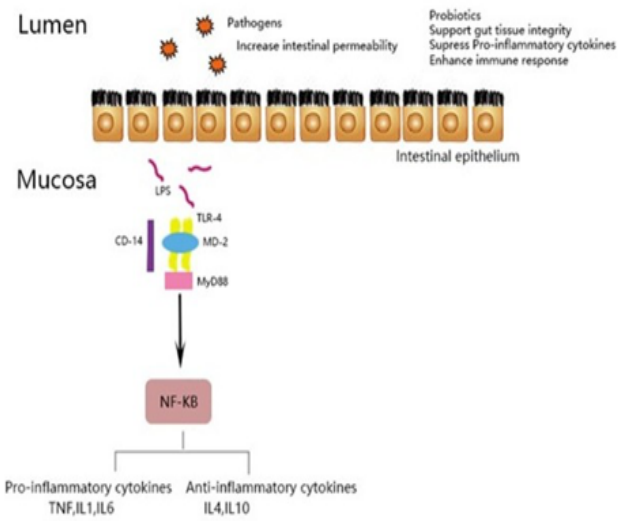

Fig 2. Effect of probiotics on immune responses

Toll-like receptors 4 (TLR4) is a trans-membrane proteins, an important member of the toll-like receptors family, which detect the PRRs and activate the NF-κB (intracellular signaling pathway), which ultimately activate immune response by producing pro-inflammatory cytokines (Fig. 2) (Lebeer et al., 2010; Gu et al., 2016). Some studies exposed that IgA is the dominant immunoglobulin in the intestine and plays a key role in immunity (Mahfuz et al., 2017). IgA-producing B cells increased the gut IgA without increasing the production of CD4+ T-cells (Vitini et al., 2000; Vinderola et al., 2005). The probiotics increased the production of IL-6 by the gut epithelia which caused in variation of B-cells for producing IgA and IgM (Vinderola et al., 2005). Therefore, this phenomenon of IgA plays a key role in the eradication of harmful bacteria via combined with the gut-mucins. On the other hand, it is difficult to completely conclude that probiotics contribute significantly to the immune system of the host as they are not intended to eradicate invasive pathogens in the gastrointestinal tract. Therefore, such positive effects are always compromised due to the animals immunological status (Patil et al., 2015). The available data and previous findings reported that some combination of probiotic strains have generated positive results in the various animal studies (Yirga, 2015).

SAFETY AND RISK ASSOCIATED WITH PROBIOTICS APPLICATION

Safety Factors related to Probiotics

Probiotics have excellent effects throughout the gastrointestinal tract. All these microbes are of natural origin; thus any deleterious effect is highly questionable. But probiotic registration plays a significant role in environmental safety and it has better safety records than antibiotics feed additives. Several studies have been conducted with no adverse

effects being reported on animal health. Concisely, they are not transmitted from the gut to the body of animal. They are safe, have no food transmission from animal origin to human, and do not cause residual effects (Kubiszewska et al., 2014; Bajagai et al., 2016). Most of the scientific data is available on the safety of probiotics based *Lactobacillus* and *Bifidobacterium* (Hempel et al. 2011; Shanahan 2012). Thus more exploration is needed for safe application of probiotics. Specifically, Bajagai et al. (2016) have reported that probiotics formulator should emphasize 4 factors to avoid the recent allegation made on probiotic safety (Bajagai et al. 2016).

i. Probiotic strains cannot be considered as 100% safe or with zero risk, like in case of drugs.
ii. The risk of probiotics application depends on immunity and health status of animal. Therefore, probiotics may be safe in one animal (healthy) but may not be safe in another (immune deficient).
iii. Each specific probiotic species cannot be evaluated based on other probiotics, as each product has their own safety and risk evaluation plan based on each case study.
iv. Lack of public awareness to hazardous effects of probiotics, so there is need to inform the consequences of probiotic risk to general public.

Risk Factors related to Probiotics

However probiotics based microorganisms are generally safe in animal feed, but preventive measurement should be taken to prevent humans, animals, and the environment from unsafe microorganisms. Specifically, probiotics associated risks in animal diets should be assessed as follows (Marteau, 2001; FAO/WHO, 2002; Doron and Snydman, 2015; Bajagai et al., 2016):

i. Infection (gut or systemic) of the animal fed probiotics.
ii. Transmission of antibiotic resistance from probiotics to pathogenic microbes.
iii. Transfer of infectious agents to the environment from the animal production system.
iv. Infection (gut or systemic) of the handlers of animal/feed.
v. Toxic effects on the host due to transfer of toxins (entero and emetic toxins) from probiotics microbes.
vi. Hyper-activation of the immune response of animals.
vii. Infection (gut or systemic) of the humans ingesting animal products produced from probiotics given to animals.
viii. Sensitization (skin, eye or mucus membrane) of the probiotics handlers.

CONCLUSION AND RECOMMENDATION

Probiotics plays a beneficial role in domestic animals via stabilizing the gut morphology, gut function and gut pH as well as modulation of immune response. It may also reduce the incidence of calf diarrhea; calf morbidity/mortality thereby supporting the animal industry to the threat of economic losses. Recently, the effect of probiotics as nutraceuticals on the gut health of domestic animals was explored showing amazing results. In this circumstance, good management of probiotic supplementation ideally maintains the gut ecosystem of domestic animals and protects them from enteric pathogens. Furthermore, these probiotic products have been documented as relatively safe compared to antibiotic growth promoters. But personal precautions should be taken before using it in animal nutrition to avoid hazardous effects of human health associated with it. Probiotics influence the intestinal microbiota and augments the humoral and cellular immunity, which could successfully develop natural antibodies. On the other side, researchers have allowed genetic manipulations of probiotics strains to improve the development of new advantageous microbes. The available findings have provided us with adequate data on probiotics containing *Lactobacillus* and *Bifidobacterium* strains but lacking data on other probiotic microorganisms. Therefore, there is need to explore each microorganism on the strain level to confirm their potential effects on animal health. In addition, probiotic bacteria should not have the ability to produce antibiotic resistance genes; otherwise these will not be suitable for animal industry.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Abbreviations

WHO: world health organization; VFAs: Volatile fatty acids; EE: Ether extract; NFE: Nitrogen free extract; IgG: ImmunoglobulinG; IgM: ImmunoglobulinM; IgA: ImmunoglobulinA; APC: Antigen presenting cells; IL-1β: Interleukin-1 beta; TGF-β: Transforming growth factor beta; Anti-OVA: Anti-ovalbumin; CE: Competitive exclusion; ETEC: Enterotoxigenic Escherichia coli (ETEC) IPEC-J2: Intestinal porcine epithelial cell- jejunum; LAB: Lactic acid bacteria; YC: Yeast culture; PAMP: Pathogen-associated molecular patterns; MAMPs: Microbe-associated molecular patterns; PRRs: Pattern recognition receptors; DC: Dendritic cells; LPS: Lipopolysaccharide; DNA: Deoxyribonucleic acid; mRNA: Messenger ribonucleic acid; TLR4: Toll-like receptors 4; DM: Dry matter; WG: Weight gain; ADG: Average daily gain; DMI: Dry matter intake; FI: Feed intake; FCR: Feed conversion ratio; FEE: Feed efficiency ratio; EAA: Essential amino acids; AGPs: Antibiotic growth promoters; DGGE: Denaturing gradient gel electrophoresis; PUFA: Polyunsaturated fatty acids; IL-2: Interleukin-2; IFN: Interferon; C-C: Carbon-Carbon; EU: European union; FAO: Food and Agriculture Organization; FOS: Fructo-oligosaccharides; GOS: Galacto oligosaccharides; MOS: Mannanoligosaccharides; XOS: XOS: Xylo-oligosaccharides; IMO: Isomaltooligosaccharides; SCFA: Small chain fatty acids;

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Research article
Ερευνητικό άρθρο

Clinical importance of lipid profile in neonatal calves with sepsis

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ABSTRACT. In this study, it was aimed to determine of diagnostic importance of blood lipid levels in neonatal calves with sepsis. The study was carried out on a total of 70 calves, 60 with sepsis and 10 healthy calves. The calves with sepsis were included in the study, according to clinical and hematological findings. The blood samples were taken from the V. jugularis for hematological, lipid profile and biochemical analyzes after the routine clinical examinations of the calves. There were significantly ($P < 0.05$) decrease in body temperature, increase in respiration rate and capillary refill time in the calves with sepsis compared to control group. The levels of blood urea nitrogen, creatinine concentrations of calves with sepsis were significantly higher ($P < 0.05$), however, levels of total cholesterol, HDL and LDL concentrations were significantly lower ($P < 0.05$) than control group. In addition, blood triglyceride and VLDL concentrations of calves with sepsis were higher than control group, however there was no statistical difference.
In conclusion, serum total cholesterol, HDL and LDL in neonatal calves with sepsis could be used in evaluation of the sepsis in calves.

Keywords: Sepsis, calves, lipid profile

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INTRODUCTION

The disease of calves are the most important causes of economic losses in the cattle industry (Ortiz-Pelaez et al., 2008). The important part of the calf morbidity and mortality is observed in the neonatal period (Guzelbektes et al., 2007; Radostits et al., 2007; Basoglu et al., 2014). Sepsis is defined as a combination of focal or generalized infection (suspicious infection) and systemic inflammatory response to the infections (Radostits et al., 2007). Sepsis is the most common cause of morbidity and mortality in newborn (House et al., 2011). Mortality rate at high levels in sepsis because the process is progressing fast. For this reason, early diagnosis and treatment have great importance in order to reduce sepsis mortality (Aldridge et al., 1993; Radostits et al., 2007; Fecteau et al., 2009; Basoglu et al., 2014).

Biomarkers play an important role in understanding the diagnosis, prognosis and pathogenesis of sepsis. For this reason, biomarkers such as lipid profile are still being an investigation for an early diagnose of sepsis. It has been reported that significant changes in plasma lipid and lipoprotein concentration, composition and function during inflammation and infections have been reported in humans (Wendel et al., 2007; Barati et al., 2011) and in calves (Civelek et al., 2007) and dogs (Yilmaz and Senturk 2007). These changes have been reported to be induced by released cytokines (Khovidhunkit et al., 2000; Murch et al., 2007; Lekkou et al., 2014). Lipoproteins in the circulation play very important role in the pathophysiology of infectious diseases. Many studies reported that the serum level of total cholesterol, LDL and HDL decreased, and the serum triglyceride level increased in patients with inflammatory response. These changes were reported to be independent of the underlying disease or infectious agents (Alvarez and Ramos 1986; Fraunberger et al., 1999; Wendel et al., 2007; Barati et al., 2011).

The purpose of the study was to determine of diagnostic importance of blood lipid profile levels in neonatal calves with sepsis.

MATERIALS AND METHODS

Study design and animals

The study was carried out on a total of 70 calves, 60 with sepsis mean of age (days) was 13.13 ± 1.23) at brought to Large Animal Clinic of Faculty of Veterinary Medicine of Selcuk University from different farms by owner and 10 Holstein healthy calves (mean of age (days) was 12.60±2.25) were belong to Faculty farm. Breeds of calves with sepsis were Holstein 45, Simmental 10, and Montofon 5. Routine clinical examinations of all the calves were performed. Laboratory and clinical findings as described by Fecteau et al. (2009) and Lofstedt et al. (1999) were used for the diagnosis of sepsis in the calves. Along with the presence or suspected of infection and the SIRS criteria were evaluated as sepsis. A diagnosis of SIRS was made if at least two of the following criteria were fulfilled: leukopenia or leukocytosis (reference value, 4–12 10^3/μL), hypothermia and hyperthermia (reference value; 38.5–39.5˚C), bradycardia or tachycardia (< 90 or > 120 beats per minute), and tachypnea (> 36 breaths per minute). Blood samples for leukocyte count (tubes with K_3EDTA) and biochemical analyses (tubes without anticoagulant) were collected from the vena jugularis and the tubes without anticoagulant were kept at room temperature and coagulated. Serum was removed by centrifugation for 5 min at 2500 g. Serum samples were stored at -20 ˚C until analyzed. Leukocyte levels in blood with K_3EDTA of the calves were determined using a hematologic analyzer (Hemocell Counter MS4e, Melet Schloesing Laboratories, France). Serum samples were analysed for triglyceride, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), blood urea nitrogen (BUN) and creatinine. The analyses were performed on an automated analyser (BS-200, Mindray, China) and VLDL levels were calculated by the following formula: triglyceride/5 (Tietz 1995, Sevinc et al., 2003).

Statistical analysis

All data were presented as mean and standard error of mean (Mean ± SEM). Power analysis was performed and sample size of the groups was determined as statistically appropriate. Independent samples t-test was used to assess the significance of the differences between the groups. The level of statistical significance was at $P < 0.05$. Receiver Operating Characteristics (ROC) curves were used to determine the cut-off values of total cholesterol, HDL and LDL.

The likelihood ratio value for the cut-off threshold was calculated and the highest calculated value was considered as the optimum cut-off point. The SPSS software program (Version 18.0, SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

It was determined that hypothermia or hyperthermia, tachypnea, dehydration, tachycardia or bradycardia, depression, lack of suction reflex, cold in the mouth and cooling in the extremities, and capillar refill time was prolonged in calves with sepsis. Leukocyte count was significantly higher in calves with sepsis than in control group. There were significantly ($P < 0.05$) decrease in body temperature, increased in respiration rate and capillary refill time in the calves with sepsis, compared to control group (Table 1). In the sepsis group, 48 of the calves had enteritis, 7 calves had pneumonia, but in 5 calves the origin of the sepsis could not be determined.

The changes in lipid profile and biochemical parameters of sepsis and healthy calves are presented in Table 1. The levels of blood urea nitrogen and creatinine concentrations of calves with sepsis were significantly higher ($P < 0.05$) compared to control group. However, levels of total cholesterol, HDL and LDL in calves with sepsis were significantly lower ($P < 0.05$) than the control group. In addition, blood triglyceride and VLDL concentrations of calves with sepsis were higher than control group, but there was no statistical difference.

The results of ROC analysis of total cholesterol, HDL and LDL are given in Table 2 and Figure 1. The optimal cut-off values of total cholesterol, HDL and LDL were 67, 51.2, and 9.53 mg/dL, respectively. The specificity was 90% of all the parameters in these cut-off values and the sentivities were 86.7, 88.3 and 66.7%, respectively.

DISCUSSION

Until now, this is the first study to evaluate lipid profile parameters in calves with sepsis. In the present study, we demonstrated that the serum lipid profile has the potential use for diagnosis of sepsis in calves. An increase in the level of triglyceride and a decrease in the levels of total cholesterol, HDL and LDL have been observed in patients with sepsis and SIRS (Alvarez and Ramos 1986; Barati et al., 2011). Sepsis is usually accompanied by a significant decrease in cholesterol levels (Cirstea et al., 2017).

Table 1: The levels of serum lipid profil and some biochemical parameters in calves with sepsis and healty calves (Mean ± SEM)

Parameters	Sepsis n = 60	Healthy n = 10	P levels
Total cholesterol (mg/dL)	43.37 ± 3.87	100.50 ± 11.00	**< 0.001**
Triglyceride (mg/dL)	21.12 ± 3.25	15.17 ± 2.51	0.155
HDL (mg/dL)	30.42 ± 3.00	71.86 ± 6.57	**< 0.001**
LDL (mg/dL)	6.82 ± 0.73	22.84 ± 5.44	**0.017**
VLDL (mg/dL)	4.22 ± 0.65	3.03 ± 0.50	0.155
BUN (mg/dL)	40.50 ± 2.60	10.20 ± 0.98	**< 0.001**
Creatinine (mg/dL)	3.33 ± 0.43	1.59 ± 0.06	**< 0.001**
Leukocyte count (10^3/μL)	21.58 ± 1.67	9.34 ± 0.88	**0.003**
Heart rate (min)	99.54 ± 4.78	93.33 ± 7.94	0.514
Respiratory rate (min)	41.96 ± 2.72	30.14 ± 2.01	**0.001**
Temperature (˚C)	37.74 ± 0.23	38.90 ± 0.13	**0.023**
CRT (sec)	4.13±0.17	1.89±0.11	**<0.001**

HDL: high-density lipoprotein, LDL: low-density lipoprotein, VLDL: very low density lipoproteins, BUN: blood urea nitrogen, CRT; capillary refill time

Table 2. Cut-off, sensitivity and specificity values of total cholesterol, HDL and LDL in calves with sepsis

Parameters	AUC	Cut-off values	Sensitivitiy (%)	Spesifity (%)	P value	SEM
Total cholesterol (mg/dL)	0.932	67	86.7	90	<0.001	0.030
HDL (mg/dL)	0.937	51.2	88.3	90	<0.001	0.031
LDL (mg/dL)	0.876	9.53	66.7	90	<0.001	0.052

HDL: high-density lipoprotein, LDL: low-density lipoprotein

The pathophysiological mechanisms associated with hypocholesterolemia during the sepsis process are not fully understood (Barati et al., 2011). Different mechanisms including the imbalance between the synthesis and use of plasma lipids, the use of lipids to replace damaged cell membranes, and the interaction of lipids with bacterial toxins and cytokines are still being discussing (Akgun et al., 1998; Levels et al., 2001; Levels et al., 2003; Esteve et al., 2005; Morin et al., 2015). Clinical and experimental studies have shown that high levels of circulating cytokines to reduce cholesterol levels in patients with severe infection (Murch et al., 2007; Lekkou et al., 2014; Morin et al., 2015). In contrast, lipoproteins have the ability to regulate cytokine production during the inflammatory response. Therefore, the reduction in circulating levels of cholesterol plays a crucial role in the pathophysiology of sepsis (Hardaróttir et al., 1994; Fraunberger et al., 1999). In the present study, total cholesterol level in calves with sepsis was significantly lower than the control group. The possible cause of low cholesterol in calves with sepsis is caused by cytokines release to circulation in response to inflammation (Hardaróttir et al., 1994; Fraunberger et al., 1999). In some studies (Akgun et al., 1998; Fraunberger et al., 1999; Gordon et al., 2001; Lekkou et al., 2014), have been reported that inflammation with high cytokine level may be associated with hypocholesterolemia. El-Bahr and El-Deep (2013) reported that cytokine levels in bronchopneumonic water buffalo calves were significantly higher than healthy calves while serum total cholesterol, HDL and LDL levels were significantly lower. It has also been reported that cytokine (TNF-α and IL-6) levels are increased while circulating levels of cholesterol are decreased in inflammatory conditions (Akgun et al., 1998; Gordon et al., 2001; Lekkou et al., 2014).

It has been reported that lipopolysaccharide (LPS) is neutralized by lipoproteins. It is stated that an important mechanism causing the decrease in HDL is consumed by LPS and other endotoxins (Levels et al., 2001; Levels et al., 2003; Wu et al., 2004; Esteve et al., 2005; Barati et al., 2011; Morin et al., 2015). Thus, it is thought that HDL and LDL are important regulators of the host immune response during endotoxemia and have the potential to treat patients with gram-negative sepsis (Wendel et al., 2007; Barati et al., 2011). In addition, it has been reported that HDL induces various anti-atherogenic, anti-inflammatory and anti-oxidative effects, independent of changes in cholesterol metabolism (Khovidhunkit et al., 2000; Gordon et al., 2001; Barter et al., 2004; Murch et al., 2007; Barati et al., 2011). It has been reported that total cholesterol and HDL levels are significantly decreased in calves with bronchopneumonia (Civelek et al., 2007; Joshi et al., 2015) and dogs with parvoviral enteritis (Yilmaz and Senturk 2007). It is reported that low HDL levels in septic patients are significant-

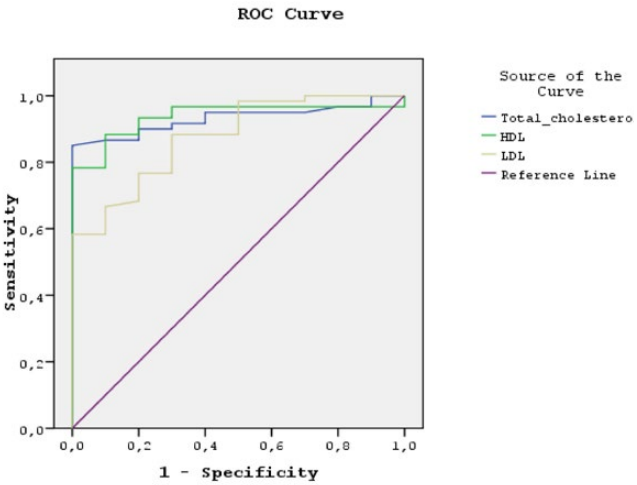

Figure 1. Plot of receiver operating characteristic (ROC) curve for total cholesterol, HDL and LDL variables

ly associated with mortality and the development of adverse clinical outcomes (Chien et al., 2005; Lekkou et al., 2014; Cirstea et al., 2017). In this study, it was observed that serum HDL and LDL levels in calves with sepsis were significantly lower than control group ($P < 0.001$, $P < 0.05$, respectively). It has been showed that the low level of HDL and LDL may be used as diagnostic criterias in evaluation of sepsis. Nassaji and Ghorbani (2012) reported that acute bacterial infections are associated with low serum total cholesterol and HDL levels and they indicate that changes in plasma lipid levels may be an important indicator of acute bacterial infections.

Sepsis causes hypertriglyceridemia in humans and animals, and this increase is due to the induction of hepatic and adipose tissue lipolysis and the increase in VLDL production (Alvarez and Ramos 1986; Civelek et al., 2007). In another study, have been suggested that as the cause of hypertriglyceridemia is to diminished conversion of VLDL to LDL by inhibition of lipoprotein lipase activity (Feingold et al., 1992; Gouni et al., 1993). Civelek et al. (2007) reported that VLDL and triglycerides levels of calves with bacterial lower respiratory tract infections were significantly higher than healthy ones. Another study reported that triglyceride concentration was higher in children with bacterial pharyngitis than in healthy children but this difference was not statistically significant (Iscan et al., 1998). In this presented study, similar to current studies, serum TG and VLDL levels were increased in calves with sepsis but this increase was not statistically significant (Table 1).

In humans, studies are being conducted on the diagnostic and prognostic value of dislipidemia in critical diseases such as sepsis and SIRS (Lüthold et al., 2007; Lekkou et al., 2014; Zou et al., 2016). However, our study has limitation. Unfortunately, the prognostic significance of this study has not been established, as there is insufficient information about whether or not the calves survived. The missing part of this study is that the prognostic follow-up of the calves with sepsis is not performed and the parameters are not considered as a mortality factor. However, we think that changes in the lipid profile may give an idea of the diagnostic value. For this purpose, ROC analysis for total cholesterol, HDL and LDL was performed to determine the optimal cut-off value and sensitivity and specificity of the relevant parameters according to this cut-off value. According to ROC analysis results, the cut-off values of total cholesterol, HDL and LDL were 67, 51.2, and 9.53 mg/dL, respectively. Despite the high specificity (90%) of all the parameters in these cut-off values, the sentivities were 86.7, 88.3 and 66.7% respectively. According to these results, it has been assumpted that total cholesterol and HDL can be used as parameters for diagnosis inflammatory response of sepsis in the calves. However, LDL is not a suitable parameter because it has low sensitivity.

CONCLUSIONS

In conclusion, it has been shown that the decrease in serum total cholesterol and HDL levels may be a sign of intense inflammatory response and that these changes in lipid levels (especially total cholesterol and HDL) can be used to detect inflammatory response in calves with sepsis. We could be said that serum total cholesterol and HDL may be used as a diagnostic indicator for sepsis in calves. However, further studies are needed to evaluate serum total cholesterol and HDL as prognostic and mortality indicators in calves with sepsis.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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Research article
Ερευνητικό άρθρο

Associations of periparturient β-hydroxybutyric acid and non-esterified fatty acids blood serum concentrations with milk yield, milk composition and milk somatic cells count of intensively managed Chios dairy ewes

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ABSTRACT. This research paper addresses the hypothesis whether in dairy ewes: periparturient β-hydroxybutyric acid (BHBA) and non-esterified fatty acids (NEFA) concentrations are associated with milk yield, composition and udder halves with increased somatic cell counts (SCC $\geq 0.5 \times 10^6$ cells/mL). A total of 186 Chios ewes reared under intensive system were used for this research. Serum BHBA and NEFA concentrations were measured before lambing (-30d, -15d), and BHBA concentrations after lambing (+7d, +15d, +30d, +45d). Milk samples were collected at 15, 30 and 45days in milk (DIM). Total milk yield (MY) of the first 30, 60 and 90 DIM and total fortnightly milk yield (FMY) produced from 15 to 59 DIM were recorded. Positive associations between BHBA at +7d and MY of the first 30, 60 and 90 DIM were revealed ($P< 0.001$). For every increased unit of BHBA at +15d, +30d and +45d, FMY was decreased (DIM: 15 – 29 and 30 – 44) ($P = 0.001$ and $P = 0.015$, respectively) or increased (DIM: 45 – 59) ($P < 0.001$). BHBA before lambing (-30d, -15d) affected the number of halves presented SCC $\geq 0.5 \times 10^6$ cells/mL at 15 and 30 DIM ($P = 0.011$, $P = 0.014$, $P = 0.009$, $P = 0.096$, respectively). Finally, for every increased unit of BHBA during lactation (+15d, +30d, +45d) a decrease in the concentration of milk in proteins, solids not fat and lactose was noted ($P< 0.001$). This work demonstrates the associations of periparturient blood biochemical parameters (BHBA, NEFA) with milk yield and specific milk production characteristics.

Keywords: dairy sheep, milk yield, milk composition, somatic cell count, β-hydroxybutyric acid

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ΠΕΡΙΛΗΨΗ. Στην παρούσα εργασία διερευνήθηκε εάν οι συγκεντρώσεις του β-υδροξυβουτυρικού οξέος (ΒΗΒΑ) και των μη εστεροποιημένων λιπαρών οξέων (NEFA), κατά την περιγεννητική περίοδο, συσχετίζονται με το ύψος της γαλακτοπαραγωγής, με τη χημική σύνθεση του γάλακτος, καθώς και με τον αριθμό των ημιμορίων του μαστικού αδένα που εμφανίζουν υψηλό αριθμό σωματικών κυττάρων (ΑΣΚ ≥ 0,5 × 10^6/mL). Για τις ανάγκες της έρευνας χρησιμοποιήθηκαν 186 προβατίνες φυλής Χίου μίας εντατικής εκτροφής. Οι συγκεντρώσεις των ΒΗΒΑ και ΜΕΛΟ προσδιορίστηκαν πριν από τον τοκετό (-30d, -15d), ενώ μετά τον τοκετό προσδιορίστηκαν οι συγκεντρώσεις του ΒΗΒΑ (+7d, +15d, +30d, +45d). Δείγματα γάλακτος ελήφθησαν τη 15η, την 30η και την 45η ημέρα της γαλακτικής περιόδου. Υπολογίστηκε η συνολική ποσότητα γάλακτος (ΣΠΓ) που παράχθηκε κατά τις πρώτες 30, 60 και 90 ημέρες της γαλακτικής περιόδου, καθώς και η ενδιάμεση ποσότητα γάλακτος (ΕΠΓ) που παράχθηκε κατά τα δεκαπενθήμερα χρονικά διαστήματα 15 – 29, 30 – 44 και 45 – 59 ημερών της γαλακτικής περιόδου. Παρουσιάστηκε θετική συσχέτιση μεταξύ της συγκέντρωσης του ΒΗΒΑ την ημέρα +7d με τη ΣΠΓ των πρώτων 30, 60 και 90 ημερών της γαλακτικής περιόδου (P< 0.001). Η αύξηση, κατά μία μονάδα, της συγκέντρωσης του ΒΗΒΑ στις ημέρες +15d, +30d και +45d, συσχετίστηκε με μείωση (ημέρες: 15 – 29 και 30 – 44, P = 0,001 και P = 0,015, αντίστοιχα) ή αύξηση (ημέρες: 45– 59, P< 0,001) της ΕΠΓ. Η συγκέντρωση του ΒΗΒΑ πριν από τον τοκετό (-30d, -15d) συσχετίστηκε θετικά με τον αριθμό των ημιμορίων του μαστικού αδένα που παρουσίασε ΑΣΚ ≥ 0.5 × 10^6/mL κατά τις ημέρες +15dκαι +30d (P = 0,011, P = 0,014 και P = 0,009, P = 0,096, αντίστοιχα). Τέλος, η αύξηση, κατά μία μονάδα, της συγκέντρωσης του ΒΗΒΑ στις ημέρες +15d, +30d και +45d, συσχετίστηκε με μείωση της περιεκτικότητας του γάλακτος σε πρωτεΐνες, σε στερεό υπόλειμμα άνευ λίπους και σε λακτόζη (P< 0.001). Η παρούσα έρευνα καταδεικνύει τη συσχέτιση συγκεκριμένων ενεργειακών παραμέτρων (ΒΗΒΑ, NEFA) με το ύψος της γαλακτοπαραγωγής, καθώς και με κάποια ποιοτικά χαρακτηριστικά του γάλακτος.

Λέξεις κλειδιά: φυλή Χίου, γαλακτοπαραγωγή, σύνθεση γάλακτος, αριθμός σωματικών κυττάρων, β-υδροξυβουτυρικό οξύ

INTRODUCTION

Milk yield, composition and somatic cells count (SCC) are of high importance in dairy sheep industry due to their contribution to milk products, determining both their quality and quantity. Dairy sheep selection over the last decades has focused towards improvement of both milk yield and composition (Barillet et al. 2001). This led in the development of highly productive dairy breeds housed and managed under intensive, zero-grazing conditions (Milan et al. 2011, Gelasakis et al. 2012). Among the indigenous Greek sheep, Chios breed is considered to be of high milk yield and prolificacy (Gelasakis et al. 2012).

High producing dairy breeds are better adapted in intensive farming systems, where ewes can express their genetic potential (Milan et al. 2011, Gelasakis et al. 2012). However, breeds or individuals with higher potential in milk production, may confront increased difficulty in controlling energy balance, especially during the period around parturition (Bizelis et al. 2000). Although periparturient period in dairy ewes is not precisely defined, its importance for health and productivity has been noted (Charismiadou et al. 2000, Theodorou et al. 2007, Mavrogianniand Brozos 2008, Karagiannis et al. 2014).

Data regarding associations of blood BHBA and NEFA concentrations with milk yield, milk composition and SCC in dairy sheep, are scarce in the accessed literature. However, in dairy cows increased prepartum blood BHBA and NEFA concentrations were associated with milk loss across the first 120 DIM (Ospina et al. 2010, Chapinal et al. 2012). High BHBA during early lactation had a negative short-term impact on milk yield across the first 30 DIM (Duffield et al. 2009, Chapinal et al. 2012), while for longer term milk yield, contradictory results have been published (Duffield

et al. 2009, Ospina et al. 2010, Chapinal et al. 2012). Prepartum blood BHBA and NEFA concentrations have been associated with SCC (Nyman et al. 2008), while postpartum BHBA were not associated (Al-Rawashdeh 1999). Finally, elevated postpartum BHBA have been correlated with increased milk fat and decreased milk protein percentage (Miettinen and Setala 1993, Duffield et al. 2009).

Objective of this study was to investigate whether periparturient BHBA and pre-lambing NEFA concentrations are associated with milk yield, milk composition and the count of the udder halves with increased SCC during lactation, in intensively managed Chios dairy ewes.

MATERIALS AND METHODS

1. Animals

The study was performed in an intensively managed purebred Chios dairy sheep farm. Two hundred clinically healthy pregnant ewes were initially enrolled: 40/200 ewe-lambs (pregnant animals that were going to lamb for the 1st time), 40/200 primiparous ewes (animals that had already lambed at the start of the study and would lamb again for 2nd time during the study) and 120/200 multiparous ewes (animals with more than 2 lambings at the start of the study). The average litter size of the 200 ewes was 1.98 ± 0.83. Ninety-four out of the 200 ewes lambed in November and 106/200 in January. In both occasions, lambings took place within 10 days, due to -previously applied- estrus synchronization programs.

During the first week of lactation, 14 ewes were excluded from the study due to very low milk yield (in average < 0.65 L/d). Therefore, data from the remaining 186 ewes were finally taken into account for the statistical analysis. Moreover, for various reasons, 9/186 ewes were removed from the study between the 7th and the 45th DIM and 10/186 between 45th and 90th DIM. Data from the last 19 ewes were thereafter not included in the statistical analysis.

2. Housing and nutrition

All animals were kept indoors and fed with a controlled ration throughout the year. Dry period lasted approximately two months. The ewes were fed according to National Research Counsil (1985) recommendations. During the first month of the dry period, each ewe received daily a ration containing 0.5 kg alfalfa hay and 0.5 kg concentrate mixture (corn 52%, barley 7%, wheat 8%, soybean 15%, wheat bran 6%, fat 2%, vitamins and minerals 10%) and grass hay on *ad libitum* basis. During the second month of the dry period, an additional 0.3 kg of concentrate mixture, plus 10 gr of sodium-propionate were added. After lambing, ewes received a total mixed ration consisting of 1.1 kg corn silage, 1 kg alfalfa hay and 1.55 kg of a mixture of concentrate feed (corn 35.5%, barley 22%, wheat 8%, soybean 22%, wheat bran 9%, fat 1%, vitamins and minerals 2.5%) per day and grass hay on *ad libitum* basis.

3. Blood sampling and analysis

Blood samples, for serum BHBA and NEFA measurements, were collected 30 ± 3 (-30d) and 15 ± 3 (-15d) days before the expected lambing (0d), as well as at 7 (+7d), 15 (+15d), 30 (+30d) and 45 (+45d) days after lambing, by jugular vein puncture from each animal into 10 mL plain glass tubes without anticoagulant (BD Vacutainer®, Plymouth, United Kingdom). The sampling procedure has been described by Oetzel (2004). Blood serum was separated by centrifugation (1600 x g for 15 minutes), transferred into plastic vials and frozen at -20°C until assay. BHBA concentrations were measured at -30d, -15d, +7d, +15d, +30d and +45d, while NEFA at -30d and-15d.

Serum BHBA concentrations were analyzed using the D-β-HB-dehydrogenase method (Williamson and Mellanby 1974). Serum NEFA concentrations were assayed using a commercially available spectrophotometric analytic kit (NEFA kit, WAKO Chemicals GmbH, Neuss, Germany).Thirty-one blood samples collected from ewes at -30d (19 for NEFA and 12 for BHBA) were not analyzed due to technical problems at processing.

4. Milk yield recording, milk sampling and analysis

Milking of ewes started 3 days after lambing, when lambs were removed from their dams. Ewes were milked three times per day and individual milk yield was electronically recorded on a daily basis (ALPRO™ software, DeLaval, USA, North Congress Ave. Kansas City Missouri, 64153). Total milk yield (MY) produced during the first 15, 30, 45, 60 and 90 DIM and total

fortnightly milk yield (FMY) produced from 15 to 29, 30 to 44 and 45 to 59 DIM were recorded.

Individual milk samples were collected aseptically from each mammary half of all ewes prior to the morning milking, based on standard sampling protocols (Fthenakis 1994). Milk samples from halves with clinical mastitis (defined as presence of milk clots or abnormal mammary discharge) were excluded from the study. In total, three milk samplings were performed in each ewe, on 15, 30 and 45 DIM. Samples were maintained at 4°C during transportation to the laboratory.

SCC were measured by the Fossomatic method (Gonzalo et al. 1993), using the Fossomatic® 9000 (A/S N. Foss Electric, Hillerød, Denmark). Samples with ≥ 0.5 × 10^6 cells/mL were considered indicative of inflammation (Berthelot et al. 2005). Finally, proportional (%) milk composition (fat, protein, lactose, solids-not-fat) was determined by using automated midrange infrared spectroscopy (MilkoScan FT 120, Foss Electric, Hillerød, Denmark). The analysis for the milk components was based only on the halves that presented SCC below the threshold of 0.5 x 10^6 cells/mL. This was decided because milk composition is affected by subclinical mastitis (Olives et al. 2013).

5. Statistical analysis

Univariate analysis was carried out by descriptive statistics and results were expressed as mean (*M*), standard deviation (*SD*), median (*Mdn*), minimum (*min*) and maximum (*max*) at the examined times (days) before and after parturition. In all tests statistical significance was declared at $P < 0.05$. The examination of the relationship between BHBA (-30d, -15d, +7d) and NEFA concentrations (-30d, -15d) with MY during 30, 60 and 90 DIM, as well as average milk SCC of each udder half on DIM 15 and 30 was performed through the non-parametric Spearman's correlation coefficient. The Generalized Linear Models (GLM) under Poisson distribution were used to evaluate the effect of BHBA and NEFA concentrations before lambing (-30d,-15d) on the number of the halves with SCC ≥ 0.5 × 10^6 cells/mL at 15 and 30 DIM. Regarding post-lambing period, the Generalized Linear Mixed Models (GLMM), under Poisson distribution, were used to evaluate the effect of BHBA concentrations after lambing (+15d, +30d and +45d) and Time (days) on the number of the halves with increased SCC (≥ 0.5 × 10^6 cells/mL) at 15, 30 and 45 DIM. In addition, Linear mixed effects (LME) modeling (Pinheiro and Bates, 2006) was used to assess the effect of BHBA concentrations on milk components (fat, protein, solids-not-fat, lactose) at +15d, +30d, and +45d and on FMY during the 15-days intervals (15 – 29, 30 – 44 and 45– 59 DIM, respectively). All statistical analyses were conducted using the statistical language R (R Core Team, 2013) and the function lme from package nlme (Pinheiro and Bates 2006).

RESULTS

1. Associations of periparturient BHBA and pre-lambing NEFA serum concentrations with milk yield

The descriptive statistics for BHBA (days: -30d, -15d, +7d, +15d, +30d, +45d) and NEFA (days: -30d, -15d) serum concentrations, as well as MY (15, 30, 45, 60 and 90 DIM) are shown in Table 1, respectively; BHBA concentrations are also depicted in Figure 1.

The Spearman's correlation coefficient revealed a statistically significant and weak positive correlation between BHBA serum concentration at -30d and MY produced during the first 30 DIM ($r_s = 0.185$, N=155, P = 0.021). Moreover, BHBA concentration at +7d was significantly positive correlated with MY produced during the first 30 ($r_s = 0.339$, N=182, $P < 0.001$), 60 ($r_s = 0.401$, N=171, $P < 0.001$) and 90 DIM ($r_s = 0.396$, N=167, $P < 0.001$). In contrast, there was not detected any statistically significant correlation between NEFA serum concentrations before lambing (-30d,-15d) and MY for any of the studied time periods.

With regard to the post-lambing period, the LME models fitted on the raw data revealed significant violations of the homogeneity of variance assumption and for this reason the measurements (BHBA and FMY) were logarithmically transformed using the natural logarithm. The final model indicated statistically significant main effects of both BHBA ($P = 0.042$) and Time ($P < 0.001$) on the FMY, and a significant interaction term (BHBA × Time) ($P < 0.001$). The parameters of the model are presented in Table 2. Interpreting the LME model with the interaction effect, the coefficient of BHBA at +15d ($\beta = -0.188$, $P = 0.001$) indicates that one percentage change in BHBA results in a 0.188 % decrease for FMY of the next 15-days period (15 – 29 DIM). Similarly, for one percentage change in BHBA at +30d, a

Table 1. Descriptive statistics for serum β-hydroxybutyric acid (BHBA) and non-esterified fatty acids (NEFA) concentrations during the study (days: -30d, -15d, +7d, +15d, +30d, +45d), for body condition score (BCS) (-30d, 0d, +30d), for total milk yield (MY) of ewes during the first 15, 30, 45, 60 and 90 days in milk and for the ewes according to the count of halves (0, 1 or 2 halves) that found with milk somatic cells count (SCC) ≥ 0.5×10⁶ cells/mL (days: +15d, +30d, +45d).

		Days (before/after lambing)								
		-30d	-15d	0d	+7d	+15d	+30d	+45d	+60d	+90d
N (ewes)		155	186	186	186	185	182	177	171	167
BHBA (mmol/L)	*Mean (± SD⁺)*	0.89 (±0.55)	1.03 (±0.74)	-	0.67 (±0.40)	0.63 (±0.33)	0.54 (±0.17)	0.64 (±0.18)	-	-
	Median [min, max]	0.76 [0.35, 4.40]	0.78 [0.44, 4.60]	-	0.61 [0.28, 5.20]	0.58 [0.27, 4.00]	0.54 [0.26, 1.60]	0.61 [0.23, 1.10]	-	-
NEFA (mmol/L)	*Mean (± SD⁺)*	0,43 (±0.37)	0,44 (±0.43)	-	-	-	-	-	-	-
	Median [min, max]	0.31 [0.005, 2.00]	0.30 [0.005, 2.30]	-	-	-	-	-	-	-
BCS (scale 1–5)	*Mean (± SD⁺)*	2.87 (±0.58)	-	2.36 (±0.69)	-	-	2.35 (±0.60)	-	-	-
Total Milk Yield (L)	*Mean (± SD⁺)*	-	-	-	-	26.89 (±9.54)	60.59 (±18.65)	98.34 (±27.33)	135.87 (±34.72)	202.36 (±47.39)
	Median [min, max]	-	-	-	-	26.00 [7.00, 58.00]	60.00 [20.00, 109.00]	98.00 [39.00, 168.00]	133.00 [50.00, 237.00]	199.00 [100.00, 344.00]
Count of halves with SCC ≥ 0.5 x 10⁶ cells/mL	*0*	-	-	-	-	148 (80%)	148 (81.3%)	145 (82%)	-	-
	1	-	-	-	-	28 (15.1%)	28 (15.4%)	28 (15.8%)	-	-
	2	-	-	-	-	9 (4.9%)	6 (3.3%)	4 (2.2%)	-	-

⁺Standard deviation

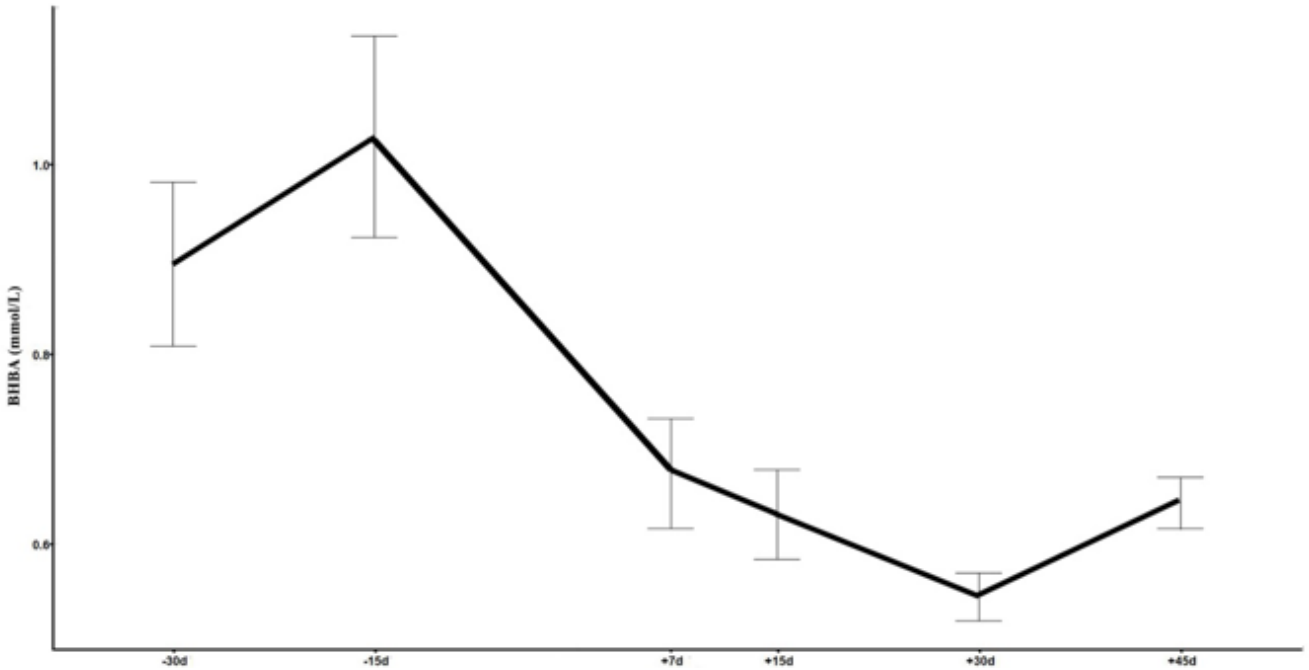

Figure1. Serum β-hydroxybutiric acid (BHBA) concentrations (95% Confidence Interval) during the study (days from lambing: -30d, -15d, +7d, +15d, +30d, +45d).

Table 2. Linear mixed effects models for fortnightly milk yield (FMY) during 15 – 29, 30 – 44 and 45 – 59 days in milk (DIM) and for milk protein, solids not fat and lactose at +15, +30 and +45 days in milk, with β-hydroxybutyric acid (BHBA) (log-transformed), Time and interaction term logBHBA × Time as fixed effects.

	Estimate	SE	*t*	*P*
Model 1 [Fortnightly Milk Yield (log-transformed)**]**				
Intercept	3.350	0.040	84.202	< 0.001
BHBA (log-transformed)	-0.188	0.055	-3.420	0.001
Time: [30 – 45 DIM]	0.184	0.055	3.699	< 0.001
Time: [45 – 60 DIM]	0.206	0.045	4.559	< 0.001
logBHBA×Time: [30 – 45 DIM]	0.177	0.072	2.452	0.015
logBHBA×Time: [45 – 60 DIM]	0.295	0.078	3.805	< 0.001
Model 2 [Milk Protein (log-transformed)**]**				
Intercept	1.570	0.007	211.734	< 0.001
BHBA (log-transformed)	-0.033	0.008	-4.219	< 0.001
Time: +30d	-0.025	0.003	-7.240	< 0.001
Time: +45d	-0.030	0.005	-5.954	< 0.001
Model 3 [Milk Solids Not fat (log-transformed)**]**				
Intercept	2.383	0.004	648.033	< 0.001
BHBA (log-transformed)	-0.020	0.004	-5.070	< 0.001
Time: +30d	-0.007	0.002	-4.351	< 0.001
Time: +45d	-0.007	0.002	-2.779	0.006
Model 4 [Milk Lactose (log-transformed)**]**				
Intercept	1.654	0.004	393.612	< 0.001
BHBA (log-transformed)	-0.007	0.004	-1.727	0.085
Time: +30d	0.010	0.002	5.591	< 0.001
Time: +45d	0.013	0.002	5.488	< 0.001
Note: Time: +15d is the reference category				

decrease of 0.011 % in FMY (β = 0.177, $P = 0.015$) of the next 15-days period (30 – 44 DIM) was noted. In contrast, for one percentage change in BHBA at +45d, an increase of 0.107 % FMY (β = 0.295, $P < 0.001$) of the next 15-days period (45 – 59 DIM) was found.

2. Associations of periparturient BHBA and pre-lambing NEFA concentrations with the count of the halves with increased SCC

Table 1 shows the distribution of the ewes according to the count of the halves (0, 1 or 2 halves) that found to have SCC ≥ 0.5 × 10^6 cells/mL at 15, 30 and 45 DIM. The GLM revealed a statistically significant main effect of BHBA concentration at -30d on the count of the halves with SCC ≥ 0.5 × 10^6 cells/mL at +15d ($P = 0.011$) and at +30d ($P = 0.009$). Concerning BHBA concentration at -15d, a statistically main positive effect on the count of the halves with SCC ≥ 0.5 × 10^6 cells/mL was detected at +15d ($P = 0.014$), while was not detected at +30d. The GLM indicated a marginally significant positive main effect of NEFA concentration at -30d on the count of the halves with SCC ≥ 0.5 × 10^6 cells/mL at +15d ($P = 0.051$). On the other hand, there was not detected any main effect of NEFA concentrations at -30d on the count of the halves with increased SCC at +30d ($P = 0.116$) and NEFA concentrations at -15d on the count of the halves with increased SCC at +15d ($P = 0.179$) and at +30d. Finally, the GLMM model for the examination of the effect of BHBA concentrations and Time on the count of the halves with SCC ≥ 0.5 × 10^6 cells/mL at 15, 30 and 45 DIM did not reveal any statistically significant main or interaction effects for the post-lambing period.

3. Associations of post-lambing BHBA concentrations with milk composition

Table 2 also presents the findings of the LME models that were fitted in order to study the effects of the covariate BHBA and factor Time on milk composition parameters. Due to violations of the homogeneity of variance assumption, both the covariate (BHBA) and the set of dependent variables (milk protein, solid not fat and lactose) were logarithmically transformed. BHBA presented a statistically significant main effect on milk protein and solid not fat parameters ($P < 0.001$) and a marginally significant effect on milk lactose ($P = 0.085$). The negative coefficients for covariate BHBA revealed a negative correlation between BHBA and milk composition parameters. Regarding the factor Time, there were noted statistically significant differences between the mean values of milk composition parameters between +15d and +30d periods and between +15d and +45d periods. In contrast, the Tukey's HSD procedure did not reveal anysignificant difference between the mean values of the examined parameters for +30d and +45d periods.

DISCUSSION

BHBA concentration 30 days before parturition was weakly positively correlated with milk yield produced until 30 DIM. It was previously reported that, blood BHBA concentration increases during late gestation and may reach its peak before or around lambing (Raoofi et al. 2013; Karagiannis et al. 2014). It is possible that the ewes with potential for higher milk production could have higher energy metabolism before lambing, expressed as increased BHBA concentrations. However, this plausible explanation requires further investigation. BHBA concentration at +7d was positively correlated with milk yield of the first 30, 60 and 90 DIM, implying that a moderate ketone metabolism in early lactation may be beneficial for achieving higher milk yield. However, a rise of BHBA concentrations at +15d had a negative short-term effect in the cumulative milk yield of the next 15-days (15 – 29 DIM). As lactation progressed (at 30 DIM), BHBA short-term effect on the FMY of 30 – 44 DIM was still negative, but weaker, while high levels of BHBA at +45d had a positive effect on FMY of the next 15-days (45 – 59 DIM). It has been shown that ewes with greater potential for increased milk yield confront a longer period of NEB during early lactation (Bizelis et al. 2000). It could be assumed that increased BHBA concentrations during early lactation (until 30 DIM) could cause a short-term decrease in milk production. The finding that +45d BHBA concentration positively affected FMY of the next 15 days (45 – 59 DIM) probably implies that energy balance was restored or may became positive around the 45th day of lactation. Under this hypothesis, increased BHBA at the beginning of lactation may indicate a potential for high milk production during the first 90 DIM (longer-term effect), including though a possible short-term negative effect, especially when NEB is not timely restored. Relevant information correlating BHBA concentrations after lambing with short and longer-term milk yields are lacking in the accessed literature for dairy ewes.

High levels of BHBA and NEFA before lambing predispose to several periparturient health disorders, such as clinical mastitis (Karagiannis et al. 2014). The results of the present study indicated that increased pre-lambing BHBA (-30d, -15d) and NEFA (-30d) concentrations were positively correlated with the count of the halves with increased SCC after lambing (15 and 30 DIM). The negative effect of subclinical pregnancy toxemia and elevated BHBA and NEFA concentrations to immune function was previously documented (Sartorelli et al. 2000, Lacetera et al. 2001) and could explain the early postpartum intramammary infections and increased SCC found in the present study. Recently, Bouvier-Muller et al. (2016) outlined that after a dietary-induced energy restriction during lactation, NEFA and BHBA concentrations were higher in mastitis-susceptible ewes compared with mastitis-resistant ones, implying a genetic association between energy metabolism and mastitis susceptibility. This could explain the positive effect of increased pre-lambing BHBA and NEFA concentrations on the count of the halves with increased SCC postpartum.

In the current study, increased post-lambing BHBA did not have any effect on the count of the halves with elevated SCC. The fact that BHBA concentration during lactation was much lower compared with late gestation (Table 1), could imply that BHBA did not reach the required levels to impair the immune function, as previously described. Interestingly, no difference in SCC was reported, after inflammation by Staphylococcus-associated ligands, between ewes con-

fronting dietary-induced NEB and ewes in a positive energy balance (Bouvier-Muller et al. 2016).

After lambing, an increase in BHBA concentrations at 15, 30 and 45 DIM was associated with a decrease in milk protein and milk lactose percentage, while no association was found with milk fat. Although relevant information for dairy ewes is lacking in the accessed literature, research in dairy cows has indicated that increased milk fat content and decreased milk protein were associated with elevated postpartum BHBA concentrations (Duffield et al. 2009). The increase of BHBA concentrations after lambing was associated with the reduced milk protein percentage. It has been reported that milk protein is an indicator of energy balance (Grieve et al. 1986). The association of increased BHBA concentrations with decreased milk lactose percentage could be explained by the negative correlation between blood BHBA and glucose concentrations, demonstrated in dairy sheep (Panousis et al. 2012).

Chemical composition of milk was also affected by time after lambing. More specifically, a reduction in milk fat, protein and SNF percentage was detected between 15 and 30, and between 15 and 45 DIM. Dairy ewes reared under intensive system peak milk yield at about 27 – 45 DIM (Gootwine and Pollott 2000, Pollott and Gootwine 2000;). Therefore, the recorded reduction in milk components (fat, protein and SNF) seems reasonable, due to a dilution effect (Gonzalo et al. 1994, Ochoa-Cordero et al. 2002). However, an expected increase in milk lactose was noticed between 15 and 30, and between 15 and 45 DIM, since increasing daily milk yield (until 45 DIM) is positively correlated with lactose percentage (Ploumi et al. 1998, Ochoa-Cordero et al. 2002).

CONCLUSIONS

The present study investigated the potential associations of periparturient BHBA and NEFA concentrations with milk yield, milk composition and the count of the udder halves with increased SCC. It has been shown that BHBA concentration 30 days before lambing had a weak positive impact on milk yield of the first 30 DIM. BHBA during early lactation had a positive long-term (first 90 DIM), but a negative short-term (15 – 29 and 30 – 44 DIM) effect on milk yield. Increased BHBA concentrations before lambing were correlated with a rise in the count of the halves that presented increased SCC during lactation. Moreover, BHBA increase during the first 45 DIM was negatively associated with milk protein percentage, but not with milk fat percentage.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Evaluation of *Bacillus* Strains as Probiotic Based on Enzyme Production and In Vitro Protective Activity against Salmonellosis

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ABSTRACT. Probiotic strains of *Bacillus* spp. are used in industrial poultry production because of their ability to produce enzymes enhancing the absorption of nutrients and to reduce the risk of *Salmonella* spp. infection. The aim of this study was to isolate native potential probiotic *Bacillus* spp. with the ability to produce enzymes and adhere to intestinal epithelial cells in order to prevent *Salmonella* Typhimurium infection. First, 25 samples of chicken feces were collected from 7 industrial poultry farms in Golestan province located in Northern Iran. *Bacillus* species from samples were isolated on nutrient agar. These strains were evaluated for the ability of producing amylase and phytase and their probiotic characteristics such as bile salt, acid and antibiotic resistance, the ability to attach to intestinal epithelial cells and inhibit *Salmonella* Typhimurium invasion. Then selected isolates were identified based on *16S rDNA*. Results showed that from 86 isolated, 4 *Bacillus* strains had desirable characteristics such as the ability to produce phytase and amylase and having suitable probiotics features. We identified K03, K02, and K20 isolates as *Bacillus tequilensis* and K20 as *Bacillus subtilis*. *Bacillus tequilensis* K03 showed the highest attachment ability to intestinal epithelium cells and could inhibited *Salmonella* Typhimurium attachment.

Keywords: Attachment, *Bacillus*, Poultry, Probiotic, *Salmonella*

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INTRODUCTION

Chicken intestinal infections result in decreased production, increased mortality and cause decline in food economics and safety (Natsos *et al*., 2016). Using probiotics can improve feed conversion ratio (FCR) by preventing intestinal diseases in chicken (Papatsiros *et al*., 2013; Knarreborg *et al*., 2008). The World Health Organization (WHO) defined probiotics as "live micro-organisms that when administered in adequate amounts, confer a health benefit on the host" (Jorgen, 2005).

The characteristics of a bacteria that could be identified as probiotic are: non-pathogenic, resistant to gastric acidity and bile salts while passing through the digestive system, having the ability to produce digestive enzymes, facilitative for the digestion and absorption of nutrients, competitive with important pathogenic bacteria such as *Salmonella* spp. in attachment to intestinal epithelium cells, and being able to neutralize internal toxins (Gaggìa *et al*., 2010).

Among the probiotics used in chicken diets, the *Bacillus* spp. can withstand environmental conditions and survive for a long time by producing spores (Nicholson, 2002). It has been shown that species of the genus *Bacillus*, in addition to naturally occurring in soil, are found at high levels in chicken feces (Nicholson, 2002). The bacteria can remain stable in the gastrointestinal tract of the chicken and have probiotic beneficial effects (Nicholson, 2002).

Some of the *Bacillus* spp. is capable of producing biofilm in animal intestines helping the bacteria to resist changes and stresses and also to protect the intestine against attachment of the pathogenic bacteria such as *Salmonella* spp. to the intestinal epithelial cells (Thirabunyanon and Thongwittaya, 2012).

The *Bacillus* spp. have many applications by producing enzymes such as α-amylase and phytase (Lee *et al*., 2012). These enzymes can decompose anti-nutrient agents in food, increase nutrient bioavailability, break down some chemical bonds, strengthen the indigenous enzymes and finally increase production efficiency (Lee *et al*., 2012).

The major source of phosphorus in plant-derived foods, especially in cereal grains, is phytate phosphate (Askelson *et al*., 2014). It accounts for 50 to 80 percent of total phosphorus in grain and legumes, respectively (Askelson *et al*., 2014). Phytase can help releasing the phosphorus in chicken's intestinal track and making it available for absorption (Askelson *et al*., 2014).

Starch is the most abundant combination of carbohydrates in cereals, such as wheat and corn (Latorre *et al*., 2016). The presence of amylase producing bacteria in broiler diets helps the digestion of insoluble starch (Latorre *et al*., 2016).

The *Salmonella* spp. is one of the major foodborne pathogen in poultry industry which also causes infection in humans (Mouttotou *et al.,* 2017).

Using probiotics capable to attach to intestinal epithelial cells and compete with *Salmonella* spp. is a safe alternative method to antibiotic therapy (Thirabunyanon and Thongwittaya, 2012).

The purpose of this study was to isolate and identify potential native and suitable probiotic *Bacillus* spp. bacteria in Golestan province of Iran with the ability to produce enzymes and high affinity to intestinal epithelial cells. These selected native probiotic *Bacillus* spp. bacteria could be used in poultry diets to improve the production quality, reduce antibiotic usage, lower the incidence of various related diseases and to prevent *Salmonella* Typhimurium infection in human and poultry.

MATERIALS AND METHODS

Sampling and Isolation of *Bacillus* strains

Twenty five samples of chicken feces were collected from 7 farms located in Golestan province in Northern Iran, with the capacity of rearing 20,000 chicken each farm. These farms were under windowless management with full automatic feeding system and were not using any kind of antibiotics in their diet. The specimens were collected in peptone water and were transferred to the laboratory within 2h under sterile condition. In order to omit vegetative and non-spore forming bacteria, samples were put in 90˚C bain-marie for 30 minutes. *Bacillus* isolates were insulated on nutrient agar (QueLab-393506, Canada) plates, after 48 hours of incubation at 37˚C. Isolates were evaluated by Gram staining, sporulation staining, catalase and hemolysis tests (Barbosa *et al*., 2005).

Determination of α-amylase enzyme activity

All *Bacillus* strains were grown in tryptic soy broth (TSB, Merck-105459-0500, Germany) at 37˚C for 24 hours. Then 10 µl with 10^8 cfu/mL of each strain were

placed at the center of starch agar (Merck-1.01252.1000, Germany). After incubation, the α-amylase producer strain was selected by starch solubilisation zone around the colony (Latorre *et al*., 2016). Amylase activity was measured by DNS method. The reaction mixture containing 1% soluble starch, 0.1 M Tris/HCl buffer (pH 8.5), 0.5 ml enzyme solution was incubated at 37˚C for 30 minutes. The reaction was stopped by the addition of 3,5-dinitrosalicylic acid (DNS) then heated for 10 minutes in boiling water and cooled in 4˚C. For estimating the enzyme activity, glucose standard curve was drown (Nwokoro and Anthonia, 2015).

Determination of phytase enzyme activity

The ability to produce phytase enzyme was screened by using phytase specific medium (PSM). The pure cultures were placed at the center of PSM agar and incubated at 37˚C for 62 h. After incubation, the strains with the ability to produce phytase enzyme were selected by a clear zone around the colony (Kumar *et al*., 2013). For phytase activity measurement, 0.1 ml of enzyme solution, 0.9 ml of 2 mM sodium phytate and 0.1M Tris-HCl buffer (pH 7) were used. Incubation was done at 37˚C for 10 minutes. The reaction was stopped by adding 0.75 ml of 5% tricholoroaccetic acid. The released phosphate was measured by adding 1.5 ml of color reagent containing 2.5% ammonium molybdate solution, 5.5% sulfuric acid and 2.5% ferrous sulfate solution (Demirkan *et al*., 2014).

Bile salts and acid resistance

Resistance of selected bacteria to bile salts and acid were measured according to Razmgah *et al.* (2016) and Cenci *et al.* (2006) respectively.

Antibiotic resistance of *Bacillus* strains (MIC method)

The antibiotic resistance pattern of selected *Bacillus* strains was determined according to Sorokulova *et al.* (2008).

Attachment and invasion assay of *Bacillus* spp. and *Salmonella* Typhimurium to the intestinal epithelial cells

Cells of the intestinal epithelial cell line (Caco2) were cultured in dulbecco´s modified Eagle´s minimal medium (DMEM; Sigma-Aldrich, USA) adding 10% fetal calf serum inactivated by heating at 56ºC for 30 min, 1% (v/v) L-glutamine and 1% Streptomycin (10 mg/ml and 10-103 IU/ml). Cells were incubated in 5% CO_2 at 37ºC and attachment and colonization tests were done according to Thirabunyanon and Thongwittaya (2012).

The ability of *Bacillus* spp. to inhibit *Salmonella* Typhimurium attachment to Caco-2 cells was assayed by two methods of exclusion and competition, after Caco-2 monolayers at 90% confluence in a 12-well plate were washed twice with PBS (phosphate buffered saline, pH 7.4) (Zhang *et al*., 2010).

In the exclusion assay, Caco-2 monolayers were inoculated with 300 µl of *Bacillus* spp. suspension (10^7 CFU/well) in DMEM medium and incubated in 5% CO_2 at 37ºC for 1 h then 100 µl of *Salmonella* Typhimurium suspension (10^7 CFU/well) in DMEM medium was added. Finally incubation was done in 5% CO_2 at 37ºC for 1 h.

In the competition assay, Caco-2 monolayers were inoculated with 400 µl of *Bacillus* spp. suspension (10^7 CFU/well) and *Salmonella* Typhimurium suspension (10^7 CFU/well) in DMEM medium and incubated in 5% CO_2 at 37ºC for 1 h.

Molecular identification based on *16S rDNA*

The DNA of selected *Bacillus* strains was extracted using the lysozyme enzyme digestion according to Araújo *et al.* (2004). For amplification of a 1500 bp fragment from *16S rDNA* region was performed with universal primers, 27F-5/ -AGAGTTTGATCCTGGCTCAG-3/ and 1492R-5/ - ACGGCTACCTTGTTACGACTT -3/.The 25 µl PCR reaction mixture consisted of 200 ng template DNA, 2.5 µl of PCR 10x Buffer, 200 µM dNTP, 1.5 mM $MgCl_2$, 10 pmol of each primers and 1 unit of Taq DNA polymerase enzyme and distilled water. Amplification cycling included primary denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 45 seconds, annealing of primer at 58 °C for 1 minute, extension at 72 °C for 30 seconds, and final extension at 94 °C for 5 minutes. Then, the PCR products were electrophoresed on 1.5% agarose gel. The 100 bp molecular weight marker (Sina-Clon, Tehran, Iran) was used as a molecular marker. After purification of the PCR product from the agarose gel, the samples were sent for sequencing to Bioneer Company (Daejeon, Republic of South Korea) (Araújo *et al*., 2004).

Statistical and phylogenetic analysis

Data obtained from sequencing were edited by bioinformatic software of Chromas Pro. and saved in FASTA format. Then GenBank of EzTaxon database was used to identify the bacteria (Chun *et al*., 2007). The phylogenetic tree was drawn by MEGA 5 software and neighbor-joining method (Saitou *et al*., 1987., Tamura *et al*., 2011). The statistical data analysis was done using GraphPad Prism v7.03 statistical software for selected *Bacillus* strains.

RESULTS

Isolation of *Bacillus* spp.

Spore-forming bacteria were selected by heat treatment of chicken feces. Thirty-four pure colonies were selected for further analysis. These strains were named K1_K34. All of the isolates were catalase-positive, oxidase-positive and non-hemolytic.

α-amylase enzyme assay

K03 strain was the only isolate with a significant zone of clearance on starch agar and the ability to produce α-amylase enzyme (Figure 1). Using the equation, the percentage of starch solution by selected *Bacillus* strain was calculated. Also, by drawing the standard curve (Figure 2), the linear regression equation (Equation 2) was used to calculate the production of α-amylase by the strain (Equation 3) (Table 1).

% soluble starch = (OD control-OD sample) / OD control * 100 (Equation 1).

Y = 0.0001234 * X + 0.05034 (Equation 2).

Where Y = OD supernatant, X = starch concentration remain.

X-total starch: starch concentration consumption

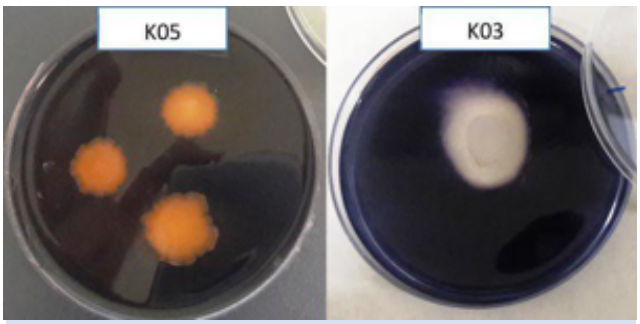


Fig 1: *Bacillus* spp. with the ability to produce the α-amylase enzyme: K03 (+), K05 (-)

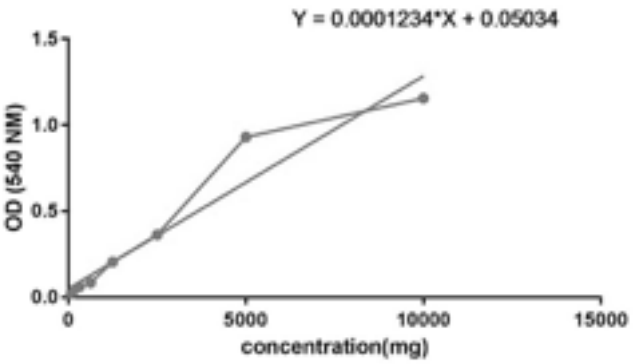


Fig 2: α-amylase enzyme standard curve

Table 1. Amount of α-amylase enzyme production

Starch solution (%)	Produced α-amylase enzyme (u/ml)	Strain
60.62	36.7±1.3	K03

Amount of amylase (u/ml) = Starch concentration consumption / 10min (Equation 3).

Phytase enzyme assay

Four *Bacillus* strains of K03, K02, K20 and K10 showed a clear zone in plate assay.

Percent of dissolution capacity of phosphorus was investigated in these strains (Equation 4). The standard curve was drown by standard phytase (Figure 3) and the linear regression equation (Equation 5) was prepared and the rate of phytase production by strains (Equation 6) was calculated (Table 2).

Percent of dissolution capacity of phosphorus = (OD control-OD sample)/OD control*100 (Equation 4)

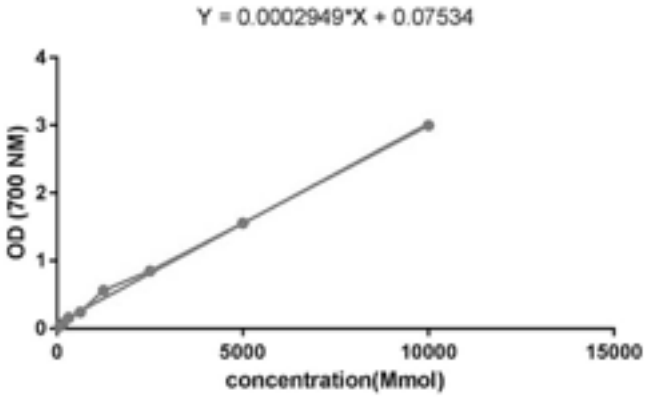


Fig 3: Phytase enzyme standard curve

Table 2. Amount of phytase enzyme production

Phosphorus solution (%)	Phytase production (u/ml)	Strain
45.97	22.33±1.2	K10
33.15	15.95±1.8	K02
30.28	14.53±2.3	K20
10.25	4.56±1.1	K03

Standard linear regression formula: Y = 0.0002949 * X + 0.07534 (Equation 5)

Y = OD supernatant

X = Calcium phytate concentration remains

Total Calcium Phytat-X = Calcium phytate concentration consumption

Amount of phytase (u/ml) = Calcium phytate concentration consumption / 30min (equation 6).

Tolerance to bile salts and acid

All strains which produced enzymes, were resistant to bile salts. Among these strains, the 2 strains of K03 and K20 showed the highest resistance to bile (Figure 4).

Table 3. Influence of pH on growth parameters of *Bacillus* strains

pH			Strain
7	4	2	
7.246±0.2457	6.554±0.04846	6.322±0.2803	K03
7.540±0.06247	6.253±0.2526	6.429±0.1276	K02
8.517±0.4375	7.751±0.02694	6.301±0.3010	K20
8.246±0.2457	7.440±0.1617	6.246±0.2457	K10

All strains of *Bacillus*-producing enzymes grew in pH 2, pH 4 and pH7 (Table 3).

Antibiotic resistance analysis

Bacillus strains were sensitive to all antibiotics listed by EFSA in 2012 (Table 4).

Adhesion capability of *Bacillus* spp. to intestinal epithelial cells

The adhesion ability of *Bacillus* spp. to intestinal epithelial cells is shown in Figure 5. Affinity ranged between 1.3-1.9 log CFU/well among the isolates. K03 strain showed the highest adherence ability to intestinal epithelial cells (Figure 6).

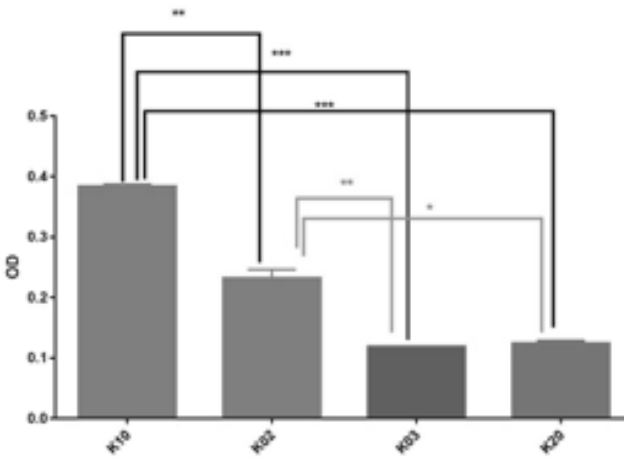
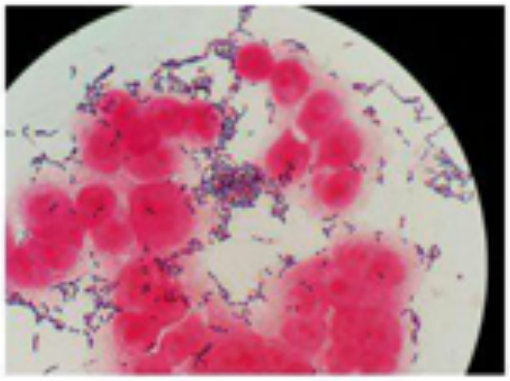


Fig 4: Bile tolerance of selected *Bacillus* spp.
*: P ≤ 0.05; **: P ≤ 0.01; ***: P ≤ 0.001

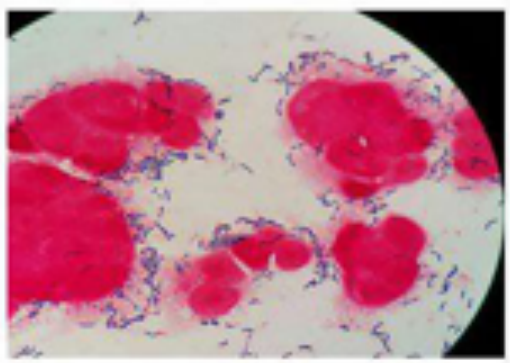
Table 4. Antibiotic resistance of *Bacillus* strains (MIC method)

Antibiotic (μg)	K02	K03	K10	K20	EFSA 2012 *Bacillus* spp. mg/L
Ampicillin (10)	16	15	16	14	n.r.*
Vancomycin (30)	1	2	1	1	4
Gentamycin (10)	1	1	1	1	4
Kanamycin (30)	3	2	4	2	8
Streptomycin (10)	4	4	3	4	8
Erithromycin (15)	2	1	1	2	4
Clindamycin (2)	1	1	3	2	4
Tetracyclin (2)	2	2	1	1	8
Chloramphenicol (30)	4	3	4	1	8

* not required



A



B

Fig 5: Attachment ability of *Bacillus* spp. to intestinal epithelial cells (A:K03/B:K02)

Inhibitory capability of *Bacillus* spp. against *Salmonella typhimurium* adherence to intestinal epithelial cells

The results showed that all the selected *Bacillus* spp. have inhibitory effects on the attachment of *Salmonella* Typhimurium to Caco-2 cells. Among them K03 strain showed more inhibitory strength than the others in both methods of exclusion and competition assay (Table 5).

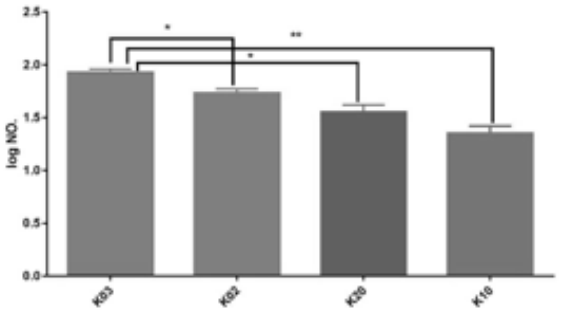


Fig 6: The adherence activity of Bacillus spp. to intestinal epithelial cells *: $P \leq 0.05$; **: $P \leq 0.01$

Phylogenetic identification of *Bacillus starins*

The analysis of 16S rDNA gene similarity showed K10 strain had the highest similarity to *Bacillus subtilis* and K03, K02 and K20 strains closely belonged to *Bacillus tequilensis* (Figure 7). Both strains are probiotic bacteria (Thirabunyanon and Thongwittaya, 2012; Parveen *et al*., 2016).

DISCUSSION

In vivo models of probiotic applications of *Bacillus* spp. have shown that these bacteria can enhance the absorption of food and have protective effects against infections (Ouwehand *et al*., 2002). In this study *Bacillus* strains were isolated from chicken feces with the specific ability of enzyme production and inhibition of invasive *Salmonella* Typhimurium.

Table 5. Evaluation of inhibition of *Salmonella* Typhimurium attachment to Caco-2 cells by exclusion and competition methods

Strain	Exclusion experiment *S.*Typhimurium (CFU/well *10^5)	Competition experiment *S.*Typhimurium prevented from adherence to Caco-2 (%)	*S.typhimurium* (CFU/well *10^5)	*S.*Typhimurium prevented from adherence to Caco-2 (%)
*S.*Typhimurium (control)	2.46±0.18		2.53±0.37	
K03	1.16±0.23*	53	2.18±0.18*	14
K02	1.64±0.31*	34	2.29±0.29	10
K20	1.93±0.12*	22	2.28±0.19	10
K10	1.95±0.23*	21	2.30±0.21	10

Data are mean standard deviation of three independent experiments, *S.* Typhimurium alone served as a control. Asterisk (*) indicates means which were significantly different from the control value ($p \leq 0.05$). % prevented from adherence to Caco2 cells = (control- test)/control

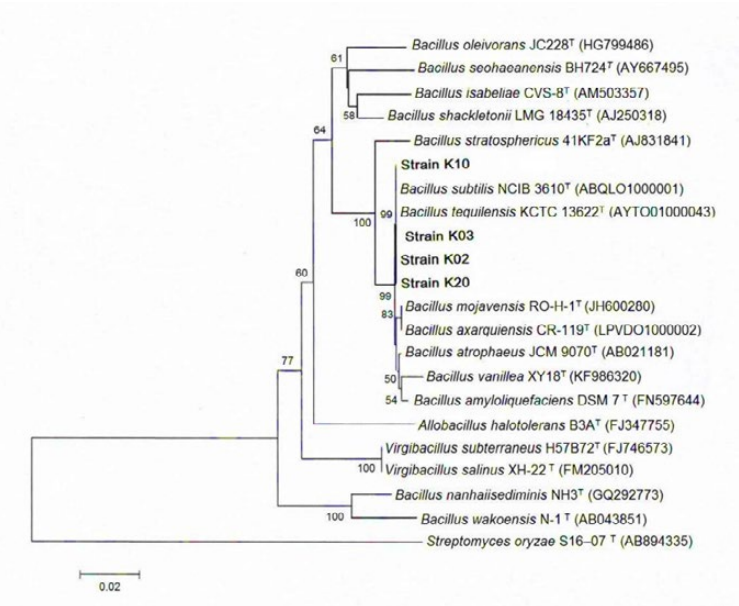


Fig 7: The phylogenic tree of *Bacillus* spp. Neighbour-joining phylogenetic tree based on 16S rDNA gene sequences, showing the position of Bacillus strains and other related genera. GenBank accession numbers are given in parentheses. The 16S rDNA gene sequence of the Streptomyces oryzae S16-07T was used as outgroup. Bootstrap values (%) are based on 1000 replicates. Bar, 0.02 substitutions per nucleotide position.

Khusro et al. (2017) isolated α-amylase producing *Bacillus* strains from chicken, and increased the amount of amylase enzyme production to 136.71 IU/ml by optimizing the culture conditions.

Latorre et al. (2016) isolated 31 strains of *Bacillus* from chicken, which could produce amylase, phytase, protease and lipase enzyme. In the current study, 4 *Bacillus* isolates from chicken feces with the ability to produce phytase enzyme were isolated. From these strains K03 was also able to produce amylase enzyme. Among them the K10 strain had the highest ability to produce phytase enzyme at 22.33 ± 1.2 IU/ml and K03 strain was the superior bacterium in production of 4.56 ± 1.1 U/ml phytase and 36.7 ± 1.3 U/ml α-amylase enzymes.

Seeber et al. (2015) reported that among 69 *Bacillus* isolated from broiler chickens, only three isolates were able to tolerate bile salts with a concentration of 0.037% and acidic conditions (pH 2.0).

Mingmongkolchai and Panbangred (2017) isolated 187 *Bacillus* strains from fresh milk of cattles, pigs and calves, and reported that 7 strains had the ability to produce phytase, cellulase, xylanase enzymes and had the most compatibility with intestinal conditions. The results of the present study showed that all strains of *Bacillus*-producing enzymes were able to withsand bile salts with a concentration of 0.03% and acidic conditions (pH 2.0, 4.0), which were consistent with mentioned studies.

Khusro and Aarti (2015) isolated the strains of amylase producing *Bacillus* from chicken feces. The 16S rDNA identification showed that 5 strains that had the ability to produce amylase enzyme were closer to *Bacillus tequilensis*, *Bacillus subtilis* and *Bacillus licheniformis*, which were further selected to optimize the production of amylase enzyme. In this investigation, phylogenetic studies showed isolated *Bacillus* strain had the highest relationship with *Bacillus subtilis* and *Bacillus tequilensis.*

Thirabunyanon and Thongwittaya (2012) reported that among 117 bacilli isolated from chicken intestines, 10 isolates had inhibitatory abilities against 7 pathogenic bacteria, including *Salmonella* spp. and also had the ability to attach to Caco-2 cells in a variety of conditions ranging from 2.8-4.9 logCFU/well. In the present study a novel probiotic *Bacillus tequilensis* K03 had the highest attachment ability to Caco-2 cells and showed the highest inhibition of *Salmonella* Typhimurium, up to 53% compared to control. According to Kizerwetter-Swida and Binek (2006) the bacteria with a high attachment ability to the Caco-2 cell has a higher ability to inhibit pathogenic bacterial attachments. These results are consistent with a study done by Jankowska and Laubitz (2008) that found a new probiotic bacteria belonged to *Bacillus tequilensis* FR9 acquired from chicken digestive tract, which was capable of inhibiting *Listeria monocytogenes*.

CONCLUSION

In the present study, we isolated a novel starin of *Bacillus tequilensis* K03 from chicken feces that was a potent producer of amylase and phytase with efficient protection activity against *Salmonella* Typhimurium infection *in vitro*. It was resistant to bile and acidic environment of intestinal track. Further investigation is needed, to evaluate its in vivo use as a native probiotic in chicken diets in order to improve the production

quality, reduce antibiotic consumption, lower the incidence of salmonellosis in broiler chickens and human and its native to the region.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

The effect of intrastromal PRP and oral doxycycline in corneal wound healing after alkali burn

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ABSTRACT. Ocular chemical burns are among the most critical and true ocular emergencies in dogs and cats, with destructive consequences on the ocular surface and intraocular tissues. Platelet alpha granules are an important reservoir of growth factors that can stimulate chemotaxis of inflammatory cells, mitosis, migration and differentiation of cells. On the other hand, tetracyclines and their derivatives exhibit non-antimicrobial properties, such as affecting inflammation, immunomodulation, cell proliferation and angiogenesis. The objective of this study is to investigate the effect of intrastromal platelet rich plasma (PRP) in combination with oral doxycycline in the healing process of the cornea. Thirty six New Zealand rabbits were used in this experimental study. Alkali burns were created by applying a round filter paper soaked in NaOH. PRP was prepared by centrifugation of autologous blood. The rabbits were allocated to four groups (9 animals each). Group 1 served as the control group without any treatment. Group 2 received doxycycline (5 mg/kg b.w.) orally once a day throughout the study. Group 3 received an intrastromal injection of PRP (0.5 ml) in the cornea, and group 4 received intrastromal PRP combined with oral doxycycline. Treatments had varied levels of success, with PRP combined with oral doxycycline producing the best results regarding corneal healing. Animals in the control group had epithelial defects for the whole duration of the study, and 3 animals presented corneal perforation. Groups 3 and 4 had smaller mean defect area, compared to groups 1 and 2. On the 7th day, neovascularisation was lower in treatment groups compared to the control group. Groups 3 and 4 also had less corneal oedema compared to the control group on day 3 and 7. Group 4 exhibited the best wound healing, with less neovascularization and better collagen arrangement, as shown by the histopathological evaluation. To the authors' knowledge this is the first experimental study that intrastromal PRP is combined with oral doxycycline for the management of corneal chemical burns. This combination is a simple, safe and economical therapeutic approach that promotes corneal healing.

Keywords: Platelet rich plasma, PRP, doxycycline, corneal alkali burn, cornea

ΠΕΡΙΛΗΨΗ. Τα χημικά εγκαύματα του κερατοειδούς συγκαταλέγονται ανάμεσα στα πιο σοβαρά επείγοντα οφθαλμολογικά περιστατικά, ενώ συχνά έχουν καταστροφικά αποτελέσματα για την όραση και τον βολβό. Στα α-κοκκία των αιμοπεταλίων υπάρχουν μεγάλες ποσότητες αυξητικών παραγόντων, που ελέγχουν τη χημειοταξία των φλεγμονωδών κυττάρων, τη μίτωση, τη μετανάστευση και τη διαφοροποίηση των κυττάρων. Επιπλέον, οι τετρακυκλίνες και τα παράγωγά τους επηρεάζουν τη φλεγμονή, την ανοσορύθμιση, τον κυτταρικό πολλαπλασιασμό και την αγγειογένεση. Στην παρούσα μελέτη διερευνήθηκε πειραματικά η επίδραση του εμπλουτισμένου σε αιμοπετάλια πλάσματος (PRP) χορηγούμενου ενδοστρωματικά, σε συνδυασμό με συστηματικά χορηγούμενη δοξυκυκλίνη σε χημικά εγκαύματα κερατοειδούς σε κονίκλους. Χρησιμοποιήθηκαν συνολικά 36 κόνικλοι Νέας Ζηλανδίας. Σε όλα τα πειραματόζωα προκλήθηκε χημικό έγκαυμα με την εφαρμογή διηθητικού χαρτιού διαμέτρου 6 mm εμποτισμένο με NaOH στο κέντρο του κερατοειδούς. Η παρασκευή του PRP έγινε μετά από φυγοκέντρηση αυτόλογου αίματος. Τα πειραματόζωα χωρίστηκαν σε 4 ομάδες των 9 ζώων. Στην ομάδα 1, η οποία αποτελούσε την ομάδα ελέγχου, δεν δόθηκε καμία φαρμακευτική αγωγή. Στη ομάδα 2 χορηγήθηκε δοξυκυκλίνη στη δόση των 5 mg/kg, ημερησίως από το στόμα. Στη ομάδα 3 έγινε ενδοστρωματική έγχυση 0,5 ml PRP και στην ομάδα 4 έγινε ενδοστρωματική έγχυση PRP και παράλληλα χορηγήθηκε δοξυκυκλίνη (5 mg/kg). Οι ομάδες που έλαβαν θεραπευτική αγωγή παρουσίασαν βελτίωση σε μερικές από τις παραμέτρους που ελέγχθηκαν, ενώ η ομάδα που έλαβε συνδυασμό PRP και δοξυκυκλίνης είχε την καλύτερη εξέλιξη στην επούλωση του κερατοειδούς. Συγκεκριμένα, τα πειραματόζωα που άνηκαν στην ομάδα ελέγχου είχαν ελλείμματα στον κερατοειδή σε όλη τη διάρκεια του πειραματισμού και σε 3 από αυτά παρατηρήθηκε διάτρηση του κερατοειδούς. Οι ομάδες 3 και 4 παρουσίασαν ελλείμματα κερατοειδούς με μικρότερη έκταση σε σχέση με τις ομάδες 1 και 2. Την 7η μέρα μετά την πρόκληση του χημικού εγκαύματος, η νεοαγγείωση ήταν μικρότερης έκτασης στις ομάδες που έλαβαν θεραπευτική αγωγή συγκριτικά με την ομάδα ελέγχου. Στις ομάδες 3 και 4 παρατηρήθηκε λιγότερο οίδημα του κερατοειδούς σε σχέση με την ομάδα ελέγχου την 3η και 7η μέρα. Στην ομάδα 4, σύμφωνα με τα ευρήματα της ιστοπαθολογικής εξέτασης, παρατηρήθηκε καλύτερη επούλωση του κερατοειδούς, με μικρότερης

έκτασης νεοαγγείωση και καλύτερη διάταξη των ινών του κολλαγόνου. Η παρούσα εργασία αποτελεί την πρώτη πειραματική μελέτη της ενδοστρωματικής χορήγησης PRP σε συνδυασμό με συστηματικά χορηγούμενη δοξυκυκλίνη για την αντιμετώπιση ενός χημικού εγκαύματος κερατοειδούς. Ο συνδυασμός αυτός αποτελεί μία απλή, ασφαλή και αποτελεσματική θεραπευτική προσέγγιση, που προάγει την επούλωση του κερατοειδούς.

Keywords: Πλάσμα εμπλουτισμένο σε αιμοπετάλια, PRP, δοξυκυκλίνη, αλκαλικό έγκαυμα κερατοειδούς, κερατοειδής

INTRODUCTION

Ocular chemical burns represent potentially blinding ophthalmic injuries in humans (Dua et al., 2001), and could be among the most critical and true ocular emergencies in dogs (Christmas, 1991) and cats (Şenel and Ergİn, 2014). Alkali burns, that can be caused by ammonia, lye or potassium hydroxide (McCulley, 1987), are more common than acid ones since alkalis are components of commonly used cleaning products and civic construction materials (Wagoner, 1997; Williams et al., 2002; Busse et al., 2014), with soaps being the most common source in dogs and cats (Christmas, 1991).

Alkali agents can cause severe damage to the eye, as they have both hydrophilic and lipophilic properties, allowing them to quickly penetrate the cell membranes and enter the anterior chamber (Dua et al., 2001). The damage to the cornea is related to pH change, ulceration, proteolyses and collagen synthesis defects. Alkalis are deposited within the ocular surface and can cause saponification reaction. Besides, the damaged tissue secretes proteolytic enzymes as part of an inflammatory response, which leads to further damage (Singh et al., 2013). Moreover, the production of free radicals has been implicated in the pathogenesis of an alkali burn cornea damage and the cornea becomes more susceptible to the harmful effects of reactive oxygen radicals (Gunay et al., 2015).

After an ocular chemical burn, in dogs, the presenting sings include blepharospasm, conjunctival hyperaemia, chemosis and conjunctival ischemia in injured areas (Busse et al., 2014). Other symptoms include tear film deficiency, corneal neovascularization, ulceration and uveitis (Christmas, 1991). Limbal stem cell deficiency is also noticed, if the damage of the corneal and conjunctival epithelium involves the limbus. The loss of goblet cells and inflammation of conjunctiva can result in tear film inadequacy. In addition, if the alkaline material penetrates the anterior chamber, the result may be cataract formation, and damage of the ciliary body and the trabecular meshwork (Singh et al., 2013). In general, chemical injuries of the cornea are followed by four phases: immediate, acute, early reparative and late reparative (McCulley, 1987). Limbus loss, inflammation and neovascularization take place during the acute phase and the clarity of the cornea is compromised by the slow epithelialisation, the persistent ulceration, corneal perforation and neovascularization (Kuo, 2004). In this phase, anti-inflammatory and anti-angiogenic treatments, as well as treatments that promote corneal healing are proposed (Bakunowicz-łazarczyk and Urban, 2016).

Various medical means such as sodium hyaluronate 1% (Chung et al., 1996), autologous serum (Salman and Gundogdu, 2010; Gunay et al., 2015), mesenchymal stem cells (Almaliotis et al., 2015), amniotic membrane therapy (Fish and Davidson, 2010), and platelet rich plasma (PRP) (Márquez-de-Aracena et al., 2007; Khaksar et al., 2013) have been proposed for the promotion of the biological healing process after a chemical burn. Furthermore, several collagenase inhibitors have been studied for the management of chemical burns, such as acetylcysteine (Khaksar et al., 2013), EDTA, synthetic peptides, tetracyclines, sodium citrate, cysteine (Burns et al., 1989), aprotinin (Stuart et al., 1989), ascorbic acid (Levinson et al., 1976) and citric acid (Pfister et al., 1988). Also a variety of anti-angiogenic agents have been recommended for the prevention of corneal neovascularization including steroids (Crum et al., 1985), angiostatin (Ambati et al., 2002), methotrexate (Joussen et al., 1999) and ascorbic acid (Peyman et al., 2007).

Healing of the cornea after a trauma is a complex biological process, in which growth factors play a critical role (Schultz et al., 1992). Corneal wound healing is a unique process due to the fact that cornea

is an avascular tissue. Stimulation and regulation of healing rely on growth factors which can reach the cornea through the tears, aqueous humor and the limbic vessels (Swank and Hosgood, 1996). Platelet alpha granules are an important reservoir of proteins and growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), insulin growth factor (IGF), fibroblast growth factor 2 (FGF-2), and vascular endothelial growth factor (VEGF). Growth factors can stimulate chemotaxis of inflammatory cells, mitosis, migration and differentiation of cells, angiogenesis, and the production and regulation of other growth factors (Nurden, 2011). Platelets can release growth factors upon activation (Borzini and Mazzucco, 2005) and it has been demonstrated that a great amount of growth and wound healing factors can be stored as concentrated in PRP. It has been found that nonactivated PRP to whole blood ratio is 4.6 for EGF and 1.9 for IGF-I (Frechette et al., 2005). PRP has been used experimentally and in clinical trials for the treatment of several ocular conditions, such as corneal epithelial wounds (Tanidir et al., 2010), chemical burns (Khaksar et al., 2013; Márquez-de-Aracena et al., 2007), dry eye (Alio et al., 2007b), corneal ulcers caused by neurotrophic keratitis or trauma (Geremicca et al., 2010) and other ocular surface disorders (Alio et al., 2015).

Apart from the antibiotic properties of tetracyclines and their derivatives, it is known that they exhibit non-antimicrobial properties, such as affecting inflammation, immunomodulation, cell proliferation and angiogenesis (Federici, 2011). It is suggested that tetracyclines can bind essential Zn2+ in collagenase and thus inhibit collagenase activity by this mechanism (Burns et al., 1989). Previous studies suggest that tetracyclines can inhibit the gene expression of neutrophil collagenase (Suomalainen et al., 1992) and epithelial gelatinase (Nip et al., 1993), and also reduce degradation of 1-antitrypsin (Sorsa et al., 1993). Additionally, tetracyclines can inhibit collagenase activity by the scavenging of reactive oxygen species thus diminishing the amount of neutrophil procollagenase that is activated (Ramamurthy et al., 1993). It has been reported that among tetracyclines, doxycycline is the most potent corneal collagenase inhibitor (Burns et al., 1989). Other properties of tetracyclines are also their ability to inhibit the formation of arachidonic acid (Vadas et al., 1991) and the synthesis of prostaglandin E2 (El Attar et al., 1988).

Considering the destructive consequences of alkali chemical burns on the ocular surface and intraocular tissues (Singh et al., 2013), as well as the poor, already known treatment outcomes, there is a great interest for an effective, easy and safe treatment (Bakunowicz-łazarczyk and Urban 2016). The aim of this study is to experimentally investigate the effect of intrastromal injection of PRP in combination with oral doxycycline, after an alkali burn, and to propose an alternative treatment acting directly to the lesion site, accelerating more sufficiently the healing process.

MATERIALS AND METHODS

Thirty six New Zealand rabbits, weighing 2.7-3.0 kg, were used in this experimental study, obtained from a licensed rabbit farm (EL08RAB8Y). All rabbits were kept in a well-ventilated room with a standard 12-hour light dark cycle and stable temperature and humidity. Animals had free access to food and water and everything was performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research (ARVO, 2016). All procedures and experimental designs of the study were reviewed and approved by the Ethics Committee of the Aristotle University of Thessaloniki as well as by the local Committee of the Department of Veterinary Medicine of Thessaloniki (licence number: 74188/485, date issued: 30/3/2015). The study was conducted in the Companion Animal Clinic, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki (facility licence number: EL54BIO18).

A complete and thorough ophthalmic examination was performed in all rabbits to ensure that they were free of any ocular pathologic conditions. The rabbits were then randomly allocated into four groups of nine animals each.

Preparation of PRP

For the preparation of PRP the method described by Gimeno et al. (2006) was followed. A 10 ml syringe, preloaded with 1.3 ml of Anticoagulant Citrate Dex-

trose solution (ACD citrate-dextrose solution, Sigma-Aldrich, U.S.A.) to avoid coagulation, was used to draw 8.7 ml of blood under strict aseptic conditions from the jugular vein. One millilitre was set apart for cell counting. Two centrifugations were made at 4oC. After centrifuging the whole blood at 72 g for 15 minutes, the 6 ml plasma layer was aspirated and centrifuged for a second time at 1006 g for 5 minutes. After removing the upper part (platelet poor plasma, PPP), the lower part consisting of 0.5 ml was the platelet rich plasma. PRP was aspirated in a sterile 1 ml syringe and kept at 4^{o}C until use (Gimeno et al., 2006).

PRP that was prepared from animals that were allocated in the control group was used for platelet count that was performed using the ADVIA 120 haematology system (Siemens, Germany). A veterinary software package was run and "rabbit" was chosen as the default species in all cell-counting procedures. Enrichment percentage was calculated as described by Efeoglu et al. (2004).

Chemical burn

All animals were anaesthetized with dexmedetomidine (Dexdomitor, Zoetis Hellas) 0.075-0.1 mg/kg b.w., intramuscularly, and ketamine (Imalgene 1000, Merial, France) 15 mg/kg b.w., intramuscularly, and 1-2 drops of topical anaesthetic (proxymetacaine hydrochloride, Alcaine, Alcon Laboratories Hellas) were installed. The ocular surface and the conjunctival fornix were cleansed and disinfected with a mild antiseptic solution containing aqueous 0.5% povidone–iodine, and an eye lid retractor was placed. The alkali burn was induced only in one eye of each animal for ethical purposes, as described by Khaksar et al. (2013). A round filter paper, 6.0 mm in diameter, pre-soaked in sodium hydroxide (1 M NaOH) was placed on the central cornea for 60 seconds (Khaksar et al., 2013). After the disc removal, remnants of the corneal epithelium on the lesion were removed with a sterile swab and the ocular surface was rinsed with physiological saline for 2 minutes.

Treatment groups

The rabbits were allocated to four groups of 9 animals each. Group 1 was the control group and received no treatment. Group 2 received doxycycline (Novadox oral suspension, AST Farma, Netherlands) at a dose of 5 mg/kg b.w. orally once a day throughout the study. Group 3 received 0.5 ml of PRP, which was injected with the aid of a 22mm, 30g sterile ophthalmic microsurgical cannula at 3 sites in the anterior corneal stroma, through pockets around the lesion, created by a 15° knife. Group 4 received both intrastromal PRP and oral doxycycline, as described above (Fig. 1).

All animals received tobramycin (Tobrex eye drops solution, Alcon Laboratories Hellas) every 6 hours for the first postoperative day, and meloxicam (Metacam, Boehringer Ingelheim, Germany) was given for 5 days (0.2 mg/kg b.w., subcutaneously, SID). Throughout the study, a tear film substitute with dexpanthenol (Corneregel eye gel, PharmaSwiss Hellas) was used three times a day and an Elizabethan collar was placed to prevent self-trauma.

On the 3rd and 7th postoperative days 2 animals of each group were euthanatized while the remaining 5 animals of each group were euthanatized on the 14th day. For euthanasia, a mixture of dexmedetomidine (0.1 mg/kg b.w., intramuscularly) and ketamine (15 mg/kg b.w., intramuscularly) followed by a high dose of iv propofol and potassium chloride was used. Enucleation was performed and the eye globes were fixed for histopathological analysis.

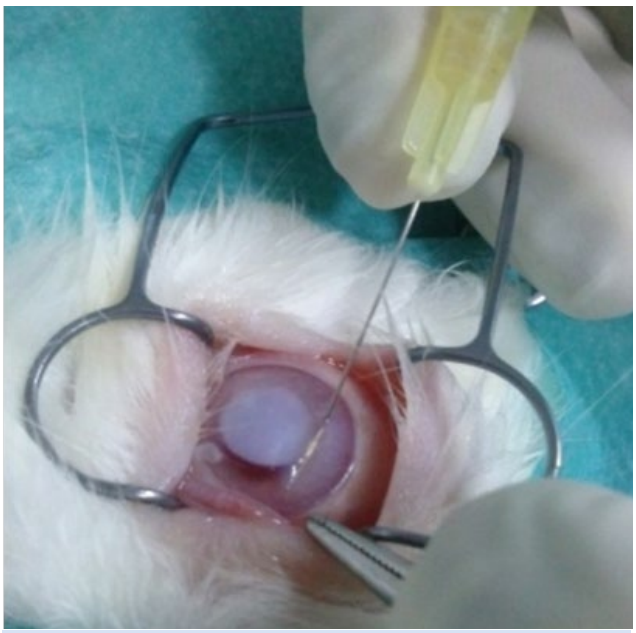

Figure 1. Intrastromal injection of PRP in groups 3 and 4

Clinical evaluation

The outcome was monitored daily by detailed clinical evaluation of the eyes, with the use of a portable slit lamp biomicroscope and photographs were taken. Corneal opacity and neovascularisation were recorded, and fluorescein dye test was performed to detect epithelial defects. Photographs were analyzed by ImageJ 1.31v (an image-processing software), examining the area of oedema, neovascularization and epithelial defect, and the lesion areas were determined in terms of mm or mm2 on digitized photographs. Conjunctival congestion, ocular discharge and the presence of uveitis were scored using a scale from 0 to 3. Grade 0 represented no abnormalities (normal conjunctiva, absence of ocular discharge, and normal iris and anterior chamber), grade 1 represented mild symptoms (mild conjunctival congestion, serous discharge, mild iris hyperaemia and inflammation), grade 2 represented moderate symptoms (moderate conjunctival congestion, mucoid ocular discharge, miosis, iris hyperaemia and inflammation) and grade 3 represented severe symptoms (severe conjunctival congestion, copious purulent discharge, and miosis, aqueous flare and iris inflammation respectively). Corneal sensitivity was measured at the centre and the periphery of the cornea using a handheld Cochet-Bonnet esthesiometer (quantitative method). To determine the eye dryness, Schirmer tear test 1 (STT) was performed. Intraocular pressure was not measured due to the deleterious effects of local anaesthetics on the healing process (Bisla and Tanelian, 1992).

Histopathological examination

The enucleated eye globes were fixed in formaldehyde and prepared for paraffin embedding. Paraffin sections (5 mm thick) were obtained with a microkeratome and the samples were subjected to routine hematoxylin and eosin staining. Histopathological analysis was carried out by a single pathologist blinded to the experimental groupings.

Statistical evaluation

Data were analyzed using SPSS version 22.0 software. Statistical analysis between the groups for corneal oedema, corneal vascularisation, corneal sensitivity and STT was performed by the nonparametric Kruskal-Wallis test. Pair-wise comparison between each group and control was performed using a Mann-Whitney U test. Chi-squared test was performed for corneal reepithelialisation, conjunctival congestion, ocular discharge and the presence of uveitis. P values of <0.05 were considered to be significant.

RESULTS

Platelet count

The mean value of platelet counts in venous blood was 642.330/μL, in PRP it was 4950.400/μL and in PPP it was 196.850/μL. The mean enrichment percentage was 670%.

Clinical evaluation

Corneas in all animals became cloudy immediately after the chemical burn, turned opaque with a ground-glass appearance and were distinct from the rest of the normal tissue of the cornea. Comparison between groups (Fig. 2) showed that groups 3 and 4 had statistically less corneal oedema compared to the control group on day 3 (P: 0.001 for group 3 and P: 0.003 for group 4) and 7 (P: 0.004 for group 3 and P: 0.018 for group 4). Corneal opacity revealed no significant differences on the 14th postoperative day between groups ($P>0.05$).

The new vessels were fine and superficial, and emerged from the limbus on the 3rd and 4th day after the chemical burn in most animals from all groups. Statistical analysis for corneal vascularisation (Fig. 3) between groups on days 3 and 7 showed significant difference ($P<0.05$), but there were not any statistical differences for day 14 ($P>0.05$). More specifically, on the 3rd day groups 1 (P: 0.012) and 3 (P: 0.012) had less vascularization compared to group 4, and on the 7th day vascularisation was lower in groups 2 ($P<0.001$), 3 ($P<0.001$) and 4 (P: 0.001) when compared to the control group; but comparison between treatment groups was not statistically significant.

Comparison between groups (Fig. 4) showed that groups 3 and 4 had smaller mean defect area and greater wound healing. When considering as "corneal healing" the healing of 80% of the initial wound

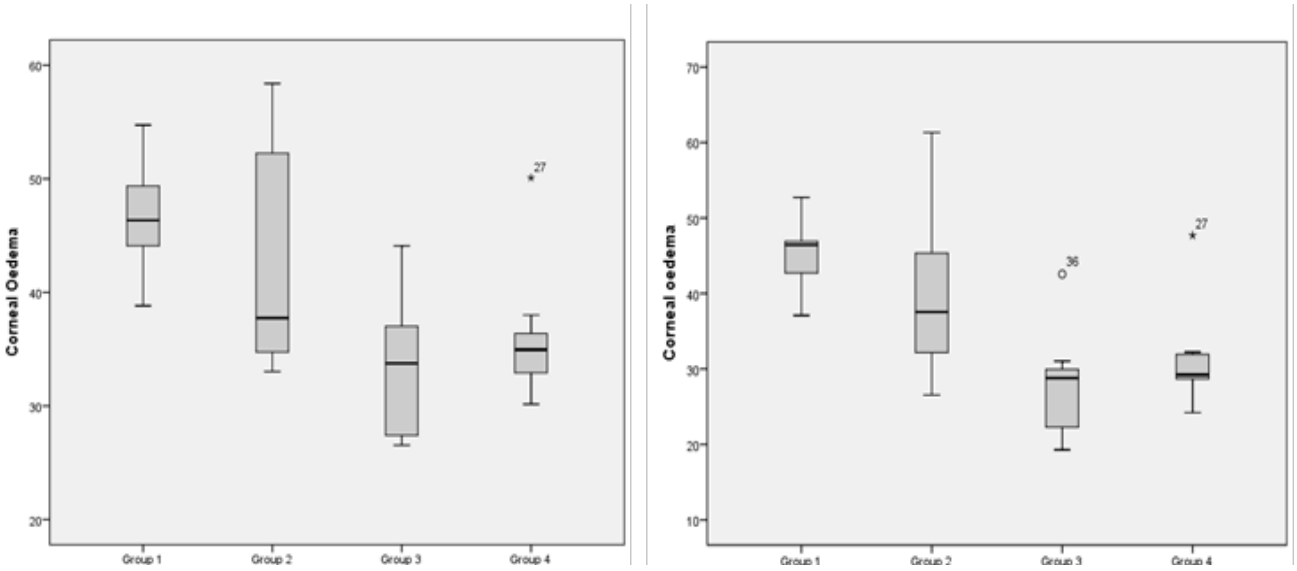

Figure 2. Corneal opacity measured in mm2 on the 3rd (left) and 7th (right) day after the chemical burn in all groups.

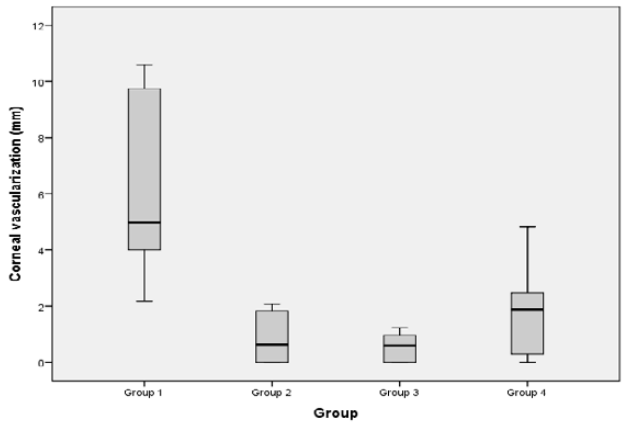

Figure 3. Corneal neovascularization (mm) on the 7th day after the chemical burn in groups 1, 2, 3 and 4.

surface (Table 1), there were statistically significant differences between all groups on days 3, 7 and 14 (P <0.05). On the 3rd day 55% of the animals in group 3 and 77% of the animals in group 4 had a corneal defect whose surface was less than 20% of the initial defect area on the 1st day. On the 7th and 14th all animals in group 3 and 4 had less than 20% of the initial defect area, compared to 20% of the animals in group 2. Animals in the control group had epithelial defects greater than the 20% of the initial surface for the whole duration of the study. Furthermore, corneal perforation was noticed only in 3 animals that belonged to the control group on the 10th (N=2) and on the 14th (N=1) day after the chemical burn.

Conjunctival congestion was significantly different (P <0.001) on days 7 and 14, showing more intense congestion in animals in the control group compared to the treatment groups.

On the 3rd day, ocular discharge was statistically significantly (P <0.001) less in group 3 compared to the other groups. On the 7th (P: 0.01) and 14th day (P: 0.012) control group had statistically significantly more discharge compared to the other groups.

For the presence of uveitis there were statistically

Table 1: Percentage (%) of corneal healing of the 80% of the initial wound surface on days 3, 7 and 14 after the chemical burn

Day	Wound healing	Group 1	Group 2	Group 3	Group 4
3rd	>80%		11.1	77.8	55.6
P: 0.002	<80%	100	88.9	22.2	44.4
7th	>80%			100	100
P: 0.000	<80%	100	100		
14th	>80%		20.0	100	100
P: 0.000	<80%	100	80.0		

Table 2: Percentage (%) of severity of uveitis on days 3, 7 and 14 after the chemical burn

Day	grade*	Group 1	Group 2	Group 3	Group 4
3rd	0	33.3	100	88.9	100
P: 0.001	1	22.2		11.1	
	2	44.4			
	3				
7th	0		100	85.7	100
P: 0.000	1				
	2	14.3		14.3	
	3	85.7			
14th	0		80.0	100	100
P: 0.001	1		20.0		
	2	20.0			
	3	80.0			

*grade 0: normal iris and anterior chamber, grade 1: mild iris hyperaemia and inflammation, grade 2: miosis, iris hyperaemia and inflammation, grade 3: miosis, aqueous flare, severe iris hyperaemia and inflammation.

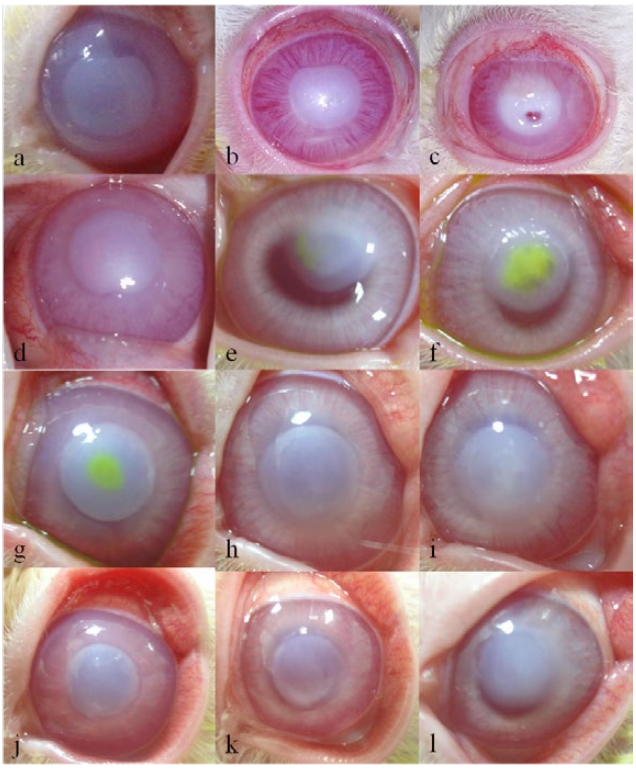

Figure 4.. Postoperative photos in group 1 (a-c), group 2 (d-f), group 3 (g-i) and group 4 (j-l) at days 3, 7 and 14. In group 1 intense conjunctival congestion is seen, along with iris inflammation, corneal opacity, neovascularization and perforation. In group 2 conjunctival congestion and corneal opacity is shown. Group 3 had complete corneal healing and limited corneal opacity, as group 4, where conjunctival congestion was not present.

significant differences between groups (P <0.05) for days 3, 7 and 14 (Table 2). More specifically, on the 3rd day 66.7% of the animals in the control group showed signs of uveitis, compared to 11.1% of animals in group 3 and none of the animals in groups 2 and 4. On the 7th day all animals in the control group had signs of uveitis, compared to 14.3% of animals in group 3 and none of the animals in groups 2 and 4. Lastly, on the 14th day all animals in the control group and 20% of animals in group 2 had uveitis, whereas none of the animals in groups 3 and 4 showed signs of uveitis.

Corneal sensitivity in the periphery of the cornea (Fig. 5) was significantly higher in groups 2 (P: 0.03) and 4 (P: 0.045) compared to the control group on the 3rd day. On the 7th and 14th day no statistically significant differences were noticed. Also, corneal sensitivity measurements in the centre of the cornea showed no statistically significant difference (P>0.05) between groups.

The STT showed no statistically significant differences in tear production between groups for the whole duration of the study.

Histopathological evaluation

In the control group, histopathological evaluation on the 3rd day revealed superficial and deep neovascularization of the cornea, moderate corneal oedema,

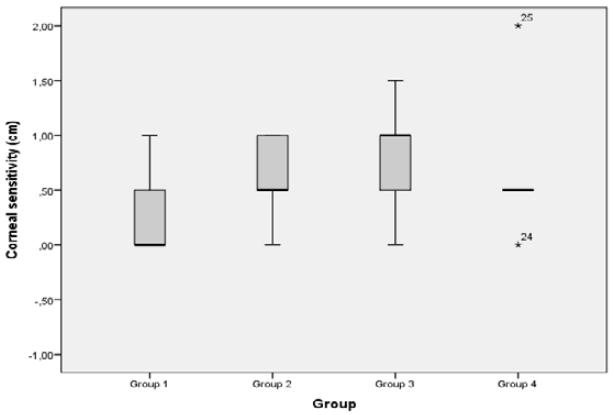

Figure 5. Corneal sensitivity (cm) in the periphery of the cornea, on the 3rd day after the chemical burn.

and infiltration of inflammation cells (mostly lymphocytes and some neutrophils). On the 7th and 14th day, intense neovascularization, corneal oedema and epithelial hyperplasia were noticed. In group 2, corneal oedema and infiltration were lower compared to the control group, but neovascularization was present on the 3rd, 7th and 14th day. In group 3 neovascularization and corneal oedema were lower compared to groups 1 and 2. On the 7th and 14th day mild corneal oedema was recorded. In group 4 superficial vascularization of the cornea was noticed on the 3rd day, but on days 7 and 14 it was limited. Corneal epithelium appeared normal, without any hyperplasia and with less corneal oedema; furthermore collagen arrangement was better when compared to the other groups (Fig. 6).

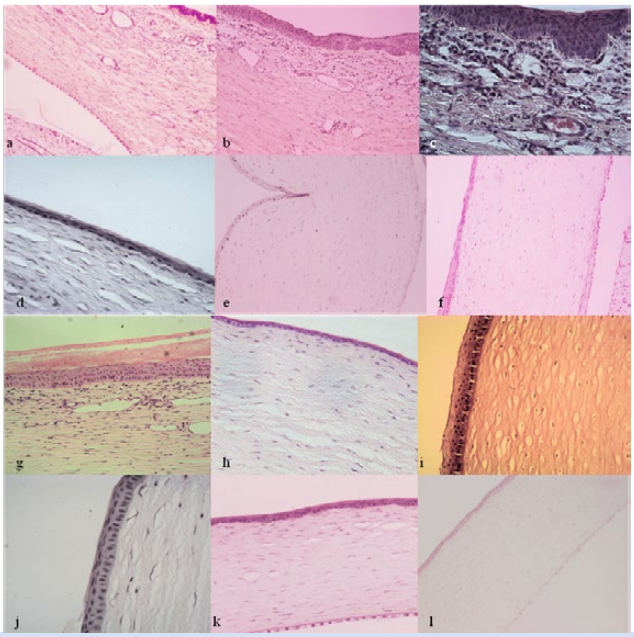

Figure 6. Histopathological examination in group 1 (a-c), group 2 (d-f), group 3 (g-i) and group 4 (j-l) at days 3, 7 and 14. In group 1 intense superficial and deep vascularization are noticed, along with epithelial hyperplasia, stromal oedema and the presence of inflammatory cells. In group 2 there is less corneal oedema, but neovascularization is present. In group 3, vascularization is seen on the 3rd day, and on the 7th and 14th mild corneal oedema is noticed. In group 4, mild corneal oedema, less vascularization and better collagen arrangement is recorded, without any epithelial hyperplasia.

DISCUSSION

In the present study the therapeutic efficacy of intrastromal PRP injection, oral doxycycline treatment or combination of them were evaluated on corneal alkali burns in New Zealand rabbits.

In the present study, for the preparation of PRP the method described by Gimeno et al. (2006) was followed. The same method has been used in other experimental protocols in rabbits regarding ocular surface conditions (Gimeno et al., 2010; Tanidir et al., 2010; Khaksar et al., 2013). Intracardiac blood sampling was not performed, and blood was drawn from the jugular vein as it can be well tolerated by rabbits, no anaesthesia was required and the resulting blood collection was without clumps. The mean enrichment percentage was greater compared to previous studies (Efeoglu et al., 2004; Gimeno et al., 2006) resulting in greater platelet number in PRP, possibly due to the higher platelet concentration in the whole blood.

In previous studies PRP has been administered by subconjunctival application (Márquez-de-Aracena et al., 2007; Tanidir et al., 2010; Khaksar et al., 2013) or as topical eye drops (Alio et al., 2007a,b). In this experimental study PRP was delivered in the form of intrastromal injections, a route previously used in corneal chemical burns in rabbits for the administration of stem cells (Almaliotis et al., 2015). In our study intrastromal injections were preferred as they can increase the concentration of growth factors in the deep layers of the cornea in a minimally invasive way.

In corneal chemical burns one of the major treatment goals is control of the balance between collagen synthesis and collagenolysis (Singh et al., 2013). In

this study doxycycline was used as a MMP inhibitor. Among tetracyclines, doxycycline has been shown to be the most potent corneal collagenase inhibitor (Burns et al., 1989). Perry et al. (1993) examined the effects of orally administered doxycycline in rabbits after a chemical burn and their findings suggest that the dose of 5 mg/kg once a day of doxycycline can promote corneal reepithelization compared to 1.5 mg/kg. The same dosage was used in this study for groups 2 and 4.

It is noteworthy that animals that received PRP (groups 3 and 4) had less corneal oedema compared to the control group on the 3rd and 7th day after the chemical burn. In another study the effect of subconjunctival PRP in combination with topical acetylcysteine on corneal alkali burns in rabbits was investigated. The findings of that study revealed prominent corneal oedema in all groups for the first week (Khaksar et al., 2013). The reduction of corneal oedema in our study may be related to the more direct application of PRP, as an intrastromal route was used for the delivery of growth factors into the cornea. Furthermore, in the study of Khaksar et al. (2013) the group that received only PRP had smaller mean defect area and greater wound healing compared to the group that received PRP and acetylcysteine. The retarded healing effect in that group was due to acetylcysteine's poor ability to penetrate the corneal stroma and its relative toxicity (Wagoner, 1997). In our study, the collagenase inhibitor agent (doxycycline) was administered systemically to avoid any topical toxicity effects.

In a study carried out by Dan et al. (2008), the efficacy of oral doxycycline compared to oral and topical dexamethasone for inhibiting corneal neovascularization after an alkali burn in rats was investigated. In the group that was treated with oral doxycycline, corneal neovascularisation was less compared to the untreated control group, and the epithelial healing was significantly more rapid. It was concluded that oral doxycycline can inhibit neovascularization without the harmful side-effects associated with oral or topical dexamethasone use (Dan et al., 2008). These results are in accordance with our results concerning the significantly less neovascularization of the groups which received doxycycline compared to the control group. One possible explanation for the inhibition of neovascularization is the inhibition of MMPs, but further investigation is needed (Dan et al., 2008).

Regarding the healing process, animals that received PRP (groups 3 and 4) had smaller defect area compared to control group and group 2. These findings are in accordance with a study by Tanidir et al. (2010), where the effect of PRP was examined in corneal epithelial wound healing in rabbits. After the creation of a 7-mm diameter central epithelial defect, a single dose of subconjunctival PRP was injected, with or without concurrent antibiotic treatment. Animals that received PRP had a better healing process compared to the control group (Tanidir et al., 2010). In our study topical antibiotics were used only for the 1st day after the chemical burn, to avoid any retarded healing effect. In the study of Tanidir at al. (2010), it was pointed out that the group that received PRP in combination with topical antibiotic had a delay in the epithelial healing, and it should be due to corneal toxicity caused by the antibiotic or the preservatives included in the antibiotic solutions, or by possible distraction on the balanced process during cornea healing (Tanidir et al., 2010).

In group 2 (where only doxycycline was administered), 20% of the animals had less than 20% of the group's initial defect area on the 14th day, and no corneal perforations were reported compared to the control group. This effect of doxycycline is probably related to the drug's ability to inhibit collagenase activity. Tetracyclines have been studied in corneal chemical burns in rabbits (Seedor et al., 1987; Perry et al., 1993) and rats (Dan et al., 2008) for their effect on corneal healing and angiogenesis. It is reported that corneal tetracycline levels are directly correlated to the dose administered systemically, and eyes with higher levels of tetracycline in ocular tissues are less likely to ulcerate (Seedor et al., 1987).

Animals that received only doxycycline (group 2) had less conjunctival congestion and less ocular discharge compared to the control group. Also, groups 2 and 4 (doxycycline and doxycycline+PRP groups) had fewer sings of uveitis when compared to the control group and the group that received PRP only. These findings may be the result of the anti-inflammatory and immunemodulatory properties of doxycycline (Federici, 2011).

In the present study corneal sensitivity was greater in groups that received oral doxycycline. Further-

more, animals that received combination of doxycycline and PRP (group 4) had increased corneal sensitivity on the 3rd day compared to the group that received only PRP (group 3). This effect can be due to the anti-inflammatory properties of doxycycline (Sapadin and Fleischmajer, 2006). Also doxycycline can have neuroprotective effect to the sensory (trigeminal) nerve fibers, like the neuroprotective features of tetracycline (Uckun et al., 2015) and minocycline (Sanchez Mejia et al., 2001) to the brain tissue, which can be attributed to the increased corneal sensitivity; however further studies are needed.

Among the late complications that occur usually three weeks after a chemical ocular injury are xerophthalmia and dry eyes (Singh et al., 2013). In the present study no statistically significant differences were noted among groups regarding tear production, maybe due to the relatively short duration of the study (14 days). On the other hand, normal STT values for rabbits may be useful only for the evaluation of increased values correlated with ocular irritation, rather than for the determination of decreased values associated with keratoconjunctivitis sicca (Abrams et al., 1990).

A study was carried out by Campos et al. (2003), with experimentally induced corneal chemical ulcers in dogs. For the treatment, topical applied autogenous serum and 10% acetylcysteine were used. It was noticed that when compared to the control group, no differences were found in the healing process of the cornea, whereas in the present study the animals that received PRP had smaller defect area and better wound healing. These results may be due to the fact that blood serum contains lower concentrations of growth factors compared to PRP (Frechette et al., 2005). Therefore, the efficacy of PRP and doxycycline may have beneficial results in other species than rabbits, as dogs and cats experiencing ulcerative keratitis with MMP involvement.

CONCLUSIONS

This is the first experimental study that uses PRP with intrastromal injection to the cornea. We believe that despite the local destruction of the cornea's architecture, the close proximity of growth factors to the lesion site can have beneficial results to the outcome of a chemical burn, or other severe corneal lesions with collagenase activity. Despite the fact that PRP alone had better results in corneal healing on the 3rd day after the chemical burn, on the 7th and 14th day the combination of PRP and doxycycline resulted in better corneal healing, with better collagen arrangement and less corneal oedema in histopathological examination. Further studies are needed to examine the effect of intrastromal PRP in other corneal conditions. Doxycycline on the other hand, is a potent collagenase inhibitor, with minimal side effects and thus a safe additional treatment option for chemical burns. According to our findings, doxycycline can reduce the clinical symptoms caused by chemical burns, resulting in less intense uveitis, ocular discharge and conjunctival congestion. Therefore, the above combination is a simple, safe and more economical therapeutic approach to promote quick corneal healing, so anti-inflammatory agents can be used when the epithelium becomes intact without any undesirable side effects.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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Research article
Ερευνητικό άρθρο

Effects of GnRH or hCG on day 11 after artificial insemination in cows luteal activity

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ABSTRACT. In order to optimize luteal function, human chorionic gonadotrophin (hCG) or gonadotrophin releasing hormone (GnRH) were used on day 11 after artificial insemination (AI). 33 cows synchronized by the Ovsynch and divided into 3 groups according to the type of treatment: 1) hCG (1500 IU, n=11); 2) GnRH (100 µg, n=11) and 3) control (2 mL of saline, n=11). Blood samples were collected from all animals every 3 days from day 5 to day 23 to determine progesterone concentration. Ultrasonography was used to monitor the luteal surface structures at the time of blood sample collection. An accessory corpus luteum (CL) formed in 63.63% of cows treated with GnRH or hCG, resulting in an increase in the total luteal tissue area compared with the controls. Compared with the controls, the principal CL area was increased by hCG but not by GnRH. Additionally, compared with the control group, hCG-treated cows had increased progesterone concentrations ($p<0.0001$), while GnRH-treated cows had P4 similar to that of controls cows.

Keywords: hCG, GnRH, Progesterone, Accessory corpus luteum, Cow.

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INTRODUCTION

Early embryonic loss is the principal cause of low pregnancy rate (Inskeep and Dailey 2005). Indeed, embryonic and foetal mortalities are estimated to be 50% in dairy cows and between 70 and 80% of these losses occur in the first 16 days after AI (Diskin et al., 2011). One of the causes of embryonic loss is related to delayed embryo development, which reduces signalling for the maternal recognition of pregnancy (Mann and Lamming 2001). This condition may be due to a lower progesterone concentration in dairy cows (Mann and Lamming 2001; Wiltbank et al., 2011). Progesterone plays an important role in regulating changes in the uterine environment conducive to the development of the embryo and maintenance of pregnancy (Geisert et al., 1992). Increased milk production in dairy cows is connected with increased general metabolism as well as with increased progesterone metabolism in the liver, which decreases progesterone concentration in peripheral blood (Rhinehart et al., 2009). Progesterone insufficiency can be an important factor responsible for the decrease of the conception rate and is associated with abnormal early embryo development (Mann and Lamming 2001), while high progesterone concentration favours embryo development and interferon-τ secretion (Lonergan 2011). Several approaches have been used to increase the concentration of progesterone in the blood in order to reduce the occurrence of embryo death (Campanile et al., 2007). In different studies, progesterone has been administered via implants or intra-vaginal devices with inconsistent results (Wiltbank et al., 2014). Other studies have shown that administration of natural sequence GnRH, GnRH agonists or hCG after AI can stimulate CL function which induces accessory CL formation, increases progesterone concentration, and reduces oestradiol production with a consequent positive effect on embryonic survival (Thatcher et al., 2003). Administration of GnRH, a GnRH agonist or hCG after AI at specific times, coincident with the presence of the dominant follicle of the first and second follicular waves, may stimulate CL function, induce accessory CL formation, increase progesterone concentration (Schmitt et al., 1996a, Stevenson et al., 2007) and reduce oestrogen production with a consequent positive effect on pregnancy rate or embryo survival (Thatcher et al., 2003; Šuluburić et al., 2017). Several studies have investigated the effects of GnRH administered during mid-estrous cycle (day 12, 13 or 14 after AI) (Szenci et al., 2006; Lopez-Gatius et al., 2006). Beneficial effects of treatment with GnRH at day 11-12 after AI on fertility have been observed in lactating dairy cows during heat stress (Lopez-Gatius et al., 2006), but very few studies that have attempted to compare GnRH and hCG injected in the mid-cycle to improve luteal function after AI. We hypothesized that cows receiving GnRH in day 11 of estrous would have concentrations of progesterone closer to cows receiving hCG. The objective of this study was to compare the effects of GnRH and hCG administration at day 11 after AI on CL development and function, in terms of progesterone secretion in cows.

MATERIALS AND METHODS

Ethical approval

Experimental procedures were approved by the Institutional Animal Care Committee of the National Administration of the Algerian Higher Education and Scientific Research (Ethical approval number: 98-11, Law of August 22, 1998).

Animals and treatments

The experiment was undertaken at a commercial dairy farm in Mitija (longitude 36° and latitude 3°), Algeria from January to April. Animals were housed in free-stall barns and fed a diet consisting of grass and clover supplemented with a commercial concentrate (18% digestible raw protein), as well as roughly crushed maize grains, soybean meal, barley and vitamin–mineral mixture. Cows were selected by clinical examination before AI and only those in a healthy reproductive status were included in the study. A total of 33 primiparous Holstein lactating cows (ranging in age from 2-3 years old) were selected. Cows were synchronized by the Ovsynch protocol initiated with Gonadorelin (Cystoreline® CEVA, France) followed by an injection of prostaglandin PGF2α analogue at day 7 (Enzaprost® CEVA, France), and two days later, the cows received a second Gonadorelin injection. All animals were inseminated 12–16 h after the second injection of Gonadorelin (day 0). Eleven days after AI (day 11), cows were randomly assigned to one of the three treatment groups, administered a single injection of 1500 IU hCG (hCG group, n=11, Chorulon® Intevet Laboratories

Ltd Holland given i.v.), 100 μg GnRH (GnRH group, n=11, Cystoréline® CEVA France given i.m.) or 2 mL of 0.9% saline (n=11, control group).

Blood collection procedures and progesterone quantification

Blood samples were collected via venipuncture of the median caudal vein or artery into 5 mL evacuated tubes (VACUETTE® Blood Collection Tubes) every 3 days from day 5 to day 23 after timed AI (Fig 1) for later analysis of serum progesterone; the first sample collected immediately before administration of treatments. Blood samples were allowed to clot for 24 h at 4°C, then were centrifuged (1,935×g for 15 min), and serum was harvested and stored at −20°C until assayed for progesterone using a solid-phase, no-extraction radioimmunoassay.

Ultrasonography

Ultrasound examination of the ovaries was performed, using a Chison 600VET Ultrasound (Japan) equipped with a linear array 5 MHz transrectal transducer, to determine ovulatory response to Ovsynch and post-insemination treatments. Ultrasound examinations were performed at day -10 and at day 0 and day 1 after AI to confirm ovulation. Cows were considered to have synchronized ovulation after the second GnRH injection of Ovsynch when 1 or more follicles ≥10 mm were present at AI and were absent at an ultrasound examination conducted 2 days later. Ultrasound examinations were continued every 3 days from day 5 to day 23 after AI (Fig 1). Pregnancy status was determined at day 45 after AI by transrectal ultrasonography.

Statistical analysis

Because progesterone and CL area were measured over time, a repeated measures approach using ANOVA with mixed linear models (fixed effects of treatment, day and their interaction, random effect of cow) was used in SAS (Version 9.1.3; SAS Institute Inc., Cary, NC). For clarity, corresponding means ± SEM of the non-transformed data are presented in the results. Additionally, a mixed general linear model was fitted using the MIXED procedure of SAS (random effect of cow) to evaluate the effect of treatment on pregnancy rate. A Pearson correlation coefficient between progesterone concentrations and total area of luteal tissue and between dominant follicle diameter on day 11 and accessory CL area at day 17 after AI ware calculated by the PROC CORR procedure in SAS.

RESULTS

Pregnancy rate

The pregnancy rate was 45.45% (5/11) in either control or GnRH-treated cows. However, this rate was 54.54% (6/11) in hCG-treated cows. There was no significant difference ($p > 0.05$) between groups.

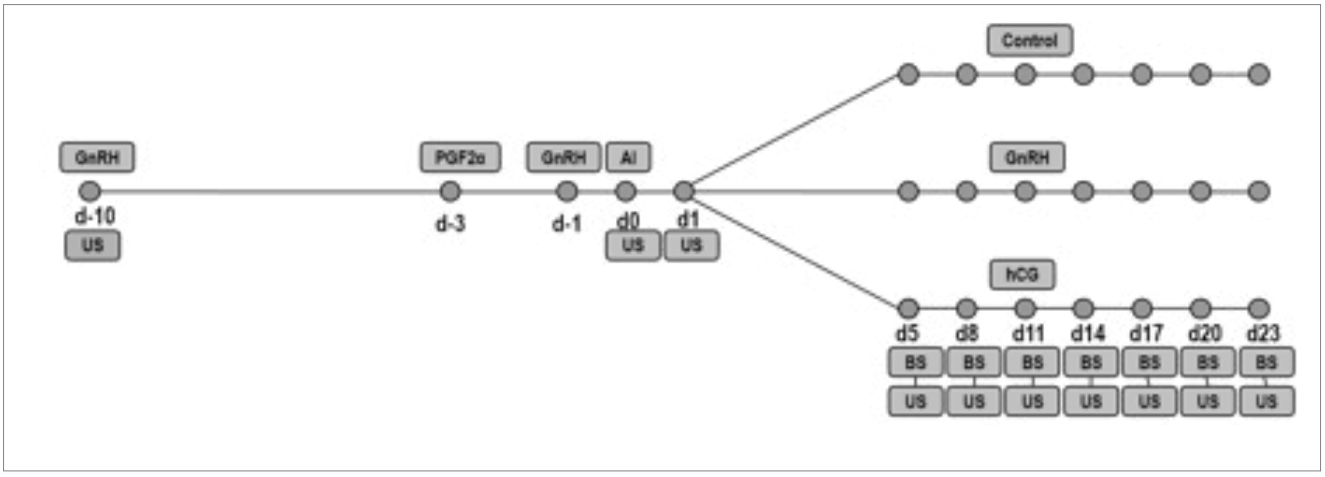

Fig 1: Schematic diagram of activities and treatment schedules of control group (Saline), day 11 GnRH group (gonadoréline 100 μg) day 11 hCG group (hCG, 1500 IU) in cows (AI: artificial insemination; BS: blood sample d: day; PG: prostaglandin; US: ultrasonography).

Ovarian status of cows at day 11 after AI

The ultrasound examination at day 11 after AI (the day of GnRH or hCG injection to induce ovulation or luteinisation of the dominant follicle) revealed the presence of one CL (no double ovulations were detected after AI) and one dominant follicle. The mean diameter of the dominant follicle on day 11 after AI was 11.74 ± 1.34, 12.06 ± 1.31 and 11.86 ± 1.31 mm in the hCG group, GnRH group and control group, respectively. In cows treated with GnRH or hCG, the mean (± S.E.M.) diameter of the dominant follicle for cows that subsequently formed an accessory CL (14/22) was 14.52± 0.61 mm (maximum diameter: 18.6 mm, minimum diameter: 10 mm). The mean follicular diameter for cows that have not formed an accessory CL (8/22) was 7.28 ± 0.4 mm (maximum diameter: 9 mm, minimum diameter: 5 mm). There was a significant difference in the diameter of dominant follicle between cows that have formed an accessory CL and cows that have not formed an accessory CL (p=0.004).

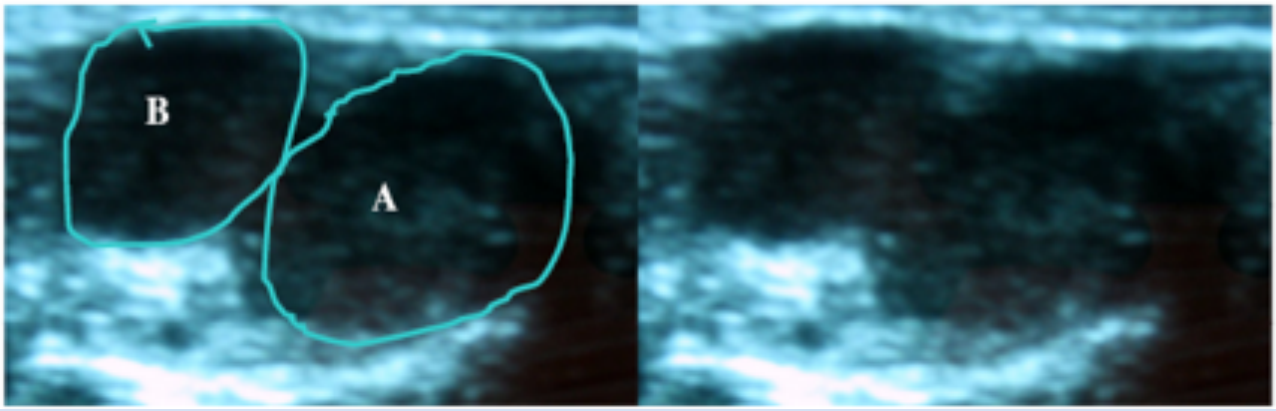

Fig 2:Sonogram image of an ovary at day 24 post AI showing two CL after GnRH treatment: (A) principal CL, (B) GnRH-induced accessory CL

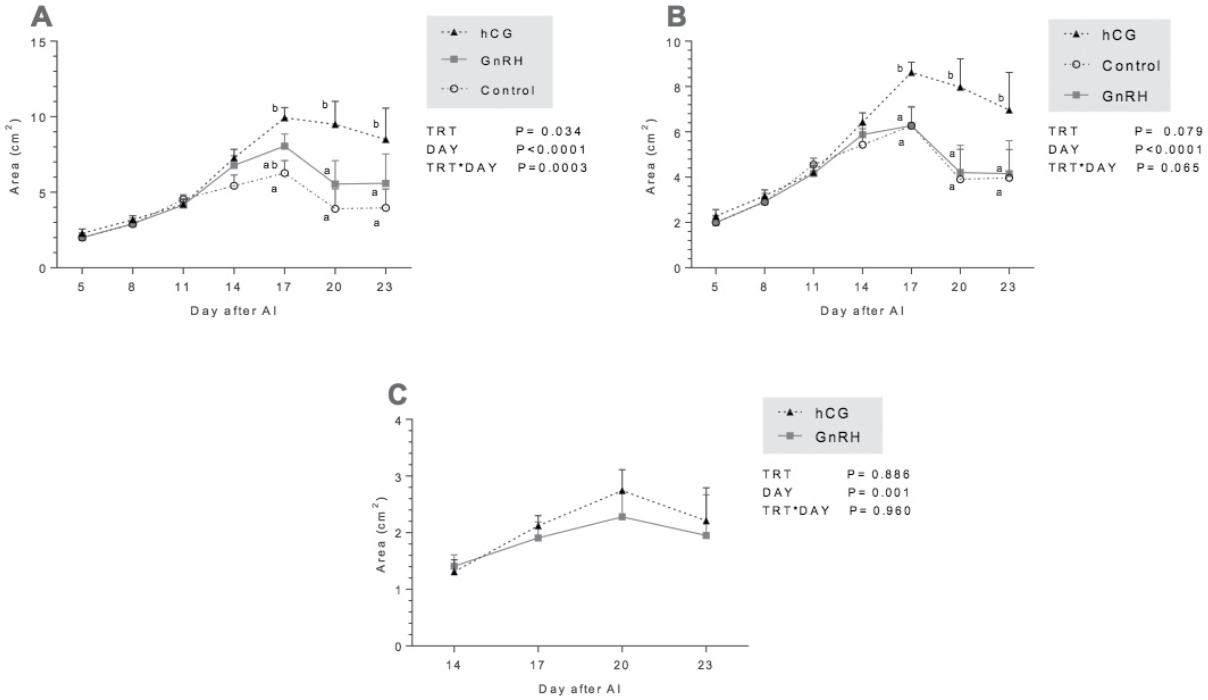

Fig 3: Mean (± SEM) of area of luteal tissue (cm2) in control cows that did not receive treatment, in cows that received 1500 IU of human chorionic gonadotrophin (hCG) and in cows treated with GnRH. (A) Mean (± SEM) of total area of luteal tissue (accessory CL+ principal CL. (B) Mean (± SEM) of principal CL area. (C) Mean (± SEM) of accessory CL area. (TRT: type of treatment, DAY: day of measurement, TRT*DAY: interaction treatment by day).

Corpora lutea

Treatment at day 11 induced ovulation of the dominant follicle and formation of an accessory CL in 63.63% (7/11) of cows treated with GnRH and in 63.63% (7/11) of cows treated with hCG. However, none of the control cows formed an accessory CL (Fig 2). Thus, there was an acute disappearance of the dominant follicle and subsequent appearance of an accessory CL at the same location on the ovary in GnRH and hCG treated cows. Total CL areas was significantly higher at day 20 and 23 in hCG-treated cows compared to GnRH and control groups (Fig 3.A). Furthermore, principal CL areas was higher at day 17, 20 and 23 in hCG group compared to GnRH and control group (Fig 3B). There were significant effects of measurement time on total and principal CL areas ($p<0.0001$) (Fig 3. A and B), and there was an effect of group-by time interaction ($P=0.0003$) for total CL area (Fig. 3A). However, the accessory CL area induced by GnRH and hCG showed no significant change through the time and no group-by time interaction was observed (Fig 3. C). A significant correlation ($r=0.83$, $p< 0.001$) was found between dominant follicle diameter on day 11 and accessory CL area at day 17 after AI.

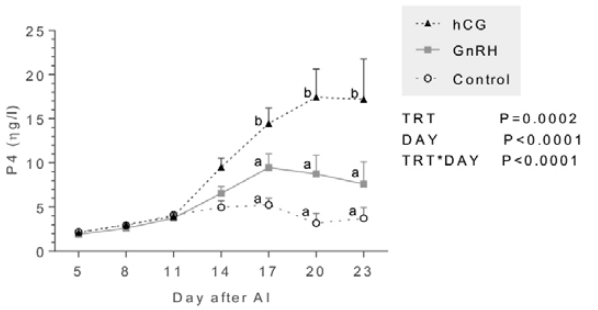

Fig 4:Mean (± SEM) of progesterone concentration in cows (ng/mL) .

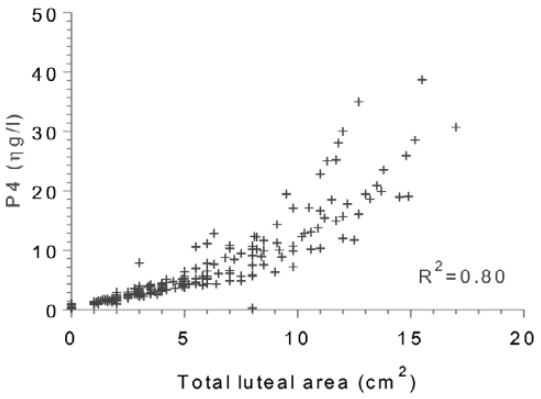

Fig 5. Correlation between progesterone concentrations and total area of luteal tissue. Both principal and accessory CLs were taken into account

Plasma concentration of progesterone

Cows treated with hCG presented significantly increased circulating progesterone concentrations from day 17 to day 23 after AI relative to the control and GnRH groups (Fig 4). There was an effect of group-by time interaction ($p<0.0001$). A significant correlation ($r=0.89$, $p< 0.001$) was found between progesterone level and total CL area (principal CL+ accessory CL) for each day (Fig 5).

DISCUSSION

Previous studies showed that administration of GnRH, a GnRH agonist or hCG after AI at specific times coincident with the presence of the dominant follicle of the first and second follicular waves may stimulate CL function, induce accessory CL formation, increase progesterone concentration and reduce oestrogen production with a consequent positive effect on embryo survival (Stevenson et al., 2007).

In the present study, the diameter of the dominant follicle was higher in cows that formed an accessory CL compared to those that did not ($p=0.004$). Similarly to our results, Musilová et al (2014) observed that the incidence of two CL was higher in cows bearing the largest follicles from 10 to 20 mm in diameter compared to cows bearing only follicles ≤ 9 mm. Whether cows respond to GnRH may depend upon the characteristics of follicular growth; a follicle must reach 10 mm in diameter to ovulate in response to LH (Sartori et al., 2001). In the current study, 63.63% (n=14/22) cows treated with hCG or GnRH at day 11 after AI had formed an accessory CL; these results were higher than that (33.5%) reported by Musilová et al (2014) following a al t on the fact that others have used different dose of hCG? GnRH injection on day 11-13. Stevenson et al (2007) found an almost similar (60% for GnRH-treated cow) or grater (77.5% for hCG-treated cows) accessory CL formation after treatment of the cows between day 4 and day 9 after AI. This finding may be explained by the timing of treatment.

The type of hormone (hCG or GnRH) injected at day 11 after AI did not really influence the size of the accessory CL (p=0.88). However, the principal CL area was significantly larger in cows treated with hCG compared to that of cows treated with GnRH and the control cows. There was no significant difference between the area of principal CL in cows treated with GnRH and control cows. Despite difference in doses, our results match with those found by Stevenson et al (2007), Stevenson and Pulley (2012) and Rizos et al (2012). Previous studies in dairy cows have reported similar frequencies of newly formed luteal structures when hCG was administered post AI at doses ranging from 1,000 to 3,300 IU (Santos et al., 2001; Stevenson et al., 2007). The increase of luteal tissue area arises from the LH-like action of hCG on luteal cells (Schmitt et al., 1996a). In the present study, administration of hCG not only resulted in ovulation of the dominant follicle present on day 11 and the formation of an accessory CL but also stimulated the principal CL in treated cows, leading to an increase in the area of luteal tissue in that CL, in addition to an increase in luteal tissue associated with the presence of the accessory CL.

The main objective of administration of GnRH or hCG is to improve the fertility of post-partum dairy cows by increasing progesterone concentration (Santos et al., 2001; Stevenson et al., 2007; Rizos et al., 2012; Maillo et al., 2014). In the present study, injection of hCG on day 11 increased plasma concentrations of progesterone compared with that of controls or GnRH treated cows. Several authors (Franco et al., 2006; Cruz et al., 2009, Ataman et al., 2011; Musilová et al., 2014) detected higher progesterone concentrations in dairy cows after induction of an accessory CL with GnRH or hCG administered between day 11 and 15 after AI. It is worth mentioning that the stimulation of progesterone secretion by the principal CL after treatment with hCG was higher compared with that of GnRH. This finding is supported by the observation of an increase of principal CL area in hCG- treated cows compared with GnRH-treated cows with a strong positive and close correlation between the total luteal area and the progesterone level in the current study ($r=0.89$, $p<0.0001$) and in previous studies (Kastelic et al., 1990; Herzogn et al., 2010) between the total luteal area and the progesterone level. Indeed, Mann (2009) showed that progesterone level is related to the diameter of the CL that secretes progesterone until it reaches its final size.

The longer half-life of hCG in the blood and the slower turnover of the hCG-LH receptor complex on the surface of granulosa cells are probably responsible for an increased gonadotropic stimulation on the day 5 ovulatory follicle (De Rensis et al., 2010; Lonergan 2011) and its subsequent differentiation into a principal CL with a greater progesterone secreting capacity. The half-life and therefore the LH-like effect of hCG on the ovarian cells may last for 30 h after treatment (Schmitt et al., 1996a); in contrast, administration of 10mg buserelin increases LH concentrations in serum for approximately 5 h (Chenault et al., 1990). Moreover, the CL formed after ovulation induced by GnRH may not be fully functional (Santos et al., 2001; Schmitt et al., 1996b). Thus, hCG treatment usually elevates progesterone concentrations more than GnRH treatment does (Schmitt et al., 1996a; Stevenson et al., 2007).

It has been suggested that the increase in progesterone after hCG administration is due to the progesterone produced by the induced CL. In the study of Schmitt et al (1996b), plasma progesterone did not differ between the control and hCG groups after removal of the accessory CL on day 13. Thus, other evidence indicates that the increased progesterone cannot be entirely attributed to the induced CL. For example, administration of hCG leads to an increase in the area of luteal tissue in the principal CL in addition to the increase in total luteal tissue associated with the presence of the accessory CL (Rizos et al., 2012).

In conclusion, the injection of 1500 IU hCG or 100 μg GnRH in dairy cows at day 11 after AI results in the formation of an accessory CL, in cows with a dominant follicle with a diameter ≥ 10 mm. Compared with the control group, only hCG-treated cows had increased progesterone production.

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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 PRID administration post-insemination on the establishment of pregnancy of dairy cows under commercial farm conditions

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ABSTRACT. The aim of this study was to test the efficiency of progesterone supplementation post insemination (p.i.) in the fertility of dairy cows under commercial farm conditions. At initiation of the study, 405 cows from three farms -irrespective of their open days- were bred after natural or synchronized estrus. Subsequently, the cows were randomly allocated to receive a progesterone-releasing intra-vaginal device (PRID®) between D5 and D17 p.i (P4+, n=213), or to remain untreated (P4-, n=192). Pregnancy per artificial insemination (P/AI) did not differ between P4+ (42.3%) and P4- (41.2%, P = 0.82) groups. However, the treatment improved P/AI in cows enrolled in the study after the second p.p. insemination (46.8 vs. 25.5, P = 0.02). In the farm that showed the less days to insemination before treatment, progesterone supplementation tended to raise the P/AI (48.2 vs. 23.5, P = 0.08). However, in the farm with the greatest fertility the P4+ group had significantly lower P/AI than the P4- group (37.7 vs. 57.4, P = 0.03). Conclusively, the beneficial effect of the post-insemination administration of PRID is mainly apparent only after the second p.p. insemination and probably during early lactation when fertility is suppressed.

Keywords: dairy; cow; fertility; progesterone supplementation

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INTRODUCTION

Early and late embryonic mortality is considered a common problem of high yielding dairy cattle and can lead to poor reproductive performance (Bilodeau-Goeseels & Kastelic 2003; Lopez-Gatius 2012). Progesterone (P4) during the early stages of gestation plays a key role in embryo survival. It facilitates and sustains a uterine environment, which is friendly for embryo growth, implantation and development (Green et al. 2005; Forde et al. 2009; Mullen et al. 2012). Elevated concentrations of progesterone have been associated with increased pregnancy retention during early gestation (Starbuck et al. 2004). However, according to Stronge et al. (2005) almost 75% of modern dairy cows will suffer from sub-optimal P4 concentrations during the early luteal stage. Dairy cows that have low concentrations of progesterone may have a higher metabolic clearance rate of P4 in the liver due to a higher dry matter intake and a higher liver blood flow (Wiltbank et al. 2006; Lemley et al. 2008). Moreover, low concentrations of progesterone after insemination has been implicated in the pathogenesis of repeat breeding and poor reproductive performance (Ferguson et al. 2012). Research has been directed towards the development of different hormonal treatments during early pregnancy that could enhance embryo survival. Administration of GnRH (Franco et al. 2006), hCG (Torres et al. 2013), or progestagen releasing intravaginal devices (PRID®, CIDR®) (Lopez-Gatius et al. 2004; Forro et al. 2012) seem to contribute to P4 concentration and consequently, facilitate embryonic survival. CIDR® or PRID® are usually placed 4-5 days post insemination (p.i) (Yan et al. 2015) and remain in situ until 17-18 day p.i. (Forro et al. 2012). However, P4 supplementation can have both embryotrophic and luteolytic effects (O'Hara et al. 2014). PRID® administration before D5 seems to have a negative effect on CL development, as it inhibits LH secretion (Mann et al. 2006; O'Hara et al. 2014). Additionally, it has been reported that the administration of P4 as early as D1-D3 of the estrus cycle, could lead to an earlier return to estrus (O'Hara et al. 2014).

The beneficial effects of such treatments have to be cost effective and relevant to the everyday farm conditions. P4 supplementation with PRID® or CIDR® alone between D5 to D18 in order to improve pregnancy rates has been previously tested with beneficial (Larson et al. 2007; Forro et al. 2012) or inconsistent results (Villarroel et al. 2004; Stevenson et al. 2007). Based on a recent meta-analysis from Yan et al. (2015), there are conditional effects instead of an overall positive effect from progesterone supplementation p.i. Specifically, it seems that the administration of progesterone post insemination had a positive effect only in cows of lower fertility and after natural estrus (Yan et al. 2015).

We hypothesized that the administration of a progesterone-releasing intra-vaginal device between Day 5 and Day 17 after insemination would improve pregnancy per AI (P/AI). The overall purpose of the study was to investigate the amplitude and consistency of this benefit under commercial farm conditions.

MATERIALS AND METHODS

2.1. Animals, experimental design and treatment

This study was performed on three commercial dairy farms of approximately 100, 120 and 150 mature lactating dairy cows in central and north Greece over an approximately 2-year period. Mean annual milk production of the herds for this period ranged from 9,800 to 10,500 kg per cow. The cows were milked three times daily and were housed in freestall barns.

The health status of all cows was assessed during the first 3 days after parturition. Moreover, the reproductive management of farms included a routine examination of the reproductive tract of the cows to monitor uterus involution and ovarian activity within four weeks after parturition. Any detected clinical abnormality was treated according to health protocols and only cows without abnormal vaginal discharge were subjected to artificial insemination (AI). The voluntary waiting period (VWP) in the three farms ranged between 55 and 65 days in milk (DIM). Artificial insemination was performed by the owner of each farm and according to farm's previous data the effectiveness in all three farms was similar. At initiation of the study, not pregnant cows -irrespective of their open days- showing estrus (in all three farms cows were observed twice daily, for estrus detection) during the first fifteen days were inseminated following the am-pm rule. The remaining cows were examined and, a) in the case of absence of a CL, were enrolled into the Ovsynch protocol (Pursley et al. 1995) followed by timed artificial insemination (TAI) or b) if a CL was palpated, were

injected with PGF2α and were inseminated after heat detection. In case of estrus absence 5 days after the PGF2α administration animals were also enrolled into the Ovsynch protocol. The commercially available frozen semen used for the insemination was of proven fertility. Following the insemination, the cows of the three farms were randomly allocated to receive a progesterone-releasing intra-vaginal device (PRID®, containing 1.55g of progesterone, CEVA) on D5 post insemination and remove it on Day 17 (P4+ group, n=213), or to remain untreated (P4- group, n=192). In every cow PRID® was applied only once. Pregnancy check was performed by transrectal palpation at a time point between 38 and 45 days p.i. and confirmed at 68-75 days p.i.

2.2. Statistical analysis

Statistical analysis was conducted using the Statistical Analysis System V9.3 (SAS Institute Inc., Cary, NC, USA). The Shapiro–Wilk test was performed in all outcome variables to test for the underlying distribution of the data. In order to detect differences between farms regarding productive and reproductive parameters Kruskal-Wallis one way ANOVA and Wilcoxon's two sample test were applied. Where univariate analysis was used, differences in binary variables were evaluated with the use of chi-squared analysis. For the effect on pregnancy, analysis was run by the application of a linear logistic model with a binary response variable (Proc GLIMMIX). A set of variables were used in the statistical model for their effect on pregnancy, namely number of inseminations that the cow had received (1, 2, 3, 4, greater than 4), days to insemination (up to 80, between 81 and 200, greater than 200 days), milk yield on the day of the insemination divided in 4 equal quartiles (<24kg, 24-<31kg, 31-<36kg and greater or equal to 36kg), parity (first, second, third, greater or equal to forth), season (summer, from April to September, vs. winter), and progesterone supplementation (yes vs. no). Herd was included in the model as a random effect. Effects with P-values >0.15 were removed in a stepwise backward elimination process. Progesterone supplementation was forced in all models and all two way interactions between the above mentioned parameters and PRID were tested. All analyses were considered to be statistically significant at $P < 0.05$. Quantitative data are presented as the mean ± SEM.

RESULTS

The average daily milk yield on the day of the insemination was 29.7 ± 9 kg. The average lactation number, days to insemination and number of inseminations were 2.3 ± 1.4, 192 ± 121 and 2.5 ± 1.8, respectively. However, there were significant differences between the 3 farms (Table 1). Specifically, the first farm had the lowest daily milk yield and the greatest number of inseminations, as animals of higher parity animals compared to the other two farms. On the other hand, the third farm had the greatest milk yield and the lowest days to insemination. The second farm had better P/AI than the third farm (48.1 vs. 34.2, $P = 0.05$).

Cows that received the progesterone supplementation after the second p.p. insemination showed improved P/AI compared to the untreated group (46.8 vs. 25.5, $P = 0.02$, Fig 1a). There was no significant effect from P4 supplementation in any other level of this variable. In cows with less than 80 days open the P4+ group had numerically higher P/AI compared to the P4- group (41.4 vs. 25.7, respectively, $P = 0.18$, Fig 1b). There was no significant effect from P4 supplementation in any level of milk yield, parity or season (Fig 1c, 1d, 1f). In the third farm progesterone supplementation tended to raise the P/AI (48.2 vs. 23.5, $P = 0.08$, Fig 1e). However, in the second farm the P4+ group had lower P/AI than the P4- group (37.7 vs. 57.4, $P = 0.03$, Fig 1e).

In the final model, there was no effect of open days, daily milk yield, parity, season and PRID on P/AI. However, there was a significant effect of number of insemination on P/AI ($P = 0.02$), as there was a gradual increase on the P/AI from the first to the fourth insemination (Fig 1a). Moreover, the interaction be-

Table 1: Production and reproductive data of the 3 farms (Mean ± SD)

Item	Farm 1	Farm 2	Farm 3
n	200	129	76
Parity	2.6 ± 1.5 [a]	2.0 ± 1.0 [b]	2.1 ± 1.1 [b]
Milk yield	25.5 ± 8.4 [a]	32.5 ± 7.6 [b]	36.3 ± 7.6 [c]
Days to insemination	202 ± 126 [a]	207 ± 127 [a]	138 ± 72 [b]
No. of inseminations	2.9 ± 2.0 [a]	2.3 ± 1.6 [b]	2.1 ± 1.4 [b]

Within rows, values with different superscripts differ ($P < 0.05$)

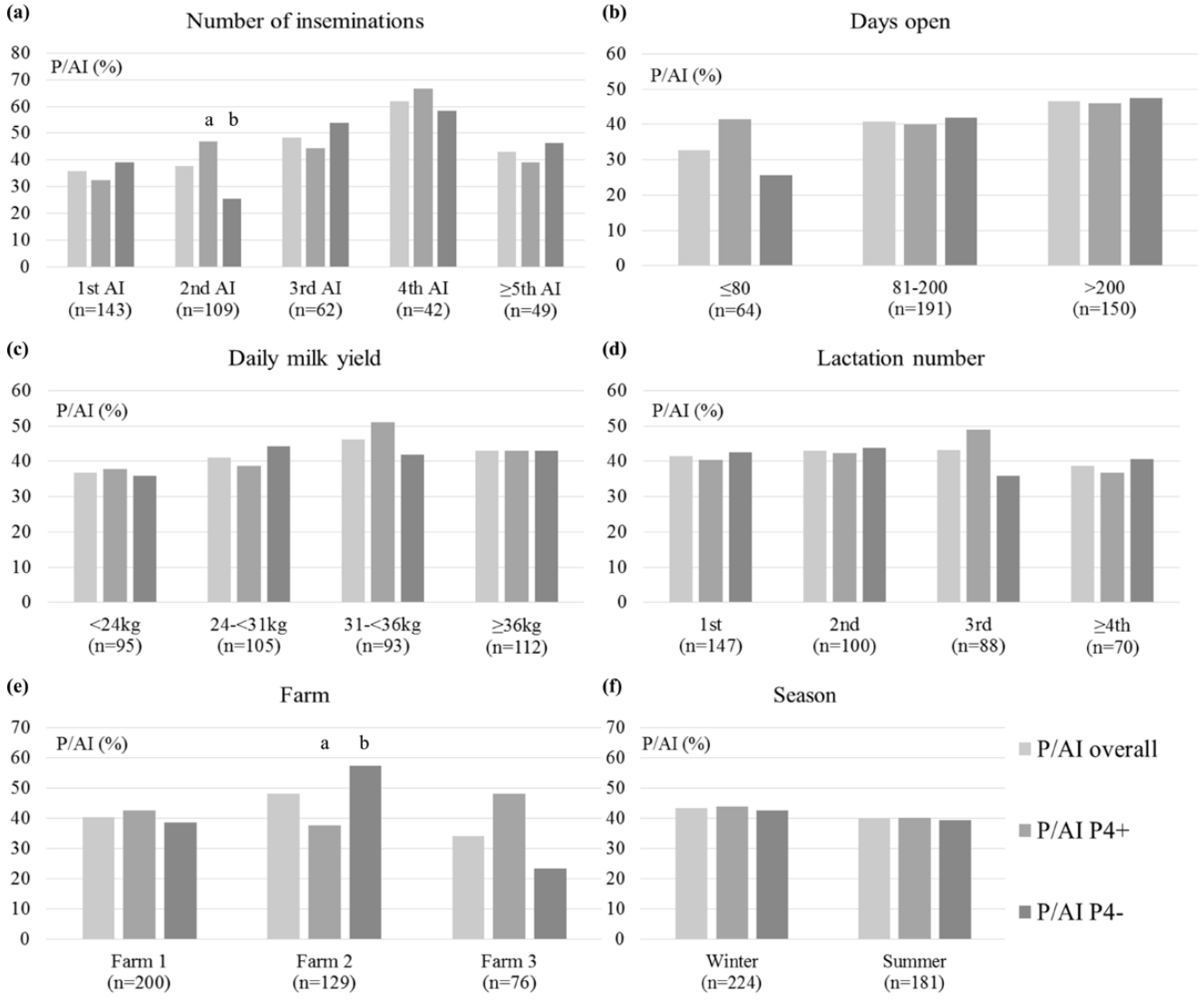

Figure 1. Effect of progesterone (P4) supplementation on pregnancy per AI (P/AI) of Holstein Friesian cows under different number of insemination (a), open days (b), daily milk yield (c), parity (d), farm (e) and season (f) levels. Values within category and level with different superscript differ ($P < 0.05$).

tween the number of insemination and PRID satisfied the cut off level for entry in the final model ($P = 0.15$).

DISCUSSION

The need of adequate progesterone levels post insemination on embryo development and survival has led to the implementation of protocols that supplement P4 post insemination in everyday farm practice. However, the response to such protocols varies widely. To our knowledge this is the first study that estimated the effect of such an intervention under commercial farm conditions.

According to Wiltbank et al. (2014) higher milk yields can lead to decreased circulating P4 concentrations due to increased feed intake and higher P4 metabolism. However, the results from the present study indicate that under farm conditions no decision for the selective PRID administration can be made based on daily milk production data. This leads to the indirect inference that PRID supplementation for the increase of pregnancy rate is not directly influenced by milk production. High milk production and negative energy balance (NEB) are reported to have a key role in em-

bryo survival (Santos et al. 2009; Senosy et al. 2012). This effect is both direct and indirect and although NEB is noticed during the first weeks postpartum, its consequences seem to have a carryover effect on ovary activity (Roth et al. 2001; Butler 2003). Follicles containing oocytes available for fertilization have initiated their differentiation 60-80 days before estrus (Leroy et al. 2008) and it was postulated that NEB affects the steroidogenic capacity of follicles. Under such circumstances their ability for survival is diminished. Nevertheless, results from a recent research indicate that high-producing cows can still have high reproductive performance providing a successful adaptation to lactation due to effective nutritional management (Drackley & Cardoso 2014).

It has been previously reported that several environmental conditions may be associated with low plasma progesterone concentrations. Heat stress is one factor recognized as a cause of low fertility but the mechanism of action remains obscure. The role of summer in progesterone insufficiency has been reported and could be one possible mode of action (Howell et al. 1994; Sartori et al. 2002). On the other hand, it has recently been reported that repeat-breeder cows have low fertility during summer and this is related to a low oocyte competence to develop into blastocysts (Ferreira et al. 2011). This is in accordance to our findings, since the potential beneficial effect of PRID administration is expressed after blastocyst formation. In addition, it seems possible that despite the hot summer conditions in the region, the microenvironment of the farms was sufficiently supported (fans and sprinkles) to overcome any possible detrimental effect of heat stress on progesterone concentration.

Based on our results, the effect from progesterone supplementation p.i. varies from farm to farm and is diverse depending on insemination number and open days level. The animals of the farm with the smaller calving-to-PRID administration interval and, most importantly, the worst P/AI in the control group profited more from P4 supplementation compared to the other two farms. This finding implies that farms with more fragile fertility could benefit more from such an intervention. On cow level, the positive effect from P4 supplementation was evident in the second insemination and numerically in cows that were early (<80 days) in lactation, although the latter difference was not significant (P = 0.18). These cows had also the poorest fertility in the control group and were at greater risk for negative energy balance. Under commercial farm practices synchronization protocols are implemented later in lactation. These findings are in accordance with those of Yan et al. (2015) that reported a positive effect from P4 supplementation only in cows with low fertility and after natural estrus. On the other hand, it is difficult to explain the apparent negative effect noticed in the second farm. Yan et al. (2015) reported very wide variations across studies (-40% to +50% change) that supplemented P4 post insemination. Additionally, Yan et al. (2015) actually found a significant fall in the pregnancy rate of farms with high fertility after P4 supplementation. Nevertheless, it is more likely that there was an unexplained exceptional P/AI of almost 60% in the control group rather than a true negative effect in the treatment group that showed P/AI of almost 40%.

Conclusively, our results suggest that progesterone supplementation post insemination can improve the pregnancy rates of cows and farms at risk for low fertility. Well managed farms or cows after the period of negative energy balance are not expected to profit from such an intervention.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Seroprevalence of *Mycoplasma bovis* in grazing dairy cows from five different areas in Serbia

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ABSTRACT. *Mycoplasma bovis* infection in grazing dairy cows has not been reported in the Republic of Serbia to date. It is important to monitor its seroprevalence on the field. The presence of specific antibodies against *M. bovis* in the blood serum of grazing dairy cows is investigated in the present study. A total of 131 blood serum samples of clinically healthy dairy cows were examined. Sampling was performed during 2013 from five different areas in Serbia: Zasavica, Pozarevac, Gruza, Novi Sad and Banatski Karlovac. A commercial ELISA kit for diagnosis of *M. bovis* antibodies in blood serum samples, manufactured by Bio-X Diagnostics, Belgium, was used. Specific antibodies against *M. bovis* were identified in 13 out of 131 samples (9.92%) from 4 locations; the only negative location was the most southern Gruza. The revealed seroprevalence is evidence for the presence of *M. bovis* in grazing dairy cows in different locations of Serbia.

Keywords: *Mycoplasma bovis*, ELISA, serology, grazing cows, Serbia

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INTRODUCTION

The genus *Mycoplasma* (class *Mollicutes*, family *Mycoplasmataceae)* includes prokaryotic microorganisms that are capable of self-reproduction (Razin, 1998). Pathogenic species for cattle include *Mycoplasma bovis* (which is the main causative agent for infection) and *Mycoplasma mycoides* subsp. *mycoides SC*, causing contagious pleuropneumonia, which was included in the list for 2018 of the Office International des Epizooties (Pfutzner and Sachse, 1996; OIE, 2018). *Mycoplasma bovis* was first isolated in 1961 from the udder of cows suffering from mastitis (Hale et al., 1962).

In cattle, *M. bovis* can cause pneumonia, mastitis, arthritis, meningitis, otitis, keratoconjunctivitis and genital disorders (vaginitis and abortion) (Gonsales et al., 1983; Stipkovits, 1993; Pfutzner and Sachse, 1996; Gagea et al., 2006). The infection is transmitted mainly airborne, but can occur also via milk or sexually. Semen for artificial insemination can introduce the infection in a herd (Haapala et al., 2018). *Mycoplasma bovis* infection is of major importance in cattle production in European countries and the United States of America (USA), causing large economic losses (Tenk, 2005).In Europe, the damage caused annually by mycoplasmosis in general in cattle is estimated at 576 million Euros(Nicholas et al., 2000) and is responsible for one third of all pneumonia cases in calves. In the last decade *M. bovis* infection has been expanded to new geographical areas, including Ireland (Blackburn et al., 2007; Bell et al., 2012) and Greece (Filioussis et al., 2007), where it was not previously registered, as well as on other continents: parts of South America (Cerdá et al., 2000) and Australia (Al-Farha et al., 2017).

The methods used for serological diagnosis of *M. bovis* infection are indirect hemagglutination, film inhibition and indirect enzyme-linked immunoabsorbent assay (ELISA). The ELISA test, which uses the entire bacterial particle as the antigen, was applied for the first time in 1979 (Onoviran and Taylor-Robinson, 1979). Afterwards, ELISA test for the detection of antibodies against *M. bovis* present in milk of cows suffering from mastitis was developed (Byrne et al., 2000).

To the best of the authors' knowledge, there is no published data about *M. bovis* seroprevalence in grazing dairy cows in Serbia. Existing data indicated the presence of *M. bovis* antibodies (seropositivity of 2.57 and 4.81%), but only in calves so far (Vojinovic et al., 2014; Vidic et al., 2014).

Since the main route of *M. bovis* excretion is via milk, dairy cows were selected as the target animals for this study. Due to the close contact between animals from different farms and locations at grazing, animals are at increased risk to *M. bovis* infection compared to indoors intensively reared dairy cows. Extensive cattle breeding in pastures from early spring to late autumn is a re-emerging practice in Serbia, as a closer step to organic farming.

This study was performed in order to assess the exposure of grazing dairy cows to *M. bovis* in several parts of Serbia.

MATERIALS AND METHODS

The study was performed in compliance with Serbian Law on Animal Welfare (Official Gazette of the Republic of Serbia No 41/09).

Sampling material originated from 131 clinically healthy grazing dairy cows. Blood was collected during year 2013 from coccygeal vein, using 0.8mm needles (BD Vacutainer Precision Glide 0.8 x 38mm), into 5mL tubes with increased silica act clot activator, silicone-coated interior (BD Vacutainer serum tube). Cows were randomly selected from unvaccinated against *M. bovis* herds, in the age-range of 2 to 5 years and originated from 5 different areas of Serbia: Zasavica, Gruza, Pozarevac, Novi Sad and Banatski Karlovac (Fig. 1). Concerning cows' breeds, Simmental crossbreeds were sampled in Zasavica and Gruza, while Holstein-Friesians were sampled in the other 3 areas.

Blood samples were allowed to clot at room temperature. Serum was obtained, transferred into plastic vials and stored at +4°C until analysis. A commercial *M. bovis* ELISA indirect double well test kit for the serological (antibody) diagnosis of blood serum and milk was used (Bio-X Diagnostics, Belgium). ELISA plates washing was performed on ELISA PW41 Micro plate (Bio–Rad Laboratories, France) with optical values reading on ELISA reader (TEKAN, Austria), using a 450 nm filter. All procedures were carried out according to the manufacturer's instructions.

RESULTS

A total of 13 out of 131 (9.92%) sera samples were positive for the presence of specific *M. bovis* antibodies (Table 1). The number of seropositive samples ranged from 4.4% (Pozarevac) to 14.3% (Zasavica, Novi Sad). All the 15 cows from Gruza area were seronegative for antibodies against *M. bovis*.

Table 1. Number and percentage of examined and seropositive to specific *Mycoplasma bovis* antibodies blood sera samples from grazing dairy cows of 5 different areas of Serbia.

Area	Number of examined samples	Number of positive samples	Percentage %
Zasavica	14	2	14.3
Pozarevac	23	1	4.4
Gruza	15	-	-
B. Karlovac	58	7	12
Novi Sad	21	3	14.3
Total	**131**	**13**	**9.9**

DISCUSSION

Obtained results show, for the first time, preliminary data concerning specific *M. bovis* antibody prevalence in blood sera of clinically healthy grazing dairy cows in different locations of Serbia. Our previous reports showed seroprevalence of 2.57% in 5603serum samples of clinically healthy calves from 3 farms (Vojinovic et al., 2014). That emphasized the need for determination of seroprevalence in adult dairy cows, because the occurrence of clinical disease on the field would be expected (Vojinovic et al., 2014). Vidic et al.(2014) determined the seroprevalence to be 4.81% in 3,777 fattening calves during period 2012-2013 in the Republic of Serbia. The higher seroprevalence in the present study could be due to the lower number of examined samples, to different management systems and because the tested animals were older. It would be possible for older cattle to come in contact more times with carriers or infected cattle, having thus increased possibilities to be infected by *M. bovis*. Moreover, at pasture commingling of cattle from different herds could increase the possibility of cross-infection.

During a 10-year period, from 1990 to 2000 in the Weybridge Veterinary Laboratory, Great Britain, *M. bovis* was the most abundant microorganism isolated from cattle with pneumonia and occasionally in cattle with arthritis and mastitis (Ayling et al., 2004). Several studies suggest that synergisms between *M. bovis* and other microorganisms might exacerbate disease outcome of bovine mycoplasmosis, thus careful screening should routinely be performed (Burgi et al., 2018).

Le Grand et al. (2002) examined 32,197 cattle blood sera from 824 randomly selected herds, originating from 8 different French provinces. Using the ELISA method, they found 2 to 13% of positive herds within each province and an animal infection rate ranging between 10 to 20% within individual infected herds.

Mycoplasmatic infections are considered to be endemic in Poland. Examination of 3,670 cattle blood sera samples from 16 provinces in the period from 2007 to 2010 by ELISA showed high seroprevalence (76.1%), but there was no clinical manifestation of the disease. After correction of the test sensitivity, the prevalence was decreased to 28.2%, which was similar to other European countries. In Hungary, 595 cattle blood sera were examined and 10.9% were found seropositive to *M. bovis* (Tenk, 2005). In Bosnia and Herzegovina, from 2002 to 2010, *M. bovis* was isolated 8 times from nasal swabs and lung samples of cattle (Maksimovic and Rifatbegovic, 2012), indicating that there was active infection in Serbia`s neighboring country. Comparing available literature, we can conclude that the obtained results of 9.92% seropositivity are similar to surrounding and other European countries.

Apart from the fact that grazing cattle are an especially sensitive population to the development of bacterial and viral infections due to every day close contact between animals (many times from different farms), they are also more prone to parasitic infections. Those can affect nutrient intake, leading to deficiencies that compromise protective immunity against *Mycoplasma* spp. and other agents (Fekete and Kells, 2007). Thus, more intensive monitoring of these animals' health status and further studies are needed in order to establish measures for *M. bovis* infection control and eradication.

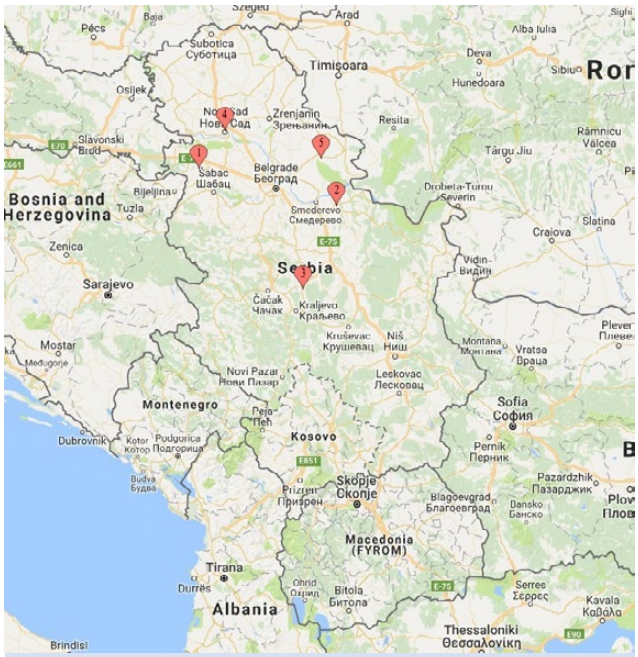

Figure 1: Location of the 5 different areas of Serbia where grazing cows were sampled for *Mycoplasma bovis* serology: 1 -Zasavica, 2 - Pozarevac, 3 - Gruza, 4 - Novi Sad and 5 - Banatski Karlovac (MapCustomizer, https://www.mapcustomizer.com/).

CONCLUSIONS

Specific antibodies against M. bovis were identified in 9.92% of the grazing cows from 4 areas; the only negative was the most southern region, Gruza. The revealed seroprevalence is the first evidence for the presence of M. bovis in grazing dairy cows in different locations of Serbia.

CONFLICT OF INTEREST STATEMENT

The authors have nothing to disclose.

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Research article
Ερευνητικό άρθρο

Iodine Intoxication in Beef Cattle in Turkey - Clinical, Hematological and Biochemical Evaluation

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ABSTRACT. Iodine is an essential trace element for humans and animals. The incidence of iodine poisoning in cattle is low. In the present study, we evaluated the clinical findings, serum glucose and cholesterol, thyroid hormone and urine iodine levels in cattle exposed to excess iodine. All of the clinical data were determined following the addition of potassium iodide to the drinking water. Inappetence, cough, and hyperthermia were notable clinical findings. We detected a very high iodine level (470 µg /L) in an analysis of the drinking water samples. A biochemical analysis revealed that the serum cholesterol levels in the affected cattle were significantly lower ($p<0.05$) than in healthy cattle. However, the serum glucose in the affected cattle was significantly higher ($p<0.05$) compared to healthy cattle. The iodine concentration in the urine of the affected animals was also significantly higher ($p<0.05$) than in the healthy animals. Importantly, a hematological analysis indicated leukocytosis with neutrophilia. Several clinical signs, including hyperthermia, tachycardia, alopecia, and a naso-oral discharge, based on suspected history can suggest iodine intoxication. In addition, biochemical parameters, such as urine iodine, serum glucose and cholesterol levels, were observed to be different between healthy and affected cattle. The thyroid function in affected cattle should also be studied.

Keywords: iodine toxicity, beef cattle

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INTRODUCTION

Iodine is an essential trace element that is primarily obtained from plants and is involved in the structure of thyroid hormones. Thyroid hormones are extremely important in cell and tissue growth and development (Hetzel and Dunn, 1989). In addition, thyroid hormones are necessary for many metabolic processes, such as lipolysis, glucose metabolism and erythrocyte production (Brent, 2012; Bustad and Fuller, 1970). The recommended iodine levels for a mature lactating dairy cow in the ration dry matter have been set in a range from 0.6 ppm (National Research Council, 2001) to 0.8 ppm (British Agricultural Research Council, 1965), which is equivalent to 9 to 12 mg for a 500 kg cow that consumes 20-25 kg dry matter daily. Bustad and Fuller (1970) estimate that on the basis of the thyroxine secretion rate (TSR) and radioiodine metabolism data, the daily iodine requirement for a 500 kg cow would be 5.5 to 11 mg.

The incidence of iodine poisoning in cattle is seen less frequently compared to iodine deficiency. Thyroiditis, goiter, hypothyroidism, hyperthyroidism and hypersensitivity may follow excess iodine intake in humans (Pennington, 1990). In iodine intoxicated cattle, cough, hyperthermia, depression, tachycardia, alopecia, dermatitis, exophthalmus and susceptibility to infectious diseases may be observed (Olson et al., 1984; Paulíková et al., 2002). Changes in serum biochemical parameters, such as hypocholesterolemia and hyperglycemia, and changes in hematological parameters, such as leukocytosis, may occur in cattle with iodine intoxication (Paulíková, 2002). The best method to detect an iodine deficiency or an iodine excess is to measure the urine iodine level (Herzig et al., 1996). In a study by Hilman and Curtis (1980), the urine iodine levels in healthy and intoxicated cattle have been reported as 1.87 ppm and 6.81 ppm, respectively.

Iodine deficiency is a well-known issue, but limited knowledge exists regarding excess iodine intake in cattle, which causes systemic toxicity. Thus, the aim of this study was to evaluate the clinical symptoms of excess iodine intake and changes in the serum glucose and cholesterol levels and in the thyroid hormones and the urine iodine levels following iodine exposure in the drinking water.

MATERIALS AND METHODS

The study was conducted on a Simmental breeding farm consisting of 600 cattle near the town of Luleburgaz in Edirne, Turkey. Potassium iodide was added by a local farmer to the drinking water of only one paddock (n: 100) that included two different age groups because of an erroneous belief that this could be helpful against respiratory diseases. The potassium iodide was added once, and the duration of the iodated water consumption was 7 days. A farm owner and a local practitioner said that 35 kg of potassium iodide was added to 100 tons of drinking water. However, the amount of iodine that the affected animals received with their food is unknown. Clinical signs were apparent 3 days after the animals consumed the iodine-containing drinking water. A detailed clinical examination of animals showing tachypnea and mental depression in the paddock (n:100) was performed in a cattle crush.

The detailed clinical examination included body temperature, heart rate and respiration rates, the peripheral pulse quality (4- Bounding, 3-Increased 2-Normal 1-Weak 0-Absent or nonpalpable-using the coccygeal artery) and the mental status, which was scored as 0: clinically normal, 1: mild signs of depression (standing), 3:moderate signs of depression (able to stand but with a dull status) 4: severe (recumbent with no alertness) (Modified from Walker et al., 1998). The ages of the affected animals ranged from 4 to 22 months old. These animals included fattening beef cattle aged 4 to 8 months and pregnant heifers which were in their 7^{th} – 8^{th} month of gestation.

Healthy animals were determined by a clinical examination and selected (Simmental beef cattle, 6 – 22 months of ages) (n=10) randomly via a lottery of the herd list of different groups.

Blood samples were collected for the measurement of the biochemical parameters from the coccygeal vessels using plain vacuum tubes, (Becton Dickinson and Company, Franklin Lakes, NJ) from healthy animals (n:10) and from animals showing clinical signs (n:10). One hemolyzed sample was excluded from the study. Urine samples were also collected from healthy animals (n:10, showing clinical signs (n:10, 8M-2F) using a urine catheter. The serum was collected from blood samples (15 minutes at 1000 g) that were centrifuged on the farm. Water samples

for iodine analysis were collected in 100-mL deiodinated plastic tubes. Blood and urine samples were sent to the laboratory on dry ice (Düzen laboratory, Ankara, Turkey). The biochemical parameters were determined by a reflectance photometry method using a commercially available blood biochemical measurement device (Reflotron Plus System, Roche Diagnostics®, Switzerland). The iodine concentration in the water and in the urine was measured with an ion chromatography device (ICS 3000, Dionex Corp., USA). The urine specimens were sent to the laboratory immediately after they were taken.

The hematological parameters, including the total white blood cell count (WBC), hematocrit (HCT), hemoglobin (Hgb), erythrocyte (RBC) and platelet counts (PLT), were measured by an automatic hemogram analyzer (Vetscan HM5 Abaxis®, USA). In addition, a serum hormonal profile analyses included triiodothyronine (T3), and thyroxine (T4) measured with a commercial kit (Immulite 2000 Total T3/ L2KT36; Immulite 2000 Total T4/L2KT46; Siemens, Italy).

The affected animals were provided with a supply of fresh water. Ascorbic acid (25 mg/kg, IV, for 3 days), vitamin E + selenium (single dose, 4 to 8 cc, IM), acetyl cysteine (1200 to 2400 mg/per animal, IV, for 3 days) and ceftiofur (2.2 mg/kg, IM for 5 days) were also given.

All data passed a normality test. Differences between cattle suffering from iodine intoxication and healthy cattle were analyzed by Student's t-test for each parameter. The results were expressed as the mean ± standard deviation (SE), and a P-value less than 0.05 was considered to be statistically significant.

RESULTS

Water iodine analysis

The water analysis showed that the level of iodine was very high (47 µg/L).

Clinical signs

In animals considered intoxicated (n:10), clinical signs, including inappetence, hyperthermia (39.5-41.6 C), naso-ocular discharge, cough, increased heart rate (110-148/beats/minute), increased respiratory rate (52-68 breaths /minute), hypersalivation, severe mental depression and decreased ruminal movements, were observed in varying degrees (Table 1).

Serum biochemical results and urine iodine analysis

The serum biochemical results and the iodine level (Mean±SE) in the urine are presented in Table 2. The serum cholesterol concentration in the iodine intoxicated cattle was significantly lower ($p<0.05$) than

Table 1. Clinical findings in 10 cattle with Potassium iodide toxicity

Variable	Finding	Number of cattle (n:10)	Reference range
Appetite	Decreased	3	
	Absent	7	
Ruminal motility	Reduced	2	
	Absent	8	
Salivation	Severe	7	
	Mild	3	
Alopecia	Mild	2	
	Marked	2	
	Absent	6	
Cough	Mild	3	
	Persistent-severe	7	
Naso-ocular discharge	Mild	3	
Body temperature	Normal	0	37.8 - 39.2°C
	High	10	
	Low	0	
Heart Rateb (reference range:	Normal	0	60 -80/beats/ minute)
	Bradycardia	0	
	Tachycardia	10	
Respiratory Rate	High	10	10 - 30 breaths/ minute)
Mental depression	Severe	10	
Exophthalmos	Present	2	
	Absent	8	

Table 2: Biochemical and urine iodine results in healthy and cattle affected by potassium iodine toxicity

Parameters	Cattle with KI toxicity (n=10) (Mean±SE)	Healthy Cattle (n=10) (Mean±SE)
Cholesterol (mg/dL)	43.0±4.8^{A}	139.1±15.1^{B*}
Triglyceride (mg/dL)	16.1±1.4	20.0±1.8
Potassium (mEq/L)	4.6±0.2	4.3±0.9
BUN (mg/dL)	17.9±3.9	10.1±0.4
Glucose (mg/dL)	88.7±2.6^{A}	74.4±3.7^{B*}
Triiodo thyronine (**T3**)	1.6±0.1	1.5±0.1
Thyroxine (**T4**)	7.2±0.8	6.9±0.1
Urine iodine (μg/dL)	45.7±13^{A}	12.0±0.8^{B*}

*$p<0.05$
A, B: define statistically importance within a row
KI: Potassium iodide

in healthy cattle (Table 2). We also detected an elevated serum glucose concentration (75.4-104.1 mg/dL) in iodine intoxicated cattle compared to healthy animals ($p<0.05$). More importantly, the urine iodine concentration (13.4-113.8 μg/dL) in the intoxicated animals was significantly higher ($p<0.05$) compared to healthy animals, and the BUN values (31.3-38.3 mg/dL) of three animals were observed to be high. No statistically significant differences were found in the remaining biochemical parameters between animals exposed to potassium iodide (n:10) and healthy animals (n:10) (Table 2).

Hematological analysis

A hematological examination revealed leukocytosis 19.8±1.6 x 10^9/L (reference range 4.0 to 12.0 x 10^9/L) together with neutrophilia 16.4±0.1 x 10^9/L (reference range 0.7 to 6.0 x 10^9/L) associated with a suppurative infection, which might have been related to a previous respiratory infection.

DISCUSSION

Many researchers have described the clinical signs of iodine intoxication, including a persistent cough, hyperthermia, naso-ocular discharge, inappetence, depression, dermatitis, alopecia, tachycardia, nervousness, weight loss, exophthalmos, susceptibility to infectious diseases, respiratory stress and death (Olson et al., 1984; Döcke et al., 1994). The clinical signs of iodine toxicity in ruminants observed in the present study except for nervousness were consistent with those in previously reported studies.

The most appropriate method to detect an iodine deficiency or an iodine excess is to determine the urine iodine level (Herzig et al., 1996). Renal iodine clearance is related to the glomerular filtration rate (GFR) or creatinine clearance, the sex and pregnancy (Soldin, 2002; Stilwell et al., 2008). Stilwell et al. (2008) reported that urine iodine excretion was increased during early lactation and that excretion decreased in subsequent stages of lactation In a study conducted by Herzig et al. (1996), the urine iodine levels in 672 dairy cows from 22 herds were determined. They reported that the urine iodine levels were less than 2 μg/dL, 2 to 5 μg/dL, 5 to 10 μg/dL and more than 10 μg/dL in 27.5, 24.6, 16.8, and 31.3% of the cows, respectively. The same authors reported that the mean urine iodine concentration was 9.48 μg/dL at the peak of the lactation period and 2.3 μg/dL immediately before drying off. To the best of our knowledge, no study of urine iodine levels in beef cattle has been conducted. In a study reported by Meyer et al. (1996), the mean serum iodine level was 15.3 mg/dL in cattle fed a ration with 0.5 mg iodine per kg dry matter added.

In our study, the mean urine iodine concentration in healthy cattle was 12.0 ± 0.8 μg/dL, while in cattle suffering from iodine intoxication, it was 45.7 ± 13 μg/dL. Moreover, the BUN levels were within the reference limits for healthy and intoxicated animals,

but were numerically higher in the latter than in the former.

The daily iodine requirements of beef cattle should be 0.4 mg/kg dry matter (NRC, 2001 In this study, 35 kg of potassium iodide was added to 100 tons of water. Furthermore, it is not known how much iodine the affected animals received via the feed. Technical problems prevented an analysis of the amount of iodine in the diet. However, high concentrations of urine iodine in the affected animals indicate that they received excessive amounts of iodine. The iodine level in the drinking water in Turkey varies geographically. The mean iodine level in the drinking water in the Aydın region in Turkey was 270 μg/L (Özkan, 2008). Compared to that study, the amount of iodine in the drinking water in our study (470 μg/L) was very high. These results indicate that the drinking water was the source of the iodine poisoning.

Biochemical and hematological changes may occur in animals subjected to iodine poisoning. Hillman and Curtis (1980) noted that the serum glucose and BUN values were increased, but on the contrary, the serum cholesterol concentration was decreased. In the present study, the serum cholesterol values in cattle affected by potassium iodine toxicity was significantly lower ($p<0.05$) than in healthy cattle (Table 2). These results are in agreement with Hillman and Curtis (1980) and Paulíková (2002), who reported hypocholesterolemia. Thyroid hormones induce triglyceride mobilization from the adipose tissue for lipolysis and are responsible for the decrease in the blood cholesterol level. Additionally, iodine-induced thyroid hormones activate LDL clearance in the serum and reduce intestinal absorption (Shin et al.,2003; Galman et al., 2008). In the presence of thyroid hormone, an increase in NEFA and glycerol levels is also observed (Nikitin and Babenko, 1989; Pucci et al., 2000). In the present study, the serum glucose level in cattle affected by potassium iodine toxicity was significantly higher (88.7±2.6 mg/dL) than in healthy cows (74.4±3.7 mg/dL) ($p<0.05$). This effect may explain the decreased serum cholesterol level in mammals affected by iodine intoxication.

In a study by Minelli et al., (1997), iodine was given to humans with hepatitis B and C who had previously received interferon-alpha. In the study, the thyroid hormone levels varied, either increasing, decreasing or normal. This variation may have been caused by cytokines (Minelli, et al., 1997). In our study, a concurrent respiratory disease may have caused the release of cytokines which might have resulted in normal thyroid levels (Minelli et al., 1997). In our study, some samples from intoxicated animals had high thyroid levels. Furthermore, a surge in the thyroid hormone level may also be related to the blood collection time and the variance in the samples, differently than implied by the above explanation. We assumed that repeated blood analysis can remove the variability of thyroid hormone levels in future studies. High thyroid hormone levels increase glucose concentration in the blood and trigger hepatic insulin resistance (Klieverik et al., 2009; Ferrannini et al., 2017). In a study conducted by Hillman and Curtis (1980), no difference was found between the thyroid hormone concentrations in cows fed a high or normal iodine diet ((32.66 ± 3.3 ng/ml vs 32.29 ±2.0 ng/ml). In the same study, it was concluded that the serum glucose and cholesterol levels in cattle intoxicated with iodine were respectively elevated or decreased.

In the present study, the serum T3 and T4 levels were observed to be numerically higher in animals affected by iodine intoxication compared to healthy animals, although these results were not statistically significant. However, cholesterol levels were significantly lower (43.0±4.8 mg/dL) in cattle intoxicated with iodine than in healthy animals (139.1±15.1 mg/dL). High levels of thyroid hormones induce lipolysis from adipose tissue. Increased lipolysis promotes hepatic insulin resistance (Brent, 2012; Rachel et al., 2014). It can be interpreted that the increase in the serum glucose concentration in sick animals may be related to hepatic insulin resistance in accordance with the high thyroid hormone levels.

Derscheid et al. reported that the use of potassium iodide decreased the severity of a BRSV infection in the cattle *in vivo* (Derscheid et al., 2014). In our study, KI was used against respiratory problems by a practitioner. However, it has been observed that respiratory system infections were much more severe in cows affected by iodine toxicity. In the present study, neutrophilic leukocytosis was detected in cattle affected by iodine intoxication. These results are comparable with those reported by Hillman and Cur-

tis (1980) and might be related to the suppression of cellular and humoral activity caused by an excessive iodine uptake (Olson et al., 1984). An excessive iodine intake may also cause lymphopenia, and neutrophilia along with the dysfunction of the T and B lymphocytes, as well as a decrease in the phagocytic function of macrophages (Haggard et al., 1980). In addition, changes in the hemogram may be related to respiratory problems.

The thyroid gland has a limited capacity for iodine storage. In humans, excessive iodine uptake caused differences in the thyroid function. Excessive iodine intake may cause increased thyroid hormone synthesis and its release to the circulation in humans (Koukkou et al., 2017). As a result, excess iodine supplementation can cause hyperthyroidism in susceptible humans. (Leung and Braverman, 2014). Another study (Schnur, 2015) reported that excessive amounts of dietary iodine intake led to hyperthyroidism associated with autoimmune changes in the thyroid. Contrarily, Hillman and Curtis (1980) have reported that long-term iodine uptake did not lead to changes in the thyroid function in animals. However, changes in the thyroid function were observed when a 500-fold excess of the daily amount of normal iodine required by calves was given (Leung et al., 1980). Excess iodine can lead to iodopeptid formation, which might be related to the transient blocking of enzyme reactions and protein synthesis in the thyroid gland (Leung and Braverman, 2014; Koukkou et al., 2017). In the present study, clinical signs associated with hyperthyroidism, such as exophthalmos, tachycardia, and an increased respiratory rate, were also identified. In addition, we detected an increase in serum cholesterol level and a rise in the glucose level. In conclusion, the determination of the iodine level in the urine of an animal showing signs of iodine intoxication will be decisive for diagnosis. Future studies might also evaluate the urine iodine concentration together with the urine creatinine clearance or the GFR ratio. In addition, future detailed studies are necessary to reveal the effect of excessive iodine exposure on the thyroid function.

Iodine intoxication should be considered in the cattle with clinical signs that include respiratory problems, hyperthermia, tachycardia, dermatitis, alopecia, coughing, and naso-oral discharge following excessive iodine supplementation.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Developing an Antiviral Drug Screening System for Anti-Bovine Viral Diarrhea Virus (BVDV) Therapies

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ABSTRACT. Bovine viral diarrhea virus (BVDV) is an economically important animal pathogen affecting cattle. Despite the use of vaccination, test and slaughter practices, BVD remains a serious problem of cattle breeding. This study was conducted in order to develop a cell line that expresses some of BVDV sub-replicons. BVDV-NADL NS3 and 5'UTR were cloned in pWPI-linker B lentiviral plasmid at the upstream of EGFP gene. Consequently, lentiviral vectors containing BVDV-NS3 and BVDV-5'UTR were produced by using the second-generation lentiviral packaging system. By these lentivectors, MDBK cells expressing BVDV-5'UTR and BVDV-NS3 partial fragments were prepared. The efficiency of the infection was evaluated by fluorescence microscopy, western blotting, and RT-PCR. The results indicated that the development of MDBK cell line expressing these transgenes provides a very sensitive antiviral drug screening system for anti-bovine viral diarrhea virus (BVDV) therapies.

Keywords: Packaging system, BVDV, Lentiviral vectors, Cell line.

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INTRODUCTION

Bovine viral diarrhea virus (BVDV) is one of the most important viral pathogens in cattle, causing economic losses all over the world. BVDV is an enveloped, positive sense, single stranded RNA virus that is classified in the genus pestivirus of Flaviviridae family (Li et al., 2010). BVDV can induce lifelong persistent infection in cattle populations (Li et al., 2010; Carmona et al., 2012), and it can lead to a severe disease involving the respiratory, enteric, reproductive, immune, and endocrine systems (Keyvanfar and Hemmatzadeh, 2000; Hemmatzadeh et al., 2006; Lambeth et al., 2007). This virus has two biotypes which are termed cytopathic (cp) and non-cytopathic (ncp). Furthermore, based on the difference in the nucleotide sequences, it has two genotypes: BVDV-1 and BVDV-2 (Spurgers et al., 2008; Khaliq et al., 2010; Li et al., 2010). Although commercial BVDV vaccines are available, its antigenic diversity is one of the reasons for the relative failure in vaccination programs. In addition, despite the extensive use of eradication and slaughter strategies after screening tests, the disease caused by BVDV remains prevalent in cattle herds all over the world (Wilson et al., 2005; Khaliq et al., 2010; Li et al., 2010).

BVDV genome is approximately 12 Kb in length and is flanked by a 3' and a 5' untranslated region (UTRs). The BVDV genome encodes a single open reading frame (ORF) with approximately 4000 codons (Houe, 2003; Henry et al., 2006; Zemke et al., 2010; Fan and Bird, 2012). This ORF encodes a poly-protein precursor which can simultaneously or post-translationally be cleaved by viral or cellular proteases to produce 12 or 13 proteins in the following order:

NH2-Npro-C-Erns-E1-E2-p7-NS2-NS3 (NS23)-NS4A-NS4B-NS5A-NS5B-COOH.

NS3 is known as a cp-BVDV marker with serine protease activity. Its cleavage sites in the genome of the virus are the following: NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B. Moreover, NS3 has RNA helicase and nucleoside three trihosphatase activities. The expression of NS3 or NS23 is necessary for the formation of infectious virions. The protease activity of NS3 can induce apoptosis in the infected cells (Xu et al., 1997; St-Louis et al., 2005; Pankraz et al., 2009).

The 5'UTR region with 370 b length, is the most conserved region of the viral genome, and it includes an internal ribosome entry sequence (IRES). The nucleotide sequence and secondary structures of 5'UTR is important for the translation of ORF, the regulation of gene transcription, and the expression of genes associated with the virulence of BVDV (Poole et al., 1995).

Considering the specific functional role of these two parts of BVDV genome, we produced a cell line, after the insertion of these parts, in order to evaluate the therapeutic strategies against this virus (Henry et al., 2006).

For that purpose, in this study, lentiviral vectors expressing NS3and 5'UTR were produced by second-generation lentivirus packaging system, to infect Madin-Darby bovine kidney (MDBK) cells, permanently.

During their life cycle, lentiviruses can integrate their genomes into the host genome; therefore, they can be suitable tools for long-term expression of a transgene. Lentiviral vectors are modified so that only a limited number of lentiviral genes are required for the engineered viral vectors. Furthermore, the stable integration of viral DNA into the host genome can be provided by these essential genes. By this strategy, the desired recombinant gene can be expressed frequently, but the infectious virus cannot be produced. To create such a lentiviral vector, one of the newest methods is the cloning of the essential genes of lentivirus and the transgenes in three or four different plasmids. Consequently, the co-transfection of these plasmids can produce a lentivector in a host cell line.

Due to the persistent lentiviral vector infection and its ability to enter different cell lines (Salmon and Trono, 2006), in the present study, we employed these vectors for the production of MDBK cell lines to express BVDV NS3 partial genes and BVDV 5'UTR. That system has been tested by our group for BVDV gene therapies (unpublished data).

MATERIALS AND METHODS

Cloning of *BVDV NS3* and *BVDV 5'UTR* in pWPI-Linker B lentiviral vector

2.1.1- RT-PCR

BVDV was inoculated in Bovine testicular cell line (BT). BT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, America, Catalog No. 116- 12800) supplemented with 10% fetal bovine serum (FBS) (GIBCO, America, Catalog No. 106- 10270) and 1x penicillin streptomycin (Sigma, America Catalog No. 116-12800). Cultures were incubated for 24 hours at 37 ºC with 5% CO2. Consequently, BVDV-NADL (NC-001461) from the Sekans animal health laboratory, Ankara University (a generous gift from Dr. Faraji) inoculated to BT cells. RNA was isolated 48 h after BVDV infection using QIAzol (QIAGEN, Germany, Catalog Number: 79306) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed with TaqMan Reverse Transcription kit (Invitrogen, Germany, Catalog Number: 8080234N) according to the manufacturer's instructions. PCR was carried out using the primers designed for apart of *NS3* gene (3790-4000) and *5'UTR* (first 386 nucleotides (of BVDV-NADL (ac.no: NC00146). The sequences of primers were as follows: BVDV NS3 F: 5'-CGC CAC A GA TCT ACC GC AAG AGT ACT GG GGA A-3'and BVDV NS3 R: 5'-CGC CAC AGA TCT ACC CA TCA GTA GG TTA TAG TC-3' with *BglII* restriction sites. BVDV 5'UTR F: 5'- CGC GGA TCC ACC A CTCGTAT ACG TAT TGG GC -3' with *BamHI* restriction site and BVDV 5'UTR R: 5'- GTT CCCCGG GC GT CCA TGT ACA CA GAG AT -3' with *SmaI* site. The PCR thermal cycle reactions were consisted of denaturation at 95 º C for 30 s followed by 30 cycles at 95 º C for 15 s, 68 º C (for NS3) and 69 º C (for 5'UTR) for 35 s and 72 º C for 40 s, followed by a final extension at 72 º C for 5 minutes. The positive and negative controls prepared from Animal Health Veterinary Laboratories Agency (AHVLA) (a generous gift from Dr. Steinbakh and Dr. Dastjerdi) were used in each test.

Restriction enzyme digestion, ligation and colony PCR

The pWPI-Linker B lentiviral Plasmid had been prepared by the Research Center for Bio-Medical (IMIM) in Spain (a generous gift from Fabien Delaspre) and digested by *BamHI* (Roche, Germany, Catalog Number: 10220612001) and *SmaI* (Roche, Germany, Catalog Number: 10220566001) for cloning of 5'UTR. This plasmid had been also digested by *BamHI* for cloning of NS3. Therefore, 1 µl of 1 µg / µl plasmid, 1µl enzyme, 2 µl 10x enzyme buffer and nuclease-free water were used in a 20 µl reaction, after overnight incubation at 37 º C for single digestion and 20 º C for double digestion. Ligation reaction was performed with 2µl of T4 10x ligation buffer (Fermentas, Germany, Catalog Number: B69), 2µl of PEG-4000 50% (Fermentas, Germany), 1 µl of T4 DNA ligase (Fermentas, Germany, Catalog Number: EL0013), 150 ng of the inserting DNA and nuclease-free water up to 20µl. Then ligation products were transformed separately in the appropriate competent cell (Stbl4) by heat shock protocol. Briefly, 10 µl of ligation product was added to 100 µl of Stbl4, and the mixture was incubated for 30 minutes at 4 °C, 90 seconds at 42 °C and 5 min at 4 ° C. Consequently, 900 µl of LB medium was added and shaked at 200 rpm for 45 minutes. After centrifugation at 6000 rpm for 3 min, the pellet was cultured in LB agar with 100 mg / ml ampicillin. Plates were incubated at 37 ºC for 16-14 hours. In order to determine the presence or absence of the inserted DNA in plasmid constructs, 10 individual colonies from each plate were selected for PCR reaction and extraction of plasmid by using Mini preparation of DNA method. After electrophoresis in 0.8% agarose, the recombinant plasmids were sent for sequencing.

Lentiviral transduction of MDBK cells

The second-generation lentivirus packaging system (Addgene cat no. 12260 and 12259) was used to generate *BVDV NS3* and *BVDV 5'UTR* lentiviruses. Briefly, 293Tcells (3×106 cells per10 cm dish)

were co-transfected with a mixture of 4 μg of pMD2.G plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) envelope, 7.5 μg of the psPAX2 packaging plasmid, 11 μg of the BVDV NS3 - pWPI-Linker B and BVDV 5'UTR - pWPI-Linker B, using calcium phosphate-DNA precipitate method. The following day, the medium was replaced with 7ml of DMEM (Invitrogen) and further cultured for 72h. The supernatant was collected 48h and 72 h after transfection, cleared by centrifugation (3000 rpm, 15 min) and it was kept in -70 °C until the infection of MDBK cells. For the titration of the produced BVDV NS3 and BVDV-5′UTR lentiviral particles, the 293T cells (3×105 cells per each well of a 6-well plate) were transduced by 1 ml of suspensions containing these lentiviral particles. Then, the cells were analyzed for eEGFP expression using fluorescent microscopy and the viral titer was calculated. Consequently, 10^5 trypsinized MDBK cells were infected in a 6-well plate by lentiviral vectors (MOI = 0.8), diluted in 1ml of DMEM medium supplemented by 10% FBS and polyberene (8 μg/ml). The medium was replaced with 7ml of DMEM (Invitrogen) 12h after the infection. MDBK cells were examined 48 h and 72 h after infection, by using a fluorescent microscope.

RT- PCR to validate infection of MDBK cells

To confirm the expression of *BVDV-NS3*and *BVDV-5'UTR* in MDBK cells, RNA was isolated from infected cells and following treatment with DNase, RT- PCR was carried out using the designed primers. The sequences of primers were as follows: BVDV NS3 F: CAT AGG TAG GCG TGA CCC AAC -3'and BVDV NS3 R: 5'- TCA GTG ACC CTC AGT GCT GC -3'. BVDV 5'UTR F: 5'- AGG GTA GTC GTC AGT GGT TC -3' with and BVDV 5'UTR R: 5'- AGG TTA AGA TGT GCT TTG GG -3'. The PCR materials and methods were the same with the ones described in section 2.1.1. The RT-PCR thermal cycle programs were consisted of denaturation at 95 ° C for 2 min, followed by 30 cycles at 95 ° C for 30 s, 60 ° C (for *NS3*) and 55 ° C (for *5'UTR*) for 30 s and 72 ° C for 30 s, including a final extension step at 72 ° C for 5 minutes. The positive (MDBK infected with BVDV) and negative (MDBK cells without any infection) controls were employed in each test.

Western blotting

For western blotting analysis, protein extracts were prepared from cell cultures in lysis buffer (50 mM Tris–HCl pH = 8.0, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS,1 mM PMSF) for 20 min on ice. Protein extracts from positive and negative controls (MDBK cells infected with BVDV- NADL and MDBK cells without any lentiviral vector, respectively) were extracted too. All samples were vortexed for 10 min. Samples were centrifuged at 1000 g for 10 min at 4 ◦C. After Bradford and Lowry protein assay, 50 μg from the supernatant were added to sample buffer (Tris-HCl 62.5 pH = 6.8 mM, 15% SDS, 0.01% Bromophenol blue, 10% glycerol, mercaptoethanol 25%, water), boiled for 3 min and loaded in polyacrylamide Resolving [3.5 mL of Deionized water, 4.0 mL of 30% acrylamide:bis-acrylamide (29:1), 2.5 mL of 1.5 M Tris-HCl, 0.4% SDS, pH 8.8, 100 μL of 10% ammonium persulfate and 10 μL TEMED] and Stacking [2.1 mL of Deionized water, 0.63 mL of 30% acrylamide:bis-acrylamide (29:1), 1 mL of 0.5 M Tris-HCl, 0.4% SDS, pH 6.8, 30 μL of 10% ammonium persulfate and 7.5 μL TEMED] SDS - PAGE gels and then electrophoresed. After SDS-PAGE, proteins were transferred to PVDF membranes. Viral proteins were detected with a mouse anti *BVDV-NS3* monoclonal antibody (1:100) (Santa Cruz, America, Catalog Number: 101592sc) and rabbit anti-mouse IgG antibody conjugated with HRP (Santa Cruz, America, Catalog Number: 358914sc-) (1:10 000). As a positive cellular control, alpha tubulin was detected with primary anti alpha tubulin mouse monoclonal antibody (Sigma, America, Catalog Number: 9026T). To develop Western blot, we used an ECL detection system (Amersham, Piscataway, NJ) according to the manufacturer's directions.

RESULTS

RT-PCR

The presence of *BVDV- 5'UTR* and *BVDV- NS3* fragments was detected using RT-PCR. Positive PCR products and positive control sample in the PCR test for detecting BVDV- NS3, were in the size of 223 bp, while the size of the *BVDV- 5'UTR* -specific band was 380 bp. The validity of pWPI- Linker B digestion was tested by electrophoresis in 1% agarose gel (Fig 1).

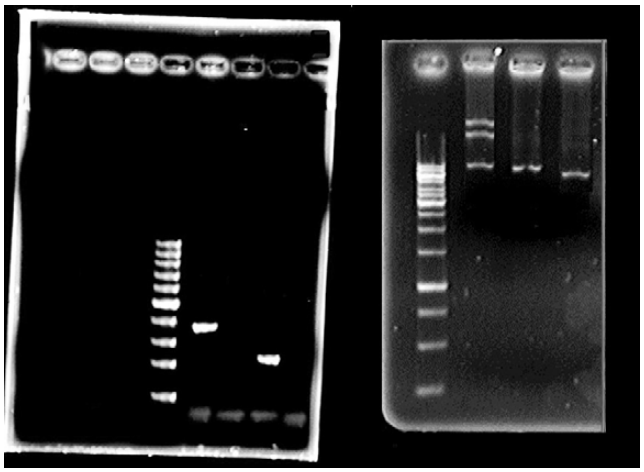

Fig 1. amplification of BVDV-NS3, BVDV – 5'UTR and digestion of pWPI-Linker B: Right: RT-PCR for amplification of BVDV-NS3 and BVDV – 5'UTR after inoculation of the virus into BT cell line: left to right: negative control for NS3, negative control for 5'UTR, 5'UTR band (380bp),Gene ruler(100bp), NS3 band(223bp).
Left: Single and double digestion of pWPI-Linker-B: left to right: double digestion of pWPI-Linker B, single digestion of pWPI-Linker B, pWPI-Linker B, Gene ruler(1Kb)

Colony PCR

After the overnight incubation of the transformed Stbl4 as well as the controls (positive and negative) on plates containing LB agar + ampicillin at 37 ° C, recombinant plasmid containing colonies and positive control colonies (containing pWPI-LinkerB plasmid resistant to ampicillin that confirmed in previous experiments) were observed, while in the negative control plate (only Stbl4) there weren't any colonies present.

The presence of *BVDV- 5'UTR* and *BVDV- NS3* fragments was detected in transformed colonies and positive control samples by using PCR (BVDV-NS3 bands were in the size of 223 bp, while the size of the BVDV- 5'UTR -specific band was 380 bp)

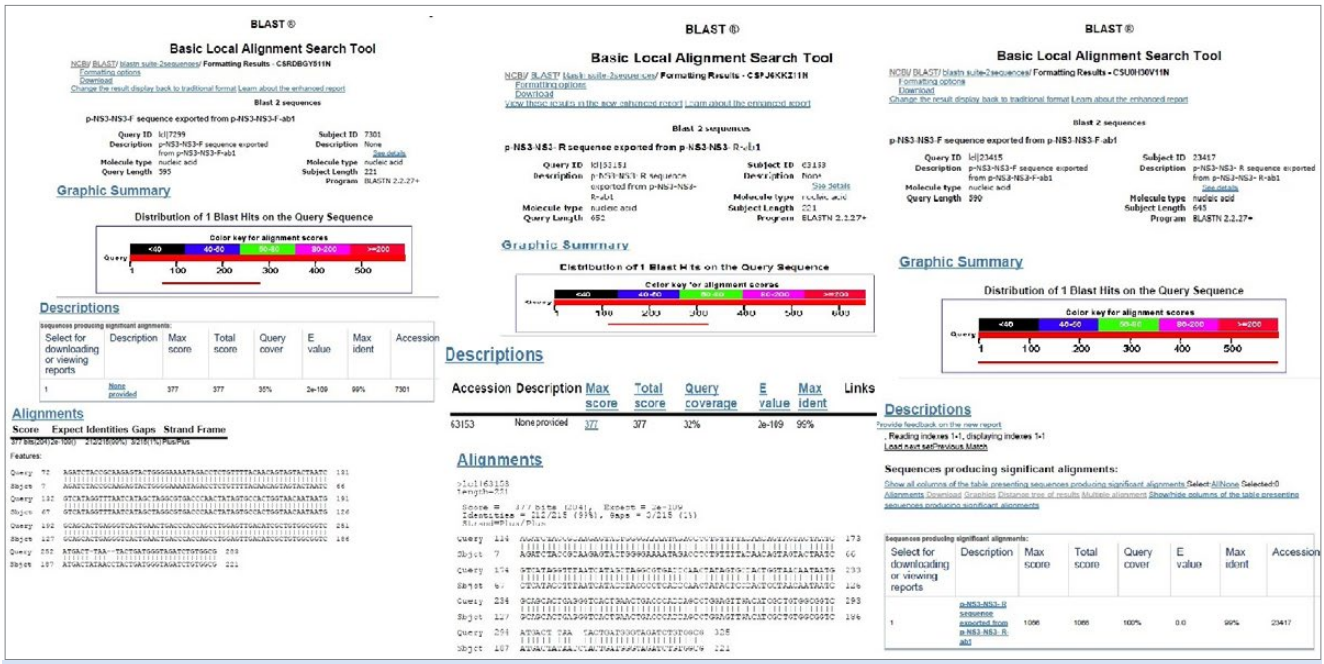

Fig 2. Alignment of the NS3 sequence derived from sequencing with the desired amplicon.Left: Single and double digestion of pWPI-Linker-B: left to right: double digestion of pWPI-Linker B, single digestion of pWPI-Linker B, pWPI-Linker B, Gene ruler(1Kb)

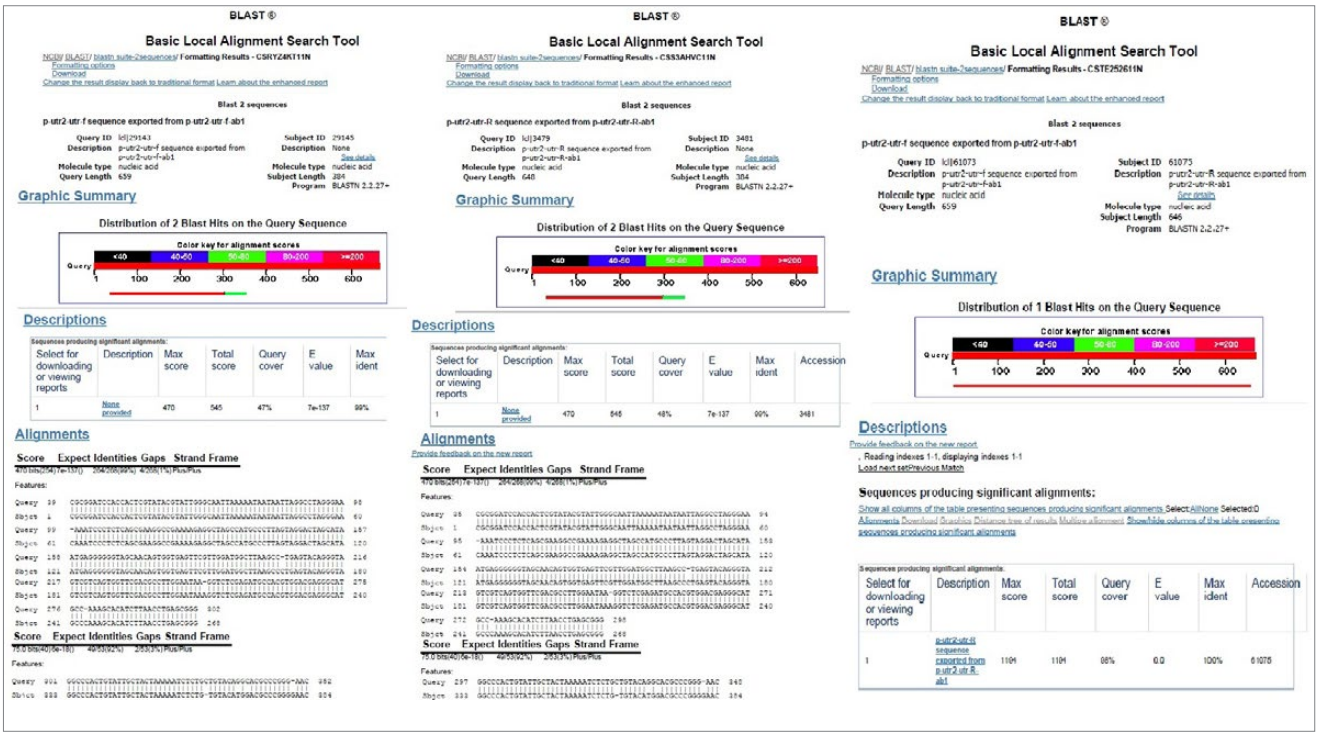

Fig 3. Alignment of the 5'UTR sequence derived from sequencing with the desired amplicon.

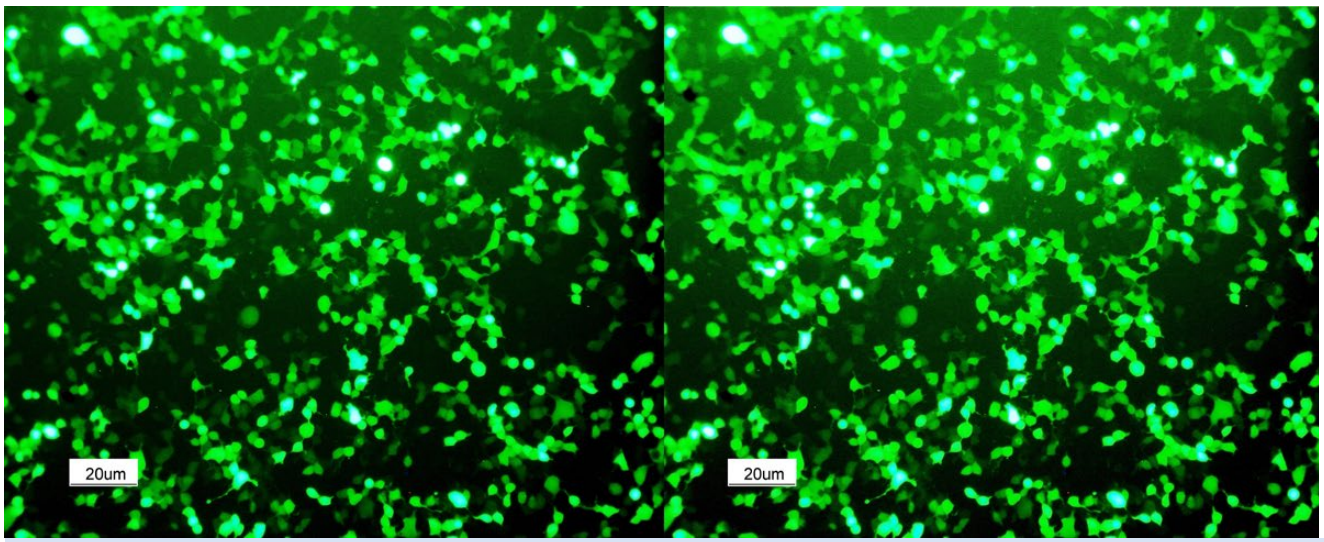

Fig 4. transfection of 293T cells with the lentiviral plasmids expressing the BVDV NS3 and BVDV 5'UTR
Right: EGFP levels observed after transfection of pWPI-Linker B-5'UTR in a second-generation lentiviral packaging system.
Left: EGFP levels observed after transfection of pWPI-Linker B- NS3 in a second-generation lentiviral packaging system.

After plasmid extraction from positive colonies, the validity of plasmid extraction and the presence of BVDV- NS3 and BVDV- 5'UTR fragments in the recombinant plasmids, were confirmed by digestion and electrophoresis. Sequence analysis of the recombinant plasmids revealed the presence of *BVDV- NS3*and *BVDV- 5'UTR* (Figs 2 and 3).

entivirus production and infection

72 hours after transfection, the percentage of EGFP expressing 293 T cells was approximately 80-70%. That percentage suggested that the transfection effi-

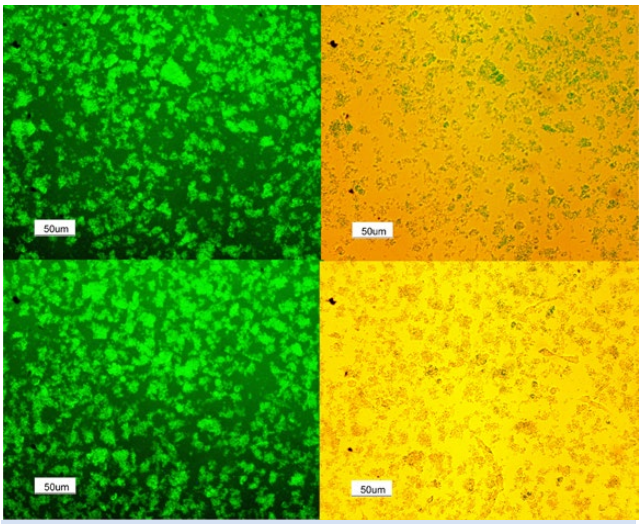

Fig 5. Infection of MDBK cells with lentiviral vectors expressing BVDV NS3 and BVDV 5'UTR:
Images above: Right: EGFP expression after infection with lenti-vector carrying BVDV NS3 - Left: The same picture by using light microscopy.
Images below: Right: EGFP expression after infection with lenti-vector carrying BVDV NS3 - Left: The same picture by using light microscopy.

Fig 6. RT-PCR to confirm the expression of BVDV 5'UTR and BVDV NS3 in MDBK cells:
Left to right: MDBK-NS3, positive control: BVDV NS3, negative control for NS3, Gene ruler (100bp), MDBK 5'UTR, positive control: BVDV 5'UTR, negative control for5'UTR

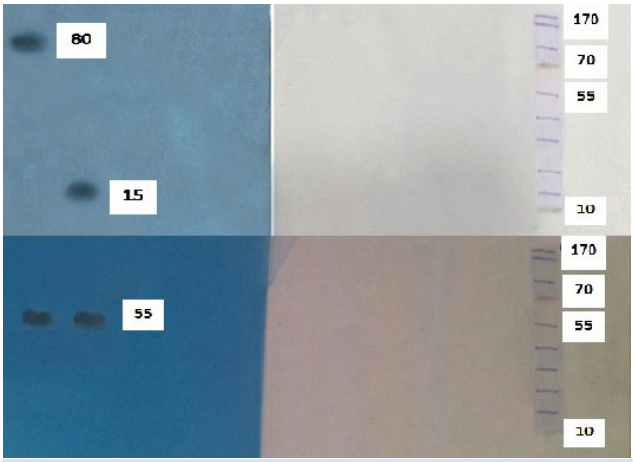

Fig 7. Western blot to confirm the expression of BVDV NS3 in MDBK cells:
Top row: BVDV-NS3 band with the size of 80 kDa (left) and MDBK-NS3 serine protease as much as approximately 15 kDa (right)
Bottom row: The 55 kDa band corresponding to alpha Tubulin in negative controls, positive controls (BVDV-NS3) and test sample (MDBK-NS3) (left to right)

ciency was appropriate for infection. By fluorescence microscopy observation, efficiency was also proved to be excellent (more than 90% EGFP expression). Thus, the MDBK cells infected with BVDV- NS3 - lentivector and BVDV- 5'UTR- lentivector were considered as cell clones expressing BVDV NS3 and BVDV 5'UTR (Figs 4-5).

RT- PCR to validate infection of MDBK cells

The expression of *BVDV- 5'UTR* and *BVDV- NS3* was detected in MDBK cells infected by lentiviral vectors expressing these transgenes and positive control (MDBK cells infected with BVDV) using RT-PCR tests (*NS3* band: 87bp and *5'UTR* band: 68bp) (Fig 6).

Western blotting

Viral product was detected with rabbit anti mouse IgG against *BVDV-NS3* (1:100). The specific band related to selected sequence of *NS3* was approximately 15 KD. As loading control, alpha tubulin was detected with a mouse monoclonal antibody (1:1000) and its specific band was 55 KD (Fig 7).

DISCUSSION

BVDV establishes a persistent infection in cattle populations worldwide, often resulting in significant economic impacts. Although the control measures for eradication, namely selective test and slaughter as well as vaccination, are widely used, BVDV remains prevalent due to its diversified antigenicity. The efficiency and the specificity of RNAi are attractive therapeutic characteristics that may prove useful for the development of antiviral therapies (Lambeth et al., 2007; Zemke et al., 2010; Jordao et al., 2011). Constant changes in the antigenic regions of BVDV structure, particularly C, Erns, E1, and E2 antigens and the formation of scape mutants that evade the host immune response, have made the production of an effective vaccine difficult. The majority of BVDV vaccines are designed against the previously mentioned glycoproteins and proteins, and this is a crucial factor for the medium or low success to control or eradicate the disease (Meyers and Theil, 1996; Lambeth et al., 2007; Zemke et al., 2010; Jordao et al., 2011; Ni et al., 2012).

The aim of this study was to provide a suitable cell line expressing the genes of BVDV in order to evaluate the new antiviral treatment strategies, demonstrate their effectiveness and satisfy the methods for controlling or treating BVDV.

Gene therapies performed with the aim of antiviral treatments against Flaviviridae were targeted at almost all regions of the viral genome; however, due to the conservation of the 5'UTR sequences during the viral evolution and the critical tasks of NS2-3, NS3, NS5A, and NS5B, these parts have received more attention. Considering the previous studies with respect to the inhibitory efficacy of targeting each of these Flaviviral genomic sequences, NS3 and 5'UTR were selected for the cloning into lentiviral plasmid (Lv et al., 2003; Randall et al., 2003, Randall et al., 2004; Lambeth et al., 2007; Gamlen et al., 2010).

Moreover, NS3 and 5'UTR were selected because of their conservation during virus evolution and due to their specific biological roles in cell proliferation, survival, and virulence of the virus. In addition, for stable and long-term expression of the desired genes in the target cells, the lentiviral vector was utilized for transduction. Transfection of plasmid vectors has also been used by researchers to induce gene expression in the target cells, but with this method, the duration of gene expression is about 4-10 days. Lentiviral vectors are specific types of retroviruses that can infect both dividing and non-dividing cells because their pre-integration complex (virus "shell") can get through the intact membrane of the nucleus of the target cell. Lentiviruses can be used to provide highly effective gene therapy, as they can change the expression of their target cell's gene for up to six months. They can also be utilized for non-dividing or terminally differentiated cells, such as neurons, macrophages, hematopoietic stem cells, retinal photoreceptors, muscle and liver cells, to which the previous gene therapy methods could not be applied. Employing lentiviral vectors (LVs) offers multiple advantages for gene therapy, because they encompass the efficient delivery, the ability to transduce the proliferating and resting cells, the capacity to integrate into the host chromatin to provide the stable long-term expression of the transgene, the absence of any viral genes in the vector, and finally the absence of interference from pre-existing viral immunity. In addition, LVs can transfer large nucleotide sequences (3000 bp). As a result, they are preferred to other viral vectors such as adenoviral vectors, retroviral vectors, and AAVs. Moreover, due to the lower frequency of integration at or near the cellular proto-oncogenes, the possibility of mutagenesis and carcinogenesis as a result of gene induction by lentiviral vectors, is low (KuÈmmerer et al., 2000; Haga et al., 2006; Henry et al., 2006; Salmon and Trono, 2006; Tiscornia et al., 2006).

The genetic and antigenic diversity of BVDV is a major concern for the development and the efficacy of current vaccines. Thus, anti-BVDV drugs might be an alternative strategy to control BVD. Over the past decade, in vitro anti-BVDV therapies have been conducted on cell systems

with either sub-genomic replicon or infectious viral particle of BVDV as the target. The BVDV sub-genomic replicon contains only non-structural regions, and the anti-viral therapeutic intervention of the structural region of BVDV cannot be studied. As for the infectious viral particles of BVDV, the time-consuming, labor-intensive, quantitative reverse transcriptase PCR methods have to be applied. A screening tool targeting both structural and non-structural proteins of BVDV and supporting high through-put anti-BVDV drug screening can be a monocistronic reporter virus with an enhanced green fluorescent protein (eEGFP) as a marker. The positive-oriented, single-stranded RNA viruses can be reconstituted, using reverse genetics strategy that provides a powerful tool for investigation of many aspects of the viral life cycle and pathogenesis. Numerous recombinant viruses and sub-genomic replicons have been generated by Flaviviridae family from viral cDNA clones and have been extensively employed as tools for antiviral drug screening. As for BVDV, efforts have been made by a few research groups to achieve a stable, easy-handling, reverse genetics system. Over the past decade, several milestones have been reached in the development of cell-based generation of recombinant BVDV. For example, the employment of a Bacterial artificial chromosome (BAC) vector stabilizes the viral genome during the construct replication in bacteria. It was previously found that BVDV is a suitable viral vector for the expression of heterologous proteins such as eEGFP when inserted between viral Npro and capsid proteins. In the study done by Fan and Bird in 2012, a stable, reverse genetic system was created on the basis of an infectious BAC cDNA clone (pBSD1) that has an NCP BVDV strain SD-1 background. A recombinant reporter BVDV was produced, stably expressing eEGFP protein as inserted between viral NS3and NS4A proteins. Despite the huge advantages of monocistronic reporter BVDV for screening anti-BVDV drugs, this screening system has some disadvantages and limitations as well. First, it takes time to design and produce this screening system accurately, and great bioinformatics and biotechnological knowledge are essential to prepare it. The selection of an appropriate insertion site for the marker gene is critical for the development of a recombinant reporter BVDV for antiviral drug screening, because the genotype and the phenotype of the recombinant virus should not be impaired by eEGFP or any other reporter gene insertion. Furthermore, the reporter gene must not affect the replication of the recombinant virus, which shows a same level of RNA and viral protein expression as the parent virus; in addition, the peak yield of progeny virus of the recombinant should not be less than the parent virus. The replication kinetics of the recombinant reporter BVDV and that of the parent virus should be similar. The reporter gene expression and its fluorescent signal intensity are correlated with the replication of the recombinant reporter BVDV. In many cases due to weaknesses in the design of monocistronic reporter virus, the fluorescence intensity is less than the amount approved for careful screening of antiviral drugs. Moreover, owing to the diversity and abundance of genetic changes that occur in the genome of RNA viruses, it is necessary to have the valid parent virus or its cDNA clone in order to produce the reporter virus (KuÈmmerer et al., 2000; Wilson et al., 2005; Carmona et al., 2012; Fan and Bird, 2012). Because of the limitations and difficulties mentioned in the design and the production of recombinant monocistronic viruses, lentiviral vectors were utilized to induce the expression of target genes of BVDV.

CONCLUSIONS

Therefore, in this study, MDBK cells expressing BVDV NS3 and BVDV 5´UTR were produced, using lentivector transduction. The results indicated that the development of MDBK cell line expressing these transgenes, acted very sensitive for anti-BVDV therapies screening. That cell line has been tested by our group for gene therapies based on RNAi against BVDV (unpublished data).

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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

First report of *Angiostrongylus vasorum* in red foxes (*Vulpes vulpes*) in Serbia

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ABSTRACT. Angiostrongylosis caused by *Angiostrongylus vasorum* is an emerging disease in Europe and the red fox (*Vulpes vulpes*) is considered as a main reservoir species for this parasite. Since there have been no reports of *A. vasorum* in red foxes in Serbia at the time of carrying out our investigations, the aim of the investigations was to explore the role of red foxes in South Banat (northern Serbia) as reservoirs for *A. vasorum*. Legally hunted foxes were autopsied in the Veterinary Specialised Institute "Pančevo". The heart, lungs and pulmonary artery were examined macroscopically for evidence of gross lesions and for the presence of adult specimens of *A. vasorum*. Impression smears of the changed lung tissue were examined microscopically for the presence of first stage larvae of *A. vasorum* and histopathological examination was performed on lung samples. Out of 24 examined foxes hunted in different locations, 13 had lesions manifested in the lungs, which were suspected to be indicative of angiostrongylosis. In the majority of the foxes distal parts of the pulmonary lobes were swollen, firm, and discoloured to dark-red, dark-yellow and dark-brown. The characteristic lesions in distal parts of the pulmonary lobes were completely consistent with the presence of adult parasites in the right heart and pulmonary arteries, and with the presence of the first stage larvae in the impresion smears. The present finding contributes to the knowledge of geographic distribution of angiostrongylosis in red foxes in Europe and provides valuable information that should raise awareness in veterinarians to consider this parasitosis in dogs with signs of cardiopulmonary diseases.

Keywords: *Angiostrongylus vasorum*, angiostrongylosis, red foxes, Serbia

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INTRODUCTION

Angiostrongylosis caused by metastrongyloid nematode *Angiostrongylus vasorum* (the French heartworm) is an emerging disease in Europe (Traversa et al., 2010). *Angiostrongylus vasorum* has an indirect life cycle, residing in its adult form in the pulmonary arteries and right heart of domestic dogs (*Canis lupus familiaris*) and wild carnivores of the family Canidae, in particular the red fox (*Vulpes vulpes*), recognized as an important reservoir of the parasite (Bolt et al., 1994).

The oviparous female lays eggs that hatch to the first-stage larvae (L1). The L1 penetrate into the alveoli and are coughed up, swallowed and excreted into the environment with the feces, where they can potentially infect intermediate hosts. The intermediate hosts are snails and slugs, in which the L1 larvae develop to the third-stage larvae (L3), infective for definitive and paratenic (transport) hosts (Guilhon, 1963; Morgan et al., 2005; Koch and Willesen, 2009; Elsheikha et al., 2014). Definitive hosts acquire the infection through ingestion of an intermediate or paratenic host or through ingestion of free L3 present in the environment (Elsheikha et al., 2014).

Although *A. vasorum* was discovered for the first time in 1853 (Serres, 1854), reports of the disease in Europe have been increasing only over the past three decades. Possible reasons should be found in the growing awareness among practitioners and researchers, and in the increased prevalence of the parasite (Traversa et al., 2010). These first reports of angiostrongylosis in red foxes from European countries came from France, Ireland, Switzerland, and Italy (Poli et al., 1984). Currently, *A. vasorum* has a worldwide distribution, although it usually maintains in small enzootic foci (Helm et al., 2010). Canine angiostrongylosis is considered endemic in certain areas of Europe, including regions of Denmark, Germany, Hungary, Finland, France, Ireland, Italy, the Netherlands, Poland, Slovakia, Spain, Sweden, Switzerland, Turkey and the United Kingdom (Elsheikha et al., 2014).

The distribution of *A. vasorum* in red foxes in Europe is well investigated in some countries (Saeed et al., 2006; Morgan et al., 2008; Schug et al., 2013; Eleni et al., 2014; Härtwig et al., 2015; Santoro et al., 2015; Tolnai et al., 2015; McCarthy et al., 2016) while on the other hand, there are still many uninvestigated regions. At the time of carrying out our investigations, the disease was reported in Serbia in a dog in the region of Srem (Simin et al., 2014) and in a golden jackal in the region of South Banat (Gavrilović et al., 2017). Since there have been no reports of *A. vasorum* in red foxes, the aim of the investigations was to explore their role as reservoirs for *A. vasorum* in two selected hunting grounds in South Banat (northern Serbia).

MATERIAL AND METHODS

The investigation area

The investigations were conducted in the area around the City of Pančevo (100,000 inhabitants), located at the confluence of the Tamiš and Danube rivers, and only 17 km distant from Belgrade, the capital of Serbia. Pančevo is the administrative center of South Banat, one of the Administrative Districts of Vojvodina Province. A northern part of Serbia was chosen as the target area because in previous investigations carried out in the Scientific Veterinary Institute of Serbia, during a two year period from 2015 to 2017, 192 specimens of red foxes, golden jackals and wolves from central and southern Serbia tested negative for the presence of *A. vasorum* (Pavlović, unpublished data).

Animals

Twenty four foxes legally hunted at different locations around the City of Pančevo during a three month period from December 2016 to February 2017 were delivered to the Veterinary Specialised Institute "Pančevo", the Department of Pathology and Parasitology within the Programme for Control and Eradication of Rabies in Serbia. The foxes originated from two hunting grounds: Nadel, whose area is 38,682.47 ha and Stari Tamiš, with an area of 33,211.23 ha. Twelve foxes from each hunting ground were presented for examination.

Methods

Twenty four foxes were necropsied and the heart, pulmonary artery and lungs were examined macroscopically for evidence of gross lesions and the presence of adult specimens of *A. vasorum*. Impression smears of the macroscopically changed lung tissue were examined microscopically for the presence of the first stage larvae of *A. vasorum*. The adult parasites and first stage larvae were identified on the basis of the characteristic

morphological features described by Guilhon and Cens (1973) and McGarry and Morgan (2009), respectively.

Macroscopically changed lungs were sampled for histopathology. For histopathological examination tissue samples were fixed in 10% buffered formalin. After processing in an automated tissue processor, the samples were embedded in paraffin blocks. Approximately 5 μm thick paraffin sections were stained using the standard haematoxylin-eosin (HE) method.

RESULTS

Of the 24 examined foxes, hunted in different locations around Pančevo, in 13 of them lesions suspected to be indicative of angiostrongylosis were found in the lungs. Distal parts of the pulmonary lobes were swollen, firm, and discoloured to dark-red, dark-yellow and dark-brown and occasionally with whitish areas (fibrosis). The cut surface of the changed lung tissue was dry and granular. In the majority of the foxes lesions were seen in the periphery of the lung lobes, while in one fox the majority of the right diaphragmatic lobe was grossly affected (Fig. 1a).

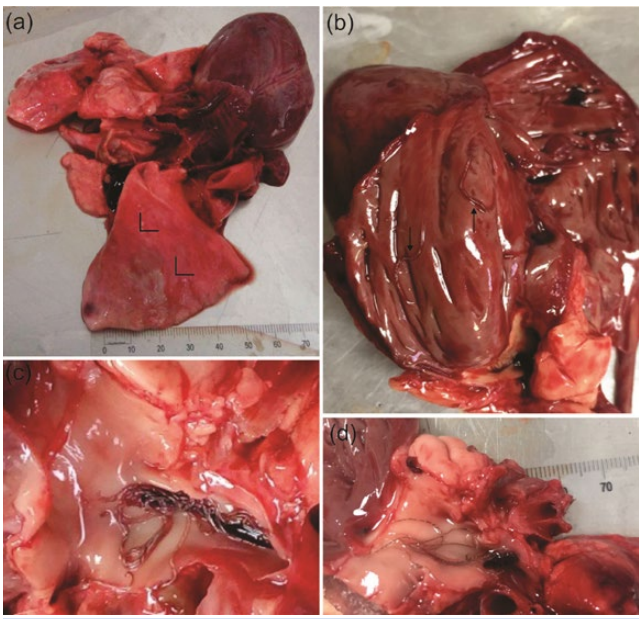

Fig 1: Gross lesions in the lungs and the presence of adult *Angiostrongylus vasorum* in the heart and pulmonary artery: (a) The majority of the right diaphragmatic lung lobe is swollen and discoloured; (b) Two adult parasites in the right heart chamber; (c) Numerous adults of *A. vasorum* in the pulmonary artery curled up around each other; (d) Adult females in the pulmonary artery recognized by the "barber pole" appearance

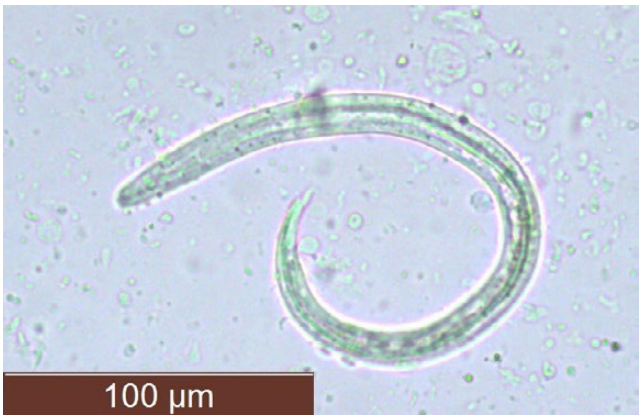

Fig 2: A first stage larva of *Angiostrongylus vasorum* from the lungs of an infected fox

Adult parasites were found in the right ventricle (Fig. 1b) and pulmonary arteries (Fig. 1c, d) in all the 13 foxes that had gross lesions in the lungs. In the other foxes, in which the lungs were without gross lesions, no adult parasite was found in the right heart or pulmonary arteries. Microscopy of impression smears of the changed lung tissue found the first stage larvae of *A. vasorum* (Fig. 2).

Histopathology revealed parasitic larvae and eggs in the lung tissue (Fig. 3). Histopathological lesions included vascular damage, haemorrhage, haemosiderosis, multifocal granulomas, generation of fibrous tissue in the alveolar septa, and hyperplasia and hypertrophy of the *tunica muscularis* of the arteries containing adult parasites.

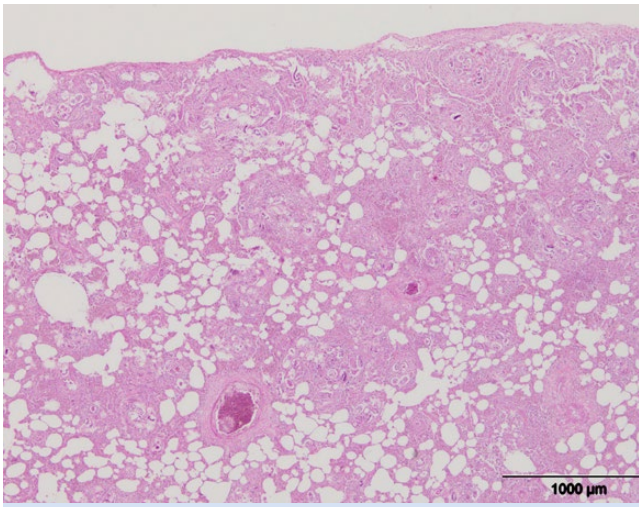

Fig 3: Histopathological lesions in the lungs of a fox infected with *Angiostrongylus vasorum*. Eggs and larvae of the parasite in the lung tissue (HE staining)

DISCUSSION

The present finding is, to the best of our knowledge, the first report of enzootic angiostrongylosis in red foxes in Serbia. The disease was diagnosed in the majority of foxes from both investigated hunting grounds. As the agent was found in 13 of the 24 examined foxes (54.17%) the results indicate a high prevalence of the infection in the area of investigation.

In countries neighboring Serbia, a prevalence of 17.9% has been reported in the fox population in Hungary (Tolnai et al., 2015), and less than 5% in the fox population in Croatia (Rajkovic-Janje et al., 2002). The overall prevalence of 7.3% has been reported in the UK fox population varying widely between regions, from 0% in Scotland and northern England to 23% in south-east England (Morgan et al., 2008). In the Republic of Ireland the incidence of infection was found to be 39.9% with positive samples occurring in each of the country's 26 counties (McCarthy et al., 2016). In Denmark, recognized as an endemic area, *A. vasorum* was recorded in 48.6% of foxes (Saeed et al., 2006). The disease has recently been diagnosed in German red foxes, showing regionally differing prevalences of 8.4 %, 19.1 %, 27.3%, and 9% for Thuringia, Hesse, Rhineland-Palatine, and the Federal State of Brandenburg, respectively (Schug et al., 2013; Härtwig et al., 2015). In Portugal, *A. vasorum* was found in 16.1% of 62 red foxes (Eira et al., 2006). A prevalence of 23.88% has been found in red foxes in Tuscany (Poli et al., 1984), 43.5% in Central Italy (Eleni et al., 2014), 33.3% in southern Italy (Santoro et al., 2015), and 78.2% in north-west Italy (Magi et al., 2015) . Prevalence in red foxes in Poland in the Forest District Głęboki Brod in Augustowska Primeval Forest has been estimated at 5.2% (Demiaszkiewicz et al., 2014). The prevalence was estimated based on necropsy and examination of the heart and lungs in all the studies except for the study by Härtwig et al. (2015) who used real-time PCR.

Pathomorphological lesions are comparable to those described by Poli et al. (1984) who found in the lungs large wedge-shaped areas of reddish-brown coloration with increased consistency. Similarly, Santoro et al. (2015) described reddish-brown and yellow-brown foci with increased resistance to slicing and scattered areas of emphysema. Eleni et al. (2014) found severe pulmonary lesions in 25.9% of examined dead foxes in Italy. In three foxes, almost the whole diaphragmatic lobes have been consolidated, similar to our finding for one fox in which the majority of the right diaphragmatic lobe was consolidated. Unlike the present study, the samples originated from dead animals in which the lesions would have been much more pronounced.

The red fox is the natural reservoir of *A. vasorum*, capable of sustaining the infection without serious clinical consequences (Eleni et al., 2014). However, considering the severe gross lesions in the lungs (Fig 1a) and the high level invasion in some of the animals examined in the present study (Fig 1c), angiostrongylosis should be considered as a disease that may affect the health status of the red fox population. Such an opinion is supported by Eleni et al. (2014) who found remarkable gross lesions in the lungs of dead foxes in Italy and, at least for three specimens, angiostrongylosis was considered the cause of death.

Out of 24 foxes examined in this study, 13 were found to have the first stage larvae of *A. vasorum* in the lungs which implies that they would have excreted larvae and transmitted the infection to their habitats. The Baermann's diagnostic method which would prove this statement was not used in the study. As the red fox frequently comes close to cities and urban areas in which there are dogs, there is a high probability for this parasitosis to be transmitted to dogs in which the infection can be fatal (Benda et al., 2017). Dogs from Pančevo and nearby places including Belgrade - the capital of Serbia - should, therefore, be considered under the high risk from angiostrongylosis. Stray dogs and hunting dogs visiting the habitats of red foxes are under a higher risk of infection.

Pathomorphological findings observed in the lungs of infected foxes show that the infection has a chronic course, and confirm the role of the red fox as an important reservoir and transmitter of angiostrongylosis in the target area of northern Serbia. Until 2013, when angiostrongylosis was diagnosed in a dog from the region of Srem, there had been no data related to the presence of *A. vasorum* in Serbia (Simin et al., 2014). Considering the high prevalence in red foxes as the main reservoir species in the area of investigation, it may be assumed that the disease is prevalent and neglected in Serbia. A lack of awareness among practitioners in Serbia of the presence of the agent could have resulted in past misdiagnosis of the disease in dogs. Such an opinion is supported by the findings of Schnyder et al. (2015) who

found 1.36 % *Angiostrongylus* antibody- and antigen-positive dogs in Hungary, bordering Serbia in north. On the contrary, some authors believe that relatively high level of pathogenicity associated with *A. vasorum* infection makes it unlikely that clinical cases of the disease have been missed in the past years (Traversa et al., 2010). Based on the present investigations it is very possible for the disease to have existed in Serbia, at least in red foxes, for many years before the first case was diagnosed in a dog in 2013.

The trend towards a higher incidence of angiostrongylosis observed in some European countries and the concomitant expansion in foxes strongly indicate that the parasite is actually emerging in Europe (Morgan et al., 2009; Helm et al., 2010; Traversa et al., 2010). Recently, the first case of aelurostrongylosis in a kitten was reported in the investigation area (Gavrilović et al., 2017). This is also a gastropod-borne disease with a similar life cycle that completes in the cat as the definitive host. The finding of aelurostrongylosis in a cat, coupled with the finding of angiostrongylosis in red foxes, leads to the conclusion that gastropod-borne parasitoses are emerging in South Banat which seems to provide suitable conditions for the completion of the life cycle of both *A. abstrusus* and *A. vasorum*. The life cycle and the dynamics and activity of the population of gastropod intermediate hosts are mainly influenced by temperature, moisture and water availability (Traversa et al., 2010; Traversa and Di Cesare, 2014).

CONCLUDING REMARKS

The present investigations confirmed that the target area in South Banat, northern Serbia should be considered as an enzootic focus of angiostrongylosis, contributing to the knowledge of geographic distribution of the disease in red foxes in Europe. The paper provides strong evidence for veterinarians to consider angiostrongylosis in dogs with signs of cardiopulmonary disease in Serbia and other uninvestigated countries. Further investigations should be carried out in order to assess the true prevalence of *A. vasorum* in red foxes, other wild canids and domestic dogs in Serbia.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Association of polymorphisms in the *CSN2*, *CSN3*, *LGB* and *LALBA* genes with milk production traits in Holstein cows raised in Turkey

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ABSTRACT. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) test was performed on DNA samples extracted from blood samples of 189 Holstein Friesian cows to detect genotypic distribution of polymorphic markers in the bovine beta-casein (*CSN2*), kappa-casein (*CSN3*), beta-lactoglobulin (*LGB*) and alfa-lactalbumin (*LALBA*) genes responsible for milk production traits. Statistical analysis was carried out using least square methods of the general linear model (GLM) procedure. *CSN2* locus was significantly associated with the following traits: 305-day milk yield, days before peak milk production, fat percentage and protein yield. There was no significant effect of *CSN3*, *LGB* and *LALBA* markers on the traits analysed. These results suggested that *CSN2* marker may be evaluated in selection programmes regarding not only milk content but also milk production.

***Keywords*:** cattle, polymorphic marker, milk production, association

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INTRODUCTION

Advances in molecular technics, will ultimately provide researchers and breeders a better knowledge of the locations and effects of major loci, and evaluating of their associations with phenotypic traits that contribute to variation in quantitative traits (Spelman and Garrick, 1997). In this context, the use of genetic markers to increase the frequency of desired genotypes in herds, based on selection in breeding programmes, can be used to determine properties that are essential to achieve economical benefits in dairy cattle. The development of genotypic selection has enhanced the use of genome-wide dense marker data to reduce generation intervals depends mainly on the accuracy of genomic breeding values (Habier et al., 2010).

In recent years, genes associated with milk production traits have been identified and single nucleotide polymorphisms (SNPs) of many candidate genes have been specifically determined. On the one hand, some studies have focused on associations between phenotypes of cows and their corresponding genotypes and on the other hand, some of them evaluated the relationships between estimated breeding values of bulls and their respective genotypes (Boettcher et al., 2004). Bovine milk protein largely (± 90 % wt/wt) consists of six major milk proteins that are products of the α-LA, β-LG, αS1-CN, αS2-CN, β-CN, and κ-CN genes (Heck et al., 2009). Composition of bovine milk is highly affected by season, stage of lactation, feeding, and health status of the cow but is also is predominantly determined and regulated by genomic structure (Bobe et al., 1999). Therefore, candidate gene studies have examined the effects of the casein (*CN*), beta-lactoglobulin (*LGB*) and alfa-lactalbumin (*LALBA*) genes on milk production traits. Among them, *CN* genes, including β-CN and κ-CN, are located at chromosome 6 within a region of about 200 kb (Ferretti et al., 1990; Ikonen et al., 2001). The *CSN2* gene encodes the β-casein which is a major milk protein in most mammals and this gene is generally induced by lactogenic hormones bound to its promoter. In addition, the expression of the *CSN2* can be enhanced by signal transducers and activators of transcription (STAT) and glucocorticoid receptor (GR) (Lee et al., 2008). *CSN2* gene has 12 known genetic variants in the coding sequence of the gene including A, B, C (Aschaffenburg, 1961), A1, A2, A3 (Kiddy et al., 1966), D (Aschaffenburg et al., 1968), E (Voglino, 1972), F (Visser et al., 1991), X (Visser et al., 1995), G (Chin and Ng-Kwai-Hang, 1997), H (Han et al., 2000), I (Jann et al., 2002). The most common variants of β-casein in dairy cattle breeds are A1 and A2, while B is less common, and A3 and C are rare (Farrell et al. 2004; Keating et al. 2008). A1 variant differs from the A2 variant at position 67 where a histidine replaces a proline (Miluchova et al., 2009). Multiple genome scans have reported presence of significant quantitative trait loci (QTL) in this region that affect milk productions traits (Ikonen et al., 2001; Olsen et al., 2002; Viitala et al., 2003, Huang et al., 2012). The total size of the *CSN3* gene is about 13 kb and this gene has 12 genetic variants including A, B, B2, C, E, F, F1, G, H, I, A(1), and J (Kaminski, 1996; Prinzenberg et al., 1999; Sulimova et al., 2007; Sulimova et al., 1992). However, most of these variants are rare; they are found in only a few breeds and usually at low frequencies (Sulimova et al., 2007). A and B are the most common genetic variants in dairy cattle (Mitra et al., 1998). Variants A and B of the *CSN3* gene differ from each other in two amino acid substitutions: threonine (Thr)/isoleucine (Ile) at position 136 and aspartic acid (Asp)/alanine (Ala) at position 148 (Grosclaude et al., 1972). The *CSN3* gene encodes milk protein that is important for the structure and stability of casein micelles (Alexander et al., 1988). Alleles A and B of the gene, have been reported to be associated with milk yield, milk protein content and milk quality (Boettcher et al., 2004; Caroli et al., 2004; Kucerova et al., 2006). The *LGB* gene is located on bovine chromosome 11 and encodes the main protein of whey (Eggen and Fries, 1995). This gene has a total of 15 variants with alleles A and B being the most frequent (Matejicek et al., 2007). The mentioned variants differ by two amino acid substitutions in the polypeptide chains and 2 single nucleotide substitutions in the bovine *LGB*. In this context, variant A has aspartic acid (GAT) and valine (GTG) at positions 64 and 118, respectively, whereas variant B has glycine (GGT) and alanine (GCG) (Patel et al., 2008). Results of studies on the effect of *LGB* genotypes on milk production traits have been rather more consistent compared to studies of other milk protein polymorphisms. Thus, this gene was suggested as a genetic factor influencing mainly milk composition and milk quality and especially B allele was recognized as superior for milk quality in European cattle breeds. Allele A has been associated

mostly with milk yield parameters (Strzalkowska et al., 2002; Matějíček et al., 2007; Czerniawska-Piatkowska et al., 2011). In addition, the AA genotype of *LGB* has been shown to have a significant effect on protein yield (Sabour et al., 1996; Lunden et al., 1997), whereas the BB genotype has been associated with fat content (Ron et al., 1994; Tsiaras et al., 2005; Czerniawska-Piatkowska et al., 2011). The bovine *LALBA* gene, mapped to chromosome 5, is considered a candidate gene for milk performance traits and technological properties of milk (Kaminski et al., 2002). *LALBA*, which encodes one of the major milk whey proteins, has two genetic variants namely A and B. Variant A differs from B by a glutamine (Gln) for arginine (Arg) substitution (Sashikanth and Yadav, 1998). Variations in the *LALBA* gene have been associated with lactose concentration, milk yield, protein yield, and fat yield (Bleck, 1993; Bleck and Bremel, 1993; Voelker et al., 1997; Bleck et al., 1998; Martins et al., 2008).

Among known genes that affect milk production, milk proteins have received considerable attention in recent years because of possible associations between the genotypes and economically important traits in dairy cattle (Peñagaricano and Khatib, 2012). It is important to note that studies on the significance of milk protein polymorphisms have been carried out by many authors and inconsistencies in the outcomes, however, have prevented clear conclusions from being made. Further genetic studies focusing on milk yield and quality are needed to achieve efficient selection procedures. Therefore, the aim of the study was to investigate the effects of *CSN2*, *CSN3*, *LGB* and *LALBA* genes on milk production traits in Holstein cows raised in Turkey.

MATERIALS AND METHODS

Animals and management

Data from 189 purebred Holstein cows randomly selected from a commercial herd located in South Marmara region (40° 15' 09.5" N and 28° 17' 59.9" E) were used in the present study. The study protocol was approved by the ethics committee of by the Uludag University Local Ethics Committee for Animal Research (Approval number: 2010-08/06). The animals were housed in three free-stall barns and fed with the same diets according to standard commercial practices. All cows were milked three times a day when allowed free access to milking parlors in which they were equipped with electronic devices that automatically recorded the quantity of milk. Milk yield for each cow at each milking was recorded using DeLaval's Alpro Herd Management System (DeLaval International, Tumba, Sweden). Lactation milk yield, 305-day milk yield, days before peak milk yield and peak milk yield were evaluated to test possible associations of *CSN2*, *CSN3*, *LGB* and *LALBA* gene polymorphisms with milk production traits. The phenotypic data set was defined based on milk production traits availability in a way that would describe a complete picture of a productive history for a cow for four lactations. 305-day milk yield was calculated for each cow based on total milk yield (Lucy et al., 1993). Milk samples, obtained thrice a day seperately, were analyzed monthly for fat protein, and lactose content by infrared analysis with a Bentley 2000 midinfrared instrument (Bentley Instruments, Chaska, MN, USA). Milk fat yield, lactose yield, protein yield and total milk solids were calculated individually based on the milk production levels obtained from the analysis.

Markers Used and Genotyping

In order to perform polymorphism analysis and genotyping, initially, DNA was isolated from 4 mL blood samples obtained from the vena jugularis of each cow and collected in K_3EDTA tubes (Vacutest Kima, SRL, Italy) by a phenol-chloroform method as described by Green and Sambrook (2012). The amount and purity measurements were carried out on a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All PCR reactions were carried out in a final volume of 50 μL. The reactions consisted of: 33.5 μL ddH_2O, 5 μL 10x buffer (100 mM Tris HCI at pH 8.3, 500 mM KCI, and 0.01% gelatin, Mg-free), 5 μL (25mM) $MgSO_4$, 1 μL dNTPs (2.5 mM), 2.5 U Taq DNA polymerase (Biomatik, Cambridge, Canada, A1003-500U, 5U/μL), 1 μL (0.025 μM) of each primer, and 3 μL template DNA (approximately 80 – 150 ng). Detailed information about the markers selected and the original citation for PCR-RFLP are shown in Table 1. Gene-specific primer sequences for *CSN2* locus were as follows: forward primer 5′-CCTTCTTTCCAGGATGAA CTCCAGG-3′ and reverse primer 5′-GAGTAAGAGGAGGGAT GTTTTGTGGGAGGCTCT-3′. For determining the *CSN3* genotypes, primers were used to amplify the 379 bp (10791-11170) fragment of the gene containing a part of exon 6 and intron 5.

Gene-specific primer sequences for *CSN3* locus were as follows: forward primer 5'-CACGTCACCCACACC CACATTTATC-3' and reverse primer 5'-TAATT-AGCCCATTTCGCCTTCTCTGT-3'. The bovine *LGB* A variant differs from the B variant by two amino acids (Asp and Val) at positions 64 and 118. The primers used for primer of a 247 bp fragment of the *LGB* gene with the following specific primer sequences: forward primer 5′-TGTGCTGGACACCGACTACAAAAA-3′ and reverse primer 5′-GCTCCCGGTATATGACCACCC TCT-3′. The bovine *LALBA* variant A differs from B by a single amino acid substitution of glutamine (Gln) for arginine (Arg) at position 10. Gene-specific primer sequences for amplifying a 309 bp fragment of *LALBA* locus were as follows: forward primer 5'-TTGGTTT-TACTGGCCTC TCTTGTCATC-3' and reverse primer 5'-TGAATTATGGGACAAAGCA AAATAGCAG-3'.

The DNA amplification reactions were performed in a Thermal Cycler (Palm Cycler GC1-96, Corbett Reseach) with thermal conditions as follows:

(a) *CSN2* locus: 95°C for 5 min, followed by 30 cycles of 95°C for 40 sec, 58°C for 1 min, and 72°C for 1.5 min, followed by a final extension step at 72°C for 10 min.

(b) *CSN3* locus: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension step at 72°C for 10 min.

(c) *LGB* locus: 95°C for 5 min, followed by 30 cycles of 94°C for 45 sec, 60°C for 1 min, and 72°C for 5 min, followed by a final extension step at 72°C for 5 min.

(d) *LALBA* locus: 95°C for 5 min, followed by 30 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec, followed by a final extension step at 72°C for 10 min.

Following PCR amplification, polymorphic sites were genotyped using Restriction Fragment Length Polymorphism (RFLP) analysis. PCR amplicons of the *CSN2*, *CSN3*, *LGB* and *LALBA* were subjected to restriction enzyme digestion with *Dde*I, *Hin*dIII, *Hae*III and *Msp*I restriction enzymes (Biomatik, Cambridge, Canada), respectively, according to the suppliers instructions. Briefly, enzyme-buffer mix was prepared by mixing 2 µL of restriction enzyme with 8 µL of the respective buffer. Reaction mix was prepared by mixing 10 µL PCR product with 2 µL of enzyme buffer mix. Volume was made up to 20 µL with autoclaved MilliQ water. Afterwards, these reaction mixtures were incubated at 37°C for 16 h. The digestion products were electrophoresed in 3 % agarose gels (Sigma Aldrich, Steinheim, Germany) containing ethidium bromide (0.4 µg/mL) under 85 – 90 V for about 1 h. Electrophoresis was performed in 1x TBE buffer (108 g Tris, 55 g boric acid, and 40 mL 0.5 M EDTA in 1,000 mL for 10x concentrated stock solution at pH 8). Visualisation of the gels was performed by a gel imaging system (DNr-Minilumi, DNR Bio-Imaging Systems, Israel). Genotypes were assigned by multiple operators in accordance with authors designations of observed patterns of RFLP bands.

Statistical Analysis

Frequency and distribution of genotypes and alleles were calculated by the standard procedure as described by Falconer and Mackay (1996). Hardy-Weinberg equilibrium (HWE) was tested as a genotyping quality control procedure using the χ^2 statistic, with expected frequencies derived from allele frequencies. All markers were evaluated on the basis of their population genetic indices including gene heterozygosity (He), effective allele numbers (Ne) and polymorphism information content (PIC) as described by Nei and Roychoudhury

Table 1. Detailed information about the markers selected, the original citation and RFLP method, as well as the amplicon sizes and annealing temperatures for PCR amplification.

Gene	NCBI Gene ID	Reference	PCR amplicon (bp)	Chromosomal Location	Allele	Annealing temperature (°C)	Enzyme for RFLP
CSN2	281099	Miluchova et al. (2009)	121	6q31	A1/A2	58	*Dde*I
CSN3	281728	Mitra et al. (1998)a	379	6q31	A/B	55	*Hind*III
LGB	280838	Strzalkowska et al. (2002)	247	11q28	A/B	60	*Hae*III
LALBA	281894	Mitra et al. (1998)b	309	5q21	A/B	60	*Msp*I

CSN2: beta-casein; *CSN3*: kapa-casein; *LGB*: beta-lactoglobulin; *LALBA*: alfa-lactalbumin

(1974) and Botstein et al. (1980). The general linear model (GLM) procedure of Minitab (Minitab Inc., State College, PA, USA, v17.1.0) was used to perform association analysis and Levene's test was used to test for homogeneity of the variances. Differences between the genotypes were evaluated using the following statistical model:

$$Y_{ijklmno} = \mu + W_i + X_j + Z_k + AG_l + BG_m + CG_n + DG_o + e_{ijklmno}$$

where: $Y_{ijklmno}$ = the studied traits; μ = the overall mean; W_i = the fixed effect of lactation season (i = autumn, winter, spring and summer); X_j = the fixed effect of service period (j = ≤50, 51-80, 81-110, 111-140, 141≤); Z_k = the fixed effect of lactation rank (k = 1, 2, 3, 4); AG_l = the fixed effect of the *CSN2* genotype (l = A1A1, A1A2, A2A2); BG_m = the fixed effect of the *CSN3* genotype (m = AA, AB, BB); CG_n = the fixed effect of the *LGB* genotype (n = AA, AB, BB); DG_o = the fixed effect of the *LALBA* genotype (o = BA, BB) and $e_{ijklmno}$ = the random residual effect.

Post hoc contrasts were conducted with Tukey's multiple comparison test ($P < 0.05$).

RESULTS

We have amplified the 121 bp fragment for the *CSN2* gene (Fig 1). Digestion of the PCR product with the *Dde*I nuclease resulted in two bands (121 bp and 86 bp) for heterozygote genotype (A1A2) and a single band (86 bp) for the A2A2 genotype. The DNA amplified from A1A1 animals remained undigested (121 bp) with the corresponding restriction enzyme (Fig 2). The amplified fragment (379 bp) of the *CSN3* gene (Fig 3) showed three genotypes including AA, AB and BB by digestion with the restriction enzyme *Hin*dIII and the AB genotype was cleaved into three bands of 379 bp, 225 bp and 154 bp, while AA genotype remained undigested. The BB genotype was characterized by fragment sizes of 225 bp and 154 bp (Fig 4). The electrophoresis pattern of PCR amplification for the *LGB* gene is shown in Fig 5. The cleavage of a 247 bp PCR product by *Hae*III yielded two fragments of 148 bp and 99 bp and was diagnostic for the AA genotype in the *LGB* assay. Heterozygote genotype was cleaved into three bands of 148 bp, 99 bp and 74 bp. Besides the DNA amplified from BB animals was characterized by fragment sizes of 99 bp and 74 bp (Fig 6). The electrophoresis pattern of PCR amplification for the *LALBA* gene is shown in Fig 7. Digestion of 309 bp amplified fragment was performed by using *Msp*I restriction enzyme. Typing *LALBA* allelic variation by PCR-RFLP showed a separation of two different genotypes including BB and AB. Hence, the AA genotype, which forms a single band of 309 bp, was not present in this study. Restriction digests of BB and AB animals yielded two (220 and 89 bp) and three (309, 220 and 89 bp) fragments, respectively (Fig 8).

The genotypic and allelic frequencies, population genetic indices including He, Ne and PIC and compatibility with the HWE are shown in Table 2. Results

Table 2. Allele and genotype frequencies of polymorphisms in *CSN2, CSN3, LGB and LALBA* genes, population genetic indices (He, Ne, PIC) and compatibility with the Hardy-Weinberg equilibrium.

	CSN2			***CSN3***			***LGB***			***LALBA***		
Genotypes	A1A1	A1A2	A2A2	AA	AB	BB	AA	AB	BB	AA	AB	BB
N	30	106	53	7	50	132	35	101	53	0	3	186
%	15.88	56.08	28.04	3.70	26.46	69.84	18.52	53.44	28.04	0	1.59	98.41
Allele Frequency	A1		A2	A		B	A		B	A		B
	0.439		0.561	0.169		0.831	0.452		0.548	0.008		0.992
He		0.4925			0.2809			0.4953			0.0158	
Ne		1.9707			1.3906			1.9817			1.0161	
PIC		0.3713			0.2414			0.3727			0.0157	
χ2(HWE)		3.63			0.67			1.17			0.01	
P*		0.06			0.41			0.28			0.91	

N: number of experimental cows, MAF: minor allele frequency, He: heterozygozity, Ne: effective allele number, PIC: polymorphism information content, χ^2(HWE): Hardy-Weinberg equilibrium χ^2 value, * P>0.05 – not consistent with HWE

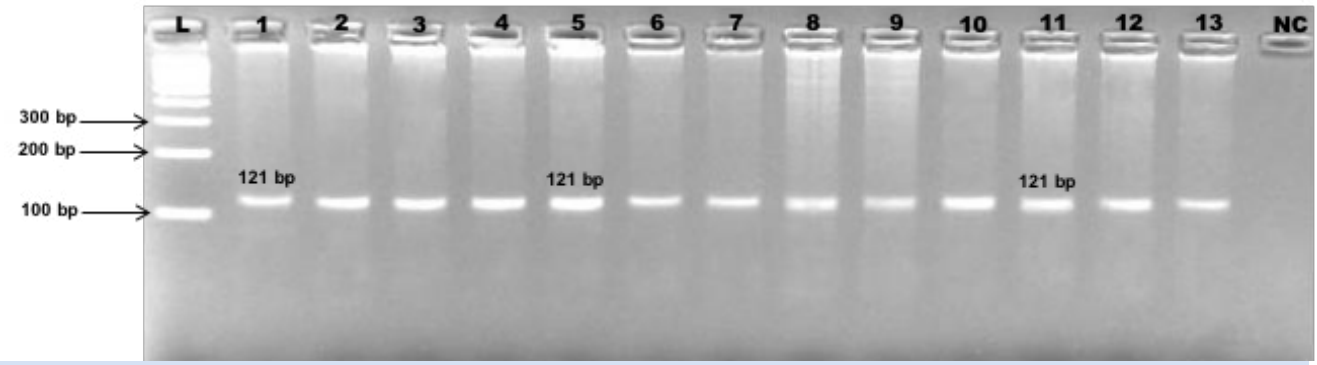

Figure 1 The electrophoresis pattern of PCR amplification for the bovine CSN2 gene (L: DNA Ladder-amplicon length 100–1500 bp; NC: Negative control).

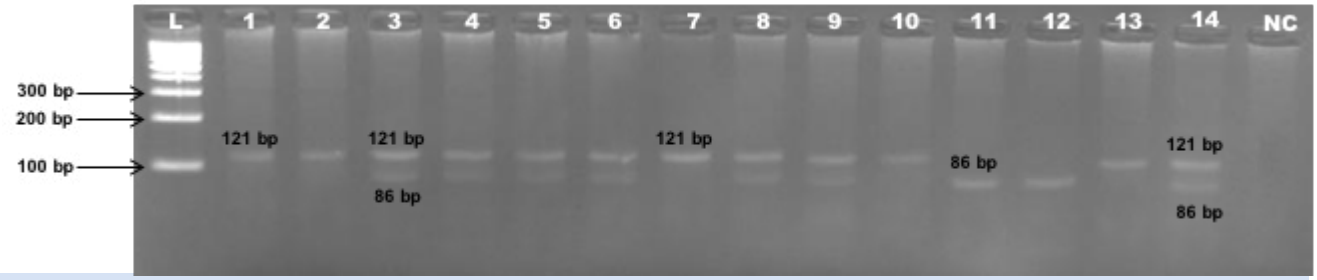

Figure 2 The electrophoresis pattern of restriction enzyme digestion of PCR product with DdeI for the bovine CSN2 gene (L: DNA Ladder-amplicon length 100–1500 bp; Lanes 1, 2, 7, 10 and 13: A1A1; Lanes 3-6, 8, 9 and 14: A1A2; Lanes 11 and 12: A2A2; NC: Negative control).

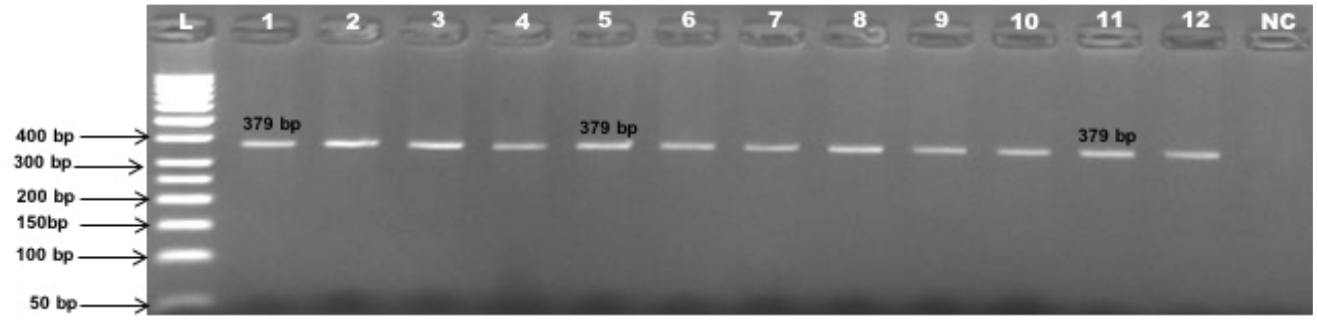

Figure 3 The electrophoresis pattern of PCR amplification for the bovine CSN3 gene (L: DNA Ladder-amplicon length 50–1000 bp; NC: Negative control).

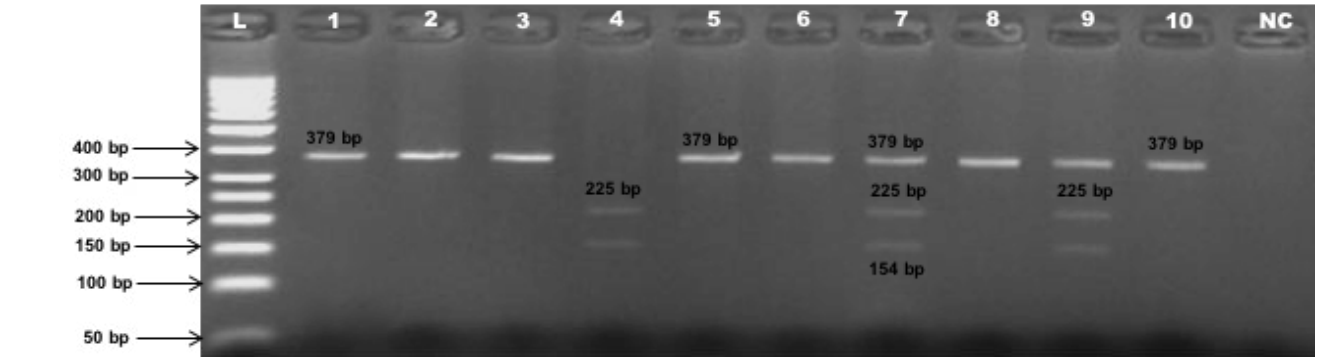

Figure 4 The electrophoresis pattern of restriction enzyme digestion of PCR product with HindIII for the bovine CSN3 gene (L: DNA Ladder-amplicon length 50–1000 bp; Lanes 1-3, 5, 6, 8 and 10: AA; Lanes 7 and 9: AB; Lane 4: BB; NC: Negative control).

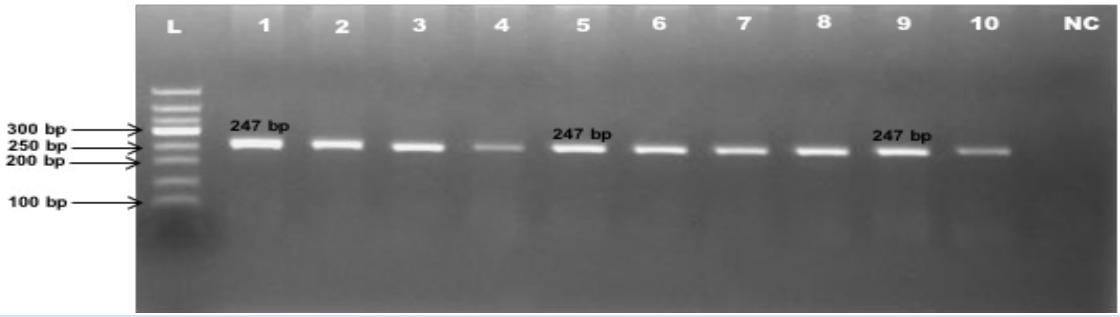

Figure 5 The electrophoresis pattern of PCR amplification for the bovine LGB gene (L: DNA Ladder-amplicon length 50–600 bp; NC: Negative control).

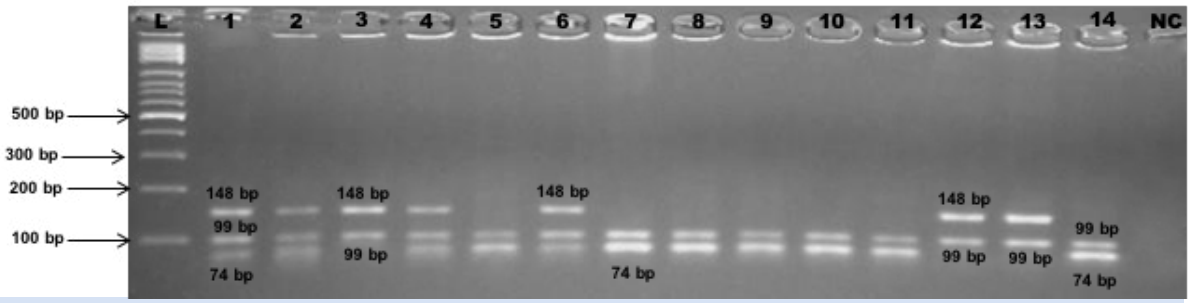

Figure 6 The electrophoresis pattern of restriction enzyme digestion of PCR product with HaeIII for the bovine LGB gene (L: DNA Ladder-amplicon length 100–1500 bp; Lanes 3, 12 and 13: AA; Lanes 1, 2, 4 and 6: AB; Lanes 5, 7-11 and 14: BB; NC: Negative control).

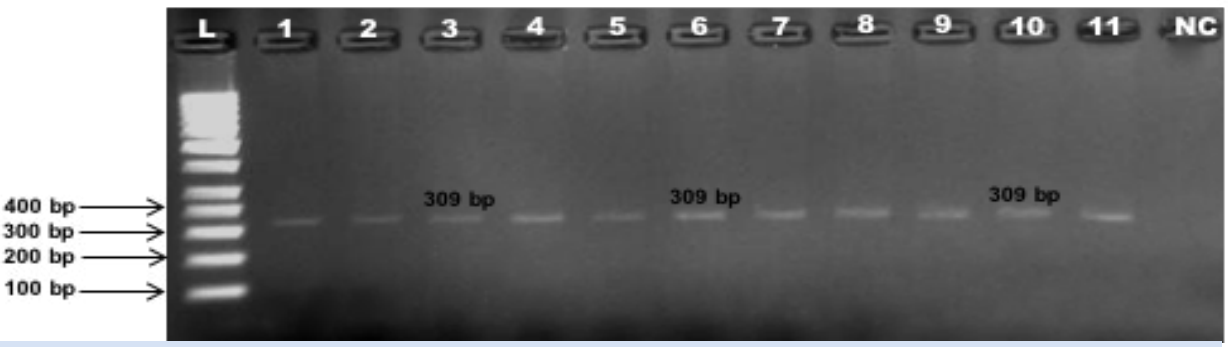

Figure 7 The electrophoresis pattern of PCR amplification for the bovine LALBA gene (L: DNA Ladder-amplicon length 100–1500 bp; NC: Negative control).

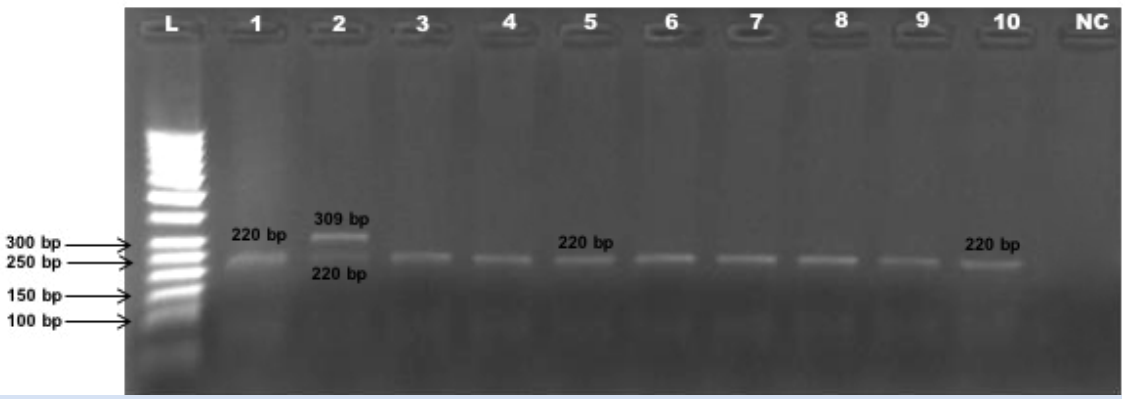

Figure 8 The electrophoresis pattern of restriction enzyme digestion of PCR product with MspI for the bovine LALBA gene (L: DNA Ladder-amplicon length 50–1000 bp; Lane 2: AB; Lanes 1, 3-10: BB; NC: Negative control).
Note: The AA genotype was not present in this study.

Table 3. Effects of *CSN2, CSN3, LGB and LALBA* gene polymorphisms on milk production traits in Holstein cows.

	TMY (kg)	305-dMY (kg)	DBP (days)	PMY (kg)	MFY (kg)	MFC (%)	MPY (kg)	MPC (%)	MLY (kg)	MLC (%)	TMS (kg)	MSC (%)
CSN2												
A1A1	8389	8235^{b}	55.83^{a*}	33.90	293.60	3.58^{a}	261.70^{b}	3.25	401.20	4.93	994.20	12.13
A1A2	8412	8147^{b}	41.71^{ab*}	33.93	274.00	3.36^{ab}	256.60^{b}	3.19	396.00	4.86	980.30	11.99
A2A2	9155	8969^{a}	34.89^{b*}	35.31	283.00	3.20^{b}	284.70^{a}	3.22	430.20	4.84	1049.80	11.80
CSN3												
AA	8513	8430	43.16	32.30	283.60	3.40	274.20	3.36	403.60	4.89	1004.60	12.10
AB	8927	8633	41.98	35.03	287.80	3.33	271.70	3.16	419.30	4.86	1029.30	11.91
BB	8517	8287	47.30	35.82	279.20	3.39	257.10	3.14	404.40	4.88	990.30	11.92
LGB												
AA	8942	8814	39.15	34.50	292.60	3.41	274.40	3.19	422.70	4.88	1040.80	11.98
AB	8694	8497	46.63	34.88	286.60	3.39	268.40	3.22	410.20	4.85	1014.20	11.95
BB	8320	8039	46.65	33.76	271.30	3.33	260.20	3.24	394.50	4.90	969.30	11.99
LALBA												
BA	7881	7389^{1}	34.43	34.66	262.30	3.44	253.20	3.36	370.70	4.90	925.00	12.13
BB	9424	9511^{1}	53.87	34.10	304.70	3.31	282.20	3.08	447.60	4.85	1091.20	11.81
PSE	773.42	662.54	10.90	2.07	30.98	0.23	21.95	0.10	37.27	0.07	88.83	0.29

TMY: total milk yield, 305-dMY: 305-day milk yield, DBP: days before peak milk production, PMY: peak milk yield, MFY: milk fat yield, MFC: milk fat content, MPY: milk protein yield, MPC: milk protein content, MLY: milk lactose yield, MLC: milk lactose content, TMS: total milk solids, MSC: milk solid content, PSE: Pooled Standard error

^{a,b} Different superscripts within a column indicate significant difference (P<0.05)

* represents significance level at P<0.01

^{1} represents a tendency P = 0.085

indicated that the population were determined to be compatible for *CSN2*, *CSN3*, *LGB* and *LALBA* genotypes in the Hardy-Weinberg equilibrium. The minor allele frequencies ranged from 0.008 to 0.452 and all markers in the present study were polymorphic except for *LALBA*, according to the classification reported by Menezes et al. (2006), because the frequency of the BB genotype at the *LALBA* locus was extremely high (98.41 %). The evaluation of population genetic parameters indicated that He values ranged from 0.0158 to 0.4953, PIC values ranged from 0.0157 to 0.3727 and Ne values ranged from 1.0161 to 1.9817.

Least squares means and pooled standard errors for *CSN2*, *CSN3*, *LGB* and *LALBA* genotype effects on milk production traits are shown in Table 3. The results indicated that *CSN2* had significant effects on 305-day milk yield (P<0.05), days before peak milk production (P<0.01). A2A2 genotype was associated with higher 305-day milk yield and fewer days before peak compared to alternative genotypes. In addition *CSN2* showed associations with milk content. Significant effect of the A1A1 genotype on fat percentage were found (P<0.05). Moreover, A2A2 genotype was associated with higher protein yield (P<0.05). There was no significant effects of the *CSN3*, *LGB* and *LALBA* genotypes on milk production traits in the current study. However, a tendency was observed (P = 0.085) for *LALBA* genotypes to be associated with 305-day milk yield, as shown in Table 3.

DISCUSSION

In the present study, the effects of *CSN2*, *CSN3*, *LGB* and *LALBA* genes on milk yield and content were evaluated in Holstein cows. These genes were chosen because they have been shown to be strongly associated with milk production traits in various cattle breeds. The population-based analyses showed a consistence with HWE for all markers. However, an unbalanced genotypic distribution was observed for *LALBA* resulting in low genetic variabilities of He, Ne and PIC compared

to other markers. Botstein et al. (1980) reported that a marker with a PIC value higher than 0.5 is considered to be very informative, whereas values between 0.25 and 0.5 are mildly informative, and values lower than 0.25 are low informative. According to this classification the *CSN2* and *LGB* markers were mildly informative whereas the *CSN3* and *LALBA* markers were low informative. It is worth noting that the major allele frequencies of *CSN3* and *LALBA* were extremely high, 0.831 and 0.992 respectively. Low values of population genetic parameters including He, Ne and PIC may be explained by high level of inbreeding or high selection pressure.

The *CSN2* gene encodes milk protein that is important for the structure of casein micelles (Kucerova et al., 2006) and there are many studies about the association of *CSN2* genotypes with milk production traits in the literature (Ng-Kwai-Hang et al., 1984; Boettcher et al., 2004; Miluchova et al., 2009). However, the effects of alleles within the CN cluster determined by different authors in different breeds are sometimes conflicting (Caroli et al., 2009). In the current study, three genotypes (A1A1, A1A2, A2A2) of *CSN2* locus were determined in the genotyped animals and significant differences between the genotypes of this locus were found for 305-day milk yield, days before peak milk production, fat percentage and protein yield. Animals with the A2A2 genotype had the highest milk yield (+734 kg and +822 kg higher than A1A1 and A1A2, respectively) and protein yield (+23 kg and +28.1 kg higher than A1A1 and A1A2, respectively) and in addition they reached the peak faster (20.94 days and 6.82 days earlier than A1A1 and A1A2, respectively) compared to alternative variants. Besides, the present results indicated that the genotype A1A1 was associated with the highest milk fat percentage (+0.38 % and +0.22 % higher than A2A2 and A1A2, respectively). Similarly, Freyer et al. (1999) reported that the genotype A1A1 is superior regarding fat and protein percentage. Consistent with our results, Heck et al. (2009) also found that cows with the A1 allele had a lower protein yield than cows with the A2 allele, resulting from decreased milk production. Kucerova et al. (2006) reported that significant differences between the genotypes of *CSN2* locus were found in breeding values for milk yield, protein yield, fat yield and fat content. However, their results indicated that, conversely, the genotype A1A1 was associated with the highest breeding value for milk yield and the breeding values of animals with genotype A2A2 were negative for yield parameters but positive for content parameters compared to the animals with genotype A1A1. Comin et al. (2008) also found that *CSN2* locus was associated with milk and protein yields. As mentioned above, results of the studies about associations between *CN* genes and milk production traits are often inconsistent (Zepeda-Batista et al., 2017). On the one hand, in most cases, the reasons for this situation may be due to differences in population size, breed of cows, genotypic distribution, methods of expressing traits, the power of statistical models considering other factors such as age of cow, parity number, season, stage of lactation, health status, and effects of other genetic variants (Ng-Kwai-Hang et al., 1990). On the other hand, *CSN1S1* and *CSN2* are also located on chromosome 6, within a region of about 200 kb that includes *CSN3* (Ferretti et al., 1990). This closeness in physical location makes it difficult to separate the effects of different *CN* genes (Boettcher et al., 2004). Hence, because of the tight linkage between the two loci, the composite genotypes, or haplotypes, may provide more adequate outcomes than the single-locus genotypes before considering them in marker-assisted selection (Comin et al., 2008). Similar to *CSN2*, the *CSN3* gene encodes milk protein that plays an important role in providing an essential structure and stability of casein micelles (Alexander et al., 1988). However, the *CSN3* genotypes had no significant effect on milk production traits in this study, which is in agreement with previous studies reported by Aleandri et al. (1990), Davoli et al. (1990), Ikonen et al. (1999) and Comin et al. (2008). Moreover, the present results indicated that, this marker was not associated with milk content traits, which is consistent with the results reported by Comin et al. (2008). A possible explanation for the result could partially depend on the unbalanced genotypic distribution observed in this study. Besides, as mentioned above, tight linkage between *CN* genes on bovine chromosome 6 should be considered to perform an adequate evaluation of the association of this marker with phenotypic traits.

Although there were evident differences in milk yield with different *LGB* genotypes, they were not significant in the present study. The association between the *LGB* and breeding values for milk production parameters

has been investigated by several authors. Kucerova et al. (2006) reported that non-significant differences were observed in the average breeding values of animals with different *LGB* genotypes. Conversely, Kaminski et al. (2002) reported the relation between *LGB* genotypes and breeding values for protein yield. Czerniawska-Piatkowska et al. (2011) found that cows with the *LGB* AA genotype were characterized by the highest milk yield in all lactations and cows with the *LGB* BB genotype was associated with the highest milk fat yield and content for the three lactations. Neamt et al. (2017) reported that the AB genotype was associated with a higher milk production and the BB genotype was associated with a higher fat percentage in milk compared with the other two genotypes. Neither did Litwinczuk et al. (2003) and Micinski et al. (2008) find any association between *LGB* genotypes and milk production traits. The *LGB* gene encodes mainly whey protein and this gene has been shown to be effective on formation of physicochemical properties of milk. However, the results are not always concurrent and sometimes they are even contradictory. This justifies the necessity of continuing the research on the utilization of this marker in evaluating the genetic basis of milk production traits (Czerniawska-Piatkowska et al., 2011). The results indicated that there was no significant relationship between *LALBA* marker and any of the selected traits in the present study. However, there was a tendency for 305-day milk yield and animals with the BB genotype seemed to have higher milk production. Only two genotypes, BA and BB, for locus *LALBA* were detected in the genotyped animals, and moreover, there were only three animals with genotype BA in the examined group of animals. Hence, futher genetic studies should be performed to confirm the present results and to draw conclusions.

CONCLUDING REMARKS

The significant effects of *CSN2* genotypes, A1A1 and A2A2, was detected in the observed population of Holstein breed raised in Turkey. The genotype A1A1 affected fat content in milk, whereas, the genotype A2A2 was associated with 305-day milk yield, days before peak milk production and protein yield. The findings contribute to a better understanding of the role of the *CSN2* locus in dairy breeds and help with the application of the information to breeding process and effective selection procedures.

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CONFLICT OF INTEREST STATEMENT

None of the authors of this article has any conflict of interest.

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Research article
Ερευνητικό άρθρο

Aflatoxin M1 occurrence in Serbian milk and its impact on legislative

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ABSTRACT. Serbia is a country which has repeatedly changed aflatoxin M1 (AFM1) legislation in milk. As a country that clearly has aspiration toward the EU membership, Serbia implemented the EU legislation for this mycotoxin at level of 0.050 μg/kg. However, due to high occurrence of AFM1 in milk, legislation has been changed several times in the past few years as an effort to preserve domestic milk production.

This paper presents the results of four years monitoring of different milk types taken from Serbian market and from Serbian farmers. The samples were analyzed by liquid chromatography on ODS Hypersil column with fluorescence detector (FLD), after cleanup on immunoaffinity column. Limit of quantification was 0.005 μg/kg, while obtained mean value for trueness was 95.1%, respectively. Average AFM1 levels in 2013, 2014, 2015 and 2016 were 0.205, 0.127, 0.238. and 0.269 μg/kg, respectively. Overall occurrence of AFM1 was 80.9%, with the average content of 0.216 μg/kg (ranged from 0.005 to 5.078 μg/kg). According to this, 49.1% of samples were above the EU regulation.

In years to come, Serbia will have a challenge to produce the milk that is in compliance with the permitted level of AFM1. Especially, when it is known that in not so distant future, an increase in temperature as a result of the certain climate changes is expected.

Keywords: aflatoxin M1, high-performance liquid chromatography, legislation, milk, Serbia

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INTRODUCTION

Aflatoxins (AFs) in food and feed are recognized as a public health problem of considerable importance. Williams et al. (2004) estimated that 4.5 billion of the world's population is exposed to AFs. Because security blankets in crops at pre-harvest and post-harvest level are not as strict as in developed countries, populations of developing countries are the most susceptible to aflatoxicosis illness (Williams et al., 2004). The same problem occurs with milk derivates in developing countries for the reason that they have not accepted and assumed amenities as quick as developed countries (Lizárraga-Paulín et al., 2011).

Aflatoxin losses to livestock and poultry producers from aflatoxin-contaminated feeds include death and the subtler effects of immune system suppression, reduced growth rates, and losses in feed efficiency. Other adverse economic effects of AFs include lower yields of food and fiber crops (Anon, 1989). Among all AFs, the aflatoxin B1 (AFB1) is genotoxic and is considered to be the most potent hepatocarcinogenic substance (Van Egmond and Jonker, 2004; Zein, 2011).

Aflatoxins M1 (AFM1) and M2 (AFM2) are thermo-resistant hydroxylated metabolites produced by lactating animals consuming aflatoxin contaminated feeds (Lizárraga-Paulín et al., 2011). In farm animals, AFB1 and aflatoxin B2 (AFB2) are converted into AFM1 and AFM2 metabolites with the ratio of 1–3% between AFB1 and AFM1 (Ali et. al., 1999; Herzallah, 2009). Cows can convert AFB1 into AFM1 within 12-24 hours after ingestion of contaminated feed and the highest levels are reached after a few days (Ayar, 2007). After exclusion of contaminated feed from diets, the AFM1 concentration in the milk decreases to an undetectable level after 72 hours (Van Egmond, 1989; Gimeno, 2004; Özdemir, 2007). However, AFs carry-over from feed into milk is exponentially increased (Britz et al., 2013). These authors suggested that in the case of high yielding cows with the average milk production of 45 kg and daily intake of 25 kg dry matter (DM), aflatoxin B1 needed to be below 1.4 μg/kg to ensure milk production with AFM1 levels lower than 0.05 μg/kg (Britz et al., 2013).

To reduce the risk of exposure, many countries have regulated the maximum level (ML) of AFB1 in feed (and have set or proposed ML of AFM1 in milk). Currently, the legal limits of AFB1 in feedstuffs are highly variable from the European Union (EU) countries to other countries (the EU has a limit of 5 μg/kg for dairy feed) (European Commission, 2003). In Serbia, proposed ML of AFB1 is harmonized with EU since the April 2014 (Serbian Regulation, 2014). Regarding the regulation of AFM1, European Union has established ML in raw milk of 0.05 μg/kg (European Commission, 2006). This level was also set in Serbia (Serbian Regulation, 2011) but since then, it has been changed several times.

The aim of this paper was to investigate the occurrence of AFM1 in different types of milk during 2013-2016, in Serbia and its impact on Serbian legislation. Such data would certainly be a contribution to food safety assessment of this very important foodstuff, and could surely assist to finally determine ML of AFM1 in milk, on a long-term period in Serbia.

MATERIALS AND METHODS

Samples

Milk samples were randomly collected in Serbia during four-year period (2013-2016). Samples were then divided into four groups, regarding year 2013 (raw milk, pasteurized milk, UHT milk, and samples of organic pasteurized milk), three groups in samples originating from year 2014 (raw milk, UHT milk, and organic pasteurized milk) while in samples from 2015, only raw milk samples were analyzed. In the last year of monitoring, samples of raw and pasteurized milk were tested. Samples of raw milk were collected from small dairy farms; pasteurized milk samples were collected from small and big milk producers while UHT and organic milk samples were collected from big milk producers. All milk samples were produced in Serbia. Immediately after collection samples were transported to the laboratory and analyzed.

Reagents

Acetonitrile was purchased from Sigma Aldrich (Buchs, Switzerland) while n-hexane was obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) was purchased from Thermo Fisher Scientific (Cheshire, United Kingdom).

Sample preparation for high-performance liquid chromatography (HPLC) analysis was done using AflaStar™ M1 R-Immunoaffinity Columns (IAC) (Romer Labs Inc., Union, MO, USA). Deionized water (electric conductivity, < 3.5 µS/cm) from reverse osmosis filtration system DS – 83 (Amtast, USA), was used. Nitrogen gas was obtained from Messer (Belgrade, Serbia).

AFM1 standard with certified concentration of 10 µg/ml was purchased from Sigma Aldrich (Buchs, Switzerland). Standard stock solutions were prepared in acetonitrile and stored at – 18 °C. These solutions were used for solvent based calibration. The standard solutions were stored under refrigerator conditions (4 °C).

Sample preparation

Fifty ml of warm milk (35 – 37 °C) was filtered through a quantitative filter paper for fast filtration (Filtros Anoia, Barcelona, Spain) and applied to the IAC. Flow rate of milk was approximately 1-3 ml/min. After the milk completely passed, IAC was rinsed with 20 ml of deionized water. The AFM1 was eluted with 4 ml of acetonitrile. Eluate was collected and evaporated to dryness at 50 °C using gentle stream of nitrogen.

Since AFM1 in milk samples occur in small concentrations, post derivatization step for HPLC-FLD analysis is required to enhance its fluorescence (Chen et al., 2005). This was achieved by adding 200 µl of TFA and the same volume of n-hexane to the residue from the evaporated acetonitrile eluate or to the AFM1 working standards, vortexed for 30 s, and kept in the dark for 10 min at 40 °C. Further, after evaporation 300 µl of water:acetonitrile (75:25, v/v) mixture was added to the vials and vortexed for 30 s.

HPLC determination

The HPLC instrument was an Agilent 1260 (Agilent Technologies Inc., USA) system equipped with a Chemstation Software (Agilent Instrument Utilities, ChemStation for LC 3D systems, Rev. B.04.03), fluorescence detector (FLD), a binary pump, a µ-degasser, an auto sampler and Agilent column (Hypersil ODS C18, 4.6 x 100 mm, 5 µm). The mobile phase consisted of an isocratic mixture of water:acetonitrile (75:25, v/v) and flow rate was 1.0 ml/min. Twenty microliters of standards and samples were injected into the HPLC column. The fluorescence detector was set to an excitation and emission wavelengths of 360 and 423 nm, respectively. The retention time was around 2.1 min.

Analytical quality control

Calibration curves used for quantitative determination were constructed on the basis of the area under the AFM1 chromatographic peaks, using seven AFM1 working standard solutions. Analytical quality control was implemented according to the Commission Regulation (European Commission, 2002). The linearity of the method was assessed by standard ranging from 2.5–50 ng/ml. The correlation coefficient was 0.9999. The limit of quantification (LOQ) for liquid chromatography determination based on ten times the ratio of the standard deviation of intercept and slope of the calibration curve, was 0.25 ng/ml of AFM1, which is equivalent to 0.005 µg/kg of AFM1 in sample. Method accuracy was investigated by analyzing certified reference material MI1142-1/CM (Progetto Trieste, Padova, Italy) in six replicates and the mean value for trueness was 95.1%.

RESULTS

In this study, a total of 423 milk samples were analyzed to determine concentration of AFM1. Obtained results were summarized in Table 1. Overall occurrence of AFM1 was 80.9%, with the average content of 0.216 µg/kg (0.005 – 5.078 µg/kg). For easier interpretation, the results were classified, based on the contamination level, into five groups: <0.005 µg/kg, 0.005-0.05 µg/kg, 0.05-0.25 µg/kg, >0.25 µg/kg and >0.5 µg/kg. According to this, 49.1% of samples were above the EU regulation (European Commission, 2006).

As can be seen, in all 4 groups of samples from 2013 a very high level of contamination was established, ranging from 80.0 to 100.0%, respectively. The highest contamination with AFM1 was found in pasteurized milk (100.0%), as well as the highest mean value of this mycotoxin (0.270 µg/kg). Similar average content of AFM1 was found in raw milk (0.231 µg/kg), and slightly lower in UHT milk (0.145

Table 1. Occurrence of AFM1 in Serbian milk during four-year period (2013–2016)

Year	Category	No.	Positive samples	Range of conc. (µg/kg)					Average±SD	Min-Max
				<0.005	0.005-0.05	0.05-0.25	>0.25	>0.5		
2013	Raw	64	55 (85.9)	9 (14.1)	29 (45.3)	18 (28.1)	8 (12.5)	4 (6.3)	0.231±0.719	0.005–5.078
	Pasteurized	22	22 (100.0)	0 (0.0)	1 (4.5)	14 (63.6)	7 (31.8)	2 (9.1)	0.270±0.268	0.037–1.215
	UHT	39	37 (94.9)	2 (5.1)	8 (20.5)	22 (56.4)	7 (17.9)	0 (0.0)	0.145±0.107	0.007–0.411
	Organic	5	4 (80.0)	1 (20.0)	3 (60.0)	1 (20.0)	0 (0.0)	0 (0.0)	0.044±0.039	0.016–0.101
	Total	**130**	**118 (90.8)**	**12 (9.2)**	**41 (31.5)**	**55 (42.3)**	**22 (16.9)**	**6 (4.6)**	**0.205±0.508**	**0.005–5.078**
2014	Raw	47	39 (83.0)	8 (17.0)	23 (48.9)	11 (23.4)	5 (10.6)	4 (8.5)	0.153±0.329	0.008–1.486
	UHT	16	14 (87.5)	2 (12.5)	8 (50.0)	6 (37.5)	0 (0.0)	0 (0.0)	0.051±0.038	0.010–0.114
	Organic	1	1 (100.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.008	0.008
	Total	**64**	**54 (84.4)**	**10 (15.6)**	**32 (50.0)**	**17 (26.6)**	**5 (7.8)**	**4 (6.3)**	**0.127±0.288**	**0.008–1.486**
2015	**Raw**	**126**	**98 (77.8)**	**28 (22.2)**	**25 (19.8)**	**46 (36.5)**	**27 (21.4)**	**17 (13.5)**	**0.238±0.346**	**0.006–2.613**
2016	Raw	68	53 (77.9)	15 (22.1)	19 (27.9)	21 (30.9)	13 (19.1)	9 (13.2)	0.353±0.716	0.008–3.928
	Pasteurized	35	19 (54.3)	16 (45.7)	17 (48.6)	2 (7.7)	0 (0.0)	0 (0.0)	0.035±0.049	0.007–0.233
	Total	**103**	**72 (69.9)**	**31 (30.1)**	**36 (35.0)**	**23 (22.3)**	**13 (12.6)**	**9 (8.7)**	**0.269**±0.630	**0.007–3.928**
Total		**423**	**342 (80.9)**	**81 (19.1)**	**134 (31.7)**	**141 (33.3)**	**67 (15.8)**	**36 (8.5)**	**0.216**±0.470	**0.005–5.078**

No.: number of samples
Positive samples: number (percentage)
Range of concentrations: number (percentage)
Average ± SD: average concentration (µg/kg) ± standard deviation (µg/kg)
Min–Max: minimum and maximum concentrations (µg/kg)

µg/kg). Maximum concentration of AFM1 (5.078 µg/kg) was found in a raw milk sample. Particularly worrying fact was that even 95.5% of pasteurized milk and 74.4% of analyzed UHT milk samples established AFM1 concentration greater than ML defined by EU Regulation (European Commission, 2006) and Serbian Regulation (Serbian Regulation, 2011) that was in force at the time of analysis.

As for the samples obtained in 2014, the situation is considerably different. Although the presence of AFM1 remained very high (> 80%), the number of samples containing AFM1 in concentration higher than EU and Serbian MLs (European Commission, 2006; Serbian Regulation, 2011) was significantly smaller. Maximum concentration of AFM1 was again found in sample of raw milk, but it was significantly lower (1.486 µg/kg) compared to the previous year. An encouraging fact is the significantly lower average content of AFM1 in UHT milk (0.051 µg/kg), which is slightly above the ML defined by EU Regulation.

During 2015, a high percentage of raw milk samples containing AFM1 over the current regulations was established once more. This number is higher than in 2014 or even in relation to 2013, when it comes to raw milk. However, these results cannot be associated with the entire 2015. To be precise, in the period from January to mid-September 2015, only one sample (4.3%) exceeded the current regulations, at a concentration of 0.073 µg/kg. During this period was found both, the lowest presence of AFM1 (17.4%) and its lowest mean value (0.034 µg/kg). In samples analyzed in period from mid-September 2015 to November 2015, we started establishing the above mentioned very high levels of AFM1. Differences in results between the two periods in 2015 are shown in Table 2.

In 2016, there was still a problem with AFM1 contamination since 69.9% of samples contained levels above LOQ. Also, a high proportion (34.9%) of samples containing AFM1 above European ML persisted. The average AFM1 levels were 0.716 µg/kg (raw milk) and 0.049 µg/kg (pasteurized milk). This is encouraging, since pasteurized milk is used for human consumption.

DISCUSSION

Presence of AFM1 in milk depends primarily on the presence of AFB1 in feed. Hot and dry weather

Table 2. Differences in occurrence of AFM1 in Serbian milk during 2015

Year	Period	No.	Positive samples	Range of conc. (µg/kg)					Average±SD	Min–Max
				<0.005	0.005-0.05	0.05-0.25	>0.25	>0.5		
2015	Jan – Sep 15	23	4 (17.4)	19 (82.6)	3 (13.8)	1 (4.3)	0 (0.0)	0 (0.0)	0.034±0.030	0.008–0.073
	Sep 15 – Nov	103	94 (91.3)	9 (87.4)	22 (21.4)	45 (43.7)	27 (26.2)	17 (16.5)	0.247±0.351	0.006–2.613
Total	**126**	**98 (77.8)**	**28 (22.2)**	**25 (19.8)**	**46 (36.5)**	**27 (21.4)**	**17 (13.5)**	**0.238±0.346**	**0.006–2.613**	

No.: number of samples
Number of positive samples: number (percentage)
Range of concentration: number (percentage)
Average ± SD: average concentration (µg/kg) ± standard deviation (µg/kg)
Min–Max: minimum and maximum concentrations (µg/kg)

conditions during maize growing season 2012 were favorable for *Aspergillus* molds growth and AFs productions. Many authors from Serbia confirmed the presence of AFs in maize from mentioned growing season. Jajić et al (2013) investigated the occurrence of aflatoxin in 44 samples of maize and established presence of 63.6% with the average value of 74.5 µg/kg. Levels of AFs exceeded the ML established by European Regulation (European Commission, 2006) in 45.5% analyzed samples. Kos et al. (2013) detected AFs in 68.5% of maize samples with the mean level of 36.3 µg/kg. High levels of AFs in maize (69.9%), as well as in maize silage (38.0%), which is primarily used for dairy cattle diets, were found by Lević at al. (2013). Since maize is mainly used as a component of animal feed, it is most likely the reason for the appearance of AFM1 in milk and milk products. Particularly interesting is the period September-November 2015 (Table 2), because the low presence of AFs in maize from 2015 harvest (unpublished results) could not indicate a potential occurrence of AFM1 in milk. Regardless, the AFM1 contamination of milk in Banat region was very important. According to unofficial data of Serbian Ministry of Agriculture, the cause of milk contamination was shortened period for preparation of silage intended for cows. This situation was particularly evident in Banat region, from where the contaminated samples originated. The authors of this study did not have the possibility to analyze the mentioned silage samples, because that was under the authority of the Ministry of Agriculture.

Presence of AFM1 in milk in an earlier period in Serbia was published in few reports. Janković et al. (2009) analyzed 23 milk samples using ELISA method, and in 3 (13.0%) samples concentration of AFM1 was higher than 0.05 µg/kg. In 70 cow's milk samples, Polovinski-Horvatović et al. (2010) used TLC method after immunoaffinity column clean-up to determine AFM1 and have found that none were contaminated with AFM1 in concentration greater than 0.05 µg/kg. Lower occurrence of AFM1 contamination reported in these reports from Serbia, compared to the results obtained in this and subsequent studies, can be explained with the absence of AFs in maize and other feed material in Serbia in previous years (Kokić et al., 2009; Jakšić et al., 2011; Kos 2013). In all the reports, ELISA test kit was used for AFs determination. During this period in the neighboring Republic of Croatia, Bilandžić et al. (2010), by applying ELISA method, analyzed AFM1 in 61 raw milk samples and found that only 1.6% of samples was contaminated with AFM1 in concentration greater than 0.05 µg/kg.

A few years later, the situation in the Republic of Serbia and the whole region, when it comes to AFM1 in milk, appeared to be completely different. In period February-May 2013 in Republic of Serbia, Škrbić et al. (2014) analyzed 50 samples of sterilized, pasteurized and raw milk using UHPLC-MS/MS method. They found that 76.0% of analyzed samples were above the maximum allowed limit set by European legislation. The highest average level of AFM1 was found in raw (0.49 µg/kg), while the lowest average level was found in pasteurized milk (0.19 µg/kg). The authors also found that the average level of AFM1 was decreasing during the February-

April-May period. Kos et al. (2014) investigated the occurrence of AFM1 in larger number of samples of cow's milk (150) by applying ELISA method. AFM1 was detected in 98.7% of analyzed cow's milk samples in concentrations ranged from 0.01 to 1.2 μg/kg. Further, even 129 (86.0%) cow's milk samples contained AFM1 in concentration greater than ML of 0.05 μg/kg defined by European Union (EU) Regulation. Vuković et al. (2013) investigated 111 milk samples and detected AFM1 in 95 (85.6%) samples. However, 62 (55.9%) samples were contaminated at a level above the ML accepted by the European regulation and 4 (3.6%) above Serbian ML for AFM1, which was in force at the time. The authors applied HPLC-FLD method for the AFM1 determination. Torović (2015) determined AFM1 levels, using HPLC-FLD method, in 80 samples of milk and 21 samples of infant formulae. Samples were contaminated in the range 0.02–0.32 μg/kg, whereby AFM1 exceeded European ML in 75.0% of the samples.

Practically at the same time, in the immediate surroundings, in neighboring Croatia, Bilandžić et al. (2014) analyzed a very representative number of samples of raw milk (3736) and UHT milk (706) collected in the period from February to July 2013 in Croatia, using ELISA method. AFM1 levels exceeded the EU ML values in 27.8% of raw and 9.6% of UHT milk samples.

The results of our tests, and tests of researchers from the Western Balkans region certainly contributed to significant changes in the Serbian regulations in the last 4 years. Namely, with the first reports of high presence and content of aflatoxin in feed, and then in milk, in the Republic of Serbia was in force legislation which prescribed the aflatoxin ML of 50 μg/kg in maize and 10 μg/kg in complete mixtures for dairy cows (Serbian Regulation, 2010) as well as 0.050 μg/kg of AFM1 in milk (Serbian Regulation, 2011). As the Ministry of Agriculture, based on the initial results, found that most of the milk present in the Serbian market contained quantities of AFM1 greater than those prescribed in Serbian Regulation at that time (Serbian Regulation, 2011), it adopted a new regulation that prescribed ML of AFM1 in milk at 0.500 μg/kg (Serbian Regulation, 2013). In April 2014 the Ministry, to contribute to the reduction of AFM1 in milk, tightened regulation that prescribed the content of aflatoxin in feed: at 30 μg/kg in maize and 5 μg/kg in complete mixes for dairy cows (Serbian Regulation, 2014). At the same time, new regulation (Serbian Regulation, 2014a) returned the ML of AFM1 in milk to 0.050 μg/kg, which entered into force on July 1, 2014. However, the test results indicated that milk was still contaminated with AFM1 at levels higher than 0.050 μg/kg in high percentage of milk samples (> 30% in our tests). The Ministry reacted very quickly by adopting new regulations in which the maximum content of AFM1 in milk was raised to 0.250 μg/kg (Serbian Regulation, 2014b). This legal act placed specified ML of AFM1 in milk until the end of 2014, and as of 01.01.2015 ML returned to the European level of 0.050 μg/kg. During September, in samples of milk the concentration of AFM1 is significantly increased, which is indicated by our results. During this period, the analysis of 103 milk samples showed that 91.3% of samples contained AFM1 and 69.9% of samples exceeded the ML. The Ministry, probably under pressure from producers and milk processors, acted again, raising the ML of AFM1 in milk at 0.250 μg/kg (Serbian Regulation, 2015).

One of the first EU countries facing the problem of AFM1 in milk was Italy (EFSA, 2004). Extensive measures have been taken to prevent recurrence of the problem. Several studies showed how the implementation of aflatoxin M1 monitoring plan in milk reduced this problem (Nachtmann et al., 2007; Schirone, 2015). Unfortunately, even with the all actions taken to prevent the problem, AFM1 has been found in milk and milk products from Italy in the recent period, which is presented on the RASFF (six notifications in 2016).

CONCLUSION

It was predicted that a temperature increase of +2°C in Europe will probably cause a problem with aflatoxin B1 in the years to come (Battilani, 2016). Serbia and the surrounding countries should expect a struggle with AFB1 and AFM1 in the future. A high occurrence of AFM1 in 2016 may lead to a conclusion that Serbia, and probably the entire region, would still have a problem with this mycotoxin. Therefore, the high level of AFM1 in investigated

samples of milk confirmed that constant monitoring throughout the milk production chain is necessary to minimize health risks related to the presence of this toxin in milk.

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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

Comparative effects of addition of superoxide dismutase and reduced glutathione on cryopreservation of Sahiwal bull semen

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ABSTRACT. The present study aimed to investigate effects of superoxide dismutase (SOD) and reduced glutathione (GSH) on the quality of frozen-thawed semen of Sahiwal bulls. Semen was collected twice a week for 8 weeks by artificial vagina from six Sahiwal bulls, kept at the Semen Production Unit Qadirabad, Sahiwal-Pakistan. After gross and microscopic evaluation, qualifying semen ejaculates were divided into 10 equal aliquots and diluted in extenders enriched with no antioxidants (control); or supplemented with either SOD (50, 100 and 200 IU/mL), or GSH (0.5, 1 and 2 mM) or their combinations (50 IU/mL SOD and 0.5 mM GSH, 100 IU/mL SOD and 1 mM GSH and 200 IU/mL SOD and 2 mM GSH). Samples were then frozen and stored in liquid nitrogen at -196°C for 24 h. The following parameters were evaluated for semen quality: post-thawed sperm motility, viability, acrosome and membrane integrity. According to the results, sperm motility, viability, acrosome and membrane integrity were significantly ($P<0.05$) higher in samples treated either with 100 IU/mL of SOD; 1 mM and 2 mM of GSH or 50 IU/mL of SOD plus 0.5 mM of GSH. In conclusion, semen quality might be improved by supplementing semen extenders with 100 IU/mL of SOD; 0.5 and 1 mM of GSH and combination of 50 IU/mL and 0.5 mM of SOD and GSH, respectively

Keywords: Semen, Superoxide Dismutase, Reduced Glutathione, Sahiwal Bull

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INTRODUCTION

It is over debated that repeated cooling and freeze-thawing process induce oxidative stress in semen samples, leading to poor viability and fertility of spermatozoa (Stradaioli *et al*., 2007). Lead factor of oxidative stress is the excessive reactive oxygen species (ROS) generation (Budai *et al*., 2014) that profoundly dissolves lipids, proteins and DNA. Since sperm cells are surrounded by protective polyunsaturated fatty acids membrane (Choudhary *et al*., 2010) and thus, provide an opportunity for the ROS to break them. Another key factor that spread the oxidative stress (OS) is the presences of lipids double bonds sperm membranes (Uysal and Bucak, 2007). Antioxidant system of spermatozoa is also compromised during semen manipulation and due to excessive production of oxygen radicals (Stradaioli *et al*., 2007). Sperm dispose of most of their cytoplasm during terminal stages of differentiation, so they lack endogenous enzymatic defense mechanisms, resulting in overall stress situation (Beheshti *et al*., 2011).

In order to counteract the deleterious effect of OS, semen is naturally provided with SOD that can neutralize OS and defend spermatozoa. It is demonstrated in some previous studies that SOD supplementation can improve sperm parameters in different species (Cocchia *et al*., 2011; Asadpour *et al*., 2012; Perumal, 2014). Morever, GSH, a non-enzymatic antioxidant, has exhibited similar physiological function as does SOD. As reported earlier by many researchers (De Oliveira *et al*., 2013; Ismail and Darwish, 2011; Kaeoket *et al*., 2008), GSH can neutralize the negative impacts of OS on semen quality. However, there is a huge gap in literature regarding the optimum dose effect of SOD and GSH on cryopreservation of Sahiwal bull semen. Therefore, the present study was conducted to monitor the effects of SOD and GSH alone and in combinations on frozen-thawed Sahiwal bull semen.

MATERIALS AND METHODS

The stock extender contained tris-hydroxymethyl-aminomethane (2.42%; w/v), citric acid (1.34%; w/v), fructose (0.1%; w/v), glycerol (7%; v/v), egg yolk (20%; v/v), streptomycin sulphate (1mg/mL), procaine penicillin (400 IU/mL), and benzyl penicillin (500 IU/mL). Ten experimental extenders were prepared as shown in Table 1. The groups S1, S2 and S3 were supplemented with 50, 100 and 200 IU/mL of SOD; the R1, R2 and R3 groups were added with 0.5, 1 and 2 mM of GSH; while SR1, SR2 and SR3 groups included 50 IU/mL SOD and 0.5 mM GSH, 100 IU/mL SOD and 1 mM GSH and 200 IU/mL SOD and 2 mM GSH, respectively. CSR group was kept as a control (no antioxidant added). The antioxidants used in this study were purchased from Sigma–Aldrich Chemicals, St. Louis, MO, USA.

Semen ejaculates were collected in graduated plastic tubes using artificial vagina (42˚C) twice a week for a period of eight weeks. Semen ejaculates having acceptable color (creamy white/yellow), volume >2.0 mL, mass activity >3+, sperm motility percentage >60% and sperm concentration >500 × 10^6/mL were selected. Qualifying semen ejaculates were split into10 aliquots and diluted in 10 different experimental extenders so that each of diluted semen contained a concentration of 50 × 10^6 motile spermatozoa per mL. Diluted semen samples were cooled to 4˚C within 2 h and equilibrated

Table 1: Different doses of superoxide dismutase and reduced glutathione added in Tris- based extender

Serial No	Group	Antioxidant	Dose
1	S1	Superoxide dismutase	50 IU/mL
2	S2	Superoxide dismutase	100 IU/mL
3	S3	Superoxide dismutase	200 IU/mL
4	R1	Reduced glutathione	0.5 mM
5	R2	Reduced glutathione	1.0 mM
6	R3	Reduced glutathione	2.0 mM
7	SR1	Superoxide dismutase + Reduced glutathione	50 IU/mL + 0.5 mM
8	SR2	Superoxide dismutase + Reduced glutathione	100 IU/mL + 1.0 mM
9	SR3	Superoxide dismutase + Reduced glutathione	200 IU/mL + 2.0 mM
10	CSR	Superoxide dismutase + Reduced glutathione	No antioxidant

at 4°C for 4 h. Then semen samples were filled in 0.5 mL French straws (IMV, France) with suction pump in a cold cabinet. Semen straws were kept in liquid nitrogen vapors for 10 min and then plunged and stored in liquid nitrogen (-196°C). After 24 h of freezing, semen straws were thawed in a water bath (37°C for 30 sec) and assessed for sperm motility, viability, acrosomal integrity and plasma membrane integrity.

Sperm with progressive motility were assessed using a phase contrast microscope at 200X by placing semen sample on a pre-warmed (37°C) glass slide and covered with a cover slip. The sperm viability was determined by eosin-nigrosin stain as per method of Salisbury and Van-Demark, 1978. It was assessed by counting 200 spermatozoa under phase contrast microscope (400X). Sperm plasma membrane integrity was evaluated by hypo-osmotic swelling test (HOST) as described by Andrabi *et al*. (2008). The sperm acrosomal integrity was judged by mixing 500 µl of semen with 50 µl of 1% formaldehyde citrate in a test tube and observing a drop of sample under phase contrast microscope at 1000X as described by Asr *et al*. (2011).

The obtained data was analyzed using one-way analysis of variance. The differences in groups were compared by Duncan's Multiple Range Test using SPSS (version 20.0, IBM Corp. Armonk, NY). Value having $P<0.05$ was considered statistically significant.

RESULTS

The results of present study showed that SOD, GSH and their combination efficiently improved sperm motility, viability, acrosome integrity and membrane integrity. The analysis of data revealed that S2, R1, R2 and SR1 groups had significantly ($P<0.05$) higher frozen-thawed motility as compared to control as shown in Table 2. The highest motility was achieved in R2 as compared to all other groups ($P<0.05$). However, non-significant difference was present between S2 and SR1 groups. The viability percentages were significantly ($P<0.05$) higher in S2, R1, R2 and SR1 groups as compared other groups, while R1, R2 and SR1 groups had non-significant difference among each other.

The acrosomal integrity of S2, R1, R2 and SR1 groups were significantly ($P<0.05$) higher than other groups. The highest acrosome integrity was seen in R2 group ($P<0.05$). However, the difference of acrosome intergrity was non-significant between R1 and SR1 ($P>0.05$). The functional membrane integrity was significantly ($P<0.05$) high in S2, R1, R2 and SR1 groups as compared to all other groups. Moreover, the membrane integrity of spermatozoa was not different in R1, R2 and SR1 groups ($P>0.05$).

DISCUSSION

In the current study, we have evaluated effects of different concentrations of SOD and GSH along with their combinations on post thaw semen quality parameters (motility, viability, acrosomal integrity and membrane functional integrity) of Sahiwal Bulls. These parameters are known as important indices for the evaluation of

Table 2: Mean (±SE) values for motility, viability, membrane integrity and acrosomal integrity of post-thawed Sahiwal bull semen

Treatment	Motility %	Live Sperm %	HOST %	Acrosomal Integrity %
S1	44.00 ± 0.408 ^{e}	54.69 ± 0.514 ^{d}	46.69 ± 0.514 ^{cd}	65.00 ± 0.612 ^{d}
S2	50.31 ± 0.313 ^{c}	65.31 ± 0.313 ^{b}	50.31 ± 0.514 ^{b}	68.31 ± 0.514 ^{c}
S3	45.00 ± 0.408 ^{e}	50.00 ± 0.408 ^{e}	44.69 ± 0.313 ^{d}	59.69 ± 0.717 ^{f}
R1	52.69 ± 0.514 ^{b}	69.00 ± 0.612 ^{a}	51.00 ± 0.408 ^{ab}	72.00 ± 0.408 ^{b}
R2	55.00 ± 0.408 ^{a}	70.31 ± 0.514 ^{a}	52.69 ± 0.514 ^{a}	75.00 ± 0.408 ^{a}
R3	44.38 ± 0.625 ^{e}	54.69 ± 0.514 ^{d}	45.06 ± 0.544 ^{d}	63.00 ± 0.612 ^{de}
SR1	51.69 ± 0.514 ^{c}	69.06 ± 0.739 ^{a}	51.00 ± 0.612 ^{ab}	70.00 ± 0.612 ^{bc}
SR2	47.38 ± 0.625 ^{d}	57.31 ± 0.514 ^{c}	45.69 ± 0.514 ^{d}	62.00 ± 0.612 ^{ef}
SR3	40.00 ± 0.408 ^{f}	49.06 ± 0.739 ^{e}	39.69 ± 0.120 ^{e}	57.06 ± 0.544 ^{g}
CSR	47.69 ± 0.514 ^{d}	59.00 ± 0.612 ^{c}	48.00 ± 0.612 ^{c}	64.69 ± 0.514 ^{d}

^{abcdefg} values within same column sharing similar superscripts are statistically not different ($P>0.05$).

semen fertility and suggested as primary markers for epididymal maturation and spermatogesis (Morakinyo *et al.,* 2010).

The spermatozoa are susceptible to OS due to cold shock, which reduces the motility of spermatozoa due to decrease in ATP production (Dandekar *et al*., 2002). The present study demonstrated that 100 IU/mL of SOD had significantly (P<0.05) improved motility of post-thawed semen as compared to control. The results of study were almost similar to those reported by El-Sisy *et al*., (2008) and Shoae and Zamiri, (2008).

In present study, the addition of 0.5 and 1.0 mM of GSH in Tris-citric acid extender improved the motility of post-thawed semen. These findings were in accordance with the reports of Ansari *et al*. (2011) and Munsi *et al*. (2007) in bull semen. The post-thaw motility was also significantly higher in 50 IU/mL of SOD plus 1.0 mM of GSH supplemented group as compared to control. The probable reason for increased motility might be due to counteraction of ROS by the antioxidants added in the extender (Bilodeau *et al*., 2001).

The present study revealed that % viability was significantly (P<0.05) higher in 100 IU/mL of SOD supplemented group, which is similar to the results previously reported in buffalo bull semen (El-Sisy *et al*., 2008). Furthermore, sperm viability was significantly higher in 1.0 mM of GSH treated group as compared to control (P<0.05). This is in agreement with findings of previous reports in buffalo bull (Ansari *et al*., 2012) and stallion semen (Khlifaoui *et al*., 2005). The viability was significantly enhanced in combination group containing 50 IU/mL of SOD and 1.0 mM of GSH. Sperm plasma membrane contains high contents of unsaturated fatty acids which are at risk of lipid peroxidation by the oxygen radicals (Nair *et al*., 2006). This lipid peroxidation may damage sperm plasma membrane and may lead to sperm death (Ansari *et al*., 2011). The prevention of freezing damage to the spermatozoa by fortification of antioxidants in extender might be due to limiting the process of lipid peroxidation by the antioxidants.

The plasma membrane prevents spermatozoa from harmful effects of OS and intact plasma membrane is regarded as an index of fertilizing potential of spermatozoa (Jeyendran *et al*., 1984). The results of present study demonstrated higher membrane integrity of spermatozoa in groups containing 100 IU/mL of SOD, 0.5 mM and 1.0 mM of reduced glutathione and 50 IU/mL SOD plus 0.5 mM of GSH, as compared to other groups. The results of this study are in harmony with some previous reports (Perumal *et al*., 2011; Perumal, 2014; Farouzanfar *et al*., 2013 and Ansari *et al*., 2012).

It is well established that oxygen radicals, produced during freezing process, have high affinity to unsaturated fatty acids of sperm plasma membrane. Reaction of oxygen radicals with sperm plasma membrane can cause lipid peroxidation and sperm death (Uysal and Bucak, 2007). However, the antioxidants added in semen extenders can counteract with oxygen radicals and can prevent injury to sperm plasma membrane.

Acrosome integrity is an indication of functional membrane status of spermatozoa (Silva and Gadella, 2006). Acrosome is a secretory organelle derived from golgi/endoplasmic reticulum which contain hydrolytic enzymes. The presence of intact acrosome is needed to facilitate the acrosome reaction of spermatozoa and is essential for the process of fertilization. Along with total antioxidant potential, freeze-thawing process decreases the intact acrosome of spermatozoa (Anzar *et al*., 2010). In the present study, the percentages of spermatozoa with normal acrosomes were significantly (P<0.05) higher in extenders containing 100 IU/mL of SOD than control. Our results were in line with the previous findings on the acrosome integrity of frozen-thawed ram semen (Farouzanfar *et al*., 2013 and Silva *et al*., 2012).

A high number of sperm with normal acrosome were found in 0.5 mM and 1.0 mM of GSH supplemented groups than control. These results are similar to the findings of Funahashi and Sano (2005) and Gadea *et al*., (2007). Similarly, the acrosome integrity was significantly (P<0.05) higher in combination group containing 50 IU/mL of SOD and 0.5 mM of GSH. The ROS produced during freeze-thaw process interact with the sperm plasma membrane and cause hyper-activation along with pre-mature capacitation of spermatozoa. Hence, it seems that the disruption of sperm acrosomes might be reduced by the addition of exogenous antioxidants in treatment extenders.

In conclusion, supplementation of semen extenders with SOD and GSH in various concentrations can improve the post-thawed semen quality of Sahiwal bulls. However, higher concentrations of these antioxidants have no beneficial effects on semen. Moreover,

the routine inclusion of these antioxidants in semen extenders could be recommended only after performing fertility trials.

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CONFLICT OF INTERESTS

The authors report no conflict of interests.

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LIST OF ABBREVIATIONS

Superoxide dismutase – SOD

Reduced glutathione – GSH

Reactive oxygen species – ROS

Oxidative stress – OS

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Reseach article
Ερευνητικό άρθρο

Prevalence and distribution of staphylococcal enterotoxin genes among *Staphylococcus aureus* isolates from chicken and turkey carcasses in Algeria

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ABSTRACT. This study is aimed to determine the prevalence of *staphylococcus aureus* (*S.aureus*) by biochemical tests in poultry carcasses. It is also intend to detect the distribution of genes for classical staphylococcal enterotoxins A, B, C, D and E (*sea, seb, sec, sed* and see) and for gene *femA*, specific for *S.aureus* species, using multiplex PCR.

A total of 385 samples of neck skins from fresh poultry carcasses were collected during the period 2012-2013 from 16 different slaughterhouses located in the region of Algiers, Algeria.

The overall prevalence of *S.aureus* in freshly slaughtered poultry carcasses was 41.56%, with an individual prevalence of 40.63% and 45.71% for chicken and turkey respectively. From the 95 strains of *S.aureus* identified by biochemical tests, 82 (86.32%) isolates were *femA* positive using multiplex PCR. The investigation has also revealed the presence of both enterotoxins B and D, with a predominance of *seb* (13.33%) followed by *sed* (1.67%), in the chicken carcasses while in turkey only *sed* was detected (4.55%)

It has been found that strains of *S.aureus* of poultry origin can be enterotoxigenic with the predominance of genes encoding for enterotoxins *seb* in chicken and *sed* in turkey. As enterotoxins can be produced in adequate amounts to induce foodborne illnesses, these potential dangers must be considered in terms of a real risk to public health.

Keywords: *Staphylococcus aureus*, Poultry carcasses, Staphylococcal entérotoxine, Algiers.

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INTRODUCTION

Recent studies have revealed that high risk of foodborne diseases is often related to the contamination of poultry carcasses by pathogens micro-organisms (Escudero-Gilete et al., 2007). Among the pathogen bacteria that maybe present in poultry carcasses is *Staphylococcus aureus*. Its presence in food indicates poor hygiene and improper storage conditions (Gundogan et al., 2005).

S.aureus is an opportunistic pathogen that can colonize the skin and mucous membranes particularly in the nose of both healthy humans and animals (Nader et al., 2016). In addition to colonizing various hosts, it can also be the origin of a wide range of different infections in poultry ranging from septicemia, pneumonia, endocarditis, and arthritis (Smyth and McNamee, 2008). Amongst the foodborne pathogens *S.aureus* is a major one contaminating meat products. It is considered the third largest cause of food related illnesses throughout the world (Achi and Madubuik, 2007; Aydin et al., 2011; Sasidharan et al., 2011).

S.aureus is characterized by its ability to produce a large variety of Staphylococcal enterotoxins (SEs) (A, B, C, D, E, G, H, I, J, K, L, M, N, O, P, Q, R and U), but 95 % of food poisoning are caused by theenterotoxins A, B, C, D and E (Letertre et al., 2003). It is notable that SEs are heat stable toxins. Indeed, the heat used in cooking and pasteurization is insufficient to destroy them. Moreover, they are difficult to be perceived in food due to the lack of taste and food appearance (Aycicek et al., 2005). For the case of food ingestion contaminated with SEs, food poisoning occurs shortly after, 30 min to 8 hrs while infected individuals usually recover from the toxicity within 24 - 48 hours(Argudín et al., 2010).

Based on recent published studies, just a little is known about enterotoxigenic *S.aureus* strains from poultry. Particularly in Algeria, the number of studies discussing the prevalence of enterotoxins genes of *S.aureus* in poultry meats is really limited. Therefore, the present study is aimed to determine the prevalence of *staphylococcus aureus* (*S.aureus*) by biochemical tests in poultry carcasses. It is also intend to detect the distribution of genes for classical staphylococcal enterotoxins A, B, C, D and E (*sea, seb, sec, sed* and see) and for gene *femA*, specific *for S. aureus* species, using multiplex PCR.

MATERIALS AND METHODS

Sample collection and microbiological analysis

A total of 385 samples of neck skins from fresh poultry carcasses were collected from 16 different slaughterhouses located in the region of Algiers, Algeria. These samples has been examined during 2012-2013. They consist of chicken carcasses (n=315), and turkey carcasses (n = 70). All samples were placed in sterile plastic bags and brought to the laboratory in cold chain and analyzed within the following 3 h. Microbiological analysis in this study has been carried out according to the international standard NF EN ISO 6888-1/A1(ISO, 2004).Twenty-five portions of the samples were weighed into sterile stomacher bags diluted with 225 ml sterile buffered peptone water (BPW; Oxoid CM 509) and homogenized in a stomacher (Seward 400) for 2 min. The samples were diluted with BPW, and 0.1 ml portions of dilution levels were streaked on Baird-Parker (BP) agar (Oxoid CM 275) supplemented with egg yolk-tellurite emulsion (Oxoid SR 54) and incubated at 37ºC for 24 - 48 h.

The colonies suspected for Staphylococci positive coagulase were cultured on 5% blood agar and identified by catalase, coagulase tests. *S. aureus* was identified by Staphaurex (Bio-Rad). The reference strain used for microbiological analysis was *S.aureus* ATCC 25923. The pure isolates were then stored at −20 °C in TSB-broth and 10% glycerol for future studies.

Statistical Analysis

Data collected from the detection of *S.aureus* on turkey and chicken were analyzed by SPSS Statistics 20 software, to test the significance of differences among the biochemical tests and the Polymerase-Chain Reaction (PCR), and the toxigenic potency of *S.aureus* between the two species (turkey and chicken).

Genomic DNA Extraction

The methodology of DNA extraction is taken fromSambrock and Russel, (2001). For nucleic acid isolation, 95 strains of Staphylococcus species isolated from poultry carcasses were activated on trypticase soya agar (TSA) (BioMérieux, France). After overnight incubation at 37°C, one to two colonies for each strain was re-suspended on 300 µl of TNE buffer (Tris- Nacl-EDTA-SDS) and then vortexed, 10 µl of lysozyme was added plus 200 µl of SETS (Sodium- EDTA- Tris- SDS). The mix was incubated at 37°C for 1 hour. After incubation

Table 1. Oligonucleotide primers sequences used for PCR amplification of *S. aureus's* enterotoxins (SEs) genes.

Gene Primer	Oligonucleotide sequence	Size of amplified product (bp)	
Sea	GSEAR-1	GGTTATCAATGTGCGGGTGG	102
	GSEBR-2	CGGCACTTTTTTCTCTTCGG	
Seb	GSEAR-1	GTATGGTGGTGTAACTGAGC	164
	GSEBR-2	CCAAATAGTGACGAGTTAGG	
Sec	GSEAR-1	AGATGAAGTAGTTGATGTGTATGG	451
	GSEBR-2	CACACTTTTAGAATCAACCG	
Sed	GSEAR-1	CCAATAATAGGAGAAAATAAAAG	278
	GSEBR-2	ATTGGTATTTTTTTTCGTTC	
See	GSEAR-1	AGGTTTTTTCACAGGTCATCC	209
	GSEBR-2	CTTTTTTTTCTTCGGTCAATC	
femA	GSEAR-1	AAAAAAGCACATAACAAGCG	132
	GSEBR-2	GATAAAGAAGAAACCAGCAG	

each tube was vortexed once every 15 min. Then, 10 µl of proteinase- K (Vivantis Technologies, Malaysia) and 100 µl of TNE were added. The final solution was vortexed and incubated at 50°C for 1 h. After that each microcentrifuge tube was vortexed once every 15 min. A volume of 150 µl of NaCl (5M) was added and vortexed. The obtained DNA was harvested by centrifugation at 10.000 rpm for 10 min at room temperature. The supernatant was transferred to a new microcentrifuge tube for washing by 99% cold ethanol and then stored at -20°C overnight. A second washing of DNA was done by 70% cold ethanol. The sample was dried for 10-30 min and re-suspend in 100 µl sterile distilled water and stored at -20°C until PCR analysis.

Multiplex PCR Conditions

The multiplex PCR has been performed as described by Mehrotra et al. (2000). The primers used in the multiplex PCR are presented in Table 2. Multiplex PCR mix contained 400 µM deoxynucleoside triphosphates (dNTPs); 5 µl of 10X reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl); 4 mM $MgCl_2$; 20 pmol of each *sea*, *seb*, *sec*, *see*, and *femA* primers; 40 pmol of *sed* primers; 2.5 U of *Taq* DNA polymerase (AmpliTaq DNA polymerase, Perkin-Elmer), and 5µl of template DNA. The final volume was adjusted by adding 50µl with sterile ultra-pure water. DNA thermocycling (CFX 96thermal cycler, Bio-Rad)was carried out with the following thermal cycling profile: an initial denaturation at 94°C for 5 min was followed by 35 cycles of amplification (denaturation at 94°C for 2 min, annealing at 57°C for 2 min, and extension at 72°C for 1 min), ending with a final extension at 72°C for 7 min. Ten microliters of the PCR products were then analyzed by electrophoresis on 2% agarose (BIOMAX) gel, stained with ethidium bromide. Note that the DNA fragments were visualized using a UV transluminator (EC3, UVP Biommaging systems, Inc (BioMax) while immigration was made by Electrophoresis Power Supply (Model EC 1000 XL Thermo Scientific, Inc.) in TBE buffer (0.09 M Tris–HCl, 0.09 M boric acid, 2 mM EDTA, pH 8.3) for 45 min at 120.Primers are shown in Table 1.

RESULTS

Table 2 illustrates the overall prevalence of *S.aureus* in freshly slaughtered poultry carcasses that are collected from 16 slaughterhouses. The prevalence was 160 of

Table 2. Isolation of *S.aureus from* chicken and turkey carcasses

Type of meat sample	**No. of samples**	***S. aureus* positive samples**	
		No.	**%**
Chicken carcasses	315	128	40.63
Turkey carcasses	70	32	45.71
Total	**385**	160	**41.56**

Table 3. Confirmation of the isolated strains as *S.aureus* by multiplex PCR

Origin of the isolates	No. of the isolates	PCR confirmed as *S. aureus* isolates	
		***FemA* positive**	**%**
Chicken carcasses	70	60	85.71
Turkey carcasses	25	22	88.00
Total	**95**	**82**	**86.32**

385 samples (41.56%), with an individual prevalence of (40.63%) and (45.71%) for chicken and turkey respectively.

Table 3 shows the confirmation of the isolated strains as *S.aureus* by multiplex PCR. The multiplex PCR has revealed that 82 (86.32%) from these isolates were *femA* positive, i.e., 60 (85.71%) in chicken and 22 (88%) for turkey. Statistical analysis showed no significant difference between the two methods (Classical method and PCR) ($p< 0.05$).

It has also been observed that 12.19% of *S.aureus* isolates encoded classical staphylococcal enterotoxins, i.e., nine (15%) from chicken and 1 (4%) from turkey carcasses. Statistical analysis showed no significant difference for the prevalence of enterotoxigenic isolates from chicken carcasses and turkey for $p< 0.05$.Moreover, detected the presence of both enterotoxins B and D in poultry carcasses with a prevalence of 9.76% and 2.44% respectively.

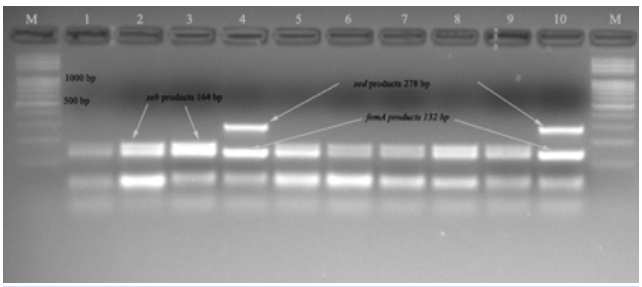

Figure1. Agarose gel electrophoresis patterns showing multiplex PCR amplification. Lanes M represent100bp DNA ladder . Lanes 1 to 7 represent, PCR amplicons from primer set A. Lanes 8 to 10 represent PCR amplicons from setB. Lanes: 1,2,3,5,6,7,8 and 9 present the characteristic band of *seb while* 4 and 10 present the characteristic band of ,*sed.*In all cases the band *femA* is present.

As indicates in Figure 1 and Table 4, isolates from chicken present both enterotoxins B and D, with a predominance of *seb* (13.33%) followed by *sed* (1.67%), while in turkey only *sed* was detected (4.55 %). It is notable that none of the isolates was positive for *sea*, *sec* or *see* genes.

DISCUSSION

The isolation of *S.aureus* from chicken and turkey carcasses (41.56%) confirms the contamination of the poultry carcasses by *Staphylococcus aureus*. Indeed, the present study is mainly focused on the determination of the prevalence of *S.aureus* in freshly slaughtered chicken and turkey, in 16 slaughterhouses in the Wilaya of Algiers. Moreover, it also aims to the molecular characterization of the isolated strains by highlighting the presence of enterotoxins genes.

The prevalence of *S.aureus* recorded for chicken (40.63%) was in good agreement with those advocated

Table 4. Distribution of *Staphylococcus aureus* enterotoxins genes in poultry carcasses

Origin of isolates	Screened isolates	Se positive (%)	Types of entero-toxin' genes(%)				
			Sea	***Seb***	***Sec***	***Sed***	***See***
Chicken carcasses	**60**	**9 (15.0)**	**0 (0)**	**8 (13.33)**	**0 (0)**	**1 (1.67)**	**0 (0)**
Turkey carcasses	**22**	**1 (4.55)**	**0 (0)**	**0 (0)**	**0 (0)**	**1 (4.55)**	**0 (0)**
Total	**82**	**10 (12.19)**	**0 (0)**	**8 (9.76)**	**0 (0)**	**2 (2.44)**	**0 (0)**

by Citak and Duman (2011) and Nader et al.(2016). In Algeria a study conducted in the region of Biskra, carried out on 60 chicken carcasses revealed a prevalence similar to that obtained in the present study (Alloui et al., 2013). However, lower prevalence rates of *S.aureus* were found by others studies (Khallaf et al., 2014; Sarrafzadeh Zargar et al., 2014).

In our study, the recorded prevalence of *S.aureus* present in turkey carcasses (45.71%) was higher than that obtained by Sarrafzadeh Zargar et al.(2014) (16.6%) and El Allaoui (2013) (41.6%).

Two other studies that have been carried out in Turkey recorded a prevalence of 9.61% (Kiliç et al., 2009) and 48% (Bystron et al., 2005) of coagulase-positive *Staphylococcus* from meat and hash meat respectively.

The high prevalence recorded in both species often indicates a lack of good hygiene practices(Lindblad et al., 2006; Pacholewicz et al., 2016) which are linked to the multiple handling of carcasses during slaughter processing. The staff represents a source of contamination spreading the germs by inadequate clothing hygiene, or by hosting (sores, angina, sinusitis and nasopharyngitis).

The lack of control of good manufacturing practices can also be at the origin of these contaminations-multiple contacts with the equipment: tables, bags, knives, towels, scalding bath and feathery (Azelmad et al., 2017; Kotula and Pandya, 1995;Mead et al., 1993).

It could also be related to the sanitary status of poultry intended for slaughter (carried by the skin and feathers, or various pathologies as osteomyelitis, arthritis and synovitis).

The variations in the prevalence between our results and other studies, that have been carried-out in other countries, can be attributed to several factors, including the size of the samples tested, the sampling mode(Whole chicken or parts of carcasses), the seasons in which the sampling was done, the methods of isolation used and the hygiene conditions of the slaughterhouse(Teramoto et al., 2016; Wang et al., 2013).

The use of PCR and biochemical identification to confirm *S.aureus* strains revealed similar results (average86.32%), which confirms the concordance between the two techniques, already reported by the study ofBenhamed (2014).

The present study shows that the poultry isolates can be enterotoxigenic (12.19%). These results are in good agreement with the previous published studies which revealed that the percentage of *S.aureus* strains producing enterotoxin A to E, bovine and avian, can vary from 0 to 15% (Bergdoll, 1991; Genigeorgis, 1989;Rosec et al., 1997). More recent studies showed that the genes encoding the classical enterotoxins, were absent or occur in less than 3% in *S.aureus* strains isolated from poultry (Hazariwala et al., 2002; Normanno et al., 2007; Smyth et al., 2005).

As for the chicken some authors note relatively higher percentages. For instance, Nader et al.(2016)show that 7 strains out of 12 (58.3%) of *S.aureus* isolated from chicken meat were enterotoxigenic.They also reported the presence of *sea*, *seb* and *sed* genes in chicken meat isolates, with percentages of 33.3%; 8.3% and 16.7% respectively.

Kitai et al.(2005) revealed that 21.7% of *S.aureus* strains, isolated from chicken carcasses were enterotoxigenic. Moreover, Nemati (2013) observed that71% of *S.aureus* strains isolated from chicken nose and cloaca were enterotoxigenic. As for the turkey isolates, Koluman et al.(2011) showed that the production of enterotoxins in turkey meat was 36%.

However, the present study demonstrated that the prevalence of enterotoxigenic isolates from chicken carcasses (15%) and turkey (4.54%) is relatively lower.

Although, there was a variability in the frequency of different enterotoxins types production, in chicken we observed the dominance of *seb* gene with a rate of 13.33% compared to the *sed gene* (1.67%).Similar results were stated by Kitai et al.(2005). Nevertheless, only type D enterotoxins (4.54%) were detected in turkey isolates. This variability between the two species had not yet been defined.

Madahi et al.(2014) showed that *S.aureus* strains isolated from chicken nuggets present staphylococcus enterotoxins genes, with values of 33.33%; 4.16%; 12.50%; 8.33%, 12.50%; 12.50% for *sea*, *seb*, *sec*, *sed*, *sea* + *sec* and *sea* + *sed* respectively. No *see* gene was detected. Another study by Nemati (2013) also reported that 33.3% of *S.aureus* strains isolated from the nose and cloaca of healthy chicken were positive for *sea* and 5% had a mixed *sea* + *egc* enterotoxins production, whereas the genes encoding *seb*, *sec*, *sed* and *see* were absent.

A study of the incidence of enterotoxigenic *S.aureus* strains in turkey meat, was carried out by Bystron et

al.(2005) presenting that from the 4 coagulase-positive Staphylococcus strains, isolated from 11 samples of turkey meat the 3 strains had enterotoxin genes of type B and one had type C. Adams and Mead (1983)isolated enterotoxigenic Staphylococci in 2 of the 3 turkey' slaughterhouses studied. They reported that in the first slaughterhouse60% of the 55 isolates produced enterotoxin C while in slaughterhouse B, 4% of the 41 isolates produced enterotoxin D and 2% produced enterotoxin F. No enterotoxigenicity was detected from the 50 isolates in the third slaughterhouse.

As type A enterotoxin is usually typical for the human origin isolates (Orden et al., 1992) and type C enterotoxin is frequently produced by bovine origin isolates (da Silva et al., 2005; Jorgensen et al., 2005; Katsuda et al., 2005)could explain their absence in poultry studies.

The variability observed in the frequency of enterotoxin production by *S.aureus* strains may be related to the origin of the studied isolates (food or other) and their geographic origin(Bergdoll, 1991; Genigeorgis, 1989; Larsen et al., 2000; Rosec et al., 1997).

Type A, B and D enterotoxins are implicated in 95% of reported foodborne illness outbreaks (Letertre et al., 2003). During the years 2001-2003 in Taiwan, enterotoxins A, B, C, and D were detected with respective percentages of 29.2%; 19.7%; 6.8% and 2.0% in patients associated with staphylococcal foodborne illness outbreaks (Chiang et al., 2008). Furthermore, more than 50% of staphylococcal food-borne infections were caused by type A enterotoxin. In addition, *sea* and *seb* enterotoxins are the two most important agents causing gastroenteritis, they are also the most implicated in foodborne illness (> 60%) in the United States and England (Kluytmans and Wertheim, 2005).

CONCLUSION

We have experimentally determined the prevalence and enterotoxins genes of *S.aureus* in fresh poultry carcasses that are collected from 16different slaughterhouses located in the region of Algiers, Algeria. The investigation has revealed that the microbial risk by *S.aureus* in poultry carcasses is not negligible. It has been found that the prevalence of *S.aureus* in 385 carcasses was 41.56% with an individual prevalence of 40.63% and 45.71% for chicken and turkey respectively. The strains of *S.aureus* of avian origin can be enterotoxigenic with the predominance of the genes encoding for enterotoxin *seb* in chicken and *sed* in turkey.

When meat is preserved under favorable conditions for germs multiplication and toxinogenese, enterotoxins can be produced in sufficient amount to trigger foodborne illnesses. These potential dangers must be considered in terms of real risk to public health. To ensure the safety and hygienic quality of meat, the application of good hygiene practice (GHP) and the implementation of HACCP in poultry slaughterhouses has become an absolute necessity to protect consumer' health.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Case Report
Κλινικό Περιστατικό

Ovarian remnant syndrome after laparoscopic ovariectomy in a cat

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ABSTRACT. The first case of accidental implantation of ovarian tissue at the port site after laparoscopic ovariectomy in a cat was described. A 2-year-old, spayed female, domestic cat showed recurrent behavioral oestrus signs (vocalization, increased playfulness, and lordosis) four months after laparoscopic ovariectomy. Abdominal ultrasound revealed bilateral endometrial hyperplasia and an inhomogeneous mass attached to the abdominal wall at the port site of previous laparoscopic ovariectomy. Ovarian remnant syndrome was supposed. Diagnostic laparoscopy confirmed the presence of a mass that was 2 cm in diameter and macroscopically similar to ovarian tissue. Laparoscopy was converted to laparotomy to remove the abnormal tissue and the uterus. Histopathological findings showed follicles of various maturation stages in the mass, and confirmed endometrial hyperplasia. Histology underscored that ovarian remnant tissue resumed function. No clinical signs consistent with ovarian remnant syndrome were reported six months after excision of the abnormal tissue. In conclusion, removal of the ovaries through the laparoscopic port site may cause fortuitous ovarian tissue implantation at the abdominal wall in the cat.

Keywords: ovarian remnant syndrome, ORS, laparoscopy, ovariectomy, cat

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CASE HISTORY

A 2-year-old, spayed female, 3.6 kg domestic cat was referred for recurrent clinical signs of oestrus (vocalization, increased playfulness, and lordosis) appeared 4 months after laparoscopic ovariectomy.

The queen's vital parameters, serum biochemical parameters and complete blood count were within normal limits. No other clinical signs (i.e. inappetence, dysuria, vomiting, and diarrhea) were reported. Mild abdominal pain was detected during abdominal palpation. Abdominal ultrasound revealed bilateral endometrial hyperplasia and an inhomogeneous mass attached to the abdominal wall. This mass measuring 2 cm in diameter was medial to the left kidney, approximatively 1.5 cm caudal to the umbilicus. ORS was supposed based on anamnesis, clinical signs and ultrasound examination. The owner did not give his consent to perform hormonal analysis and vaginal cytology. The queen underwent diagnostic laparoscopy performed by the same surgeon that carried out previous laparoscopic ovariectomy.

The queen was premedicated with methadone (Semfortan®, Dechra) 0.2 mg/kg, dexmedetomidine (Dexdomitor®, Orion Pharma Zoetis) 4 μg/kg and ketamine (Lobotor®, Acme) 2 mg/kg combined and administered intramuscularly. Anesthesia was induced with 2 mg/kg of propofol (Proposure®, Merial) intravenously (IV), and endotracheal intubation was performed with a cuffed tube. Anesthesia was maintained with isoflurane (Isoflo®, Esteve) delivered in 100% oxygen via a rebreathing circuit. Ringer's lactate solution at a rate of 10 mL/kg/h was administered IV during surgery. The breathing was supported by intermittent positive pressure ventilation to maintain end-tidal carbon dioxide tension in a range of 30−40 mmHg during surgery. The queen was administered 20 mg/kg, IV, cephazoline (Cefazolina®, Teva) every 90 minutes throughout surgery and 0.2 mg/kg, subcutaneously, meloxicam (Meloxoral®, Ati) upon extubation.

The patient was positioned in dorsal recumbency. A 5-mm cannula (T1) (Trocar X-ONE, MedLine) was inserted 1 cm cranially to the umbilicus on midline to create pneumoperitoneum using carbon dioxide by an automatic insufflator (264305 20, Karl Storz Endoscopy) with a pressure of 5-7 mmHg, and to insert a 30° telescope (Hopkins II, Karl Storz Endoscopy). The presence of a mass, shaped like ovarian tissue, was confirmed (Fig. 1). The mass was attached to the peritoneal area caudal to the umbilicus, where the laparoscopic port was inserted to perform previous laparoscopic ovariectomy. To exclude incomplete removal of ovarian tissue, each suspensory ovarian ligament was lifted using Wolf grasping forceps (Wolf Medical Instrument, Knittlingen) introduced in a 5-mm cannula (T2) (Trocar X-ONE, MedLine) inserted 2 cm caudally to the umbilicus. During these procedures, hyperplasia of both uterine horns was underscored. Laparoscopic procedure was converted to open surgery (Fig. 2) to remove the mass (Fig. 3) and to perform hysterectomy. The abdominal wall and subcutaneous tissues were closed in a continuous pattern using, respective-

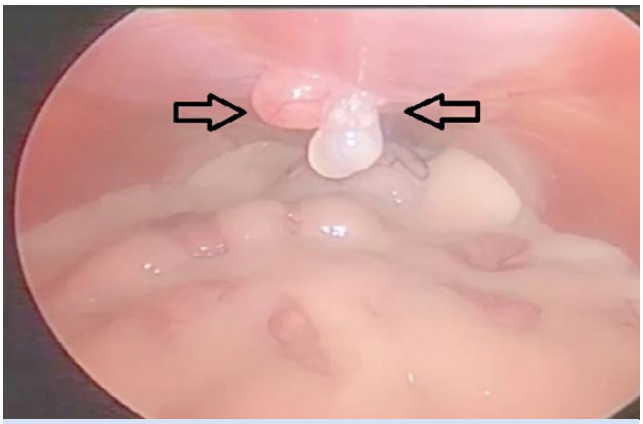

Figure 1. Laparoscopic photograph showing the presence of a mass (arrows) attached to the peritoneum

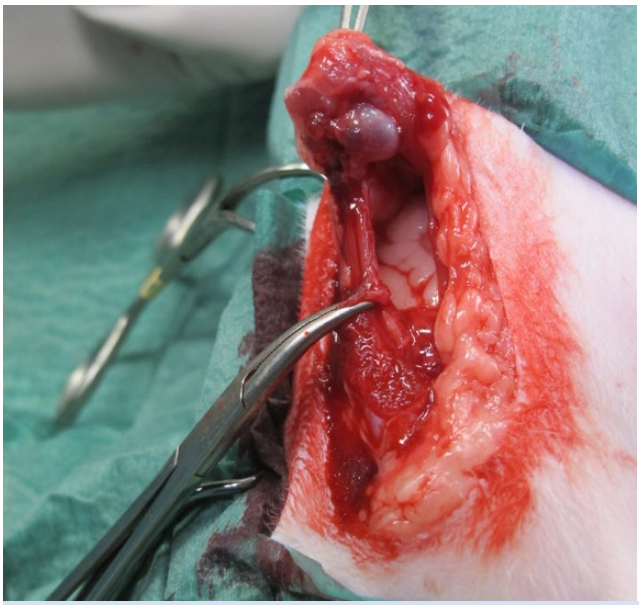

Figure 2. Photograph showing the macroscopic features of the mass

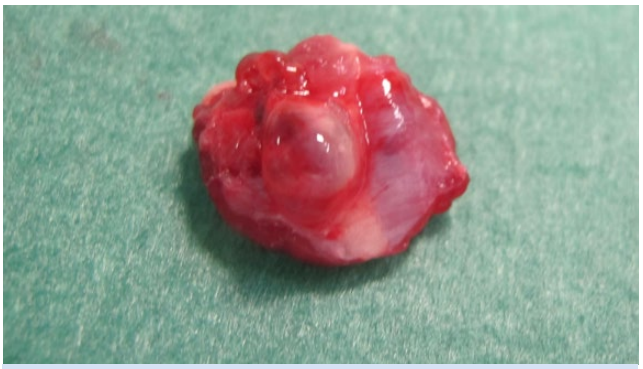

Figure 3. Photograph of the excised mass

ly, 3-0 polydioxanone (PDS®, Ethicon) and 3-0 polyglactin 910 (Vicryl®, Ethicon). The skin was closed in an interrupted pattern using 3-0 nylon (Assunyl®, Assut Europe).

Recovery from anesthesia was uneventfully. The patient was discharged within 24 hours of recovery from surgery with 0.1 mg/kg, orally, SID, meloxicam (Metacam®, Boehringer Ingelheim) and 22 mg/kg, orally, BID, amoxicillin combined to clavulanic acid (Clavaseptin®, Vetoquinol) for seven days.

Histology of the uterus showed hyperplastic endometrium and moderately hyperplastic glandular component, with the formation of multiple cystic dilatations containing eosinophil secretion. Moderate fibroplasia was observed around the proliferated glandular structures. Histopathologic studies of the mass revealed large follicles of various maturation stages (Fig. 4).

The patient did not show any clinical signs of oestrus

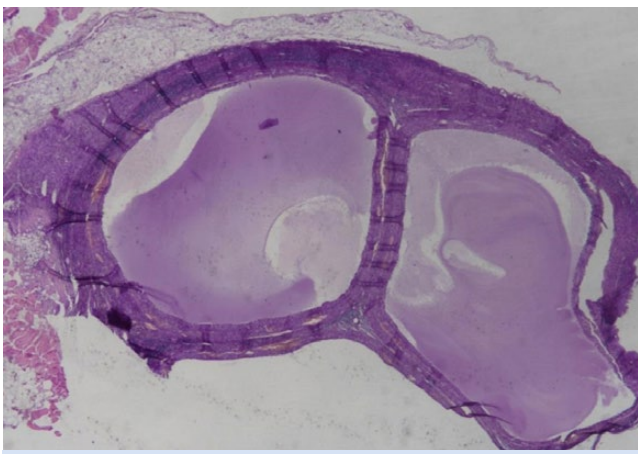

Figure 4. Photomicrograph of the excised mass. There are large follicles of various maturation stages.

in the following 6 months.

DISCUSSION

Ovarian remnant syndrome (ORS) is characterized by the presence of functional ovarian tissue after previous ovariectomy and it is considered a long-term complication of ovariectomy (Miller, 1995; DeNardo et al., 2001). ORS is commonly caused by incomplete removal of ovarian tissue, dropping of a thin piece of ovarian tissue into the abdomen, and accidental implantation of ovarian tissue (Ball et al., 2010). Remnant tissue may contain follicles, corpus lutea, luteoma and germ cells (DeNardo et al., 2001) and cats may show clinical signs of oestrus, mild abdominal pain, endometrial hyperplasia and stump pyometra (Wallace, 1991; Demirel and Acar, 2012).

In women, autograft of ovarian tissue can occur at the incision site after laparoscopic oophorectomy (Marconi et al., 1997; Chao, 2008). Surgical techniques based on laparoscopy are widely applied in dogs. Similarly, laparoscopic ovariectomy is feasible in cats (Van Nimwegen et al., 2007; Coisman et al., 2014). Compared to traditional open surgery, laparoscopic ovariectomy reduces recovery period, postoperative discomfort, pain and the incidence of infection, nevertheless some complications may occur (Van Nimwegen et al., 2007). In the veterinary literature, ORS has not been previously reported after laparoscopic ovariectomy.

The present report describes the first case of ORS after laparoscopic ovariectomy in a cat. Clinical signs of oestrus after previous ovariectomy and histopathological findings confirmed the suspected diagnosis. If ORS is supposed, vaginal cytology and hormonal assay should be performed, although the cytological and endocrine changes during feline cycle are often unpredictable (Wallace, 1991). Consequently, if clinical signs and imaging investigations suggest ORS, diagnostic laparoscopy or explorative celiotomy are recommended. Afterwards, histological findings will confirm the diagnosis of residual ovarian tissue.

This case report adds essential information about the preservation of ovarian tissue in the queen because it underscores that an unexpected and non-experimentally induced ovarian tissue implantation at the peritoneum of the abdominal wall can spontaneously revitalize

and resume function. In fact, ORS related to free ovarian tissue has been only experimentally demonstrated in the queen until today. In four cats, the ovarian cortex was implanted into the peritoneum of the abdominal wall and, after 12 weeks, all cats presented active ovarian tissue (Shemwell and Weed, 1970). Another finding reported that, in eight cats out of nine, free-floating ovarian tissue showed histopathological features of ovarian activity six months after ovariohysterectomy (DeNardo et al., 2001). In the present case report, remnant ovarian tissue resumed its function 4 months after laparoscopic ovariectomy. It is likely that a thin piece of free ovarian tissue spontaneously adhered to the peritoneum with subsequent angiogenesis into the ovarian tissue. These events were probably induced by fibroblast growth factor, transforming growth factor and vascular endothelial growth factor (Demeestere et al., 2009).

In the human literature, only three cases reported residual ovarian tissue on the abdominal wall after laparoscopic surgery (Marconi et al., 1997; Chao, 2008; Na et al., 2013). In the first case, a piece of ovarian tissue remained in the patient's abdominal wall (Marconi et al., 1997), whereas in the other two cases accidental implantation of a fragment of ovarian tissue occurred during the extraction of the ovary through the laparoscopic port (Chao, 2008; Na et al., 2013). Laparoscopic ovariectomy is commonly performed in dogs and it is similarly feasible in cats, though some complications may occur (Van Nimwegen et al., 2007). The case described here is the first report that underscores ORS after laparoscopic ovariectomy in the veterinary literature. The origin of residual ovarian tissue has not been clearly recognized; nevertheless, it is likely that ovarian tissue may be fragmented during ovary extraction through the trocar. If the size of the ovary is large compared to the diameter of the trocar, removal of the ovary through the port is quite difficult and ovarian tissue may be fragmented. Albeit fragmentation of ovarian tissue is uncommon, it is important to adopt preventive methods as follow: large-diameters trocars, retrieval plastic bags, laparoscopic morcellator and abundant irrigation of the surgical field (Nezhat et al., 2000; Chao, 2008; Na et al., 2013).

In conclusion, the present case report underlines that accidental implantation of a thin piece of ovarian tissue on the abdominal wall is a likely long-term complication of laparoscopic ovariectomy in the cat. Ovarian remnant tissue may resume function and must be excised. Consequently, it is mandatory to take preventive methods to avoid this problem.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Case Report
Κλινικό Περιστατικό

Thymoma associated exfoliative dermatitis in a rabbit with hepatic cirrhosis.

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Αποφολιδωτική δερματίτιδα λόγω θυμώματος σε κουνέλι με κίρρωση του ήπατος.

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ABSTRACT. A 6.5-year-old, male, non-castrated pet rabbit was presented with generalized exfoliative dermatitis, depression and anorexia. Skin laboratory examinations were negative for ectoparasites, bacteria and fungi whereas thoracic radiography revealed a large mediastinal mass. Complete blood and biochemical examinations did not show anything abnormal. Thymoma was suspected and therapeutic options were discussed but the owner declined. Supportive and symptomatic therapy was of no avail and severe deterioration of systemic symptoms necessitated euthanasia. Necropsy revealed a whitish-gray mass on the anterior and lower part of the mediastinum and pale voluminous liver. Histopathology confirmed the presence of thymoma and the hypothesis of thymoma-associated exfoliative dermatitis along with hepatic cirrhosis.

Keywords: Rabbit, Thymoma, Dermatitis, Paraneoplastic, Cirrhosis

ΠΕΡΙΛΗΨΗ. Ένα κατοικίδιο κουνέλι 6.5 ετών, αρσενικό, ακέραιο προσκομίστηκε με γενικευμένη αποφολιδωτική δερματίτιδα, κατάπτωση και ανορεξία. Οι εργαστηριακές εξετάσεις του δέρματος ήταν αρνητικές για εξωπαράσιτα, βακτήρια και μύκητες, ενώ στην ακτινογραφία του θώρακος παρατηρήθηκε ευμεγέθης μάζα στο μεσοπνευμόνιο. Η γενική εξέταση αίματος και οι βιοχημικές εξετάσεις δεν εμφάνιζαν αποκλίσεις από τις φυσιολογικές τιμές. Υπήρξαν ισχυρές υποψίες για θύμωμα και οι θεραπευτικές επιλογές συζητήθηκαν, αλλά ο ιδιοκτήτης αρνήθηκε. Η υποστηρικτική και συμπτωματική θεραπεία αποδείχθηκαν αναποτελεσματικές, γεγονός το οποίο, και σε συνδυασμό με την σοβαρή επιδείνωση των συστηματικών συμπτωμάτων καθιστούσαν αναγκαία την ευθανασία του ζώου. Κατά τη νεκροψία παρατηρήθηκε μάζα λευκόφαιου χρώματος στο πρόσθιο και κοιλιακό τμήμα του μεσοπνευμόνιου, καθώς και ωχρό και διογκωμένο ήπαρ. Οι ιστοπαθολογικές εξετάσεις επιβεβαίωσαν την ύπαρξη θυμώματος και έθεσαν διάγνωση παρανεοπλασματικής αποφολιδωτικής δερματίτιδας λόγω θυμώματος, σε συνδυασμό με κίρρωση του ήπατος.

Λέξεις ευρετηρίασης: Κουνέλι, Θύμωμα, Δερματίτιδα, Παρανεοπλασματική, Κίρρωση

INTRODUCTION

Exfoliative dermatitis in rabbits is a challenging multifactorial disease that can be attributed to malnutrition, ectoparasitosis, dermatophytosis, cheyletielosis, Malassezia dermatitis, sebaceous adenitis (Jassies-van der Lee et al., 2009; Miller et al., 2013), dermatosis with response to zinc (Bentley and Grubb, 1991), epitheliotropic lymphoma (White et al., 2000), erythema multiforme (Scott, 2010), or can be associated with mediastinal (Florizoone, 2005; Rostaher-Prélaud et al., 2013) and interface hepatitis (Florizoone et al., 2007). Paraneoplastic exfoliative dermatitis in rabbits is rare in veterinary medicine. Five cases have been reported to this point, from which only 4 were completely diagnosed with a post-mortem histopathological examination. One was consistent with thymic lymphoma (Rostaher-Prélaud et al., 2013) and the other 3 were thymomas (Florizoone, 2005; Rostaher-Prélaud et al., 2013). Thymoma, though uncommon, is the most common mediastinal mass in rabbits (Clippinger et al., 1998; Morrisey and McEntee, 2005). Diagnostic approach requires a prompt history, a careful clinical and dermatological examination, imaging and biopsy to confirm diagnosis. Additionally, only two exfoliative dermatitis cases have been related to hepatitis; one was described as interface hepatitis and was comparable to autoimmune hepatitis in man (Florizoone et al., 2007), and the other was of non-immune-mediated origin (Isasi et al., 2012). To the authors' knowledge, this is the first case of paraneoplastic exfoliative dermatitis in rabbit related to thymoma along with hepatic disease.

CASE HISTORY

A 6.5-year-old, male, non-castrated pet rabbit, weighing 1.7 kg, was referred to our Clinic with a history of at least one month non pruritic scaly dermatitis and blepharitis, accompanied with anorexia and depres-

sion the last week. The rabbit was not vaccinated or dewormed, fed a balanced diet, that included hay/pellet and fresh fruits and vegetables, and housed indoors with two other female rabbits, but in an individual enclosure. The other rabbits were clinically healthy, since they did not exhibit clinical signs of any type. According to the history demodicosis was suspected based on a positive skin scraping and antiparasitic therapy with ivermectin (Valaneq®, Merial) 0.4 mg/kg SC twice every 15 days was started. However, since no improvement was seen additional therapy was suggested by the referring veterinarian including fusidic acid eye ointment (Fucidin®, Leo Pharmaceutical Hellas S.A.) BID, dexpathenol cream (Bepanthol®, Bayer AG) BID and enrofloxacin (Baytril®, Bayer Animal Health GmbH) 5 mg/kg PO SID for the last three days but this also was of no avail. No medication had been given before the appearance of skin lesions.

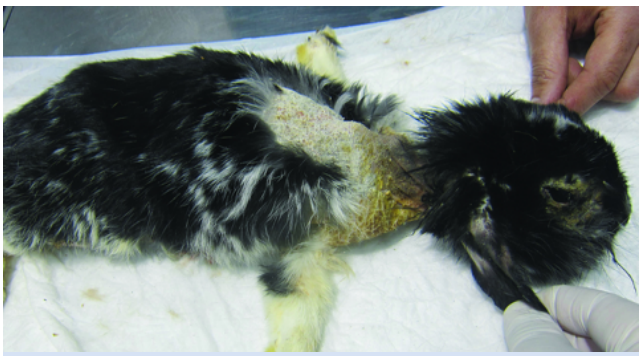

Fig 1: Hypotrichosis in the base of ear pinnae, alopecia over the neck region along with erythema and scaling.

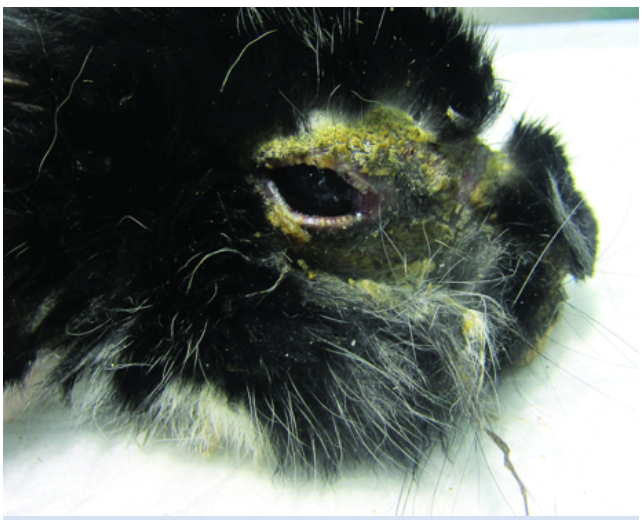

Fig 2: Symmetrical periocular lesions characterized by alopecia, crusting, scaling and erythema

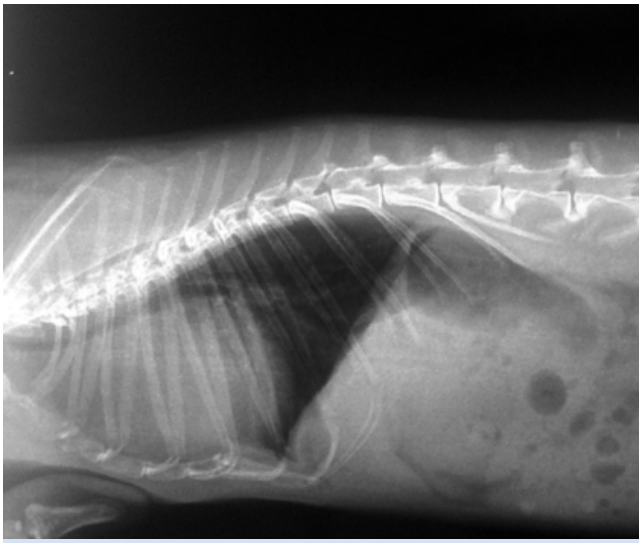

Fig 3: Lateral chest radiograph: Soft tissue mass in the cranial mediastinum. Notice the characteristic elevation of the trachea.

Upon clinical examination generalized non-pruritic dermatitis, depression, weight loss and reluctance to move were noticed. Skin lesions were characterized by hypotrichosis, alopecia, erythema, copious whitish scales, crusting and follicular casts. The main affected areas were the face, the neck, the sternum, the dorsum, the abdomen and the inner thighs (Figure 1). Severe bilateral erythroderma of the eyelids, blepharedema, periocular crusting and scaling dermatitis, blepharitis and mucopurulent ocular discharge were also observed, whereas a superficial corneal ulcer and cataract were also present in the left eye (Figure 2). Multiple superficial and deep skin scrapings from the lesional areas, acetate tape impression smears, trichograms and a DTM culture were negative for parasites, bacteria and fungi. Thorax auscultation as well as the palpation of the abdomen, appeared normal. Complete blood and biochemical examinations did not show anything abnormal. Lateral and ventrodorsal thoracic radiography revealed a space-occupying mediastinal mass in the cranial thoracic cavity (Figure 3).

A paraneoplastic exfoliative dermatosis due to systemic causes was suspected, whereas the differential diagnosis included thymoma, thymic lymphoma, epitheliotropic lymphoma and/or hepatitis. Multiple skin biopsies to confirm diagnosis and surgical removal of the mass were recommended, but the owner declined due to possible fatal side effects.

Symptomatic treatment and supportive therapy initiated

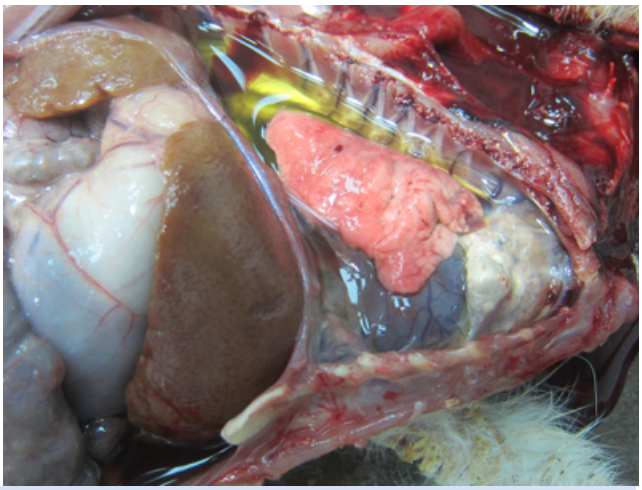

Fig 4: Non-homogeneous, greyish mass occupying the lower, anterior mediastinum, compatible with thymoma.

with rehydration fluids: Lactated Ringer's Solution iv, along with antibiotics: enrofloxacin (Baytril®, Bayer Animal Health) 5mg/kg PO BID for 15 days, meloxicam (Novacam®, AST Pharma) 0.25mg/kg SID for 7 days and probiotics (Florentero®, Candioli) 1 cc PO SID. Local ophthalmic treatment with azythromycin (Azyter® eye drops, Laboratoires Thea) in the left eye for 3 days and tobramycin (Tobrex® eye ointment, Alcon) TID for 7 days in both eyes were also administered. To improve the intense desquamation of the area, 30% propylene glycol, applications over the head were performed. Force feeding with critical care diet (Critical Care®, Oxbow), high-fiber supplements (Immune support®, Oxbow) 1tab PO SID, antiseborrheic shampoo (Douxo Séborrhée® Shampooing, Sogeval) once weekly as well as daily change of the substrate were also recommended. Upon re-examination 45 days later, severe deterioration of systemic signs and radiography findings along with severe dyspnea, necessitated euthanasia.

On necropsy, a non-homogeneous, whitish-gray mass measuring 3 x 3 x 2.5 cm was detected in the anterior and lower part of the mediastinum. Free flowing, clear liquid was present in the thoracic and abdominal cavity. The lungs were emphysematous and the liver appeared discoloured (Figure 4). Histopathology of the mass revealed a moderately cellular neoplasm composed of a dual population of polygonal and round cells arranged in small nests and trabeculae. Polygonal cells had distinct cell borders, abundant eosinophilic cytoplasm and basophilic nuclei. Round had indistinct cell borders, a variable amount of granular eosinophilic

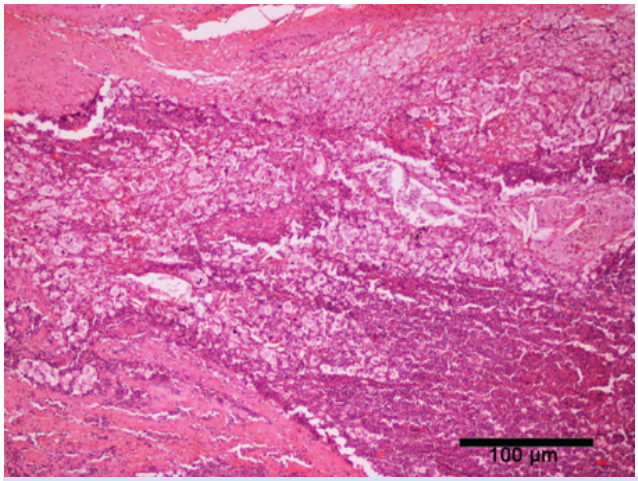

Fig 5: Histo thymus: neoplastic population of large epithelial cells arranged in small nests and trabeculae disrupt the normal thymic architecture. Haematoxylin-eosin staining, bar=100μm.

cytoplasm, round to oval nuclei with finely-stippled chromatin and one variably distinct nucleolus. Multifocally scattered throughout the neoplasm were many tingible body macrophages (Figure 5). Histopathological examination of skin samples showed transepidermal and follicular apoptosis with severe hyperkeratosis, mild to moderate lymphoplasmacytic dermatitis and generalized absence of sebaceous glands (Figure 6). Moreover, histological examination of the liver showed distortion of the hepatic architecture. Specifically, portal areas were markedly expanded and adjacent portal triads were bridged with bands of poorly cellular con-

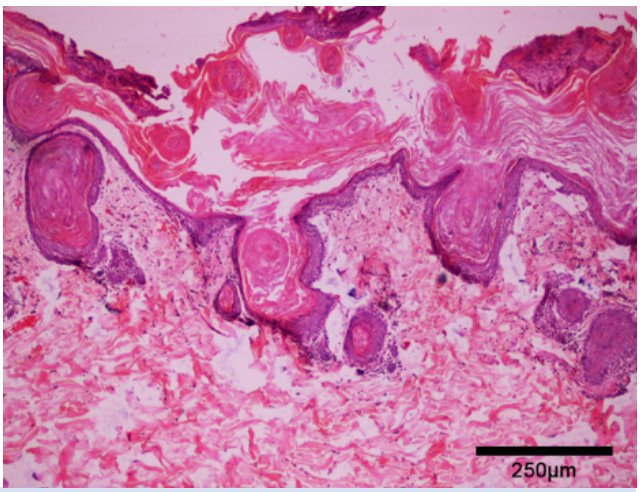

Fig 6: Histo skin: severe hyperkeratosis and mild infiltration by lymphocytes and plasma cells. Haematoxylin-eosin staining, bar=250μm.

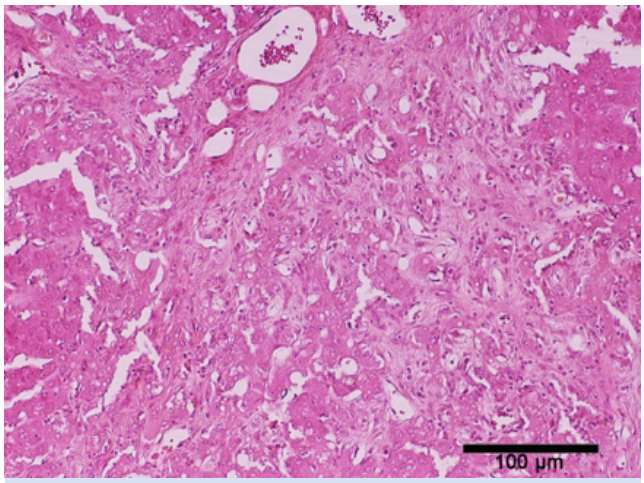

Fig 7: Histo liver: hepatic architecture is distorted due to severe fibrosis. Haematoxylin-eosin staining bar=100µm.

nective tissue, rich in collagen and blood vessels (bringing fibrosis). Multifocally, fibroblasts disrupted the limiting plate and extended into the adjacent periportal parenchyma and separated, surrounded, and replaced hepatocytes that were often degenerate with swollen vacuolated cytoplasm or necrotic with shrunken hypereosinophilic cytoplasm and karyolytic or karyorrhectic nuclei. Areas of fibrosis were multifocally infiltrated by scattered lymphocytes and neutrophils. Hepatocytes exhibited a degree of atypia (mild anisocytosis and anisocariosis, occasionally more than one prominent nucleolus) (Figure 7).

Based on the localization of the mediastinal mass and the histological findings, the diagnosis of mixed lymphoepithelial thymoma and thymoma-associated exfoliative dermatitis, along with hepatic cirrhosis was established.

DISCUSSION

Thymomas and thymic lymphomas represent the primary mediastinal masses in rabbits (Clippinger et al., 1998; Florizoone, 2005; Morrisey and McEntee, 2005). To date, they appear to be more common than previously reported, though there is still not much information about their frequency of occurrence (Künzel et al., 2012; Snook et al., 2013). Signs of disease differ from those seen in other species such dogs, cats or humans (Clippinger et al., 1998; Florizoone, 2005). Main signals include dyspnea, fatigue, depression and characteristically bilateral exophthalmos as a result of precaval syndrome (Clippinger et al., 1998; Morrisey and McEntee, 2005). Oedema of the neck, head, and forelimb, lack of appetite, coughing and bilateral prolapse of the third eyelid may also be present. Auscultation of the thorax may reveal muffled heart sounds or a heart murmur, and diminished lung sounds, due to the decreased compressibility of the chest (Florizoone, 2005; Künzel et al., 2012). Even though many of the above symptoms were present in our case, thymoma was not in the first line, since there was absence of exophthalmos, no dyspnea and auscultation findings on admission. Only upon re-examination, further distention of the mediastinal mass resulted in severe dyspnea.

Radiographs, ultrasonography and CT are used for the diagnostic imaging of mediastinal masses in rabbits. In thoracic radiographs, a soft-tissue mass in the cranial mediastinum is visualised, which causes a characteristic elevation of the trachea, findings compatible with our case. Its consistency can be verified via ultra-sonography. Ultrasound-guided FNA may be diagnostic and give good information about the mass origin, unless there are too few cells for an evaluation (Künzel et al., 2012). Cross-sectional imaging with CT provides the most detailed information regarding the full extent of the disease and consistency of the mass (Weisbroth, 1994). Diagnostic cytology may reveal variably sized well-differentiated lymphocytes and epithelial cells, as well as mast cells, eosinophils, macrophages, melanocytes, plasma cells and neutrophils (Zitz et al., 2008). Definite diagnosis of lymphoma requires fine needle aspiration or biopsy. In lymphoma, which is the primary differential for a cranial mediastinal mass in a rabbit, small, mature lymphocytes are dominant (Florizoone et al., 2005; Rostaher-Prélaud et al., 2013). In our case diagnosis was guided only by radiographs, since the owner declined any more exams in the first place.

Liver disease has been also incriminated to cause exfoliative dermatitis in rabbits. More specifically, this has been observed in one rabbit with interface hepatitis comparable to human autoimmune hepatitis (Florizoone et al., 2007), and another in a rabbit with bacterial chronic hepatitis (Isasi et al., 2012). The mechanisms of this condition are still under speculation. It appears to be related to an immune-mediated response in which T-cells, originating from the internal organs, affect the cells in the dermis and epidermis (Isasi et al., 2012). In our case hepatic cirrhosis could be attributed to several

causes. However, post mortem and histopathological examination excluded parasitic and bacterial disease as well as neoplasia and liver lipidosis. Interface hepatitis can be associated with hepatocytic damage, necrosis and progressive fibrosis that ultimately leads to liver cirrhosis. Nevertheless, at this point the cause of the fibrotic process of the liver is difficult to establish.

In affected with thymoma rabbits there aren't any significant changes in hematology, serum and biochemistry tests (Künzel et al., 2012). Hypercalcemia has been previously discussed, but a possible influence of diet and Ca metabolism on the serum concentration of calcium in rabbits has not been taken into consideration (Weisbroth, 1994; Rosenthal et al., 1995). Hyperglycemia may be present but it is considered to be stress-induced (Künzel et al., 2012). Thus far, immune disorders have not been described in rabbits with these tumours. In addition, hepatic disorders can be challenging to diagnose via basic serum biochemistry findings, as there are no liver-specific enzymes in the rabbit (Vennen and Mitchell, 2009). In our case complete blood and biochemical examinations were were not conclusive. Total bilirubin and γ-GT may be useful combined with ultrasonography (Harkness et al., 2010). However, they are usually not part of a basic serum biochemistry profile and were therefore not included in this case.

Thymomas have the potential to cause significant morbidity and mortality (Clippinger et al., 1998). Various treatment options are available depending on the case, including surgery, chemotherapy, and radiation therapy (Clippinger et al., 1998; Morrisey and McEntee, 2005; Künzel et al., 2012). They are generally considered to be benign; and there is great potential for treatment to be curative. Metastasis can occur but is quite rare (Morrisey and McEntee, 2005); only one case is reported with metastasis to thoracic organs and abdominal lymph nodes in a rabbit with a thymoma (Heatley and Smith, 2004). Consequently, the sooner the mass is detected, the better the potential outcome (Clippinger et al., 1998; Florizoone, 2005; Künzel et al., 2012). Surgical removal is the treatment of choice, with good long-term postoperative survival rates (Clippinger et al., 1998; Morrisey and McEntee, 2005; Künzel et al., 2012). The most common complication after surgical resection of thymoma in rabbits is acute perioperative death (Turek, 2003). Survival time without treatment has been documented in only 3 cases with an average survival period of 6 months (Künzel et al., 2012). In the present report, no metastasis was observed and therefore the prognosis looked fair, with the anaesthetic risk being the primary concern. The therapeutic options were discussed, but the owner hesitated due to possible fatal side effects. Later, euthanasia was recommended, since the clinical signs and radiography findings worsen.

Based on the predominant cells, thymomas are categorized as lymphocyte predominant (>50% lymphocytes), epithelial predominant (>50% epithelial cells) and mixed lymphoepithelial (50:50 cells) (Künzel et al., 2012), the latter being the category of which our case came under. It seems possible that abnormal antigen presentation of neoplastic thymic epithelial cells triggers a T-cell mediated process, resulting in autoimmune disease (Carpenter and Holzworth, 1982; Rottenberg et al., 2004).

Thymoma has a key role in causing exfoliative dermatitis in several species. The pathophysiology of thymoma-associated exfoliative dermatitis in cats has yet to be completely determined. A number of theories have been hypothesized and are focused on the role of the normal thymus in the development of the immune system (Rottenberg et al., 2004). In dogs and cats, an association is present between thymoma and myasthenia gravis and in cats between thymoma and exfoliative dermatitis (Carpenter and Holzworth, 1982; Kesler et al., 2004; Gross et al., 2005). In rabbits, the latter, though rare, is possible (Florizoone, 2005). The present case shares many characteristics with exfoliative dermatitis in cats. The lesions in cats appear as non-pruritic white scales with focal areas of alopecia, sometimes with erythema. Histologically a cell-poor interface dermatitis is found (mild lymphocytic exocytosis, apoptosis of keratinocytes in the basal cell layer and to a lesser extent in the stratum spinosum). Areas of cell-rich interface dermatitis as well as loss of sebaceous glands are typical findings (Carpenter and Holzworth, 1982). In addition, two cases of exfoliative dermatitis have been described in cats, in which no evidence of internal disease or neoplasia was found after necropsy, suggesting that this disease was an indicative reaction pattern of immunity driven by non-exclusive feline thymoma T cells (Gross et al., 2005). In human dermatology, exfoliative

dermatitis has been described very well and related to paraneoplastic syndromes (lymphoma and leukemia, but not as a thymoma-associated), adverse reactions to drugs, cutaneous manifestations of Sezary syndrome and idiopathic causes (Boyce and Harper, 2002; Isasi et al., 2012).

Demodex cuniculi is rarely found in skin scrapings taken from rabbits (Harvey, 1990). Even though its pathological significance is unknown, this parasite is considered a normal resident of the epidermis and hair follicle of rabbits (Harvey, 1990). Affected rabbits can show variable levels of pruritus (Paterson, 2006), or can be completely normal (Jenkins, 2001). On the other hand, adult onset demodectic mange in dogs and cats has been associated with systemic disease and with the administration of immunosuppressive chemicals (White et al., 1987; Mueller, 2004). In our case the absence of repetitive positive skin scrapings and the absence of parasitic elements in the histopathology preparations further support previous reports that its can be incidental. However further research is required to better understand its aetiopathogenesis in rabbits.

Conclusively, thymomas though relatively uncommon, have been related to exfoliative dermatitis in rabbits. Autoimmune interface hepatitis and non-immune-related hepatitis, have also been reported in cases with exfoliative dermatitis. Both hepatic disorders and thymic neoplasia in rabbits are challenging conditions to diagnose. Generalized scaling disorders, coupled with concurrent histologic changes of lymphocytic mural, interface dermatitis and absence of sebaceous glands should induce the clinician to search for a systemic cause, and include thymic neoplasia on the top and/or hepatic disorders in differential diagnosis.

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