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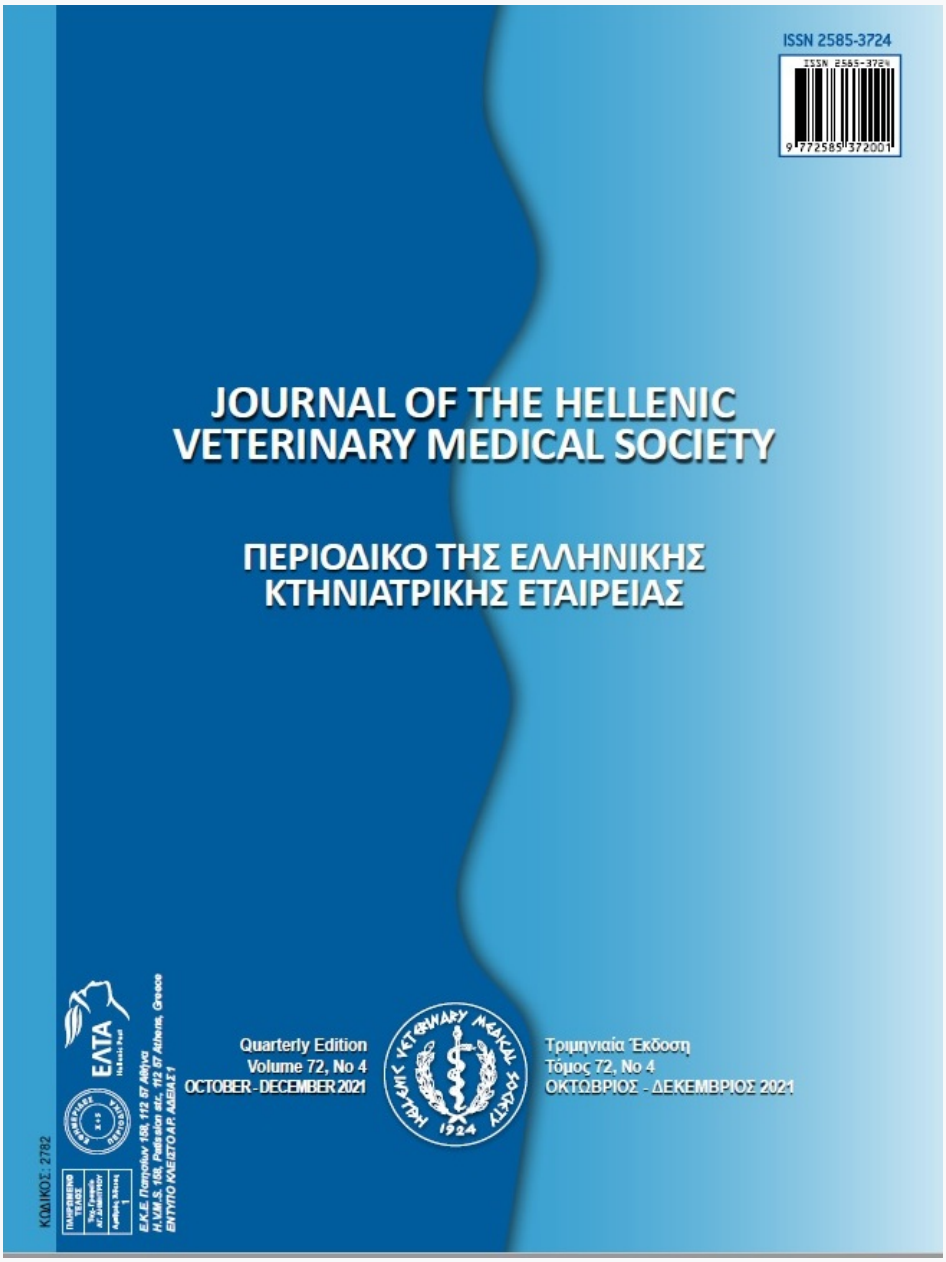

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Update: July 2019
Ενημέρωση: Ιούλιος 2019

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ΕΥΡΩΠΑΪΚΑ ΚΟΛΕΓΙΑ ΕΙΔΙΚΕΥΜΕΝΩΝ ΚΤΗΝΙΑΤΡΩΝ

				Number of specialist veterinarians active in Greece Αριθμός ειδικευμένων κτηνιάτρων εργαζόμενων στην Ελλάδα
1		ECAR	European College of Animal Reproduction	2
2		ECAWBM	European College of Animal Welfare and Behavioural Medicine	2
3		ECAAH	European College of Aquatic Animal Health	3
4		ECBHM	European College of Bovine Health Management	3
5		ECEIM	European College of Equine Internal Medicine	0
6		ECLAM	European College of Laboratory Animal Medicine	0
7		ECPHM	European College of Porcine Health Management	3
8		EPVS	European College of Poultry Veterinary Science	4
9		ECSRHM	European College of Small Ruminant Health Management	12
10		ECVAA	European College of Veterinary Anaesthesia and Analgesia	1
11		ECVCN	European College of Veterinary Comparative Nutrition	0
12		ECVCP	European College of Veterinary Clinical Pathology	1
13		ECVD	European College of Veterinary Dermatology	3
14		ECVDI	European College of Veterinary Diagnostic Imaging	2
15		ECVECC	European College of Veterinary Emergency and Critical Care	0
16		ECVIM-ca	European College of Veterinary Internal Medicine-companion animals	0
17		ECVN	European College of Veterinary Neurology	2
18		ECVO	European College of Veterinary Ophthalmology	0
19		ECVP	European College of Veterinary Pathology	0
20		ECVPH	European College of Veterinary Public Health	5
21		ECVPT	European College of Veterinary Pharmacology and Toxicology	1
22		ECZM	European College of Zoological Medicine	1
23		ECVS	European College of Veterinary Surgery	1
24		EVDC	European Veterinary Dentistry College	0
25		EVPC	European Veterinary Parasitology College	3

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History of the Hellenic Veterinary Medical Society

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

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

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

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

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

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

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

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

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

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

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

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

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

President:	Theodoros Cl. Ananiadis†
Vice-President:	Veniamin Albalas
General Secretary:	Athanassios E. Tyrpenou
Spec. Secretary:	Konstantinos Chandras
Treasurer:	Olga Sabatakou
Member:	Emmanuel Archontakis
Member:	Apostolos Rantsios

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Review article
Ανασκόπηση

Ochratoxin A occurrence, its pathological effects on poultry health and decontamination approaches

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ABSTRACT: Mycotoxins are filamentous fungi which can be seen microscopically and they impart adverse effects on poultry health. The occurrence of mycotoxins in poultry feed imparts adverse effects not only to the birds' health but also causes huge economic losses by affecting feed conversion ratio, production of meat and eggs,and also responsible for histopathological changes in lymphoid organs of chicken. More than 300 different species of mycotoxins have been reported from which the ochratoxin and aflatoxins are the most harmful for poultry industry. Among ochratoxins, especially the Ochratoxin A (OTA) is produced from *Aspergillus Ochraceus* and and *Penicillium verrucosum* species as storage fungi inside stored drains and feed ingredients. The ochratoxicity is a dose-dependent factor and hits the immune system of birds besides decreased feed intake and decreased body weight. Higher doses of OTA toxicity resulted in deterioration of egg shell quality and hatchability in layers and breeders. Histopathological profile of ochratoxicity affected birds showed severe to moderate changes in the kidney, bursa of Fabricius, liver, thymus, spleen, heart, and lungs depending on dosage. In this review article, an attempt has been made to concise the OTA associated alterations in growth pattern, production index, biochemical changes and histopathological profile of the poultry. The current approaches and agents (Vitamin-E, Silymarin), which are being used to reduce the effects of OTA toxicity in birds, have also been discussed.

***Keywords*:** Ochratoxins, Poultry, Immunity, Biochemical changes

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INTRODUCTION

The poultry sector is an important and vibrant segment of agriculture which plays important role in national economy of many countries (Zhang *et al.,* 2020; Khater *et al.,* 2020; Yasim *et al.,* 2020). However, its progress is affected by various diseases including mycotoxins (Baran *et al.,* 2020; Elmossalamy *et al.,* 2020; Shoaib *et al.,* 2020; Sugiharto *et al.,* 2020). Mycotoxins occurrence is global issue with strong economically negative impact on health of livestock, poultry and humans (Ali *et al.,* 2020; Moussa *et al.,* 2020; Lin *et al.,* 2020). Among mycotoxins, ochratoxin is a well-known toxin that survives in the agriculture and livestock sector damaging the cereal grains, poultry and livestock by-products (Ghonaim*et al.,* 2020; Imran *et al.,* 2020; Khaskheli *et al.,* 2020). Several fungi species like *Aspergillus ochraceus, Aspergillus carbonarius, Aspergillus niger* and *Penicillium verrucosum* are responsible for ochratoxin production due to un-hygienic and improper storage of products under optimal temperature and humid conditions (Kembo *et al*.,2020). Ochratoxin (OTA) is a chemically stable compound and its toxicity cannot be averted by adopting normal food storage procedures. OTA is found as a deleterious and carcinogenic substance in contrast to other live species. In animals, the kidneys is the chief target organ for OTA (Joint *et al.*, 2002; Duarte *et al.*, 2011; Kembo *et al*.,2020). Immunosuppression and immune toxic effects are also reported due to OTA (Bondy and Pestka, 2000). Other deleterious effects of OTA includes the increased lipid peroxidation, mitochondrial damage and reduced macromolecules formation (Tinelli *et al.*,2019).

During the last decade, several scientific studies have been conducted on mycotoxins to check their presence in the agriculture fields (Jarvis and Miller, 2005). Wang *et al.* (2006) confirmed different studies regarding the airborne presence of *Aspergillus* and *Fusarium* species in poultry sheds ranging from 361.3 and 74.6 CFU/m3 and taken as toxicogenic.

Toxico-pathological mechanism of *Alternaria* toxins (plants such as oilseeds and cereals) includes genotoxicity, cytotoxicity, teratogenicity and fetotoxicity which were hazardous to animal health (Kembo *et al*., 2020). Mycotoxin contaminated poultry houses were reported by researchers due to unhygienic environmental conditions taking both the birds and workers at risk by inhaling the toxic air. The adverse effects of mycotoxins have made scientists to ponder in order to abate the escalating OTA in food chain supplies to overcome the economic crises globally (Wang *et al.,* 2006). Similarly, in poultry sector the major devastating mycotoxins includes the aflatoxins (AF), ochratoxin A (OTA), fumonisins (FUM), deoxynivalenol (DON) and T2 toxin (Ghor *et al.,* 2020). Mycotoxin type-2 is considered 10 to 20 times more toxic when absorbed by the digestive system instead of inhaled, similarly 20 to 40times more toxic if absorbed through the skin (Schleibinger *et al.*, 2004). Regarding the economic losses caused by OTA in the poultry include the stunted growth, poor FCR and enhanced mortality ratio. Peanuts and cereal grains are most commonly affected by mycotoxins globally and their 100% removal is still not possible. (Vieira *et al.,* 2004; El Miniawy *et al.,* 2014; Khatoon *et al.,* 2016). The different studies through toxicity testing approved the oral median lethal dose (LD 50) of OTA as 2-4mg/kg in chicken, 5.9 in turkeys and in quails& ducks 16.5 (Elaroussi *et al.,* 2006, 2008; Santin *et al.,* 2002).

Initially the OTA was discovered at Balkan region, but currently it is present around the globe due to its favorable growth rate in storage components under optimum conditions (Bui-Klimke *et al.,* 2014; Khatoon *et al.,* 2017; Sharif *et al.,* 2018). The OTA elimination is still a major issue in poultry sector due to is capability to survive the higher temperature. However, several different approaches are now approved for reduced OTA contamination at all levels. This review article summarizes the OTA associated alterations in growth pattern, production index, biochemical changes and histopathological profile of poultry. The current approaches and agents (Vitamin-E, Silymarin), which are being used to reduce the effects of OTA toxicity in birds, have also been discussed in the following sections.

Table 1 shows International standards and codes of practice to limit exposure to mycotoxins from certain foods established by the Codex Alimentarius Commission based on JECFA assessments.

These tolerable daily intakes are used by governments and international risk managers, such as the Codex Alimentarius Commission, to establish maximum levels for mycotoxins in food. The maximum levels for mycotoxins in food are very low due to their severe toxicity. For example, the maximum levels for aflatoxins set by the Codex in various nuts, grains, dried figs and milk are in the range of 0.5 to 15 μg/kg (a μg is one billionth of a kilogram). The Codex maximum limit for patulin in apple juice is 50 μg/L.

Table 1: Approved Level of OTA Species in Poultry Feed (JECFA, 2001)

OTA- Spp.	Optimal Temp.Range (Min-Max) °C	Water Activity	Affected Food Products
Ochraceus	24-31 (8-37)	0.95-0.99	chickpeas, rapeseed, pepper, and sesame seeds, nuts, oats rice, grain, maize, wheat, flour, and grain, espresso beans.
Ochraceus carbonarius	32-25	0.82	Grapes and grape items, including table grapes, wines, and dried vine organic products.
Ochraceus niger	35-37 (6-47)	0.77	Nuts, apples, pears, peaches, citrus, grapes, figs, strawberries, mangoes, tomatoes, melons, onions, garlic, and yams.
Ochraceus verrucosum	20 (0-30)	0.80	Oat crops; cheddar, meat items.

Chemistry of Ochratoxins

Ochratoxins are toxic metabolites consisting of dihydro-isocoumarin moiety linked with a phenylalanine through an amide bond. The chemical and physical properties involve the molecular weight of 403.8, being a white, odorless and heat stable crystalline substance having a melting point of 168-173°Cresisting the 3 hours of sterilization process at 121°C (Ko″szegi *et al.,* 2013). Its partial degradation is noted at 250°C (Trivedi *et al.,*1992). The OTA exhibits a strong fluorescence due to its specific structure property and existed in non-ionic, monoanionic and dianionic forms depending upon the microenvironment (Poór *et al.,*2013; Kembo *et al*., 2020).

Pathophysiology of Ochratoxins

Ochratoxins (OTA) is the causing agent of nephropathy and increases the incidence of renal carcinomas and adenomas in rats. Pathophysiological studies revealed that OTA acts on different sites along the nephron. Acute OTA exposure leads to an impairment of postproximal nephron function, predominantly of the collecting duct, resulting in altered electrolyte and titratable acid excretion (Pozo *et al.,* 2013). After activity of poison it assimilates into alimentary canal, wherever it ties with egg whites which supplies OTA an extended half-life from few days to month, depending on plasma grouping (Roth *et al.,* 1988; Berger *et al.,* 2003; Lino *et al.,* 2008). In rabbits, OTA is extremely cytotoxic and may cause hemolysis in red blood cells (Jan *et al*., 2017). Low doses of OTA cause influence energy metabolism (amino acid, cofactors, vitamins and carbohydrates) while in high doses of OTA, different body systems are influenced like excretory, digestive, endocrine and circulatory system (Anzai *et al.,* 2010; Tinelli *et al.,* 2019).

In rodents, OTA caused effect on lymphoid organs such as enlarged kidneys and liver (Schwerdt *et al.,* 1999; Zlender *et al.,* 2009).Discharge of poison is finished by cylindrical emission and re-retention is guilty of intra amassing of poison (Leier *et al.,* 2000; Jung *et al.,* 2001). Poison may be reabsorbed (from nephron) by dynamic vehicle and distant dispersion in pH.

Clinical Signs of Ochratoxins Toxicity in Poultry

The severity of clinical signs and symptoms of OTA toxicity depends upon the dose rate and the duration of exposure. The major signs exhibited by birds include general weakness, low FCR, stunted growth, poor egg and feathering quality, increased mortality, elevated weight of visceral organs like liver, spleen, pancreas, proventiculus, gizzard and testes in male birds (Resanovic, 2009).The kidney impairment leads to a decrease in urine concentration, glomerular filtration rate, impaired proximal tubule and degenerated ultrastructural changes in renal integrity (Elaroussi *et al.,* 2008).Day-old chicks when exposed to low dose of OTA, caused marked reduction in circulating lymphocytes in contrast to the enhanced ratio of monocytes and heterophils reported. This change in inflammatory cells profile in newly hatched day old chicks is ultimately related to their inflammation response against a variety of ecological pathogens and certainly disturbs their capability to react against vaccines (Moura *et al.*, 2004).

OTA along with nephrotoxicity also affects the liver and compromises the immune response, causing histopathological lesions in vital organs (Sharif *et al.*, 2018).OTA at 20mg/kg induces severe lesions in the glomerulus and convoluted tubules of the kidney. Interstitium incorporated with elevated levels of collagen fibers ultimately increases the membrane thickness. OTA also tempts abnormal mitochondria peroxisomes, lipid droplets inside the cytoplasm and nucleus, while the epithelium of convoluted tubules can be infiltrated with round electron-dense bodies, enhanced SER, intra-cytoplasmic and intra-nuclear myelin like projections (Sugiharto *et al.*, 2020).400 and 800 µg level of OTA in poultry feed caused enlargement in renal and hepatic size along with ele-

vated levels of serum creatinine, uric acid, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase (Elaroussi *et al.*, 2008).In rabbits, teratogenic effects were found as skeleton abnormalities like incomplete ossification of ribs and skull bones, and soft tissues abnormalities due to OTA (Tinelli *et al.,*2019).

Effect of Ochratoxinson Biochemical &Histopathological Profile

Element of OTA remains beneath discussion and reported to be cancer-causing agent, toxic, immunotoxin and teratologic agent in poultry (Koszegi *et al.,* 2016). Elevated levels of ochratoxins ultimately causes adverse histopathological and biochemical changes along with reduced performance (Hassan *et al.*, 2010; Mohamed *et al*., 2019).A high level of OTA showed a significant reduction ($p<0.05$) in the antibody titers of anti-NDV by 10.4% and a non-significant reduction in mRNA cytokine IL-2 and IFR-gamma level upon induction of lipopolysaccharides. However, serum levels of these two parameters (IL-2 and interferon-gamma) significantly drop ($p<0.05$) in the OTA toxin group. Anomalies in bursa, thymus, spleen, and liver with OTA+T-2 toxin and the use of commercially available mycofix Plus as treatment, encounter its toxic effects significantly (Xue *et al.*, 2010).

Moura *et al*. (2004) worked on different combination of OTA and PBS(Phosphate-buffered saline) to check effects of OTA on broilers leucocytes and reported that exposure of day old chicks to low doses of OTA resulted in a significant decline in proportion of circulating lymphocytes with a marked increase in proportion of monocytes and heterophils.

Indresh *et al*. (2013) observed the impacts of OTA upon the general performance, immunological and biochemical profile of broilers chicks. Both toxins caused dejecting effects on serum biochemical, hematological and immunological specifications, also affected health status of birds. Bharathi *et al*. (2014) assessed the changes in biochemical and liver antioxidant profile of birds by feeding of OTA at the rate of 100 ppb for 4 weeks. Results showed a prominent reduction in globulin level and IgA to IgG ratio in OTA fed birds. Moreover, hypo-glycaemia, hypo-amylasaemia, reduced high density lipids and elevated levels of ALT, ALP, and blood urea nitrogen, triglycerides were recorded. Thus, conclusively it was considered that 100 ppb OTA in feed results in severe damage to liver and kidneys along with causing adulterations in their normal biochemical and antioxidant profile.

Hassan *et al*. (2018) studied to investigate the prevalence of OTA in animal feed by using glutathione an antioxidant in rats. Using glutathione, an increase in urea, ALT, AST and decrease in serum total protein, albumin and globulin level was recorded in OTA treated rats. Results indicated the economic importance of glutathione by addition in healthy animal feed to overcome the deleterious effects of ochratoxins.

The suppression of the immune system and impairment of the normal physiology of several constituents of the immune system is also caused by OTA toxicity (Hassan *et al.*, 2012). A study affirmed the enhanced effects of bentonite clay in broiler chicks fed with aflatoxin and Ochratoxin treated feed by evaluating the pathological changes besides alterations in the immune system of treated birds. A depressed immune reaction in combination with pathological lesions in the immune system, reduced antibody (Ab) titer against sheep red blood cells, low response to PHA-P and impaired phagocytic activity was reported. The addition of bentonite clay in the aflatoxin group treated with 0.1 and 0.2mg/kg dose rate exhibited an enriched response against immune-toxicity whereas no such prompt results were recorded in 0.6mg/kg dose rate group. The OTA treated group showed only mild ameliorative effects upon bentonite clay addition in contrast to the aflatoxin group (Bhatti *et al.*, 2017). Histopathologic findings included vacuolation of hepatocytes, megalocytosis along with hyperplastic binary epithelial cell layer and increased liver weights. In kidneys, hypertrophied proximal tubule epithelial cell layer can be found beside a solidifying glomerulus basal membrane (Santin *et al.*, 2002). Pathological alterations induced by OTA are dependent on the duration of exposure and dose rate (Hameed *et al.*, 2013).The maximum acceptable level of OTA 0.1 mg OTA/kg was established by the European Commission considered as safe and no traces can be examined on breast and thigh muscles at this level. At 0.1 mg OTA/kg level no clinical sign and symptoms were examined because the dose level is less while mild histopathological lesions can be examined in such cases (Pozzo *et al.*, 2013).Liver and kidneys involvement in body's detoxification mechanism leads to hepato-renal enlargement, swollen and tan with white urate crystals in mild and complete failure with dehydration, hyper-uricemia in advanced OTA toxicity cases (Biró *et al.,* 2002).

Activity of Silymarin, Vitamin E and Absorbents against Ochratoxins

Different types of absorbents like Bentonite clay,

activated charchol, distillarysludge, vitamin E, silymarin are used to control the activity of mycotoxins depending upon their mode of action. The use of vitamin E and selenium are beneficial to cope with the immunosuppressive effects of OTA. A study showed 11% gain in body weight caused by OTA but with less severe lesions in selenium and vitamin E treated birds (Ahmad *et al.*, 2012). 2% level of yeast sludge is more profitable in contrast to 1% and has safer zone against mycotoxins (Mujahid *et al.*, 2012). The effects of Ochratoxin A and its amelioration with silymarin and vitamin E in separate groups of white leghorn cockerels were tested. Hematological and serum biochemical parameters when evaluated in OTA treated birds showed diminish levels of PCV (packed cell volume), Hb. (hemoglobin), erythrocytic count, albumin, serum while increased levels of creatinine and urea as compared to vitamin E and silymarin treated groups (Ahmad *et al.*, 2012). Mycotoxin deactivator reduces the accumulation of ochratoxin in the organs while fecal excretion can be increased in such cases (Joo *et al.*, 2013).

Comparatively investigation of the 3 different adsorbents against Ochratoxin toxicity in birds show that the three different adsorbents effects on mycotoxin feed (a) esterified glucomannans, organic in nature, (b) modified zeolite of inorganic nature, and (c) combination of both glucomannan and zeolite. OTA treated groups and a combination of adsorbents show enhanced FCR and advanced histopathological changes in the kidney and liver. Organic and inorganic nature show high body weight and minimized histopathological changes (Nedeljković-Trailović *et al.*, 2015).Unique sugar (STOC) termed as sucrose thermal oligosaccharide caramel on hematology, particular biochemical components, and hormones, fibro-nectin and cecal gram-negative bacterial levels affect the physiology. The addition of STOC in feed leads to improve the physiology of several parameters in exposure to OTA (Khaskheli *et al.,*2020).

Abidin *et al*. (2013) focused on hematological and serum biochemical parameters in white Leghorn cockerels feeding OTA contaminated feed to study ameliorative effects of L-carnitine and vitamin E (α-tocopherol).Vitamin-E and L-carnitine alone or combination with OTA (1.0 mg/kg) can mitigate toxin produced hematological and serum biochemical specifications.

CONCLUSIONS

Conclusively, the presence of ochratoxins in poultry feed produces adverse effects on the poultry health and performance parameters. They also have immunosuppressive effects on birds. Ochratoxins also induce mild to moderate changes in the biochemical and histopathological profile. Wherever, the use of some absorbents like bentonite, silymarin, and vitamin E proved to be effective against the adverse effects caused by OTA in birds and also improve performance parameters of birds.

CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Tuberculin test errors and its effect on detection of bovine tuberculosis

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ABSTRACT: Bovine tuberculosis is an endemic disease in Egypt, a notable gap exists between limited cases identified by single intradermal tuberculin test performed through the national control program and higher detected cases at abattoirs. Therefore, this study aimed to investigate epidemiological situation and causes of the previously mentioned gap in Middle-Delta Region. A total of 25 emergency-slaughtered animals of unknown tuberculosis-status were investigated by cocktail-antigens ELISA and post-mortem examination. Five visible tuberculous-lesion cases were detected and confirmed by PCR, ELISA was sensitive and predictive of the existence of tuberculous-lesions; 4(80%) out of 5 visible-lesion cases were seropositive. True prevalence among the slaughtered animals was 27%. In addition, tuberculin-testing of 400 animals during the national control program was evaluated, many technical and procedural errors were detected, and all animals were negative.Out of them, 55 animals were tested by ELISA before the application of tuberculin test, 30 (54.5%) animals were seropositive. To confirm the effect of the reported errors on reliability of tuberculin test, reference serum of 20 tuberculosis-positive animals that were tested by standard-procedures tuberculin test and their status were confirmed by PCR after slaughtering, were tested by ELISA. A complete matching was evident, the 20 standard-tuberculin positive animals were all seropositive. In conclusion, bovine tuberculosis is endemic at high levels in the study area, reported errors of tuberculin test during national control program may be the cause for missing tuberculosis cases and not tuberculin test itself and finally, a further wide-scale funded study is required to discover the situation throughout Egypt.

Keywords: *Bovine, Post-mortem, Tuberculin, Tuberculosis, Egypt*

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INTRODUCTION

Bovine tuberculosis (TB) is one of the most serious infectious diseases affecting a wide range of domesticated and wild animals. The disease adversely affects animal health, welfare and has implications for the international trade, production of animals and animal products (Fontana et al., 2018).In addition to its negative impact on livestock, TB has a great risk to public health; in 2017 only, 6.4 million new cases of human TB were officially reported to WHO (Global Tuberculosis Report, 2018).

Most countries have official TB control programs that depend mainly on cellular immunity-based tests. The single intradermal tuberculin test (SID) and gamma-interferon assay, which is also used for diagnosis of pseudotuberculosis (Oreiby and Hegazy, 2016), are being used worldwide based on the cellular response to mycobacterial purified protein derivative (PPD).

The SID remains the international field test for TB in bovines. The test has low cost, low logistical demands, a well-documented use, high availability, long history of employing and, for a long time, the lack of alternative field methods to detect bovine TB. Therefore, the test is suitable for routine, systematic surveillance to identify *Mycobacterium* (*M.*) *bovis*-infected animals, slaughter of positive reactor animals, movement tests, epidemiologic trace-back testing, and within TB affected herds to delineate animals going to a slaughter plant versus being condemned for rendering (Bezos et al., 2014; Koni et al., 2016; Waters et al., 2017; Sahli et al., 2018).However, the SID has an imperfect performance caused by nonspecific reactions of the *M.bovis*-crossly reactive bacteria; Mycobacteria, Corynebacteria and *Nocardia* spp. are antigenically related and termed CMN group bacteria (Oreiby, 2015). In addition, the test is complex, require two visits with 72 hrs. in between, variable interpretations between operators in addition to the difficulty to be used in surveillance being requiring professionals in intradermal injection (Brito et al., 2014; Buyuk et al., 2017; Bernttz et al., 2018; Sahli et al., 2018).

Recently, the importance of humoral responses against *M.bovis* infection was highlighted (Infantes-Lorenzo et al., 2019). The pathogen predominantly triggers cell-mediated immunity during its early and intermediate phases. Then after, while the disease progresses, a decrease in the cell-mediated immunity response occurs in parallel with an increase in the production of antibodies resulting in serological responses (Casal et al., 2017; Fontana et al., 2018). The combination of recombinant antigens either as a pool or chimeric antigen (fusion protein) was expected to enable the detection of antibodies from cattle in different stages of *M.bovis* infection (Souza et al., 2012; Infantes-Lorenzo et al., 2017). Thus, great efforts have focused on finding new antigens that would help to improve the performance of the ELISA (Bezos et al.,2014).In addition to the enhanced performance, ELISA is simple and rapid. Therefore, the newly developed commercial ELISA tests, based on a cocktail of carefully selected antigens, have a potential diagnostic value for bovine TB (Riad, 2015).

In Egypt, TB is endemic (Borham et al., 2021), and its control relies on two strategies: test and cull scheme and case detection during meat inspection at slaughterhouses. Test and cull scheme is performed during the national surveillance program against both brucellosis and TB conducted by "General Organization for Veterinary Services", based on annual testing of bovines with the SID. The second strategy is the detection of the affected animals in abattoirs during the regular inspection of meat (Abdellrazeq et al., 2016). A marked unexplained gap exists between the detected TB cases by test and cull scheme, using SID, and that is during meat inspection at abattoirs; the latter is being much higher than the former.

Thus, this work aimed to investigate the prevalence of bovine TB, and to study the causes of the gap between TB-detection of the national control program SID test and that is during routine meat inspection in the study area to be considered in similar endemic countries.

MATERIAL AND METHODS

Study area, animals and sampling

This study was conducted in the Middle-Delta region; Disuk, Baltim, Sidi-Salem, EL-Reyad and Beyala districts of Kafrelsheikh Governorate as well as Tanta abattoir at Gharbia Governorate.

Animals are divided into the followings:

a- Blood samples from 25 animals (19 cows and 6 buffaloes) emergency slaughtered at Tanta abattoir were collected for ELISA examination and these animals were subjected to Post-mortem examination according to (Domingo et al., 2014) for detection of visible TB lesions.

b- A Total of 400 animals (330 cows and 70 buffa-

loes) were tested with SID during the national control program against bovine TB. Of these 400 animals, blood samples for ELISA testing were collected from 55 animals (44 cows and 11 buffaloes) before testing with SID.

c- Reference positive serum samples of 20 (10 cows and 10 buffaloes) TB-affected animals that were tested positive by standard-procedures SID, then subjected to post-mortem examination and their TB-positive status was confirmed by PCR. These serum samples belonged to the TB project (No. 2966, Science & Technology Development Fund, 2017) and were kindly supplied by the tuberculosis unit of the Animal Health Research Institute, Giza, Egypt. The samples were collected 6 months after application of SID.

Evaluation criteria of SID

The SID procedure during the national program was evaluated according to the criteria described in Bovine Tuberculosis Manual(2016). Availability of the test instrument, methods of identification of injection site, measuring the skin thickness, keeping and injection of PPD and final interpretation of the test results were recorded. ThePPD used in the program was *M. bovis* PPD (1mg/mL, Veterinary Serum & Vaccine Research Institute, Egypt). The performance of both the SID conducted during the national control program and that is of the standard SID which was done on the reference positive animals of the TB project (No. 2966, Science & Technology Development Fund, 2017) was judged by a newly developed highly sensitive cocktail-antigens ELISA.

***In Vitro*Testing by cocktail-Antigens ELISA**

The one hundred serum samples were tested using a patent antibody ELISA kit for bovine TB **(**Wuhan UnibiotestCo., Ltd, China).The kit is an indirect ELISA assay for the qualitative detection of *M. bovis* antibody in serum depending on especially selected *M.bovis* antigens that used as detector materials, allowing the detection of the antibodies in specimens with a high degree of accuracy. Briefly, 100 µl of 1:100 diluted serum samples were added into the wells of the pre-coated microplate. Similarly, 100 µl of negative and positive controls were added. The plates were incubated at 37 °C for 30 min. After successive five washing steps, 100 µl of enzyme conjugate (IgG Fc-HRP) were added to each well and incubated at 37 °C for 30 min. The washing was repeated and 50 µl of substrate A and B were added, respectively followed by shaking at 37 °C for 10 min. Finally, 50 µlof the stop solution was added, and the plates were read at 630nm after 10 min using a microplate reader (Model: Clindiag MR-96, Serial No: R2DO003) (Clindiag Systems Co., Ltd, China). The sero-status was calculated according to the following provided equation:

S/P=(Sample OD-Neg.mean)/(Pos.mean-Neg.mean)

If the $S/P \geq 0.17$, the sample was positive while, if it was < 0.17, the sample was negative**.**

Epidemiological investigation

The true prevalence (TP) of bovine TB among emergency slaughtered animals will be calculated as follows:

TP= ((AP+SP-1)/ (SP+Se -1)*100

Where AP is the apparent prevalence of bovine TB among emergency slaughtered animals using post-mortem examination (number of animals with visible lesions and confirmed by PCR/ total examined animals) *100. The Se and Sp are the sensitivity and specificity, respectively, of Post-mortem examination for bovine TB. The Se and specificity were 71% and 99%, respectively, and these estimated were obtained from (Nunez-Garcia et al., 2018).

The apparent of bovine TB among the 55 animals tested by both field SID and ELISA will be estimated as in the previous step as follows:

(The number of seropositive animals to the cocktail-antigens ELISA/55)*100

The statistical difference between apparent prevalence of TB among cows and buffaloes to cocktail-antigens ELISA was examined using Fisher's exact at level of significance $P< 0.05$.

RESULTS

Prevalence of bovine TB among the emergency slaughtered and national control program SID tested animals

Out of the 25 non-tuberculin tested emergency-slaughtered animals, 5 contained visible lesions and TB was confirmed by RTPCR (Data is published in another paper), PM lesions are shown in (Fig. 1 and 2). The true prevalence of the disease among examined animals was 27.14% (95% Confidence interval (CI): 9.74% - 44.5%). The number of true positive animals was estimated at 7 animals.

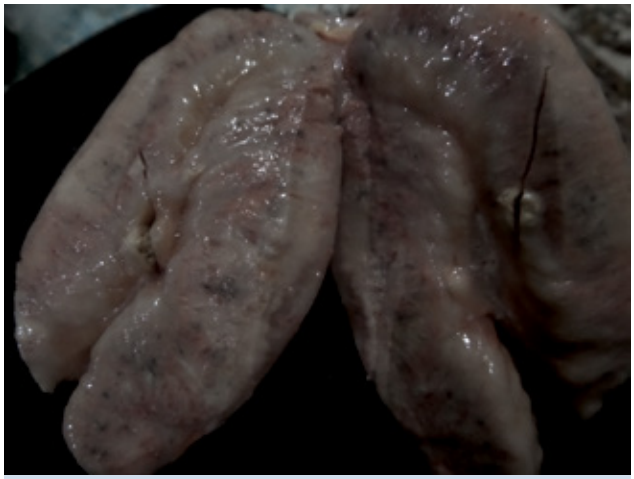

Fig. 1. Incised severely enlarged bronchial lymph node containing tuberculous lesions

Fig. 2. Milliary TB on the peritoneum "Pearls appearance"

Results of ELISA showed that 4 (80%) out of the 5 lesions-contained animals were seropositive, and one (20%) animal was seronegative. On the other hand, out of the 20 non-visible lesion animals, 11 (55%) were seronegative and 9 (45%) were seropositive. These results showed the high sensitivity of cocktail ELISA for detection of infected cases, but on the other hand it showed its low specificity due to the high number of false positive animals. The apparent prevalence among cows and buffaloes are shown (Table 1). There is no significant difference between the prevalence of the disease in cows and buffaloes ($P< 0.644$).

All the 400 SID-tested animals during the national control program were negative. The cocktail-antigens ELISA of the 55 serum-sampled animals showed 30 seropositive animals and an apparent prevalence of 54.5% (95% CI: 41.34% - 67.66%) (Table 1). There is no significant differencein the apparent prevalence among cows and buffaloes ($P< 0.2$).

Evaluation findings of SID performed through the national control program in the study area

Many procedural and technical serious faults were recorded during the official application of the SID. The veterinary units involved in the testing procedures lacked the standard test equipments: syringes, needles, callipers, dark coolers and hair clippers. Moreover, a shortage in animal handling as well as operator safety equipment were evident. The veterinarians involved in the testing process had complained from few numbers of the operators and shortage of transportation vehicles which carry them to the test location. Furthermore, they indicated that sometimes there was a conflict between the scheduled dates of SID and the vaccination campaigns against other diseases such as FMD and LSD. The veterinarians confirmed that there were not any incentives for them in regard of performing the test. They also mentioned that the simultaneous collection of blood samples from the same animals to be examined against brucellosis made much more difficult and slow performance of the SID. The veterinarians thought that the incomplete awareness of farmers about the dangerousness of the disease, lack of their interest in testing against TB and the low compensation value offered by the authority when positive cases exist are seriously affecting the success of the national control program against TB.

Table 1. Results of ELISA in relation to tuberculin test status.

	Seropositve								
	Cows +/t	AP%	95% CI	Buffaloes +/t	AP%	95% CI	Total +/t	AP%	95% CI
A	9/19	47.4	24.95 - 69.85	4/6	66.7	28.98 - 100	13/25	52.0	32.41 - 71.85
B	26/44	59.1	44.75 - 73.45	4/11	36.4	7.97 - 64.83	30/55	54.5	41.34 - 67.66
C	10/10	100	-	10/10	100	-	20/20	100	-

A are the 25 non-tuberculin tested animals.
B are the 55 tuberculin-negative animals of the national control program.
C are the 20 tuberculin-positive reference positive animals.
+/**t** is the number of positive /number of tested animals.

In addition to the previously mentioned procedural errors, several technical mistakes during the application of the test were detected. Most of the SID practitioners didn't shave and clip the hair, none of them disinfected the injection site before the application. Not all the tested animals were numbered and identified. It was noticed that most of the tested animals didn't expose to careful examination for any existing lumps, bruises, adhesions and other skin conditions at the injection site. Also, measuring of the skin thickness was performed using the opened and closed calipers by more than one operator at the same farm during the two visits. During the test, most of the practitioners didn't keep the PPD in dark coolers and it was exposed to direct sunlight. Some practitioners used too long needles not suitable for the intradermal injection. In most instances, disinfection and replacing of needles between animals or herds wasn't performed. During the test interpretation, most operators depended mainly on visual observation and palpation only. To ascertain the effect of these reported errors on the performance of SID, the performance of the cocktail-antigens ELISA was compared to the standard-procedures SID of the reference positive animals.

Comparison between the standard-procedures SID and cocktail-antigens ELISA

A complete matching was existing between the cocktail-antigens ELISA and the standard procedures-SID of the TB project No. 2966. All the 20 animals were SID and ELISA positive. These animals were slaughtered, and their status was confirmed by PCR.

The ELISA Optical density (OD) and sample values (S/P) are illustrated in (Fig. 3 and 4, respectively). In addition, the relationship between the results of the SID and of ELISA is summarized in (Table 1).

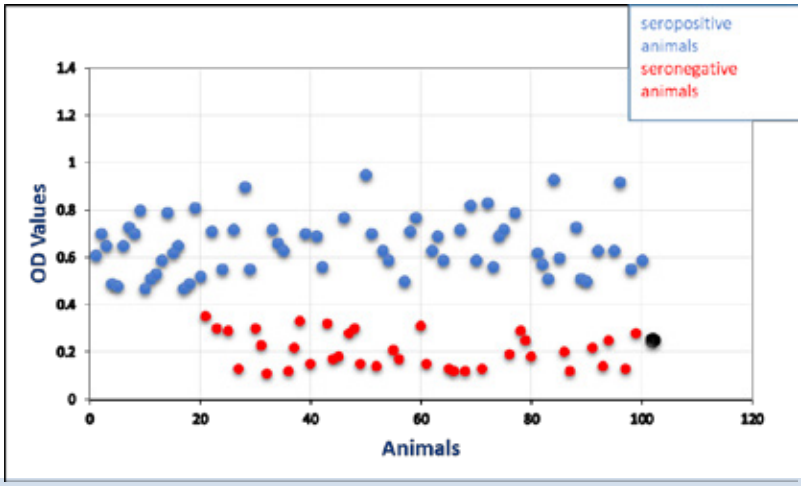

Fig. 3. Optical Density values of ELISA tested animals

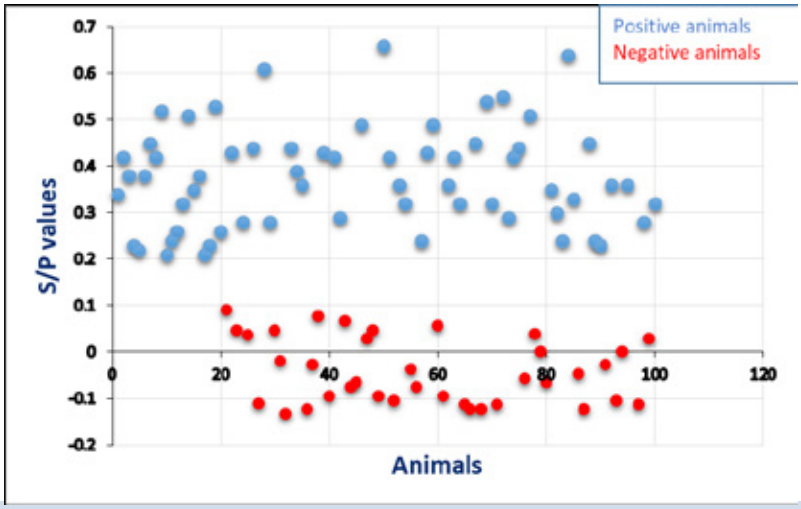

Fig. 4. S/P Values of positive and negative serum samples

DISCUSSION

Based on the official reports recorded by the "General Organization for Veterinary Services"at Kafrelsheikh Governorate in 2005, 2006 and 2007, the prevalence of tuberculin test reactors using SID among cows was 0.014%, 0.17% and 0.088, whilst it was 0.029%, 0.28% and 0.0% among the buffaloes, respectively. These reports indicate the very low percentage of positive reactors detected during the national control program in the study area and comes in harmony with the findings of the current study. In 2007, only 7 positive cases out of 7918 tested cows, and no positive animals out of 3823 tested buffaloes (Khoudair et al., 2009). On the other hand, a higher prevalence rate, 30.11% of the tested animals were positive reactors, was recorded using theSID (EL-Mahrouk and EL-Balawy, 2010). Further study during 2012-2015 in five regions within the Nile Delta had showed a prevalence of 7.3%, whereas it was as high as 45% in some farms (Amin, 2019).Prevalence of the disease in the study areais influenced by increasing animal density, unhygienic practices and stress during inter-current diseases and mass vaccination campaigns (Abou-Eisha et al., 2002). Recent data indicated that bovine TB is increasing sharply in some Egyptian governorates especially that are locatedwithin the Nile Delta and valley compared to the rest of the country being densely populated areas andthe nature of the rural communities where farmers keep the animals inside their houses (Abdellrazeq et al., 2016; Amin, 2019).

Despite of the annual diagnostic efforts for controlling bovine TB in Egypt, the disease still evident. The results obtained in this study confirmed the existence of bovine TB in the study area at a significantly high level; 27.14%. Despite that the examined animals were emergency slaughtered animals, but the findings were relatively like that of animals tested during the national control program. Reasons of such conclusions include the results obtained by ELISA on the SID-negative animals of the national control program which showed that the apparent prevalence among these animals is very high. The reported procedural and technical errors of SID performed during the national program may be responsible for the notable gap between the diagnosed TB cases by SID and those detected during routine meat inspection at abattoirs. It is well known that the SID is of unsatisfactory accuracy, which is considered the major impediment facing the control of bovine TB (Waters et al., 2017). Errors during the application of SID increases the magnitude of poor performance of the test.

In the current study, many procedural and technical errors were detected. In the same context, (Koni et al., 2016) recorded similar errors during the application of the SID which negatively affect the active surveillance against TB in Albania; untrained people, unavailability of test equipment as syringes and needles, lack of the appropriate identification and registration of animals, in addition to the cost of certified tuberculin.

Shortage of animal handling and operator safety equipment in addition to the dangerous temperament of some buffaloes explain, to some extent, the poor performance of the test in these buffaloes. The direct injection of tuberculin by most of the operators without preceding examination of the skin for lesions will affect the test interpretation, especially when the test performed during the outbreaks or even on recovered animals from diseases affecting skin such as oedematous skin disease or lumpy skin disease. Also, the improperly stored PPD, exposed to direct sunlight and the injection of sub-doses might cause false negative results (Humblet et al., 2011; Bezos et al., 2014). Tuberculin should be stored at 4 - 8 ^{0}C, never be shaken and away from sunlight. In addition, the usage of too long needles leads to the deposition of the tuberculin under the skin, especial needles of 3.9mm long must be used to confirm the intradermal injection (Bovine TuberculosisManual, 2016). Moreover, the detection of the formation of a small pea-like swelling at the injection site should be practiced because it is a sign of successful intradermal injection (Bezos et al., 2014). Furthermore, a regular check of the needle for being damaged or bent, and replacing them between animals is very important, otherwise it may act as a vector for the transmission of pathogens (Humblet et al., 2011).The interpretation of the SID without measuring the skin thickness, depending on observation and/or palpation has a negative effect on the test result as it is subjective and may vary between different operators (Bernttz et al., 2018). All these reported errors may explain the negative status of the followed-up SID-tested animals during the national scanning against bovine TB in the study area.

The comparison between the results of cocktail ELISA and that is of both SID performed during the national control program and standard-procedures SID offer a strong evidence about the effect of the reported errors on the performance of SID. The low number of detected bovine TB cases by SID performed by the veterinary authority compared to the

higher number of cases detected at slaughterhouses is mainly because of the errors practiced during the application of SID and not because of the accuracy of the SID itself. A similar study in Brazil reported a significantly greater sensitivity of ELISA than the SID; 46.1% of infected cases were detected by ELISA compared to 5% by SID (Souza et al., 2019).This may reflect the predominance of humoral immune response during the advanced stages of the disease as reported by (Waters et al., 2017) who detected 45% of non-reactor TB cases by ELISA**.** Similarly,(Hassanain et al., 2009) in Egypt, recorded that 43.5% of the tested cattle were positive by ELISA. In addition, the multi-antigen ELISAs, especially that contained a cocktail of MPB70, MPB83, CFP-10, ESAT-6, enhance the overall test sensitivity extremely greater than the single antigen ELISA such as PPD-ELISA, because the latter shares antigens of non-tuberculous mycobacteria resulting in cross reactions (Infantes-Lorenzo et al., 2017; Waters et al., 2017). Other studies reported better detection of bovine TB by the parallel interpretation of SID and serological assays compared with that of each test individually, and the increased sensitivity of ELISA by previous stimulation of the immune system by PPD injection (Koni et al., 2016;Trost et al., 2016;Casal et al., 2017).

Post-mortem examination as a gold standard to judge the performance of the cocktail-antigens ELISA revealed its ability to detect 80 % of visible lesion cases. McCallan et al. (2017) reported one serologically negative animal that was subsequently found to have visible lesions and confirmed for *M.bovis* at post-mortem which may indicate anergy or immunosuppression (Ellner, 1996). On the other hand, 8 non-visible lesion cases were seropositive which may be due to minor lesions that escape the routine PM examination,or even cross-reaction with antigenically related bacteria (Domingo et al., 2014; Picasso-Risso et al., 2019).

In conclusion, bovine TB is endemic at high level and represent a great risk for human and animal population and further wide scale funded studies are required to discover the situation throughout Egypt. On the other hand, the errors performed during the application of SID may be responsible for the gap between the low detection ability of the test and higher detected cases during routine meat inspection in the study area. Finally, cocktail-antigens ELISA is promising as it was able to detect 80% of cases with TB lesions at the slaughterhouse.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Evaluation of different types of feline blood groups in cats of Isfahan, Iran

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ABSTRACT: There is a high demand for pet veterinary care due to the increasing tendency to keep pets in Iranian households. These include blood transfusions because incompatible blood groups can lead to some negative effects such as isoerythrolysis in cats.

This study was the first attempt to evaluate the distribution of blood type of cats in Iran.

Blood samples were collected from 63 domestic short hair cats in Isfahan, Iran and blood groups were determined by the kit card agglutination method (Rapid Vet-H IC Feline kits, Agrolabo, Scarmagno, Italy). According to the results, the frequency of blood types of A, B, and AB were 96.8%, 3.2%, and 0%, respectively. Card agglutination method is a fast method with high validity. Therefore, it is recommended for determining blood types in donor and recipient in veterinary hospitals and breeding centers.

***Keywords*:** Blood transfusion; Blood type; Cat; Rapid Vet-H IC Feline kit.

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INTRODUCTION

Over the recent years, owners' willingness to keep their pets into their households has increased in Iran. Thus an increasing demand of high quality veterinary care has risen. Whole blood or blood product transfusion is frequently required in clinical practice (e.g. trauma, blood loss, hemolysis, surgery) and rapid, reliable access to safe blood product is mandatory (Balakrishnan et al., 2016, Hanson et al., 2017, Langston et al., 2017, Weingart et al., 2004). These centers must determine the blood type of the donor and recipient to prevent the post-transfusion reaction and to conduct the necessary tests to prevent the development of transmissible infectious diseases (*feline leukemia virus*, *Bartonella* species, *anaplasmosis, ehrlichiosis*) (Hegarty et al., 2015; Pennisi et al., 2015; Yagi and Holowaychuk, 2016). With appropriate selection of donors and screening for blood incompatibility, the blood transfusion (including red blood cells and frozen plasma) may increase the chance of survival and recovery (Snow et al., 2010). Unlike dogs, cats naturally have alloantibodies against the red blood cell antigens of other blood groups. Therefore, it is necessary to pay attention to the various blood types and their distribution in cat breeds and in a specific region (Bovens and Gruffydd-Jones, 2013).

In addition, recognizing the issues of blood types and antibodies in pets, including cats, is also helpful to those who use these species as a research model for human diseases. Moreover other professions such as biologists, toxicologists, and in vivo researchers are interested in the distribution of various feline blood types in a specific geographical region (Hohenhaus, 2004). Small breeding facilities, and breeding centers should also pay attention to blood types due to blood-relatedreactions and problems like feline neonatal isoerythrolysis (FNI) (Bücheler, 1999; Giger, 1991; Giger and Casal, 1997; Silvestre-Ferreira and Pastor, 2010).

In spite of these applications that indicate the importance of blood transfusion, and blood typing, there is no efficient and comprehensive research in Iran to check the distribution of blood types in small animals, especially in cats that are highlysensitive. Also, to our knowledge, no study has been conducted on the blood group of Iranian cats in context from reliable sources.The present study has evaluated Domestic Short Hair Breed (DSH) cat blood types in Isfahan, Iran to provide adequate knowledge of the distribution of the blood types in the intended breed and region to save the cats from fatal and emergency conditions.

MATERIAL AND METHODS

Sampling

This study was performed on DSH cats from different areas of Isfahan, Iran during 2018. Blood samples were obtained from the cephalic vein in 63 adult and healthy cats from veterinary clinics, boarding, breeding and support centers. The samples were transferred to the laboratory in EDTA tubes (EDTA vacuum tube, Avapezeshk, Iran) at 4 °C and the blood type was determined within the first 4 hours.

Sample preparation and blood type determination

In this study, the card agglutination method (Rapid Vet-H IC Feline kits, Agrolabo, Scarmagno, Italy) was used for the determination of the blood types in cats. The test was performed as follows: First, the cat's name was recorded on the agglutination card and the card placed on a flat surface. A drop of anticoagulated blood was added to the tube containing buffer (white cap tube) with the pipette in the package and mixed by changing the axis of the tube. 3 drops of the resulting solution were poured vertically into a circular well in the center of the card with a new plastic pipette. Then 3 drops of the buffer solution in the dropper bottle with red cap, were added to the circle well. After 5-10 minutes, the result of the kit is observed and interpreted as follow:

Blood type A: The appearance of a red line in the well, marked as type A, also in test control well

Blood type B: The appearance of a red line in the type B well, also in test control well

Blood type AB: The appearance of a red line in both wells (type A and B) and test control well

If the control well does not give a positive reaction, the test must be repeated with the new kit.

Statistical analysis

The frequencies and percentage of blood type in cats was recorded and determined by Excel software and SPSS v.24 (SPSS Inc., Chicago, IL).

RESULTS

The present study examined the blood type of 63 cats. Of the 63 cats, 61 cases (96.8%) were in the blood type A, and 2 cases (3.2%) were in the blood type B, and no blood type AB was found. The blood

types of cats are A, B and rarely AB. The highest frequency is related to blood type A while the lowest is related to blood type AB. The frequency of the blood type A, B, and AB were 96.8%, 3.2%, and 0%; respectively. Geographical location, number of animals, method used to determine the blood group, and the proportion of each blood group type in various countries presented in Table 1-2.

Table 1: Blood type frequencies in cats (pedigree and non-pedigree cats) in various countries

Country, City, Pedigree or not	Method	Total number	Blood type (%)			Reference
			A	B	AB	
UK - South east	Card method (Kit cards)	105	67	31	2	(Forcada et al., 2007)
UK _ pedigree	Card method (new desk-top feline blood typing kit)	207	54.6	40.1	5.3	(Knottenbelt et al., 1999a)
UK _ non pedigree	Card method (desk-top feline blood typing kit)	139	87.1	7.9	5	(Knottenbelt et al., 1999a)
Hungary	Agglutination method (Alloantibody testing by Triticum vulgaris (T. V.) lec-tin)	100	97	3	0	(Bagdi et al., 2001)
Turkish- Van	The direct agglutination method	78	42.3	57.7	0	(Arikan and Akkan, 2004)
Australia - Sydney_ domestic crossbred cats (short and long-haired)	Card method (RapidVet-H (Feline) desk-top blood typing kit)	187	62	36	2	(Malik et al., 2005)
Australia - Sydney_ pedigree	Card method (RapidVet-H (Feline) desk-top blood typing kit)	166	66	32.7	1.3	(Malik et al., 2005)
New Zealand - North and South Island _ non pedigree	Card method	89 (North Island = 62, South Island = 27)	79 (North Island = 77, South Island = 81)	20 (North Island = 21, South Island = 19)	1 (North Island = 2, South Island = 0)	(Cattin, 2016)
New Zealand -South Island_ non pedigree	Tube method	156	89.1	10.3	0.6	(Cattin, 2016)
England	Microplate agglutination (phenotyping) and pyrosequencing of a fragment of the cytidine monophospho-N-acetylneuraminic acid hydroxylase gene (genotyping)	112	77	17	6	(Tasker et al., 2014)
Portugal and Spain	Card method (RapidVet-H Feline Blood Typing; MDS)	1070 (Portugal = 926, Spain = 144)	96.5	3.5	0	(Vieira et al., 2017)

Table 2: Blood type frequencies in domestic and non-domesticcats in various countries

Country, City, Pedigree or not and Animal type	Method	Number	Blood type (%)			Reference
			A	B	AB	
Blood type frequencies in domestic cats in various countries						
UK	Gel method (gel column agglutination)	140	90.7	7.1	2.1	(Knottenbelt et al., 1999b)
Northern Italy_ Ragdoll cats	Gel method (gel column agglutination)	127	90.7	7.1	2.1	(Proverbio et al., 2011)
Northern Italy_ Ragdoll cats	Gel method (gel column agglutination)	61	77.1	4.9	18	(Proverbio et al., 2013)
Japan	Genotype (They investigated the distribution of AB blood group antigens, CMAH gene structure, mutation, diplotypes, and haplotypes of the cat CMAH genes)	734	95.1	4.9	0	(Omi et al., 2016)
Northern Portugal	Tube method	147	89.3	4.4	6.3	(Silvestre-Ferreira et al., 2004b)
Portugal - Lisbon	classical agglutination assay or using a cartridge assay	515	97.5	2.1	0.4	(Marques et al., 2011)
Grand Canaria Island_ non pedigree	Tube method (using Triticum vulgaris lectin)	97	88.7	7.2	4.1	(Silvestre-Ferreira et al., 2004a)
Greece	Card method (Desktop kit)	207	78.3	20.3	1.4	(Mylonakis et al., 2001)
Philadelphia - Pennsylvania	All blood samples were tested by use of GEL, SLIDE, and TUBE methods. Fifty-eight samples were also tested by use of CARD and CHROM methods.	490	83	11	6	(Seth et al., 2011)
Brazil -Rio de Janeiro	agglutination assays (using Triticum vulgaris lectin and feline anti-A serum)+high-performance thin-layer chromatography (HPTLC)	172	94.8	2.9	2.3	(Medeiros et al., 2008)
China -Beijing_ non pedigree	Tube method	262	88.2	11.4	0.4	(Zheng et al., 2011)
Spain	Card method (Rapid-Vet-H [feline]; DMS Laboratories) + autoagglutination control with saline to detect false positive reactions	100	94	5	1	(Espada, 2004)
Canada- Quebec	Tube method	207	95.2	4.3	0.5	(Fosset and Blais, 2014)
Blood type frequencies in non-domestic cats in various countries						
UK Bengal cats	Card method (Rapid Vet-H Feline Blood Type)	100	100	0	0	(Gunn-Moore et al., 2009)
zoos and wild animal parks in the United States (n = 126) and from Dubai (n = 5)	Tube method	131	80	18	2	(Griot-Wenk and Giger, 1999)

DISCUSSION

As mentioned above, this study examined the blood samples from 63 healthy DSH cats in Isfahan, Iran. Similar results have been reported from studies conducted in Portugal and Spain (2017) (Vieira et al., 2017), Japan (2016) (Omi et al., 2016), the province of Quebec of Canada (2014) (Fosset and Blais, 2014), Lisbon, Portugal (2011) (Marques et al., 2011) and Hungary (2001) (Bagdi et al., 2001).

However, it should be noted that these ratios are different based on cat breeds and different countries (Yagi and Holowaychuk, 2016). Based on the collected data and similar to our results, the most common blood type is type A followed by blood type B, while the blood type AB is very rare (Figure 1). Unlike most of recent studies, no type AB cats were found in our study.

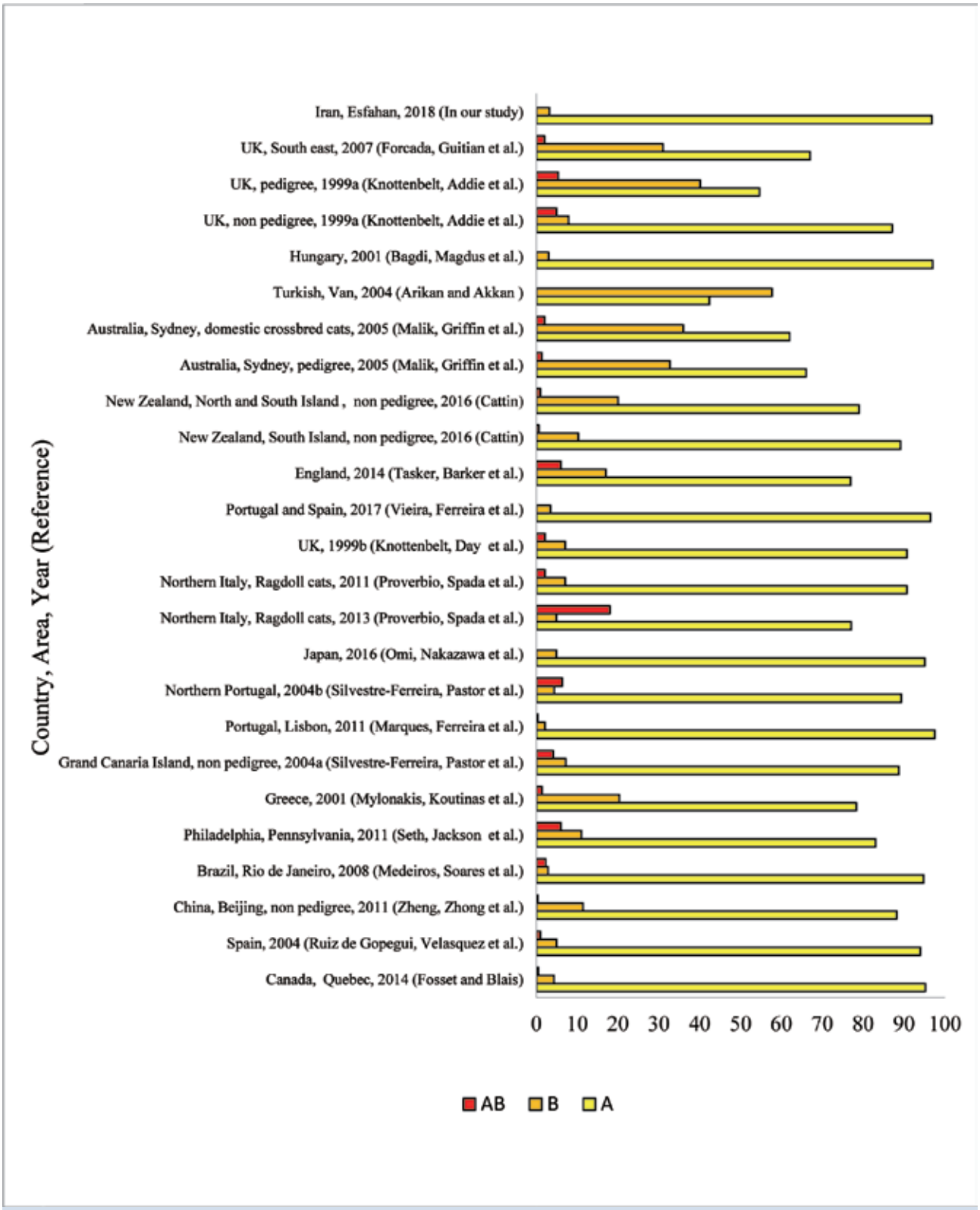

Figure 1: Comparison of the results of the present study with the other countries (%)

Nowadays in Iranian society, pets' variety and numbers have increasedand clinical emergencies have become more frequent. Blood typing, blood compatibility testing, the increasing frequency of blood type B and the possibility of adverse transfusion reactions are vital and should be considered.

The critical issue about blood and blood products in Isfahan, Iran is the lack of blood banks for animals, as well as the lack of easy access to kits and other tools for determining the blood type in various affairs. There is a lack of awareness about the importance of blood type in veterinarians, cat owners, pet clinics, and especially cat breeders which could increase the frequency of type B and results in endangering the health of cats. Unfortunately, cat breeders in Iran breed cats without considering the importance of blood type for reproduction. Axner (2014) developed a questionnaire and investigated the effect of parental blood type and isoerythrolysis in kittens. They analyzed the result of breeding the blood type B female cat with blood type A male cat, blood type A female cat with blood type A male cat. They reported that there was no significant differencein the mortality (Axnér, 2014). While in our study as in most studies, there were adverse blood complications related to the blood transfusion and isoerythrolysis in breeding. Cat owners have no desire to determine the cat's blood and consider blood test as an invasive method. The lack of awareness and treatment policy in Iran has put the life of these pets at risk. The lack of comprehensive research on cat blood type in Iran is also evident. There is no sufficient information on blood types and related complications in different parts of Iran.

Since the frequency of the blood type A is 96.8% and type B is only 3.2%, the prevalence of the blood transfusion and Feline neonatal isoerythrolysis of neonates due to blood type B in Iran is low. However, lack of knowledge about the importance of blood types could lead to an increase in type B and further complications on cats.

DNA testing for the determination of blood groups in cat breeds has not been fully evaluated. On the other hand, August (2009), found that there are inconsistent results between different serological tests in genotypic studies of Ragdoll cats. The immunochromatographic technique represents a very accurate approach for identifying A, B and AB blood types in anemia and non-anemia cases and provides higher sensitivity and accuracy than the card agglutination method. Also, this technique can be used in samples stored with anticoagulant and different methods. Immunochromatographic method can be very efficient in clinical practices (Hourani et al., 2014; Spada et al., 2016). Testing with kits and immunochromatographic is very simple and time saving. However, it is very expensive and not easily accessible.

CONCLUSION

Determining the compatibility of blood donors and recipients in the blood transfusion and breeding are very important. The card agglutination method is recommended to determine the blood type of donors, recipients and breeders' blood compatibility in the treatment centers. Establishment of blood banks specific to animals (to make blood and blood products available in emergencies and facilitating the researches on animal blood and blood products), production and exporting of these blood test products, education and awareness among cat breeders about determining the blood group of cats in blood transfusion and breeding are highly recommended. Also it is suggested that determination of blood type in cats should be performed before any blood transfusion.

The veterinarian policies should be focused on preventive programs rather than treatment of the issues resulted from incompatible blood groups. The distribution of blood types has been evaluated in many countries. This study is the first attempt that evaluated the blood types of cats in Iran. However, this study was performed only on DSH cats at a limited time and the number of cases. Therefore, further studies are required to provide clear information about the distribution of blood groups in cats of Iran.

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CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

The Effect of Curcumin on the Antioxidant System in Diabetic Rats

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ABSTRACT: The aim of this study was to determine the effect of oral administration of curcumin, also known as a highly effective antioxidant, which has been used as a spice in experimental diabetic rats with streptozotocin (STZ) on antioxidant system. In this research, 30 healthy adult male Wistar albino rats, were used. The animals were divided into four groups as control group (K), diabetes group (D), curcumin group (C), and diabetes + curcumin group (DC). In order to create diabetes, D and DC groups were administered 60 mg/kg STZ as a single dose by intraperitoneal injection. Curcumin (50 mg/kg live weight/day) was given orally to the C and DC groups. At the end of the experiment, Malondialdehyde (MDA), Superoxidedismutase (SOD), Glutathione (GSH), Catalase, and glucose levels were determined. The MDA level was significantly higher in the D group compared to the other groups. MDA level determined in DC group was found to be significantly lower from group D ($p < 0.05$) while getting closer to groups K and C. It was also found that the levels of antioxidants SOD, GSH and catalase which are known to be effective against oxidative stress, were significantly apparently lower in group D compared to control groups (K and C). Again from the perspective of these parameters, when the data in group DC were evaluated, it was observed that the obtained data were getting closer to K and C or becoming similar. The blood glucose level obtained from the D group was significantly higher than the other groups. As a result, the data obtained from the research shows that curcumin, which is used in experimental diabetes-induced rats for its antioxidative and antidiabetic effects, is very helpful thanks to the positive effect in terms of the parameters followed.

Keywords: Antioxidants, Diabetes Mellitus, Curcumin, oxidative stress, Streptozotocin

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INTRODUCTION

Diabetes Mellitus (DM), which is a metabolic disorder characterized by hyperglycemia and in which degenerated insulin secretion or insulin resistance or both are observed at the same time, recently has become a global problem (Parildar et al.., 2011, Ada, 2014). DM, which is an important disorder due to the widespread and chronic complications it generates, may bring about acute or chronic complications by causing damages, functional disorders, and insufficiencies in many organs in the long run in the organism. (Valentovic et al., 2006; Çambay, 2011, Kılıçarslan ve Dönmez 2016).

Among the complications of diabetes retinopathy, nephropathy, neuropathy, and atherosclerosis can be counted. (Kikkawa, 2000).

There are many hypotheses in explaining the outbreak of diabetes and its complications. Among them aldose reductase, Maillard products or advanced glycation end products (AGE) hypothesis, oxidative stress, changes in lipoprotein metabolism, increase in proteinase C activity, changes in growth factors and cytokine activity can be counted. Nevertheless, the most significant and on which most emphasis is made is the stress hypothesis. Hence, many researchers inform that DM originates from oxidative stress. (Kılıçarslan and Dönmez 2016). In researches, it is shown that in the formation of complications appearing in relation to diabetes, the most important factor is oxidative stress. (Koca et al., 2008; Kılıçarslan ve Dönmez, 2019). It is claimed that DM, which has many different types, generates oxidative stress through glycometabolic way in Type 1 and Type 2 diabetes (T1DM, T2DM) (Ceriello et al., 2000). It is proved that the complications come into existence, through the reactive oxygen species (ROS), by causing an imbalance between production and destruction, by autooxidation of glucose, by the increase in ROS production, and by triggering many different mechanisms. The aforementioned changes may destroy in the biomolecular extent in the organelles and membranes of cells. (Nakhjavani et al., 2010). In recent research, it is laid out that in diabetic animal experiments and diabetic individuals, lipid peroxidation products and independent oxygen radicals increased remarkably and oxidative stress plays an effective role in the formation of diabetes and its complications (Suryanarayana et al., 2007; Hamacioglu, 2017).

Curcumin derived from Curcuma longa, which is also known as turmeric and also used as a spice, has many effects such as anti-inflammatory, antioxidant, anticarcinogenic, antimutagenic, anticoagulant, antidiabetic, antibacterial, antiviral and neuroprotective (Kahkashan et al., 2017; Karlowicz-Bodalska et al., 2017). Curcumin shows antioxidant attributes because it facilitates the removal of many reactive oxygen radicals, most notably superoxide anions, nitrogen dioxide radicals and hydroxyl radicals (Kahkashan et al., 2017). It is reported that curcumin at DM shows an anti-diabetic effect through repressing oxidative stress and inflammation and executes this effect generally through inducing the last glycation products by assuming advanced glycation and by reducing the blood glucose levels and hemoglobin levels through polio path regulation (Nabavi et al., 2015). Suryanarayana et al (2007) have reported that the administration of curcumin and turmeric improves but does not completely prevent oxidative stress in streptozotocin-induced diabetes. Many researchers have reported that given its beneficial effects, safety and cost-effectiveness, curcumin could be used to treat diabetes and complications (Xie et al., 2018).

The aim of this study was to establish curcumin's oxidative effects on STZ- induced diabetic rats.

MATERIALS AND METHODS

In the research, 30 healthy male Wistar Albino rats (average weight of 280 ± 20 g) were used. The rats used in the experiment were procured from Selçuk University Center of Experimental Medicine Research and Application (SÜDAM). During the research, attention was paid for providing the recommended life conditions (23±2 ^{O}C room temperature, 50±10% relative humid room, 12/12 night/daylight) for the rats. Animals were provided with *ad libitum* standard rat forage and clean water (~50 ml/day/rat). The laboratory conditions were maintained as per the guidelines given by the SÜDAM Experiment Animals Ethics Committee. The research protocol was conducted with the authorization No 2017-11 of Selçuk University Experimental Medicine Research Center (SÜDAM) Experiment Animals Ethics Committee.

The animals used in the experiment were divided into four equal groups, intended to keep their average live weights: Control (K), Diabetes (D), Curcumin (C), and Diabetes + Curcumin (DC). In order to expose them to diabetes, 60 mg/kg streptozotocin intraperitoneal injection was dissolved in the 0.1M citrate buffer (pH: 4.5) and was applied to the rats in the D and DC groups (Akbarzadeh et al., 2007; Maciel et

al., 2013; Keshk et al., 2020). After 72 hr, induction of diabetes was verified by measuring blood glucose level via the tail vein and animals with blood glucose levels greater than 250mg /dl were considered diabetic. Curcumin at a dosage of 50 mg/kg/day was given to the C and DC groups through gavage during the study every day (Pourmahmoudi et al., 2021).

After 4 weeksend of thetrial, blood samples were taken from cardiac puncture under anesthesia, and transferred into anticoagulant tubes for determination. All animals were sacrificed by cervical dislocations. The drawn blood samples, after being centrifuged (Hermle Z380) and separated from serum and plasma were stored at - 80^0C until the analysis time to measure SOD, MDA, GSH, Catalase, and glucose.

Plasma glucose levels were detected with the use of the commercial kit in Siemens Centaur XP Immunoassay System device, oxidative stress' determinant and lipid peroxidation were established by commercial kits (Cayman) which is the product of MDA (Oxis) and GSH, SOD, Catalase and glucose levels are established by using commercial kits in Biotek ELX 800 Elisa device, in accordance with their prospectus.

Statistical Analysis

Statistical differences among the groups were tested by analysis of variance (*ANOVA*) which is followed by Duncan's test using SPSS for windows version 17.0. Significantwasconsidered as $p<0.05$.

RESULTS

Data belonging to the MDA, SOD, GSH, Catalase, and glucose that are detected in the blood samples of the research groups at the end of the 4 week-experimentation are presented in Table 1.

When the data gathered at the end of the study is examined, while it is established that in the diabetic rats (D and DC groups) the lipid peroxidation final product MDA is considerably higher than the other two experiment groups (K and C), it is observed that MDA level in D Group is significantly higher than the other three groups ($p<0.00$) (Table 1).

Yet in the study it is established that SOD, GSH, and CAT levels at diabetic group are significantly lower ($p<0.001$, $p<0,002$) (Table 1) than the other three groups (K, C, and DC), contrary to the increasing lipid peroxidation product.

In this study it is observed that in diabetic groups (D and DC) the level of plasma glucose showed a remarkable increase vis-à-vis the control groups (K and C) ($p<0.05$). When the data from the DC group is examined it is detected that while the blood glucose level decreases significantly ($p<0.00$) relative to D group, it is still distinctly ($p<0.00$) higher than K and C groups (Table 1).

DISCUSSION

Oxidative stress plays an important role in the etiology of diabetes. Therefore, it was expected that the MDA level would increase significantly from a statistical standpoint, as diabetes causes oxidative stress. Therefore, our finding is important in that it supports many previous studies (Akkaya and Çelik, 2010; Kılıçarslan and Dönmez, 2016; Kahkashan et al., 2017; Xie et al., 2018). The increase in the MDA level at diabetic rats can be originated from the increase in the free radical formation that is formed in the organism which is in line with the increase in lipid peroxidation at glucose autooxidation and glycated proteins because of the hyperglycemia. When the data gathered from the other three groups (K, C, and DC), while there could not be established a statistical difference among the groups, although it was observed that the MDA value gathered from DC Group

Table 1. Effect of the orally taken curcumin application in the diabetic rats (done so through STZ induction) on MDA, some antioxidants, and glucose level

Parameters	Control(n=6)	Curcumin(n=8)	Diabetes(n=8)	D+C(n=8)	p
MDA(nmol/ml)	0,80±0,04^{a}	0,83±0,04^{a}	1,85±0,14^{b}	1,05±0,06^{a}	0,00
SOD(U/ml)	0,46±0,03^{c}	0,42±0,01^{bc}	0,29±0,02^{a}	0,38±0,01^{b}	0,001
GSH(µM)	0,51±0,03^{c}	0,50±0,02^{bc}	0,30±0,04^{a}	0,40±0,02^{b}	0,001
Catalase(U/ml)	1,67±0,07^{b}	1,53±0,03^{b}	1,20±1,45^{a}	1,45±0,08^{b}	0,002
Glucose(mg/dl)	161,33±23,30^{a}	206,83±27,79^{a}	463,33±48,19^{c}	330,16±56,39^{b}	0,00

a,b,c; Means in the same row with different superscripts significantly differ ($P<0.05$)
Values are expressed as mean ± SD

decreased significantly ($p<0.00$) vis-à-vis Group D and it approached the data gathered from the K and C Groups, still it was higher than the values gathered from these two groups (Table 1).

In many studies carried out concerning diabetes, the prevention of oxidative stress by various antioxidants has been investigated. In the studies made to this end, the establishment of enzymatic activities of enzymes such as CAT, SOD, GSH-PX, GSH-RD, and establishment of MDA level, the oxidative stress many researches have reported that given its beneficial effects, safety and cost-effectiveness, curcumin could be used to treat diabetes and complications appeared indirectly in diabetes is established (Hamamcıoglu, 2017). In the study conducted by Aluwong et al (2016), it is established that in the diabetic rats Type 1, while probiotic+C vitamin reinforcement decreased the glucose level and oxidative stress, it increased antioxidant level. Garg et al. (2005); informs that E vitamin reinforcement to diabetic rats does not decrease plasma glucose level, but MDA level decreases and CAT, GSH-Px, GSH-RD levels approach normal level. In another study, it is observed that in the diabetic rats to which C and E vitamins along with melatonin are applied glucose and MDA levels decreased, hematological and biochemical parameters along with antioxidant levels returned to normal levels. (Allagui et al., 2014). (Garg et al., 2005; Aluwong et al., 2016).

In the study conducted by Liang et al. (2011) for establishing the effectiveness of quercetin, they detected that in diabetic rats serum SOD, CAT, GPx and GST levels significantly decreased in DM Group, however in the DM+Q Groups quercetin (groups to which 30 and 50 mg/kg were given) significantly increased SOD, CAT, GPx and GST levels.

Yet also in the study Kılıçarslan (2015) conducted in order to establish the antioxidative effectiveness of quercetin at diabetic rats, it is established that while quercetin application decreased MDA level significantly at diabetic rats, SOD and GSH levels increased. In the study conducted by Panahi et al. (2017) in order to detect the effect of curcumin in Type 2 DM patients, they inform that oxidative stress plays a key role in the pathogenesis of T2DM and vascular complications. At the end of 8 weeks of study, they detected that curcuminoids significantly ($p<0.001$) increased in diabetic patients the serum total capacity, they decreased significantly the serum MDA level.

In another study intended to detect the effect of the curcumin at rats on which Type 1 DM was established on antioxidants, it is announced that while curcumin, which was given to the rats with their diet during 21 days, significantly decreased blood glucose level and plasma MDA concentration, it increased SOD, CAT and GSH-Px levels at the end of the experiment. (Xie et al., 2018). Kahkashan et al. (2017), in their study which is intended to detect the effectiveness of curcumin against oxidative stress originating from diabetes, established a significant increase in MDA and glucose amount at the diabetic group, yet in SOD, CAT and GSH levels significant decrease ($p<0.5$). Researchers layout that these negative changes in the diabetic group to which curcumin reinforcement was carried out returned to normal and they attribute this to the fact that curcumin is a fairly effective antioxidant.

Our findings also support the above-mentioned studies carried out previously in this subject. It is known that lipid peroxidation (with respect to MDA levels) is a common determinant of oxidative modification of cell membrane damage and proteins. In the previous studies, it makes us think that the overproduction of ROS and NO - under hyperglycemic conditions - cause oxidative damage at intracellular protein molecules and damage phospholipids that are tied to the membrane through lipid peroxidation (Kahkashan ve ark., 2017). Contrary to the increase of the MDA level at diabetes group, a decrease in SOD, GSH and CAT levels also shows this. Yet in this study, the fact that data in the K and C groups are similar to each other, while the data in DC group is significantly different from D group it approaches the values in K and C group confirms the fact that curcumin has the feature of arranging the intracellular enzyme activities effectively and being an effective antioxidant. This finding makes us think that reinforcement of regularly used curcumin in diabetes increases resistance against lipid peroxidation and hence along with oxidative protein damage, it may cause a decrease in DNA damage.

The increase in glucose level we observed in the experimental groups (D, DC) in this study suggests that the β cells of the pancreas are damaged. Therefore, the finding we obtained here is an expected result, but it supports many previous studies on this subject (Tahara et al., 2008; Wu &Huan, 2008; Aluwong et al., 2016; Kahkashan et al., 2017). This is in line with insulin deficiency, penetration and entry of glucose into the body cells decrease. Besides, due to

the oxidative stress occurring some stress hormones along with due to increase of growth and glucagon hormones' secretion, events of glycogenolyses, gluconeogenesis, lipolysis and ketogenesis are also tempted and hyperglycemia is being formed (Rowland and Bar-Or, 2004). The fact that in the DC group with the reinforcement of curcumin the blood glucose level decreases significantly makes us think that it originates from the fact that curcumin displays hypoglycemic effect.

In conclusion, the results of the present study suggest that, curcumin, which was specifically used for this purpose in this study, may play an active role in the regulation of increased oxidative stress, protein glycation and glucose metabolism in cases of diabetes. In addition, different dose, duration, age and comparison of the administration routes shows that curcumin has an antidiabetic effect.

CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Epidemiology of cryptosporidiosis in dairy calves in central and eastern Algeria

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ABSTRACT: The purpose of our survey is to estimate the frequency of *Cryptosporidium* spp in dairy calves under 60 days of age, the pathogenic role of *Cryptosporidium* spp in the development of diarrhea, the intervention and association of other enteropathogens often involved in neonatal diarrhea and the possible relationship between infection and certain farming practices in order to estimate the share of risk factors, and on the other hand, to compare between the modified Ziehl-Neelsen staining method and the copro-ELISA test.

We found 35 positive samples out of 223 faecal samples analyzed by modified Ziehl-Nielson staining, with a prevalence of 15.69%, the infected calves had 4 times the risk of diarrhea (RR= 4), the age group [8-15 days] is the most susceptible to infection compared to the other age groups (31.1%; $p<0.05$).

The ELISA test revealed that 33 out of 92 calves were infected by *Cryptosporidium* spp., with a prevalence of 35.87%, showing a sensitivity and specificity of Ziehl-Neelsen staining of 51.5% and 96% respectively.

Keywords: *Cryptosporidium* spp, Calf, Ziehl-Neelsen, ELISA, Colostrums, Algeria.

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INTRODUCTION

Cryptosporidium spp are ubiquitous parasites of the digestive tract of several animal species and humans (Xiao a nd al., 2004).These protozoa are a major cause of diarrhea in young animals (Aslanova and al., 2013). Diarrhea has been shown to be the primary cause of calf mortality in dairy cattle farms in eastern Algeria (Bouzebda and al 2007). Recent studies on dairy cattle in northern Algeria show that *Cryptosporidium* spp have been isolated more particularly from diarrhoeal calves compared to non-diarrhoeal calves (Ouakli and al., 2018, Akam and al.,2009) and the calf can become infected within the first three days of its life (Khelef and al., 2007, Bendali and al., 1999). *Cryptosporidium* causes very significant economic losses related to calf mortality and treatment costs. The importance of cryptosporidiosis is considerable both economically and zoonotic (Björkman and al., 2003,Foll and Jérôme and al., 2011).

The objectives of our study were to estimate the prevalence of *Cryptosporidium* spp. in dairy calves less than 60 days of age and their distribution by age, to assess the pathogenic role of *Cryptosporidium* spp. in the occurrence of diarrhea in young cattle, to develop hypotheses on the risk factors linked to *Cryptosporidium* spp. Infection and to evaluate the diagnostic performance of the coprological method represented by comparing it with the modified Ziehl-Neelsen stain and the copro-ELISA test.

Inadequate colostrum management, unbalanced feeding of pregnant cows, intervention by other enteropathogens and collective parking of calves of different ages appear to be the most likely risk factors associated with *Cryptosporidium* spp. infection.

MATERIALS AND METHODS

Study population

Our survey concerns dairy calves under 60 days of age, belonging to dairy cattle farms selected by lot from lists of farms in the study area, the target population is mainly composed of two breeds; Prime-Holstein, Montbeliarde. To assess the age of the calf most susceptible to cryptosporidium infection we divided the study population into six (6) age classes from the first week of age, the interval between each class is two (2) weeks to one (1) month of age and two (2) weeks from the age of 30 days to the age of 60 days.

Period and study area

Our research was carried out from March 2015 to October 2018 in dairy cattle farms belonging to the following wilayas: Blida, Tipaza, Media (north-central Algeria), Tizi-Ouzou, and Setif (north-east Algeria).

Collection of samples

The fecal matters were collected as soon as they were released after the anal orifice was excited in sterile plastic vials, and then transported under a cold blanket in an isothermal cooler to the laboratory. We split each sample in two:

- The first, intended for coproscopy examination, treated freshly or stored under cover of 2.5% Potassium Dichromate (Cr2K2O7) (1volume/2volume) then stored immediately in the refrigerator (4-8°C).

- The second vial was stored at a temperature of -20°C without the addition of dichromate and will be reserved for the direct ELISA test.

A first individual sheet of the information collected during the farm visit, including the number, date of sampling, age, sex, calving conditions and clinical condition of the calves, a second sheet focused on farming practices for each farm.

Laboratory analysis

At the NVS parasitological laboratory in Algiers, 223 samples (105 healthy calves and 118 sick calves) were analyzed by the modified Ziehl-Neelsen stain following treatment with the simplified Ritchie concentration technique (Starkey and al., 2006). The ELISA test was performed on 92 samples from 52 sick calves and 40 symptomatically healthy calves where we used an ELISA kit provided by the Bio-X Diagnostics laboratory, a tetravalent kit (*Rotavirus, Coronavirus*, *E. coli F5* and *Cryptosporidium* spp) for the direct Copro-ELISA test. (Boussena et al., 2009)

Statistical analysis

The results were processed with the software statistica 06, are based mainly on descriptive statistics, and we used the Chi-square dependency test and the student test at the significance level $\alpha = 5\%$ for the analytical analyses.

RESULTS

Prevalence of *Cryptosporidium* spp (by Ziehl- Nielsen staining): Microscopic analysis of samples treated with modified Ziehl-Neelsen staining shows that 35 of 223 samples (Table 2) had at least

one *Cryptosporidium* spp oocyste, representing a prevalence of 15.69% (Table 1). Veal can be infected from the first days of life and the age group [8-15 days] is the most affected (31.11%) (Figure 1).

Impact of *Cryptosporidium* spp infection on the development of diarrhea in calves: *Cryptosporidium* spp has been excreted in sick and non-ill calves, the prevalence of cryptosporidiosis is higher in diarrheal calves compared to non-diarrheal calves (the difference is significant (p=0.04) regardless of the age of the animal, with the exception of calves aged one month and calves in the age group [46-60 days] (Table 2).

Table 1: Prevalence of cryptosporidiosis according to age

Age	Number of samples	Number of positive cases	Prevalence (CI 95%)
1-7 days	45	5	11,11%[8,2%-14,03%]
[8-15days]	45	14	31,11% [28,2%-34,03%]
[16-21 days]	23	1	4,35%[1,36%-10,05%]
[22-30 days]	40	7	17,50%[14,22%-20,78%]
[31-45 days]	30	3	10,00%[5,63%-14,37%]
[46-60 days]	40	5	12,50%[9,22%-15,78%]
Total	223	35	15,69%[15,11%-16,28%]

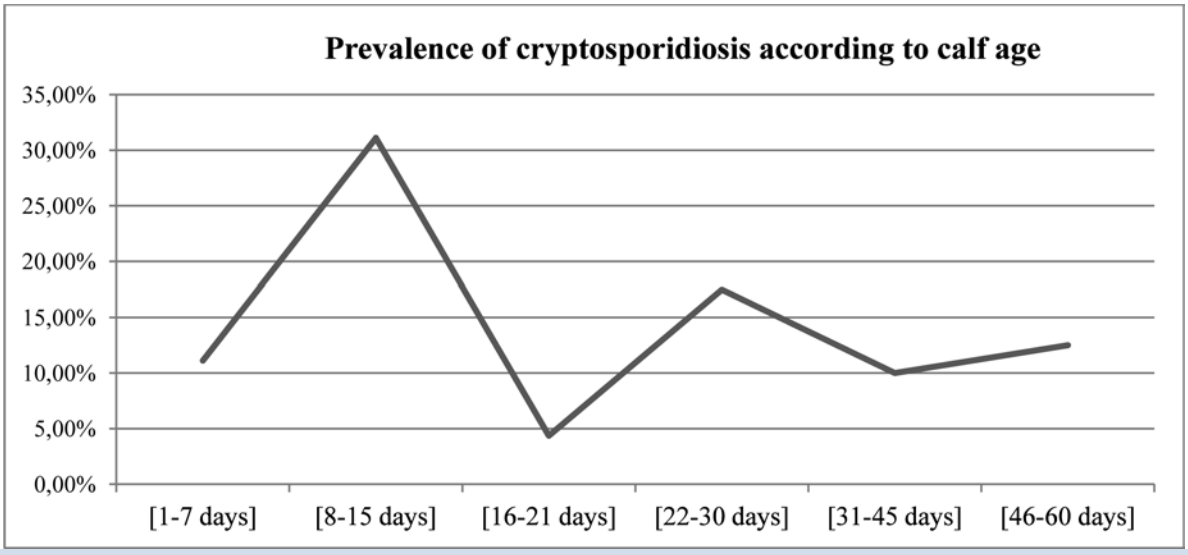

Chart 1: Prevalence of cryptosporidiosis in calves as a function of age

Table 2: Prevalence of cryptosporidiosis in diarrheal and non-diarrheal calves

	D			ND		
Age group	Number of samples	Number of positive cases	Prevalence (CI 95%)	Number of samples	Number of positive cases	Prevalence (CI%) (CI 95%)
[1-7days]	27	5	18,52%[16,71%-20,33%]	18	0	0%
[8-15days]	27	9	33,33%[31,52%-35,14]	18	5	27,78%[25,45%-30,11%]
[16-21days]	14	1	7,14% [5,33%-8,95%]	9	0	0%
[22-30days]	20	2	10,00%[8,19%-11,81%]	20	5	25%[22,67%-27,33%]
[31-45days]	16	2	12,50%[10,69%-14,31%]	14	1	7,14%[4,81%-9,47%]
[46-60days]	14	1	7,14%[5,33%-8,95%]	26	4	15,38%[13,05%-17,71%]
Total	118	20	16,95%[15,14%-18,76]	105	15	14,29%[11,96%-16,62%]

D: sampling of the sick calf (diarrhoeal stool); **ND:** sampling of non-diarrhoeal stool.

The importance of *Cryptosporidium* spp as an etiological pathogen of gastroenteritis was determined by estimating the percentage of diarrheal calf infection by *Cryptosporidium* spp in different age groups. This infection rate was compared to other rates caused by different etiological agents, whether infectious or foodborne (Table 3).

Risk factors: The analytical study showed that pregnant cow feeding, colostrum management, type of calf housing and lack of disinfection in calving facilities are the most important factors related to calf infection by Cryptosporidia (Table 4).

Prevalence of cryptosporidian infection (by ELISA test): The direct ELISA test found that *Cryptosporidium* spp is the most frequently enteropathogenic in calves under 60 days of age, of which (33/92) samples were positive and had a prevalence of 35,87%; the association of other germ was found in 5 cases with *Rotavirus* in 2,17% of cases, *Coronavirus* (1%), E. Coli F5 (2,17% of samples examined) and the association of two viruses with *Cryptosporidium* spp was detected in one sample (1,08%) (Table 5).

Table 3: The importance of cryptosporidiosis in the etiology of neonatal diarrhea

		Diarrheal calves					
Age class	**Number of calves**	**Crypto-sporid-iosis (+)**	**Percentage (CI95%)**	**Crypto-sporid-iosis (-)**	**Percentage (CI95%)**	***RR***	**CI (RR)**
[1-7 days]	27	5	18,52%[16,71%-20,33%]	22	81,48%[79,67%-83,29%]	3,57	[1,87- 6,54]
[8-15days]	27	9	33,33%[31,52%-35,14%]	18	66,67%[64,86%-68,48%]	1,37	[1,6-2,7]
[16-21days]	14	1	7,14%[5,33%-8,95%]	13	92,86%[91,05%-94,67%]	4	[1,5-10,6]
[22-30days]	20	2	10,00%[8,19%-11,81%]	18	90,00%[88,19%-91,81%]	0,6	[0,09-4,05]
[31-45days]	16	2	12,50%[10,69%-14,31%]	14	87,50%[85,69%-89,31%]	2	[0,8-4,95]
[46-60days]	14	1	7,14%[5,33%-8,95%]	13	92,86%[91,05%-94,67%]	1	[0,16-6,43]
Total	118	20	16,95%[15,14%-18,76%]	98	83,05%[81,24%-84 ?86%]	2	[1, 3- 3]

RR: relative risk. **(+) :** presence of *Cryptosporidium spp* oocyst. **(-):**negative sample.
CI (RR): 95% confidence interval of RR.

Table 4: Risk factors for *Cryptosporidium spp*

Variable	**Method**	**(%) Crpt**	**χ^2 (p)**
Pregnant cow ration supplements	Yes	8,02	0,01
	No	23,46	
Drying-up practice	Yes	14,51	0,01
	No	16,98	
Amount of colostrum drunk	Enough	7,4	0,04
	Inadequate	24,07	
Hygiene of the stable	Good	11,75	0,002
	Bad	19,73	
Systematic disinfection of calving rooms	Yes	13,89	0,04
	No	17,59	
Systematic disinfection of parking spaces	Yes	7,41	0,01
	No	24,07	
Type of parking	Collective	24,38	0.03
	Individual	7,1	

(%) crpt: prevalence of cryptosporidiosis; **p:** significance of the chi-square test (χ2).

Table 5: Prevalence of *Cryptosporidium* spp. using tetravalent ELISA test

Detected enteropathic agent	Number of samples (n = 92)	
	Number of positive cases	(%)
Negative sample	36	39,13%
Coronavirus	06	6,52%
Rotavirus	09	9,78%
E.Coli F5	08	8,70%
***Cryptosporidium* spp.**	**33**	**35,87%**
Rotavirus + ***Cryptosporidium* spp**	02	2,17%
Coronavirus + ***Cryptosporidium* spp**	01	1,08%
E.Coli F5+ ***Cryptosporidium* spp**	02	2,17%
Rotavirus+ *Coronavirus*+ ***Cryptosporidium* spp**	01	1,08%

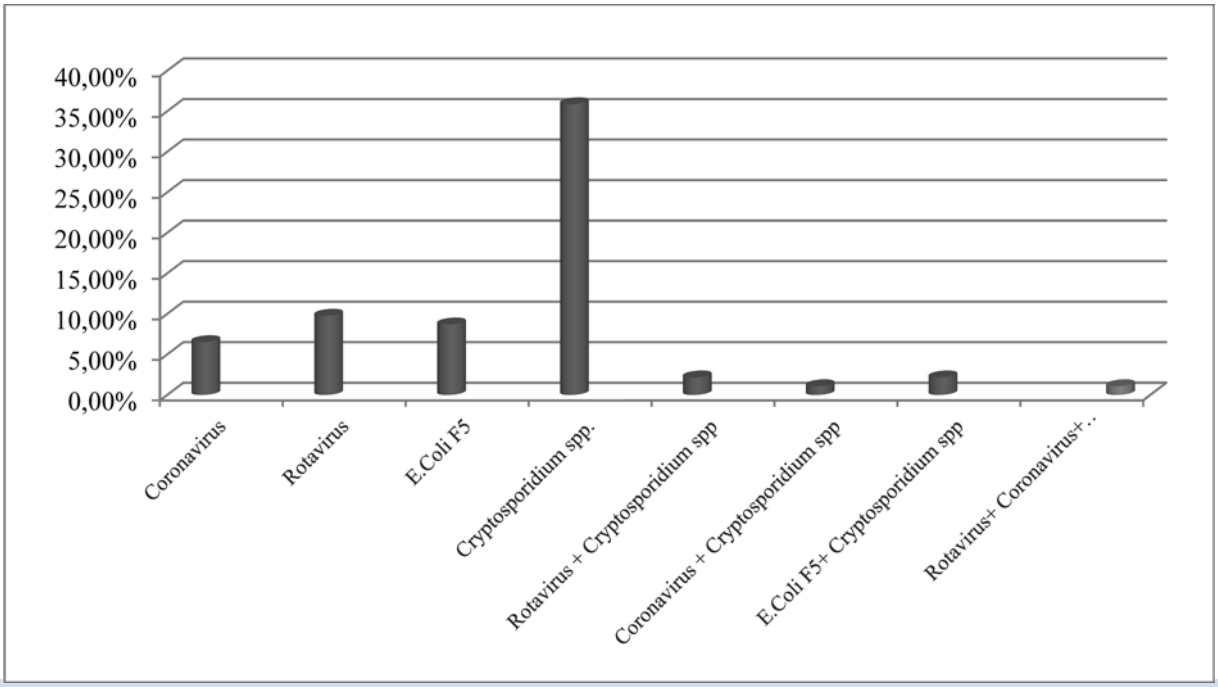

Figure 2: Prevalence of the four main pathogens responsible for neonatal diarrhea in calves using the direct Copro-ELISA test

According to Figure 2, *Cryptosporidium* spp. was detected in 35% of the samples examined with a higher prevalence compared to that found by modified Ziehl-Neelsen staining (15.69%).The association of other enteropathogens was observed in 5.4% of *Cryptosporidium* spp positive cases, the other enteropathogens can be added to the factors favoring infection by this protozoan and the development of diarrhea in calves less than 60 days of age.

Sensitivity and Specified of the modified Ziehl-Neelsen staining: To estimate the sensitivity and specificity of the Ziehl-Neelsen staining technique in the identification of *Cryptosporidium* spp oocysts in 92 samples, we used the ELISA test as a reference method and calculated the TP, FP, TN and FN to determine Sp, Se by the following formulas:

Se = TP/ TP+FN x 100 ; Sp = TN / TN+FP x 100 …. (Saul Tzipori et al .,2002)

Of which:
Se: sensitivity, Sp: specificity / FP: false positive, TP: true positive
FN: false negative, TN: true negative

Out of 92 samples we detected 19 positive cases by modified Ziehl-Neelsen staining, representing a rate of 20%, this rate is lower than that found by the ELISA test (35.8%), this indicates that positive ELISA cases were not identified by Ziehl-Neelsen staining (16 false negative cases), the copro-immunological test has more specificity because the micro-cups in the ELISA test are sensitized by specific antibodies against *Cryptosporidium* spp. from Table 5 we have observed that the Ziehl-Neelsen staining method has a sensitivity of 51% and a specificity of 96% (Table 6).

DISCUSSION

Our survey was targeted at calves under 60 days of age, which represent the population at high risk and most exposed to diarrhea, the area of study is a region with a very high number of dairy livestock farms and is one of the potential areas for dairy production in Algeria. The study period included the calving seasons to explore the role of some rearing practices during calving such as animal density, calf parking and colostrum feeding as part of Cryptosporidium spp infection (Pwaveno et al., 2006) (Quigley et al .,2001).

In our investigation we selected the targeted population by drawing lots from the source population which was also randomly included from a general population represented by the lists of approved farms in each region to ensure the representativeness of our sample. To estimate the prevalence of cryptosporidiosis and develop hypotheses on risk factors, we used Ziehl-Neelsen staining modified by Henriksen and Pohlenz (1981) to detect *Cryptosporidium* spp oocysts in the feces of diarrheal and non-diarrheal calves, it is a reference method (Millemann et al., 2009), fast, inexpensive and easy to read, with considerable sensitivity and specificity, more important than other coprology techniques (Khelef et al., 2002) (Aslanova et al., 2013).

The estimated prevalence of cryptosporidiosis in our survey 15.7% is similar to that reported by Khelef and al (2007) in the Mitidja area of central Algeria 16.9% (Khelef et al., 2007), in addition, another study was conducted in the same region by Akam in 2009 with a prevalence of 33% (Akam et al.,2009) , similar to that found by Ouchene and al in 13 dairy cattle farms located in the Setif region in 2012(22%) (Ouchene et al., 2012), a little higher than Björkman's result in Sweden in 2003 (14%) (Björkman et al., 2003), almost the same prevalence as that estimated for dairy calves in 5 French departments by Naciri in 1999 (17%) (Naciri et al., 1999) and lower than the prevalence in 140 farms in Vendee (France 2005) 32% (Quillet et al., 2005) our result is higher than that given by Boussena in 2008 in 14 farms in the Constantine area and one farm in the Setif area (5%) (Boussena et Sfakssi., 2009) The previously referenced surveys used the modified Ziehl-Neelsen stain.

For the prevalence determined after using the direct ELISA technique, our result (35,8%),which explains the high number of false negative samples reported in our study using Ziehl-Neelsen staining 35,8% vs 15,7%). It is higher than that reported by Bendali in the Midi-Pyrénées in France (15.6%) (Bendali and al., 1999), and the other shown by Ouakli in northern Algeria (15,8%) (Ouakli and al., 2018), by Fu Chen in China in 2012 (19%) (Fu Chen and al., 2012) and also by Follet in France (34%) (Follet at al., 2011) and is below that reported by Lise in south-western Ontario in Canada (40.5%) (Lisa A and al., 2005), these three surveys have used molecular biology methods (PCR-RFLP), this difference could be related to the husbandry practices in the populations studied as well as the conditions of each survey, or may be related to factors such as epidemiological parameters, the size and sampling mode and the laboratory diagnostic technique.

Table 6: Sensitivity and Specified of the modified Ziehl-Neelsen staining

	ELISA (n=92)		
Ziehl-Neelsen staining (n=92)	**Positive**	**Negative**	**Total (%)**
Positive	17	2	19 (20.6%)
Négative	16	57	73 (97,3%)
Total(%)	33 (35,8%)	59 (64,2%)	92

Se = 17 / (16+17)*100 = 51,5% / **Sp** = 57 / (2+57)*100 = 96%

As regards risk factors, there were few analytical surveys carried out in Algeria, however, at the livestock studied in our research, we found that the correct colostral management (quantity, quality and deadlines) significantly reduces the infection by *Cryptosporidium* spp oocysts ($p<0.05$), the colostrum quality is related to the feeding of pregnant cows and the practice of drying-up (Barwick et al., 2003), the latter two factors have been found significantly related to *Cryptosporidium* spp infection in the target population ($p<0.05$).

The presence of organic matter in the litter and moisture (especially urine) in the calf housing area and close contact between calves of different ages in the community yard were associated with an increased risk of excretion of *Cryptosporidium* spp oocysts ($p<0,05$), because these two factors ensure the survival, multiplication and transmission of the parasite from sick calves to newborns, as older calves and sick calves multiply the parasite and transmit it to particularly sensitive newborns.

Regarding the role of concomitant infections in calves, we found that 5% of the study population infested by *Cryptosporidium* spp was also affected by one of the three main enteropathogens Rotavirus, Coronavirus and E.coli F5, this predisposing factor requires thorough analytical studies to accurately assess its epidemiological index.

CONCLUSION

Cryptosporidiosis is a very frequent disease in dairy calves in Algeria, it may affect them from the first week of life, with a peak in excretion around 15 days of age (this moment is known as the “immune hole”, indeed at this time colostral antibodies tend to decrease and the calf has not yet fully established its own active immunity and is therefore immune deprived and vulnerable to infection., it causes considerable economic losses due to the lack of specific treatment, it is also accompanied by high mortality in the newborn after the installation of diarrhea, where we recorded a four-fold higher risk in infected calves (RR=4).

Factors related to livestock management such as stable hygiene, calf parking irrespective of age, drying-up, feeding of pregnant cows especially in the last months of pregnancy and the intervention of other enteropathogens *Rotavirus, Coronavirus* and *E. coli F5* are all major contributory factors in *Cryptosporidium* spp infection.

The copro-immunological test (ELISA-direct) reveals that *Cryptosporidium* spp is the most prevalent pathogen in the neonatal period in cattle with a significantly high frequency compared to that shown by the Ziehl-Neelsen stain modified by Henriksen and Polhenz (35% vs 16% ; $p<0,05$).

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CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

Methicillin and vancomycin resistant isolates of *Staphylococcus aureus* and *Enterococcus faecalis* recovered from bovine mastitis

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ABSTRACT: Mastitis is the most costly disease in the dairy industry. Selecting the proper antibiotic treatment is beneficial for economic and avoids the emergence of antimicrobial resistance. The objective of the present study was to investigate the prevalence of methicillin and vancomycin resistant isolates of mastitis-causing *Staphylococcus aureus* and *Enterococcus faecalis* as a probable source of transferable vancomycin resistance to staphylococci. A total of sixty-one *Staphylococcus aureus* and eight *Enterococcusfaecalis* isolates were investigated for genotypic and phenotypic antimicrobial resistance.Presence of the *mecA*, *vanA* and *vanB*genes were surveyed by PCR. The MIC (Minimum Inhibitory Concentration) of vancomycin was determined bybroth microdilution test for all the isolates.Moreover, the antibiotic resistance patterns of the isolates to the most common classes of antibiotics used in dairy cattle such as β-lactam, macrolides and tetracyclines were determinedusing the disk diffusion method. Among *Staphylococcus aureus* isolates, one MRSA (methicillin-resistant *Staphylococcus aureus*) isolate was detected while 47.5% of isolates were detected as multidrug-resistant. Furthermore, no phenotypic and genotypicvancomycin-resistance *Staphylococcus aureus* was found. Most of the *Enterococcus faecalis* isolates (6/8) showed high MIC for vancomycin (in the range of 128- 1024 µg/ml) and one *vanA*-type *Enterococcus faecalis* was observed. This study indicates thatsince the source of transferable resistance to vancomycin exists in dairy farms, there is a potential for emerging and spreading VRSA (vancomycin-resistant *Staphylococcus aureus*) in dairy cattle which is a risk to animal and human health.

Keywords: Bovine mastitis; VRSA; MRSA;VRE; MDR

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INTRODUCTION

Bovine mastitis is one of the most costly concerning in dairy farms. The principles of prevention and control programs of mastitis are the improvement of milking hygiene and antimicrobial application.To date, many bacterial pathogens are identified as causes of intra mammary infections (IMI). *Staphylococcus aureus* (*S. aureus*) is the cause of the most common types of chronic and contagious mastitis. It is also responsible for various types of infections in human and other animals (Ruegg, 2017). Antimicrobial resistance of *S. aureus* has attracted a lot of attention, thus numerous studies has been conducted all around the world to surveil it (Jamali et al., 2014; Wang et al., 2016; Li et al., 2017; Zaatout et al., 2019). As emerging of methicillin (oxacillin) resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) strains has led the therapeutic programs face a big challenge (Tarai et al., 2013), it is essential to monitor the development and expansion of MRSA and VRSA. Resistance to vancomycin in *S. aureus* is an acquired antimicrobial resistance from enterococci through the acquisition of the genes *vanA* and/or *vanB* (Courvalin, 2006). Different species of enterococci are considered as environmental mastitis-causing pathogens. To date, no report of genotypic resistance to vancomycin in *S. aureus* has been recorded in dairy cattle. Monitoring the development of antimicrobial resistance especially the acquired type is necessary in food-producing animals and dairy cattle is not an exceptional. Our purpose of the present study was to investigate the prevalence of MRSA and VRSA among *S. aureus* recovered from bovine mastitis milk. We also aimed to detect vancomycin resistance enterococci from mastitis milk as a probable and possible source of transmission of vancomycin resistance to *S. aureus*. We also covered the antibiotic resistance patterns of *S. aureus* and *Enterococcus faecalis* (*E. faecalis*) isolates to the most common classes of antibiotics used in dairy cattle such as β-lactam, macrolides and tetracyclines.

MATERIALS AND METHODS

Bacterial isolates

Sixty-one isolates of *S. aureus* and eight isolates of *E. faecalis*were investigated in the current study. The isolates belonged to subclinical bovine mastitis which were submitted to Veterinary Hospital of Ferdowsi University of Mashhad. Sampling and microbial culture were conducted according to National Mastitis Council guidelines. Conventional biochemical tests were carried out in order to confirm bacterial species (National Mastitis Council (U.S.), 2004).

DNA extraction

Bacterial DNA was extracted by GeneAll Exgene™ Cell SV kit (GeneAll, South Korea) following the manufacture's instructions.

Molecular confirmation of S. aureus

Molecular confirmation was performed by amplification of the*S. aureus*-specifi c*nuc*g ene as described by Graber et al. (2007). The primer (Macrogen, South Korea) sequenceand PCR condition are mentioned in table 1.

Molecular detection of methicillin (oxacillin) and vancomycin resistance genes

All the isolates were tested for the presence of genes *vanA* and *vanB*. To detect MRSA, all the *S. aureus* isolates were investigated for the presence of *mecA*. The primers' characteristics (Macrogen, South Korea) and PCR conditions are listed in table 1. All PCR products were analyzed by 1.2% agarose gel (w/v) (DENAzist Asia, I. R. Iran) and Green Viewer safe stain (0.01 v/v) (SinaClon, I. R. Iran).

Antimicrobial susceptibility testing

A standard agar-disk diffusion (Kirby-Bauer) was performed for all *S. aureus* and *E. faecalis* isolates according to CLSI interpretive criteria using

Table 1. PCR conditions and primers used in this study

Gene	Sequence (5' to 3')	Product size (bp)	No. of cycles T_a, Time	Ref.
nuc	CTGGCATATGTATGGCAATTGTT TATTGACCTGAATCAGCGTTGTCT	664	35 cycles 60°C, 1 min	(Graber et al., 2007)
mecA	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTGC	533	40 cycles 55°C, 30 sec	(Murakami et al., 1991)
vanA	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	1030	30 cycles 58°C, 30 sec	(Clark et al., 1993)
vanB	GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	433		

Mueller-Hinton agar plates (Merck, Germany) and antibiotic disks (Padtan Teb, I. R. Iran) for penicillin (10 units), ampicillin (10 μg), erythromycin (15μg) and tetracycline (30μg) (Bauer et al., 1968). Based on CLSI guideline, phenotypic resistance to vancomycin (Sigma-Aldrich, Germany) determined by broth microdilution method for both genus and disc diffusion test for vancomycin was carried out for *E. faecalis* isolates (CLSI, 2017). *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213 were used as quality control strains for disk diffusion method and broth microdilution method, respectively.

RESULTS

S. aureus

Sixty-one isolates were confirmed as *S. aureus* based on biochemical reactions, coagulase test and possessing the *nuc* gene. Ten different antibiotic resistance patterns were obtained according to the combination of the results of agar-disk diffusion (penicillin, ampicillin, erythromycin and tetracycline), broth microdilution method (determination of MIC for vancomycin) and molecular detection of *vanA* and *vanB* (involved in vancomycin resistance), and *mecA* (responsible for oxacillin resistance).

Only one isolate (1.6%) was detected positive for *mecA* and considered as MRSA, while no VRSA isolate was found. The MIC of all the tested isolates for vancomycin was ≤2 μg/ml and none of them carried *vanA* and/or *vanB* genes.

According to the definition of multidrug-resistance (MDR) in veterinary medicine"an isolate which is not susceptible to at least one agent in at least three antimicrobial classes" (Sweeney et al., 2018), 29 isolates (47.5%) showed multidrug-resistance while 18% of isolates were detected susceptible or resistant to one antibiotic agent. To sum up, the most frequent antibiotic resistance patterns are simultaneous resistance to penicillin and ampicillin (34.4%) and penicillin, ampicillin and erythromycin (27.8%). Antibiotic resistance patterns for *S. aureus* isolates are described in details in figure 1.

E. faecalis

Most of the *E. faecalis* isolates (6/8 isolates) were resistant to all the tested antibiotics (penicillin, ampicillin, erythromycin, tetracycline and vancomycin). From the rest, one isolate showed complete susceptibility and the other one identified as resistant to penicillin and ampicillin. The MIC of vancomycin for multidrug-resistant isolates (6/8) was high and in the range of 128- 1024 μg/ml. The other two isolates were susceptible to vancomycin according to the MIC. The *vanA* gene was only detected in one isolate (MIC: 1024 μg/ml) and no isolate was identified positive for the presence of *vanB*. The results of the study for *E. faecalis* are presented in details in figure 2.

DISCUSSION

In the current study, 61 isolates of *S. aureus* were investigated for antibiotic resistance against different classes of antibiotics such as β-lactam (penicillin, ampicillin, oxacillin), macrolides (erythromycin), tetracyclines (tetracycline) and polypeptide antibiotics (vancomycin). Phenotypic and genotypic resistance to vancomycin was studied and all the *S. aureus* isolates were found to be susceptible to vancomycin. The transferable genes *vanA* and *vanB* are responsible for inducible resistance to vancomycin and*S. aureus* acquisition of the genes from enterococci has been proved (Courvalin, 2006). To date no report of simultaneous genotypic and phenotypic resistance to vancomycin among bovine mastitis causing strains of *S. aureus* has been recorded. A probable explanation could be that the some of the reports from the presence of VRSA were based on the results of the application of agar-disk diffusion which is not acceptable today (Sharma et al., 2015). Furthermore, the majority of those studies which were applied broth microdilution test or E-test to investigate phenotypic resistance to vancomycin, targeted only *vanA* for molecular investigation which is responsible for high level of vancomycin resistance and ignore *vanB*, while the gene *vanB* involves in variable levels of vancomycin resistance (Courvalin, 2006). Only Bhattacharyya et al. reported VRSA based on application of broth microdilution test and investigating for genes *vanA* and *vanB*, although they did not detect any genotypic positive strain (Bhattacharyya et al., 2016).

Low prevalence of MRSA (1.6%) was observedin the study and it agrees with researches done by Gentilini et al. (2000) and Erskine et al. (2002), while high prevalence of MRSA and outbreaks of subclinical mastitis due to oxacillin resistant strains have been reported (23.3- 83%) (Hata, 2016; Guimarães et al., 2017). MRSA is a healthcare-acquired pathogen and its transmission between human and cows has been shown (Sato et al., 2017), thus monitoring the state of MRSA in both human and dairy cattle is necessary.

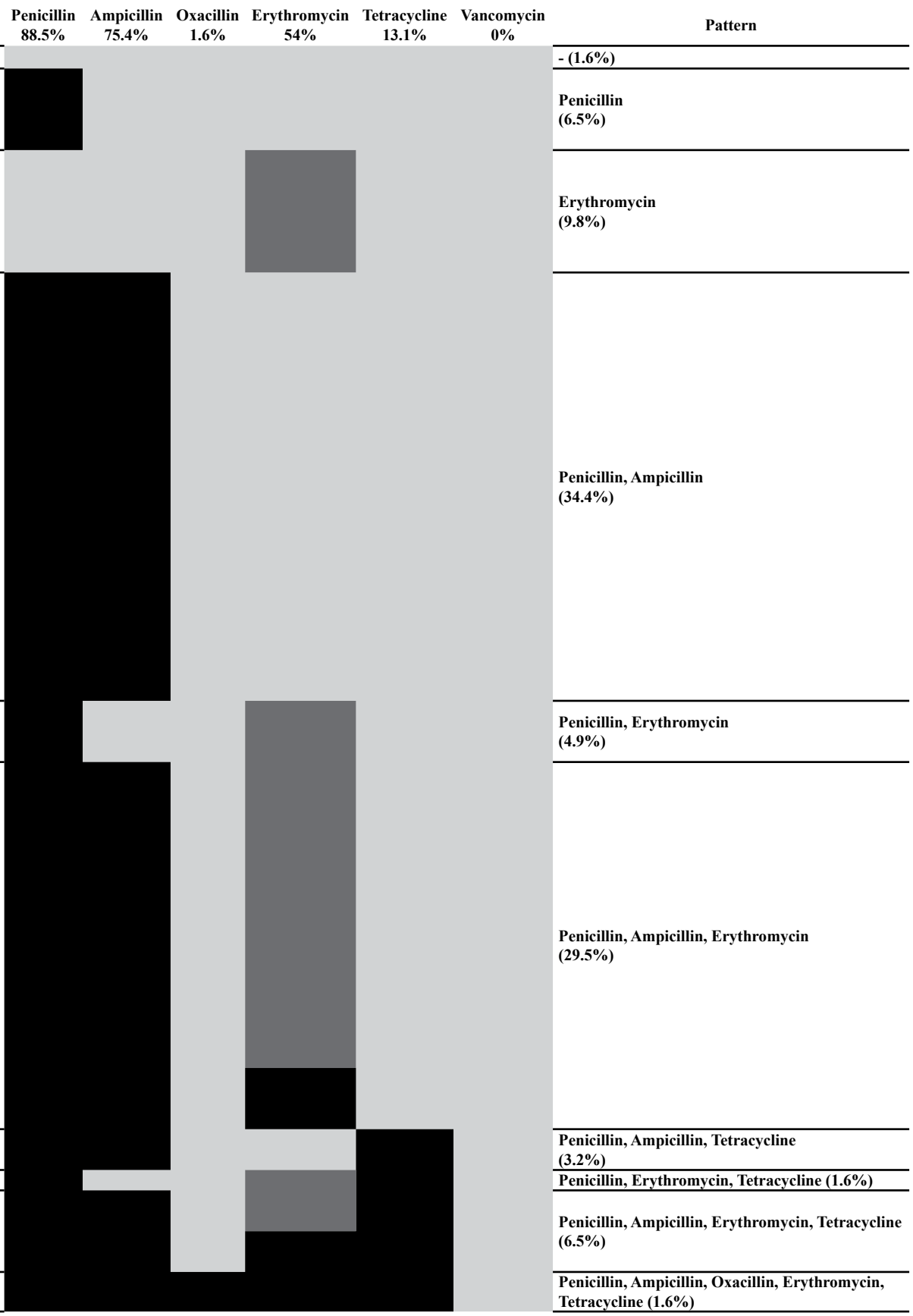

Figure 1: Antibiotic resistance patterns for *S. aureus* isolates. Black indicates resistance, dark gray indicates intermediate resistance, and light gray indicates sensitive.

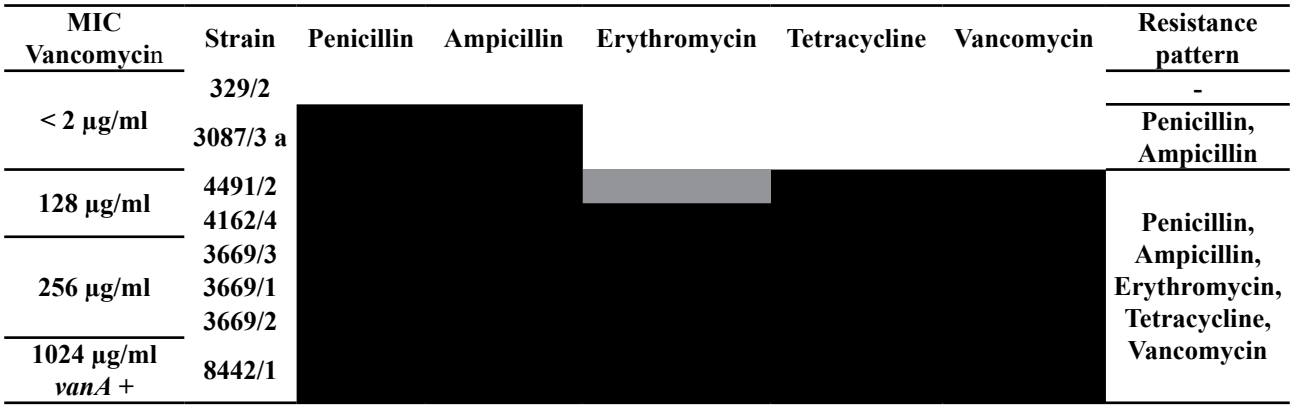

Figure 2: Antibiotic resistance patterns for *E. faecalis* isolates. Black indicates resistance, dark gray indicates intermediate resistance, and light gray indicates sensitive.

High incidence of resistance to penicillin (88.5%) and ampicillin (75.4%) were identified among *S. aureus* isolates which is as high as records (84- 94%) reported by other researchers(Wang et al., 2016; Yang et al., 2016). The high reported incidence can be the result of routine application of antibiotics for dry cow therapy and lactation therapy which cause pressure for selection of resistant strains. Prevalence of resistant isolates of *S. aureus* against erythromycin and tetracycline was determined 54% and 13.1%, respectively. Different studies reported various ranges for prevalence of resistant isolates to erythromycin. While Wang et al. (2016) reported 68.6% of erythromycin resistance, Ruegg et al. (2015) recorded 8.6%. On the contrary, there is a narrow spectrum of tetracycline resistance prevalence from 3- 17% based on different reports (Oliver and Murinda, 2012; Ruegg et al., 2015).

Monitoring the antimicrobial resistance should not be limited to contagious or prevalent causes of mastitis and pathogens which are the origin of antimicrobial resistance should also be included. The main goal of studying antimicrobial resistance of *E. faecalis* isolates in the current study was to find phenotypic and genotypic vancomycin-resistant strain as a probable and possible source of transferring of vancomycin resistance to *S. aureus*. Noticeably, most of the tested isolates (6/8) were resistant to vancomycin and one isolate carried resistance gene *vanA*. The presence of *vanA* type- *E. faecalis* is a caution that there is a potential of emerging VRSA in dairy cattle which is a risk to dairy cattle and human health. Furthermore, this type of *E. faecalis* pose a threat to human health by freely spreading of resistance gene to human enterococci that should not be ignored (Angulo et al., 2006).

High incidence of resistance against penicillin, ampicillin, erythromycin, tetracycline and vancomycin in *E. faecalis* isolates was observed during the present study. High-level resistance to erythromycin and tetracycline in enterococci acquired from bovine mastitis has been recorded from China, Korea and Poland (Nam et al., 2009; Różańska et al., 2019; Yang et al., 2019). Although the number of investigated *E. faecalis* isolatesin the current study was not high, the presence of high proportion of MDR (6/8) isolates warns us about using antimicrobial agents cautiously in dairy cattle.

CONCLUSIONS

The current study showed the existence of the source of vancomycin resistance in dairy cattle. Although no VRSA was isolated, the risk of emerging and spreading of VRSA in dairy cattle should not be underestimated. Moreover, bovine vancomycin resistant enterococci (VRE) isolates can be the source of vancomycin resistance for human enterococci and staphylococci and act as a human health hazard.

CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Diversity and prevalence of *Eimeria* species in goats of Nepal

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ABSTRACT: This study aimed to determine the prevalence of *Eimeria* and identify its different species in adult goats brought to the Kathmandu valley, Nepal, for commercial meat purposes. *Eimeria* spp. were present in 916 samples out of 991 examined samples (92.4%). A total of 15 different morphologic forms of *Eimeria* were detected, with the prevalence rates as follows: *E. ninakohlyakimovae* (83.0%), *E. alijevi* (75.2%), *E. capralis* (75.2%), *E. masseyensis* (67.2%), *E. hirci* larger form (63.2%), *E. tunisiensis* (47.3%), *E. charlestoni* (33.0%), *E. jolchejevi* larger form (32.4%), *E. arloingi* (32.4%), *E. caprina* (19.3%), *E. aspheronica* (16.5%), *E. jolchejevi* smaller form (13.5%), *E. christenseni* (9.4%), *E. hirci* smaller form (9.4%), and *E. caprovina* (8.1%). The current data support the fact that *Eimeria* species are predominant in adult populations. Therefore, coprologic diagnosis and anticoccidial treatments should be implemented in these populations before their transport to new sites to avoid the transmission.

Keywords: *Eimeria ninakohlyakimovae*; Coccidiosis; Goats; Morphometry

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INTRODUCTION

The genus *Eimeria* Schneider, 1875 (Apicomplexa: Eimeriidae) is a diverse and widespread coccidian parasite of the invertebrate and vertebrate hosts. As they are present in chicken, calves, sheep, goats, and rabbits, they are prioritized by the veterinary professionals and livestock scientists. This unicellular protozoan parasite is monoxenous because it completes its lifecycle in a single host. The host becomes infected with the consumption of food and water contaminated with sporulated *Eimeria* oocysts. Once oocysts enter the host, asexual and sexual reproductive cycles occur inside the host's intestinal epithelium. As a result, unsporulated oocysts are released in the feces. In the external environment, the oocysts undergo sporogony and become infectious. This coccidian parasite causes a disease called coccidiosis that is characterized by decreased growth, weight loss, decrease in the production of milk, wool, and hair, reduced fertility, enhanced mortality of goats, and severe economic loss (Khodakaram-Tafti and Hashemnia 2017). Therefore, the study of the role of *Eimeria* is important in livestock.

Livestock production, especially goat farming, is becoming increasingly popular in Nepal. It plays an essential role in achieving the sustainable development goals (SDG) of the United Nations, mainly Goal 1: no poverty, Goal 2: zero hunger, and Goal 3: decent work and economic growth. Although the disease caused by *Eimeria* species has been recognized as a critical problem in the goat industry in this country for many years (Khakural 2003; Ghimire 2018; Ghimire and Bhattarai 2019). However, the study of occurrences of various *Eimeria* species in goats has not been investigated here yet. The understanding of various *Eimeria* species in goats is crucial because pathogenicity caused by different species is different, and even some *Eimeria* species lead to asymptomatic infection (Sayin et al. 1980; Soe 1989; Dai et al. 1991; Dai et al. 2006). Thus, species identification of *Eimeria* should be perfectly carried out to subsequently evaluate the parasite's lethality and follow the therapeutic options. This study aims to determine the prevalence of different *Eimeria* species present in the goats in Kathmandu valley, where thousands of the goats are imported from all over the country for commercial meat.

MATERIALS AND METHODS

Sample collection

The study was conducted on naturally-infected goats brought to the goat market and butcher shops from different parts of Nepal. No experimental infection was established during this research work. Goats were not directly involved in the study. From August 2019 to February 2020, 991 fresh fecal samples of male goats were collected from the local goat markets and butcher shops in Kathmandu, Nepal. The samples were immediately placed in sterile vials containing 2.5% potassium dichromate solution and stored in a refrigerator (4 Degree Celsius, ^{o}C) until further examination.

Laboratory processing, examination, and culture

The presence of *Eimeria* oocysts in the fecal samples was examined by the flotation method using 45% NaCl (1200 revolutions per minuteX5minutes) as standardized by laboratory and described in an earlier publication (Ghimire and Bhattarai 2019). Then, sporulation assays were performed in the *Eimeria*-positive samples. The fecal samples were placed in Petri dishes and incubated at 2.5% potassium dichromate (28 ^{o}C) up to 10 days (Adhikari et al. 2020). Sporulation time was calculated as the average time required for complete sporulation of 90% of the oocysts.

Sample identification

All oocysts were observed under a light microscope (Optika Microscopes Italy, B-383PLi) at a different magnification of X100, and X400. The images were taken using SXView 2.2.0.172 Beta (Nov 6, 2014) Copyright © 2013-2014. The identification of the oocysts was carried out using the parameters which include the oocyst characteristics (size, shape, shape index, color, the thickness of the wall, and presence of polar and refractile granules), sporocyst characteristics (size, shape, shape index, sporocyst residuum), presence or absence of micropyle and its cap, size, shape, and shape index (Soe 1989; Duszynski and Wilber 1997; Koudela and Bokova 1998; Wang et al. 2010). Different shapes of the oocysts were considered while identifying the species; for example, ellipsoid/ellipsoidal meant symmetrical elliptical in shape, ovoid meant egg-shaped with the broader end remote from the micropyle/polar cap, and urn-shaped meant egg-shaped with the broader end towards the micropyle/polar cap (Soe 1989). Species description was solely based on the literature (Soe 1989; Soe and Pomroy 1992).

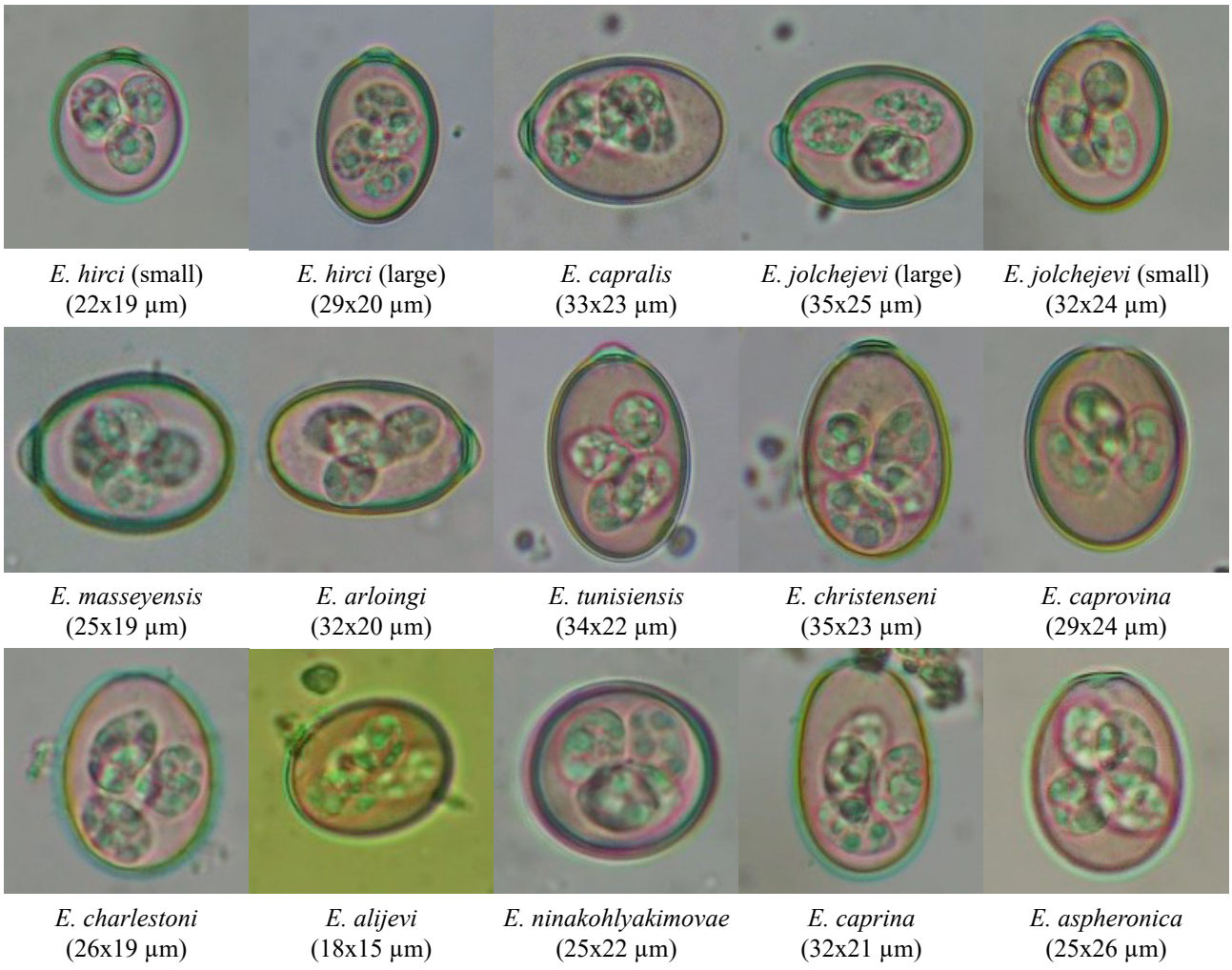

Fig 1. Different *Eimeria* species (X400) detected in goats in Kathmandu, Nepal

Data analysis

At least 100 sporulated oocysts from each species of *Eimeria* were used in the investigation of the morphometric identification of various species. The GraphPad Software, Inc (Prism 5.00, 2007) was used to calculate mean, range (minimum to maximum), and standard deviations of length and width of oocysts, sporocysts, micropylar cap and thickness of oocyst wall. Prevalence of the *Eimeria* was calculated by dividing the total numbers of *Eimeria* positive samples by whole numbers of collected samples.

RESULTS

The current study revealed a total of 916 *Eimeria* positive fecal samples (92.4%) out of 991 examined samples (Supplementary 1). We detected a total of 13 species of *Eimeria* with a total of 15 different morphometric forms (Fig 1) (Supplementary 2). The prevalence of all of these species was as follows: *E. arloingi* (32.4%), *E. christenseni* (9.4%), *E. hirci l.f.* (63.2%), *E. hirci s.f.* (9.4%), *E. jolchejevi l.f.* (32.4%), *E. jolchejevi s.s.* (13.5%), *E. masseyensis* (67.2%), *E. capralis* (75.2%), *E. tunisiensis* (47.3%), *E. alijevi* (75.2%), *E. aspheronica* (16.5%), *E. caprina* (19.3%), *E. caprovina* (8.1%), *E. charlestoni* (33.0%), and *E. ninakohlyakimovae* (83.0%) (Supplementary 1).

Interestingly, *E. ninakohlyakimovae* was the common parasite in different fecal samples; for example, it was found to be along with 11 different species in different fecal samples. There was no sample with a single infection. All positive fecal samples were concomitantly positive for different numbers, for example, with three (1.3%), four (27.7%), five (8.1%), seven (18.9%), eight (30.9%), nine (4.8%), and ten (0.7%) species of *Eimeria* (Supplementary 1).

Supplementary 1: Prevalence of each *Eimeria* sp. in the fecal samples of goats. N represents total numbers of fecal samples examined (N=991) and n represents total numbers of particular *Eimeria* sp. positive fecal samples. Prevalence rate was calculated by using 100n/N formula for particular species or concomitant infection. l.f.: large form and s.f.: small form.

Species of *Eimeria*	n	Prevalence (%)
E. ninakohlyakimovae	823	83.0
E. alijevi	745	75.2
E. capralis	745	75.2
E. masseyensis	666	67.2
E. hirci l.f.	626	63.2
E. tunisiensis	469	47.3
E. charlestoni	327	33.0
E. arloingi	321	32.4
E. jolchejevi l.f.	321	32.4
E. caprina	191	19.3
E. aspheronica	164	16.5
E. jolchejevi s.f.	134	13.5
E. christenseni	93	9.4
E. hirci s.f.	93	9.4
E. caprovina	80	8.1
Concomitant infections		
Triplet	13	1.3
Quadruplet	275	27.7
Pentuplet	80	8.1
Heptuplet	187	18.9
Octuplet	306	30.9
Nonaplet	48	4.8
Decaplet	7	0.7
Total infected	**916**	**92.4**

DISCUSSION

As per our knowledge, this is the first published report on the diversity and prevalence of *Eimeria* species in the goats of Nepal. The high prevalence rate accompanied by mixed infections up to ten species and the presence of 13 different species indicates that *Eimeria* is a dominant coccidian parasite in Nepal's goats. Although there are 17 accepted species of *Eimeria* from goats (Levine 1988; Soe and Pomroy 1992), we have detected 15 different morphologic forms. Among the 15 morphologic forms identified in this study, only four are significant for coccidiosis. These species include *E. ninakohlyakimovae, E. arloingi, E. christenseni,* and *E. caprina* that have been proved as etiologic agents of hemorrhagic diarrhea and lesions in the GI tracts in goats (Sayin et al. 1980; Aumont et al. 1984; Norton 1986; Koudela and Bokova 1998; Dai et al. 2006; Hashemnia et al. 2012). However, the former is regarded as the most pathogenic species resulting in extra-intestinal complications (Dai et al. 1991). Although the latter three species are considered to be pathogenic, *in vivo* experiments with *E. ninakohlyakimovae*, *E. christenseni,* and *E. arloingi* have resulted in sub-clinical coccidiosis which is characterized by a decrease in the intake of dry matter, momentary diarrhea or slight constipation, and a very large oocyst excretion (Aumont et al. 1984; Aumont et al. 1986). The various results indicate that these *Eimeria* species lead to both clinical and subclinical manifestations, and the effect depends on the different species involved.

Our results were obtained from the fecal samples of male goats transported from different parts of the country for commercial meat purposes. These goats appeared clinically healthy and robust, although the detection of various *Eimeria* species indicated that they might have a mild to moderate or asymptomatic infection. In this context, the goats can act as asymptomatic carriers for different *Eimeria* species. It is believed that healthy goats can resist coccidiosis without developing clinical signs; however, stress situations during transportation or underfeeding and the associated factors may disrupt the immune system, which leads to a huge effect of *Eimeria* (Chartier and Paraud 2012; Mohamaden et al. 2018). These explanations and our exploration indicate that constant monitoring of *Eimeria* should be carried out routinely in the future, especially for the adult goats that might act as transmitting means of these coccidia.

CONCLUSIONS

In conclusion, based on our laboratory findings, the current study revealed that *Eimeria* species are dominant in Nepal's goats. It has already been suggested that goat kids are severely affected by *Eimeria* with high morbidity and mortality. However, the current study detected *Eimeria* in more than 90% of the goat adult populations. Therefore, to avoid transmission, coprologic diagnosis and anticoccidial treatments should be carried out in the goat populations before transporting them to other sites.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest. The work is a part of the research proposal of the Red Book (075/076, 076/077, Planning Division, Nepal Academy of Science and Technology, NAST).

Supplementary 2: Morphologic characters of oocysts of different *Eimeria* species detected in the goats in Nepal. Length and Width are expressed as minimum to maximum (mean±standard deviation). The length of micropyle has been measured in the species without micropylar cap. l.f.: large form and s.f.: small form.

Eimeria species	Oocyst characteristics			Sporocyst			Micropyle cap			Oocyst wall		Polar granules / Refractile globules	Sporocyst Residuum
	Length (µm) x Width (µm)	*Shape index (l/b)*	*Shape of oocyst*	*Length* (µm) x Width (µm)	*Shape index (l/b)*	*Shape*	*Length* (µm) x Width (µm)	*Shape index (l/b)*	*Shape*	*Thickness*	*Color*		
With micropylar cap													
E. arloingi	26-32 (30±1.6) x 17-23 (20±1.2)	1.4-1.5	Ellipsoidal/ Ovoid	11-15 (13±1.1) x5-8 (7±0.6)	1.9-2.2	Elongated and Ovoid	5-7 (6±0.6) x 1-4 (2±0.6)	1.8-5	Mound-shaped	1-2 (1)	Greenish Brown	Present/ Present	Present
E. christenseni	34-38 (36±1.1) x 21-27 (24±1.8)	1.4-1.6	Ovoid	14-16 (15±0.7) x 7-9 (8±0.8)	1.8-2	Ovoid	6-8 (7±0.7) x 3-4 (3±0.3)	2	Mound-shaped	1-2 (2±0.1)	Green	Present/ Present	Present
E. hirci l.f.	25-31 (28±1.6) x 17-23 (20±1.3)	1.3-1.5	Ellipsoidal/ Ovoid	10-13 (12±0.9) x5-8 (7±0.7)	1.6-2	Ellipsoidal	4-7 (6±0.8) x 2-4 (3±0.5)	1.8-2	Dome-shaped	1-2 (1±0.4)	Yellow, Green, and Red	Present/ Present (Indistinct)	Present
E. hirci s.f.	18-22 (21±0.9) x 17-20 (18±1.1)	1.1	Ellipsoidal	8-11 (9±0.9) x 5-8 (6±0.9)	1.4-1.6	Ellipsoidal	3-4 (4±0.2) x 1-2 (2±0.1)	2-3	Nipple-like	1 (1±0.0)	Green and Purple	Present/ Present (Indistinct)	Present

E. jolchejevi l.f.	32-41 (35±1.8) x 23-28 (25±1.7)	1.4-1.5	Urn-shaped/ Ellipsoidal	10-17 (14±1.8) x 6-10 (7±0.9)	1.7	Elongated and Ovoid	5-8 (7±0.8) x 2-4 (3±0.5)	2-2.5	Truncated-cone shaped	1-2 (2±0.4)	Yellowish Green	Present/ Present (Prominent)	Present (Mostly aggregated)
E. jolchejevi s.f.	28-33 (31±1.3) x 19-25 (22±1.4)	1.3-1.5	Ellipsoidal / Urn-shaped	10-16 (13±1.3) x 6-10 (7±0.9)	1.6-1.7	Elongated and Ovoid	5-8 (6±0.7) x 2-4 (3±0.5)	2-2.5	Half-moon -shaped	1-2 (2±0.4)	Blue, Yellow, and Green	Present/ Present	Present (Mostly aggregated)
E. masseyensis	20-27 (25±1.3) x 16-21 (19±1.2)	1.3	Ellipsoidal or Broadly ovoid	4-13 (10±1.4) x 5-9 (6±0.7)	0.8-1.4	Elongated and Ovoid	4-6 (5±0.7) x 1-3 (2±0.2)	2-4	Dome-shaped	1 (1±0)	Greenish Yellow	Present (Fragmented)/ Present	Present
E. capralis	26-33 (29±1.6) x 18-23 (20±1.3)	1.4	Ellipsoidal	10-14 (12±0.9) x 6-9 (7±1.0)	1.6-1.7	Ovoid	4-7 (6±0.7) x 1-5 (3±0.6)	1.4-4	Dome-shaped	1-2 (1±0.2)	Dark Green	Present/ Present	Present
E. tunisiensis	30-38 (34±1.8) x 20-26 (22±1.3)	1.5	Ellipsoidal (Large)	12-16 (14±0.9) x 6-10 (8±1.2)	1.6-2	Elongated and Ovoid	5-8 (6±0.6) x 2-4 (3±0.2)	2-2.5	Dome-shaped	1-2 (2±0.3)	Greenish Yellow	Present (Fragmented)/ Present	Present (Large)
Without micropylar cap													
E. alijevi	15-22 (17±1.5) x 13-17 (15±1.0)	1.1-1.3	Ovoid/ Sub-spherical	7-11 (9±0.7) x 4-6 (5±0.7)	1.8	Elongated and Ovoid	-	-	-	1 (1±0.0)	Greenish Blue	Present/ Present	Absent

E. aspheronica	26-33 (29±2.1) x 18-25 (21±1.7)	1.3-1.4	Ovoid and flat at micropylar end	11-14 (12±1.3) x 5-8 (7±0.9)	1.8-2.2	Elongated and Ellipsoidal	4-7 (6,0.8)	-	-	1-2 (1±0.1)	Pinkish Green	Present/ Unknown	Present
E. caprina	29-37 (32±2.6) x 21-25 (23±1.1)	1.4-1.5	Ellipsoidal/ Ovoid, and flat at micropylar end	12-14 (13±0.8) x 6-8 (7±0.6)	1.8-2	Elongated and Ovoid	4-7 (5,0.6)	-	-	1-2 (1±0.4)	Green	Present (Fragmented)/ Unknown	Present
E. caprovina	23-31 (28±2.5) x 19-24 (22±1.3)	1.2-1.3	Ovoid/ Sub-spherical and flat at micropylar end	11-14 (13±1.0) x 6-8 (7±0.7)	1.8	Elongated and Ovoid	4-6 (6,0.5)	-	-	1-2 (1±0.3)	Green, Yellow & Purple	Present/ Unknown	Present
E. charlestoni	20-26 (23±1.7) x 15-19 (17±1.0)	1.3-1.4	Ellipsoidal and flat at micropylar end	10-14 (11±0.9) x 5-7 (6±0.7)	2	Ellipsoidal/ Ovoid	3-5 (4,0.5)	-	-	1 (1±0)	Light to Dark Green	Present/ Present (Prominent)	Absent/ Invisible
E. ninakohlyakimovae	20-29 (23±1.6) x 16-22 (19±1.4)	1.3	Ellipsoidal/ Ovoid	10-14 (11±0.8) x 4-9 (6±0.7)	1.6-2.5	Elongated and Ovoid	-	-	-	1-2 (1±0.4)	Purple and Green	Present (Fragmented)/ Present (Non prominent)	Present (Aggregated)

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Research article
Ερευνητικό άρθρο

In silico design of short hairpin RNA (shRNA) Molecules for DNA pol gene of Contagious Ecthyma virus (ORFV)

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ABSTRACT: Contagious ecthyma is an infectious skin disease of ruminants caused by the ORF virus (ORFV) that is a member of genus Parapoxvirus of the Poxviridae family. In addition to the significant effects on lambs and human, ORFVs have been recently shown to infect other hosts. The disease causes significant economic damages to the sheep industry, so attempts to eliminate it must be taken into.RNA interference (RNAi) is an evolutionarily conserved mechanism in which the expression of homologous target genesis is suppressed by means of double-stranded RNA molecules. Since RNAi can be considered as a therapeutic method for viral gene silencing, we tend to make the most of this capability. The present study aims to design potential shRNAs to knockdown the DNA-polymerase gene coded by ORF025.A significant number of computational methods such as clustal omega website to target alignment, BLAST-NCBI to similarity search, CLC software to secondary structure prediction, BLOCK-iTRNAi Designer and WI siRNA Selection Program and Software to design of shRNA molecules and scoring have been applied for shRNA molecules designing against the ORF025-DNA pol gene of ORFV. Then three shRNA molecules were logically designed against the ORF025-DNA pol gene. In conclusion the present study provides a strong and superior approach for achieving a validated strategy to design an antiviral shRNA molecule that meets many sequence features for efficient ORFV knockdown and treatment at the mRNA level. The efficiency of these anti ORFV shRNAs need to be tested

Keywords: In silico, knockdown, ORFV, RNAi, shRNA

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INTRODUCTION

Contagious ecthyma or orf is a life-threatening skin disease affecting ruminants worldwide (Nandi, De, & Chowdhury, 2011; Zeedan, Abdalhamed, Ghoneim, & Ghazy, 2015). The causative agent of the disease is the *ORF virus (ORFV)* which is a member of the genus *Parapoxvirus* of the family *Poxviridae*. The disease is significant, especially in young lambs. The most common sites for those orf signs occurring are around the mouth, teats and, skin surface. The lesions are proliferative and self-limiting. Notably, that contagious ecthyma is a risk to the sheep industry and threatens human health (Wang et al., 2014)

The genome of ORFV is a linear double-stranded DNA, 134-139-kb (Zeedan et al., 2015). Since ORFV-can be replicated within the host cell cytoplasm, it needs to encode its DNA transcription and replication(Fleming, Wise, & Mercer, 2015). One of the most essential proteins in the replication of ORFV is the DNA polymerase (DNA Pol) encoded by the virus and plays a key role during ORFV replication (Wang et al., 2014).In contrast, vastly efforts to discover the ORFV pathogenesis, none of the available vaccines induces complete and long-term immunity and vaccinated animals may be re-infected (Bergqvist, Kurban, & Abbas, 2017).

The potential of RNAi for efficient and specific target gene silencing has increased efforts to develop several therapeutic agents based on RNAi (Barata, Sood, & Hong, 2016). RNAi can be induced by the introduction of synthetic small interfering RNAs (siRNA) or by the intracellular generation of siRNA through vector-driven expression of the small precursor hairpin (sh)RNAs (Manjunath, Wu, Subramanya, & Shankar, 2009). There are also evidence that shRNAs may be more effective in gene silencing than siRNAs and induce lower non-specific gene expression changes (Lambeth & Smith, 2013). It noteworthy that, the most benefit shRNA than siRNA is that it is capable of cloning in to a viral vector and enter into numerous cell lines (Haussecker, 2014).

Given the high efficiency of RNAi to silence target genes and the crucial role of DNA-pol to replicate ORFV genome, the present study aimed to design potential shRNA molecules against the ORF025-DNA-pol gene of ORFV.

MATERIALS AND METHODS

Selection of ORF025-DNA pol conserved regions

The complete sequences of ORF025 (ORFV-OV-SA00 strain) were obtained using the NCBI GenBank database (accession number AY386264.1). In order to identify the conserved regions among ORF025- DNA pol gene, the complete CDS of ORF025-DNA pol was aligned with other similar strains using clustal omega website, The ORFV-OV-SA00 strain was used for shRNA designing.

The noticeable thing is that one mismatch between the target mRNA and shRNA has a profound effect on shRNA efficiency. Using this rule ensures that all the designed shRNA molecules belong to the consensus regions of transcripts. Therefore, the regions of transcripts with maximum homology are selected for the following analysis.

Design of shRNA molecules against ORF025-DNA pol gene

The design of shRNA molecules was performed by three online websitesas performed by three online websites: BLOCK-iTRNAi Designer, WI siRNA Selection Program, and Software. These websites have their advantages. All shRNAs offered by these websites were aligned with the completed CDS of ORF025-DNA pol to detect more appropriate shRNAs. After designing shRNA, both sequence and structural rules must be taken into account; however, according to the evidence, the most significant determinant of the potency of the silencing trigger is the sequences of shRNAs (Paddison, Caudy, Bernstein, Hannon, & Conklin, 2002). Briefly, several sequence rules are as follows: The target region should not be placed in 50-100 nucleotides of the start codon and the termination codon as well as intron regions or single nucleotide polymorphism (SNP) sites; The target sequence needs to have a GC content <30% or > 60%providing the vital stability for the shRNA (Protocol-online, 2009). Mcintyreet al. found it difficult to find a fixed correlation between stem length and suppressive activity, and described that core placement at the base terminus is essential for appropriate activity (Mcintyre et al., 2011). A significant number of sequence rules denoting the preference or avoidance of the specific positions belonging to both the sense or antisense strand of the duplex have been reported (Protocol-online, 2009). Structural rules characterize thermodynamic properties in terms of the secondary structures and accessibilities of shRNA/target site (Baghban-Kohnehrouz & Nayeri, 2016).

Table 1: Characteristics of Effective shRNA Molecules Targeting ORF025-DNA pol gene

Target No.	Location of Target Within mRNA	Sense strand sequence	Length	GC, %	1*conserved DNA sequence	2*unconserved DNA sequence	U at position 10 (sense)	proper regions of the RNA secondary structure	crowded regions of the RNA secondary structure
1(OrfshRNA-1)	959-980	GCACCACCTTCCACATCAACA	21	52.39	+		+	+	
2	963-985	CACCTTCCACATCAACAACAACA	23	42	+		+	+	
3	959-978	GCACCACCTTCCACATCAA	19	52.63	+		+	+	
4	960-982	CACCACCTTCCACATCAACAACA	23	47	+			+	
5(OrfshRNA-2)	894-916	GACAGTCAACTTCTGCGTGTACG	23	47	+		+	+	
6	14-36	TGGAGCTGAAATGTTTGAACTGG	23	37	+		+	+	
7	17-39	AGCTGAAATGTTTGAACTGGTTC	23	37		_	+	+	
8	2706-2728	AACGCACCACAAGAACTTCAAGT	23	47	+			+	
9	2708-2730	CGCACCACAAGAACTTCAAGTCC	23	42	+			+	
10	2705-2723	GAACGCACCACAAGAACT	18	47.37		_		+	
11(OrfshRNA-3)	1649-1671	ACGTGCTCATCTTCGACTACAAC	23	47		_	+	+	
12	1651-1670	GTGCTCATCTTCGACTACA	19	47.37		_	+	+	

*1: Linked to the entirely conserved DNA sequence
*2: Linked to unconcerved DNA sequence

On top of that using the shRNA design tool, the shRNAs-suggested were manually investigated according to the parameters proposed by Tom Tuschl'srules (Protocol-online, 2009), Mcintyre et al. (Mcintyre et al., 2011), Bofill-De Ros et al. (Bofill-De Ros & Gu, 2016)and Fakhr et al. (Fakhr, Zare, & Teimoori-Toolabi, 2016)for optimal designing.

Alignment of shRNA with sheep genomic

In order to eliminate shRNAs having off-target effects on non-targeted genomes, a BLAST homology search with sheep genomic and transcripts database (http://blast.ncbi.nlm.nih.gov/) was performed. shRNAs having a perfect match of 16 nucleotides or more with any other mRNAs of the same species were excluded from the suggested list of shRNAs(Taxman, Moore, Guthrie, & Huang, 2010).

Secondary structure prediction of ORF025-DNA pol

The ORF025- DNA pol secondary structures were predicted using CLC software (CLC Genomics Workbench). In order to detect that the shRNAs are matched with the appropriate regions of the structure, they were blasted with this predictable secondary structure. The shRNAs that were placed in stem and crowded regions were excluded from consideration.

Choosing shRNAs with the best score

Afterwards, the shRNAswhichremained characteristics such as sequence specificity, off-target effects, and G/C content, were investigated once again. Finally, three shRNAs with the highest scores were selected. Nucleotide sequences TCAAGAG, as a loop sequence, were added between the sense and antisense strands.

RESULTS

Around 11 DNA pol gene sequences from various strains of ORFV were obtained from the data base nucleotide sequence, NCBI.The clustal omega was used for detecting the consensus region by multiple sequence alignment.The shRNAs were designed based on the conserved regions of ORF025. BLOCK-iTRNAi Designer, WI siRNA Selection Program and wizard websites were used for shRNA designing. WI siRNA Selection Program was also able to show off-target effects on the seed complementarity of the target site among the related species.

Some shRNAs were obtained by BLOCK-iTRNAi Designer, the WI siRNA Selection Program and wizard server, respectively. Then they were manually and accurately investigated in order to design the most potent molecules. Using such scoring system, an acceptable score remarkably raises the eventuality for gene knockdown. Finally, the number of shRNAs were determined to stick to the scoring system rules (Table 1). Before the final scoring, all shRNAs were analyzedto demonstrate the specificity of shRNA by BLASTN. Since all the shRNAs were proprietary, this step has been deleted from the final scoring. All shRNAs retain GC content within 37% - 52%. Finally, three of the shRNAs were selected with the highest scores (Table 2).

The CLC software was used for predicting the

mRNA secondary structure and the interaction between shRNAs and mRNA, as shown in figure.1. Finally, shRNAs namely OrfshRNA1, 2, 3 have more accessibility for target recognition (Table 2)

Table 2: Characteristics shRNAs with the best score

Target Name	Sense Sequence loop sequence Antisense sequence
OrfshRNA-1	GCACCACCTTCCACATCAACATCAAGAGTGTTGATGTGGAAGGTGGTGC
OrfshRNA-2	GACAGTCAACTTCTGCGTGTACGTCAAGAGCGTACACGCAGAAGTTGACTGTC
OrfshRNA-3	ACGTGCTCATCTTCGACTACAACTCAAGAGGTTGTAGTCGAAGATGAGCACGT

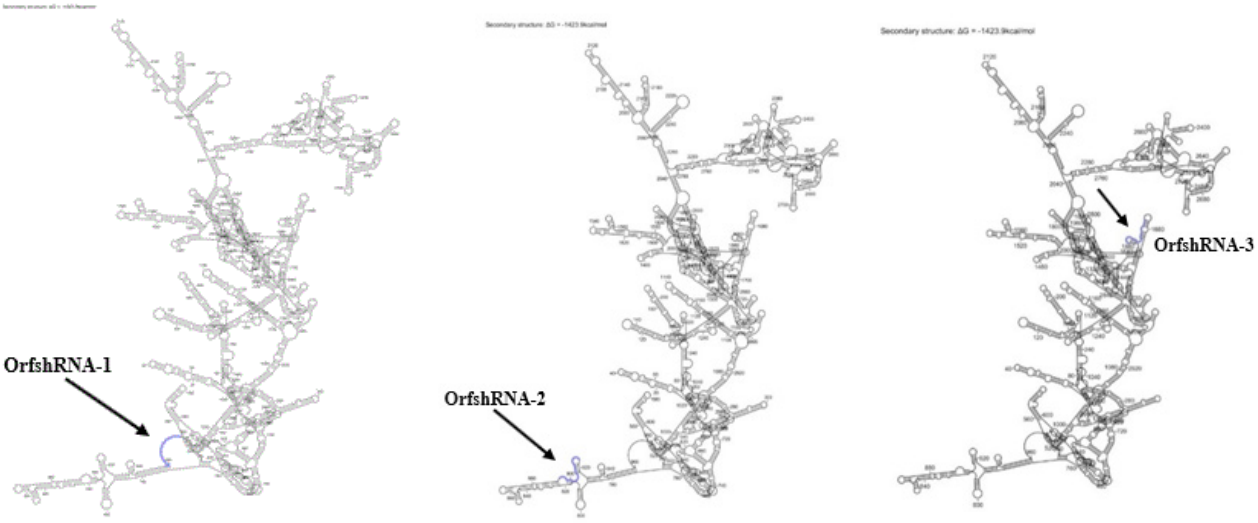

Figure 1: The arrows indicate the location of interaction shRNA with the mRNA

*shRNA Target Sites (black) on the ORF025 mRNA Secondary Structure Predicted Using CLC

DISCUSSION

Since the available vaccines are not able to provide complete protection, ORFV is capable of reinfection the same individual and that is what makes disease control very tough (Hosamani, Scagliarini, Bhanuprakash, McInnes, & Singh, 2009).

As time has passed, gene therapies through RNAi, particularly to treat viral diseases, have become more and more attractive and promising (Qureshi, Tantray, Kirmani, & Ahangar, 2018). We can benefit from shRNA technologies due to permanent integration of expression constructs for long-term expression, the use of viral vectors to infection of cell lines and tissues that is hard to target, and the temporal control of shRNA transcription by means of inducible promoters (Lambeth & Smith, 2013).

The central region of the viral genome includes the DNA pol gene that codes for a highly conserved non-structural protein playing an initial role in viral proliferation. This gene is exceptionally conserved across the species belonging to*Parapoxvirus*(Bora et al., 2011; Li et al., 2013). It can be stated that knockdown of the DNA pol gene will disturb its critical functions. The researchers have shown that the lower GC content is more effective for hybridizing the two different RNAs (Kanasty, Whitehead, Vegas, & Anderson, 2012).

The lack of effective drugs against most viruses and the definitive treatment of viral diseases are undeniable facts. The treatments are merely supportive. For this reason, the therapeutic application of RNAi is of interest to virologists since it provides exact and swift targeting of viral genes (J Blake, F Bokhari,

& AJ McMillan, 2012). Yin et al. (2010) have used shRNAs to inhibit NDV replication by targeting the viral matrix protein gene (Yin et al., 2010). Zhao et al. (2012) have carried out another study targeting the ORF095 gene of the Goatpox virus by shRNAs design (Zhao et al., 2012). Wang et al, (2014) applied siRNAs against the DNA pol gene of ORFV and displayed 73-89 % less than viral DNA in transfected cells (Wang et al., 2014). However, due to the pros of shRNA molecules over siRNA above-mentioned, in the present study, shRNA molecules were applied to prevent ORFV replication.

In this study, shRNA molecules were designed by means of BLOCK-iTRNAi Designer, WI siRNA Selection Program and wizard websites as above-mentioned. The mRNA secondary structure and the interaction between shRNAs and mRNA were predicted. Afterwards, to design the most potent molecules, we manually and accurately checked some options thanks to a proper scoring system: such as GC %, conserved DNA sequence, U at position 10 (sense), analyzing the specificity of shRNA by BLASTN , proper regions or uncrowded regions of the RNA secondary structure, etc. as above-mentioned. To sum it up, three of these molecules were elected with the highest scores. Experimental studies suggest that constructs with a pyrimidine rich loop sequence and short hairpin stems (19 nucleotides) are profoundly influential for prosperous shRNA design. The short hairpin (18-21 nucleotides); pyrimidines do not pair, and the use of uridines in the loops are the best conditions for designing effective shRNA molecules (Matveeva et al., 2010). The presence of internal repeats or palindromes in siRNA constructs may lead to internal fold-back structure formation consequently may reduce knockdown efficiency (Reynolds et al., 2004). Finally, these rules provided us with the chance to design powerful and strong shRNAs molecules as best as possible.

In the future, we tend to apply a lentiviral vector to gain a constant expression of the designed shRNAs to prevent ORFV replication for a long time.

CONCLUSION

Concerning the overall outcome of the observations, the application of new strategies for the treatment of orf disease is imperative. Due to the numerous pros of shRNAs, using these molecules provides researchers with the chance to control this contagious infection. Also, this method seems absorbing and can slow down or even stop the progression of the disease.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGMENT

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Research article
Ερευνητικό άρθρο

Study of ovine cysticercosis in two slaughterhouses in the north of Algeria

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ABSTRACT: A total of 10696 randomly selected sheep have been collected in two slaughterhouses in the north of Algeria (El Harrach and Boufarik) to determine the prevalence of ovine cysticercosis, to find out the association between prevalence and potential risk factors as well as to assess the distribution of *C. tenuicollis* and *C. ovis* in surface's muscles, viscera and cavities (abdominal, thoracic and pelvic) of slaughtered sheep. Sheep were native breedsand originated from different sub-districts within the municipality and its environs. All the slaughtered sheep carcasses were visually and carefully inspected. Ovine cysticercosis was found in 461 sheep (4.31%). There were *Cysticercus tenuicollis* and *Cysticercus ovis* with respectively, 2.25% and 2.06% prevalence. The prevalence of *C. ovis* was significantly higher ($p<0.0001$) in females than males while all positive for *C. tenuicollis*sheep were male. The prevalence of the both species increased with age, and the difference was statistically significant ($p<0.0001$). For *C. ovis*, it didn't have significant difference between season, but, the incidence of *C. tenuicollis* was significantly higher ($p<0.0001$) in autumn. All the detected cysts of *C. ovis* were non-viable, and were more frequently detected in the heart (51.82%) followed by diaphragm (30.77%) and esophagus (17.41%). The predominant localization of *C. tenuicollis* were the liver (57.71%) and the omentum (42.29%), however, no vesicle was observed in the peritoneum. This anatomical distribution of *C. ovis* and *C. tenuicollis* cysts showed a significant variation ($p<0.001$) in different predilection sites. The present study has revealed a non-negligible prevalence of ovine cysticercosis in the two slaughterhouses in the north of Algeria,suggesting that ovine cysticercosisis present in the north of Algeria.Appropriate control measures need to be introduced to reduce the prevalence of these parasites in sheep.

Keywords : ovine carcasses, slaughterhouses, *Cysticercus ovis*, *C. tenuicollis*.

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INTRODUCTION

Cysticercosis in livestock and wild animals is caused by the larval stages (metacestodes) of cestodes (taeniosis), the tape worm of humans, dogs and wild canids. Cysticercoses in sheep (in muscle, liver and peritoneal cavity) are caused by *Tænia ovis (*the adult stage of *Cysticercus ovis)*, and *Tænia hydatigena* (the adult stage of *Cysticercus tenuicollis)* of which adults develop in the intestines of dogs and wild canids (OIE, 2005).Infections with the larval stage of some species of *Taenia* are of veterinary importance because they cause economic losses. These losses are associated with total condemnation of infected offal or carcasses with generalized infestation and downgrading of carcasses which are subjected to refrigeration, in addition to the cost of refrigeration and transport (Kebede et al., 2009).Like beef and pork cysticercosis, ovine cysticercosis due to *Cysticercus ovis* is of special interest in meat inspection because it affects the musculature, that part of the animal which is at once the most valuable for food purposes and the most difficult to inspect thoroughly (Ransom, 1913). The cysticerci of *Taenia hydatigena* are responsible for a high degree of morbidity and mortality in livestock (Abidi et al., 1989). During the life cycle of the parasite, the oncosphere penetrates the small intestine and migrates to soft tissues such as the omentum and lungs. During its migration, the cyst damages the visceral organs, especially the liver (Kara, 2005),resulting in condemnation during meat inspection. The liver is very nutritious as it contains abundant glycogen and vitamin A, so condemnation deprives humans of these important health requirements (Samuel and Zewde, 2010). Also, heavy infections and traumatic hepatitis in young lambs are leading to death (Radfar et al., 2005).Different investigators around the world have reported the prevalence of cysticerci (Dada et al., 1978 ; Akinboade and Ajiboye, 1983 ; Georgieva and Yabulkarov, 1984 ; Bekele et al., 1988 ; Deka and Gaur, 1993 ;Radfar et al., 2005 ; Kanchev et al., 2008 ; Samuel and Zewde, 2010, Attindehou and Salifou, 2012 Soares et al., 2012 ; Braae et al., 2015). However, there are limited reports concerning ovine cysticercosis in slaughtered animals in Algeria. Considering the importance of these pathologies, and the lack of epidemiological information on these diseases in Algeria, the present work was conducted to determine the prevalence of cysicercosisfrom sheep in two slaughterhouses (El Harrach and Boufarik) in the north of Algeria.

MATERIALS AND METHODS

Inspection of sheep carcasses for *Cysticercus* spp. cysts

A total of 10,696 sheep carcasses slaughtered in two abattoirs in the north of Algeria (El Harrach and Boufarik) were examined during our study for *Cysticercus* spp. cysts. Sheep were native breeds and originated from different sub-districts within the municipality and its environs. The animals inspected, were of different sex and age. A few females were sampled only, because the females are kept for reproduction, the age was estimated and the animals were categorized into three age groups : ≤ 1 year, 1.5-3 years and 3 -5 years.All the slaughtered sheep carcasses were visually and carefully inspected. Visceral organs, such as liver, lung, omentum, and mesentery, as well, abdominal, thorax and pelvis cavities were investigated for the presence of *C. tenuicollis* cyst. Transparent cyst filled with clear fluid and with a long neck with white corn sized spots in the fluid were considered to be C. *tenuicollis.*For the presence of C. *ovis*, the muscles (skeletal muscle, diaphragm, esophagus, heart) were examined. Live vesicles namely translucent containing clear fluid, sometimes bloody, with a white point (protoscolex) or degenerate cyst was considered to be C. *ovis.*

Statistical analysis

For statistical analysis, we used the software program Microsoft Excel 2010. The comparison of the distribution of different populations were analysed by using Chi-Square test with the level of significance $p<0.05$.

RESULTS

Overall prevalence

Of 10 696 carcasses examined, ovine cysticercosis was found in 461 sheep (4.31%), 241 (2.25%) sheep were found to be infected with *C. tenuicollis* cysts, followed by those of *C. ovis* cysts with 220 (2.06%) carcasses. No significant difference was found between the prevalence of the two types of ovine cysticercosis (muscular and hepato-peritoneal cysts).

Prevalence of ovine cysticercosis according to the predilection sites

The rate of cysticerci in different organs is represented in Table 1.

Muscular cysticercosis (*C. ovis* cysts)

All muscular cysticercosiscysts found in sheep slaughtered were dry, in the form of hard whitish vesicles, pearlescent sometimes yellowish, about 1 cm in diameter on average, which could correspond to *C. ovis.* These cysts had a tendency to be located more in the heart (Figure 1), followed by diaphragm. However, a low percentage were found in esophagus and this difference between infections rate of heart and other organs was highly significant ($p<0.001$).

Hepato-peritoneal cysticercosis (*C. tenuicollis* cysts)

The prevalence of *C. tenuicollis* in the liver (Figure 2) was higher than in the omentum**.** However, no vesicle was observed in the peritoneum during our study. This anatomical distribution of cysticerci showed a significant variation ($p<0.001$) in different predilection sites.

Table 1: Prevalence of ovine cysticercosis according to the predilection sites

Organs	Infected organs with muscular cysticercosis of *C.ovis*		Organs	Infected organs with *C. tenuicollis*	
	N	%		N	%
Heart	128	51.82	Liver	146	57.71
Diaphragm	76	30.77	Omentum	107	42.29
Esophagus	43	17.41	Peritoneum	0	
Total	247	100	**Total**	253	100

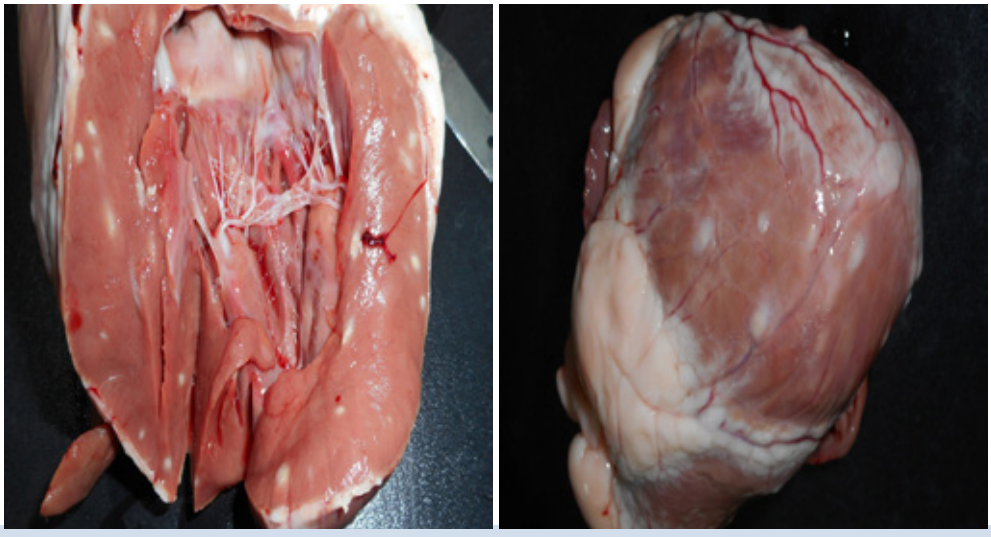

Figure 1: Degenerated *C. ovis* cysts in the heart of sheep

Figure 2: *C. tenuicollis* cyst in the liver of sheep

Intensity of parasitism by cysts of cysticercosis in the predilection sites

Muscular cysticercosis(*C. ovis* cysts)

From parasitized sheep, 493 muscular cysts were counted on inspected organs, the distribution of the cysts countedis summarized in Figure 3. The heart was the most infested organ with 354 cysts, followed by diaphragm and esophagus with 83 and 56 cysticerci respectively. The statistical test detected a highly significant difference ($p<0.0001$) between the level of parasitism by muscular cysticercosisbetween the three organs namely the heart, the diaphragm and the esophagus.

Hepato-peritoneal cysticercosis (*C. tenuicollis* cysts)

We obtained a total of 465 cysts of *C. tenuicollis* from parasitized organs. In omentum, we observed 246 cysts, while 210 cysts were counted in liver (Figure 4). The chi-square test was very significant ($p< 0.0001$) between the level of infestation with cysts of *C. tenuicollis* in the omentum and liver.

Prevalence of ovine cysticercosis according to the risk factors

The prevalence of ovine cysticercosis in relation to the season, the age and sex of sheep is shown in Table 2.

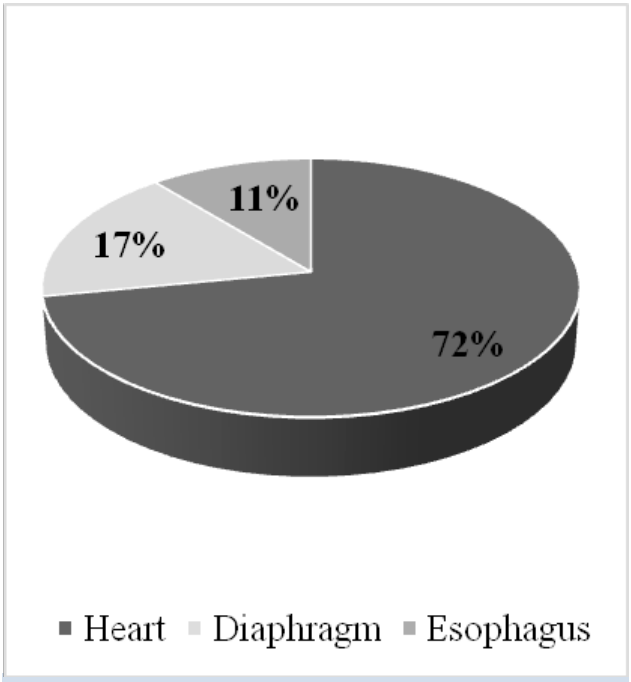

Figure 3: Intensity of parasitism by cysts of *C.ovis* in the predilection sites

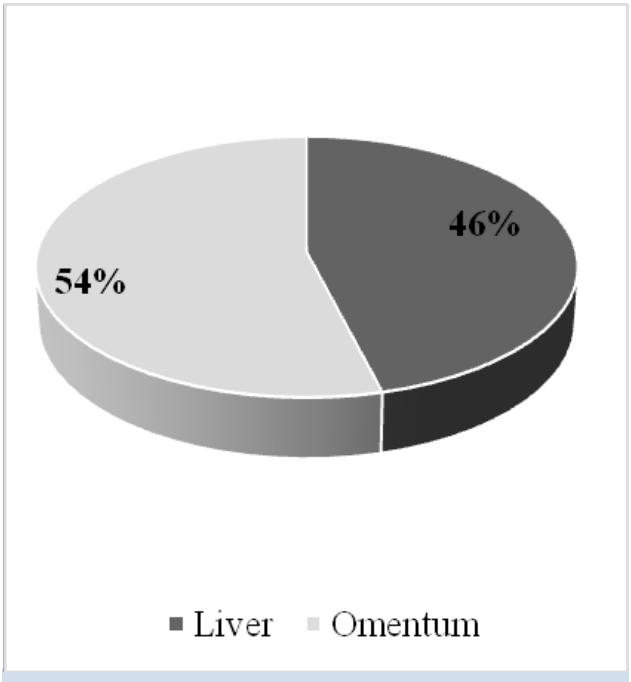

Figure 4: Intensity of parasitism by cysts of *C. tenuicollis* in the predilection sites

Table 2: Prevalence of ovine cysticercosis according to risk factors

Factors	Category	Number of sheep examined	Carcasses with muscular cysticercosis of *C. ovis*		95%CI	Degree of significance and pvalue	Carcasses with *C. tenuicollis*		95%CI	Degree of significance and pvalue
			N	%			N	%		
Gender	Male	10641	200	1.88	[1.6 - 2.10]	p< 0.0001	241	2.26	[1.98 - 2.55]	
	Female	55	20	36.36	[23.6-49.1]		0			
Age Year (s)	≤ 1	2267	5	0.22	[0 - 0.40]	p< 0.0001	31	1.36	[0.89 - 1.85]	p< 0.0001
	[1.5-3]	5269	79	1.50	[1.2- 1.8]		118	2.24	[1.84 - 2.64]	
	[3-5]	3160	136	4.30	[3.6- 5]		92	2.91	[2.33 - 3.50]	
Season	Winter	2280	56	2.46	[1.8- 3.1]	p> 0.05	34	1.50	[0.99 - 1.99]	p< 0.0001
	Spring	1439	45	3.13	[2.2 - 4]		22	1.52	[0.89 - 2.16]	
	Summer	3655	71	1.94	[1.5- 2.4]		48	1.31	[0.94 - 1.68]	
	Autumn	3322	48	1.44	[1 - 1.8]		137	4.12	[3.45 - 4.80]	

DISCUSSION

Overall prevalence

The prevalence of ovine cysticercosis during our study was 4.31%. In the world, results lower than ours were observed in Romania (1.73%) (Lezeriuc et al., 2002),andin Egypt (1.2%) (Elmonir et al., 2015). While a higher prevalence compared to our results were recorded in Greece where a prevalence of 29.41% was noted (Christodoulopoulos et al., 2008).

In an epidemiological survey conducted by Christodoulopoulos et al. (2008), the most common risk factors that may increase the risk of exposure of sheep to cysticercosis were the improper disposal of dead animals, the access of farm dogs to the offal of slaughtered sheep, the carelessness of farmers to treat farm dogs with anthelmintics, and the grazing of flocks in fields where stray dogs have free access(Christodoulopoulos et al., 2008).

In Algeria, the traditional extensive farming method used exposes the sheep to an intensive parasitism. Also, the need for frequent movement of the herd to fetch water and food, when they are lacking, weakens the animals and increases their susceptibility to infestation. Water points, wetter pastures, areas with high animal density are high places of contamination.

In the present study a prevalence of muscular cysticercosis was found to be 2.06%. Similar finding was reported in Benin (3.44%) (Attindehou and Salifou, 2012), in Bulgaria (2.4% and 3.1%) in 1980 and 1981 respectively (Georgieva and Yabulkarov, 1984), in southern Australia (2.39%) (Whiting, 1972) and in Nigeria (1 %) (Dada and Belino, 1978).

While, higher prevalence compared to current result was reported from other countries : 7% in England (Eichenberger et al., 2011) and 11.14% in USA (Jensen et al., 1975).

The role of dogs is important in the transmission of pathology. In a study done in the Netherlands, it was concluded that the abattoir dogs had introduced the parasite because the affected sheep had spent a «long time» on a field adjacent to the abattoir, and the infection was absent from the flocks of origin, but the tapeworms were present in abattoir dogs, which had been fed offal from the sheep (Borgsteede et al., 1985). In another study, Eichenberger et al. (2011) showed that the frequent use of fields by dogwalkers represented a high risk for transmission of *T. ovis* (Eichenberger et al., 2011). In Australia, White and Chaneet (1976) noted that the incidence of *C. ovis* fell from 6.9% to 0.3%, in a 4 year programme to control *Taenia ovis* cysticercosis on farms. In this program, the farmers were advised not to feed dogs any raw sheep meat or offal and were supplied with sufficient cestocide to treat all dogs every two months, recommendations were also given on the control of movement of dogs and the disposal of offal from home-killed sheep (White and Chaneet, 1976).

In our study, all the cysts detected were non-viable. Broadbent (1972) noted that 99.8% of the cysts detected were non-viable and viable cysts were found only in lambs under 12 months of age. Sheep probably have a good immune system that allows them to fight cysticercosis cysts. In effect, Euzeby (1998) noted that this evolution of the lesions is due to the immune reaction of the host.

The reason for the low prevalence of muscular cysticercosis recorded in this study may be related to the low level of environmental contamination, also, our findings were based on surveys carried out on carcasses subjected to routine meat inspection procedures. This can possibly underestimate the incidences reported. Jensen et al. (1975) noted that young cysticerci were not easy to detect. Moreover, according to Euzéby (1966) in experienced meat, inspectors could most likely miss out quite number of viable cysticerci, which blend with the pinkish-red color of the meat, or which are lodged in the intermuscular connective tissue, generally infiltrated with fat (Euzéby, 1966). Dornyet al. (2010) reported that currently the only affordable, workable and available test is visual inspection of the meat, but it is not sufficient, its sensitivity is considered low (<30%). However, it is believed that visual inspection of the meat can identify heavily infected carcasses, which also poses the greatest risk (Dornyet al.2010).

A prevalence of 2.25% for *T. hydatigena* cysticercosis was found in the examined sheep. Our observation is in agreement with those obtained in Italy by Scavone et al. (1999), who found prevalence of 2.6 %. Also, similar results were observed in Egypt by Taher and Sayed, (2011) where a incidence of 4.8% were reported. However, low prevalence was observed in the present study compared to those obtained in some localities from : Bulgaria (11.30%) (Kanchev et al., 2008), Iran (12.87%) (Radfar et al., 2005), Australia (13.6%) (Broadbent, 1972), Italy (14.6%) (Scala et al., 2015), India (20.25%) (Deka and Gaur, 1993), Nigeria (21.4%) (Dada and Belino, 1978) and (23%)

(Akinboade and Ajiboye, 1983), Belarussia (23.2 %) (Nikulin and Karasev, 1975), Brazil (35.2%) (Soares et al., 2012), Ethiopia (37.1%) (Bekele et al., 1988) and (40.0%) (Samuel and Zewde, 2010), Tanzania (51.9%) (Braae et al., 2015), Benin (55.57%) (Attindehou and Salifou, 2012), Spain (85.5%) (Peris Palau et al., 1987).

The infection is often associated with a lack of hygiene and poor farming practices. According to Nikulin and Karasev (1975), the incidence of the disease among sheep was attributed to their special grazing behaviour on pastures contaminated by infected dogs. Also, Samuel and Zewde (2010) concluded that the prevalence is influenced by the type and stocking density and husbandry practices. In Iran, from Radfar el al. (2005), the prevalence of cysticerci was reported in wild animals, this suggested that a sylvatic cycle between wild carnivores and wild herbivores occurs and that interaction between domestic cycle and sylvatic cycles may occur (Radfar et al., 2005).

Prevalence of ovine cysticercosis according to the risk factors

Muscular cysticercosis (*C. ovis* cysts)

This study used samples from abattoir where the majority of the slaughtered animals were male, and only females less than 5 years old were slaughtered in accordance with Algerian regulations because females are preserved by breeders for reproduction.This may not reflect the reality of difference between gender in the occurrence of muscular cysticercosis.

Results from our study showed an increase in prevalence of ovine muscular cysticercosis with age confirming that a major source of infection for sheep is likely to be through the consumption of *Taenia* eggs from the environment. These results are similar to those of some previous investigations (Whiting, 1972 ; Christodoulopoulos et al., 2008 ; Attindehou and Salifou, 2012) indicating that age was an important factor for being positive as a measure of the cumulated life-time risk.

In Benin, Attindehou and Salifou (2012) noted that the infection was prevalent all seasons with nevertheless high rates in rainy seasons. In USA, Jensen et al. (1975)found that the prevalence was higher in the autumn than spring. The authors assumed that the lambs reached the infection from summer pastures contaminated by *T.ovis* eggs (Jensen et al., 1975).

Hepato-peritoneal cysticercosis (*C. tenuicollis* cysts)

The difference in the occurrence of *C. tenuicollis* cysts between genders was not considered. However gender-related tendency of prevalence had been reported previously and some data had suggested that the sex was a significant factor in determining previous exposure to *C.tenuicollis* in sheep (Akinboade and Ajiboye, 1983 ; Taher et al., 2011) who indicated that female animals were more susceptible than males to infection with *C.tenuicollis*. However, according to Soares et al. (2012), it didn't have significant difference between the sexes in the prevalence of *C.tenuicollis.*

Adults were more heavily infected than young animals. These results are similar to the findings of Akinboade and Ajiboye (1983) ; Samuel and Zewde (2010) ; Attindehou and Salifou (2012). The higher rate of infection in adult animals may be attributed to age itself. Adult animals might have picked more eggs of *T. hydatigena* during their life (Samuel and Zewde, 2010). In contrast, Soares et al. (2012) noted that the frequency of parasitism was high among young animals and that cestode parasites produce significant quantities of antigens in adult animals, which protect small ruminants from infection.

Higher prevalence was observed during the autumn, this indicated that sheep picked up the infection from summer pastures contaminated by faeces of dogs infected.Our finding does not support the reports of Deka and Gaur (1993) who found high incidences during the winter. However, Akinboade et al. (1983) and Attindehou and Salifou (2012) showed that the prevalence of infection was higher in the wet season than in the dry season.

Prevalence of ovine cysticercosis according to the predilection sites

Muscular cysticercosis (*C. ovis* cysts)

The present abattoir study revealed that among the organs accessible for detailed inspection, the heart, was the most affected. This preferred predilection was similar with earlier findings reportes by Bilan and Tassin (1969); Jensen et al. (1975) and Kebede (2008).

Euzéby et al. (2005) noted that the elective muscular localizations were the heart, the masseter and pterygoid, the diaphragm and the tongue.From Kebede (2008), the distribution of lesions on the various

organs can be influenced by several factors such as muscle activity, age, and the geographic area (Kebede, 2008).

Hepato-peritoneal cysticercosis *(C. tenuicollis* cysts)

C. tenuicollis was more frequently detected in the liver followed by the omentum. Deka and Gaur (1993) found that cysts were commonly found in the liver as well as mesentery and omental fat (Deka and Gaur, 1993). In a another hand, Radfar et al. (2005) reported taht the predominant sites of *C. tenuicollis* was the omentum followed by the liver, the peritoneum, and finally the lung, heart and bladder with similar proportions (Radfar et al., 2005). Similarly, in another study, *C. tenuicollis* was more frequently detected in the omentum, followed by the liver, peritoneum and finally the lung (Samuel and Zewde, 2010). Akinboade and Ajiboye (1983) found that the commonest sites for cysts were the mesenteries and omentum (Akinboade and Ajiboye, 1983). Also, Soares et al. (2012) noted that the predominant localization of *C. tenuicollis* were the omentum, followed by the abomasal serosa (Soares et al., 2012). Howerver, Cassali and Nascimento (1994) showed that the examination of the genital system of 225 ewes revealed three cases of genital cysticercosis in different parts of the uterus and in ligamentum latum (Cassali and Nascimento, 1994).

CONCLUSION

The present study has revealed a non-negligible prevalence of ovine cysticercosis in the two slaughterhouses in the north of Algeria, suggesting a dispersion of *T. hydatigenae* and *T. ovis* eggs and parasite reservoir hosts in the environment. We noted a dominance of *C. tenuicollis* compared to *C. ovis*, *C. tenuicollis* vesicles were mainly present in the liver. All the cysts of muscular cysticerci detected were non-viable probably due to the efficiency of the immune system of the sheep; these cysts seem to have the heart as their predilection. This study highlights the impact of these parasitic diseases in the study region, further studies are needed to determine the impact of ovine cysticercosis in different regions in order to stimulate better efforts towards the control and possible eradication of these deseases.

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Research article
Ερευνητικό άρθρο

Determination of microbiological quality and histamine levels in rainbow trouts *(Oncorhynchus mykiss)*

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ABSTRACT: This study aims to determine the microbiological quality and histamine levels of fresh-marketed rainbow trouts (*Oncorhynchus mykiss*). The total mesophilic aerobic bacteria (TMAB), total psychrophilic aerobic bacteria (TPAB), coliform, fecal coliform, *Enterobacteriaceae, Pseudomonas*, *Staphylococcus-Micrococcus* and mold-yeast numbers were detected as 4.24, 4.00, 1.10, 0.17, 1.50, 3.05, 0.28 and 3.82 $\log_{10}$ cfu/g, respectively. *Yersinia enterocolitica, Listeria monocytogenes, Escherichia coli* O157:H7 and *Vibrio* spp bacteria were found in 18 (37.5%), 14 (29.1%), 8 (16.6%) and 14 (29.1%) respectively. Twenty-eight out of 51 *Listeria* isolates (54.9%) were verified through PCR analysis as *L. monocytogenes*. It was determined that the histamine level in the samples was above the detectable level (>2.5 mg/kg) in 8 of the samples (16.6%)determined using an ELISA method. It was concluded that the detection of foodborne pathogens in fresh-marketed fish samples potentially constitutes a public health hazard.

Keywords: Rainbow trout, Microbial quality, Histamine, ELISA, PCR

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INTRODUCTION

Fish and fishery products are among the main sources of proteins and functional components that are very important for human health (Han et al., 2017). In addition, fish meat contains water-soluble B and C and fat-soluble A, D, E and K vitamins in adequate and balanced amounts (Kara et al., 2020). Despite the difficulties the fish and fishery products industry faces, fish constitutes a significant part of the diet in many countries. It is appreciated throughout the world due to its high nutritional value and delicious taste. Therefore, it is very important in the international fishing industry to ensure the safety of edible fish (Han et al.,2017).Although fish is a rich nutritional source of animal origin and healthy in many aspects, it is an extremely perishable food (Junior et al.,2014). The microbial condition of the seafood after it is caught is closely related to environmental conditions, the microbiological quality, temperature and salt content of water, the distance between the areas of fecal contamination and fishing, natural bacterial growth in water, hunting methods, and cooling conditions (Feldhusen, 2000).

There are different types of bacteria that cause spoilage of fish and fishery products and such spoilage is largely connected to the processing and preserving methods. Yet, the composition of the microflora on newly caught fish may be due to the microbial content of the water the fish live in. The microflora that causes spoilage of fish consists of bacteria such as *Salmonella* spp., *Enterohemorrhagic Escherichia coli*, *Campylobacter jejuni*, *Y. enterocolitica*, *L. monocytogenes*, *Bacillus anthracis*, *Bacillus cereus, Staphylococcus aureus, Clostridium botulinum, Clostridium perfringens, Vibrio vulnificus*and *Vibrio parahaemolyticus* (Han et al., 2017).In unpreserved fish, microbial spoilage is mainly a result of Gram-negative, fermentative bacteria (such as *Vibrionaceae*), whereas psychrotolerant Gram-negative bacteria (such as *Pseudomonas spp*. and *Shewanella spp*.) tend to spoil chilled fish (Ghaly et al., 2010). Kuley et al. (2017), identified *Enterobacter cloacae, Serratia liquefaciens, Proteus mirabilis, Photobacterium damseale, Pseudomonas luteola, Pantoea spp., V. vulnificus, Stenotrophomonas maltophila, Acinetobacter lwoffii,Pasteurella spp*. and *Citrobacter spp.* from spoiled fish.Some psychrotrophic pathogens can multiply in chilled food without causing a significant change in its sensory properties. *Pseudomonas* species cause deterioration in refrigerated food products by forming the dominant microflora during cold storage. Therefore, the presence of these microorganisms in foods poses a great risk as they cause both food poisoning and food spoilage (Popelka et al., 2016).

Pathogenic bacteria associated with seafood may be examined under three groups, i.e. bacteria constituting the normal components of the marine or port environment: *Vibrio cholerae, V. parahaemolyticus, V. vulnificus, L. monocytogenes, C. botulinum* and *Aeromonas hydrophila*; enteric bacteria growing due to fecal contamination: *Salmonella* spp., pathogenic *E. coli, Shigella* spp., *Campylobacter* spp. and *Y. enterocolitica*; and bacteria growing due to bacterial contamination during processing: *B. cereus*, *L. monocytogenes*, *Staphylococcus aureus* and *C. perfringens* (Feldhusen, 2000).

Histamine, which is the most important cause of food poisoning, is a biogenic amine (Cavanah and Casale, 1993; Ehsanet al., 2015). It is mainly formed by decarboxylation of amino acids due to enzyme activities of microorganisms (Shalaby, 1996; Chen et al., 2010;Akyol et al.,2015). Biogenic amine poisoning is often caused by histamine. Histamine poisoning is also named as “scombroid fish poisoning” as it is often associated with the consumption of scombroid fish such as tunas, mackerels and sardines (Halasz et al., 1994). In fish and fishery products, biogenic amines can be considered indicators of both quality and safety. Because of its toxicity, histamine is a biogenic amine with regulatory limits for fishery products (Visciano et al., 2020). Consumption of food that is high in histamine content causes poisoning with clinical symptoms such as nausea, vomiting, diarrhea, abdominal cramps, headache, rash, asthma and hypotension (Cavanah and Casale, 1993; Maintz and Novak,2007).A highlevel of biogenic amine in food causes food poisoning. Particularly, a high level of histamine in fishery products is a significant indicator of bacterial spoilage. In this regard, the measurement of histamine level is very important in the determination of the hygienic quality of fish.

This study aims to determine the microbiological quality and histamine levels of fresh-marketed rainbow trouts.

MATERIAL AND METHODS

Samples

The samples were obtained from three different sales points (fresh fish retailers) between February and April 2016 in Kars, Turkey. A total of 48 fish fillets consisting of 16 samples from each sales point

were analyzed. The fillets were prepared by the fisherman (internal organs were removed, beheaded, filleted and washed). The fillets were collected in sterile plastic bags. The samples were brought to the laboratory under the cold chain and microbial analysis was made immediately. Samples were stored at -20 °C until used in histamine analysis.

Microbiological analysis

Twenty-five g of each fish fillet sample was measured and homogenized in 225 ml sterile physiological saline solution. After homogenization, decimal dilutions of the samples were prepared and inoculated into suitable mediums by pour plate and streak plate methods, i.e. into Plate Count Agar (PCA, Oxoid CM 325) at 30°Cfor 48 h for Total Mesophilic Aerobic Bacteria (TMAB), Plate Count Agar (PCA, Oxoid CM 325) at 7°C for 10 days for Total Psychrophilic Aerobic Bacteria (TPAB), Violet Red Bile Lactose Agar (VRBA, Oxoid CM 0107) at 37°C for 24 h for Coliform group bacteria, Violet Red Bile Lactose Agar (VRBA, Oxoid, CM 0107) at 44.5°C for 24-48 h for Fecal coliform group bacteria, VRB Glucose Agar (VRBG, Oxoid CM 485) at 35°C for 48 h for *Enterobacteriaceae*, Baird Parker Agar (Oxoid CM 275) at 37°C for 24-48 h for *Staphylococcus- Micrococcus*, Pseudomonas Agar (Oxoid CM 559) and C-F-C Supplement (Oxoid SR 103) at 30°C for 48 h for *Pseudomonas*, and Potato Dextrose Agar (Oxoid CM 139) at 22°Cfor 5-10 days for Yeast-Mold; and the incubation was conducted under the abovementioned conditions (Harrigan, 1998).

***Y. enterocolitica* isolation and identification**

Y. enterocolitica isolation was performed by the conventional inoculation method. 25 gr the fish fillet sample was homogenized in a 225 ml Yersinia selective enrichment broth (Merck 1.16701) medium and incubated at 30 °C for 24 h. From this pre-enrichment culture, inoculation was made to the Yersinia Selective Agar (Oxoid CM0653) medium involving Yersinia selective supplement (Oxoid SR0109) by streak plate method and incubated at 30°C for 24 h. Colonies with crimson red centers and transparent zones at the periphery were considered suspicious and stored in the agar slant. For the identification of the isolated suspicious colonies, inoculation into the Gram staining, simmons citrate, motility, Voges Proskauer (VP), urea, Kligler Iron Agar reaction and Triple Sugar Iron media was made and lactose, sucrose and glucose use, gas and hydrogen sulphide (H_2S) formation, lysine decarboxylase, ornithine decarboxylase, nitrate reduction, catalase, oxidase and indole tests were conducted (FDA, 2007).

***L. monocytogenes* isolation and identification**

L. monocytogenes isolation was performed by the FDA method. 25 gr the fish fillet sample was homogenized in 225 ml Buffered Listeria Enrichment Broth (Oxoid, CM 897) medium and incubated at 30 °C for 4 h. Then, a selective supplement **(**Listeria Selective Enrichment Supplement, Oxoid SR141) was added and incubated at the same temperature for 44 h. From this pre-enrichment culture, inoculation into the Listeria Selective Agar medium (LSA, Oxoid CM 856) involving Listeria Selective Supplement (Oxoid SR140) was made by streak plate method. The LSA plates were incubated at 37°C for 48-72 h. After incubation, blackish green-brown colonies 2-3 mm in diameter surrounded by a black zone and having a sunken center that grew in the medium were evaluated as *Listeria* suspected colonies and stocked at the agar slant. For the identification of the isolates, Gram staining, catalase, oxidase, indole, Methyl Red (MR), VP, nitrate reduction, motility, CAMP and carbohydrate fermentation tests were performed (Seeliger andJones,1986; Hitchins, 1992;Harrigan, 998).

***Vibrio* spp. isolation and identification**

Twenty-five g the fish fillet sample was added into225 ml alkaline peptone (Merck, 101800. 0500) medium and homogenized on the Stomacher. It was left to incubation at 35-37ºC for 8 h. Then, it was inoculated into Thiosulphate Citrate Bile Salt Sucrose Agar medium (TCBS, Merck, 110263.0500) by loop and the Petri dishes were left to incubation at 35-37 °C for 18- 24 h. After the incubation, round, yellow, green or blue-green colonies2-3 mm in diameter that grew in the TCBS agar were evaluated as suspicious colonies and stocked in the agar slant. For the identification of the isolated strains, inoculation was made into the Gram staining, oxidase (Bactident Oxidase, Merck), catalase, Triple Sugar Ironmedia, the lactose, sucrose and glucose use, gas and hydrogen sulfide (H_2S) formation, indole, VP and lycine decarboxylase tests were conducted, and the results were evaluated (FDA, 1992).

***Salmonella* spp isolation and identification**

Twenty-five g of each fish fillet sample was measured and homogenized with 225 ml buffered peptone water and incubated at 37 °C for 24 h. Following incubation, 0.1 ml was taken without enrichment and

inoculated into Rappaport Vassiliadis Broth (Oxoid, CM 669) and incubated at 42 °C for 18-24h. After incubation, inoculation was made on the selective solid media of Brilliant Green Agar (Oxoid, CM 263), Hektoen Enteric Agar (Oxoid, CM 419) and Xylose Lysine Deoxycholate Agar (Oxoid, CM 469) by streak plate method and incubated at 37 °C for 18-24 h. Ten of each typical colony that grew in the selective agars were taken and stored in the agar slant. For the identification of the isolates, urea test, acid and gas production from glucose in Triple Sugar Iron Agar, lactose and sucrose use, H_2S formation, lysine decarboxylase, VP and indole tests were conducted and the results were evaluated. For serological analysis, the Salmonella latex test (Oxoid FT 203) was performed on the isolates (Andrews and Hammack, 1995;ISO, 2002).

E. coli O157:H7 isolation and identification

Twenty-five g of each fish fillet sample was measured and homogenized with 225 ml Modified Tryptic Soy Broth (mTS, Merck 1.09205), then incubated at 35-37°C for 18 h. Following enrichment, inoculation was made into cefixime-tellurite (Merck 1.109202) added SMAC agar (Merck 109207) by streak plate method and incubated at 41-42°C for 18-24 h. Sorbitol-negative colorless colonies that grew in the medium were taken and inoculated into 4-methylumbelliferyl-D-glucuronide (Oxoid, BR0071) added MacConkey Agar (Oxoid, CM007) and incubated at 41-42°C for 18 h, then MUG-negative colonies were selected and stocked in the agar slant. Gram staining, indole, MR, VP, citrate, hydrogen sulfide formation, lactose, gas production from glucose, motility and lysine decarboxylase tests were performed on the isolates (Feng and Weagant, 2011; Harrigan,1998).

Detection of histamine

For determining the histamine levels of the trout samples, the Ridascreen Histamine ELISA test kit (R-Biopharm AG, R1601, Darmstadt, Germany) was used. The analysis was made according to the test procedure. The absorbance value was measured in the ELISA reader (Microplate reader, BioTekELx800) at 450 nm in 10 min (Ridascreen R1601, 2014). In the evaluation of the results obtained, Ridasoft Win PC Software was used.

Identification of isolates through the molecular method

Genomic DNA isolation

Genomic DNA extraction in bacteria isolates was conducted by DNA extraction kit (Qiagen-Mericon DNA Bacteria Kit and Mericon DNA Bacteria Plus Kit, Germany) for Gram-negative and Gram-positive bacteria in accordance with the recommendations of the manufacturer.

L. monocytogenes genetic identification

For genomic DNA amplification, the method recommended by Aznar and Alarcon(2003) was used in modified form. The target gene in the identification was selected as hylA. LM1: CCTAAGACGCCAATCGAA and LM2: AAGCGCTTGCAACTGCTC primers were used (Border et al.,1990). For PCR, 25 µl master mix was prepared. PCR buffer (10 mmol/L, Tris-HCI, pH 8.8; 1.5 mmol/L $MgCI_2$, 50 mmol/L KCI, 0.1%Triton X-100), 1 µmol/Lof each primer (IDT, USA), 100 µmol/L of each dNTP (Thermo Scientific, R0181), 1 U Taq DNA Polymerase (Thermo Scientific, EP0402) and 5 µl genomic DNA were used. The thermal conditions applied for the PCR were initial denaturation at 94°C for5 min, 30 cycles of denaturation at 94°Cfor 30 sec, annealing at 50°Cfor 1 min, synthesis (extension) at 72°Cfor 1 minandfinal extension at 72 °Cfor 5 min in the TProfessional Basic Thermocycler (Biometra, Model TProfessional Basic Gradient) device. In order to observe the PCR products, 0.5 X TBE (Tris-Borate-EDTA) was conducted in 1% agarose gel. *L. monocytogenes* (ATCC 7644) was used as positive control and the *hlyA* gene was visualized at 702 bp under UV illumination.

Y. enterocolitica genetic identification

For genomic DNA amplification, the method recommended by Lucero Estrada et al. (2011) was used in modified form. The target genes in the identification were selected as virF (591 bp) and ail (170 bp),and multiplex PCR was applied. virF-F: TCATGGCAGAACAGCAGTCAG virR: ACTCATCTTACCATTAAGAAG and ailF: ACTCGATGATAACTGGGGAG ailR: CCCCCAGTAATCCATAAAGG primers were used (Hussein et al.,2001). For PCR, 25 µl master mix was prepared. PCR buffer x1 (10 mmol/L, Tris-HCI, pH 8.8; 50 mmol/L KCI, 0.1% Triton X-100), 1.5 mM $MgCI_2$, 10 pmol of each primer (IDT, USA), 200 µmol/L of each dNTP (Thermo Scientific, R0181), 1 U Taq DNA Polymerase (Thermo Scientific, EP0402) and 5 µl genomic DNA were used. Thermal conditions were initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94°Cfor 30 sec, annealing at 60 °Cfor 30 sec, synthesis (exten-

sion) at 72 °Cfor 1 minand final extension at 72 °Cfor 4 minas applied in the TProfessional Basic Thermocycler (Biometra, Model TProfessional Basic Gradient) device. In order to observe the PCR products, 0.5 X TBE (Tris-Borate-EDTA) was conducted in 2% agarose gel. As a positivecontrol,*Y. enterocolitica* (ATCC 9610) was used.

*V. cholerae*genetic identification

For genomic DNA amplification, the method suggested by Sheikh et al. (2012) was used in modified form. In the identification, ompW-F: CACCAAGAAGGTGACTTTATTGTG ompW-R: GAACTTATAACCACCCGCG primers were used, and a target gene, ompW (588 bp) was selected (Nandi et al., 2000). For PCR, 25 µl master mix was prepared. PCR buffer x1 (10 mmol/L, Tris-HCI, pH 8.8; 50 mmol/L KCI, 0.1% Triton X-100), 2.5 mM $MgCI_2$, 10 pmolof each primer (IDT, USA), 2,5 mM dNTP (Thermo Scientific, R0181), 1 U Taq DNA Polymerase (Thermo Scientific, EP0402) and 5 µl genomic DNA were used. The thermal conditions were initial denaturation at 94 °Cfor 5 min, 28 cycles of denaturation at 94 °Cfor 30 sec, annealing at 64 °Cfor 30 sec, synthesis (extension) at 72°Cfor 30 secand final extension at 72 °Cfor 6 minas applied in the TProfessional Basic Thermocycler (Biometra, Model TProfessional Basic Gradient) device. In order to observe the PCR products, 0.5 X TBE (Tris-Borate-EDTA) was conducted in 1.5% agarose gel. *V. cholerae* (RSKK 751) was used as a positive control.

E. coli O157:H7 genetic identification

For amplification, the method suggested by Kim et al. (2005) was used in modified form. In the identification, the target genes were selected as stx1 (614 bp) and stx2 (779 bp). stx1-F: ACACTGGATGATCTCAGTGG, stx1-R: CTGAATCCCCCTCCATTATG and stx2-F: CCATGACAACGGACAGCAGTT, stx2-R: CCTGTCAACTGAGCAGCACTTTG primers were used (Fagan et al., 1999).For multiplex PCR,a 25 µl master mix was prepared. PCR buffer (10 mmol/L, Tris-HCI, pH 8.8; 50 mmol/L KCI, 0.1% Triton X-100), 3 mM $MgCI_2$, 1 µM of each primer (IDT, USA), 0.2 mM of each dNTP (Thermo Scientific, R0181), 1 U Taq DNA Polymerase (Thermo Scientific, EP0402) and 4 µl genomic DNA were used. The thermal conditions applied for the PCR were initial denaturation at 94 °Cfor 3 min, 35 cycles of denaturation at 94 °Cfor 20 sec, annealing at 58 °Cfor 40 sec, synthesis (extension) at 72°Cfor 90 secand final extension at 72 °C for 5 min as applied in the TProfessional Basic Thermocycler (Biometra, Model TProfessional Basic Gradient) device. In order to observe the PCR products, 0.5 X TBE (Tris-Borate-EDTA) was conducted in 1.5% agarose gel. *E. coli* (ATCC 43894) was used as a positive control.

Statistical analysis

The statistical analysis of the findings of the study was performed in the SPSS 20 software package by chi-square test.

RESULTS

Microbiological analysis and PCR results of the isolates

The microbiological analysis results of the analyzed fish samples are shown in Table 1 and Table 2. The identification of the *Y. enterocolitica* and *V. cholerae* isolates was performed by biochemical tests, and they were not genetically verified by the PCR analysis. The identification of the *E. coli* O157:H7 isolates was performed by biochemical verification tests, and none were shown by the PCR to produce Shiga toxin. Out of 51 *Listeria* isolates, 28 (54.9%) were verified as *L. monocytogenes. L. monocytogenes* was identified in 9 out of 48 (18.75%) fish samples according to the PCR result (Figure 1).

Table 1. Microbiological quality parameters (in $\log_{10}$ cfu/g) of rainbow trout samples (n: 48)

Microorganism	Mean ± SD	Min.	Max.
TMAB	4.24±0.94	2.60	7.92
TPAB	4.00±0.70	<1	4.70
Total coliform	1.10±1.29	<1	3.08
Fecal coliform	0.17±0.70	<1	3.49
Enterobacteriaceae	1.50±1.44	<1	3.68
*Pseudomonas*spp.	3.05±1.26	<1	4.60
Staphylococcus-Micrococcus	0.28±0.74	<1	2.60
Mold-Yeast	3.82±0.37	3.08	4.82

Table 2. Presence of pathogenic microorganisms in rainbow trout samples

Microorganism	Number of samples (%)	Number of positive samples (%)
Y. enterocolitica	48	18 (37.5%)
L. monocytogenes	48	14 (29.1%)
E. coli O157:H7	48	8 (16.6%)
Vibrio spp.	48	14 (29.1%)
Salmonella spp.	48	Not detected
S. aureus	48	Not detected

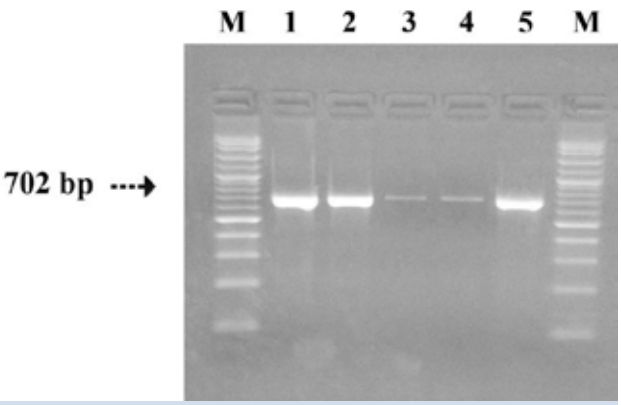

Figure 1. Amplification products obtained from the PCR. Band M :100-bp DNA ladder, Band 1-4: *L. monocytogenes* PCR amplification products, Band 5: Positive control for *L. monocytogenes*

Histamine analysis results

In the histamine analysis performed by the ELISA method, it was detected that the majority of the samples showed a histamine level below 2.5 mg/kg. Eight samples yielded numerical results. The highest histamine level was measured as 10.05 mg/kg. A statistical difference existed between the histamine levels of the samples ($P<0.05$).

DISCUSSION

In this study, microbial quality and histamine levels of fresh rainbow trout were evaluated. The numbers of TMAB, TPAB, Coliform, Fecal coliform, *Enterobacteriaceae*, *Pseudomonas*, *Staphylococcus-Micrococcus* and Mold-Yeast were found to be 4.24, 4.00, 1.10, 0.17, 1.50, 3.05, 0.28, and 3.82 $\log_{10}$ cfu/g, respectively. In terms of foodborne pathogens, *Y. enterocolitica, L. monocytogenes, E. coli* O157:H7 and *Vibrio* spp were found to be positive in 18 (37.5%), 14 (29.1%), 8 (16.6%) and 14 (29.1%) samples respectively, whereas none of the samples were positive for *Salmonella* spp and *S. aureus*. Popelka et al. (2016) reported in fillets of rainbow trout the numbers of total viable, psychrotrophic bacteria and *Pseudomonas spp*. as 4.55, 4.55 and 1.28 log cfu.g^{-1} respectively. In our study, the number of psychrotrophic bacteria and *Pseudomonas spp*. is higher than the findings of the researchers. Ucak et al. (2020) reported the numbers of initial total viable, psychrotrophic bacteria, yeast-mold and *Enterobacteriaceae* as 1.543, 1.932, 1.194 and3.390$\log_{10}$ cfu/g respectively. The findings of the researchers are quite low compared to our findings in terms of total viable, total psychrotrophic bacteria and yeast and mold numbers. The number of *Enterobacteriaceae* is higher than our findings. Arslan (2019) reported in fillets of rainbow trout the numbers of initial TMAB, TPAB, *Enterobacteriaceae* and Yeast-Mould as 4.07, 4.36, 2.00 and 2.00 $\log_{10}$ cfu/g respectively. These results are in parallel to our study findings in terms of the number of TMAB and TPAB. The number of *Enterobacteriaceae* was found to be higher than our results. The number of yeast- mold is lower than our findings. Eltholth et al. (2018) reported that the number of positive samples for *E. coli*, *L. monocytogenes*, *Salmonella* spp*, S. aureus* and *V. parahaemolyticus* were 8.0%, 7.7%, 3.3%, 13% and 12.3%, respectively. In a study analyzing different fish species, Eizenberga et al. (2015) reported that the total number of bacteria was 5.58 to 7.84 $\log_{10}$cfu cm^{-2}, 11 out of 20 fish samples were positive for *L. monocytogenes* and all samples were negative for *Salmonella*. In our study, the total number of aerobic bacteria was 4.24 $\log_{10}$ cfu/g, *L. monocytogenes*positive samples were 14 (29.1%), and all samples were negative for *Salmonella*. In a study examining the effect of the traditional marination process on certain features of rainbow trout fillets throughout cold preservation, the initial total number of mesophilic bacteria was determined as 3.9 log and psychrophilic bacteria as 3.5 log in the control group samples (Maktabi et al., 2016). In our study, the total number of mesophilic bacteria and psychrophilic bacteria were determined as 4.24 and 4.00 $\log_{10}$ cfu/g, respectively. The results of our study are inconsistent with the findings of the researches. In a study analyzing different types of fish, Onmaz et al. (2015) reported 5 (5%) *Salmonella* spp and 9 (9%) *S. aureus* infected samples. Another study reported in two of the tilapia fillet samples, coagulase-positive *Staphylococcus* and fecal contamination in the muscles (Junior et al., 2014). Again, consistently with this study, our study detected an average of 0.17 $\log_{10}$ cfu/g fecal coliform but no *S. aureus.* The differences between similar studies may be attributed to seasonal and environmental differences, microbial quality of the water in which the fish were caught, distance between the fishing area and the sales area, contamination that may occur during the transportation and marketing of fish, unsuitable cold preservation conditions and contamination that may occur due to the personnel working in manual processing.

The acceptable level of histamine is specified as 100 ppm in the Turkish Food Codex (TFC) whereas the limit value is identified as 200 ppm (TFC, 2011). According to EU Regulation (EC) No 2073/2005, the recommended histamine level in fish is 100-200 mg/kg (EC, 2005). The presence of biogenic amines above the recommended limit in foodstuff causes

serious health problems and high dosages may even cause fatalities (Akyol et al., 2015). This study was conducted to determine the presence of such compounds that are extremely significant for food quality and constitute a threat to human health, as a result of which it was determined that in all the samples, the histamine levels were below the limit considered as toxic. Emir Çoban and Patır (2008) reported that the average histamine levels in fresh-marketed anchovies, horse mackerels, mackerels, rainbow trouts, goatfish and cyprinus carpio were 24.24, 26.49, 25.81, 12.21, 11.27 and 10.33 ppm, respectively, concluding that the histamine levels did not exceed the recommended limit. Sadeghi et al. (2019)reported that the lowest and highest histamine concentration in canned tuna fish were 2.14±0.17 and 21.69±0.11 mg/100 g of fish respectively.The authorsstated that the amount of histamine in the tuna was below the standard limit (<50 mg histamine/100 g).In another study on canned tuna fish, in a total of 80 fish samples, the average histamine level was found as 10.97±9.86 mg kg^{-1} which complied with the limits permitted in the TFC (Er et al., 2014). Hosseini et al. (2014), in a study where they observed the change in the histamine concentration level during storage in ice for 18 days, reported that they did not detect histamine on days 1 and 3 of storage. As a result of a study analyzing the histamine levels in fresh and canned fish, Evangelista et al.(2016) reported no histamine in fresh tuna, tilapia and rainbow trout samples, but 44.6% of the canned tuna samples involved histamine below the allowed limits. In our study, a total of 48 samples revealed that in 8 samples (16.6%), the histamine levels were above 2.5 mg/kg whereas in 40 samples (83.3%), they were below 2.5 mg/kg. The highest rate was measured as 10.05 ppm. It was determined that the fish samples involved histamine at statistically different rates ($P<0.05$).Our results are highly consistent with the studies conducted by various researchers.

CONCLUSION

In this study, it was determined that fresh fish were contaminated with important foodborne pathogens such as *Y. enterocolitica*, *L. monocytogenes*, *E. coli* O157: H7 and *Vibrio* spp. It was concluded that this situation may pose an important public health hazard, especially due to the consumption of undercooked or raw fish. On the other hand, it has been pleasantly found that the histamine levels were below the specified limits and therefore much lower than the level that may harm human health. It is essential that the manufacturers and consumers are informed about foodborne pathogens, the manufacturers satisfy suitable cold storage conditions at sales points and the consumers are informed about the health risks associated with inadequately cooked products. It is crucial for consumer health to organize training programs for fishing industry personnel about the issues to take into consideration during the fish cleaning process and about cross contamination. As a result, it is suggested that the necessary hygienic precautions are taken at every step of the process from the hunting of the fish until putting it on the market, for the sake of protecting public health against foodborne pathogens and enabling safe food consumption.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Epidemiology of digestive parasites in buffaloes from Romania

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ABSTRACT: Digestive parasites are a constraint on buffalo productivity. Data regarding the prevalence of parasitic diseases in buffaloes are reported in developed countries, but in Romania, they are missing. The aim of this study was to evaluate the prevalence of internal parasites in buffaloes raised in households and farms from Romania. Faeces samples were collected from 180 buffaloes of different ages (calves: 0-6 months; juveniles: 6-30 months; adults:> 30 months). The faecal samples were processed by coproparasitological methods: flotation, sedimentation, and Mc-Master. Digestive parasites were detected in 57.2% of buffaloes. Most buffaloes had single species infection(42.2%) (p<0.001), and only 15.0% had infection with 2 (14.5%) or 3 (0.6%) parasites. The prevalence of digestive parasites was higher in calves (68.8%) and juveniles (76.9%) compared with adults (19.6%) (p<0.001). *Eimeria* spp. (43.3%), *Buxtonella sulcata* (1.7%), *Fasciola hepatica* (4.4%), *Paramphistomum cervi* (2.8%), *Moniezia* spp. (0.6%), *Toxocara vitulorum* (11.7%), digestive strongyles (3.3%), *Strongyloides papillosus* (5.0%), and *Capillaria* spp. (0.6%) were identified. *Eimeria* spp. and *T. vitulorum* were the most prevalent parasites in calves and juveniles (p<0.001). *Fasciola hepatica* was the most prevalent parasite in adults. *Eimeria* spp. and *S. papillosus* were the only infections diagnosed in farmed buffaloes, with a higher prevalence (p>0.05) than in household buffaloes. *Buxtonella sulcata, Moniezia* spp., *T. vitulorum* and *S. papillosus* were diagnosed only in young buffaloes, while *Capillaria* spp. only in adults. The OPG of *Eimeria* spp. was higher in young buffaloes compared with adults (p<0.001). Age was the risk factor identified for infections with *Eimeria* spp., *T. vitulorum* and *F. hepatica.*

Keywords: digestive parasites, buffaloes, coproparasitological examination, OPG.

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INTRODUCTION

The buffalo farming is continuously increasing worldwide, including in Western-European countries (Bodnar, 2017). Buffaloes are the second biggest milk-producing species in the world (Guo, 2010). Buffalo milk has a higher fat and protein content than cow's milk and it is used in the dairy industry to produce mozzarella, cream, yogurt or butter (Coroian, 2012). Romanian buffaloes are raised traditionally for milk and meat production, and to a lesser extent for draught. Romania has the second highest proportion of milk recorded buffaloes from the Mediterranean area, after Italy (Borghese, 2010). Romanian buffalo population was ranked 2nd in Europe in 2004 with 100,000 heads, but gradually decreased up to 25,000 heads in 2013 (Vidu et al.,2008; Borghese, 2013). Around 97% of buffaloes are raised in centre and north-western regions of Romania (Coroian, 2009).

Internal parasites are a constraint on productivity (Bodnar, 2017). Parasitic diseases are responsible of decreased weight gains and milk production, capricious appetite, anaemia, mortality, leading to significant economic losses (Fikruet al., 2006). Maintenance and climatic conditions are extrinsic factors that determine the presence of internal parasites (Sargison, 2016), but the intraspecific susceptibility is based on factors such as genetics, physiological, nutritional status, age and feeding mode (Bhutto et al., 2002).

Extensive data regarding parasite epidemiology is required in order to control parasitic diseases. Regular monitoring of parasites in animals is necessary to detect the early stages of infections (Gunathilaka et al., 2018).

Data on the epidemiology of internal parasites in buffaloes are reported in developed countries, howeverthe information is scarcein developing countries (Mamun et al., 2011). The prevalence of parasites in buffaloes from Romania has not been studied so far.

Taking into consideration the importance of buffalo rearing in Transylvania region, the purpose of this study was to evaluate the prevalence of internal parasites in buffaloes raised in households and farmsfrom the North-West of Romania.

MATERIALS AND METHODS

Study area

Romania is a Southeastern European country thathas a temperate continental climate, with four distinct seasons (spring, summer, autumn, and winter). The Carpathian Mountains split the territory into two groups: intra-Carpathian regions and extra-Carpathian regions. The Transylvania Depression and the western part of Romania is included in the first group and it is dominated by western moist air masses. Precipitation decreases on average from West to East and increases with altitude (A.N.M., 2008). However, in the last years, there is an increasing trend in extreme precipitation indices in north-western regions of Romania (Croitoru et al., 2016).

Animals

One-hundred-eighty buffaloes were sampled in this study. Their age varied from 2 weeks to 24.0 years with an average of 45.1 (±5.3) months (3.8 years). The buffaloes were divided into three age groupsas follows: calves (*n*=101) from 0 to 6 months old; juveniles (*n*=28) older than 6 months to 30 months (2.5 years) old; and adults (*n*=58) older than 2.5 years (> 30 months) (Table 1). The buffaloes came from five counties: Cluj, Sălaj, Bihor, Bistriţa-Năsăud and Maramureş (Fig. 1). Most of the animals were raised in household system (160/180), while the rest of the animals originated from a farm in Cluj County.

The buffaloes from households were grazingon pastures nearby forests. These pastures were used by several species of animals, and all age groups. In the cold season, the animals were kept in small shelters. Milking was done manually; calves and young buffaloes were living in the same shelter as adults.

Table 1. Distribution of the study samples by age, gender, and origin

	Number	Age [mean (±SEM)]		Gender *(n)*		Origin *(n)*	
		Months	Years	Females	Males	Household	Farm
Calves	77	4.0 (0.2)	-	44	33	67	10
Juveniles	52	11.7 (1.0)	-	32	20	42	10
Adults	51	141.4 (9.8)	11.8 (0.8)	46	5	51	0
Total	180	45.1 (±5.3)	3.8 (0.5)	122	58	160	20

Figure 1. The map of Romania showing the counties of origin of sampled buffaloes

Samples and sample analysis

Single fresh faeces samples (n=180) were randomly collected from individual buffaloes of different ages.

The faecal samples were collected in sterile plastic containers, each labelled with reference number,transported,and stored at 4 °C in the Laboratory of Parasitology and Parasitic Diseases of The University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca until further processing.

The faecal samples were processed by coproparasitological methods:salt flotation technique (specific gravity 1.28) and sedimentation, followed by examination using optical microscopy. The parasitic elements were identified based on morphological aspects (Zajac and Conboy, 2012). Ninety-nine out of 180 faeces samples were also examined by the McMaster method to determine the number of oocysts or eggs/gram of faeces (Zajac and Conboy, 2012).

Statistical analysis

Cross-sectional measurements were performed at the individual level. The diagnostic methods used to identify the positive cases were applied once. The studied parameters were not time related.

Frequency, prevalence, and its confidence interval (95% CI) were calculated online in Epitools (Sergeant, 2018) for each identified parasitic infection, overall and for single or mixed infection. All these parameters were calculated overallas well as for each age group (calves, juveniles, and adults), gender (females vs. males) and origin (household vs. farm) (Table 1). The differences recorded between groups were analysed through the chi-square test in MedCalc software version 19.1.3 (MedCalc Software bv, Ostend, Belgium; https://www.medcalc.org; 2019).

Risk factors were evaluated for those parasites with significant results in univariate analysis in MedCalc. Logistic regression model was used forage category, gender, and origin as independent variables. An independent variable was removed if p value was >0.1 and the model was considered if the overall model fit was significant.

The arithmetic mean and standard error of the mean were calculated for OPG and EPG. The normal distribution of data was checked and then, the differences in OPG and EPG values among different groups were evaluated using the non-parametric test Kruskal-Wallis (MedCalc software version 19.1.3).

A value of $p<0.05$ was considered statistically significant.

RESULTS

The prevalence of digestive parasites in buffaloes from North-West of Romania

Following coproparasitological examination, parasitic infections were recorded in 57.2% of buffaloes. The following parasitic elements were identified: *Eimeria* spp. oocysts (43.3%), *Buxtonella sulcata* cysts (1.7%), *Fasciola hepatica* eggs (4.4%), *Paramphistomum cervi* eggs (2.8%), *Moniezia* spp. eggs (0.6%), *Toxocara vitulorum* eggs (11.7%), strongyle-type eggs (3.3%), *Strongyloides papillosus* eggs (5.0%) and *Capillaria* spp. eggs (0.6%). The infections with *Eimeria* spp. (p = 0.0001) and *T. vitulorum* (p = 0.00006) were significantly more prevalent (Table 2) (Fig. 2).

Table 2. Frequency, prevalence and its 95% confidence interval for identified parasites in buffaloes (*n*=180) from North-West of Romania

Infections	Frequency	Prevalence (%)	95% CI	Age Mean in months (95% CI)
***Eimeria* spp.**	78	43.3***	36.3-50.6	11.7 (3.9-19.6)
B. sulcata	3	1.7	0.6-4.8	5.3 (1.5-9.1)
F. hepatica	8	4.4	2.3-8.5	93.0 (18.8-167.2)
P. cervi	5	2.8	1.2-6.3	52.8 (22.4-83.2)
***Moniezia* spp.**	1	0.6	0.1-3.1	2
T. vitulorum	21	11.7	7.8-17.2	5.7 (4.3-7.1)
Strongyles	6	3.3	1.5-7.1	15.0 (-8.2-38.2)
S. papillosus	9	5.0	2.7-9.2	6.8 (4.1-9.4)
***Capillaria* spp.**	1	0.6	0.1-3.1	60
Total	103	57.2	49.9-64.2	-

Chi-square *p< 0.05, **p< 0.01 and ***p< 0.001

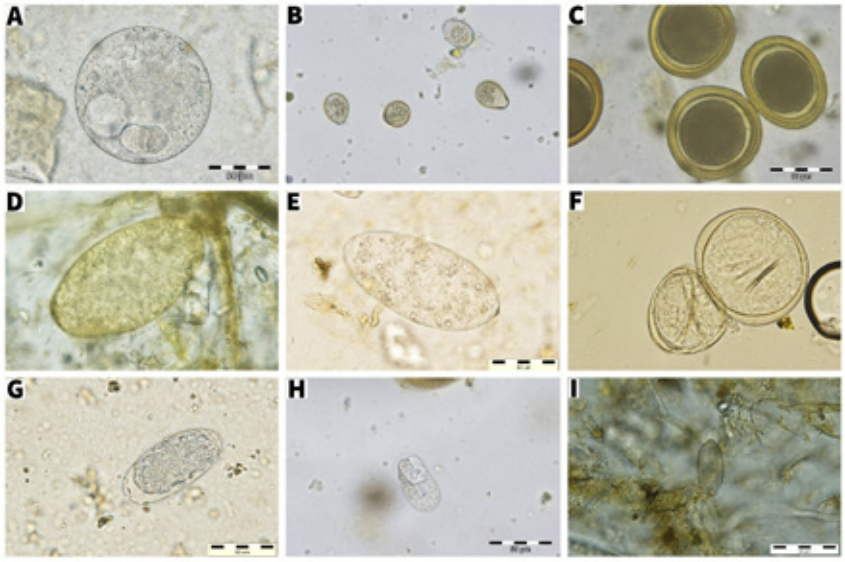

Figure 2. Parasitic elements identified in buffaloes from North-West of Romania
Legend: A - *B. sulcata* cyst; B - *Eimeria* spp. oocysts; C - *N. vitulorum* eggs; D - *F. hepatica* egg; E - *P. cervi* egg; F - *Moniezia spp.* eggs; G - strongyle egg; H - *S. papillosus* egg; I - *Capillaria* spp. egg.

The calves had a much higher prevalence of parasitic infections, than juvenile and adult buffaloes. Moreover, juveniles had a higher prevalence than adults. The infection with *Eimeria* spp. was the most prevalent in juveniles (59.6%) and in calves (57.2%) (p = 0.0001). In adults, the infection with *F. hepatica* had the highest prevalence (9.8 %) (p = 0.004) (Table 3).

The infection with *Eimeria* spp. was significantly more prevalent in males (56.9%) (p = 0.02) than in females (36.9%) (Table 3).

In both farm and household system, the infection with *Eimeria* spp. had the greatest prevalence, but without statistical significance (p>0.05). Only the infections with *Eimeria* spp. and *S. papillosus* were detected in samples from buffaloes that were raised on farm (Table 3).

Single species infections with *Eimeria* spp., *F. hepatica*, *P. cervi*, *Moniezia* spp., *T. vitulorum*, strongyles and *S. papillosus* were identified. The infection with *Eimeria* spp. was more prevalent than the other single infections (p = 0.0001) (Table 4).

The most frequent parasitic association was that of *Eimeria* spp. and *T. vitulorum* (7.2%; 95% CI 3.9-12.03). Mixed infection with *Eimeria* spp. and *S. Papillosus* was observed in 6 (3.3%) buffaloes. Mixed infection with *Eimeria* spp. and *F. hepatica* was detected in 2 (1.1%) buffaloes. Other parasitic associations identified were *Eimeria* spp. with digestive strongyles, *Eimeria* spp. with *Buxtonella sulcata*, *Eimeria* spp. with *Capillaria* spp. and *Eimeria* spp., *B. sulcata* and *T. vitulorum*, each in a single buffalo (0.6%).

Table 3. Frequency and prevalence [*n*(%)] of parasitic infections in buffaloes from North-West of Romania by age, gender and origin.

Infections	Age category			Gender		Origin	
	Calves	Juveniles	Adults	Females	Males	Household	Farm
	N=77	*N* = 52	*N* = 51	*N* =122	*N* = 58	*N* =160	*N* =20
***Eimeria* spp.**	44 (57.2)***	31 (59.6)***	3 (5.9)	45 (36.9)	33 (56.9)*	66 (41.3)	12 (60.0)
B. sulcata	2 (2.6)	1 (1.9)	0	2 (1.6)	1 (1.7)	3 (1.9)	0
F. hepatica	0	3(5.8)	5 (9.8)**	6 (4.9)	2 (3.4)	8 (5.0)	0
P. cervi	0	2 (3.9)	3 (5.9)*	4 (3.3)	1 (1.7)	5 (3.1)	0
***Moniezia* spp.**	1 (1.3)	0	0	1 (0.8)	0	1 (0.6)	0
T. vitulorum	14 (18.2)**	7 (13.5)**	0	13(10.7)	8 (13.8)	21 (13.1)	0
Strongyles	3 (3.9)	2 (3.9)	1 (2.0)	5 (4.1)	1 (1.7)	6 (3.8)	0
S. papillosus	4 (5.2)	5 (9.6)	0	7(5.7)	2 (3.4)	7 (4.4)	2 (10.0)
***Capillaria* spp.**	0	0	1 (1.96)	0	1 (2.0)	1 (0.6)	0
Total	53 (68.8)***	40 (76.9)***	10 (19.6)	65 (53.3)	38 (65.5)	91 (56.9)	12 (60.0)

Chi-square *p< 0.05,**p< 0.01 and ***p< 0.001

Table 4. Frequency and prevalence of single and mixed parasitic infections in buffaloes from North-West of Romania.

	Frequency	Prevalence (%)	95% CI
Single infections	**76**	**42.2**	**35.2-49.5**
Eimeria spp.	53	29.44	22.9-36.68
F. hepatica	5	2.78	0.91-6.36
P. cervi	4	2.22	0.61-5.59
Moniezia spp.	1	0.56	0.01-3.06
T. vitulorum	5	2.78	0.91-6.36
Strongyles	5	2.78	0.91-6.36
S. papillosus	3	1.67	0.35-4.79
Mixed infections	**27**	**15.0**	**10.5-20.9**
Eimeria spp. + *B. sulcata*	1	0.56	0.01-3.06
Eimeria spp. + *Fasciola* spp.	2	1.11	0.13-3.96
Eimeria spp. + *Capillaria* spp.	1	0.56	0.01-3.06
Eimeria spp. + *T. vitulorum*	13	7.22	3.9-12.03
Eimeria spp. + *S. papillosus*	6	3.33	1.23-7.11
Eimeria spp. + digestive strongyles	1	0.56	0.01-3.06
B.coli + *T. vitulorum*	1	0.56	0.01-3.06
F. hepatica+ *P. cervi*	1	0.56	0.01-3.06
Eimeria spp. + *B. coli* + *T. vitulorum*	1	0.56	0.01-3.06

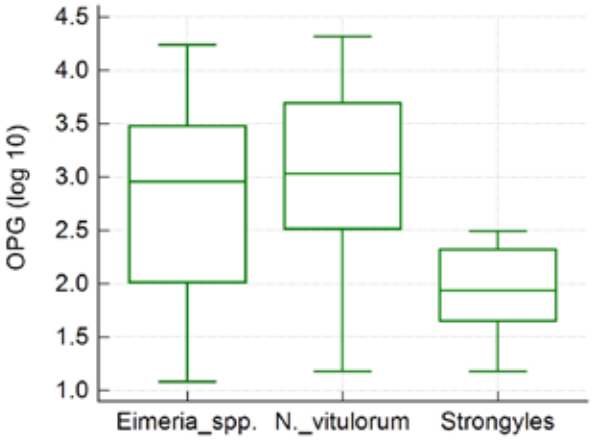

Figure 3. Mean number of parasitic elements/gram of faeces of *Eimeria* spp, *T. vitulorum* and digestive strongyles shed by buffaloes from North-West of Romania

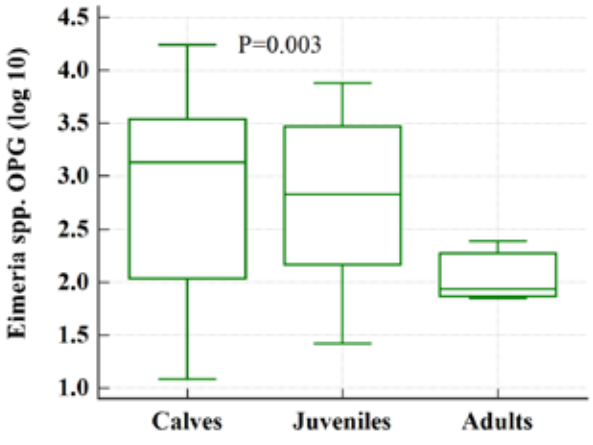

Figure 4. Mean number of parasitic elements/gram of faeces **of** *Eimeria* spp, shed by buffaloes from North-West of Romania by age category

OPG/EPG

The faecal egg and oocysts count per gram of faeces (EPG and OPG) are presented in Figure 3 and Figure 4: average (SE) *Eimeria* spp. 2176.6±395.1; *N. vitulorum* 4348.2 (±1643.9); strongyles 126.3 (±45.8). High values of *T. vitulorum* EPG and of *Eimeria* spp. OPG were recorded. *Eimeria* spp. OPG was significantly higher in calves (2679.8±631.8) and juveniles (1677.1±386.4) compared with adult buffaloes (132.7±54.9) ($p< 0.01$) (Fig. 3).

Risk factors

The age was identified as a risk factor for infection with digestive parasites (Table 3). The overall infection in young buffaloes was 2.7 (1.8-4.1) times more likely than in adult buffaloes. The same risk factor was identified for the infection with *Eimeria* spp. (young buffaloes OR 2.8; 95% CI: 1.8-4.3), *T. vitulorum* (young buffaloes OR 3.1; 95% CI: 1.5-6.5) and *F. hepatica* (adult buffaloes OR 3.8; 95% CI: 1.1-12.5).

DISCUSSION

In buffaloes, the parasites have a major economic impact through the high morbidity and mortality they cause. Thus, in order to design and apply effective surveillance programs, the prevalence of parasitic infections must be known (Sreedevi and Hafeez., 2014).

In the present study, the prevalence of internal parasites in buffaloes from North-Western Romania was 57.2%. In the study of Alam et al. (2016), 85.0% of examined animals were diagnosed with parasitic infections. Biswas et al. (2014) obtained a similar prevalence (84.9%). Jyoti et al. (2012) identified a prevalence of 73.6%, with coccidiosis the most prevalent infection detected.

Global prevalence of coccidiosis varies between 30 and 60%. The highest prevalence was reported in Iran (100%), Italy (100%) and Brazil (100%) (Bahrami and Alborzi, 2013; Fusco et al., 1997; Cringoli et al. 1998; Barbosa et al., 1992). In the present study, in buffaloes from North-West of Romania, the infection with *Eimeria* spp. had the highest prevalence (43.3%) from all detected infections, with significantly higher values in juveniles (59.6%) and calves (57.2%). In Brazil, a similar prevalence of coccidiosis was obtained (43.6%) (Rebouças et al., 1994). Coccidiosis mostly occurs in subclinical forms in bovines and its OPG value can vary considerably in different animals. An OPG greater than 1000 is considered as moderate infection, whilst in high infections the cattle shed more than 1 x 10^4 oocysts per gram of faeces (Koutny, 2012). In the present study the OPG of buffaloes was greater than 2 x 10^3, suggesting a moderate infection that can have an impact on health and productivity.

The data are scarce with respect to the infection with *B. sulcata*. Prevalence of *B. sulcata* infection was reported in Nepal (27.0%) and India (35.0%) (Adhikari et al., 2013; Kumar et al., 2017). In the present study *B. sulcata* was detected only in 1.7% of samples.

The prevalences of fasciolosis (4.4%) and paramphistomosis (2.8%) obtained in the present study are similar to other results obtained in Pakistan (4%), India (2.0%) and Italy (2.1%) (Bhutto et al., 2002; Condoleo et al., 2007; Sreedevi and Haffez, 2014).

Similar results in terms of prevalence of infection with *Capillaria* spp. and *Moniezia* spp. were obtained in studies conducted in Bangladesh (*Capillaria* spp.: 0.4%; *Moniezia* spp. :0.6%) (Biswas et al., 2014), Italy (*Moniezia* spp.: 0.2%) (Condoleo et al., 2007) and India (*Moniezia* spp.: 0.6%) (Sreedevi and Haffez, 2014).

Global prevalence of *T. vitulorum* infection varies between 1.4% and 33.0%. The highest prevalence was reported in Laos (25.5%), Egypt (28.4%) and Pakistan (33.0%) (Rast et al., 2012; Abdel-Rahman and El-Ashmawy, 2013; Bhutto et al., 2002), but the prevalence value in the present study (11.7%) was much lower than these values. A faecal egg count greater than 1000 is considered highly infectivefor livestock (Leahy, 2017). In the present study, the buffaloes shed more than 4000 *T. vitulorum* eggs per gram, suggesting that this parasitic infection was highly damaging. Moreover, the zoonotic potential of *T. vitulorum* remains to be clarified, as *Toxocara canis* and *Toxocara cati,* parasites of carnivores, are responsible for visceral larva migrans in humans (Leahy, 2017).

Literature data on infestation with *S. papillosus* and digestive strongyles are numerous and different prevalence have been reported depending on the age category studied and geographical area. The prevalence of *S.papillosus* infection from the present study (5.0%) is similar to that obtained in Pakistan (5.9%) (Azam et al., 2002).The prevalence of infection with digestive strongyles (3.3%) is similar with that obtained in India (5.0%) (Das et al., 2017).

In the present study, calves had a much higher infectionrate than juveniles and adults. Gunathilaka

found also that adults were less susceptible to parasitic infections, but the yearlings were more susceptible than calves (Gunathilaka et al., 2018).

The parasitism was higher in males than in females buffalo in the present study, but statistically significant only for the infection with *Eimeria* spp. Gunathilaka et al. observed a higher prevalence of gastro-intestinal parasites in males compared to females. According to these authors, this might be due to the attitude of farmers that focus more on female's health as they provide milk (Gunathilaka et al., 2018).

The prevalence of internal parasites was higher in buffaloes raised in household system because there is a permanent source of contamination (Radostits et al., 1994). Raising buffaloes on farm could be a method of prophylaxis that could lead to a decrease in the number of contaminated animals, and special attention should also be paid to deworming.

CONCLUSIONS

Internal parasites were detected in 57.2% of the samples of buffaloes from North-West of Romania. *Eimeria* spp., *B. sulcata*, *F. hepatica*, *P. cervi*, *Moniezia* spp., *T. vitulorum*, digestive strongyles, *S. papillosus*, and *Capillaria* spp. were the parasitic infections identified. The highest prevalence was recorded for *Eimeria* spp. and *T. vitulorum*. The buffaloes from households had greater infection rates. The calves had higher parasitic infection prevalence than juveniles and adults. Single infections were more prevalent than mixed infections.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Expressions and Distributions of Leucine-enkephalin, Delta, Mu, and Kappa Opioid Receptors in Four Systems of the Octopus, *Octopus ocellatus* by Comparative Immunohistochemical Method and ELISA

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ABSTRACT: We have investigated the expressions and distributions of leucine-enkephalin (Leu-enk), delta (δ), mu (μ), and kappa (κ) opioid receptors immunoreactivities in the respiratory, circulatory, excretory and reproductive systems of the Octopus, *Octopus ocellatus*, by comparative immunohistochemical method and enzyme-linked immunosorbent assay (ELISA). The results showed that δ opioid receptor and Leu-enk immunoreactivities were both detected in the branchia, ventricle, branchial heart, ovary and oviducal gland, while δ opioid receptor was additionally detected in the branchial gland and kidneys in less extend. The majority of the examined tissues presented weak immunoreactivities of δ opioid receptor and Leu-enk, with the notable exception of the white body and spermary tissues that were found negative. No labellings of μ and κ opioid receptors were observed in the respiratory, circulatory, excretory and reproductive systems of *O. ocellatus*. The combined results of δ opioid receptor and Leu-enk immunoreactivities indicated that they may be involved in the regulations of respiration, circulation, reproduction and endocrine together with other hormones or neurotransmitters in the *O. ocellatus*' body. The different densities of δ opioid receptor and Leu-enk in the respiratory, circulatory, excretory and reproductive systems of *O. ocellatus* may be related to the different functions.

Keywords: *O. ocellatus*, leucine-enkephalin, opioid receptors, respiratory system, circulatory system, excretory system, reproductive system

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INTRODUCTION

Among the family of endogenous opioid peptides, Enkephalin (Enk) is considered to play an important role in the regulations of cardiovascular, digestive, endocrine, and immune systems of animals (Eccles 1990; Makman 1994). Enk mainly includes leucine-enkephalin (Leu-enk), methionine-enkephalin (Met-enk), met-enkephalin-Arg^6-Gly^7-Leu^8, met-enkephalin-Arg^6-Phe^7, and is widely distributed in the nervous system of higher animals. So far, three kinds of opioid receptors have been identified in mollusk and higher animals, namely delta (δ), mu (μ) and kappa (κ) opioid receptors. Delta opioid receptor is involved in the analgesic effect of the endogenous opioid peptides of spinal cord, and participates in the regulations of emotion, incretion and cardioprotective effect through ischemic preconditioning. Because of the strong interaction with δ opioid receptor, Enk is considered to be the endogenous ligand of the receptor (Stefano et al. 1993a; Sha et al. 2012).

The opioid receptors and Enk have been found in mollusk. First reports originated from Stefano and Catapane (1979), who investigated the regulation of dopamine levels in the central nervous system (CNS) of *Mytilus edulis* by exogenous opioids. Then, Leung and Stefano (1984) isolated and sequenced Leu-enk and Met-enk from the pedal ganglia of *M. edulis*, and sequential amino acid analysis showed that these peptides share the same primary structure as that of vertebrates. The above-mentioned authors purified a heptapeptide from these fractions of invertebrates by high-pressure liquid chromatography under isocratic conditions. Sequential amino acid analysis demonstrated that this heptapeptide share the same primary structure as the met-enkephalin-Arg^6-Phe^7 of invertebrates. In addition, Stefano et al. (1989, 1996) have shown that there were two kinds of δ opioid receptors in the immunocytes and the nervous tissue of *M. edulis*, those are δ-1 and δ-2. Moreover, the participation of δ, μ and κ opioid receptors has been demonstrated in the digestive regulation of *Limax maximus* (Kavaliers et al. 1986), as well as in pain modulation of *Cepaea nemoralis* (Thomas 1997). More recently, Cadet (2004) have detected the μ transcripts from the pedal ganglia of *M. edulis*. These results indicate that invertebrates such as *M. edulis* possess enkephalinergic systems similar to those found in higher organisms.

In our previous study, we identified the presence of δ opioid receptor and Leu-enk in mantles and feet of *O. ocellatus*, using immunohistochemical techniques (Sha et al. 2012). The aim of the present study was to investigate the expressions and distributions of Leu-enk, δ, μ, and κ opioid receptors immunoreactivities in the respiratory, circulatory, excretory and reproductive systems of the Octopus, *Octopus ocellatus*. The results will provide baseline information for future investigation regarding the roles of Leu-enk and opioid receptors of δ, μ, and κ in the respiratory, circulatory, excretory and reproductive systems of adult mollusk.

MATERIALS AND METHODS

All mentioned procedures were carried out in agreement with Chinese legislation on experimental animals, after approval by the Ethic-Scientific Committee for Experiments on Animals of Chongqing Three Gorges University.

Experimental Animals

A batch of adult male and female Octopuses, *O. ocellatus* (n = 10 for each sex), ranging from 15 to 21 cm in length, were purchased from a commercial farm in Yantai (China). They were kept for 3 days in large tanks with aerated circulating seawater maintained at 20-21°C and fed ad libitum with living crabs and shrimps.

Preparation of the Respiratory, Circulatory, Excretory and Reproductive Systems Tissues in Immunohistochemistry

The tissues of branchia, branchial gland, ventricle, white body, branchial heart, kidneys, ovary, oviducal gland and spermary were dissected and fixed for 5-7 h in a solution of 4% paraformaldehyde (the chemicals of preparation part were purchased from Sinopharm Chemical Reagent Co., Ltd., China). The tissues were washed three times in 0.05 M phosphate-buffered saline (PBS, pH 7.4, 2% sodium chloride) every 30 minutes. Cryostat sections (20 μm thick) of the tissues were placed on chromalum-gelatin-coated glass slides and rehydrated in 0.05 M PBS containing 0.3% Triton X-100 (PBST, pH 7.4). After dried, the frozen sections were stored in the refrigerator at 4°C.

Immunohistochemistry

The immunohistochemical SABC reaction program was carried out according to our previous studies (Sha et al. 2012, 2013). The sections were incubated with rabbit anti-mouse Leu-enk, δ, μ, or κ opioid receptors (Sigma Chemical Co., USA, diluted 1:400

in blocking buffer), washed with PBST (Sinopharm Chemical Reagent Co., Ltd., China), then incubated with goat anti-rabbit IgG (Santa cruz Biotechnology, Inc., CA, USA, diluted 1:200) and streptavidin biotin peroxidase complex protein (AB composite liquid, Santa Cruz Biotechnology, Inc., CA, USA) in turn. Finally, the reaction was visualized using the diaminobenzidine (DAB, Fluka Chemie AG, Switzerland), and the nuclei were counterstained with hematoxylin (Sigma Chemical Co., USA).

Imaging

The slides were observed under a BX50 Light Microscope (Olympus Corporation, Japan) and digital images were obtained using a DP70 Camera System (Olympus Corporation, Japan) and processed by Adobe Photoshop CS (Adobe Systems Inc., CA, USA). The immunoreactivities of Leu-enk, δ, μ, and κ opioid receptors were based on the appearance of brownish-red staining corresponding to the presence of DAB deposition in the tissues. The nuclei was blue, the cytoplasm and background were not colored or yellowish.

Preparation of the Branchia, Ventricle, Branchial Heart and Ovary Tissues in Enzyme Linked Immunosorbent Assay (ELISA)

The tissues of branchia, ventricle, branchial heart and ovary were collected postmortem *O. ocellatus* and washed with precooled PBS (0.01M, pH = 7.4), in order to remove the residual blood, and final tissues were harvested after weight measuring. The collected tissues were sliced and placed in 9-fold PBS. After adding protease inhibitor (Sigma Chemical Co., USA), 10% tissue homogenate was prepared with ice water bath homogenate for 10min. Finally, the homogenate was centrifuged at 5000 rpm for 8 min, and the supernatant was collected for detection.

ELISA

ELISA was performed according to the Leu-enk ELISA kit instruction provided by Shanghai Jingkang Bioengineering Co., Ltd, Shanghai, China. Stop solution was added to each well for 15 min and the OD value was measured at 450nm using an iMark Microplate Reader (Bio-Rad, USA). Then, the OD value of the sample was substituted into the linear regression equation, and the Leu-enk concentration of the sample was calculated.

RESULTS

Immunohistochemistry

Distributions of Mu and Kappa Opioid Receptors Immunoreactivities in the Respiratory, Circulatory, Excretory and Reproductive Systems

No labellings of μ and κ opioid receptors were observed in the respiratory, circulatory, excretory and reproductive systems of *O. ocellatus*.

Distributions of Leu-enk and Delta Opioid Receptor Immunoreactivities in the Respiratory System

Branchia

The branchia lies in the anterior part of the mantle, composed of a pair of feathery gills. The branchia comprises of the mantle cavity and three types of tissues: a single layer of respiratory epithelium lying atop a dense irregular connective tissue layer and the adjacent inner layer consists of muscle tissue. The results indicated that individual immunopositive cells of Leu-enk and δ opioid receptor were revealed in the respiratory epithelium and connective tissue of the branchia (Fig. 1-A, 2-A, 2-B).

Branchial Gland

The vascular branchial gland is a pair of adjacent glands of the branchia, which extends along the entire dorsal gill's length, without presenting ducts and being quite separate from the tissue of the gills. And each, suspended from the overlying mantle and closely attached to the dorsal surface of the gills, is contained in a capsule consisting of an external epithelial layer of columnar cells upon connective tissue which is well vascularized, and contains longitudinal and oblique muscle fibres. There was no Leu-enk immunoreactivity in the branchial gland (Fig. 1-B). Weak immunoreactivity of δ opioid receptor was detected in the apical part of the columnar epithelial cells and connective tissue of the branchial gland (Fig. 2-C, 2-D).

Distributions of Leu-enk and Delta Opioid Receptor Immunoreactivities in the Circulatory System

Ventricle

The flavescent orbicular-ovate ventricle lies between the two atria, the structure of the ventricle comprises of three layers: a single layer of flattened epithelium lying atop a dense irregular connective tissue layer and the adjacent inner layer consists of myocardium. The results indicated that a small amount of δ opioid receptor and Leu-enk immunoreactive ma-

terial was observed in the epithelium and connective tissues (Fig. 1-C, 2-E). The muscle tissue was found immunonegative for both δ opioid receptor and Leu-enk.

White Body

The white body is a pair of multilobed organs, attached to the medial external surface of each eye and is responsible for hemocyte production by *O. ocellatus*. No labellings of δ opioid receptor and Leu-enk were observed in the white body (Fig. 1-D).

Branchial Heart

The flavescens saccate branchial heart lies at the end of the branchia, the efferent vein leaves the branchial gland to enter the branchial heart, whence the blood passes via the gills to the systemic heart. The structure of the branchial heart comprises of three layers: epithelial tissue, connective tissue and muscle. There were weak stained granules of δ opioid receptor and Leu-enk in the connective tissue of the branchial heart (Fig. 1-E, 2-F). No immunoreactivities were seen in the epithelium and muscle tissues. The epithelial and muscle tissues of the branchial heart were found immunonegative for both δ opioid receptor and Leu-enk.

Distributions of Leu-enk and Delta Opioid Receptor Immunoreactivities in the Excretory System

Kidneys

The renal appendage is a gland-like, highly branched structure protruding in the renal sac. They are formed of continuous sheets covered of two layers of cuboid epithelial cells separated by blood sinuses derived of the vena cava system (Budelmann et al. 1997). No Leu-enk immunopositivy was detected in the kidneys (Fig. 1-F), but weak immunoreactivity of δ opioid receptor was presented in the connective tissue of the kidneys (Fig. 2-G).

Distributions of Leu-enk and Delta Opioid Receptor Immunoreactivities in the Reproductive System

O. ocellatus is dioecious animal. The female reproductive system consists of a single ovary with two oviducts opening on either side of the mantle cavity. The male reproductive system consists of an unpaired testis and a duct opening into the left side of the mantle cavity. The duct is composed of the vas deferens proximal to the testis, followed by the first spermatophoric gland (seminal vesicle), the second spermatophoric gland (prostate), the Needham's sac and the penis.

Ovary

The ovary is located at the posterior part of the visceral mass, which was formed by the epithelial development of the body cavity. The structure of the ovary comprises of two layers: epithelial tissue and connective tissue. The results indicated that weak Leu-enk's immunoreactivity was revealed in the connective tissue of the ovary, and weak immunoreactivity of δ opioid receptor in the epithelial tissue of the ovary (Fig. 1-G, 2-H).

Oviducal Gland

The oviducal gland is the enlarged part at the bottom of the fallopian tube, which is about half the size of the fallopian tube. It is a compact ovoid-shaped gland that exhibits two different sectors, an outer sector and an inner one, and it is covered with a thin connective-muscular capsule. The gland appears organized in compact lobes of branched glandular epithelial tubules, separated by thin connective septa. Diffuse Leu-enk immunoreactivity was scattered in the gland ciliary cells of central gland (Fig. 1-H) and general or weak immunoreactivity of δ opioid receptor was detected in the gland cells of peripheral gland's epithelial tissue of the oviducal gland (Fig. 2-I), the other examined tissues were found immunonegative.

Spermary

The spermary lies in back of the visceral mass, which was also formed by the epithelial development of the body cavity. The spermary was found immunonegative for both δ opioid receptor and Leu-enk (Fig. 1-I).

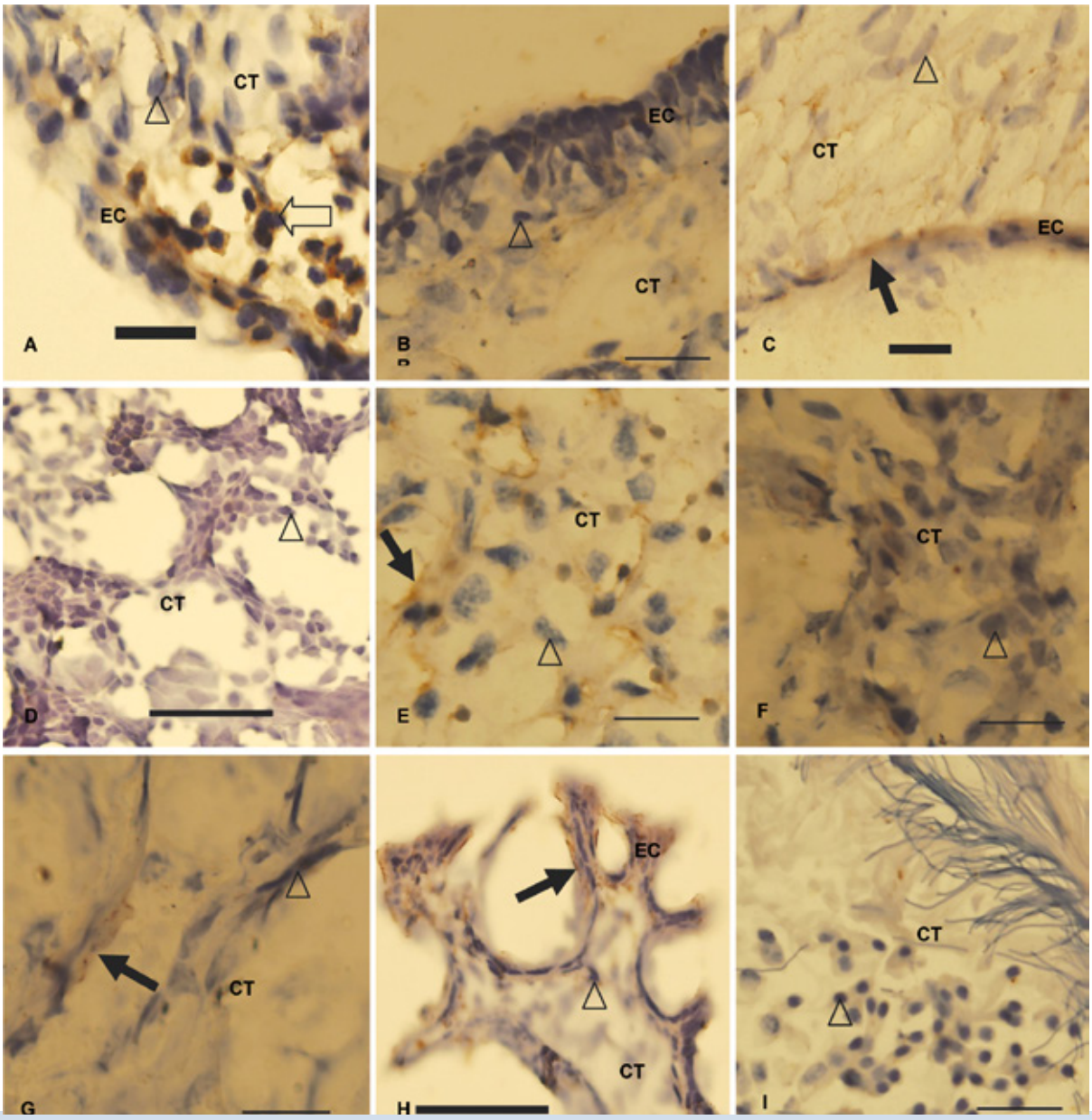

Fig.1 Distribution of Leu-enk Immunoreactivity in the respiratory, circulatory, excretory and reproductive systems

A. Distribution of Leu-enk Immunoreactivity in the branchia; B. Distribution of Leu-enk Immunoreactivity in the branchial gland; C. Distribution of Leu-enk Immunoreactivity in the ventricle; D. Distribution of Leu-enk Immunoreactivity in the white body; E. Distribution of Leu-enk Immunoreactivity in the branchial heart; F. Distribution of Leu-enk Immunoreactivity in the kidneys; G. Distribution of Leu-enk Immunoreactivity in the ovary; H. Distribution of Leu-enk Immunoreactivity in the oviducal gland; I. Distribution of Leu-enk Immunoreactivity in the spermary. Scale bars in D and H=50μm, the others=20μm.
Black arrows-positive Leu-enk granules; white arrows-positive cells; open triangles-karyon; EC-epithelium; CT-connective tissue.

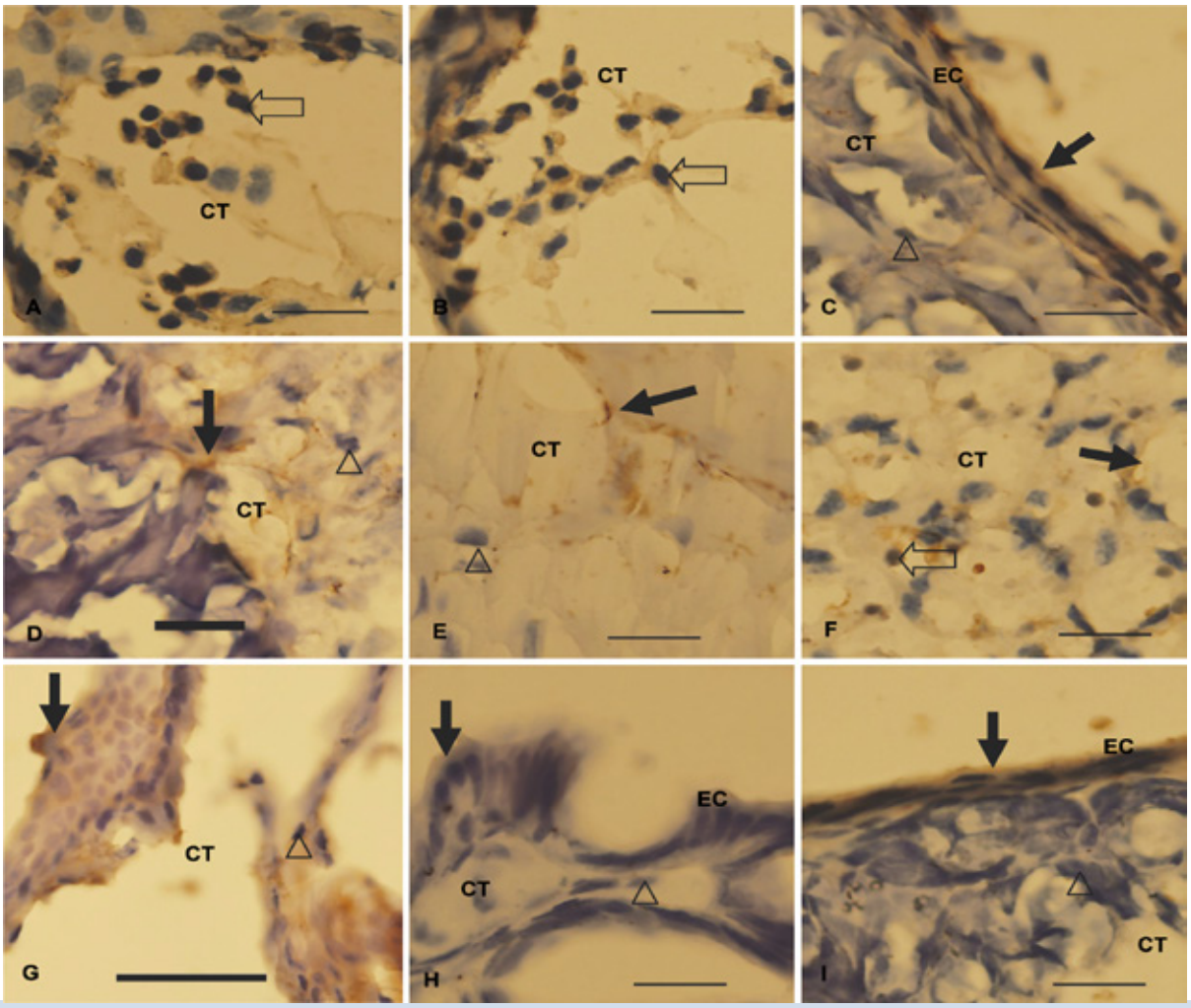

Fig.2 Distribution of delta opioid receptor Immunoreactivity in the respiratory, circulatory, excretory and reproductive systems
A~B. Distribution of delta opioid receptor Immunoreactivity in the branchia; C~D. Distribution of delta opioid receptor Immunoreactivity in the branchial gland; E. Distribution of delta opioid receptor Immunoreactivity in the ventricle; F. Distribution of delta opioid receptor Immunoreactivity in the branchial heart; G. Distribution of delta opioid receptor Immunoreactivity in the kidneys; H. Distribution of delta opioid receptor Immunoreactivity in the ovary; I. Distribution of delta opioid receptor Immunoreactivity in the oviducal gland. Scale bars in G=50μm, the others=20μm.
Black arrows-positive delta opioid receptor granules; white arrows-positive cells; open triangles-karyon; EC-epithelium; CT-connective tissue.

Table 1 Expression of Leu-enk in the Branchia, Ventricle, Branchial Heart and Ovary by ELISA ($\bar{\chi}$±s, n=9)

Tissues	Branchia	Ventricle	Branchial Heart	Ovary
Leu-enk concentration (pg/ml)	8.26±0.94	1.20±0.18	3.15±0.41	0.69±0.07

Expression of Leu-enk in the Branchia, Ventricle, Branchial Heart and Ovary by ELISA

As shown in Table 1, the highest concentration of Leu-enk was located at branchia (8.26±0.94 pg/ml), followed by branchial heart (3.15±0.41 pg/ml). The above mentionned organs are both related to the respiration of *O. ocellatus*. On the contrary, Leu-enk concentration was very low in both ventricles (1.20±0.18 pg/ml) and ovaries (0.69±0.07 pg/ml).

DISCUSSION

Enk and opioid receptors have been observed in the circulatory system of mollusk. Stefano and Catapane (1979) have detected the leu-enkephalin-like and the met-enkephalin-like in the pericardial cavity of *M. edulis*. Using a highly specific antibody, Martin et al. (1979) detected immunoreactivity of met-enkephalin-like in large granules of numerous distinct cells that are embedded in a layer of secretory terminals

inside the vena cava of *Octopus variabilis*. Using indirect immunocytochemistry, Ewadinger et al. (1996) found immunoreactive individual cells and cell clusters of met-enkephalin-like in the CNS, as well as to fibers in the atrium of the heart of the freshwater snail *Lymnaea stagnalis*. Using immunohistochemistry, the immunoreactivities of leu-enkephalin-like, δ, μ, and κ opioid receptors were localized in haemolymph of the scallop *Chlamys farreri* (Liu 2008; Liu and Sun 2010). In our study, the ELISA results indicated the presence of Leu-enk in the ventricle (1.20±0.18 pg/ml) and branchial heart (3.15±0.41 pg/ml) of *O. ocellatus*. In addition, the immunohistochemical method localized both δ opioid receptor and Leu-enk in the epithelium and connective tissue of ventricle, as well as in the connective tissue of branchial heart. However, the muscle tissue was found immunonegative for both δ opioid receptor and Leu-enk, suggesting that further studies are required in order to clarify their contribution in the regulation of the *O. ocellatus*' spontaneous rhythmicity and myocardial contractility.

Mantione et al. (2006) found that morphine regulated gill ciliary activity via binding with μ3 opiate receptor and nitric oxide released in *M. edulis*. The results of Liu and Sun (2010) demonstrated that μ, δ, and κ opioid-like receptors were present in the central axis and gill filaments of the scallop *C. farreri*, indicating that endogenous opioid peptides and receptors may play a significant role on controlling of ciliary activity of the scallop. Our results are consistent with the above results. In our study, Leu-enk was detected using ELISA method in the branchia (8.26±0.94 pg/ml) and branchial heart (3.15±0.41 pg/ml) of *O. ocellatus*. Moreover, the immunohistochemical method localized both δ opioid receptor and Leu-enk in the respiratory epithelium and connective tissue of branchia, as well as in the connective tissue of branchial heart. Our results are further supporting the role of Leu-enk via coupling with δ opioid receptor on controlling of ciliary activity, herein observed in the *O. ocellatus*.

In the present study, Leu-enk was detected in the ovary using ELISA and immunohistochemical method. In addition, δ opioid receptor immunopositivity was also observed in the epithelial tissue of the ovary of *O. ocellatus*. Moreover, δ opioid receptor and Leu-enk immunoreactivity were also localized in the gland cells of the oviducal gland. Since there are no available data regarding Leu-enk and opioid receptors in the reproductive system of cephalopods, the present study herein demonstrates their presence, suggesting that Leu-enk may play a weak role in reproduction of *O. ocellatus*, via coupling to δ opioid receptor. Further studies are required about the specific role and mechanism of δ opioid receptor and Leu-enk in the ovary and oviducal gland of *O. ocellatus*, and whether Leu-enk and opioid receptors are presented in the reproductive system of other cephalopods. The densities of δ opioid receptor and Leu-enk may be related to the physiological functions in different parts of *O. ocellatus*, awaiting further study in future. The absence of immunoreactivities of κ and μ opioid receptors in the respiratory, circulatory, excretory and reproductive systems of *O. ocellatus*, indicates that Enk has weak μ and κ opioid receptors selectivity and further supports its consideration as the endogenous ligand of δ opioid receptor.

Endogenous opioid peptides have been shown to be involved in the mobilization, directed movement, adherence of immunoreactive cells and in several related immune processes via coupling to opioid receptors. There is evidence for the presence of opioids in lymphoid cells of *M. edulis* and human and for their release into the circulation in response to stress (Hughes et al. 1991; Stefano et al. 1990, 1993b, 1995). In invertebrates, these same peptides induce chemotaxis and the release of mammalianlike cytokines, including tumor necrosis factor-α and interleukin-1, -6 (Stefano et al. 1991a, 1991b; Osman et al. 2003). By combining with δ opioid receptor, Enk could up-regulated the immunity of the mollusk (Stefano et al. 1993a). In our previous study, we found that the immunoreactivities of δ opioid receptor and Leu-enk were presented in the mantles and feet of *O. ocellatus* (Sha et al. 2012). The mantles and feet, besides their relation to animal movement, are also related to mucosal immunity and external defense of *O. ocellatus*, since they are in direct contact with the external environment (Wang et al. 2005). In the present study, the immunoreactivities of δ opioid receptor and Leu-enk were additionally presented in the branchia and branchial heart of *O. ocellatus*. These results may indicate that the δ opioid receptor and Leu-enk in these regions could also participate in the mucosal immunity, mucus secretion, and external defense of *O. ocellatus*, since these organs are also in direct contact with seawater.

In conclusion, the results showed that δ opioid receptor and Leu-enk immunoreactivities were both detected in the branchia, ventricle, branchial heart,

ovary and oviducal gland, while δ opioid receptor was additionally detected in the branchial gland and kidneys. The majority of the examined tissues presented weak immunoreactivities of δ opioid receptor and Leu-enk and the quantitative comparison results of Leu-enk ELISA in branchia, ventricle, branchial heart and ovary were consistent with the results of immunohistochemistry. The combined results of δ opioid receptor and Leu-enk immunoreactivities indicated that they may be involved in the regulations of respiration, circulation, reproduction and endocrine likely along with other hormones or neurotransmitters in the *O. ocellatus*' body.

CONFLICT OF INTEREST

The authors have declared that there were no conflicts of interest.

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Research article
Ερευνητικό άρθρο

Isolation and antimicrobial resistance of vancomycin resistant *Enterococcus* spp. (VRE) and methicillin-resistant *S. aureus* (MRSA) on beef and chicken meat, and workers hands from slaughterhouses and retail shops in Turkey

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ABSTRACT: The objectives of this study were to determine the presence and antimicrobial resistance of Methicillin Resistant *Staphylococcus aureus* (MRSA) and Vancomycin Resistant Enterococci (VRE) on beef and chicken carcasses and meat, and workers hands' at processing time from a cattle and a poultry slaughterhouse, and beef and chicken meat at retail level. Disk diffusion method was used to determine the antimicrobial resistance profile of the *Enterococcus* spp. and *S. aureus* isolates. Minimum Inhibitory Concentration (MIC) values were determined for vancomycin and oxacillin resistance. Finally, conventional PCR was performed to determine the presence of the *mec*A and *van*A resistance genes in isolates classified resistant to oxacillin and vancomycin according to MIC values. *S. aureus* and *Enterococcus faecium* isolated from 17 (17%) and eight (8%) samples, respectively. *E. faecalis* was not detected in any sample. The highest resistance rates were to ampicillin (3/5, 60 %) and penicillin G (5/5, 100 %) in MRSA and tetracycline (4/5, 80 %) in VRE isolates. While the *mec*A gene was detected in all MRSA isolates, *van*A gene was not detected in any of the phenotypically vancomycin resistant *E. faecium* isolates. The present study provides data for multiple antimicrobial resistance and presence of VRE and MRSA isolated from an ongoing surveillance in humans, livestock and poultry in Turkey.

Keywords: MRSA, VRE, chicken, beef, slaughterhouse, workers

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INTRODUCTION

Staphylococcus aureus is a well known foodborne bacterial pathogen related to foodborne intoxications (Peacock and Paterson, 2015; Haaber et al., 2017). Food handler carriers of enterotoxin-producing *S.aureus* are regarded as the main contamination source of food, via direct manual contact or respiratory secretions. The emergency of *S. aureus* in recent decades, especially the relation between livestock is required to highlight the contamination ways through the food chain starting from slaughtering of animals. Some strains have virulence characteristics which ensure their adaption to different environmental conditions, causing various life-threatening infections and gaining antibiotic resistance (Lowy, 2003). The emergence of methicillin resistance in *S. aureus* strains has become a serious international concern in the treatment and control of Staphylococcal infections. There are several studies reported the presence of MRSA on meat-producing animals including beef and chicken. The scientific report of EFSA (2015), declared that food-producing animals may be contaminated with methicillin-resistant *S. aureus* (MRSA) due to close contact with livestock or by foods of animal origin and lead human illnesses. Unlike penicillinase-related resistance, methicillin resistance affects a broad spectrum of antibiotics, such as the β-lactams, which includes penicillins, cephalosporins, and carbapenems (Chambers and DeLeo, 2009).

Enterococcus species are part of the normal microbiota of humans and warm-blooded animals. Enterococci are found in many foods of animal origin and are able to survive for long time on inanimate surfaces because of their ability to survive in adverse environmental conditions. The most common species identified in food animals are *E. faecium, E. cecorum, E. faecalis* and *E. hirae* (Ahmed and Baptiste, 2018).

Vancomycin-resistant enterococci (VRE) have been an increasing problem worldwide since VRE were first identified in 1980s and *van*A-type VRE was first reported in 1993. The use of avoparcin, a vancomycin analogue as a growth-promoting feed additive, has been linked to an increase in vancomycin-resistant enterococci in food animals (Birkegard et al., 2019). However, 25 years after the ban of avoparcin as a growth promoter in feed, a continuing resistance has been observed to vancomycin in a Danish pig farm (Birkegard et al., 2019). There are different vancomycin resistance mechanisms including acquired resistance (eg. *van*A, *van*B, *van*D, *van*E, *van*G and *van*L) and intrinsic resistance (*van*C in *E. gallinarum* and *E. casseliflavus* / flavescens species) (Çetinkaya et al., 2013). Although *van*A is responsible for the most cases of vancomycin-resistant *Enterococcus* (VRE) in the world, *van*B is emerging in recent years (O'Driscoll and Crank, 2015). Food producing animals carrying VRE have been regarded as a probable source of VRE infections in humans. Besides, there are several studies on the presence of VRE on chicken carcasses and commercial meat products indicates the VRE contamination risk via the food chain.

The aim of this study was to determine the presence and antimicrobial resistance of VRE and MRSA on beef and chicken carcasses, workers' hand surfaces in slaughterhouses and beef and chicken meats at retail.

MATERIAL AND METHODS

Sample collection

Samples (n = 100) were collected between February 2018 and March 2019. The carcass excision (n=10 for each) and the swab samples (n=10 for each) from the brisket of beef cattle and wings of chicken at the pre-chilling stage and workers hands' surfaces (n=10 poultry, n=10 cattle slaughterhouses) were collected at processing time. The samples were obtained from a vertically integrated commercial poultry slaughterhouse where more than 1,000,000 poultry are slaughtered and sold in a year and a large-scale cattle slaughterhouse (with a daily capacity of at least 40 cattle, according to the classification of Turkish slaughterhouses). Retail beef (n=20) and chicken (n=20) meat samples were also purchased from different retailers: 20 samples from 9 modern butcher shops, 12 samples from 8 supermarkets and 8 samples from 6 districts retailers. Swab sampling was performed by modifiying the swabbing methods described by Arthur et al. (2004) and Gill et al. (2005) with slight modification. Accordingly, the cotton swabs (Lp Italiana, Italy) moistened with sterile Buffered Peptone Water (BPW) were used to cover an area of 10x10 cm (5 horizontal and 5 vertical passes). Carcass excision samples were taken from brisket of beef cattle and wing of chicken by cutting an area of approximately 5 cm^2 and 2.5 cm^2, respectively (Fromm 1959, Pearce and Bolton 2005). The samples were excised using a sterile scalpel and a sterile forceps and then placed into the sterile stomacher bags. Carcass samples of beef cattle and chicken were taken at the post-intervention stage. The hand surface samples of slaughter-

house workers' were voluntarily taken by swabbing the palm of the right hand as described by Sammarco et al., (1997). The collected samples were immediately transported to the laboratory in a cool box containing ice cubes and analyzed within 2 h.

Isolation and identification of *S.aureus, E. faecium* and *E. faecalis*

Isolation of *S. aureus* was performed in accordance to the procedure for the identification of *S. aureus* in animal feed and food published by the International Organization for Standardization (ISO 6888-3: 2003). Accordingly, the excision samples, and 25 g of retail samples weighed into sterile stomacher bags (VWR, 432-3123) were suspended in 100 ml and 225 ml of BPW, respectively and stirred in a stomacher (Interscience, France) then transferred to sterile glass pyrex bottles. After pre-enrichment at 35 ± 2 º C overnight, 100 µl volume of broth and the swab samples were streaked on to the Baird Parker Agar (Merck 105406) containing 5% Egg Yolk Tellurite (Oxoid, SR0054) and Mannitol Salt Agar (Oxoid, CM 0085) and incubated at 37 ºC for 24-48 hours under aerobic conditions. The suspected colonies were evaluated for gram staining, catalase, oxidase, coagulase and then API Staph (Biomerieux, Ref. 20500) test kit was used for identification of the isolates.

Isolation and identification of *E. faecium* and *E. faecalis* was performed as reported by Klein et al. (1998) with modification. Briefly, the homogenate was prepared as mentioned in *S. aureus* isolation. Then, 100 µl of the homogenate was streaked onto Slanetz Bartley Agar (Oxoid CM 0377) and incubated at 35ºC for 24-48 hours. After the incubation, five suspicious colonies were selected and subcultured onto Bile Esculin (BEA) Agar (Oxoid CM 0888) to discern Enterococci based on it's potential to hydrolyze esculin then incubated at 35 ºC for 24 hours. Colonies with bright black, round, convex shape grown on BEA were considered as suspected *Enterococcus* spp.. Gram staining, catalase, growth in 6.5% NaCl Brain Heart Infusion Broth (BHI, Merck 110493), L-pyrrolidonyl arylamidase activity, motility, hemolysis and API 20 Strep (Biomerieux, Ref. 20600) test were performed to typical colonies.

Antimicrobial susceptibility test

Antimicrobial susceptibility of *E. faecium* isolates to 10 antimicrobials [amikacin (AK-30 µg, Oxoid CT0107B) ampicillin (AMP-10 µg, Oxoid, CT 0003B), erythromycin (E-15 µg, Oxoid, CT 0020B), gentamicin (CN-120 µg, Oxoid, CT 0794B), chloramphenicol (C-30 µg, Oxoid, CT 0013B), penicillin G (10 units, Oxoid, CT 0043B), ciproflaxocin (CIP-5 µg, Oxoid, CT 0425B), streptomycin (S-300 µg, CT 1897B), teicoplanin (Oxoid-CT 0647B-TEC 30 gg), tetracycline (TE-30 µg, Oxoid, CT 0054B)] and *S. aureus* isolates to 11 antimicrobials [amikacin (AK-30 µg, Oxoid CT0107B), amoxicillin / clavulanic acid (AMC-30 µg, Oxoid, CT 2223B), ampicillin (AMP-10 µg, Oxoid, CT 0003B), erythromycin (E-15 µg, Oxoid, CT 0020B), gentamicin (CN-120 µg, Oxoid, CT 0794B), chloramphenicol (C-30 µg, Oxoid, CT 0013B), penicillin G (10 units, Oxoid, CT 0043B), ciproflaxocin (CIP-5 µg, Oxoid, CT 0425B), clindamycin (DA-10 µg, Oxoid CT0015B), (tetracycline (TE-30 µg, Oxoid, CT 0054B), trimethoprim / sulfamethaxol (SXT-25 µg, Oxoid, CT 0052B)] were determined by disk diffusion method. Accordingly, the isolates were cultured in Mueller Hinton Broth (MHB, Oxoid, CM0405) and the optical density was adjusted to 0.5 Mc Farland (DEN-1B McFarland Densitometer). The broth culture was streaked on to the surface of Mueller Hinton Agar (MHA, Oxoid, CM0337) using a sterile cotton swab. The antibiotic disks were placed on top of the agar surface with sterile forceps. Inhibition zone diameters were measured and evaluated according to the antimicrobial susceptibility testing procedure reported by the Clinical Laboratory Standards Institute (CLSI, 2020) for ampicillin, teicoplanin, tetracyclin, penicillin, erythromycin and chloramphenicol in Enterococcus isolates. The other antibiotics in *Enterococcus* spp. and all the *S. aureus* isolates were evaluated according to European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2020). *E. faecalis* ATCC 29212, *S. aureus* ATCC 25923, *S.aureus* ATCC 29213 were used as the control strains.

Determination of MICs to oxacillin and vancomycin

Minimum Inhibition Concentration (MIC) values to vancomycin (Carbosynth, FV11352) and oxacillin (Carbosynth, AO61591) were determined using microdilution method. Accordingly, antibiotic dilutions were prepared at 10 ml volumes in tubes, diluted at 1/1, 1/2,1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512 µg/ml concentrations and then dispensed into the ELISA microplate wells as 100 µl. The bacterial suspensions were adjusted to 0.5 McFarland turbidity standard in MHA tubes (supplemented with 2 % w/v NaCl for *S. aureus* isolates). The suspensions were di-

luted 1:20 and added as 10 µl to each well to yield the concentration approximately 5 x 10^5 CFU / ml. The inoculated microplates were covered and incubated for 24 h at 35 ± 2 ºC under aerobic conditions. The microplates were read on a Spectrophotometric Elisa Reader (MWGt Lambda Scan 200, Bio-Tek Instruments, Winooski, VT, USA) at 600 nm. MIC values were evaluated according to CLSI (2020), and EUCAST (2020).

Determination of the *mec*A and *van*A genes

DNA extraction

DNA extraction was performed according to the manufacturer's instructions with DNeasy PowerFood Microbial Kit (Qiagen, 21000-100) to the isolates that optical density was adjusted to the Mc Farland 4 in Tryptone Soy Broth.

PCR Mix

A commercially available PCR mix (MyTaq PCR Premix) that consisted of DNA polymerase, dNTP set, reaction liquid, $MgCl_2$, stabilizer and tracking dye was used for the PCR assay. Volumes of 2.5 µl template DNA and 1 µl forward and reverse primers were included to the reaction mix and the total volume was adjusted to 25 µl with nuclease free water (Sigma-Aldrich, LSKNF0500, Germany).

Primers

Forward (5'AAA ATC GAT GGT AAA GGT TGG C 3') and reverse (5'AGT TCT GCA GTA CCG GAT TTG C 3') *mec*A primers were used according to Murakami et al. (1991) to detect the *mec*A gene. Both primers amplify a region of 533 bp length. Primer sequences of *van*A Forward (5'-CAT GAA TAG AAT AAA AGT TGC AAT A 3') and *van*A Reverse (5'-CCCCTTTAACGCTAATACGATCAA-3') were used. The primers amplify a gene region of 1033 bp in length (Kariyama et al., 2000).

Reference strains

S. aureus ATCC 25923, *E. faecium* ATCC 51559 and *E. faecalis* ATCC 29212 were used for quality control strains of antimicrobial susceptibility testing and PCR assays.

RESULTS

In our study, *S. aureus* was isolated from 17 samples (17%). *S. aureus* isolates were isolated from cattle slaughterhouse workers' hands (4), poultry slaughterhouse workers' hands (4), beef cattle carcass swab (1), chicken carcass swab (2), retail beef meat (4) and retail chicken meat (2). *S. aureus* contamination rates of beef and chicken samples were 18% and 16% respectively (Table 1).

Among *S. aureus* isolates the highest antibiotic resistance was found to ampicillin (82.35 %) and penicillin G (94.11%). However, all isolates were susceptible to amoxicillin / clavulanic acid, and chloramphenicol. According to the MIC test to determine oxacillin resistance, five isolates (29.41%) showed resistance at concentrations ranging from 16-128 µg/ml (Table 2).

The oxacillin resistant isolates, according to their MIC value, were found to harbour the *mecA* gene using conventional PCR (Figure 1). These isolates were isolated from beef cattle carcass swab (1), chicken carcass swab (2), retail beef meat (1) and retail chicken meat (1) (Table 1).

E. faecium was isolated from eight (8 %) samples. *E. faecalis* was not detected in any of the samples. *E. faecium* was isolated from cattle slaughterhouse workers' hand (1), poultry slaughterhouse workers' hand (1), chicken carcass swab (2), retail beef meat (1) and retail chicken meat (3), (Table 1).

The highest antibiotic resistance of *E. faecium* isolates was to tetracycline (87.5%) (Table 4). However, most of the isolates were susceptible to ampicillin (62.5 %), penicillin G (75 %) and teicoplanin (75 %). According to the MIC test to determine vancomycin resistance, five isolates (62.5%) displayed resistance at concentrations ranging from 32-64 µg/ml (Table 3 and Table 4). These isolates were obtained from chicken carcass swab (2) and retail chicken meat (3). None of the isolates was determined to harbour the *van*A gene using conventional PCR assay (Figure 2).

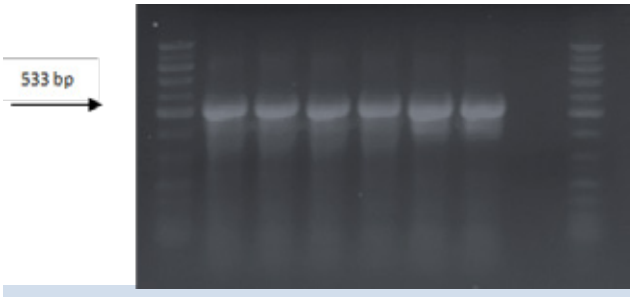

Figure 1. Image of rhe *mec*A gene positive isolates on agarose gel 1:Ladder (100 bp), 2: Positive control (*S. aureus* ATCC 43300), 3-8: Positive isolates, 9:Ladder (100 bp)

Table 1. Distribution of isolates in sample groups (%)

Origin of samples			Number of samples	*S. aureus*	MRSA	*mec*A	*E. faecium*	VRE	*van*A
Cattle	Slaughter-house	Carcass swab	10	**1** (10 %)	**1** (10 %)	**1**(10 %)	-	-	-
		Carcass excision	10	-	-	-	-	-	-
		Workers	10	**4** (40 %)	-	-	**1** (10 %)	-	-
	Retail		20	**4** (20 %)	**1** (5 %)	**1** (5 %)	**1** (5 %)	-	-
Poultry	Slaughter-house	Carcass swab	10	**2** (20 %)	**2** (20 %)	**2** (20 %)	**2** (20 %)	**2** (20 %)	-
		Carcass excision	10	-	-	-	-	-	-
		Workers	10	**4** (40 %)	-	-	**1** (10 %)	-	-
	Retail		20	**2** (10 %)	**1** (5 %)	**1** (5 %)	**3** (6 %)	**3** (6 %)	-
Total			100	**17**(17 %)	**5** (5 %)	**5** (5 %)	**8** (8 %)	**5** (5 %)	-

Table 2. Antimicrobial resistance and oxacillin MIC values of *S. aureus* isolates

Isolate number	Origin of Sample	OX MIC (µg/ml)	VA MIC (µg/ml)	P	AK	AMC	AMP	CIP	DA	E	CN	C	SXT	TE
1	Bcs 1	16	≤2	R	R	S	R	S	S	S	R	S	S	R
2	Cw 1	≤2	≤2	R	S	S	R	S	R	R	R	S	I	R
3	Cw 2	≤2	≤2	R	S	S	R	S	R	R	S	S	S	R
4	Cw 3	≤2	≤2	R	S	S	R	S	R	R	R	S	S	I
5	Cw 4	≤2	≤2	R	S	S	R	S	S	S	S	S	S	R
6	Br 1	≤2	≤2	S	S	S	S	S	S	S	S	S	S	S
7	Br 2	≤2	≤2	R	S	S	R	S	R	R	R	S	I	R
8	Br 3	≤2	≤2	R	S	S	R	S	R	R	R	S	I	R
9	Br 4	64	≤2	R	S	S	S	R	R	R	S	S	S	S
10	Ccs 1	128	≤2	R	S	S	S	I	R	R	S	S	S	S
11	Ccs 2	32	≤2	R	S	S	R	R	S	S	S	S	S	S
12	Pw 1	≤2	≤2	R	S	S	R	S	S	S	S	S	S	R
13	Pw 2	≤2	≤2	R	S	S	R	S	S	S	S	S	S	S
14	Pw 3	≤2	≤2	R	S	S	R	S	R	R	R	S	I	R
15	Pw 4	≤2	≤2	R	S	S	R	S	R	I	S	S	I	R
16	Cr 1	≤2	≤2	R	R	S	R	S	R	R	S	S	R	R
17	Cr 2	64	≤2	R	R	S	R	R	S	S	R	S	R	R

* R: Resistance, I: Intermediate, S: Sensitive, OX: oxacillin, AK: amikacin, AMC: amoxicillin / clavulanic Acid, AMP: ampicillin, CIP: ciprofloxacin, DA: clindamycin, E: erythromycin, CN: gentamicin, C: chloramphenicol, P: penicillin G, SXT: sulfamethoxazole / trimethoprim, TE: tetracycline, VA: vancomycin.

**Bcs: Cattle beef carcass swab; Cw: Cattle slaughterhouse workers' hand surface; Br: Retail beef; Ccs: Chicken carcass swab; Pw: Poultry slaughterhouse workers' hand surface; Cr: Retail chicken

Table 3. Antimicrobial resistance and vancomycin MIC values of *E. faecium* isolates

Isolate Number	Origin of Sample	VA MIC (µg/ml)	AK	AMP	CIP	E	CN	C	P	TEC	TE	S
1	Cw 1	≤4	S	S	I	R	R	S	S	S	R	R
2	Br 1	≤4	R	R	S	R	R	S	S	S	R	R
3	Ccs 1	64	R	S	R	R	R	R	S	S	R	R
4	Ccs 2	32	R	S	S	R	I	R	S	S	R	S
5	Pw 1	≤4	S	R	R	I	S	R	R	R	R	S
6	Cr 1	32	S	S	S	I	S	S	R	R	S	S
7	Cr 2	64	S	S	I	I	S	R	S	S	R	S
8	Cr 3	32	I	R	R	R	S	I	S	S	R	S

*R: Resistance, I: Intermediate, S: Sensitive, VA: vancomycin, AK: amikacin, AMP: ampicillin, CIP: ciprofloxacin, E: erythromycin, CN: gentamicin, C: chloramphenicol, P: penicillin G, TEC: teicoplanin, TE: tetracycline, S: streptomycin.

** Cw: Cattle slaughterhouse workers' hand surface; Br: Retail beef; Ccs: Chicken carcass swab; Pw: Poultry slaughterhouse workers' hand surface; Cr: Retail chicken

Table 4. Antimicrobial resistance rates (%) of *S. aureus* and *E. faecium* isolates

Antibiotic	*S. aureus*							*E. faecium*					
	Cattle		Poultry					Cattle		Poultry			
	Slaughterhouse			Slaughterhouse				Slaughterhouse		Slaughterhouse			
	Carcass swab (n:1)	Workers (n:4)	Retail Beef (n:4)	Carcass swab (n:2)	Workers (n:4)	Retail chicken (n:2)	Total (n:17)	Workers (n:1)	Retail Beef (n:1)	Carcass swab (n:2)	Workers (n:1)	Retail chicken (n:3)	Total (n:8)
VA	0	0	0	0	0	0	0	0	0	1 (50%)	1 (100%)	3 (100%)	5 (62.5%)
P	1 100(%)	3 (75%)	4 (100%)	2 (100%)	4 (100%)	2 (100%)	16 (94.1%)	0	0	1 (50%)	0	1 (33.3%)	2 (25%)
AK	0	0	0	0	2 (50%)	1 (50%)	3 (17.6 %)	0	1 (100%)	1 (50%)	1 (100%)	0	3 (37.5%)
AMP	1 100(%)	3 (75%)	4 (100%)	2 (100%)	2 (50%)	2 (100%)	14 (82.3%)	0	1 (100%)	1 (50%)	0	1 (33.3%)	3 (37.5%)
CIP	0	0	0	0	1 (25%)	2 (100%)	3 (17.6 %)	0	0	2 (100%)	0	1 (33.3%)	3 (37.5%)
CN	1 (100%)	1 (25%)	2 (50%)	1 (50%)	1 (25%)	1 (50%)	7 (41.1%)	1 (100%)	1 (100%)	1 (50%)	0	0	3 (37.5%)
C	0	0	0	0	0	0	0	0	0	2 (100%)	1 (100%)	1 (33.3%)	4 (50%)
SXT	0	0	0	0	1 (25%)	1 (50%)	2 (11.7%)	-	-	-	-	-	-
TE	1 (100%)	2 (50%)	3 (75%)	2 (100%)	2 (50%)	1 (50%)	11 (64.7%)	1 (100%)	1 (100%)	2 (100%)	1 (100%)	2 (66.6%)	7 (87.5%)
OX	0	0	0	0	3 (75%)	2 (100%)	5 (29.4%)	-	-	-	-	-	-
AMC	0	0	0	0	0	0	0	-	-	-	-	-	-
DA	1 (100%)	2 (50%)	2 (50%)	2 (100%)	3 (75%)	0	10 (58.8%)	-	-	-	-	-	-
E	1 (100%)	2 (50%)	2 (50%)	1 (50%)	3 (75%)	0	9 (52.9%)	1 (100%)	1 (100%)	1 (50%)	1 (100%)	1 (33.3%)	5 (62.5%)
TEC	-	-	-	-	-	-	-	0	0	1 (50%)	0	1 (33.3%)	2 (25%)
S	-	-	-	-	-	-	-	1 (100%)	1 (%100)	1 (50%)	0	0	3 (37.5%)

VA: vancomycin, P: penicillin G, AK: amikacin, AMP: ampicillin, CIP: ciprofloxacin, CN: gentamicin, C: chloramphenicol, SXT: sulfamethoxazole / trimethoprim, TE: tetracycline, OX: oxacillin, AMC: amoxicillin / clavulanic Acid, DA: clindamycin, E: erythromycin, TEC: teicoplanin, S: streptomycin

DISCUSSION

Presence of *S. aureus*

In our study, overall *S. aureus* prevalance was 17% (Table 1) and the distribution of the positive samples was 16% in chicken and chicken-related sources, and 18% in beef and beef related samples. Percentage distribution was in line with the study of Hanson et al. (2011) in the United States with the rate of 16.36%, lower of the of Lim et al. (2010) in Korea and Kitai et al. (2005) in Japan, with rates of 43.3% and 65.8%, respectively. These results were far below those observed by Bystron et al. (2005) with no coagulase-positive staphylococci contamination out of 65 samples of chicken parts in Poland. The variability of the contamination rates is thought to be due to factors such as geographical locations, sample size, sampling season, samples analyzed (whole carcasses, parts, different species of animals, etc.) and differences in isolation methods.

Oxacillin resistance and carriage of the *mec*A gene

In our study, MRSA was detected in 5% of the samples. Although the highest *S. aureus* contamination rate in sample groups was noted in the workers' hands both in cattle and poultry slaughterhouses, none of the isolates was MRSA.

The MIC test displayed resistance to oxacillin at different ratios (16-128 μg/ml) among the isolates. There are studies that differ from the present study in terms of sample size and the results of the samples analyzed that MRSA contamination rates were reported lower in Korea, (0.6 %, Lim et al., 2010), Spain (1.6 %, Lozano et al., 2009), Jordan (2.3 %, Quddoumi et al., 2006), and higher in Denmark (16 %, Agersø et al., 2012) in Netherlands (11.9 %, de Boer et al., 2009) and Germany (37.2 %, Feßler et al., 2011).

Determination of the *mec*A gene in all the MRSA isolates was comparable with a study carried out in Germany by Feßler et al. (2011) reported that all MRSA isolates from chicken and turkey products have the *mec*A gene and exhibit oxacillin MICs between 4 - 32 μg/ml. A more recent study conducted in Turkey by Sırıken et al. (2016) reported that 4 of 44 (9.09%) *S. aureus* isolates from beef samples were detected to be MRSA according to their MIC values and all of the isolates confirmed to have the *mecA* gene. On the contrary the researchers reported that the *mec*A gene was not detected in milk isolates which were resistant to oxacillin according to their MIC values.

A high resistance was displayed in *S. aureus* isolates against tetracycline (64.7 %), ampicillin (82.3 %) and penicillin G (94.1 %) antibiotics (Table 4) and a high sensitivity (100 %) against amoxicillin / clavulanic acid, chloramphenicol and vancomycine. Our results were in good agreement with Abdalrahman et al. (2015) in United States of America, which was reported that two of the *S. aureus* (2/114, 1.8%) isolates from retail chicken and turkey meats were determined as MRSA and displayed an antimicrobial resistance against ampicillin (94.6%), tetracycline (72 %) and penicillin (70.8 %). The highest antimicrobial resistance in MRSA isolates against tetracycline with a rate of 100 % was in a similar pattern with Lin et al. (2009) in Taiwan and Momtaz et al. (2013) in Iran stated that *S. aureus* strains from chicken processing plants and raw chicken meats were highly (100% and 97.56%, respectively) resistant to tetracycline. Besides, all the MRSA isolates were sensitive to amoxicillin/clavulanic acid, chloramphenicol and vancomycin. Relatively similar patterns were observed among the methicillin-sensitive *S. aureus* isolates that all of them was sensitive against amoxicillin-clavulanic acid, ciprofloxacin, chloramphenicol and vancomycin. This result was in accordance with a previous study conducted by Osman et al. (2016) in Egypt except the vancomycin resistance (74.1 %) declared to be determined in chicken breast samples.

Resistance rate of MSSA isolates was 91.6 % (11/12) to ampicillin and penicillin but both MSSA and MRSA strains were sensitive against amaoxicillin / clavulanic acid. These results were in accordance with the reports of Peacock and Paterson (2015), suggesting that the most of the MRSA isolates express resistance against β-lactam group heterogeneously. Furthermore, Foster (2017) stated that some isolates would display a high level of resistance could be expressed homogeneously. Conversion of this heterogeneous construct to homogeneously expressed resistance occurs as a result of chromosomal mutations in transcription of the *mec*A gene and PBP2a levels.

Presence of *E. faecalis* and *E. faecium*

Presence of *E. faecium* in eight samples (8 %) seem to be consistent with other research which found that *E. faecium* was detected in varying percentages (Boulianne et al., 2016; Donado-Godoy et al., 2015; Hidano et al., 2015; Kasımoglu-Dogru et al., 2010; Kim et al., 2018; Rehman et al., 2018; Stępień-Pyśniak et al., 2016). *E. faecalis* was not detected in any of the samples. However, there are several con-

trary studies (Donado-Godoy et al., 2015; Hidano et al., 2015; Kasımoglu-Dogru et al., 2010) conducted with poultry and beef samples reported to isolate the microorganism. This difference is thought to be due to the sampling area, sampling method, seasonal variations and individual differences.

Vancomycin Resistance and *van*A Gene

Vancomycin resistance in chicken samples according to MIC values (32-64 μg/ml) in spite of the absence of the *van*A gene was in accordance with the findings of other studies conducted in poultry meats, poultry products, poultry slaughterhouses (Boulianne et al., 2016 in Canada; Donado-Godoy et al., 2015 in Colombia; Hidano et al., 2015 in Japan; Kasımoglu-Dogru et al., 2010 in Turkey; Kim et al., 2018 in Korea; Rehman et al., 2018 in Canada; Stępień-Pyśniak et al., 2016 in Poland) cattle products and cattle slaughterhouses (Çetinkaya et al., 2013 in Turkey; Hayes et al., 2003 in United States of America; Liu et al., 2013 in China; Guerrero-Ramos et al., 2016 in Spain; Yılmaz et al., 2016 in Turkey) report the different resistance profiles and vancomycin MICs of *E. faecium* and *E. faecalis*. The lower values were observed by Hidano et al. (2015) that they stated 1 *E. faecalis* and 1 *E. faecium* isolates were detected as vancomycin resistant but MIC test showed a low resistance level (8μg/ml) in both isolates.

Absence of the *vanA* gene in phenotypically resistant isolates were in aggrement with several studies (Gousia et al., 2015; Raafat et al., 2016; El-Tawab et al., 2019; Onaran et al., 2019). In this context, Gousia et al. (2015) in Greece found that eight out of 30 *E. faecium* and one *E. faecalis* vancomycin-resistant isolates did not harbour the *van*A, Raafat et al. (2016) reported that only one of the 10 vancomycin-resistant isolates was *van*A-positive in foods of animal origin, El-Tawab et al. (2019) reported that *van*A was detected in three out of nine vancomycin-resistant strains isolated from milk and milk products in Egypt and in a recent study by Onaran et al. (2019) with the absence of the *van*A gene in 14 of 20 phenotypically positive VRE strains. Vancomycin resistance is known to be mediated by several van types as *van* A, *van* B, *van* C, *van* D, *van* E etc. although *van*A and *van*B are the most commonly detected clusters. In this context, a major source of limitation in our study is thought to be related to the expression of the resistance by the other van types.

The current data displayed a high resistance to tetracycline in *E. faecium* and VRE isolates (87,5 %, 80 %, respectively, Table 4), while all isolates were highly susceptible to ampicillin (62.5 %), penicillin G (75 %) and teicoplanin (75 %). A similar pattern of multiple antibiotic resistance was obtained in Kasımoglu-Dogru et al. (2010) in Turkey, Liu et al. (2013) in China, Boulianne et al. (2016) in Canada. In a study conducted by Yılmaz et al. (2016), *Enterococcus* isolates from chicken meat (96 %) and red minced meat (63 %) were resistant to at least one of the 12 tested antibiotics and the highest resistance rate was observed against tetracycline (53%-89.5%). These results were in agreement with the results of the present study as well as Pesavento et al. (2014) in Italy.

For many years β-lactams have been used as one of the first choices in Enterococcal infections, including, ampicillin and penicillin G used in the study showed a relatively lower resistance with rates 37.5% and 25%, respectively. Although ampicillin resistance is generally expressed as rare in *E. faecalis*, it is mostly related to the hospital-associated *E. faecium* isolates which is the result of enhanced production of PBP5 or polymorphisms of this protein (Gagetti et al., 2019).

Another antibiotic class, aminoglycosides are also used generally but resistance against Enterococcal species is alarming over the last few decades (Pesavento et al., 2014). Consistent with the literature, this research found that two of the *E. faecium* (one VSE and one VRE) isolates (25%, 2/8) were resistant to all antibiotics tested of aminoglycoside group (amikacin, gentamicin, streptomycin). These findings were in line with previous findings of Hayes et al. (2003) in USA, Osuka et al. (2016) in Japan and Khodabandeh et al. (2018) in Iran. On the contrary, the relatively lower resistance profiles were determined by Trivedi et al. (2011) in Czechia and Kim et al. (2019) in South Korea.

CONCLUSION

Detection of MRSA and VRE phenotypically and/or genotypically in chicken and beef cattle carcasses and retail products is a noteworthy point for public health surveillance programmes running for antimicrobial resistance. Besides, determination of multiple resistant isolates were also considered to be highly risky in terms of public health. Future research is needed to clarify in monitoring programs whether antibiotic resistant bacterial strains are personnel or animal origin in the slaughtering line and final product.

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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

Environmental factors affecting milk composition in Holstein cattle breed

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ABSTRACT: The incidence of mastitis in dairy herds is one of the main difficulties faced by dairy farmers, with a negative effect on the productivity of the herd and the welfare of the animals. Somatic cell count in milk is an indicator of udder health and frequency of clinical and subclinical mastitis incidence in dairy herds, and it is also often used to determine quality payments to dairy producers. Milk urea can be an indicator of the nutritional status of the dairy cows. The interpretation of these parameters assists in making important management decisions with regards to the health status and nutrition of dairy cattle. The objective of this study was to identify and evaluate environmental factors (farm, season, parity and stage of lactation) which affect the milk production and composition of Holstein breed, using field data. The presented research included 25,460 individual milk samples which were analysed as part of the Dairy Herd Improvement program, from 11 Holstein dairy farms in the region of Vojvodina, Northern Serbia. Analyses of raw milk samples were carried out on the FOSS instruments - CombiFoss^{TM}FT+, a combination instrument consisting of the MilcoScan^{TM}FT+ and the Fossomatic^{TM}FC. Statistical data processing was carried out by applying General Linear Model procedure, Statistics 13. Farm, season of milk control, parity and stage of lactation were included in the models as fixed effects. Significant differences in milk urea concentrations and somatic cell count were observed between farms ($P<0.01$), seasons ($P<0.01$), parity ($P<0.01$) and stage of lactation ($P<0.01$). Results showed that there were a highly significant ($P<0.01$) positive relationships between milk urea (MU) concentration and milk yield, MU and milk fat content, and between MU and solids non-fat (SNF), also between somatic cell count (SCC) and milk fat content, between SCC and protein content, as well as in between SCC and SNF. Highly significant negative relationships were found between milk urea and protein content and SCC, and between SCC and daily milk yield and lactose content. Proper analyses and interpretation of obtained results of milk samples obtained within the Dairy Herd Improvement program could contribute to better health management on the farms and it could have a positive impact on composition and nutritional value of milk, as well as on milk safety. It would be important to carry out further research in order to facilitate the detection of subclinical mastitis with MU as a potential indicator.

Keywords: somatic cell count; milk urea; season; parity; stage of lactation

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INTRODUCTION

The incidence of mastitis in dairy cattle herds is one of the main difficulties faced by dairy farmers, with a negative effect on the productivity of the herd and the welfare of the animals. Infection of the mammary gland is among the most important diseases of cows, causing high economic losses (Boboš et al., 2013). Somatic cell count (SCC) is often used to determine the milk price paid to dairy producers. The delivery control of milk with high SCC was established by the Regulation of the European Union 853 in 2004 for dairy cattle, which requires that bulk tank milk used for production of dairy products should have SCC levels below 400,000 cells/ml.

Milk urea (MU) and SCC are important parameters which can be used as indicators for formulating preventive and corrective measures for nutrition and health management in the herd. Somatic cell count in milk is an indicator of udder health and frequency of clinical and subclinical mastitis incidence in dairy herds. MU can be used as an indicator of the nutritional status of the herd. The interpretation of these milk parameters can assist in making important management decisions regarding the health status and nutrition of dairy cattle herds.

Urea as a part of the non-protein fraction of nitrogen in milk represents the final product of protein metabolism in the rumen of ruminants. Via the portal bloodstream, toxic ammonia is transported into the liver where it is transformed into urea, which later gets into milk through the bloodstream. This urea then can be measured in the bloodstream and milk (Rajala-Schultz et al., 2001). When milk samples are taken as a part of regular Dairy Herd Improvement (DHI) program, sampling involves no extra labor, and it is cheaper than sampling and analyzing blood. Nutrition and content of crude protein in the diet have the greatest influence on the milk urea content. Milk urea nitrogen (MUN) can be used as a tool to monitor protein feeding efficiency and dietary protein - energy ratio in dairy cows. Some other paragenetic factors, such as season, can have an influence on the milk urea content in addition to feeding, milk yield, stage of lactation, parity, breed, body weight, etc. (Godden et al., 2001; Hojman et al., 2004; Wattiaux et al., 2005; Fatehi et al., 2012).

The aim of this study was to identify and evaluate environmental factors (farm, season, parity and stage of lactation) that influence MU, SCC and milk production traits in Holstein dairy herds and to determine associations between SCC and MU (mg/dl) and milk production traits (milk yield - MY (kg), milk fat (%) and protein (%)).

MATERIALS AND METHODS

The study included 11 dairy farms (each with over 120 dairy cows) located in Vojvodina, Northern Serbia, with a total of 4,057 Holstein cows. Milk recording control was performed by AT4 method (ICAR, 2014). A total of 39,313 individual milk samples were collected at monthly DHI milk tests between February 2014 and January 2016. However, in accordance with the ICAR's Protocol (2020) for the Evaluation of Milk Analysers and in order to exclude additional factors that affect milk composition (as improper sampling and some health problems in cows), 13,853 samples were excluded from the study due to at least one of the following reasons: SCC < 50,000 or SCC ≥ 1,000,000; samples with milk fat < 2% or greater than 6% and milk protein < 2% and greater than 5.5%; thus, this research included a total of 25,460 individual milk samples of Holstein cows.

Analyses of raw milk samples were carried out on FOSS instruments - CombiFoss^{TM}FT+. This device is a combination instrument consisting of the MilcoScan^{TM}FT+ and the Fossomatic^{TM}FC. To convert MU into MUN, the following conversion formula was used: MUN (mg/dl) = MU (mg/dl) × 0.4667 (Oudah EZM, 2009). The principle of analyzing of raw milk samples is based on the methodology by mid - infrared spectrometry method (ISO 9622 /2013). Milk and liquid milk products - Guidelines for the application of mid-infrared spectrometry) and flow cytometry (ISO 13366-2 /2006) Milk - Enumeration of somatic cells. Part 2: Guidance on the operation of fluoro-opto electronic counters).

For the statistical analysis of SCC data the absolute values were transformed into somatic cell linear scores (Log 2 SCC) by applying the following equation (Sant' Anna and Paranhos da Costa, 2011): Log 2 SCC = log2 (SCC/100.000) + 3.

Logarithmic transformations are the most appropriate for the SCC data because they yield normality and homogeneity of the variances, enabling the execution of statistical analysis taking into account the above assumptions (Ali and Shook, 1980).

Dataset included: farm code, date of test (season), days in milk (DIM - interval between date of calving and milk test day), daily milk yield, milk fat, milk

protein, lactose, and SNF content, MU concentration (mg/dl), somatic cell count (cells/ml) and parity. According to the season of sampling, milk samples were divided into four groups: 1 - winter (December - February), 2 - spring (March - May), 3 - summer (June - August) and 4 - autumn (September - November). For the analysis, cows were grouped in five categories regarding their parity status (first, second, third, fourth and fifth+). Lactation was divided into 4 DIM intervals (I - 30 to 100 days, II - 101 to 200 days, III - 201 to 300 days and IV - greater than 300 days). The average values and variability of examined traits (daily milk yield - DMY, milk fat - MF, protein - P, lactose - L, solid non fat - SNF, milk urea - MU and somatic cell count - SCC) as well as the effect of factors on mentioned traits were studied by means of the PROC UNIVARIATE and PROC GLM procedures within the Statistic software package (ver. 13 Stat Soft Company 2016). Post-hoc analysis (Duncan test) was used to determine the statistically significant differences between the mean values of different classes, with a significance level at $P<0.05$ and $P<0.01$. The model equation used for the evaluation was as follows:

$$\mathbf{Y_{ijkl} = \mu + Si + Fj + Pk + DIMm + eijkl}$$

Legend:
Yijkl - MU, MF, P, L, SNF, SCC and DMY (dependent variable) value of dependent variable;
μ - mean value of dependent variable;
Si - fixed effect the season of sampling i (i = 1, 2, 3, 4);
Fj - fixed effect the farm, j = 1(Farm 1), 2 (Farm 2), …, 11 (Farm 11);
P_k - fixed effect the parity, k = 1st, 2nd, 3rd, 4th, 5th+;
DIMm - fixed effect of the stage of lactation (days in milk), m = 1, 2, 3 and 4;
eijkl - other random effects.

Finally, the correlation between SCC and MU concentration and production variables was performed using the correlation procedure (Statistic. 13). For all parameters, model effects were declared significant at $P<0.05$ and $P<0.01$.

RESULTS

The average results for milk fat, total protein, lactose and SNF percentages, DMY, SCC and MU concentration are presented in Table 1.

The influence of the farm was included in the model as a fixed effect and as expected, management of the farm had a great influence on the content of milk urea, SCC and other examined parameters, Table 2.

According to the data in Table 3., season had a significant effect on SCC and MU concentration and other examined traits (the values of F-test in all cases are highly significant) in Holstein cows.

Table 4. shows that stage of lactation had a significant effect on SCC, MU concentration and other examined traits in Holstein cows (the values of F-test in all cases are highly significant).

The concentration of MU was significantly ($P < 0.01$) lower in the first 100 DIM (24.65 mg/dl) and after 300 days of lactation (24.64 mg/dl).

The content of SCC, MU, milk components and daily milk yield, were significantly influenced by the number of lactations (Table 5.). The high values of the F - ratios are the proof of the important influence of the parity on the examined variables.

Cows in the first lactation had a higher milk fat content (3.78%), cows in the second lactation had higher protein content (3.33%) and in the third lactation had a higher daily milk yield (27.75 kg). Cows in the first lactation had the lowest SCC (255,300/ml) and SCC was increased with increased number of lactation, the highest SCC were cows in the fifth and greater lactation (308,430/ml).

Table 1. Means, minimum, maximum, standard deviation and coefficient of variation of analyzed variables

Trait	N	Mean	Minimum	Maximum	SD	CV
Fat (%)	25460	3.76	2.00	6.00	0.85	22.61
Protein (%)	25460	3.31	2.00	5.43	0.41	12.39
DMY (kg)	25460	26.75	2.00	67.20	9.88	36.93
SNF (%)	25460	8.74	5.59	10.98	0.47	5.38
Lactose (%)	25460	4.62	2.35	5.44	0.23	4.98
MU (mg/dl)	25460	25.49	10.00	92.00	8.11	31.82
SCC (10^3/ml)	25460	274.84	50.00	1000.00	227.25	82.68
Log 2 SCC	25460	3.99	2.00	9.62	1.17	29.32

Legend: DMY - daily milk yield; SNF - solid non fat; MU - milk urea; SCC - somatic cell count; N - total number of individual cow milk samples; SD - standard deviation; CV - coefficient of variation

Table 2. Effect of farm on milk traits

Farm	N	Fat (%)	Protein (%)	DMY (kg)	SNF (%)	Lactose (%)	MU (mg/dl)	SCC (10^3/ml)	Log 2 SCC
1	5174	3.96^{a}	3.44^{a}	22.34^{a}	8.74^{a}	4.52^{a}	23.81^{a}	210.25^{a}	3.63^{a}
2	482	3.74^{b}	3.38^{b}	26.60^{b}	9.04^{b}	4.68^{bc}	22.52^{b}	256.02^{bc}	3.96^{b}
3	1014	3.85^{c}	3.20^{ce}	27.36^{c}	8.68^{c}	4.55^{d}	30.50^{c}	243.93^{bd}	3.82^{c}
4	4859	3.56^{d}	3.32^{d}	27.52^{c}	8.80^{d}	4.66^{be}	25.44^{d}	364.96^{e}	4.50^{d}
5	3495	4.09^{e}	3.23^{c}	27.26^{bc}	8.64^{e}	4.60^{f}	26.31^{ef}	312.84^{f}	4.18^{e}
6	2025	3.73^{b}	3.37^{b}	30.08^{d}	8.90^{f}	4.68^{bgh}	26.44^{e}	268.85^{c}	3.97^{b}
7	996	3.71^{b}	3.43^{a}	27.50^{c}	8.94^{f}	4.69^{cg}	23.32^{a}	233.64^{dg}	3.80^{c}
8	2997	3.45^{f}	3.23^{c}	27.84^{c}	8.75^{a}	4.66^{eh}	23.92^{a}	267.92^{c}	3.97^{b}
9	1956	3.25^{g}	3.22^{c}	23.59^{e}	8.74^{a}	4.65^{eh}	25.57^{df}	244.24^{bg}	3.81^{c}
10	1702	3.97^{a}	3.18^{e}	32.72^{f}	8.58^{g}	4.61^{f}	29.13^{g}	253.22^{bc}	3.91^{bc}
11	760	4.15^{h}	3.31^{d}	29.30^{g}	8.37^{h}	4.60^{f}	26.81^{e}	241.28^{bg}	3.82^{c}
F		267.7**	130**	242.5**	165**	188**	135.2**	148.8**	170.5**

Legend: N - total number of individual cow milk samples; DMY - daily milk yield; SNF - solid non fat; MU - milk urea; SCC - somatic cell count; Means within the same column with different superscripts ($^{a,b,c...h}$) differ significantly ($P < 0.01$); significant differences: * $P < 0.05$; ** $P < 0.01$

Table 3. Effect of season of sampling on SCC, milk urea concentration, daily milk yield and milk components

Season	N	Fat (%)	Protein (%)	DMY (kg)	SNF (%)	Lactose (%)	MU (mg/dl)	SCC (10^3/ml)	Log 2 SCC
1	5737	3.81^{a}	3.43^{a}	27.51^{a}	8.85^{a}	4.63^{a}	25.86^{a}	276.65^{a}	4.02^{a}
2	4932	3.74^{b}	3.25^{b}	27.87^{b}	8.79^{b}	4.65^{b}	27.34^{b}	283.71^{a}	4.04^{a}
3	6645	3.64^{b}	3.16^{c}	26.73^{c}	8.59^{c}	4.60^{c}	27.47^{b}	260.81^{b}	3.91^{b}
4	8146	3.83^{a}	3.38^{d}	25.54^{d}	8.75^{d}	4.59^{c}	22.49^{c}	279.61^{a}	4.02^{a}
F		69.3**	605**	73.8**	357**	93**	638.5**	12.30**	17.6**

Legend: Season: 1 - Winter; 2 - Spring; 3 - Summer; 4 - Autumn; N - total number of individual cow milk samples; DMY - daily milk yield; SNF - solid non fat; MU - milk urea; SCC - somatic cell count; Means within the same column with different superscripts (^{a,b,c,d}) differ significantly ($P < 0.01$); significant differences: * $P < 0.05$; ** $P < 0.01$

Table 4. Effect of stage of lactation on SCC, milk urea concentration, daily milk yield and milk components

Lactation stage	N	Fat (%)	Protein (%)	DMY (kg)	SNF (%)	Lactose (%)	MU (mg/dl)	SCC (10^3/ml)	Log 2 SCC
1	5845	3.62^{a}	2.99^{a}	32.75^{a}	8.47^{a}	4.68^{a}	24.65^{a}	255.45^{a}	3.86^{a}
2	8257	3.63^{a}	3.22^{b}	29.51^{b}	8.67^{b}	4.65^{b}	26.68^{b}	265.82^{a}	3.93^{b}
3	7058	3.84^{b}	3.45^{c}	23.52^{c}	8.85^{c}	4.58^{c}	25.31^{c}	280.73^{b}	4.04^{c}
4	4300	4.06^{c}	3.68^{d}	18.56^{d}	9.05^{d}	4.51^{d}	24.64^{a}	308.84^{c}	4.22^{d}
F		328.1**	4267**	2915.2**	1747**	672**	98.0**	52.50**	91.2**

Legend: N - total number of individual cow milk samples; DMY - daily milk yield; SNF - solid non fat; MU - milk urea; SCC - somatic cell count; F - values of F-test; Means within the same column with different superscripts ($^{a.b.c.d}$) differ significantly ($P < 0.01$); significant differences: * $P < 0.05$; ** $P < 0.01$

Table 5. Effect of parity on SCC, milk urea concentration, daily milk yield and milk components

Parity	N	Fat (%)	Protein (%)	DMY (kg)	SNF (%)	Lactose (%)	MU (mg/dl)	SCC (10^3/ml)	Log 2 SCC
1	10046	3.78^{a}	3.30^{ab}	25.61^{a}	8.80^{a}	4.68^{a}	25.58^{a}	255.30^{a}	3.88^{a}
2	7242	3.72^{b}	3.33^{a}	27.53^{b}	8.74^{b}	4.60^{b}	25.37^{a}	276.81^{b}	4.00^{b}
3	4462	3.76^{ab}	3.32^{ab}	27.75^{b}	8.69^{c}	4.56^{c}	25.25^{a}	296.38^{cd}	4.12^{c}
4	2107	3.75^{ab}	3.29^{b}	27.28^{bc}	8.64^{d}	4.53^{d}	25.32^{a}	290.05^{bc}	4.09^{c}
5	1603	3.74^{ab}	3.26^{c}	26.82^{c}	8.64^{d}	4.53^{d}	26.32^{b}	308.43^{d}	4.20^{d}
F		5.6**	11.0**	58.2**	91.0**	422.0**	6.0**	40.10**	53.4**

Legend: N - total number of individual cow milk samples; DMY - daily milk yield; SNF - solid non fat; MU - milk urea; SCC - somatic cell count; F - values of F-test; Means within the same column with different superscripts (^{a,b,c,d}) differ significantly ($P < 0.01$); significant differences: * $P < 0.05$; ** $P < 0.01$

Table 6. Coefficient of correlation between milk composition components, DMY, SCC and MU

Trait	Protein (%)	DMY (kg)	SNF (%)	Lactose (%)	MU (mg/dl)	SCC (10^3/ml)
Fat (%)	0.3362**	-0.2942**	0.1731**	-0.1477**	0.1106**	0.0571**
Protein (%)		-0.4663**	0.7551**	-0.2000**	-0.0270**	0.1260**
DMY (kg)			-0.2877**	0.2753**	0.0614**	-0.1231**
SNF (%)				0.2429**	0.0934**	0.0103^{NS}
Lactose (%)					0.0082^{NS}	-0.1983**
MU (mg/dl)						-0.0412**

Legend: DMY - daily milk yield; SNF - solid non fat; MU - milk urea; SCC - somatic cell count; Significant differences: * $P < 0.05$; ** $P < 0.01$; ^{NS} - Non Significant

Intensity of the correlation between analyzed parameters in milk and determined statistical significance is presented in Table 6. It can be noticed that MU concentration negatively correlated ($P < 0.01$) with protein (%), but positively correlated with milk fat (%), SNF (%), lactose (%) and DMY (kg).

SCC also negatively correlated ($P < 0.01$) with DMY (kg) and lactose (%), but positively correlated with milk fat and protein.

DISCUSSION

Mean values for milk fat (3.76%) and protein contents (3.31%) determined in this study were a higher than average values for total Holstein population in Vojvodina in the year 2015 (milk fat 3.71%, protein 3.25%, total milk yield 9,177 kg) given by Main breeding organization (2016).

In this research, the mean MU concentration (25.49 mg/dl) was within the optimal values of 15 to 30 mg/dl given by Carlsson and Pehrson (1993). Average MU concentration was higher than values reported in studies of Hof et al. (1997) and Kohn et al. (2004), but lower than values reported by Wattiaux and Karg (2004), Zadeh-Hossein and Ardalan (2011) and Fatehi et al. (2012) for Holstein dairy cows.

The average SCC (274,840 cells/ml) was higher than that reported by Konjačić et al. (2010), but was lower than that found by Yoon et al. (2004). Also, Sadeghi-Sefidmazgi and Rayatdoost-Baghal (2014) report an average SCC that is very similar to the result of this research.

The highest content of MU, 30.50 mg/dl, was found on Farm 3. On the contrary, the lowest MU content was estimated on Farm 2 (22.52 mg/dl). The effect of farm on MU and other examined parameters is related to the different ratio of energy and protein in feeding (Table 2.). Mean value of SCC on all examined farms was below 400,000/ml. The lowest SCC was on Farm 1 (210,250/ml) and the highest was on Farm 4 (364,960/ml).

Statistically significant differences in MU content between farms are reported by others (Wattiaux Met al., 2005; Konjačić et al., 2006). The effect of the farm is a very complex factor which reflects the action of numerous different systematic and non-systematic environmental influences, such as nutrition, type and quality of housing facilities, health status of cows, climatic conditions and farm management.

MU was lower in autumn (22.49 mg/dl) and highest during summer (27.47 mg/dl). Similar results have been reported by Hojman et al. (2004) and Fatehi et al. (2012). The highest values of SCC were evidenced during the spring (283,710/ml) and lowest values in the summer (260,810/ml). Ivanov et al. (2017) reported similar results, in which SCC showed significant elevation in the autumn-winter period compared to the spring and summer period. Some other authors found the lowest count of somatic cells in winter and highest during the summer (Wells and Ott, 1998; Memiši et al., 2011). Ferreira and De Vries (2015) evidenced higher SCC in warmer months (August, September and October) than the average SCC in colder months (February, March and April). According to Syridion et al. (2012), there was evidenced significantly higher SCC during the summer months compared to both autumn and winter seasons.

As presented in Table 4., the peak of lactation was in the first 100 days after calving. Some authors found that the peak of lactation was between 4 and 8 weeks after calving (Čobić and Antov, 1996; Park and Lindberg, 2004), but Piccardi et al. (2014) reported the peak of lactation around 122 days after calving. The highest MU level was evidenced between 101 and 200 DIM (26.68 mg/dl), this was a signal of the excess protein in diet of cows in the period after the

peak of lactation. Similar results were reported in other studies (Hojman et al., 2004; Fatehi et al., 2012).

The SCC was lowest in the first 100 days of lactation (255,450/ml) and after it increased, reaching the highest value at the end of lactation (308,840/ml). The reports by Campos et al. (2006) showed that lactation curves of the content of somatic cells and milk yields usually show opposite patterns. Syridion et al. (2012) and Sitkowska (2008) also concluded that SCC increased with lactation progressing.

A lower MU concentration (25.58 mg/dl) was found in cows in the third lactation and mean MU concentration of cows in the fifth and greater lactations (26.32 mg/dl) was higher and differs significantly from other lactations (Table 5.). The overall differences between lactations are numerically small. Contrary to our results, Godden et al. (2001) recorded the highest MU concentration in cows in the first lactation. Hojman et al. (2004) found lower MUN content in the first lactation cows than the second or later lactation animals.

According to reports of Godden et al. (2001) and Hojman et al. (2004) negative correlation was found between the milk protein content and MU concentration. Contrary, Bendelja et al. (2011) found a positive correlation between milk protein and MU.

Milk fat content increased with the increasing MU level. Bendelja et al. (2011) reported a positive correlation between milk fat content and MUN. Hojman et al. (2004) explained that higher content of neutral detergent fibres in forage may increase milk fat content and at the same time caused an increased urea concentration, due to the high degradability of its proteins. A negative relation between milk fat and MUN was reported by Konjačić et al. (2010). A positive association between daily milk yield and MUN has also been reported by Godden et al. (2001) and Konjačić et al. (2010). Hojman et al. (2004) determined the correlation coefficient between the above stated parameters (r=0.17). A positive correlation between daily milk yield and MU was expected because cows with higher milk production were fed diets richer in protein component.

Some studies have examined urea transfer from milk to blood by measuring disappearance of injected labelled urea in the mammary gland of dairy cattle (Spek et al., 2016). Roy et al. (2001) claim that MU concentration decreased as intensity of infection increased from mild to moderate. Licata (1985) reported that udder quarters positive to California mastitis test was 0.45 mM lower in urea content than that from healthy quarters. It could be assumed that infections of the mammary gland, which cause increased permeability of the udder tissue, also increase MU transfer from milk into bloodstream.

A negative correlation has been evidenced between SCC and milk yield, as reported by Coffey et al. (1986). There was a negative correlation between SCC, fat/protein contents and milk yield in the report published by Yoon et al. (2014).

Very little research has been conducted concerning the relation between the SCC and the MU concentration. In the present research, a significant and negative correlation coefficient ($r = -0.0412$) between these parameters was determined. Increased somatic cell count was followed by reduced urea concentration in milk, also reported by Hojman et al. (2004) and Bendelja et al. (2011). Yoon et al. (2004) show that by increasing SCC, milk yield was reduced and MU level was increased.

CONCLUSION

Based on the present research results, the following conclusions can be drawn:

- The farm, season, parity and stage of lactation had significant effects ($P<0.01$) on SCC, MU concentration, milk fat and protein content and daily milk yield.
- MUN concentration and SCC should be eval-

uated considering the parity, season and stage of lactation.

- There are positive and statistically significant correlations between the MU concentration and milk fat and lactose content, as well as between MU concentration and milk yield; also between SCC and milk fat and protein content.

- Negative and statistically significant correlations were found between MU concentration and protein content and SCC, and between SCC and milk yield and lactose content.

Proper analyses and interpretation of obtained results could contribute to better health management on the farms and it could have a positive impact on composition and nutritional value of milk, as well as on milk safety. Moreover, individual milk samples can be taken easily, involving almost no extra labor and without causing stress to dairy cattle. Since there is no clear correlation between MU concentrations and occurrence of mastitis, it would be important to carry out further research on this topic in order to facilitate the detection of subclinical mastitis with MU as a potential indicator.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Genetic parameter estimates of chick quality, growth, and carcass characteristics in Japanese quail

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ABSTRACT: The aim of this study was to estimate the heritabilities of chick quality and growth traits and their genetic relationships with some slaughter traits. Chick quality was determined using both Tona and Pasgar score methods. All birds were slaughtered at 8 weeks of age to measure carcass yield (CY), and percentage of breast (BY), leg (LY), wing (WY), abdominal fat (AFY). Heritability estimates for chick quality scores of Tona and Pasgar were found 0.08, and 0.09, respectively. Heritability estimates for growth curve parameters and inflection point coordinates of the Gompertz model were low to moderate, with values ranging from 0.17 to 0.26. Low heritability estimates for CY, BY, LY, and WY were found 0.04, 0.14, 0.09, and 0.07, respectively. Genetic and phenotypic correlations between chick quality and BW-growth traits were determined low and statistically non-significant (P>0.05). Similarly, genetic and phenotypic relationships between chick quality and carcass yield, and between chick quality and percentages of carcass parts were found low and statistically non-significant (P>0.05). As a result, it is possible to say that applying multitrait selection, including chick quality, will not affect other yield characteristics positively or negatively. However, in virtue of the chick quality is a very low heritable trait, environmental improvement of chick quality trait may be considered rather than genetic improvement.

Keywords: Chick quality, Genetic correlation, Heritability, Pasgar score, Tona score

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INTRODUCTION

Thanks to the genetic improvement studies carried out in the last 50 years, there have been significant developments in the rapid growth, muscle development and feed utilization characteristics of the fast-growing genotypes. In the selection studies carried out for many years, more than 25 traits from the ultimate pH of breast meat to angle of sternum have been used for the development of broilers. Environmental improvement such as feed quality, rearing systems, thermal conditions, air quality, equipment technology, and flock management gained importance in this period. Today, all environmental conditions at every stage of broiler production in poultry houses are under control. As a result of all these genetic and environmental improvements, the products with two kilograms of carcass weight at the age of 40 days are obtained and offered on the market. In addition to all these genetic and environmental features, one-day-old chick quality determined by various methods is also important because it directly affects the amount of salable chick (Leksrisompong et al., 2007; Tona et al., 2003). However, chick quality traits have never been incorporated in breeding indexes of applied genetic improvement schemes.

Starting broiler production with chicks of high quality reduces losses and increases profitability during the fattening period. All of the studies in which chick quality is measured by quantitative and qualitative methods are concerned with the effects of environmental factors on yield characteristics, and studies discussing the phenotypic relationships between chick quality and yield traits. Chick quality, like other quantitative characteristics, is affected by both genetic and environmental factors. Environmental factors affecting chick quality can be listed as age, nutrition and health status of breeder flock, egg storage conditions, position, and rotation of eggs, and thermal conditions in incubator. In the literature, there is no study focusing on the heritability of chick quality, and genetic relationships between other yield characteristics and chick quality. This study aims to estimate the heritability of chick quality and genetic correlations between yield characteristics and chick quality. Japanese quail, a commercially important and model animal for other poultry, was used in the study.

MATERIAL AND METHODS

The experiment was conducted at the Animal Science Department, Akdeniz University, Turkey. The care and use of animals were in accordance with laws and regulations of Turkey and approved by the Ministry of Food, Agriculture and Livestock (decision number 22875267-325.04.02-E.3706253) and Animal Experiments Local Ethics Committee of Akdeniz University (decision number B.30.2.AKD.0.05.07.00/59). Japanese quail (*Coturnix coturnix Japonica*) was used as animal material in the research. A total of 42 male and 126 female breeders were used at the Akdeniz University livestock facilities in order to create a base population which had not been selected before. The breeder females were housed in cages with five floors and ten individual compartments on each floor, with a stocking density of 275 cm^2/bird in individual pens. The breeder flock was formed in the family structure. Families consisted of 3 females and 1 male to avoid full and half-sib mating, and males were shifted through the 3 female cages daily. A breeder diet containing 2,800 kcal of ME/kg and 19% CP/kg was given. All adult birds were kept under constant artificial lighting for 18 h/d. Eight hundred and sixty-seven chicks randomly selected from hatchlings obtained from 1200 hatching eggs of the base population were used as experimental animals.

The hatched chicks were kept until dry, and then the wing numbers were attached. Thanks to this process the pedigree records were created, weekly live weights and other measurements were performed by matching the pedigree records during the trial. After 420 h of incubation, all hatched chicks were examined by experienced operators to determine Tona and Pasgar chick quality score of chicks as previously described by Tona et al. (2003) and Boerjan (2002). Tona and Pasgar methods are qualitative scoring systems that assess total score index of 100 and 10, respectively, based on a wide variety of visual parameters, such as activity, appearance, retracted yolk, eye condition, leg and feet condition, navel deformities, and status, remaining egg membrane, beak condition, and remaining yolk (Tona et al., 2003; Boerjan 2002). Quail chicks were housed in brooding cages (90 cm^2/quail) for 3 wk before being feather-sexed, then they were transferred to fattening cages (160 cm^2/quail), and they were housed here until the slaughtering age of 56 days. A grower diet containing 24% CP and 2,900 kcal of ME/kg for the first 21 d, and a fattening diet containing 21% CP and 2,800 kcal of ME/kg were used. Ad libitum feeding, water, and a 23-hours/daylighting program were applied from hatch to the end of the study. The cumulative mortality was found 1.38% in the study.

At 8 weeks of age, the BW of all birds was de-

termined 4 hours after feed withdrawal and slaughtered in an experimental processing plant. The birds were slaughtered, bled out, scalded (55°C, 2 min), defeathered with equipment, manually eviscerated, and the abdominal fat pad (from the proventriculus surrounding the gizzard down to the cloaca) was taken, chilled in an ice-water tank, and drained (Narinç et al., 2014). The next day, after carcass dissection, breast with bone and the remaining abdominal fat on cold carcasses were weighed using an electronic digital balance with a sensitivity of 0.01 g. Slaughter and dissection were performed by the same experienced operators. Cold carcass, breast, leg, wing, and total fat pad yields were calculated in relation to body weight at 8 weeks of age.

To obtain the estimates of individual growth curve parameters, all quail were weighed weekly from hatching to 8 weeks of age. The Gompertz non-linear regression model (1) was used to estimate the growth curve of each quail.

$$y_t = \beta_0 e^{(-\beta_1 e^{-\beta_2 t})} \quad (1)$$

where y_t is the weight at age *t*, β_0 is the asymptotic (mature) weight parameter, β_1 is the scaling parameter (constant of integration) and β_2 is the instantaneous growth rate (per day) parameter (Akbaş and Yaylak, 2000; Narinç et al., 2010b). The Gompertz model is characterized by an inflection point in a manner such that β_0/e of the total growth occurs prior to it and the remainder occurring after. The coordinates of the point of the inflection, age, and weight at inflection point (IPA and IPW, respectively), were obtained as follows:

$$IPA = ln(\beta_1)/\beta_2 \quad (2)$$

$$IPW = \beta_0/e \quad (3)$$

The descriptive statistics and Kolmogorov-Smirnov normality tests of the traits were obtained using UNIVARIATE procedure of SAS 9.3 statistics software.

The restricted maximum likelihood (REML) estimator was used to estimate variance-covariance components for following multi-trait model;

$$y=X\beta+Zu+e$$

Where **y**, a vector of observations for the trait; **β**, a vector of fixed effects for the trait; **u**, a vector of random animal effects for the trait; **e**, a vector of random residual effects for the trait; and **X** and **Z** are incidence matrices relating records of the trait to fixed and random animal effects, respectively (Narinç et al., 2014). The sire, dam, and residual variance components and additive genetic and environmental covariance matrices for multivariate analysis were estimated from the mixed-model equations by SAS PROC MIXED. Heritabilities (h_i^2) and genetic correlations ($r_{g(ii')}$) were calculated from the variance and covariance parameters as follows:

$$h_i^2 = \frac{\sigma_{iu}^2}{\sigma_{iu}^2 + \sigma_{ie}^2}$$

$$r_{g(ii')} = \frac{\sigma_{ii'a}}{\sigma_{iu}^2 + \sigma_{i'a}^2}$$

where *i* and *i'* represents the trait(s) of interest and σ_{iu}^2 and σ_{ie}^2 are the diagonal elements of $\mathbf{G_0}$ and $\mathbf{R_0}$ matrices, respectively. Also, $\sigma_{ii'a}$ stands for the additive genetic covariance between the traits *i* and *i'*. Genetic correlation and heritability estimates and their approximate standard errors of the traits were obtained by SAS interactive matrix language (IML) procedure.

RESULTS

The descriptive statistics of TS, PS, BW6, BW8, β_0, β_1, β_2, IPT, IPW, CY, BY, LY, WY, and AFY are presented in Table 1. In addition, the results of the independent sample t-test conducted to determine the sex effect are also given in Table 1. The significant effects of sex of the birds were not observed for chick quality traits. But, there were significant differences between males and females for traits of BW6, BW8, β_0 parameter, IPT, and IPW, and all slaughter-carcass traits. Females showed higher mean values than males for BW6, BW8, β_0 parameter, IPT, IPW, AFY traits ($P<0.05$ for all mentioned characteristics). The coefficient of determination R2 of the estimated growth curves were found to be above 0.99.

The estimates of genetic parameters (heritabilities and genetic relationships) for chick quality traits and growth characteristics are presented in Table 2. Heritability estimates for slaughter-carcass traits are presented in Table 3. Also, the genetic correlations between chick quality traits and slaughter-carcass characteristics are presented in Table 3. There are phenotypic correlation coefficients between all traits and their statistical significances in Table 2 and Table 3. In the study, the genetic correlation estimates among all characteristics were found higher than the phenotypic correlation coefficients (Table 2 and Table 3).

Table 1. The descriptive statistics and effect of sex on studied traits

Trait	N	Mean	SE	CV %	Min	Max	Sex Effect
TS	855	98.69	0.17	5.14	54.33	100.00	NS[1]
PS	855	9.80	0.02	6.41	5.00	10.00	NS
BW6 (g)	858	114.59	1.25	32.24	35.15	210.43	*
BW8 (g)	855	150.71	1.35	26.43	43.12	234.02	*
β_0[1] (g)	855	253.25	2.45	28.49	54.34	559.83	*
β_1[2]	855	3.25	0.02	17.99	1.81	11.67	NS
β_2[3]	855	0.038	0.001	39.22	0.012	0.126	NS
IPT[4] (d)	855	35.60	0.48	40.10	9.49	119.11	*
IPW[5] (g)	855	93.37	0.92	29.07	19.99	259.75	*
CY (%)	855	74.86	0.21	8.08	61.93	88.74	*
BY (%)	855	28.52	0.14	14.24	20.85	41.14	*
LY (%)	855	17.20	0.08	13.45	2.28	27.91	*
WY (%)	855	5.36	0.02	13.56	3.33	9.28	*
AFY (%)	855	1.08	0.02	53.63	0.08	2.94	*

TS= Tona score; PS= Pasgar score; BW6 and BW8= Body weight at 6 and 8 wk of age; β_0= Asymptotic BW parameter; β_1= Shape parameter; β_2= Instantaneous growth rate parameter; IPT and IPW = age and weight at inflection point, CY= Carcass yield, BY= Breast yield, LY=Leg yield, WY= Wing yield, AFY= Abdominal fat yield.

[1] NS= Non-significance, P>0.05. *=Statistically significance, P<0.05.

Table 2. Heritability estimates (on diagonal), genetic correlation estimates (below diagonal) and SE (in parentheses), phenotypic correlations (above diagonal) and P values (in parentheses) of chick quality and growth traits (N=855 for all traits)

	TS	PS	BW6	BW8	β_0	β_1	β_2	IPT	IPW
TS	0.08	0.81*	0.03	0.08	0.00	-0.04	0.00	-0.01	0.01
	(0.02)	(0.001)	(0.388)	(0.466)	(0.889)	(0.209)	(0.916)	(0.765)	(0.845)
PS	0.82	0.09	0.02	0.05	0.02	-0.03	-0.01	0.01	0.03
	(0.01)	(0.02)	(0.605)	(0.315)	(0.486)	(0.367)	(0.856)	(0.879)	(0.453)
BW6	0.14	0.06	0.46	0.55	0.05	0.02	0.69*	-0.60*	0.05
	(0.03)	(0.04)	(0.01)	(0.001)	(0.549)	(0.876)	(0.001)	(0.001)	(0.225)
BW8	0.13	0.09	0.69	0.51	0.34*	-0.18*	0.68*	-0.61*	0.11
	(0.01)	(0.01)	(0.01)	(0.01)	(0.005)	(0.002)	(0.001)	(0.001)	(0.091)
β_0	0.38	0.35	0.31	0.35	0.17	0.35*	-0.57*	0.66*	0.99*
	(0.03)	(0.02)	(0.02)	(0.02)	(0.02)	(0.008)	(0.001)	(0.001)	(0.001)
β_1	0.56	0.28	-0.21	-0.21	-0.08	0.27	-0.08	0.40*	0.35*
	(0.02)	(0.03)	(0.03)	(0.03)	(0.05)	(0.02)	(0.221)	(0.001)	(0.001)
β_2	-0.08	-0.22	0.88	0.84	-0.17	-0.07	0.26	-0.93*	-0.58*
	(0.05)	(0.03)	(0.01)	(0.02)	(0.03)	(0.04)	(0.02)	(0.001)	(0.001)
IPT	0.28	0.32	-0.85	-0.65	0.20	0.33	-0.95	0.26	0.67*
	(0.02)	(0.02)	(0.01)	(0.02)	(0.03)	(0.02)	(0.01)	(0.02)	(0.001)
IPW	0.39	0.35	0.29	0.43	0.97	-0.06	-0.20	0.24	0.19
	(0.02)	(0.01)	(0.04)	(0.03)	(0.01)	(0.05)	(0.04)	(0.03)	(0.02)

TS= Tona score; PS= Pasgar score; BW6 and BW8= Body weight at 6 and 8 wk of age; β_0= Asymptotic BW parameter; β_1= Shape parameter; β_2= Instantaneous growth rate parameter; IPT and IPW = age and weight at inflection point

Table 3. Heritability estimates (on diagonal), genetic correlation estimates (below diagonal) and SE (in parentheses), phenotypic correlations (above diagonal) and p values (in parentheses) of chick quality and slaughter traits (N=855 for all traits)

	TS	PS	CY	BY	LY	WY	AFY
TS	0.08	0.81*	-0.03	-0.05	-0.02	0.03	0.03
	(0.02)	(0.001)	(0.768)	(0.257)	(0.816)	(0.374)	(0.881)
PS	0.82	0.09	-0.02	-0.03	-0.03	0.01	0.07
	(0.01)	(0.02)	(0.254)	(0.128)	(0.375)	(0.278)	(0.554)
CY	-0.05	-0.12	0.04	0.66*	0.70*	0.38*	0.28*
	(0.03)	(0.04)	(0.05)	(0.001)	(0.001)	(0.001)	(0.000)
BY	-0.11	-0.14	0.14	0.14	0.46*	0.11*	-0.07
	(0.06)	(0.03)	(0.03)	(0.03)	(0.001)	(0.041)	(0.356)
LY	-0.12	-0.12	0.78	0.42	0.09	0.32*	0.13
	(0.05)	(0.05)	(0.03)	(0.03)	(0.03)	(0.001)	(0.094)
WY	-0.07	-0.08	0.61	0.10	0.57	0.07	0.12
	(0.04)	(0.03)	(0.03)	(0.03)	(0.03)	(0.04)	(0.196)
AFY	0.22	0.31	0.33	0.18	0.33	0.27	0.21
	(0.03)	(0.03)	(0.04)	(0.01)	(0.01)	(0.01)	(0.01)

TS= Tona score; PS= Pasgar score; CY= Carcass yield, BY= Breast yield, LY=Leg yield, WY= Wing yield, AFY= Abdominal fat yield.

DISCUSSION

The mean values of Tona score and Pasgar Score (98.69 and 9.80) were in agreement with those reported for broiler chickens by Willemsen et al. (2008), Van de Ven et al. (2012), and Bergoug et al. (2015), which range from 95.6 to 90.4 and 9.84 to 9.52, respectively. In the current study, the mean values of chick quality scores of birds were higher than those published by Tona et al. (2004) and Sözcü and İpek (2015) who reported Tona scores of 90.4-68.9 and Pasgar scores of 8.9-7.1 in broiler chickens. Chick quality varies considerably in studies conducted depending on factors affecting chick quality. In the studies where optimum conditions were provided, the Tona score and Pasgar score were over 90 and 9, respectively. In this study, chick quality scores obtained for Japanese quails were found to be compatible with the averages obtained under optimum conditions in these studies.

The mean values of BW at 6 and 8 weeks of age were detected at 114.59 and 150.71 g, respectively. Comparable results (91.63 to 114.76 g) have been reported for body weight at 6 weeks of age by Aggrey et al. (2003), Tarhyel et al. (2012), Raji et al. (2014), and Rocha et al. (2020). In another study conducted by Daikwo et al. (2013), it was found that the live weight at 8 weeks of age was 133.76 g. However, some researchers (Akbaş and Yaylak, 2000; Balcıoğlu et al., 2005; Narinç et al., 2014b) reported much higher mean values for body weight (170.9-184.4 g) even at six weeks of age in random-bred control lines. Minvielle (2004) reported that discrepancies among studies in the literature are attributed to the adaptation of these birds to cage conditions from immigrant life and the genetic improvement that has been applied. Sex differences for BW6 and BW8 in Japanese quail have been reported previously by Aggrey et al. (2003) and Narinç et al. (2010a), who reported that the body weights of females were heavier.

The mean value of β_0 parameter (253.25 g) was in agreement with the mean values reported by Beiki et al. (2013) and Narinç et al. (2014a). Similarly, the mean values for β_0 parameter of Gompertz function in Japanese quails were estimated in the range of 242-276 g by Karabağ et al. (2017), Hyankova et al. (2001), and Kizilkaya et al. (2005). The estimations of integration coefficient parameter (β_1) of the Gompertz model for growth of Japanese quail were found to be 3.39 and 3.31 by Akbaş and Yaylak (2000) and Narinç et al. (2010a), which is in agreement with the mean value (3.25) of β_1 parameter in this study. The mean value of instantaneous growth rate parameter (β_2) was found to be 0.038 and this value is in agreement with the values (from 0.032 to 0.046) reported by Akbaş and Yaylak (2000), Narinç et al. (2014a), and Alkan et al. (2009). As a result of studies conducted by various researchers (Alkan et al., 2009; Akbaş and Oğuz, 1998; Kaplan and Gürcan, 2018), quite different results (ranged from 15 to 35 days of age) have been found for the age at inflection point of the growth curve. The results for IPW have been reported by Alkan et al. (2009), Kızılkaya et al. (2006), and Kaplan and Gürcan (2018) that ranged from 88.13 to 105.84 g.

The percentages of carcass, breast, leg, wing, and abdominal fat were 74.86, 28.52, 17.20, 5.36, 1.08 %, respectively, which were higher than those previously reported by Narinç et al. (2010c), Lotfi et al. (2011), Narinç et al. (2013), and Raji et al. (2014). The significant effects of the sex of the birds were observed for all slaughter-carcass traits. The average percentages of slaughter-carcass traits (BY, LY, and WY), except AFY, were higher in males compared with females. Similar sex differences for slaughter-carcass characteristic in Japanese quail have already been reported for BY, LY, and WY (Narinç et al., 2010c; Shokoohmand et al., 2007; Khaldari et al., 2010).

Heritability estimates for the chick quality traits measured using Tona and Pasgar methods were found to be 0.08 and 0.09, respectively (Table 2). There are no studies on scientific literature for the genetic structure of chick quality traits of poultry species. In terms of chick quality, 4.06 % of the phenotypic variance for the Tona score and 4.48% of the total variance for the Pasgar score could be explained by genetic factors, while the remaining environmental variance rates were 95.94% and 95.52%, respectively. This situation supports the view reported by Tona et al. (2005) that "although there are many genetic and environmental factors affecting chick quality, environmental factors have a larger share".

Heritabilities for BW at 6 and 8 weeks of age were estimated at 0.46 and 0.56, respectively (Table 2). Consistent with the findings in the current study, many researchers found high heritabilities (0.40-0.69) for both traits (Akbaş and Yaylak, 2000; Narinç et al., 2010b; Sezer 2007; Taraco et al., 2019; Sarı et al., 2011). The estimates of heritability for the Gompertz model parameter β_0 (0.17), β_1 (0.27), β_2 (0.26), and live weight (0.26) and age (0.19) at an inflection point of growth function were close to those reported in a previously published study in Japanese quail (Akbaş and Yaylak, 2000). Akbaş and Yaylak (2000) reported that the heritability estimates for β_0, β_1, β_2 parameters and point of inflection coordinates (IPT and IPW) of Gompertz model were low to moderate (0.18, 0.33, 0.32, 0.32 and 0.18, respectively).

The current estimate (0.04) of the heritability of carcass percentage (Table 3) was close to previously reported low estimates for Japanese quail which range from 0.12 to 0.21 (Lotfi et al., 2011; Sarı et al., 2011; Daikwo et al., 2013; Akbernejad et al., 2015). In the present study, heritabilities for BY, LY, and WY were found at 0.14, 0.09, and 0.07, respectively (Tablo 3). These estimates were in agreement with estimates reported by Vali et al. (2005), Akşit et al. (2003), Lotfi et al. (2011) ranged from 0.09 to 0.19. The estimated heritability for AFY (0.21) was low to moderate. In other studies involving quail, low to moderate heritabilities (0.23-0.29) were also reported for AFY trait (Akşit et al., 2003; Narinç et al., 2010c; Lotfi et al., 2011). However, Narinç et al. (2013) and Le Bihan Duval et al. (1998) reported higher heritability estimates for AFY in Japanese quail and chickens, which range from 0.35 to 0.84.

In the present study, the genetic correlation coefficients among all characteristics were estimated higher than the phenotypic correlation coefficients (Table 2 and Table 3). Cheverud (1988) reported that genetic correlation estimates were slightly larger than their phenotypic counterparts. The correlation patterns were strikingly similar in all studies using data sets based on appropriate effective sample sizes. Hence, Cheverud (1988) claimed that most of the difference between phenotypic and genetic correlation estimates was the imprecise estimates of genetic correlations.

The coefficients of phenotypic and genetic correlation between the Tona and Pasgar chick quality traits were 0.81 and 0.82, respectively. A positive and strong relationship between chick quality scores showed that these measurements were performed consistently and accurately. Positive and strong genetic and phenotypic correlations between weekly live weights (BW6 and BW8) were estimated to be 0.99 and 0.91, respectively (Table 2). These estimates were in agreement with estimates reported by Sezer (2007), Akbaş et al. (2004), and Sarı et al. (2011) and ranged from 0.80 to 0.96. The genetic relationships between the β_0-β_1, β_0-β_2 and β_1-β_2 parameters of the Gompertz model were negatively estimated. The low value of the parameter β_2 and long growing period resulted in high mature weight in birds as a consequence of the correlation between parameters β_0-β_2. These results were in agreement with similar research findings (Akbaş and Oğuz, 1998; Akbaş and Yaylak, 2000; Narinç et al., 2010b). Genetic correlations between parameter β_0, β_2, and weekly live weights were positive, and genetic correlations between β_1 and weekly live weights were negative. These estimates were similar to those reported by Akbaş and Oğuz (1998), Akbaş and Yaylak (2000), and Narinç et al. (2014a). This is the fact that an increase in the asymptotic (mature) body weight results in a decrease in the parameter β_2, which denotes the average rate of maturing. According to Ta-

ble 2, there were negative genetic correlations (-0.85 and -0.83) between body weight traits and IPT. These results agreed with the estimates of Mignon-Grasteau et al. (2000) who found a strong negative relationship (-0.60) between BW and IPT traits. Genetic correlations between IPT and IPW were estimated to be positive. Similar findings for these genetic correlations Narinç et al. (2010b) have also been reported.

Phenotypic and genetic relationships between chick quality traits determined by Tona and Pasgar methods in one-day-old chicks and yields of cold carcass and carcass parts were found to be low (genetic correlations between -0.14 and 0.13, phenotypic correlations between -0.05 and 0.05) and statistically insignificant. Phenotypic and genetic correlations between whole carcass yield, breast yield, thigh yield, wing yield were generally statistically significant and positive and generally moderate (Table 3). It was determined that the highest phenotypic and genetic correlations were between cold carcass and breast and thigh (0.61-0.78). Lotfi et al. (2011) reported similarly that the genetic relationships between cold carcass yield and percentage of breast and, between cold carcass yield, and the percentage of abdominal fat were 0.46 and 0.43, respectively.

CONCLUSION

Considering the results obtained from the study, it is not possible to say that Tona and Pasgar chick quality scores in Japanese quail have important genetic and phenotypic relationships with most yield characteristics. Since the chick quality is both low heritable trait and low genetic correlated trait with other yield characteristics, there is no requirement to be included directly in the selection index in a poultry breeding scheme, where improvement of the chick quality is also taken into account. In virtue of the chick quality is a very low heritable trait, environmental improvement of chick quality trait may be considered rather than genetic improvement.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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Research article
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Investigation of bovine interleukin-6 gene polymorphism and its association with *Cryptosporidium* infection in calves

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ABSTRACT: Interleukin-6 (IL-6) is associated with inflammatory diseases, but its connection with *Cryptosporidium* in Holstein calves remains unknown. This study aimed to investigate the effect of single nucleotide polymorphisms (SNPs) of IL-6 on the resistance and susceptibility to *Cryptosporidium* in calves and to prepare a phylogenetictree in order to show the relation between *Cryptosporidium* species. Seventy-two samples were studied from healthy and infected with *Cryptosporidium* calves and genotyped using the tetra amplification refractory mutation system (ARMS). The phylogenetic tree was constructed by the neighbor-joining method using the MEGA 7.0 software. The results showed a frequency of 76.40% for T allele and 23.60% for C allele in the healthy calves, while the results showed a frequency of 73.60% for T allele and 26.40% for C allele in calves infected with *Cryptosporidium.* The results did not reveal a significant difference between healthy and infectious animals according to the allele frequency (P=0.637). The phylogenetic tree demonstrated that *C. parvum* (HQ259589.1) with an 81% bootstrap were clustered with *C. hominis* (KM012041.1). The results also indicated that *C. parvum* (HQ259589.1) and *C. hominis* (KM012041.1) had a common ancestor with *C. cuniculus.* Additionally, *C. andersoni*(HQ259590.1) with an 88% bootstrap of support was placed in the same clade of *C. muris* (L19069.1), and both of them had a common ancestor with *C. serpentis*(KF240618.1). Further studies are required to investigate the relation between SNPs of IL-6 in other regions and the resistance or susceptibility to *Cryptosporidium* in calves.

Keywords: SNP, interleukin-6, phylogenetic tree, calves

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INTRODUCTION

Intestinal parasites are one of the most common challenges for health in countries with poor economy (Saki and Asadpouri, 2017). The species infect various animals and humans (Firoozi et al., 2019). *Cryptosporidium* species are observed in humans and animals and cause high water-borne prevalence all over the world (Alsmark et al., 2018). *Cryptosporidium* species are the most important species in inducing infection in calves and are a potential reservoir for zoonotic infection (Saki and Asadpouri, 2017). Intestinal epithelial cells are the initial cellular sites for *Cryptosporidium* infection in animals (Thomson et al., 2017). The induction of infection by *Cryptosporidium* species increases the expression of TLR2 and TLR4 that activate NFκB and some interleukins (Yang et al., 2015). Interleukins are commonly produced by macrophages, dendritic cells, and lymphocytes during response to infections (Diez-Fraile et al., 2003). Interleukin-6 (IL-6) increases the production of antibodies in response to inflammation, regulates leukocyte populations, and promotes the T cell production (Dienz and Rincon, 2009). The interleukins, particularly IL-6, are commonly considered a marker for early inflammation and prognosis in calves (Rincon, 2012). Lacroix et al. (2001) reported the key role of IL-6 in *C. parvum* infection, showing the increased expression level in adult knockout mice. The changes in some sequences and/or single nucleotide polymorphisms (SNPs) are related to *Cryptosporidium* infection (Widmer et al., 2012). SNPs could change the expression and activity of the genes and the related gene products. SNPs as the genetic markers can be used in the dairy industry, and farmers can raise genetic lines with lower susceptibility to *Cryptosporidium* infection. The association between IL-6 and inflammatory diseases is well known, but its connection with infected calves from *Cryptosporidium* is still unknown. Therefore, this study aimed to investigate the genetic polymorphism of the bovine IL-6 gene. The study also investigated the effect of the identified SNP of IL-6 on the resistance and/or susceptibility to *Cryptosporidium* in infected calves.

Furthermore, the use of phylogenies based on larger datasets of sequences from multiple genes provides greater resolution of phylogenetic relationships between organisms (Naushad et al., 2015a, 2015b). We also prepared genome sequencing for *Cryptosporidium* isolates and utilized the data to construct broad and well-resolved phylogenetic trees based on all genes in the core genome of *Cryptosporidium*. We aimed to prepare a highly reliable base to understand interspecies relatedness among *Cryptosporidium* species.

MATERIALS AND METHODS

Animals

The present study was conducted on 72 calves of the Holstein-Friesian breed, comprising 36 healthy calves and 36 calves infected with *Cryptosporidium* diarrhea. The samples were collected from preweaned calves (≤2 months) in the town of Shahrood (Semnan-Iran). The calves were randomly selected from different dairy farms. It is essential to mention that dairy farms used artificial insemination and did not use conventional methods for mating, resulting in a lower rate for inbreeding.

DNA extraction and Tetra ARMS

Blood samples (5 mL per animal) were collected from the jugular vein into EDTA containing vacutainer tubes. The samples were stored at -91°C until future use. DNA was extracted by the commercial kits of CinnaGen Company. The quality and quantity of the isolated DNA were investigated by agarose gel electrophoresis (2%) and NanoDrop spectrophotometer (GE Healthcare) prior to use in PCR and DNA sequencing. To investigate the DNA quality, purified DNA was run in agarose gel, and the OD 260/280 ratio for all the samples was between 1.8 and 2.

The PCR conditions were as follows: denaturation at 95°C for 5 minutes, denaturation at 95°C for 45 seconds, annealing at 58°C for 30 seconds, extension at 72ºC for 30 seconds and final extension at 72°C for 10 minutes. To amplify the IL-6 gene, specific primers were designed using the Primer3 software (http://frodo.wi.mit.edu/; NW_001494874.2). The specified forward and reverse sequence primers used were compared to other bovine DNA sequences by the basic alignment search tool (BLAST) option from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by the input of a DNA reference sequence. One SNP was observed at amplicon E +519, and primers were genotyped at individual cow DNA at this locus (c.+472-152C>T). Table 1 shows the used primers.

The samples were genotyped and conducted by Tetra ARMS primers generated using a publicly available software program (Ye et al., 2001).

Table 1: The primers used for the Tetra ARMS PCR

Tetra ARMS Primer Sequence	Temperature	bp size
Forward inner primer C allele: 5 GGGCTCAGAGCAGAGGACCTCCCACC-3	67.80	225
Reverse inner primer T allele: 5-GCCACTGGCCTTGACTGCCCAGCTA-3	68.20	255
Forward outer ARMS primer: 5-AGGCCCCCGAAGAACCCATTAAAATGCCT-3	65.30	428
Reverse outer ARMS primer: 5- TCCAGCAGGTCAGTGTTTGTGGAG-3	65.60	428

Phylogenic tree

The sequence for *Cryptosporidium* species was downloaded from the NCBI'sGene Bank database. The alignments were used for phylogenetic analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. The analysis involved 17 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 516 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura et al., 2013).

Statistical methods

The results for genotypic and allelic frequencies were analyzed by the FREQ procedure of the SAS software (SAS Institute Inc., v. 9.2). The Hardy-Weinberg equilibrium of the mutation was investigated by the chi-square test.

RESULTS

Figure 1 presents the results for genotype frequency in healthy calves and those with *Cryptosporidium*. The results indicated that TT genotypic frequency was 52.80% (n=19), and the CT genotype frequency was 47.20% (n=17) in healthy calves. The genotypic frequencies for the CT genotype (n=19) and the TT genotype (n=17) in infected calves were 52.80% and 47.20%, respectively. The CC genotype was not observed in the calves. The frequencies were 23.60% and 76.40% of C and T alleles, in healthy calves,respectively, while, the frequencies were 26.40% and 73.60% of C and T alleles, in infected calves, respectively (Figure 2). The results did not show a significant difference between the allelic frequencies in healthy and infected calves (X^2=0.222, P=0.637). This result indicates a lack of association between polymorphism of the IL-6 gene and the occurrence of *Cryptosporidium*.Figure 3 depicts the amplicons +472-152C>T SNP of genotype determination by gel electrophoresis.

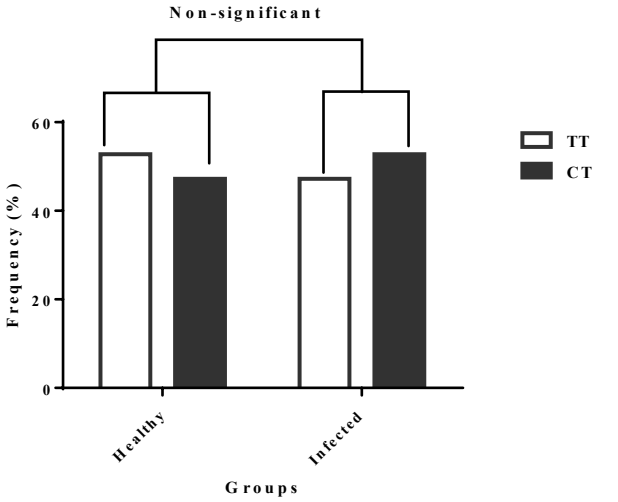

Figure 1: The frequency of genotypes in healthy and infected calves. The results did not show a significant difference between healthy and infected calves

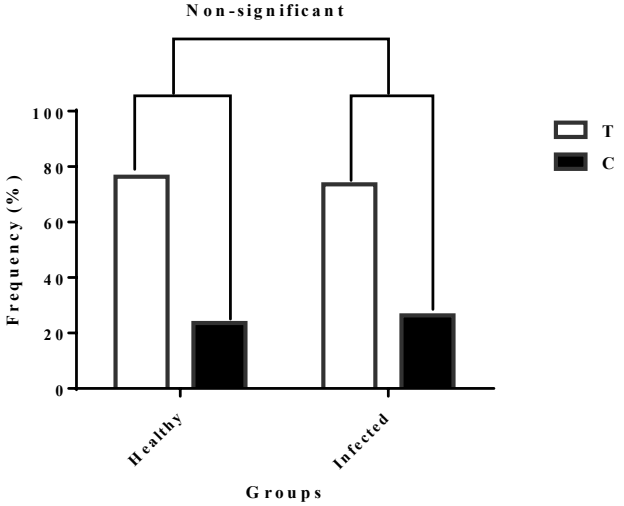

Figure 2: The frequency of alleles in healthy and infected calves. The results did not show a significant difference between healthy and infected calves

Figure 4 displays the phylogenetic relationships of *Cryptosporidium* parasites. The phylogenetic analysis showed that our samples were grouped with identity values ranging from 80.2 to 100%. *C. parvum*, *C. bovis*, *C. andersoni* and *C. muris* were distinguished.

The results showed that *C. parvum* (HQ259589.1) with an 81% bootstrap was clustered with *C. hominis* (KM012041.1). The results also revealed that *C. parvum* (HQ259589.1) and *C. hominis* (KM012041.1) had a common ancestor with *C. cuniculus.* In addition, *C. andersoni*(HQ259590.1) with an 88% bootstrap of support was placed in the same clade of *C. muris* (L19069.1), and both had a common ancestor with *C. serpentis*(KF240618.1).

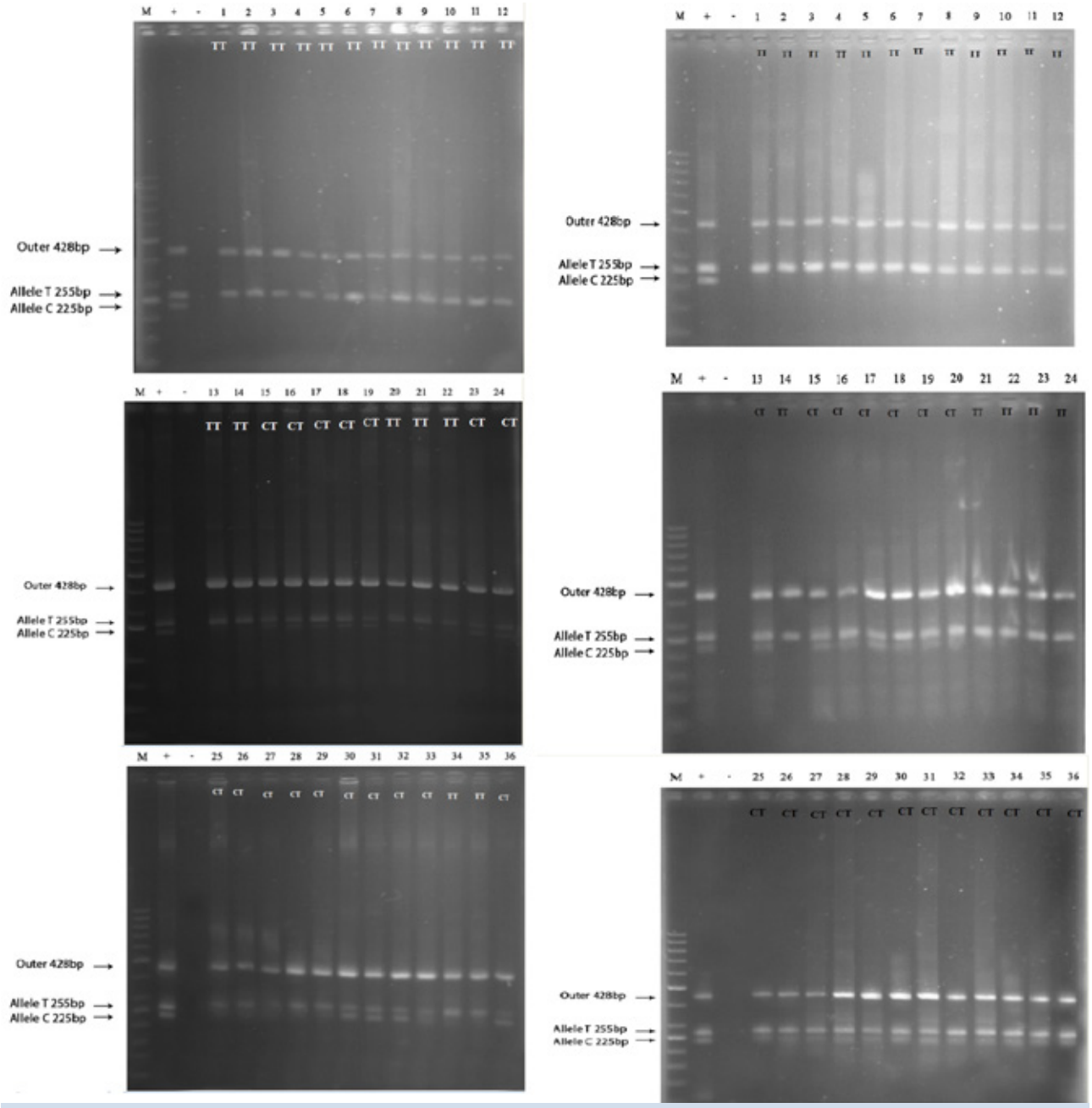

Figure 3: Gel electrophoresis of IL-6 SNP genotyping by specific Tetra-ARMS primers. M shows molecular weight markers, where CC and CT show the calves genotype at the IL-6 c.+472-152 C>T SNP locus. At the left column gels are shown the PCR amplicons of healthy and at the right column gels the PCR amplicons of infected calves

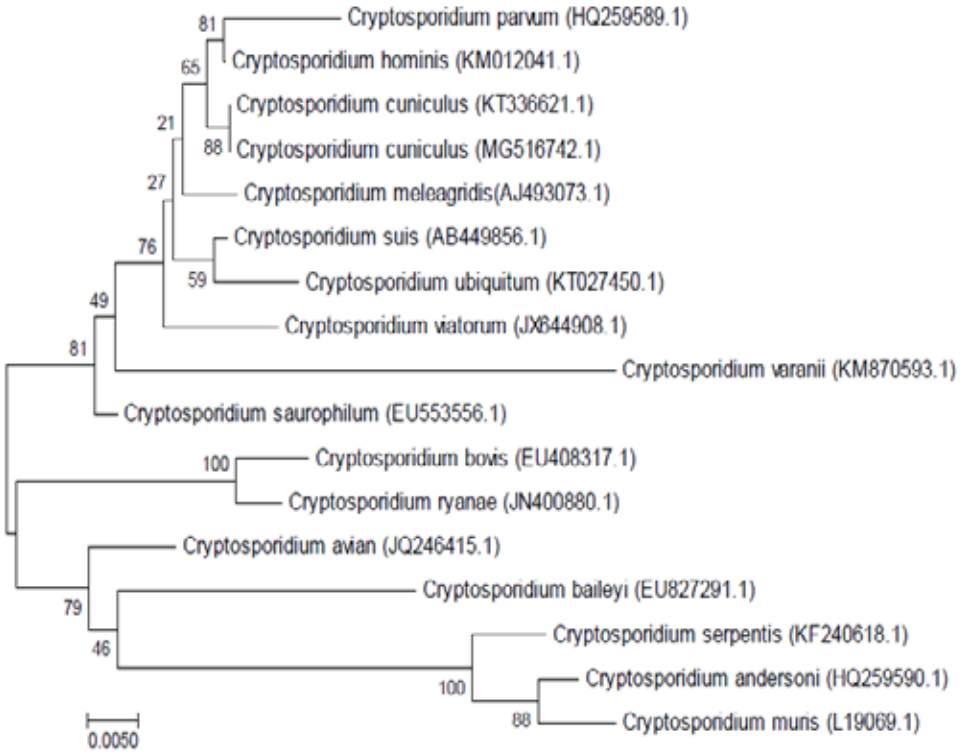

Figure 4: Phylogenetic relationships of Cryptosporidium parasites

DISCUSSION

The current study investigated polymorphism in the bovine interleukin-6 gene and its association with *Cryptosporidium* in calves in Iran. Until now, studies have not investigated SNPs in the bovine interleukin-6 gene and their association with *Cryptosporidium* infection. However, some studies have shown the relation between other interleukins and *Cryptosporidium* (Yang et al., 2015). The relation between IL-6 and *Cryptosporidium* in mice has been reported (Lacroix et al., 2001). Some studies have reported IL-6 as a prognostic marker associated with a specific pathogen in people like children with hemolytic uremic syndrome(Karpman et al., 1995), shigellosis (de Silva et al., 1993) and viral diarrhea (Jiang et al., 2003; Azevedo et al., 2006). The diseases have been related to intronic polymorphisms or nucleotide repeats (Stangl et al., 2000; Sanghera et al., 2004; Kumar and Ghosh, 2008). However, our results did not show any association between the polymorphism of bovine IL-6 and *Cryptosporidium* in calves. It might be attributed to the location of the SNPs. SNPs are potential diagnostic and therapeutic biomarkers for some diseases. The location of SNPs influences resistance and sensitivity to diseases (Deng et al., 2017). Ghavimi et al. (2016) showed that polymorphisms within the promoter or other regulatory regions of cytokine genes only influence the transcriptional activity.Donath &Shoelson(2011) reported that sensitivity and resistance to inflammatory diseases depend on the location of SNPs of IL-6. It could be said that studied region has not any relation with *Cryptosporidium*but the relationship might be in different regions of IL-6. Genetic diversity could be increased by selecting calves with unrelated pedigrees to the maternal and paternal grandsire for *Cryptosporidium*. Our results demonstrated that the T allele frequency was significantly higher than the C allele frequency. It is considered that SNP may have recently emerged in Holstein calves, and the C allele is probably new for the population or that the T allele is more appropriate. The results did not show any frequency for the CC genotype. The absence of CC might indicate that this genotype is genetically inappropriate.The results obtained in the current study do not follow Mendalian genetics. Blake et al. (2009) reported similar results by indicating the lack of a relationship between polymorphisms of IL-6 and mastitis. In sum, our results also suggest the lack of a relationship between polymorphisms of IL-6 and *Cryptosporidium* in Holstein calves.

Several studies have conducted phylogenetic analyses of *Cryptosporidium* parasites. Contrary to our findings, an initial SSUrRNA sequence analysis showed more than 99% identity between *C. parvum* and *C. muris*(Cai et al., 1992), while other studies failed to

separate *C. parvum*, *C. muris*, and *C. baileyi*(Tzipori and Griffiths, 1998). The differences among species suggest the biological differences between *Cryptosporidium* parasites. The two *Cryptosporidium* groups are differentiated from each other, since *C. parvum* and *C. baileyi*initially infect the small intestine and the respiratory tract, while *C. muris*and *C. serpentis*commonly infect the stomach (Cai et al., 1992).

CONCLUSION

In conclusion, the results suggest the lack of an association between polymorphisms of bovine IL-6 and *Cryptosporidium* infection. These findings suggest that alleles in IL-6 in the studied region cannot play a role in protecting against *Cryptosporidium* infection in calves. The phylogenetic analysis showed that the sequence analysis showed more than 99% identity between *C. bovis, C. parvum* and *C. muris*. The major limitation of the current study is that it was limited to calves; therefore, the results cannot be used for other animals. We recommend that more studies be conducted on other animals to investigate the relation between polymorphisms of bovine IL-6 and *Cryptosporidium* infection.

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Research article
Ερευνητικό άρθρο

Cloning of the xylanase gene from soil *Streptomyces* into *Escherichia coli* for the poultry industry application

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ABSTRACT: *Streptomyces* are gram-positive aerobic strains that are isolated from soil, water, sediments, and various sources. The bacteria are capable of producing secondary metabolites, such as enzymes that sometimes play unique functional roles in industry, and are one of the important bio-control agents. This study aimed to isolate and clone the*xylanase* gene from soil *Streptomyces*. Soil samples were collected from Markazi Province, Iranafter specific biochemical examinations, isolation of bacteria, and DNA extraction. PCR was then performed to identify the strains containing the *xylanase* gene. The gene from the positive strains was cloned into an *E.coli* host-vector by TA cloning technique and finally, the expression of genes in *E. coli origami* was measured by Real-Time PCR technique. ClustalX and Mega5 software were used to draw the phylogenetic tree. A total of twelve *Streptomyces* isolates were identified from the soil samples. Among all the isolates, threeones had the *xylanase* gene. After cloning the *xylanase* genes, the cloned strains were isolated. To confirm the DNA cloning, Real-Time PCR was performed, and finally, the PCR product where sequenced. In this study, *Streptomyces* was identified as a native strain for the expression of xylanase after generating recombinant plasmid and TA cloning. It can be stated that cloning of the xylanase gene from soil *Streptomyces* in *E. coli* can be used in the poultry industry.

Keywords: *Streptomyces, Xylanase*, TA-Cloning

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INTRODUCTION

Cellulose and hemicellulose arethe most abundant renewable polymers in plant cell walls. Xylan is the most crucial compound in lignocellulosic agro-residues (Verma et al., 2013).It is composed of β-1,4-linked xylosyl residues and different groups (arabinosyl, acetyl,and glucuronosyl) in its side chains. Xylan is a significant compound in hemicellulose that comprises 20-40% of total plant biomass. This compound has an essential role in maintaining the cell wall integrity through the creation of covalent and non covalent bonds with cellulosic fibers and lignin (Li et al., 2008).Xylanasesare glycosidases that catalyze the endohydrolysis of 1, 4-β-D-xylosidic linkages in xylan to shorten xylooligosaccharides (Li et al., 2008). Xylan needs a complex of xylanolytic enzymes for degradation, such as xylanase (Emam et al., 2016).Regarding the amino acid sequence homologies and hydrophobic cluster analysis, xylanases are divided into two families of glycosylic hydrolyses (I) family for GH 10 and (II) family G or GH 11 (Kishishita et al., 2014).Xylanase gene is commonly isolated from different microorganisms and expressed in *Escherichia coli*. It was reported that xylanase is not only produced at lower activity levels in bacteria compared to fungi that are restricted to the intracellular or periplasmic fractions (Ahmed et al., 2009).

Streptomyces are aerobic, filamentous Gram-positive soil bacteria that produce extracellular enzymes able to degrade natural polymers such as xylan (Sevillano et al., 2016).Their use as hosts for the production of the enzyme can improve problems encountered with other systems. Bacteria have many advantages compared to other microorganisms such as requiring less growth time.Also, bacterial xylanase is more functioning at high temperature and alkaline conditions, which are more appropriate for most applications (Subramaniyan et al., 2001). Some xylanase genes have been cloned and expressed in *E.coli* (Akyol et al., 2009; Guo et al., 2009).

The arabinoxylan present in cell walls of poultry feeds has an anti-nutrient effect on poultry. Xylan in soluble form increases the viscosity of the ingested feed, influencing the mobility and absorption of other components (Mirzaie et al., 2012).Dietary inclusion of xylanase in poultry feeds may improve the digestion of nutrients in the initial part of the digestive tract, and improve energy utilization (Polizeli et al., 2005). Regarding the importance of xylanase in the poultry industry, for the first time, we aimed to isolate the xylanase gene from soil *Streptomyces* into *Escherichia coli* for application in the poultry industry.

MATERIALS AND METHODS

Microorganism isolation and culture

We isolated *Streptomyces* sp. from soil samples obtained in Arak, Iran. To separate and identify proteolytic microorganisms, skim milk agar medium, and biochemical tests were used. To isolate Streptomyces sp., heat and dried treatments were used, and dilution was conducted. To dilute soil samples, 10 g aseptic soil was added to 90 ml sterilized distilled water. The samples were mixed, and then1 ml of sample was transferred into a tube containing 9 ml physiological serum. An amount of 0.2 ml of the mixture was transferred into skim milk agar medium, homogenously distributed by glass spread under aerobic condition, incubated at 30 ˚C for 72 h, and investigated for growth. *Streptomyces* sp. were obtained by biochemical tests and morphological properties in specific cultures. To keep a short time and long time, the isolates were prepared for the microbial bank and screening step. All the strains were transferred into tubes after purification. The samples were cultured, incubated, and observed at 2-5 ˚C. The samples were investigated for survival after 30 days.

Identification of bacterial isolates, and DNA extraction

To determine bacterial isolates based on morphological properties, microscopic and biochemical properties were used, including motility, indole, methyl red, VP, citrate, pigment production, gelatin hydrolysis, nitrate reduction, oxidase, catalase, casein hydrolysis, starch hydrolysis, sucrose, and SH2 production tests were used.

To extract bacterial DNA, a DNA extraction kit (Iran Genetic Reservoir Center) was used. Mini column extraction was conducted by the kit.

PCR reaction

For cloning of the obtained xylanase gene, the gene fragment was amplified by polymerase chain reaction (PCR). The primers were (F, 5′-*CCCGCTAGCATGACAGCGAGTTTGAGGAAGA*-3′; R, 5′-*CCCCTCGAGTTACGGCGTGTTTCCGTAGC*-3′). 10 µl PCR Master Mix 2x, 1 µl primers with concentration of 10 pm/µl, 4 µl template DNA(150 ng), and 4 µl distilled water were added. The PCR was set up as follows, initial denaturation at 95 ˚C for 5 min, 35 cycles of de-

naturation at 95 ˚C for the 30s, annealing at 60 ˚C for 30s, replication at 72 ˚C for 1 min, and final replication at 72 ˚C for 10 min. To conduct DNA electrophoresis, 1.5% agarose gel was prepared. For the post-run staining, Ethidium bromide was used.

Cloning

For the cloning the PCR product PCR TA-Cloning package of CinnaGen Company (Tehran, Iran) was used. Based on the company's guidelines, a linear vector of PTG19-T was used. Application of linear vector of PTG19-T caused a direct connection between PCR product and cloning vector. T4 DNA ligase enzyme created a covalent bond to the linear vector, and it did not need any enzymatic digestion. PCR TA-Cloning has 5 steps, including 1) Production of PCR product by Taq DNA polymerase, 2) Ligation of the PCR product in the PTG19-T cloning vector, 3) Transformation of vector to *E.coli* XL1-Blue host, 4) Approval of the cloning, and 5) Identification of positive clones. The approval of the cloning, was performed through four steps, 1) Conducting PCR by vector primer, 2) Colony selection by Blue/White screening and growth in presence of ampicillin, 3) Gene expression by Real-Time PCR, and 4) Sequencing junctions. To conduct ligation, a 10 µl mixture was prepared, including 2 µl PTG19-Tcloning vector, 1 µl T4 ligase enzyme, 1 µl buffer, 1.5 µl PCR products, and 4.5 µl water. The mixture was transferred into a vial at 22 ˚C and heated by a hotplate. To transform the vector to *E. coli*, liquid, and solid LB Broth were used.

Preparation of competent cells

E. coli uragami was obtained from Tehran University, and cultured for 16-17 hours at 37 ˚C and centrifuged at 10000 rpm for 10 min.the upper solution was removed and 100 µl buffers was added to the pellet and centrifuged at 12000 rpm for 1 min. Then, 50 µl buffers was added as well and placed on ice for 5 min. It was centrifuged at 12000 rpm for 1 min, and 500 µl buffers were added again. It was placed at 42 ˚C for 90 s and placed on ice for 10 min. finally, 400 µl of culture media was transferred into 1.5 ml vials and transfer materials were added and placed at an incubator for 45-60 minutes. Ampicillin was also added to the medium. The mixture was centrifuged at 650 rpm for 1 min, the upper solution was removed and the bacterial pellet was cultured on an LB plate, incubated at 37 ºC for 2 days, to approve the cloning. The cloning vector of PTG19-T contained the Lac z gene. Since the medium contained ampicillin, colony growth could show resistance to ampicillin as a result of plasmid transfer.

Recombinant bacteria were incubated before PCR and extracted after 15h incubation. RNA was extracted after 15h by RNase micro-kit (Qiagen Co, Germany). The bacterial suspension (Bacteria resistant to xylanase gene) were in the logarithmic phase (OD_{600}=0.4-0.6).

Quantitative and qualitative assessment

The extracted RNA was assessed for qualitative and quantitative evaluations. To evaluate RNA, light absorption was used in 260 and 280 nm. 2 µl extracted RNA was positioned in nano drop and absorption was investigated in 260 and 280 nm.

The cDNA was obtained by Reverse AMV enzyme (Roche Co, Switzerland) in the concentration of 25 µl/unit. The extracted RNA was incubated at 65˚C for 3 mins. Reverse transcription was performed at 42˚C for 60 mins by using 2 µl Random Primer, 8 µlAMV Reverse Transcriptase, 2 µl dNTP (10 mmol), 1 µl RNase inhibitor, and 2 µl 10x buffer of AMV enzyme. AMV enzyme was incubated at 99 ˚C for 5 minutes and inactivated.

Real-Time-PCR was performed in a volume of 20 µl by GeNetBio Cat.No: Q9210 (South Korea). To conduct Real-Time-PCR, 10 µl Prime Qmaster mix (2x) with cyber green, 5 µl Depc water, 1 µl forward primer, 1 µl reverse primer, 1 µl Rox dye, and 2 µl cDNA were used. Fragment replication was conducted in Corbet apparatus by using initial denaturation in 95 ˚C for the 30s, followed by 35 cycles of 59 ˚C for 40s, and 72 ˚C for 60s. 16srRNA housekeeping gene was used as an internal control. To calculate the expression of xylanase, and illustration of figures, specific software was used. The expression analysis was conducted compared to the standard strain.

Illustration of the phylogenetic tree

The analyses for sequencing were investigated by BioEdit software and ordered by DNA Baser. The obtained sequences were compared with registered sequences in NCBI. Each sequence was searched by Blast and sequences were set by W Cluster. Phylogenetic analysis was illustrated by W Clustral by using 5.10 MEGA software in the Neighbor-Joining method.

RESULTS

Screening and identification of *Streptomyces* sp.

Twelve isolates of *Streptomyces* sp. were isolated

based on morphological, microscopical, and biochemical tests. They absorbed theGram staining (Gram positive). Also, the other results were as follows: citrate, casein, oxidase, and catalase tests were positive, but nitrate reduction and VP tests were negative.

PCR

PCR was performed for the xylanase gene by the mentioned primers. The results showed that three strains had the xylanase gene. (Figure 1).

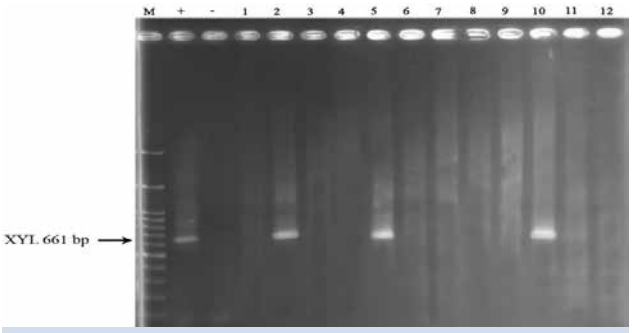

Figure 1. Xylanase gene PCR result

Xylanase gene cloning

After cloning of xylanase genes (blue/white), cloned strains were isolated (Figure 2). The results for RNA extraction confirmed RNA extraction as well (Figure 3).

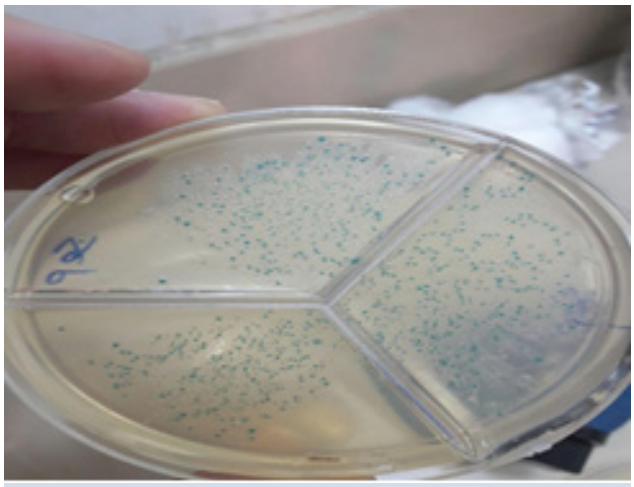

Figure 2. Colony selection(blue/white colony) of the xylanase enzyme

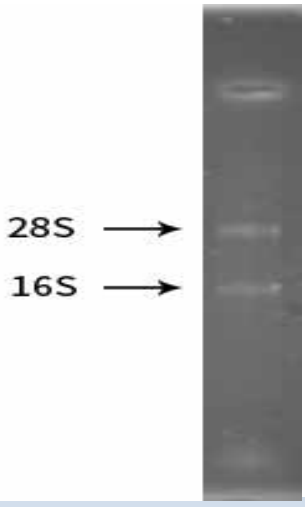

Figure 3. RNA extraction confirmation

Expression of the cloned gene by Real-Time PCR

To approve RNA cloning, cDNA was produced and investigated by Real-Time PCR for the expression of the xylanase gene (Figure 4). The results for the melting curve of PCR are shown in Figure 5.

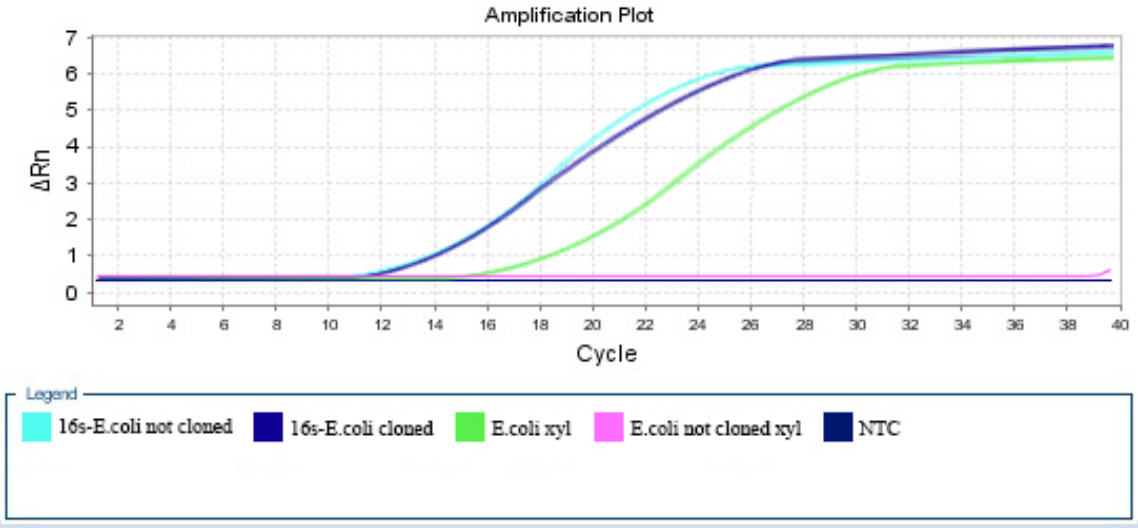

Figure 4. Real-Time PCR results

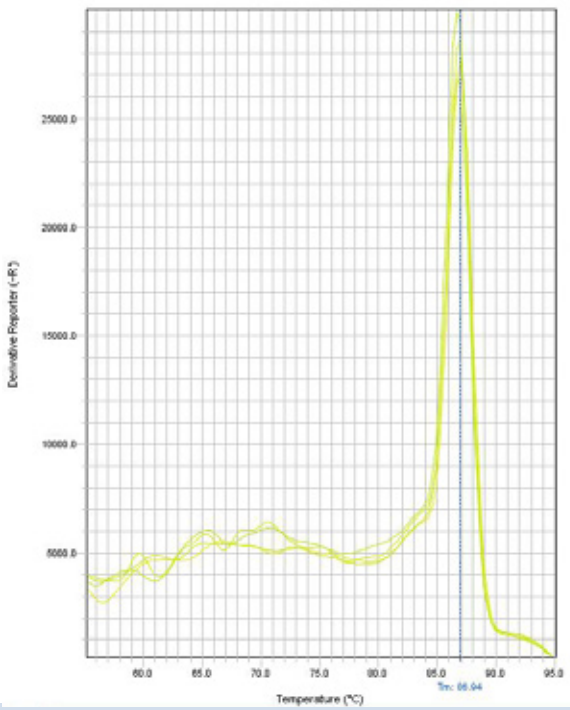

Figure 5. Melting curve of Real-Time PCR

Identification of molecular characteristics of *Streptomyces* sp.

To characterize *Streptomyces sp.,* 16SrRNA was used (Figure 6), and the PCR product was sent to Bioneer Company, South Korea for investigation of BLAST (Figure 7).

The results for the phylogenic tree by neighbor-joining method showed that BPSEAC7 *Streptomyces sp.* was in the same cluster with MI02-7b *Streptomyces* that shows a closed relation between them.

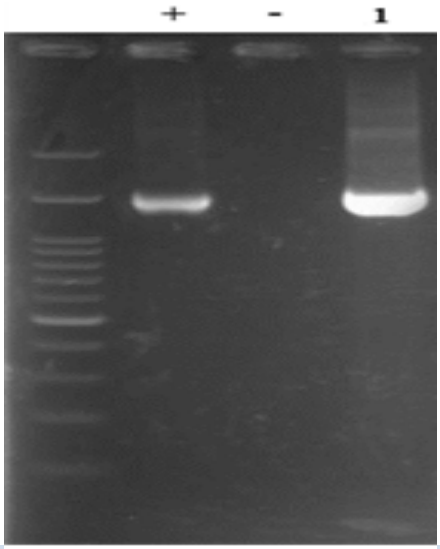

Figure 6. PCR test by 16S rRNAto identify molecular characteristics of *Streptomyces sp.*

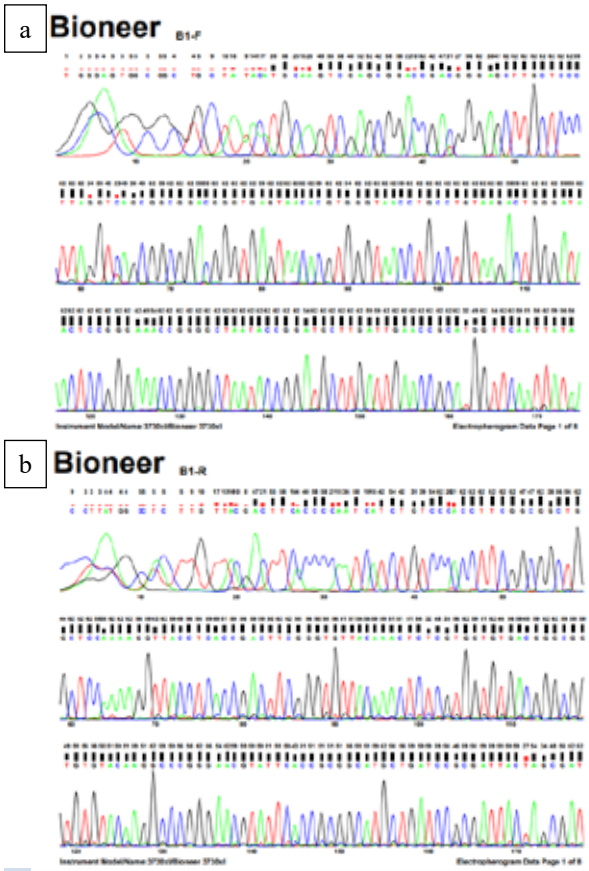

Figure 7. Sequencing and BLAST of forward (a) and reverse (b) of xylanase

DISCUSSION

This study was conducted for cloning the xylanase gene from soil *Streptomyces* into *E. coli* for application in the poultry industry. Xylanase gene was cloned by blue/white screening, and all the strains were isolated, and the PCR product sequence of xylanase gene expression in the bacteria *E. coli origami* was endorsed. Several xylanase genes were previously isolated from various microbial organisms and expressed in *E. coli* (Goswami et al., 2014). It was reported that cloning and expression of a GH11 xylanase gene from *Aspergillus fumigatus* MKU1 in *Pichia pastoris* (Jeyaet al., 2009). Hwang et al. (2010) performed the cloning of xylanase, KRICT PX1 from the strain *Paenibacillus sp*. HPL-001. Wang et al. (2011) showed direct cloning, expression, and enzyme characterization of a novel cold-activexylanase gene (*Xyn*GR40). In another study, Lin et al. (2013) showed cloning and expression of a thermostable *xylanase* from *Bacillus halodurans* C-125 (C-125 *xylanase* A).Kishishita et al. (2014) showed cloning and expression of cellulose inducible endo-β-1, 4-*xylanase* (*Xyl*10A) from the mesophilic fungus *Acremonium cellulolyticus*. The

isolates of this study showed similarity and closeness to *Streptomyces* species.In this work, we isolated a novel indigenous *Streptomyces* strain with the capability to express the xylanase enzyme. The novelty of our study is about transferring the enzyme gene to *E.coli origami* through the recombinant plasmid of PTG19. As the plasmid carried an ampicillin resistant gene so it was a perfect match for transferring xylanase gene by as the *E.coli* is sensitive to ampicillin; growing in presence of ampicillin proves the successful transfer of the xylanase gene. Also, as the plasmid carried *lac* gene, the blue-white screen was another way to ensure the success in cloning. The next step we are taking is to characterize the enzyme and trying to improve the expression through genetic engineering. from soilfor the first time, the xylanase gene was isolated from *Streptomyces sp.*and cloned in *E. coli* origamiby recombinant plasmid of PTG19and TA cloning.The expression of cloned xylanase gene in the extracellular medium of *E. coli*not only approved the successful cloning process but also draws a promising image for future commercial applications in the poultry industry.

CONFLICT OF INTEREST

There is no conflict of interest to be declared.

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Research article
Ερευνητικό άρθρο

Digestibility and Silage Quality of Potato Pulp Silages Prepared with Different Feedstuff

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ABSTRACT: The objective of this study was to determine silage quality and digestibility of potato pulp ensiled with different feedstuffs (straw, alfalfa hay and wheat bran). A total of 5 different potato pulp silages (PPS) were prepared with 5% ground barley straw, ground alfalfa hay, wheat bran alone or combination of 2.5 % wheat bran+ground straw, and wheat bran+ground alfalfa hay on a fed basis.These silages were ensiled in 2-L jars. After 49d of ensiling, all silages were opened and the chemical composition, pH, organic acids, ammonia-N contents, *in situ* organic matter (OM) and starch degradabilities and *in vitro* OM digestibility (IVOMD) of the silages were determined. Then, energy values were calculated.The highest dry matter (DM) and OM concentrations were observed in PPS prepared with wheat bran alone ($P<0.05$). Potato pulp silage containing ground barley straw alone had the lowest crude protein (CP) concentration ($P<0.05$) and silage containing wheat bran alone had the lowest neutral detergent fiber (NDF) and acid detergent fiber (ADF) concentrations among PPS ($P<0.05$). Potato pulp with wheat bran alone had the highest lactic acid concentrations among all silages ($P<0.05$). Acetic acid concentrations of silages were similar ($P>0.05$). The *in situ* OM degradability and IVOMD were significantly different ($P<0.05$) among all silages. However, in situ starch digestibility was similar among PPS ($P>0.05$). It can be concluded that PPS prepared with different feedstuffs at a 5% level had good fermentation properties and high degradability values. Even a high quality PPS can be obtained with the addition of 5% ground barley straw alone.

Keywords: *In vitro* digestibility; potato pulp; silage quality.

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INTRODUCTION

The highest cost of animal husbandry expenses in Turkey constitutes feed costs (Boğa and Çevik, 2012). Among the feed costs, the most serious problem is experienced in obtaining high quality roughage at the right price.

Potato farming has been successfully carried out in Turkey for many years. However, there is no clear data on how much of the potatoes are consumed fresh and how much of them are processed in Turkey. In recent years, Turkey's potato industry has developed rapidly and potatoes are offered for consumption as chips and frozen potatoes. In addition, another usage field of potatoes is starch production and quantity of waste pulp (Yang et al., 2018; 2019). Hundreds thousands of tonnes potato pulp produced every year in the world from starch factories (Kurnik et al., 2015). The production of starch from potato in Turkey started in a factory established in Konya by private sector initiative. The factory was established in 2013, made trial production in 2014 and potato starch production started in 2015 by contracting planting with the farmers in the region. Potato pulp, a by-product obtained after potato starch production, has the potential to be a feed source that can be utilized by animals (Cheng et al., 2019).

By-products obtained from potato enterprises (chips products, frozen potato products, potato shells) were generally reported to contain 3.7% to 27.1% CP, 3% to 55.9% starch, 20% to 65.2% NDF, 6.2% to 31.2% ADF, 2.9% to 6.9% ether extract (EE) on DM basis (Okine et al., 2005; Aibibula et al., 2007; Nelson et al., 2010; Valadares et al., 2019).

Potato processing products (especially potato pulp) is not much known in Turkey. However in countries where potato production is high, these are considered as animal feed (Wang et al., 2010). Indeed; Wang et al. (2010) have utilized potato pulp fermented in solid form as a poultry feed, Aibibula et al. (2007) have used PPS instead of ground corn in ruminants. These studies display that this product can make a significant contribution to the national economy if properly utilized in animal nutrition.

The preservation of potato pulp, which is a wet product, is possible by either drying or silage making. However, drying is probably not an economically viable alternative due to the high energy cost (Okine et al., 2005). Silo water loss is the main problem in ensiling of high moist materials. This problem can be eliminated by adding absorbent to PPS. Indeed, it has previously been reported that absorbents have been used successfully in ensiling of high moist roughage to reduce DM loss and increase silage quality (Jones et al., 1990; Zhang et al., 2012; Mohamadian et al., 2016).

In the light of available information, it was aimed to determine silage quality and digestibility of potato pulp by ensiling with different feedstuffs (barley straw, alfalfa hay and wheat bran) used as moist absorbent.

MATERIALS AND METHODS

The potato pulp used in the trial was obtained from Konya sugar potato starch production facilities owned by Konya Sugar Company. Barley straw, ground alfalfa hay and wheat bran used as an absorbent in silage making were obtained from the Ankara region. The nutrient contents of pulp and absorbent substances are given in Table 1.

The samples taken from Konya sugar potato starch production facilities with plastic bags were brought to Lalahan International Center for Livestock Research and Training (ICLRT) and mixed thoroughly to prevent water loss during transportation due to high humidity of potato pulp. In this way, 5 different potato pulp silage were prepared in 2-L jars using potato pulp brought to ICLRT.

For this purpose; (treatment)

the mixtures of 7600 gr potato pulp, 200 gr wheat bran and 200 gr barley straw (BSP), 7600 gr potato pulp, 200 gr wheat bran and 200 gr ground alfalfa hay (BAP), 7600 gr potato pulp, 400 gr ground alfalfa hay (AP), 7600 gr potato pulp, 400 gr wheat bran (BP), 7600 gr potato pulp, 400 gr barley straw (SP) were prepared, then 4 replicates have been ensiled for each treatment groups in 2-L jars.

Table 1. Nutrient contents of PP and different feedstuff used in the experiment, DM %

Items	DM	CA	CP	NDF	ADF
Potato pulp	16.05	3.18	5.04	35.08	17.33
Barley Straw	91.8	6.1	3.4	72.3	55.4
Alfalfa hay	89.3	9.39	19.49	33.97	28.85
Wheat bran	91.5	2.62	12.6	29.63	7.96

DM: Dry matter, CA: Crude ash, CP: Crude protein, NDF: Neutral Detergent Fiber, ADF: Acid Detergent Fiber.

These mini silos were opened at the end of 49 days ensiling period and silage pH were determined. For this purpose, 25 g samples were mixed with 100 ml distilled water for 5 minutes in the mixer. pH measurement was made from silage filtrate (Bingöl et al., 2008). The silage filtrates were stored at -18 °C until ammonia nitrogen and organic acid analyzes were performed. In order to determine the DM of silage samples, 1 kg sample was weighed in clean aluminium containers and left to dry for 6 days at 49 °C until samples were completely dried (Kutlu, 2008). After DM determination of the samples, the samples were ground to pass 2 mm screen and prepared for the subsequent analysis.

To determine the *in situ* nutrient degradation of silages, 3 Holstein cattles (8 year-old, about 600 kg live weight) with rumen cannula were used (International Center for Livestock Research and Training Ethics Committee, 30.11.2015/117). Before initiation of the experiment, animals were treated with Detomax® and Anaverm® to eliminate the internal and external parasite. Cattles consumed alfalfa hay 10 days before the initiation of the experiment until the end of experiment. Cattles had free access to clean water and vitamin-mineral blocks throughout the experiment.

Approximately 4 grams of silage samples, which were dried and ground to pass 2 mm scren, were placed in nylon (Dacron) bags with a pore size of approximately 45µ. Each sample were placed into the rumen of each animal as duplicate for each given times. The mouth of the bags was tightly tied with the package rubber and placed into 20x40 nylon nets with a pore size of 0.3 cm, containing marbles to keep the bags in the ventral part of the rumen. Samples were incubated in the ventral part of rumen for 0, 2, 4, 8, 12, 24 and 48-hours (Tuncer et al., 1989). At the end of incubation time, the bags were removed from the rumen and washed in running tap water to remove the remaining feed particles from the bags. The bags were kept under running water until the color of the water became clear. Then, the bags were dried at 65 °C for 24 hours (Çetinkaya, 1992), the weight of samples remaining in the bags was recorded. OM and starch contents of the residue were determined. Nutrient degradability (OM and starch) of feed was calculated according to the following formula (Orskov and Shand, 1997);

Nutrient degradability (OM and starch) = $a+b(1-e^{ct})$

a=Represents the immediately soluble fraction
b=The insoluble but slowly rumen degradable fraction
a+b =The potential degradation
c=The rate constant of degradation of b
t=The time of incubation

Silage samples were run to determine dry matter (DM), ash, and crude protein (CP; AOAC, (1990)) neutral detergent fiber (NDF; Van Soest and Robertson, (1979) and acid detergent fiber (ADF; Goering and Van Soest, (1970)). *In vitro* organic matter (OM) digestibility of silage samples were determined according to Tilley and Terry (1963) method modified by Marten and Barnes (1979) using Daisy incubator (ANKOM®, USA). Energy contents of samples were calculated according to formulas reported by Bingöl et al. (2008)

Organic acid contents of silages were analyzed by HPLC according to Tjardes et al., (2000). Ammonia nitrogen determination of samples was made by distillation method as indicated by Filya (2003).

The data obtained in the study were subjected to variance analysis according to completely randomized design SAS (1995). The difference between the means was determined by Duncan test (Steel and Torrie, 1980).

$Yin = \mu + Ai + ei$

Y_{in} = observation in trial i.
μ = the overall mean.
A_i = the fixed effect of trial i.
E_i = random error.

RESULTS

The nutrient content of 5 potato pulp silages prepared with different feedstuffs is presented in Table 2. When the table was examined, it was noted that PPS containing wheat bran alone had higher DM, OM, and starch levels compared to PPS silages containing alfalfa and barley straw alone ($P<0.05$). At the same time, PPS containing wheat bran alone had lower ash, NDF and ADF levels compared to PPS silages containing alfalfa and straw alone ($P<0.05$). The CP contents of silages containing wheat bran and alfalfa alone were similar and this value was higher than silage containing straw alone ($P<0.05$).

Data on fermentation parameters of silages are given in Table 3. The silage pH was the highest in PPS containing alfalfa hay alone (4.18) and the lowest in PPS containing straw + bran (4.01; $P<0.05$). The level of lactic acid was higher in silages containing bran alone than others, while propionic acid was the highest in PPS containing bran and alfalfa ($P<0.05$).

The acetic acid, butyric acid and ammonia nitrogen levels of silages were statistically similar (P>0.05).

The data regarding OM degradation of PPS are given in Table 4 and OM fractions are given in Table 5. Based on these tables; PPS containing bran alone had the highest OM degradation values at 0-h (water-soluble fraction) and after 48-h ruminal incubation (P<0.05). Therefore, PPS containing bran alone has the highest water-soluble OM and the lowest non-degradable and potentially degradable OM fractions compared to other silages. The highest level of non-degradable OM fraction was observed in silage containing straw alone. While the levels of starch degradation after 48h ruminal incubation were similar (P>0.05), the highest water soluble and the lowest potentially degradable starch fractions were observed in groups containing bran and straw alone (Tables 6 and 7; P<0.05).

In vitro OM digestibility values of silages varied between 76.75% - 80.76%. The highest *in vitro* OM digestibility and energy values were observed in silage containing wheat bran alone (Table 8; P<0.05).

Table 2. Nutrient contents of PPS prepared with different feedstuff, DM %

Items	DM	CA	OM	CP	NDF	ADF	Starch
PBS	25.03±0.079^{b}	4.01±0.093^{ab}	95.99±0.093^{ab}	7.24±0.196^{b}	49.66±2.355^{a}	18.32±0.482^{ab}	47.82±073^{b}
PBA	25.29±0.158^{b}	4.01±0.07^{ab}	95.99±0.07^{ab}	8.37±0.254^{a}	49.95±3.75^{a}	18.08±0.642^{ab}	47.67±0.86^{b}
PA	25.38±0.285^{b}	4.96±0.104^{a}	95.04±0.104^{b}	8.51±0.163^{a}	49.11±4.013^{a}	20.52±0.915^{a}	46.52±0.92^{b}
PB	26.32±0.009^{a}	3.87±0.031^{b}	96.13±0.031^{a}	8.41±0.139^{a}	35.26±1.206^{b}	15.53±0.185^{b}	49.62±0.16^{a}
PS	25.34±0.153^{b}	4.61±0.35^{a}	95.39±0.035^{b}	6.69±0.183^{c}	52.35±2.614^{a}	19.53±0.51^{a}	47.29±0.69^{b}

DM: Dry matter, CA: Crude ash, OM: Organic matter, CP: Crude protein, NDF: Neutral Detergent Fiber, ADF: Acid Detergent Fiber, PBS: potato pulp+bran+straw, PBA: potato pulp+bran+alfalfa, PA: potato pulp+alfalfa, PB: potato pulp+bran, PS: potato pulp+straw, $^{a, b, c}$: letters with differet superscript at same column indicate statistical differences (P<0.05).

Table 3. Fermentation parameters of PPS prepared with different feedstuff, DM %

Items	pH	LA	AA	PA	BA	NH_3-N
PBS	4.01±0.0091^{c}	4.702±0.0878^{b}	1.281±0.383	-	-	0.55±0.068
PBA	4.04±0.0075^{bc}	4.226±0.2357^{c}	0.897±0.06	0.247±0.1372^{a}	-	0.56±0.017
PA	4.18±0.0123^{a}	4.261±0.2958^{c}	0.936±0.08	0.073±0.0707^{b}	0.033±0.033	0.55±0.016
PB	4.04±0.0126^{bc}	4.813±0.097^{a}	0.825±0.087	-	0.0057±0.0034	0.55±0.016
PS	4.06±0.0048^{b}	4.228±0.042^{c}	0.852±0.0377	-	0.0058±0.0058	0.52±0.0043

LA: Lactic acid, AA: Acetic acid, PA: Propionic acid, BA: Butyric acid, NH_3-N: Ammonia nitrogen
PBS: potato pulp+bran+straw, PBA: potato pulp+bran+alfalfa, PA: potato pulp+alfalfa, PB: potato pulp+bran, PS: potato pulp+straw, $^{a, b,c}$: letters with differet superscript at same column indicate statistical differences (P<0.05).

Table 4. *In situ* OM degredation of PPS prepared with different feedstuff, OM %.

Items	0. h	2. h	4. h	8. h	12. h	24. h	48. h
PBS	28.35±0.749^{b}	32.54±1.881^{ab}	31.53±1.498^{ab}	54.43±0.816^{ab}	63.05±3.092	79.6±1.639	85.45±0.457^{bc}
PBA	31.65±0.655^{b}	32.31±2.124^{ab}	34.19±2.694^{ab}	51.44±2.398^{bc}	59.7±3.23	79.4±2.157	85.78±1.591^{bc}
PA	30.78±1.364^{b}	29.61±1.736^{b}	26.74±5.754^{b}	45.68±1.46^{c}	64.63±2.506	78.97±2.427	87.78±0.294^{ab}
PB	35.47±0.828^{a}	35.38±2.059^{a}	38.92±2.144^{a}	55.22±2.787^{a}	63.47±2.73	79.77±3.104	89.34±0.297^{a}
PS	30.03±1.21^{b}	28.81±1.455^{b}	34.3±1.428^{ab}	49.41±1.149^{bc}	60.19±0.22	77.45±2.437	84.17±0.425^{c}

PBS: potato pulp+bran+straw, PBA: potato pulp+bran+alfalfa, PA: potato pulp+alfalfa, PB: potato pulp+bran, PS: potato pulp+straw, $^{a, b,c}$: letters with differet superscript at same column indicate statistical differences (P<0.05).

Tabe 5. Organic matter fractions of PPS prepared with different feedstuff, OM%

Items	Water Soluble	Potantially Degradable	None- Degradable
PBS	28.35±0.749^{c}	57.10±0.631^{a}	14.45±0.457^{ab}
PBA	31.65±0.655^{b}	54.13±0.913^{ab}	14.22±1.591^{ab}
PA	30.78±1.364^{bc}	57.00±0.833^{a}	12.22±0.294^{bc}
PB	35.47±0.828^{a}	46.13±0.563^{b}	10.66±0.297^{c}
PS	30.03±1.21^{bc}	54.14±0.613^{ab}	15.83±0.425^{a}

PBS: potato pulp+bran+straw, PBA: potato pulp+bran+alfalfa, PA: potato pulp+alfalfa, PB: potato pulp+bran, PS: potato pulp+straw, $^{a, b,c}$: letters with differet superscript at same column indicate statistical differences (P<0.05).

Table 6. *In situ* starch degredation of PPS prepared with different feedstuff, Starch %

Items	0. h	2. h	4. h	8. h	12. h	24. h	48.h
PBS	28.06±2.18 [b]	35.06±3.91	35.02±3.48	64.87±1.54	75.02±5.12	93.18±1.34	97.22±0.21
PBA	30.77±1.63 [b]	31.63±5.26	33.24±6.69	56.31±5.28	65.58±6.76	91.20±2.26	96.29±1.01
PA	29.89±3.38 [b]	28.20±4.33	38.39±3.10	50.49±3.26	73.97±4.52	93.00±1.98	97.62±0.14
PB	33.14±2.10 [a]	33.34±5.20	33.29±5.74	58.46±6.33	68.24±5.82	89.05±4.11	96.92±0.21
PS	33.33±2.82 [a]	30.26±3.49	39.97±3.20	59.80±2.23	73.71±6.03	92.63±1.95	97.27±0.18

PBS: potato pulp+bran+straw, PBA: potato pulp+bran+alfalfa, PA: potato pulp+alfalfa, PB: potato pulp+bran, PS: potato pulp+straw, [a, b]: letters with differet superscript at same column indicate statistical differences (P<0.05).

Table 7. Starch fractions of PPS prepared with different feedstuff, Starch %

Treatments	Water Soluble	Potantially Degradable	None- Degradable
PBS	28.06±2.18 [b]	69.16±2.04 [a]	2.78±0.21
PBA	30.77±1.63 [b]	65.53±2.00 [ab]	3.71±1.01
PA	29.89±3.38 [b]	67.73±3.39 [ab]	2.38±0.14
PB	33.14±2.10 [a]	63.77±2.88 [b]	3.08±0.21
PS	33.33±2.82 [a]	63.94±2.82 [b]	2.73±0.18

PBS: potato pulp+bran+straw, PBA: potato pulp+bran+alfalfa, PA: potato pulp+alfalfa, PB: potato pulp+bran, PS: potato pulp+straw, [a, b]: letters with differet superscript at same column indicate statistical differences (P<0.05).

Table 8. *In vitro* OM digestibility and energy values of PPS prepared with different feedstuff

Items	IVOMD, %OM	ME, Mcal/kg	NEL, Mcal/kg
PBS	76.75±0.381[d]	3.384±0.0168[d]	1.76±0.009[d]
PBA	79.9±0.521[ab]	3.523±0.023[ab]	1.838±0.013[ab]
PA	77.29±0.613[cd]	3.408±0.027[cd]	1.774±0.015[cd]
PB	80.76±0.129[a]	3.561±0.006[a]	1.859±0.003[a]
PS	78.59±0.354[bc]	3.465±0.016[bc]	1.806±0.009[bc]

IVOMD: *In vitro* organic matter digestibility, ME: Metabolic energy, NEL: Net energy for lactation
PBS: potato pulp+bran+straw, PBA: potato pulp+bran+alfalfa, PA: potato pulp+alfalfa, PB: potato pulp+bran, PS: potato pulp+straw, [a, b,c,d]: letters with differet superscript at same column indicate statistical differences (P<0.05).

DISCUSSION

Since DM level of potato pulp alone was very low for silage making, different feedstuff used as water absorbers to improve DM content of PPS in this study. Among the potato silages prepared with different water absorbers, the PPS containing wheat bran alone had the highest DM level with 26.32%. In the study, it was noted that the DM level of PPS varied according to feedstuffs used as absorbent. It seemed that wheat bran additions into silages relatively increased the DM level. In the literature, DM levels of PPS prepared alone or with different feedstuff ranged from 15.9 to 27.69% (Okine et al., 2005; Aibibula et al., 2007; Nelson, 2010). The DM levels obtained in the current study were close to the upper edge of the values reported in the literature. The reason for the DM differences between the studies was thought to be due to differences in levels of feedstuff and initial DM levels of the potato pulps used in the silages.

In terms of OM content of silages, the highest OM level was observed in PPS containing wheat bran alone with 96.13 (P<0.05). OM values observed in the current study were similar to those of Sugimato et al. (2008, 2009, 2010), higher than that of Pen et al. (2005), however less than those of Okine et al. (2005), Sugimoto et al. (2007), and Zunong et al. (2009). These differences in OM levels were thought to be due to excessive amount of soil and field residues and not doing a good pre-wash. Again, it seemed that the OM levels of feedstuff used as absorbent also affected the OM content of PPS.

The lowest CP content was observed in PPS containing barley straw alone with 6.69% whereas the highest CP content was found in silage containing alfalfa hay with 8.51 in the study. This was due to the low CP content of the feedstuffs. Silages containing bran and alfalfa hay alone had higher CP content than silage containing straw alone (P <0.05). Again, it was thought that the CP contents of alfalfa hay and bran were higher than that of straw (NRC, 2001). Crude

protein values of the current study were similar to the data reported by Zhang et al. (2012), but higher than those of Okine et al. (2005), Sugimoto et al. (2007), and Zunong et al. (2009). These differences were thought to be due to the ratio of the other feedstuff and the protein contents of the other feedstuff used for silage making in the studies.

When the NDF and ADF of the silages were examined, the lowest NDF and ADF contents were observed in silage with wheat bran alone ($P < 0.05$). The NDF and ADF contents of silages except silage containing wheat bran alone were similar to that of Zhang et al. (2012), higher to Omer et al. (2011) and Dhingra et al. (2013) but the NDF and ADF content of silage with wheat bran alone was lower than that of Omer et al. (2011), Zhang et al. (2012), Dhingra et al. (2013) and Ncobela et al. (2017). Since the NDF and ADF content of wheat bran was much lower than that of straw and alfalfa hay (NRC, 2001), these low levels of NDF and ADF resulted from wheat bran in silage containing wheat bran.

The starch content of silages ranged from 46.52% to 49.62%. Since wheat bran contains some starch, it was thought that PPS prepared with wheat bran alone has a higher starch content than others. In previous studies, PP has been reported to contain starch between to 17.7 and 43% levels (Aibibula et al., 2007; Ncobela et al. 2017). This confirms the low levels of starch in the current study.

In the study, the pH values of silages were in the range of 4.01-4.18 and the highest pH values were observed in the group containing alfalfa hay alone ($P<0.05$). These pH values were very close to the values (pH: 3.5 - 4) previously reported in the literature (Sugimoto et al., 2007, 2008; Zhang et al., 2012) for PPS prepared with different additives. The pH values obtained in the study were in the 3.8-4.2 pH range, which is considered ideal for silage (Ergün et al., 2002) indicating very good fermentation. The high pH level of alfalfa hay silage was thought to be due to a higher CP content of alfalfa hay. As it is known, ammonia is alkaline and has high buffering power. Therefore, the pH level of silages made from legumes is generally higher than silages made from cereal grains.

While the silage containing wheat bran alone had the highest lactic acid content (4.813; $P<0.05$), acetic acid contents were similar in all silages ($P>0.05$). The level of butyric acid, which is important for silage quality and indicates poor fermentation, was either absent or negligible levels in all silages. The fact that 70% or more of the total amount of organic acid in all silages was lactic acid and the amount of butyric acid was not at significant levels, suggesting a good fermentation. The amount and profile of organic acid released by lactic acid bacteria as a result of fermentation in silages were related to the sugar and moisture contents, and buffering capacity of the silage product (Rotz and Muck, 1994). Zhang et al. (2012) have reported the lactic acid values of 3.22%, 2.73%, 2.74%, and acetic acid values of 0.54%, 0.42% and 0.55% in the PPS prepared with 20% rice straw, corn cob and bean cob. Both lactic acid and acetic acid levels obtained in this study were lower than the values obtained in the current study. The reason for the difference between the studies may have been due to type and the levels of feedstuff used in the silages.

Silage ammonia nitrogen was similar in all groups and ranged from 0.52 to 0.56% DM ($P>0.05$). Zhang et al. (2012) reported the ammonia nitrogen values in the range of 1.71 - 2.5% of total nitrogen for PPS prepared with rice straw, corn residue, and dried bean residue, which was lower than ammonia nitrogen values observed in the current study, but the ammonia nitrogen levels (1.06 %M) reported by Baytok et al. (2005) for maize silage was higher than the ammonia nitrogen levels in the current study.

In situ OM degradation values of all PPS used in the study were calculated in the range of 82.32-89.34% after 48 hours of ruminal incubation. Among PPS, the highest *in situ* OM degradation values were observed in PPS with wheat bran alone ($P<0.05$). Percentage of DM degradation for PPS prepared with different feedstuff ranged from 56.1 to 75.5% in the literature (Sugimato et al., 2006; Sugimato et al., 2007; Sugimato et al., 2008; Zunong et al., 2009; Sugimoto et al.,2010). Organic matter degradation values obtained in the current study was similar to that of Zunong et al.,(2009) but higher than those of Sugimato et al., (2006, 2007, 2008, 2009, 2010). The difference between the studies was thought to be due to the differences in the nutrient content of the pulp and the additives involved and also levels of additives used in PPS.

Among the OM fractions, water soluble OM level was the highest (35.47%) in wheat bran-added silage, while it was the lowest in wheat bran+straw-added PPS with 28.35%. Potentially degradable OM fractions of PPS were similar. The non-OM fractions in PPS ranged from 10.66% to15.83 ($P<0.05$).

When the *in situ* starch degradation values were examined, it was observed that the starch degradation values were similar in all silages and were at approximately 97% level after 48 hours incubation. It was an expected result since the source of starch in silages was PP, except the bran added silage. Starch present in wheat bran has a high digestibility like starch in potato so that degradation rate in silage containing wheat bran was also similar.

In vitro OM digestion values for PPS varied from 76.75 to 80.76%. Among the PPS, the highest OM digestion and energy values were observed in wheat bran-added silage, while wheat bran + straw-added silage had the lowest OM digestion and energy values. Since, energy values were calculated based on OMD values, energy values were also parallel with OMD values. In a study conducted by Zunong et al (2009), the DM digestion of PPS was 75.5%. The OM digestion values obtained for PPS in the current study were similar to that reported by Zunong et al. (2009). The level of digestion of PPS is expected to vary depending on the nature of the additive involved.

CONCLUSIONS

The result of the study, the nutritional content of the PP was very good in terms of carbohydrates, which was demonstrated by the high starch content. Although silages did not contain enough DM for ideal silage production, all of the silages have very good fermentation properties. In particular, the lactic acid levels in all silages was more than 70% of the total organic acids and the butyric acid level was either negligible or absent, which was a desirable form of fermentation in silages. It can be thought that due to the good compressibility of the potato pulp and its high digestible carbonhydrate content. All these positive properties were reflected in both *in situ* and *in vitro* digestion values, and thus, highly digestible silages have been obtained.

It can be concluded that PPS prepared with different feedstuffs at 5% level had good fermentation properties and high digestion and degradation values. Even PPS prepared by adding barley straw at 5% level has very high digestibility, indicating that can be good alternative feedstuff for ruminant animals.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
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Proteolytic activities and safety use of *Enterococcus faecalis* strains isolated from Turkish White Pickled Cheese and milk samples

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ABSTRACT: In this study, *Enterococcus faecalis* proteolytic strains which have the potential to degradation of bovine milk proteins were isolated from Turkish White Pickled Cheeses and milk samples. *E. faecalis* strains were found to have strong caseinolytic activity. The extracellular protease enzymes produced by *E. faecalis* strains from 60 different samples were analyzed in the pattern of bands on a stained SDS-PAGE gel. The highest proteolytic activity of *E. faecalis* isolates were determined at pH 7.0 and 40 °C for 24 h. In addition, antimicrobial resistance and the presence of selected virulence genes of isolates were investigated for microbiological safety. These findings further emphasize that the *E. faecalis* isolates can be effective in the degradation of bovine milk proteins.

***Keywords*:** *Enterococcus faecalis*, milk samples, Turkish White Pickled Cheeses, protease.

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INTRODUCTION

Enterococci, which include pathogen and non-pathogen strains, are commensal bacteriathat can be found in the environment as well as in normal gout microbiota in humans (Dubin & Pamer, 2014). Nevertheless, the genus of some lactic acid bacteria (LAB) including *Enterococcus* spp. play a positive role in improving the characteristic organoleptic properties of various dairy products (Bhardwaj et al., 2008). It has been accepted for a long time that they contaminated the milk as a result of fecal contamination. However, studies have shown that these microorganisms are a part of normal food microflora and that their use in traditional food processes is considered to be natural (Franz et al., 2011). In addition, the bioavailability of bioactive compounds (i.e.,Bac-21) and antimicrobial agents such as enterocin has been demonstrated by recent studies (Foulquié Moreno et al., 2006; Hanchi et al., 2018; Worsztynowicz et al., 2019). In this regard, *E. faecalis* (i.e., Symbioflor1®) is currently usedto maintain microbial balance in the intestinal flora in cases of diarrhea (Franz et al., 2011). Despite ongoing research, no common perspective of the genes and biological processes involved in the pathogenicity of enterococci has been identified on a global basis. Since *E. faecalis* (or Enterococcus spp.) has biotechnological potentials, there is no case of lack of research on probiotic and food preservation applications (Hanchi et al., 2018).

Enterococci contribute to the fermentation process by providing casein degradation especially in fermented dairy products with their proteolytic activities. LAB is a bacterium that grows in need of an exogenous amino acid or peptide source, which is provided by proteolysis of casein in large quantities in milk (Erdoğan & Baran, 2012). Basically, degradation of caseins is initiated by cell envelope proteinase (*CEP, PrtP*), which cleaves the proteins produced by lactic acid bacteria into oligopeptides. The obtained oligopeptides are transported through cell-specific peptide transport systems (*DtpT, Dpp* and *Opp*) and are broken down into shorter peptides and amino acids by joint action of various intracellular peptidases. Proteolysis, one of the most important biochemical reactions observed during ripening, also takes place in Turkish White Pickled cheese, which has an almost similar production process to feta cheese. The milk of cows, sheep, goats or their mixtures are used in the production of Turkish White Pickled Cheese. They are ripened in brine at the end of the production process. Processing conditions such as temperature and pH have not been optimized in terms of proteolysis. Despite proteolytic properties known to have *Enterococcus* species, studies on Turkish white pickled cheese mostly focused on *Lactobacillus* and *Lactococcus* species. However, studies on enterococci in dairy products have shown that *E. faecalis* is the most active bacterium with proteolytic activity (Christensson et al., 2002; Savijoki et al., 2006).

Despite the aforementioned benefits, enterococci have been considered as the cause of nosocomial infections such as bacteremia, endocarditis, and meningitis. In addition, resistance against some antimicrobial agents and virulence properties cause the biotechnological use of these microorganisms to be questioned. All of these adverse effects require investigation of whether enterococci are safe for human health in determining the biotechnological usability on dairy products.

The aim of this study was to investigate the proteolytic activities of isolated and identified proteolytic *E. faecalis* strains in Turkish White Pickled Cheese and raw milk samples. In addition, antimicrobial resistance and the presence of selected virulence genes were investigated for microbiological safety of the strains.

MATERIAL AND METHODS

Isolation and identification of proteolytic *E. faecalis* strains from raw milk and Turkish White Pickled Cheese samples

Raw milk and Turkish White Pickled Cheese samples (30 cheese and 30 raw milk samples) were collected from different local markets in eastern-Turkey (Erzurum province). Ten g of cheese samples (10ml for milk) were taken and homogenized for 2 min. using a masticator stomacher blender (IUL Instruments, Barcelona, Spain) in sterile filtered bags containing 90 ml of sterile ¼ ringer solution (MERCK, 115525). Tenfold serial dilutions from this homogenate were prepared in sterile ¼ ringer solution. The detection of isolates showing proteolytic enzyme production was performed as reported by Graham et al. (2017). For this purpose, Kanamycin Skimmed Milk Aesculin Azide Agar (KSMEA) prepared by combining Kanamycin Aesculin Azide Agar (KAA, Merck KGaA, Darmstadt, Germany; selective medium for enterococcus; concentration of Kanamycin - 20 mg / l) with Reconstituted Skim Milk (RSM) (10%) in a ratio of 1: 1 was used. A loopful of homogenate was streaked onto KSMEA. After incubation for 48 h at 37 °C, pro-

teolytic enzyme producing strains were determined by evaluating the media for growth, proteolysis (transparent zone surrounding colonies) and aesculin hydrolysis. Presumptive colonies of *Enterococcus* spp. (colonies displaying black halo with a zone of proteolysis) were confirmed with a 99% probability identification by VITEK2 Compact system (bioMerieux, Marcy l'Etoile, France) using GP ID cards. The isolates were stored in cryogenic vials containing Brain Heart Infusion (BHI) broth (Oxoid USA, Inc., Columbia, Md.) with 30% glycerol at -20 °C until further analysis.

Isolation of genomic DNA

Genomic DNA of the isolates were extracted from 2 ml overnight culture according to the manufacturer's recommendation using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA USA).

Molecular identification

Multiplex PCR was used to confirm *E. faecalis* and *E. faecium* using specific primers including internal control (16 sRNA) (Table 1). PCR amplifications were performed in 25 µL reaction mixtures containing 1 µL of genomic DNA, 2.5 µl 10X reaction buffer, 1.5 mM $MgCl_2$, 1 U of Taq DNA polymerase, 200 µM of dNTPs mix (Vivantis Tech., Malaysia), 10 pmol of each primer, and water. Amplification was performed in a Bio-Rad thermal cycler (Bio-Rad Laboratories, USA) with following steps: initial denaturation at 94 °C for 1 min.; 30 cycles of 95°C 30 s., 55°C 30 s. and 72°C 60 s.; and a final extension at 72°C for 10 min (Kariyama et al., 2000). PCR products were analyzed by electrophoresis at 90 V for 1 h using 1% w/v agarose gel (containing 0.5 µg/ ml ethidium bromide) in TAE (Tris, acetate and EDTA) buffer and bands were visualized under UV light (Gel Doc™ XR, Bio-Rad Laboratories, USA).

The PCR products of strains were made Sanger sequencing using primer sequence (*efcs*) specific to *E. faecalis*. The phylogenetic tree was generated with Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.01207194 was shown. There was a total of 858 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. Afterwards, the partial sequence results were deposited in GenBank.

Table 1. PCR Primers used in this study

Target genes	Specifity	Oligonucleotide sequences (5′-3′)	Annealing temp (°C)	Product size (bp)	Reference
asa1	Aggregation substance (*asa1*)	Fw: GCACGCTATTACGAACTATGA Rv: TAAGAAAGAACATCACCACGA	56	375	(Vankerckhoven et al., 2004)
gel	Gelatinase (*gelE*)	Fw: TATGACAATGCTTTTTGGGAT Rv: AGATGCACCCGAAATAATATA	56	213	(Vankerckhoven et al., 2004)
cyl	Cytolysins (*cyl*)	Fw: ACTCGGGGATTGATAGGC Rv: GCTGCTAAAGCTGCGCTT	56	688	(Vankerckhoven et al., 2004)
esp	Enterococcal surface protein (*esp*)	Fw: AGATTTCATCTTTGATTCTTGG Rv: AATTGATTCTTTAGCATCTGG	56	510	(Vankerckhoven et al., 2004)
hyl	Hyaluronidase (*hyl*)	Fw: ACAGAAGAGCTGCAGGAAATG Rv: GACTGACGTCCAAGTTTCCAA	56	276	(Vankerckhoven et al., 2004)
ace	Adhesin of collagen protein (*ace*)	Fw: GGAATGACCGAGAACGATGGC Rv: GCTTGATGTTGGCCTGCTTCCG	58	616	(Creti et al., 2004)
efa	Cell wall adhesion (*efaA*)	Fw: CGTGAGAAAGAAATGGAGGA Rv: CTACTAACACGTCACGAATG	56	499	(Shankar et al., 1999)
efcs	*E. faecalis*	Fw: ATCAAGTACAGTTAGTCTTTATTAG Rv:ACGATTCAAAGCTAACTGAATCAGT	54	941	(Kariyama et al., 2000)
efcm	*E. faecium*	Fw: TTGAGGCAGACCAGATTGACG Rv: TATGACAGCGACTCCGATTCC	54	658	(Kariyama et al., 2000)
rrs	16s RNA	Fw: GGATTAGATACCCTGGTAGTCC Rv: TCGTTGCGGGACTTAACCCAAC	54	320	(Kariyama et al., 2000)

Assessment of antimicrobial susceptibility

Antibiotics susceptibility in *E. faecalis* isolates was evaluated using the Kirby-Bauer disc diffusion method, as recommended by the Clinical and Laboratory Standards Institute (CLSI) (2019) with 12 antibiotics: gentamycin (10 μg), tetracycline (30 μg), cefepime (30 μg), chloramphenicol (30 μg), ampicillin (10 μg), streptomycin (10 μg), trimethoprim (5 μg), ciprofloxacin (5 μg), penicillin G (10 U), erythromycin (15 μg), fosfomycin (200 μg), and vancomycin (30 μg). Antibiotic disks were purchased from Liofilchem (Teramo, Italy). Results were interpreted according to the observed cutoff level recommended by CLSI (2019) (resistant, intermediate or susceptible). Control strains included *E. faecalis* ATCC 51299 (vancomycin resistant) and 29212 (vancomycin susceptible) were used in this study.

Detection of virulence genes

The presence of virulence genes of *E. faecalis* strains (*asa*, aggregation substance; *gel*E, gelatinase; *cyl*, cytolysins; *esp*, enterococcal surface protein; *hyl*; hyaluronidase; *ace*, adhesin of collagen protein; and *efa*A, cell wall adhesion) (Table 1) were investigated by PCR based on previously published protocols (Creti et al., 2004; Kariyama et al., 2000; Shankar et al., 1999; Vankerckhoven et al., 2004).

Analysis of proteolytic activities of isolates

Proteolytic activities of all isolates were measured by casein digestion method. For this purpose, casein substrate solution (1%, w/v) was prepared in Tris-HCl buffer (0.1 M, pH = 7.0). This solution was allowed to stand in a hot water bath for 30 min. and it was chemically stable for seven days.

For activity measurement; the reaction was initiated by adding extracellular enzyme solution (0.5 ml) produced from *E. faecalis* isolates to 1 ml casein solution. Then it was incubated at 40 °C for 20 min. and the reaction was stopped by the addition of trichloroacetic acid (3 ml, 5% per volume). After 1 h, the nondigestible proteins were separated by centrifugation (10 000 x*g* for 5 min.). The supernatant was then filtered and the protein therein was subjected to further experiments. An enzyme unit (U) was calculated as the amount of protein digested by the enzyme per minute (Fadiloğlu, 2001).

Proteolytic activities in non-proliferative cells system

Coagulation of milk using protease enzyme of *E. faecalis* strains

The modified Berridge method was used to determine the coagulation of the milk against the blank sample used as the control sample. For this purpose, milk coagulation of protease enzyme from culture supernatants of *E. faecalis* strains was monitored by changing the temperature (40 and 60 ºC) and time (1 to 24 h) parameters. At the end of 24 h, no clotting was observed in the control tube, but time was recorded when the protease enzyme caused clotting in the test tube.

Effect of some compounds on protease activity of *E. faecalis*

Analyzes made after this part were carried out on *E. faecalis* ERZ-ATA7, which showed the highest proteolytic activity to represent all isolates. The effect of thiol-specific inhibitors, activators and various non-specific compounds on *E. faecalis* protease activity was determined. Briefly; 0.5 ml of protease enzyme, which was produced extracellularly from *E. faecalis* bacteria, was incubated in the presence of 10 mM concentration of thiol reagents, 1.5 ml final volume of Tris-HCl buffer (0.1 M, pH = 7.0) using casein as substrate.

Compounds such as phenylmethanesulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), β-mercaptoethanolsodium dodecyl sulfate, (SDS), 1,10-Phenanthroline (phen), EDTA (Ethylenediaminetetacetic acid) were used at a concentration of 10 mM. Enzyme activity measurements were calculated by comparing against control experiment using no inhibitor and distillated water and the gained activity was given as 100%.

SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to determine the subunits of the protease enzyme after extracellular production of *E. faecalis* strains. It was performed at concentrations of 3% and 10% acrylamide for agglomeration and elution gels, respectively, each containing 0.1% SDS according to Laemmli's method. Samples (20 mg) from the strains were applied to electrophoresis gel medium. Then the samples were run at 150V for 1 h 20 min. The gels stained with 0.1% Coomassie Brilliant Blue R-250 dye were washed several times

with a solution of methanol (50% (v/v)) and acetic acid (10% (v/v)) and the resulting bands were photographed.

RESULTS AND DISCUSSION

A total 15 *E. faecalis* isolates with proteolytic properties (transparent zone on agar), cultured from 30 milk and 30 Turkish White Pickled Cheese samples in eastern-Turkey (Erzurum province) were used in this study. Isolates were recovered from 4 of raw milk and 11 of cheese samples. The isolates were named *E. faecalis* ERZ-ATA1 to 15. In addition to the colony morphology of the isolates, Vitek-2 Compact system (bioMérieux, Marcy l'Etoile, France) identified that they were *E. faecalis* with a level 99% probability. Also, all isolates were submitted to amplification and sequencing of *E. faecalis* specific primer (*efc*s), which resulted in 99% identity with the reported for *E. faecalis* strains and then designated under GenBank accession number MN856126 to MN856140. For phylogenetic comparison, 15 of *E. faecalis* strains isolated in this study and *E. faecalis* ATCC 29212 in the NCBI database were selected (Figure 1). The phylogenetic tree displayed two main clades.

Enterococci have become one of the most common nosocomial infectious agents after their clinical significance has been reported. Virulence factors are bacteria-associated molecules that are required for *Enterococcus* spp. to cause infection. They play an important role in the pathogenicity of enterococcal strains have been extensively investigated in the last decade (Barbosa et al., 2010). In addition to that, resistance of *Enterococcus* spp. to antimicrobial agents, mainly vancomycin, is an important public health problem today (Mundy et al., 2000). The bacteria can be transmitted from person to person, as well as by consuming contaminated food and water. Hence, determination of virulence factors is important to enable their use in biotechnology. The most commonly reported that enterococci harbored *asa1*, *gel*E, *cyl*, *esp*, *hyl*, *ace*, and *efa*A virulence genes. PCR results showed that all of the isolates carried *gel*E, 14 out of 15 carried *asa1* and *ace* and none of them carried *cyl, esp, hyl* and *efa*A (Table 2). The *cyl* virulence gene, encoding bactericidal and hemolytic activity, is associated with lysing cells with peptide and lipid surfactant structure. Presence of *cyl* virulence gene, which is one of the most important features contributing to the

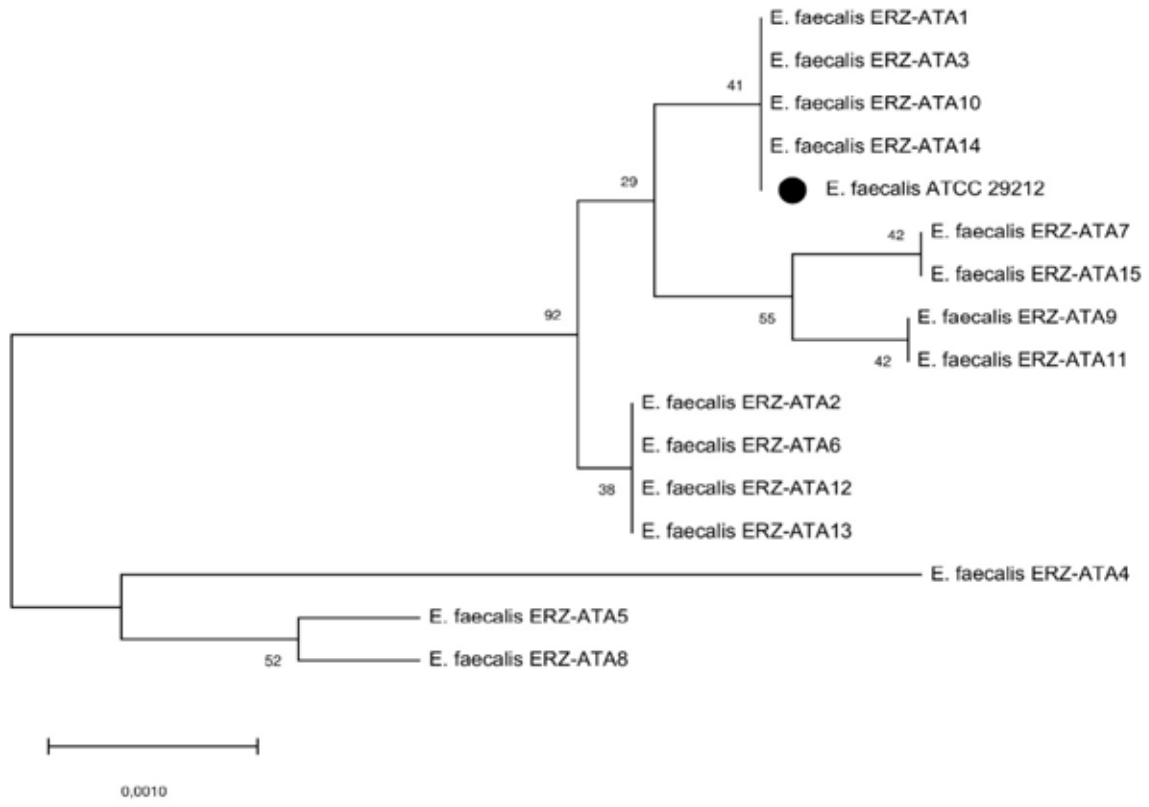

Figure 1. Phylogenetic tree of *E. faecalis* isolates

pathogenicity of enterococci strains, is recommended in both hemolytic and non-hemolytic *Enterococcus* species (Endo et al., 2015). None of the enterococci strains isolated in the current study carried this virulence gene, which is important for public health. On the other hand, *asa1* and *ace* virulence genes encoding adhesion-associated proteins were found in all strains except one sample (ERZ-ATA4 for *asa1*, ERZ-ATA11 for *ace*). Presence of these two virulence genes is thought to be associated with milk and dairy products as reported previously (Ahmadova et al., 2011; Bíscola et al., 2016). However, these gene regions could not be clearly and precisely related to the clinical strains.

The *gel*E virulence gene, encoding the gene region responsible for extracellular Zn-metallo-endopeptidase (gelatinase) production and biofilm formation, was detected in all isolates although the importance of which is not yet fully understood in the virulence of enterococci (Del Papa et al., 2007; Franz et al., 2011). In addition, the detection or failure of the *gel*E virulence gene in *E. faecalis* isolates does not completely indicate that this endopeptidase may be present phenotypically (Qin et al., 2001). The researchers (Ahmadova et al., 2011; Bíscola et al., 2016) reported that *gel*E virulence gene was frequently found in proteolytic strains isolated from milk and meat products. It shows that *gel*E gene has an important function in the metabolism of bacteria. On the other hand, Archimbaud et al. (2002) reported that they could not find a relation between the presence of gelatinase, cytolysin and aggregation agent and the adhesion of *E. faecalis* strains to the heart cells.

It has been suggested that enterococci have low pathogenic properties due to the presence of microbiota in the body and their long-term use as probiotics (Archimbaud et al., 2002). Of note, they have the important virulence and antibiotic resistance genes, hence, demonstration of the antimicrobial susceptibility profiles of *E. faecalis* strains isolated in the current study was important (Table 2).

The emergence of antibiotic resistance is thought to be due to the use of them for treatment or prophylaxis purposes in animals and humans. Therefore, there is a surging trend to limit the use of antibiotics in food animals and human medicine worldwide (Adıguzel et al., 2020). Antimicrobial susceptibility of *E. faecalis* strains was determined using disk diffusion methods according to the Clinical and Laboratory Standards Institute (CLSI) (2019) guidelines with 12 commercial discs (Table 2). All isolates tested in this study were susceptible to chloramphenicol, ampicillin, streptomycin, trimethoprim, ciprofloxacin, and fosfomycin. ERZ-ATA6, ERZ-ATA2 and ERZ-ATA10 isolates were found to be resistant to gentamicin, while other isolates were susceptible. Although ERZ-ATA7 and ERZ-ATA11 were found to have intermediate

Table 2. Antibiotic susceptibility and virulence genes of *E. faecalis* strains

Isolate ID	Antibiotics susceptibility[a]												Virulence genes[b]						
	GEN	TE	FEP	C	AMP	S	W	CIP	P	E	FOS	VA	*asa1*	*gel*	*cyl*	*esp*	*hyl*	*ace*	*efaA*
ERZ-ATA8	S	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA6	R	S	S	S	S	S	S	S	S	IM	S	S	+	+	-	-	-	+	-
ERZ-ATA13	S	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA2	R	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA4	S	S	S	S	S	S	S	S	S	S	S	S	-	+	-	-	-	+	-
ERZ-ATA15	S	S	S	S	S	S	S	S	S	IM	S	S	+	+	-	-	-	+	-
ERZ-ATA3	S	S	S	S	S	S	S	S	S	IM	S	S	+	+	-	-	-	+	-
ERZ-ATA10	R	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA9	S	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA7	S	IM	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA5	S	S	S	S	S	S	S	S	S	IM	S	S	+	+	-	-	-	+	-
ERZ-ATA1	S	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA14	S	S	S	S	S	S	S	S	S	IM	S	S	+	+	-	-	-	+	-
ERZ-ATA11	S	IM	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	-	-
ERZ-ATA12	S	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-

GEN gentamicin, TE tetracycline, FEP cefepime, C chloramphenicol, AMP ampicillin, S streptomycin, W trimethoprim, CIP ciprofloxacin, P penicillin, E erythromycin, FOS fosfomycin, VA Vancomycin

a "S": sensitive; "R": resistance; "IM": Intermediate, b"+": gene is present; "-": gene is absent

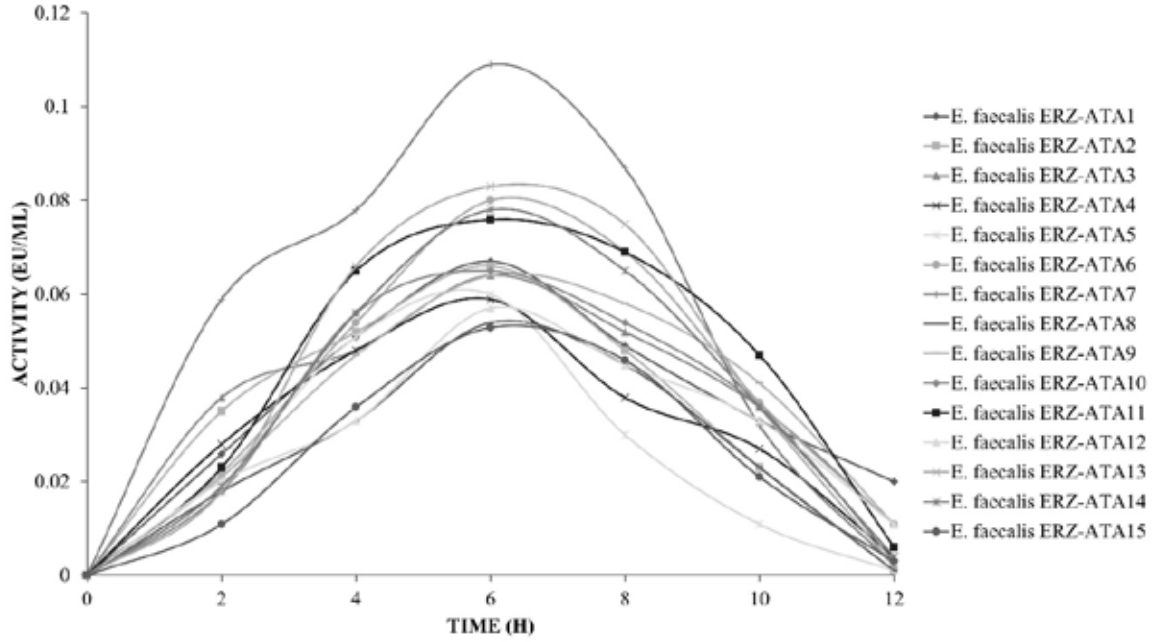

Figure 3. The effect of time on the production of extracellular protease enzyme from some *E. faecalis* strains

resistance, 13 isolates were susceptible to tetracycline. On the other hand, ERZ-ATA6, ERZ-ATA15, ERZ-ATA6, ERZ-ATA5 and ERZ-ATA14 isolates showed intermediate resistance to erythromycin, while others were susceptible. Milk and cheese isolates tested in this study were all susceptible to beta lactam antibiotics and vancomycin. This result is consistent with other studies on enterococci (Ahmadova et al., 2011; Archimbaud et al., 2002; Worsztynowicz et al., 2019)

The effect of protease enzyme on extracellular production from *E. faecalis* isolates was investigated for 12 h. The protease enzyme activity was performed in the samples taken from the reaction medium every two h (Figure 3). Our results showed that the all isolates tested in this study reached the peak level for proteolytic activity at the 6th hours, however, the ERZ-ATA7 strain had the highest proteolytic activity between isolates. In their study, Waters et al. (2003) reported that the optical density (630 nm) of the *E. faecalis* strains, in which they followed the absorbent during the first 6 hours in the medium, increased continuously, and that there was a decrease in bacterial growth as a result of the proteolytic activity and due to the nutrient decrease in the subsequent follow-up process. In their study, Waters et al. reported that the optical density (630 nm) of the *E. faecalis* strain, in which they followed the absorbent during the first 6 hours in the medium, increased continuously, and that there was a decrease in bacterial growth as a result of the proteolytic activity and due to the nutrient decrease in the subsequent follow-up process. Findings are consistent with the data of our current study.

The maximum protease activity for *E. faecalis* ERZ-ATA7 strain was determined in the pH range of 2 to10 increasing gradually (each time one pH value) by using casein substrate. The optimum pH was found to be 7.0 (Figure 4), while it had high proteolytic activity in pH 5 to 9. As a matter of fact, several researchers have reported that neutral pH is optimal for protease production for *E. faecalis* strains (Ahmadova et al., 2011; El-Gaish et al., 2010). Also, low and high pHs values had an inert effect on the protease enzyme activity of *E. faecalis* ERZ-ATA7, which provides a great advantage in food processes.

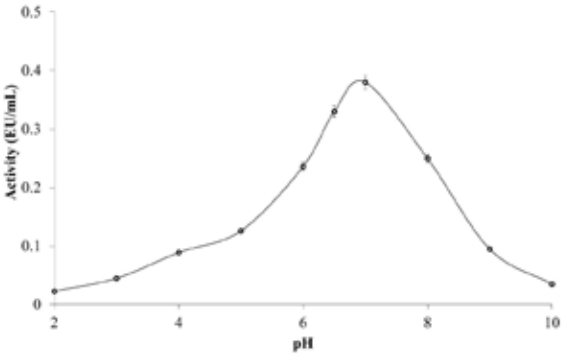

Figure 4. The effect of pH on the activity of protease from *E. faecalis* ERZ-ATA7

Similar to our finding, in a study, testing the hydrolytic activity of *E. faecalis* VB63F strain using casein substrate, has been reported the whole protein was hydrolyzed at pH 6.5 in the medium (Biscola et al., 2016). it has been reported that *E. faecalis* strains were more effective at pH 6.5 - 7 (Kiriliov et al., 2011; El-Ghaish et al., 2010). Similarly, *E. faecalis* ERZ-ATA7 strain also had the highest hydrolytic activity at pH 7 (Figure 4).

The protease enzyme activity of *E. faecalis* ERZ-ATA7 strain was investigated in the temperature range from 0 to 90 °C. The temperature was increased by increased gradually (each time 10 °C) from 0 to 90 °C, and the optimal temperature was found to be 40 °C (Figure 5). It has been reported that *L. acidophilus* BGRA43 and *L. delbrueckii* BGPF1 strains showed the highest proteolytic activity at 45 and 40 °C, respectively (Fira et al., 2001). This finding further emphasize *E. faecalis* ERZ-ATA7 strain has an excellent choice enzyme for both allergenicity and flavor development processes in the food industry.

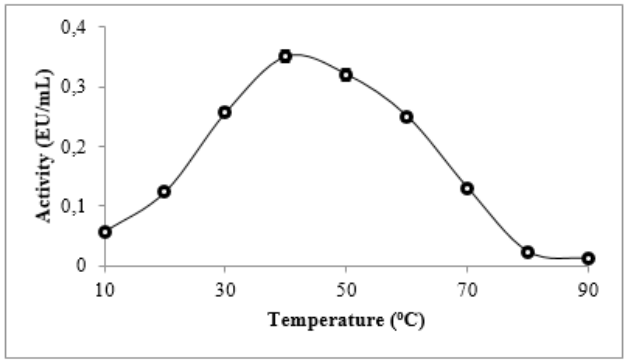

Figure 5. The effect of temperature on the activity of protease from *E. faecalis* ERZ-ATA7

The extracellular hydrolysis capacity of protease enzyme gained from *E. faecalis* ERZ-ATA7 is shown in Table 3. The extracellular hydrolysis capacity of the protease enzyme obtained from *E. faecalis* ERZ-ATA7 is shown in Table 3. The results showed that *E. faecalis* ERZ-ATA7 protease was not able to hydrolyze hemoglobin and azoalbumin, while it was able to hydrolyze substrates of gelatin (highest rate), azocasein (highest rate) and casein. Biscola et al. (2016) stated in their study that the optimum temperature value for *E. faecalis* strain was 42 °C. In addition, Nešuta et al. (2017) suggested that they detected higher protease activity when bacteria were incubated at 37 °C.

Table 3. Determination of *E. faecalis* ERZ-ATA7 protease activity (EU/ml) values for different substrates

	Activity
Substrate	**(EU/ml)**
Serum albumin	0.85±0.02
Hemoglobin	nd
Azoalbumin	nd
Gelatin	1.89±0.05
Azocasein	3.21±0.11
Casein	1.56±0.03

nd=not detected

When *E. faecalis* isolates were evaluated in terms of substrate specificity, it was found that they did not hydrolyze hemoglobin and azoalbumin, however, serum albumin, casein, gelatin, and azocasein were revealed to increase substrate specificity, respectively. The findings suggest that the protease enzyme active center is formed by L-leucine (Leu), L-phenylalanine (Phe), or Isoleucine (Ile) and is an endopeptidase (Makinen et al., 1989). It is also clear that *E. faecalis* strains isolated in this study had gelatinase enzyme activity. The extracellular protease enzyme from *E. faecalis* strains isolated was examined by SDS-PAGE. The gel image of the protease enzyme of *E. faecalis* strains tested in this study carried out against the standard protein. SDS-PAGE result indicated that the protein of representing protease enzyme was located on a ~34.4 kDa.

The results of the effects of some chemical compounds on protease activity of the *E. faecalis* ERZ-ATA7 strain are shown in Table 4. Many of these compounds are specific inhibitors of certain types of proteases, such as serine (DIPF and PMSF), metal- (PHT and EDTA), and cysteine protease (iodoacetamide and some PMSF).The results showed that EDTA strongly inhibits the protease enzyme, indicating that the protease of the *E. faecalis* ERZ-ATA7 strain is metalloprotease. In general, the inhibition of protease enzyme activity by EDTA indicates that the protease is metalloprotease. In particular, the fact that EDTA or similar chemicals, which have the ability to form chelates, inhibit the enzyme, proves that they are metalloproteases, which are certain to have metal ions in the active center (Kitamura and Shimada 2009). On the other hand, studies (Ahmadova et al., 2011; El-Gaish et al., 2010) have revealed that *E. faecalis* strains produce metalloproteases in accordance with our current study. Also, it has been suggested that this enzyme played a minor role in the hydrolysis of milk protein fractions in the presence of other types of pro-

teases. However, from the findings; there was also a decrease in the proteolytic activity of some *E. faecalis* strains in the presence of PMSF and iodoacetamide chemicals. Similar findings for PMSF and iodoacetamide was reported by Ahmadova et al (2011). In addition, SDS and m-mercaptoethanol compounds, which are known to have an effect on the three-dimensional chemical structure of the enzyme, were also found to reduce activity (Table 4).

When the cow milk and randomly selected three different *E. faecalis* strains tested in this study were incubated, the best coagulation was detected to occur at 60 °C for 20 min in the tubes. As the congealed part was filtered and then kept in the refrigerator for 24 h, it was observed that milk was transformed into good quality cheese.

CONCLUSIONS

Findings from current study further point out *E. faecalis* strains can produce active proteases that can be used in many different fields such as coagulation of milk in the dairy industry and the separation of proteins from whey. *E. faecalis* strains isolated in this study had strong caseinate activity. SDS-PAGE electrophoresis of all strains tested in this study showed that the protein of the protease enzyme was at ~34.4 kDa. Characterization of the enzyme was performed by selecting ERZ-ATA7 strain which was determined to have the highest activity. The optimum pH and temperature values of the enzyme were determined as 7.0 and 40 °C, respectively. The extracellular protease enzyme was determined to coagulate milk and it was determined that the protease enzyme gained from *E. faecalis* ERZ-ATA7 strain further emphasizing the potential for rennet.

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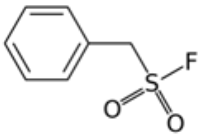
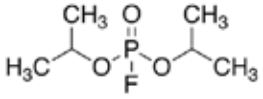
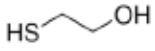
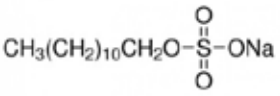
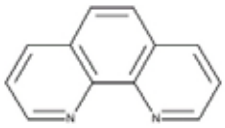
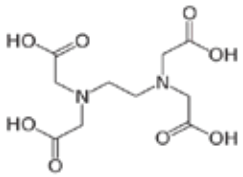
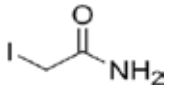
Table 4. The effect of some chemical compound on protease activity

Chemical Compound	Chemical structure	Concentration (mM)	Protease Activity(%)
Control	-	-	100
PMSF		5	95.36
		10	55.32
DIPF		5	112.10
		10	98.32
β-mercaptoethanol		5	43.15
		10	0
SDS		5	38.12
		10	0
PHT		5	125.19
		10	102.11
EDTA		5	12.45
		10	0
Iodoacetamide		5	83.63
		10	60.14

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Research article
Ερευνητικό άρθρο

A 30 year topic analysis of Veterinary Medicine literature

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ABSTRACT: In the present study Latent Dirichlet allocation (LDA) was used as a generative probabilistic model to extract major topics in interdecadalresearch for the Veterinary Medicine scientific literature. A total of 22 topics were extracted during the 1991-2000 period, 23 topics during 2001-2010 and 60 topics during 2011-2020. Three different algorithms were used to validate the model: perplexity, silhouette clustering and gradient boosted trees. All three validation metrics showed that LDA performed well in extracting topics. Each decade was characterized by unique topics as well as common topics which existed throughout periods. The most frequent topics were identified and trends were quantified with the use of indexes. A list of the 30 most frequent and most associated with the term Veterinary Medicine words is provided. A shift in scientific thinking probably occurred during the 30-year-period in the process of incorporating the fields related to Veterinary students, antimicrobial resistance and animals' behavior.

***Keywords*:** Veterinary Medicine, latent Dirichlet allocation, topic modeling, model validation, literature trends

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INTRODUCTION

The more classical approach of collecting information and exploratory analysis in scientific literature includes qualitative research methods, which offer high flexibility and focus well on understanding a problem. However, there are faster and less subjective methods to study the literature. Quantitative research methods offer solutions as they include easy data collection and analysis procedures and are not affected by the subjectivity of the researcher (Queirós et al., 2017).

The Latent Dirichlet allocation (LDA) is a generative probabilistic Bayesian model for collecting of discrete data such as text corpora (Blei et al., 2003). In LDA, text documents are used as a collection of words to identify underlying topics. Unsupervised machine learning techniques such as LDA, require little prior work from the researcher and are able to categorize big amount of data. However, LDA can also generate ambiguous topics which are hard to interpret and to classify as discussed previously in Web analysis research (Nanni, 2017). LDA has been successfully applied in the past in various fields such as in biology, biodiversity, climate change and animal communities (Zhang et al., 2019).

Studies in the literature of Veterinary Medicine with the use of LDA are scarce. However other advanced statistical techniques of machine learning have been applied on necropsy reports for detecting emerging diseases (Bollig et al., 2020) and in a literature review of urothelial cancer (Lin et al., 2020). Machine-learning-based literature mining may analyze large collections of documents, identify patterns in a dataset using statistical and computational methods or make predictions based on the discovered patterns (Lin et al., 2020). It is useful in summarizing key research themes and trends (Lin et al., 2020).

Previous works on text information extraction of literature mainly used text mining processesto study several subfields of Veterinary Medicine such as poor animal welfare (Contiero et al., 2019), epidemiology (Van der Waal et al., 2017), studies in livestock animals (Sahadevan et al., 2012), parasitology (Ellis et al., 2020), studies in antimicrobial prescribing practices (Welsh et al., 2017) or in geographic trends of science (Christopher andMarusic, 2013). Furthermore, another aspect of extracting information from text is the construction of automated electronic surveillance systems in order to predict emergency situations regarding disease outbreak (Lustgarten et al., 2020;Dórea et al., 2015), companion animal syndromes (Anholt et al., 2014), temporal and spatial features of diseases (Bollig et al., 2020) or in decision support frameworks (Jones-Diette et al., 2019).

Although many information collection techniques have been applied in Veterinary Medicine in the past for the purposes of surveillance systems, historical studies on an extended temporal scale has not been conducted. This is the first attempt that aims at clarifying the major scientific terms of literature during a 30 year period, from 1991to present. Topic extraction using LDA modeling is the main purpose of the present study to reveal the diachronically major topics in the field of Veterinary Medicine and to ascertain possible interdecadal differences in trends. Simultaneously, a lack of validation processes in related literature has been observed, thus a three-way validation approach was followed in this study to calculate the accuracy of the LDA model in predicting topics.

MATERIALS AND METHODS

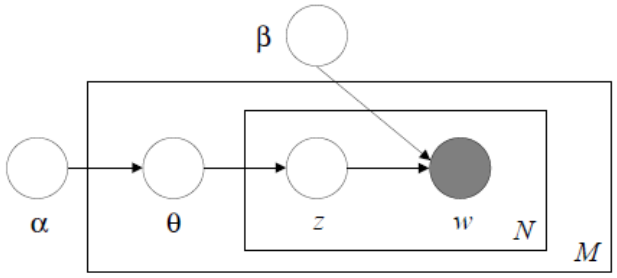
Abstracts of publications related to Veterinary Science were extracted from the Web of Science database. For this purpose the term “Veterinary Science” in quotes was entered in the search engine to extract only the Abstracts belonging to the category of Veterinary Sciences. Research articles, Reviews and Conference Proceeding papers were selected as they are the source of Abstracts and the study was planned at the level of decade; thus three decades 1991-2000, 2001-2010 and 2011-2020 were used as a filter to extract 857, 1.732 and 3.256abstracts respectively. Abstracts were stored in three separate files representing decades.

At first, a pre-processing stage transformed Abstract texts into words: special characters, symbols, numbers and articles were excluded from the analysis with the “stop words” procedure which was common for all three decades. KH coder (Higuchi, 2016) is able to analyze English data by grouping derivatives based on a built-in dictionary (it is called lemmatization and uses the Stanford POS Tagger toolkit) or by cutting the last letters and grouping words by their stem (it is called stemming and uses the Snowball Stemmer toolkit). For instance, the term “veterinary” during the stemming process becomes “veterinari”, terms “veterinarian/ veterinarians” become “veterinarian” and terms “tumor/ tumors/ tumorous/ tumoral” become “tumor”. Both toolkits were tested for their ability to group derivatives and to extract a large number of words. After the data preparation process, a pre-processing command was used to segment the word file

into words. This is a necessary internal process to organize the results in a SQL database form, to carry out searching and tabulating (Higuchi, 2016).

Quantitative analysis of text data followed. Words in the documents were counted to obtain the number of appearances. A word frequency table was constructed with the 30 most frequent words in each decade. A comparison of term frequencies was done to study differences between decades. For this purpose word frequencies were normalized as documents contained a different number of abstracts. A Word Association Table was constructed for each decade using the Jaccard coefficient to determine the associations between words and the term "Veterinary Science". The Jaccard coefficient emphasizes whether or not specific words co-occur, and is suitable for analyzing sparse data and is also calculated irrespectively of the term frequency (Higuchi, 2016). The values of Jaccard coefficient vary between 0 and 1. In KH Coder, words that appear frequently in the same sentence/paragraph are considered to be closely associated, and in that case the Jaccard coefficient reaches 1.

The Latent Dirichlet allocation (LDA) was used as a generative probabilistic model to extract topics from documents. LDA is based on the idea that documents represent a distribution of words which surround a topic. The model assumes that we predefine the number of topics into a document (k), one parameter for the distribution of topics into a document (α) and one parameter for the distribution of words into topics (β). These two parameters were set as α = 50/N and β = 0.1 in all runs of the algorithm. A plate notation from (Blei et al., 2003) of LDA variables is presented below, where:



M denotes the number of Abstracts
N is number of words in a given Abstract (Abstract i has N_i words)
α is the parameter of the Dirichlet prior on the per-Abstract topic distributions
β is the parameter of the Dirichlet prior on the per-topic word distribution
θ_i is the expected topic proportion of Abstract M, which is generated by a Dirichlet distribution parameterized by parameter α
z is the topic for the *n*th word in Abstract M and
w is the word in the *n*th position word of Abstract M.

Three measurements were used to evaluate the effectiveness of the LDA modeling those of perplexity, gradient boosted trees (GBT) and silhouette clustering. Validating LDA is a hard procedure as unsupervised machine learning uses data without pre-existing labels. For this purpose perplexity was used to define the ideal number of topics into each document. GBT were used to evaluate the performance of LDA in identifying topics and silhouette clustering with a manual labeling procedure was used in order to validate topics extracted with LDA.

Perplexity is a measurement of how well a probability model predicts a sample.In language modeling, perplexity decreases in the likelihood of the test data. A lower perplexity score indicates a better generalization performance (Blei et al., 2003). Due to the large volume of words collected during the 2011-2020 period, perplexity tends to get minimized at over 150 topics which makesit difficult to interpret such a large number of topics. Considering that word frequency of the endmost topics was very low only the 60 most important topics were finally presented concerning this decade in terms of word frequency.

$$perplexity\ (Document) = exp\left\{-\frac{\sum_{d=1}^{M} log\ p(w_d)}{\sum_{d=1}^{M} N_d}\right\}$$

M denotes the number of Abstracts
N is number of words in a given Abstract (Abstract i has N_i words)
p is the probability distribution of the model
w is aword-level variable.

Gradient boosted trees is an ensemble consisting of a set of alternative models using multiple learning algorithms to produce a more accurate classifier than that of the standard classifier (Opitz and Maclin, 1999). In this case the whole dataset (5.845 Abstracts) was divided in two random subsets for a total of10 random times to compare the model performance. These subsets were used in combinations of two to produce 45 different trees to evaluate the performance of LDA in identifying topics. Each subset was used to extract 40 topics, thus 80 topics were compared each time (40 topics from the training set and 40 topics from the prediction set) which were manually labeled. Manual labeling is a time-consuming process and rules have to be followed in order to obtain meaningful topics for a neutral individual. Three rules were applied to label topics: at least two words are necessary in order to label a topic meaningful; if there were more than two common words between topics, the label was given according to a third

word etc., and words in a similar context were considered as belonging to the same topic. For instance, the words tumor, tumour, malign, lymphoma, neoplasm, cancer were all identified under the topic of cancer and the words echocardiography, cardiac, pulmonary, valve, doppler, heart, pressure, arrhythmia, myocardium were all identified under the topic of heart-related problems. The weight of each word was used as an advisory index but not as a criterion in the identification of common topics between training and prediction sets. An example of labeling is given in Table 1.

Table 1. Example of the manual process followed to give labels to topics according to their meaning. Topics with the same context were given same labels (Topic 1 was the label for tumor related context) while topics with different context were given different labels (Topic 2 and 3)

Training set		**Prediction set**	
Word	Weight	Word	Weight
Topic1		Topic1	
tumor	394.0	tumor	284.0
cell	316.0	cell	256.0
histopatholog	150.0	tumour	153.0
tumour	144.0	lymphoma	104.0
histolog	115.0	cytolog	99.0
Topic2		Topic3	
effect	87.0	vaccin	198.0
dog	72.0	infect	182.0
agent	70.0	antibodi	101.0
chemotherapi	57.0	immun	81.0
treatment	56.0	virus	63.0

An indirect use of silhouette clustering was used to validate the results of LDA. Silhouette clustering is a method of interpretation and validation of consistency and cohesion within clusters of data. The average silhouette width can be used to evaluate cluster validity (Rousseeuw, 1987). As LDA extracted topics without any labels applied, the same manual procedure described above was used to give labels to each topic. An agglomerative hierarchical clustering of the two sets combined (each set consisted of 40 topics * five most frequent words of each topic = 200 words * two sets = 400 words) was used to identify groups of topics at the level of sets (training and prediction). Silhouette clustering was then applied to measure the consistency and the cohesion of 45 different combinations of clusters. A silhouette value (score) of one data point can be calculated with the formula:

$$s(i) = \frac{b(i)-a(i)}{\max\{a(i),b(i)\}}, \quad -1 \le s(i) \le 1$$

where a(i) is the dissimilarity 'within' a cluster and b(i) is the dissimilarity 'between' clusters.

The range of silhouette scores varies between +1 for objects that classified well in the predefined clusters (those of training and prediction sets) and -1 for objects that have been misclassified. In our case an accurate model would lead to small 'between' and 'within' distance dissimilarities between the training and the prediction sets, thus s(i) score would tend to zero. The overall average silhouette width for the entire plot (the average of the s(i) for all objects i belonging to the whole dataset)(Rousseeuw, 1987), was calculated with the Euclidean distance as a metric to indirectly calculate the accuracy of the model.

Three measures of topic diagnostic information were used to highlight the most frequent topics of each decade those of document entropy, document burstiness and corpus distance. Entropy is the degree of probability of a topic to occur in different documents (Abstracts in our case). The concept of entropy in information systems has been introduced by Shannon (1948) and has been widely used in topic extraction analysis. A topicof higher entropy is possible to have been extracted by a high number of several documents. Burstiness in natural language documents is the property of the most common words to represent a large number of topics (Boyd-Graber et al., 2014). This relationship of a few words representing a majority of topics and vice versa seems to follow a Zipfian distribution, an empirical law previously observed in social and physical sciences (Boyd-Graber et al., 2014). The combination of entropy and burstiness gives us a measure of the most common words and topics of literature. Finally the distance of topics from the corpus of each decade was measured with the Kullback-Leibler divergence distribution (Kullback andLeibler, 1951). A lesser distance from a corpus indicates that a topic is closely related to Veterinary medicine. Closely related topics consist of the most frequent words of the corpus. On the other hand, more distant topics are more distinct from the main corpus. A three-decade comparison was carried out to ascertain trends in corpus-related topics. A list of the most frequent words appearing before and after selected terms is presented in the Supplementary material, aiming not only to help but also to furtherly promote the interpretation of the topics and the comprehension of their position into the text.

Three open source free software were used for the procedures of document preprocessing, topic extraction and silhouette clustering: KH coder v. 3.Beta.01a (Higuchi, 2016), Orange v. 3.26.0 (Demsar et al., 2013) and RapidMinerv. 9.7.2+ (Mierswa et al., 2006).

RESULTS

Word stemming extracted almost the same number of words compared with the lemmatization procedure and was preferred for its better grouping ability (Table 2). From the total number of terms approximately half of them were excluded from the analysis (Table 2). The majority of words were common between decades with a different order of appearance (Table 3). Word association analysis revealed 14 strongly associated unique words in the between decade comparison (Table 3). Four words (dure, test, drug, system) were unique during 1991-2000, four words (provide, patient, student, medic) during 2001-2010 and six words (associ, dog, perform, compare, product, group) during 2011-2020. Normalized word frequency comparison revealed an increase in the use of common words such as dog, cat, pig, antibiotics and tumor and a decrease in words such as substance, vaccine, market, public and epidemiology during the decade 2011-2020. A detailed list of comparisons is given in Table 4.

Table 2. Comparison of two different algorithms for their ability to extract words from documents. The Tagger algorithm uses a built-in dictionary while the Stemmer algorithm groups words by their stem and then cuts their last letters

	1991-2000	2001-2010	2011-2020
	Stemmer		
Tokens in total	122627	314517	762825
Tokens in use	67094	175453	431440
	Tagger		
Tokens in total	126876	326773	797047
Tokens in use	67264	175970	433491

Table 3. List of the 30 most frequent and most associated words with the term Veterinary Medicine for each decade. Numbers correspond to frequencies and to Jaccard coefficient (JC).Words that appear frequently in the same abstract are closely associated thus JC reaches 1

Term Frequency						**Associated Words**					
1991-2000		**2001-2010**		**2011-2020**		**1991-2000**		**2001-2010**		**2011-2020**	
veterinari	1527	veterinari	3779	veterinari	6528	medicin	0.8672	medicin	0.8486	medicin	0.8609
medicin	1164	medicin	2538	medicin	4561	anim	0.2721	anim	0.3165	studi	0.4569
anim	813	anim	1833	anim	3809	clinic	0.2053	clinic	0.2841	anim	0.3992
dog	483	dog	1406	studi	3704	result	0.1787	studi	0.2726	result	0.3336
clinic	396	clinic	1192	dog	3521	studi	0.1759	human	0.2249	clinic	0.3129
diseas	378	studi	1121	clinic	2540	develop	0.1726	result	0.2195	human	0.2695
studi	358	case	962	treatment	2104	human	0.1725	univers	0.2047	increas	0.2440
result	309	human	948	result	2020	diseas	0.1713	diseas	0.1889	differ	0.2413
human	285	diseas	914	group	1989	import	0.1521	develop	0.1875	signific	0.2378
effect	281	student	723	diseas	1936	year	0.1448	year	0.1793	evalu	0.2332
develop	271	result	692	effect	1846	effect	0.1367	includ	0.1757	treatment	0.2312
treatment	269	develop	666	human	1846	increas	0.1287	increas	0.1681	associ	0.2293
test	268	health	648	signific	1760	method	0.1265	case	0.1632	effect	0.2200
drug	267	treatment	634	differ	1660	includ	0.1259	evalu	0.1570	includ	0.2169
method	246	resist	611	increas	1656	practic	0.1251	differ	0.1567	diseas	0.2118
case	243	report	600	case	1583	univers	0.1240	effect	0.1529	dog	0.1998
differ	226	increas	599	evalu	1583	treatment	0.1169	import	0.1501	perform	0.1967
year	219	effect	573	cell	1501	differ	0.1167	practic	0.1442	develop	0.1896
practic	206	year	569	student	1451	examin	0.1088	treatment	0.1440	report	0.1884
system	205	differ	564	perform	1377	review	0.1074	examin	0.1437	import	0.1860
cat	202	cat	552	report	1372	case	0.1066	provid	0.1330	year	0.1830
increas	202	practic	552	associ	1330	time	0.1044	patient	0.1324	compar	0.1829
import	201	evalu	547	cat	1321	dure	0.1028	signific	0.1312	case	0.1773
group	200	univers	533	resist	1307	test	0.1022	report	0.1279	product	0.1736
signific	200	examin	512	product	1216	signific	0.1005	method	0.1259	method	0.1725
veterinarian	193	includ	508	test	1189	determin	0.0995	review	0.1246	determin	0.1712
infect	188	veterinarian	508	compar	1182	evalu	0.0993	time	0.1239	time	0.1645
evalu	186	patient	491	includ	1177	articl	0.0978	student	0.1231	univers	0.1629
examin	186	hors	485	control	1146	drug	0.0968	articl	0.1215	examin	0.1623
includ	185	infect	481	sampl	1146	system	0.0955	medic	0.1177	group	0.1573

Table 4. Normalized word frequency comparisons of selected words presented in subcategories. Arrows indicate a frequency value over [⇧], below [⇩] or equal [▭] to the average of the 1991-2020 study period. Red gradient was used to highlight higher word frequency values of each subcategor

Animals	dog		cat		hors		pig		bird		sheep		domest		mammari		cow		bovin		breed			
	⇩	14,61	⇩	5,95	⇩	5,03	⇩	1,27	⇩	1,18	⇩	1,08	⇩	0,99	⇩	0,62	⇧	2,04	⇧	1,24	⇩	2,50		
	▭	21,20	▭	8,19	⇧	7,17	⇩	1,68	⇩	0,55	⇩	0,87	⇧	1,36	⇧	0,90	⇩	1,56	⇩	1,03	⇩	2,72		
	⇧	30,77	⇧	11,42	▭	6,41	⇧	5,15	⇧	2,81	⇧	1,56	⇧	1,53	⇩	0,68	⇧	1,90	⇧	1,25	⇧	3,30		
Diet	feed		milk		dairi		nutrit		Other		infecti		care		chronic		protocol		routin		altern			
	⇩	1,58	▭	1,42	⇩	0,65	⇧	1,48			⇩	1,14	⇩	1,21	⇩	0,87	⇩	0,81	⇩	0,81	⇩	1,33		
	⇩	0,97	⇩	0,98	⇧	1,16	⇩	0,94			⇧	1,26	▭	2,72	▭	1,32	⇩	0,81	▭	0,97	⇩	1,09		
	⇧	6,37	⇧	2,16	⇧	1,37	▭	1,14			⇧	1,24	⇧	4,00	⇧	2,12	⇧	1,69	⇧	1,16	⇧	2,01		
Immune system	antibiot		antibodi		vaccin		antimicrobi		antigen		serum		plasma		immun		virus		metabol		dna			
	▭	3,73	⇧	1,48	⇩	2,47	⇩	1,67	⇩	0,53	⇩	2,72	⇩	1,45	⇩	1,24	⇩	1,14	⇩	0,90	⇩	0,56		
	⇩	2,55	⇩	0,69	⇧	3,31	▭	5,29	▭	0,69	⇩	2,86	▭	1,82	⇩	1,47	⇧	1,83	⇩	0,80	⇩	0,58		
	⇧	5,41	⇧	1,78	⇩	2,27	⇧	8,84	⇧	0,86	⇧	4,96	⇧	2,37	⇧	2,54	⇧	1,65	⇧	1,72	⇧	1,03		
Professions	human		student		administ		econom		safeti		market		owner		materi		public							
	⇩	8,51	⇩	2,84	⇩	0,93	▭	0,93	⇩	0,87	⇧	0,81	⇩	0,81	⇩	0,62	⇩	2,62						
	⇧	14,22	⇧	10,79	⇩	1,18	⇩	0,84	▭	1,18	⇩	0,51	▭	1,99	▭	1,64	⇧	5,70						
	⇧	16,04	⇧	12,56	⇧	1,95	⇧	1,04	⇧	1,34	⇩	0,48	⇧	3,45	⇧	2,55	⇩	2,69						
Pathology / Diseases	surgeri		gastrointestin		dose		tumor		disord		therapeut		joint		pressur		pharmacokinet		salmonella		respiratori			
	⇩	2,87	▭	0,87	⇩	2,32	⇩	1,91	⇧	1,85	⇩	1,76	⇧	1,73	▭	1,54	⇧	1,48	⇩	0,56	⇩	1,33		
	⇩	2,58	⇩	0,45	⇩	2,69	⇧	3,69	⇩	1,62	⇩	2,02	⇩	0,60	⇩	1,29	⇩	1,03	▭	0,69	⇧	1,41		
	⇧	3,60	⇧	1,11	⇧	3,81	⇧	4,03	⇧	1,78	⇧	2,87	▭	1,34	⇧	1,83	▭	1,31	⇩	0,59	⇧	1,41		
	renal		patholog		surgic		bone		pathogen		heart		cardiac		diagnos		mastiti		biochem		diabet			
	▭	1,51	⇩	1,45	⇩	1,42	⇩	1,36	⇩	1,36	⇩	0,68	⇩	0,65	⇩	1,30	⇧	0,59	⇩	0,68	⇧	0,68		
	⇩	1,13	⇧	3,22	▭	2,75	▭	2,35	⇧	2,43	▭	1,09	▭	1,04	▭	2,70	⇩	0,43	⇩	0,60	⇧	0,78		
	⇧	2,06	⇧	2,82	⇧	3,79	⇧	3,18	⇧	2,82	⇧	1,81	⇧	1,41	⇧	3,75	⇧	0,65	⇧	1,43	⇩	0,32		
	epidemiolog		physiolog		strain		genet		mechan		oral		arteri		enrofloxacin		pharmacolog		urinari		anaesthet			
	⇧	1,30	⇧	1,27	⇩	1,27	⇩	1,24	⇩	0,96	⇩	1,11	⇩	1,05	⇧	1,02	▭	0,77	⇩	0,71	⇧	0,68		
	⇩	1,15	⇩	1,18	⇧	2,93	⇩	1,21	▭	1,61	⇧	2,35	⇩	0,92	▭	0,80	⇧	1,10	⇩	0,81	▭	0,39		
	⇩	1,11	⇩	1,14	⇧	2,80	⇧	1,56	⇧	2,27	⇧	2,14	⇧	1,60	⇩	0,63	⇩	0,50	⇧	1,16	⇩	0,00		

LDA successfully extracted 22 topics during 1991-2000, 23 topics during 2001-2010 and 60 topics during 2011-2020 (Figure 1-3) as indicated by the perplexity score (Figure 4). Topics including the term "veterinari" differed between decades. Six topics with this term were extracted during 1991-2000, seven topics during 2001-2010 and six topics during 2011-2020 in a relevant different volume of literature Abstracts. During the first decade the terms study, animal-veterinarian, student-education, school, surgery-techniques and drugs-use-effects co-appeared with the term veterinary. During the second decade the terms practice, student-education-program, clinic-examination, human-treatment, study-group, faculty-school and animal-health co-appeared with the term veterinary. Finally the terms clinic-case, university-research, animal-human-health, disease-human-patient, clinic-studies and student co-appeared with the term veterinary during the third decade. Apart from the topics directly related to the overly generalized terms "Veterinari" and "medicine" which were common for all three decades, several other topics were extracted many of which were very specific. For instance, the topics "ultrasound-arteri-pregnanc-fetal-Doppler", "cell-tumor-tumour-dog-carcinoma" and "resist-antimicrobi-isol-strain-antibiot" refer to artery examination, cancer in dogs and antimicrobial resistance respectively.

GBT modeling indicated that LDA is able to extract topics with a root-mean-square error (RMSE) of 2.759 ±0.053. This means that proportionally with the 40 topics extracted from each subset during the validation process, a mean of 2.759 topics were not predicted correctly. Cluster analysis of the 45 different combinations of sets grouped the 80 topics into two clusters at the level of training and prediction sets indicating the degree of correctly predicted (or respectively incorrectly predicted) topics. Silhouette scores of each cluster analysis indicated the number of topics from the training set that was predicted incorrectly (Figure 5).An overall average of s(i) = 0.079864 (±2σ of 0.02429) means that there are small 'between' and 'within' dissimilarities between training and prediction sets, thus the model is accurate.

Each decade consisted of different topics of high entropy and high burstiness (Figure 1-3). Topics "dog-cat-case-clinic-examin", "studi-data-age-veterinari-sampl" and "veterinari-anim-medicin-veterinarian-develop" were most frequent during the first decade. Topics "veterinari-student-medicin-educ-pro-

gram", "veterinari-medicin-use-practic-inform", "clinic-examin-case-veterinari-medicin" and "veterinari-human-medicin-treatment-diseas" were most frequent during the second decade. Finally topics "student-veterinari-medicin-studi-survey", "anim-veterinari-medicin-health-human", "diet-group-feed-supplement-fed","veterinari-medicin-diseas-human-patient" and "case-dog-veterinari-clinic-report" were most frequent during the third decade. A distance from the corpus revealed that "antimicrobial resistance" and "veterinary students" were more distinct topics from the corpus of Veterinary medicine during 1991-2000 while "animal behavior problems" and "surgeries" were more distinct during 2011-2020 (Figure 6).

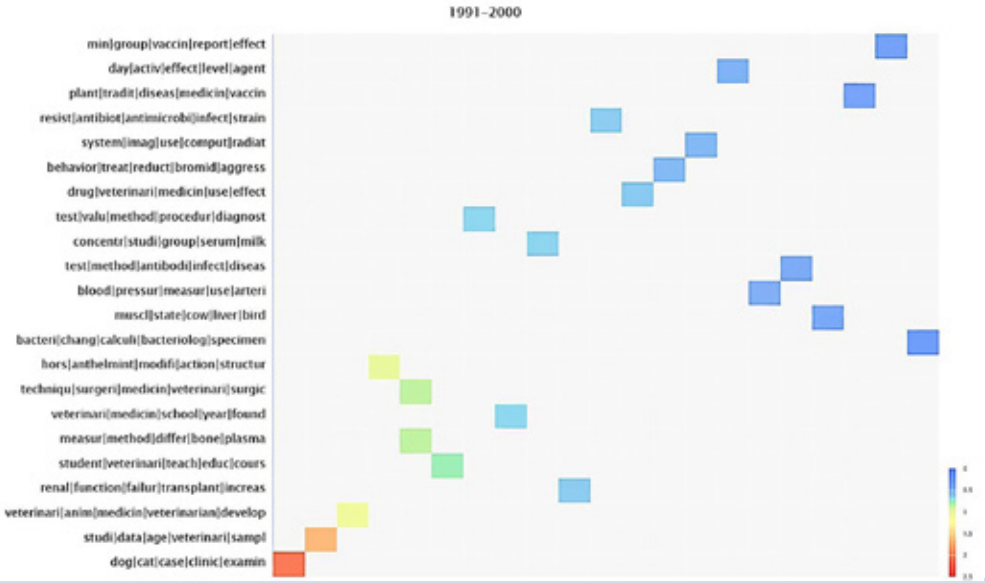


Figure 1. Topics extracted from the period of 1991-2000 with the LDA analysis. The combination of entropy and burstiness measures was used as an index to quantify the most frequent topics of literature (red color)

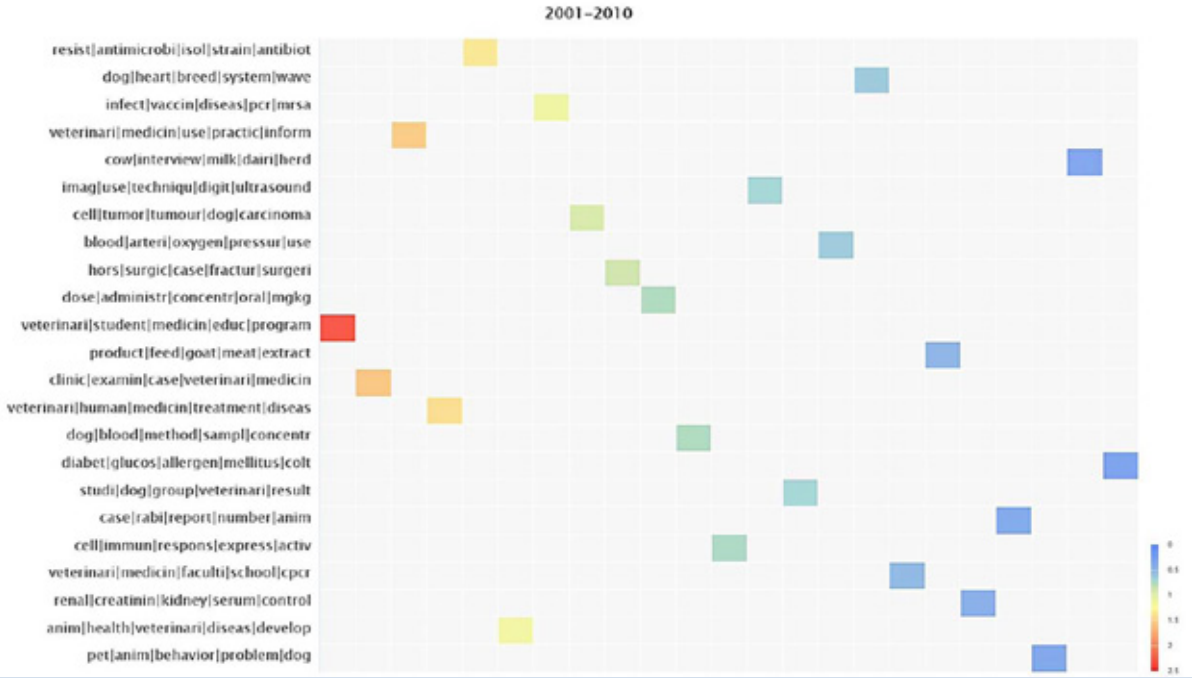


Figure 2. Topics extracted from the period of 2001-2010 with the LDA analysis. The combination of entropy and burstiness measures was used as an index to quantify the most frequent topics of literature (red color)

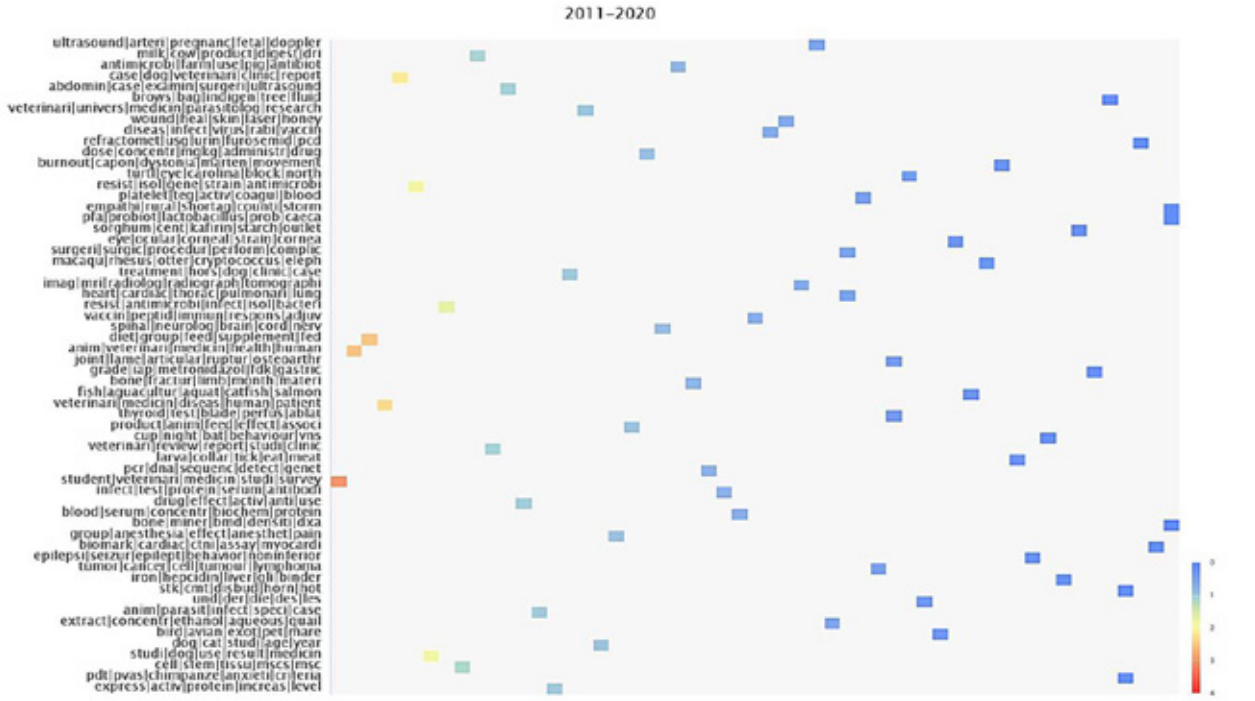


Figure 3. Topics extracted from the period of 2011-2020 with the LDA analysis. The combination of entropy and burstiness measures was used as an index to quantify the most frequent topics of literature (red color)

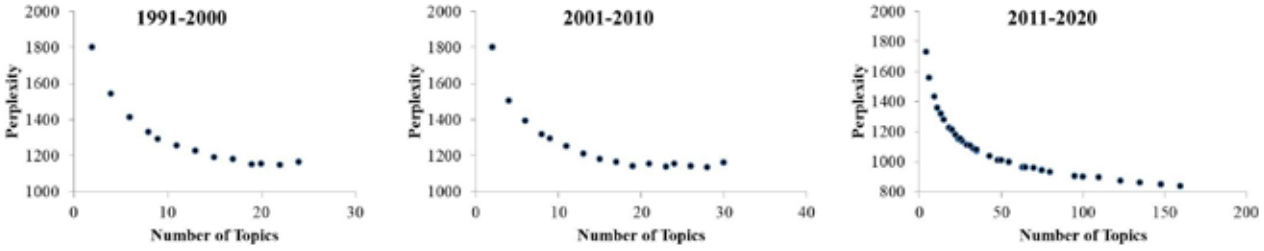


Figure 4. Perplexity is a measure of how well the LDA model predicted a sample. A lower perplexity score indicates better generalization performance of the model

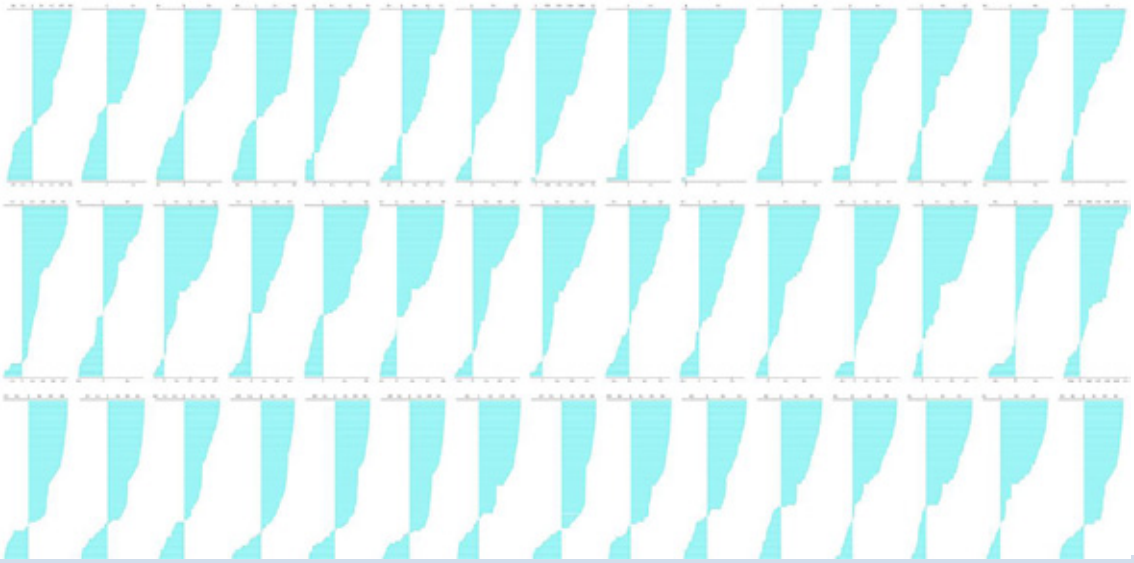


Figure 5. Silhouette graphs showing the consistency and the cohesion of 45 different combinations of training and prediction sets. Small 'between' and 'within' dissimilarities between training and prediction sets, give a small silhouette score [s(i) close to zero] and would imply that the model is accurate. The overall average width for each plot is calculated with the Euclidean distance to give the average s(i)

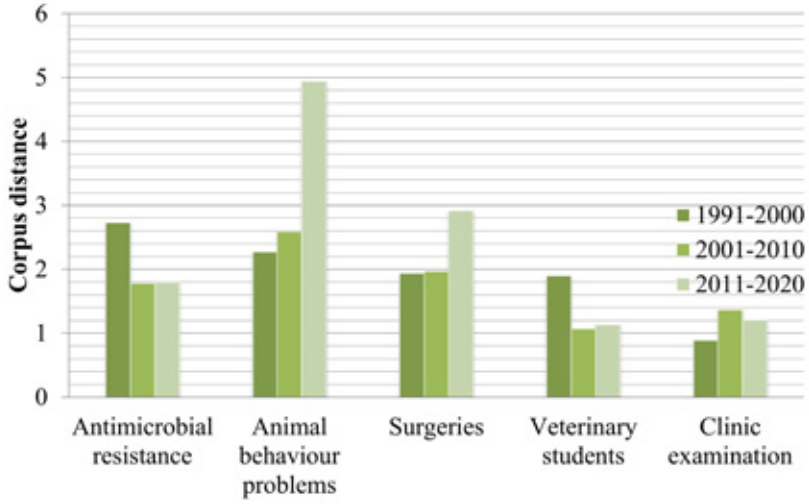


Figure 6. Comparison of selected topics described in terms of their context. Distance of topics from the corpus ofVeterinary medicine-was measured with the Kullback-Leibler divergence distribution. Lower distance from a corpus indicates that a topic is closely related with Veterinary medicine

DISCUSSION

The interpretation of topics is not an easy mission due to the generality of some topics or because many of their words do not match in meaning. The extraction of ambiguous topics has been reported in the past (Nanni, 2017). However, the LDA model managed to uncover a satisfying number of topics, many of which are well interpretable. The term "veterinari" was included in a number of topics during each decade, showing that these topics were not able to acquire a special meaning. Apart from these strongly linked and sometimes overgeneralized topics, other more specific and more meaningful topics existed. The basic analysis in extracting the meaning of a document by giving us a number of topics is the LDA modeling. The interpretation process is subjective and shall focus on the more meaningful topics. To avoid this subjectivity the indexes of topic diagnostic information, document entropy, document burstiness and corpus distance, will constitute a more quantitative approach which helps us objectively interpret results in contrast with the direct subjective human interpretation. They can provide a useful automated summary of topic quality (Boyd-Graber et al., 2014).

LDA analysis constitutes the main technique applied in the present study to extract the major scientific topics of Veterinary literature. At first, the three measures of topic diagnostic information are discussed combined to analyze the relation between persistently present topics during the three decades and the main corpus of literature. Secondly, an effort is made to identify major shifts occurred in the overall topic rank. For this purpose, a simplification is used to shortly describe one topic (Supplementary material). For instance the topics "student|veterinari|teach|educ|cours" and "veterinari|student|medicin|educ|program" are both described as "students" and the topics "resist|antibiot|antimicrobi|infect|strain" and "resist|antimicrobi|isol|strain|antibiot" are both described as "antibiotic resistance" because they refer to students and antibiotics resistance respectively. Thirdly, trends of selected words representing topics are discussed with the use of word frequencies (collocation statistics - Supplementary material) to further facilitate the interpretation of topics under a more analytical aspect.

The use of document entropy and document burstiness helped to identify and rank the most frequent topics. A focus on two animals (dog and cat) during the first time period, a focus on students' educational programs during the second period and a focus on students surveys during the third period indicate different hot topics of each period. Simultaneously, student-related topics were less distinct from the corpus during the last two periods. This means that veterinary student topics became more frequent and were progressively incorporated in the main body of scientific literature. It is possible that a shift in scientific thinking had occurred during the 30-year-period. Students' attitude, learning, motivation, competence in science, learning in practice are some of the aspects of the use of this term in literature (Mich et al., 2010; Jones et al., 2019). A relevant incorporation of a topic into the main corpus of Veterinary Medicine occurs with the topic of antibiotic resistance. The latter is less frequent during the first period and becomes a hot topic less distanced from the corpus during the past two pe-

riods. It is possible that scientists working on antibiotics take time to find novel solutions to this problem or they are aware of microorganisms' resistance to drugs (Toutain et al., 2016) thus the reporting of problems is progressively accumulated in literature.

The topic of animal behavior is extracted in all the three periods from the LDA model but it does not belong to the most frequent ones. This probably shows that veterinarians have already incorporated a perception that behavior problems are equally important with others topics such as clinical examination even before the nineties. The large distance from the corpus during 2011-2020 indicates that during the past few years this topic is linked toseveral words other than the most common ones. These different words probably reflect a variety of new subjects introduced in larger quantities into scientific community which were not strongly present in the past. Indeed, abstracts collected during the present study, contained the term behavior in the context of behavior of aggressive dogs (Csoltova et al., 2017), changes of behavior under a specific therapy (Packer et al., 2016), behavior studies, behavior alterations or behavior abnormalities (Tynes and Sinn, 2014).

A diachronically increasing interest of scientific literature for the topics of students and antimicrobial resistance and a decreasing interest for the topics of surgeries and animal behavior have been observed (Fig. 1-3 and Supplementary Material). On the contrary, the topic of clinical examination constantly seems to be of high interest among publications as it shows up in the five most frequent topics during the 30-years-period. During the 1991 - 2000 period the most frequent topics included clinical examination, were followed by topics including surgeries, students, antimicrobial resistance and animal behavior (Supplementary material). During the 2001 - 2010 period a shift was noticed in topic frequencies rank: the most frequent topic included students while clinical examination, antimicrobial resistance, surgeries and animal behavior followed. Lastly, during the 2011 - 2020 period the most frequent topics included students followed by topics including clinical examination, antimicrobial resistance, surgeries and behavior. The frequencies of these topics show their relative position in the overall ranking of each decade and probably reflect similar shifts in the interests of each decade. Furthermore, during the last period topics including clinical examination, antimicrobial resistance and surgeries were extracted in two different versions. It is possible that a broadening occurred regarding their discussion in the scientific literature. Other topics emerge during specific time periods such as tumors during 2001 - 2020, vaccinations during 2001 - 2010, dosage (dose, mg/kg, concentration) during 2001 - 2020, dairy (milk, cow) during 1991 - 2000 and 2011 - 2020 and blood pressure during 1991 - 2010. It is difficult to tell whether there is a specific incident that provoked these shifts in topic ranking (for instance a pandemic) or if advances in technology have promoted the interest of scientists (for instance new tumor confrontation techniques). It is possible that a reporting increase in the national veterinary registration systems of each country acts as a signal to activate further scientific research of specific diseases.

Collocation statistics retrieved with KH coder (Supplementary material) contributed to the objective interpretation of selected words representing topics. Four of these words, those of "behavior", "resistance", "student" and "surgery", were selected as representatives of the topics that were firmly extracted during the three-decade-period and displayed changes intothe topic ranking of each decade (animal behavior, antimicrobial resistance, students and surgeries respectively). Words appearing before or after these selected terms are directly depended on them (they display greater weight). It is more probable to see the word "veterinary" and "female" one word left from "student" and word "educ" one word right from "student" during 1991 - 2000. All these words combined thus indicate that they are connected under this point of view: veterinary student's education or female student's education. During 2001 - 2010 other words co-appear with the word student such as medicine, learning, graduate, interest, evaluation, training, participation or experience. All these words combined thus indicate that they are connected under the following point of view: veterinary student's learning, veterinary student's training etc. During 2011 - 2020 new words appear close to the term student: performance, perception, studies, assessment, training, attitude, experience or improvement. All these words combined indicate that they are connected under this point of view: veterinary student's performance or veterinary student's perception etc. It is possible that a shift in the scientific thinking occurred during 1991 - 2020 from the simple perspective of veterinary student's education (Heath et al., 1996) to a more competitive aspect including performance, assessment of their experience etc (Zenner et al., 2005).

It is more probable to encounter the words "veterinary", "animal", "invas", "cardiovascular" one word left from "surgeri" and the words "procedur" and "depart" one word right from "surgeri" during 1991 - 2000. All these words combined thus suggest that they are connected under this point of view:veterinary surgeries or invasive surgeries or surgeries procedure. During 2001 - 2010 new words appear close to the word surgeries such as "dure", "clinic", "laparoscop", "abdomin", "convent", "hors", "colic" etc. All these words combined suggest that they are connected under this point of view:veterinary surgeries or laparoscopic surgeries or conventional surgeries etc. During 2011 - 2020 new words appear close to the word surgeries such as "dog", "perform", "course", "spinal", "open". All these words combined suggest that they are connected under this point of view:surgeries duration or veterinary surgeries or surgeries performance or dog surgeries etc. It is possible that the scientific field of surgeries is constantly evolving and dealing with ever-changing topics.

It is more probable to see the words "problem"," animal", "therapy", "pet", "pharmacotherapy" close to the word "behavior" during 1991 - 2000. New words appear close to "behavior" during 2001 - 2010 those of "medicin", "veterinary" and "cours". During 2011 - 2020 words that appear close to "behavior" include "problem", "change", "relat", "dog" and "intervent". All these words combined suggest that they are connected under this point of view: animal behavior problem during 1991 - 2020, but with some differentiations between decades from behavior therapy/pharmacotherapy to behavior course and then to behavior change. It is possible that scientists make efforts to intervene into the animal behavior problems through therapies. However during 2001 - 2020 an effort is made to attribute biological interpretations into the aggressive behavior of animals. Some types of agitated behavior indeed have a strong genetic basis (Grandin and Deesing, 2014).

It is more probable to see the words "antibiot", "bacteri" and "pathogen" during 1991 - 2000, the words "antimicrobi", "isol" and "methicilin" during 2001 - 2010 and the words "antimicrobi", "antibiot" and "multidrug" during 2011 - 2020close to the term "resist". All these words combined suggest that they are connected under this point of view: antibiotic resistance or antimicrobial resistance or isolates of bacteria to study the antibiotic resistance etc. Many of the terms closely present with "resist" are common between the three decades. However it is possible that each decade is characterized by different priorities regarding research on antibiotic resistance as new terms stand out during the 2001 - 2020 period, those of multidrug, *Staphylococcus aureus* and gene nevertheless this is not so evident and cannot be distinctly supported by the specific results.

Previous text mining works have revealed a common number of terms also described in the present study. The subject of public awareness regarding the way in which farm animals are kept, the use of antimicrobials to increase animal performance and animal welfare have been reported before (Contiero et al., 2019). The subject of infectious diseases transmitted from animals to humans (or the opposite) and antimicrobial resistance due to prescriptions and results in human health have also been reported in the past (Lustgarten et al., 2020).

The validation of the LDA model carried out with several ways to verify that the accuracy in extracting the same topics was good at a satisfactory level. Each time the LDA model is applied a number of less frequent and hard to interpret topics is extracted (Nanni, 2017). We have to take into consideration that a part of the topics that LDA failed to correctly extract is these nonessential topics. On the other hand the LDA model is suitable in correctly identifying the most important of them over a large period of time. More specialized queries in the future may contribute to revealing possible trendsof less studied topics of the scientific literature.

CONCLUSIONS

LDA managed to reveal the most frequent topics of three continuous decades. The number of topics extracted during each period increases proportional to the volume of scientific literature. Differences throughout decades occur and may reflect perceptions of researchers. Topics related with veterinary students and antibiotic resistance are probably incorporated into the main corpus of literature during the 2001 - 2020 period while topics related with animal behavior were probably enriched with a variety of new subfields not recorded in the past. Quantitative literature research is an appropriate tool in identifying trends in topics.

ACKNOWLEDGEMENTS

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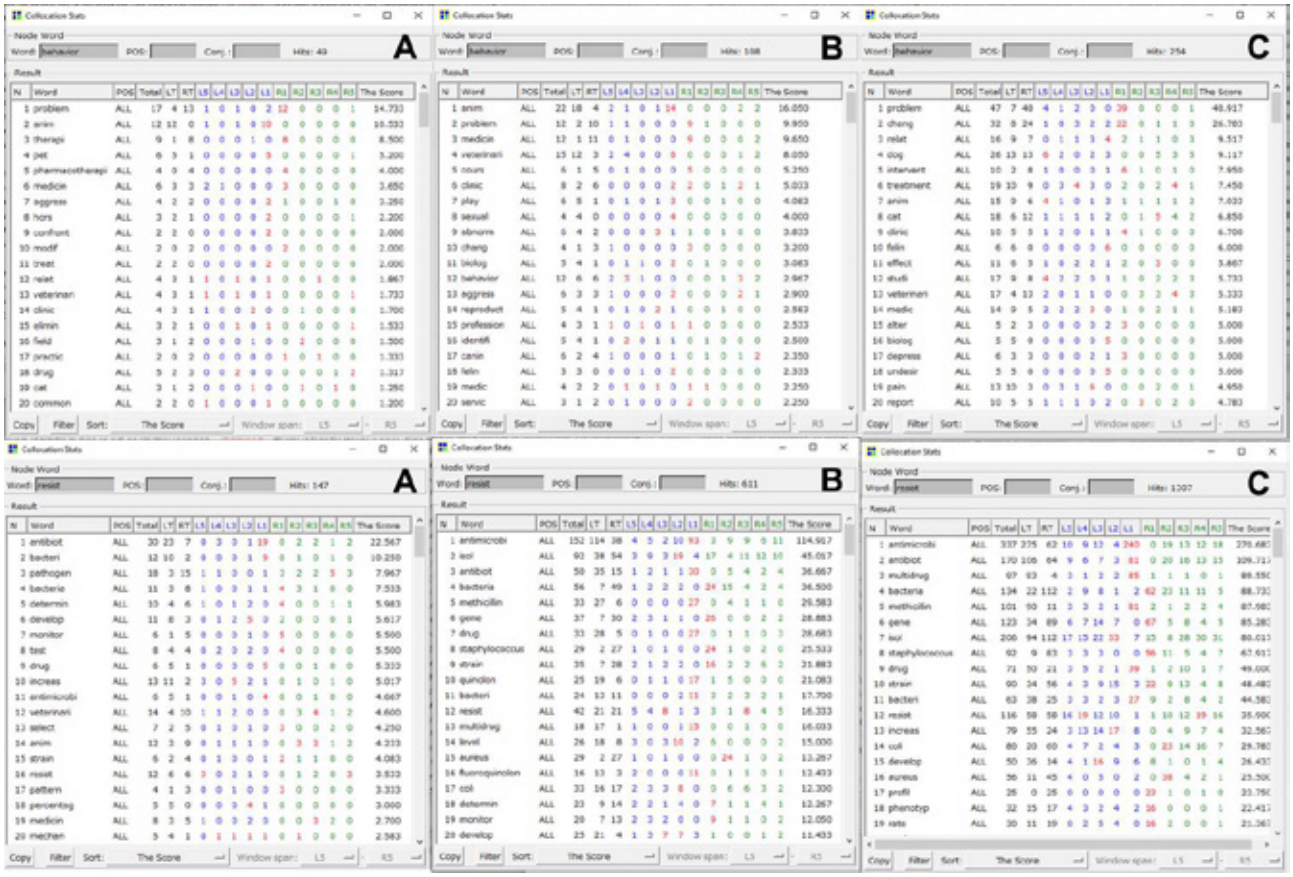
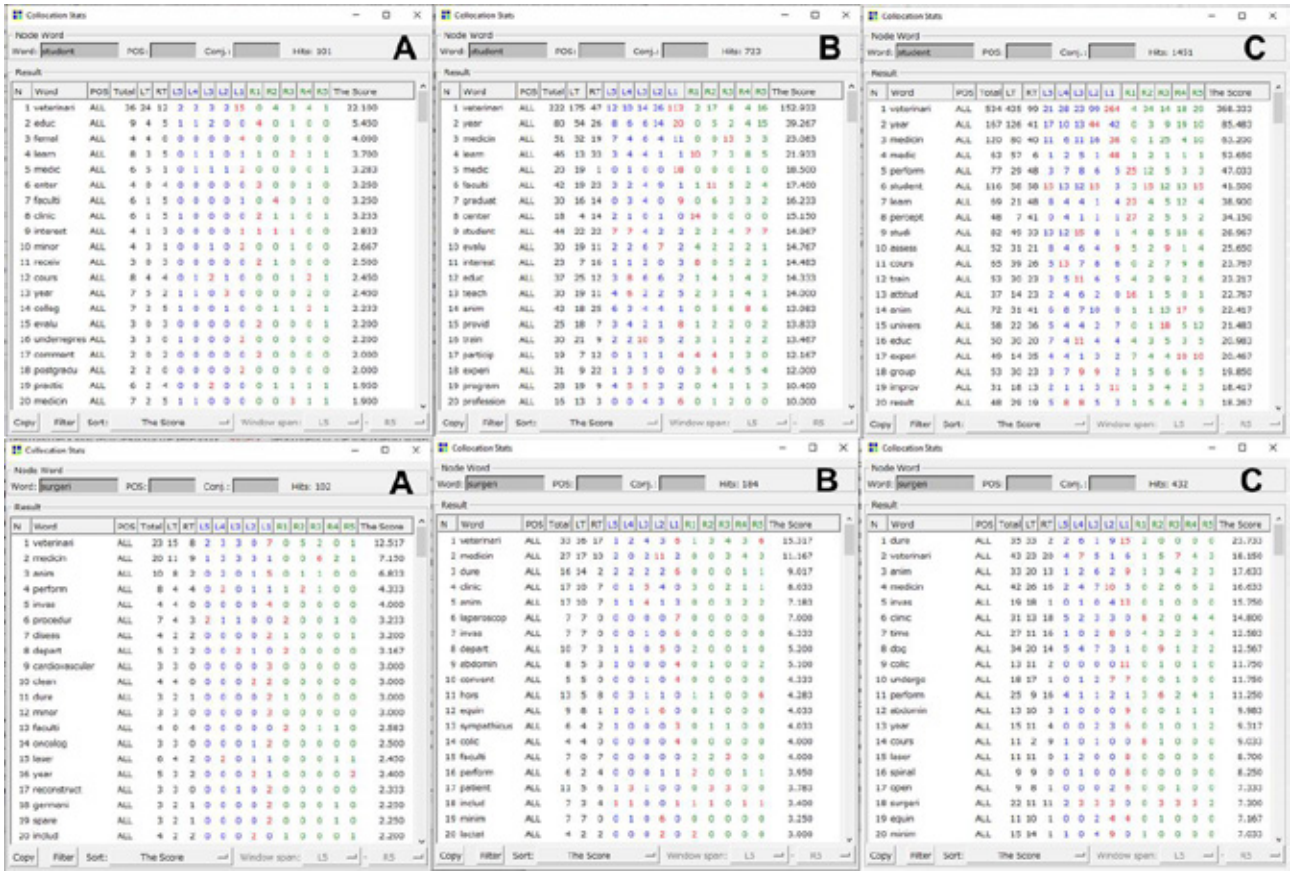
SUPPLEMENTARY MATERIAL

Collocation statistics retrieved with KH coder. Word frequencies appearing before (L) and after (R) a node word are presented. The list shows the words that directly depend on the node word. Words that appear closer to the node words display greater weight, thus a higher Score. Four examples of strongly connected words with "Veterinary", persistently present in all the 1991 - 2020 period are shown here those of "behavior", "resistance", "student" and "surgery".[A = 1991 - 2000, B = 2001 - 2010, C = 2011 - 2020]





List of the topics extracted during 1991 - 2020 with the use of the LDA model. Topics are presented in descending order with the use of entropy and burstiness as indexes to measure the most common words and topics of literature. Abbreviations of selected topics were used to help track their order during each time period.

	1991 - 2000		2001 - 2010		2011 - 2020			
1	dog|cat|case|clinic|examin	CE	veterinari|student|medicin|educ|program	ST	student|veterinari|medicin|studi|survey	ST	imag|mri|radiolog|radiograph|tomographi	
2	studi|data|age|veterinari|sampl		clinic|examin|case|veterinari|medicin	CE	anim|veterinari|medicin|health|human		ultrasound|arteri|pregnanc|fetal|doppler	
3	veterinari|anim|medicin|veterinarian|develop		veterinari|medicin|use|practic|inform		diet|group|feed|supplement|fed		extract|concentr|ethanol|aqueous|qual	
4	hors|anthelmint|modifi|action|structur		veterinari|human|medicin|treatment|diseas		veterinari|medicin|diseas|human|patient		surgeri|surgic|procedur|perform|complic	SU
5	techniqu|surgeri|medicin|veterinari|surgic	SU	resist|antimicrob|isol|strain|antibiot	AR	case|dog|veterinari|clinic|report	CE	heart|cardiac|thorac|pulmonari|lung	
6	measur|method|differ|bone|plasma		anim|health|veterinari|diseas|develop		resist|isol|gene|strain|antimicrobi	AR	platelet|tegl|activ|coagul|blood	
7	student|veterinari|teach|educ|cours	ST	infect|vaccin|diseas|pcr|mrsa	VA	studi|dog|use|result|medicin		tumor|cancer|cell|tumour|lymphoma	TU
8	test|valu|method|procedur|diagnost		cell|tumor|tumour|dog|carcinoma	TU	resist|antimicrob|infect|isol|bacteri	AR	joint|lame|articular|ruptur|osteoarthr	
9	veterinari|medicin|school|year|found		hors|surgic|case|fractur|surgeri	SU	cell|stem|tissu|mscs|msc		thyroid|test|blade|perfus|ablat	
10	concentr|studi|group|serum|milk	DA	dose|administr|concentr|oral|mgkg	DO	milk|cow|product|digest|dri	DA	turt|eye|carolina|block|north	
11	renal|function|failur|transplant|increas		dog|blood|method|sampl|concentr		veterinari|review|report|studi|clinic	CE	und|der|die|des|les	
12	resist|antibiot|antimicrobi|infect|strain	AR	cell|immun|respons|express|activ		abdomin|case|examin|surgeri|ultrasound	SU	bird|avian|exot|pet|mare	
13	drug|veterinari|medicin|use|effect		imag|use|techniqu|digit|ultrasound		drug|effect|activ|anti|use		eye|ocular|corneal|strain|cornea	
14	behavior|treat|reduct|bromid|aggress	AB	studi|dog|group|veterinari|result		anim|parasit|infect|speci|case		fish|aquacultur|aquat|catfish|salmon	
15	system|imag|use|comput|radiat		blood|arteri|oxygen|pressur|use	BP	express|activ|protein|increas|level		macaqu|rhesus|otter|cryptococcus|eleph	
16	day|activ|effect|level|agent		dog|heart|breed|system|wave		treatment|hors|dog|clinic|case		burnout|capon|dystonia|marten|movement	
17	blood|pressur|measur|use|arteri	BP	veterinari|medicin|faculti|school|cper		veterinari|univers|medicin|parasitolog|research		larva|collar|tick|eat|meat	
18	test|method|antibodi|infect|diseas		product|feed|goat|meat|extract		dog|cat|studi|age|year		epilepsi|seizur|epilept|behavior|noninferior	AB
19	muscl|state|cow|liver|bird		renal|creatinin|kidney|serum|control		group|anesthesia|effect|anesthet|pain		cup|night|bat|behaviour|vns	
20	plant|tradit|diseas|medicin|vaccin	VA	case|rabi|report|number|anim		product|anim|feed|effect|associ		iron|hepcidin|liver|gli|binder	
21	min|group|vaccin|report|effect	VA	pet|anim|behavior|problem|dog	AB	dose|concentr|mgkg|administr|drug	DO	sorghum|cent|kafirin|starch|outlet	
22	bacteri|chang|calculi|bacteriolog|specimen		cow|interview|milk|dairi|herd	DA	spinal|neurolog|brain|cord|nerv		grade|iap|metronidazol|fdk|gastric	
			diabet|ghcos|allergen|mellitus|colt		antimicrobi|farm|use|pig|antibiot		brows|bag|indigen|tree|fluid	
					bone|fractur|limb|month|materi		pdt|pvas|chimpanze|anxieti|criteria	
					pcr|dna|sequenc|detect|genet		stk|cmt|disbud|horn|hot	
					infect|test|protein|serum|antibodi		refractomet|usg|urin|furosemid|pcd	
					blood|serum|concentr|biochem|protein		biomark|cardiac|ctni|assay|myocardi	
					vaccin|peptid|immun|respons|adjuv	VA	empathi|rural|shortag|count|storm	
					diseas|infect|virus|rabi|vaccin	VA	pfa|probiot|lactobacillus|prob|caeca	
					wound|heal|skin|laser|honey		bone|miner|bmd|densit|dxa	

Abbreviations

Clinical Examination	**CE**	**Tumor**	**TU**
Surgeries	**SU**	**Vaccinations**	**VA**
Students	**ST**	**Dosage**	**DO**
Antimicrobial Resistance	**AR**	**Dairy**	**DA**
Animal Behavior	**AB**	**Blood Pressure**	**BP**

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Research article
Ερευνητικό άρθρο

The phylogenetic analysis of RNA-dependent RNA polymerase gene of chronic bee paralysis virus in Turkey

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ABSTRACT: Chronic bee paralysis virus (CBPV) is a major contributor to global bee colony losses. However, among the RNA viruses infecting honeybees, CBPV is still not classified. In this study, samples of asymptomatic adult honey bees were randomly collected from 45 apiaries in three provinces (Burdur, Antalya, and Isparta) in Turkey. Five of the 45 samples were determined to be positive by reverse transcription polymerase chain reaction (RT-PCR). In addition, three positive samples from Burdur, Isparta, and Antalya were confirmed by sequencing. We constructed a phylogenetic tree that was divided into five main groups based on the RNA-dependent RNA polymerase gene region. The Turkish strains TUR/BUR/CBPV1, TUR/ISPT/CBPV2, and TUR/ANT/CBPV3 and the Turkish strains from obtained previous studies formed a different cluster. The sequence homology results of a phylogenetic analysis showed that the Turkish strains shared 97-98.9% of their nucleotide identity and had 96.9-99% similarity rates with each other. The strains obtained in this study and the Turkish strains detected in previous studies were also 81-84.6% similar to European, Chinese, and Uruguay strains. This research underlines the presence of CBPV in apparently healthy Turkish bee colonies and the remarkable differences in Turkish CBPV strains. Further investigation is needed to identify the molecular characterization, complete genome sequence, and pathogenesis of Turkish CBPV strains.

***Keywords*:** honey bee, chronic bee paralysis virus, RT-PCR, phylogenic analysis, Turkey

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INTRODUCTION

Honey bees, which contribute greatly to agriculture and economics, are one of the most important pollinators in the world (Morse and Colderone, 2000; Chauzat et al., 2003; Celli and Maccagni, 2003). However, the number of honey bee colonies has decreased significantly in recent years because of a large number of biotic and abiotic stressors (vanEngelsdorp et al., 2011; Cornman et al., 2012). Bacteria, parasites, and viruses are some of the main threats to honey bee health, among which viruses have caused significant colony losses (Brutscher et al., 2016; Gisder et al., 2016; Tehel et al., 2016).

CBPV is one of the most common viruses affecting honey bee colonies. First detected by Bailey et al. (1963), CBPV is an infectious and contagious disease that causes a high mortality ratein adult honey bees. CBPV is transmitted by two main routes: through physical contact between adult healthy bees and infected individuals (Bailey et al., 1983) and oral contact between healthy bees and infectious particles in the feces of paralyzed bees (Ribiere et al., 2007). In particular, this virus causes individual black, hairless bees to stand at the hive entrance and leads to clusters of trembling, flightless, crawling bees (Chen and Siede, 2007; Dittes et al., 2020). Bees with symptoms from CBPV die within 6 days, and inapparent virus infections can persist in colonies (Youssef et al., 2015).

The symmetry, size, and genomic structure of CBPV are completely different from other bee viruses. For example, CBPV has anisometric particles that measure 20 nm in width and 30-60 nm in length (Bailey et al., 1968). In addition, it is an unclassified polymorphic, segmented, non-enveloped, positive single-stranded RNA virus, with a viral particle composed of two major RNAs (RNA1 [3674 nt] and RNA2 [2305 nt]) and three minor RNAs (RNA 3a, 3b, and 3c). It also has seven overlapping open reading frames (ORFs), with three on RNA1 and four on RNA2. The RNA-dependent RNA polymerase is encoded by RNA1-ORF3 (Olivier et al., 2008). The structural proteins hSP and pSP are thought to be encoded by RNA2-ORF2 and RNA2-ORF3, respectively (Chevin et al., 2015). To date, CBPV has not been classified by the International Committee on Taxonomy of Viruses (ICTV) (http://www.ictvonline.org). It was stated by Oliver et al (2008) that only ORF3 on RNA1 showed similarities with the Nodaviridae and Tombusviridae families. However, Lake Sinai Virus, which is a novel honey bee virus, and CBPV are thought to belong to a new viral family (Schuster et al., 2014).

CBPV is commonly detected in bee colonies worldwide, such as France (Tentcheva et al., 2004), Uruguay (Antunez et al., 2005), Australia (Berenyi et al., 2006), Hungary (Forgach et al., 2008), Denmark (Nielsen et al., 2008), China (Wu et al., 2015; Li et al., 2017), Iran (Ghorani et al., 2017) and Turkey (Cagirgan et al., 2020; Kalayci et al., 2020). In addition to agarose gel immune-diffusion assay (AGID) (Ball, 1999), RT-PCR (Ribiere et al., 2002, Blanchard et al., 2007a), multiplex RT-PCR (Sguazza et al., 2013; Cagirgan and Yazici, 2020), and real-time RT-PCR (Blachard et al., 2007b) are used for the diagnosis of CBPV. However, it is possible to detect CBPV by RT-PCR test, even in the case of inapparent infections (Tentcheva et al., 2004; Blanchard et al., 2007a; Cagirgan et al., 2018).

This study aims to investigate the presence of CBPV in asymptomatic adult honey bees in the southern cities of Burdur, Isparta, and Antalya in Turkey and construct its phylogenetic tree based on the RNA-dependent RNA polymerase gene region.

MATERIAL AND METHODS

Ethics Statement

No ethics committee approval document was required for this study. The study involved the *Apis mellifera* which is neither a protected nor endangered species.

Samples and Preparation

Samples of alive asymptomatic adult bees were randomly collected from 45 apiaries in three provinces (Antalya [n=17], Isparta [n=8], and Burdur [n=20]) of Turkey (Figure 1). The samples were collected between March and November 2019. The samples were taken from three hives from each apiary. A pool consisting of 30 adult bees was created for each apiary. These pools were homogenized with 9 ml of Eagle's Minimum Essential Medium (Sigma Aldrich, UK). Then, the homogenates were centrifuged at 5000 rpm at 4ºC for 30 minutes.

Following this step, 200 µl of the supernatant were taken for RNA extraction after centrifuging the homogenates. This step was carried out using the High Pure Viral RNA Kit (Roche, Germany) according to the manufacturer's instructions. The extracted RNA was stored at -20°C until testing.

Figure 1. The geographical location of the three proviences of Turkey where honeybee samples were collected.

RT-PCR

An RT-PCR for the partial genes was conducted using the oligonucleotide sense and antisense primers (5'-TCAGACACCGAATCTGATTATTG-3' 1921-1933 and 5'-CCGGAGACAAAGGTCATCAT-3' 3445-3426) targeting a 1525-bp fragment of the RNA-dependent RNA polymerase gene of the CBPV, as described by Blanchard et al. (2009). For the amplification, an Xpert One-Step RT-PCR Kit (Grisp Research Solutions, Porto, Portugal) was used. The total reaction volume was 25 µl, and the final concentration of the primer was 0.4 mM.

The thermocycler was programmed with the following steps and cycling times. First, one cycle at 45ºC for 15 minutes was performed for the reverse transcription, followed by the initial denaturation at 95ºC for 3 minutes. This step was followed by another denaturation at 95ºC for 10 seconds, annealing at 55ºC for 10 seconds. Finally, an extension at 72ºC for 15 seconds was sustained for 35 cycles, with a final extension at 72ºC for 1 minute. The final PCR products were analyzed in a 1.5% agarose gel electrophoresis (containing ethidium bromide) and visualized under a UV light source.

The PCR products were sequenced in the Forward and Reverse directions by a Microsynth AG (Balgach, Switzerland). A phylogenetic analysis of the partial RNA-dependent RNA polymerase gene region targeting a 1290-bp region was performed using 32 CBPV sequences from Turkey and other geographical regions of the world. These additional sequences were taken from National Center for Biotechnology Information (NCBI). The nucleotide sequence results were analyzed and assembled, and the obtained sequenc-

es were aligned using DNADynamo DNA Sequence Analysis Software. To interpret the results of the phylogenetic analysis, an SDT a stand-alone program was used (Muhire et al., 2014). A graphical port demonstrated pairwise identity scores using a color-coded pairwise-identity matrix and enabled the visualization of the proximity among sequences in a dataset. Phylogenetic analyses were constructed the MEGA X (Kumar et al., 2018) with Maximum likelihood trees based on the Tamura-Nei parameter (TN93+G) model with 1,000 bootstrap replicates for the determination of genetic distances between nucleotide sequences. GenBank accession numbers were taken for three different strains obtained in this study.

RESULTS AND DISCUSSION

The results revealed that 5 of the 45 samples were CBPV positive. The basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI) was used for searching the obtained nucleotide sequences. The TUR/BUR/CBPV1, TUR/ISPT/CBPV2, and TUR/ANT/CBPV3 strains detected in this study and the Turkish strains detected in previous studies formed a separate branch in phylogenetic tree (Figure 2). Based on the non-structural RNA-dependent RNA polymerase gene region, the tree was divided into five main groups. According to phylogenetic analysis, the sequence homology results showed that the Turkish strains shared 97-98.9% of the nucleotide identity and 96.9-99% similarity rates with each other (Figure 3). All of the strains obtained in this study and the Turkish strains detected in previous studies showed 81-84.6% similarities with European, Chinese, and Uruguayan strains.

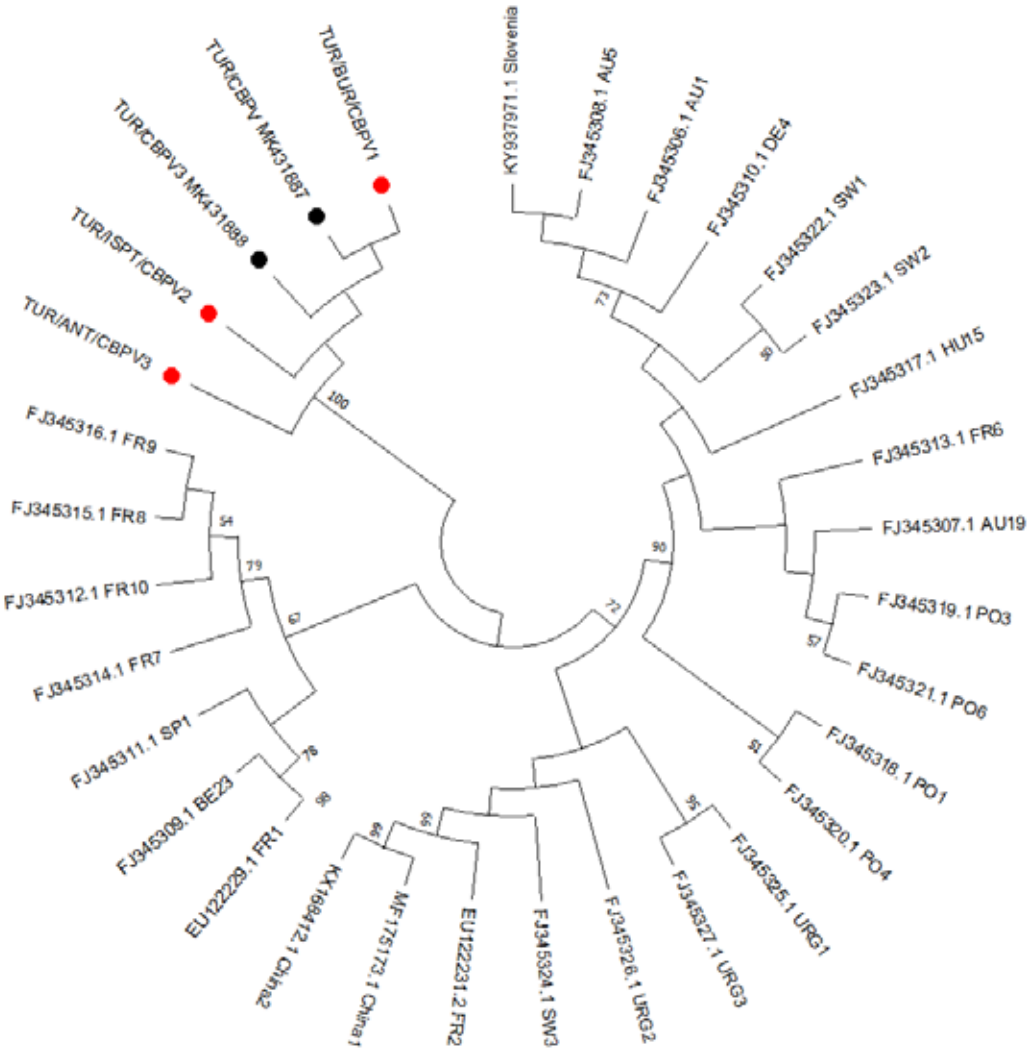

Figure 2. The phylogenetic tree constructedusing Maximum Likelihood (ML) method with Tamura-Nei parameters, included in the MEGA-X software, on the aligment of the 1290 bp of RNA-dependent RNA polymerase sequences of 32 CBPV strains.

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
	Identity Rate																															
TUR/CBPV_MK431887		97,4	97,9	97,4	97	86,7	85,4	87	85	85,8	87	86,9	87,2	87,2	87,2	86,2	86,8	86	87	87	86	87,3	86,8	87	87	87	87	87	88	87	87
TUR/CBPV3_MK431888	97,3		97,5	98,7	98,1	86,7	85,4	86,7	84,8	85,4	87,0	87,3	87,2	87,2	87,0	86,9	86,5	86,8	86,8	86,5	86,2	87,2	86,9	87,2	86,9	86,5	87,2	87,0	86,6	87,1	86,8	86,7
TUR/BUR/CBPV1	97,9	97,5		97,9	97,5	87,2	86,0	87,8	85,5	86,2	87,2	87,9	88,1	88,1	87,9	86,5	87,5	86,5	87,2	86,9	86,7	87,6	87,2	87,7	87,2	87,0	87,3	87,2	87,1	87,5	87,8	87,8
TUR/ISPT/CBPV2	97,3	98,7	97,8		98,9	87,0	85,8	87,1	85,1	85,8	86,6	87,5	87,5	87,6	87,4	87,0	87,1	86,8	87,2	86,8	86,4	87,5	87,2	87,5	87,2	86,8	87,5	87,1	86,9	87,2	87,2	87,1
TUR/ANT/CBPV3	96,9	98,1	97,5	99,0		86,8	85,8	87,2	85,1	85,8	86,8	87,7	87,7	87,7	87,5	86,9	87,2	86,7	87,1	86,7	86,4	87,4	87,2	87,4	87,2	86,7	87,4	87,0	86,8	87,2	87,4	87,2
KY937971.1_SLO	83,4	83,4	84,3	84,0	83,7		92,8	92,3	91,9	92,6	97,2	91,7	92,1	92,3	92,3	93,7	94,5	93,7	97,9	97,9	93,5	98,5	98,2	97,2	97,9	97,1	97,2	97,0	97,4	98,1	92,3	92,3
MF175173.1_CN1	81,0	81,0	82,2	81,8	81,7	91,8		91,3	97,8	96,1	92,8	91,2	91,5	91,5	91,8	93,1	93,6	92,1	93,2	92,5	93,7	93,1	92,8	93,1	93,2	93,1	92,8	93,2	92,4	92,6	91,4	91,5
EU122229.1_FR1	83,9	83,3	83,2	84,0	84,2	91,1	89,8		91,4	91,6	92,5	96,7	97,1	96,9	96,7	91,4	92,8	91,4	92,5	92,4	92,1	92,2	92,1	92,3	92,3	92,5	92,3	92,6	92,1	92,7	99,1	100,0
KX168412.1_CN2	81,1	80,0	81,5	80,7	80,7	90,5	97,7	89,8		95,8	92,0	90,6	90,9	90,7	90,9	91,8	93,4	91,1	92,3	92,0	93,5	92,1	92,0	92,6	92,4	92,4	92,1	92,7	91,8	92,1	91,3	91,3
EU122231.2_FR2	82,0	81,1	82,7	81,9	81,8	91,5	95,8	90,1	95,5		92,1	91,6	91,7	91,9	92,1	92,7	93,7	92,2	93,1	93,1	93,2	92,7	92,7	92,5	93,4	92,9	92,7	92,8	92,4	92,5	91,8	91,6
FJ345313.1_FR6	83,2	83,9	84,3	83,8	83,6	97,1	91,8	91,2	90,6	91,6		92,1	92,3	92,4	92,6	93,8	93,7	93,9	97,5	97,5	93,2	97,4	96,9	97,5	97,5	97,2	97,6	97,4	97,5	97,2	92,3	92,4
FJ345314.1_FR7	83,7	84,3	85,2	84,6	84,9	90,4	89,6	96,4	88,8	90,2	90,9		98,4	98,0	97,8	91,2	91,8	91,6	91,7	91,9	92,1	92,1	91,6	92,1	91,8	92,4	92,6	91,9	91,7	92,0	96,8	96,6
FJ345315.1_FR8	84,2	84,1	85,6	84,8	85,0	90,9	90,1	96,9	89,2	90,4	91,1	98,4		99,2	98,9	91,6	92,3	91,9	92,1	92,2	92,0	92,3	91,8	92,3	92,0	92,2	93,0	91,9	92,0	92,0	97,1	97,1
FJ345316.1_FR9	84,2	84,2	85,7	84,9	85,0	91,2	89,9	96,7	88,9	90,6	91,3	97,9	99,2		98,8	92,1	92,3	92,4	92,2	92,4	92,1	92,0	91,9	92,5	92,3	92,5	92,9	92,1	92,2	92,2	97,1	96,9
FJ345312.1_FR10	84,1	83,8	85,4	84,5	84,7	91,1	90,5	96,5	89,1	90,9	91,5	97,7	98,9	98,7		92,1	92,3	92,4	92,4	92,4	92,2	92,3	91,9	92,2	92,3	92,6	92,9	92,2	92,2	92,2	97,1	96,7
FJ345326.1_URG1	82,4	83,7	82,9	83,9	83,7	93,0	92,0	89,5	90,1	91,5	93,1	89,4	90,0	90,7	90,6		95,9	98,4	93,5	94,0	93,6	93,7	93,8	93,5	93,6	93,4	94,0	93,8	93,2	93,5	91,6	91,4
FJ345326.1_URG2	83,8	83,2	84,7	84,1	84,3	94,0	92,7	91,7	92,4	93,0	93,0	90,3	91,1	91,1	91,1	92,9		93,6	94,5	94,7	94,4	94,4	94,1	94,0	94,7	94,0	93,8	94,4	93,7	94,4	92,5	92,7
FJ345327.1_URG3	82,4	83,5	82,9	83,5	83,3	92,9	90,7	89,5	89,1	90,8	93,2	89,8	90,4	91,1	91,0	98,4	92,6		93,8	94,1	93,1	93,7	94,0	93,2	93,7	93,4	94,1	94,0	93,2	93,5	91,6	91,3
FJ345322.1_SW1	83,4	83,6	84,2	84,2	84,0	97,9	92,3	91,3	91,0	92,2	97,5	90,3	90,8	91,1	91,2	92,5	93,9	93,1		98,2	93,4	97,9	97,9	97,3	98,0	97,0	97,6	97,2	97,6	97,9	92,3	92,4
FJ345323.1_SW2	82,9	82,9	83,7	83,5	83,3	97,8	91,3	91,1	90,5	92,1	97,4	90,5	91,0	91,2	91,2	93,2	94,1	93,5	98,2		93,3	97,6	97,5	97,6	98,0	97,5	97,3	97,3	97,5	97,6	92,2	92,4
FJ345324.1_SW3	83,1	82,6	83,5	83,0	83,0	92,8	92,9	90,7	92,7	92,3	92,4	90,8	90,7	90,8	90,9	92,8	93,8	92,1	92,7	92,5		93,4	93,3	93,7	93,8	93,8	93,3	93,8	93,0	93,4	92,2	92,0
FJ345306.1_AU1	84,4	84,1	84,9	84,7	84,5	98,5	92,1	90,9	90,8	91,7	97,3	90,8	91,2	90,8	91,2	92,8	93,9	93,0	97,9	97,6	92,7		98,2	97,5	97,9	97,0	97,5	97,2	97,5	98,2	92,1	92,1
FJ345308.1_AU5	83,5	83,7	84,3	84,3	84,1	98,2	91,7	90,8	90,5	91,5	96,8	90,1	90,4	90,6	90,6	92,9	93,5	93,3	97,9	97,4	92,4	98,2		97,2	97,7	96,8	97,3	96,8	97,2	97,9	92,0	92,0
FJ345307.1_AU19	84,3	84,1	85,0	84,7	84,5	97,1	92,2	91,1	91,4	91,3	97,4	90,8	91,2	91,4	90,9	92,3	93,3	92,2	97,2	97,5	93,1	97,4	97,0		97,7	97,3	97,6	96,6	97,5	97,2	92,1	92,3
FJ345317.1_HU15	83,8	83,7	84,3	84,3	84,1	97,8	92,3	91,1	91,1	92,5	97,5	90,4	90,7	91,2	91,1	92,7	94,2	92,8	98,0	98,0	93,2	97,8	97,6	97,6		97,7	97,4	97,5	97,7	97,5	92,1	92,3
FJ345318.1_PO1	83,5	83,0	83,9	83,6	83,3	97,0	92,0	91,3	91,1	91,9	97,1	91,2	90,9	91,4	91,6	92,4	93,3	92,5	96,9	97,5	93,1	96,9	96,7	97,2	97,6		97,3	97,7	97,5	97,2	92,4	92,4
FJ345319.1_PO3	83,3	84,1	84,4	84,7	84,5	97,1	91,7	91,1	90,8	91,5	97,6	91,5	92,1	91,9	91,9	93,2	93,1	93,3	97,6	97,2	92,5	97,4	97,2	97,5	97,3	97,2		96,9	97,9	97,2	92,1	92,3
FJ345320.1_PO4	84,3	83,8	84,1	84,0	83,9	96,9	92,2	91,4	91,7	91,7	97,3	90,5	90,6	90,8	91,0	92,9	93,9	93,2	97,1	97,2	93,2	97,1	96,6	96,7	97,3	97,6	96,8		97,4	97,5	92,2	92,5
FJ345321.1_PO6	83,2	83,2	84,1	83,8	83,6	97,3	91,2	90,8	90,4	91,3	97,4	90,3	90,8	91,0	91,0	92,2	93,0	92,3	97,6	97,4	92,1	97,4	97,0	97,4	97,6	97,4	97,9	97,3		97,6	91,9	92,0
FJ345310.1_DE4	84,6	83,9	84,8	84,3	84,1	98,1	91,4	91,7	90,7	91,4	97,1	90,9	90,8	91,0	91,0	92,5	93,8	92,6	97,9	97,6	92,7	98,2	97,9	97,0	97,4	97,0	97,0	97,5	97,6		92,5	92,7
FJ345311.1_SP1	85,9	85,5	85,2	84,2	84,4	91,1	89,9	99,1	89,7	90,5	91,1	96,6	96,9	96,9	96,9	89,9	91,4	89,9	91,1	91,0	91,0	90,9	90,8	90,9	90,9	91,3	90,9	91,0	90,6	91,5		99,0
FJ345309.1_BE23	83,9	83,3	85,2	84,0	84,2	91,1	89,8	100,0	89,8	90,1	91,2	96,4	96,9	96,7	96,5	89,5	91,7	89,5	91,3	91,1	90,7	90,9	90,8	91,1	91,1	91,3	91,1	91,4	90,8	91,7	99,1	

Similarity Rate

Figure 3. Detailed nucleic acid identity and similarity rates for 32 CBPV strains

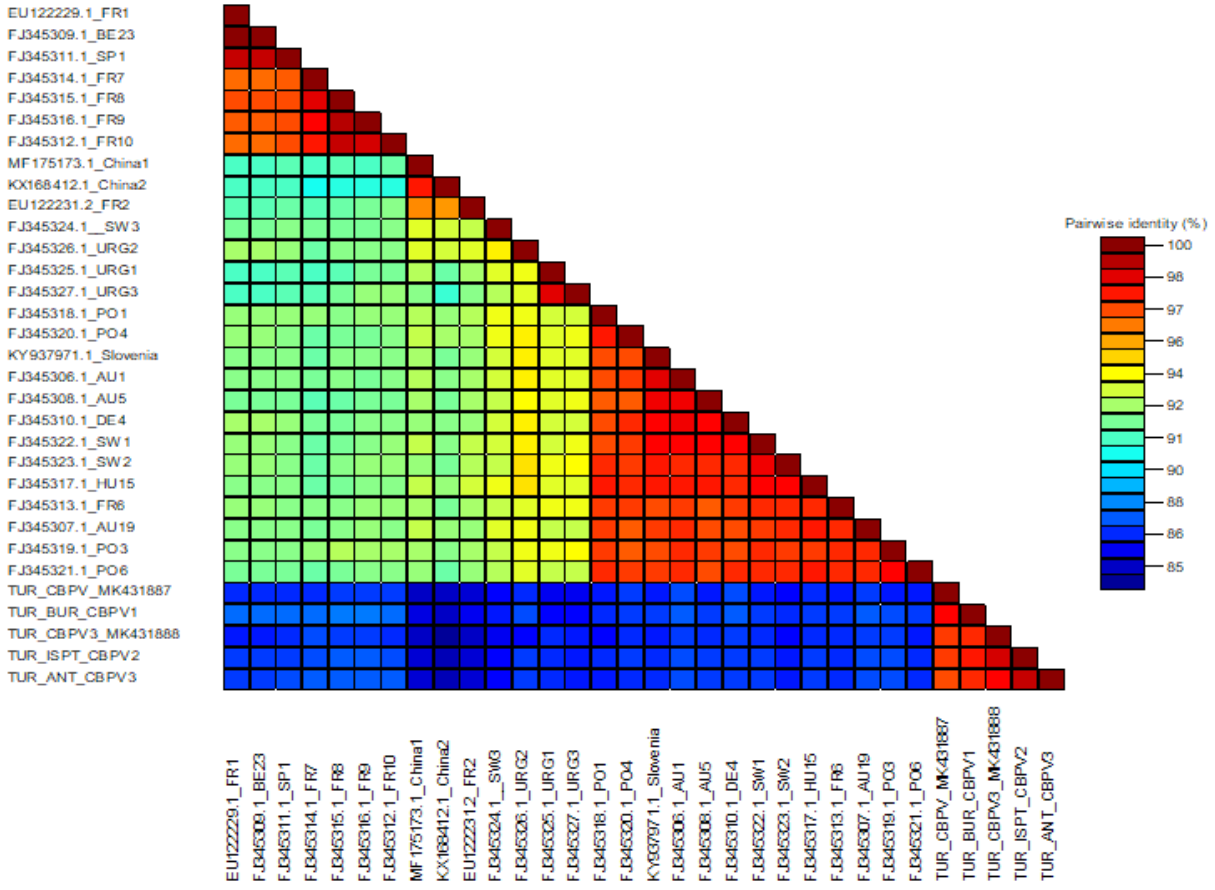

Figure 4. SDT color-coded matrix of nucleotide pairwise identity scores performed by alignment of a 1290 bp fragment RNA-dependent RNA polymerase gene for 32CBPV strains. Each colored square symbolizes a percentage identity score between two sequences

A total of 32 CBPV strains were analyzed using the Sequence Demarcation Tool (SDT) program. The nucleotide pairwise identity changed between the interval of 100% to 85%. The identity showed in different colors: from maroon to dark red, to red, orange-red, orange, yellow, yellow-green, aquamarine, turquoise, and blue to navy blue. The CBPV strains reported in this study largely showed as blue (~86-88%) and dark blue (~85%) (Figure 4). According to the SDT graphic, the Turkish CBPV strains exhibited high differences compared to European, Uruguayan, and Chinese strains.

Honeybees play an important role in pollination and, as such, offer both ecological and economic contributions. Turkey, with its 7.9 million honeybee colonies (FAOSTAT, 2018), is one of the most important honey producers in the world. One of the major challenges that Turkish beekeepers face is viruses. CBPV is common in Turkish bee colonies and the cause of many colony losses (Kalayci et al., 2020).

In France, CBPV was sporadically detected in adult bees sampled from 360 healthy colonies using the RT-PCR diagnosis method. CBPV was found in 28% of 36 apiaries (Tentcheva et al. 2004). Previous studies in Europe show that the prevalence of this virus was low in Austria, Denmark, and Croatia, respectively (Berenyi ve ark., 2006; Nielsen ve ark., 2008; Gajger ve ark., 2014). Ghorani et al. (2015) reported a finding of CBPV in 3 out of 89 (3.3%) apiaries in Iran.

The first molecular detection of CBPV in Turkey was reported by Gumusove et al. (2010), who detected the virus in 7 of 28 (25%) samples from the Black Sea region in Turkey. CBPV was previously reported in 1.8% of 111 apparently healthy colonies in seven provinces of the Aegean Region (Cagirgan, 2018). In addition, CBPV was identified in 14 of 76 (18.4%) apiaries complaining of colony losses in Turkey (Kalayci et al., 2020). In this study, CBPV was detected in 5 of 45 (11.1%) apiaries.

The nucleotide similarity rates of the three CBPV strains obtained from the Burdur, Isparta, and Antalya provinces were 96.9-99%. Their similarities with the strains obtained from different countries are shown in Figure 3, which details that the strains obtained from different countries and the Turkish strains ranged in similarity from 81-84.6%. This range can be explained by the fact that CBPV is a segmented and variable virus. In addition, the genomic variability of honeybee viruses, such as the Sacbrood virus in Turkey has been previously described (Kalayci et al., 2019; Yildirim et al., 2020). The genetic variability of CBPV was also reported in a previous study by Cagirgan (2018).

The phylogenetic tree based on the RNA-dependent RNA polymerase gene region was first constructed by Blanchard et al. (2009). In the phylogenetic evaluation, CBPV strains formed four different groups: A, B, C, and D. Strains obtained from the same countries took place in different branches on the phylogenetic tree. Strains of the same country were included in different groups. Turkish strains formed the E group in the study conducted by Cagirgan (2018). In that study, the Turkish strains were separated into a different branch. This difference suggests that Turkish CBPV strains generate a new group in addition to the groups formed by Blachard et al. (2009). In a study based on the RNA-dependent RNA polymerase gene region, the strains from Iran formed Group 1 and Group 2 (Ghorani et al., 2015). While CBPV reveals genetic differences even in the same countries, the Turkish strains were not separated into different groups.

In conclusion, this study underlines the presence of CBPV in apparently healthy colonies in Turkey and remarkable differences between Turkish CBPV strains, which divide into different branches of the phylogenetic tree, from the CBPV strains obtained in other countries. Further investigation of CBPV strains is necessary to reveal their geographical distribution and genetic variability, to evaluate the global distribution of this virus, and to investigate the role of CBPV in disease outbreaks in Turkey.

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Research article
Ερευνητικό άρθρο

Detection of antibodies to *Toxoplasma gondii* in neotropical primates from São Paulo state, Brazil

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ABSTRACT: Toxoplasmosis is a life-threatening disease in neotropical primates. The aim of the present study was to determine the frequency of antibodies to *Toxoplasma gondii* in neotropical primates from São Paulo state (SP), Brazil. The modified agglutination test (MAT, cut-off: 25) was used in 49 neotropical primates upon or after their admission to Associação Mata Ciliar (Jundiaí, SP, Brazil). Eight of the 49 animals (16.3%) were seropositive. The genus *Sapajus* had the highest antibody titer (12,800), followed by the genus *Callithrix* (3,200). No association ($p > 0.05$) was found between seroprevalence and genera (*Alouatta*, *Callicebus*, *Callithrix*, and *Sapajus*), sex or age. The three positive primates of the genera *Allouata*and the one of the genera C*allithrix* died, whereas the two seropositive *Sapajus* were alive. Further studies on the epidemiology of *T. gondii* infection are necessary in a larger sample size of captive and wild neotropical primates.

Keywords: Brazil; MAT; neotropical primates;*Toxoplasma gondii*

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INTRODUCTION

Toxoplasmosis is a zoonotic disease with worldwide distribution affecting homeothermic animals, including humans. Its aetiological agent, *Toxoplasma gondii*, is an obligatory intracellular protozoan parasite, having wild and domestic felids as definitive hosts. Intermediated hosts are mainly infected by ingestion of food or water contaminated with oocysts or by consumption of infective tissue cysts in meat or viscera (Tenter et al., 2000; Dubey, 2010). In some areas of Brazil *T. gondii* is estimated to have one of the highest human seroprevalences (Sepúlveda-Arias et al., 2014).

In New World primates, toxoplasmosis is a critical and life-threatening disease (Salant et al., 2009), and according to Catão-Dias et al. (2013) it is the most prevalent cause of death from acute infectious disease in captive neotropical primates. Wild animals may remain in rescue and recovery centers for long periods or indefinitely and are subject to stressful situations which can lead to a weakened immune system, thus becoming more susceptible to opportunistic infections and diseases including fatal toxoplasmosis (Gyimesi et al., 2006; Bouer et al., 2010). The aim of the present study was to assess seroprevalence of *T. gondii* in sera of neotropical primates at or after admission to Associação Mata Ciliar (AMC), a Rescue Center located in São Paulo state (SP), Brazil.

MATERIALS AND METHODS

Geographical area of the study and samples

This study was developed at AMC, in the city of Jundiaí, SP, Brazil. AMC was established in 1987 with the purpose of welfare and conservation of animal species as well as their habitat. Between April 2015 and December 2017, blood samples were collected from 49 neotropical primates from SP upon (n = 29 free-living) or from 1 week after (n = 20 captive) admission to AMC. Information on genus (taxonomy), sex (female or male) and age group (juvenile or adult) were also obtained. After centrifugation at 10,000 rpm for 3 minutes, serum samples were stored at -20 °C until analysis.

All samples used were surplus of clinical sera. All procedures within the scope of this study followed the ethical standards of the relevant national and institutional guidelines.

Serological testing for antibodies to *T. gondii*

Serum samples were assayed for *T. gondii* IgG antibodies by the modified agglutination test (MAT, cut-off: 25), as described by Dubey& Desmonts (1987). Samples were tested in serial dilutions on the basis two, starting at 1:25 (Valentini et al., 2004), and positive and negative control samples were included in each testing plate.

Data analysis

Exact binomial 95% confidence intervals (CI) were established for prevalence values. The Fisher's exact test was used to compare seroprevalence values between categories of the independent variables genus, sex and age. Pairwise comparisons between categories of the same independent variable incorporated Bonferroni's correction (Petrie and Watson, 2013). A probability (p) value < 0.05 was considered as statistically significant. Analyses were performed with the SPSS 26.0 program for Windows.

RESULTS

Eight (16.3%; CI: 7.3–29.7) out of 49 neotropical primates were seropositive to *T. gondii*, 5 (10.2%) had a titer of 25 (2 *Alouatta guariba*, 2 *Callicebus personatus* and 1 *Sapajusapella*); 1 (2.0%) a titer of 50(*A. guariba*); 1 (2.0%) a titer of 3,200 (*Callithrix penicillate*); and 1 (2.0%) a titer of 12,800 (*S.apella*).

Of the seropositive animals 62.5% (5/8) entered AMC due to urban rescue, 25% (2/8) were run over by vehicles and 12.5% (1/8) were free-living orphans.

Five of the 8 seropositive animals (62.5%) presented physical alterations. Sixty percent (3/5) of these animals had prostration or apathy, with one of them also presenting foamy nasal discharge, dyspnea and bloating, and another one having jaundice. However, 40% (2/5) of these animals did not show clinical signs compatible with toxoplasmosis, as one animal had a spine fracture and the other one a mandible fracture. No association was found between seroprevalence and all the variables analyzed (Table 1).

DISCUSSION

The frequency of primates positive to antibodies to *T. gondii* at or from 1 week after the admission in the AMC was 16.3% (IC 7.3-29.7).In neotropical primates, toxoplasmosis poses a conservation concern, since it may be the cause of acute disease and high mortality (Casagrande et al., 2013; Pardini et al., 2015; Nishimura et al., 2019). Clinical manifestations include general malaise, dyspnea, hypothermia, foamy or serosanguinolent nasal secretion, and abdominal distension (Epiphanio et al., 2003; Casagrande et al., 2013).

Table 1. *Toxoplasma gondii* infection in neotropical primates admitted to Associação Mata Ciliar, Brazil, according to genus, gender and age

Variable/categories	Nº of primates tested (%)	Number of seropositive (%)	CI (%)
Genus/species			
Alouatta guariba	31 (63.3)	3 (9.7)	2.0-25.7
Callicebus personatus	4 (8.2)	2 (50.0)	6.8-93.2
Callithrix spp.	12 (24.5)	1 (8.3)	0.2-38.5
Sapajus apella	2 (4.1)	2 (100)	15.8-64.3
		$p \geq 0.114^{*}$	
Sex			
Female	18 (36.7)	2 (11.1)	1.4-34.7
Male	27 (55.1)	5 (18.5)	6.3-38.1
ND	4 (8.2)	1 (25.0)	0.6-80.6
		$p = 0.684^{\dagger}$	
Age			
Juvenile	11 (22.5)	1 (9.1)	0.2-41.3
Adult	36 (73.5)	7 (19.4)	8.2-36.0
ND	2 (4.1)	0 (0.0)	0.0-84.2
		$p = 0.659^{\phi}$	
Total	49 (100)	8 (16.3)	7.3-29.7

*pairwise comparisons incorporating Bonferroni's correction (i.e. multiplying each *p* value by the number of comparisons; ND category not included); †calculated only between female and male; ϕcalculated only between juvenile and adult; CI: 95% confidence interval; ND: not determined.

Information on *T. gondii* infection in neotropical primates in Brazil is limited, with most reports from captive animals (Epiphanio et al., 2003; Antoniassi et al., 2011; Minervino et al., 2017). In addition to high susceptibity of some species of primates to clinical toxoplasmosis, they can act as sentinels to *T. gondii* infection (Leite et al., 2008).

In all genera of primates surveyed here, at least one animal was seropositive. Although seroprevalence was higher in genus *Sapajus*, no statistically significant difference was detected. According to Garcia et al. (2005) and regarding wild animals, the genus *Sapajus* (formerly *Cebus*) presented higher seroprevalence than the genus *Alouatta*. This higher value may be justified by the more terrestrial behavior of those animals, which increases the probability of contact with *T. gondii* sporulated oocysts. A lower value (9.7%) in the genus *Alouatta* is probably due to their arboreal way of life and to the essentially folivorous-frugivorous food.

Also by using the MAT, but in captive animals, Minervino et al. (2017) found a higher occurrence (52.2%) in New World non-human primates from the Amazon region. On the other hand, no antibodies to *T. gondii* were found in recently captured neotropical primates from Niterói, Rio de Janeiro state, Brazil studied by Molina et al. (2017). Hence, the scarcity of information on the prevalence of *T. gondii* in free-living primates and the use of different methods to diagnose specfic antibodies, makes it difficult to compare the results of the present study with other published studies worldwide (reviewed by Dubey, 2010).

In the genus *Callithrix* a seronegativity of 91.7% suggests that these animals had no contact with *T. gondii* or died of acute toxoplasmosis, with no time to produce IgG antibodies (Catão-Dias et al., 2013). The causes of mortality of the animals that arrived dead or that died at AMC have not been confirmed and therefore we cannot infer about the real pathogenic role of *T. gondii*.

Primates can be sentinels for human infection, as they have access to human sites and eat the same food potentially contaminated with soil oocysts. In addition, in some geographical areas, primates are hunted and used as food themselves, which represents another mode of transmission (da Silva et al., 2014).

CONCLUSIONS

Considering the life cycle of *T. gondii* and the high susceptibility of neotropical primates, it is relevant to emphasize the need for strict sanitary measures to protect captive animals that may be under the threat of extinction. Fighting for the conservation of these primates is imperative, since they are subject to sev-

eral diseases that are potentially fatal to them and can drastically reduce their populations(Santos et al., 2014).

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Research article
Ερευνητικό άρθρο

Effect of hempseed meal on performance, egg quality and egg yolk fatty acids content in laying quails

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ABSTRACT: In this study, the laying performance, external egg quality, egg yolk colour and fatty acids profile of quails (*Coturnix coturnix japonica*) fed on diets containing hemp seed meal (HSM) were determined. During 8 weeks trial, a total of 150, 10-weeks-old laying quails were used. Five diets were formulated to contain HSM at the level of 0 (control),5, 10, 15, and 20% that represented as 0 HSM, 5 HSM, 10 HSM, 15 HSM and 20 HSM, respectively.The performance parameters were not significantly (P>0.05)influenced by the dietary HSM contents. Eggshell ratio, eggshell breaking strength, egg shape index, egg yolk index and egg yolk colour values were not significantly (P>0.05) influenced by the dietary HSM, whereas eggshell thickness was significantly (P<0.05) affected. The albumen index has been significantly (P<0.05) increased by increasing in the HSM level in the diets. The HSM supplementation to the diets was effective on fatty acid composition and total saturated fatty acids, total mono unsaturated fatty acids and total polyunsaturated fatty acids content of the egg yolk depending on the addition level. In conclusion, HSM can be used to increase egg total monounsaturated fatty acids and especially omega-3 fatty acids without unfavourable effects on the performance and egg quality parameters.

***Keywords*:** Quail; performance; egg yolk colour; eggshell quality; omega-3

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INTRODUCTION

Hemp (*Cannabis sativa L.*) is an important food and fiber source, and also used in drug production due to psycohotropic compound tetrahydrocannabinol content(Cromack 1998).Hemp seed is a rich source of some nutrients such as protein (25%), fat (34%), carbohydrates (34%), as well as high levels of some vitamins and minerals content. It also contains an amount of phenolic compound and tocopherol that are beneficial for human health (Oomah et al. 2002; Kriese et al. 2004; Chen et al. 2012).Phytic acid, tannins, and trypsin inhibitors are called anti-nutritional factors and found in cannabis seed in small amounts(Russo and Reggiani 2015). Hemp seed meal is a product obtained by pressing the seeds and removing the oil from them. The product contains 30-50% protein in dry matter depending on the hemp variety used and the oil extraction methods(Malomo et al. 2014; Wang and Xiong 2019).Protein and amino acid content of HSM has high digestibility (Callaway et al. 2005; Yu et al. 2005; Hu et al. 2008; Girgih et al. 2011) The major fatty acids of hempseed oil are linoleic (60%) and alpha-linolenic (19%) acids and its total polyunsaturated fatty acids ratio is around 75 - 80% (Callaway 2004; House et al. 2010). The cultivation of hempseed has been limited by the regulations in many countries including Turkey for a long year(Anonymous 1990). Hempseed and its by-products have not subjected to the numerous researches of animal nutrition due to the restricted production. However, in some countries (Canada) with the releasing of cannabis agriculture, hempseed by-products have become able to use as animal feed(Callaway 2004; Karche 2019). The nutrient values of hemp seeds and its products are valuable enough to be a source of feed in poultry(Wang and Xiong 2019). Since poultry especially need high quality protein sources in the diet, HSM is a feed raw material that needs to be emphasized to meet their needs. In addition, it is reported that its contents of oil is quite rich in terms of unsaturated fatty acids, and it will be effective in changing the egg fatty acid content in laying hens(Gakhar et al. 2012; Konca et al. 2014).Research has shown that HSM in laying hens diet increases the amount of omega-3fatty acids found in eggs(Silversides and Lefrancois 2005; Neijat et al. 2014). Japanese quail (Coturnix coturnix japonica) is used as a model animal and is one of the smallest birds used for its egg and meat production (Shokoohmand 2008; Narinc et al. 2013). Quail provides more advantages than the chicken such as the low maintenance cost associated with its small body size (80-300g) coupled with its short generation interval, resistance to diseases and high egg production (Mills et al. 1997). Lately, quail meat has gained much popularity among consumers (Ikhlas et al. 2011). It is an ideal food for all ages due to its high meat yield, less shrinkage during cooking, being more effortless to cook, and being more easy to serve (Mountney 2012). For this purpose, it will be important to increase the studies examining the use of hemp meal in poultry. In this study, the effects of using different levels of HSM in laying quail diets on production performance, egg's internal and external quality and egg yolk fatty acids content were investigated.

MATERIAL AND METHODS

A total of 150, 10 - weeks - old laying quails (*Coturnix coturnix japonica*) were used in this study. The laying quails were divided into 5 treatment groups with 6 replicates (5 quails, each). The laying quails were reared in 33 × 40 ×28 cm size cages under the semi-controlled periphery terms (ventilation controlling system) with 16-h light - 8 h dark illumination period. Quails were provided with feed and water *ad-libitum* for 8 weeks experimental period. Quails were experimental diets were formulated to meet the nutrient requirements of National Research Council(Council 1994) (Table 1). Hemp seeds were provided from a local supplier. The seeds were pressed in a cold press machine (Karaerler Machine, NF 100 model, Ankara) at 45-50 °C and HSM was obtained by removing the oil. The HSM used in experimental diets contained 30.4% crude protein and 10.9% crude oil. Experimental diets were formulated as the control (basal diet) and the diets containing 5, 10, 15, and 20% HSM. All treatment diets were adjusted as to be isocaloric and isonitrogenic.

The measured parameters:

Quails were weighed at the first and at end the last day of the trial and the body weight changes were determined. Feed intake as determined on the 28th and 56th days of experiment. Feed conversion ratio was calculated with the ratio of feed intake (g) and egg mass (g). Egg production was recorded daily and calculated on the 28th and 56th days of experiment.

The average egg percentage (%) was detected with the formula of the (total eggs/ total quail) × 100. Egg mass was determined from the ratio of egg production (%) to egg weight (g).A total of 480 eggs (96 eggs

Table 1. The nutrients composition of experimental diets

Ingredients	Dietary hempseed meal (HSM) levels, %				
	0	5	10	15	20
Corn	50.10	47.70	44.85	42.35	39.90
Hempseed meal (30.4 %CP)	0	5.00	10.00	15.00	20.00
Soybean meal (45% CP)	36.00	33.10	30.30	27.40	24.50
Vegetable oil	6.20	6.50	7.00	7.40	7.75
Limestone	5.10	5.10	5.15	5.15	5.15
Dicalcium phosphate	1.80	1.80	1.85	1.85	1.85
Salt	0.25	0.25	0.25	0.25	0.25
Premix[1]	0.25	0.25	0.25	0.25	0.25
L-Lysine	0.10	0.10	0.15	0.15	0.15
DL-Methionine	0.20	0.20	0.20	0.20	0.20
Calculated nutrients%					
Crude protein	20.01	20.02	20.04	20.04	20.05
Energy, kcal ME/kg	2904	2900	2902	2903	2902
Calcium	2.50	2.50	2.52	2.52	2.52
Available phosphorus	0.35	0.35	0.36	0.36	0.36
Lysine	1.05	1.04	1.05	1.04	1.03
Methionine	0.48	0.48	0.49	0.50	0.50
Methionine + Cystine	0.84	0.85	0.85	0.86	0.86

[1]: Premix provided the following per kg of diet: retinyl acetate, 4.0 mg; cholecalciferol, 0.055 mg; DL- α-tocopheryl acetate, 11 mg; nicotinic acid, 44 mg; calcium-D-pantothenate, 8.8 mg; riboflavin sodium phosphate, 5.8 mg; thiamine hydrochloride, 2.8 mg; cyanocobalamin, 0.66 mg; folic acid, 1 mg; biotin, 0.11 mg; choline, 220 mg; Mn, 60 mg; Fe, 30 mg; Cu, 5 mg; I, 1.1 mg; Se, 0.1 mg

for each group) were collected in the last two days of each 14- days period. Egg samples selected randomly from each sub-group and weighed and then determinated to have eggshell breaking strength, eggshell thickness, eggshell weight, egg shape index, egg yolk index and albumen index. Egg yolk height and albumen height were determined by digital height caliper. Egg yolk diameter, egg and egg albumen length and width were determined by digital caliper (Mitutoyo Inc., Japan). Egg shape(Anderson et al. 2004), egg yolk index and albumen index (Romanoff and Romanoff 1949) were computed by following formulas respectively; [Egg width/ Egg length) x 100], [(yolk height/yolk diameter) x 100], [(albumen height/(average albumen length and width)) x 100]. Eggshell breaking strength was measured using the compression test module and resistance of eggshell broad pole to pressure was determined (Orka Food Technology Ltd., Ramat Hasharon, Israel). The cracked eggshells were washed and dried, then weighed using a 0.01 g precision scale. Eggshell weight ratio was calculated using the formula: Eggshell weight (%) = [eggshell weight (g)/egg weight (g)]/100. The thickness of the egg shell (with membrane) was determined as the average of the measurements made from the blunt end with two points on the equatorial axis of the egg using a micrometer(Mitutoyo Inc., Japan).

The yolk colour was determined using Minolta CR-410 colorimeter (Konica Minolta, Osaka, Japan). The L*, a* and b* parameters correspond to the lightness (-100/+100, dark/white), redness (-100/+100, green/red) and yellowness (-100/+100, blue/yellow), respectively.

Fatty acid profile of a total 150 egg yolk (30 eggs for each group) was detected and oils were extracted by the solvent method (Ethanol/chloroform solvent) (Kovalcuks and Duma 2014). Fatty acid methyl esters of the egg yolk oils were obtained according to the method of the recommendation of the European Union (EU) regulation 2568/91(Regulation 1991). Egg yolk oils were weighed (0.10 g) into the screw-cap glass tubes and dissolved within 10.0 mL hexane. Following, 100 µL 2N potassium hydroxide solution in the methanol was added to the tubes and shaken vigorously for 30 s. The tubes centrifuged at 2500 × g for 5 min and the upper layer was taken to a small vial and stored at 0°C till analysing date(Ayyildiz et al. 2015).The fatty acid composition was detected by gas chromatography (GC) device (Shimadzu GC-2010 Plus, Japan) which had the FID detector and HP-88 column (100m ×250 µm×0.20 µm id). The temperature at the injection block was 250 °C and the column oven heat program was adjusted as 2 min at 50 °C, 4 min between 50 °C -250 °C, and 10 min at

250 °C. The carrier gas was Helium with 1.3 mL/min flow rate. Fatty acids were detected by using retention time (min) and area (%) data of identified peaks and classed with standards of fatty acids and were presented as a percentage.

The trial was designed as a complete randomized model and data were analysed by using the ANOVA procedure with Minitab (Minitab 2000). Duncan's multiple range test was used to determine the differences among treatments which found significantly different ($P<0.05$).

RESULTS AND DISCUSSION

The effects of hempseed meal on performance parameters are shown in Table 2. The results showed that the effect of dietary different levels of HSM on the initial and final body weight and body weight changes of quails were not statistically significant ($P> 0.05$).

Similar body weight gain among quails groups indicated that HSM had no negative effect and it meet the nutrient requirements needs of birds. Konca et al. (2014) reported that different dietary levels of hempseed for quails had no effect on final body weight and body weight changes. Neijat et al. (2014) found that there was no significant difference among treatments in terms of body weight changes of laying hens, after using of dietary hempseed at 10, 20 and 30% levels. Similarly, Gakhar et al. (2012) and Silversides and Lefrancois (2005)demonstrated that laying diets containing up to 20% HSM did not significantly affect on the body weight at the end of the experiment. Contrary results were obtained by Khan et al. (2010) who stated that addition of 20% HSM to the diet increased the body weight of broilers, as well as Gakhar et al. (2012) who recorded that feed intake and feed efficiency were not affected by the addition of hempseed up to 20% to the diet in broilers.

This study revealed that adding different levels of HSM to the laying quail's diets induced no significant ($P> 0.05$) effect on egg production, feed intake, feed conversion rate, egg weight and egg mass. Supplementation of diets of laying hens with 10, 20 and 30% levels of HSM had no significant effection egg production and egg weight (Neijat et al. 2014).Moreover, laying diets supplemented with 20% HSM did not significantly affect the egg production, feed intake and feed conversion ratio (Silversides and Lefrancois 2005).Konca et al. (2014)detected that different levels of hempseed (5, 10 and 20%)didn't significantly affect the egg production, egg weight and egg mass. Whereas 15% raw hempseed(RHS)and 15% heat treated hempseed(HHS) added to the laying hen diets did not affect the feed conversion rate and egg yield, the feed consumption of the RHS group was less compared to the HHS and control groups. Egg weight and egg mass were not significantly different in RHS and HHS groups compared to the control group (Konca et al. 2019).

The present work demonstrated that the used levels of HSM induced no significant ($P> 0.05$) effects regarding the egg shell ratio and egg shell breaking strength. The egg shell thickness was significantly ($P <0.05$)affected by addition of HSM to the diets as level up to 5-20% decreased the shell thickness compared with control group (Table 3). Previously, Neijat et al. (2014) recorded that the egg shell weight and thickness of laying hens were not affected by diets containing HSM at levels of 10, 20 and 30%. Another study on laying hens showed that egg shell thickness was similar in groups fed with 0, 10 and 20% hempseed(Gakhar et al. 2012). In addition, Konca et al. (2019) noticed that the egg shell weight, egg shell ratio and eggshell thickness were not significantly affected with raw and heat-treated meals with hemp seeds.

Here, giving laying quails diets containing different levels of HSM didn't significantly ($P> 0.05$) affect on the egg shape and yolk index, while their effect on the albumin index was significant ($P <0.05$). The albumen index was statistically higher in groups containing 15 and 20% HSM than control group, but was statistically similar to those treated with 5 and 10% (Table 3). It has been documented that there was no significant difference in yolk and albumen height among groups fed with diets containing 4, 8 and 12% HSM (Gakhar et al. 2012).Albumin height was not affected by raw and heat-treated hemp seeds(Konca et al. 2019).

As seen in Table (3), diets containing different levels of HSM induced no statistical significant ($P> 0.05$) effect on egg yolk colour of quails.

Xanthophyll pigments and oxy-carotenoids are the main factors that determine the egg yolk colour, which affects consumer preferences(Vuilleumier 1969). Skřivan et al. (2019) noticed that addition of 3% hempseed to the diet of laying hens increased the egg yolk redness, while level of 9% produced adverse

Table 2. Effect of different levels of dietary HSM on performance in Japanese quail

Performance Parameters	0 HSM	5 HSM	10 HSM	15 HSM	20 HSM	*SEM*
Initial body weight, g	219.73	217.00	219.30	208.70	218.57	*3.507*
Final body weight, g,	266.12	268.36	262.30	260.61	259.40	*5.632*
Body weight change, g	46.38	51.36	43.00	51.91	40.83	*5.855*
Egg production, %	90.81	90.90	91.79	91.87	90.18	*1.516*
Feed intake, g	27.42	27.45	27.12	28.49	27.85	*0.478*
Feed conversion ratio, (Feed/Egg mass)	2.45	2.51	2.40	2.43	2.44	*0.052*
Egg weight, g	12.35	12.14	12.37	12.79	12.65	*0.158*
Egg mass, g	11.21	10.96	11.36	11.76	11.40	*0.225*

SEM: Pooled standard error of means. Diets contain hemp seed meal at the level of 0 (control), 5, 10, 15, and 20% for experimental groups named as 0HSM, 5HSM, 10HSM, 15HSM and 20HSM respectively

Table 3. Effect of different levels of dietary HSM on eggshell quality in Japanese quail

Egg Quality Parameters	0 HSM	5 HSM	10 HSM	15 HSM	20 HSM	*SEM*
Eggshell weight ratio, %	7.97	8.45	8.09	8.56	8.26	*0.173*
Eggshell thickness, mm	0.292^{b}	0.316^{a}	0.318^{a}	0.324^{a}	0.319^{a}	*0.005*
Eggshell breaking strength, kg	1.54	1.57	1.60	1.55	1.51	*0.045*
Egg shape index	76.99	78.35	77.65	76.60	78.01	*0.551*
Egg yolk index	43.70	42.60	43.48	43.09	43.53	*0.337*
Albumen index	8.64^{b}	9.39^{ab}	9.19^{ab}	9.71^{a}	9.67^{a}	*0.221*
Egg Yolk Colour Value						
L*	53.95	52.39	51.84	52.49	52.14	*0.690*
a*	13.49	14.60	14.50	13.78	14.04	*0.566*
b*	41.99	41.36	39.79	42.31	41.76	*0.697*

$^{a, b}$: Differences between the averages are significant in the same column with different letter (P<0.05). SEM: Pooled standard error of means. Diets contain hemp seed meal at the level of 0 (control), 5, 10, 15, and 20% for experimental groups named as 0HSM, 5HSM, 10HSM, 15HSM and 20HSM respectively

Table 4. Effect of different levels of dietary HSM on egg yolk fatty acids content in Japanese quail

Egg yolk fatty acids content (%)	0 HSM	5 HSM	10 HSM	15 HSM	20 HSM	*SEM*
Myristic acid (14:0)	0.355^{a}	0.345^{ab}	0.288^{abc}	0.275^{bc}	0.260^{c}	*0.0163*
Palmitic acid (16:0)	22.97^{a}	23.07^{a}	20.20^{b}	19.39^{b}	19.83^{b}	*0.2426*
Palmitoleic acid (16:1)	3.49^{a}	2.66^{b}	2.64^{b}	2.42^{b}	2.40^{b}	*0.1537*
Stearic acid (18:0)	9.26	10.23	10.55	10.49	10.33	*0.4483*
Oleic acid (18:1)	39.85^{ab}	37.41^{b}	41.18^{a}	40.09^{ab}	39.67^{ab}	*0.6350*
Linoleic acid (18:2)	18.93^{b}	21.57^{a}	16.60^{b}	18.09^{b}	18.28^{b}	*0.6010*
α-Linolenic acid (18:3, n3)	1.68^{b}	1.29^{b}	5.62^{a}	6.17^{a}	6.17^{a}	*0.1726*
Arachidic acid (20:0)	0.473^{a}	0.460^{a}	0.310^{b}	0.413^{ab}	0.415^{ab}	*0.0330*
Eicosapentaenoic acid (EPA; 20:5)	0.044^{b}	0.039^{b}	0.247^{a}	0.246^{a}	0.254^{a}	*0.0177*
Erucic acid (22:1)	1.84^{a}	1.89^{a}	1.18^{b}	1.21^{b}	1.24^{b}	*0.0713*
Docosahexaenoic acid (DHA; 22:6)	0.200^{b}	0.228^{ab}	0.250^{a}	0.265^{a}	0.258^{a}	*0.0114*
ΣSFA	33.29^{ab}	34.33^{a}	31.59^{bc}	30.81^{c}	31.08^{c}	*0.5001*
ΣMUFA	45.18^{a}	41.96^{b}	45.00^{a}	43.75^{ab}	43.27^{ab}	*0.6813*
ΣPUFA	20.86^{b}	23.10^{ab}	22.71^{ab}	24.75^{a}	24.95^{a}	*0.6529*
Σ omega−3	1.93^{b}	1.54^{b}	6.12^{a}	6.67^{a}	6.67^{a}	*0.1950*

$^{a, b, c}$: Differences between the averages are significant in the same line with different letter (P<0.05). SEM: Pooled standard error of means. Diets contain hemp seed meal at the level of 0 (control), 5, 10, 15, and 20% for experimental groups named as 0HSM, 5HSM, 10HSM, 15HSM and 20HSM respectively.

effect on the color. The egg yolk colour did not increase linearly with increasing HSM levels. Chickens egg yolk colour was higher in group fed with raw hempseed than those of control and heat-treated hempseed groups(Konca et al. 2019).In the study of Goldberg et al. (2012), addition of hempseed oil to the laying hens diets induced significant decrease in egg yolk lightness(L*) but significant increase in redness(a*) and yellowness(b*) and the largest changes were observed in the group with 20% hempseed. The effect of diets containing different levels of HSM in laying quail on egg yolk fatty acid profile is presented in Table (4).

The highest level of saturated fatty acid in egg yolk oil was palmitic acid, while the unsaturated fatty acid was oleic acid. The addition of HSM caused significant ($P<0.05$) decrease in the content of palmitoleic acid. In a comparison with control group, addition of HSM at levels 10% and above induced lower palmitic acid and levels of 15% and above produced significant ($P<0.05$)reduction in myristic acid content. Oleic and linoleic acid contents were significantly ($P<0.05$) influenced by the addition of HSM to the diet. The highest oleic acid content was found in group treated with 10% hempseed whereas the highest linoleic acid in 5% hempseed treated birds. The contents of α-linolenic acid, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA)increased significantly($P<0.05$) when the level of HSM in the diet was 10% and above. This result showed that HSM significantly ($P<0.05$) enriched the yolk fatty acid composition in favour of omega-3 fatty acids. In addition, when the level of HSM in the diet was 10% and above, the level of erucic acid decreased significantly. The effect of adding HSM to the diet on total unsaturated fatty acids, total monounsaturated fatty acids and total polyunsaturated fatty acids contents was significant. When the level of HSM in the diet was 15% and above, the content of ΣSFA decreased while ΣPUFA content increased compared to the control group.

Inclusion of HSM in to laying quails diet significantly changed fatty acid content of egg yolk ($P<0.05$). Result of the current study showed that dietary HSM may altered the fatty acid profile of egg yolk. Similar results were obtained in quails (Konca et al. 2014; Konca et al. 2019) and in chickens(Shahid et al. 2015). Raza et al. (2016) reported on a significant reduction in palmitic acid content of the egg yolk of laying hens as a result of addition of 25% hempseed to the ration. The results of the studies showed that with the addition of hempseed to the diet, the overall decrease in saturated fatty acids(SFA) in egg yolk is due to the high content of unsaturated fatty acids of hemp seeds(Konca et al. 2014; Shahid et al. 2015; Raza et al. 2016; Konca et al. 2019). According to the results of this study, the major change in fatty acid composition in egg yolk was the increase in PUFA (α-Linolenic acid, EPA and DHA). Reducing the SFA content and increasing the omega-3 amount provides an advantage for the health of consumers(Ayerza and Coates 2000). Goldberg et al. (2012) described significant increase in the amount of EPA in the egg yolk of hempseed fed hens compared to the control group. Although hempseeds do not contain docosahexaenoic-rich content, the amount of this fatty acid has been increased in the eggs of quail fed with HSM. The fatty acid docosahexaenoic is gained in eggs by two ways. It may be taken directly by diet or synthetized from α-linolenic acid(Yalçın and Ünal 2010). Similar study by Konca et al. (2019) revealed an increase in the synthesis of DHA as a result of high accumulation of α-linolenic acid. This work denotes an increase in omega-3 fatty acids in groups containing 10% and above HSM. Silversides and Lefrancois (2005) and Gakhar et al. (2012) stated that the eggs content of omega-3 fatty acids was increased with increasing the dietary hempseed. Furtheremore, Goldberg et al. (2012) found that the total omega-3 content in egg yolk was higher than the control group after addition of 12% hempseed oil to the laying hens diets.

CONCLUSION

According to the results of this study, addition of HSM to laying quail diets did not induce any negative effects on body weight change, egg production, feed intake, egg weight and egg mass. In addition, supplementation of HSM did not cause significant changes in the egg shell quality characteristics and egg yolk colour. However, 10% or more levels of HSM decreased the egg yolk SFA content but increased the MUFA and omega-3 fatty acid content. These results showed that HSM can be used to increase egg total MUFA especially omega-3 fatty acid. Comprehensive studies on the utilization of hempseed and its by-product in poultry nutrition are needed.

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Research article
Ερευνητικό άρθρο

Some testicular characteristics of Şavak Akkaraman rams and their relationship with live weight

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ABSTRACT: In this study, relationships between body weight and testicular characteristics were investigated in Şavak Akkaraman rams. Measurements taken from 60 rams in 7 traditional breeding farms were subjected to variance analysis to determine effects of the ram's age and the farm, and correlations between traits were calculated. Live weight (LW) average in rams was determined as 74.74±1.33 kg. Scrotum circumference (SC), scrotum width (SW), scrotum length (SL), scrotum thickness (ST), scrotum-ground distance (SGD), right testicular length (RTL), left testicular length (LTL), right testicular diameter (RTD) and left testicular diameter (LTD) values were calculated as 32.98±0.28 cm, 11.27±0.11 cm, 19.11±0.28 cm, 0.597±0.20 cm, 26.30±0.41 cm, 14.31±0.14 cm, 13.91±0.14 cm, 7.15±0.07 cm, 6.78±0.07 cm, respectively. Positive and significant correlations were determined between the body weight of the rams and testicular characteristics, except for SGD measurement. Correlation coefficients between LW and SD (0.620), RTL and LTL (0.541 and 0.524), RTD and LTD (0.676 and 0.561) were high. This study is the first study to determine the relationship between body weight and testicular characteristics in Şavak Akkaraman rams. Due to the limited number of studies on Şavak Akkaraman rams; considering the correlation findings between testicular measurements and live weight, it can be said that morphometric testis measurement results can contribute to future studies as a preliminary information and a descriptive study.

***Keywords*:** Şavak Akkaraman, body weight, testicular characteristics, correlation

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INTRODUCTION

There has been the privileged place of the sheep farming in Turkey until today from the past. Therefore, sheep breeding has been common in the livestock sector. Turkey, ovine animals for the presence among world countries takes 8th place (FAOStat, 2020). Turkey had about 23.974 million sheep assets in 2008, and this presence rose to 37.276 million sheep in 2019. The number of sheep has increased by about 60% in the last ten years. However, in the same period, sheep milk production increased by approximately 80% and reached 1 344 000 tons (TÜİK, 2019). The number of sheep in Turkey has rapidly increased in recent years, therefore, the improvement works in the sheep farming have been gained more importance. Many sheep breeds are grown in Turkey. Akkaraman sheep breed constitutes 47% of its sheep assets in Turkey (Kaymakçı, 2016). There are many varieties of the Akkaraman sheep breed. One of these varieties is the Şavak Akkaraman sheep, which is locally bred in Erzincan, Elâzığ and Tunceli provinces (Yağcı, 2017).

The Şavak Akkaraman sheep takes its name from the Şavak Tribe that breeds them. The Şavak Tribe is a community that continues its life with sheep farming in a semi-nomadic system from past to present. The people of Şavak, their animals which have adapted to the geography and climatic conditions in which they live, have been raised them with their own traditional methods in order to obtain more milk and produce their stocks. The Şavak Akkaraman sheep are generally considered to have a smaller size and a more delicate body than Akkaraman and Kangal Akkaraman sheep (Yağcı, 2017). Although the fleece covers are longer and brighter, there are more stains on their faces. The vast majority of milk is used in cheese making and a very good income is obtained from the cheese produced. Cheese, a special local product, has a geographical indication certificate and is called "Şavak Tulum Cheese" (Yağcı et al., 2018).

Şavak Akkaraman sheep is one of the important local gene resources that has been supported by the Ministry of Agriculture and Forestry since 2011 within the scope of the " National Domestic Animal Genetic Resources Conservation and Improvement Project". With this project, primarily by protecting the populations of Şavak Akkaraman, the best breeding rams have been selected and high-yielding herds have been tried to be obtained (Daşkıran et al., 2015). In this sense, it is important to determine the various characters of the rams to be selected as breeders, which are important for breeding. This is also necessary to improve economic gain by increasing productivity in the population (Elmaz ve ark., 2008).

Determining breed-specific characters in sheep breeding has an important effect on increasing performance (Abbasi and Kesbi, 2011). In this context, it is especially important to determine the reproductive characteristics that ensure the continuity of the species. There have been many studies on the reproduction characteristics of sheep in different breeds (Koyuncu et al., 2000; Kulaksız et al., 2010; Yakubu et al., 2013; Al-kawmani et al., 2014; Babashani, 2015; Adjibode et al., 2016). Except this, relationship studies between reproductive traits and different characters were also made. In a study examined the relationships between live weight and testicular diameter, testicular length, scrotum length, scrotum circumference and scrotum volume in Karayaka yearlings; all correlations were found to be significant except these between body weight and testicular length, and between scrotum length and testicular diameter and scrotum circumference. In addition, the coefficients of determining testicular measurements of live weight were found to be significant except for testicular length (Koyuncu et al., 2000). In a study conducted on Kıvırcık rams, the effect of age of yearling lambs and body weight on testicular measurements was found to be significant (Koyuncu et al., 2005). Similarly, it was reported that there was a positive correlation between live weight and testicular measurements for Najdi male lambs by Al kawkani et al. (2013), and for rams in Djallonke and Ouda breeds raised in northern Benin by Benoit et al. (2017). As can be understood from the studies conducted, especially the scrotum circumference that is one of testicular measurements, sperm production and quality are important indicators for reproductive potential (Bourdon and Brinks, 1986; Hahn et al., 1969). These testicular features can also be used as selection criteria (Toe et al., 2000). For this reason, it is important to determine testicular characteristics and to reveal their relationships with other characters.

Due to the reproductive performance of Şavak Akkaraman sheep is low, it is necessary to determine and improve the reproductive performance of this breed (Yağcı, 2017). Studies on Şavak Akkaraman sheep are limited, and no study has been found on testicular characteristics in rams. Conducting research on reproductive traits of Şavak Akkaraman sheep which is one of varieties of Akkaraman breed which is indigenous

genetic resource cultivated extensively in Turkey, will provide data / contribution to improvement of reproductive traits in breeding studies.

In this study, it was aimed to investigate some testicular measurements and the relationship between these testicular measurements and live weight in Şavak Akkaraman rams.

MATERIALS AND METHODS

Location

This study was carried out in Erzincan Province, Turkey where Şavak Akkaraman sheep is widely breed. The equatorial location of the region is between 39 ° 02′ - 40 ° 05′ north latitudes and 38 ° 16′ - 40 ° 45′ longitudes and its surface area is 11903 km². The city center is 1.185 meters above sea level (Karadeniz and Altınbilek, 2016). Erzincan has a continental climate characteristic (Anon, 2019).

Collection of Data

The animal material of the study consisted of 60 rams between the ages of 2 and 6, in similar conditions, randomly selected from 7 different farms in the Şavak Akkaraman Sheep Public Breeding Project in central and Tercan districts of Erzincan, Turkey. Similar practices were made for the rams within the scope of the project in the studied farms. The rams were generally grazed on the pasture, and care and feeding were carried out in the pen in winter. Hay straw and barley were given to the rams in the pen. No other supplementary feeding program was applied to the rams during the mating season. In the farms where the research was conducted, the participation of rams was carried out for approximately 60-70 days at the beginning of October and the free-mating method was applied. Testicular characteristics measured together with body weight in rams were performed once in August, at the beginning of the mating season.

On Şavak Akkaraman rams; live weight (LW), scrotum circumference (SC), scrotum width (SW), scrotum length (SL), scrotum thickness (ST), scrotum-ground distance (SGD), right testicular length (RTL), left testicular length (LTL), right testicular diameter (RTD) and left testicular diameter (LTD) were measured. Live weight measurement was made with a sensitive electronic scale up to 100 g (Ovine Livestock Scale, IMMAX, EB-600). Testicular measurements were made by gently holding the hand. TD, TL and ST were determined with metal caliper precision to 0.01 mm. SC was measured with a tape measure, SW and SGD were measured with a tape measure.

Statistical Analysis

Statistical evaluation of the data obtained in the study was performed using the Least-Squares analysis method in the statistical package program of IBM SPSS Version 23.0 (IBM Corp. Released, 2015). Statistical model used in analysis;

$$Y_{ijl}=\mu+a_i+b_j+e_{ijl}$$

In the model; Y = the value of any ram in terms of the attribute considered at any sub-level of any factor, μ = the expected average of the population in terms of the investigated trait, a = the age of the ram, b = the farm that grown the ram, e = normal, independent, chance error (0, σ2).

RESULTS

Least squares means, standard errors, significance test results of live weight and testicular characteristics determined in Şavak Akkaraman rams were given in Table 1, and the relationship between live weight and testicular characteristics was given in Table 2.

The average live weight of Şavak Akkaraman rams was 74.74 ± 1.33 kg. Among testis properties, SC, SW, SL, ST, SGD, RTL, LTL, RTD and LTD values were calculated as 32.98 ± 0.28 cm, 11.27 ± 0.11 cm, 19.11 ± 0.28 cm, 0.597 ± 0.20 cm, 26.30 ± 0.41 cm, 14.31 ± 0.14 cm, 13.91 ± 0.14 cm, 7.15 ± 0.07 cm, 6.78 ± 0.07 cm, respectively

The farm factor was found to be high significant ($P<0.01$) for LW, SC, SW, RTD and LTD. In the farm with the highest average live weight of rams (83.80 kg), testis characteristics such as SC (34.92 cm), SW (11.98 cm), RTL and LTL (15.16 and 14.47 cm) and RTD (7.61 cm) were the highest calculated. The farm with the highest average live weight also had the second highest value in terms of SL (19.92 cm) and LTD (6.99 cm) measurements. The SGD (25.88 cm) value detected in this weight group indicated that the testicles were neither too droopy nor too close to the body.

In the study, since there were no rams from every age group in every farms, the age effect was examined by dividing the rams into two age groups, which can be called younger and older ones. The average body weight (67.92 kg) of the 2 and 3-year-old rams (37 heads) used in the study was calculated 13.63 kg lower than the average body weight (81.55 kg) of the 4, 5 and 6 aged rams.

Table1. Least squares means, standard errors and significance test results of live weight and testicular measurements in Şavak Akkaraman rams

	n	Live Weight (kg) $\bar{X} \pm S_{\bar{x}}$	Scrotum Circumference (cm) $\bar{X} \pm S_{\bar{x}}$	Scrotum Widht (cm) $\bar{X} \pm S_{\bar{x}}$	Scrotum Lenght (cm) $\bar{X} \pm S_{\bar{x}}$	Scrotum Thickness (cm) $\bar{X} \pm S$	Scrotum Height (cm) $\bar{X} \pm S_{\bar{x}}$	Testicular Lenght		Testicular Diameter	
								Right (cm) $\bar{X} \pm S_{\bar{x}}$	Left (cm) $\bar{X} \pm S_{\bar{x}}$	Right (cm) $\bar{X} \pm S_{\bar{x}}$	Left (cm) $\bar{X} \pm S_{\bar{x}}$
Overall mean	60	74.74±1.33	32.98±0.28	11.27±0.11	19.11±0.28	0.60±0.02	26.30±0.41	14.31±0.14	13.91±0.14	7.15±0.07	6.78±0.07
Farms		**	**	**	ns	ns	ns	ns	ns	**	ns
1	10	65.92±3.15	30.14±0.67	10.25±0.25	17.22±0.65	0.65±0.05	27.92±0.97	13.65±0.34	13.73±0.33	6.41±0.16	6.33±0.17
2	9	64.33±3.21	32.48±0.68	11.32±0.26	18.92±0.66	0.55±0.05	25.46±0.98	14.60±0.34	14.28±0.34	6.95±0.16	6.70±0.17
3	10	80.99±3.15	33.61±0.67	11.16±0.25	19.41±0.65	0.68±0.05	25.58±0.97	13.78±0.34	13.45±0.33	7.45±0.16	6.85±0.17
4	11	74.28±2.92	31.96±0.62	11.14±0.23	19.28±0.61	0.62±0.04	25.95±0.90	14.09±0.31	13.51±0.31	6.95±0.15	6.62±0.16
5	6	83.80±3.95	34.92±0.84	11.98±0.32	19.92±0.82	0.61±0.06	25.88±1.21	15.16±0.42	14.47±0.41	7.61±0.20	6.99±0.22
6	8	77.83±3.47	34.76±0.73	11.84±0.28	20.32±0.72	0.54±0.05	25.69±1.07	14.87±0.37	14.44±0.36	7.52±0.18	7.23±0.19
7	6	76.01±4.03	33.02±0.85	11.17±0.32	18.67±0.84	0.53±0.06	27.59±1.24	14.03±0.43	13.52±0.42	7.16±0.21	6.76±0.22
Ages		**	**	ns	ns	ns	ns	**	**	**	**
2 and 3	37	67.92±1.64	31.96±0.35	11.09±0.13	18.09±0.34	0.56±0.03	27.16±0.50	13.84±0.17	13.41±0.17	6.86±0.08	6.57±0.10
4, 5 and 6	23	81.55±2.19	34.01±0.46	11.44±0.18	20.12±0.46	0.63±0.03	25.43±0.67	14.79±0.23	14.42±0.23	7.44±0.11	7.00±0.12

*:P<0.05, **: P<0.01; ns : non significant

Table 2. Correlation coefficients and significance levels between body weight and testicular measurements detected in Şavak Akkaraman rams

Measurments		LW	SC	SW	SL	ST	SGD	RTL	RTD	LTL
SC	**Corr.**	**.620****								
SW	**Corr.**	**.490****	**.871****							
SL	**Corr.**	**.439****	**.550****	**.540****						
ST	**Corr.**	**.395****	.214	.203	.253					
SGD	**Corr.**	-.203	-.335	-.338	-.563	-.144				
RTL	**Corr.**	**.541****	**.672****	**.607****	**.649****	**.294***	-,370			
RTD	**Corr.**	**.676****	**.893****	**.702****	**.458****	**.260***	-,326	**.645****		
LTL	**Corr.**	**.524****	**.623****	**.550****	**.657****	**.362****	-,416	**.913****	**.565****	
LTD	**Corr.**	**.561****	**.835****	**.695****	**.491****	.218	-,309	**.665****	**.861****	**.651****

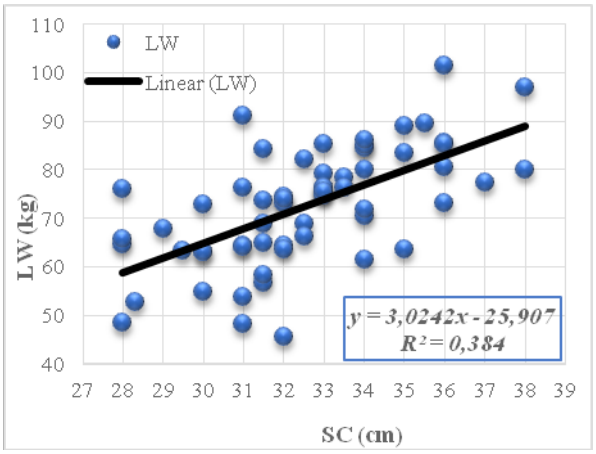
*: significant (P<0.05). **: high significant (P<0.01); Live weight (LW), Scrotum circumference (SC), scrotum width (SW), scrotum length (SL), scrotum thickness (ST), scrotum-ground distance (SGD), right testicular length (RTL), left testicular length (LTL), right testicular diameter (RTD) and left testicular diameter (LTD)

The effect of age group on live weight was found to be high significant (P<0.01). Likewise, the effect of the age group on testicular features such as SC, RTL and LTL, RTD and LTD was high significant (P <0.01). Except for SGD feature, the values in 4, 5 and 6 year old rams were calculated higher than the values calculated for 2 and 3 year old rams (Table 1). The low value of this group in terms of SGD essentially showed that the testicles were more developed and the distance to the ground was less.

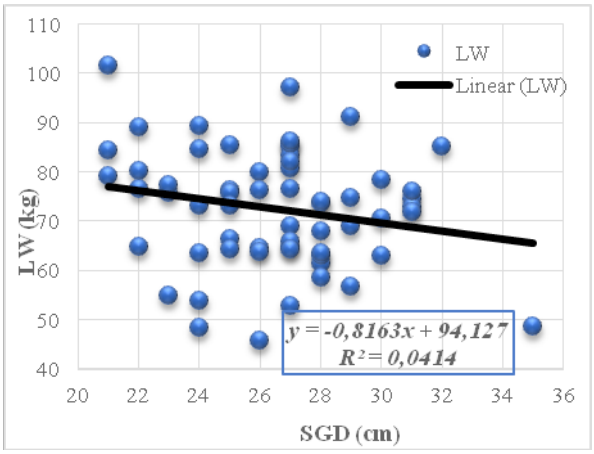
The relationships between live weight and testicular characteristics in Şavak Akkaraman rams were given in Table 2. Positive, high and very significant (P<0.01) correlations were determined between live weight and testicular characteristics, except SGD.

Correlation coefficients calculated between live weight and SC, RTL and LTL, RTD and LTD (0.620, 0.541, 0.524, 0.676, 0.561, respectively) indicated very strong relationships between live weight and these characteristics. The weak negative correlation determined between body weight and SGD meant that as body weight increases, the distance of the scrotum to the ground decreases, which indicated that the testicles were more developed.

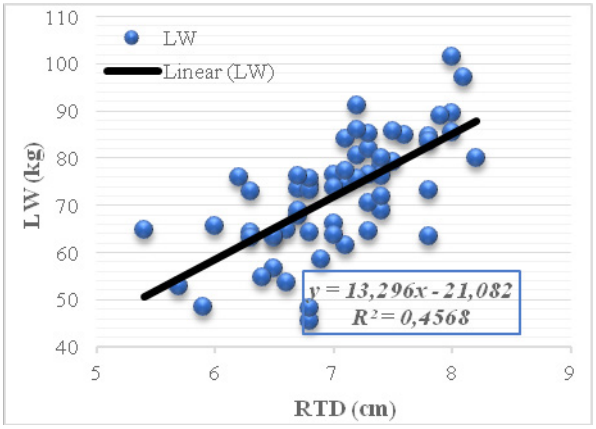
The scatter chart showing the relationships between the live weights of the rams and some testicular measurements was given in Figure 1 and the regression equations in Table 3.



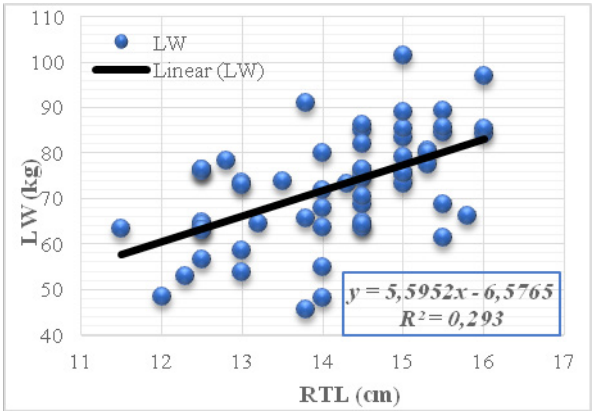
Scrotum circumference



Scrotum - ground distance



Right testicular diameter



Right testicular lenght

Figure 1. Scatter plot of some testicular measurements in Şavak Akkaraman rams

Table 3. Regression equations and determination coefficients of body weight according to some testicular measurements in Şavak Akkaraman rams

Live Weight = Constant + Regression Coefficient x Testis Size	R^2 (%)
Y = (-25.907) + 3.0242 x SÇ	38.4
Y = (-2.2803) + 6.7027 x SG	23.98
Y = 27.057 + 2.417 x SU	19.31
Y = 54.028 + 31.083 x SK	15.64
Y = 94.127 + (-0.8163) x SYAM	4.14
Y = (-21.082) + 13.296 x Sağ TÇ	45.68
Y = (-6.5765) + 5.5952 x Sağ TU	29.3

DISCUSSION

Scrotum circumference was determined as 32.98 cm in Şavak Akkaraman rams. This calculated value was similar to the values reported for genotypes such as Chios (32.1 cm) (Öztürk et al., 1996), and 2-4 years old Karayaka rams (32.5) (Aslan et al., 2019), and were higher than the values reported for genotypes such as Akkaraman (30.7 cm) (Öztürk et al., 1996), 206 days-old Kıvırcık male lambs (26.88 cm) (Özdemir and Altın, 2002), Dağlıç (30.8 cm), Awassi (31.8 cm) measured pre-mating (Gündoğan et al., 2003), 480 days-old Norduz male lambs (29.74 cm) (Yılmaz and Cengiz, 2006), Karayaka rams (29.80 cm) (Kulaksız et al., 2010)

Scrotum circumference value found in this research was lower than the values reported for the genotypes such as Akkaraman (33.75 cm) (Öztürk et al., 1996) and Karya (33.98 cm) (İnce & Karaca, 2009),

18-20 month-old Pırlak rams (35 cm) measured in October (Yeni and Gündoğan, 2018). Same time; the findings of this research were higher than the values of Balami (31.25 cm) (İbrahim et al., 2012), Najdi male lambs (25 cm) (Al-kawmani et al., 2014), Yankasa rams (26.8 and 26.34 cm) (Babashani, 2015), Djallonke male lambs (23.26 and 21.08 cm) (Adjibode et al., 2016), Djallonke and Ouda rams (21.41 ve 24.51 cm) (Benoit et al., 2017), and lower than the values of Uda (38 cm) and Yankasa rams (35.25 cm) (Yakubu et al., 2013).

Scrotum length in Şavak Akkaraman rams was determined as 19.11 cm. This value is higher than the genotypes of Acıpayam (3.67 cm) (Kaymakçı et al., 1988), Morkaraman (10.2 cm) and Awassi (17.63 cm) (Odabaşıoğlu et al., 1992), Karakaş male lambs (9.27 cm and 8.31 cm), Konya Merino (12.33 cm) (Aygün and Karaca, 1995), Akkaraman rams (18.03 cm), Awassi rams (17.63 cm) (Öztürk et al., 1996), Dağlıç (16.69 cm) and Karayaka (18.7 cm) (Gündoğan, 1999).

Scrotum thickness in Şavak Akkaraman rams was found to be 0.59 cm. This value was similar to the values reported for genotypes such as Akkaraman (0.6 cm) (Gündoğan et al., 2003), Karayaka (0.57 cm) (Kulaksız et al., 2010), and lower than Chios (0.7 cm), and was higher than rams of Dağlıç (0.4 cm), Awassi (0.5 cm) (Gündoğan et al., 2003).

Right and left testicular diameters found in Şavak Akkaraman rams were 7.15 and 6.78 cm. This values were found higher than values of the genotypes of Akkaraman (6.44 cm) and Awassi (5.86 cm) (Öztürk et al., 1996), Kıvırcık male lambs (4.34 and 4.22 cm) (Özdemir and Altın, 2002), Kıvırcık (4.20 and 4.11 cm) Karya (5.64 cm), Çine Çapar (5.19 cm) (İnce and Karaca, 2009), Karayaka (5.9 cm) (Kulaksız et al., 2010).

The right and left testicular length values found in this study were 14.31 and 13.91 cm. These values were found higher than the values in the research on some sheep breeds such as Akkaraman (12.17 cm), Awassi (11.95) (Öztürk et al., 1996), Kıvırcık male lambs (10.24 and 10.12 cm) (Özdemir and Altın, 2002), Akkaraman rams (9.7 and 9.9 cm) (Gündoğan et al.,2003), Kıvırcık male lambs (5.94 cm) (Koyuncu et al., 2005), Karayaka (12.31 cm) (Kulaksız et al., 2010), and Balami (12.63 cm), Uda (12.75 cm) Yankasa (12.25 cm) in Nigeria (Yakubu et al., 2013).

It can be said that among the reasons for the values found as a result of the study being higher or lower than the literature values, factors such as the breed, age, care and feeding of the rams used in the research, the climatic conditions of the region where they were raised or the season in which they were measured (Gündoğan et al., 2003; Yılmaz and Aygün, 2001). The rams used in this study were grazed in the pasture under the conditions of the breeders, and in the winter season, care and feeding were carried out in the pen and care was fed with hay straw and barley crushed. If the rams are provided with better care and feeding conditions, it can be predicted that they will reach better condition and show higher performance. In this context, considering the positive relationships between testicular measurements and reproductive performance, if the existing care feeding conditions are improved, it can be expected that the reproductive traits of rams with better condition will also be superior.

CONCLUSION

In this study, the body weight and testicular characteristics of Şavak Akkaraman rams were defined and the effects of environmental factors such as the age of the rams and the farms where they were raised were determined. At the same time, it has been observed that body weight significantly affects testicular characteristics. Considering the significant correlation coefficients between live weight and testicular measurements of Şavak Akkaraman rams raised in the Erzincan region, Turkey, it was concluded that testicular characteristics and body weight values can be used as a criterion in breeding selection.

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CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Effect of the addition of bee pollen to the diet on performance, eggshell quality and serum parameters in layer quails

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ABSTRACT: This study was carried out to determine the effect of bee pollen addition to quail diets on performance, eggshell quality and serum biochemical properties. In the experiment, 120 quails aged 70 days were randomly distributed to 6 treatment groups with 4 replicates. The quails were fed for 10 weeks with 6 trial diets with 0, 2, 4, 6, 8 and 10 g/kg bee pollen added. The body weight change, egg production, egg weight, egg mass, feed conversion ratio, damaged eggs, eggshell weight, eggshell thickness and serum glucose, creatinine, albumin, globulin and total protein levels of quails were not affected by the addition of bee pollen to the diet ($P>0.05$). The addition of 10 g/kg bee pollen to the diet significantly decreased feed intake compared to the control group (0 g/kg). Eggshell breaking strength was decreased by adding bee pollen to the diet and the lowest value was observed at the group fed the diet containing 6 g/kg bee pollen.Serum cholesterol concentration was minimized when 10 g/kg bee pollen was added to the diet($P<0.05$). In addition, serum calcium and phosphorus contents were increased compared to the control (0 g/kg) group and these parameters reached the maximum at the 10 g/kg bee pollen level ($P<0.01$).According to the results of the experiment, the addition of bee pollen to the diet had a positive effect on serum cholesterol, calcium and phosphorus levels in layer quails, however, it had a negative effect on eggshell breaking strength.

***Keywords*:** Bee pollen, quail, performance, eggshell quality, cholesterol.

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INTRODUCTION

In recent years, the use of alternative additives to antibiotics has increased to improve the immunity and performance of animals. One of the candidate products for these additives is bee pollen (Farag and El-Rayes, 2016).

Bee pollen is a bee product formed by mixing flower pollen collected by worker bees with digestive enzymes such as beta-glycosidase (Carpes et al., 2008). Bee pollen is rich in carbohydrates (13-55%), protein (10-40%), fatty acids (1-20%), vitamins (0.02-0.10%) and minerals (0.5-3%) as well as flavonoids (0.04-3%) (Villanueva et al.,2002;Carpes et al., 2007; Isidorov et al.,2009; Taha, 2015).However, the nutrients and active substances contained in bee pollen vary according to available flowers or, in other words, to the flora around the hive (Taha, 2015).

Bee pollen is reported to have anti-microbial, anti-inflammatory (Kacaniova et al.,2012;Pascoal et al., 2014), anti-fungal (Garcia et al.,2001), anti-allergic (Moita et al., 2014), anti-viral, hypolipidemic, hypoglycaemic and immune-enhancing effects (Komosinska-Vassev et al., 2015).Desoky and Kamel (2018) stated that the addition of bee pollen (2 g/kg) to the diet increased feed intake (FI), egg mass (EM), eggshell thickness and serum total protein, albumin and globulin levels, and decreased feed conversion rate and serum cholesterol level in laying quails. Rizk et al. (2018) reported that the feed efficiency of laying hens was maximized by adding 1000 mg/kg of bee pollen to their diets, however, the addition of 500 mg/kg to diet was more economically appropriate.Similarly, in another study, it was reported that with the supplementation of bee pollen to the diet, feed efficiency in laying hens enhanced and as a result, producer profit increased (Demir and Kaya, 2020). However, it has been reported that supplementation of bee pollen (2.5, 5.0 or 7.5 g/kg) to the diet does not affect performance in laying quails (Al-Hamdani and Al-Douri, 2018). In addition, some research results showed that the addition of bee pollen to the diet was effective in changing blood biochemistry parameters, especially lowering serum cholesterol level (Fazayeli-Rad et al.,2015; Farag and El-Rayes, 2016; Rizk et al., 2018; Demir and Kaya, 2020).

Research results show that addition of bee pollen to the diet has a positive effect on poultry, but the number of studies on the effect of addition of bee pollen to the diet in layer birds is very few, and the optimum level of bee pollen in the diet is still uncertain. Therefore, it is necessary to increase the number of studies.

This research was carried out to determine the effect of bee pollen additionto layer quail diets on production performance, eggshell quality and serum biochemical parameters.

MATERIALS AND METHODS

Ethical Approval

The animal care practices were used in the experiment in consistency with animal welfare rules stated in Article 9 in government law in Turkey (No. 5996).

Animals and Feed Materials

In the experiment which lasted 70 days, 120 female Japanese quails at the age of 10 weeks were used. The quails were assigned to six treatment groups with four replicates, each with five quails. The six treatment diets used in the experiment were prepared by adding bee pollen at the 0, 2, 4, 6, 8 or 10 g/kg levels to the control diet (Table 1) which was formulated according to NRC (1994) nutrient requirements for layer quails. The bee pollen used in the experiment was collected from Konya region, and it contains 14.3% moisture, 2.1% crude ash, 19.4% crude protein, 7.4% crude fat, 13.2% fiber and 56.9%carbohydrate (Başdoğan et al., 2019).During the experiment, feed and water were given to quails *ad-libitum*and a lighting program of 16 hours/day was applied.

Determination of performance parameters

Body weights of quails were determined by group weighing at the beginning and the end of the experiment, and body weight change (BWC) was determined from these values. At the end of the experiment, feed intake (FI) was calculated as g/day/quail. Egg production (EP) was recorded daily and the percentage of egg production per quail was calculated for the total experimental period.Egg weight (EW) was obtained by weighing all eggs collected in the last three days of the research. Egg mass was determined using this formula: EM = EP (%) x EW) / 100.The feed conversion ratio was calculated by dividing FI (g/d/quail) by the EM (g/egg/quail).

Determination of eggshell quality parameters

All eggs collected in the last three days (on days 68-70) of the experiment were used to determine the eggshell quality. The collected eggs were firstly weighed, and then the eggshell breaking strength (kg)

Table 1. Basal diet and its calculated nutrient contents

Ingredients	g/kg	Nutrient contents	g/kg
Corn	542.0	Metabolisable energy, kcal ME/kg	2902
Soybean meal (47%)	270.0	Crude protein	200.9
Sunflower meal (36%)	70.0	Calcium	25.1
Sunflower oil	43.0	Available phosphorus	3.5
Limestone	56.0	Lysine	10.0
Dicalcium phosphate	11.5	Methionine	4.5
Salt	3.5	Cystine	3.7
Premix[1]	2.5	Methionine + cystine	8.2
DL methionine	1.5		
Total	1000.0		

[1]Premix provide per kg of diet: manganese: 80 mg, iron: 60 mg, copper: 5 mg; iodine, 1 mg, selenium: 0.15 mg, Vitamin A:8.800 IU, Vitamin D3: 2.200 IU, Vitamin E: 11 mg, Nicotine acid: 44 mg, Cal-D-Pan: 8.8 mg, Riboflavin: 4.4 mg, Thiamine: 2.5 mg, Vitamin B12: 6.6 mg, Folic acid: 1 mg, Biotin: 0.11 mg, Choline: 220 mg.

Table 2. Effects of bee pollen addition to diet on performance parameters in layer quails

Parameters	Bee Pollen, g/kg						SEM*	*P value*
	0	2	4	6	8	10		
BWC, g	14.33	26.08	17.08	18.54	20.83	13.00	5.728	0.762
EP, %	81.28	88.01	88.49	86.98	82.18	80.66	2.348	0.096
EW, g	13.09	12.89	12.64	12.54	12.58	12.86	0.344	0.895
EM, g/egg/quail	10.64	11.34	11.16	10.91	10.34	10.38	0.390	0.441
FI, g/d/quail	32.51[a]	32.76[a]	33.18[a]	32.65[a]	31.17[ab]	30.47[b]	0.610	0.050
FCR, g feed/g egg	3.06	2.90	2.98	3.00	3.03	2.96	0.105	0.936

BWC: Body weight change, EP: Egg production, EW: Egg weight, EM: Egg mass, FI: Feed intake, FCR: Feed conversion ratio.
*Standard error means.
[ab]Values bearing different superscript in rows are statistically significant (P<0.05).

was measured by applying supported systematic pressure (Egg Force Reader, OrkaFoodTechnology, Israel) to the blunt of the eggs. Afterwards, the eggs were emptied and the eggshells were washed so that no egg whites remained in tap water and dried at room temperature (24 °C) for three days. The dried eggshells were weighed by a precision scale with 0.01 g delicate, divided into the egg weight, and the eggshell rate was calculated as% of the egg weight.Membrane eggshell thickness (µm) was determined with a micrometer by taking three measurements from blunt, middle and pointed sections of eggshells.

Determination of serum parameters

At the end of the experiment, one quail from each subgroup (24 quails in total) was randomly selected and used for blood sampling by the method of heart puncture.Blood samples were centrifuged at 3000 rpm for 5 minutes to obtain serum samples that were stored at -20 °C until analysed. The evaluation of serum biochemical constituents was made by an auto-analyser (Beckman LX-20 Coulter, Ireland) and using commercial kits (Beckman, Ireland).

Statistical analysis

The variance analyses have been applied to all variables obtained from the trial groups (Minitab, 2000), and the differences between means of the groups were determined by the Duncan (Duncan, 1955) multiple comparison test.

RESULTS AND DISCUSSION

Performance Parameters

The effects of bee pollen addition to the diets on performance parameters in layer quails are given at Table 2. The supplementation of bee pollen to quail diets did not statistically affect BWC, EP, EW, EM and FCR (P> 0.05).

Feed intake was significantly affected by bee pollen supplementation to the diet, and the FI was significantly lower in the group that received bee pollen at a high level (10 g/kg) than in the groups supplemented with 0 (control group), 2, 4 and 6 g/kg bee pollen (P = 0.05). Similarly, Demir and Kaya (2020) reported that the addition of adding 0.5, 1.0 or 1.5% bee pollen to laying hen diets caused a linear decrease in FI.

In a study conducted with broilers, it was stated that FI declined by adding 0.2, 0.4 or 0.6% bee pollen to the diets (Farag and El-Rayes, 2016). Hosseini et al. (2016) also reported that the supplementation of bee pollen (20 g/kg) to broiler diets reduced FI.Rizk et al. (2018) demonstrated that there was no difference between the groups in terms of FI by adding bee pollen at levels of 0, 500, 1000 or 1500 mg/kg to laying hen diets. Similar results were reported in another study in which 0.5% palm pollen was used in diets (Mousa et al., 2018). However, Desoky and Kamel (2018) showed that the addition of 2 g/kg level of bee pollen to the diet increased FI in laying quails. Similar results were reported by Canoğulları et al. (2009) in growing quails. It is seen that some studies are in agreement with the results of current research in terms of FI (Farag and El-Rayes, 2016; Hosseini et al., 2016; Demir and Kaya, 2020), but some studies results in the literature are not compatible with present research (Canoğulları et al., 2009; Mousa et al., 2018;Rizk et al., 2018). The decrease in feed intake when high levels of pollen (10 g/kg) are added to the diet may be due to the low palatability of the pollen.

Eggshell Quality Parameters

The effects of adding different levels of bee pollen to the diets on eggshell quality parameters in layer quails are demonstrated at Table 3.

The treatments did not statistically affect the eggshell quality parameters (P>0.05), except for eggshell breaking strength (P<0.05). The highest eggshell breaking strength was obtained in the control (0 g/kg) group, and only a difference between this group and the group that was supplemented with 6 g/kg bee pollenwas observed.Demir and Kaya (2020) stated that the addition of 1.5% bee pollen to laying hen diets did not affect eggshell parameters, including eggshell breaking strength.Similar results were also reported by Rizk et al. (2018).Also, Desoky and Kamel (2018) noted that addition of 2 g/kg level bee pollen to the diet did not affect the eggshell weight of the eggshell quality parameters, but increased the eggshell thickness in laying quails. These results are not in agreement with the current study results. Differences in animal material used and diet pollen level can be listed as possible causes of incompatibility.

Serum Parameters

The effects of adding different levels of bee pollen to the diets on serum biochemical constituents in layer quails are demonstrated at Table 4. The effect of bee pollen addition to the quail diets was statistically insignificant on serum glucose, creatinine, albumin, globulin and total protein (P> 0.05).

Table 3. Effects of bee pollen addition to diet on egg quality parameters in layer quails

Parameters	Bee Pollen, g/kg						SEM*	*P value*
	0	2	4	6	8	10		
Damaged eggs, %	1.68	0.00	0.97	1.21	1.71	2.44	0.996	0.768
Eggshell breaking strength, kg	1.52^{a}	1.46^{ab}	1.38^{ab}	1.24^{b}	1.37^{ab}	1.33^{ab}	0.048	0.022
Eggshell weight, % of EW	7.91	7.64	7.70	7.55	7.79	7.96	0.197	0.777
Eggshell thickness, µm	198	193	196	197	202	201	3.803	0.594

***** Standard error means.

^{ab}Values bearing different superscript in rows are statistically significant (P<0.05).

Table 4. Effects of bee pollen addition to diet on serum biochemical constituents in layer quails

Parameters	Bee Pollen, g/kg						SEM*	*P value*
	0	2	4	6	8	10		
Glucose, mg/dL	274	286	305	282	276	280	6.4	0.066
Cholesterol, mg/dL	213^{ab}	239^{a}	196^{ab}	221^{ab}	179^{ab}	153^{b}	13.6	0.019
Total protein, g/dL	4.27	4.37	4.35	4.27	4.20	4.70	0.261	0.829
Creatinine, mg/dL	0.33	0.34	0.34	0.32	0.32	0.32	0.010	0.624
Albumin, g/dL	1.43	1.47	1.37	1.42	1.37	1.60	0.062	0.172
Globulin, g/dL	2.83	2.90	3.07	2.85	2.65	3.10	0.186	0.696
Calcium, mg/dL	22.50^{B}	25.17^{AB}	26.32^{A}	23.00^{AB}	23.80^{AB}	25.95^{A}	0.731	0.007
Phosphorus, mg/dL	5.47^{b}	5.87^{b}	5.83^{b}	5.48^{b}	6.87^{ab}	8.30^{a}	0.592	0.040

***** Standard error means.

^{AB}Values bearing different superscript in rows are statistically significant (P<0.01).

^{ab}Values bearing different superscript in rows are statistically significant (P<0.05).

Serum cholesterol reduced as the level of bee pollen added to the diets increased and it was minimal in the group fed diets containing 10 g/kg bee pollen. In terms of serum cholesterol levels, the difference between the groups supplemented with 2 g/kg and 10 g/kg bee pollen was found statistically significant ($P<0.05$), while the serum cholesterol levels of the other groups were similar to these two groups. Desoky and Kamel (2018) indicated that the supplementation of bee pollen at the level 2 g/kg to the diet decreased serum cholesterol level of laying quails. Similarly, Demir and Kaya (2020) reported that serum cholesterol decreased linearly in laying hens by adding bee pollen (0, 0.5, 1.0 or 1.5%) to the diet. Similar results were stated in the results of researches conducted with broilers (Fazayeli-Rad et al., 2015; Farag and El-Rayes, 2016), roosters (Abuoghaba and Ismail, 2018) and laying hens (Rizk et al., 2018). In the present study, although there was no difference with the control group, there was a high decrease in serum cholesterol level with the addition of bee pollen at levels of 8 and 10 g/kg to the diet, 15.96% and 28.17%, respectively.Rizk et al. (2018) reported that there was a decrease in serum cholesterol levels with the addition of bee pollen to the diets due to the high levels of unsaturated fatty acids such as oleic, linoleic and linolenic acids contained in bee pollen.

The effect of diet supplementation with bee pollen on serum calcium level was statistically significant ($P<0.01$). The serum calcium levels of quails that received 4 and 10 g/kg bee pollen were the highest while the respective level in quails of the control group was the lower. The effect of bee pollen supplementation on serum phosphorus levels in the diets of laying quails was statistically significant ($P<0.05$). The highest serum phosphorus level was detected in the group supplemented with 10 g/kg bee pollen and was statistically significant compared to values of groups supplemented with 0, 2, 4 and 6 g/kg bee pollen.In previous studies, it was stated that the serum calciumlevel was not affected by the addition of pollen to the diet (Desoky and Kamel, 2018; Demir and Kaya, 2020), and the serum phosphorus level was not affected (Desoky and Kamel, 2018) or decreased (Demir and Kaya, 2020) in layer birds. These results are in disagreement with the results of current research. Farag and El-Rayes (2016) stated that the bee pollen contains 0.35% calcium and 0.03% phosphorus. It can be said that this increase in serum calcium and phosphorus levels may have been caused by the calcium and phosphorus contained in bee pollen. However, it should be noted that the increase in serum mineral level does not reflect positively on the eggshell quality.

CONCLUSION

The addition of bee pollen to laying quail diets negatively affected feed intake (10 g/kg) and eggshell breaking strength (6 g/kg), and positively affected serum calcium and phosphorus levels. Since bee pollen tends to decrease the serum cholesterol level in quails, it can be said that it is beneficial to conduct further researches on the pollen-cholesterol relationship.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Epidemiology, burden and seasonal variation of fasciolosis determined through faecal examination and excretory/secretory antigens based ELISA

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ABSTRACT: The study was conducted to evaluate predominance and the related risk factors of sheep fasciolosis in Faisalabad district, Punjab, Pakistan. In addition, comparison of the coprological examinationand excretory/secretory antigens (ES Ag)-based ELISA was also performed to determine the variation in the prevalence of fasciolosis. Oft-he1200 faecal and blood samples,128 (10.67%) and 241 (20.08%) samples were found positive for *Fasciola* infection and anti-*Fasciola* antibodies respectively.The prevalence of fasciolosis was significantly ($P < 0.05$) higher in female and adultanimals. Most of the animals showed moderate (55.47%) infection which was significantly higher than mild (28.91%) and severe (15.63%) infections. The highest prevalence of fasciolosis was determined in December (26.00% through faecal examination; 42.00% through ELISA) with a mean EPG of 842.3 while lowest in May (02.00% through faecal examination; 07.00% through ELISA) with a mean EPG of 650. The frequency distribution of fasciolosis was significantly ($P < 0.05$) highest in the winter (faecal examination 20.67%; ELISA 34.67%) followed in order by the monsoon (faecal examination 09.00%; ELISA 18.67%), spring (faecal examination 08.33%; ELISA 17.33%) and summer (faecal examination 04.67%; ELISA 09.67%). The highest mean EPG of fasciolosis was determined in the spring (894) followed in order by the winter (851.67), summer (654.67) and monsoon (616.33). Although the faecal examination is the gold standard and the "best method" in the diagnosis of sheep fasciolosis; however, the serological tests should not be excluded especially the home-made ES Ag-based ELISA which might be preferred and more affordable risk assessment tool in the field epidemiology.

***Keywords*:** Coprology; ELISA; Excretory/secretory antigens; EPG; Fasciolosis;

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INTRODUCTION

Fasciolosis, an important, emerging food and water-borne parasitic zoonosis occurring in human communities, is considered among the significant public health problems in several countries (Intapan et al., 2003). Fasciolosis is a significant problem of animals and the infection is brought about by the Digenean trematodes of the genus *Fasciola*, generally called as the liver flukes. Both juvenile and adult developmental phases of the parasite are responsible for pathogeneses and production losses in the final hosts. In temperate areas without enormous occasional climatic variations, for example Ireland, the management factors unequivocally impact the spatial distribution of the *Fasciola* sp. (Munita et al., 2016). Fasciolosis causes an enormous financial loss of approximately US$ 3.2 billion around the world (Ahmad et al., 2017; Khan et al., 2017).

The conclusive analytic test for *Fasciola* sp. is the liver necropsy which gives a profoundly exact diagnosis of the fasciolosis when bile ducts have the parasite (Ahmad et al., 2017). However, this cannot be a preventive management tool for the flock and/or herd health monitoring system because we can only apply this on the post-mortem examination (Mazeri et al., 2016). Oftenly utilized ante-mortem test for the diagnosis of fasciolosis is the identification of eggs in the faeces by the sedimentation method followed by the faecal egg counts (FEC) which is reported to have a higher specificity. The later i.e. FEC is the best tool for the detection of current infection and to determine a higher precision of the burden of the parasitic infection (Rizwan et al., 2017; 2019; Ahmad et al., 2020). However, FEC does not prove to be a sensitive diagnostic tool in cases of lower magnitude of parasitism or when the juvenile (non-reproducing) stages of the flukes are in the process of internal migration towards the predilection site, i.e. the bile duct (Brockwell et al., 2013).

The technique which depends on the antibody (Ab) detection will overwhelmingly be the favorable technique for the immunodetection of *Fasciola* sp. This is due to the overall simplicity of the method and an early sero-conversion during the premature disease. Thus, most of the specialists are currently using enzyme-linked immunosorbent assay (ELISA) for the immuno-detection of *Fasciola* sp. (Rizwan et al., 2016; Acici et al., 2017; Munita et al., 2019).The excretory/secretory antigens (ES Ag)-based ELISA is explored which not only detects infection but also gives a better picture of the infection as compared to the conventional diagnostic techniques in the preventive management strategy of the *Fasciola* sp. in the livestock. The current study was performed to (a) compare the prevalence of fasciolosis through the classical coprology and ES Ag-based ELISA owing to their capabilities to detect the exposure of the sheep population to the *Fasciola* sp., (b) determine the quantitative magnitude of the parasitic load through the eggs per gram (EPG) of *Fasciola* sp., and (c) analyze an association of season as an extrinsic determinant with the prevalence and EPG of the *Fasciola* sp.

MATERIALS AND METHODS

Collection and Processing of Samples

The study was conducted in the district Faisalabad, Punjab, Pakistan which is the third largest city of the country. The faecal samples (5 g) and blood samples (5 mL) were collected from the randomly selected 100 sheep/month of the pastoral communities for one year aseptically with the owners' consent. Following criteria was used for the selection of animals: (a) clinically health, (b) normal physiological parameters, and (c) no history of the fasciolicidal treatment over the past three months. The collected faecal samples from 519 young animals, 681 adult, 713 male and, 487 female animals were processed to identify the *Fasciola* sp. infection through the standard indirect qualitative faecal examination assay (sedimentation technique) as given by Hayat and Akhtar (2000). Eggs were identified following the standard identification keys (Soulsby, 1982).

Quantitative faecal examination for the assessment of parasitic burden was performed through the "Modified McMaster Test" as given by Mazeri et al. (2016). The animals found positive for the *Fasciola* sp. were categorized as mildly, moderately and severely infected according to the FEC of 100 to 600, 700 to 1000, and over 1000 per gram of faeces, respectively. The blood samples were collected from the jugular veins into the gel-clot-activator vacutainers and indorsed to clot for 35 to 40 minutes and shipped to the Department of Parasitology Lab., following the starndard operating procedures of the transportation. The supernatant was centrifuged (2500 rpm for15 minutes), pipetted, transferred to the pre-labeled eppendorf tubes, and stored at -20°C till further use.

Preparation of *Fasciola* Excretory/Secretory Antigens

The technique depicted by Anderson et al. (1999)

was followedfor the preparation of ES Ag of the *Fasciola* sp. as described elsewhere (Rizwan et al., 2016).Briefly, flukes in groups of around 20 were placed into a phosphate buffer saline (PBS) solution. Early regurgitates containing bile, debris and blood were removed just after twirling the flasks containing parasites and the procedure was revised multiple times. After proper washing of the parasites, 50 mL fresh PBS was added and incubated at 37°C for 6 to 8 hours. Then, the contents were centrifuged (2500 rpm) for 15 minutes, filtered through the 0.22 µm filter, and kept at -20°C. Spectrophotometery was applied to determine the protein (ES Ag) concentration at A280 through the Bradford technique.

Enzyme Linked Immunosorbent Assay

The ELISA test protocol as described by Rizwan et al. (2016) was followed. Briefly, 50 µL of the ES Ag (1/200 dilution) was added into each of the wells of a micro-titer plate and incubated overnight at 4°C.The plates were washed multiple times with 0.05% Tween 20 in a phosphate buffer saline (PBS) solution. For blockage of the nonspecific binding, 5% skimmed milk in PBS was added and incubated at 37°C for 60 minutes. Then, 50 µL serum with 1/100 dilution was added into the wells and incubated at 37°C for 60 minutes. Subsequently, 50 µL HRP-labeled rabbit anti-sheep IgG with 1/5000 dilution was added to each of the wells and incubated for 60 minutes at 37°C. Between each progression, plates were washed multiple times with 0.05% Tween 20 in PBS. After this, tetra-methyl benzidine was added and incubated for 10 minutes. Then, 50 µL of 0.6 N Sulphuric acid was added to stop the reaction and the optical densities were measured through an ELISA reader at 450 nm wavelength.

Data Analyses

Differences between independent variables (breed, sex, age and season) concerning the prevalence of *Fasciola* sp. were explored using the Chi-square test (categorical variables). Data were analyzed using Minitab 17 software.

RESULTS

The overall prevalence of fasciolosis determined by the faecal examination (FE) was 10.67%, and that determined by ES Ag-based ELISA was 20.08%. The frequency distribution of fasciolosis was significantly higher in females than males and in adults than young stock (Table 1). Most of the animals showed moderate (55.47%) infection which was significantly higher than mild (28.91%) and severe (15.63) infections (Table 2). The highest prevalence of fasciolosis was determined in December (26.00% through the FE; 42.00% through ELISA) with a mean EPG of 842.3.In contrast, the lowest prevalence of fasciolosis was determined in May (02.00% through the FE; 07.00% through ELISA) with mean EPG of 650 (Fig. 1). The prevalence of fasciolosis was significantly higher in winter followed in order by the monsoon, spring and summer (Table 3).The highest mean EPG of fasciolosis was determined in spring (894.00) followed in order by winter (851.67), summer (654.67) and monsoon (616.33).

Table 1. Frequency distribution of fasciolosis in sheep population of Faisalabad district, Punjab, Pakistan determined by the faecal examination and excretory/secretory antigens-based ELISA

Variable	Level	Faecal Examination					ES Ag based ELISA				
		Examine	Positive	Prevalence	Chi-Square	P-Value	Examine	Positive	Prevalence	Chi-Square	P-Value
Breed	Kajli	497	58	11.67	1.657	0.437	497	105	21.13	1.065	0.587
	Thali	327	29	8.87			327	61	18.65		
	Lohi	376	41	10.90			376	75	19.95		
Age	Young	519	30	5.78	22.915	0.000	519	84	16.18	20.308	0.000
	Adult	681	98	14.39			681	157	23.05		
Sex	Male	713	50	7.01	24.618	0.000	713	103	14.45	28.019	0.000
	Female	487	78	16.02			487	138	28.34		

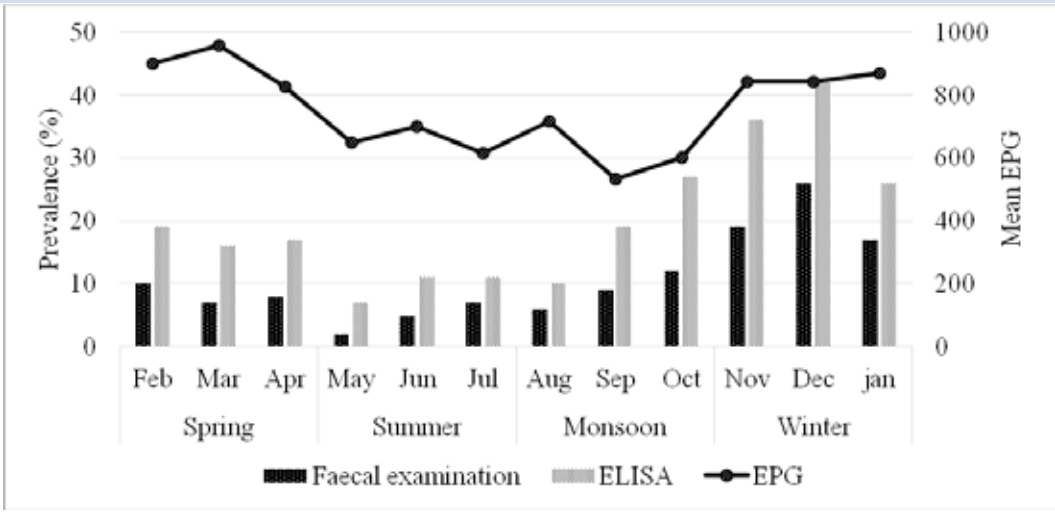
Table 2. Faecal egg count-based severity of the sheep fasciolosis in district Faisalabad, Punjab, Pakistan

Severity	Examined	Positive	Prevalence (%)	Mean Number of Eggs Per Gram (EPG)	Std. Dev.	Minimum	Maximum
Mild	128	37	28.91	364.9	170.3	100	600
Moderate	128	71	55.47	836.6	103.1	700	1000
Severe	128	20	15.63	1320	220.3	1100	1800

Table 3. Comparative prevalence of the sheep fasciolosis through the faecal examination and excretory-secretory Ag-based ELISA in Faisalabad district, Punjab, Pakistan

Diagnostic tool	Spring	Summer	Monsoon	Winter
Faecal examination (mean eggs per gram; EPG values)	8.33 (894.00)	4.67 (654.67)	9.00 (616.33)	20.67 (851.67)
Excretory-secretory Ag- based ELISA	17.33	9.67	18.67	34.67

Figure 1. Mean EPG and month-wise prevalence of fasciolosis determined by faecal examination and excretory/secretory antigens based ELISA



DISCUSSION

Fasciola sp. has an across-the-border dispersion in Africa, South America, Eastern Europe, Middle East, Eastern and South Asia (Matanovic et al., 2007). Liver fluke is a significant parasite affecting6 to 7 million ruminants around the world every year (Coma et al., 2009). The death rates due to fasciolosis rely upon the type of illness, whether chronic or acute with a high death rate being recorded in the acute form (Reddington et al., 1986). The subclinical and chronic type of fasciolosis can lead to the reduced performance and the shrouded financial losses. In chronic infections, the economic losses occur due to the delayed puberty, lowered fertility rates, milk yield, meat production, birth weight, and quantity and quality of fleece (Soulsby, 1986; Ahmad et al., 2017).

The snail acts as an intermediate host of *Fasciola* sp. which propagates in the marshy areas close to the ponds and lakes. Fasciolosis has been reported in various areas of Pakistan by many researchers in different periods (Anjum et al., 2014; Rizwan et al., 2016; Ahmad et al., 2017; Zafar et al., 2019; Rizwan et al., 2019), however, the prevalence and association of fasciolosis with various factors in sheep population have not been well-documented in the district Faisalabad.

The prevalence of fasciolosis determined by Utuk et al. (2012) was 4.9% through faecal examination, 15.6% through an ES Ag-based ELISA and 16.2% through the commercial ELISA kit. The outcomes of the serological tests were seen to coincide while faecal assessment yielded a lower efficacy rate consistent with the review of literature. Besides, 41.8% of sheep recognized negative by sedimentation were seen as positive by sandwiched ELISA test. In a study conducted by Gonenc et al. (2004)in the Central Anatolia, all the seropositive animals (through western blot analysis) presented *Fasciola* sp. eggs during the faecal examination. This was decoded by specialists that the juvenile flukes serologically gave a positive response while moving to the liver parenchyma and as they were juveniles, no egg was found in the faecal samples. In our investigation, the contrasts between the faecal assessment and serological tests can be associatedwith the primary stage of infection.

The variation in the prevalence of *Fasciola* sp. may be due to the method used for the identification, availability of snails, temperature (> 9.5°C),location, sample size, annual rainfall rate, flooding during rainy season, humidity, soil moisture and outdated pasture management practices (Coma et al., 2009).The variations in the grazing practices (grazing near the marshy areas)and agro-climatic conditions also have sound

effects on the frequency distribution of *Fasciola* sp. in animals. All these factors directly or indirectly affect the propagation of *Fasciola* sp. Besides, adjustment of unhygienic measures, irrational utilization of the medicine and indiscriminate dealing of the livestock can contribute in the development of infection (Khan et al., 2011; 2013).

Similar to the present study, Hassan et al. (2011) and Rizwan et al. (2016) found a higher incidence of fasciolosis in adult animals than in the young stock. The higher predominance in adult sheep may be because of their standard grazing close to the marshy areas, compromised immunity of the host, prolonged contact with the infectious agents and long inactive stage in the final host (around 5 to 6 months). However, Anjum et al. (2014) and Zafar et al. (2019) recorded a higher rate of infection in younger animals. The higher predominance in young sheep may be expected due to the low level of immunity development in young sheep which are not completely grown up.

The females were seen as more prone to fasciolosis than males in other studies as well (Khan et al., 2013; Rizwan et al., 2016; Zafar et al., 2019).However, in some investigations directed by Khan et al. (2011) and Gebeyehu et al. (2014),an insignificant association betweenthe sex and abundance of fasciolosis was found. The possible reasons for higher prevalence of fasciolosis in females are: rearing of females for a long time increasing the exposure of infection, consistent change in physiological parameters especially during pregnancy and lactation leading to immunosuppression, unavailability of proper nutrition especially during production and reproduction phase of life and nonstop grazing of sheep in and around the marshy areas (Najib et al., 2020).

In the present study, breed of host showed insignificant association with fasciolosis; however, Anjum et al. (2014) in Pakistan and Munita et al. (2019) in Ireland found a significant association. Anjum et al. (2014) documented a significantly higher frequency in Kajli (46.32%) breed followed in order by Lohi (37.43%), Cholistani (8.24%) and Thalli (7.90%). In Pakistan, still there is very few documented data available regarding the susceptibility of fasciolosis in different breeds of sheep. However, the insignificant association of different breeds of sheep with fasciolosis might be due to the rearing and grazing of different breeds together (Anjum et al., 2014).

The range of EPG of flock determined by Martínez - Valladares et al. (2013) was 0-154 with a mean of 17.5± 33.9. About 21% of flocks showed a medium/high level of severity with mean EPG higher than 40. A study conducted by Duthaler et al., (2010) found 46.0% of animals infected with moderate infection, 45.1% infected with heavy infection and 3.5% with low infection. An insignificant association of severity of infection was determined by Carneiro et al. (2018) i.e. about 68%, 59.36% and65.88% animals showed mild, moderate and heavy infections, respectively. This variation in the severity of infection might be due to differences in grazing patterns, contamination of grazing areas, rate of rainfall and availability of snails.

In Iran, the highest prevalence of fasciolosis was determined in spring (8.3%) while the lowest (4.0%) in summer (Khanjari et al., 2014).In Pakistan, Anjum et al. (2014) noted a significant influence of time of year on the occurrence of fasciolosis i.e. highest in winter and lowest during summer. Because, the development of intermediate host snail and hatching of eggs of parasites require suitable temperature (> 9ºC), high humidity and rainfall (Taylor, 2012). In Pakistan, such environmental condition can be found in the monsoon when numerous parasites eggs hatch, propagation of snail's increases and afterward cercariae are produced, and are discharged on wet fields before encystation onto herbage. The frequency distribution of fasciolosis in different seasons has also been determined by various scientists (Khanjari et al., 2010; Ali et al., 2011; Rizwan et al., 2016). *Fasciola* sp. Inclines towards the temperate climatic regions as its 18-30-weeksof the lifecycle requires mild temperature and high moisture both for the propagation of the intermediate hosts and the free-living stages of the parasite (Relf et al., 2011; Ducheyne et al., 2015). The necessity of explicit climatic conditions for the completion of its lifecycle leads towards the cyclic variation in the animal disease (Bloemhoff et al., 2015).

In conclusion, although faecal examination is the "best method" and the gold standard in the diagnosis of sheep fasciolosis; however, serological tests must not be excluded especially the home-made ES Ag based ELISA which might be preferred and more affordable (Utuk et al., 2012).Likewise, the propagation of the snail, rainfall, soil moisture, and temperature are the significant elements affecting the growth of *Fasciola* sp. from egg to miracidium development. Even these physiological factors are directly influencing the rate of development of the fluke inside the snails and/or the final hosts (Najib et al., 2020). Hence, local and yearly

differences in the frequency distribution of *Fasciola* ap. are related to the fundamental climatic conditions. The diagnosis of fasciolosis by home-made ES Ag-based ELISA is effective and more affordable risk assessment tool in the field epidemiology. There is need to find the specific antigens from the ES product to reduce the chances of false positive results.

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Research article
Ερευνητικό άρθρο

A report on less-severe, long-duration persistent hind-limb ischemia surgical rabbit model

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ABSTRACT: Development of a persistent hind limb ischemia rabbit model without excessive adverse symptoms and compromised animal welfare.

New Zealand White (n=18, male and female) rabbits of 3-4 months of age and 3.0± 0.1 Kg body weight were used. The surgical technique for ischemia avoided the ligation of vessels above the inguinal ligament and included only the ligation and complete excision of the common and superficial femoral arteries along with all their branches up to popliteal and saphenous arteries. Study duration was 84 days.

All animals completed the study period uneventfully. The activity of the animals remained unaffected throughout the study except for the first post-operative day. Adverse symptoms of other models such as loss of limb due to necrosis, loss of nails and necrosis of skin were not observed while successful ischemia was confirmed. There was a significant decrease (P=0.0381) in ischemic right limb circumference.Terminal angiography by abdominal aortic cannulation in the animals demonstrated negligible amount of angiogenesis at the distal ischemic thigh in comparison to the control limb (P=0.001).

This study reports successful development of a refined chronic hindlimb ischemia rabbit model.

***Keywords*:** Hind Limb Ischemia, Rabbit Model, Welfare, Thigh muscle atrophy, Angiography

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INTRODUCTION

In hindlimb ischemia, the obliterated blood supply to the legs results in intermittent ischemic claudication pain, rest pain, ulceration, and gangrene in human patients. In a normal healing process, spontaneous angiogenesis and revascularization ensure adequate reperfusion of blood to tissues in cases of ischemia in younger individuals and in cases of less severity in the aged. However, the occurrence of revascularization is obviously nonexistent in adult humans with severely aggravated ischemic condition. Unlike human patients, animals have the potential to spontaneously recover from ischemia (Dragneva et al., 2013) since they show an extraordinary capability to form collaterals and they regain normal arterial perfusion within a short period of time. Therefore, the symptoms as well as recovery at the cellular level differs between animal models and humans.This fact makes it difficult to produce animal models of chronic persistent ischemia.

For studies of hindlimb ischemia, researchers tried to develop animal models with ischemia in various animal species (Klausner et al., 1988; Belkin et al., 1989; Belkin et al., 1990; Blebea et al., 1990; Freischlag & Hanna 1991; Sternbergh III & Adelman 1992; Sternbergh III et al., 1994; Blaisdell, 2002) with highly varying results. Mice, rabbits, and pigs are preferred for peripheral arterial ischemia studies owing to a large extend of baseline collateral vasculature and in their closer resemblance to human vascular anatomywhilerats,cats, rabbits,and dogs are used due to their relative simplicity to produce models(Waters et al., 2004). Ischemia produced by the simple ligation of arteries isinadequate for a treatment study as this results in only a partial ischemia (Seifert et al., 1985; Hendricks et al., 1990) for a short period. To overcome this, experimental animal models were created by inducing ischemia in the hindlimb by ligation or excision of the iliac artery (Skjeldal et al., 1991), femoral artery (Kalka et al., 2000; Milia et al., 2002) or both the femoral and saphenous arteries (Kanno et al., 1999; Byun et al., 2001). Occlusion of both the femoral artery and vein by strangling the thigh with a tourniquet to produce hindlimb ischemia was also reported in some studies (Wiersema et al., 2000; Messina et al., 2002). Aortic ligation produced models of high severity and the contralateral limb could not be used as the control (Weiss, 1974) since it affects both the hindlimbs.

Most of these above demonstrated models cause highly damaging side effects with mortality and acute lesions rendering a model of high-severity and clinical signs. Hence, most of the studies that use ligation of femoral arteries and its branches enumerate clinical symptoms as part of observations. Influx arterial ligation and excision at the level above inguinal ligament renders the animals with skin necrosis of hind limb (Varnavas et al., 2010), blackened toenails and limping (Baffour et al., 1992), nonfunctional hind limb (Pu et al., 1994) or limb necrosis (Kyriakides et al., 2001). Reports of mortality are also made by various groups owing to uncontrollable damage and complications resulting by the generation of models of high-severity (Waters et al, 2004).

There exists a necessity to report models of mild to moderate severity and to deliver chronic hind-limb ischemia to study proof of concepts in preclinical research. The model is required to be of high survival rate to aid research and at the same time with milder symptoms to benefit its welfare. In this study, we report the development of a persistent ischemic model and its confirmation in New Zealand White rabbits without any notable clinical symptoms with a 100% survival rate. This safe, simple and effective technique was postulated to withdraw unnecessary animal suffering to create a rabbit model of hind limb ischemia with mild severity to suit pre-clinical studies to evaluate angiogenesis.

MATERIALS AND METHODS

Animals

Eighteen New Zealand white rabbits of either sex (nine males and nine females) with an age of 5-6 months and weight of 3.0±0.1 kg were selected randomly from the colony. Resource equation method was used to find the sample size for a non-blocked study design in which the animals underwent a single treatment. Pre-clinical studies that aim to scrutinize potential therapies are widely encouraged to see the results in both males and females to extrapolate and rule out any sex-influence in interpretation of the results. Hence this study also used both the sexes of animals. Each individual animal formed an experimental unit in this study. Blinding of groups was not applicable since there was only one group involved and the investigators were scoring the clinical signs of all animals in the study. The animals were bred and housed in the Division of Laboratory Animal Science (DLAS) of Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Bio Medical

Technology Wing (BMT Wing), Thiruvananthapuram, Kerala, India. Health monitoring was carried out in accordance with the FELASA guidelines (Nicklas et al., 2010) and the animals were free from any diseases.Ethical clearance was obtained from the competent authority (Institutional Animal Ethics Committee).

Housing Conditions

The animals were housed individually by following the floor space recommendations proposed in "Guide for the Care and use of Laboratory Animals" (NRC,2010) which is in unison with the recommendations of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) Guidelines, Ministry of Fisheries, Animal Husbandry and Dairying, Government of India. The animals were fed with *ad libitum* standard rabbit pelleted feed (Amrut Laboratory Animal Feeds, India) and U.V sterilized drinking water. Treats were provided daily with carrots, sprouted Bengal gram, fresh chopped green grass and cabbages; all washed and cleaned with potassium permanganate solution (1 in 1000). A 12/12 h automated lighting schedule was provided with an intensity not exceeding 325 Lux at 1-meter height from the floor. The temperature of 22±2°C and relative humidity of 30-70% with 12-15 air exchanges per hour were provided in the experimental animal rooms and the supplied fresh air was HEPA filtered. Cage changes were done in every alternate day. Faecal and urine trays were removed daily and replaced with a cleaned one. All the experiments were carried out between 9.00am and 5.00pm.

Surgical technique

The animals were anesthetized with an intramuscular injection of xylazine (5 mg/kg) and ketamine (50 mg/kg). Pre-emptive analgesia and antibiotics were provided an hour before the surgery using a single dose each of meloxicam0.5mg/kg subcutaneously and cephtriaxone 15 mg/kg intramuscularly respectively.Under aseptic precautions, the entire right limb was shaven cleanly and swabbed with povidone iodine solution, and draped with sterile window drapes. The right femoral artery was exposed through a longitudinal skin incision in the medial thigh that extended from the inguinal ligament to the knee. At first, the common femoral artery was dissected along its total length and blunt dissection enabled the separation of artery from vein and nerve. This was followed by dissection of all of its branches distal to the inferior epigastric artery (deep femoral, lateral circumflex, and superficial epigastric arteries). Then, the dissection of the popliteal and saphenous arteries was done distally and all arteries were ligated. Finally, the ligated arteries with all branches were resected and removed (Fig 1). After muscle apposition with 3-0 Vicryl suture, the skin was closed with 3-0 braided silk. The animals were left to recover in a quiet area and upon complete recovery, the animals were left back to their respective home cages. All the animals received cephtriaxone 15 mg/kg intramuscularly once daily for 5 days. Meloxicam injection was given once daily at 0.5 mg/kg subcutaneously for 5 post-operative days and povidone iodine ointment was applied on the wound site until the skin sutures were removed on the 12^{h} -14^{th} post-operative day.

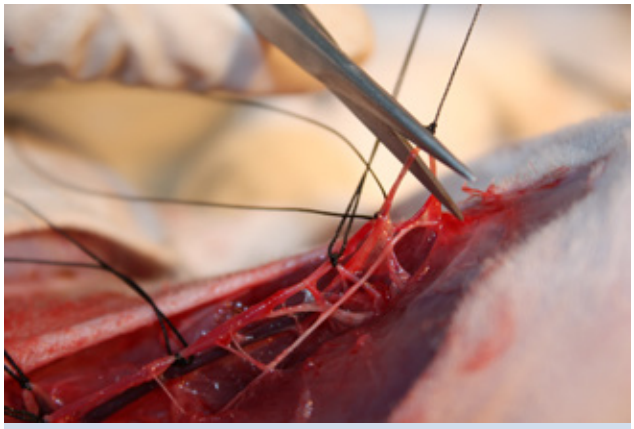

Fig. 1: Ligation and complete excision of the common and superficial femoral arteries along with all its branches up to popliteal and saphenous arteries

Clinical Assessments

All the assessments and procedures were performed by well experienced and trained personnel. Both the hindlimbs of rabbits were clinically evaluated daily until the end of the study. The state of surgical wounds, limping, gait and changes if any in movement patterns, changes of hair and skin, nails, and toes for 6 weeks after the operation were assessed using an objective score-sheet. Thigh circumference on day of surgery of both limbs was obtained and it was compared with the same limb post-operatively to assess muscular atrophy.

Home cage activity assessment

The animals after the intervention for the induction of ischemia were housed individually. Using a camera, (D-Link Surveillance system) five-minute videos were recorded and time of activity during this period was calculated. The observation was conduct-

ed one day before the day of surgery to obtain the baseline value, and then on 1st, 10th and 42nd and 84th post-operative days. Comparison was done with each animal's baseline value with respect to these post-operative days. Activity was defined as time spent on active grooming, drinking and eating, walking and rearing in the cage. Rest comprised of the time of sitting idle in the cage without exhibiting any of the activities enlisted above.

Angiographic analysis

Angiography was done on the 84th post-operative day. The animals were premedicated with xylazine at the rate of 5 mg/kg, intramuscularly and ketamine at the rate of 50 mg/kg, intramuscularly to induce the anesthesia.When the animals were under deep surgical anesthesia, under aseptic conditions laparotomy was performed with a mid-ventral incision towards the lower part of the abdomen and the abdominal aorta was exposed below the level of kidneys. Heparinisation was done with Heparin (Nuparin®, Troikaa, Gujarat, India) at the rate of 1.0 mg/ kg, intravenously ten minutes prior to catheterization of abdominal aorta to prevent thromboembolism during the vascular procedure.After looping the aorta with umbilical tape, arterial cannulation was done using 20G intravenous cannula with injection valve (Mediflon®, Eastern Medikit Ltd., India) and the cannula was secured *in situ* with 3-0 braided silk sutures. The aorta was ligated anteriorly to prevent the retrogradeflow of the dye while angiography was performed. The animal was positioned dorsoventrally to obtain angiography of both the hind limbs simultaneously. Patency and blood flow to thigh region and shank regions were assessed using 7 ml of Iohexol (Non-Ionic dye) "Omnipaque-350" (GE Healthcare, Ireland). For each rabbit, angiography was performed using Fluoroscopic "C" arm (Powermobil, Germany). An angiogram of four seconds duration was recorded at the level ofthe medial thigh and arterial count was done to compare the right and left thighs. The number of contrast-opacified arteries crossing over circles and the total number of circles encompassing the medial thigh area were counted in a single blind fashion. An angiographic score was calculated for each film.

Statistical analysis

The statistical tests were performed using GraphPad Prism 8.4.3 (686) (GraphPad Software, San Diego, CA, USA). All data are expressed as the Mean± SD. Normality of data was tested using D'Agostino & Pearson test and normal data was analyzed with repeated measures-one-way ANOVA whereas a non-parametric test, Friedman's test was used to analyze the non-normal data. Comparisons between pre-surgical and post-surgical right hindlimb and left hindlimb thigh circumferences were performed using repeated measures One-way ANOVA, with baseline value as control for respective limbs. For comparing the activity scores on days 1, 10, 42 and 84, the baseline value of activity of each animal was used as the control and a repeated measures ANOVA was done to find out differences in activity levels. Sidek's multiple comparison test was used to assess post-hoc pairwise differences when repeated measures one-way ANOVA was employed whereas Dunn's multiple comparison test was used for Friedman's test. For comparing the angiographic score, each animal's right limb was compared against its left limb using a student's *t*-test. A value of $P<0.05$ was considered to be statistically significant in the study.

RESULTS

Clinical Assessments

All the animals completed the study period uneventfully and are included in the analysis. After surgery, all the animals regained righting reflexes in 15±3 minutes after skin suture and recovered completely from anesthesia in 180±32 minutes after skin closure. Complete wound healing was observed in 12 ± 2days after surgery. All the animals showed general weakness of the right hind limb on the first postoperative day. No animals exhibited any visible clinical signs indicative of toe or skin necrosis owing to ischemia. No changes of hair and skin, nails, and toes could be observed during the entire study period.

Thigh Circumference Measurements

Pre-operative and post-operative thigh muscle circumference measurements of left limb (Fig. 2a) showed significant increase on days 10, 42 and 84 with respect to the measurements obtained as baseline showing an increase in size of growth attributable to the non-operated and normally well perfused limb ($P<0.0001$, $F(17, 51) = 1.477$). A significant decrease of the ischemic right thigh circumference was noticed on days 10, 42 and 84 (Fig 2b) with respect to the baseline circumference ($P=0.0381$, $F(17, 51) = 1.915$). The trends on left and right limbs with respect to the baseline thigh circumference of animals are given as Fig. 2c.

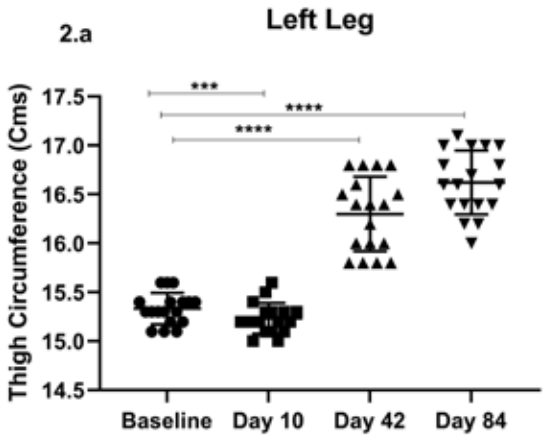

Fig 2a: Left thigh circumference (cm) over time. *** = P<0.001, ****=P<0.0001

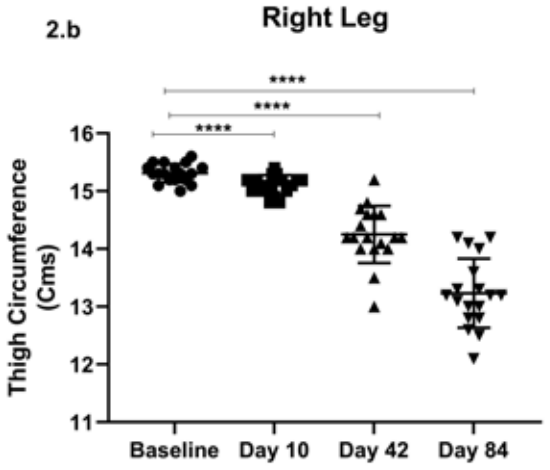

Fig 2b: Right leg circumference (cm) over time. ****=P<0.0001.

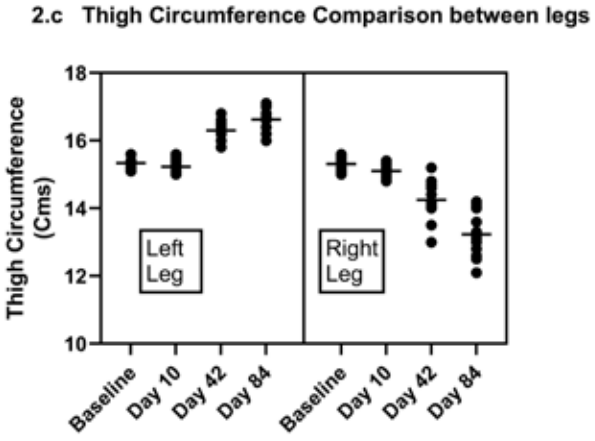

Fig 2c: The graph depicting the loss of thigh circumference of the perfusion deprived right leg with respect to the contra-lateral left leg showing increase in circumference during the entire period of the study

Home cage activity assessment

Significant decrease in activity was noticed on day 1 post-operatively in activity of the animals (P<0.0001, Friedman Statistic= 34.71) and no differences could be observed at any of the other time points (Fig 3).

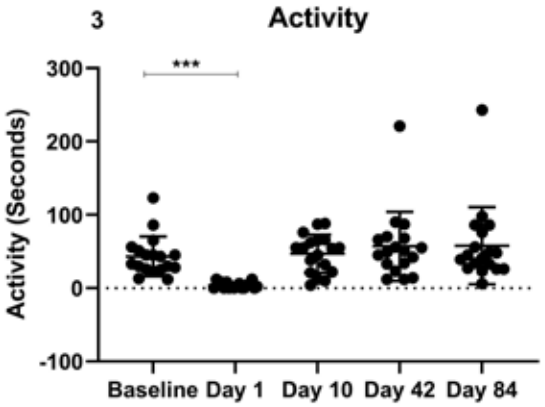

Fig 3: Reduced light phase-home cage activity levels on immediate post-operative day. The animals showed difference in repeated measures ANOVA in comparison to the control (baseline) value on the first post-operative day. *** = P<0.001

Angiographic analysis

The number of patent blood vessel counted differed between the right and the left limbs with consistently higher number of vessels in the left limb (P=0.001, t=21.31, df=34) (Fig 4). Hence this terminal angiography suggests a successful animal model which is consistent and persistent in the perfusion loss of the ischemic hind limb (Fig. 5). This method is a simpler one neither exposing the animal to procedures like cardiac puncture and radioactive substances exposure for the assessment and confirmation of animal model development nor requires repeated general anesthesia within the study period.

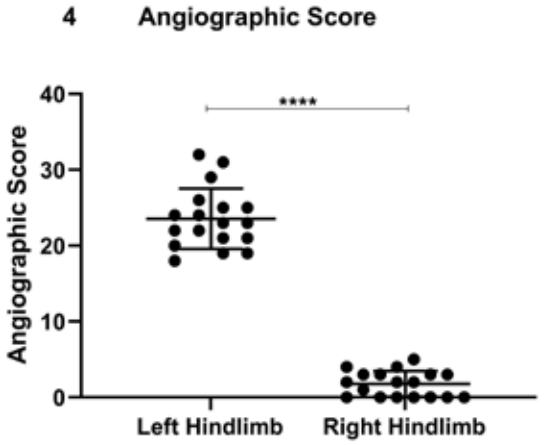

Fig 4: Angiographic score (patent-arterial count) on day-84 showing a significant loss of supply arteries in the ischemic right limb in animals. ****=P<0.0001

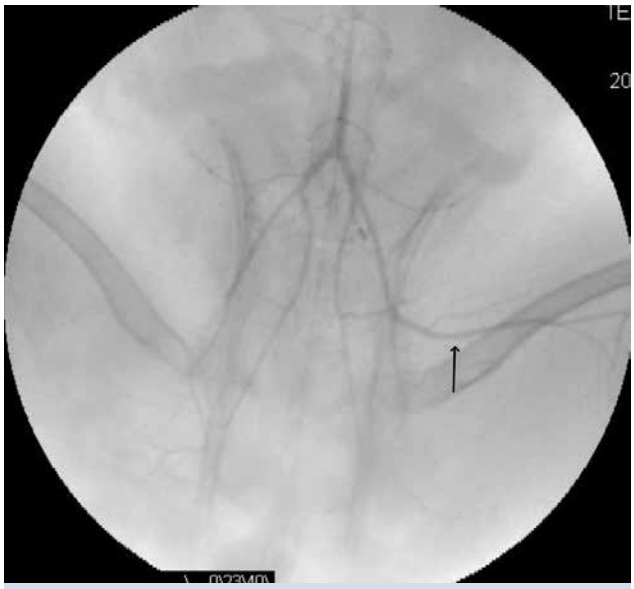

Fig 5: Angiograph on day-84 with contrast material showing loss of patent vasculature in the right limb in comparison with the intact left limb. Patent artery of the left leg is indicated with arrow head

DISCUSSION

Many animal species such as dogs exhibit widely established hind limb collateral pathways (Sunder-Plassmann et al., 1984) necessitating complicated procedures like identifying as many as 14 branches of iliac artery and their subsequent ligation to develop the animal ischemia model. There exist at least three collateral routes that take care of the supply when the femoral artery is surgically obstructed in species such as rats making it a difficult surgical model as well (Seifertet al., 1985). Specieswise differences of femoral arterial anatomy is very well documented and in comparison to murine hindlimb, rabbit femoral anatomy is different(Kochi et al., 2013). Limited baseline arterial network in hind legs and lesser collateral network make species such as mice, rabbits and pigs excellent animal models to study hindlimb ischemia to closely simulate human vascular anatomy (Waters et al., 2004). Of these favorable species, rabbits are widely used because of the optimum size of the animal unlike mice and pigs. Apart from ligation and excision of arteries, multiple techniques had been tried in the past decades in animal models like occlusion using pharmacologic and chemical agents resulting in high mortality of experimental animals (Longland, 1953). External tourniquet application was also a widely practiced technique in a wide range of species such as mice, rats, rabbits, cats, dogs and in non-human primates (Barie & Mullins, 1988). However, muscular necrosis occurring at around 6 hours post-ischemia and loss of the entire limb renders the technique less adoptable. Further, tourniquet application never results in chronic ischemia even though it produces acute ischemia making the animal model useful only to limited applications. To overcome these shortfalls, an animal model of surgical ligation followed by excision of major supply artery closer to the aorta at the level of the common iliac artery and the ablation of potential sources of collateral supply to produce profound chronic effects rather than performing simple distal occlusion of common femoral artery is demonstrated previously in rabbits (Gao et al., 2020; Waters et al., 2004). This technique documents a persistent model preventing short-bridging collateral vessel formation by ligating above the level of inguinal ligament. Clinically noticeable ischemia with limping of the affected limb on the first post-operative day and after the 10th post-operative day with marked atrophy has been reported (Pu et al., 1994). Superficial tissue necrosis and non-functional hindlimb and mortalities have been reported previously with this technique (Pu et al., 1994) andvarious groups had reported highly adverse clinical symptoms in rabbits ranging from dysfunctional leg and necrosis of skin and loss of nails (Waters et al., 2004).

For proofof concept studies, molecular and local evidence of persistent ischemia is the targeted outcome rather than adverse effects that affects animal's well-being. To achieve this, we planned to spare the influx arteries at the level of aorta and above inguinal ligament and distally ligate and resect local arteries to develop an animal model with effective loss of tissue vascularity. It was postulated that this approach could minimize or avoid the severity of symptoms.

The present work documents a method that could maintain hind limb ischemia successfully up to 12 weeks (84 days) with a modified technique by avoiding influx arterial ligation and excision but at the same time equally persistent in duration when compared to previous reports (Pu et al., 1994; Hong et al., 2001). However, in contrast to several previously reported studies, it was noticed that there existed no readily observable clinical signs in the animals except for a chronic reduction in the thigh circumference, hence resulting in an effective but less severe disease model. This model avoided common iliac artery ligation, which required a laparotomy and is less effective in producing ischemia.

Rabbit models have the ability of forming collaterals and regain the normal arterial perfusion with-

in a short period of time in three to seven days after occlusion of arteries (van Bemmelen et al., 2007). Dissection of the complete femoral artery and its side-branches produced deep distal ischemia in this study.When compared to the established methods (Waters et al., 2004), the present study involved more refined techniques by not involving radioactive chemicals, redundant anesthesia to the animals within the study period and procedures like cardiac puncture under general anesthesia. These techniques ifrepeated within the study period could affect the percentage of survival and there are possibilities that the increased stress levels can adversely affect the efficacy of treatment adopted in studies of angiogenesis.

The right leg exhibited ischemic atrophy and progressively lower thigh circumference measurements. Interestingly, a significant increase in left thigh circumference was also noticed which is due to compensatory hypertrophy since the left limb was more used to bear weight predominantly after surgery. The home cage activity reduction observed on the first post-operative day may beattributable more to the surgical pain rather than to an effect of arterial perfusion of the operated leg since this phenomenon could not be observed on other post-operative days. Since analgesia was administered daily, the adequacy of coverage of meloxicam needs to be probed into in rabbits in similar surgeries. Multimodal analgesia shall be tried to bring in better welfare in similar procedures. This work demonstrates that less tedious confirmatory measures can be adopted to replace repeated invasive techniques that are stressful to the animal model. Animal activity as a measure of welfare is often overlooked by scientists. This parameter can be of great utility since it is totally non-invasive. It could be useful in analyzing the difference between groups in terms of limb functionality and as a measure of general physical well-being. The terminal angiographic scoring which is a non-recovery procedure avoids the exposure of the animal to invasive diagnostic procedures or radioactive substances for the confirmation of model development and also avoids repeated general anesthesia within the study period.

CONCLUSION

Less severe animal models that are equally effective and validated are a necessity to evaluate proof of concept in preclinical studies involving hind-limb ischemia. Report on loss of hind limb blood supply and establishment of atrophy with no obvious adverse clinical signs by a modified surgical approach in a group of experimental rabbits is presented. This technique avoids laparotomy and minimizes the chances of animal mortality and at the same time ensures the effectiveness in providing an animal model useful to study hind limb ischemia.

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CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

Effects of *Alchemilla vulgaris* on haematology and antioxidant status of heat-stressed quails during the late laying period

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ABSTRACT: The effects of *Alchemilla vulgaris* (AV) on haematology and serum, liver, and ovarian antioxidant status of heat-stressed quail in the late laying period were observed in this study. A 2×3 factorial design was used with 0, 1 and 3% AV fed in thermoneutral (TN) and heat stress (HS) conditions. A total of 150 quails were randomly assigned to six groups. The quails were located in temperature controlled rooms. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and platelet distribution width (PDW) obtained in quail fed 1% AV were higher than in 3% AV under both TN and HS conditions. Comparing 3% AV to 1% AV, the concentration of MCH obtained for 1% AV was higher in HS and lower in TN conditions. Besides, quails fed for 1% AV had a lower procalcitonin (PCT) value in HS than 3% AV but this PCT value was the same in TN. The serum malondialdehyde (MDA) was lower in 1% AV than 3% AV in both HS and TN. The ovarian MDA was lower in TN than HS. In both TN and HS conditions, the ovarian MDA value was determined higher for 1% AV than for 3% AV. The liver glutathione (GSH) value was higher in 1% AV than 3% AV in both TN and HS conditions. The Total Oxidant Capacity (TOS) value was found higher for 3% AV in TN and 1% AV in HS. The serum GSH, TOS, and oxidative stress index (OSI) values were lower for 3% AV compared to 1% AV for both TN and HS conditions, whereas for MDA value this was the opposite. The ovarium MDA and TOS values were lower for 3% AV than for 1% AV in both TN and HS. Also, the liver MDA, GSH, and Total Antioxidant Capacity (TAS) values were lower for 3% AV than for 1% AV in both TN and HS conditions. Finally, dietary AV has been shown to have a partial antioxidative effect on the defense system and also has effect on red blood cell profiles and platelet counts rather than white blood cell profiles.

Keywords: Blood parameters, *Coturnix coturnix japonica*, lady's mantle, oxidative stress

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INTRODUCTION

Eggs have an important place in human nutrition (Tunsaringkarn et al., 2013). High environmental temperature causes severe problems in poultry eggs' production (Melesse et al., 2011). Heat stress (HS) arising from high environmental temperature not only adversely affects the production of laying hens but it also inhibits their immune function (Mashaly et al., 2004) compromises their welfare, and impinges upon food quality and safety (Shane, 1988; Lara and Rostagno, 2013). Thus, understanding and controlling environmental conditions is crucial to success in the meat and egg production in the poultry breeding systems (Lara and Rostagno, 2013).

When the environmental temperature (ET) exceeds the limits of the thermoneutral zone (TN) (16-25 °C), the body temperature of poultry increases (Melesse et al., 2011). High environmental temperature causes oxidative stress (OS) in the body, causing the depletion of antioxidant substances. Moreover, imbalance in the body due to excessive production of free radicals and excessive lack of antioxidants in the event of OS leads to damaged proteins, lipids, and DNA, as well as numerous diseases (Sakac & Sakac, 2000; Dalle-Donne et al., 2006). As a result, under the HS conditions yield losses, high mortality rates, and nominal profitability can occur (Sahin & Kucuk, 2003; Akdemir et al., 2015).

Antioxidant substances or natural herbs can be added to poultry diets for protecting the cells from the damaging effects of oxidative stress (Shane, 1988). Indeed, it has been demonstrated by many previous studies (Oktyabrskay et al., 2009; Akdemir et al., 2015). The degree of oxidative stress is indicated by some specific parameters such as malondialdehyde (MDA), total antioxidant capacity (TAS), total oxidant capacity (TOS), and oxidative stress index (OSI). Also, MDA is the specific biomarker of lipid peroxidation (Lykkesfeldt, 2007; Singh et al., 2014). Therefore, antioxidant parameters can be used as a meaningful indicator in poultry exposed to HS.

Alchemilla vulgaris (AV) is a biologically active herb important for humans as an immunomodulatory agent, which is rich in phyto components. It is widely referred to as Lady's mantle, Bear's foot, or Lion's foot, and used as a public medicine in European countries to a significant extent (Al-Osaj, 2016). The bioactive composition of AV is given in Table 1.

Table 1. The amounts of selected phenols in *Alchemilla vulgaris* (Vlaisavljevic et al., 2019).

Compounds	Amounts (μg/g dry weight)
p-Hydroxybenzoic acid	135.45
Protocatechuic acid	255.94
2,5-Dihydroxybenzoic acid	45.74
p-Coumaric acid	470.21
Gallic acid	2465.79
Esculetin	353.03
Caffeic acid	1138.54
Ferulic acid	346.87
Genistein	94.01
Apigenin	501.97
Naringenin	15.40
Luteolin	638.09
Kaempferol	364.00
Catechin	8144.98
Chrysoeriol	222.63
Quercetin	4541.70
Chlorogenic acid	23.13
Apigenin-7-O-b-glucoside	141.6
Luteolin-7-O-b-glucoside	329.71
Quercitrin	70.37
Kaempherol-3-O-glucoside	1038.52
Quercitrin-hexosides	2274.85

*Phenolic compounds were quantified in ethyl-acetate.

AV is a member of plants with antioxidant properties, has important biological efficacy such as antimicrobial, anticancer, antidiarrheal, antiarthritis, diuretic, depurative, intestinal antiseptic, and improving menopausal irregularities (Spiridonov et al., 2005; Neagu et al., 2015). AV is widely used by the public as a phytotherapeutic agent for the treatment of various disorders (Havsteen, 2002; Spiridonov et al., 2005; Neagu et al., 2015). Moreover, Havsteen (2002) reported that the biological effect of AV is largely due to its content of flavonoids. They are the most forceful and abundant ingredients of AV, which activate the antioxidant enzymes in OS conditions (D'agostino et al., 1998; Oktyabrskay et al., 2009).

AV has antioxidant, antibacterial, antifungal, and anti-inflammatory activities, also the remarkable biological activity of its extracts, as well as their full biocompatibility with fibroblasts and keratinocytes (Vlaisavljevic et al., 2019). The high amount of phenolic compounds in methanolic and ethyl-acetate extracts of above ground parts and roots of AV especially they were rich in condensed tannins (Boroja et al., 2018; Vlaisavljevic et al., 2019). AV is recommended to protect against hepatotoxicity in rats, and this effect is dependent on the antioxidant content of AV and its free radical scavenging effect (El-Hadidy et al., 2018). It is reported that the Rutin (is a member of flavonoids) contained in AV, increases the lymphocyte levels of broiler under normal environmental conditions (Hassan et al., 2018). Besides, there are some studies on the effects of flavonoids or flavonoid-containing plants on poultry defense systems and antioxidant parameters (Surai 2013; Ma et al., 2014).

Oxidative stress accelerates the aging process by causing changes in body functions, and aging-related diseases and complications are prevented by changes in antioxidant support or antioxidant enzyme systems (Kregel et al., 2007). The serum total antioxidant capacity of elderly individuals is lower than that of adult subjects. Serum oxidant status and oxidative stress index of elderly subjects were found to be significantly higher in adults (Yalçın, 2018).

In a study on the potential for mitigating effects of heat stress through dietary AV supplementation during the late laying period of Japanese quail (Coturnix coturnix japonica); in HS quail supplemented with 1% AV, egg production was reduced and FCR was increased compared with the other treatments. Dietary AV was found to reduce egg production in TN conditions, but 3% AV supplementation in the HS group prevented decreased egg production and improved FCR. Various indicators of egg quality were significantly affected by supplementation with AV at certain times during the experiment. Most effects of HS on egg quality were manifest in the first 15 days of ET regimes. Although HS significantly decreased eggshell weight until 31-45 days, AV supplementation improved it on the 45th day and then maintained it through the end of the experiment. Thus, AV may mitigate some effects of HS by partially preventing decreased egg production and increased FCR during the late laying period of Japanese quail (Akdemir et al., 2019).

Although there have been many studies on the use of AV in the field of biological and metabolic disturbances, no studies have been found on the effects on the haematological parameters and serum, liver, and ovarian antioxidant parameters in poultry. Therefore, considering that antioxidant capacity decreases more with age, the present study aimed to determine the effects of dietary AV supplementation on the haematological parameters and serum, liver, and ovarian antioxidant status in the late laying period of heat stressed quails.

MATERIALS AND METHODS

One hundred and fifty 20-wk old Japanese quails (purchased from Insanay Kanatli Hayvan Uretim Paz. Tic. Inc., Elazig, Turkey) were used in this 75 days experiment. After an adaptation period (10 days), birds with an average body weight of 197.8 ± 2.3 g were randomly assigned to 6 groups of 25 birds, and each group was subdivided into 5 replicates with 5 birds per cage. The quails in TN group were housed at 22 ± 2 °C/24 hours/day, and the quails in HS group were housed at 22 ± 2 °C /8 hours/day between 09:00-17:00 hours, at 34 ± 2 °C /16 hours/day for the rest of the day, in temperature-controlled rooms throughout the experiment. The experiment was approved (approval document no 2018/A-22) by the Committee on Animal Research at Inonu University, Malatya, Tyrkey, and conducted to Akcadag Vocational School Division of Inonu University.

In this study, 2 (ET; TN and HS) x 3 (basal diet supplemented with AV at 0, 1, and 3 %) factorial design was used. These doses have been determined considering the use of herbal additives used in similar studies in close or similar doses. Also, it was deemed appropriate to handle low doses since the effect of AV was not fully known. Quails were fed one of three

diets (Table 2), namely a basal diet or the basal diet supplemented with 1% or 3% of *Alchemilla vulgaris* in powder form (Altinterim Co., Elazig, Turkey). They were housed in cages providing 100-120 cm^2 floor area per bird. The birds were exposed to a 16 L:8 D illumination cycle for 75 days. Diets and freshwater were offered for *ad libitum* consumption throughout the experiment.

Table 2. Ingredients and nutrient composition of the basal diet (%)[1] fed to Japanese quail during the late laying period

Ingredient	Amount (%)
Corn	54.34
Soybean meal	28.91
Soy oil	4.96
Salt	0.31
DL-methionine	0.19
Limestone	9.26
Dicalcium phosphate	1.68
Vitamin and mineral premix[2]	0.35
Nutrient composition (%, dry matter basis)	
Crude protein	18.09
Calcium	3.73
Phosphorus	0.63
Methionine[3]	0.42
Lysine[3]	1.04
Calculated metabolizable energy kcal/kg[3]	2912

[1]1% or 3% of *Alchemilla vulgaris* was added to basal diet at the expense of corn for the supplemented diets

[2] Per kilogram, retinyl acetate: 1.8 mg, cholecalciferol: 0.025 md, dl-tocopheryl acetate: 1.25 mg, menadione sodium bisulfite: 2,5 mg, thiamine-hydrochloride: 1.5 mg, riboflavin: 3 mg, niacin: 12.5 mg, d-pantothenic acid: 5 mg, pyridoxine hydrochloride: 2.5 mg, vitamin B12: 0,0075 mg, folic acid: 0.25 mg, choline chloride: 125 mg, manganese ($MnSO_4$-H_2O): 50 mg, iron ($FeSO_4$-$7H_2O$): 30 mg, zinc (ZnO): 30 mg, copper ($CuSO_4$-$5H_2O$): 5 mg, cobalt ($CoCl_2$- $6H_2O$): 0.1 mg, iodine as KI: 0.4 mg, selenium (Na_2SeO_3): 0.15 mg

[3]Calculated value according to tabular values listed for the feed ingredients (Jurgens, 1996).

Chemical analyses of the basal diet for crude protein (988.05), ether extract (932.06), crude fiber (962.09), crude ash (936.07), Ca (968.08), and P (965.17) were done in triplicate using the methods described by the AOAC International (1990). Energy and amino acid (methionine and lysine) ingredients were computed from tabular values for the feedstuffs (Jurgens, 1996). Feed consumption was measured on the 25th, 50th, and 75th days of the experiment.

At the end of the study, a total of 36 birds were killed by cervical dislocation, including 6 birds per group. One part of the blood samples was put into additive-free vacutainers. They were centrifuged (Remi, R-8C BL R-8M) at 3,000 g for 10 min at 4 °C and aliquots were transferred to microfuge tubes. Serum, liver, and ovary samples were kept on ice and protected from light to avoid oxidation during sampling and were then stored at −80 °C until analyses. The other part of the blood samples was taken into anticoagulant tubes. Haematological parameters; white blood cell (WBC), lymphocytes (LYM), monocytes (MON), granulocytes (GRAN), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT) and Platelet larger cell ratio (P-LCR) was immediately measured with haematological analysis device (PROCAN pe-6800 VET).

MDA and GSH were determined according to Mihara & Uchiyama (1978) and Ellman (1979). The serum, liver, and ovarium TAS and TOS were determined according to Erel (2005) with a spectrophotometer (Konika Minolta CM-5). The OSI was defined as the ratio of the TOS level to the TAS level. Specifically, OSI (arbitrary unit) = TOS (μmol H2O2 Eq/L)/TAS (μmol Trolox Eq/L).

Data were analyzed by two-way ANOVA using the GLM procedure (SPSS, 2015). The following model was applied: $y_{ijk} = \mu + ET_i + AV_j + (\text{ET*AV})_{ij} + e_{ijk}$, where y = response variable, μ = population mean, ET = effect of environmental temperature (1: 22 ± 2

°C for 24 h/day (TN), 2: 34 ± 2 °C for 8 h/day (HS; between 09:00-17:00 hours) followed by 22 ± 2 °C for 16 h/day throughout the experiment), AV = effect of AV supplementation (0: 0% AV, 1: 1% AV *and 3:* 3% AV*),* (ET*AV) = effect of interactions between E and AV*,* and e = residual error [*N* (σ, μ; 0, 1)]. The differences among treatments were evaluated by Duncan Multiple Range Test. Also, statistical significance was considered at P < 0.05 (SPSS, 2015).

RESULTS AND DISCUSSION

The study aims to determine the effects of dietary AV supplementation on the haematological parameters and serum, liver, and ovarian antioxidant status in the late laying period of heat stressed quails. High environmental temperature primarily causes oxidative stress in animals. It has been largely known that increased environmental temperature causes significant yield losses, increased mortality rates, and low profitability in poultry (Lu et al., 2007). It is emphasized that the supplementation of natural herbal additives to animal feed can effectively reduce these negative effects of heat stress.

In the present study, white and red blood cell profile and platelets were analyzed to determine the effect of AV in quail under different ET conditions (Table 3). The effects of ET on PDW and PCT were significant (P <0.05). In the HS, PDW level was lower but PCT level was higher than TN. In terms of the 0% AV, GRAN was the lowest (0.53, 10^3/μL) in TN and the highest (1.20, 10^3/μL) in HS. MCH and PCT values were the highest (32.82, Pg and 0.06, %, respectively) in TN, MCHC was the lowest (43.62, g/dl) in HS. MCV, and PDW were the highest (74.00, fL and 18.83, %, respectively) in HS. In comparisons between 1% AV and 3% AV the GRAN for 1% AV was higher (1.27, 10^3/μL) in the TN than the 3% AV (0.70, 10^3/μL), but was similar (0.80, 10^3/μL and 0.80, 10^3/μL, respectively) in the HS (Table 3).

Table 3. Effects of dietary *Alchemilla vulgaris* supplementation (%) on haematological parameters in late laying period of heat-stressed quails (Mean ± SE)

Variables	n	WBC (10^3/μL)	LYM (10^3/μL)	MON (10^3/μL)	GRAN (10^3/μL)	RBC (10^6/μL)	HG (g/dl)	HCT (%)	MCV (fL)
					ET[a]				
TN[b]	18	36.91±0.95	34.64±0.35	1.50±0.12	0.83±0.09	3.78±0.10	12.00±0.28	27.59±0.72	71.48±1.21
HS[c]	18	37.38±0.50	34.18±0.40	1.46±0.08	0.93±0.09	3.98±0.09	12.54±0.32	27.35±0.61	70.51±1.43
					AV[d]				
	0	6	34.88±1.44	35.17±0.56	1.28±0.10	0.53±0.02^{A}	3.63±0.22	11.88±0.58	28.50±1.71	72.75±2.03^{BC}
TN	1	6	39.07±1.45	34.30±0.64	1.83±0.13	1.27±0.19^{C}	3.82±0.14	12.07±0.36	27.98±0.73	73.72±1.53^{C}
	3	6	36.77±1.81	34.47±0.69	1.38±0.28	0.70±0.6^{A}	3.88±0.15	12.05±0.55	26.28±0.12	67.97±2.19^{AB}
	0	6	38.97±0.58	33.82±0.49	1.53±0.13	1.20±0.22^{BC}	4.09±0.12	13.20±0.38	28.93±0.52	74.00±0.62^{C}
HS	1	6	36.88±0.77	33.67±0.99	1.41±0.17	0.80±0.13^{AB}	3.95±0.19	12.78±0.47	27.77±0.91	73.65±1.66^{C}
	3	6	36.31±0.94	35.07±0.44	1.43±0.13	0.80±0.09^{AB}	3.89±0.15	11.65±0.66	25.35±0.20	63.87±2.09^{A}
ET			-	-	-	-	-	-	-	-
AV			-	-	-	*	-	-	-	*

Variables	n	MCH (Pg)	MCHC (g/dl)	PLT (10^3/μL)	MPV (fL)	PDW (%)	PCT (%)	P-LCR (%)
					ET			
TN	18	31.83±0.46	44.71±0.46	43.50±1.93	11.99±0.19	17.64±0.50	0.05±0.00	28.58±0.71
HS	18	31.51±0.44	44.89±0.49	43.61±1.53	12.17±0.14	16.23±0.37	0.06±0.00	29.85±0.49
					AV			
	0	6	32.82±0.59^{B}	45.30±1.00^{BC}	43.17±2.52	12.00±0.15	17.35±0.66^{BC}	0.06±0.01	28.25±1.09
TN	1	6	31.65±0.61^{AB}	43.05±0.31^{A}	40.33±1.76	12.35±0.19	17.75±0.33^{BC}	0.04±0.01	30.23±0.32
	3	6	31.03±1.03^{AB}	45.77±0.45^{C}	41.83±2.34	11.62±0.52	16.35±1.14^{AB}	0.04±0.01	27.25±1.71
	0	6	32.25±0.39^{B}	43.62±0.52^{AB}	48.33±4.70	12.52±0.10	18.83±0.68^{C}	0.06±0.01	31.37±0.67
HS	1	6	32.45±0.60^{B}	44.20±0.69^{BC}	39.33±2.84	12.20±0.11	16.45±0.55^{AB}	0.05±0.01	28.67±0.99
	3	6	29.82±0.76^{A}	43.85±0.75^{AB}	48.33±1.05	11.78±0.34	14.88±0.23^{A}	0.06±0.01	29.52±0.50
ET			-	-	-	-	*	*	-
AV			*	*	-	-	*	-	-

-:P >0.05, *: P <0.05, ^{A,B,C}: Means with a common superscript do not differ at P<0.05).

ET: Environmental temperature; TN:: Thermoneutral; HS: Heat stress; AV: *Alchemilla vulgaris*.

MCV, MCH and PDW values for 1% AV were higher in both TN (73.72, fL, 31.65, Pg and 17.75, %, respectively) and HS (73.65, fL, 32.45, Pg and 16.45, %, respectively). The MCHC value for 1% AV was higher (44.20, g/dl) in the HS and lower (43.05, g/dl) in the TN (Table 3).

PCT value for 1% AV was lower (0.05, %) in the HS and same (0.04, %) with %3 AV in the TN. Also, GRAN, MCV, PDW in terms of 1 and 3% AV and MCH in terms of 3% AV was calculated lower under HS conditions (P <0.05; Table 3).

No statistically significant difference was observed in WBC, LYM, MON, RBC, HG, HCT, PLT, MPV, and P-LCR levels of the groups 1 and 3% AV supplementation under TN and HS conditions. Also, the effects of interaction between ET x AV levels were not significant on the blood haematological profiles in quails exposed to heat stress in the study (P>0.05) (Table 3).

A study on broilers showed that; AV has been shown to significantly affect certain blood parameters (MON (%), MON (10^3/μL), PLT, and PCT) (p <0.05, p <0.01). It was noteworthy that monocyte levels increased significantly because monocytes are phagocytic cells of the blood and support the immune system of the body by killing pathological microorganisms. This suggests that the addition of AV to the diets may contribute to the immune system and the resistance to diseases (Köseman et al., 2020).

Acute HS caused changes in the proportions of circulating leucocyte components but it was determined that acute HS did not affect the hematocrit levels or eosinophil proportion (Altan et al., 2000). In another study, it was found that hematocrit levels decreased in birds exposed to heat stress (Altan et al., 2003). The hematocrit level obtained in our study was consistent with Altan et al., (2000), but did not similar to Altan et al., (2003). During the acclimation period, some blood parameters such as basophil, heterophil, and H/L ratio increased in the high temperature group. Exposure to an acute heat temperature of chickens at 42 d resulted in a significant increase in basophil, heterophil, and H/L ratio in both groups. High temperatures caused a decrease in monocyte and lymphocyte proportions, whereas the proportion of eosinophil was not affected (Erköse and Akşit, 2009).

In the present study, suggested that GRAN (eosinophils, basophils, neutrophils) were lower in HS than the control group in supplemented with 1 and 3% AV because of neutropenia is the low occurrence. Neutropenia occurs in congenital and immune/nonimmune reasons and it is less than 1500/mm^3 neutrophil count in humans (Kaya, 2013). The level of neutropenia in quails is unknown. Therefore, it is considered that the decrease in GRAN may be a positive feature of AV since WBC, LYM, and MON levels determined in this study do not show a statistically significant change.

The lower blood MCV, MCH, and PDW levels in stressed quails supplemented with AV suggest that AV supplementation may affect red blood cell profiles. MCV is a measure of the average size of RBC and is also biochemically associated with MCH and MCHC. Pathologically low MCV level is occurred parallel with microcytic erythrocyte, while low MCH and MCHC levels have occurred parallel with hypochromic erythrocyte cases. Low levels are caused by severe blood loss, hemoglobinopathy, microcytic anemia, and iron deficiency (Kaya, 2013). However, there was no decrease in RBC, HG, and HCT levels or statistical significance related to this study. Therefore, low MCV and MCH levels are thought to be caused by a factor other than AV supplemented.

The PDW test is used to measure the difference between the sizes of the platelets and is interpreted taking into account the other levels obtained from the test. An unstable distribution may indicate serious deficiencies in the body or various diseases. Only low platelet count; platelet agglutination (EDTA-dependent), platelet satellitism (EDTA-dependent and polymorph or around the other cell), platelet neutrophil agglutination (EDTA-dependent), giant platelet, clotted sample, over-filled tube results from the study (Kaya, 2013). In this study, the PDW levels for 1% AV were higher in both TN and HS from 3% AV.

Besides in the present study, the effect of AV on ET conditions was determined by analyzing MDA, GSH, TAS, TOS, and OSI levels in blood serum of quail fed ration supplemented with 1 and 3% AV (Table 4). The effect of AV on serum MDA levels was determined significant (P <0.05; Table 4). The differences were not found significantly for GSH, TAS, TOS, and OSI levels in the blood serum of the quails fed with 1 and 3% AV supplementation under TN and HS conditions (P >0.05; Table 4). However, in terms of AV; MDA in the blood serum was significant (P <0.05; Table 4). The effects of interaction between ET x AV factors were not significant on the serum antioxidant parameters in quails exposed to heat stress (P>0.05) (Table 4).

Table 4. Effects of dietary *Alchemilla vulgaris* supplementation (%) on serum antioxidant status in late laying period of heat-stressed quails (Mean ± SE)

Variables		n	MDA (µmol/L)	GSH (µmol/L)	TAS (mmol Trolox Equiv/L)	TOS (µmol H_2O_2 Equiv/L)	OSI (TOS/TAS)
					ET[a]		
TN[b]		18	7.70±0.57	18.97±1.11	1.11±0.10	13.95±1.22	13.64±1.07
HS[c]		18	8.61±0.70	18.57±0.55	1.00±0.07	12.44±0.71	13.72±1.77
					AV[d]		
TN	0	6	8.09±1.20^{A}	17.18±0.97	1.06±0.18	11.25±1.02	12.83±1.60
	1	6	7.33±0.66^{A}	20.93±2.70	1.06±0.18	15.52±2.36	15.45±2.01
	3	6	7.69±1.16^{A}	19.14±2.01	1.20±0.21	14.07±2.75	12.65±2.03
HS	0	6	11.27±0.92^{B}	16.60±1.12	0.89±0.11	12.75±0.92	17.35±4.88
	1	6	6.89±1.26^{A}	19.07±10.6	1.15±0.11	13.27±0.83	12.12±1.44
	3	6	7.69±0.65^{A}	19.03±0.68	0.97±0.14	11.30±1.79	11.70±1.47
ET			-	-	-	-	-
AV			*	-	-	-	-

-:P >0.05, *: P <0.05, ^{A,B}: Means with different superscripts are significantly different (P <0.05).
[a]: Environmental temperature; [b]: Thermoneutral; [c]: Heat stress; [d]: *Alchemilla vulgaris*.

A study on broilers showed that; AV known to have anti-inflammatory, antioxidant, and anti-microbial effects did not affect serum MDA, GSH, TAS, TOS, and OSI values (p> 0.05). Although not statistically significant, MDA levels and suppressed lipid peroxidation in a dose-dependent manner decreased in the serum (Köseman et al., 2020).

In terms of the 0% AV group, the MDA value in the blood serum was the highest in both TN (8.09, µmol/L) and HS (11.27, µmol/L) (Table 4). In the study, when comparing MDA levels in the blood serum in 1% AV and 3% AV, MDA levels were found lower in both HS and TN in 1% AV groups (Table 4). The GSH, TOS and OSI values in blood serum for 3% AV were lower in both TN (19.14, µmol/L, 14.07, µmol H_2O_2 Equiv/L and 12.65, TOS/TAS, respectively) and HS (19.03, µmol/L, 11.30, µmol H_2O_2 Equiv/L and 11.70, TOS/TAS, respectively) whereas MDA was higher (7.69, µmol/L for TN, 7.69, µmol/L for HS) (Table 4).

The effects of AV on ET conditions were presented for MDA, GSH, TAS, TOS, and OSI levels in the liver of the quails in Table 5. The effect of ET on TOS in liver was significant (P <0.05; Table 5). The effect of AV on the liver GSH, and TOS levels were found significant (P <0.05; Table 5). In terms of AV; GSH and TOS levels in the liver, and also in terms of ET; TOS levels in the liver were significant (P <0.05; Table 5).

Table 5. Effects of dietary *Alchemilla vulgaris* supplementation (%) on liver antioxidant status in late laying period of heat-stressed quails (Mean ± SE)

Variables		n	MDA (nmol/g wet tissue)	GSH (nmol/g wet tissue)	TAS (mmol Trolox Equiv/L)	TOS (µmol H_2O_2 Equiv/L)	OSI (TOS/TAS)
					ET[a]		
TN[b]		18	87.61±5.02	894.22±43.12	1.34±0.04	24.94±1.51	23.27±1.43
HS[c]		18	90.89±3.68	835.50±28.73	1.30±0.05	31.30±2.17	19.56±1.27
					AV[d]		
TN	0	6	90.00±12.01	901.67±36.27^{AB}	1.45±0.09	28.57±2.30^{AB}	24.32±2.46
	1	6	88.00±9.41	830.33±61.05^{A}	1.41±0.03	20.83±1.60^{A}	25.25±2.65
	3	6	84.83±4.62	774.50±41.83^{A}	1.35±0.07	25.42±3.06^{A}	20.27±2.23
HS	0	6	100.17±6.27	814.67±35.60^{A}	1.26±0.08	30.61±3.31^{AB}	20.05±2.01
	1	6	87.50±7.06	1042.50±101.54^{B}	1.30±0.10	35.72±3.88^{B}	16.40±1.48
	3	6	85.00±4.75	825.50±27.94^{A}	1.15±0.06	27.57±3.89^{AB}	22.23±2.61
ET			-	-	-	*	-
AV			-	*	-	*	-

-:P >0.05, *: P <0.05, ^{A,B}: Means with different superscripts are significantly different (P <0.05).
[a]: Environmental temperature; [b]: Thermoneutral; [c]: Heat stress; [d]: *Alchemilla vulgaris*.

Table 6. Effects of dietary *Alchemilla vulgaris* supplementation (%) on ovarium antioxidant status in late laying period of heat-stressed quails (Mean ± SE)

Variables		n	MDA (nmol/g wet tissue)	GSH (nmol/g wet tissue)	TAS (mmol Trolox Equiv/L)	TOS (μmol H_2O_2 Equiv/L)	OSI (TOS/TAS)
				ET[a]			
TN[b]		18	63.78±2.56	837.78±41.97	0.97±0.08	17.16±1.29	27.51±8.25
HS[c]		18	64.94±4.35	797.94±37.31	1.04±0.09	17.75±1.29	22.86±4.95
				AV[d]			
TN	0	6	61.50±4.19^{A}	888.67±85.06	1.01±0.12	16.91±2.76	28.28±2.92
	1	6	69.17±5.05^{AB}	903.67±57.28	1.02±0.08	20.29±1.52	20.50±2.46
	3	6	60.67±3.86^{A}	721.00±57.72	0.91±0.15	14.30±1.84	23.75±1.22
HS	0	6	79.50±9.23^{B}	880.50±55.71	0.96±0.18	18.16±2.35	18.88±2.88
	1	6	62.83±5.44^{A}	730.50±47.41	0.99±0.13	18.12±2.63	20.70±4.72
	3	6	52.50±1.93^{A}	782.83±80.82	1.11±0.23	16.97±2.07	28.98±1.41
ET			-	-	-	-	-
AV			*	-	-	-	-

-:P >0.05, *: P <0.05, ^{A,B}: Means with different superscripts are significantly different (P <0.05).

[a]: Environmental temperature; [b]: Thermoneutral; [c]: Heat stress; [d]: *Alchemilla vulgaris*.

The GSH value in the liver was highest (901.67, nmol/g wet tissue) in TN, and the lowest (814.67, nmol/g wet tissue) in HS (Table 5). In the study, while TOS levels in the liver in 1% AV in TN were found to be lower but it was found to be higher in 1% AV in HS (P <0.05) (Table 5). MDA, GSH, and TAS values for 3% AV in the liver were lower in both TN and HS (Table 5). The effects of interaction between ET x AV factors were not significant on the liver antioxidant parameters in quails exposed to heat stress (P>0.05) (Table 5).

In this study, the effects of AV on ET conditions for MDA, GSH, TAS, TOS, and OSI levels in ovarium of quail fed ration supplemented with 1 and 3% AV were determined (Table 6). The effect of AV on ovarian MDA levels was significant (P <0.05; Table 6). However, the differences were not found significant for GSH, TAS, TOS, and OSI levels in the ovaries of the quails fed with 1 and 3% AV supplementation under TN and HS conditions (P >0.05; Table 6). The differences between 1% AV and 3% AV for MDA levels in the ovarium were significant in both HS and TN (P <0.05; Table 6).

In the study, TOS was the highest (28.57, μmol H_2O_2 Equiv/L) in TN. MDA value in the ovary was found to be the highest value (79.50, nmol/g wet tissue) in HS. MDA levels were found higher in 1% AV groups in both HS and TN (Table 6). The effects of interaction between ET x AV factors were not found significant on the ovarium antioxidant parameters in quails exposed to heat stress (P>0.05) (Table 6).

The decrease in MDA levels in plasma or tissues indicates decreased lipid peroxidation. In the case of OS, GSH activity decreases due to excessive consumption of GSH like other antioxidant enzymes (Singh *et al*., 2014). In light of these findings, it can be said that the different ratio of AV has a different effect on stress parameters. Different levels of the same stress parameters in the blood serum, liver, and ovarium are evaluated as the effect of AV on different organs is different. Also, the lack of statistical significance in most parameters is thought to be the destruction of polyphenols and flavonoids in AV content.

If all of the data are evaluated together, data express that the HS caused general OS in the quails and dietary AV supplementation reduced the OS by exhibiting antioxidant effect. Besides, there is no record coping with dietary AV supplementation on antioxidant status in late laying periods of heat stressed quails for comparing our data. But flavonoids were determined that antioxidant activity. Besides, flavonoids significantly increased some antioxidant enzymes such as GSH, while lowering MDA levels (Mahmoud *et al*., 2012). The outcomes of the present study are in agreement with our study.

CONCLUSIONS

In conclusion, some hematological profiles and serum, liver and ovarian antioxidant parameters are impressed in a heat stress exposured in late laying period of quails. Supplementation 1% and 3% AV is partially improved the antioxidant defense system with its antioxidant effect and also, incompletely positively affected these parameters. Besides, 3% AV supplementation is more effective than 1% AV on some haematological and antioxidant parameters. However, more research including different doses and durations is needed to better define the effects of AV on quails exposed to heat stress.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Research article
Ερευνητικό άρθρο

Garlic powder attenuates pulmonary hypertension in broiler chickens: an electrocardiographic-based study

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ABSTRACT: One of the most severe health-threatening issues in broiler chickens is believed to be pulmonary hypertension,characterized by inadequate oxygen levels in blood, elevated workload of the cardiopulmonary network, right ventricle hypertrophy, and death. A total of 180 one-day-old male chickens were dividedinto four equal groups, with 3 replicates per group (45 birds per group, 15 birds per replicate). To induce pulmonary hypertension syndrome, triiodothyronine was added to their diet. A corn-soy based feed was formulated for all treatment groups, and birds were treated with different doses of dietary garlic powder (0.2%, 0.6%, and 1%) for 49 days.Right ventricle/total ventricles (RV/TV), RV/body weight, TV/body weight ratio,and electrocardiographic records were assessed. RV to TV ratio was significantly reduced in all treatment groups at 49 days of age ($P < 0.05$). The S-wave amplitude was decreased significantly (0.2% and 1% garlic powder groups, lead II; and all treatment groups, lead aVF) at 49 days of age. R-wave amplitude showeda significant reduction at 49 days of age (0.6% and 1% garlic powder receivers, lead aVR; all treatment groups, lead II) ($P<0.05$). QT interval showed a significant increase at 14 days (0.2% garlic powder group, lead III) and at 49 days (0.6% garlic powder group, lead II) ($P<0.05$). RR interval at 49 days of age (0.6% and 1% treatment groups, lead II; and1% garlic powder receivers, leads III, aVR, and aVF) showed a significant increase compared with controls ($P<0.05$).According to our study, garlic powder canimprove the electrocardiographic patterns and reversethe detrimental effects offree radicals and oxidative stress in birds withpulmonary hypertension.

***Keywords*:** garlic, hypertension, electrocardiogram, broilers, waves

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INTRODUCTION

Overthe pastthree decades, one of the most concerning issues known aspulmonary hypertension syndrome (PHS) has led to difficulties in movement, fast growth, increased metabolic processes of the cardiopulmonary system,and death in broiler chicken (Tankson et al., 2001). PHS is also known as ascites or "water belly" and is frequently imposed by insufficient oxygen to the lungs causing systemic arterial hypoxemia.High altitude is the most conspicuous environmental factor that plays an essential role in PHS formation in broilers. Stressful conditions such as cold temperature,feeding irregularities, and lighting alsoresult in PHS (Hassanpour et al., 2011). Ascites syndromes include pulmonary edema, aggregation of fluid in the pericardial sac, epicardial fibrosis, heart dilation, and right ventricular hypertrophy (Baghbanzadeh and Decuypere, 2008) which are all associated with the reactive oxygen species (ROS) accumulation. The elevated levels of ROS in the birds with PHS potentially contribute to disease development and increased mortality numbers (Tankson et al., 2001;Baghbanzadeh and Decuypere, 2008). Cellular dysfunction resulting from lipid and protein changes caused by ROS aggregation plays an important role in the deterioration of the cardiopulmonary system in ascites syndromes (Nain et al., 2008).

Nutritious foods are one of the most important and modifiable determinants in life. To mitigate the progression of various disorders such as PHS and support the metabolic requirements, appropriate nutritionalapproachesshould be applied, as mentioned by many investigations (Callejo et al., 2020;Wideman et al., 2007).Nemati and colleagues (2017) reported thata combination of vitamin C (300 mg/kg) and coenzyme Q10 in the daily diet of birds with PHS induced by cold temperature could significantly improve the body weight, feed conversion ratio and reduce the mortality rates (Nemati et al., 2017). Behroojet al. (2012) found that mortality rates would increase as a low-protein diet was added. Conversely, when high-protein meals were added to their diets, mortality rates reduced significantly (Behrooj et al., 2012).

In the challenging condition, proper nutrition, including antioxidant-based interventions, is supposed to inhibit the onset of ascites in chickens (Khajali and Wideman, 2016).Potent therapeutic effects of antioxidants have introduced them as a rightcandidate for preventing and/or treating and treating different diseases (Pham-Huy et al., 2008). Among these, Garlic (*Allium sativum* L.), a traditional medicinal plant, has gained a global reputation for treating different diseases such as cardiovascular disorders, hypertension, and lung diseases (Bayan et al., 2014). Garlic is a rich source of beneficial nutrients andcontainsbioactive constituents such as alliin, allicin, diallyl sulfide, diallyl disulfide, diallyl trisulfide, S-allyl-cysteine, and ajoene (Zeng et al., 2017; Shang et al., 2019).Organic sulfides, saponins, phenolic compounds, and polysaccharides are also known as other beneficial garlic compounds, which all have made it a usefulherb for many centuries (Shang et al., 2019).

Many studies have illustrated that the expressions of IL-6, IL-10, and TNF-α can be regulated by dietary garlic (Shang et al., 2019;Li et al., 2017;Rabe et al., 2015). Garlic is prepared in two forms including, solid form containing dried powders and aliquid form containing aqueous, oil, and solvent extracts. The main components of powder from crushed and dried garlic are appeared to be alliin and diallyl disulfide (DADS) (Trio et al., 2014).Due to having numerous compounds, dietary garlic has been demonstrated to have cardioprotective, antibacterial, antioxidant, antifungal, immunomodulatory, anti-carcinogenic, anti-apoptotic and anti-inflammatory properties against a wide variety of diseases (Schäfer and Kaschula, 2014;Lee et al., 2016). Taken together, we aimed at understanding the possible useful effects of garlic powder on electrographic parameters of birds with pulmonary hypertension syndrome induced by T_3.

MATERIALS AND METHODS

Animals, management and treatments

A total of 1801-day old chickens (Ross 308) were separately divided into 4 groups including 1 control and 3 treatment groups with 3 replicates per group (45 birds per group, 15 birds per replicate), as mentioned below:

Control group: Basic diet receivers over the test period; 3 Treatment groups: Receiver of basic diet + 0.2%, 0.6% and 1% garlic powder over the test period.To induce PHS, from day 7 onwards, 1.5 mg / kg triiodothyronine (T_3) (Sigma Chemical Co.) was added to the diet of birds.

Birds were reared for 49 days. From day 1 of age, all chickens were kept under a 23-h light and 1-h without light. They were fed with standard feed mixture and water *ad libitum* (starter: 3200 kcal metabolizable energy/kg of diet, 23% crude protein; grower:

3200 kcal metabolizable energy/kg of diet, 20% crude protein; finisher: 3200 kcal metabolizable energy/kg of diet, 18% crude protein) according to the National Research Council (NRC) recommendation for the broilers (NRC, 1994).

Electrocardiographic recordings

At 14 and 49 days of age, 8 chickens from each group were randomly selected, and electrocardiograms were recorded with an automatic recorder (Cardiomax FX- 2111, Fukuda, Japan) and standardized at 10 mm = 1 mV with a chart speed of 50 mm/s. Leads I, II, III, aVR, aVL, and aVF recorded for each bird. The amplitude of T, R, and S waves, the intervals of QRS, QT, RR, and ST, and the mean electrical axis (MEA) were measured.

Dissection and assessment of right ventricular hypertrophy

After the recording of electrocardiograms, the chickens were weighed and euthanized. Then, they were killed by decapitation, and right ventricle hypertrophy was evaluated, as previously described by Cueva et al.(1974).

The heart was dissected, and to the plane of the atrial ventricular valves, the atria were removed, and the total ventricles (TV) were weighed. The right ventricular (RV) wall was dissected from the left ventricle (LV) and septum. The RV was weighed, and the RV-to-TV ratio was calculated. Over our trial, pulmonary hypertension syndrome was defined as RV-to-TV ratio greater than 0.28 (Wideman, 2001).

Statistical analysis

All results were represented as mean ±SEM. Comparisons were made by one-way ANOVA using SPSS 22.0 (Chicago, IL, USA), with $P < 0.05$ accepted as significant.

RESULTS

Compared with controls, asignificant reduction of R-wave amplitude at 49 days of agein 0.6% and 1% garlic powder group (lead aVR) was observed ($P<0.05$). Besides, lead II in all three treatment groups (0.2%, 0.6% and 1% garlic powder), at 49 daysof age, showed a significant decrease ($P <0.05$) compared with the controls (Table 1, Figure1).

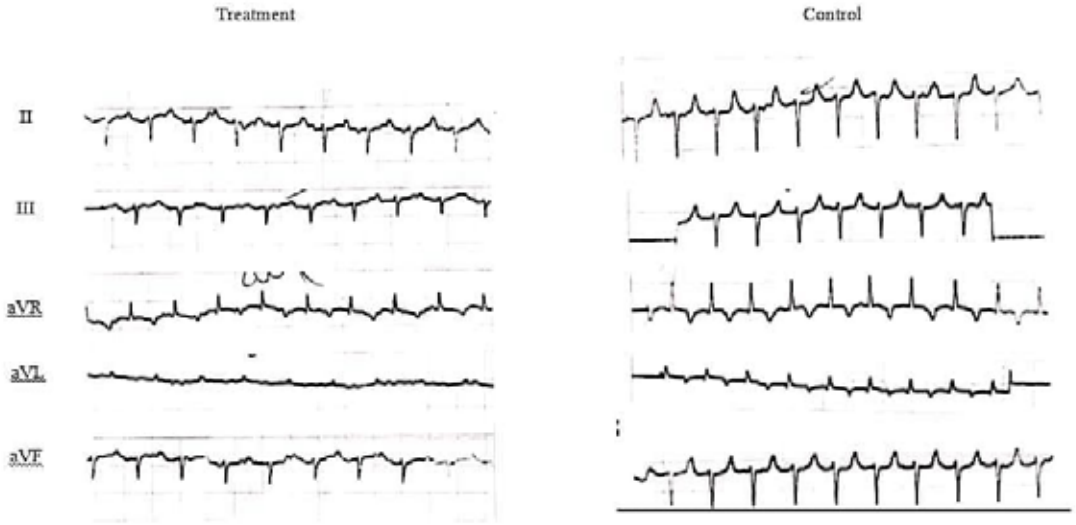

Figure 1: Samples of different electrocardiographs in 2 groups of hypertensive broilers (control and 1% garlic powder groups) at 49 days. Standardization, 10 mm = 1 mV; chart speed, 50 mm/s. Garlic powder in treatment group decreased the high amplitudes of R and S waves seen in control group (chickens with PHS).

Table 1: Amplitude of the electrocardiographic waves in the different groups

Age (days)	Lead Group	R (mV)				S (mV)			T (mV)			
		Lead II	Lead III	Lead aVR	Lead aVF	Lead II	Lead III	Lead aVF	Lead II	Lead III	Lead aVR	Lead aVF
14	Control	0.17±0.07	0.13±0.05	0.14±0.03	0.18±0.02	0.24±0.05	0.21±0.05	0.18±0.05	0.09±0.00	0.08±0.01	0.08±0.01	0.12±0.02
	T (0.2%)	0.14±0.03	0.09±0.01	0.17±0.02	0.19±0.08	0.16±0.03	0.18±0.02	0.17±0.04	0.08±0.03	0.08±0.02	0.10±0.02	0.09±0.06
	T (0.6%)	0.19±0.03	0.12±0.02	0.18±0.05	0.17±0.02	0.25±0.01	0.23±0.02	0.23±0.04	0.08±0.03	0.07±0.02	0.08±0.06	0.13±0.02
	T (1%)	0.22±0.02	0.16±0.03	0.15±0.02	0.21±0.04	0.21±0.05	0.26±0.01	0.21±0.06	0.09±0.02	0.09±0.03	0.10±0.02	0.08±0.04
49	Control	0.51±0.05	0.38±0.05	0.45±0.03	0.28±0.04	0.42±0.05	0.25±0.05	0.37±0.04	0.15±0.01	0.13±0.01	0.13±0.02	0.12±0.01
	T (0.2%)	0.17±0.04*	0.25±0.04	0.29±0.02	0.21±0.03	0.21±0.04*	0.16±0.04	0.15±0.03*	0.17±0.04	0.13±0.04	0.12±0.02	0.10±0.03
	T (0.6%)	0.21±0.03*	0.28±0.02	0.12±0.02*	0.24±0.03	0.26±0.03	0.23±0.03	0.18±0.03*	0.12±0.03	0.12±0.02	0.12±0.02	0.09±0.03
	T (1%)	0.18±0.04*	0.21±0.03	0.17±0.06*	0.23±0.03	0.18±0.04*	0.20±0.03	0.19±0.03*	0.11±0.04	0.13±0.01	0.10±0.04	0.11±0.03

T = treated; *significantly different vs. control (P < 0.05);

Table 2: Intervals of the electrocardiographic waves in the different groups

Age (days)	Lead Group	QT (seconds)				ST (seconds)		
		Lead II	Lead III	Lead aVR	Lead aVF	Lead II	Lead III	Lead aVF
14	Control	0.09±0.00	0.08±0.01	0.10±0.00	0.09±0.01	0.03±0.00	0.02±0.00	0.03±0.00
	T (0.2%)	0.10±0.00	0.11±0.00*	0.10±0.00	0.11±0.00	0.03±0.00	0.03±0.01	0.03±0.00
	T (0.6%)	0.10±0.00	0.10±0.00	0.09±0.01	0.09±0.00	0.03±0.00	0.03±0.00	0.02±0.00
	T (1%)	0.10±0.00	0.10±0.00	0.09±0.00	0.10±0.00	0.03±0.00	0.02±0.01	0.03±0.00
49	Control	0.09±0.00	0.10±0.01	0.11±0.00	0.09±0.00	0.04±0.00	0.03±0.00	0.04±0.00
	T (0.2%)	0.11±0.00	0.12±0.00	0.10±0.00	0.12±0.00	0.03±0.01	0.04±0.00	0.05±0.01
	T (0.6%)	0.13±0.01*	0.12±0.00	0.12±0.00	0.13±0.00	0.04±0.00	0.04±0.00	0.05±0.00
	T (1%)	0.12±0.00	0.12±0.01	0.11±0.00	0.12±0.00	0.03±0.00	0.05±0.01	0.05±0.00

T = treated; *Significantly different vs. control (P < 0.05)

Table 3: Intervals of the electrocardiographic waves in the different groups

Age (days)	Group	QRS (seconds)			RR (seconds)			
		Lead II	Lead III	Lead aVF	Lead II	Lead III	Lead aVR	Lead aVF
14	Control	0.03±0.00	0.02±0.00	0.03±0.00	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.01
	T (0.2%)	0.03±0.00	0.03±0.01	0.03±0.00	0.11±0.00	0.12±0.00	0.11±0.00	0.11±0.00
	T (0.6%)	0.03±0.00	0.04±0.00	0.03±0.00	0.11±0.00	0.11±0.00	0.11±0.01	0.11±0.01
	T (1%)	0.03±0.00	0.03±0.01	0.03±0.01	0.12±0.01	0.11±0.00	0.12±0.01	0.11±0.00
49	Control	0.04±0.00	0.05±0.01	0.04±0.00	0.13±0.00	0.13±0.00	0.13±0.00	0.14±0.00
	T (0.2%)	0.04±0.01	0.04±0.00	0.04±0.01	0.14±0.00	0.14±0.00	0.15±0.00	0.15±0.00
	T (0.6%)	0.03±0.00	0.04±0.00	0.04±0.00	0.16±0.00*	0.15±0.00	0.16±0.00	0.16±0.00
	T (1%)	0.04±0.01	0.04±0.00	0.04±0.00	0.16±0.00*	0.17±0.01*	0.16±0.00*	0.17±0.00*

T = treated; *Significantly different vs. control (P < 0.05)

Table 4: Cardiac indices and mean electrical axis (MEA) in the different groups

Age	Groups	MEA (°)	RV/TV	%TV/BW
14	Control	-53.25±42.96	0.19±0.00	0.43±0.00
	T (0.2%)	-6.60±23.53	0.17±0.00	0.36±0.00
	T (0.6%)	-31.11±25.69	0.19±0.00	0.38±0.01
	T (1%)	-54.00±36.00	0.18±0.00	0.41±0.01
49	Control	77.66±12.57	0.31±0.00	0.35±0.00
	T (0.2%)	-17.60±35.41	0.23±0.00*	0.33±0.00
	T (0.6%)	51.40±57.70	0.21±0.00*	0.30±0.00
	T (1%)	45.60±50.33	0.21±0.01*	0.31±0.00

T = Treated; *Significantly different vs. control (P < 0.05)

However, there were no significant differences-between treatmentand control groups in leads III and aVF (Table 1).At 49 days of age, a significant reduction of S wave (leads II, 0.2% and 1% treatment groups) and (lead aVF, all three treatment groups)was observed (P <0.05), while there were no significant differences between control and treatment groups in T wave amplitude (Table 1).

According to table 2, in treatment groups, mean ST intervals did not show any significant differences compared with control group.The mean QT interval (leads III) at 14 days of age in the treatment group receiving 0.2% garlic powder, and also at day 49 of age in the 0.6% garlic powder group (lead II) showed a significant increase (P <0.05) compared with the control group (Table 2, Figure 1).

By comparing control with treatment groups, RR and QRS intervals showed no significant differences at 14 days of age, while RR intervals in treatment groups (0.6%; Lead II, and 1%; all leads) at 49 days of age showed a significant increase (Table 3, Figure 1).

Compared to the control group, the mean RR interval at day 49 of age in the treatment group (1% garlic powder, all leads) and in the treatment group (0.6% garlic diet, lead II) showed a significant difference (P

<0.05) (Table 3). Our results also showed no significant differences in MEA (Table 4).

Evaluation of right ventricle hypertrophy

Compared to the controls, the mean RV/ TV ratios in the treatment groups were decreased in both ages, but it was significant for birds at 49 days of age($P<0.05$). There was no significant difference between the treatments than the control group in the mean TV/BW ratio. Our results showed no significant differences in electrocardiographic patterns among 3 treatment groups.

DISCUSSION

Environmental factors such as high altitude leading to oxygen loss mainly contribute to the PHS progression (Hassanpour et al., 2011). Cold, growth rate, and consumption of high-energy diets that increase the incidence of ascites via promoting the metabolic activity of chickens directly affect thyroid hormones, especially T_3. It has been revealed that 1.5 mg / kg T_3 can result in right ventricular hypertrophy, ascites, and increased mortality (Hassanzadeh et al., 2000).In the current study, the aim of adding T_3 to the broilers diet was to accelerate metabolism and consequently increase oxygen consumption and metabolic activities.

High demand for oxygen causes tissue hypoxia, which increases cardiac output to meet tissue requirements.Increased pulmonary blood flow leads to accelerated pulmonary blood pressure and eventually ascites (Julian, 2000). Increased oxygen consumption induced by T_3 causes cardiovascular disorders and reduced systematic vascular resistance (Hafe et al., 2019).

Previously it has been revealed that nutritional interventions can attenuate the ascites-related cardiovascular disorders and improve different electrocardiographic parameters in broilers. Ahmadipour et al. (2019) found that flavonoids extracted from hawthorn due to having potent antioxidant and free radical scavenging activities would effectively alleviate the NO serum levels in the numbers of PHS birds. Flavonoids (0.1 ml/L and 0.2ml/L) could significantly improve the amplitudes of S and T waves. Heart weight, RV:TV, RV:BW, and TV:BW ratios were also significantly reduced at receivers of 0.1 and 0.2 ml/L flavonoids (Ahmadipour et al., 2019). In a trial by Hassanpour and colleagues (2011), it was reported that 0.4g/L acetic acid would significantly alleviate S amplitudesat the age of 36 (leads II and III) and 45 days (lead aVF) in the birds with PHS. Acetic acid also reduced T amplitudes at 28 (leads aVR and aVL) and 36 days (lead aVL) of age. Besides, they reported a significant elevation of QRS and QT intervals in these broilers (Hassanpour et al., 2011).

In a similar trial by Hassanpouret al. (2009), 1.5 g/l citric acid significantly decreased S amplitude (leads II, III, aVF at 45 days), and T amplitude (lead aVR, at 28 days and lead aVL at 36 days). A significant reduction of R-wave amplitude was also reported. A significant increase in QRS, QT and RR intervals in some leads in all citric acid groups was observed (Hassanpour et al., 2009). Furthermore, in a different investigation on birds with PHS, they also reported that at 3 different doses (400, 800 and 1200ppm) of ascorbic acid after 36 and 45 days, RV/RT ratio was significantly decreased. S amplitude reduction in all 3 treatment groups was also reported and QRS, QT and RR intervals were increased significantly (Hassanpour et al., 2008). These studies are all consistent with our results on reducing wave amplitude and increase of wave intervals by antioxidant properties of garlic powder.

Garlic has been used as a herbal medicine for more than 4 thousand years, and the potentantioxidant traits of garlic have been represented by accumulating evidence (Shang et al., 2019; Locatelli et al., 2017). Bioactive substances of garlic such as ethyl linoleate have been demonstrated to have anti-inflammatory responses. Through the NO synthase (iNOS) and cyclooxygenase-2 (COX2) gene suppression, they can inhibit the generation of nitric oxide (NO) and prostaglandin E-2 (Park et al., 2014). It has been revealed that 14-kDa protein isolated from garlic prevents NO, tumor necrosis factor-α (TNF-α), and interleukin (IL) -1β, and enhances the adenosine monophosphate-activated protein kinase (AMPK). Another useful compound of garlic, allicin, cantackle potently with inflammatory responses (Shang et al., 2019;Rabe et al., 2015;Metwally et al., 2018).

Dietary garlic by scavenging free radicals can cope effectively with the pathogenesis of vascular diseases. They canimpede oxidative stress, systolic blood pressure, aortic NAD(P)H oxidase activity, and vascular disorders (Vazquez-Prieto et al., 2010). Varmaghany and colleagues (2015) found that after 42 days, 5 g/kg dietary garlic bulb reduced the incidence of ascites in broilers. The anti-hypertensive property of the garlic bulb also decreased PHS- associated

mortality (Varmaghany et al., 2015). Ogbuewuet al. (2019) showed that garlic-active components strongly affected livestock and poultry and reduced their blood lipid and cholesterol content. Garlic could stimulate the gastrointestinal defense system, promote glutathione concentration, and reduce the free radical attack (Ogbuewu et al., 2019).

Javandel et al. (2008) showed that 2% garlic meal for birds at 42 days of age would significantly reduce their daily weight and improve feed conversion ratios (Javandel et al., 2008).Elagibet al. (2013) reported that after 42 days,3% garlic powder in broilersimproved feed consumption ratios and growth performance without any side effects on blood biomarkers (Elagib et al., 2013).Bahadoran et al. (2016) showed that after 42 days, 2 different doses of garlic supplement (0.6% and 1%)could significantly improve the duodenal, jejunal and ileal villus length and width in chickens with PHS (Bahadoran et al., 2016).In this study, the beneficial effects of garlic on electrocardiographic parameters of broilers with PHS have been approved.

CONCLUSION

Our results showed that after 49 days of treatment with dietary garlic,R and S waves were significantly decreased, and RR and QT intervals were significantly increased in some treated broilers showing improved ventricular hypertrophy.RV-to-TV and RV-to-BW ratios were improved in treated birds.We also had low-mortality rates among the birds that consumed dietary garlic compared to controls. It is deduced that garlic powder can modulate pulmonary hypertension, hypertrophy, and arrhythmia of ventricles induced by T_3.

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CONFLICT OF INTEREST

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

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Research article
Ερευνητικό άρθρο

Ameliorative effect of tocotrienol and selenium yeast against the adverse effect of florfenicol in broilers' liver

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ABSTRACT: The prolonged use of florfenicol can lead to detrimental side effects in poultry. This work focuses on the role of tocotrienol and selenium yeast to mitigate the adverse effects of florfenicol in broilers' liver. One hundred and fifty, one-day-old Cobb broiler chicks where equally divided in 5 experimental groups according to the following experimental design: Group (1) control group chicks fed to a balanced diet only. Group (2) Chicks treated with florfenicol (20 mg/kg b.w.) per bird for 3 successive days and the florfenicol was administered in other groups by the same dose and for the same period of time. Group (3) chicks treated with florfenicol and tocotrienol (170 mg/kg b.w.) for 7 successive days. Group (4) Chicks treated with florfenicol and selenium yeast (0.15mg /kg b.w., on feed) for 7 successive days. Group (5) Chicks treated with a combination of florfenicol, tocotrienol and selenium yeast. Chickens treated with florfenicol exhibited an increased level in hepatic malondialdehyde (MDA), as well asa decreasedlevel in hepatic superoxide dismutase (SOD) and reduced glutathione (GSH). Tocotrienol and selenium yeast decreased the MDA and increased SOD and GSH in hepatic tissue as well as return ALP, cholesterol, triglyceride and VLDL to their normal levels. Treated chicks with tocotrienol returned serum (ALT) to normal activity but serum total protein and albumin levels were increased. Theselenium yeast treated groups showed an increase of serum total globulin. Histopathologically, florfenicol treated group had focal hepatic leukocytic infiltration and focal coagulative necrosis of hepatocytes but chickens with the combination of tocotrienol and selenium yeast had activated Kupffer cells and revealed less evident necrotic changes in liver. In conclusion, tocotrienol and selenium yeast administrated alone or in combination highlightedimproved antioxidant effects and mitigated the lipid peroxidation in broilers' liver treated with florfenicol. Thus, tocotrienol and selenium yeastcan improve the safety of using florfenicol in broiler chickens under experimental conditions.

***Keywords*:** Florfenicol, Tocotrienol, Selenium yeast, Liver, Antioxidants, Broilers.

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INTRODUCTION

Florfenicol, a synthetic broad -spectrum antibiotic, contains a fluorine atom at the 3-carbon position, instead of the hydroxyl group found in thiamphenicol used in veterinary practice to treat most gram-positive and gram-negative bacteria (Chang et al., 2010). Florfenicol action is mainly bacteriostatic by inhibition protein synthesis of bacteria by binding to 50S and 70S subunits ribosome and abolishes the activity of peptidyl transferase (Khalil et al., 2012). Ronette (2012) reported that florfenicol is used for the treatment of many microorganisms which affect poultry such as *Escherichia coli, Klebsiella, Ornithobacterium rhinotracheale, Pasteurella multocida, Mannheimia haemolytica, Enterobacter cloacae, Haemophilus somnus, pneumonia, Salmonella typhi, Shigella dysenteriae* and *Staphylococcus aureus*. Florfenicol usually made biotransformation effect (more concentrations of reactive oxygen species) with biochemical changes after entering into cells because it is a highly lipophilic drug. Florfenicol damage changes differ according to the dosage of the drug, time of administration and animal (Ren et al., 2014). Florfenicol administration can produce oxidative stress in broiler's liver by inhibiting the expression of antioxidant proteins nuclear factor-erythroid 2-related factor 2 (Nrf2), hemeoxygenase-1 (HO-1) and NAD(P)H dehydrogenase quinone-1 (Han et al.,2020). In addition Wang et al. (2020) reported that administration of florfenicol by different doses (0.15, 0.3, 0.6, 1.2 and 1.8 g/L) in drinking water of broilers for five successive days produced oxidative stress effect on chicks through inhibition of the expression of related factors in Nrf2.

Antioxidants are substances that interact with unstable free radicals and prevent there damage effects (Sies, 1997; Olayinka et al., 2012). The antioxidants can be classified to natural and synthetic.Natural antioxidants are vitamin E, organic selenium,vitamin C and beta carotene. Synthetic antioxidants are butylated hydroxyanisole and butylatedhydroxytoluene (Hurrell, 2003).

Tocotrienols are the primary form of vitamin E which has α, β, γ, and δ forms. The main sources of tocotrienols are palm oil extract from *Elaeis guineensis* (African oil palm) contain more than 800 mg/kg of tocotrienol and cereal grains such as wheat, barley, rice and differ from tocopherol by the presence of three trans-double bonds in the hydrocarbon tail. Tocotrienols have hypo cholesterolemic, neuroprotection and anticancer effects (Sen et al., 2006). Tocotrienol rich fraction from palm oil can protect cellular membranes from damage effect and it can inhibit protein oxidation and lipid peroxidation in rat liver microsomes (Kamat et al., 1997). Palmitate oil (tocotrienol containing diet) displayed significant decrease inmalondialdehyde MDA level with raises superoxide dismutase SOD activity and reduced glutathione GSH level (Khan et al., 2011).Selenium yeast is one of the best organic sources of selenium for poultry and other farmed animals which was approved for chicken consumption in June 2000 by the United States Food and Drug Administration US-FDA, and has a potent antioxidant effect (Wangand Xu,2008). Elevation selenium level in the diet was led to lowering lipid peroxidation products, free radical elimination and protecting cell membranes (Fan et al., 2009). Diets supplemented with selenium yeast improve activity of hepatic glutathione peroxidase and help for production of oxidized glutathione then stimulate glutathione reductase, which prevent its deactivation by NADPH (Upton et al. 2009).The prolonged use of florfenicol can lead to detrimental side effects in poultry. This work focuses on the role of tocotrienol and selenium yeast alone or their combination to mitigate these side effects in broilers' liver.

MATERIAL AND METHODS

Animals

One hundred and fifty, one-day-old, unsexed Cobb broiler chicks were used and purchased from El-Watania Poultry Company - Cairo - Egypt. The birds were allocated in separate units of metal wire-floored battery for five successive weeks. The study was approved by the Ethical Committee for care and use of animals at Animal Health Research Institute Benha Branch, Egypt (25/3/2019).

Drugs

Three drugs were used in this experimental model. Firstly, florfenicol 10% (Floricol®, PharmaSwede Co., Egypt) was administrated for 3 successive days at 20 mg/kg according to the instructions of manufacture. Each mlof the product contained 100 mg of florfenicol base. Secondly, tocotrienol 50% (Tocovid®, Hovid Company, Malaysia) was administrated for 7 successive days, at the recommended dose of 170 mg/kg. Finally, seleniumyeast 0.2% (Bio-SEL 2000®,IBEX International Co. LTD, Egypt)was administrated at 0.15mg/kg for 7successive days.

Experimental design

One hundred and fifty (150), apparently healthy, one-day-old, unsexed, Cobb broiler chicks were used. The chicks were housed in clean and disinfected enclosure, with controlled environmental temperature, and fed with a well-balanced ration throughout the experimental period of five successive weeks. The chicks were equally divided into five groups of 30 chicks each. Group 1, was the negative control group, including chicks which fed with balanced diet only. Chicks in Group 2 where treated with florfenicol(20 mg/kg) for 3 successive days (15^{th} - 17^{th} day of age) by drinking water. Group 3 was treated with florfenicol and tocotrienol (170 mg/kg, per os) for 7 successive days (15^{th} -21^{st}day of age). Group 4 was treated with florfenicol and selenium yeast (0.15 mg /kg, in feed) for 7 successive days (15^{th} - 21^{st}day of age). Group 5 was treated with the combination from florfenicol, tocotrienol and selenium yeast for 7 successive days (15^{th} - 21^{st} day of age).

Blood samples were collected from jugular vein of five birds of each group on the 18^{th}, 25^{th} and 35^{th} days of age. The liver of chicks was divided into two parts. One part was obtained immediately for fixed with 10% formalin solution for 48h and the another part was stored in at -20°C for determination of antioxidant /oxidant statusin the hepatic tissue on the 18^{th}, 25^{th} and 35^{th} days of age.

Biochemical analysis (Liver function tests)

Blood samples were collected and sera were separated. The sera were stored -20°C until examinationto evaluatealanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Varliy, 1974), alkaline Phosphatase ALP (Belfield and Goldberg,1971), total protein (TP) (Domas,1975), serum albumin (Doumas,1971), serum total globulin (Coles, 1974), serum total cholesterol (Flegg, 1973), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) (Gordon et al.,1977), and low-density lipoprotein cholesterol (LDL-C) (Friedewald et al.,1972) were estimated. Very low-density lipoprotein cholesterol (VLDL-C) was calculated by dividing triglycerides value by 5 (Tietz, 1976). Low-density lipoprotein cholesterol (LDL-C) was calculated by the following equation: LDL-C = (Total cholesterol) - (HDL-C) - (VLDL-C) (Ashayerizadeh et al., 2009).

Evaluation of antioxidant and oxidant status in hepatic tissue

Liver tissues from each chick were collected immediately and stored in low temperatures for reserve. Prior to dissection, perfuse hepatic tissues with phosphate buffered saline solution (PH 7.4) containing heparin (0.16 mg/ml) to remove any blood cells and clots. Homogenize the tissue in 5 -10 ml cold buffer (I, e, 50mM potassium phosphate, pH 7.5.1 mM EDTA) per gram tissue, using tissue homogenizer. Centrifuge at 4000 rpm for 5 minutes at 4°C. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at - 80°C. The sample will be stable for at least one month. The prepared samples were ready to evaluate superoxide dismutase (SOD) activity (Nishikimi et al., 1972), reduced glutathione **(**GSH) concentration (Beutler et al., 1963) and malondialdehyde (MDA) level (Ohkawa et al., 1979).

Histopathological examination of liver tissue

Liver samples were fixed in10% formalin solution for 48 hours for further histopathological examination following standard methodologies to obtain hematoxylin and eosin-stained slides (Bancroft and Stevens, 1977).

Statistical analysis

Statistical analysis was conducted with the Statistical Package for Social Science (SPSS Inc. Released, 2009) to determine if variables differed between groups, according to Snedecor and Cochran, 1989. The Shapiro-Wilk test was used to test the normal distribution of the data before statistical analysis was performed. Analysis of variance was conducted by one-way ANOVA and compare between means were conducted by Duncan's multiple range test (Duncan, 1955). Probability values of less than 5 % ($P < 0.05$) were considered a significant finding.

RESULTS

The blood biochemical parameters (liver enzymes) in broilers treated with florfenicol showed a significant ($P < 0.05$) increase in serum ALT on the 25^{th} day of age and no significant ($P > 0.05$) differences on the 35^{th} day of age while a significant ($P < 0.05$) increase in serum ALP activity when compared with normal group. Other treated group returned serum ALT and ALP activities to normal levels as shown in Table1.

On the 25^{th} day of age, florfenicol treated group showed significant ($P < 0.05$) decreases in serum TP, albumin and serum total globulin when compared with control group. Other groupsdisplayed significant ($P < 0.05$) increases in serum TP and albumin

when compared with florfenicol treated group. Selenium yeast and florfenicol treated group alone and the group treated with its combination with tocotrienol displayed a significant ($P < 0.05$) increase in serum total globulin when compared with florfenicol treated group as shown in Table1.

Florfenicol and tocotrienol treated group showed a significant ($P < 0.05$) decrease in serum cholesterol and LDL levels on the 18th day of age with no significant ($P > 0.05$) differences on the 25th day of age but triglycerides returned to normal on the 18th and 25th day of age when compared with control group. Selenium yeast and florfenicol treated group alone and the group treated with its combination with tocotrienol returned to normal lipid profile levelsas shown in Table1.

Florfenicol increased MDA level in hepatic tissue, but significantly ($P < 0.05$) decreaseSOD activity and GSH concentration in hepatic tissue when compared with the control group. Florfenicol, tocotrienol and selenium yeasteither alone orin combination showed a significant ($P < 0.05$) decrease in MDA as well assignificant ($P < 0.05$) increases in SODand GSH in hepatic tissue when compared with florfenicol treated group as shown in Table2.

For histopathological examination five samples from 30 birds (5/30) per groupwere collected on the 18th, 25th and 35th days of age.As shown in Fig.1A, the liver section of the negative control group revealed normal hepatic architecture. Liver sections of florfenicol treated group showed focal leukocytic infiltration in the hepatic tissue and focal coagulative necrosis of hepatocytes represented by deeply eosinophilic cytoplasm of the nuclei with karyorrhexis and karyolysis in the liver section of chicks (Fig.1B). Liver sections of florfenicol with tocotrienol treated group displayed fatty degeneration of some hepatic cells, with eccentric location of the nuclei on the 35th day of age and focal mononuclear cell infiltration of the hepatic tissue and activation of the Kupffer cells on the 35th day of age(Fig.1C).Liver sections of florfenicol and selenium yeast treated group revealed focal coagulation hepatocellular necrosis with karyolysis of the nuclei and ballooning degeneration represented by empty cytoplasm and centrally located nuclei in the adjacent hepatocytes(Fig.1D). Liver sections of florfenicol, tocotrienol and selenium yeast treated group showed activation of Kupffer cells and less evident necrotic changes (Fig. 1E).

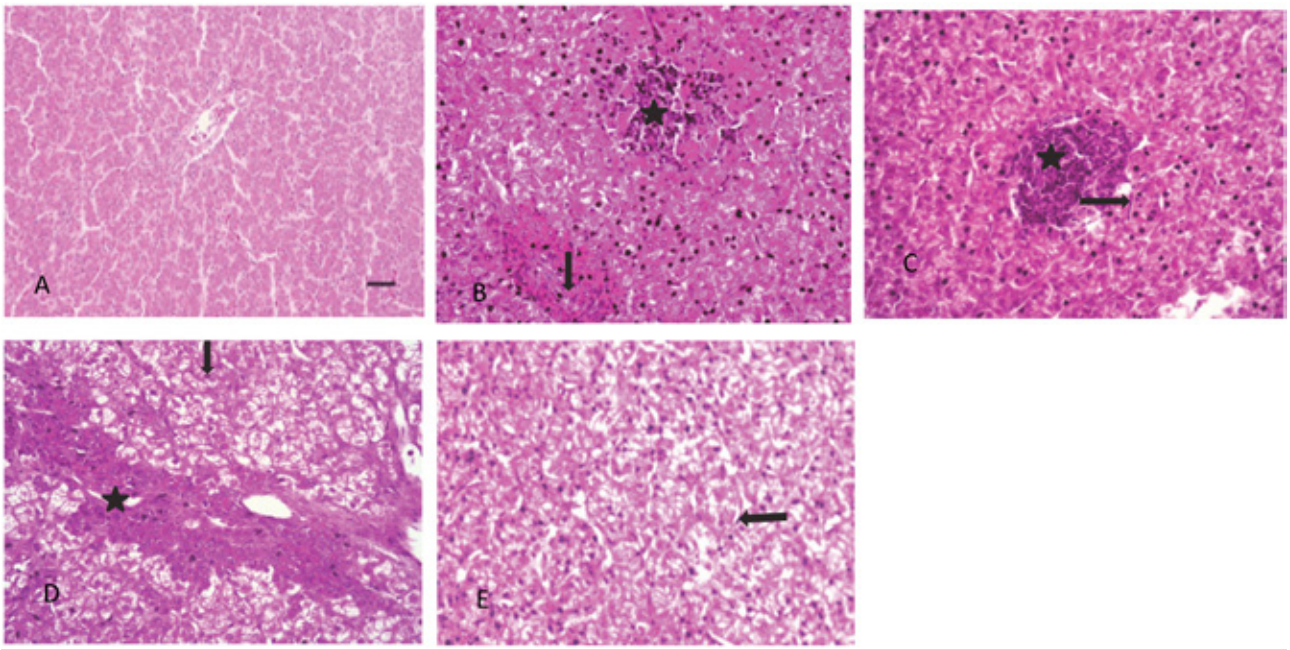

Figure 1. Histopathological analysis of liver tissue. Note: Liver tissues were fixed and stained with H&E. A) Control group 200X, B) Florfenicol treated group showed focal leukocytic infiltration in the hepatic tissue (star) focal coagulation necrosis of hepatocytes (black arrow)400X, C) Florfenicoland tocotrienol treated group showed activations of kupffer cells (black arrow) focal mononuclear cell infiltration of the hepatic tissue (star) 400X, D) Florfenicol and selenium yeast trated group showed focal coagulation necrosis (star) and ballooning degeneration (black arrow) 200X, E) Florfenicol, tocotrienoland seleniumyeast treated group showed activation of Kupffer cells (black arrow) and less evident necrotic changes. 200X

Table 1: Effect of oral administration of tocotrienol and, selenium yeast on blood biochemical parameters of broilers exposed to adverse effect of florfenicol. (Mean ± SEM)

Parameters	Experimental Periods	Groups				
		1	2	3	4	5
ALT (U/L)	18 day	11.00±.58^{a}	6.50±.29^{b}	5.00±.58^{b}	10.50±.29^{a}	10.00±.58^{a}
	25 day	4.50±.29^{c}	10.00±.29^{ab}	3.50±.29^{c}	9.00±1.15^{b}	11.50±.29^{a}
	35 day	12.50±.29^{b}	15.00±1.15^{ab}	12.00±1.73^{b}	16.50±.87^{ab}	17.50±2.02^{a}
AST (U/L)	18 day	12.50±.87^{a}	12.00±.00^{a}	12.00±.58^{a}	12.00±58^{a}	12.50±.29^{a}
	25 day	16.00±1.73^{bc}	11.00±1.15^{c}	14.00±2.31^{bc}	21.00±3.46^{ab}	25.00±2.89^{a}
	35 day	21.5±.87^{a}	22.00±1.73^{a}	23.00±.58^{a}	27.50±3.75^{a}	27.50±4.33^{a}
ALP (U/L)	18 day	522.50±17.03^{b}	608.00±23.67^{a}	600.00±10.39^{ab}	595.00±42.15^{ab}	672.00±20.21^{a}
	25 day	660.00±7.50^{b}	735.50±18.76^{a}	733.50±10.68^{a}	680.00±1.15^{b}	680.00±9.81^{b}
	35 day	618.50±3.50^{ab}	708.50±20.50^{a}	540.50±22.50^{b}	594.00±158.00^{ab}	519.00±158.00^{b}
Total Protein (g/dl)	18 day	3.55±.043^{a}	3.55±.043^{a}	3.33±.04^{ab}	3.25±.07^{b}	3.49±.14^{ab}
	25 day	4.27±.12^{b}	3.25±.12^{d}	3.62±.05^{c}	3.99±.08^{b}	4.79±.04^{a}
	35 day	3.60±.058^{ab}	3.35±.09^{b}	3.45±.09^{b}	4.00±.23^{a}	4.00±.17^{a}
Albumin (g/dl)	18 day	1.89±.07^{a}	1.78±.012^{ab}	1.70±.017^{bc}	1.63±.003^{c}	1.80±.055^{ab}
	25 day	2.26±.04^{a}	1.63±.04^{c}	2.03±.12^{b}	2.00±.017^{b}	2.13±.032^{ab}
	35 day	2.00±.06^{a}	1.80±.06^{a}	1.95±.03^{a}	2.15±.20^{a}	2.15±.14^{a}
Globulin (g/dl)	18 day	1.65±.02^{a}	1.75±.01^{a}	1.63±.02^{a}	1.62±.07^{a}	1.69±.08^{a}
	25 day	2.01±.075^{b}	1.61±.08^{c}	1.58±.06^{c}	1.99±.06^{b}	2.66±.07^{a}
	35 day	1.60±.00^{b}	1.55±.03^{b}	1.50±.11^{b}	1.85±.03^{a}	1.85±03^{a}
A/G ratio	18 day	1.15±.05^{a}	1.02±.00^{b}	1.04±.00^{ab}	1.00±.05^{b}	1.07±.02^{ab}
	25 day	1.12±.02^{ab}	1.01±.03^{b}	1.29±.13^{a}	1.00±.03^{b}	0.8 ±.03^{c}
	35 day	1.25±.03^{a}	1.16±.02^{a}	1.32±.12^{a}	1.16±.09^{a}	1.16±.05^{a}
Total Cholesterol (mg/dl)	18 day	129.00±3.46^{a}	138.00±9.23^{a}	102.50±.29^{b}	127.00±1.15^{a}	123.50±2.02^{a}
	25 day	91.00±10.97^{ab}	63.50±.87^{b}	69.50±3.17^{b}	91.00±15.58^{ab}	95.00±2.88^{ab}
	35 day	55.00±1.73^{c}	61.00±4.04^{bc}	75.50±9.53^{ab}	73.00±4.04^{ab}	69.50±3.75^{abc}
Triglycerides (mg/dl)	18 day	101.50±7.79^{b}	133.00±5.77^{a}	100.50±3.18^{b}	101.00±1.15^{b}	94.50±1.44^{b}
	25 day	77.50±4.90^{a}	46.00±1.73^{b}	67.50±3.18^{a}	64.00±7.51^{a}	63.50±4.33^{a}
	35 day	75.50±2.50^{b}	69.50±1.44^{b}	71.50±6.06^{b}	79.00±2.30^{ab}	78.50±2.02^{b}
HDL-C(mg/dl)	18 day	41.00±.58^{a}	39.00±1.15^{ab}	42.00±1.73^{a}	41.50±3.18^{a}	34.50±.29^{b}
	25 day	38.50±.87^{a}	33.00±2.31^{ab}	34.50±2.02^{ab}	32.00±2.31^{b}	31.50±.87^{b}
	35 day	38.00±.58^{b}	41.00±.58^{ab}	31.50±1.44^{c}	42.50±.29^{a}	37.00±.58^{b}
LDL-C (mg/dl)	18 day	67.70±2.30^{a}	72.40±16.00^{a}	40.40±4.600^{b}	65.30±7.100^{a}	70.10±3.500^{a}
	25 day	37.00±15.80^{ab}	21.30±6.10^{b}	21.50±.90^{b}	46.20±20.4^{a}	58.80±8.00^{a}
	35 day	13.90±1.50^{b}	15.10±5.50^{b}	29.70±11.90^{a}	14.50±5.70^{b}	16.80±4.80^{b}
VLDL-C (mg/dl)	18 day	20.30±2.70^{b}	26.60±2.00^{a}	20.10±1.10^{b}	20.20±.40^{b}	18.90±.50^{b}
	25 day	15.50±1.70 ^{a}	9.20±.60^{b}	13.50±1.10^{a}	12.80±2.60^{a}	12.70±1.50^{a}
	35 day	15.10±.50^{b}	13.90±.50^{b}	14.30±2.10^{b}	17.00±.80^{ab}	15.70±.70^{b}

^{ab} Mean values within the same row with different superscript letter are statistically different at $P \leq 0.05$. SEM = Standard Error of Means.1) Control group 2) Florfenicol treated group 3) Florfenicol and Tocotrienol treated group 4) Florfenicol and Selenium yeast treated group 5) Florfenicol and Tocotrienol with Selenium yeast treated group.
(ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline Phosphatase,A/G: Albumin/Globulin ratio, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, VLDL: Very low-density lipoprotein).

Table 2: Effect of oral administration of tocotrienol and selenium yeast on hepatic antioxidant/oxidant Status in broilers exposed to adverse effect of florfenicol. (Mean ± SEM) (n=5)

Parameters	Experimental Periods	Groups				
		1	2	3	4	5
Hepatic SOD (mg/dl)	18 day	80.39±7.33^{a}	46.72±2.76^{cd}	62.57±1.76^{bc}	60.71±2.33^{bc}	68.79±6.58^{ab}
	25 day	77.29±8.75^{a}	47.78±6.17^{b}	80.85±15.67^{a}	82.55±2.63^{a}	85.68±4.24^{a}
	35 day	53.61±.002^{a}	39.28±5.18^{b}	61.42±1.91^{a}	53.56±.26^{a}	57.10±4.41^{a}
Hepatic GSH (mg/dl)	18 day	4.76±.06^{a}	3.88±.069^{c}	4.68±.037^{a}	4.62±.133^{a}	4.18±.032^{b}
	25 day	4.14±.26^{b}	3.36±.020^{c}	3.58±.254^{c}	4.70±.069^{a}	4.66±.092^{ab}
	35 day	4.83±.060^{d}	4.03±.012^{e}	5.56±.057^{bc}	5.66±.10^{a}	5.32±.008^{b}
Hepatic MDA (mg/dl)	18 day	7.82±.10^{b}	9.95±.32^{a}	7.95±.40^{b}	5.95±.46^{c}	7.00±.72^{bc}
	25 day	6.25±.144^{b}	7.50±.69^{a}	7.67±.043^{a}	5.83±.13^{b}	6.85±.086^{ab}
	35 day	5.20±.12^{c}	6.18±.10^{a}	5.56±.057^{bc}	5.48±.21^{bc}	5.97±.30^{ab}

^{ab}Mean values within the same row with different superscript letter are statistically different at P≤ 0.05. SEM = Standard Error of Means. 1) Control group 2) Florfenicol treated group 3) Florfenicol and Tocotrienol treated group 4) Florfenicol and Selenium yeast treated group 5) Florfenicol and Tocotrienol with Selenium yeast treated group.
(SOD: Superoxide dismutase, GSH: Reduced glutathione, MDA: malondialdehyde).

DISCUSSION

Florfenicol administration has a damage effect in liver of broiler chickens so tocotrienol and selenium yeast used to mitigate these side effects. In serum biochemical examination,ALP activity returned to normal level in all groups except in the florfenicol treated group. This result was previously explained by Hasan et al. (2018) who stated that tocotrienol derived from seeds of *Bixa orellana* enhance bone structure and bone strength with decrease bone resorption and improve bone formation by increased activity of ALP. Moreover, Norazlina et al. (2002) reported that tocotrienol plays an important role in bone calcification. ALP has a critical role in bone calcification. Therefore, tocotrienol may improve the ALP activity. Our results related to selenium yeast were in accordance with those obtained by Yang et al. (2012) and Invernizzi et al. (2013) in maintaining ALP activity within normal**.** On the other hand, a significant increase in serum ALP activity of rats fed ontocotrienol (120-130 mg/kg b.w.) for 13 weeks was previously reported by Nakamura et al., 2001. This elevation may be caused by cholestasis or bone remodeling.

Albumin is one of main protein sources formed in the liver to maintain plasma osmotic pressure, providing energy and repairing tissue. Albumin is considered a carrier of nutrients to maintain the body tissue protein dynamic balance (Ahmed et al., 2002). In our results on the 25th day of age, all treated groups showed significant increases in serum TP and serum albumin when compared with the florfenicol treated group. These results were agreed by who reported with Shi et al., (2018) stating that diets supplemented with selenium can improve metabolism of major elements due to elevation of total protein and globulin. Our results related totocotrienol disagreed with those previously described by Tasakia et al. (2008) and Shibata et al. (2012), while our results regarding selenium yeast agreed with those previously described by El-Demerdash and Nasr (2014) and Shi et al. (2018) and disagree with those previously reported by Attia et al. (2010), Invernizzi et al. (2013) and Liu et al.(2020) in serum albumin characterization.

On the 25th day of age, the florfenicol treated group showed significant decreases in serum total globulin when compared with the control group, which is in agreement with Shaheen and El-Far **(**2013). The immunosuppressive effects of florfenicol could be attributed to its protein inhibition. However, our results disagree with those previously published by Allam et al. **(**2014) who reported a significant increase in serum total globulin of Pekin ducklings.

The selenium yeast treated group alone and combinedwith tocotrienoldisplayed a significant increase in serum total globulin when compared with the florfenicoltreated group. This resultagree with that mentionedby Shi et al. (2018) who proved that elevation

of serum globulines enhance immune system of animals. In addition, these results were unconformity with those of Yang et al. (2012) who showed no significant difference in serum total globulinin broilers fed on 3 ppm selenium yeast from 0 to 3 weeks of age.

The tocotrienol treated group showed a significant decrease in serum cholesterol and LDL levels on the 18th day of age when compared withthe control group. The selenium yeasttreated group alone and combined with tocotrienol retuned the lipid profile levels to normal. The hypocholesterolemic effect of tocotrienol may be attributed to the decrease activity of β-hydroxy-β-methylglutaryl coenzyme A reductase (Yuet al., 2006). These results referred to tocotrienolagreed with those previously described by Qureshi and Peterson (2001), Yu et al. (2006) and Budin et al. (2009). Our results involving selenium yeast are similar with those published by Sevcikova et al. (2008) and Yang et al. (2012). Our results were not in agreement with Hasselwander et al. (2001) who reported that tocotrienol had little effect on serum lipid levels. Attia et al. (2010) showed significant decreases in serum cholesterol level and triglycerides after selenium yeast treatment.

Florfenicol treated group showed a significant increase of MDA level and significant decrease of SOD activity and GSH concentration in hepatic tissues in comparison with the control group.These results attributed to the ability of florfenicol to inhibit the expression of antioxidant proteins Nrf2, HO-1 and NQO-1 resulted in decrease antioxidant factors SOD and GSH (Han et al., 2020). Firozian et al. (2020) reported that elevation of lipid peroxidation is usually joined by decrease GSH and SOD antioxidant factors. GSH depletion can cause oxidative damage but SOD can directly inhibit reactive oxygen species (ROS) formations which they are considered an important antioxidant defense enzymes. These results are in agreement with those described by Farombi et al. (2001) who reported that MDA level was elevated and glutathione was lowered in liver of rats, which received 28.6 mg/kg chloramphenicol. On the other hand, Elia and Pacini, (2016) reported an increase in liver glutathione levels of rainbow trout treated with a dose of 7.5 and 15 mg/kg b.w. florfenicol. Glutathione levels were 1.5 fold higher in elevated dose of florfenicol attributed to protect liver cells from oxidative damage of florfenicol.

Other treated groups such as the tocotrienol and selenium yeast alone and combination of them showed significant decreases in MDA level while significant increases in SOD activity and GSH concentration in hepatic tissue when compared with the florfenicol treated group. The result of SOD activity in liver tissue with the addition of tocotrienol was attributed to the disturbance of defense mechanism of liver tissue which stimulates production of superoxide anion radicals which prevent lipid peroxide formations (Lee et al., 2009). Selenium yeastmay improve the antioxidant status of broilers by increasing the activity of antioxidant enzymes and inhibiting lipid peroxidation (Jiang et al., 2009; Yang et al., 2012**).** Gladyshev and Hatfield, (1999) reported that selenium inter in the form of amino acid called selenocysteine (one of selenoprotein) which have important enzymatic functions associated with antioxidant activity. Selenium is important in sulphur amino acid metabolism. In this way, the sulphur amino acids methionine and cystine can spare selenium through their antioxidant role.

Our results referred totocotrienol of liver tissue were in accordance to those explained by Khan et al. (2011). Moreover, Palozza et al. (2006) reported that tocotrienols decrease MDA in rat liver microsomes (obtained from tissue homogenization then *in vivo* added tocotrienols to suspension) by inhibition of 2,2 -azobis 2-amidinopropane (AAPH) which induced MDA production.This results related to in selenium yeastwere also observed by several authors (Mahmoud and Edens, (2003); Petrovič et al., 2006; Baowei et al., 2011; Li et al.,2016; Hamidet al., 2018) who concluded that selenium yeast restored or increased the liver antioxidant defense. The antioxidant capacity of selenium is an integral component of glutathione peroxidase (GPx) which plays a crucial role to reduce cellular damage by ROS (Kong et al., 2017). Our results disagreed with those previously explained by Lee et al. (2005) who reported SOD activity in liver of rats was lowered with age when theses rats were fed with palmvitee (palm oil). This decrease may be due to the compensating effect of palmvitee which replaces antioxidant enzyme activities.Our results in selenium yeastalso disagreed with those described by Holovská et al. (2003) and Chenet al. (2013) who showed no significant differences in SOD activity of hepatic tissue in chickens fed on selenium yeast.

Liver sections of the florfenicol treated group displayed focal leukocytic infiltration in the hepatic tissue and focal coagulative necrotic foci represented by deeply eosinophilic cytoplasm of the nuclei with karyorrhexis and karyolysis in the liver section

of chicks. Our findings were in correlation to those described by Yue and Li-hai (2009) who reported fatty degeneration and necrosis in the livers of seventh day old broiler chicks, which were fed diet containing different doses of florfenicol (200, 400, 800 and 2000 mg/kg) for 14 days. In addition, Reda et al. (2013) noted diffused hydropic degeneration in the liver examined in Nile tilapia (*Oreochromis niloticus*) which received 5 mg/kg of florfenicol for 12 weeks, whereas Isa et al. (2020) described coagulative necrosis with karyopyknosis and karyorrhexis in livers of 8-week-broiler chicks which treated with 250 mg/kg chloramphenicol from the 1st day.

Liver sections of the tocotrienol treated group showed fatty degeneration of some hepatic cells with eccentric location of the nuclei on the 35th day of age and focal mononuclear cell infiltration of the hepatic tissue and activation of the Kupffer cells on 35th day of age. These findings are in agreement with those of Qureshi et al. (2011) who stated that mild chronic inflammation was observed in liver of chickens fed with diet containing 50 ppm δ-tocotrienol and Wong et al. (2012) who observed mild fatty changes in the liver of rats which fed a high carbohydrate diet, contained 240 mg TRF/mL palm olein, for 8 weeks. On the other hand, no histopathological changes were observed in the liver tissue of mice and rats treated with different doses of tocotrienol (Husain et al., 2009; Shibataet al., 2012).

In our study, treatment with selenium yeast revealed focal hepatocellular coagulative necrosis with karyolysis and ballooning degeneration represented by empty cytoplasm and centrally located nuclei in the adjacent hepatocytes.

Our results are in agreement with those previously published by Attia et al. (2010) who stated that breeding hens fed on selenium yeastshowed mildfocal necrosis in hepatic tissue. Moreover, Hamid et al. (2018) concluded that rats treated with selenium-enriched yeast showed ballooning of liver hepatocytes, mild fatty changes, and mild degree of centrilobular necrosis with partial infiltration of inflammatory cells.

On other hand, Mirjana et al. (2004) reported that chickens orally fed on selenized yeast showed varying degree of intracellular edema and fatty changes in the liver of sacrificed chicks..

Broilers treated with the combination of tocotrienol and selenium yeast revealed focal mononuclear cell infiltration in the fibrous connective tissue of the portal area and activation of the Kupffer cells with little evidence of necrotic changes in the liver. This means that the addition of tocotrienol with selenium yeast mitigated the damage caused by florfenicol on liver tissue.

CONCLUSION

The addition of tocotrienol, selenium yeast alone or their combination can improve the antioxidant effect and mitigate lipid peroxidation in the livers of treated broiler. Therefore, tocotrienol and selenium yeast can improve the safety of using florfenicol in broilers under experimental conditions.

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CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

Clinical and bacteriological analysis of respiratory tract infections in sheltered dogs and determination of antibacterial treatment options*

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ABSTRACT: After canine infectious respiratory disease complex-CIRDC, only bacterial pneumonia or accompanied with CIRDC has a higher proportion in respiratory disease in stray dogs. Management of these respiratory problems in terms of both treatment and prevention in shelters has a big importance for animal welfare. With this purpose, the present study evaluates bacterial pneumonia in terms of clinical, bacteriological and the antibacterial treatment options in 100 sheltered dogs with respiratory tract infection symptoms. In all dogs, status of respiratory disease and treatment efficacy were evaluated by haematological analysis and clinical scores. Haematological analyses showed that all of the dogs suffered leucocytosis before treatment. Health status of all animals before, during and after treatment were evaluated according to nine clinical scores include clinical condition, body temperature, respiratory and heart rates per minutes, nasal discharge, tracheal sensitivity, mucous membranes, coughing, auscultation.For bacteriological analysis and antimicrobial susceptibility tests, Bronchoalveolar lavage-BAL fluids were obtained from all dogs, twice. The bacterial agents isolated in the present study were *Bordetella* spp. (38.98%), *Mycoplasma* spp. (21.19%), *Klebsiella* spp. (16.10%), *E. coli* (5.93%), *S. aureus* (5.08%) and *Pasteurella* spp. (4.24%). Susceptibility tests were performed by using the disc diffusion method for Enrofloxacin (ENR), Trimethoprim/Sulpha (TS), Chloramphenicol (C), Amoxicillin clavulanate (AC), and Erythromycin (E) in all cases. *Bordetella* spp. isolated from 46 cases were found to be most susceptible to ENR (21/46 = 46%), TS (12/46 = 26%), and C (11/46 = 24%). *Mycoplasma* spp. were isolated from 25 cases and were found to be susceptible to C (14/25 = 56%), TS (8/25 = 32%), and ENR (3/25 = 12%). *Klebsiella* spp. were isolated from 19 cases and the antibiotics most effective were ascertained as C (9/19 = 47%), ENR (9/19 = 47%), and TS (1/19 = 5%).The results showed that clinical scores could be useful in the diagnosis and monitoring of respiratory tract diseases in sheltered dogs. Besides, in the light of the findings of presented study, enrofloxacin, chloramphenicol, and trimethoprim/sulpha were proven efficient against to bacterial isolates in sheltered dogs in the treatment of bacterial pneumonia. Antibacterial therapy should be conducted by antibiotic sensitivity test. But, in the cases this is not possible, antibiotic choice may contain enrofloxacin, trimethoprim/sulpha. If it is not forbidden to use for companion animals by administrations, chloramphenicol may also be thought as alternative.

***Keywords*:** Antimicrobial susceptibility, Bacterial pneumonia, Aetiology, Sheltered dog, Treatment

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INTRODUCTION

One of the most common health problem encountered in dogs which are housed in crowded environments such as shelters and pet shops, is respiratory disease (Kennerman et al., 2000; Maden et al., 2000; Mochizuki et al., 2008; Litster et al., 2011; Ayodhya et al., 2013b). Canine infectious respiratory disease, which is primarily caused by viruses (i.e. distemper virus, canine parainfluenza virus-CPIV) (Mochizuki et al., 2008; Priestnall et al., 2010), can also be of bacterial origin (Kennerman et al., 2000; Ayodhya et al., 2013a). Opportunistic bacteria, including *Bordetella bronchiseptica*, *Mycoplasma* spp., *Klebsiella* spp., *Escherichia coli*, *Pasteurella multocida*, *Streptococcus* spp., *Staphylococcus* spp. and *Pseudomonas* spp., are frequently isolated in cases of bacterial pneumonia (Hawkins, 2005; Gonul et al., 2010; Vieson et al., 2012).

During diagnosis of respiratory diseases, history as well as general and specific physical examinations are used to detect the probable cause and localization of the disease (Peeters et al., 2000; Kuehn, 2005). Bronchoalveolar lavage is the most frequently used for the diagnosis of respiratory diseases (Maden et al., 2001; Silverstein and Drobatz, 2005; Gonul et al., 2010) andis also the safest with the least complications (Maden et al., 2000; Hawkins, 2005; Silverstein and Drobatz, 2005).

The treatment of respiratory diseases requires the control of the infection, the maintenance of the airways in an open state, and the acceleration of the cleaning of the airways (Peeters et al., 2000; Gonul et al., 2010; Vieson et al., 2012). For better efficiency, it is recommended to apply antibacterial treatment based on bacterial culture results (Ettinger and Kantrowitz, 2005; King, 2010a; King, 2010b). In this study, the clinical and bacteriological analysis of bacterial respiratory diseases in sheltered dogs, and the determination of antibacterial treatment options were aimed.

MATERIALS AND METHODS

This study was conducted pursuant to approval numbered with 2011/032 of the Ethics Board of Selçuk University, Faculty of Veterinary Medicine.

Animals

The study materials were 100 dogs in different breeds, age (6 months-7 years) and sex, which were kept at the shelter of the Konya Metropolitan Municipality and showed signs of respiratory disease. The inclusion criteria for dogs showing signs of respiratory disease selection were made on the basis of physical examination, haematology, blood gas analysis and BAL fluid analysis. Dogs with serous eye and nasal discharge and/or sneezing, expectoration, wet/dry cough, signs of abnormal lung auscultation, and general signs of infection such as high fever, weakness, and anorexia were included in the study. Dogs with comorbid diseases other than respiratory tract disease and determined to be treated before were excluded from the study.

All of the dogs with respiratory disease were evaluated according to the results of the treatment as recovered and non-recovered which were defined after 5 days of treatment on the basis of clinical scores and BAL fluid analysis.

Clinical Examination

All of the dogs were physically examined for their general clinical condition, body temperature, heart and respiratory rates, mucous membranes, nasal discharge, tracheal sensitivity, and coughing. Auscultation was also performed. These clinical parameters were scored as shown in Table 1, and were evaluated and recorded on a daily basis throughout the treatment period.

Laboratory Analyses

For to evaluate both the presence of infection in the sick dogs and the state of ventilation of the animals, blood samples were collected from each dog before and after treatment into anticoagulant-coated tubes (BD EDTA K2, BD Diagnostics, USA) for complete blood count (MS™4E, France) and heparin-coated syringe (BD Preset™ Syringe, BD Diagnostics, USA) for venous blood gas analyses (GEM®Premier 3000, USA).

Collection and Examination of Bronchoalveolar Lavage Fluid

For the collection of BAL fluid samples, the animals were anaesthetized with a combination of ketamine hydrochloride (2-4 mg/kg, b.w., i.m., Ketasol® 10%, Interhas, Wels-Austria) and xylazine (1-2 mg/kg, b.w., i.m., Alfazyne® 2%, Ege Vet, Holland). A sterile endotracheal tube, the size of which was selected according to the size of the dog (No: 4.5/7/8 mm, Bıçakçılar®, İstanbul) was inserted into the trachea. Next, a sterile propylene catheter, measuring 2.67 mm x (8 ch) x 500 mm (Feeding tube, Bıçakçılar®,

Table 1. Clinical Scoring

PARAMETER	EVALUATION/SCORES				
	1	2	3	4	5
Clinical Condition	***Normal***	***Mild*** has food and water intake, and environmental relation	***Moderate*** No appetite, has water intake, poor environmental relation	***Severe*** No appetite, water intake too low, depressive	
Mucous Membranes	***Normal***	***Mild*** Less hyperemia	***Moderate*** Diffuse hyperemia	***Severe*** Diffuse dark hyperemia, plumped conjunctival vessels	
Tracheal Sensitivity	***None***	***Available***			
Nasal Discharge	***None***	***Serous***	***Seromucous***	***Mucous***	***Purulent***
Auscultation	***Normal***	***Mild*** Hardened vesicular and bronchial sounds	***Moderate*** Wet rales, crackling and rustling sounds	***Severe*** Dry rales and wheezing / friction sounds	
Cough	***None***	***Mild*** with long intervals	***Moderate*** with short intervals	***Severe*** Continuous	

İstanbul), was passed through the lumen of the endotracheal tube and pushed forward up to the carina region. At this point, 20 cc of saline was injected into the carina and was immediately aspirated. BAL fluid samples were collected twice, before and after treatment.

Microbiological Analyses

The BAL fluid samples taken before treatment were used for the isolation of bacteria and the antimicrobial susceptibility tests. Blood agar, MacConkey agar, Bordet-Gengou agar, Mycoplasma agar and Sabouraud dextrose Agar (SDA) for fungi (OxoidTM, Thermo Fisher Scientific Inc. Basingstoke, England) were used as growth media. The growth media were prepared according to the manufacturers' instructions. After the inoculation of BAL samples, SDA was incubated aerobically at room temperature for 5-7 days while other media except Mycoplasma agar were incubated at 37°C for 24-48 hours. Mycoplasma agar was incubated at 37°C for 24-48 hours in microaerophilic conditions. The colonies that were grown at the end of incubation were identified based on colony morphology, microscopic morphology and biochemical characteristics (Winn et al, 2006). The BAL fluid samples taken after treatment were also inoculated into growth media to check for the presence of bacteria. Biochemical tests were used catalase test, coagulase test, optochin sensitivity test, CAMP test, nitrate test, motility test, haemolysis on blood agar, growing on Mannitol Salt Agar, Bile Esculin Agar for gram positive bacteria and oxidase test, gas production, Methyl Red / Voges-Proskauer (MR/VP) test, urease test, growing on MacConkey agar, Kliger's Iron Agar (KIA), Sulfur Indole motility media for gram negative bacteria. In addition, the germ tube test with human serum performed for the identification of yeasts. Fungi were identified according to microscopic morphology and colony morphology. The antimicrobial susceptibility of the identified bacterial strains was determined using antibiotic discs (OxoidTM, Thermo Fisher Scientific Inc. Basingstoke, England) and the disc diffusion method with Mueller-Hinton agar (OxoidTM, Thermo Fisher Scientific Inc. Basingstoke, England) (Bauer et al, 1966). Results were evaluated according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2015).

Establishment of the Treatment Protocol

The treatment was initiated with an antibacterial drug selected according to the results of the antimicrobial susceptibility test, and a non-steroidal anti-inflammatory drug (NSAID). The antibacterial drug to which the highest susceptibility level was detected was selected for cases, in which a single microorganism was isolated, whilst the antibiotic with the broadest spectrum was selected for cases, in which more than one microorganism was isolated. Depending on the health status of the patient and the course of the disease, supportive medications (i.e. use of expectorants, bronchodilators) were included in the treatment protocol. The appropriate antibacterial drug was selected

according to the BAL fluid culture and antimicrobial susceptibility tests. The dosage and duration for drugs used in the treatment of animals were determined according to manufacturer recommendations described in the drug package insert and administered to rules of shelter management as generally, single-dose use and the period of five days. Ketoconazole was added to the treatment protocol in the cases isolated the fungal agents along with bacteria. The drugs used and their administration doses were determined according to text books (Boothe, 2005; Yazar, 2012; Papich, 2016) and presented in Table 2.

Statistical Analyses

For non-parametric data, Mann-Whitney U test was used and for parametric data, independent *t*-test was used for the evaluation of difference between the groups. The difference between the groups for the treatment results (recovered/non-recovered), the scores of clinical condition, mucous membranes, nasal discharge, tracheal sensitivity, auscultation and cough were analysed with the Mann-Whitney U test (SPSS, 2007). Data related to fever, heart rate and respiration rate were analysed with the independent *t*-test (SPSS, 2007).Within the groups, for the evaluation of difference, daily data collected during the observation of the animals for 5 days was firstly evaluated with the Friedman test. Later, the difference determined by the Friedman test was investigated with Wilcoxon *t-test* for non-parametric data and paired *t-test* (SPSS, 2007) for parametric data. Differences were considered significant when $P<0.05$.

Table 2. Antimicrobial drugs used and their respective dose

ACTIVE INGREDIENT	APPLICATION DOSAGE / WAY
Enrofloxacin (Killoxacin® %5, BaVET, Turkey)	10 mg/kg b.w.,SID, i.m., for five days
Chloramphenicol (Gemysetinsuksinat® 1 gr im/iv lyofilize enjektabl, Deva İlaç, Turkey)	50 mg/kg b.w.,BID, i.m., for five days
Trimethoprim-sulfamethoxazole (Primoksal®, Alke®, Turkey)	25 mg/kg b.w.,SID, i.m., for five days
Amoxicillin-Clavulanic acid (Klavil®, Vilsan, Turkey)	8,75 mg/kg b.w., SID,i.m., for five days
Erythromycin (Apirocin-F®, Teknovet, Turkey)	10 mg/kg b.w.,SID, i.m., for five days

RESULTS

The state of recovery of the sick dogs was assessed on the basis of 9 clinical scores (6 non-parametric and 3 parametric). The clinical scores of the dogs that recovered and did not recover significantly differed for their examination results of general clinical condition ($P<0.05$), the mucous membranes ($P<0.001$) and nasal discharge ($P<0.001$) on the first day of treatment; for pulmonary auscultation findings ($P<0.05$), coughing ($P<0.001$) and respiratory rate ($P<0.05$) on the second day of treatment; for heart rate ($P<0.05$) and tracheal sensitivity ($P<0.001$) on the third day of treatment; and body temperature ($P<0.001$) on the fourth day of treatment (Table 3 and Table 4).

Haematological analyses performed before and after treatment showed that all of the dogs suffered leucocytosis before treatment. While leukocyte counts were observed to normalize in the dogs that recovered upon treatment, the dogs that did not recover continued to suffer from leucocytosis (Table 5). Blood gas analyses demonstrated that pO2 levels, which were low before treatment ($P<0.05$), normalized after treatment in the cases that recovered but remained close to pre-treatment levels in the animals that did not recover (Table 6).

In the microbiological analysis of BAL fluids of all dogs, bacterial agents (n:70), *Aspergillus* spp. (n:3) and in one dog both bacterial agent and *Candida* spp. were isolated (Figure 1).The bacterial agents isolated in the present study were *Bordetella* spp. (38.98%), *Mycoplasma* spp. (21.19%), *Klebsiella* spp. (16.10%), *E. coli* (5.93%), *S. aureus* (5.08%) and *Pasteurella* spp. (4.24%) (Figure 1).

Bordetella spp. isolated from 46 cases were found to be most susceptible to ENR (21/46 = 46%), TS (12/46 = 26%), and C (11/46 = 24%). *Mycoplasma* spp. were isolated from 25 cases and were found to be susceptible to C (14/25 = 56%), TS (8/25 = 32%), and ENR (3/25 = 12%). *Klebsiella* spp. were isolated from 19 cases and the antibiotics they were most susceptible to were ascertained as C (9/19 = 47%), ENR (9/19 = 47%), and TS (1/19 = 5%) (Table7). Treatment with the drugs selected according to the results of the antimicrobial susceptibility tests, and supportive therapy resulted in the clinical recovery of 57 out

of the 74 dogs with bacterial respiratory disease. In ten of these dogs, despite clinical recovery, *Bordetella* spp. and *Klebsiella* spp. were isolated from the BAL fluid samples taken after treatment (Figure 2). The clinical efficacy of drugs used for treatment, on the basis of the antimicrobial susceptibility test results, are given in Table 8. Accordingly, the overall rate of clinical recovery achieved with the treatment of bacterial respiratory disease was determined as 77.02% (74/57), whilst the overall clinical efficacy of drugs used for treatment was ascertained as 63.51% (74/47). Post-treatment evaluation demonstrated that the overall clinical success rates achieved with the drugs used were 76.08% for *Bordetella* spp., 80% for *Mycoplasma* spp. and 73.68% for *Klebsiella* spp. infections.

Table 3. Daily recovery status according to treatment outcomes (non-parametric clinical scores)

			Daily Median of Clinical Scores					
Parameters	Results of treatment	n	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Clinical Status	+	57	3	3	2	2	1	1
	-	17	4	4	4	3	3	3
	P		0.363	0.002	0.001	0.001	0.001	0.001
Mucous Membrane	+	57	4	3	2	2	1	1
	-	17	4	4	4	4	3	3
	P		0.555	0.001	0.001	0.001	0.001	0.001
Tracheal Sensitivity	+	57	2	2	2	1	1	1
	-	17	2	2	2	2	2	2
	P		0.475	0.475	0.866	0.001	0.001	0.001
Nasal discharge	+	57	5	4	4	3	3	2
	-	17	5	5	5	5	5	4
	P		0.105	0.001	0.001	0.001	0.001	0.001
Auscultation	+	57	3	3	2	2	2	2
	-	17	3	3	3	3	3	3
	P		0.886	0.325	0.002	0.001	0.001	0.001
Cough	+	57	3	3	2	2	1	1
	-	17	3	3	3	3	3	3
	P		0.499	0.080	0.001	0.001	0.001	0.001

Clinical condition, mucous membrane, auscultation and cough were scored with in 1 to 4. Nasal discharge was scored with in 1 to 5. And, tracheal sensitivity was scored as none (1) or available (2) (Table 1). +: Recovered, -: Non-recovered, *P*: Significance value of between groups, recovered or non-recovered.

Table 4. Daily recovery status according to treatment outcomes (parametric clinical scores)

			Daily Means of Clinical Scores ($\bar{x}$) ± SEM					
CS	Results of treatment	n	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
T (°C)	+	57	39.177 ± 0.106	39.053 ± 0.084	38.849 ± 0.070	38.667 ± 0.068	38.454 ± 0.047	38.204 ± 0.040
	-	17	39.088 ± 0.166	39.164 ± 0.188	39.000 ± 0.211	38.965 ± 0.191	39.141 ± 0.185	39.159 ± 0.155
	P		0.680	0.545	0.383	0.070	0.001	0.001
PL	+	57	92.684 ± 2.870	94.263 ± 2.324	94.877 ± 1.864	92.386 ± 1,485	93.737 ± 1.288	92.070 ± 0.974
	-	17	97.177 ± 6.515	98.412 ± 5.058	100.235 ± 5.477	100.706 ± 5.156	101.529 ± 4.388	99.706 ± 3.607
	P		0.481	0.416	0.240	0.036	0.023	0.005
R	+	57	26.825 ± 1.345	25.737 ±1.039	25.018 ± 0.919	23.737 ± 0.735	23.807 ± 0.612	22.228 ± 0.524
	-	17	27.118 ± 2.602	28.824 ± 2.456	30.059 ± 2.396	31.118 ± 2.14	32.647 ± 2.174	35.824 ± 2.187
	P		0.918	0.188	0.020	0.001	0.001	0.001

+: Recovered, -: Non-recovered, T: Body temperature, PL: Heart Rate/min, R: Breath/min, $\bar{x}$: Mean, SEM: Standard error of means, *P*: Significance value of between groups, recovered or non-recovered.

Table 5. Comparative haematology analysis before and after treatment

Parameter	Treatment result	n	BEFORE ($\bar{x}$) ± SEM	*P*	AFTER ($\bar{x}$) ± SEM	*P*
WBC (x10³/mm³)	-	17	23.132±1.931	0.912	21.582±0.777	0.001
	+	57	22.874±1.135		14.311±0.432	
RBC (x10⁶/ mm³)	-	17	6.248±0.308	0.935	5.988±0.291	0.019
	+	57	6.220±0.163		6.761±0.154	
HCT (%)	-	17	38.171±1.815	0.794	37.506±1.776	0.046
	+	57	37.619±1.011		41.679±0.991	
HB (g/dl)	-	17	12.914±0.768	0.673	12.865±0.650	0.241
	+	57	12.612±0.315		13.563±0.260	
MCV (fL)	-	17	59.594±1.133	0.963	59.276±0.723	0.003
	+	57	59.509±0.940		62.209±0.489	
MCH (Pg)	-	17	20.182±0.538	0.676	20.612±0.503	0.203
	+	57	20.653±0.588		21.907±0.529	
MCHC (g/dL)	-	17	33.471±0.585	0.613	33.506±0.858	0.251
	+	57	34.386±0.964		35.588±0.945	
RDW (%)	-	17	11.453±0.440	0.132	12.853±0.527	0.004
	+	57	10.775±0.205		11.482±0.203	

-: Non-recovered, +: Recovered, $\bar{x}$: Mean, SEM: Standard error of means, *P*: Significance value of between groups, recovered or non-recovered.

Table 6. Comparative venous blood gas analysis before and after treatment

Parameter	Results of treatment	n	BEFORE ($\bar{x}$) ± SEM	*P*	AFTER ($\bar{x}$) ± SEM	*P*
pH	-	17	7.325±0.014	0.593	7.292±0.010	0.001
	+	57	7.315±0.010		7.363±0.004	
pCO2 (mmHg)	-	17	43.294±1.419	0.178	48.941±1.273	0.001
	+	57	41.088±0.778		38.281±0.520	
pO2 (mmHg)	-	17	40.941±2.240	0.026	52.647±1.768	0.001
	+	57	48.509±1.688		59.000±0.964	
HCO3 (mmol/L)	-	17	22.388±1.180	0.242	19.588±0.985	0.001
	+	57	20.998±0.539		24.058±0.254	
BEecf (mmol/L)	-	17	-3.482±1.346	0.240	-7.412±1.171	0.001
	+	57	-5.153±0.658		-1.118±0.263	

-: Non-recovered, +: Recovered, $\bar{x}$: Mean, SEM: Standard error of means, *P*: Significance value of between groups, recovered or non-recovered.

Table 7. Results of antimicrobial susceptibility tests

Agents	n	TS	ENR	C	E	AC
Mycoplasma spp.	74/25	25/8	25/3	25/14		
Bordetella spp.	74/46	46/12	46/21	46/11	46/1	46/1
Pasteurella spp.	74/5		5/5			
Klebsiella spp.	74/19	19/1	19/9	19/9		
E. coli	74/7		7/3	7/4		
Staphylococcus spp.	74/2		2/1			2/1
Streptococcus spp.	74/2			2/2		
S. aureus	74/6	6/1	6/1	6/1	6/2	6/1
*Staphylococcus*coagulase -	74/1		1/1			
Trueperella pyogenes	74/1					1/1

TS: Trimethoprim sulfamethoxazole, ENR: Enrofloxacin, C: Chloramphenicol, E: Erythromycin, AC: Amoxicillin / clavulanate.

Table 8. Percentage results of clinical efficacy after antimicrobial treatment

	ENR			C			TS			AC			E		
	+	-	%	+	-	%	+	-	%	+	-	%	+	-	%
Mycoplasma spp.	2	1	66.66	12	2	85.71	6	2	75						
Bordotella spp.	13	8	61.90	10	1	90.90	11	1	91.66	0	1	0	1	0	100
Pasteurella spp.	5	0	100												
Klebsiella spp.	6	3	66.66	7	2	77.77	1	0	100						
E. coli	0	3	0	4	0	100									
Staphylococcus spp.	1	0	100							1	0	100			
Streptococcus spp.				2	0	100									
S. aureus	1	0	100	1	0	100	1	0	100	1	0	100	2	0	100
*Staphylococcus*coagulase -	1	0	100												
T. pyogenes										1	0	100			

+: Effective, -: Non-effective, TS: Trimethoprim sulfamethoxazole, ENR: Enrofloxacin, C: Chloramphenicol, E: Erythromycin, AC: Amoxicillin / clavulanate. %: Percentage of clinical efficacy of drugs chosen according to results of antimicrobial susceptibility tests.

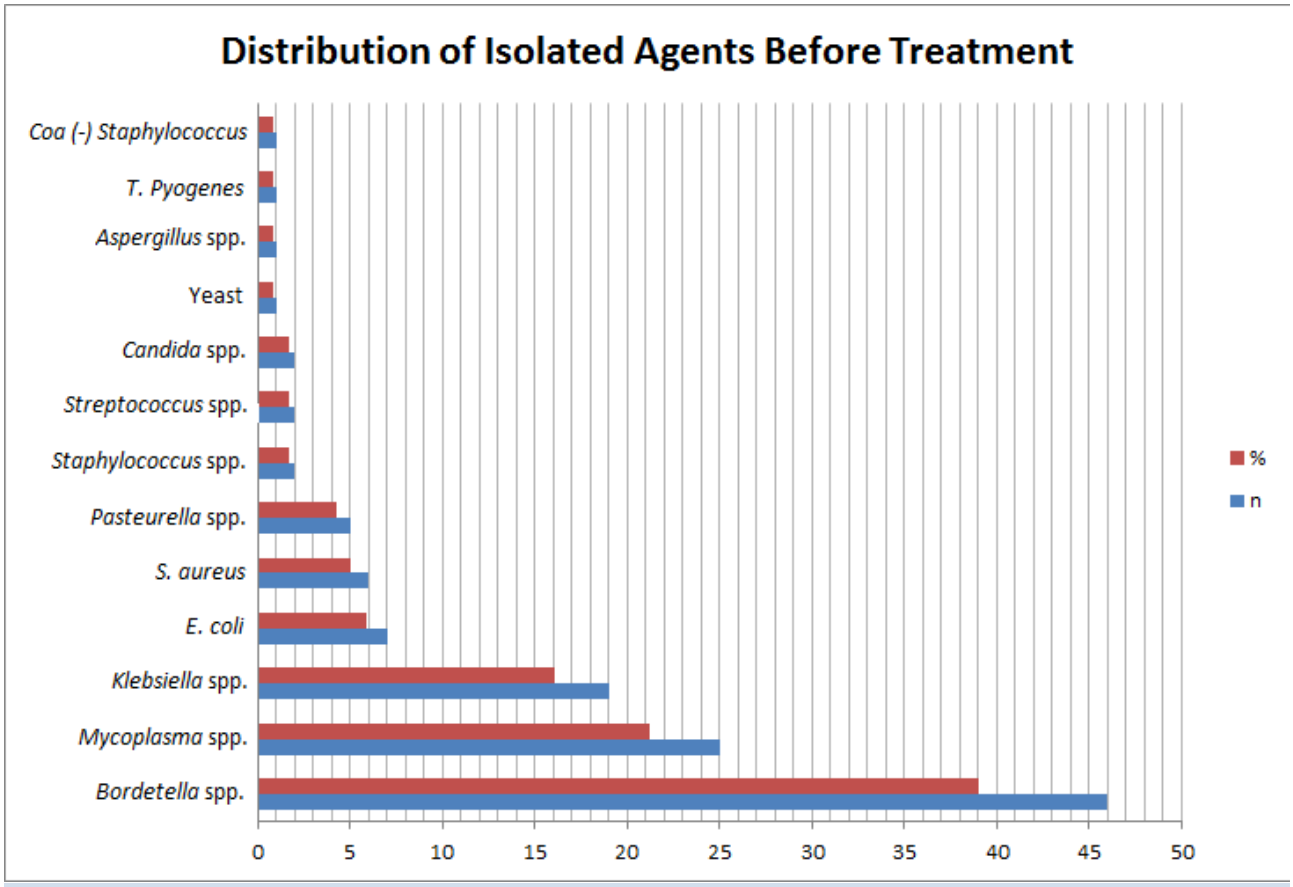

Figure 1.

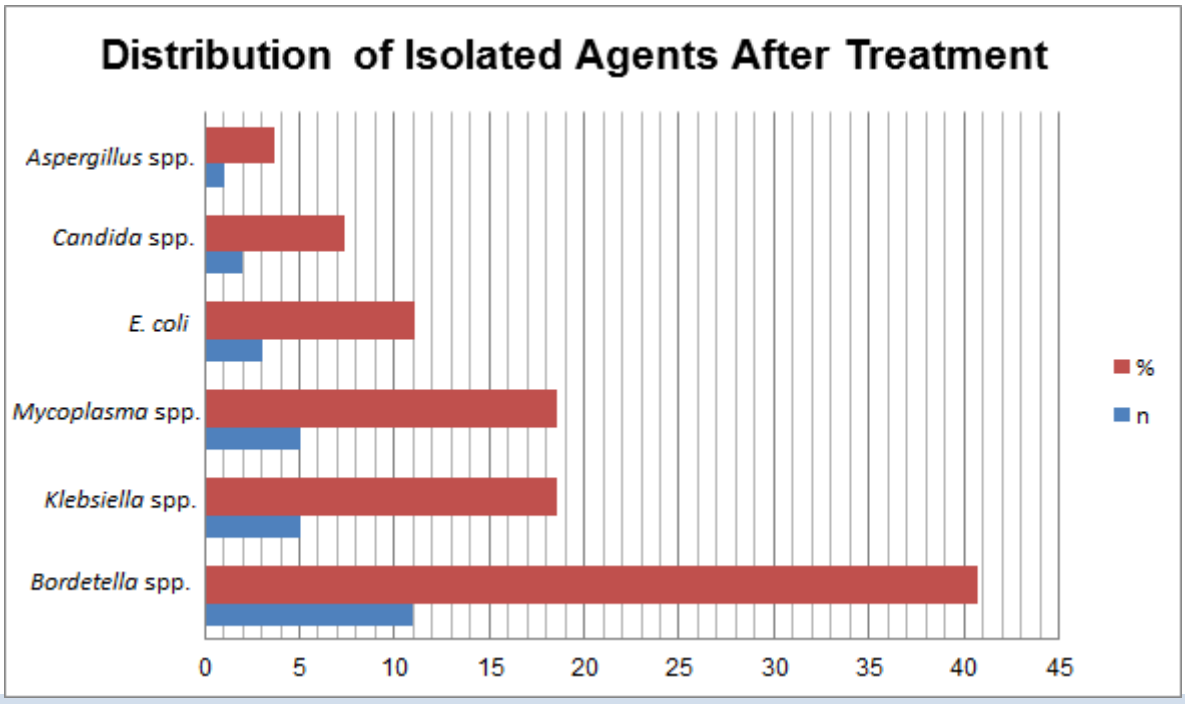

Figure 2.

DISCUSSION

General clinical symptoms such as lethargy and anorexia, which were accompanied with coughing, fever, nasal discharge and tracheal sensitivity, may be seen in the dogs suffering from respiratory disease (Maden et al., 2000; Maden et al., 2001; Chalker et al., 2003a). Clinical scoring for animals in the respiratory infection has been made between 1-5 according to clinical findings such as coughing, nasal discharge, depression and/or anorexia, and symptoms of lower respiratory tract infection (Chalker et al., 2004). In a study, clinical findings were scored based on the severity of respiratory disease as mild (dry cough, serous nasal discharge, normal appetite, no depression), moderate (dry or moist cough, mucous nasal discharge, mild dyspnoea, anorexia, mild fever of 39.05 ± 0.055), and severe (dry or moist cough, mucopurulent nasal discharge, severe dyspnoea, dyspepsia, fever of 40.25 ± 0.056) (Ayodhya et al., 2013a). Also, clinical scoring was made based on the characterization of ocular and nasal discharge, and according to the existence of coughing, sneezing, dyspnea, depression, and body temperature (Jirjis et al. 2010). In our study clinical scores as described before (Table 1) were used for the evaluation of treatment success and monitoring the prognosis of dogs. The results demonstrated that clinical scores contribute to the assessment of treatment and the prognosis in animals suffering from respiratory disease, and suggest that particularly general clinical condition, mucous membrane, nasal discharge, auscultation and body temperature scores, which showed significant differences as from the start of treatment, could be used for the monitoring of dogs with respiratory disease. Also, literature knowledge shows that considering the clinical score assessments in this area, there are differences between the studies (Chalker et al., 2003a; Chalker et al., 2003b; Jirjis et al. 2010; Weiser, 2012; Ayodhya et al., 2013a).

A complete blood count is a useful diagnostic tool that is considered not specific, they point out the existence of inflammation for animals showing signs of respiratory disease (Priestnall and Erles, 2011; Weiser, 2012). The cases of bacterial pneumonia are characterized by inflammatory leucogram results (Dear, 2014). In previous researches carried out in dogs with respiratory infection, total leucocyte numbers were observed to increase in parallel with the severity of the disease (Maden et al., 2000; Zeugswetter et al., 2007; Ayodhya et al., 2013b), high WBC counts were determined to suggest active inflammation (Maden *et al.*, 2000) and increased leucocyte concentrations were considered to be associated with the bacterial respiratory infection (Priestnall and Erles, 2011; Ayodhya *et al.*, 2013b). In the present study, on the basis of the haematological data of the dogs diagnosed with respiratory disease, the presence of leucocytosis

was interpreted as an indicator of active inflammation and bacterial infection. The decrease observed in this parameter after treatment in the dogs that recovered demonstrated the success of the treatment applied. The erythrocyte profile determined in the present study was found to fall within the reference range (Rizzi et al., 2010; Khan et al., 2011), and similar findings have been reported in previous research on respiratory diseases (Maden et al., 2000; Ayodhya et al., 2013b). Blood gas analyses not only aid in determining the severity and prognosis of the disease, but also provide data on the first interventions that need to be made to the patient (serum therapy, oxygenation, electrolyte administration etc.) (Irizarry and Reiss, 2009; Gonzalez and Waddell, 2016). Venous blood gases provide data on the acid-base state and ventilation (Irizarry and Reiss, 2009; Waddell, 2013). With insight of the blood gas analysis results of the study presented, the alleviation or elimination of the respiratory inflammatory disorder with treatment was considered to be an indicator of the improvement of ventilation in the dogs that recovered. Thus, it is suggested that the venous blood gas analyses can be used for respiratory diseases.

Reports indicate that several infectious agents (viral, bacterial, parasitic, fungal, etc.) are involved in canine lower respiratory tract diseases (Kennerman et al., 2000; Vieson et al., 2012; Ayodhya et al., 2013a), and most of these diseases are reported to be of bacterial origin (Kennerman et al., 2000; Peeters et al., 2000; Ayodhya et al., 2013a; Ayodhya et al., 2013b; Lavan and Knesl, 2015). Johnson et al. (2013)reported that the agents most frequently isolated from dogs with lower respiratory tract infection were *Mycoplasma* spp. (30%), *B. bronchiseptica* (22%), *Pasteurella* spp. (21%) and 20% *Enterobacteriaceae* (*E. coli* 17%, *K. pneumoniae* 2%, *Proteus* spp. 2%). Battersby (2014) indicated that *B. bronchiseptica*, *Mycoplasma* spp. and *Streptococcus* spp. are frequently isolated from cases of infectious tracheobronchitis. Meyer and Rawton (2010) reported beta-haemolytic streptococci, *S. intermedius*, and *Klebsiella* spp. to be commonly isolated from sick dogs. In another study carried out in dogs with respiratory disease, the most common infectious agents were determined as *Pasteurella* spp. (25%), *B. bronchiseptica* (11%), *E. coli* (11%), and *K. pneumoniae* (4%) (Epstein *et al.*, 2010). In their study on sheltered dogs with respiratory disease, Chalker et al. (2003b) reported to have isolated *B. bronchiseptica* from the post-mortem pulmonary fluid at a rate of 47%, and indicated that this agent was isolated at a rate of 39% from clinically healthy sheltered dogs. In another study conducted by the same researchers in sheltered dogs with respiratory disease (Chalker *et al.*, 2004), *M. cynos* was isolated at rates of 9.7% and 23.9% from the tracheal wash fluid of healthy and sick dogs, respectively, and at rates of 9.7% and 21.7% from the bronchial lavage samples of healthy and sick dogs, respectively. Thus, these researchers suggested that this species could be involved in the aetiology of canine respiratory diseases. Maden et al. (2000) reported to have isolated *B. bronchiseptica* and *E. coli* (24%), *Pasteurella* spp., coagulase (+) *Staphylococcus* spp. and *Corynebacterium* spp. (12%); *Mannheimia haemolytica* and *Enterobacter* spp. (8%), and *S. aureus*, *Streptococcus* spp., *Pseudomonas* spp., *Bacillus* spp., *Proteus* spp. and *Klebsiella* spp. (4%) from BAL fluid cultures. Durgut et al. (2003) indicated that the infectious agents isolated from transtracheal aspiration and pharyngeal swab samples were *Pasteurella* spp. (16/54, 29.62%), *K. pneumoniae* (12/54, 22.22%), *E. coli* (8/54, 14.81%), beta-haemolytic streptococci (7/54, 12.96%), enteric bacteria (6/54, 11.11%), and coagulase-positive staphylococci (5/54, 9.25%). In their study in dogs with lower respiratory tract disease, Gonul et al. (2010) isolated *E. coli* (4/30, 13.33%), *Staphylococcus epidermidis*, *K. pneumoniae*, *Mycoplasma* spp. and *Enterobacter cloacae* (2/30, 6.66%) from BAL fluid samples. On the basis of data obtained in the present study and previous research, the most common bacteria isolated from sheltered dogs with respiratory disease are *B. Bronchiseptica* (Maden et al., 2000; Epstein et al., 2010; Johnson et al., 2013), *Mycoplasma* spp. (Chalker et al., 2004; Sumner et al., 2011; Johnson et al., 2013) and *E. coli* (Epstein et al., 2010; Sumner et al., 2011; Ayodhya et al., 2013a). In the present study, the three most common bacteria isolated from sheltered dogs with respiratory disease both before and after treatment were *Bordetella spp., Mycoplasma spp*., and *Klebsiella spp*. (Figures 1, 2).

The comparison of the results of the present study and previous research demonstrate that while the first two most common bacteria isolated by Johnson et al. (2013) are the same, the third most common bacteria differs in some studies (Epstein et al., 2010; Ayodhya et al., 2013a). While Sumner et al. (2011) identified other agents as the first two most common bacteria, the third most common bacteria they isolated (*Klebsiella* spp., 20%) is in agreement with the results of the present study (16.10%). The prevalence determined for *Bordetella* spp. in the present study (38.98%) is

close to that reported in Chalker et al. (2003b) (47%) and higher than Johnson et al. (2013) (22%). The prevalence determined for *Mycoplasma* spp. in the present study (21.19%) is close to (21.7%) (Chalker et al., 2003b) and lower than (30%) (Johnson et al., 2013). The results obtained in the present study for the isolation of *Klebsiella* spp. (16.10%) are close to (20%) (Sumner et al., 2011) and higher than (2%) (Johnson et al., 2013). As regards *E. coli*, the prevalence detected in the present study (5.93%) is lower than the isolation rates previously reported, such as (75%) (Sumner et al., 2011) and (17%) (Johnson et al., 2013). Rycroft et al. (2007) isolated *Mycoplasma* spp. at a rate higher (46%) than that detected in the present study (21.19%). The results of the previous research referred to above and the present study demonstrate that bacterial agents and their prevalence vary in different regions. It should also be taken into consideration that the prevalence of respiratory diseases and the bacterial species involved in the aetiology of these diseases may be influenced by environmental factors and living conditions.

It is emphasized that the selection of the treatment method for bacterial infections should be based on causative agent isolation and antimicrobial susceptibility test results using diagnostic samples (Vieson et al., 2012). It is suggested that, depending on in vivo factors (host, causative agent and pharmaceutical), the clinical efficacy of antibiotics may differ from in vitro susceptibility test results (Carbone et al., 2001). However it was stated that empiric antimicrobial therapy should be based on the most likely agent to be present. Broad-spectrum antimicrobials are more suitable for empirical use if a bacterial infection is doubtful of to be secondary to an underlying viral infection (Reagan and Sykes, 2020). Reports indicate that the options for the empirical treatment of infectious tracheobronchitis could be fluoroquinolones, chloramphenicol (Murphy *et al.*, 2012), trimethoprim/sulphonamides and tetracyclines (Battersby, 2014). In a previous study carried out with *B. bronchiseptica* cultures, susceptibility levels to several antibiotics including enrofloxacin (100%), sulfadiazine (81%), and trimethoprim (73%) were detected (Speakman *et al.*, 2000). According to the results of antimicrobial susceptibility tests, *Klebsiella* spp. are reported to be susceptible to cefotaxime (100%), and enrofloxacin (90%) (Kruth, 2006). Grobbel et al. (2007) determined that *Klebsiella* spp. were resistant to ampicillin (53-67%), sulfamethoxazole (19-29%), trimethoprim/sulfamethoxazole (19-24%), and enrofloxacin (29%). Durgut *et al.* (2003) ascertained that cultures of transtracheal aspiration and pharyngeal swab samples were susceptible to amikacin, gentamycin, amoxicillin/clavulanic acid, chloramphenicol, tetracycline and ticarcillin/clavulanic acid. Johnson et al. (2013) detected that most of the *B. bronchiseptica* strains they isolated were resistant to some antimicrobial agents (such as cefazolin, ceftiofur, ceftizoxime, marbofloxacin) and thus, indicated that a specific recommendation for the treatment of cases confirmed or suspected to be caused by *Bordetella* strains could not be made. In the same study, *Bordetella* strains were found to be susceptible to chloramphenicol (>90%), enrofloxacin (<70%), and trimethoprim/sulfamethoxazole (<55%). *In vitro* antibacterial efficacy against *Bordetella* spp. has been reported as 48-100% for ENR (Speakman et al., 2000; Carbone et al., 2001; Epstein et al. 2010; Johnson et al., 2013), 16-100% for TS (Speakman et al., 2000; Johnson et al., 2013), and 57-95% for C(Epstein et al. 2010; Johnson et al., 2013). In vitro antibacterial efficacy against *Klebsiella* spp. has been reported as 48-90% for ENR (Kruth, 2006; Epstein et al. 2010; Johnson et al., 2013), 57-90% for C (Epstein et al., 2010; Johnson et al., 2013), and 76-100% for TS (Grobbel et al., 2007; Johnson et al., 2013). The in vitro antibacterial efficacy of ENR and C against *Mycoplasma* spp. has been reported as 47.05% and 5%, respectively (Chandler and Lappin, 2002). In view of the results of previous field studies and the present study, it is obvious that over time, bacteria develop resistance to antibiotics under the influence of various factors, including among others environmental factors, geographical conditions, the immunological status of the host, the virulence of bacterial agents (cell wall structure, ability to form biofilms etc.), and erroneous dosage.

This study has some limitations such as inability to evaluate the presence of viral agents in the aetiology of the canine respiratory disease complex, not performing radiographic examinations in the shelter conditions, and not knowing the detailed disease history in stray dogs. The findings and treatment results of our study, only bacterial and fungal agents of respiratory tract diseases in shelter dogs are related and have been made according to limited shelter conditions. Although the results of this study provide useful information for veterinary medicine practice on the aetiology and the treatment options in shelter dogs with respiratory disease, it was also evaluated that detailed studies including viral agents, supportive treatments, dosage regimen, and treatment period are needed.

CONCLUSION

In conclusion, it was determined that in sheltered dogs, bacterial lower respiratory tract infections are mostly caused by *Bordetella* spp., *Mycoplasma* spp. and *Klebsiella* spp. in this study. The drug selection and the treatment should be made on the basis of BAL fluid bacterial culture and antimicrobial susceptibility tests (ENR, TS and C against *Bordetella* spp.; C, TS, and ENR against *Mycoplasma* spp.; C, ENR, and TS against *Klebsiella* spp.). It is suggested that in the event of emergency cases and outbreaks of respiratory diseases until the antimicrobial susceptibility tests are completed, by taking into consideration etiological agents, the treatment protocol can be arranged with these antimicrobial drugs.

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CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Research article
Ερευνητικό άρθρο

Analysis of factors influencing the qualitative characteristics of equine synovial fluid

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ABSTRACT: The objective of the present study was to investigate the viscoelastic properties in synovial fluid between normal horses and horses with naturally occurring OA and to detect factors affecting synovial fluid viscosity. In total, 105 horses were included in this study. Synovial fluid samples were obtained from 60 mature horses with mild to moderate osteoarthritis in the 2nd interphalangeal, the metacarpophalangeal or the intercarpal joint. Forty-five horses were used as controls. Full rheological sample characterization was performed in order to measure the elastic G' and viscous G'' moduli. For determining hyaluronic acid concentrations a commercially available ELISA kit was used. The results of the linear mixed effect (LME) model revealed statistically significant ($p < 0.001$) effect of HA concentration, on the mean values of logG' and logG'' measurements. The ANOVA findings of the final model revealed statistically significant effect of joint type ($p < 0.001$) on the mean values of viscoelastic measurements. Interpreting

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the coefficients of the covariates osteoarthritis ($p < 0.001$) and age ($p = 0.013$), a negative correlation was detected on the response logG' and logG" measurements. Geldings seemed to present lower viscous properties compared to mares. To the authors' knowledge this is the first multivariate study to quantitatively evaluate the several factors that affect the viscoelastic properties of equine synovial fluid. Horses with osteoarthritis seemed to present lower viscoelastic properties compared to the healthy subjects that are joint type dependant. Finally, considering the multifactorial nature of osteoarthritis, one should expect an emerging need of personalized disease-modifying treatments.

Keywords: horse, osteoarthritis, synovial fluid, rheological properties

INTRODUCTION

Osteoarthritis (OA) is one of the most common challenges in equine orthopedics causing career-limiting or career-ending lameness (Ferris et al., 2009; Ross and Dyson, 2010). It is a chronic, progressive disease, characterized by cartilage degeneration, subchondral bone sclerosis and osteophyte formation (Trotter and McIlwraith 1996). Synovial fluid (SF) constituents, like hyaluronic acid (HA), contribute both at physiologic and pathophysiologic status, to the boundary lubrication of apposing articular cartilage surfaces (Schmidt et al., 2007).

In healthy individuals, articular cartilage and an easily sheared film of SF provide a protective barrier between joint surfaces in relative motion (Neu et al., 2008). There is evidence that the rheological properties of SF alter in OA, exhibiting decreased viscoelasticity in both human and canine SF (Goudoulas et al., 2010; Madkhali et al., 2018), eventually affecting joint homeostasis. A thorough elucidation of the viscoelastic properties of equine SF is necessary in order to better understand its role in joint lubrication.

Rheology, including the elastic modulus or G' (reflecting the energy stored in the elastic structure of the material) and the viscous modulus or G" (representing the amount of energy dissipated in the material) is a convenient method to evaluate the viscoelastic behavior of SF (Morrison, 2001). Ogston and Stanier (1953) first described normal viscosity in ox SF. The results indicated that hyaluronan molecules may primarily contribute to SF viscosity, and hence to the boundary lubrication of articular cartilage. The rheologic behavior of commercially available HA solutions is strictly related to the molar mass and HA concentration (Vitanzo and Sennett, 2006). In early studies, although an attempt was made to separate HA molecules from SF, obtaining samples of HA free of proteins proved impossible (Bingöl et al., 2010).

The objectives of the present study are i) to investigate the viscoelastic differences of SF between normal horses and horses with naturally occurring OA, and ii) to detect factors affecting SF viscosity between different breeds and joint types.

MATERIAL AND METHODS

Animals

A total of 105 horses were included. Synovial fluid samples were obtained from 60 mature Warmblood and English Thoroughbred horses, aged from 5 to 18 years (median 8 years). Horses had mild to moderate lameness (grade 1-3 on the AAEP lameness scale) attributable to OA in the 2nd interphalangeal (coffin), the metacarpophalangeal (fetlock) joint or the intercarpal (carpus) joint. Moreover, 45 healthy Warmblood and English Thoroughbred horses aged from 4 to 17 years (median 8 years) were used as controls. Diagnosis of OA was based on a comprehensive lameness examination and a positive response to intra-articular anaesthesia. For radiographic assessment standard views specific for each joint type were used. Radiographs were scored blindly by two experienced radiologists (board-certified, ECVDI). Radiographs were assessed for swelling, presence and size of osteophytes, narrowing of the joint space, sclerosis or lysis of the bone underlying the joint cartilage. Lameness was required to be present for at least 3 months prior to enrolment in order to exclude horses with synovitis. Horses were included if the owner provided written consent and was deemed to be willing and capable of complying with the requirements of the study.

Synovial fluid analysis

Synovial fluid was directly aspirated from the joints by use of a 21-gauge needle in a routine sterile manner, as previously reported (Moyer et al., 2007). Samples were placed in tubes containing ethylenediaminetetraacetic acid (EDTA) for routine SF analysis. None of the affected joints had undergone intra-articular analgesia or treatment in the month prior to SF aspiration.

Biochemical analysis

Smears of SF that has been placed in EDTA immediately after collection were prepared for differential cell counts. The total nucleated cell count (NCC) of each aliquot was determined using an automated cell counter (scil Vet abc Plus(+)). Refractometry (ATAGO T2-NE CLINICAL, Atago Ltd, Tokyo, Japan) was also used to measure total solids as an estimate of total protein (TP).

For determining HA concentrations in equine SF samples, a commercially available ELISA kit (TECO Hyaluronic Acid, TECO medical AG®) was used. The assay was based on a HA-specific binding protein. The susceptibility of errors related to high dilution and high viscosity of SF samples was determined by repeated dilution of three samples and calculation of variations. SF concentrations of HA are reported in μg/mL.

Rheological analysis

The rheological behavior of most SF samples was evaluated within 6 hours after aspiration. For cases where the measurement could not be performed within 6 hours, aspirated SF was put in a refrigerator at approximately 4°C for testing within 24 hours

The viscoelastic properties of the samples were determined by via steady state and dynamic experiments in order to measure the shear viscosity η and the elastic G' and viscous G'' moduli respectively, at horse's body (37.5 ºC) temperature. The investigation of the rheological behavior was carried out on a TA Instruments AR-G2 controlled stress CMT Rheometer via dynamic and steady state experiments. Steady state flow steps were performed with a shear rate from 1 to 400 s^{-1} at 25°C and 37.5 °C. Average viscosity values were calculated for shear rates from 18 - 75 s^{-1}. Data for lower and higher shear rates were not included due to noise and viscous heating respectively.

Statistical analysis

In order to study the effect of factors and covariates on the mean values of the elastic modulus (G'), and the viscous modulus (G"), the Linear Mixed Effects (LME) modeling were used. Graphical validation was used to assess the underlying assumptions of homoscedasticity and normality of residuals of the selected models. All statistical analyses were conducted using the statistical language *R* (Team RC, 2013) and the function *lmer* from package lme4. In addition, the function step from package *lmerTest* (Kuznetsova et al., 2015) was used in order to perform backward elimination of all effects of the examined LME. The p-values for the fixed component of the model were calculated from F test based on Kenward-Roger approach in order to get approximate degrees of freedom. In all tests a difference was considered as statistically significant when p-value (significance) was less than 0.05. All the tests conducted were two-tailed (non-directional) in the sense that the alternative hypothesis is that the measures tested are not equal.

RESULTS

Descriptive Statistics

Biochemical analysis

The results of univariate analysis for TP, NCC, G', G" parameters were expressed as mean (M), standard deviation (SD), median (Mdn), minimum (min) and maximum (max). Mean NCC at normal and pathological joints was 111.40±40.66 cells/μl and 231.47±59.91 cells/μl respectively. Mean TP levels at normal and pathological joints were 1.26±0.21 g/dl and 1.74±0.28 (Table 1).

Mean HA concentration at normal and pathological joints was 939±188 μg/ml and 389±176 μg/ml respectively in the two groups. Horses with OA presented statistically significant ($p<0.05$) lower HA concentration, compared to the healthy subjects.

In order to graphically explore the distributions of G', G" parameters boxplots were constructed for each level of factor breed (Figure 1).

The effect of HA concentration

The simultaneous effect of several covariates and factors on G', G'' values was tested for subjects that there were available measurements for HA parameter. Sex and age were inserted as control variables in the models, so as to examine their potential effects on the response variables.

The ANOVA findings revealed statistically significant ($p < 0.001$) effect of HA concentration, on the mean values of logG' measurements $F(1, 58) = 38.162$ and on the mean values logG" measurements, $F(1, 58) = 71.676$. More significantly, a positive effect of HA on the response logG' measurements (b = 0.003, SE = 0.0005, p < 0.001) and logG" measurements ((b = 0.003, SE = 0.0003, p < 0.001) was detected.

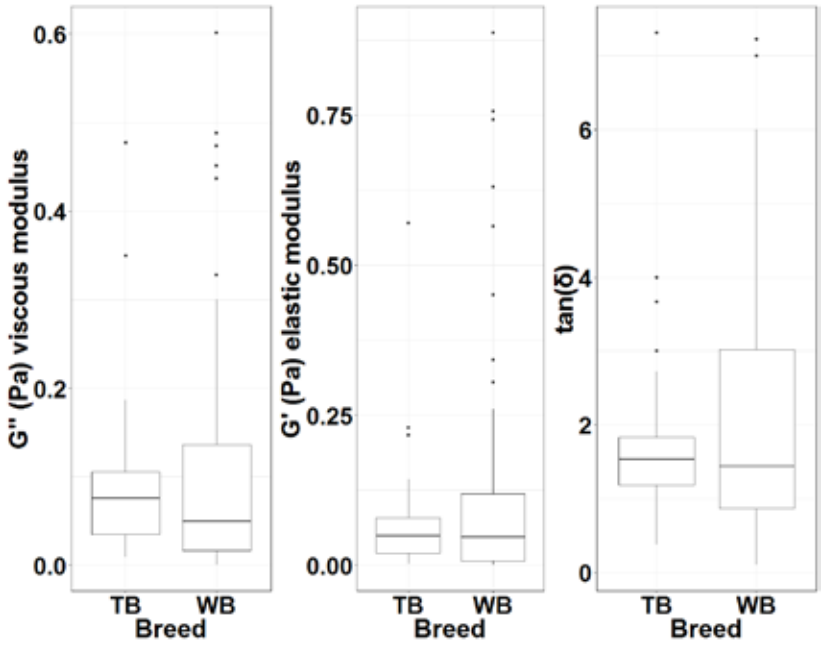

Figure 1. Boxplots. Distributions of G", G' values for Thoroughbred and Warmblood breed

Table 1. Descriptive statistics for categorical variables of the study

Parameter		Breed	N	M	*SD*	*Mdn*	*min*	*max*
Total Protein (g/dl)	Normal	TB	15	1.37	0.15	1.40	1.20	1.60
		WB	20	1.20	0.21	1.20	0.80	1.50
		Total	35	1.26	0.21	1.25	0.80	1.60
	OA	TB	30	1.82	0.28	1.80	1.60	2.60
		WB	40	1.63	0.24	1.60	1.30	2.20
		Total	70	1.74	0.28	1.65	1.30	2.60
Total nucleated cell count (cells/μl)	Normal	TB	15	130.00	54.41	125.00	70	200
		WB	20	102.08	30.44	100.00	55	153
		Total	35	111.40	40.66	100.00	55	200
	OA	TB	30	256.84	52.71	250.00	180	370
		WB	40	199.33	53.91	180.00	120	320
		Total	70	231.47	59.91	210.00	120	370
G' (Pa) elastic modulus		TB	45	0.069	0.092	0.049	0.001	0.570
		WB	60	0.122	0.204	0.047	0.001	0.887
		Total	105	0.099	0.167	0.047	0.001	0.887
G'' (Pa) viscous modulus		TB	45	0.085	0.085	0.076	0.010	0.477
		WB	60	0.109	0.139	0.050	0.001	0.601
		Total	105	0.099	0.119	0.055	0.001	0.601

Abbreviations: M, average; Mdn, median; min, minimum; max, maximum; N, number of samples; OA, osteoarthritis; SD, standard deviation; TB, Thoroughbred; WB, Warmblood

The effects of Age, Breed, Sex, Joint Type and Joint status

In this section, the results derived from the analysis conducted in order to examine the simultaneous effect of several covariates and factors on G', G'' parameters are presented.

G' (elastic modulus)

The ANOVA findings of the final model revealed statistically significant effect of Joint Type, $F(2, 95) = 7.718$, $p = 0.006$, Age, $F(1, 70) = 6.474$, $p = 0.013$ and OA, $F(1, 92) = 23.603$, $p < 0.001$ on the mean values of logG' measurements.

Interpreting the coefficients of the covariates OA, (b = -0.423, SE = 0.085, p < 0.001) and Age (b = -0.110, SE = 0.043, p = 0.013), a negative correlation was detected on the response logG' measurements. Horses with OA seem to present lower elastic properties, gradually decreasing with age.

G" (viscous modulus)

The same analysis was used in order to fit a multivariate model for the response variable G". The results did not reveal statistically significant interaction terms for Breed×Radiographic OA, $F(1, 84) = 0.183$, $p = 0.670$ and Breed×Joint Type, $F(1, 90) = 1.322$, $p = 0.253$ and they were removed from the final model. Moreover, marginally significant main effects of Age, $F(1, 70) = 10.734$, $p = 0.056$ and Sex, $F(2, 65) = 3.022$, $p = 0.069$ were noted and for this reason, we decided not to omit them from the final model.

Once again, the ANOVA findings revealed that logG" measurements vary depending on joint type, F(2, 86) = 5.374, p = 0.006 and Sex, F(2, 67) = 3.150, p = 0.049 while main effects of Age, F(1, 76) = 4.226, p = 0.043 and OA, F(1, 80) = 10.062, p = 0.002 were also detected.

Interpreting the coefficients of the covariates OA, (b = -0.350, SE = 0.107, p = 0.002) and Age (b = -0.053, SE = 0.025, p = 0.043), the negative sign indicates a negative correlation on the response logG" values. Horses with OA seem to present lower viscous properties, gradually decreasing with age. Regarding the effect of Sex and osteoarthritis, depending on joint type on logG" measurements the results are presented in Figure 2. Based on these results, it is concluded that geldings presented lower viscous properties (means of logG") compared to mares. Moreover, it is noted that SF from coffin joints seems to present lower viscous properties compared to the one obtained from fetlock and intercarpal joints.

DISCUSSION

In this study, a multivariate analysis was used in order to further investigate the several factors that can possibly affect the complex nature of equine SF. The clearly significant differences in the concentration of HA between normal joints and joints with OA indicate that there are differences in the metabolism of joint tissues. However, in a previous study by Venable et al. (2008) no differences were reported between concentrations of the experimentally induced-OA group and the control group. In our study, we detected significantly lower HA concentrations in osteoarthritic than in normal joints. These results are supported by other recent studies that investigated the HA concentration and molecular weight distribution with OA progression (Plickert et al., 2013; Band et al., 2015). A shift in the molecular weight distribution of SF HA toward lower values is associated with an increased risk for rapid OA progression (Band et al., 2015).

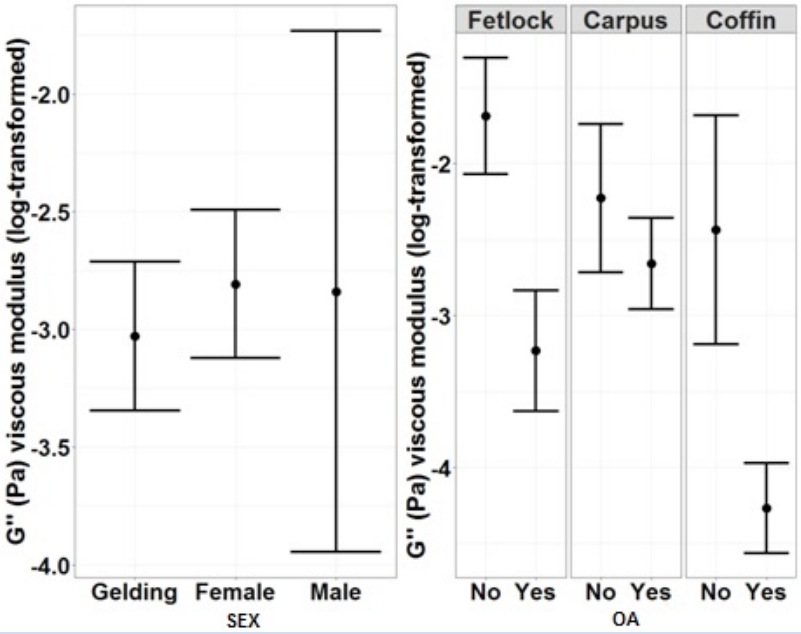

Figure 2. Means of G" measurement (log-transformed) with 95% CI for factors Sex and JointType×Radiographic. Geldings present lower viscous properties compared to mares. Synovial fluid from coffin joints presents lower viscous properties compared to the one obtained from fetlock and intercarpal joints

The concentration of HA has been reported to vary not only between animals but between joints within the same animal as well (Auer et al., 1980). Reduced levels of HA have been reported in joints following immobilisation, prompting the suggestion of mechanical control of SF HA concentration (Pitsillides et al., 1999). The decrease of HA concentrations in osteoarthritic SF has been attributed to degradation, fragmentation, alteration in joint metabolism and synthesis, and dilution by joint effusion (Henderson et al., 1991; Kuroki et al., 2002). However, HA concentrations in urea-adjusted and unadjusted SF samples were found to be similar, so that dilution effects were considered unlikely to explain the reduction of HA concentrations (Budsberg et al.,2006). In this report, the decreased HA concentration in diseased joints, in line with the fact that a significant effect was detected on the viscoelastic properties (in terms of logG" and logG' measurements), support this hypothesis. However, results from recent studies indicate that boundary lubrication is a result of interaction between HA and other macromolecules, such as lubricin (Bonnevie et al., 2015). Further research is required to investigate whether this interaction is purely a mechanical entanglement or it is dictated by the hydrophobic and hydrophilic nature of these molecules.

In a study of Temple-Wong and colleagues (Temple-Wong et al., 2016) the effect of age and stage of joint degeneration on the concentrations of protein and HA distribution were assessed. The concentration decrease of HA in SF with age, in the absence of OA, and the association of lower HA in SF with increased friction between cartilage surfaces, suggest that this relationship may be an important factor in the age-related deterioration of articular cartilage (Squires et al., 2003).

In this multivariate study a negative effect of age on the viscoelastic properties of equine SF was detected [mean values of logG' measurements ($p < 0.001$) and logG'' measurements ($p = 0.013$)]. In human patients, the most prominent hypothesis linking aging to OA is that chondrocytes undergo premature aging due to several factors, such as excessive mechanical load or oxidative stress (Squires et al., 2003; Courties et al., 2015). The risk factors for development of OA in humans has been classified into two fundamental mechanisms related either to the adverse effects of 'abnormal' loading on 'normal' cartilage or of 'normal' loading on 'abnormal' cartilage, and similar pathways have been described in horses (McWraith, 1996). Considering the fact that horses included in this study were all active athletes, the repeated mechanical forces to the joints could explain the negative correlation between viscoelastic properties and aging.

Moreover, it was noted that sex presented a significant effect on the mean values of logG", with geldings presenting significantly lower viscous properties compared to the ones obtained from mares. Unlike humans, where women seem to be slightly more prone to OA than men (O'Connor, 2007; Hanna et al., 2009; Blagojevic et al., 2010) there is no evidence to support the argument of sex-related susceptibility to OA in horses. This result could be attributed to the fact that geldings are overrepresented in this study compared to mares. Still, more research is needed in order to extrapolate more accurate results, regarding sex predisposition to OA.

The investigation of SF properties has been of considerable interest, mainly due to its viscoelastic character. Viscosity measurements have been previously performed on SF and other polymer solutions (Yu et al., 2014) while it has been proposed that G' and G" values mainly depend on shear rate, temperature, pressure, and concentration (Giap, 2010). However, there is limited evidence investigating the possible variations in the viscoelastic properties of SF between equine normal joints and joints with naturally occurring OA, while most of these studies focus on the *in vitro* rheological behavior (Borzacchiello et al., 2010). In the study reported here an effort was made to detect the effect of several factors (breed, age, sex, joint type and joint status) in SF rheological properties with respect to the mean levels of G' and G" values. In our report a statistically significant effect of horses with OA was detected on the mean values of logG' measurements ($p<0.001$) as well as on the mean values of logG" measurements ($p=0.002$). Horses with OA seem to present lower logG' measurements and logG'' measurements compared to the healthy subjects. Hence, it is concluded that, in OA, SF tends to lose both its elastic and viscous properties. It is hypothesized that compromise in viscoelasticity leads to the diminished rheological properties and to unmitigated forces transmitted to the cartilage and intercellular matrix.

This study was designed to investigate the several factors that contribute to changes in synovial fluid viscoelasticity. The statistical analysis results indicated that the mean values of rheological parameters are joint type dependent. This can be attributed to the

fact that the equine forelimb exhibits a wide variety in biokinematic variables in terms of ground force and stride adaptation (Back et al., 1996). Hence, depending on joint type, different adaptation to ground forces can lead to alterations in viscoelastic properties. An early report by Clayton and colleagues (Clayton et al., 2011) presented a full kinetic analysis of the relative motion of equine forelimb joints at the trot. It was reported that during stance phase, most of the energy absorbed was by fetlock joint, while only about 6% of the total energy absorbed was by coffin joint. Moreover it was concluded that during stance phase, coffin joints generate energy while the carpus and fetlock joints absorb energy. All Thoroughbred horses in our study were flat racehorses, trained to race over short or middle distances, while Warmblood horses were used for low-level show jumping. Considering that viscoelastic properties of SF alter, depending on joint type and ground forces, the different type of exercise may explain that fact.

CONCLUSIONS

To the authors' knowledge this is the first multivariate study to quantitatively evaluate and compare the several factors that affect the viscoelastic properties of equine SF. Human OA is a multifactorial process in which systemic risk factors like age, sex, mechanical trauma, obesity and genes determine the susceptibility of an individual. In our study an approach was made to investigate the impact of some of these factors in an equine model. Based on our results, it can be summarized that HA concentrations are positively correlated with the viscoelastic properties of equine SF, while there seem to be significantly lower to OA horses compared to the normal ones. Moreover, viscoelastic properties of equine SF seem to reduce, during aging, and present variation depending on joint type. The present results can provide extent knowledge to further understand the complex role of SF, in order to maximize the potential to novel treatment strategies in OA.

CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Effect of enzyme and yeast-based feed additives on growth, nutrient digestibility, meat quality and intestinal morphology of fattening rabbits

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ABSTRACT: Non-antibiotic feed additives are nowadays increasingly used in animal feed industry as more and more reports are surfaced on antibiotic resistance. This study quantified the effects of enzymes and yeast supplementation, individually or in combination, on growth performance, diet digestibility, carcass quality, and ileum morphometry of fattening rabbits. Forty-eight indigenous male weaned rabbits (age, 37 ± 2 days; average body weight (ABW)) 340 ± 3.2 g) were assigned into four dietary treatments (16 replicates; 3 rabbits/replicate)). The four diets were (i) a basal (control) diet (without additives), containing concentrate (75% of the feed) and wilted mulberry leaves (25% of the total feed); (ii) basal diet plus enzyme blend (driselase-1®; cellulase, amylase and protease; 2 g kg^{-1} feed); (iii) basal diet with the addition of *Saccharomyces cerevisiae* (dry yeast TR 100; 2 g kg^{-1} feed); (iv) basal diet with the addition of mixture of the enzymes blend and *S. cerevisiae* (1 g each kg^{-1} feed). All rabbits had a*d-libitum* access to clean drinking water and experimental diets, with 16 h light for seven-weeks. The results revealed that all additives improved ($P < 0.05$) weight gain (WG) and feed conversion ratio (FCR) but did not alter feed intake than the control group. Although all additives increased ($P < 0.05$) neutral detergent fibre (NDF) digestibility, the dry matter digestibility (DMD) was increased ($P < 0.05$) only by the addition of enzymes blend. Furthermore, all additives increased ($P < 0.05$) carcass yield without any negative effects on meat physical (pH, water release and cooking loss) and chemical (moisture, protein, fat and ash) properties ($P < 0.05$). Moreover, villus height and crypt depth values were improved ($P < 0.05$) with the incorporation of additives. This study demonstrates that addition of enzymes blend and yeast-based additives alone or in combination in rabbit diet improved nutrients digestion and intestinal health, which subsequently improved their growth and carcass quality.

***Keywords*:** Enzymes, yeast, mulberry leaves, growth performance, digestibility, meat quality, rabbit

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INTRODUCTION

Antibiotic growth promoters (AGPs) are still commonly used in animal rations in developing countries, such as Pakistan, with the aim to improve animal performance and reduce the rate of early mortality. However, recent studies show that the use of AGPs can cause cause serious health issues such as antibiotic resistance (Ali et al., 2017), and subsequently, the European Union banned AGPs in January 2006 (Kabploy et al., 2016). This has triggered enormous research into the exploration of alternate feed additives in an effort to improve gut health, animal performance and to overcome the emerging problem of antibiotic resistance (Wu et al., 2011). In recent past, considerable interest has been established in the use of enzymes and yeast as feed additives (Falcao-e-Cunha et al., 2007). Exogenous enzymes and yeast supplementation in the rabbit diet have been reported to improve growth performance and health status (Chandra et al., 2014). In addition, dietary enzyme supplementation into rabbit diets has been reported to improve nutrient utilization (Falcao-e-Cunha et al., 2007).

Mulberry (*Morus alba*) leaves are getting growing attention in the face of higher price of concentrates ingredients in rabbits diets due to their excellent nutritional value, such as high protein (15-35%), Ca (2.42-4.71%) and P (0.23-0.97) contents (Ayasan and Baylan, 2016), and improved *in vitro* DM digestibility (62.5%; Khan et al., 2019). This has led to a renewed interest in feeding of *Morus alba* leaves to rabbits to improve their growth performance (Khan et al., 2020) and reduce the cost of production and farm profitability.

Rabbit production has increased in Pakistan because it is a good source of affordable meat. In this respect, medium and large scale (semi)-commercial rabbit production systems have gained popularity for providing cheap and high-quality animal protein to the people (Khan et al., 2016). Recent study shows that total meat production and production efficiency of indigenous rabbits can be enhanced by improving feeding methods and management practices (Khan et al., 2017a, 2017b). Just after weaning, rabbits are particularly exposed to enteric diseases particularly diarrhea causing economic losses in terms of mortality; reduce growth and poor feed utilization efficiency due to the transition from feeding on mother's milk to solid food (Bivolarski and Vachkova, 2013). The negative effects of weaning on rabbit health and production could be reduced by the dietary inclusion of non-antibiotic feed additives, such as probiotics and enzymes (Trocino et al., 2005; Kritas et al., 2008). Literature is available on the beneficial impact of non-antibiotic feed additives on production performance of farm animals. However, literature on the impact of additives (non-antibiotic) on rabbit growth performance is limited. Thus, the present research was executed to find out the effects of inclusion of enzymes blend (cellulase, amylase and protease) and yeast (*Saccharomyces cerevisiae*) on the growth, nutrient digestibility, carcass yield, meat physicochemical properties and ileum morphology of indigenous rabbits.

MATERIAL AND METHODS

Management of rabbits and experimental diets

A total of forty-eight healthy male rabbits [age, 37 ± 2 days; average body weight (ABW) 340 ± 3.2 g] were randomly distributed to four dietary groups (16 replicates (3 rabbits per replicate)). Each replicate group was kept in separate cage in the same shed. Each diet (iso-caloric and iso-nitrogenous) was assigned to the 4 replicate groups.

Table 1. Nutrient profile of concentrate and mulberry leaves fed to the rabbit

Nutrients[1]	Concentrate[2]	Mulberry leaves[3]
Dry matter	92.1	20.8
Crude protein	18.5	21.8
Neutral detergent fiber	37.7	35.5
Acid detergent fiber	23.4	23.8
Ether extract	4.50	3.31
Total Ash	9.30	15.3
Digestible energy (MJ/Kg)	10.9	11.3

[1], % of dry matter, unless otherwise stated

[2]Concentrate contained 15% maize grain, 25 % wheat bran, 8% soybean meal, 15% cotton seed cake, 15% maize gluten, 15% palm oil cake, 5% molasses, 1% Di-Ca-Phosphate, 0.1% DL-Methionine, 0.5% sodium chloride, 0.5% vitamin and mineral premix (g/kg: premix provided per kg of diet: 2000 IU Vit. A, 150 IU Vit. D, 8.33 g Vit. E, 0.33 g Vit. K, 0.33 g Vit. B1, 1.0 g Vit. B2, 0.33g Vit. B6, 8.33 g Vit. B5, 1.7 mg Vit. B12, 3.33 g pantothenic acid, 33 mg biotin, 0.83 g folic acid, 200 g choline chloride, 11.7 g Zn, 12.5 g Fe, 16.6 mg Se, 16.6 mg Co, 66.7 g Mg and 5 g Mn)

[3]Mulberry leaves fed to the rabbits are in the green wilted form

The rabbits were fed a basal diet, comprising of concentrate (75%) and wilted mulberry leaves (25%) of the total feed, formulated to fulfil all the nutrients requirements of growing rabbits (Table 1; NRC, 1977). The total amount of basal feed required for the

trial was mixed and divided in to four equal portions before pelleting. One portion of the basal diet was taken as the control feed (without feed additives). The remaining three portions were supplemented either with (ii) enzyme blend (driselase-1®; cellulase, amylase and protease; 2 g kg^{-1} feed); (iii) *S. cerevisiae* (dry yeast TR 100;2 g kg^{-1} feed); or (iv) with a mixture of the enzymes blend and *S. cerevisiae* (1 g each kg^{-1} feed) to get three test diets. Driselase-1 contained cellulase (1,000,000 IU), amylase (700,000 IU) and protease (450,000 IU). Fubon (dry yeast TR 100; live yeast count ≥ 10 billion/g) is live yeast product and is provided by Angel Yeast Co. LTD. The four diets were separately pelleted with pellet size of 2 to 2.3 mm. All rabbits received green wilted mulberry leaves (25% portion of the daily feed offered) along with a basal diet. Rabbits had *ad libitum* (measured quantity) access to water and pelleted diets throughout the experiment, with 16 h light during the experimental period. The trial continued for 7 weeks period including a week of acclimatization period to the diets. This study was approved by the Departmental Board of Studies on Ethics, Methodology and Welfare, SBBU Sheringal, KP, Pakistan.

Growth performance and digestibility assay

On the first day of the trial, body weight (BW) of individual rabbits were noted before morning feeding, and then at the end of each week to compute the daily weight gain (g/day).The feed offered to all experimental rabbits were weighed, and the left-over feed was weighed next morning (8:00 am) to calculate the daily feed intake (FI; g/day). The feed conversion ratio (FCR) was computed for the fattening period. Morbidity and mortality were recorded daily. Morbidity was measured by examining all clinical signs of digestive troubles or sickness of individual rabbit.

A digestibility assay for 6 consecutive days (40 to 46 days of fattening trial) was conducted using 6 randomly selected rabbits from each group. The weight of feed offered, feed refused and fresh hard faeces produced during 24 h were recorded. Before weighing, the faeces were sorted and soft faeces were discarded. Representative faeces samples were collected from each animal, subsampled for dry matter (DM) analysis. The remaining faeces were pooled over 6 days and stored in a freezer (-20 ˚C) for onward chemical analysis. The experimental diets and rabbit faecal samples were ground (~ 1 mm particle size). The ground samples were examined for the contents of DM, crude protein (CP; N × 6.25), ether extract (EE) and acid detergent fibre (ADF) according to AOAC (2000). Method of Van Soest et al. (1991) was used for the determination of NDF content.

Carcass traits and meat quality variable

On day 49 of the fattening trial, from each dietary group 6 rabbits were sacrificed for measurement of carcass traits. Prior to slaughter, rabbits were fasted for 10 h, and had free access to clean drinking water. Pre-slaughter weight was recorded. The carcass dissection procedures were followed as suggested by the World Rabbit Science Association recommendation described by Blasco and Ouhayoun (1996). Samples were taken from *longissimus lumborum* muscle (10 g) 24 h post-mortem in triplicate, homogenized with distilled water (50 ml), and analysed for pH value. The water release (%) was calculated using standard method of Grau and Fleischmann, (1957), while the cooking loss were measured using procedure prescribed by Boccard et al. (1981). The chemical profile of *longissimus lumborum* muscle was determined using standard method as per guidelines of AOAC (2000). The vital organs such as liver, heart, kidney and spleen were weighed on digital balance.

Ileum morphology

Segments (~ 3.0 cm in length) from the ileum were taken from 3 randomly selected rabbits of each dietary treatment, to determine villus height and crypt depth. For this study, three cross-sections from each ileum specimens were fixed by neutralised formalin (10%; v/v), washed with running water, dehydrated, fixed in paraffin wax, and then divided into 5 μm thick segments, and finally marked with hematoxylin-eosin stained (Yan et al., 2017).

Statistical analysis

The impact of feed additives on growth traits, carcass yield, diet digestibility, ileum morphometry and meat physicochemical properties of rabbits were evaluated by the procedure of PROC MIXED of the Statistical Analysis System (SAS Institute, 2009). For parameters with significant ($P < 0.05$) treatment effect the pairwise differences between mean values were determined by post-hoc test analysis using the Duncan's Multiple Range test.

RESULTS

Data showing effects of the additives on feed intake (FI), weight gain (WG) and FCR of rabbits is presented in Table 2. Additives in the rabbit diet in-

creased (P < 0.05) final BW. Notably, the additives had no effect (P > 0.05) on the FI of rabbits. However, the efficiency of feed utilization, as reflected by FCR, was improved (P < 0.05) due to addition of the additives in rabbit diets. No mortality was reported in the present study throughout the experimental period.

Data on effects of different feed additives on total tract nutrients digestibility are presented in Table 3. All feed additives increased (P < 0.05) NDF digestibility. However, DM digestibility increased (P < 0.05) only with the addition of enzymes blend. On the other hand, CP, EE and ADF digestibility did not alter (P > 0.05) due to addition of the additives in rabbit diet.

Table 4 summarises data on effects of the additives on carcass yield, dressing percentage and organs weight. Hot and chill carcass yield was higher (P < 0.05) in the additives supplemented groups. Similarly, dressing percentage also increased (P < 0.05) due to incorporation of the additives in the control diet. Additives did not alter (P > 0.05) the organs weight, except for kidney, which was higher (P < 0.05) in yeast supplemented group. Incorporation of additives in the diet did not alter (P > 0.05) the physical properties (pH, water release and cooking loss) and chemical profile (moisture, protein, fat and ash) of rabbit meat (Table 5).

Table 2. Effects of enzyme and yeast-based diet on growth performance of fattening rabbit

Body Weight, BW, g	Diets[1]				SEM	Significance
	Control	Enzyme	Yeast	Enzyme-yeast		
Initial, day 42	425 ± 3.54	428 ± 2.21	427 ± 1.56	424 ± 2.60	0.183	ns
Weight, day 56	611.8 ± 3.67	628.7 ± 3.47	633.6 ± 2.15	621.9 ± 3.27	0.453	ns
Weight, day 77	1030^{b} ± 4.96	1063^{a} ± 4.34	1059^{a} ± 4.26	1047^{ab} ± 4.17	1.684	**
Final, FW, day 90	1206^{b} ± 6.56	1247^{a} ± 3.22	1244^{a} ± 6.11	1243^{a} ± 3.51	4.143	***
Average daily gain, ADG, g						
During days 43-56	13.34 ± 0.23	14.34 ± 0.25	14.76 ± 0.17	14.14 ± 0.19	1.667	ns
During days 57-77	19.94^{b} ± 0.43	20.68^{a} ± 0.42	20.26^{a} ± 0.38	20.23^{a} ± 0.32	1.189	**
During days 78-90	13.5^{b} ± 0.16	14.15^{a} ± 0.23	14.22^{a} ± 0.18	15.09^{a} ± 0.25	2.196	**
During days 43-90	16.27^{b} ± 0.05	17.06^{a} ± 0.06	17.02^{a} ± 0.17	17.06^{a} ± 0.14	1.345	**
Feed intake, FI, g/d						
Concentrate	54.3 ± 0.115	56.4 ± 0.125	57.1 ± 0.267	55.8 ± 0.345	1.189	ns
Mulberry leaves	37.55 ± 1.45	32.79 ± 1.56	33.13 ± 1.67	33.68 ± 1.78	3.445	ns
Total intake, g/d	91.85 ± 2.13	89.19 ± 2.45	90.23 ± 3.15	89.48 ± 2.78	2.834	ns
Total intake during d 43-90	4409 ± 5.48	4281 ± 5. 81	4331 ± 6.12	4295 ± 4.89	0.021	ns
F/G ratio	4.99^{a} ± 0.030	4.62^{b} ± 0.025	4.69^{b} ± 0.141	4.66^{b} ± 0.061	0.065	**

In the same row, values carrying different superscript letter $^{a, b}$ means significant difference at P < 0.05; SEM, standard error of mean, F/G, feed to gain ratio; ns, non-significant; ** for P < 0.01; ***, P < 0.001; [1]Control, basal diet (blend of concentrate (75%) and wilted mulberry leaves (25%) without the inclusion of enzyme and yeast; Enzyme, kg^{-1} basal diet contained 2 g enzyme blend (cellulase, amylase and protease); Yeast, kg^{-1} basal diet contained 2 g *Saccharomyces cerevisiae*; Enzyme-yeast, kg^{-1} basal diet contained blend of enzyme (cellulase, amylase and protease) and *S. cerevisiae* (1 g each)

Table 3. Effects of enzyme and yeast-based diet on total tract apparent nutrient digestibility of fattening rabbit

Nutrients	Diets[1]				SEM	Significance
	Control	Enzyme	Yeast	Enzyme-yeast		
DM	69.8^{a} ± 0.45	71.9^{b} ± 1.37	71.7^{ab} ± 0.21	70.9^{ab} ± 0.44	0.623	**
CP	55.5 ± 0.85	54.4 ± 0.35	55.0 ± 0.13	55.4 ± 0.14	0.174	ns
EE	82.8 ± 0.12	80.9 ± 0.05	80.6 ± 0.05	81.8 ± 0.43	0.138	ns
NDF	43.5^{a} ± 0.09	48.5^{b} ± 0.55	49.6^{b} ± 0.07	48.7^{b} ± 0.47	0.729	***
ADF	33.5 ± 0.11	34.6 ± 0.83	35.5 ± 0.15	35.2 ± 0.13	0.117	ns

Values are presented as mean with standard deviation
In the same row, values carrying different superscript letter $^{a, b}$ means significant difference at P < 0.05
DM, Dry matter, CP, crude protein, EE, ether extract; NDF, neutral detergent fibre; ADF, acid detergent fibre
SEM, standard error of mean; ns, non-significant; ** for P < 0.01; ***, P < 0.001
[1]Control, basal diet (blend of concentrate (75%) and wilted mulberry leaves (25%) without the inclusion of enzyme and yeast; Enzyme, kg^{-1} basal diet contained 2 g enzyme blend (cellulase, amylase and protease); Yeast, kg^{-1} basal diet contained 2 g *Saccharomyces cerevisiae* ; Enzyme-yeast, kg^{-1} basal diet contained blend of enzyme (cellulase, amylase and protease) and *S. cerevisiae* (1 g each)

Table 4. Effects of enzyme and yeast-based diet on carcass weight (CW), dressing percentage and organs weight of fattening rabbit

	Diets[1]				SEM	Significance
	Control	Enzyme	Yeast	Enzyme-yeast		
Hot CW, g	$692^{b} \pm 6.56$	$734^{a} \pm 6.85$	$735^{a} \pm 4.58$	$739^{a} \pm 9.29$	5.732	**
Chill CW, g	$643^{b} \pm 6.07$	$685^{a} \pm 6.42$	$686^{a} \pm 4.54$	$689^{a} \pm 9.20$	5.529	**
Reference CW, g	$543^{b} \pm 6.56$	$585^{a} \pm 6.85$	$586^{a} \pm 4.72$	$589^{a} \pm 9.29$	5.751	**
Dressing percentage	$57.4^{b} \pm 0.23$	$58.9^{a} \pm 0.36$	$59.1^{a} \pm 0.23$	$59.4^{a} \pm 0.69$	0.342	**
Liver, g	57.9 ± 0.21	57.1 ± 0.15	56.8 ± 1.15	57.1 ± 0.40	0.509	ns
Kidney, g	$8.82^{ab} \pm 0.07$	$8.69^{b} \pm 0.05$	$8.84^{a} \pm 0.02$	$8.51^{c} \pm 0.05$	0.043	**
Heart, g	4.03 ± 0.01	4.01 ± 0.02	4.12 ± 0.12	3.99 ± 0.02	0.05	ns
Lungs, g	8.78 ± 0.45	$8.86 \pm .067$	8.98 ± 0.47	8.82 ± 0.33	0.298	ns
Spleen, g	0.68 ± 0.01	0.67 ± 0.02	0.69 ± 0.02	0.67 ± 0.01	0.012	ns

In the same row, values carrying different superscript letter [a, b, c] means significant difference at $P < 0.05$. ns, non-significant; **, $P < 0.01$, SEM, standard error of mean

[1]Control, basal diet (blend of concentrate (75%) and wilted mulberry leaves (25%) without the inclusion of enzyme and yeast; Enzyme, kg^{-1} basal diet contained 2 g enzyme blend (cellulase, amylase and protease); Yeast, kg^{-1} basal diet contained 2 g *Saccharomyces cerevisiae* ; Enzyme-yeast, kg^{-1} basal diet contained blend of enzyme (cellulase, amylase and protease) and *S. cerevisiae* (1 g each)

Table 5. Effects of enzyme and yeast-based diet on physical properties and chemical profile of *longissimus lumborum* muscles of rabbit

Physical properties	Diets[1]				SEM	Significance
	Control	Enzyme	Yeast	Enzyme-yeast		
pH24	5.76 ± 0.03	5.75 ± 0.04	5.76 ± 0.03	5.70 ± 0.10	0.096	ns
Release water (%)	14.5 ± 0.14	14.9 ± 0.19	15.2 ± 0.12	15.0 ± 0.08	0.45	ns
Cooking loss (%)	34.8 ± 0.17	34.5 ± 0.20	34.5 ± 0.23	34.8 ± 0.74	0.44	ns
Chemical profile (%)						
Moisture	72.1 ± 0.04	71.9 ± 0.07	72.0 ± 0.03	71.9 ± 0.02	0.774	ns
Crude protein	21.4 ± 0.48	21.5 ± 0.03	21.2 ± 0.18	21.3 ± 0.21	0.37	ns
Fat	1.82 ± 0.03	1.87 ± 0.02	1.89 ± 0.03	1.89 ± 0.04	0.023	ns
Ash	1.29 ± 0.12	1.26 ± 0.11	1.27 ± 0.10	1.28 ± 0.12	0.987	ns

pH24, ultimate pH determined at 24 h post-mortem; SEM, standard error of mean; ns, non-significant

[1]Control, basal diet (blend of concentrate (75%) and wilted mulberry leaves (25%) without the inclusion of enzyme and yeast; Enzyme, kg^{-1} basal diet contained 2 g enzyme blend (cellulase, amylase and protease); Yeast, kg^{-1} basal diet contained 2 g *Saccharomyces cerevisiae* ; Enzyme-yeast, kg^{-1} basal diet contained blend of enzyme (cellulase, amylase and protease) and *S. cerevisiae* (1 g each)

Table 6. Effects of enzyme and yeast-based diet on the ileum morphometry of fattening rabbit

Diets[1]	**Villus height, µm**	**Crypt depth, µm**	**Villus height crypt depth ratio**
Control	$840.8^{a} \pm 3.95$	$137.1^{a} \pm 1.22$	$6.13^{a} \pm 0.06$
Enzyme	$1192^{b} \pm 2.18$	$117.3^{b} \pm 1.63$	$10.2^{c} \pm 0.15$
Yeast	$1177^{b} \pm 10.3$	$119.2^{b} \pm 1.57$	$9.88^{bc} \pm 0.22$
Enzyme-yeast	$1178.4^{b} \pm 2.26$	$120.6^{b} \pm 0.93$	$9.77^{b} \pm 0.06$
SEM	4.676	1.115	0.114
Significance	***	***	***

In the same column, values carrying different superscript letter [a, b, c] means significant difference at $P < 0.05$, SEM, standard error of mean, ***, $P < 0.001$

[1]Control, basal diet (blend of concentrate (75%) and wilted mulberry leaves (25%) without the inclusion of enzyme and yeast; Enzyme, kg^{-1} basal diet contained 2 g enzyme blend (cellulase, amylase and protease); Yeast, kg^{-1} basal diet contained 2 g *Saccharomyces cerevisiae* ; Enzyme-yeast, kg^{-1} basal diet contained blend of enzyme (cellulase, amylase and protease) and *S. cerevisiae* (1 g each)

Data on effects of the additives on ileum morphology are shown in Table 6. Incorporation of additives increased ($P < 0.05$) villus height and reduced ($P < 0.05$) crypt depth. Nevertheless, villus height to crypt depth ratio only increased in enzymes blend supplemented group ($P < 0.05$).

DISCUSSION

The AGP use in the animal feed has been widely practiced in developing countries including Pakistan for decades. Research has established that the addition of AGP in animal diets, maintain healthy gut ecosystem and improves the animal performance (Cas-

tanon, 2007). However, due to growing incidence of antibiotic resistance (Falcao-e-Cunha et al., 2007; Ali et al., 2016),the antibiotic use has been discouraged in animal feeds. Alternatively, non-antibiotic feed additives, mostly enzymes and probiotics are increasingly used in animal feeds to maintain gut health, improve nutrient utilization and promote animal performance (Mateos et al., 2010; Abudabos et al., 2015; Ayasan and Inci, 2019). This study provides a comprehensive insight on the effects of enzymes (cellulase, amylase and protease) blend and yeast-based additives on growth performance, carcass quality traits, nutrients utilization, ileum morphology and meat physicochemical quality of native rabbits in Northern Pakistan.

Our results showed that inclusion of enzymes blend, yeast or their mixture in the rabbit diet increased WG and improved FCR as compared to the control group (without feed additives), highlighting their potential as an alternative to AGP in rabbit diets. The observed improvement in growth and FCR could be attributed to the positive effects of enzymes and yeast probiotics on the microflora of gut and cecum, such as due to the higher production of beneficial volatile fatty acids in the cecum (Guedes et al., 2009; Pinheiro et al., 2009). The increased production of these volatile fatty acids, particularly butyrate, suppresses the pathogenic bacteria in the gut (Mourao et al., 2006), and improve feed digestibility and gut health. Our findings are in accordance with Chandra et al., (2014), who found significant effects of supplementation of probiotics (*Saccharomyces boulardi* and *Pediococcus acidilacticii* 50% each) and Kemzyme HF (containing fibrolytic, proteolytic and lipolytic enzymes) alone or in combination on BW gain of the rabbits. Considering the carcass yield, in the present study probiotics and/or enzyme blend supplemented groups supported higher carcass weight and dressing percentage than the control group. Shanmuganathan et al. (2004) reported that exogenous enzymes (cellulases and proteases) and yeast culture (Yea-Sacc1026 at 200 ppm) remarkably increased (24.7%) the carcass yield of rabbit, which could be attributed to better nutrient utilization. In the present study, only weight of the kidney is higher in supplemented diet, while Shanmuganathan et al. (2004) reported that enzyme and yeast supplements increased the weights of liver, pancreas and caecum of rabbits. However, the reasons for heavier organs observed with additives are not clear.

In the present study, the physical properties (pH, cooking loss and water release), and chemical profile of meat were not altered by enzymes and yeast inclusion in the diets. Rotolo et al. (2014) found that live yeast (*S. cerevisiae var. boulardii*) supplementation in rabbit diet did not affect meat physicochemical traits. Similarly, Simonova et al. (2009) reported that bacteriocinogenic and probiotic strain supplemented diet had no significant effects on physicochemical properties of rabbit meat. Studies have established that rabbit meat is of high nutritional value due to high protein (21.2%; Khan et al., 2016) and linolenic acid (C18:3n-3) contents (Khan et al., 2018). Nonetheless, the chemical composition reported in the present study fell within normal range of rabbit meat.

It is an established fact that unhealthy digestive tract has been linked with gastro-intestinal diseases, inefficient digestive functions and lower feed efficiency (Tang et al., 2009). Intestinal villus height and crypt depth are vital indices for the intestinal health status (Jia et al., 2010). Morphometric variations such as shorter villi in the small intestine are linked with lower absorption of the nutrients, feed utilization efficiency and animal performance (Xu et al., 2003). In our database, the supplementation of enzymes and yeast in the rabbit diet resulted in longer villi, lower crypts depth and higher villus height to crypt depth ratio in the ileum, which may have contributed to the improved feed efficiency and growth performance as compared to the control group.

The favourable effects of the yeast-based diet on maintenance of gut health contributed towards improvement in nutrient digestibility (Mateos et al., 2010). In the present study, enzymes and yeast-based supplemented groups supported higher DM and NDF digestibility, suggesting more efficient utilization of these nutrients in the intestinal tract, which contributed to an improved FCR as compared to the control group (without feed additives). Our findings are consistent with Bhatt et al. (2016), who reported that digestibility of NDF improved with the dietary dry yeast addition in rabbit diet. The significant positive effects of probiotics on nutrient digestibility, growth performance and feed efficiency in combination with maintenance of healthier intestinal morphology are expected to have practical implication for rabbit's productivity and meat quality.

CONCLUSIONS

The results of this study report the effects of en-

zymes (cellulase, amylase and protease) blend and yeast (*S. cerevisiae*)-based probiotic supplementation on growth performance, nutrients digestibility, carcass quality and ileum morphology of rabbits. Supplementation of *S.cerevisiae* and enzymes blend alone or in combination supported better ileum morphometry, and increased DM and NDF digestibility. As a result, feed conversion efficiency and weight gain of rabbits significantly improved with supplementation of the additives. These results suggest that inclusion of probiotics can improve intestinal health, feed utilization efficiency and rabbit productivity, which can potentially improve the profitability of rabbit farms.

CONFLICT OF INTEREST

None declared.

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Research article
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The effects of cGnRH on gonadotropin secretion and hatching traits in Japanese quail

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ABSTRACT: The aim of this study was to measure the effect of chicken Gonadotropin Releasing Hormone-I (cGnRH-I) on male fertility potential. Male and female quails were placed in cages(1 male and 5 females) and devoted into four experimental groups: a) control group - no injection, b) negative control group - 200 µl standard saline was injected, c) 5µg cGnRH group - 5µg cGnRH-I was injected and d) 20µg cGnRH group - 20µg cGnRH-I was injected. Each group was consisting of scattered six cages. In each cage only males were subcutaneously injected under the wing (3 injections - 1 week apart from each other). A fourth injection was administered at the end of egg collection and one hour later blood was collected and serum concentrations of Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), prolactin and testosterone were measured. Egg fertility, hatchability and weekly egg production per hen were also measured. Injection of cGnRH-I did not increase pituitary gonadotropin secretion (LH or FSH); however, serum LH concentration non-significantly reduced in negative control group. A significant decrease in serum testosterone concentration was observed in negative control group compared to 20 µg cGnRH injected group. Fertility and hatchability of total set eggs were lower in negative control group compared to other groups. Egg production in control group was significantly decreased, probably due to the non-significant suppression of prolactin. Embryonic mortality (hatchability of fertile eggs) non-significantly increased in control and negative control groups compared to GnRH injected groups. It seems that cGnRH has a positive effect on fertility and hatchability; however, more studies are needed with older males to confirm our findings.

***Keywords*:** Egg, Fertility, Hatchability, Quail, Testosterone

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INTRODUCTION

Naturally, quails are seasonal breeders. They breed when there is enough food, water, light, temperature and security for the mother to raise the offspring (Ubuka et al., 2013). Gonadal development occurs in spring, in response to increasing day length. If the day length is over 11.5 h, rapid gonadal development occurs. Reproductive cycle terminates in late summer when the day length decreases below 14.5 h causing the complete regression of gonads (Nicholls et al., 1988). If the short day length is artificially increased (16h light and 8h dark) then a complete return to maturity occurs (Nicholls et al., 1988). Quails remain in breeding continuously by keeping them on unchanging 16h light and 8h dark cycle after the short days.

Quails use photoperiodic information directly through photoreceptors located within the mediobasal hypothalamus, which regulates the synthesis of Gonadotropin Releasing Hormone (GnRH) (Dawson, 2015). GnRH regulates reproduction through the regulation of the pituitary gonadotropins secretion *via* GnRH receptors on the gonadotropes in anterior pituitary gland (Kaprara and Huhtaniemi, 2018).

In birds, two distinct forms of cGnRH (cGnRH-I and cGnRH-II) have been characterized, and a third immune-reactive form similar to lamprey GnRH-III has also been reported (Bédécarrats et al., 2006). Chicken GnRH-I was first chromatographically isolated from chickens' hypothalamus (Miyamoto et al., 1982). Its structure and amino acid sequence were further determined (King and Millar, 1982; Miyamoto et al., 1983). It was shown that cGnRH-I differs from the mammalian GnRH-I by the substitution of Arg with a Gln at position 8. Miyamoto et al., (1984) identified a second form of GnRH (cGnRH-II) from chicken hypothalamic extracts. ThecGnRH-II differs from cGnRH-I at position 5, 7 and 8 (Bédécarrats et al., 2006).

In quails and chickens, in vitro as well as in vivo experiments have shown that both cGnRH-I and II stimulate the release of LH and FSH (Hattori et al., 1986). In contrast to mammalians, LH and FSH are synthesized from different gonadotropes in anterior pituitary gland, in poultry (Bédécarrats et al., 2006). Both LH and FSH act on the gonads to stimulate gametogenesis and sex steroid secretion (Ubuka et al., 2013).

It was reported that photo-stimulation causes an increase in GnRH release within 23 h in in vitro condition in quails (Millam et al., 1984; Perera and Follett, 1992). Photo stimulation causes substantial increase in LH and FSH secretion in male quails leading to the increase in steroid synthesize and the attainment of the puberty in about 5 weeks (Ubuka et al., 2013). According to Follet (1976), LH, FSH and testosterone could be detected in birds at short-day but their concentrations were greatly increased following photo-stimulation. In quail, LH pulses were observed only after exposure to long days with a frequency of 1 pulse per 80 min (Urbanski, 1984). In spring, LH levels first rose when the day length reached 11.9 h; however, in late summer the first signs of a decrease were detectable when the photoperiod decreased to 14.7 h and levels became basal when it reached the14 h (Robinson and Follett, 1982).

Aging in male quails and chickens causes reduction in GnRH-I release (Ottinger et al., 2004; Avital-Cohen et al., 2013). There is significant decrease in quail egg fertility and hatchability from the week 56 of age and onwards (Ottinger et al., 1983). According to Ottinger et al. (2002) aging in quails causes morphological abnormalities in the testes and the decrease in LH and FSH receptor expressions leading to the loss of fertility. However, in young photo-regressed males, testosterone implants stimulated the increased LH binding; whereas they did not affect FSH binding. The response to testosterone treatment was reduced by aging (Ottinger et al., 2002).

Reduction in GnRH-I secretion, in ageing roosters, has been associated with the lowest LH and FSH secretion and a reduction in plasma testosterone concentration (Ottinger et al., 2002; Weil et al., 1999). As a result, testes weight, semen volume and sperm concentration found generally lower compared to that of young roosters (Avital-Cohen et al., 2013). Reduced blood concentrations of FSH in ageing roosters have been deemed responsible for diminished daily sperm production, which is in turn strongly associated with decreased egg fertility (Rosenstrauch et al., 1994; Vizcarra et al., 2010).

Egg fertility constitutes an economically important factor in the poultry industry due to its effect on chick hatching. Reduction in egg fertility leads to diminished hatching rate and causes the removal of the males from the flock. Poultry industry is current-

ly suffering from a shortage of breeder males. Therefore, keeping genetically superior breeder males within the flock for a longer period of time, without any decrease in fertility, is economically important. Thus, the main aim of this study was to evaluate the effects of cGnRH-I injection on fertility of male Japanese quails (*Coturnix japonica*).

MATERIAL AND METHODS

Animal, housing, feeding and adaptation period

Japanese quails (*Coturnix Japonica*) used in this study were housed and treated according to the animal right committee act no 5199, which was published in 25509 numbered official paper of the state, on 01 July 2004. The related certificate from Selçuk University, School of Agriculture, Animal Right Committee allowing animal experimentation was obtained (Certificate no: 2019/2-003). On 5th of December 2019, fortyweeks old male (n=24, average body weight= 213.20 ± 6.35) and female quails (n=120, average body weight= 238.31 ± 10.28) were randomly selected and housed in numbered (From 1 to 24) cages (25 x 35 x 48 cm) under 16L: 8D light-dark cycle. Birds were placed in cages as one male and five females in the poultry yard of the School of Agriculture, Selçuk University. Along the course of the study, birds were fed *ad libitum* with diet containing 20% CP, 2901 kcal/kg metabolic energy, 2.5%Ca, 0.35% available P, 1.055% LIMS and 0.45% M. Water was freely available.

Preparation of cGnRH-I

Five mg cGnRH-I (Cat: ab143495, Abcam, Discovery Drive, Cambridge Biomedical Campus, Cambridge, CB2 0AX,UK) was dissolved in pre-prepared isotonic saline (OSEL Pharmacy and Trade Incorporation, Beykoz, İstanbul-Turkey) and then its concentration was arranged to 5 or 20 µg/200 µl. The amino acid sequence of injected cGnRH-I was different from the mammalian GnRH-I at position 8 as the Arg has been substituted with the Gln. The amino acid sequence of cGnRH-I used in this study was pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH2.

Grouping and injection

Along the study, birds were kept in numbered cages. Four weeks before the first injection on 30th of January2020,groups are randomly formed. Each group was consisting of six scattered cages. Two weeks before the start of injections, all groups were compared with each other to see if there were any differences in terms of egg laying, fertility and hatchability parameters. No statistically important differences were found. In each group, only males were subcutaneously injected under the wing. Birds were devoted into four experimental groups: a) control group -which was received no injection, b) negative control group, which was subcutaneously injected with 200 µl standards aline, c) 5µg cGnRH group, which injected with 200 µl standard saline containing 5 µg cGnRH and d) 20 µg cGnRH group, which injected with 200 µl standard saline containing 20 µg cGnRH. Birds were injected four times. The first injection was conducted when birds were 48 weeks old at 10:00 am on 30thJanuary 2020, the second injection was conductedat10:00 am on 6thFebruary and the third injection was conducted at 10:00 am on 13th February 2020. After the completion of egg collection and incubation works, the fourthnjection was donet 10:00 am on 3rd April 2020 and one hour later blood was collected for serum extraction and hormone analysis.

Egg collection, incubation and the measurement of egg laying performance

Eggs were daily collected at 09:30 am and weekly incubated (from Friday to Thursday), from the 7th of February 2020 to 3rd of April 2020, and the cage number was plotted on each egg collection viol without knowing which viol belongs to which group. Egg viols were daily placed in an egg collection cupboard (YMK-FN-685722,Qualitec, Fevzi Çakmak Mahallesi, Modesa Sanayi Sitesi 10735 Sk. No:13 Karatay/Konya-Turkey) in which temperature and humidity set to 15 ºC and 75%. Eggs were daily kept in this condition until the weekly incubation on Fridays. Two hours before the incubation the temperature of egg collection cupboard was brought to 25 ºC. Eggs were weekly incubated on Friday at 4pm in an incubator (T1600 S, Cimuka, 1214. Sokak 21/3 Ostim, Yenimahalle/Ankara-Turkey); temperature and humidity set to 37.6 ºC and 57% for 14 days. After 14 days, eggs were incubated in a hutching machine (T2400 H, Cimuka); temperature and humidity set to 37.2 ºC and 74% for 3 days. The time period between the incubation and the hatching named as one period and there were eight periods in total. The numbers of eggs collected and incubated in each week have been shown on Table 1. Daily egg number from each cage was recorded and weekly egg production for each cage and per quail calculated.

Table 1. The numbers of collected and incubated eggs in each week

Weeks	Number of egg collected	Number of egg incubated
Week-I	523	488
Week -II	472	439
Week -III	462	422
Week -IV	537	489
Week -V	531	490
Week -VI	522	483
Week -VII	530	488
Week -VIII	507	470

The determination of the number of hatched, fertilized and unfertilized eggs

After each period, the number of hatched eggs was determined by counting shells of hatched eggs. The number of unfertilized eggs and the number of not hatched eggs bearing an embryo were determined after breaking the eggs. The eggs not containing an embryo were accepted as unfertilized. The number of fertile eggs was determined by counting the number of hatched eggs plus the number of not hatched eggs bearing an embryo.

Measurement of embryonic mortality

The number of eggs not hatched, but bearing an embryo at different developmental stages were designated as mortal embryos. Embryonic mortality was devoted into three distich stages as mentioned below (Aygun et al., 2012).

Embryos dead on 1-9 days of incubation: Distinguished by the formation of eye and the absence of feather. The embryos bearing without feather were considered as dead between 1-9 days of incubation.

Embryos dead on 10-16 days of incubation: Distinguished by the presence of feather and yolk outside. Embryos bearing feather and the yolk outside the body were considered as dead between 10-16 days of incubation.

Embryos dead on 17-18 days of incubation: Distinguished by well-developed embryo and the absence of yolk outside.

Measurement of fertility and hatchability

The number of fertile Eggs: F

The number of hatched eggs: H

The number of not hatched eggs bearing an embryo: E

The number of total incubated eggs: I

$$F = H + E$$

Fertility (%) = (F/I) x100

Hatchability of total set eggs (%) = (H/I) x 100

Hatchability of fertile eggs (%) = (H/F) x100

Measurement of weekly egg production

Eggs produced from numbered cages were daily recorded and weekly egg produced from each cage divided by 5 to calculated weekly egg production per hen.

Measurement of weekly food consumption

The amount of food daily added to feed box was weighted by using a balance (DS-30, 99 Reagent Lane, Fair Lawn, NJ 07410, USA). The amount of food added was recorded along a week and subtracted from the reaming food in food box then divided by 6 to calculate weekly food consumption per bird.

The fourth injection, blood collection and serum extraction

The forth injection was carried out subcutaneously under the wing on Friday3rd April 2020 at 10:00 a.m. One hour later, birds were bought to the abattoir beside to the poultry yard. Birds were individually placed in a transparent plastic box attached to a CO_2 cylinder, equipped with a manometer. Gas flow rate to box arranged to 10L/min and covered by the lid. After 60-65 second bird was become unconscious and taken out form the box for bleeding. A small cut was given on the neck to see the jugular vein. A sterile 2 mL single use syringe provided with a sterile 22G hypodermic needle was used for blood collection from the jugular vein (Setecoject, Lot: A1606105, Set Medical Equipment Manufacturing and Trade Corporation, Mareşal Fevzi Çakmak Caddesi. No:18, 34522 Esenyurt/İs-

tanbul- Turkey). About 2 mL blood was collected and placed in a 5 mL sterile vacuumed blood tube containing gel and clot activator (Disera Medical Equipment Corporation, Lot: 2365.0043.19, İbni Melek OSB Mahallesi, TOSBİ Yol 4 Sokak No: 29 Tire Organize Sanayi Bölgesi 35900 Tire/ İzmir-Turkey). The tubes were transferred to the biotechnology lab of Animal Science department for serum extraction within 2 hours. Serum extracted by centrifugation at 4500 R.P.M for 20 minutes by using a bench top centrifuge (Nüve, Serial no: 02-0090, Esenboğa yolu, Akyurt o6287 Ankara- Turkey). Extracted serum was placed in sterile cryovials (Tarsons, Lot: 059D-10-200619, Jasmine Tower, Suite 213 31 Shakespeare Sarani, Kolkata-700 017, India) with cage number printed on and kept in a deep freezer set at -30ºC (Raypa, Serial no: 344 1/021, Avenida Del Vallès, 322 Polígono Industrial 'Els Bellots'08227 Barcelona-Spain) until analysis.

Hormone analysis

Serum concentration of hormones was quantitatively determined in accord to the procedure supplied with the related ELISA Kits (My BioSource, Inc. P.O. Box 153308 San Diego, CA 92195-3308 USA). Optical density values were measured by using an ELISA plate reader set to 450 nm wavelength. Optical densities were corrected by discarding the blank and then a standard curve was drawn; the curve was fit by using four parameter logistic regressions. Unknowns were compared with standard curve and their concentrations were plotted as ng/mL of blood.

Serum testosterone concentration was determined by using chicken testosterone (T) ELISA Kit (Inc. Cat: MBS703019). Serum LH concentration was measured by using chicken LH ELISA kit (Inc. Cat:MBS281782). Serum prolactin concentration was measured by using chicken prolactin ELISA kit (Inc. Cat: MBS739982) and serum FSH concentration was determined by using chicken follicle stimulating hormone (FSH) ELISA kit (Inc. Cat: MBS260290).

Statistical analysis

The data was analyzed by analysis of variance (ANOVA) by using Minitab statistical software. All data are presented as mean ±SEM. Pairwise comparisons were conducted according to the Tukey test, with 95% confidence intervals.

RESULTS

Serum hormone concentrations, fertilities of the incubated eggs, hatchability of the total set eggs, hatchability of the fertile eggs, weekly egg production per hen and weekly food consumption per bird are presented on Tables 2, 3, 4,5, 6 and 7 respectively. Injection of cGnRH-I did not increase pituitary gonadotrophin secretion (Table 2). The fertility of incubated eggs and the hatchability of total set eggs through the period I-VIII was lower in saline injected group (Table 3 and 4). There were no differences in hatchability of fertile eggs between the groups (Table 5). Through the weeks I to VIII, weekly egg production was lower in control group (Table 6). Weekly food consumption was higher in saline injected group compared to 5 µg cGnRH injected group (Table 7).

Table 2. Measured serum hormone concentrations (ng/mL) in experimental groups after the injections

	Experimental Groups				
Hormones	Control	Negative Control	5 µg cGnRH	20 µg cGnRH	*P-value*
LH	0.22 ± 0.06^{A}	0.08 ± 0.02^{A}	0.15 ± 0.04^{A}	0.22 ± 0.07^{A}	0.176
FSH	0.02 ± 0.00^{A}	0.03 ± 0.00^{A}	0.03 ± 0.00^{A}	0.03 ± 0.01^{A}	0.241
Testosterone	2.53 ± 0.52^{AB}	1.21 ± 0.32^{B}	2.79 ± 0.47^{AB}	3.38 ± 0.74^{A}	0.058
Prolactin	18.54 ± 6.89^{A}	39.35 ± 7.58^{A}	23.11 ± 4.99^{A}	25.39 ± 8.05^{A}	0.209

*Data were displayed on the table as Mean ± SEM

A,B Data with different superscripts in the same line are statistically different (P=0.058)

Table 3. The fertility of incubated eggs (%)

	Experimental Groups				
Periods	Control	Negative Control	5 µg cGnRH	20 µg cGnRH	*P-value*
Period-I	64.91 ± 12,08^{A}	65,52 ± 7,92^{A}	73,61 ± 4,39^{A}	84,31 ± 4,92^{A}	0.302
Period-II	74.68 ± 7.00^{AB}	53,02 ± 6.38^{B}	77.74 ± 5.69^{A}	78.29 ± 3.12^{A}	0.016
Period-III	73.50 ± 5,27^{A}	55.36 ± 12,24^{A}	81.84 ± 5.39^{A}	74.34 ± 5.60^{A}	0,129
Period-IV	75.82 ± 7.57^{A}	57.76 ±6.33^{A}	75.94 ± 7.56^{A}	69.49 ± 6.76^{A}	0.256
Period-V	59.08 ± 11.11^{A}	53.68 ± 10.35^{A}	77.24 ± 6.70^{A}	68.20 ± 12.28^{A}	0.410
Period-VI	75.29 ± 8.22^{A}	64.48 ± 9.69^{A}	69.80 ± 9.50^{A}	73.94 ± 9.08^{A}	0.837
Period-VII	74.95 ± 6.15^{A}	56.27 ± 5.47^{A}	73.42 ± 8.89^{A}	73.88 ±11.16^{A}	0.344
Period-VIII	79.72 ± 6.96^{A}	57.97 ± 10.38^{A}	76.65 ± 7.13^{A}	61.57 ± 13.46^{A}	0.334
Period I- VIII	72.24 ± 2.87^{A}	58.01 ± 2.97^{B}	75.78 ± 2.37^{A}	73.00 ± 3.08^{A}	0.000

*Data were displayed on the table as Mean ± SEM
^{A,B} Data with different superscripts in the same line are statistically different

Table 4. The hatchability of total set eggs (%)

	Experimental Groups				
Periods	Control	Negative Control	5 µg cGnRH	20 µg cGnRH	P-Value
Period-I	55.78 ± 10.73^{A}	59.94 ± 8.15^{A}	60.30 ± 6.68^{A}	70.98 ± 5.42^{A}	0.588
Period-II	59.73 ± 8.54^{A}	47.42 ± 6.56^{A}	62.62 ± 9.63^{A}	62.03 ± 6.58^{A}	0.505
Period-III	44.85 ± 4.93^{AB}	40.45 ± 9.06^{B}	70.68 ± 5.98^{A}	57.85 ± 5,93^{AB}	0.019
Period-IV	68.80 ± 7.22^{A}	42.64 ± 9.52^{A}	57.19 ± 10.33^{A}	54.82 ± 3.63^{A}	0.438
Period-V	52.42 ± 8.77^{A}	40.18 ± 8.37^{A}	63.8 ± 8.80^{A}	60.34 ± 10.60^{A}	0.299
Period-VI	59.17 ± 9.15^{A}	51.65 ± 10.22^{A}	61,38 ±7.74^{A}	62.57 ±7.10^{A}	0.810
Period-VII	64.38 ± 6.83^{A}	42.90 ± 3.54^{A}	60.32 ± 8.61^{A}	52.01 ± 9.53^{A}	0.217
Period-VIII	70.53 ± 7.61^{A}	36.23 ± 8.11^{B}	74.7 ± 3.74^{A}	51.6 ±11.73^{AB}	0.013
Period I- VIII	58.46 ± 2.86^{A}	45.18 ± 2.86^{B}	63.89 ± 2,71^{A}	59.02 ± 2.75^{A}	0.000

*Data were displayed on the table as Mean ± SEM
^{A,B} Data with different superscripts in the same line are statistically different

Table 5. The hatchability of fertile eggs (%)

	Experimental Groups				
Periods	Control	Negative Control	5 µg cGnRH	20 µg cGnRH	P-Value
Period-I	88.08 ± 4.76^{A}	90.99 ± 4.11^{A}	80.67 ± 5.03^{A}	84.05 ± 3,27^{A}	0.379
Period-II	77.50 ± 6.10^{A}	89.72 ± 4,72^{A}	78.17 ± 9.84^{A}	78.61 ± 6.67^{A}	0.576
Period-III	61.16 ± 6.38^{B}	77.08 ± 4.96^{AB}	86.69 ± 6.00^{A}	78.64 ± 7.22^{AB}	0.056
Period-IV	79.18 ± 4.18^{A}	71.86 ± 12.00^{A}	79.79 ± 6.38^{A}	80.34 ± 3.35^{A}	0,827
Period-V	77.56 ±5.74^{A}	74.75 ± 3.67^{A}	80.97 ± 8.83^{A}	90.40 ± 2.88^{A}	0.277
Period-VI	76.81 ± 4.73^{A}	77.77 ± 5.91^{A}	89.61 ± 3.34^{A}	86.16 ± 3.53^{A}	0.154
Period-VII	85.64 ± 3.96^{A}	77.26 ± 3.72^{A}	81.63 ± 5.69^{A}	72.34 ± 8.08^{A}	0,398
Period-VIII	90.13 ± 4.19^{A}	59.79 ± 5.23^{B}	89.37 ± 2.02^{A}	86.24 ± 5.70^{A}	0.000
Period I- VIII	79.51 ± 2.07^{A}	77.40 ± 2.41^{A}	83.36 ± 2.17^{A}	82.10 ± 1.94^{A}	0.208

*Data were displayed on the table as mean ± SEM
^{A,B} Data with different superscripts in the same line are statistically different

Table 6. Weekly egg production per quail

	Experimental Groups				
Weeks	Control	Negative Control	5 µg cGnRH	20 µg cGnRH	P-Value
Week-I	4.23 ± 0,63^{A}	4.47 ± 0.42^{A}	4.37 ± 0.28^{A}	4.37 ± 0.45^{A}	0.998
Week -II	3.60 ± 0.54^{A}	3.93 ± 0.35^{A}	4.13 ± 0.46^{A}	4.07 ± 0.44^{A}	0.845
Week -III	3.00 ± 0.32^{A}	4.17 ± 0.31^{A}	4.00 ± 0.39^{A}	4.23 ± 0.45^{A}	0.102
Week -IV	3.80 ± 0.40^{A}	4.43 ± 0.38^{A}	4.67 ± 0.48^{A}	5.00 ± 0.13^{A}	0.168
Week -V	3.93 ± 0.46^{A}	4.57 ± 0.29^{A}	4.63 ± 0.42^{A}	4.57 ± 0.26^{A}	0.512
Week -VI	3.30 ± 0.47^{B}	4,53 ± 0.29^{AB}	4.63 ± 0.24^{A}	4.93 ± 0.28^{A}	0.012
Week -VII	3.77 ± 0.57^{A}	4.43 ± 0.19^{A}	4.77 ± 0.29^{A}	4.70 ± 0.31^{A}	0.233
Week -VIII	3.70 ± 0.45^{A}	4.17 ± 0.56^{A}	4.57 ± 0.30^{A}	4.47 ± 0.29^{A}	0.470
Week I- VIII	3,67 ± 0.17^{B}	4.34 ± 0.12^{A}	4.45 ± 0.13^{A}	4.57^{A} ± 0.11	0.000

*Data were displayed on the table as mean ± SEM
^{A,B} Data with different superscripts in the same line are statistically different

Table 7. Weekly food consumption per quail (gr)

	Experimental Groups				
Weeks	Control	Negative Control	5 µg cGnRH	20 µg cGnRH	P-Value
I	248.72 ± 5.80^{A}	249.79 ± 7.67^{A}	231.78 ± 5.43^{A}	245.15 ± 3.60^{A}	0.139
II	272.49 ± 11.71^{A}	263.04 ± 10.80^{A}	253.94 ± 11.30^{A}	260.99 ± 9.19^{A}	0.672
III	296.17 ± 15.79^{A}	298.64 ± 20.27^{A}	271.22 ± 16.11^{A}	292.49 ± 23.43^{A}	0.736
IV	269,83 ± 12.20^{A}	274.58 ± 9.86^{A}	242.91 ± 13.24^{A}	261.25 ± 15.07^{A}	0.335
V	235.15 ± 18.07^{A}	265.28 ± 18.88^{A}	234.95 ± 10.86^{A}	248.55 ± 9.75^{A}	0.449
VI	242.53 ± 14.15^{A}	276.07 ± 17.01^{A}	239.05 ± 5.88^{A}	250.33 ±3.27^{A}	0.133
VII	247.51 ± 12.20^{A}	282.20 ± 26.07^{A}	237.08 ± 6.31^{A}	250.12 ± 7.50^{A}	0.212
VIII	230.65 ± 11.16^{AB}	272.88 ± 16.48^{A}	226.60 ± 6.39^{B}	251.97 ± 7.16^{AB}	0.028
I- VIII	255.38 ± 5.23^{AB}	272.80 ± 5.83^{A}	242.19 ±3.84^{B}	257.61 ± 4.33^{AB}	0.000

*Data were displayed on the table as mean ± SEM
^{A,B} Data with different superscripts in the same line are statistically different

DISCUSSION

In the present study, injection of cGnRH-I did not increase LH and FSH secretion. Several studies have shown a little or no in vivo effect of cGnRH-I on FSH secretion in non-photo-stimulated immature intact chickens (Bruggeman et al., 1998; Dunn et al., 2003; Proudman et al., 2006) or in young cockerels (Krishnan et al., 1993). However, both cGnRH-I and II significantly increased LH secretion and cGnRH-II was more potent than cGnRH-I (Proudman et al., 2006). In this study, serum concentration of LH is measured. If the pulse frequency and the pulse amplitude of a hormone change, it is reflected as a change in serum concentration of that hormone. It is impossible to take more than one blood sample from a quail. In this study, male quails were injected in order from cage 1 to cage 24. Blood was collected in order from cage 1 to cage 24 one hour after the last quail was injected. The transport of quails to the abattoir, the preparation for bleeding and the blood collection gave enough time for a LH pulse to occur. In this study, LH secretion did not change ($P > 0.05$) by cGnRH-I injection. This could be because we applied long day photoperiod (8 hours dark and 16 h light) rhythm. Long day photoperiod rhythm caused an increase in endogen cGnRH-I release. In culture condition, it was reported that GnRH release increased from super-fused hypothalamic slices in photo-stimulated quail (Millam et al., 1984; Perera and Follett, 1992). Therefore, cGnRH concentration was already high in their blood and injecting extra cGnRH did not cause significant differences in blood concentrations of LH and FSH at the time of blood collection.

Serum testosterone concentration was increased ($P = 0.058$, close to significant) after the injection of 20 µg cGnRH as compared to that of negative control group. This might be a result of a non-significant decrease in serum LH concentration in negative control group, which has not been treated with cGnRH. There were no significant differences in serum prolactin concentrations among the experimental groups, but serum prolactin concentration was numerically

higher in negative control groups as compared to other groups. This is not a consequence of a decreased serum testosterone concentration due to the saline injection, since plasma prolactin concentrations were found to be similar in intact and castrated male quail (Boswellet al., 1995). Furthermore, an inverse relationship was reported between serum LH concentration and serum prolactin level in European quail *(Coturnix coturnix japonica)* (Camper and Burke, 1977). In the negative control group, serum LH concentration was non-significantly reduced, and this might be a reason for a non-significant increase in serum prolactin concentration.

The fertility of incubated eggs and the hatchability of total set eggs through the period I-VIII were lower in saline injected negative control group compared to other groups. However, these significant differences were obvious only in few cases (fertility - period II or hatchability period III and VIII between negative control and 5μg GnRH groups) probably because of increased SEM. The decrease in fertility and hatchability might be due to non-significant decrease in serum concentration of LH, and to the significant decrease in serum testosterone concentration in negative control group compared to that of 20 μg cGnRH injected group. A strong positive correlation between serum concentration of LH and testosterone was found previously (Ottinger et al., 1983). Therefore, the reason for decreased serum testosterone concentration ($P < 0.05$) might be the reflection of the non-significant decrease in serum LH concentration due to the lack of GnRH injection in negative control group. Hatching is depending on whether an egg is fertilized or not fertilized. Therefore, it is a broadly male dependent phenomenon. The decreased fertility might affect hatchability and it could be due to numerically increased serum prolactin concentration in negative control group. The adverse effect of prolactin on egg fertility in Japanese quail has already been reported (Renzoni, 1970).

The hatchability of fertile eggs was similar to all groups through I-VIII period. Decrease in hatchability of fertile eggs is a consequence of increased embryonic mortality. Increase in embryonic mortality might be a result of decreased sperm quality rather than the number of sperm inseminated. In broiler breeder hens, no relation has been reported between inseminated sperm number and embryonic mortality (Van Wambeke, 1984). A correlation has been reported between semen quality and the testosterone response to GnRH in cockerels (Barna and Mézes, 1994). Therefore, it could be assumed that decrease in sperm quality in control group may lead to the increase in the number of dead embryos, which caused the decrease in hutching. However, in this study this was obvious only in few periods; it seems that hatchability of fertile eggs did not affected by the injection of GnRH in the present study.

In control (natural) group, egg production was significantly lower through the period I-VIII and only in period VI compared to other groups. This might be a result of non-significant decrease of prolactin in control group. In birds, a positive relation between serum prolactin concentration and egg production has been reported (Goldsmith and Hall, 1980; Jensen et al., 2019). In control group, males received no injection. Therefore, prolactin concentrations weren on-significantly reduced. Semen was transferred to females via the mating and this probably resulted the significant reduction in egg laying. Follicle-stimulating hormone is crucial for steroidogenesis, follicular recruitment, growth, and selection (Li et al., 2011). There is a positive relation between FSH concentration and egg laying. Blood concentration of FSH increases during the laying period? (Li et al., 2011). Weekly food consumption per quail increased in negative control group as compared to 5 μg cGnRH injected group. The increased appetite in negative control group might be a result of suppressed GnRH secretion. The role of cGnRH in food intake was studied in the goldfish. Intracerebroventricular administration of cGnRH induced a decrease of food consumption in a dose-dependent manner (Matsuda et al., 2008). Non-significant reduction in serum LH concentration could be an indication that GnRH secretion was suppressed in negative control group.

CONCLUSION

According to our results, the injection of cGnRH significantly increased egg fertility compared to the negative control group. Furthermore, the hatchability of total set eggs was lower in negative control group, as compared to other groups, due to the decreased egg fertility. This is probably caused by the reductions in serum LH (non-significant) and testosterone (significant) concentrations. The decrease in hatchability of fertile eggs is a reflection of embryonic mortality. In overall, through the Period-I-VIII, no significant differences, in terms of embryonic mortality, were noted among the groups. In this study, we injected cGnRH at 48 weeks old males; thus, it is difficult to say that

cGnRH injection increases fertility potential of older males. More studies are needed with older males to confirm.

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CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Identification of serum proteins in dogs naturally infected with *Anaplasma phagocytophilum* and *Borrelia burgdorferi*: a pilot study

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ABSTRACT: Serum proteomic analysis would aid in better understanding the pathophysiology of several diseases. The aim of this study was to identify the serum proteomes of dogs with anaplasmosis and Lyme disease using a proteomic approach. Diseases were diagnosed by a commercial rapid in-clinic ELISA. *Borrelia* antibodies were evaluated by IFAT. Four groups were designated: symptomatic dogs with anaplasmosis (n=5), dogs with Lyme disease (n=5), dual-positive dogs (n=5), and healthy control dogs (n=5). Serum samples were collected before treatment. Two-dimensional electrophoresis of pooled samples in each group were run in triplicate. Ten out of 57 differentially expressed spots between groups were evaluated for identification by mass spectrometry.

Compared to those of controls, levels of vitamin D-binding protein (VDBP), glycoprotein-9 (GP9) and kininogen-1 (KGN-1) decreased, while haptoglobin (Hp) and immunoglobulin (Ig) heavy chain levels increased in dual infection group. Serum apolipoprotein-A1 (Apo-A1) levels decreased in dogs with anaplasmosis, Lyme disease and dual infections compared to those in control dogs. Serum clusterin levels decreased in dogs with anaplasmosis but were not differentially expressed in dogs with Lyme disease or dogs with dual infections compared to those in control dogs. Calpain-3 decreased in dogs with anaplasmosis and Lyme disease.

This study showed that many protein levels might be changed in dogs with naturally acquired anaplasmosis and Lyme disease. Understanding the role of these proteins in different biological processes can provide information of interest for diagnostic and therapeutic approaches for these clinical conditions.

Keywords: Anaplasmosis, Lyme disease, proteomic, dog

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INTRODUCTION

Anaplasma phagocytophilum (*A. phagocytophilum*) and *Borrelia burgdorferi* (*B. burgdorferi* s.l.) are two tick-borne diseases that affect humans and animals in Africa, the Middle East, Europe and North America. *A. phagocytophilum* is a gram-negative obligate organism that is the causative agent of anaplasmosis in dogs, humans, cats and horses as well as tick-borne fever in ruminants. *B. burgdorferi* s.l. causes Lyme disease, also called borreliosis, which is the most frequently diagnosed vector-borne disease (VBD) in humans and dogs (Kybicova et al, 2009; Mc Causland et al, 2011; Hovius, 2015).

Coinfections are common, with these VBDs transferred by the same ticks to mammals and showing clinical symptoms within 20 days to 3 months after getting bitten by an infected tick. Increased body temperature, lethargy, lameness, and painful joints are frequently observed symptoms. Diagnosis of Lyme disease in daily practice can be challenging (Hovius, 2005); however, Lyme arthritis is seen on the extremity close to the tick bite area (Ettinger and Feldman, 2010). Hepatomegaly and increased serum liver enzyme levels can be seen with exposure to both organisms. In cases of anaplasmosis, pale mucous membranes and bleeding disorders due to thrombocytopenia, renal damage accompanied by proteinuria, haematuria, azotaemia (Greene, 2012), neurologic and cardiologic abnormalities (hyperesthesia, posterior paresis, epilepsy, meningoencephalitis, arrhythmia and cardiomyopathy) can also be seen (Agudelo et al, 2011; Janus et al., 2014; Kybicove et al, 2009; Schanilec et al 2010). Although serum analysis of specific antibodies (immunoglobulins) and PCR tests are more suitable for reliable diagnosis of Lyme disease and anaplasmosis, ELISA-based in-clinic rapid tests are commonly used as a diagnostic test with high specificity and sensitivity for both diseases (Stillman et al, 2014).

Screening of the disease in detail gives rise to an understanding of the complex pathophysiological mechanism in dogs and humans. Proteomic analysis can identify biomarkers that elucidate the pathophysiological changes associated with the infection and may have implications for better knowledge of the pathology of these VBDs. To the authors' knowledge, there are no data of serum proteomes on these VBDs and modifications in canine expressional proteomics of serum samples. Thus, the aim of this study was to investigate potential serum proteomes (bioindicators) in dogs with anaplasmosis, Lyme disease and dual infections from both pathogens by using a proteomic approach.

MATERIALS AND METHODS

Animals and sampling

For this study, 4 groups were created; dogs with anaplasmosis (Group-1), dogs with Lyme disease (Group-2) and dogs with dual infection (Group-3), and healthy control dogs (Group-4). These groups consisted of a total of 20 dogs (5 dogs in each) with 6 different breeds (Golden Retrievers, Terriers, Boxers, German Shepherds, Cavalier King Charles Spaniels and mixed breed dogs). The mean age was 6.2 ± 2.4 yrs in Group-1, 8.4 ± 3.2 yrs in Group-2, 7.5 ± 2.6 yrs in Group-3 and 6.5 ± 2.0 yrs in Group-4.

The symptoms that were observed in our study population, which were compatible with a vector-borne disease were inappetence (2/Group-1, 3/Group-2, and 2/Group-3), lethargy (1/ Group-1, 2/ Group-2, and 1/ Group-3), fever (3/Group-1, 2/Group-2, and 1/Group-3), joint swelling (3/Group-2, 2/Group-3), and/or thrombocytopenia (5/Group-1, 4/Group-2, and 5/Group-3) and serum samples of the patients were collected for further diagnostic and evaluation steps. Diagnosis was based on a seropositive test result of a commercial rapid in-clinic ELISA for the qualitative detection of *Dirofilaria immitis* antigen, anti-*Ehrlichia canis* antibody, anti-*Borrelia burgdorferi* antibody and anti-*Anaplasma phagocytophilum/Anaplasma platys* antibody in canine serum, plasma or whole blood (Anigen Rapid CaniV-4 Test Kits, Bionote). The sensitivity (Anaplasma: 88.5%, Lyme: 93%) and specificity (Anaplasma: 97.1%, Lyme: 98%) of the rapid ELISA test kit were found to be sufficient for diagnostic accuracy. However, there is limitation of detecting *Borrelia* antibodies using a commercial rapid in-clinic ELISA test, *Borrelia* antibodies were evaluated by an immunofluorescent antibody testing (IFAT- MegaScreen Fluoborrelia, MegaCor Diagnostik GmbH, Austria) and cutoff titer was accepted as a 1:64 (sensitivity, 90%; specificity, 98.6%). In the control group, dogs were considered healthy based on clinical, haematological and serum biochemistry profiles as well as negative test results of the rapid test.

Blood samples were collected from the cephalic vein before treatment and then placed into special tubes with and without anticoagulant (BD Vacutainer CAT and K3E 3.6 Mg, Plymouth, UK) to perform haematological (complete blood cell count with VetS-

can® HM5, Abaxis) and serum biochemistry analyses (serum C-reactive protein [CRP], haptoglobin [Hp] and ferritin levels). The samples were centrifuged, and the serum samples were separated into cryo tubes (CryoClear™ Crygenic Vials, Globe Scientific, USA). Then, frozen samples were stored (-80 °C) until proteomic analysis, which was performed in less than three months after the final patient sampling.

All the procedures made were approved by the Local Ethical Committee of Bursa Uludag University (24.09.2013/2013-14/07).

CRP, Hp and Ferritin Measurements

Frozen-packed serum samples were sent to the Laboratory of Clinical Pathology, Faculty of Veterinary Medicine, University of Murcia, Spain, for C-reactive protein (CRP), haptoglobin (Hp), and ferritin measurements with the same techniques as reported previously (Karnezi et al, 2016; Martinez-Subiela et al, 2005).

Proteomic Analysis

These procedures of two-dimensional protein electrophoresis (2D-PAGE), high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and protein identification were performed as reported in detail in our previous article (Escribano et al, 2016). These procedures are briefly explained below.

2D-PAGE and image analysis

Pooled serum samples from each group were prepared. The Bradford assay (Sigma-Aldrich, USA) was used to measure protein concentrations in serum samples to standardize the total protein concentrations of each animal in each pool. Isoelectric focusing was performed in a Protean IEF Cell (Bio-Rad). Two-dimensional protein electrophoresis (SDS-PAGE) was run using 12% polyacrylamide gels prepared for the Protean II XL Multi Cell (Bio-Rad). Protein separation was performed with the following 2-step program. Scanning of the stained gels was performed in an ImageScanner II (GE Healthcare Europe GmbH) and evaluated using specific 2D software (Image Master 2D Platinum 7.0, GE Healthcare Europe GmbH). Images of all patient samples were digitalized and aligned to identify differentially expressed protein spots (ANOVA $P < 0.05$) between groups.

HPLC-MS/MS analysis

Spots were cut out and in-gel digested with trypsin, as described in a previous study (Pantchev, 2010). The separation and analysis of the tryptic digests of the samples were performed with an HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, USA) equipped with a μ-well plate auto sampler and a capillary pump and connected to an Agilent Ion Trap XCT Plus Mass Spectrometer (Agilent Technologies, USA) using an electrospray (ESI) interface.

Protein identification

The data analysis program for Liquid Chromatography/Mass Selective Detector (LC/MSD) Trap Version 3.3 (Bruker Daltonik, GmbH, Germany) and Spectrum Mill MS Proteomics Workbench (Rev A.03.02.060B, Agilent Technologies, USA) were used to perform MS data processing. Raw data were extracted under default conditions as follows: unmodified or carbamidomethylated cysteines; sequence tag length>1; [MH]+ 50-7000 m/z; maximum charge +7; minimum signal-to-noise (S/N) 25; and finding 12C signals. The MS/MS search against the appropriate and updated NCBInr database from *Canis* sp. was performed with the following criteria: identity search mode; tryptic digestion with 3 maximum missed cleavages; carbamidomethylated cysteines; peptide charges +1, +2, and +3; monoisotopic masses; peptide precursor mass tolerance 2.5 Da; product ion mass tolerance 0.7 amu; ESI ion trap instrument; minimum matched peak intensity 50%; and STY phosphorylation, oxidized methionine, and N-terminal glutamine conversion to pyroglutamic acid as variable modifications.

Statistics

Descriptive statistical procedures and software (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA) were used to perform statistical analysis. The CV of the assay was calculated as SD divided by the mean value of analysed replicates (100%). Linear regression analyses were performed to assess linearity under dilution. Because of the small sample size, the changes in analytes among healthy dogs and dogs with anaplasmosis, Lyme disease and dual infection were evaluated by the nonparametric Kruskal-Wallis test. $P < 0.05$ was considered significant.

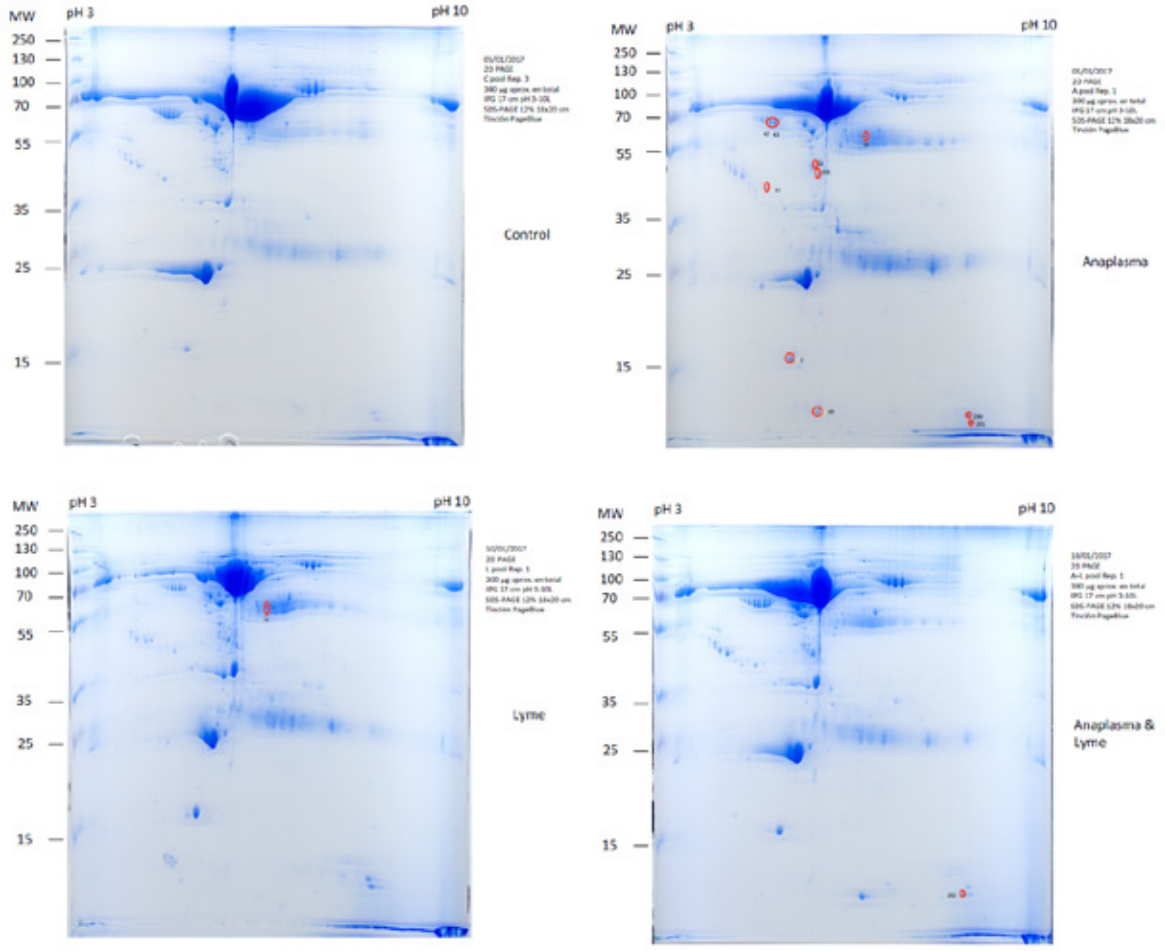
RESULTS

Selected haematological and acute phase proteins were evaluated for all groups (Tables 1 and 2, respectively). CBC results except platelet count in infected

dogs did not differ statistically compared to those of controls (Table 1). The platelet count was significantly lower (P<0.01) in infected dogs (Group-1, Group-2 and Group-3) than in healthy controls. The decrease in platelet count was more severe in dogs with anaplasmosis (120 ± 22 × 10^3/μL) than in dogs with Lyme disease (185 ± 33 × 10^3/μL) and dual infected dogs (140 ± 28 × 10^3/μL) (Table 1).

Serum levels of CRP, Hp and ferritin in infected dogs were significantly higher (P<0.05 - 0.001) than those in control dogs (Table 2). There were no statistically significant differences in these parameters among the three groups of infected dogs.

2DE Gel spots of differentially expressed proteomes were evaluated in dogs with Anaplasmosis, Lyme Disease and dual infection of both, and healthy dogs. 2DE image analysis showed 57 differentially expressed protein spots between infected animals and controls. Of those, 10 spots were evaluated for identification by MS (Figure 1). Compared to those in control dogs, VDBP, GP9 and KGN-1 levels decreased, while Hp and Ig heavy chain levels with different spots increased in dogs with both diseases. Apo-A1 levels decreased in dogs with anaplasmosis, Lyme disease and dual infections compared to those in control dogs. Serum clusterin levels decreased in dogs with anaplasmosis but were not differentially expressed in dogs with Lyme disease or dual infections compared to those in control dogs. Calpain-3 content decreased in dogs with anaplasmosis and Lyme disease. In dual infection dogs, levels of VDBP, GP9 and KGN-1 decreased, but alpha-1-acid glycoprotein (AGP), Hp and Ig heavy chain levels increased compared to those in control dogs (Table 3).



Vitamin D binding protein: 105, Hemopexin: 34, Apolipoprotein A1: 15, Ig Heavy chain: 25, Haptoglobin-like: 54, Calpain 3: 104, Glycoprotein 9: 42, Kininogen-1: 43, Clusterin: 13, Alpha-1-acid glycoprotein: 46.

Figure 1. 2DE Gel spots of differentially expressed proteomes in dogs with Anaplasmosis, Lyme Disease and dual infection of both, and healthy dogs

Table 1. Selected hematological and physiological values of the dogs in each group (mean ± SEM) of this study

Parameters	Dogs with Lyme Disease (n=5)	Dogs with Anaplasmosis (n=5)	Dual Infected dogs (n=5)	Healthy Control (n=5)
WBC×103/µl	11.3 ± 1.4	9.8 ± 1.7	10.2 ± 1.8	17.2 ± 3.4
RBC ×109/µl	6.5 ± 0.4	6.3 ± 0.4	5.9 ± 0.8	7.3 ± 0.5
HCT %	44.2 ± 0.2	38.2 ± 2.5	42.3 ± 2.1	43.9 ± 1.7
PLT ×103/µl	185 ± 33*	120 ± 22**	140 ± 28**	315 ± 17
BW (kg)	17.0 ± 2.5	24.4 ± 2.2	19.2 ± 2.8	17.2 ± 3.4

Compared to Control Group * P<0.01 and ** P<0.001
WBC: White blood cell, RBC: Red blood cell, HCT: Hematocrit, PLT: Platelet, BW: Body weight

Table 2. Selected acute phase protein concentrations of the dogs in each group (mean ± SEM) of this study

Parameters	Dogs with Lyme Disease (n=5)	Dogs with Anaplasmosis (n=5)	Dual Infected dogs (n=5)	Healthy Control (n=5)
CRP µl/ml	30.6 ± 15.2***	25.9 ± 4.8*	25.5 ± 4.3*	2.0 ± 0.2
Hp mg/ dL	3.8 ± 2.4*	3.5 ± 1.8*	3.9 ± 1.8 *	1.5 ± 0.5
Ferritin µg/L	379 ± 165**	453 ± 165**	153 ± 54	113 ± 40

Compared to Control Group * P<0.05 ** P<0.01 *** P<0.001
CRP: C reactive protein, Hp: Haptoglobin

Table 3. Identification and characterization of the spots differentially expressed in dogs with Anaplasmosis, Lyme Disease and dual infection of both, and healthy dogs

Match ID	Region ID	Dogs with anaplasmosis	Dogs with Lyme Disease	Dual infected dogs	Biological Function
105	VDBP	Decrease***	Decrease***	Decrease***	Vitamin D transport and storage
34	Hemopexin	Increase***	Increase***	ND	Inflammation and hemoglobin metabolic process
15	Apo-A1	Decrease**	Decrease**	Decrease**	Immunity, inflammation, apoptosis, lipid metabolism
25	Ig heavy chain	Increase*	Increase*	Increase*	Inflammation
54	Haptoglobin-like	Increase*	Increase*	Increase*	Inflammation, antioxidant activity and hemoglobin binding molecular function
104	CAPN3	Decrease***	Decrease***	ND	Cellular response to sodium and calcium ions
42	GP9	Decrease*	Decrease*	Decrease*	Coagulation
43	KNG-1	Decrease*	Decrease*	Decrease*	Negative regulation of blood coagulation
13	Clusterin	Decrease*	ND	ND	Inflammation and protein stabilisation
46	Alpha-1-acid glycoprotein	ND	Increase*	Increase*	Regulation of acute phase inflammatory response and negative regulation of interleukin-6 production

Compared to Control Group * P<0.05 ** P<0.01 *** P<0.001
VDBP; Vitamin D binding protein, Apo-A1; Apolipoprotein A1, CAPN3; calpain 3, GP9; Glycoprotein 9, KNG-1; Kininogen-1, ND; not determined.

DISCUSSION

This study showed that many serum proteins might be expressed, upregulated or downregulated, in dogs naturally infected with *A. phagocytophilum* (anaplasmosis) and *B. burgdorferi* (Lyme disease). These proteins could be used to understand the detailed pathophysiology of the diseases and provide advantages as possible biomarkers revealing the diagnostic and therapeutic steps of the cases.

In this study, thrombocytopenia and an activated serum acute phase reaction (elevated CRP, Hp, and ferritin levels) were observed in dogs with anaplasmosis and Lyme disease. Compared to those in control dogs, the increase in the serum CRP level was more severe (max 15-fold) than the increase in serum Hp and ferritin levels (maximum 2-3-fold) in infected dogs. In previous studies, serum CRP levels increased in dogs with Anaplasma (Pantchev, 2010; Dyachenko

et al, 2012; Baric Rafaj et al., 2013) and Babesia-infected dogs (Kuleś et al, 2016). We also observed that dual infection did not aggravate thrombocytopenia and inflammation in this study. These results showed that the severity of thrombocytopenia and the increase in serum acute phase proteins, especially CRP, could not be used to discriminate between VBD and dual infections.

2DE of pooled samples showed 57 differentially expressed protein spots between infected animals and controls. Of those, 10 proteins were evaluated by MS, while others, such as Hp, VDBP and Apo A-1, were already identified in previous reports (Ecsribano et al, 2016; Kocatürk et al, 2016; Kuleś et al, 2014; Miller et al, 2014).

Hp is one of the positive acute phase proteins in dogs with a status of inflammation that also has antioxidant activity and haemoglobin-binding molecular function. Increased oxidative stress in dogs with both VBDs may have a positive role in increased haemoglobin uptake in the bloodstream, triggering Hp upregulation (Vickers et al., 2010). In this study, the observed increases in serum Hp levels showed the activation of the inflammatory response of the host organism against vector-borne pathogens, compatible with a previous study carried out in dogs with ehrlichiosis (Escribano et al., 2017).

In this study, the serum level of VDBP decreased in dogs with anaplasmosis, Lyme disease or both. VDBP is expressed in the liver and plays important role(s) in vitamin D transport and storage. Decreased VDBP content in dogs with VBDs may be related to decreased synthesis with/or excessive use of proteins in the liver that can have an impact on circulating vitamin D levels, leading to negative effects on the immune response of the host against infections, such as VBDs (Uysal et al., 2015). Possible tissue and/or organ injury as well as coagulation activation during both infections in dogs can cause alterations in serum VDBP levels (Bottari et al., 2016; Li et al., 2016). The overuse of vitamin D to neutralize the overproduction of ferritin and transferrin in response to VBDs may also be the reason for the decrease in VDBP in this study (Escribano et al., 2017, Kules et al, 2014; Kuleś et al., 2016).

Serum Apo-A1 has multiple roles in immunity, inflammation, and apoptosis and plays a vital role in cholesterol-binding and transport activities and cellular cholesterol homeostasis (Kuleś et al., 2016, Mangaraj et al., 2015). In the present study, decreased levels of serum Apo-A1 in dogs with Lyme disease were considered a host response to the pathogen. Apo-A1 has been documented as a potential biomarker in treatment monitoring in canine leishmaniasis (Escribano et al., 2016). Excessive use of Apo-A1 via the immune response and organ damage may play a role in the decreased level of serum Apo-A1 in response to anaplasmosis, Lyme disease and dual infections in dogs.

AGP is one of the regulators of the acute phase inflammatory response and negatively regulates interleukin-6 production. In previous studies, an increase in serum AGP was reported to be a useful marker to assess inflammatory status (Escribano et al., 2016, Hagman, 2011) and to differentiate from acute to chronic stages of inflammatory disease (Yuki et al., 2010). In parallel with the literature, in this study, an increased level of serum AGP was detected in both the single and dual infection groups compared to that in the healthy control group (Escribano et al., 2016, Hagman, 2011, Yuki et al., 2010).

KNG-1 binds to kallikrein to release the proinflammatory mediator bradykinin, a proinflammatory peptide, and has antithrombin function and antiadhesive properties (Yousef et al., 2003; Bryant et al., 2009). However, kallikrein is a chemotactic factor for neutrophils (Kaplan et al., 1972) and monocytes (Gallin et al., 1974) shown to cause neutrophil aggregation (Schapira et al., 1982). In our study, decreased levels of GP9 were seen in dogs with dual and single infections. GP9 functions as a von Willebrand factor (vWF) receptor and mediates vWF-dependent platelet adhesion to blood vessels (Ware et al., 2002; Ruggeri et al., 2003; Garcia-Martinez et al., 2012). To the authors, downregulation of both KNG-1 and GP9 might signal an impaired inflammatory response and coagulation cascade due to vector-borne diseases evaluated in our study.

Clusterin (apolipoprotein J) is one of the chaperone proteins secreted under stress conditions, such as cell death, tumour progression and neurodegenerative disorders. It has been reported that increases in serum and urine clusterin content are early diagnostic tools to evaluate renal function (Garcia-Martinez et al., 2012, Zhou et al., 2014). In our study, decreased serum clusterin levels were detected in dogs with anaplasmosis compared to those in control dogs. As mentioned in a previous study (Miller et al.,2014), the possible reason for the decreased level of serum

clusterin in Group-1 might be the excessive use of the protein due to the inflammatory oxidative stress status in VBDs.

CAPN3, which is found within muscle cells in structures called sarcomeres, belongs to the calpain superfamily and is predominantly expressed in skeletal muscle. In human medicine, a previous report showed that CAPN3 has an active role in type 2 diabetes mellitus (Harris et al., 2006). CAPN3 was also shown to be highly expressed by melanoma cells, causing cell proliferation and stimulating cell death (Weeraratna et al., 2004). However, downregulation of serum CAPN3 has been reported in dogs with mitral valve disease (Locatelli et al., 2017). In our study, downregulation of serum CAPN3 was seen in dogs with only anaplasmosis or Lyme disease. Unlike in human studies, CAPN3 seems to behave differently in dogs with inflammation; therefore, additional studies are needed to elucidate the behaviour of this proteome constituent.

Serum proteomic changes pre and post treatment were not evaluated in this study. Investigators may further evaluate this hypothesis to compare the diseased and treated expressional serum proteome status of dogs with anaplasmosis and/or Lyme disease. Serum AGP content may be a reliable biomarker of inflammatory disease in dogs and may be useful to differentiate acute and chronic phases of disease.

A major limitation of this study is the small number of patients in the groups, limiting the potential to identify proteins due to biological inter-individual variation. Nevertheless, the proteome is commonly studied in a minimum of three biologically similar samples in order to adequately determine the biological variations in the proteome in veterinary studies (Westermeier et al., 2008). Even in larger numbers of patients, the probability of false positive and false negative results due to biological variation could occur when samples are pooled. For that reason, only proteins identified in all five samples were chosen for statistical evaluation and bioinformatic analysis to lower the inter-individual variation in this study (Sadiq et al., 2008; Kristensen et al., 2014). The main advantage of pooling samples is to find the main biomarkers differentiating each group of patients. Pooling samples from healthy and diseased groups reduces the sample size while maintaining a high degree of confidence in the data; however, it also eliminates the estimation of inter-individual variation within each group and can mask outliers that can reduce the applicability of the biomarker upon validation (Orton and Douchette, 2013). Therefore, this study may be considered a pilot (initial) study using pools, and in a future study, these findings should be validated by individual sample analysis. Another limitation of our study is the use of a commercial rapid in-clinic ELISA (Anigen Rapid CaniV-4 Test Kits, Bionote) despite having sufficient sensitivity (Anaplasma: 88.5%, Lyme: 93%) and specificity (Anaplasma: 97.1%, Lyme: 98%) for the diagnosis of those two diseases. Besides, *Borrelia* antibodies were evaluated by an IFAT in this study (sensitivity, 90%; specificity, 98.6%). However, panellists of the ACVIM-updated consensus statement of Lyme borreliosis in dogs and cats did not recommend whole-cell ELISA, IFAT or Western blot testing as a result of possible cross-reactions with other spirochetal infections or IgM versus IgG antibody testing due to not showing acute illness before seroconversion in dogs (Littman et al, 2018). Therefore, in this study, a multiplex assay detecting OspC antibodies might be accepted as a more sensitive and reliable test indicating recent exposure or re-exposure to Lyme borreliosis in dogs. This study focused on changes in serum proteomes differentiating between healthy and diseased dogs; however, serum protein analysis could have been performed before and after treatment to monitor treatment efficiency.

Proteomics studies of VBD are important for supplying additional information for a better understanding of pathology and for the evaluation of the outcome of these diseases. This study showed that the levels of many proteins might be changed in anaplasmosis and Lyme disease, and determining the role of these proteins in many biological processes (acute phase response, immunological reactions, transport, oxidative stress, apoptosis, and calcium, iron and lipid metabolism) can supply benefits during diagnostic and therapeutic procedures for both.

CONFLICT OF INTEREST

None of the authors has any financial or personal relationships that could inappropriately influence the content of the paper.

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Case report
Κλινικό περιστατικό

A Case Report of *Echinococcus granulosus sensu stricto* (G1) in a Domestic Cat in Turkey

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ABSTRACT: *Echinococcus granulosus sensu lato* is a zoonotic helminth with a life cycle that include of definitive hosts (dogs and wild carnivores) and intermediate hosts (usually the even-toed ungulates, Artiodactyla). Intermediate hosts become infected by ingesting the parasite eggs in contaminated food and water.Accidental intermediate hosts acquire infection in a similar way as other intermediate hosts. A two-year-old female cat was presented to the Van (Turkey) Animal Care and Rehabilitation Center with abdominal tension. Multiple intraperitoneal vesicles, which were found to be *E. granulosus* (s.1.) metacestodes, were observed during the ultrasound imaging. Then, the animal was laparotomized. Phylogenetic analysis based the partial cytochrome c oxidase 1 (pcox1) mitochondrial gene region was performed on metacestode samples (hydatid cysts). The isolate was identified as sensu stricto genotype G1, which is most commonly found in Turkey.

Keywords: *Cat, Cystic echinococcosis, Echinococcus granulosus, Mitochondrial gene, Turkey*

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INTRODUCTION

Cystic echinococcosis (CE) is a neglected and zoonotic disease as it includes domestic animal species as definitive and intermediate hosts. This disease is caused by infection with *Echinococcus granulosus sensu lato* (s.l). *E.granulosus s.l* (Rudolphi, 1801) is classified under the kingdom Metazoa, phylum Platyhelminthes, class Cestoda, subclass Eucestoda, order Cyclophyllidea, family Taenidae and comprise different genotypes: *E. granulosussensu stricto* (genotypes G1-G3), *E.equinus* (G4), *E.ortleppi* (G5), *E.canadensis* (G6-G10) and *E. felidis* (lion strain) (Scott et al., 1997; Nakao et al., 2007; Thompson, 2008; Romig et al., 2015). A large number of reports on the incidence of *E. granulosus (s.l)* have displayed that G1 (common sheep strain) is the most common genotype in worldwide (Bonelli et al., 2018). Turkey is also located in the Mediterranean countries which are widespread of genotype G1-G3 (Cardona and Carmena, 2013). Especially, the incidence of CE in humans, sheep and cattle is more prevalence in Van, where the livestock industry is important (Oğuz and Değer, 2013; Sayır and Çobanoğlu, 2013).

The life cycle of the genus *Echinococcus* mainly occurs between domestic/wild carnivores and herbivorous animals, with humans and cats being accidental intermediate hosts involved in this cycle. Domestic and wild carnivores harbour the mature tapeworm in their small intestine. Intermediate hosts, usually herbivores, are infected by ingesting the parasite eggs which are spread in the stool of the definitive host. In a study on whether cats were final hosts or not, it was reported that *E. granulosus* was not fully adult worm (Konyaev et al., 2012). On the contrary, it was reported in a study that domesticated cats might be definitive hosts of *E. multilocularis* (Kapel et al., 2006). There are several case reports in which cats were intermediate hosts and which resulted in cystic echinococcosis (CE) disease (Burgu et al., 2004; Konyaev et al., 2012; Armua-Fernandez et al., 2014; Bonelli et al., 2018). This case report provides on *E. granulosus sensu stricto* (genotype G1) in a domestic cat in Turkey.

CASE HISTORY

Abdominal enlargement, loss of appetite, and loss of weight were determined in the physical examination of a 2-year old stray cat brought to the Van province . Animal Care and Rehabilitation Center, eastern Turkey, in January 2020 (Fig. 1C). It was observed in abdominal palpation that the cat had pain and multiple round masses were felt by hand. Ultrasound imaging showed an image characterized by an anechoic content that contained a multi-leaf structure and multiple intraperitoneal vesicles at different sizes (Fig. 1A) and limited by a hyperechoic edge. After laparotomy, nearly 100 hydatid cysts (measured 0.58 cm and 2.96 cm) were removed, most of which were secondary cysts (translucent and thin-walled) that were commonly found in the entire abdominal cavity (Fig. 1B). Also, one burst primary cyst was seen in liver. The cat was alive after the operation.

The fertility of hydatid cysts was determined by the microscopic presence of protoscolexs, confirming that the parasite was able to complete full development in cats (Fig. 2B). DNA isolation from protoscolex was performed using genomic DNA tissue kit (GeneJET Genomic DNA Purification Kit, Thermo Scientific, Waltham). Genomic DNA extracts from one cyst samples (average number of protoscolex was 80 in 100 µl cyst liquid) were subjected to the JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT -3′) and JB4.5 (5′- TAAAGAAAGAACAT AATGAAAATG -3′) specific primer PCR reactions amplifying the partial mitochondrial cox1 gene region approximately 450 bp of *E. granulosus* (Bowles et al., 1992). The reaction mixture was prepared at the final concentration of 25µl (Bowles et al., 1992). PCR was carried out in a final volume of 25 µL, containing 7.5 µL DNase- and RNase-free steril distilled water (Biobasic, Canada), 10 µL 5X MyTaq Reaction buffer, 1 µL of each primer (20 pmol), 5 µL of template DNA (100-200 ng), and 0.5 µL of Taq DNA polymerase (1.25 IU) (MBI Fermentas, Lithuania). The PCR conditions were as follows: 5 min at 94 °C (initial denaturation), 35 cycles of 30 s at 94 °C, 45 s at 50 °C, 35 s at 72 °C, and finally 10 min at 72 °C (final extension). The PCR products were separated on agarose gels (1.5 %), stained with ethidium bromide and visualized and photographed on an gel documentation system (Avegene, Taiwan). The positive sample was purified using a commercial purification kit (High Pure PCR Cleanup Micro Kit, Roche, Germany) before index analysis, and then, was subjected to capillary electrophoretic separation in a private laboratory (Sentebiolab, Ankara, Turkey) and index analysis of the products was carried out. Sequence chromatograms were checked and arranged using the BioEdit software (Hall, 1999). “Nucleotide BLAST” (National Center for Biotechnology Information, www.ncbi.nlmn.nih.gov/BLAST) analysis was applied to the final consensus sequences of the isolates in the GenBank database and similarity rates

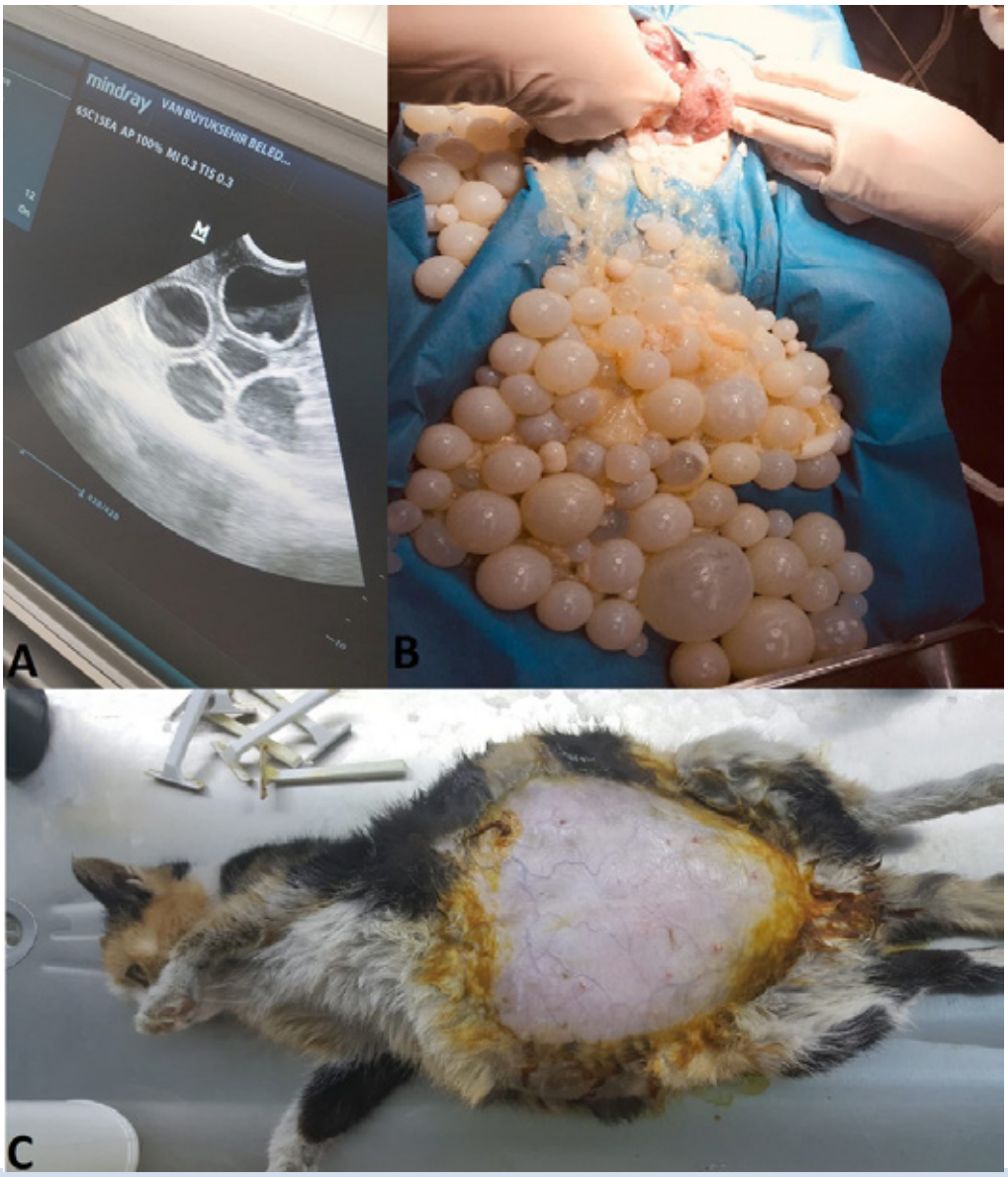

Figure 1. A: Ultrasound image of hydatid cysts, B: Hydatid cysts removed from the abdominal cavity, C: Abdominal distension due to peritoneal cystic echinococcosis

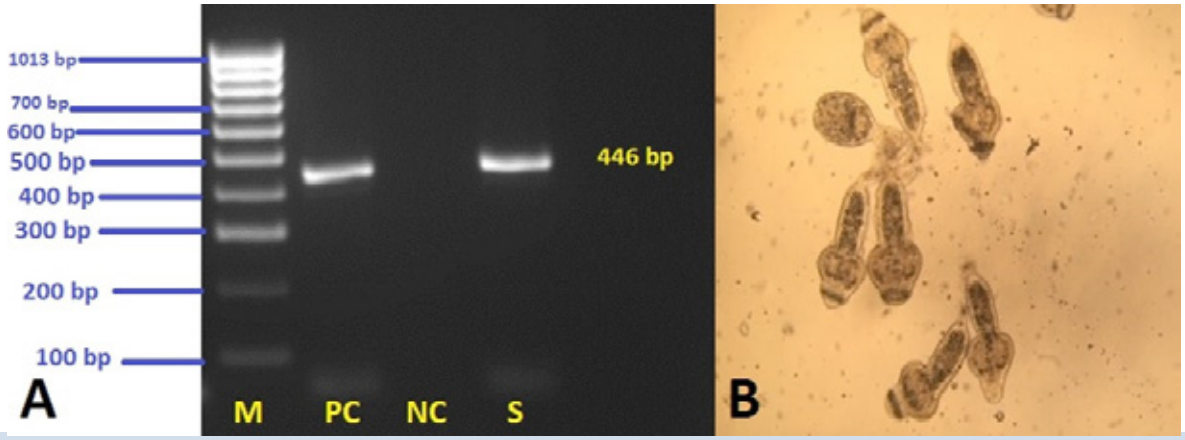

Figure 2. A: Gel electrophoresis from cox1-PCR amplification of *E. granulosus*. (M; molecular marker 100 bp DNA ladder (Hyper-Ladder, Bioline, London, United Kingdom), NC; negative control (no DNA), PC; positive control, S; specific product for *E. granulosus* isolated from cat cyst. B: Fertile cysts were indicated by the presence of protoscoleces

of the isolates were compared with the ones reported from different countries. The partial Cox 1 phylogenetic analysis data set was composed of nucleotide sequences of a total of 23 isolates. *Taenia multiceps* (AB792725) was used as the "outgroup." About a portion of 450 bp was used for phylogenetic analysis.

Phylogenetic analyses and tree creation were carried out using the "maximum likelihood" method with a 1000-iteration bootstrap in the MEGA 7.0 (Kumar et al., 2016) software. The nucleotide sequence obtained in the study was recorded in the GenBank as MN990735. *E. granulosus* positive control DNAs and negative control, which are available in our laboratory, were used for all processes. ~450 bp-bands expected for *Echinococcus granulosus* were successfully amplified (Fig. 2A). Phylogenetic analysis (Fig. 3) revealed that the sequence isolated from the cat belonged to the G1 cluster unlike the other genotypes and was %100 similar to common haplotypes (MG722980 and MF544127) that had been previously reported to be dominant in Europe and Turkey.

DISCUSSION

Cystic Echinococcosis (CE) is one of the most common zoonotic diseases and occurs in people who have close contact with livestock and dogs, especially in rural areas (Orsten et al., 2018). While the lifecycle of *Echinococcus granulosus sensu lato* is observed between dogs and sheep in Europe, Spain, Italy, former Yugoslavian countries, Greece, and Turkey according to the geographic distribution of the animals that are intermediate hosts; it is observed between dogs and horses in Western Europe and Ireland and

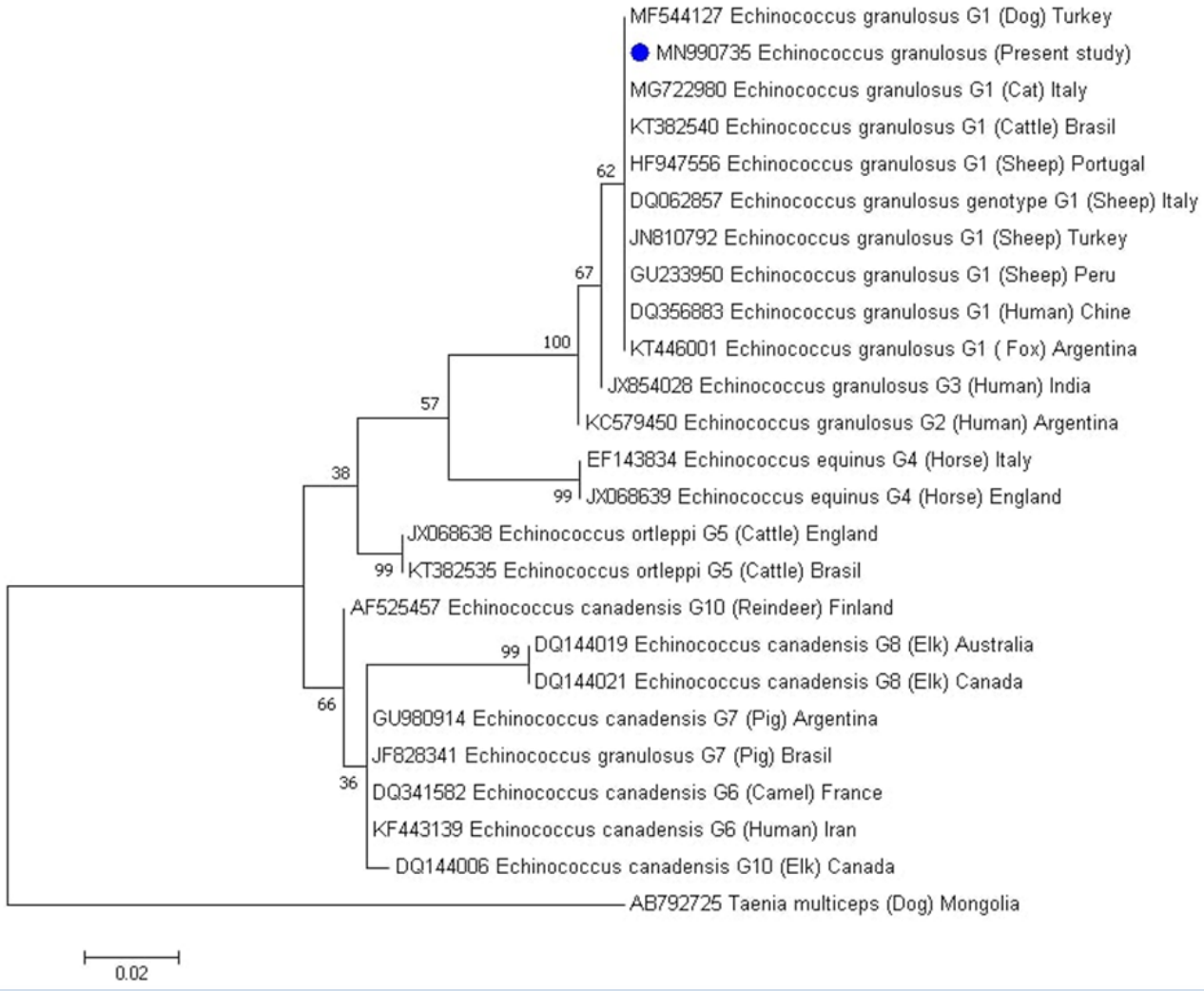

Figure 3. Phylogenetic analysis of *Echinococcus granulosus* cat isolate from Van, Turkey (Blue dot) and reference sequences for *E. granulosus* and *Taenia multiceps* as the outgroup. The relationships were inferred based on phylogenetic analysis of partial cox1sequence data using Maximum likelihood

mostly between dogs and cattle in Belgium, Germany, and Switzerland (Grosso et al., 2012; Otero-Abad and Torgerson, 2013; Ozcel et al., 2007). In the studies conducted in South Africa and Africa, wild cats were reported to be the definitive hosts of *E. felidis* and *E. oligarthrus* species (Lizardo-Daudt et al., 1993; Kapel et al., 2006). Studies on domestic cats proved that they are intermediate hosts and could not be definitive hosts. However, it was found for *E. multilocularis* that adult tapeworm that could lay eggs at low rates could be formed (Lizardo-Daudt et al., 1993; Kapel et al., 2006).

Some researchers could not detect primary cysts or any scars in cats with cystic echinococcosis during operations (Von der Ahe, 1967; McDonald and Campbell,1993). Also, Armua-Fernandez et al. (2014) claim that cysts that are observed in the free hydatid cyst during operations are secondary cysts. They stated that the reason for this was that primary cysts burst during climbing and jumping of cats and in this way, protoscoleces in primary cysts produce secondary cysts. In our case, a burst primary cyst was observed in the liver of the cat, and similarly, we think that secondary cysts that were detected were formed in consequence of the bursting of this cyst. We did not carry out any morphological examination of secondary cysts related to the cyst wall (such as cuticular or germinal layer).

The diagnosis of cystic echinococcosis rests mostly on imaging (ultrasound images) for humans (Higuita et al., 2016).However, cat ultrasounds have been in more use throughout the veterinary medical community for many decades.Ultrasounds use sound waves to examine and photograph internal organs in real time. To determine whether the cat infected with cystic echinococcosis (CE), the cat's abdominal region was analyzed for hydatids of large dimension by Bonelli et al., (2018).The study demonstrated that multiple intraperitoneal vesicles of different dimensions and anechoic content by ultrasound imaging . According to the results of our study, such imaging systems the can be used to determine free hydatid larvae.

Until now, G1, G3, G4, G5, G6 and G7 genotypes have been isolated in Turkey (Utuk and Simsek, 2008; Simsek et al., 2011; Oguz et al., 2018; Avcioglu et al., 2021). G1 is the dominant strain also in Turkey, and in consequence of the DNA sequence analyses of the mitochondrial cox1 regions of the isolates obtained from sheep, goats, cattle, camels, humans, and dogs, G1 strain was identified in all of these hosts (Utuk and Simsek, 2008). In all of the case reports in which domestic cats are intermediate hosts in Russia, Italy, and Uruguay, domestic sheep strain of *E. granulosus*, i.e., genotype G1 was found. The fact that genotype G1 was found in also our study shows again that we are in accord with the literature in both Turkey and the rest of the world and it is the most common strain. Also, genotype G1 of *E. granulosus* was reported in a study on dogs in the Van province of Turkey, where coincides with this case report (Oguz et al., 2018).

In conclusion, this report of a clinical case of cystic echinococcosis in a dometic cat. Molecular analysis suggest that sequence isolated from the cat is 100% identical to the common haplotype (MG722980 and MF544127) previously reported as the *E. granulosus* G1 genotype.Cystic echinococcosis still continues to be a major public health problem and environmental contamination.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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