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Research Article

The Neuroprotective Effect of Pioglitazone on NB2a Mouse Neuroblastoma Cell Culture

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Abstract

Pioglitazone (PGT) is a PPAR-γ activator that has neuroprotective properties via different mechanisms. It is thought to be neuroprotective in both acute and chronic use. Chlorpyrifos (CPS) is an Organophosphate insecticide that leads to attention deficit and cognitive problems in children and its neurotoxic effects are well known. This study aims to investigate the neuroprotective effects of PGT on CPS neurotoxicity in NB2a cell in the culture medium. We investigated the cell viability and proliferation using MTT assay and the percentage of neurite inhibition was analysed by measuring neurite outgrowth. Apoptosis was evaluated using the apoptotic index in TUNEL staining. Cell proliferation was found to be significantly reduced by CPS (25 µM), and this concentration-based reduction was prevented by PGT. Neurite outgrowth was inhibited by CPS (25 µM), whereas PGT significantly reversed neurite inhibition at and above 10 µM concentrations. The apoptotic index, which was increased using CPS (25 µM), was observed to reduce using PGT, depending on the concentration. Organophosphate is harmful to human health, and to our knowledge, there is no treatment. In individuals exposed to chlorpyrifos toxicity, acute toxic effects on neurons may be prevented or treated by PGT.

Keywords: *NB2a, Neurotoxicity, Pioglitazone, Neuroprotective, Neurite outgrowth, Apoptosis, Chlorpyrifos*

Pioglitazonun NB2a Fare Nöroblastoma Hücre Kültürüdeki Nöroprotektif Etkisi

Öz

Pioglitazon (PGT), farklı mekanizmalar yoluyla nöroprotektif özelliklere sahip bir PPAR-γ aktivatörüdür. PGT akut ve kronik kullanımının nöroprotektif olduğu düşünülmektedir. Klorpirifos (CPS) çocuklarda dikkat eksikliği ve bilişsel problemlere yol açan bir organofosfat insektisit olup nörotoksik etkileri iyi bilinmektedir. Bu çalışmada, kültür ortamında fare kaynaklı NB2a kanser dizin hücrelerinde CPS nörotoksisitesi üzerine PGT'nin nöroprotektif etkileri olup olmadığının araştırdık. MTT analizini kullanarak hücre proliferasyonu üzerine etkilerini ve ılımlı nörotoksik etkinin bir göstergesi olan Nörotoksisite Tarama Testi ile % nörit inhibisyonu incelendi. Apopitoz, TUNEL boyamada apopitotik indeks kullanılarak değerlendirildi. CPS (25 µM) konsantrasyonda uygulandığında NB2a hücre proliferasyonu azalttı. PGT ise konsantrasyon bağlı olarak klorpirosa bağlı azalmayı önlendi. Klorpirifon 25 µM konsantrasyonda tama yakın Nörit uzamasını inhibe ederken, PGT 10 µM ve üzerindeki konsantrasyonlarda nörit inhibisyonunu önemli ölçüde engelledi. CFS (25 µM) ile artan apopitotik hücre sayısını, PGT 10 uM konsantarasyondan itibaren anlamlı düzeyde azalttığı görüldü. Organofosfatlar insan sağlığına zararlıdır ve toksik etkilerini önlemek için bildiğimiz bir tedavisi yoktur. Klorpirifos toksisitesine maruz kalan bireylerde, nöronlar üzerindeki akut toksik etkileri PGT ile önlenebilir veya tedavi edilebilir.

Anahtar sözcükler: *NB2a, Nörotoksisite, Pioglitazone, Nöroprotektif etki, Nörotoksisite tarama testi, Apoptioz, Klorpirifos*

INTRODUCTION

People may be exposed to toxic effects of many chemicals throughout their lives. Some of these chemicals have a neurotoxic effect. Although neurons are highly developed cells, they cannot protect themselves and regenerate in case of damage [1].

Some of them have a neurotoxic effect. Although neurons are highly developed cells, they cannot protect themselves and to be regenerated in case of damage [1].

Chlorpyrifos (CPS: chlorpyrifos, O, O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothionate, O, O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothionate, chlor-

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pyrifos-ethyl) is one of the most extensively used organophosphate (OP) insecticides. CPSwas introduced into the market in the 1960s and is still used widely in agriculture and the home. Following its entry into the body, CPS is metabolically converted into its oxygen or oxon analogue, in which theSulphur of its P=S group is replaced by oxygen. This biotransformation reaction is carried out mainly in the liver by the cytochrome P450 (CYP)-dependent monooxygenase system. CPS activation to CPS-oxon is the main cause of moderate acute toxicity in mammals.CPS, have neurotoxic effects in susceptible species and cause neurodegenerative changes in central and peripheral nerves [2]. Therefore, we decided to use this compound to produce moderate acute toxicity.

Thiazolidinedione group drugs used in the treatment of diabetes act by increasing insulin response in the target cell. Cellular effects occur via the activation of peroxisome proliferator-activated receptors (PPARs) [3]. Activation of PPARγ is known to regulate inflammatory responses.and reduce the expression of many proinflammatory genes (COX-2, iNOS and cytokines), and it is also associated with inflammation in neurodegeneration [4]. Pioglitazone (PGT), a member of this group, is thought to have neuron-protective property through several mechanisms by increasing the gene transcription with the agonistic effect of PPARγ. However, it is not known whether it has a protective effect against neuronal damage in acute and long-term use [5]. PGT is known to have antidiabetic and adverse effects on the heart [6,7]. One of the findings of PGT use is that the medication may have a neuroprotective effect [3].

The differentiation of neurons in culture (evidenced by neurite outgrowth) is a physiological process that is a general indicator of cellular well-being. Its measurement, therefore, provides a useful in vitro model for the assessment of neurotoxicity and has been successfully used to demonstrate the neurotoxic potential of a wide range of agents, including excitatory amino acids. Neurite outgrowth is a specific structural end-point unique to the nervous system and depends upon a number of critical cellular processes, such as axonal transport. The inhibition of neurite outgrowth is only one marker of neurotoxicity that involves differentiating cells; thus, it may be of more relevant to exposure of the developing nervous system, rather than the mature nervous system. The mouse NB2a neuroblastoma cell has been determined to be a sensitive predictor of neurotoxicity, and its relative ease of culture and reproducibility suggested it was suitable for further use and development of experiments involves the assessment of interactions [8]. NB2a is a cell line and is frequently used in the examination of neurotoxic effects on the neuronal cells. The neurotoxic effect of the drugs is evaluated by % inhibition of neurite. If the damage is moderate, the nerve cell withdraws the neurite outgrowth [9].

The aim of this study is to show neuroprotective property of PGT on the OP toxicity. In the damage caused by CPS, the neuroprotective effect created by PGT is an important condition, whichcould be used clinically. The findings will also contribute to understanding the behavioural mechanisms of the nerve cell in pathological conditions.

MATERIAL and METHODS

Ethical Approval

Ethical Approval was given by the Medical Ethics Committee of Manisa Celal Bayar University (No: 10.05.2018 - 20.478.480).

Materials

Mouse NB2a neuroblastoma cells were provided from. European Collection of Cell Cultures (ECACC) (cell line: 89121404). All the chemicals used in the experiment were obtained from Sigma (St. Louis, MO, USA). Tissue culture flasks and culture plates were supplied from Falcon/ Fred Baker (Runcorn, Cheshire, UK) and gentamicin was provided from I. Ethem (Genta® 20 mg ampul, I. Ethem, Istanbul, Turkey). In all experiments, the solvent was evaluated for its effect on culture cells. PGT was dissolved in ethyl alcohol at 1/5 (0.2%) final concentration and the cells were incubated for a further 24 h.

Cell Culture

Neuroblastoma cells were proliferated in culture flasks with high glucose Dulbecco's Modified Eagle Medium (DMEM) with Glutamax-1, containing 5% fetal calf serum, 5% horse serum, 1% penicillin/streptomycin solutions (10000 U/10 mg) and 25 µg/mL gentamicin within the incubator humidified with 37°Cand 5% CO_2.

Cell Viability

The MTT assay, reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product, was used to estimate cell viability and growth. PGT at concentrations of 1, 3, 10 and 30 µM and CPS at 25 µM concentrations for measurement. Cell suspensions were first prepared at densities of 5×10^4/mL cells per each well of 96-well culture dishes and plated in triplicate for each concentration. Medium (100 µL) without PGT was used as a positive control, and only medium which did not contain any cells and PGT was used as a negative control.

Cells were treated with the concentrations previously mentioned for 24 and 48 h. Cells were incubated in humidified 5% CO_2 (in air) at 37°C with MTT in the last 4 h of the culture period tested. The medium was then decanted and 200 µL dimethylsulfoxide (DMSO, Sigma-Aldrich) was added to each well to ensure dissolving of the formazan salts. The absorbance was immediately determined at 570 nm in an UV-visible spectrophotometer multiplate reader (Versa Max, Molecular Device, Sunnyvale, CA) [10].

Measurement of Neurite Outgrowth

In order to measure neurite outgrowth, NB2a cells were plated in the proliferation medium on to 24 well culture plates at a cell density of 15.000 cells/mL. Twenty-four hours after, the cells were induced to differentiate and generate neurites in the presence of the PGT with the following method: the culture medium within each well were poured and replaced with serum-free medium plus 0.5 mM dibutyryl cyclic AMP containing PGT at concentrations of 1, 3, 10 and 30 µM and CPS at 25 µM concentrations for measurement of neurite outgrowth [11]. Ethanol was added in 1/5 (0.2%) final concentration and the cells were incubated for a further 24 h. Fixed cells with 4% (w/v) formaldehyde.in phosphate buffered saline (PBS) for 10 min at temperature of 24°C, then were stained for 3 min with Coomassie. Blue cell stain (0.6% [w/v] Coomassie Brilliant Blue G in 10% [v/v] acetic. acid, 10% [v/v] methanol, and 80% [v/v] PBS), washed with PBS. Three blinded observers took photograph of samples by using the Olympus BX-40 (Olympus, Tokyo, Japan) light microscope with a video camera (JVC-TK-C 601, Tokyo, Japan) for digital imaging. Image analyses were made by Image-Pro Plus image analyser (5.1.259, Bioscience Technology, Bethesda, MD, USA). Than, for drug and control, 10 different fields with approximately 10 cells were selected. A software routine was written using the functions of the image analyser to enable the automatic measurement of the total length of neurites (in pixels) for the cells in a given field and to express the results as the average.length of neurites per cell [11].

TUNEL Assay

Terminal deoxynucleotidyl transferase-biotin nick end-labelling (TUNEL) using the DeadEnd™ Colorimetric TUNEL system (Promega, Madison, WI, USA) were used to detect apoptotic cells. After application, cells were fixed in 4% paraformaldehyde for 30 min and rinsed three times in PBS for 5 min. After then cells were incubated with 20 µg/mL Proteinase K. for 10 min and washed three times again in PBS for 5 min. For endogenous activity inhibition, cells were treated with 3% hydrogen peroxide and rinsed in PBS. Afterward, cells which were treated with equilibration buffer for 5 min incubated with Tdt-enzyme for 60 at 37°C than were proceeded with 2×SCC solution for 15 min and then washed three times in PBS for 5 min. Streptavidin-peroxidase procedure was performed for 45 min, after which cells were rinsed in PBS and incubated with DAB; Mayer's hematoxylin was performed for counterstaining. Cells were than rinsed in distilled water and mounted in the mounting medium. TUNEL-positive staining was evaluated by the blinded observer under an Olympus BX40 light microscope [12]. Percentage and intensity of the immunostaining were scored with H-scoring and showed as the ratio of positively labelled cells to all cells in the chosen fields. An immunohistochemical score (HSCORE). was calculated as the sum of the percentages of positively stained epithelial cells multiplied by the weighted intensity of staining: HSCORE = $\sum Pi\ (I + 1)$, where "*I*" represents staining intensity (0=no expression, 1=mild, 2=moderate, and 3 = intense) and "*Pi*" is the percentage of stained cells for each intensity.

Apoptotic Index

Apoptotic index was defined as the ratio of positively. labelled cells to all cells in selected fields. For TUNEL staining, each section was.counted for 100 TUNEL-positive cells from randomly chosen fields. The percentage of apoptotic cells were also checked out by a blinded observer as 0: no apoptosis, 1: 1%-10% apoptosis, 2:11%-25% apoptosis, 3: 26%-50% apoptosis, 4: 51%-75% apoptosis, and 5: more. than 75% apoptosis [12]. The apoptotic index was counted up as the percentage.of apoptotic cells relative to the total cell number.

Statistical Analysis

The results were analysed.usingGraphPad (GraphPad. Software, SanDiego, CA, USA) using one-way ANOVA with Tukey.post hoc testing and presented as mean ± SEM. Statistical significance was defined as $P \leq 0.05$ or $P \leq 0.001$.

RESULTS

Effect of Pioglitazone on NB2a Mouse Neuroblastoma Cells

NB2a cells were taken into the culture medium and left to proliferate. Then, we waited for further 24 h for the neurite outgrowth in the differentiation medium, and the cells were stained with Coomassie Blue *(Fig. 1)*. In the cell proliferation phase, PGT was applied, and analysis of neurotoxic effects was performed through MTT assay *(Fig. 2)*. PGT was left for neurite inhibition for 24 h after the differentiation *(Fig. 3)*. PGT did not show any neurotoxic effects nor caused neurite inhibition ($P > 0.05$).

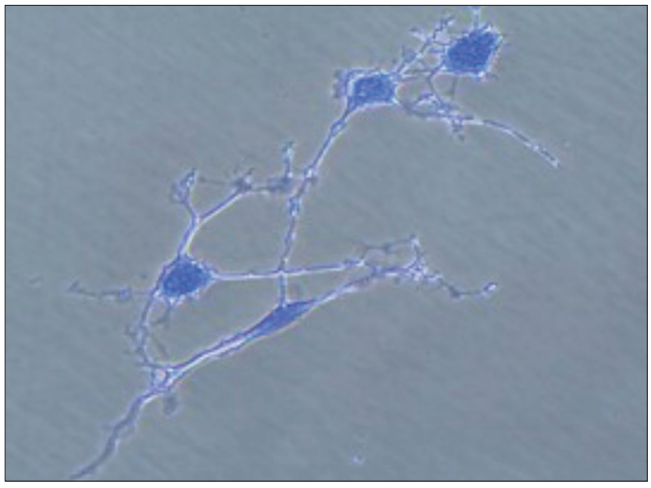


Fig 1. Differentiation NB2a cell to neurons by adding d-cAMP to serum free medium (Coomassie Blue stain, X400)

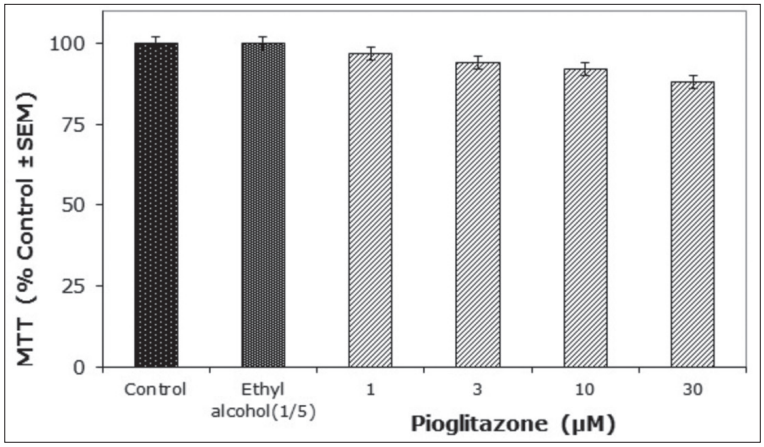
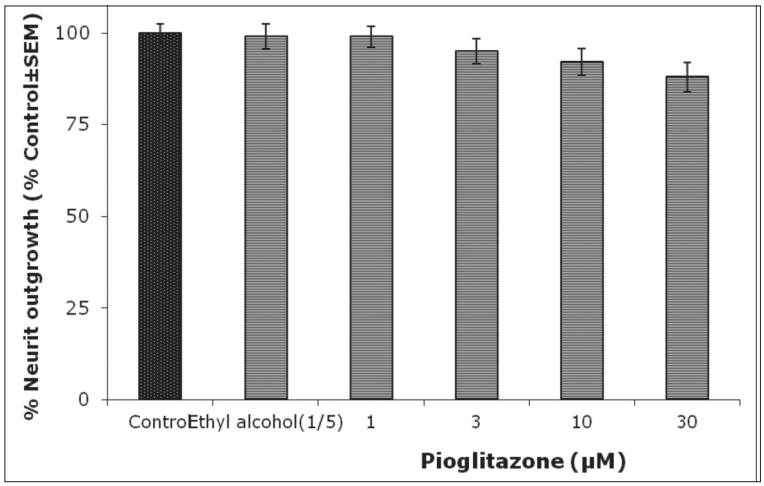


Fig 2. MTT measurements with different concentrations of PGT in NB2a cell culture Results are expressed as mean percentage compared the controls ± SEM (P>0.05)

Fig 3. The effects of PGT at different concentrations the neurite outgrowth measured by image analysis. Results are expressed as mean percentage compared the controls ± SEM (P>0.05)



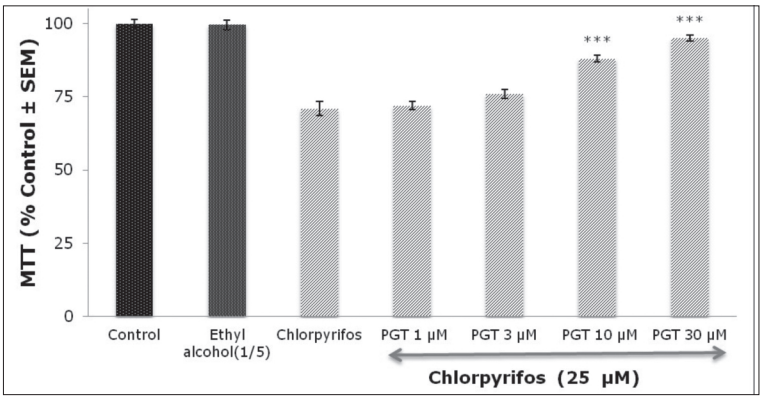


Fig 4. The effect of Chlorpyrifos administration (25 µM) with PGT at different concentrations on NB2a cells on MTT metabolism. Results are expressed as mean percentage compared the controls ± SEM (*** P<0.001)

The Neuroprotective Effect of Pioglitazone on Chlorpyrifos Induced Neurotoxicity in NB2a Cells

CPS was found to reduce the cell viability and proliferation, based on to the MTT assay results. PGT began to prevent the CPS-dependent reduction in cell proliferation at and above 10 µM concentration in the MTT assay (P<0.001) *(Fig. 4)*. PGT started to prevent the neurite outgrowth caused by CPS significantly 3 µM concentration (P<0.05) *(Fig. 5)*.

Pioglitazone is Protective Against Chlorpyrifos Induced Apoptosis in NB2a Cells

No significant difference was observed in the apoptotic cell index of PGT usage, which was analysed by TUNEL, compared to the control group of apoptosis. Depending on the dose, PGT was observed to reduce the apoptotic cell count, which was significantly increased (P<0.001) when CPS was added at a concentration of 25 µM in the culture *(Fig. 6,7)*.

DISCUSSION

OP is an important health problem, and there is not sufficient information about its chronic toxicity [13]. Information on possible chronic effects could be obtained only with the tests to be performed in the culture [14,15]. PGT is used in

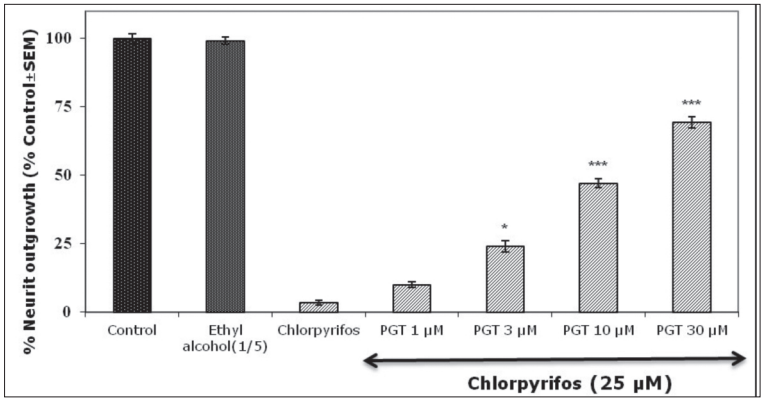
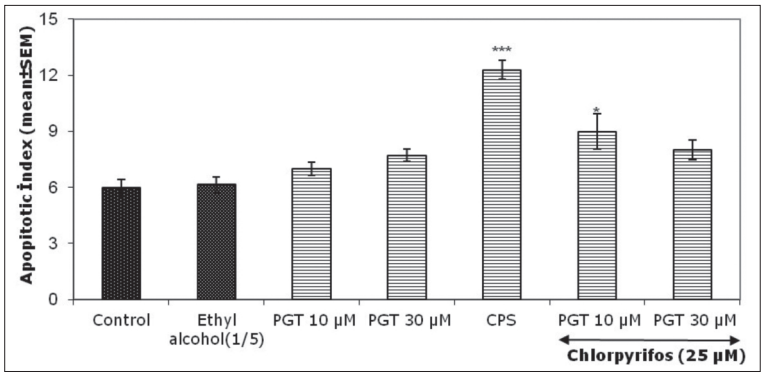


Fig 5. The effects of PGT at different concentrations on the neurite outgrowth measured by image analysis on Chlorpyrifos (25 µM) on NB2a cells. Results are expressed as mean percentage compared the Chlorpyrifos ± SEM (* P<0.05, *** P<0.001)

Fig 6. NB2a cells were observed to have quite healthy morphology with neurite outgrowths at 10 and 30 µM concentrations under PGT influence and without marked apoptosis. No statistically significant difference was observed between PG 10 and 30 µM concentrations (P>0.05), Chlorpyrifos (25 µM) produced significant apoptotic cells which were significantly (*** P<0.001) reduced by PGT at 30 µM concentrations compared to that of control



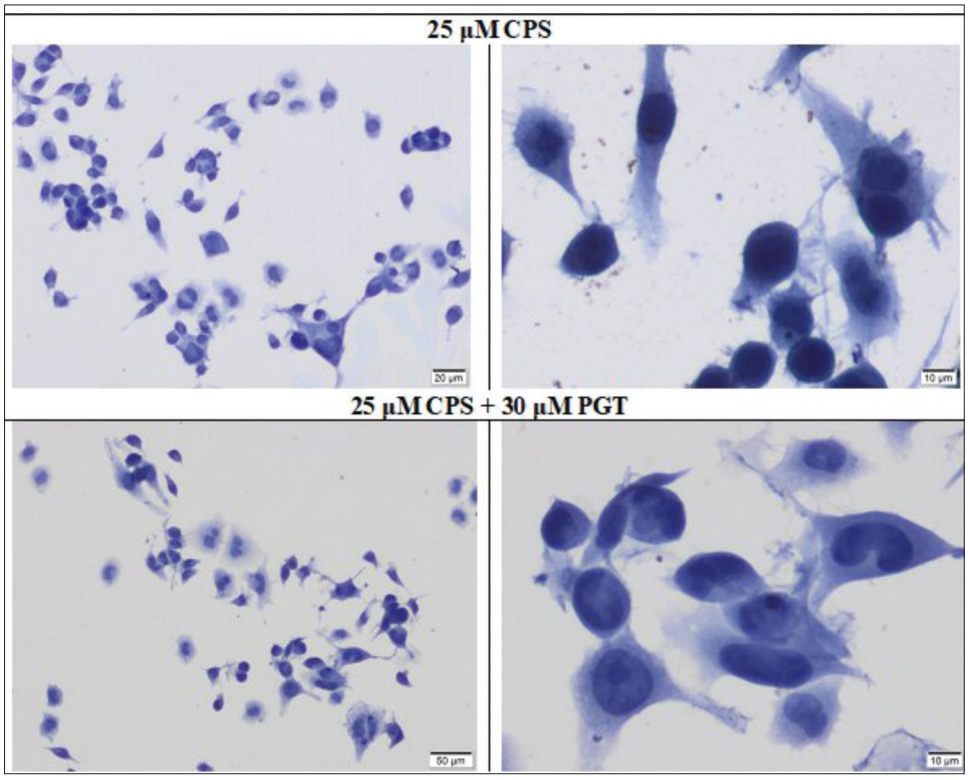


Fig 7. An increase in the number of apoptotic cells under the influence of Chlorpyrifos (25 µM) on NB2a cells was seen at low (x100) and high (x400) magnification and there was a significant decrease by PGT at 30 µM with similar magnification

the treatment of diabetes mellitus, and it is thought to have beneficial effects as well as neuroprotective effects, particularly in peripheral neuropathies, in patients with diabetes [16]. In the culture medium, OP toxicity on the neurons was shown with MTT assay. PGT showed neuroprotective effect against this toxicity. TUNEL showed that neurons underwent apoptosis in response to toxic effects, and the antiapoptotic effect was detected for PGT. MTT assay revealed that CPS provided neurite inhibition, as well as apoptosis by toxic effect, and it was blocked

by PGT. Protective effects of PGT treatment against antiproliferative, antiapoptotic and neurite retraction were revealed for the neurons in culture.

If the cell is exposed to a toxic effect, degeneration will started due to the synapse deterioration as a result of perineurium being directly affected or loss of trophic factors. The damage is determined by the severity, duration and persistence of the toxicity. Degeneration process may occur quickly or slowly depending on the mechanism. The delayed neurotoxic effect can be shown by measuring the enzyme level of neuropathy target esterase (NTE) in a culture medium [17-19]. İn our results we didn't see any toxic effect.

Neurite outgrowth, which is the primary function.of neurons *in vitro* medium, is associated with critical cellular events, such as axonal transport. The neurite outgrowth depends on specific structural elements such as the neurite-like microtubule binding protein and neurofilament protein. Neurite outgrowth can be inhibited by biological, chemical and environmental toxic substances. The neurite-developing factor, neurotropic factor and glial maturation factor have roles in the process of neurite outgrowth. Thus, monitoring of neurite outgrowth could be used to investigate the neurotoxic activities of the new molecules [11]. The mechanism by which OP reduced neurite outgrowth, whether alone or in combination is their ability to interact with acetylcholinesterase might play a role, as acetylcholinesterase has a trophic role influence in developing cells. However, this role is unrelated to the inhibition of hydrolysis of acetylcholine and therefore the relative potencies of the OP to inhibit neurite outgrowth would not necessarily be related to their short-term toxicities [15]. Our results showed that PGT did not show any significant toxic effect to our cells.

Some OP compounds, such as CPS, have neurotoxic effects in susceptible species and cause neurodegenerative changes in peripheral nerves. Accordingly, they cause organo-phosphate-induced delayed neurotoxicity. Typically, this syndrome begins with impaired coordination one or a few weeks after exposure to organic phosphorous.compounds and can progress to full paralysis of the hind legs. This condition tends to affect the distal parts of the long axons of nerve cells and manifests itself with a Wallerian-like degeneration. It is believed that there is a relationship between NTE inhibition and organophosphate-induced delayed neuropathy [20].

The main determinants in alcohol-induced neurotoxicity are mechanisms which play roles in excitotoxicity and neuroinflammation, and the protective effects of PPARy agonist seem to be associated with the inhibition of pro-inflammatory cytokines [21]. In the MPTP mouse model of Parkinson's disease, PGT reduces neuronal damage through a mechanism that involves the inhibition of the MAO-B enzyme [22]. In several studies, PPARy activation has been shown to lead to growth inhibition, apoptosis, and differentiation of a number of tumor cells [23,24].

The primary effect of thiazolidinedione (TZD) agents is to decrease the peripheral insulin resistance. They show their primary effects by activating specific receptors called PPARs. It has three subtypes: PPARα, PPARβ (δ) and PPARγ. Antidiabetic effects of TZDs have been found to be closely related to the ability to bind to and activate the PPARγ [4]. TZDs have effects on the vascular.system. Most of their vasculoprotective effects are independent from their effect on relieving the insulin resistance or antihyperglycemic effects [25]. A common function of PPAR subtypes is the suppression of oxidative stress and inflammatory processes. In this way, PPAR agonists have been shown to have neuroprotective effects in various disorders of the central nervous system [26,27]. We showed that PGT may protect the cells from toxic effect of CPS.

AT1 receptor blockage activates the nuclear receptor PPARγ, which is an important neuroprotective system. Telmisartan or losartan are thought to exert neuroprotective effects by PPARγ -activations [28,29]. Telmisartan has a neuroprotective effect in apoptosis through AT1 receptor blockage and PPARγ activation [29]. Additionally, PPARγ activation may promote neuroprotection against glutamate-mediated neurotoxicity and may also reduce neuronal damage in neurodegenerative diseases [27].

The modulation of PPARγ activity and peroxisomal function for PGT was found to relieve the NO, hydrogen peroxide-mediated neuronal damage and axonal damage. It has been suggested that PGT is a new therapeutic approach for the neurodegenerative changes associated with neuro-inflammation [30]. In the MPTP-induced rodent model of Parkinson's disease, the possible neuroprotective effect of fenofibrate and PGT has been shown to be mediated by NF-κB activation, which plays a role in neuroinflammation. MPTP treatment has been shown to activate caspase-3, resulting in apoptosis-associated neuronal death. On the other hand, treatment with neuroprotective drugs has been shown to inhibit caspase-3 activity and reduce neuronal damage [5]. PGT has been shown to be neuroprotective against decreased locomotion and rearing frequencies and to reverse hypolocomotion following intranigral infusion of MPTP. Administration of PGT at a dose of 30 mg/kg has been shown to elicit a partial neuroprotective effect against the neurotoxic effect of MPTP [31]. These results and mechanisms also support our results for neuroprotection of PGT.

Lee Et al reported that CPS attenuated PPAR-γ expression and NF-κB played a proapoptotic role in CPS-induced neuroblastoma cells death [32]. PGT is PPAR-γ agonist which has also neuroprotective effects and is able to inhibit the proinflammatory factors. These anti-inflammatory effects might.be involved in preventing the neuronal cell death caused by CPS.

AMP-activated protein kinase (AMPK) regulates signalling pathways related to cell survival and apoptosis and plays a role in increasing hippocampal neurogenesis [33]. It has been argued that AMPK activation directly inhibits β-amyloid accumulation *in vitro* [34,35]. PGT, by AMPK induction, has been shown.to improve neuronal apoptosis and β-amyloid accumulation in monosodium glutamate (MSG) neurotoxicity, which develops secondary to AMPK depletion by [36]. Our TUNEL results were similar with these studies.

Intracellular antioxidant activity caused by TZDs is important and responsible for some of the cellular protective effects. They do not have a direct antioxidant effect on free radicals. However, they exhibit protective effects by blocking the mechanism involved in the formation of the several hyperglycemic conditions causing oxidative stress [37,38]. In a study performed on hippocampal neurons, Calcium channel functions of which were improved in the culture medium, were shown to live.much longer than known. TZDs modulate Calcium dependent pathways in the brain and have different inhibitory profiles on two major Calcium sources, potentially conferring neuroprotection to an area of the brain that is particularly vulnerable to the effects of ageing and/or Alzheimer's disease. TZD may have potential applications in conditions associated with impaired learning and memory [39]. Neuroprotective effect of PGT has been.observed at doses in the range of 5-10 uM. This range is comparable.to plasma concentration of PGT when it is used in humans [40]. These studies also support for the effect of PGT which could be use for similar disease.

In vitro methods are known to be effective in demonstrating in-vivo neurotoxicity. This property can also be used to determine if an agent may be protective and preventive in case of acute damage. Neurite inhibition is an important indicator that could provide information about the long-term consequences of moderate toxicity-related damages. MTT, on the other hand, indicates severe toxic effects on cells. Using these methods, we found a significant protective effect of PGT in CPS-induced neurotoxicity and that apoptosis was involved in this damage, which can be alleviated by PGT. Absence of an adverse effect of PGT in culture environment and presence of neuroprotective effects highlights the efficacy and safety of PGT in the clinical use for many disease.

Conflict of Interest

The authors declare no conflict of interest

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Research Article

DGAT1, CAST and IGF-I Gene Polymorphisms in Akkaraman Lambs and Their Effects on Live Weights up to Weaning Age

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Abstract

Early live weight in sheep is important for lamb survival and average weight gain until slaughter. The aim of the present study was to investigate association between *CAST-Msp*I, *DGAT1-Alu*I and *IGF-1-Bsp*143II polymorphisms and early live weights between birth and weaning age in Akkaraman lambs. A total of 374 lambs were genotyped for *CAST-Msp*I, *DGAT1-Alu*I and *IGF-1-Bsp*143II polymorphisms by the polymerase chain reaction and restriction length polymorphism (PCR-RFLP) method. The results of PCR-RFLP analysis showed that the SNPs had three genotypes of *CAST-Msp*I polymorphism, two genotypes of *DGAT1-Alu*I polymorphism and one genotype of *IGF-1-Bsp*143II polymorphism of these, *CAST-Msp*I-MM, *DGAT1-Alu*I-CC and *IGF-1-Bsp*143II-BB were the predominant genotypes in the Akkaraman sheep breed. The result of Chi-square analysis indicated that the Akkaraman sheep breed was in Hardy-Weinberg equilibrium for the investigated polymorphic genes. At the *DGAT1* locus, the CT genotype showed significantly heavier birth weight (P=0.044) compared to CC genotype. *CAST* gene did not show any association for the investigated traits. The results of this study demonstrate that the CT genotype had a positive effect on birth weight in Akkaraman sheep. We concluded that further investigations are needed in *DGAT1-Alu*I polymorphism and live weight at different ages in sheep.

Keywords: *Birth weight, DGAT1, Polymorphism, RFLP, Sheep*

Akkaraman Kuzularında DGAT1, CAST ve IGF-I Gen Polimorfizmleri ve Sütten Kesim Yaşına Kadarki Canlı Ağırlık Artışı Üzerine Etkileri

Öz

Bu çalışmada, Akkaraman ırkı kuzularda, *CAST-Msp*I, *DGAT1-Alu*I ve *IGF-1-Bsp*143II polimorfizmleri ile bu polimorfizmler ve bazı canlı ağırlıklar arasındaki ilişkilerin araştırılması amaçlanmıştır. PCR-RFLP yöntemi ile toplam 374 kuzu *CAST-Msp*I, *DGAT1-Alu*I ve IGF-*1-Bsp*143II polimorfizmleri yönünden genotiplendirilmiştir. Akkaraman ırkı koyunların, *CAST-Msp*I ve *DGAT1-Alu*I polimorfizmleri yönünden polimorfik iken *IGF-1-Bsp*143II polimorfizmi yönünden monomorfik oldukları görülmüştür. Akkaraman ırkı koyunlarda *CAST-Msp*I-MM, *DGAT1-Alu*I-CC ve *IGF-1-Bsp*143II-BB genotiplerinin en yaygın genotipler olduğu görülmüştür. Ki-kare test sonuçlarına göre Akkaraman ırkı koyunların *CAST-Msp*I ve *DGAT1-Alu*I polimofizmleri yönünden Hardy-Weinberg (HW) dengesinde oldukları görülmüştür. *DGAT1* lokusu yönünden TC genotipli kuzular, diğer genotiplilerle karşılaştırıldığında bu genotipteki kuzuların daha yüksek doğum ağırlığına (P<0.05) sahip oldukları görülmüştür. Bu çalışma sonunda Akkaraman koyun ırkında TC genotipinin doğum ağırlığı üzerine pozitif etkisinin olduğu görülmüştür. Çalışma sonunda *DGAT1-Alu*I polimorfizminin farklı yaşlardaki kuzularda canlı ağırlıkla arasındaki ilişkilerin araştırıldığı çalışmaların planlanmasına ihtiyaç olduğu düşünülmüştür.

Anahtar sözcükler: *Doğum ağırlığı, DGAT1, Polimorfizm, RFLP, Koyun*

INTRODUCTION

The United Nations Food and Agriculture Organization (FAO) estimates that food demand in the coming 50 years will double globally [1]. Also, the global meat demand in 2030 is projected to be 68% more than that of 2000 [2]. Therefore, it is suggested that it will be advantageous to determine specific genes in order to choose animals

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bearing the desirable alleles to improve existing breeds or populations [3].

According to FAO data, Turkey ranks 7th globally in terms of total sheep population with a stock of approximately 31 million [4]. Of the current sheep population of Turkey, 45% consists of a fat-tailed breed called the Akkaraman, which is bred in the central Anatolia [5]. The Akkaraman breed meets most of the mutton demand in Turkey in spite of low-quality pasture and poor climatic conditions, to which the breed has adapted well [6]. Studies report lamb birth weights ranging from 3.81 kg [6] to 4.56 kg [7] in Akkaraman sheep breed. They also-demonstrate that the Akkaraman breed manifests considerable variations in terms of birth weight. Therefore, studies aimed at improving the lamb birth weights and growth characteristics will be important for this breed.

Quantitative genetic studies done on sheep demonstrated that some genetic factors have an effect on lamb birth weight. The heritability of lamb birth weight in diverse sheep breeds is reported to be between 0.15 and 0.24 [8]. Therefore, the application of genomic selection is considered as a potentially successful approach for the improvement of lamb birth weights [9]. Despite the minor effect on livestock breeding, some candidate genes to be used in the improvement of some polygenic characteristics such as growth have been reported to assist in the accurate estimation of the genetic value of different livestock species including sheep [10,11].

Diacylglycerol acyltransferase1 *(DGAT1),* is an enzyme, which takes part in the synthesis of triglycerides in adipocytes [12]. The *DGAT1* gene, encoding this enzyme is expressed in many tissues but predominantly in the adipose tissue and in the small intestine [13]. Studies have reported an association between the *DGAT1* gene and fat accumulation in sheep and cattle carcasses [14,15]. *DGAT1* gene has been found as a putative candidate gene for the milk fat content in sheep [16]. However, studies investigating the association between the SNPs in the *DGAT1* gene and mutton productivity are scarce. In one of these studies with the native Moghanian Iranian sheep breed, an association between the polymorphism in the 17th exon of the *DGAT1* gene and carcass weight was reported previously [17].

For livestock breeding, the calpastatin *(CAST)* gene has been stated to warrant attention in studies on improving live weight gain and meat quality [18]. The calpain-calpastatin system, consisting of three members, namely μ-calpain, m-calpain and calpastatin, takes part in many crucial processes in various tissues including muscle development [19,20]. Therefore, the *CAST* gene is considered to be a notable candidate gene for muscle growth and meat quality improvement in livestock breeding [21]. The *CAST* gene, located on the autosomal 5th chromosome of sheep, was reported to be associated with growth and live weight gain in a variety of sheep breeds [22].

The *IGF-I* protein, a member of the superfamily of insulin-like growth factor (IGF), is an important protein involved in the fertility, embryogenesis, and growth of mammalians [23,24]. In cattle [23], pigs [25] and goats [26], an association between the *IGF-I* gene and live weight gain was reported. However, studies examining the relationship between the *IGF-I* gene and live weight gain are relatively limited. *IGF-I* gene was proposed as a candidate gene for growth and meat yield traits in the Makui sheep breed [24].

To the best of our knowledge, there is no study on the association between the *CAST*, *DGAT1* and *IGF-1* genes and early live weight in Akkaraman sheep breed which is commonly reared in Turkey. The aim of the present study was to investigate association between *CAST-Msp*I, *DGAT1-Alu*I and *IGF-1-Bsp143*II polymorphisms and early live weight traits in the Akkaraman sheep breed.

MATERIAL and METHODS

Animals, Phenotypes and Genotyping

A total of 374 Akkaraman male lambs were used in this study. Phenotypes were recorded in the same farm. Lambs were born from the ewes those were in age two. The lambs were weighted at birth, and on the 30th, 60th and 90th day (which was classed as the weaning day) and blood samples were collected at 90th day from *Vena jugularis* in K_3EDTA tubes. All experimental procedures were performed according to the guidelines of the Local Ethics Committee for Animal Experiments at Erciyes University (13.11.2013 and #13/130). For the PCR processes, genomic DNA was obtained from whole blood by the phenol-chloroform extraction method.

PCR mixtures for the *CAST*, *DGAT1* and *IGF-1* genes were prepared as 25 μL volumes using 1.5 mM $MgCl_2$, 200 μM dNTPs, 200 μM primers (primers sequences in *Table 1)*, 1×PCR buffer, 1 U Taq polymerase and 50 ng genomic DNA. The PCR conditions for the *CAST* gene consisted of pre-denaturation (at 95ºC for 5 min), followed by 35 cycles consisting of denaturation at 95ºC for 1 min, annealing at 62ºC for 1 min, 72ºC for 2 min and post-elongation at 72ºC for 8 min. The PCR products (622 bp) were digested using 5 U of appropriate restriction enzyme. The PCR conditions for the *DGAT1* gene consisted of pre-denaturation (at 95ºC for 5 min), followed by 35 cycles consisting of denaturation at 95ºC for 30 sec, at 60ºC for 30 sec, at 72ºC for 30 sec. Finally, a post-elongation at 72ºC for 10 min was performed. The PCR products (309 bp) were digested using 5 U of appropriate restriction enzyme. The PCR condition for the *IGF-1* gene consisted of pre-denaturation (at 94ºC for 6 min), followed by 30 cycles consisting of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, at 72ºC for 30 sec. Finally post-elongation at 72ºC for 10 min was performed. The PCR products (294 bp) were digested using 5 U of appropriate restriction enzymes.

Table 1. *Primer sequences and restriction enzymes*

Gene	Primer Sequence	RE	Reference
CAST	F: 5′-TGG GGC CCA ATG ACG CCA TCG ATG-3′ R: 5′-GGT GGA GCA GCA CTT CTG ATC ACC-3′	*Msp*I	[19]
DGAT1	F: 5′-GCA TGT TCC GCC CTC TGG-3′ R: 5′-GGA GTC CAA CAC CCC TGA-3′	*Alu*I	[27]
IGF-1	F: 5′-TGA GGG GAG CCA ATT ACA AAG C-3′ R: 5′-CCG GGC ATG AAG ACA CAC ACA T-3′	*Bsp143*II	[28]

RE: Restriction enzymes

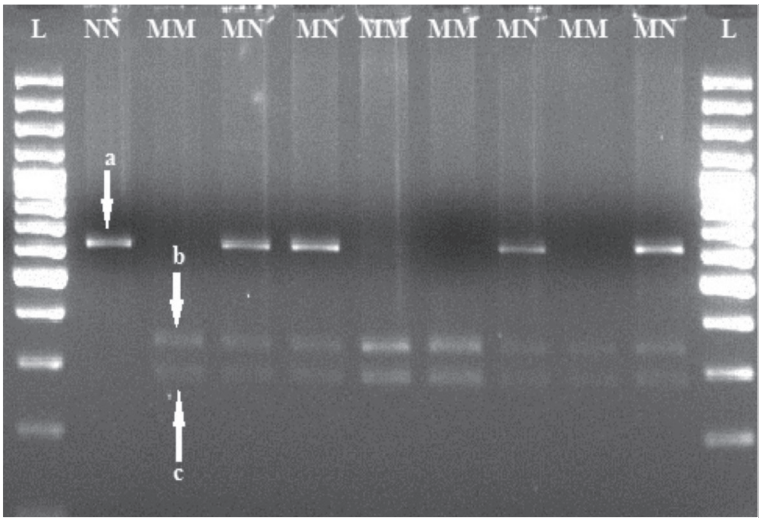

Fig 1. *CAST-Msp*I polymorphism pattern in 2% agarose gel electrophoresis (L; 100 bp DNA ladder; a: 622 bp band; b: 336 bp band; 286 bp band)

Statistical Analysis

Sheep phenotypic data were checked for normality before analyses with the UNIVARIATE procedure in SAS v9.0. (2004). Allele and genotype frequencies and Hardy-Weinberg equilibrium were calculated using the ALLELE procedure in SAS v9.0. (2004). Genotype-phenotype association was analysed with a generalized linear model using the GLM procedure in SAS v9.0 (2004). The effects of farm, feeding regime and age of ewes were not built into the linear model, because all animals were raised on the same farm therefore lambs did not expose to different environmental conditions. The reduced model included fixed effects of birth type (single or twin) and genotype. Genotypic comparisons were reported following Tukey-Kramer adjustment, and $P \leq 0.05$ was considered as significant.

The statistical model used as follows:

$$Y_{ij} = \mu + S_j + G_i + e_{ij}$$

Where Y_{ij} is the observation of the birth weight, 30th day, 60th day and 90th day live weight traits; μ is the overall mean for each trait, S_j is the fixed effect of j^{th} birth type, G_i is the fixed effect of i^{th} genotype for the relevant polymorphism and e_{ij} is the random residual error.

RESULTS

A 622 bp fragment for the *CAST* gene was amplified by PCR. After the digestion of PCR products with *Msp*I enzyme, three restriction patterns were obtained for the *CAST-Msp*I polymorphism. Two fragments (336 and 286 bp) were observed for the first pattern, which is called the MM genotype. For the second pattern, called the MN genotype, three fragments (622, 336 and 286 bp) were observed, while for the third pattern, only one fragment (622 bp) was observed for the NN genotype *(Fig. 1)*. The MM genotype frequency (0.81) was found to be the highest, while the NN genotype frequency (0.01) was the lowest. The M allele frequency (0.9) was higher than the N allele frequency *(Table 2)* and the examined Akkaraman population was found in Hardy-Weinberg equilibrium (HWE) for *CAST-Msp*I polymorphism *(Table 2)*.

A 309 bp fragment for the *DGAT1* gene was amplified, and PCR products were digested by *Alu*I enzyme. Two genotypes (CC and CT) were obtained for the *DGAT1-Alu*I polymorphism in the Akkaraman sheep breed in Turkey. Only one band (309 bp) was observed in the CC genotype. On the other hand, three bands (309, 272 and 37 bp) were expected in the CT genotype, but the 37 bp band could not be seen on 3% agarose gel electrophoresis because it was too small. However, two bands (309 and 272 bp) were found to be sufficient for genotyping of individuals *(Fig. 2)*. It was found that CC had the highest genotype frequency (0.91), whereas the TT genotype was not found in the investigated Akkaraman lambs. The population was found in HWE for the *DGAT1-Alu*I polymorphism *(Table 2)*.

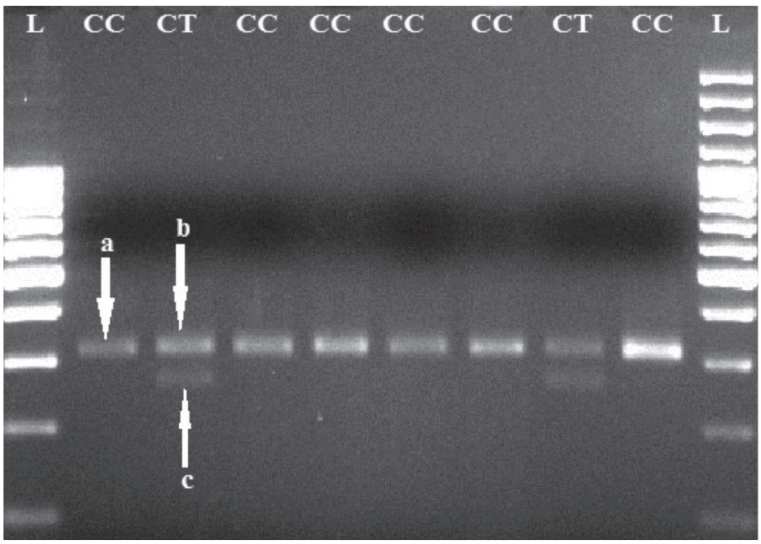

Fig 2. *DGAT1-Alu*I polymorphism pattern in 3% agarose gel electrophoresis (L; 100 bp DNA ladder; a: 309 bp band; b: 272 bp band)

Fig 3. *IGF-1-Bsp*143II polymorphism pattern in 3% agarose gel electrophoresis (L; 100 bp DNA ladder; a: 294 bp band)

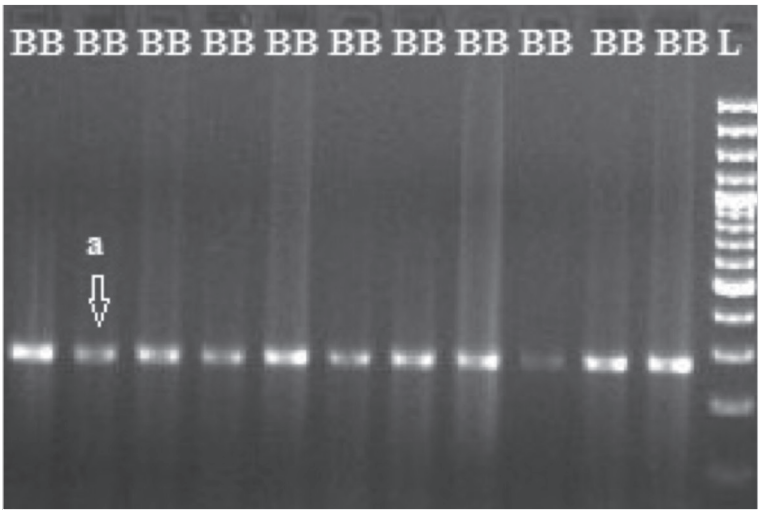

Table 2. *Allele and genotype frequencies of CAST, DGAT1 and IGF-1 in Akkaraman lambs*

Gene	n	Genotype						Allele Frequency		Chi-squared (df=1)
CAST	374	MM		MN		NN		M	N	X^2=0.93^{NS} P=0.3355
		Obs (Exp)	F	Obs (Exp)	F	Obs (Exp)	F	0.9	0.1	
		302 (303.66)	0.81	70 (66.68)	0.18	2 (3.66)	0.01			
DGAT	374	CC		CT		TT		C	T	X^2=0.75^{NS} P=0.3874
		Obs (Exp)	F	Obs (Exp)	F	Obs (Exp)	F	0.96	0.04	
		342 (342.68)	0.91	32 (30.63)	0.09	0 (0.68)	0			
IGF-1	374	AA		AB		BB		A	B	-
		Obs (Exp)	F	Obs (Exp)	F	Obs (Exp)	F	0	1	
		0	0	0	0	374	1			

Obs: *Observed genotype;* ***Exp:*** *Expected genotype;* ***F:*** *Frequency;* ***df:*** *degree of freedom;* ***X²:*** *Chi-square;* ***^{NS}:*** *Non-significant (P<0.01)*

A fragment of 294 bp was successfully amplified for the *IGF-1* gene and PCR products were digested with *Bsp*143II restriction enzyme. After digestion, only the B allele and BB genotype were found in the Akkaraman lambs *(Fig. 3)*.

Association analysis revealed that a significant difference was found among lambs with *DGAT1-Alu*I genotypes (CC and CT) in terms of birth weights (P=0.044). Lambs with *DGAT1-Alu*I-CT genotypes had higher birth weight compared to CC genotype individuals. On the other hand,

Table 3. *Means, standard errors of means (SEM), and statistical significance with CAST and DGAT1 genotypes for different age weight (kg) in Akkaraman lambs*

Gene	Genotype	Age Weight			
		Birth X±SEM	**30th day X±SEM**	**60th day X±SEM**	**90th day X±SEM**
CAST	MM	4.730±0.081	10.922±0.203	18.593±0.368	28.264±0.512
	MN	4.825±0.120	10.920±0.300	18.500±0.544	27.638±0.758
	NN	4.387±0.082	9.681±0.206	19.768±0.374	31.766±0.520
	P	**0.636**	**0.818**	**0.934**	**0.533**
DGAT1	CC	4.489±0.275	10.530±0.689	19.118±1.252	29.250±1.743
	TC	4.805±0.312	10.443±0.782	18.790±1.418	29.196±1.975
	P	**0.044**	**0.824**	**0.645**	**0.956**

no significant association was found between *CAST-Msp*I genotypes and early live weight traits in Akkaraman lambs *(Table 3)*.

DISCUSSION

Growth is a quantitative characteristic controlled by many genes. These genes can be linked to genetic markers and are segregated together [14]. An ideal marker is favourable if it is polymorphic, has a simple inheritance pattern and is easily identified [29]. This study investigated the relationship of *CAST-Msp*I, *DGAT1-Alu*I and *IGF-1-Bsp143*II polymorphisms, which are considered as potential markers for early live weight in sheep.

This study showed that genotype CC was the most common genotype (0.91) in terms of *DGAT1-Alu*I polymorphism and also revealed that the genotype TT was not present in Akkaraman lambs. The C allele frequency (0.96) was observed to be higher than that of T allele frequency, and the investigated samples were found to be in HW equilibrium for *DGAT1-Alu*I polymorphism. Similar to findings of this study, the genotype CC was reported to be the most common genotype in Imroz and Chios sheep breeds (0.68 and 0.52, respectively), which are bred in Turkey [30]. Additionally, while frequency of genotype CC was reported highest, genotype TT was found to be lowest in Indian [31], Iranian [32], Romanian [33], Chinese [27] and US [34] sheep breeds. Unlike our findings, there are a few studies reporting that the frequency of the TT genotype is higher than those of other genotypes [17].

The *DGAT1* gene has been extensively studied for the quality of meat in various livestock species. Studies examining the relationship of the *DGAT1* gene with characteristics of growth and live weight gain are relatively rare. One of these studies reported that individuals with *DGAT1-Alu*I-CC genotype had higher hot carcass weight and higher carcass yields than other genotypes in fat-tailed Lori-Bakhtiari sheep and in short-tailed Zel sheep bred in Iran [14]. It was reported that individuals with CC genotypes from the Moghani sheep breed in Iran were superior to those with other genotypes in terms of hot carcass weight and hot dressing percentage [17]. In the present study, it was found that lambs with CT genotypes had higher birth weights compared to other genotype in the examined animals (P=0.044). Birth weight is an important trait for the survival of lambs [35]. Lambs with birth weights of 4.36-4.77 kg were reported to have the maximum survival rates in the period from birth until weaning age [35].

In the current study genotype frequency of *CAST-Msp*I-MM genotype was found highest in Akkaraman sheep breed. *CAST-Msp*I-MM genotype frequencies was found also highest in other Turkish sheep breeds such as Akkaraman, Kıvırcık, Karacabey Merino, Imroz, İvesi and Çine Çaparı breeds, whereas, frequency of genotype MN was highest in the Sakız and Karya sheep breeds [36,37]. Similar to our findings *CAST-Msp*I-MM frequency was also found highest in different sheep breeds among the world [19,20,38-40].

Due to its role in the development of muscle cells, the *CAST* gene is considered to be an important candidate gene in monitoring the growth of livestock animals. The associations between *CAST* gene polymorphisms and live weights at different time points have been investigated. In lambs of the Romney breed raised in New Zealand, it was reported that there was a relationship the *CAST* gene and birth weight, but not with daily live weight gain [41]. A study reported the relationship between *CAST* genotypes and lamb birth weight and daily live weight gain [42]. In the Egyptian Barki sheep breed, an association between the *CAST* gene and fat free carcass yield was reported [43]. In the native Balkhi and Kajli sheep breeds in Pakistan, it was reported that individuals with the "MN" genotype were found to have more favourable daily live birth weights compared to those with other genotypes [22]. In the native Kıvırcık sheep breed in Turkey, individuals with the NN genotype were reported to have less favorable results in terms of daily live birth weight gains than those with different genotypes [36]. In the Russian breeds of the Soviet Merino and Salks, individuals with the MN genotype were reported to have more favorable results in terms of weaning weight and mean daily weight gain compared to

those with other genotypes [38]. However, there are studies reporting no association of the *CAST* gene with growth traits in diverse breeds of sheep [18,20]. In agreement with these findings, this study investigating the Turkish native Akkaraman breed demonstrated no relationship between *CAST-Msp*I polymorphism and birth weight or with weights at selected time points in the period after birth till weaning (day 90) in the examined samples.

The *IGF-I* gene has been reported to be a candidate gene for the growth traits of livestock animals [26]. Therefore, in this study, our aim was to investigate the relationship of the *IGF-1-Bsp*143II polymorphism with early live weight. However, unlike other genes, which were examined in this study, all of the 374 lambs of the Akkaraman breed in this study were found to have the monomorphic BB genotype in terms of the *IGF-1-Bsp*143II polymorphism. Therefore, the relationship of the *IGF-I* gene with weights at different time points could not be investigated. Unlike our study, it was reported that three different genotypes, namely AA, AB and BB, were determined in Han and Hu sheep breeds of China; AA and AB genotypes were present in the Texel breed and only AA genotype was present in the Dorset breed [28].

This is the first study investigating the *CAST-Msp*I, *DGAT1-Alu*I and *IGF-1-Bsp143*II polymorphisms altogether in Akkaraman sheep breed. It was determined that *CAST-Msp*I and *DGAT1-Alu*I polymorphisms are persistently present and are in HW equilibrium in the Akkaraman breed. It was also determined that the Akkaraman breed is monomorphic in terms of *Bsp143*II polymorphism. These results demonstrate that genetic variabilities persist in the Akkaraman breed. On the other hand, this study demonstrated the association between the *DGAT1-Alu*I polymorphism and birth weight in the Akkaraman sheep breed. However, it did not determine any association of the *CAST-Msp*I polymorphism with any of the investigated items in the study. Therefore, *DGAT1-Alu*I polymorphism is considered to be a potential molecular marker in the improvement of lamb birth weights in the Akkaraman sheep breed.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

Pharmacokinetic Studies of the Recombinant Bovine Interferon-alpha in Cattle

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Abstract

In order to evaluate the pharmacokinetics of recombinant bovine interferon-alpha (rBoIFN-α) in cattle, which has potential for its antiviral and immunomodulatory activities, 12 animals of 6-month age were classified into 4 groups (n=3) to receive rBoIFN-α through IV, IM or SC routes at a dose of 5.0×10^3 IU/kg. Serum rBoIFN-α titer was evaluated using cytopathic effect (CPE) inhibition bioassay. Then, the standard pharmacokinetic parameters were calculated using the DAS (Drug and statistics) software. The concentration-time profiles of serum rBoIFN-α following IM administration, SC administration and IV administration were characteristics of the 1-, 1-, and 2-compartment open models, respectively. After a single dose of IV administration, the drug rapidly dispersed and was rapidly eliminated from the body ($T_{1/2\alpha}$=0.15±0.02 h, $T_{1/2}$=6.48±0.49 h). After IM and SC administrations, the drug is rapidly absorbed and slowly eliminated from the body (For IM administration, T_{max}=6.12±0.32 h, $T_{1/2}$=8.19±0.74 h) (For SC administration, T_{max}=4.06±0.56 h, $T_{1/2}$=7.29±0.55 h). The bioavailability of rBoIFN-α after IM administration is 53.74%, which is higher than the bioavailability of SC administration (27.96%). Therefore, the results showed that the drug administration effect can be preferably obtained following a single dose IM injection using the rBoIFN-α aqueous preparation. We hope that this study will provide valuable information for the clinical application of rBoIFN-α as an potential antiviral agent.

Keywords: *Recombinant bovine interferon-α, Cytopathic effect inhibition assay, Bioavailability, Pharmacokinetic study*

Sığırlarda Rekombinant Bovine İnterferon-alfa Üzerine Farmakokinetik Çalışmalar

Öz

Bu çalışma potansiyel antiviral ve bağışıklık düzenleyici fonksiyonlara sahip olan rekombinant bovine interferon-alfa (rBoIFN-α)'nın sığırlarda farmakokinetik özelliklerini değerlendirmek amacıyla yapılmıştır. Çalışmada 6 aylık 12 hayvan 4 gruba ayrılmış (n=3), hayvanlara IV, IM ve SC yollarla 5.0×10^3 IU/kg dozda rBoIFN-α verilmiştir. Serum rBoIFN-α titresi, sitopatik etki inhibisyon biyotesti kullanılarak değerlendirilmiştir. Sonrasında, standart farmakokinetik parametreler DAS (Drug and statistics) yazılımı kullanılarak hesap edilmiştir. İntramusküler, SC ve IV yollarla rBoIFN-α verilmesi sonrası konsantrasyon-zaman profili sırasıyla 1-, 1- ve 2-kompartman açık model özelliklerini göstermekteydi. Tek doz IV uygulama sonrası ilaç hızlı bir şekilde dağıldı ve hızlıca vücuttan elimine edildi ($T_{1/2\alpha}$=0.15±0.02 s, $T_{1/2}$=6.48±0.49 s). İlaç IM ve SC uygulama sonrasında hızlıca absorbe edildi ve yavaşça vücuttan elimine edildi (IM uygulama için T_{max}=6.12±0.32 s, $T_{1/2}$=8.19±0.74 s) (SC uygulama için T_{max}=4.06±0.56 s, $T_{1/2}$=7.29±0.55 s). rBoIFN-α'nın IM uygulama sonrası biyoyararlanımı %53.74 olup bu değer SC uygulamadaki değerden (%27.96) daha yüksek olarak tespit edildi. Elde edilen sonuçlar, ilaç uygulama etkisinin tercihen tek doz IM rBoIFN-α sıvı preparasyon enjeksiyonu ardından elde edilebileceğini göstermiştir. Bu çalışmanın, potansiyel bir antiviral ajan olarak rBoIFN-α'nın klinik uygulaması için değerli bilgiler sağlayacağı düşüncesindeyiz.

Anahtar sözcükler: *Rekombinant Bovine interferon-α, Sitopatik etki inhibisyon testi, Biyoyararlanım, Farmakokinetik çalışma*

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INTRODUCTION

Interferon (IFN) belongs to the large-scale protein family with anti-viral, cell proliferation regulatory and immuno-regulatory activities [1,2]. It was originally reported by Isaacs and Lindenmann in 1957, that the influenza virus infected chicken cells could produce a soluble factor, which was endowed with the resistance to homologous and heterologous viruses [3]. Currently, IFN is generally classified into three categories, including type I, type II and type III IFNs [4]. Among them, type I IFN includes IFN-α, IFN-β, IFN-ω, IFN-τ and IFN-κ that play important roles in suppressing virus replication and cell growth [5,6], exerting their biological effects through the growth of co-receptor IFNAR [7]. Type II IFN only consists of one member IFN-γ [8], which plays a key role in adaptive immune responses, and is crucial for activating macrophages and natural killer (NK) cells [9,10]. Type III IFN are constituted by IFN-λ and interleukin (IL)-28/29 [4,11,12]. Typically, type I IFN and Type III IFN have anti-viral activities [13]. Some effects of various IFNs may overlap, however, in comparison with other types of IFN, type I IFN has displayed the strongest anti-viral activity [14].

IFN-α, which belongs to type I IFN, has been used as one of the most effective therapeutic drug to prevent or treat specific viral diseases. For example, human IFN-α shows favorable prospect in treating hepatitis B, hepatitis C, viral hemorrhoids, multiple sclerosis, and other diseases [15-17]. Porcine IFN-α has been gradually adopted to treat some viral infections, such as PRV [18], PRRSV [19] and CSFV [20]. Bovine IFN-α (BoIFN-α) has also been proved to have anti-viral effect on the infection of bovine viral diarrhea virus (BVDV) [21], Foot and Mouth Disease Virus (FMDV) [22]. Moreover, the recombinant bovine IFN-α (rBoIFN-α) has been generated in the yeast expression system through Molecular Biology technology [23]. This technique allows rBoIFN-α to move further toward practical application in preventing and controlling the viral diseases in bovine industry.

In order to elucidate the pharmacokinetic profiles of recombinant interferon-α, many studies have been performed in human or animals, such as the pharmacokinetic profile of *Escherichia coli*-derived human interferon type alpha in mice [24], Recombinant Human Interferon Alpha2b Formulations in healthy human volunteers [25], recombinant leukocyte A interferon in patients with disseminated cancer [26], recombinant interferon alpha-C in patients with metastatic renal cell carcinoma [27], recombinant human interferon-alpha I in African green monkeys [28], recombinant alpha A interferon in African green monkeys [29], human recombinant interferon (Re-IFN-alpha A) in cynomolgus monkeys [30], recombinant leukocyte A interferon in beagle dogs [31], recombinant human interferon-alpha 2C in rat and marmoset [32], recombinant feline interferon in cats [33], recombinant chicken interferon-α in broiler chickens [34]. However, till now, to the best of our knowledge, no information on the pharmacokinetic characteristics of rBoIFN-α has been reported in scientific literatures. Moreover, due to the natural aspect of species-specificity in IFN [35], the bovine viral diseases can only be treated with bovine IFN and cannot be treated with human IFN. Therefore, our study aims to investigate the pharmacokinetic characteristics of rBoIFN-α in cattles by calculating the serum rBoIFN-α bio-activities at different time points using cytopathic effect (CPE) inhibition bioassay. Our study is original and provides the detailed evaluation of the parameters of rBoIFN-α pharmacokinetics. We hope that this study will provide scientific contributions to the research on rBoIFN-α.

MATERIAL and METHODS

Animals and Materials: In this study, twenty four 6-month-old cattles were used, including 12 males and 12 females. All cattles were derived from the commercial cattle farm at the age of 5 months. All animals were fed *ad libitum* with commercial diet for a month in the Experimental Animal Research Center of Anhui Province (Hefei, Anhui, China). The animals weight from 186.5 kg to 226.2 kg (200±26.3 kg) and were randomly classified into 4 groups, with 6 animals per group.

The rBoIFN-α freeze-dried powder for animal injection was offered by Anhui Jiuchuan Biotech Co., Ltd (batch number: 20151024, Wuhu, Anhui, China), which was produced through yeast *(Pichia pastoris)* foreign gene expression method as previously described [23]. The rBoIFN-α was obtained following the procedure of protein purification, sterilization and freeze-drying. The product titer equaled 1.0×10^6 IU/vial. Within the four cattle group, group 1 was given IV injection of rBoIFN-α at a dose of 5.0×10^3 IU/kg. Group 2 and group 3 were given IM injection and SC injection of rBoIFN-α at the same dose, respectively. Group 4 (the normal control group) was also injected with normal saline through the same way.

Sample Collection: Blood samples (5 mL) were collected by jugular venipuncture (using contra-lateral vein from that to which the IV dose was administered) at 0 (just prior to treatment), 0.25, 0.50, 1, 2, 3, 4, 6, 8, 12, 24 and 48 h following treatment administration [36]. Samples were collected using a 1-inch by 20-gauge sterile needle and were then deposited into blood tubes. The blood tubes were placed in an ice bath, and protected from light, allowing to clot. After that, they were centrifuged at approximately 3.000×g for 10 min within 2 h after blood coagulation, and the supernatant was transferred by pipette into duplicate plastic tubes. The obtained sera were stored at approximately -70°C prior to assay. The animal experimental protocol performed in this study was approved by the Institutional Ethics Committee of Anhui Medical University (approval number: LLSC20170364).

rBoIFN-α Analysis in Serum: The IFN titers in Madin-Darby bovine kidney (MDBK) cell line, which was infected by

vesicular stomatitis virus (VSV), were determined through the cytopathic effect (CPE) inhibition bioassay. In brief, the MDBK cells were inoculated into the 96-well microtest plates at the density of 3×10^4 cells per well and incubated in DMEM containing 3% fetal calf serum (FCS) at 37°C and 5% CO_2 humid air for 12 h. The monolayers of MDBK cells were treated with 100 µL of 4-fold serial diluted rBoIFN-α liquid. After 24-h incubation, cells were attacked by VSV at the volume of 100$TCID_{50}$/well (50% tissue culture infection dose) and continued to be cultured until the appearance of 100% CPE in the virus-infected cells (virus control well without rBoIFN-α treatment). Prior to plaque counting, the culture was stained with crystal violet. One IFN unit was defined as the highest dilution of rBoIFN-α that inhibited 50% CPE in the case of 100% CPE was observed in the non-IFN treated wells. The rBoIFN titers (IU) was expressed as the reciprocal of the dilutions resulting in 50% cell lysis through the computation with Reed-Muench method [37]. A recombinant human IFN-α (rhIFN-α1, 3×10^6 IU/mL, Lot number 97/04) was provided by the China Food and Drug Inspection Institute (Beijing, China) and was used as a positive control for CPE inhibition bioassay. The precision of the IFN standard, expressed in % RSD, was 2.1%; and the accuracy of IFN standard, expressed in relative mean error (RME), was ≤9.35%. In the current work, both the precision and accuracy values met the requirements.

Data Processing and Statistical Analysis: The mean ± standard deviation (X±SD) was adopted to explore the results about the rBoIFN-α titers. The data of serum rBoIFN-α concentrations at all time points following IV, IM and SC administrative injections were computed through the curve fitting formula using the DAS (Drug and statistics) software (Version 2.0, Wenzhou Medical University, Wenzhou, Zhejiang, China) [38], along with the adoption of noncompartmental analysis. The standard pharmacokinetic parameters included plasma concentration-time related area under curve (AUC [IU/L×h]), clearance rate [CL (L/h]), maximal plasma concentration (C_{max} [$\times10^4$ IU/L]), elimination half-life ($t_{1/2}$ [h]), time to reach peak concentration (t_{max} [h]), and mean retention time (MRT [h]). These serum concentration data were uniformed with the animal's body weight by a comparable analysis. The AUC values after subcutaneous administration was computed through the linear-up/log-down trapezoidal method. To calculate $AUC_{0-\infty}$ and CL, a terminal rate was determined with the slope to 48 h. Moreover, non-paired tailed t test were adopted to compare the data of the anti-viral activity in sera collected from the group of rBoIFN-α-treated animals with the group of normal-saline-treated control animals in each day. The statistical significance level was set to $P<0.05$.

The formula for bioavailability calculation was according to the following equation:

Bioavailability = F = (AUCs.c. or i.m. × D i.v.)/(AUCi.v. × Ds.c. or i.m.) × 100%

RESULTS

The experimental cattles were given IV, IM or SC injections of rBoIFN-α at the dose of 5.0×10^3 IU/kg, and the blood rBoIFN-α efficacy was determined through the antiviral activity in the VSV-infected MDBK cell lines.

Table 1 showed the pharmacokinetic results of rBoIFN-α in the tested animals. The pharmacokinetic features of intravenous injection of rBoIFN-α conformed to the two-compartment open model, which was associated with

Table 1. *The main pharmacokinetic parameters of rBoIFN-α for injection (n=3, X±SD)*

Parameters	Intravenous Injection Group	Intramuscular Injection Group	Subcutaneous Injection Group
T_{max} (h)	-	6.12±0.32	4.06±0.56
C_{max} (IU/L)	2400.32±128.48	1205.42±104.32	975.36±84.49
AUC (0-t) (IU/L×h)	17717.22±1421.38	12377.34±983.32	8023.41±628.29
AUC (0-∞) (IU/L×h)	29443.22±1562.47	15815.12±1014.26	8232.19±643.36
CL (L/h)	33.98±1.76	-	-
C0 (IU/h)	3049.35±486.32	-	-
MRT (h) Mean Residence Time	9.44±0.45 h	12.76±0.69	11.73±0.58
$T_{1/2\alpha}$ (h)	0.15±0.02	-	-
$T_{1/2\beta}$ (h)	6.65±0.44	8.96±0.85	7.69±0.66
$T_{1/2ka}$	-	1.12±0.27	1.81±0.34
$T_{1/2}$ (h)	6.48±0.49	8.19±0.74	7.29±0.55
Bioavailability (F)	-	53.74%	27.96%
Vdss (L)	128.64±6.86	-	-
k10 (1/h)	5.33±0.76	-	-
k12 (1/h)	0.19±0.04	-	-
k21 (1/h)	0.14±0.02	-	-

first-order elimination. Besides, the elimination half-life ($T_{1/2}$) of intravenous injection was (6.48±0.49) h *(Fig. 1)*.

The pharmacokinetic features of intramuscular injection and subcutaneous injection of rBoIFN-α conformed to the one compartment open model, Their T_{max} were (6.12±0.32) and (4.06±0.56) h, respectively, and their elimination half-life ($T_{1/2}$) were (8.19±0.74) and (7.29±0.55) h, respectively *(Fig. 2* and *Fig. 3)*. The bioavailability of rBoIFN-α from the group of intramuscular administration was 53.74%.

Gender differences among all animal pharmacokinetic parameters were not statistically significant at any dosing level (data not shown). Moreover, no possible safety issues were observed at any dosing level during or after rBoIFN-α treatment in this study.

DISCUSSION

So far, the clinical application of interferon (IFN) is hindered due to the incomplete knowledge about its mechanisms of action. Nevertheless, some supportive evidence indicates that, the route of administration, namely, the pharmacokinetic behavior of drugs, is a significant factor affecting the efficacy of treatment. The pharmacokinetic characteristics of human IFN have been fully described. Its blood concentration will be rapidly decreased soon after IV administration, and the distribution volume is close to 20-60% of body weight. Animal study suggests that, the catabolism type of IFN belong to the category of the natural processing of proteins. The Clearance value of the entire IFN family varies from one to another (range: 4.8-206 L/h), which may reflect the natural digestion and the regeneration of proteins. The terminal elimination half life of IFN-α is 4-16 h. In comparison, IM and SC administration would render prolonged but really good absorption of IFN-α, which was more than 70% [39].

With regard to bovine interferon, Gillespie et al.[40] and

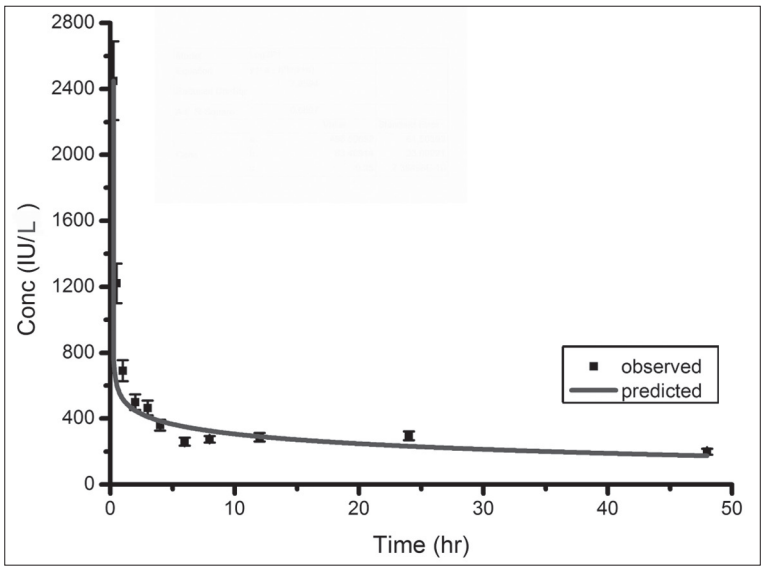
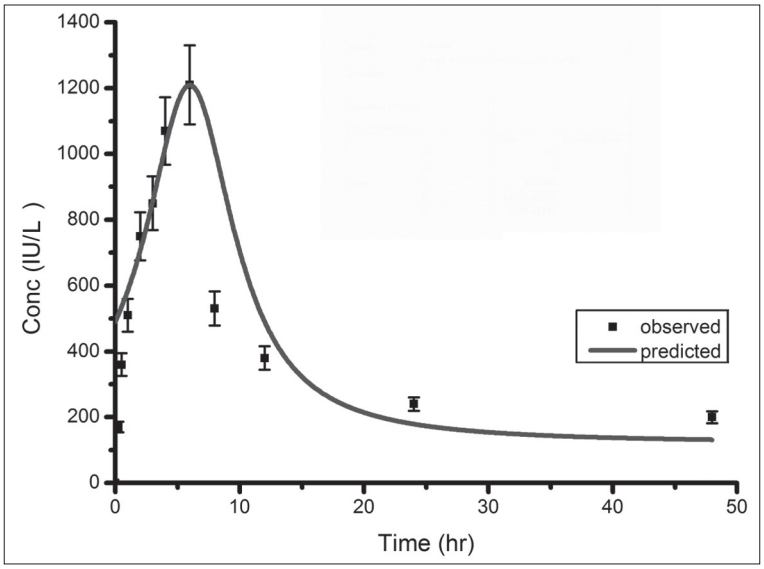


Fig 1. Serum IFN titer-time curve in the rBoIFN-α intravenous administration group, where the X-axis represented time and the Y-axis stood for the titer of rBoIFN-α. The scattered squares stood for the average value, while the Y error bars indicated standard deviations. The intravenous injection group was tested for 3 times

Fig 2. Serum IFN titer-time curve in the rBoIFN-α intramuscular administration group, where the X-axis represented time and the Y-axis stood for the titer of rBoIFN-α. The scattered squares stood for the average value, while the Y error bars indicated standard deviations. The intramuscular injection group was tested for 3 times



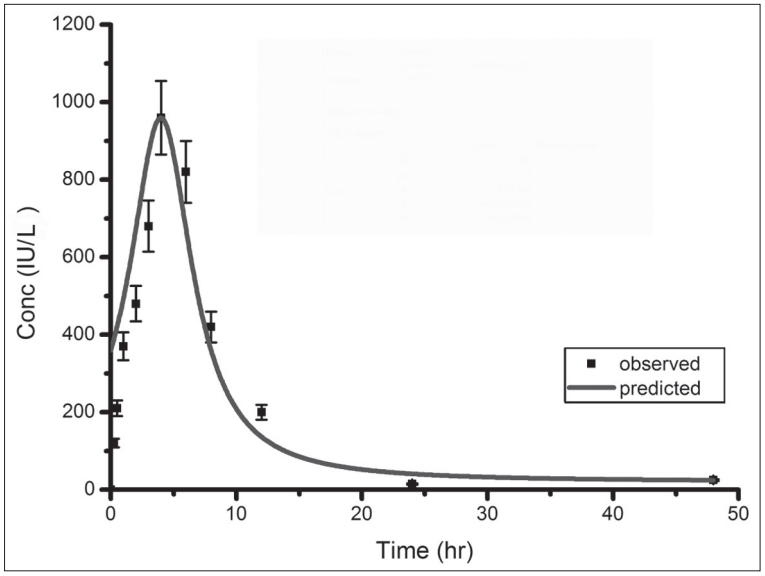


Fig 3. Serum IFN titer-time curve in the rBoIFN-α subcutaneous administration group, where the X-axis represented time and the Y-axis stood for the titer of rBoIFN-α. The scattered squares stood for the average value, while the Y error bars indicated standard deviations. The subcutaneous injection group was tested for 3 times

Gillespie et al.[41] first reported the antiviral effects of *E. coli*-derived bovine recombinant interferon-α against bovine diarrhea virus and its application levels in the blood serum of dairy calves in 1986. rBoIFN-α has also been suggested to have a prophylactic effectiveness in controlling bovine respiratory disease. The administration of rBoIFN-α into growing calves resulted in reduced mortality and incidence of respiratory diseases. Specifically, rBoIFN-α-treated calves affected by respiratory disease showed less severe clinical symptoms, shortening of sick days and less recurrence of respiratory disease [42]. Because the research report on the pharmacokinetic assessment of rBoIFN-α is unavailable, therefore, we performed this study to investigate the pharmacokinetic characteristics of rBoIFN-α following a single injection of IV or SC or IM administrations.

Following IV administration, serum concentrations of rBoIFN-α rapid declined exponentially. rBoIFN-α serum concentration verses time data can be best fitted to a two-compartment open model, which was supported by disposition characteristics of recombinant IFN-α reported in human [25] and animals [28,30-33]. The elimination half-life ($t_{1/2\beta}$) (6.65±0.44 h) determined in the present study is longer than that reported in cats (about 0.51±0.08 h) [33], rats (2.8-6.3 h) [32], beagle dogs (about 4.5 h) [31], African green monkey(about 4.0 h) [28], but shorter than that determined in marmosets (Callitrix jacchus) (10-14 h) [32]. Clearance of rBoIFN-α observed in cattles (33.98±1.76 L/h) in the present study is faster than those reported in cats (2.56±0.61 L/h) [33], rats (1.34L/h) [32], beagle dogs (1.59±0.15L/h) [31], African green monkey (about 5.34 L/h) [28]. Besides, the clearance of the drug is also faster than that has been reported in marmosets (Callitrix jacchus) (12L/h) [32]. The drug is widely distributed in the body as determined by apparent volume of distribution Vdss (128.64±6.86 L) observed in the present study. This is in similar with that reported in marmosets [32], suggesting wider distribution of drug into the tissues of cattles.

Following IM administration, the pharmacokinetics of rBoIFN-α were well described by a classic one-compartment open model. After rBoIFN-α was injected IM, peak serum rBoIFN-α concentration (1205.42±104.32 IU/L) was achieved at 6.12±0.32 h (Tmax), which is lower than the peak recombinant IFN-α concentrations observed in African green monkey [28] and healthy human volunteers [25]. Elimination half-life following IM injection of the drug in the present study is longer than that reported in beagle dogs (about 4.7 h) [31], African green monkey (about 7.0 h) [28], healthy human volunteers (7.8±3.5 h) [25]. The absolute bioavailability (F) of rBoIFN-α following IM injection observed in the present study indicates that there was moderate good absorption of the drug from the IM injection site (F=53.74%). This value is similar to that reported in marmosets (ranged from 40-80%) [32] and slight higher than that reported in beagle dogs (42%) [31].

Following SC administration, the pharmacokinetic parameters of rBoIFN-α were slightly lower than those determined through the route of IM injection. Based on the observed serum drug concentration following IM administration of the drug in the present study, IM injection of rBoIFN-α may be used as a therapeutic route in cattles.

ELISA and cytopathic effect (CPE) inhibition bioassay are commonly used to quantitatively measure IFN concentration. Usually, ELISA is a rapid and simple way to quantitatively measure the protein concentration. Nevertheless, regarding to IFN-α, it can not determine the serum bioactivity of rBoIFN-α in due course. More significantly, Cytopathic effect (CPE) inhibition assay is a widely-used routine titer determination system for biological activity determination of human interferon. Furthermore, at the present time, no stable and reliable BoIFN-α ELISA kit approved by the competent authority is available. Consequently, CPE inhibition bioassay is employed in the current study to

quantitatively detect the bioactivity of rBoIFN-α in animal serum according to the descriptions of experimental protocols reported by several published articles [43-45].

The cytopathic effect (CPE) inhibition method employed in our study is a well-established and widely recognized method for analysis of interferon [46] and was referred to the 2015 edition of the pharmacopoeia of the People's Republic of China. Most of the interferon bioassays rely on the same biological end-point: quantification of a viral cytopathic effect of host cells [47]. Host cells and the virus selected may differ depending on the interferon of interest In general, biological fluid containing interferon is added 10 plates seeded with monolayers of host cells and incubated; the medium from each well is aspirated, followed by a washing of the cells. The cells are then challenged with a cytopathic virus. The interferon titre is read as the reciprocal of the dilution in which 50% of the cell monolayer is protected, determined by visual inspection [47,48] or spectrophotometric detection [43,49]. The method's principle was based on IFN protect cells against virus attacks ability to compute the titer of IFN, the determination results were expressed by the international units (IU).

In conclusion, rBoIFN-α was well tolerated in this study. It disseminated and disposed rapidly following a single dose of IV injection, while it was quickly adsorbed and slowly metabolized after a single-dose IM injection in cattles. Based on these data, rBoIFN-α has a potential for the treatment of viral infections without alteration of the dose and dose intervals in cattles. rBoIFN-α may be beneficial and potentially applicable in cattle industry. However, in the future, more detailed studies concerning effective dosages, timing of administration and characterization of the condition should be done to access the efficacy of rBoIFN-α in clinical practices.

Author Disclosure Statement

The authors declare that they have no competing interests in this study.

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Research Article

Analysis of Chromosome Karyotype and Banding Patterns of Chicken, Quail, and Their Hybrids

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Abstract

To explore the incompatibility of hybrids between chickens and quails at the chromosome level, in the present study, chickens, quails, and their hybrids were selected and their chromosome karyotype and banding patterns were analyzed. The methods used comprised pre paring chromosomes from air-dried peripheral blood lymphocytes, the embryo method, G-banding, and C-banding techniques. The result revealed that the number of chromosomes (2n) of chicken, quail, and their hybrids was 78, with 10 pairs of macrochromosomes and 29 pairs of microchromosomes; however, there were some remarkable differences in chromosome morphology. There were significant differences in G-banding patterns between chickens, quails, and their hybrids, among which chickens chromosomes were divided into 32 zones with 155 bands, including 71 positive bands. The quails were divided into 28 zones, with 138 bands, including 61 positive bands. C-band analysis showed that the C-band of chickens, quails, and their hybrids were present on all W-sex chromosomes in all female fission phases and were deeply stained. The combined analysis of the karyotypes and different genotypes of chickens, quails, and their hybrids could provide a reference to accelerate the breeding process.

Keywords: *Chicken, Quail, peripheral lymphocyte culture, Karyotype analysis, G-banding C-banding*

Tavuk, Bıldırcın ve Hibritlerinin Kromozom Karyotipleri ve Bantlanma Modeli Analizi

Öz

Tavuk ve bıldırcınlar arasındaki hibritlerin kromozom düzeyinde uyumsuzluğunu araştırmak için, bu çalışmada, tavuklar, bıldırcınlar ve melezleri seçilmiş ve bunların kromozom karyotipi ve bantlanma modelleri incelenmiştir. Çalışmada, havada kurutulmuş perifer kan lenfositlerinden kromozomların hazırlanması, embriyo metodu, G-bantlanma ve C-bantlanma teknikleri kullanıldı. Tavuk, bıldırcın ve hibritlerinin kromozom sayılarının (2n), 10 çift makrokromozom ve 29 çift mikrokromozoma sahip olmak üzere 78 olduğu ancak kromozom morfolojileri bakımından bazı önemli farklılıkların olduğu belirlendi. Tavuk, bıldırcın ve hibritleri arasında G-bantlanma modelinde anlamlı fark olduğu tespit edildi. Tavuk kromozomları 71 pozitif bant içeren toplam 155 banta sahip 23 bölgeye ayrılmaktaydı. Bıldırcınlarda 61 pozitif bant içeren 138 banta sahip 28 bölge bulunmaktaydı. C-bant analizi, tavuk, bıldırcın ve hibritlerinin C-bantlarının tüm dişi füzyon fazında tüm W-seks kromozomlarında mevcut olduğunu ve derinlemesine boyandığını gösterdi. Tavuk, bıldırcın ve hibritlerinin karyotip ve farklı genotiplerin birlikte analizi yetiştiricilik sürecinde referans olarak kullanılabilir.

Anahtar sözcükler: *Tavuk, Bıldırcın, Perifer lenfosit kültürü, Karyotip analizi, G-bantlanma C-bantlanma*

INTRODUCTION

Chickens and quails belong to the family Aves, *Neognathae*, *Galloanserae*, Galliformes, Phasianidae, *Phasianinae*, Gallus and *Coturnix* in the Phasianidae family; which are the same family but different genera. Chickens have a larger body than quails; however, long-term high-intensity breeding has resulted in chicken meat lacking flavor. Quails have a smaller body, and their meat is nutrient-rich, delicious, aromatic, and contains the bioflavonoid rutin, which has some therapeutic value. In our country, quail meat is known as "animal ginseng", and the cholesterol content of quail eggs is lower than that of chicken eggs, which can lower blood pressure. Chicken and quail are different genera, and hybridization between them represents a typical distant hybridization, providing good resources for studies of gene function and comparative genomics. Their hybrids may form a new population showing dominant

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traits based on excellent traits such as body size, meat production, and meat quality of the parents, and might add new flavors and delicacies to the human diet. As early as 1964, Mcfarquhar reported the hatching of chicken and quail hybrids [1]. The orthogonal combination of chicken (♂) and quail (♀) was then reported in the United States (1985), Japan (1983), and Malaysia (1989). Chunmei et al.[2], performed chicken and quail hybridization experiments (orthogonal combination) with some success. They used artificial insemination to enhance the hatching rate, and the results revealed that intergeneric hybridization allows the first filial generation to obtain the excellent features of both parents at the same time, including the characteristic of the rapid growth of the male parent, and the genetic characteristic of precocious maturing. However, there has been little study of sex identification in the early development of hybridized poultry embryos. These distant hybridizations can not only enrich the breeding material, but also provide an excellent resource. The products of distant hybridization are incompatible (i.e., hybrid combinations do not produce offspring), and this is true of chicken and quail hybrids, as follows: female hybrids all die during the early embryonic stage, only male individuals survive, and hybrid glands do not show meiotic activity. At present, there is no explanation for the mechanism of incompatibility of distant hybridization in birds, and no detailed cytogenetic studies have been carried out on the hybrids between these two species.

In the present study, karyotype analysis was carried out on chickens, quails, and chicken-quail hybrids using embryo methods and the peripheral lymphocyte culture techniques. G-banded patterns were obtained using trypsin and Giemsa. The karyotypes, G-band, and C-band results for chickens, quails, and their hybrids were compared to determine discuss their similarities and differences. The results provide a valuable reference for research on hybrid incompatibility and hybrid sterility between chickens and quails.

MATERIAL and METHODS

Ethics Statement

This study was approved by the Ethical Committee of Animal Experments, Animal Science and Techonology College, Shihezi University (Number: 2011098). All samples were collected in strict accordance with the committee's guidelines. During the experiment, every effort was made to minimize suffering by the animals.

Test Animals

Fifty adult male chickens and 100 female Korean quails, all with healthy bodies and similar weights, were selected. Wannan three-yellow chicken, which has the characteristics of wild grazing, strong feeding ability, wide adaptability, resistance to rough feeding and strong disease resistance. It can be adapted to various feeding forms (herding chickens, large-scale breeding, etc.) based on grazing, and can be adapted to be raised in most provinces in China. Under various feeding forms, the survival rate of Wannan three-yellow chicken is over 90%, and the production performance is normal. The hybrids of chickens (♂) × quails (♀) were obtained by artificial insemination at five time points in the first seven days of incubation. During the time, the number of alive hybrids was observed and recorded by using an egg light to check the development of hybrid embryos. The fertilized egg embryos develop normally, the blood vessels are radially distributed, the color is bright and red; the dead embryo eggs are light in color, there are irregular blood arcs, blood rings, no radial blood vessels; no sperm eggs are bright, no vascular network, only see the shadow of the yolk.The incubation conditions were 37.8±0.5°C, and humidity control at 60-70% relative humidity (RH). Ninety embryos were harvested and 20 male hybrids were hatched for testing. The animals were tested at the Experimental Station, Academy of Animal Science and Technology, Shihezi University.

Reagents Used

Roswell Park Memorial Institute (RPMI1640, GIBCO, USA); Heparin (Hua Mei, He Bei, China); Colchicine (Tiangen, Beijing, China); Giemsa powder (Hua da, Beijing, China); Inactivated calf serum (Hua Mei).

Chromosome Specimen Preparation Method

The chromosome preparations were treated in 0.2N hydrochloric acid for 30 min and air-dried after rinsing with distilled water. The slides were placed in a 5% barium hydroxide solution at 60°C for 5 to 10 min. The slides were taken out of the barium hydroxide solution and quickly rinsed in 0.1N hydrochloric acid to remove the surface barium hydroxide precipitate and then rinsed with distilled water. The slides were incubated in 55-60°C in 2 × SSC for 45-60 min, rinsed with distilled water, and air-dried. The slides were then incubated in 1:9 Giemsa phosphate buffer (pH 6.8) for 10 min, rinsed with distilled water, air-dried, examined microscopically, and photographed.

Chromosome Analysis Method

Giemsa stained chromosome sections were counted under a microscope. The diploid chromosome number was counted under the microscope using a good chromosome spread and mitotic phase of transparent appearance was determined (50 male and 50 female). The three metakinesis phases of a good transparent chromosome spread was for each poultry and photograph under an immersion objective. The long and short arms of first 10 pairs chromosomes were measured using Photoshop image-processing software to calculate the relative length and arm ratio, the centromere index of each chromosome, and their average value in accordance with the following formula.

Relative length = (The length of the chromosome)/(The total length of chromosomes1-10chromosomes [including w chromosome]) ×100

Arm ratio = (The length of the long arm)/(The length of the short arm)

Centromere index = (Length of the short arm)/(The total length of the chromosome)×100

All the animals were killed by the method of heart oppression. A photograph was taken of a good G-band metakinesis phase of chicken, quail and a hybrid. The number of bands, the relative position, the shade of color, and the width of the chromosomes were observed and recorded under a microscope. The number of bands of the first 10 pairs of chromosomes in each cell were counted; and the frequency statistics for the band mode were determined. After that, the medium-term C band under different alkali treatments was observed under a microscope. Well-processed, well-colored metaphase fission micrographs were selected, and the band characteristics and distribution of the C-band were analyzed. Finally, we observed the morphology and bands of the W chromosome.

Statistical Analysis

The associations of the parameters of the macro-chromosomes among chicken, quail and the hybrid were evaluated using chi-square test. The Data were expressed as the mean±the standard error and all statistical analysis were performed with SPSS for Windows (version 19.0).

RESULTS

Karyotype Analysis of Chicken, Quail, and Their Hybrids

The Number of Chromosomes (2n) of Chickens, Quails, and Their Hybrids: Chromosome sections of chicken, quail and their hybrid were carried out using conventional Giemsa staining of 100 selected samples showing good disintegrated phasing for microscopy to determine the statistics of the diploid chromosomes; the results shown in *Table 1*.

From *Table 1*, the number of somatic chromosomes in cells was 2n=78 for chickens, quails and the hybrid, which was the case for 84, 82, and 81% of the total of cells observed, respectively. This demonstrated that the chromosome number of chickens, quail, and their hybrid was 2n=78. The hybrid embryos were selected at five times points (embryonic day 3-7) to detect the sex of early hybrid embryos. The results showed that there were live 70 embryos among the 90 early embryos *(Table 2)*. The ratio of females to males was compared with the theoretical value ($P<0.05$), and there were significantly more males than females. The mortality rate of early female embryos was significantly higher than that of males ($P<0.05$), which further confirmed that the sex determination methods of hybrids were ZZ (♂) and ZW (♀). As hatching proceeded, the sex ratio of the male and female embryos gradually became more unbalanced.

Karyotype Analysis Among Chickens, Quails and Their Hybrids: *Table 3* shows that there are 10 pairs of macro-chromosomes and 29 pairs of microchromosomes among chicken and quail chromosomes. The microchromosomes are all telocentric chromosomes. Chicken chromosomes 3, 5, 7, and 9 are t-type; chromosomes 1, 2, and 8 are m-type; and chromosomes 4 and 6 are sm-type. In quail, chromosome 1 was sm-type, chromosome 2 was m-type, chromosome 4 was st-type, and all other chromosomes were t-type. The Z chromosomes of chickens and quails were all m-type and all were the fifth macrochromosomes. The chicken W chromosome is m-type, its length was equal to that of chromosome 8, while the quail W chromosome is t-type, with a length between that of chromosome 7 and 8. The results of *Table 4* showed that each chromosome of the hybrid was identical to one from chicken or quail, and the method of gender determination was ZZ (♂) and ZW (♀).

Table 1. *The number of chromosomes (2n) of chicken, quail, and their hybrids*

Variety	Distribution of the Chromosomes (2 n)							Total Cellular Score	2 n Model Number	2 n=78 Frequency (%)
	<75	75	76	77	78	79	80			
Chicken	3	2	3	5	84	2	1	100	78	84%
Quail	3	2	4	4	82	3	2	100	78	82%
Chicken-Quail hybrids	2	5	3	4	81	2	3	100	78	81%

Table 2. *Sex and number of live hybrid embryos at different times*

Sex	Brood Days				
	3 days	4 days	5 days	6 days	7 days
Female	4	4	2	3	1
Male	10	10	12	12	12
c^2-test	3.324 ($P<0.05$)	2.421 ($P<0.05$)	3.142 ($P<0.05$)	2.068 ($P<0.05$)	2.073 ($P<0.05$)

Table 3. *The parameters of the macrochromosomes of chicken and quail (X ± SD)*

NO	Chicken				Quail			
	Relative Length	Leverage	Kinomere Index	Kinetochore Location	Relative Length	Leverage	Kinomere INDEX	Kinetochore Location
1	22.09±1.54	1.52±0.11	38.95±0.02	m	21.98±0.79	2.24±0.08	32.04±1.24	sm
2	18.19±1.64	1.59±0.18	38.29±0.06	m	18.21±0.49	1.42±0.06	41.53±1.24	m
3	12.76±0.49	∞	0	t	11.58±0.91	∞	0	t
4	11.07±0.38	2.78±0.42	25.98±0.12	sm	11.14±0.52	5.68±0.16	14.78±0.75	st
5	7.93±0.59	∞	0	t	7.51±0.42	∞	0	t
6	6.29±0.49	1.88±0.37	34.22±0.03	sm	6.14±0.41	∞	0	t
7	5.23±0.39	∞	0	t	4.98±0.58	∞	0	t
8	4.88±0.32	1.09±0.26	46.96±0.12	m	4.42±0.36	∞	0	t
9	3.99±0.31	∞	0	t	3.58±0.26	∞	0	t
Z	9.78±0.81	1.15±0.11	47.49±0.06	m	9.93±0.59	1.14±0.08	47.95±0.41	m
W	4.81±0.21	1.97±0.07	48.49±0.05	m	4.58±0.28	∞	0	t

Table 4. *The parameters of the macrochromosomes of hybrids (X ± SD)*

No	Moiety Chromosome Same as Chicken Karyotype				Moiety Chromosome Same as Quail Karyotype			
	Relative Length	Leverage	Kinomere Index	Kinetochore Location	Relative Length	Leverage	Kinomere Index	Kinetochore Location
1	24.06±1.76	1.56±0.13	39.06±0.04	m	22.24±0.56	2.16±0.03	31.64±1.34	m
2	19.76±1.64	1.59±0.18	38.29±0.06	m	18.21±0.37	1.42±0.01	41.53±1.32	m
3	12.99±0.49	∞	0	t	11.58±0.88	∞	0	t
4	10.91±0.38	2.86±0.42	25.79±0.12	sm	11.14±0.59	5.68±0.19	14.84±0.75	st
5	7.63±0.59	∞	0	t	7.51±0.26	∞	0	t
6	6.54±0.49	1.81±0.37	35.19±0.03	sm	6.14±0.38	∞	0	t
7	5.32±0.39	∞	0	t	4.98±0.47	∞	0	t
8	4.89±0.32	1.23±0.26	46.49±0.12	m	4.42±0.41	∞	0	t
9	3.97±0.31	∞	0	t	3.58±0.31	∞	0	t
Z	9.91±0.81	1.11±0.11	48.11±0.06	m	9.93±0.61	1.14±0.11	47.95±0.38	m
W					4.58±0.29	∞	0	t

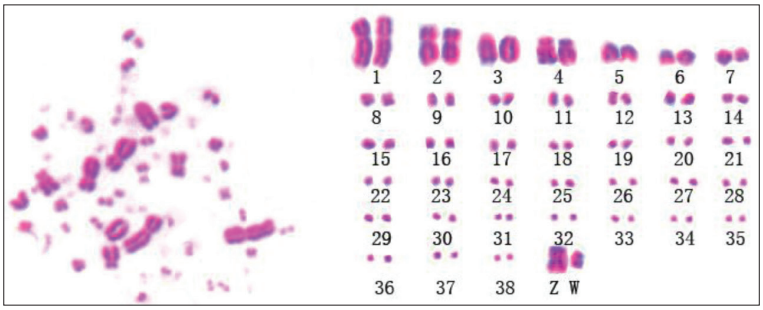

Fig 1. Metaphase chromosomes and idiogram of chicken (♀)

Sex Distribution of Early Embryos: According to the statistical results in *Table 3*, the karyotype maps of the chickens, quails, interspecific hybrids and intergeneric hybrids were drawn *(Fig. 1-4)*. In *Fig. 3* and *Fig. 4*, the chromosome on the left of each pair of chromosomes comes from the chicken, and that on the right comes from the quail

Analysis of Chromosome G-banded Patterns and C-banded Patterns of Chicken, Quail and Their Hybrids

Analysis of Chromosome G-banding: We observed the G-band split phase between chicken, quail, and their hybrids. After treatment with the trypsin-Giemsa method, macrochromosomes showed a relatively clear G band pattern and were rich in bands, homologous chromosome banding patterns are basically the same *(Fig. 5-7)*; most of the microchromosomes had fewer bands, generally only 1-2, and some had no bands and were difficult to identify. There were marked differences between the G banding of chickens and quail chromosomes, which was mainly reflected in the number of bands and the width of the banding pattern. For chromosomes 1 and 2, in chicken, there were seven deep streaks on the q arm and nine deep streaks on arm q, while in quail, there were 5 deep streaks on the p arm

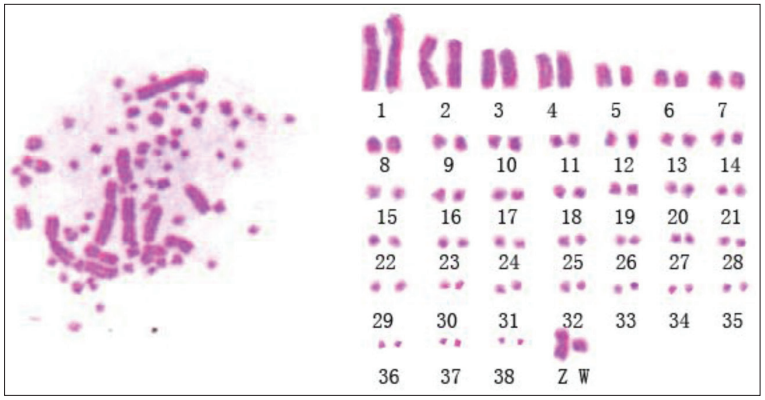

Fig 2. Metaphase chromosomes and idiogram of quail (♀)

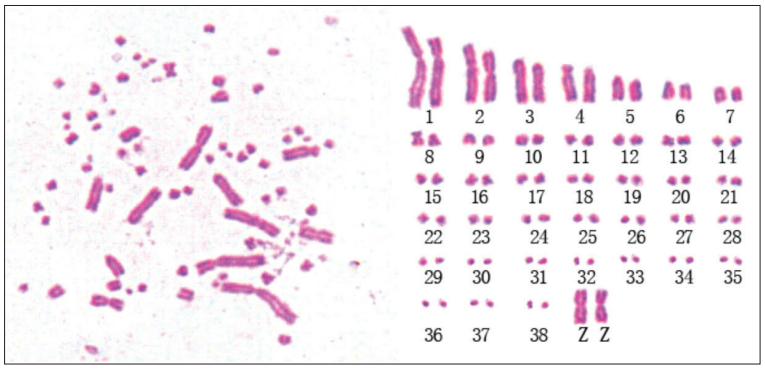

Fig 3. Metaphase chromosomes and idiogram of hybrid (♂)

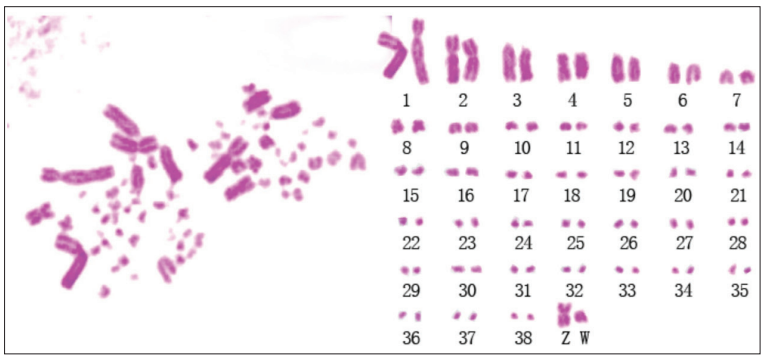

Fig 4. Metaphase chromosomes and idiogram of hybridization embryos (♀)

and 10 deep streaks on the q arm. In chicken, there are two arms on the p arm of chromosome 2 with wider deep-dyed ribbons, while the quail p- and q-arms have a wide deep-dyed band. Chromosomes of the hybrids comprised chromosomes derived from chickens and quails, each of which has the G band characteristic of chicken or of quail. In accordance with the G band pattern, the chicken could be divided into 32 zones, with a total of 155 bands, of which 71 were positive; quail could be divided into 28 zones, with a total of 138 bands, of which 61 were positive. After G-banding, homologous chromosomes showed the same light and dark stripes, and with the help of these stripes, each pair of chromosomes could be accurately paired. Meanwhile, in addition to gene mapping, chromosomal disorders can be diagnosed using G-bands of the disease-associated regions and can be used to explore the correlation between G-bands and production performance on a cytogenetic basis.

Analysis of Chromosome C-banding

We observed the metaphase C-banding of chicken, quail, and their hybrid chromosomes *(Fig. 8)*. Many of the microchromosomes showed deep-staining C-bands, whereas the macrochromosomes did not show C banding in the centromere region. All W chromosomes were darky stained, with strong repeatability, making them easy to identify. Meanwhile, we observed that the C banding was most affected by alkali treatment; when the alkali treatment time was too short (1-2 min), none of the chromosomes showed a C band. As the alkali treatment time increased, the C bands appeared on the W chromosome first, whereas the centromere of the macrochromosome showed a weaker C band, followed by the C band of the macrochromosome telomere, and the minute chromosomes. Analysis of the chromosomal C bands among chicken, quail and their hybrid represented a feasible method to identify the gender of a bird.

DISCUSSION

Chromosomal karyotype not only reflects the germplasm characteristics of a species, but also is useful for breeding studies. Chickens have a large number of microchromosomes; therefore, it is inconvenient to count and describe the chromosomal morphology. Some studies have demonstrated that the incomplete karyotypes of the first 10 chromosomes in birds can represent a species-specific karyotype [3]. Consequently, in the present study, the first 10 pairs of macrochromosomes were analyzed. Thorneycroft et al.[4] performed karyotype analysis of sparrows distributed in the Mudanjiang region and found that their top 10 pairs of chromosomes had the same morphological structure, and the number of chromosomes with 2n=78 accounted for 93.28% of the total observed cells. The criteria for having a diploid chromosome number is that more than 75% of the observed cells should have that chromosome number. Kuchta et al.[5] studied of chicken chromosome karyotypes and found that the chicken chromosome number 2n=78 cells accounted for 85% of the total observed cells. Clagett et al.[6] conducted a chromosomal karyotype analysis of Nick Red Chicken and found that the number of cells with

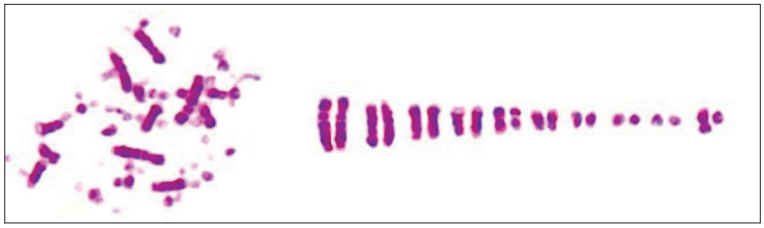

Fig 5. Chromosome G-banding of chicken

Fig 6. Chromosome G-banding of quail

Fig 7. Chromosome G-banding of hybrid

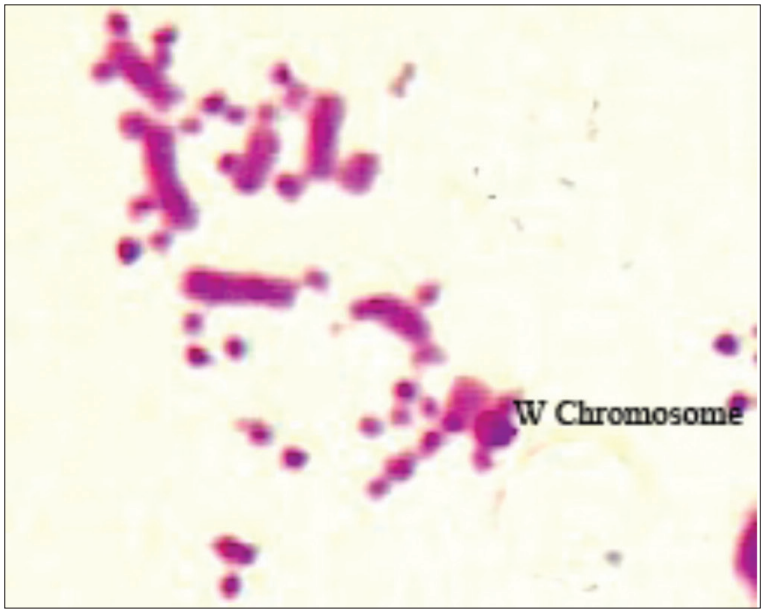

Fig 8. Chromosome C-banding of hybrid

2n=78 chromosomes accounted for 78% of the total observed cells. In the present study, 84%, 82%, and 81% of the total number of observed cells had 2n=78 chromosomes in chickens, quails, and their hybrids, respectively, which was consistent with previous studies. In the cases where 2n≠78, this may reflect the small number of chromosomes in the poultry karyotype or the occurrence of Robertsonian translocations in microchromosomes [7]. Shi et al.[8] compared the karyotype of quail and chickens, and showed that the number of chromosomes were 2n=78; however, the shape and relative length of the chicken chromosomes were significantly different from those of quail ($P<0.05$). Xu et al.[9] analyzed the chromosome karyotype of quail, and found that 2n=78 accounted for 78% of the total number of cells analyzed. In quail, the number of copies of chromosomes 2, 4, and 6 are slightly different, Which was similar to the results of Shi at al.[8] and Xu et al.[9].

It is generally believed that the rate of karyotype evolution in birds is very slow, the number of chromosomes is well conserved, and closely related species have basically the same or similar karyotypes. Chickens and quails are two organisms of the same family but different genera. Their chromosomal relative length is almost the same, but there are marked differences in chromosome morphology, mainly for chromosomes 1, 4, 6, 8 and the W chromosome, indicating a cytogenetic basis for their assignment to different genera [10,11]. The results revealed that the evolutionary trend of karyotype is, from more to less for the small chromosomes, from less to more for the large chromosomes, and from the end to the middle in the development of the centromere type [12]. Species with more subterminal/terminal (st/t) chromosomes in bird karyotypes may be relatively primitive species, whereas species with more sumedian/median (sm/m) type chromosomes are relatively specialized. Quail has significantly more t-chromosomes than chickens. According to the modern theory of bird evolution, the *Galliformes* and the chest type are preserved more than the original karyotype of birds [13], and single arm chromosome inversion through arm evolution to a two-armed chromosome is one of the major forms of bird karyotype evolution [14]. Taking these observations in to account, we hypothesized that quails may have evolved to a lesser extent than chickens.

G-banding is a chromosomal banding technique

in which metaphase chromosomes are treated with trypsin and then stained with Giemsa, which produces patterns of light and dark phases. To date, there have been few successful reports in the domestic literature of clear G-band maps, and the G-banding technique for bird chromosomes is more difficult compared with that for mammals. Xu et al.[9] mapped the G-band pattern of the top 10 quail chromosomes (including the Z and W chromosomes) and found that the main reason for the unsuccessful G-band pattern might the higher degree of spiraling of the metaphase chromosomes, such that the stripes are often combined with thick, fuzzy features. In birds, there are large length differences between the chromosomes; an early metaphase split phase; chromosomes 1, 2 and 3 are too long and easy to wrap around one another or overlap, which affects the zonation effect, and other shorter chromosomes are easily over-digested, all of which create further difficulties in the interpretation of G banding results [9]. Albertson et al.[15] analyzed the first 10 pairs of chromosomes (including the Z and W chromosomes) in chicken, and indicating that there was a band like displacement on chromosomes 1 and 2, which might have been caused by inversion between the arms. In the present study, we found that the macrochromosomes from chickens, quails, and their hybrids showed a clear G band pattern with abundant bands. The bands on the homologous chromosomes were basically the same [16]. A few lacked obvious stripes, making it difficult to identify the pairs. In addition, chromosomes 1 and 2 showed band displacement, which was consistent with the data of Albertson et al.[15].

C-banding is created by treatment with strong acids and bases, followed by visualization with Giemsa. Currently, barium hydroxide treatment is commonly used to visualize the location of structural heterochromatin [17]. There have been few reports about Chromosome C banding or the analysis of the chromosomes of livestock and birds. Christensen et al.[18] analyzed C-band patterns in pigs and found that in cells undergoing mitosis, at the centromere and its vicinity, the amount of structural heterochromatin is constant. In addition, the mid-term chromosome C band size was highly reproducible, and in the same individual during different periods, the C-band sizes were similar. Takuma et al.[2] showed that in Taihe Silky Fowl, chromosome C banding treatment revealed that all sex chromosomes in the hen's split phase had C bands, and were deeply stained and easy to identify. The results of the present study showed that the vast majority of microscopic chromosomes showed deep C-bands, whereas large chromosomes had single shallow C bands or none at all. Reproducibly, C-banding of the W-chromosome appeared first, with the entire chromosome being stained. This result is similar to those reported by Liu et al.[2]. Our results further confirmed that the centromeric region of chicken miniature chromosomes contains more heterochromatin, whereas the large chromosomes contain little or no heterochromatin [5,7,8,19]. Wojcik et al.[7] showed that a large number of microstructural heterochromatic chromosomes might be more prone to Robertson's translocation. There are many microchromosomes in the poultry genome, and the W chromosome is just 1/5–1/2 of the size of Z chromosome, and is usually difficult to identify accurately. Thus, in non-banding specimens, identifying the W chromosome of birds is always harder than in mammals, making chromosome identification much more difficult. Wang et al.[20] used C-banding technology to successfully identify the male and female *Nipponia nippon*. In that experiment, C-banding was performed using the peripheral blood lymphocyte division phase, and the W chromosome was successfully identified, allowing the sex of the early embryos of intergeneric hybrids to be determined. In the present study, we used C-banding to identify W chromosomes, in combination with the morphological identification of Z chromosomes, which greatly improved the accuracy of sex determination of birds, providing a safe and reliable sex identification test for certain rare avian species [21,22].

Karyotype analysis illustrated that males and females were present in the hybrid embryos at 3-5 days after inoculation, while the adult hybrids were all male, indicating that all female hybrids died at the embryonic stage and only male individuals survived. Why did this occur? The mechanism of early death of female embryos in distant hybrids of poultry remains unclear. Chromosome analysis suggests that early embryonic death might be caused by variations in the number of chromosomes and structural abnormalities. The sex of the silkworm is decided in the same way as that of the poultry, and all of them are male ZZ or female ZW. A recessive lethal gene in the Z chromosome causes all the embryonic female individuals to die [23,24]. If the Z chromosome has the recessive lethal gene, when the sex chromosomes are of ZW type, gene expression from the unpaired lethal gene leads to the death of female embryos. However, interspecific hybrid infertility and cell chromosomes are linked: If the two parents differ greatly in terms of their chromosomal characteristics, meiosis will be blocked such that the hybrid cannot produce normal germ cells and cannot reproduce [25]. Du et al.[26] analyzed the somatic chromosome karyotypes of Muscovy duck, Strabian duck, and their interspecific F1 hybrid. Compared with their parents, the F1 hybrids had the same number of chromosomes, but a different number of arms. The first and second pairs of autosomes had a centromere index and arm ratio with intermediate values between those of the two parents, the two sister siblings were homologous to the paternal and maternal preference respectively. Sex chromosome centromeres with double characteristics can be used for identification of the authenticity of hybrids. Mank et al.[25] found that the karyotypes of chromosomes 1 and 2 in Muscovy duck and domestic duck were the main causes of F1 sterility in the F1 hybrid. In the present study, hybrid embryos were selected for embryo

development for 3-7 days to detect the sex of early hybrid embryos. The results found that there were among the 90 early embryos, 70 were viable. The ratio of females to males diverged significantly from the theoretical value ($P<0.05$), and there were more males than females. The mortality rate of early female embryos was significantly higher than that of males ($P<0.05$), which further confirmed the sex determination of hybrids as ZZ (♂) and ZW (♀). As hatching progressed, the gender ratio became gradually more unbalanced.

In summary, chromosomes of chickens, quails, and their hybrids were selected and analyzed. Chickens and quails had the same chromosome number (2n=78), but there were significant differences in chromosomal morphology, mainly for chromosomes 1, 4, 6, 8, and the W chromosome. Their chromosome karyotype parameters showed certain differences, and there were differences in the number of bands and the width of the bands in the G-banding experiment. These differences disrupted the inherent balance between the hybrid chromosomes, thereby undermining the meiotic process and increasing the frequency of reproductive failure. The problem of sterility after distant hybridization is a complex biological issue, and comprehensive studies from the aspects of cell biology, morphology, physiology, molecular biology, and immunology are required to determine the mechanisms.

Competing Interest

The authors declare that they have no competing interests.

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Research Article

The Mortality Effects of Some Entomopathogenic Fungi Against *Helicoverpa armigera, Spodoptera littoralis, Tenebrio molitor* and *Blattella germanica* [1]

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Abstract

Laboratory bioassay studies were conducted to determine the effects of 9 isolates of *Beauveria bassiana* and one isolate of *Metarhizium anisopliae,* isolated from the soils in Isparta, Turkey, against *Helicoverpa armigera* (Hübner), *Spodoptera littoralis* (Hübner) (Lepidoptera: Noctuidae), *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) and *Blattella germanica* (L.) (Blattodea: Blattellidae). Concentrations of $2x10^7$ conidia/mL were applied on insects by hand sprayer. All treated insects were incubated at 26±1°C, 70±5% relative humidity for 12 days. All entomopathogenic fungi were infectious to all tested insects but infection rates were different. Isolates of *B. bassiana* and *M. anisopliae* showed very low infection rates on *B. germanica* (3.3-6.7%). Other insects had different mortality rates. *H. armigera, S. littoralis* and *T. molitor* had 53.3-73.3%, 56.7-66.7% and 73.3-80.0% infection rates, respectively. As all fungal isolates were not very effective on *B. germanica* under laboratory conditions but showed effectiveness on other insects.

Keywords: *Biological control, Entomopathogenic fungi, Helicoverpa armigera, Spodoptera littoralis, Tenebrio molitor, Blattella germanica*

Bazı Entomopatojenik Fungusların *Helicoverpa armigera, Spodoptera littoralis, Tenebrio molitor* ve *Blattella germanica'ya* Karşı Öldürücü Etkileri

Öz

Isparta topraklarından izole edilen 9 Beauveria bassiana ve bir *Metarhizium anisoplia* izolatının *Helicoverpa armigera* (Hübner), *Spodoptera littoralis* (Hübner) (Lepidoptera: Noctuidae), *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) ve *Blattella germanica* (L.) (Blattodea: Blattellidae) karşı laboratuvar şartlarında patojeniteleri belirlenmiştir. Fungal izolatlardan $2x10^7$ conidia/mL konsantrasyonları hazırlanarak böceklerin üzerine el spreyi ile uygulanmıştır. Uygulama yapılan böcekler, 12 gün boyunca 26±1°C'de,% 0±5 nispi nemde inkübe edilmiştir. Tüm entomopatojenik funguslar, test edilen tüm böceklere etki göstermiş, ancak enfeksiyon oranları farklılık göstermiştir. *B. bassiana* ve *M. anisopliae* izolatları, *B. germanica* (3.3-6.7%) üzerinde çok düşük enfeksiyon oranı göstermiştir. Diğer böceklerde ise farklı ölüm oranları görülmüştür. *H. armigera, S. littoralis* ve *T. molitor* sırasıyla %53.3-73.3, %56.7-66.7 ve %73.3-80.0 enfeksiyon oranlarına görülmüştür. Bütün fungal izolatlar *B. germanica* üzerinde laboratuvar koşullarında çok etkili olmamış, diğer böcekler üzerinde etkinlik göstermiştir..

Anahtar sözcükler: *Biyolojik mücadele, Entomopatojenik funguslar, Helicoverpa armigera, Spodoptera littoralis, Tenebrio molitor, Blattella germanica*

INTRODUCTION

Many researchers agreed on the importance of entomopathogenic fungi (EPF) for biological control of insect pests [1,2]. Many species of EPF are used to regulate insect pests in glasshouse and field crops, orchards, ornamental, stored products, and forest area. These biological control agents are also practiced for reduction of pest and vector

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insects of veterinary and medical importance [3]. *Beauveria bassiana* (Bals.) Vuill. (Deuteromycotina: Hyphomycetes) and *Metarhizium anisopliae* (Metsch.) Sorokin (Hypocreales: Clavicipitaceae) are the most common EPF found and grow naturally in soils throughout the world and act as a parasite on various insects species [4,5]. EPF are recognized to be an attractive alternative method to chemical pesticides. Several advantages of using EPF for pest control. They are safe for humans and other non-target organisms, no pesticide residues after spraying to target pests, and increases biodiversity in managed ecosystems [3].

The German cockroach, *Blattella germanica* (L.) can be a serious vector of some microorganisms such as pathogenic bacteria that contaminate the foods [6]. Mostly these insects can be observed in houses, apartments, restaurants, markets, hospitals, and bakeries. Because of rapid growing of these insects cause human health problem such as allergies, asthma, and other respiratory diseases. Chemical insecticides have been mostly used to control cockroaches but control failures due to insecticide resistance and chemical contamination of environment have led some researchers to focus on the other alternative control methods. Many researches have been conducted to effect of entomopathogens on cockroaches and some of them reported infections [7]. The most promising of these pathogens are EPF, such as *B. bassiana* [8] being pathogenic to cockroaches [9].

Spodoptera littoralis (Hübner) and *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), are the pests of field crops and *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) is pests at stored products [10]. EPF have been used and tested to control a wide range of insect pests including *Spodoptera* species [11-15]. The EPF, *M. anisopliae* and *Beauveria* spp. were infectious to *T. molitor* [16].

This study aimed to evaluate the mortality effect of locally isolated *B. bassiana* and *M. anisopliae* isolates against *H. armigera*, *S. littoralis, T. molitor,* and *B. germanica* under laboratory condition.

MATERIAL and METHODS

Insect Rearing

The German cockroach, *Blattella germanica* (L.) was collected from locally at some restaurant and cafe from Erzincan, Turkey and produced at laboratory conditions for bioassay study [17] *Helicoverpa armigera* was first collected in the field crops at Erzincan providence and maintained under laboratory conditions using semi-synthetic diet described by Singh and Rembold [18]. *Tenebrio molitor* reared at laboratory using the wheat bran as well as the straw were obtained from the common wheat *(Triticum aestivum* L.) planted in the Lunar Palace [19,20]. *Spodoptera littoralis* were kindly provided by Dr. Umut Toprak from Ankara University, Faculty of Agriculture, Plant Protection Department.

Fungal Species and Their Cultures

The EPF used in this study were isolated from agricultural soil [21] and *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) in Isparta, Turkey. Detail information of the EPF were presented in *Table 1*. Pathogenicity of these isolates were tested to *Galleria mellonella* (L.) in previous studies [21].

Fungal cultures were maintained on sterilized potato dextrose agar (PDA) at 25±2°C. Each EPF isolates of conidia were harvested from the surface of 2 to 3 week-old culture and suspended in 30 mL sterile distilled water with 0.3% Tween-80. Conidial suspensions were filtered through sterile muslin cloth to remove debris. Spore concentration was determined using a haemocytometer and final concentrations of $2x10^7$ conidia/mL were prepared for each fungi isolates [22].

The viability of conidia of each isolate was determined by inoculating $1x10^5$ conidia/mL^{-1} spore suspension on PDA and evaluating the germination after 24 h of incubation at 25±2°C. Percentage germination was determined by counting approximately 100 spores for each plate. The viability was above 90-95% for all isolates.

Application of EPF on Insects

The ten 3rd instar larvae of *H. armigera, S. littoralis T. molitor,* and five adult of *B. germanica* were placed in Petri plates (9 cm diameter) and covered with filter paper. The $2x10^7$ conidia/mL of the final concentration conidial suspension of each isolate were sprayed two times from 30 cm distance with hand sprayers on the insects placed in the petri plates. After drying in room temperature, the treated insects were transferred to new Petri plates with diet for each insect. Each assay consisted of 3 replicates with 10 insects. Control larvae and adult were treated with sterile distilled water with a 0.3% Tween-20. Insects were maintained in an incubator at 26±1°C, 70±5% relative humidity for 12 days.

Statistical Analysis

Larval and adult mortalities were corrected according to Abbott's formula and percent mortality rates were calculated. The data were subjected to ANOVA and subsequently to Tukey test to compare each treatment against controls. Data were analyzed using SPSS version 17.0 software (SPSS Inc., Chicago, IL).

RESULTS

This study was a preliminary virulence test of ten fungal isolates *(Table 1)* to investigate potential as a biocontrol agent against to *H. armigera, S. littoralis T. molitor,* and *B. germanica*. *Table 2* shows the infection rates of the ten EPF isolates ($2x10^7$ conidia/mL) on different larval stages of *H. armigera, S. littoralis* and *T. molitor* and adult form of *B. germanica* within 12 days post inoculation. Isolates of

Table 1. *Fungal isolates, species name, host and source of origin and geographic origin of the EPF isolates used for bioassay study*

Fungal Isolates[a]	Species Name	Host or Source of Origin	Geographic Origin
BMAUM-LDE-001	*Beauveria bassiana*	*Leptinotarsa decemlineata*	Isparta, Turkey
BMAUM -LDE-002	*B. bassiana*	*L. decemliniata*	Isparta, Turkey
BMAUM -K1-001	*B. bassiana*	Soils	Isparta, Turkey
BMAUM -M3-001	*B. bassiana*	Soils	Isparta, Turkey
BMAUIM-M1-001	*B. bassiana*	Soils	Isparta, Turkey
BMAUM-M6-001	*B. bassiana*	Soils	Isparta, Turkey
BMAUM -A4-001	*B. bassiana*	Soils	Isparta, Turkey
BMAUM -A6-001	*B. bassiana*	Soils	Isparta, Turkey
BMAUM -K6-001	*B. bassiana*	Soils	Isparta, Turkey
BMAUM -U3-002	*Metarhizium anisopliae*	Soils	Isparta, Turkey

[a] *BMAUM: Biological Control Research and Development Center, A: Atabey, M: City Center, U: Uluborlu, K: Keçiborlu, LDE: Leptinotarsa decemlineata adult*

Table 2. *Corrected percentage mortality of entomopathogenic fungi isolates (at spore concentration: $2x10^7$ conidia/mL) on Helicoverpa armigera, Spodoptera littoralis Tenebrio molitor, and Blattella germanica (% ± SE) 12 days post inoculation*

No	Entomopathogenic Fungi Species	Isolate No[a]	Percentage Mortality of Insects at 12 Days[b] (%± SE)			
			B. germanica	*S. littoralis*	*H. armigera*	*T. molitor*
1	*B. bassiana*	BMAUM-LDE-001	3.3±3.3[a]	54.2±5.7[b]	63.9±1.4[d]	77.7±0.1[b]
2	*B. bassiana*	BMAUM-LDE-002	3.3±3.3[a]	40.5±6.3[b]	59.7±5.0[cd]	77.7±6.4[b]
3	*B. bassiana*	BMAUM-K1-001	3.3±3.3[a]	44.6±9.8[b]	51.9±1.9[bc]	81.5±7.4[b]
4	*B. bassiana*	BMAUM-M3-001	3.3±3.3[a]	49.4±6.5[b]	63.9±1.4[d]	70.4±3.7[b]
5	*B. bassiana*	BMAUM-M1-001	6.7±3.3[a]	50.0±4.1[b]	68.1±3.7[d]	70.4±3.7[b]
6	*B. bassiana*	BMAUM-M6-001	3.3±3.3[a]	45.2±2.4[b]	68.1±3.7[d]	66.7±0.0[b]
7	*B. bassiana*	BMAUM-A4-001	3.3±3.3[a]	43.5±3.6[b]	63.9±1.4[d]	70.4±3.7[b]
8	*B. bassiana*	BMAUM-A6-001	3.3±3.3[a]	54.4±2.4[b]	63.9±1.4[d]	77.8±0.0[b]
9	*B. bassiana*	BMAUM-K6-001	3.3±3.3[a]	50.0±4.1[b]	63.9±1.4[d]	74.1±3.7[b]
10	*M. anisopliae*	BMAUM-U3-002	6.7±3.3[a]	40.5±6.3[b]	44.0±3.6[b]	70.4±3.7[b]
11	Control	dH_2O/Tween 20	0.0±0.0[a]	26.7±3.3[a]	16.7±3.3[a]	10.0±0.0[a]

[a] *BMAUM: Biological Control Research and Development Center, A: Atabey, M: City Center, U: Uluborlu, K: Keçiborlu, LDE: Leptinotarsa decemlineata adult;*
[b] *Means within columns with the same letter are not statistically different (Tukey's test at P≤0.05)*

B. bassiana and *M. anisopliae* showed very low infection rates on *B. germanica* (3.3-6.7%). Other insects had different mortality rates. The entomopathogenic fungi used in the bioassay were all infectious to *H. armigera*, *S. littoralis* and *T. molitor*. Mortality rates were statistically similar for each isolates on each insect species *(Table 2)*.

DISCUSSION

Adults of *B. germanica* were not very susceptible to EPF isolates in this study. The treatments of EPF showed that mortality level was not significantly different as compared to the control (3.3%-6.7%). Susceptibility of *B. germanica* to EPF isolated from Argentina were shown by Gutierrez et al.[23]. The nymphs and adults of *B. germanica* and the smokybrown cockroach, *Periplaneta fuliginosa* Serville (Blattodea: Blattidae) were tested with isolates of *M. anisopliae* (CEP 085) and *B. bassiana* (CEP 077) using bait and direct contact methods. *Metarhizium anisopliae* caused 60 and 93% mortality in nymphs and adults of *B. germanica,* respectively and 80% mortality on adults by direct contact method. Results showed differences in susceptibility between the two species of cockroaches and between nymphs and adults of the same insects. They also indicated that application methods of EPF are also important factor for insect susceptibility. The other study conducted by Davari et al.[24] to evaluate the toxicity of *B. bassiana* (PTCC5197) and *L. muscarium* (PTCC 5184) against *B. germanica*. Both fungi species were toxic to German cockroach but *B. bassiana* was significantly more effective than *L. muscarium*.

Spodoptera littoralis (Hübner) is a widely distributed polyphagous pest for many economically important crops, such as cotton, tomato, lettuce, cabbage, and so on. It is difficult to control this pest because of its cryptic habitat

and high rate of infestation [10, 25]. Several studies have been conducted to test EPF isolates for potential use as biological control agents. The potential of entomopathogenic fungi often vary among fungal species and strains [26-28]. Our study showed that all EPF isolates tested were infectious to *S. littoralis* but percentage mortality was low not exceeded 50% *(Table 2)*.

A lepidopteran insect pest, *H. armigera*, causes more than 50% loss in yield of important crops such as cotton, vegetables and sunflower. In recent years, due to high levels of insecticide resistance in *H. armigera,* yield loss became more vulnerable. To evaluate alternative methods for the control of this import agricultural pest, Revathi et al.[29] tested, *M. anisopliae, B bassiana,* and *Nomuraea rileyi* in field conditions. The *M. anisopliae* and *B. bassiana* isolates displayed 70% mortality and these species shows higher enzymes (chitinase, protease and lipase) production when compared with *N. rileyi* isolates. Other study also conducted to test susceptibility of third instar *H. armigera* to seven strains of three entomopathogenic fungal species (*M. anisopliae, B. bassiana* and *Paecilomyces fumosoroseus*) under laboratory conditions using the larval immersion method [30]. The mortality was ranging from 68 to 100% in treatments with *B. bassiana* and *P. fumosoroseus* strains. They concluded that all three fungal species, especially *P. fumosoroseus*, have a high potential for biocontrol of *H. armigera* larvae. The EPF isolates tested in this study were infectious to *H. armigera*, mortality ranging 44%-68% and *B. bassiana* isolates statistically more effective than *M. anisopliae* isolate.

In conclusion, **s**everal side effects of chemical pesticides such as development of resistance and negative impact on the environment has encouraged several researchers to investigate alternative control methods on important agricultural pests. In consequence, the development of biopesticides that are effective, biodegradable and no harmful side effect on the environment, turn out to be priority of these studies. Based on our studies, all entomopathogenic fungi isolates were infectious to *H. armigera*, *S. littoralis* and *T. molitor* larvae under laboratory conditions but not to *B. germanica* adults. Furthermore, more detailed studies will be conducted to test the control efficacy of these fungal isolates in greenhouses and different stages of insect pests.

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Research Article

Evaluation of Cellulases and Xylanases Production from *Bacillus* spp. Isolated from Buffalo Digestive System

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Abstract

Cellulases and xylanases have high industrial demand due to their paramount importance in biological processes. The present study was aimed to evaluate the fiber degrading potential of *Bacillus* spp. isolated from buffalo digestive system. A total of fourteen isolates from rumen and eight isolates from dung were screened on carboxymethyl cellulose (CMC; 1%) agar plates, showing clear zone of CMC hydrolysis. All screened isolates were confirmed by targeting the 16S rRNA gene, sequencing and phylogenetic analysis. The enzyme activity index (EAI) of all screened isolates was calculated and it was observed that BR28 *(Bacillus subtilis),* BR96 *(Bacillus amyloliquefaciens),* BD69 (Bacillus tequilensis) and BD92 *(Bacillus sonorensis)* exhibit 2.11, 2.05, 2.56 and 2.45 EAI, respectively. The isolates with high EAI (>2) were selected for further enzyme production studies. The results of enzyme activities showed that BD92 had higher endoglucanse (carboxymethyl-cellulase, CMCase, 240.76±4.12 U/L) and avicelase (153.56±7.28 U/L) activities after 72 h of incubation. However, BR96 showed highest xylanase (3379.27±10.58 U/L) activity than others isolates and previously reported studies. This study provides a helpful insight into the identification of *Bacillus* spp. from the buffalo digestive system with higher production of cellulases and xylanases for their application in the animal feed industry.

Keywords: *Cellulase, Xylanase, Bacillus spp., Phylogenetic analysis, Buffalo digestive system*

Bizon Sindirim Sisteminden İzole Edilen *Bacillus* spp.'den Selülaz ve Ksilanaz Üretiminin Değerlendirilmesi

Öz

Selüloz ve ksilanaza biyolojik süreçlerdeki önemlerinden dolayı yüksek endüstriyel talep vardır. Bu çalışmanın amacı, bizon sindirim sisteminden izole edilen *Bacillus* spp.'nin lifleri parçalama potansiyelinin değerlendirilmesidir. Rumenden on dört ve dışkıdan sekiz izolat karboksimetil selüloz (CMC; %1) agar plakasında CMC hidrolizin açık renkli bölge gösterme durumu bakımından incelendi. İncelenen tüm izolatlar 16S rRNA gen hedeflemesi, sekans ve filogenetik analizlerle onaylandı. İncelenen izolatların tümünün enzim aktivite endeksi (EAE) hesaplandı ve BR28 (*Bacillus subtilis),* BR96 *(Bacillus amyloliquefaciens),* BD69 *(Bacillus tequilensis)* ve BD92 *(Bacillus sonorensis)*'un sırasıyla 2.11, 2.05, 2.56 ve 2.45 EAE gösterdiği belirlendi. Yüksek (>2) EAE değerine sahip olan izolatlar daha sonra enzim üretim çalışmalarında değerlendirildi. Enzim aktiviteleri, 72 saat inkübasyon sonrasında BD92'nin daha yüksek endoglukanaz (Karboksimetil selülaz, 240.76±4.12 U/L) ve aviselaz (153.56±7.28 U/L) aktivitelerine sahip olduğunu gösterdi. Ancak, BR96 diğer izolat ve daha önceden rapor edilenlerden daha yüksek ksilanaz (3379.27±10.58 U/L) aktivitesine sahipti. Bu çalışma, hayvancılık endüstrisinde uygulama bulabilecek bizon sindirim sisteminden. yüksek düzeyde selülaz ve ksilanaz üretebilecek *Bacillus* spp. üretimine faydalı bir bakış sunmaktadır.

Anahtar sözcükler: *Selülaz, Ksilenaz, Bacillus spp., Filogenetik analiz, Bizon sindirim sistemi*

INTRODUCTION

Plants produce 10-50 billion tons of cellulose annually [1] which is earth's most plentiful and attractive raw material for producing many industrially important commodity products. Cellulases and xylanases have an important role in biological degradation of plant cell wall polysaccharides. These plant cell wall polysaccharides comprised of cellulose (35-50%), hemicellulose (20-35%) and lignin (5-30%). Cellulases consist of a group of enzymes such

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as endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.74) and β-glucosidase (EC 3.2.1.21) [2]. The endoglucanase also known as carboxymethyl-cellulase (CMCase) acts on reducing or non-reducing ends of the amorphous cellulose and releases glucose directly or the cellobiose dimer. The exoglucanase also known as avicelase randomly attacks at arbitrary internal amorphous sites (O-glycosidic bonds) of crystalline cellulose and cleaves the polysaccharide chain by inserting a water molecule in the 1,4-β-bond resulting in glucan chains of different lengths. The β-glucosidase hydrolyzes the cellobiose dimers and the cellodextrins of various lengths to glucose [3]. Similarly, xylanase (EC 3.2.1.8) is responsible for degradation of linear polysaccharide β-1,4-xylan into xylose. These fiber degrading enzymes (cellulases and xylanases) have attracted much interest due to their vast applications in food, animal feed, textiles, fuel, chemical and pharmaceutical industry [4,5]. The global market for these enzymes was valued at 899.19 million dollars in 2014 and is projected to reach 1371.03 million dollars by 2020, at an annual growth rate of 7.3% from 2015 to 2020. Cellulases contribute 8% of total worldwide industrial enzyme demand with an expectation of 100% increase in this demand in coming years [6] that can be fulfilled by screening and identifying novel microbes with the highest enzyme production.

Numerous microorganisms that are able to degrade cellulose include bacteria and fungi. Most of the commercially available fiber degrading enzymes have been isolated from fungi, but isolation, screening and characterization of novel fiber degrading enzymes from bacteria is still a highly active research area. As, bacteria have a higher growth rate and more complex glycoside hydrolases providing synergy with higher potency and organismal diversity of extreme niches than fungi, leading to greater production of enzymes. The bacteria belong to the genera *Clostridium*, *Cellulomonas*, *Cellulosimicrobium*, *Thermomonospora*, *Bacillus*, and *Ruminococcus* have been isolated from the variety of sources such as composting heaps, decaying agricultural wastes, elephant feces, gastrointestinal tract of buffalo and horse, soil, and extreme environments like hot-springs [7,8]. Among these, the most attractive medium in which these enzymes are present in the ruminant's digestive system because ruminants have an efficient digestive system with a unique microbial symbiosis owing to the diet of the ruminants which consists of high amounts of fibrous matter. There are at least 30 predominant bacterial species at a total concentration of 10^{10} to 10^{11} bacteria/ml of rumen liquor [9] that plays a critical role in fiber degradation [10,11]. Most attempts have been made to isolate anaerobic microbes from rumen samples that require strict anaerobic conditions and are difficult to maintain. Consequently, isolation, screening and characterization of superior aerobic/facultative thermophilic microbes is of high interest because of easy handling, maintenance and enzymatic production.

Keeping in view above mentioned significance of cellulases and xylanases, the current study was aimed to evaluate superior aerobic/facultative bacterial isolates from the buffalo digestive system (rumen and dung samples).

MATERIAL and METHODS

Sample Collection and Bacterial Isolation

The rumen fistulated adult buffalos were fed a high fiber diet (fresh fodder and wheat straw) with free access to water. The experiments were performed after approval from the institutional animal ethics committee. Representative buffalo rumen content (solid and liquid, 200 g) and dung (150 g) samples were collected using presterilized containers and spatula/plastic bags, respectively. The samples were immediately transferred to the laboratory in an ice box at 4°C. One gram of each sample was then serially diluted up to 10^{-7} in 10 mL of sterile normal saline. Each 100 µL of dilution was inoculated on enrichment medium agar plates containing (g/L) carboxymethyl cellulose (CMC; 10.0), K_2HPO_4 (1.0), K_2HPO_4 (1.0), $MgSO_4.7H_2O$ (0.2), NH_4NO_3 (1.0), $CaCl_2$ (0.02), and agar (20.0) [12]. The plates were incubated at 37°C for 48 h under aerobic conditions. The bacterial colonies were further purified by streaking onto new CMC agar plates.

Qualitative Screening of Fiber Degrading Bacterial Isolates

Each bacterial isolate was inoculated on CMC agar plate for qualitative screening using the Congo-red overlay method [13]. In this method, plates were flooded with 0.3% Congo-red for 20-25 min followed by de-staining with 1 M NaCl solution for 15-20 min or until the clear zones around the colonies were visualized. Colonies showing discoloration of Congo-red were taken as positive fiber degrading microbial colonies. Enzyme activity index (EAI) was calculated using the formula as follows:

$$\text{Enzyme activity index (EAI)} = \frac{\text{(Diameter of zone - Diameter of bacterial colony)}}{\text{Diameter of bacterial colony}}$$

Physiological and Biochemical Characterization of Screened Bacterial Isolates

The bacterial isolates which showed clear zones, were further identified based on morphological and biochemical characteristics using Bergey's manual of systemic bacteriology [14]. Gram's staining was done for morphological identification of bacteria, whereas biochemical identification was done using catalase, citrate utilization, indole, methyl red (MR), oxidase, starch hydrolysis, Voges-Proskauer (VP) and sugars (D-xylose, fructose, lactose, maltose, sorbitol and sucrose) fermentation tests by standard methods [14]. Further, thermo-philicity of the bacterial isolates was also checked at different temperatures (37-60°C).

DNA Extraction and Amplification of 16S rRNA Gene

The DNA was extracted from bacterial cells with conventional phenol-chloroform-isoamyl alcohol method [15] and 16S rRNA gene was amplified using universal primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′) [16]. Each reaction contained 25 µL DreamTaq Green PCR master mix, 2 µL of each primer, 2 µL isolated DNA (50-100 ng), and nuclease free water until the final volume reaches 50 µL. The PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles with denaturation at 94°C for 1 min each, annealing at 55°C for 30 s, extension at 72°C for 1 min, and a final elongation at 72°C for 10 min. Amplified product was checked for size and purity on 1% (w/v) agarose gel.

Bioinformatics Analysis and Phylogenetic Tree Construction

PCR products were sequenced through commercial services provided by Macrogen, Korea. The data of 16S rRNA gene sequences was analyzed using Seq Scanner 2 software and compared with National Center Biotechnology Information (NCBI) database using BLASTN with GenBank (http://www.ncbi.nlm.nih.gov) and the closest matches to bacterial isolates were obtained. The 16S rRNA gene sequences were aligned and a phylogenetic tree was constructed using MEGA 7.0.9 software with neighbor-joining method at 1000X bootstraps [17].

Quantitative Enzyme Assays for Fiber Degrading Bacterial Isolates

Potential fiber degrading isolates (BR28, BR96, BD69, and BD92) showing maximum EAI (>2) on CMC agar plates were further cultured in broth medium containing (g/L) CMC (10.0), yeast extract (5.0), tryptone (10.0), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $MgSO_4.7H_2O$ (0.2), NH_4NO_3 (1.0) and $CaCl_2$ (0.02), incubated at 37°C and 120 rpm for 5 days. Enzyme production during cultivation was assayed in triplicates at 12 h intervals. The supernatants were collected, centrifuged at 10000 × g for 10 min at 4°C and used as a crude enzyme for the enzyme assays. CMCase, avicelase, and xylanase activities were determined using the 3,5-dinitrosalicylic acid (DNS) method [18]. The reducing sugars were estimated spectrophotometrically using glucose and xylose as standards [19,20]. The reaction mixtures were prepared as follows: 500 µL of crude enzyme mixed with 500 µL of 1% (w/v) CMC for determining the CMCase activity; 500 µL of enzyme mixed with 1 mL of 1% (w/v) avicel for determining the avicelase activity; and 500 µL of enzyme mixed with 500 µL of 1% (w/v) birch wood xylan for determining the xylanase activity. The buffer used for dissolving or resuspending the substrates was 100 mM sodium citrate buffer (pH 5.5). The mixtures were incubated at 50°C for 30 min, 60 min and 15 min for CMCase, avicelase and xylanase assay, respectively. Then, the reactions were stopped by adding 1 mL of DNS reagent for CMCase and xylanase assay, while 2 mL of DNS reagent for avicelase assay. All the mixtures were heated in boiling water for 5-10 min for color development. The absorbance was measured at 540 nm [20].

One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of glucose/xylose equivalent per min under the assay conditions. The supernatant was also used for the estimation of proteins by Lowry method [21].

RESULTS

Isolation and Screening of Bacterial Isolates

Isolation and screening of fiber degrading microbes is of immense importance due to their huge demand in industrial applications. In present study initially, a total of 101 isolates were purified from enriched cultures based on shape, size and color. Two third (69) of these isolates were from rumen samples while one third (32) were from dung samples. Out of these, fourteen and eight isolates from the rumen and dung, respectively, were screened for the production of fiber degrading enzymes on CMC agar plates as showed in *Fig. 1*. EAI based on the diameter of the zone of hydrolysis and colony diameter is very useful for predicting the enzyme yield, as an aid to select isolates with a high level of fiber degrading activities. Based on EAI (mm), fiber degrading potential isolates was classified as low (0.1-0.9), medium (1-1.9) and high (>2). Two isolates from rumen samples (BR28, BR96) and two from dung samples (BD69, BD92) have high EAI (>2) as showed in *Table 1*.

Physiological and Biochemical Characterization

The physiological and biochemical characterization of selected isolates (BR28, BD69 BD92 and BR96) have been shown in *Table 2*.

Phylogenetic Analysis

Phylogenetic analysis of all the isolates and their closest related isolates by comparing the contig regions with NCBI GenBank database using BLAST algorithm (http://www.ncbi.nlm.nih.gov) showed that the isolates were the members of two major phyla namely Firmicutes and Actinobacteria with three families Enterococcaceae, Bacillaceae and Streptomycetaceae as presented in *Table 1*. Phylogenetic tree was constructed from selected bacterial isolates (BR28, BR96, BD69 and BD92) and their closest related strains from the GenBank using the neighbor-joining method in MEGA 7.0.9 software at 1000X bootstraps as presented in *Fig. 2*. BD69 and BD92 were found novel being reported for the first time as having fiber degrading activity isolated from buffalo dung samples.

Estimation of Enzyme Activities

The fiber degrading potential of promising isolates was tested by estimating enzyme activities for CMCase, avicelase,

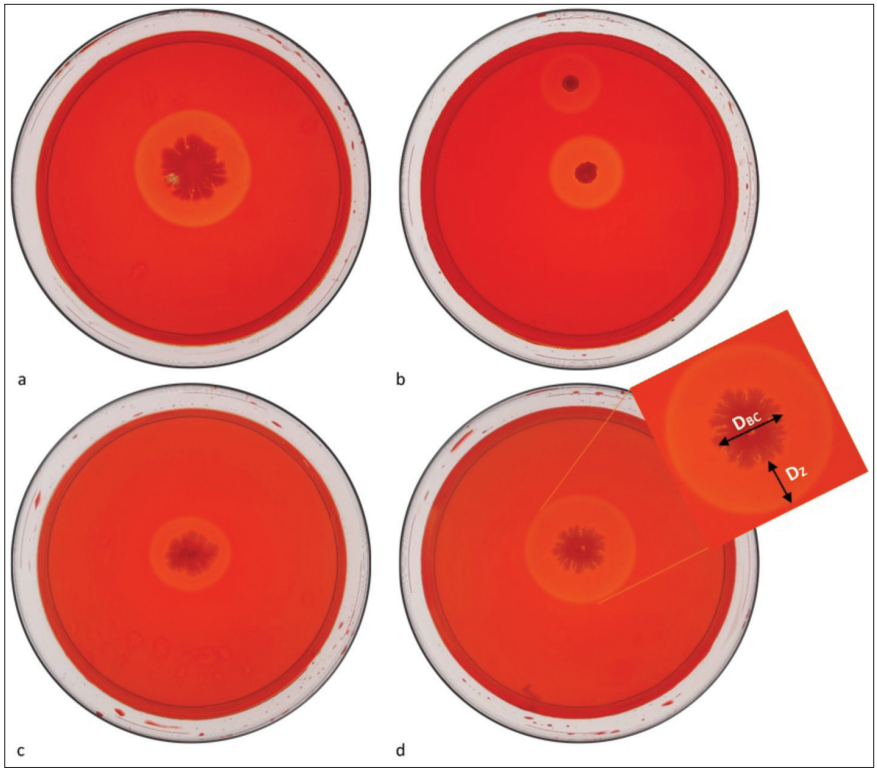

Fig 1. Plate (CMC; 1%) based screening of isolates incubated at 37°C for 48 h. (a) BR28, (b) BD69, (c) BD92 and (d) BR96 (D_{BC} = diameter of bacterial colony; D_Z = diameter of zone) after Congo-red staining (0.3%) showing zones of hydrolysis around the colonies

Table 1. *Identification of bacterial isolates based on 16S rRNA gene homology analysis and EAI*

Type	Isolate's ID	Accession Numbers	Phylum/Family	Closest Related Strains from the GenBank	D_{BC} (mm)	D_Z (mm)	EAI
Buffalo rumen samples	BR4	MF767882	Firmicutes/Bacillaceae	*Bacillus subtilis*	5.0	11.0	1.2
	BR9	MF767883	Firmicutes/Bacillaceae	*Bacillus subtilis*	15.5	23.5	0.52
	BR14	MF767884	Firmicutes/Bacillaceae	*Bacillus subtilis*	19.0	23.0	0.21
	BR17	MF767885	Firmicutes/Bacillaceae	*Bacillus subtilis*	17.5	26.5	0.51
	BR20	MF767886	Firmicutes/Bacillaceae	*Bacillus subtilis*	12.5	24.5	0.96
	BR21	MF767887	Firmicutes/Bacillaceae	*Bacillus subtilis*	6.5	17.0	1.62
	BR28	MF767888	Firmicutes/Bacillaceae	*Bacillus subtilis* subsp. *spizizenii*	9.0	28.0	2.11
	BR38	MF767889	Firmicutes/Bacillaceae	*Bacillus tequilensis*	14.0	29.5	1.11
	BR80	MF767896	Firmicutes/Bacillaceae	*Bacillus cereus*	21.0	31.5	0.5
	BR81	MF767897	Firmicutes/Bacillaceae	*Bacillus subtilis*	9.5	26.5	1.79
	BR88	MF767898	Firmicutes/Bacillaceae	*Bacillus sp.*	5.0	14.5	1.90
	BR90	MF767899	Firmicutes/Bacillaceae	*Bacillus sp.*	18.0	36.5	1.03
	BR96	MF767901	Firmicutes/Bacillaceae	*Bacillus amyloliquefaciens* subsp. *plantarum*	10.0	30.5	2.05
	BR98	MF767902	Firmicutes/Bacillaceae	*Bacillus subtilis*	9.5	26.5	1.79
Buffalo dung samples	BD49	MF767890	Firmicutes/Bacillaceae	*Bacillus subtilis* subsp. *spizizenii*	8.5	21.5	1.53
	BD55	MF767891	Firmicutes/Enterococcaceae	*Enterococcus casseliflavus*	14.0	33.0	1.36
	BD63	MF767892	Firmicutes/Bacillaceae	*Geobacillus sp.*	6.5	8.5	0.31
	BD69	MF767893	Firmicutes/Bacillaceae	*Bacillus tequilensis*	4.5	16.0	2.56
	BD73	MF767894	Firmicutes/Bacillaceae	*Bacillus subtilis*	9.5	25.5	1.68
	BD77	MF767895	Firmicutes/Bacillaceae	*Bacillus subtilis*	13.5	31.5	1.33
	BD92	MF767900	Firmicutes/Bacillaceae	*Bacillus sonorensis*	11.0	38.0	2.45
	BD99	MF767903	Actinobacteria/ Streptomycetaceae	*Streptomyces sp.*	16.5	28.5	0.73

D_{BC} = diameter of bacterial colony; D_Z = diameter of zone; EAI = enzyme activity index

and xylanase using CMC, avicel and birch wood xylan as substrate, respectively. The mean CMCase activities ranges from 22.24-55.82, 17.56-66.19, 48.60-240.76 and 38.46-118.62 U/L for BR28, BD9, BD92 and BR96, respectively as presented in *Fig .3a*. Whereas, BD92 showed highest mean CMCase activity which increases to its maximum (240.76±4.12 U/L) at 72 h of incubation while BR28, BD69 and BR96 showed maximum mean activities 55.82±2.22, 66.19±3.62 and 118.62±5.65 U/L at 24, 36 and 60 h of incubation, respectively. The mean avicelase activities ranges from 31.54-90.32, 37.16-116.03, 54.70-153.56 and 31.58-65.88 U/L for BR28, BD69, BD92 and BR96, respectively as presented in *Fig .3b*. The isolate BD92 showed highest mean activity (153.56±7.28 U/L) at 72 h of incubation while BR28, BD69 and BR96 showed maximum mean activities 90.32±4.34, 116.03±4.01 and 65.88±3.88 U/L at 60, 72 and 48 h, respectively, of incubation. Similarly, the mean xylanase activities ranges from 7.65-955.74, 23.32-1009.57, 771.54-2921.54, and 167.97-3379.27 U/L for BR28, BD69, BD92 and BR96 respectively as presented in *Fig. 3c*. BR96 showed highest mean activity (3379.27±10.58 U/L) at 24 h of incubation when compared with BR28, BD69 and BD92 which showed highest mean activity 955.74±7.52, 1009.57±11.57 and 2921.54±59.12 U/L at 24, 24 and 36 h of incubation, respectively.

Table 2. *Identification of physiological and biochemical characteristics of promising bacterial isolates*

Characteristic Features	Isolate's ID			
	BR28	BD69	BD92	BR96
Gram's staining	+	+	+	+
Growth on MacConkey	-	-	-	-
Morphology	B	B	B	B
Spores	+	+	+	+
Methyl red	-	-	-	-
Voges-Proskauer	+	+	+	+
Aerobic	+	+	+	+
Catalase	+	+	+	+
Oxidase	-	-	-	-
Starch hydrolysis	+	+	+	+
Indole	-	-	-	-
Citrate utilization	+	+	+	+
Growth at 50°C	+	-	+	-
Growth at 60°C	-	-	+	-
Lactose	+	+	+	+
D-xylose	+	+	+	+
Fructose	+	+	+	+
Maltose	+	+	+	+
Sucrose	+	+	+	+
Sorbitol	+	+	+	+

B = bacilli; - negative; + positive

DISCUSSION

Approximately 70% of plant biomass is locked up in 5 and 6 carbon sugars, which are found in lignocellulosic biomass comprised of mainly cellulose (35-50%), lesser hemi-celluloses (20-35%) and least of all lignin (5-30%) [22]. The degradation of plant cellulosic material is not an easy task. There are a lot of chemical, physical and biological pretreatment methods available for this purpose, but these methods are not environmentally safe, they generate toxic substances inhibitory to fermentation that makes process environmentally unfavorable and uneconomical [23]. Degradation of plant cellulosic materials using microbial enzyme systems is an economical and environment friendly process. There has been increasing interest for the degradation of plant cellulosic material using bacteria because of their faster growth than fungi. Habitats that contain plant-based substrates are the best sources to find these microorganisms. The buffalo digestive system has been selected as a source for obtaining novel desirable fiber degrading microbes because there is a rich assemblage of fiber degrading microbes owing to the diet of the ruminants that primarily consists of huge amounts of cellulosic matter.

In the present study, out of 101 isolates, 14 from rumen

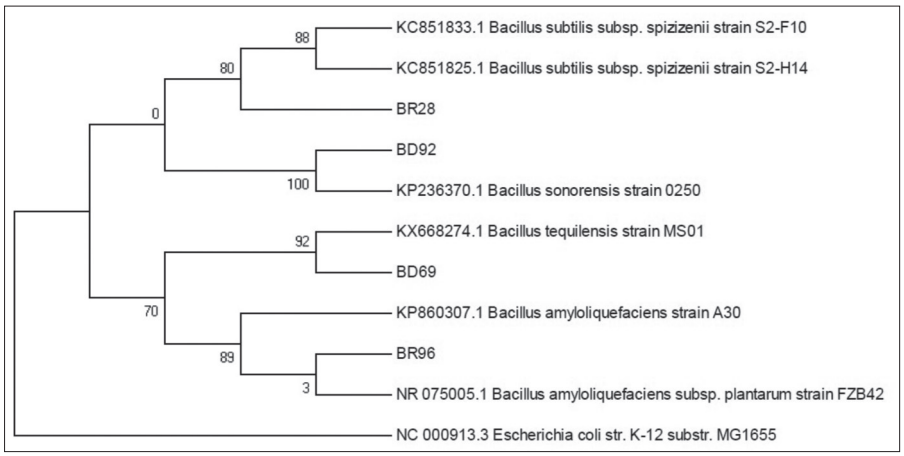

Fig 2. Phylogenetic tree of 16S rRNA gene sequences from promising fiber degrading bacterial isolates and their closest related strains from the GenBank. The tree was generated by using the neighbor-joining method in MEGA 7.0.9 software at 1000X bootstraps

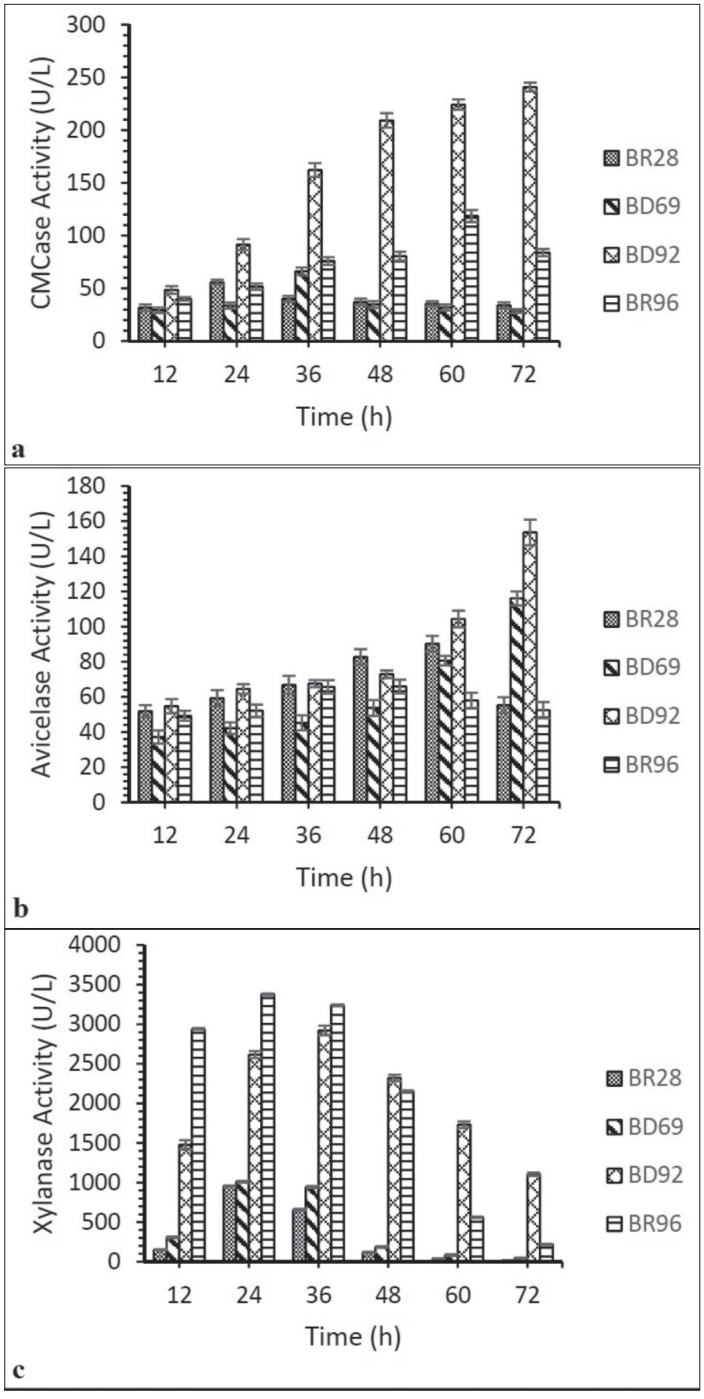

Fig 3. Mean fiber degrading enzyme production (n=3) profile of BR28, BD69, BD92 and BR96 isolates in time course of 120 h showing (a) CMCase, (b) avicelase and (c) xylanase activities

and 8 from dung samples were found positive for fiber degrading potential on CMC agar plates using Congo-red staining. Congo-red interacts with cellulose in agar medium, but when bacteria secrete fiber degrading enzymes that degrade cellulose into cellobiose/glucose and organic acids which lowered the pH of medium [3]. This pH difference affected the color of the medium and form a clear zone around the colony indicating enzyme production. EAI is a very useful parameter for predicting the enzyme yield [24]. In the present study, the isolates showed EAI >2 proved to be more prolific producers of these enzymes. Our results on the morphological/ biochemical characteristics were similar to those reported by Ozkan and Ahmet [8], who reported *Bacillus* strains from rumen samples. While, it has also been reported that buffalo has higher fiber degrading bacteria than other farming animals may be due to the fact that buffalo used fibrous feed more efficiently as compared to other farming animals. The fiber degrading potential of lower tract microbial population is high as compared to rumen microbial population which would affect the type of fermentation and the end-products [25].

Molecular characterization was done using 16S rRNA gene sequencing because 16S rRNA region is highly conserved and protected from mutation in the evolutionary period. Results confirmed that the isolates were members of two major phyla namely Firmicutes (95.65%, the most dominant culturable rumen and dung microbiota) and Actinobacteria (4.35%) and three families Streptomycetaceae, Enterococcaceae and Bacillaceae. Our findings were parallel to those in earlier studies on microbiota from the gastrointestinal tract of ruminants [8,26]. Our results were also consistent with previous studies those isolated *Bacillus* species from the rumen, cow dung and Tibetan pig's intestine [27,28]. There are continuous efforts to find novel and efficient microbial isolates with fiber degrading potential. To our best knowledge we have isolated two *Bacillus* spp., BD69 *(Bacillus tequilensis)* and BD92 *(Bacillus sonorensis)* from dung samples with the highest EAI reported first time, which indicated their ability to produce fiber degrading enzymes. Moreover, the isolate BD92 *(Bacillus sonorensis)* has the ability to survive up to 60°C which highlights its industrial importance.

The enzyme production in liquid culture medium has been determined at different time intervals using the DNS method. In this method, free carboxyl groups were released by the oxidation of aldehyde groups of the glucose molecule, which is formed by the action of fiber degrading enzymes. These reduce 3,5-DNS (yellow) to 3-amino-5-nitro salicylic acid (orange) under alkaline conditions [18]. In the present study, BD92 has higher mean CMCase (240.76±4.12 U/L) and avicelase (153.56±7.28 U/L) activity at 72 h, when compared to BR28, BD69 and BR96. Liang et al.[29] reported CMCase activity of 0.01 U/mL from *Bacillus subtilis* which was lower than the CMCase activity of the isolates in present study. They also showed that enzyme activity could be improved by optimizing carbon and nitrogen components in the media as ME27-1 *(Paenibacillus terrae)*. They improved production from 0.17 to 2.08 U/mL when using wheat bran and NH_4Cl as carbon and nitrogen source,

respectively in their study. The production of fiber degrading enzymes generally depends on a variety of growth parameters which include inoculum size, pH, temperature, medium additives (carbon and nitrogen sources), aeration, growth and time [30-32] and also on the presence of various metal ions as activators and inhibitors [33].

In present study, it was proved that *Bacillus* spp. have capability to produce CMCase and avicelase which was comparable to another study conducted by Ladeira et al.[34] whereas, Fukumori et al.[35] reported that alkalophilic *Bacillus subtilis* strains 1139 and N-4 were capable of hydrolyzing CMC, but could not degrade avicel significantly. Xylanase is also an important fiber degrading enzyme, BR96 produces high mean xylanase activity in this study, which was comparable to previously reported studies [36,37]. This proves that novel subspecies of *Bacillus* isolated in this study showed higher fiber degrading enzyme production compared to previously reported studies [7,22,29,38,39]. Further, growth optimization and enzyme characterization of these novel microbes are necessary for large scale production and offer a promising approach for its possible use in the animal feed industry.

In conclusion, present study demonstrates that the buffalo digestive system contains a novel community of fiber degrading isolates which play an important role in the degradation of fibers. Moreover, we have reported *Bacillus* spp. which demonstrates that the rumen and dung have a great potential to be a source of fiber degrading microbes.

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Research Article

The DNA Vaccine Combining the Adjuvant Porcine IL-12 with the Spike Gene of Transmissible Gastroenteritis Virus Enhances the Immune Response in Swine

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Abstract

Transmissible gastroenteritis (TGE), cause by transmissible gastroenteritis virus (TGEV), is an acute digestive and highly infectious disease in piglets. In this study, porcine interleukin-12 immunological adjuvant combined with a DNA vaccine bearing the TGEV-S1 gene was used to immunize piglets, and assessed the immune response of piglets. The results showed that $CD4^+$ and $CD8^+CD28^+$ T lymphocytes were significantly increased in immunized piglets compared to control groups. In addition, the observed increase of IFN-γ suggested that T helper cells tended to convert to Th1 cells during immune response. HE stain and indirect immunofluorescence assays indicated obvious differences between the immunized piglets and the controls, suggesting that immunized piglets suffered less pathological changes relative to piglets in the control groups. Moreover, the levels of anti-TGEV and neutralizing antibodies in serum also indicated an effective immune response in the immunized piglets by which the pathogen was rapidly controlled. Altogether, our results suggest that the combination of pVAX1-(pIL-12) and pVAX1-(TGEV-S1) in a vaccine is capable of producing a significantly enhanced immune response in piglets.

Keywords: *Transmissible gastroenteritis virus, Spike protein, DNA vaccine, porcine interleukin-12 gene, Immunologic adjuvant*

Adjuvant Domuz IL-12 ile Kombine Transmissible Gastroenteritis Virüs Spike Geni Taşıyan DNA Aşı Domuzlarda Bağışıklık Yanıtı İyileştirir

Öz

Transmissible gastroenteritis virüs (TGEV)'un neden olduğu Transmissible gastroenteritis (TGE) akut sindirim sistemi hastalığı olup domuzlarda oldukça enfeksiyöz bir hastalıktır. Bu çalışmada, TGEV-S1 geni taşıyan DNA aşısı ile kombine olarak domuz interlökin-12 immunolojik adjuvant domuzları immunize etmek amacıyla kullanıldı ve domuzlarda meydana gelen immun yanıt değerlendirildi. Sonuçlar, kontrol grubu ile karşılaştırıldığında immunize edilen domuzlarda CD4+ ve CD8+CD28+ T lenfositlerin anlamlı derecede arttığını gösterdi. Ayrıca, artan IFN-γ immun yanıt süresince yardımcı T lenfositlerin Th1 hücrelerine dönüştüğüne işaret etmekteydi. HE boyaması ve indirek immunofloresans tekniği immunize edilenler ile kontrol arasında bariz farkların olduğunu gösterdi. Bu durum immunize edilen domuzların kontrol grubundakine oranla daha az patolojik değişikliklere maruz kaldığına işaret etmekteydi. Serumdaki anti-TEGV seviyesi ve nötralize edici antikorların miktarı immunize edilen domuzlarda daha etkili immun yanıtın oluştuğunu ve patojenin hızlı bir şekilde kontrol altına alındığını gösterdi. Elde edilen sonuçlar, pVAX1-(pIL-12) ve pVAX1-(TGEV-S1)'nin aşıda kullanılmasının domuzlarda immun yanıtı anlamlı oranda iyileştireceğini göstermiştir.

Anahtar sözcükler: *Transmissible gastroenteritis virüs, Spike protein, DNA aşı, domuz interlökin-12 geni, İmmunolojik adjuvant*

INTRODUCTION

Transmissible gastroenteritis (TGE), cause by transmissible gastroenteritis virus (TGEV), is an acute digestive and highly infectious disease in pigs, with vomiting, severe diarrhea, and dehydration being classical symptoms. Previous study showed that pigs of all breeds and ages are susceptible, but TGEV mainly infects suckling piglets

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and being frequently accompanied by other pathogens. Consequently, the mortality rate of suckling piglets might reach 100%. The mortality rate in older pigs will be lower, the absence of effective preventive methods greatly affected the pig industry, and causing significant economic loss [1-3].

TGEV is an enveloped coronavirus of the subfamily *Coronavirinae* of the family *Coronaviridae*. The subfamily *Coronavirinae* consists of four genera: the alpha-, beta, gamma-, and delta-coronavirus. The transmissible gastroenteritis virus (TGEV) belongs to the alphacoronavirus genus, which is characterized by a single-stranded positive-sense RNA genome [4,5]. The alphacoronavirus genome encodes for four structural proteins: the spike (S), nucleocapsid (N), membrane (M), and envelope (E) proteins and for three non-structural proteins (by the open reading frames ORF1, ORF3 and ORF7). The virion surface is composed of a number of spike (S) proteins which contain four antigenic sites. In this study, the TGEV-S1 gene was used in our DNA vaccine against TGEV. This gene encodes for a spike (S) protein, which contains antigen sites known to induce the production of neutralizing antibodies [6,7].

Currently, several countries have carried out monovalent and combined vaccines against TGE, but the inactivated vaccine does not induce an effective immune response and the attenuated vaccine has relatively high risks associated with virulence factors. Thus, a novel and effective vaccine to prevent TGEV needs to be developed [5]. The use of DNA vaccines may be an effective approach to protect swine from TGEV, and number of studies have suggested that the eukaryotic expression of plasmids could induce the immune response [8,9]. Moreover, many reports have also suggested that the activity of cytokine adjuvant can further enhance this effect. Cytokine interleukin (IL)-12 is known to regulate and promote cellular immunity and therefore, has attracted the attention of researchers as a potential immune-adjuvant [10,11]. In this study, the potential of the DNA vaccine bearing the TGEV S1 gene and the adjuvant pIL12 to prevent TGEV infection have been assessed in piglets, that may provides a theoretical basis for further research on DNA vaccines combined with immune-adjuvant.

MATERIAL and METHODS

Plasmid Construction

The full-length S gene of TGEV strain PUR46 was used as PCR template. PCR was carried out using a forward primer (a) (5′-GGGGAAGCTTGCCACCATGAAAAAACTATTTGTG-3′), and a reverse primer (b) (5′-CCCCGAATTCTTAGTTAGTTT GTCTAATA-3′) which contained a *Hin*dIII (a) and *Eco*RI (b) restriction enzyme site (underlined), respectively. The PCR parameters for TGEV-S1 gene amplification (primers P1/P2) were as follows: 95°C for 5 min, 30 cycles of 94°C for 1 min, 45.7°C for 1 min, and 72°C for 2 min followed by a final extension of 72°C for 10 min. Products were purified and subjected to restriction enzyme digestion, then ligated into the appropriate pVAX1 (Invitrogen, USA) eukaryotic expression vector. Similarly plasmid pVAX1-(pIL-12) ligated into the pVAX1 (Invitrogen, USA) eukaryotic expression vector by polymerase chain reaction (PCR), splicing by overlap extension (SOE)-PCR and restriction enzyme digestion, that had been constructed in our previous study [12].

Immunofluorescence Assays

The pVAX1, pVAX1-(pIL-12), or pVAX1-(TGEV-S1) were diluted at 1 μg/μL by 0.1M PBS (phosphate buffer saline), and the plasmids (1 μg) were transfected into BHK-21 cells by Lipofectamine2000 (Invitrogen) respectively, then cultured about 24h (5% CO2, 37°C), according to the method as described previously [12], the cells were fixed with 4% paraformaldehyde (w/v) and then incubated with anti-pIL-12 (p40) or anti-TGEV-S1 antibody (1:200). Finally the cells were incubated with fluorescein isothiocyanate-labelled (FITC) goat-anti rabbit immunoglobulin (1:200) in darkness, then, the green fluorescences were detected by fluorescence microscope (Leica, Germany).

Immunization

The seven-day-old piglets divided into five groups (n=4, *Table 1)*. All piglets were injected with 500 μL 0.2% lidocaine hydrochloride in the cervical muscle. After 15 min, the piglets were injected with 500 μL PBS (no treatment control group), or 500 μg plasmid (pVAX1, empty vector control group), (pVAX1-(pIL-12), pIL-12 treatment group), (pVAX1-(TGEV-S1), TGEV-S1 treatment group) in 500 μL

Table 1. *Experimental groups and immune interval*

Group	Number of Piglets	Vaccine Category	Immunizing Dose	Interval of Treatments
A	4	PBS	500 μL	7-days-old, 21-days-old, 35-days-old
B	4	pVAX1	500 μg	7-days-old, 21-days-old, 35-days-old
C	4	pVAX1-(pIL-12)	500 μg	7-days-old, 21-days-old, 35-days-old
D	4	pVAX1-(TGEV-S1)	500 μg	7-days-old, 21-days-old, 35-days-old
E	4	pVAX1-(pIL-12) + pVAX1-(TGEV-S1)	500 μg 500 μg	7-days-old, 21-days-old, 35-days-old

The five groups include PBS (A), pVAX1 (B), pVAX-pIL-12 (C), pVAX1-(TGEV-S1) (D) and pVAX1-(pIL-12) + pVAX1-(TGEV-S1) (E). number of piglets, vaccine category, and Immunizing dose are indicated

PBS, or 500 µg plasmid pVAX1-(pIL-12) + 500 µg plasmid pVAX1-(TGEV-S1) (combined pIL-12-TGEV-S1 treatment group) in 500 µl PBS respectively. The piglets were injected three times at 2-week intervals (7-days, 21-days, 35-days of age). At 42-days of age, the piglets were exposed to a virus challenge after which blood samples were collected for analysis [13,14], and collected the samples of blood and histology at 47-days. The animal study was performed in compliance with institutional guidelines and animal welfare. All animal experiments were approved by the Animal Ethics Committee of Northeast Agricultural University of China (Protocol number: SRM 16).

Analysis of T lymphocytes

After the lymphocytes were separated from peripheral blood, a lymphocyte proliferation assay was undertaken, as previously described [15,16]. In summary, the lymphocytes were diluted in RPMI1640 (Gibco, USA) and added to 96-well plates (10^6 cells in 50 µL/well). Then, recombinant TGEV-S1 protein (20 µg/mL) and concanavalin A (Sigma, Germany) were added as stimulating agents. After 48 h, WST-1 (6.5 mg/mL, 10 µL/well, Boster, USA) was added to each well. At this stage, samples were left to incubate for 4 h at 37°C in 5% CO_2, after which the viability of lymphocytes was assessed at OD 450 nm.

The peripheral blood lymphocytes (PBL) were re-suspended to 1×10^7 cells/mL and incubated separately with an anti-CD4+ T cell antibody (FITC-labelled) (BD, USA) and an anti-CD8+ T cell antibody (PE-labelled) (BD, USA), and simultaneously with both anti-CD8+ T cell antibody (PE-labelled) and an anti-CD28+ T cell antibody (APC-labelled). The incubation period lasted for 30 min (5% CO_2, 37°C). The specific lymphocytes subgroups were detected with flow cytometric analysis (BD, USA).

Detection of TGEV Antibody

The TGEV-specific antibody (IgG, IgA) from serum of vaccinated piglets were detected by enzyme-linked immunosorbent assay (ELISA), after conjugation with recombinant TGEV S1 protein (50 µg/ml diluted with 0.05 M $NaHCO_3$) in the ELISA plate overnight at 4°C [17], blocking for 1 h at 37°C and incubated with sera samples from piglets for 1 h at 37°C, then added Horseradish peroxidase -labelled goat anti-pig IgG (Proteintech, China) and the Horseradish peroxidase -labelled goat anti-pig IgA (Proteintech, China) respectively for 1 h at 37°C [18-20]. Plates were measured at OD 490 nm.

The efficacy of the TGEV-neutralizing antibodies in the serum of vaccinated piglets was determined by a viral neutralization test [21]. In summary, piglets' sera samples (double dilution from 1:20 to 1:160) were mixed with TGEV viral diluent (1 mL) of ($10^{-4.67}$/0.1 mL TCID50) at 37°C. Samples were left to incubate for 1 h, then added to a monolayer of swine testis (ST) cells in 24-well plates (5% CO2, 37°C) to assess the cytopathic effects during a period of 48 to 60 h.

Detection of Cytokines

Levels of IL-4 and interferon-γ in serum were detected with test kits, in accordance with the manufacturer's instructions (YuanYe, China) [22,23]. Standard curve using a dilution series of IL-4 (256 pg/mL, 128 pg/mL, 64 pg/mL, 32 pg/mL, 16 pg/mL, 0 pg/mL) and IFN-γ (400 pg/mL, 200 pg/mL, 100 pg/mL, 50 pg/mL, 25 pg/mL, 0 pg/mL) were prepared. These solutions were then incubated in ELISA plates for 1 h at 37°C alongside sera samples from the piglets. Horseradish peroxidase-labelled goat anti-pig immunoglobulin was added to each well and measured at OD 450 nm. The serum concentrations of IL-4 and IFN-γ were estimated using the standard curves.

Histological Analysis

Frozen sections of tissues were analyzed using indirect immunofluorescence and hematoxylin and eosin staining (HE) assays. In summary, samples were embedded in optimal cutting temperature (OCT) compound and sectioned (7 µm thick). The indirect immunofluorescence assay was based on the use of the polyclonal antibody of TGEV and fluorescein isothiocyanate-labelled (FITC) goat-anti rabbit immunoglobulin, and nucleuses were dyed by propidine iodide (PI). The HE stain was applied according to the standard method [24]. Tissue sections were observed under a microscope (Leica DM2000, Germany).

Statistical Analysis

Data analysis was performed by SPSS V13.0 software; "p" was defined by one-factor analysis of variance statistically.

RESULTS

The recombine pVAX1-(pIL-12) and pVAX1-(TGEV-S1) plasmids could expressed in mammalian cells, through observed the green fluorescences in *Fig. 1*A,B,C. The proliferation and differentiation of T lymphocytes were an important stage in immune response, so the T lymphocyte proliferation assay could reflect an immune state of organism. Results showed that *(Fig. 2A)*, T lymphocytes were stimulated with specific antigen TGEV-S1 protein and non-specific antigen ConA (not shown in the figure as a comparison), the TGEV-S1 and the pIL-12 + TGEV-S1 treatment groups had significantly increased, which might be due to a beneficial reaction and a favorable state of T lymphocytes.

The $CD4^+$T and $CD8^+$T lymphocytes were very valuable in immune system, and have different marker on the surface of T cells. The changes in $CD4^+$T and $CD8^+$T lymphocytes in peripheral blood from piglets in all experimental groups were evaluated by flow cytometry *(Fig. 3)*. The number of $CD4^+$T lymphocytes in the pIL-12 + TGEV-S1 group

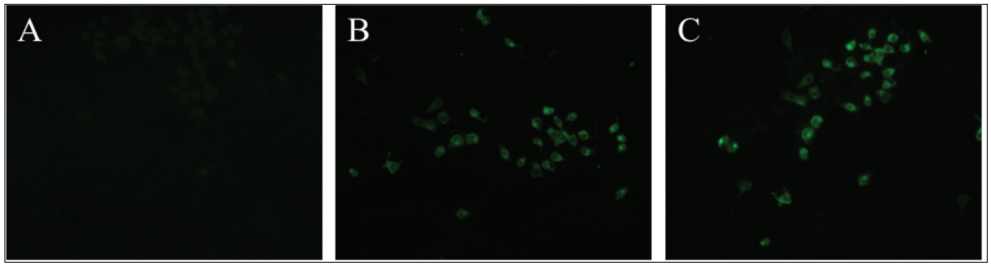

Fig 1. Immunofluorescence analysis of BHK-21 cells transfected with recombinant plasmids. BHK-21 cells were transfected with pVAX1-(pIL-12) and pVAX1-(TGEV-S1) respectively. (A) pVAX1 vector control, (B) pVAX1-(pIL-12) plasmids, (C) pVAX1-(TGEV-S1) plasmids. Transient expression of proteins were detected with anti-pIL-12 or anti-TGEV-S1 antibody. The green fluorescences indicated positive protein expression

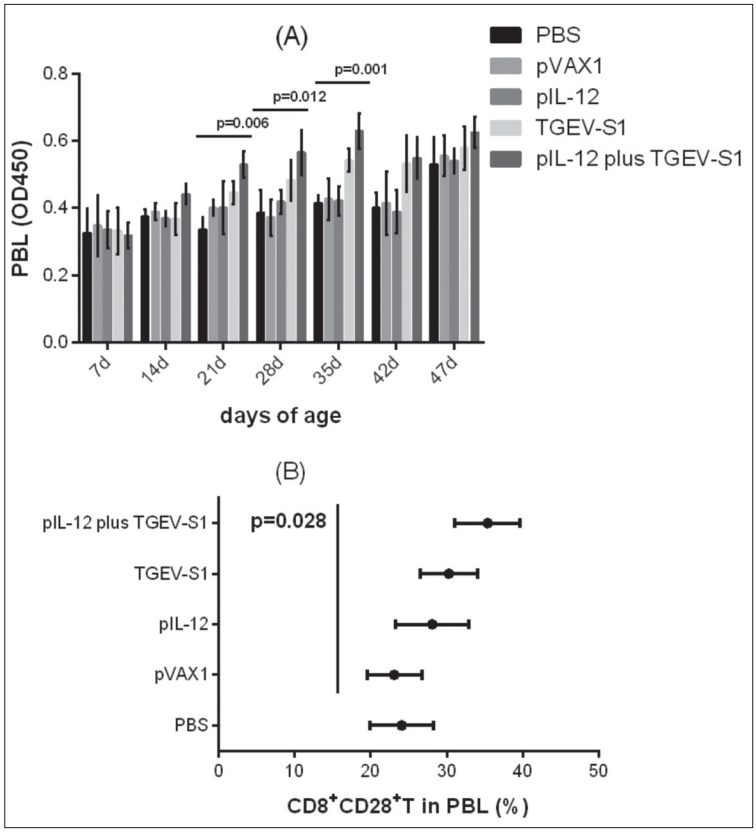

Fig 2. The proliferation of T lymphocytes and the quantity of $CD8^+CD28^+$T in peripheral blood. T lymphocytes proliferate in peripheral blood (PBL) of piglets (A) were analyzed by WST-1 assay. The y-coordinate represents the lymphocyte proliferate index (OD 450nm) in peripheral blood. The quantities of CD8+CD28+T in peripheral blood were determined by flow cytometry (B)

was considerably increased at 28 and 42 days of age. The number of $CD8^+$T lymphocytes, at 35 days of age, in the pIL-12 + TGEV-S1 group was also significantly increased, and at day 47, after virus challenge the number of $CD8^+$T lymphocytes in all groups were increased obviously, it would be an effective immune response by cytotoxic lymphocyte when the organism was infected by the virus.

Changes in $CD8^+CD28^+$T lymphocytes in peripheral blood from experimental animals at 35 days of age were also evaluated by flow cytometry *(Fig. 2B)*. The number of $CD8^+CD28^+$T lymphocytes in the pIL-12, TGEV-S1 and pIL-12 + TGEV-S1 groups showed a tendency to increase and the pIL-12 + TGEV-S1 group had a number of $CD8^+CD28^+$T lymphocytes significantly higher than those of the control groups.

The levels of TGEV-specific antibodies in the serum of immunized piglets were examined using an indirect ELISA assay *(Fig. 4A,B)*. In general, starting from 21 days of age, the TGEV-S1 and pIL-12 + TGEV-S1 groups showed a significant increase in IgG antibodies: Additionally, the IgG antibodies increased sharply in the same groups at day 47, after the exposure to the virus challenge test. However, the pIL-12 + TGEV-S1 and the TGEV-S1 groups did not differ significantly, between them regarding the IgG response in piglets. The IgA antibodies from the serum of immunized piglets at 35 days of age were significantly induced in the TGEV-S1 and the pIL-12 + TGEV-S1 groups *(Fig. 4B)* compared to other groups, although no difference was detected between the two treatment groups.

The levels of neutralizing antibody in serum of immunized piglets at 35 days of age were detected using virus neutralizing assays. The TGEV-S1 and pIL-12 + TGEV-S1 groups showed a more effective neutralizing antibody production *(Fig. 4C)*. Moreover, there was a significant difference between the TGEV-S1 and the pIL-12 + TGEV-S1 groups. The neutralizing antibody titers were 1:40.5 and 1:114.5 in the TGEV-S1 and pIL-12 + TGEV-S1 groups, respectively.

The levels of IFN-γ and IL-4 in different samples of serum, as analyzed by the ELISA method, are shown in *Fig. 5*. Starting at 21 days of age, the IFN-γ serum levels in the pIL-12, TGEV-S1 and pIL-12 + TGEV-S1 groups were significantly increased, The pIL-12 + TGEV-S1 group showed a noticeable increase in the IFN-γ serum level at day 35 and 42. The concentration of IL-4 did not vary significantly among groups.

The results of the HE stain and indirect immunofluorescence assays on frozen sections suggested significant differences

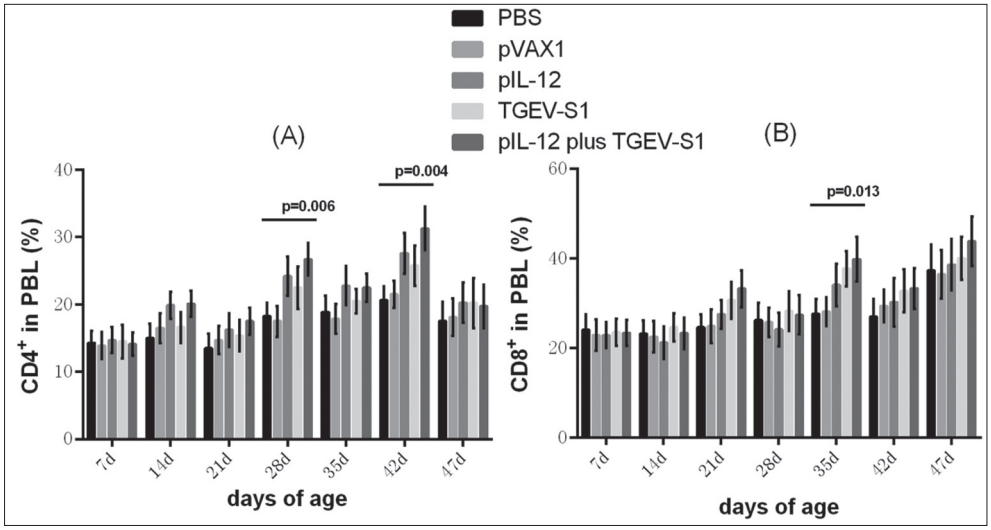

Fig 3. Changes of CD4⁺T and CD8⁺T in peripheral blood. Lymphocytes from the peripheral blood of piglets immunized with pVAX1-(TGEV-S1) and pVAX1-(pIL-12) were collected and subjected to flow cytometry to evaluate the proportions of CD4⁺T and CD8⁺T cells in peripheral blood lymphocytes (PBL)

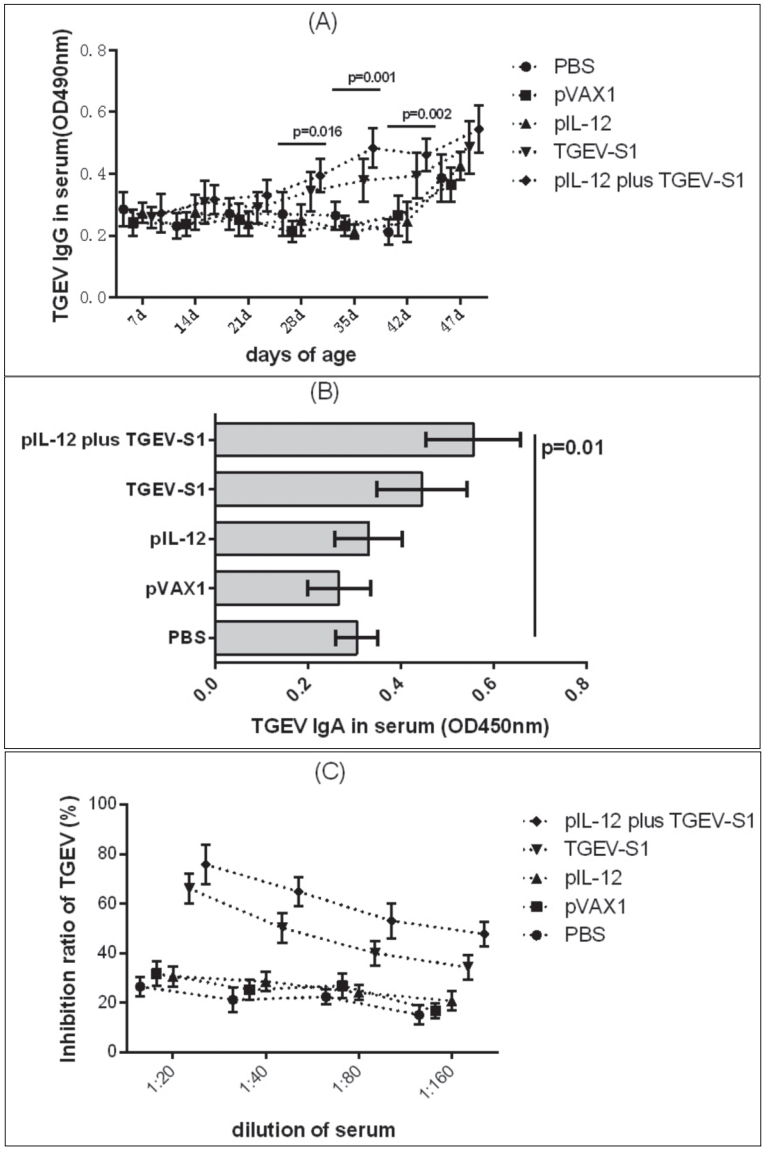

Fig 4. The specific antibody concentration of humoral immunity. The levels of Anti-TGEV IgG (A) and IgA (B) antibody in serum samples were detected by indirect ELISA. The levels of neutralizing antibodies in serum (C) were detected by virus neutralizing assays

(Fig. 6) regarding pathological change and viral replication rate after viral infection. The small intestine epithelium denaturation and endochylema vacuolization, some nucleus concentrate and smash or lysis, some inflammatory cells infiltration are conspicuous in HE stain of the control groups, and these pathological changes show an obvious weakening trend from A to E in *Fig. 6*. Moreover, these groups also have a different fluorescence signal obviously in the results of the indirect immunofluorescence, the red fluorescence represents nucleuses, and the green fluorescence indicated the content of viruses in the intestine, which also had an obvious decreasing trend from F to J in *Fig. 6*.

DISCUSSION

The S protein of TGEV has four antigenic epitopes near the N terminal portion (S1) [25]. The S1 gene has been reported to induce more impactful immune responses than the full-length S gene of TGEV in piglets [6]. Furthermore, the cytokine IL-12 is known to promote the differentiation of naive CD4⁺T cells into Th1, enhancing the activity of T cells and natural killer cells [26]. The cytokine IL-12 might also increase the production and activation of Th1-associated immunoglobulin (such as IgG2a) directly or through the effects of type-1 cytokines (such as IFN-γ) [27], and thus it has been widely used in nucleic acid vaccine research as an immunologic adjuvant [23,28,29]. In our study, the DNA vaccine for TGEV combining the TGEV S1 gene with pIL-12 as an immunologic adjuvant assessed its effect on the immune response in piglets.

T lymphocytes are main effector cells of the cellular and humoral immune response, which

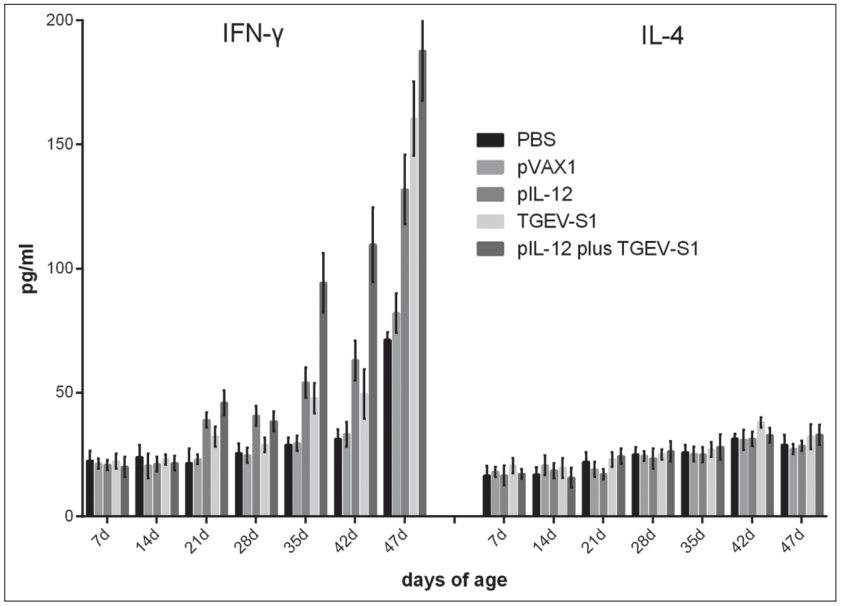

Fig 5. Changes of IFN-γ and IL-4 in serum. The levels of IFN-γ and IL-4 in serum samples were detected by ELISA

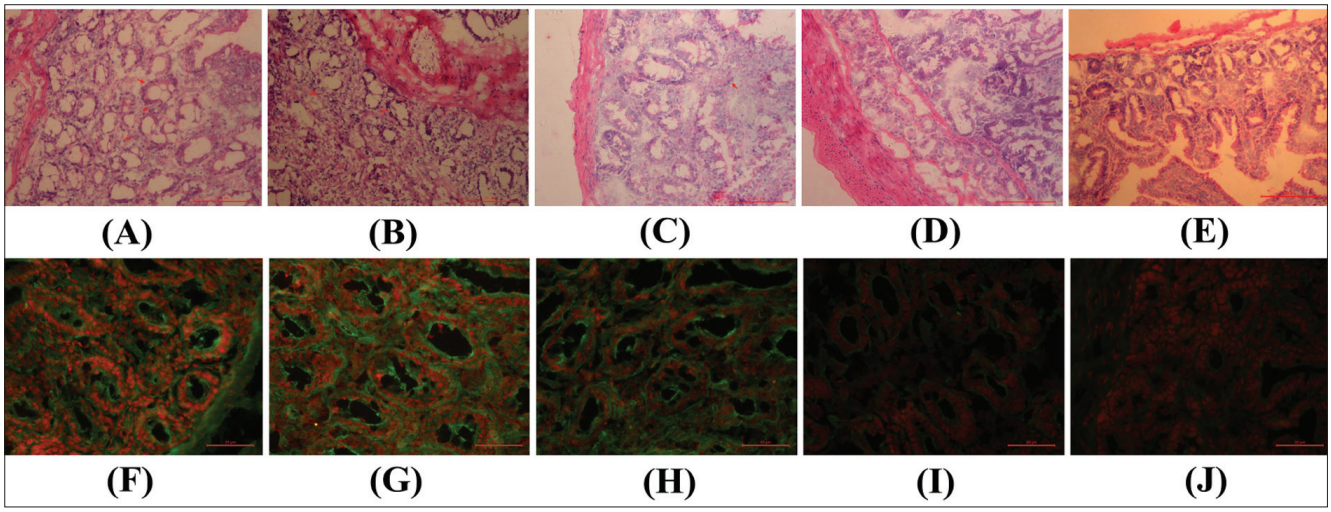

Fig 6. The result of froze sections in HE stain and indirect immunofluorescence. The froze sections of small intestines were treated with HE stain in PBS **(A)**, pVAX1 **(B)**, pIL-12 **(C)**, TGEV-S1 **(D)** and pIL-12 + TGEV-S1 **(E)** groups, and observed using 10×stage eyepiece and 20×stage objective. The froze sections of small intestines were detected by indirect immunofluorescence in PBS **(F)**, pVAX1 **(G)**, pIL-12 **(H)**, TGEV-S1 **(I)** and pIL-12 + TGEV-S1 **(J)** groups, and observed using 10×stage eyepiece and 40×stage objective

play an important role in the immunological regulation, including in the activation of effector T cells, helper T cells (Th) and cytotoxic T cells (Tc). The level of proliferation of T lymphocytes reflects the immunity level of an organism, after specific antigen stimulation. In this study, there was a conspicuous increase in the T lymphocytes in the TGEV-S1 and pIL-12 + TGEV-S1 groups.

$CD4^+$T lymphocytes are critical to the immune system. $CD4^+$T lymphocytes play a positive role in defense against the pathogen. The $CD8^+$T lymphocytes are another important subgroup. These are the pathogen "killer" of the immune system, which clean up the infected cells directly and mainly express in Tc cells. The T lymphocytes surface markers $CD8^+$ and $CD28^+$ represent the Tc cells [30,31]. All these T lymphocytes subgroups showed a degree of change in our study, among them, the increase in pIL-12 + TGEV-S1 group was particularly obvious. The $CD4^+$T and $CD8^+$T lymphocytes appeared a relative change when immune response attained a diverse stage. Furthermore, the $CD4^+$T lymphocytes could differentiate into Th1 and Th2, and secrete different cytokines. The IFN-γ and IL-4 proteins are markers for the Th1 and Th2 cells, respectively. In our study, the level of IFN-γ in the immunized piglets increased significantly, especially in pIL-12 + TGEV-S1 group, which did not occur with the level of IL-4. Consequently, Th1 cells were predominant in immune response [32,33].

The production of specific antibodies are important for humoral immune response. The specific antibodies might combine with the pathogen directly leading to pathogen devitalization or activate other defense pathways. In this

study, the levels of IgG and IgA antibodies indicate a more effective immune response in the TGEV-S1 and the pIL-12 + TGEV-S1 groups. The level of neutralizing antibodies also increased significantly in these two groups, especially the pIL-12 + TGEV-S1 group, to prevent pathogen spread.

In our report, the pIL-12 + TGEV-S1 group had the best cellular and humoral immune responses, except regarding the serum level of IL-4. The IFN-γ result indicates that the pIL-12 adjuvant mainly enhances the helper T cells differentiation into Th1. Th1 cells might induce the macrophage production and help the cytotoxic T lymphocytes ($CD8^+$ $CD28^+$) to eliminate intracellular pathogens. Moreover, the pIL-12 adjuvant showed increased levels of IgG3 and IgG2a antibody production in the serum [34], which may explain the high contents of specific antibody and neutralizing antibody in the serum of the pIL-12 + TGEV-S1 group. After the virus challenge, which initiated immune response, the pIL-12 + TGEV-S1 showed the most effective immune response to TGEV infection. It follows that pIL-12 was a advantageous immune adjuvant which could enhanced immune stimulation of antigen gene, induced the IFN-γ production, enhance the generation of cytotoxic T lymphocytes, promoted the cellular and humoral immune responses of piglets, and it could effectively improve the ability of TGEV S1 DNA vaccine to resist virus invasion, and contribute further application of DNA vaccine and pIL-12 immune adjuvant.

In summary, our study suggests that this newly developed DNA vaccine combining the adjuvant porcine IL-12 and the TGEV-S1 gene could enhance the immune response and prevent pathogen spreading through cellular and humoral immunity, that would be developed an effective means to prevent TGEV in swine.

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Research Article

Development and Validation of LC-MS/MS Method for Determination of Ten Beta Agonists in Bovine Urine

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Abstract

The use of β-agonists in livestock production is prohibited in many countries because the residues of β-agonists in food pose a potential risk to human health. The present work describes the development and validation of the LC-MS/MS method for detection of ten β-agonists in bovine urine according to Commission Decision 2002/657/EC requirements. Linearity of the method resulted with coefficient of correlation >0.990. The decision limits (CCα) ranged from 0.127 ng/mL to 0.646 ng/mL, and the detection capability (CCβ) resulted in the range 0.140 ng/mL to 0.739 ng/mL. The observed recoveries in the fortified bovine urine samples were satisfactory at every fortification level with values from 73.67% to 118.80%. The coefficient of variation (CV, %) at three fortification levels for each β-agonist was in complete agreement with the requirements from Commission Decision 2002/657/EC. The CV for intraday precision varied from 1.619% to 15.472% and the CV for interday precision varied from 2.695% to 10.441%. From the obtained validation results the proposed method is an appropriate method for determination of β-agonists in bovine urine.

Keywords: *β-agonists, Bovine, Urine, Validation, LC-MS/MS*

Sığır İdrarında 10 Beta Agonist Varlığının Belirlenmesi İçin LC-MS/MS Metodu Geliştirilmesi ve Validasyonu

Öz

Bir çok ülkede β-agonistlerin hayvancılıkta kullanılması yasaklanmıştır, çünkü gıda ürünlerinde bulunan β-agonist kalıntıları insan sağlığı için risk oluşturmaktadır. Bu çalışma, komisyon kararı 2002/657/EC ye uygun olarak, sığır idrarındaki 10 adet β-agonistin tespitini sağlayan metod gelişimini ve validasyonunu içermektedir. Doğrusallık korelasyon katsayısı >0.990 ile sonuçlanmıştır. Karar limitleri (CCα) 0.127 ng/mL ile 0.646 ng/ml arasında ve tespit kapasitesi (CCβ) 0.140 ng/mL ile 0.739 ng/ml arasında bir değer almıştır. Geri kazanım %73.67 ile %118.80 arasında bulunmuştur. Korelasyon varyasyonu (CV, %) her üç konsantrasyon seviyesi için Komisyon Kararı 2002/657/EC 'ye tamamen uygun olarak yapılmıştır. Gün içi kesinlik CV değeri %1.619 ile %15.472 arasında ve günler arası CV kesinlik değeri %2.695 ile %10.441 arasında değişmektedir. Bu çalışmada elde edilen validasyon değerlerine göre geliştirilen metod sığır idrarında β-agonist tespiti için uygundur.

Anahtar sözcükler: *β-agonistler, Sığır, İdrar, Validasyon, LC-MS/MSS*

INTRODUCTION

β-agonists are a group of synthetic compounds derived from cathecholamines, such as adrenaline and noradrenaline. They are therapeutically used in human medicine for the treatment of bronchoconstrictions and as bronchospasmolytic. In the veterinary medicine β-agonist are used for treatment of bronchoconstrictions, such us chronic obstructive pulmonary disease in horses, and as bronchodilators, tocolytics and heart tonics [1-4]. Moreover, β-agonists are illegally used in livestock production as growth promoters. After the application of 5-10 times

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higher doses than the therapeutic doses they improve carcass composition with increased muscle development (live weight-gain, improvement of feed conversion and increase of the muscle to fat tissue ratio) and reduced fat deposition [4-7]. The residues from illegally used β-agonist accumulate and persist in liver, muscle, retina, pigmented and other tissues, and for these reasons they incur a risk to human health [5,7-9]. In 1990, 22 people in France were affected after consumption of veal liver with residues of clenbuterol. In Spain, the same year, 135 people were affected, and in 1992 a total of 232 cases of poisoning were registered after consumption of liver or meat with clenbuterol residues. The main symptoms found were: tremors, tachycardia, nervousness and general malaise [10,11]. The Commission of European Communities has banned the use of β-agonists as growth promoter in livestock production with Council Directive 96/22/EC, while Council Directive 96/23/EC prescribes the measures to monitor certain substances and residues thereof in live animals and animal products [12,13]. The most commonly used confirmation and identification technique for β-agonists in biological matrices is mass spectrometry performed with gas chromatography (GC-MS/MS) or liquid chromatography. The LC-MS/MS method is used more often than GC-MS/MS method, because derivatisation step is the critical step in GC-MS/MS technique, while in the LC-MS/MS method the derivatisation is not required [7,11,13]. The aim of this study was to develop and validation the LC-MS/MS method for detection of ten β-agonist (clenbuterol, brombuterol, mabuterol, cimbuterol, isoxsuprine, clenpenterol, ractopamine, salbutamol, zilpaterol and terbutaline) in bovine urine, applying thereby the analytical criteria stipulated under the Commission Decision 2002/657/EC [14].

MATERIAL and METHODS

Chemicals and Apparatus

Standards and Internal Standards: Clenbuterol, ractopamine, isoxsuprine, terbutaline and terbutalin-d9 were from Sigma-Aldrich, brombuterol, mabuterol, clenpenterol, cimbuterol, clenbuterol-d6, brombuterol-d9, mabuterol-d9, clenpenterol-d5 and cimbuterol-d9 from Witega, isoxsu-prine-d5 and ractopamine-d6 HCl from EURL, Rikilt, zilpaterol and zilpaterol d-7 from Toronto Research Chemicals Inc., sabutamol from Riedel-de Haen and salbutamol d-9 from Dr. Ehrenstorfer GmbH were supplied. All standards and internal standards that were used in this research are donations from EURL Berlin Germany.

Reagents: Ethyl acetate, acetonitrile (LC-MS grade), methanol (HPLC grade), water (LC-MS grade), sodium acetate (pro analysis (p.a.), ammonium hydroxide 32% and HCl 37% were purchased from Carlo Erba, formic, acetic acid, β glucoronidase/aryl sulphatase from Helix pomatia and potassium hydroxide (p.a) were purchased from Sigma Aldrich, while ascorbic acid (p.a) and potassium dihydrogen phosphate (p.a) were purchased from Alkaloid.

Apparatus: The LC-MS/MS system was purchased from Waters. The LC system equipped with binary pump, vacuum degasser, thermostated autosampler and thermostated column manager. The MS/MS detector is triple quadruple with ESI source. For separation of β-agonists were used C18 column from Phenomenex, with dimensions 50×2.1 mm and particle size from 2.6 µm. MassLynx software version 4.1 was used for data acquisition and calculation of results. For solid phase extraction were used Discovery® DSC-MCAX cartridges, 300 mg, 6 mL from Supelco.

Standards and Sample Preparation

For construction of calibration curve β-agonists were divided in three groups, as follows: group I (clenbuterol, brombuterol, mabuterol), group II (cimbuterol, clenpenterol, isoxsuprine) and group III (ractopamine, terbutaline, zilpaterol, salbutamol). The standards were prepared in the blank urine (matrix-matched calibration). In the first step 10 mL blank urine and 10 mL samples were spiked with internal standards at 0.5 ng/mL. After that, in the blank urine was added mix of standards from group I, II and III for construction of calibration curve. The concentration of standards for group I were: 0.05, 0.125, 0.25, 0.375, 0.5 and 0.75 ng/mL, for group II: 0.125, 0.25, 0.375, 0.5, 0.75 and 1.0 ng/mL and for group III: 0.25, 0.5, 0.75, 1.0, 1.5 and 2.5 ng/mL. 5 mL sodium acetate buffer (pH=5) was added in the urine and then 50 µL β-glucoronidasa/aril sulfatasefrom Helix pomatia were added and incubated at 37°C over night. After cooling to room temperature, 5 mL of phosphate buffer pH 6 was added, and the samples were centrifuged at 4°C, on 4000 rpm, 10 min. After centrifugation in the supernatant, 0.2 mL methanol was added. In the next step, SPE cartridges were conditioned with 2 mL of methanol, 2 mL of water and 2 mL of phosphate buffer (pH=6). The supernatant were loaded to SPE cartridges and then cartridges were washed with 1 mL of 1 M acetic acid and evaporated to dryness followed by washing with 6 mL of methanol and evaporating to dryness. The elution was performed with 6 mL of a mixture consisting of ethyl acetate and 32% ammonia at a 97:3 ratio. The samples were evaporated to dryness under stream of nitrogen at 35°C. Then residues were dissolved in 200 µL of mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid (B) in acetonitrile, at a 95:5 ratio. 10 µL of the final extract was injected into LC-MS/MS system.

LC-MS/MS Conditions

The chromatographic separation on β-agonists was performed on C18 column at 40°C and flow rate of 0.8 mL/min. The gradient elution program is given in *Table 1*.

The mass spectrometry conditions were as follows:

Table 1. *Gradient elution program for mobile phase A and B.*

Time (min)	Flow (mL/min)	Mobile Phase A (%)	Mobile Phase B (%)
Initial	0.8	95	5
1.0	0.8	80	20
4.0	0.8	60	40
8.0	0.8	95	5
12.0	0.8	95	5

electrospray ionization ESI+, capillary voltage 3.0 kV, source temperature 150°C, desolvation temperature 400°C, cone gas 100 L/h, and desolvation gas 300 L/h. The multiple reaction monitoring (MRM) mode was used for the LC-MS/MS chromatograms acquisition of β-agonists. The conditions are given in *Table 2*.

Method Validation

The method was validated according to the criteria established by the Commission Decision 2002/657/EC for banned substances. The linearity was obtained from the calibration curve in matrix. Selectivity was determined by analyzing 20 blank bovine urine samples. The blank bovine urine were obtained from untreated cattle. Decision limit (CCα) was obtained by fortificationon 18 blank urine with β-agonist standards as follow: for group I at 0.1 ng/mL, for group II at 0.25 ng/mL and for group III at 0.5 ng/mL. CCα was calculated from the corresponding concentration at the y-intercept plus 2,33 times the standard deviation of the within-laboratory reproducibility. The Detection capability (CCβ) was obtained as corresponding concentration at the decision limit plus 1,64 times the standard deviation of the within-laboratory reproducibility (2002/657/EC). The accuracy (recovery), intra- and interday precision were determined at three different levels by fortified 18 replicates (6 replicates per level) of blank urine with β-agonist standards for group I at 0.2, 0.3 and 0.4 ng/mL, for group II 0.5, 0.75 and 1.0 ng/mL and 1.0, 1.5 and 2.0 ng/mL for group III.

RESULTS

The LCMS/MS method for determination of ten β-agonists in bovine urine was developed. The calibration curves obtained for all β-agonists were linear and gave a good coefficient of correlation (R^2) >0.99 *(Table 3)*.

Blank urine samples did not contain any traces of β-agonists and there was also no contamination observed. Moreover, no interference on β-agonist identification was found owing to the highly specific MRM acquisition method and the use of appropriate internal standards. It is concluded that the methods showed good selectivity. CCα and CCβ values for target β-agonists in bovine urine were determined according to Commission Decision 2002/657/EC. The results for CCα and CCβ and recommended concentration (RC) according to CRL guidance paper are summarized in *Table 4* [15].

The CCα ranged from 0.127 ng/mL to 0.646 ng/mL, and the CCβ ranged from 0.140 ng/mL to 0.739 ng/mL. It is concluded that the methods showed relevant CCα and CCβ according to the 2002/657/EC *(Table 4)* [13]. Nielen et al.[16], in the development of the LC-MS/MS method have determined a CCα from 0.01 -0.28 µg/l, and CCβ from 0.09-0.99 µg/L for group of 18 β-agonists in bovine and porcine urine, while in the study from VanHoof et al.[17], the CCβ of zilpaterol, ritodrine, ractopamine and isoxsuprine for calf urine and faeces was lower or equal to 1 µg/kg and for formoterol, the CCβ was lower or equal to 5 µg/kg [16,17]. According to literature data, besides the urine, with LC-MS/MS method can be detected low concentration of β-agonists in other biological matrices. For example, the CCα ranged from 0.1 to 0.3 µg/kg for bovine liver, 1-3 µg/kg for bovine retina, and CCβ from 0.2-0.5 µg/kg for liver and 2-5 µg/kg for retina, while in the bovine hair CCα resulted in the range 0.2-1.0 µg/kg. Application to feed showed CCβ value of less than 5.0 µg/kg [5,17-19]. From the results obtained from this study and from literature data it can be concluded that the LC-MS/MS method shows relevant CCα and CCβ values for β-agonists in biological samples. The validation results for accuracy and precision are shown in *Table 5*. Accuracy was expressed as recovery of the method (%). The observed recoveries were satisfactory for 10 β-agonists at every fortification level with values from 73.67% to 118.80%.

The precision of the method was determined by calculating the coefficient of variation (CV). The CV for intraday precision varied from 1.619% to 15.472% and the CV for interday precision varied from 2.695% to 10.441%. The coefficient of variation at three fortification level for each β-agonist was in complete agreement with the requirements from Commission Decision 2002/657/EC, and demonstrating the excellent method precision. Most methods for detection of β-agonist with LC-MS/MS technique reported in the literature showed good accuracy, intraday and interday precision. In the method for testing of ractopamine in bovine and sheep urine the recovery ranged from 108.4 to 117.8%, and the intraday precision was 0.9% at 35 ng/mL and 5.64% at 0.25 ng/mL, while the interday precision was 0.95% at 35 ng/mL and 4.32% at 0.25 ng/mL [18]. Recovery from 50-120%, and precision around 14% were detected in the method for detection of zilpaterol in calf urine [16]. Fesser et al.[5], detected recovery from 98-118%, for samples fortified at levels between 0.5-2.0 µg/kg (liver) and 5-20 µg/kg (retina) with good precision (CV ranging from 6 to 20%). Good recovery (83-90% for salbutamol, clenbuterol and ractopamine) and precision (CV=1.5-11%) were detected in feed by Zhang et al.[20]. Also, the recovery (97-109.4%) and precision (CV=0.1-9.5%) for ractopamine in swine and cattle tissues were satisfactory [21].

Table 2. *Parameters of MRM condition and retention times of the β-agonists.*

Standards	Retention Time (min)	Monitored Reactions Precursor		Collision Energy (v)	Adequate Internal Standards	Retention Time (min)	Monitored Reactions Precursor		Collision Energy (v)
		Parent Ion (m/z)	Daughter Ions (m/z)				Parent Ion (m/z)	Daughter Ions(m/z)	
Clenbuterol	2.22	276.97	202.95 131.87 167.77	16 30 30	Clenbuterol-d6	2.29	283.03	203.56 132.19	16
Brombuterol	2.55	366.90	292.84 211.42 57.00	20 34 38	Brombuterol-d9	2.49	375.93	293.87 212.39	18
Mabuterol	2.66	310.95	236.99 216.96 57.00	18 26 30	Mabuterol-d9	2.65	320.07	237.94 66.04	18
Clenpenterol	2.60	291.00	202.92 131.89 167.79	16 30 28	Clenpenterol-d5	2.59	296.00	203.10 132.01	16
Isoxsuprine	2.50	302.04	106.96 164.01 120.95	30 16 28	Isoxsuprine-d5	2.70	308.15	168.05 107.09	16
Cimbuterol	1.49	234.03	159.98 142.94 57.00	16 28 26	Cimbuterrol-d9	1.51	243.07	160.96 143.72	16
Ractopamine	1.94	302.04	106.96 164.01 120.95	28 16 24	Ractopamine-d6	1.99	308.10	168.05 120.95	16
Salbutamol	1.27	240.03	147.96 165.98 56.94	20 14 24	Salbutamol-d9	1.29	249.08	148.59 166.99	20
Zilpaterol	1.31	262.03	185.01 202.05 156.98	24 22 32	Zilpaterol-d7	1.23	269.08	185.15 203.04	24
Terbutaline	1.26	226.00	152.00 106.97	14 30	Terbutaline-d9	1.25	235.07	152.83 66.05	16

Table 3. *Linearity of the method*

β-agonists	Matrix Match Calibration	
	Range (ng/mL)	R^2
Clenbuterol	0.05-0.75	0.995214
Brombuterol	0.05-0.75	0.993479
Mabuterol	0.05-0.75	0.996597
Clenpenterol	0.125-0.75	0.994935
Isoxsuprine	0.125-0.75	0.990097
Cimbuterol	0.125-0.75	0.995858
Ractopamine	0.25-2.50	0.993357
Salbutamol	0.25-2.50	1.000000
Zilpaterol	0.25-2.50	0.994861
Terbutaline	0.25-2.50	0.992348

Table 4. *CCα and CCβ for β-agonists in bovine urine, recommended concentration for β-agonists in urine*

β-agonist	CCα (ng/mL)	CCβ (ng/mL)	RC (ng/mL)
Clenbuterol	0.158	0.188	0.2
Brombuterol	0.144	0.170	0.2
Mabuterol	0.127	0.140	0.2
Clenpenterol	0.299	0.329	0.5
Isoxsuprine	0.29	0.320	0.5
Cimbuterol	0.259	0.282	0.5
Ractopamine	0.577	0.619	1.0
Salbutamol	0.584	0.657	1.0
Zilpaterol	0.646	0.739	1.0
Terbutaline	0.565	0.702	3.0

DISCUSSION

In the similar, but screening quantitative method for detection of β-agonists in bovine and porcine urine, feed and hair, from Nielen et al.[16], accuracy of the method for urine was from 85-111 %, recovery at 0.5 µg/L for clenbuterol was 74.0%, recovery for another β-agonists at 1.0 µg/L was from 54-85%, the interday precision of the method was from 3-26% and intraday precision was from 5-32% [16]. The use of analogue isotope-labelled internal standards

Table 5. *Accuracy and precision of the method*

β-agonists	Added Concentration (ng/mL)	Intraday Precision			Interday Precision		
		Mean Concentration (n=6)(ng/mL)	Mean Recovery (%)	CV (%)	Mean Concentration (n=6)(ng/mL)	Mean Recovery (%)	CV (%)
Clenbuterol	0.2	0.206	103.00	3.225	0.176	88.00	7.545
	0.3	0.292	97.33	12.513	0.231	77.00	5.205
	0.4	0.370	92.50	9.791	0.363	90.75	4.730
Brombuterol	0.2	0.213	106.50	11.764	0.205	102.50	7.966
	0.3	0.305	101.67	11.764	0.307	102.33	4.471
	0.4	0.470	117.5	9.831	0.458	114.50	5.888
Mabuterol	0.2	0.198	99.00	7.591	0.162	81.00	5.592
	0.3	0.277	92.33	3.957	0.249	83.00	7.301
	0.4	0.365	91.25	11.610	0.450	112.50	4.467
Clenpenterol	0.5	0.474	94.80	7.671	0.471	94.20	6.051
	0.75	0.623	83.07	2.184	0.713	95.07	4.248
	1.0	1.188	118.80	4.714	1.184	118.40	3.217
Isoxsuprin	0.5	0.385	77.00	7.365	0.410	82.00	2.695
	0.75	0.558	74.40	7.596	0.606	80.80	3.477
	1.0	0.993	99.30	4.038	0.972	97.20	6.715
Cimbuterol	0.5	0.503	100.60	7.154	0.458	91.60	4.163
	0.75	0.739	98.53	4.924	0.666	88.80	4.345
	1.0	1.122	112.2	8.638	1.052	105.20	3.889
Ractopamine	1.0	0.872	87.20	1.619	0.853	85.30	3.383
	1.5	1.321	88.07	4.148	1.317	87.80	3.235
	2.0	1.929	96.45	3.235	1.810	90.50	5.200
Salbutamol	1.0	0.849	84.90	3.648	0.873	87.30	5.296
	1.5	1.105	73.67	5.828	1.275	85.00	9.789
	2.0	1.815	90.75	11.319	1.998	99.90	4.985
Zilpaterol	1.0	0.812	81.20	8.402	0.976	97.60	8.756
	1.5	1.351	90.07	5.676	1.487	99.13	5.734
	2.0	1.782	89.10	15.472	2.060	103.00	5.467
Terbutaline	1.0	0.953	95.30	9.316	1.091	109.10	9.770
	1.5	1.490	99.33	7.613	1.597	106.47	6.043
	2.0	2.037	101.85	10.949	2.168	108.40	10.441

for all β-agonists in this study has resulted with better interday and intraday precision, as well as better recovery (>73%) compared with the study of the Nielen et al.[16] where authors used only 3 isotope-labelled internal standards which not completely correct recovery loss and ionization suppression. Moreover, in the process of identification and quantification of the substances in the analytical methods the number of transitions play important role. In this study are included 1 precursor and 3 daughter ions for all β-agonists and 1 precursor and 2 daughter ions for internal standards and terbutalin therefore the method fulfills the requirement for a minimum of 4 identification points for a confirmatory method. On the other hand in the study of Nielen et al.[16], authors used 1 precursor ion and 1 daughter ion which is suitable for screening method because in the method yield 2.5 identification points. The confirmatory methods are more accurate, more sensitive, more precise than screening methods and enable the identification and quantification of analytes. According to 2002/657/EC "confirmatory method provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest", while "screening method means methods that are used to detect the presence of a substance or class of substances at the level of interest" [13]. The results from this study showed good validation parameters for detection of β-agonists in bovine urine; therefore, this method will

be used in routine analysis for detection of β-agonists in bovine urine.

In summary, the LC-MS/MS method for detection of ten β-agonists was validated according to Commission Decision 2002/657/EC and European Union requirements. The method validation study demonstrated acceptable linearity, selectivity, CCα and CCβ, accuracy and precision. On the basis of validation results we can conclude that the method is suitable for determination of low concentration of β-agonist residues in bovine urine samples. The method will be used for monitoring the abuse of β-agonists in bovine urine and evaluating the potential risk to human health.

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Research Article

Effects of Calcium Soaps of Animal Fats on Performance, Abdominal Fat Fatty Acid Composition, Bone Biomechanical Properties, and Tibia Mineral Concentration of Broilers

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Abstract

This study evaluated the effects of a graded concentration of dietary calcium soaps of tallow (CST) supplementation on broiler performance, carcass characteristics, abdominal fat fatty acid profile, bone biomechanical properties, and bone mineral composition. One hundred and forty 11-d-old male broiler chickens were randomly allocated to 4 experimental groups with 5 replicate pens containing 7 birds per each. The birds received corn-soybean meal based diet and CST (CST0, CST10, CST20, and CST30, respectively) was included in the grower (0, 10, 20, and 30 g/kg) and finisher (0, 15, 30, and 45 g/kg) diet at the expense of vegetable acid oil and limestone. Dietary supplementations had no significant effect on BWG and FI during the entire experimental period. However, FCR was improved in CST0 and CST10 groups in comparison to those of the CST20 group from d 11 to d 42. According to the present study result, dietary supplementation of low level of CST significantly influenced intestinal microarchitecture of the jejunum and ileum by improving villus height/crypt ratio and villus height, respectively. Femur (P=0.001) and tibia (P=0.020) stiffness increased linearly with the increasing level of dietary CST. Tibia Ca (P=0.009) and P (P=0.009) concentration of CST10 and CST30 groups were lower than the CST0 group. Increasing levels of CST in diets significantly reduced the Fe and Mn concentrations in tibia samples. In conclusion, supplementation of CST has no detrimental effect on broiler performance parameters and might be used as an alternative dietary fat source in the broiler industry.

Keywords: *Acid oil, Broiler, Calcium soap, Fat, Fatty acid composition, Tallow*

Hayvansal Yağın Yağ Asitleri Kalsiyum Tuzunun Broyler Performansı, İç Yağın Yağ Asidi Kompozisyonu, Kemiklerin Biyomekanik Özellikleri ve Tibia Mineral Düzeyi Üzerine Etkisi

Öz

Bu çalışmada rasyonlara artan düzeylerde ilave edilen hayvansal yağın yağ asiti tuzunun (CST) broylerlerde performans, karkas karakteristikleri, abdominal yağın yağ asiti kompozisyonu, kemiklerin biyomekanik özellikleri ve kemik mineral kompoziyonu üzerine olan etkisi incelenmiştir. Toplamda 140 adet 11 günlük erkek broyler 4 deneme grubuna ve her biri 7 civcivden oluşan 5 tekerrür grubuna rasgele olacak şekilde ayrılmıştır. Broylerler mısır-soyadan oluşan rasyon ile beslenmiştir. Büyütme (0, 10, 20 ve 30 g/kg) ve bitirme (0, 15, 30 ve 45 g/kg) dönemi rasyonları artan düzeylerde CST (CST0, CST10, CST20 ve CST30, sırasıyla) kapsayacak şekilde düzenlenmiştir. Karma yeme ilave edilen yağın, tüm deneme periyodu boyunca, canlı ağırlık artışı ve yem tüketimi üzerine önemli bir etkisi olmadığı görülmüştür. Ancak, araştırmanın 11-42 günleri arasında, CST0 ve CST10 gruplarının CST20 grubu ile karşılaştırıldığında daha iyi yemden yararlanma değerine sahip olduğu görülmüştür. Elde edilen araştırma sonuçlarına göre, karma yeme düşük düzeyde ilave edilen hayvansal yağın jejunum villus yüksekliği/kript derinliği oranını ve ileum villus yüksekliğini arttırmak suretiyle bağırsak histomorfolojisi üzerine olumlu bir etkisi olduğu tespit edilmiştir. Femur (P=0.001) ve tibia (P=0.020) sertliği rasyonlara artan düzeyde CST ilavesi ile birlikte doğrusal bir artış göstermiştir. CST10 ve CST30 gruplarının tibia Ca (P=0.009) ve P (P=0.009) konsantrasyonu, CST0 grubu ile karşılıştırıldığında daha düşük bulunmuştur. Karma yemde CST düzeyinin artması, tibia örneklerindeki Fe ve Mn konstrasyonunu önemli ölçüde azaltmıştır. Sonuç olarak, rasyonlara ilave edilen hayvansal yağın yağ asiti tuzlarının broyler performansı üzerine olumsuz bir etkisi olmadığı ve ucuz bir alternatif yağ kaynağı olarak kullanılabileceği görülmüştür.

Anahtar sözcükler: *Asit yağ, Broyler, Hayvansal yağ, Kalsiyum sabunu, Yağ, Yağ asidi kompozisyonu*

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INTRODUCTION

Production animals have specific energy needs and numerous nutrient requirements, such as amino acids, fatty acids, vitamins, and minerals for maintenance and growth. In the modern broiler production, diets are delicately formulated by nutritionists to meet a bird's requirements in a cost effective manner. For this purpose, fats and oils are preferred as an energy source in diets to meet high energy requirements of broilers since they contain more energy in comparison to carbohydrates and proteins [1]. There are numerous types of fats and oils, of varying quality and price, including vegetable oils (soybean oil, sunflower oil), animal fats (tallow, lard, and poultry fat), acidulated soapstocks, hydrogenated fats and oils that are available to use in diets [2]. Dietary supplementation of these products also confers beneficial effects such as improved palatability, improved absorption of fat-soluble vitamins, reduced dustiness and wastage of feed during processing, and improved texture in mash diets [2,3]. In addition, dietary fats and oils increases nutrient absorption and utilization by slowing down passage rate of digesta through the gastrointestinal tract of chickens [4-6].

Almost all ingredients used in broiler diets are vegetable based and therefore the fatty acid composition of the basal diets is highly unsaturated [7]. According to previous studies, inclusion of animal fats to poultry diets, especially at low levels, resulted in increased apparent metabolisable energy (AME) values of the added fat [7-9]. Tallow is a rendering by-product of the red meat industry and commonly used as an energy source in broiler diets because of its relatively low price [10]. However, it is well documented that digestibility and metabolisable energy content of tallow are lower than vegetable oils due to the fatty acid composition, which is mostly saturated [11,12]. Previous studies suggested that the dietary fat source has no effect on performance of broilers since energy, protein, and amino acid levels are balanced, even though the tallow has a lower AME value than those of vegetable oils [13]. However, dietary inclusion level of tallow or the combined use with several vegetable oils might affect broiler performance. Tancharoenrat and Ravindran [10] indicated that broilers have had higher fat retention and ileal fat digestibility rate when fed a diet containing 4% tallow than those fed 8% tallow. Newman et al.[14] reported that dietary addition of 8% beef tallow significantly reduced broiler performance versus those fed with sunflower oil. Moreover, rather than used alone, combined use of tallow with vegetable oils was determined to have synergistic effects [12] and improved body weight (BW) and feed efficiency in broilers [3].

Calcium soaps of tallow (CST) is preferred energy sources, especially for ruminants, due to their chemical structure which makes these compounds inert in the rumen environment [15]. On the other hand, they are efficiently digested and absorbed in the small intestine. CST can be easily incorporated into diets without any specialized equipment due to their granulated-solid nature and are more resistant to oxidation than tallow [15]. Rising et al.[16] concluded that calcium soaps from animal fat were utilized efficiently by laying hens. However, as far as we know, no available data exists about the effects of CST on broiler performance.

A diverse variety of fats and oils are available but the choice of these energy products mostly depends on their price. In this manner, interest in cost-effective alternative energy sources by the modern broiler industry is growing each year. Therefore, the purpose of this experiment was to evaluate the effects of a graded concentration of CST on performance, abdominal fat fatty acid deposition and bone characteristics of broiler chickens.

MATERIAL and METHODS

Animal Care and Use

All experimental procedures were approved by The Animal Ethics Committee of the Ankara University (2015-15-166).

Birds and Management

One-day-old Ross 308 male broiler chickens were obtained from a commercial company (Beypiliç, Bolu, Turkey) and reared to 11 d of age in a broiler house under standard conditions and were fed broiler starter diets. One hundred and forty 11-day old male broiler chickens, with average BW of 365.12±4.77 g (Mean ± SD) were selected for the experiment. The chicks were randomly allocated to 4 experimental dietary groups (5 replicate floor pens, 7 birds /pen). Birds were kept under environmentally controlled room for 31 days. Temperature was adjusted to the according to the recommended conditons for Ross 308 broiler during the study [17]. The grower and finisher diets were based on maize-soybean meal and were offered to birds from 11-21, and 21-42 days of age, respectively *(Table 1)*. All diets were formulated to meet or exceed NRC [1] nutrient recommendations. Water and diets (in mash form) were provided *ad libitum* throughout the experimental period.

Calcium soap of tallow was included to grower and finisher diets as follows: Grower Phase (11 to 21 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 10 g/kg CST; CST20 = basal diet containing 20 g/kg CST; CST30 = basal diet containing 30 g/kg CST. Finisher Phase (22 to 42 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 15 g/kg CST; CST20 = basal diet containing 30 g/kg CST; CST30 = basal diet containing 45 g/kg CST. Diets were mixed individually and CST (in fine powder form) was included to diets at the expense of vegetable acid oil and limestone. Full fat soybean was used in diets as unsaturated vegetable oil source and protein source. Vegetable acid oil (VAO) was added to experimental diets,

as a substitute for CST, due to its unsaturated fatty acid nature and low energy content (7.400 kcal/kg). Moreover, due to the Ca content (analysed data, 10%) in the tallow product, limestone level was reduced in diets with the increase of dietary CST level in order to provide the same amount of Ca. All experimental diets were isocaloric and isonitrogenous *(Table 1)*. Crude protein contents in diets were determined according to AOAC [18] and metabolizable energy levels were calculated according to Carpenter and Clegg [19]. Fatty acid composition of fat sources and diets are detailed in *Table 2*. The CST product was provided by KRV Oil and Bone Inc. (Kayarlar Group, Sakarya, Turkey).

Sampling Procedures

All chicks were weighed individually and feed intake (FI) was recorded at d 21, 28, 35, and 42. Body weight gain (BWG) and feed conversion ratio (FCR) were subsequently calculated based on the performance values. At the end of the study (d 42) two birds from each replicate were selected according to average BW of each replicate. Birds were slaughtered by exsanguination and the intestinal tract was removed immediately. The tissue samples for histomorphological analysis were taken from the mid part of the duodenum, jejunum, and ileum. Afterwards, the tissue samples were flushed with saline solution to remove adherent intestinal contents and fixed in 10% neutral buffered formalin solution for 24 h [20]. Then the carcasses were manually dissected and weighed individually to calculate carcass percentage. Liver and abdominal fat were also weighed and expressed as the percentage of carcass weight. Then abdominal fat from each carcass was collected in individual sample bags and stored at -20°C

Table 1. *Composition of experimental diets*[1]

Ingredients, g/kg	Grower (11 to 21 d)				Finisher (22 to 42 d)			
	CST0	CST10	CST20	CST30	CST0	CST10	CST20	CST30
Maize	495.8	498.3	500.8	501.8	510.0	514.6	520.2	524.6
Soybean meal (CP 47%)	272.0	270.0	270.0	265.5	180.1	180.0	181.3	181.5
Soybean (full fat, CP 36%)	165.0	167.0	167.0	173.0	231.0	230.0	227.1	226.0
VAO[2]	30.0	20.0	10.0	0	45.0	30.0	15.0	0
CST[3]	0	10.0	20.0	30.0	0	15.0	30.0	45.0
Limestone	10.0	7.50	5.00	2.50	11.0	7.5	3.5	0
MCP	19.0	19.0	19.0	19.0	16.0	16.0	16.0	16.0
Salt	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
DL-methionine (98%)	1.5	1.5	1.5	1.5	1.4	1.4	1.4	1.4
L-lysine-HCl (78%)	1.2	1.2	1.2	1.2	0	0	0	0
Sodium bicarbonate	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin premix[4]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Mineral premix[5]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Total	1000	1000	1000	1000	1000	1000	1000	1000
Analysed composition								
Crude protein, g/kg	219.9	220.1	219.8	220.2	208.4	207.6	207.9	208.3
ME, kcal/kg[6]	3.168	3.164	3.158	3.159	3.342	3.339	3.322	3.319
Calculated composition								
ME, kcal/kg	3.44	3.146	3.149	3.156	3.305	3.307	3.310	3.313
Crude Protein, g/kg	225.7	225.9	226.1	226.2	206.4	206.3	206.3	206.3
Ether Extract, g/kg	84.5	83.8	83.1	83.4	110.1	109.4	107.9	106.6
Ash, g/kg	59.2	57.8	56.4	55.0	55.5	53.6	51.2	49.3
Lysine, g/kg	14.0	14.0	14.0	14.0	11.8	11.8	11.8	11.8
Meth.+Cyst., g/kg	8.90	8.90	8.90	8.90	82.4	82.4	82.4	82.4
Threonine, g/kg	9.10	9.10	9.10	9.10	83.1	83.1	83.1	83.1
Ca, g/kg	8.60	8.70	8.70	8.70	8.40	8.60	8.50	8.60
Total P, g/kg	8.30	8.30	8.30	8.30	7.50	7.50	7.50	7.50
Available P, g/kg	5.10	5.10	5.10	5.10	4.50	4.50	4.50	4.50

[1] As-fed basis; [2] Vegetable acid oil, derived from vegetable oil refining (estimated AME: 7400 kcal/kg); [3] Calcium Soaps of Tallow (estimated AME: 7000 kcal/kg; Ca, 100 g/kg); [4] Provided per kilogram of complete diet: vitamin A, 15.000 IU; vitamin D_3, 5.000 IU; vitamin E, 100 mg; vitamin K_3, 3 mg; thiamin, 5 mg; riboflavin, 8 mg; pyridoxine, 5 mg; pantothenic acid, 16 mg; niacin, 60 mg; folic acid, 2 mg; biotin, 200 µg; vitamin B_{12}, 20 µg; [5] Provided per kilogram of complete diet: Cu, 16 mg; I, 1.5 mg, Co, 500 µg; Se, 350 µg; Fe, 60 mg; Zn, 100 mg; Mn, 120 mg; Mo, 1 mg; [6] Carpenter and Clegg [19]

Table 2. *Fatty acid composition (%) of the experimental diets*

Item	Supplemented Fats			Grower[1] (d 11 to 21)				Finisher[2] (d 22 to 42)			
	FFSB[3]	VAO[4]	CST[5]	CST0	CST10	CST20	CST30	CST0	CST10	CST20	CST30
C12:0	ND	0.36	0.24	ND[6]	ND	ND	ND	ND	ND	ND	ND
C14:0	0.21	0.38	4.22	0.09	0.27	0.28	0.52	0.11	0.27	0.14	0.54
C16:0	11.44	9.92	33.42	11.89	13.29	14.27	16.91	11.53	12.61	14.45	16.66
C16:1	0.14	ND	3.07	ND	ND	ND	ND	ND	ND	ND	ND
C18:0	3.35	3.77	21.57	4.39	5.27	6.05	7.66	4.18	5.23	6.49	7.46
C18:1 cis-9	25.76	27.90	33.12	25.48	28.49	29.77	33.31	26.39	27.74	29.62	33.77
C18:2	51.45	55.63	3.75	53.68	49.30	45.84	38.66	53.28	50.15	45.87	38.79
C20:0	0.27	0.42	0.05	3.56	3.06	3.01	2.44	3.57	3.37	2.68	2.41
C18:3	5.89	0.10	0.31	ND	ND	ND	ND	ND	ND	ND	ND
Others	0.68	1.51	0.26	0.90	0.32	0.78	0.51	0.94	0.63	0.75	0.37
Unsaturated (U)	84.05	83.63	40.25	79.16	77.79	75.61	71.97	79.67	77.89	75.49	72.56
Saturated (S)	15.27	14.85	59.50	19.93	21.89	23.61	27.53	19.39	21.48	23.76	27.07
U:S	5.50	5.63	0.68	3.97	3.55	3.20	2.61	4.11	3.63	3.18	2.68

[1] Grower Phase (11 to 21 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 10 g/kg CST; CST20 = basal diet containing 20 g/kg CST; CST30 = basal diet containing 30 g/kg CST; [2] Finisher Phase (22 to 42 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 15 g/kg CST; CST20 = basal diet containing 30 g/kg CST; CST30 = basal diet containing 45 g/kg CST; [3] Full fat soybean; [4] Vegetable acid oil derived from vegetable oil refining; [5] Calcium soaps of tallow; [6] ND: Not determined

for fatty acid analysis. Left femur and tibia samples were collected and stored at -20ºC to determine bone breaking strength and bone mineral level.

Fatty Acid Analysis of Feed and Abdominal Fat

Approximately 1 g of feed sample was weighed and fat contents were extracted according to Soxhlet method [18]. Obtained fats were stored at -20ºC for fatty acid analysis. On the day of analysis, extracted fats of the feed samples were mixed with 4 mL anhydrous diethyl ether (containing 100 ppm BHT), transferred to Teflon-capped tubes, and the solvent was evaporated. Abdominal fats were thawed at 4ºC and approximately 200 mg fat sample was placed in Teflon-capped tubes. Both feed and abdominal fat samples were dissolved in 5 mL of 2N NaOH-methanol in a 60°C bath for 15 min. Subsequently, 2.175 mL of BF3-methanol (10% w/w) was added and samples were placed in a 60°C bath for 30 min. Fatty acid methyl esters were extracted with 1 mL of hexane and 2 mL of saturated sodium chloride. After centrifugation, at 4.000 rpm for 5 min, the top layer was transferred to gas chromatography vials for analysis. Supernatants were analysed using gas chromatography (Shimadzu GC-2010, Shimadzu Co., Kyoto, Japan) coupled with 30 m×0.25 mm i.d. column (SP™-2330, Supelco, Bellefonte, PA) and a flame ionization detector to determine the fatty acid methyl esters of the feed and abdominal fat samples. Conditions were as follows: injector: 250°C; detector: 250°C; oven: 160°C for 1 min, increased to 240°C (4°C/min), and held for 1 min. One microliter supernatant was injected automatically with a split of 1:100. Each fatty acid was identified in the form of a methyl ester by comparing the retention times with the F.A.M.E Mix C8-C24 (Supelco 18918-1AMP) methyl ester standard. Those data were presented as relative percentage of total fatty acids.

Histomorphological Measurements

Intestinal tissue samples were processed with ethanol and xylol and supsequently embedded in paraffin. Cross sections from intestinal segments, at a thickness of 5 μm, were prepared and stained with hematoxylin and eosin in order to determine small intestinal morphometry: villus height, width and crypt depth using a microscope (Olympus BX51-DP71, Tokyo, Japan) with Cellsens programs (CS-ST-V1.8) [21,22]. Subsequently, villus surface area was calculated according to the following geometric formula; $2\pi \times$ (villus width/2) $\times$ villus height [23].

Femur and Tibia Biomechanical Properties

Left femur and tibia samples were thawed at 4ºC and cleaned of all tissue. Length and width of femur and tibia samples were measured by using digital caliper. Afterwards bone samples were stored at -20ºC for further analyses. Femurs and tibias were subjected to the three-point bending tests until failure occurred, with Instron 5944 testing frame (Instron, Norwood, MA, USA). Loading rate was 5 mm/min. Spon length was 70 mm for bones. Load was applied to the midpoint of the shaft. Load versus displacement data was collected for each sample. Stiffness values were calculated from the slope of the linear region of the load displacement curves. Ultimate load (UL) and displacement at ultimate load (DUL) were determined from the load displacement curves as well. Yield load (YL) is the load which permanent deformation of the system begins.

Displacement at yield load (DYL) is the displacement at which permanent deformation begins [24].

Tibia Ash and Mineral Concentrations

After bone breaking strength analysis, tibia samples were defatted in chloroform and methanol (2:1) for 72 h. Subsequently, tibia samples were dried for 12 h at 105ºC and ashed overnight at 600ºC to determine dry matter and ash percentage, respectively. Tibia ash samples were crushed manually, weighed (approximately 300 mg) and mixed with 8 mL HCl and 2 mL nitric acid. Afterwards, digested tibia ash samples were diluted and concentrations of calcium, phosphorus, magnesium, potassium, copper, iron, manganese, and zinc were determined by ICP-OES (Perkin Elmer Optima™ DV 2100 Model, Dual View, Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA) [25].

Statistical Analysis

Data were analysed using the ANOVA procedure of the SPSS version 14.01 (SPSS Inc., Chicago, IL, USA) [26]. Significant differences among treatment groups were tested by Tukey's multiple range tests. The effect of graded levels of dietary CST on different variables was analysed using polynomial contrasts. Statistical differences were considered significant at P<0.05.

RESULTS

The effects of graded levels of CST on BWG, FI, and FCR are shown in *Table 3*. No significant differences were observed among the treatment groups in terms of BWG, FI, and FCR between d 11-21. Similarly, BWG and FI were not affected by dietary addition of CST between d 21-42 and also during the entire experimental period. However, dietary CST supplementation at 10 g/kg during grower and 15 mg/kg during finisher period (CST10) significantly improved FCR in comparison to CST20 between d 21-42 (P=0.007) and 11-42 d (P=0.001) of the study.

The effects of graded levels of CST on carcass characteristics are shown in *Table 4*. Dietary supplementation of CST had no significant effect on carcass, liver and abdominal fat weights and also their relative percentages to BW.

The effects of graded levels of CST on the fatty acid composition of abdominal fat are shown in *Table 5*. Fatty acid composition of the abdominal fat was significantly influenced (P<0.001) by the increasing level of CST and the ratio of unsaturated to saturated fatty acids (U:S) were reduced with the increase in the level of CST (grower phase; 0, 10, 20, 30 g/kg; finisher phase; 0, 15, 30, 45 g/kg).

Morphological measurements of the duodenum, jejunum, and ileum are shown in *Table 6*. Dietary CST supplementation had no effect on duodenum histomorphological measurements. CST10 and CST20 had shallow crypt as compared to CST0 (P=0.005). Villus height to crypt depth ratio of the jejunum was found to be higher (P=0.011) in CST10 birds in comparison to those birds in CST0 and CST30 groups. Similarly, ileum villus height (P=0.014) and surface area (P=0.047) were significantly increased in birds fed diet supplemented with 10 g/kg (grower phase) and 15 g/kg (finisher phase) in comparison to those fed CST0 diet.

The effects of graded levels of CST on femur and tibia biomechanical characteristics are shown in *Table 7*. Dietary fat treatment had no effect on tibia and femur characteristics in terms of length and width. Significant linear response in UL was observed with the increasing level of CST in femur samples (P=0.020). No differences were observed in DUL, YL, and DYL parameters in both femur and tibia samples. Stiffness of femur was significantly

Table 3. *Effects of dietary fat treatments on BWG, FI and FCR of broilers*[1]

Period	Item	Dietary Treatment[2]				Statistics		Contrast	
		CST0	CST10	CST20	CST30	SEM	*P*-value	*L*	*Q*
11 to 21 d	BWG (g)	597.4	608.4	603.8	579.2	5.38	0.242	0.219	0.104
	FI (g)	848.5	842.9	862.4	836.0	4.92	0.288	0.679	0.293
	FCR	1.422	1.386	1.430	1.445	0.01	0.262	0.236	0.237
21 to 42 d	BWG (g)	2326	2373	2268	2337	16.75	0.159	0.624	0.725
	FI (g)	3814	3844	3830	3842	19.07	0.952	0.705	0.827
	FCR	1.641^{ab}	1.619^{a}	1.689^{b}	1.644^{ab}	0.01	0.007	0.160	0.331
11 to 42 d	BW (g), on d 42	3289	3343	3237	3283	17.68	0.211	0.420	0.912
	BWG (g)	2923	2981	2871	2917	17.69	0.180	0.399	0.844
	FI (g)	4662	4686	4693	4678	20.47	0.965	0.793	0.664
	FCR	1.595^{a}	1.572^{a}	1.634^{b}	1.605^{ab}	0.01	0.001	0.038	0.732

^{a,b} Means with different superscripts in the same row are significantly different (P<0.05)
[1] Data represent mean values of 5 replicates per treatment; [2] Grower Phase (11 to 21 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 10 g/kg CST; CST20 = basal diet containing 20 g/kg CST; CST30 = basal diet containing 30 g/kg CST. Finisher Phase (22 to 42 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 15 g/kg CST; CST20 = basal diet containing 30 g/kg CST; CST30 = basal diet containing 45 g/kg CST

Table 4. *Effects of dietary fat treatments on carcass, liver, and abdominal fat parameters on d 42*[1]

Item	Dietary Treatment[2]				Statistics		Contrast	
	CST0	CST10	CST20	CST30	SEM	*P*-value	*L*	*Q*
BW, g, slaughtered	3227	3211	3212	3177	12.03	0.512	0.170	0.698
Carcass, g	2355	2356	2345	2341	8.81	0.912	0.514	0.864
Carcass, %	72.97	73.39	73.01	73.69	0.19	0.502	0.299	0.742
Liver, g	61.27	61.24	61.32	60.53	0.45	0.924	0.616	0.689
Liver, %	1.90	1.91	1.91	1.91	0.15	0.995	0.844	0.853
Abdominal Fat, g	37.51	38.72	40.37	40.15	1.22	0.837	0.400	0.778
Abdominal Fat, %	1.16	1.21	1.26	1.26	0.04	0.782	0.324	0.814

[1] Data represent mean values of 10 replicates per treatment; [2] Grower Phase (11 to 21 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 10 g/kg CST; CST20 = basal diet containing 20 g/kg CST; CST30 = basal diet containing 30 g/kg CST. Finisher Phase (22 to 42 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 15 g/kg CST; CST20 = basal diet containing 30 g/kg CST; CST30 = basal diet containing 45 g/kg CST

Table 5. *Effects of dietary fat treatments on fatty acid composition of abdominal fat on d 42*[1]

Item	Dietary Treatment[2]				Statistics		Contrast	
	CST0	CST10	CST20	CST30	SEM	*P*-value	*L*	*Q*
C14:0	0.45^{c}	0.54^{b}	0.83^{a}	0.91^{a}	0.03	<0.001	<0.001	0.651
C16:0	15.69^{b}	16.87^{b}	19.81^{a}	20.11^{a}	0.34	<0.001	<0.001	0.211
C16:1	2.12^{b}	2.69^{b}	3.67^{a}	3.89^{a}	0.14	<0.001	<0.001	0.320
C18:0	4.91^{c}	6.13^{b}	6.82^{a}	7.10^{a}	0.16	<0.001	<0.001	0.009
C18:1 cis-9	30.30^{d}	32.21^{c}	36.54^{b}	38.30^{a}	0.55	<0.001	<0.001	0.852
C18:2	43.65^{a}	38.51^{b}	30.09^{c}	27.15^{d}	1.10	<0.001	<0.001	0.109
C20:0	2.60^{a}	2.62^{a}	2.09^{b}	2.37^{ab}	0.05	<0.001	<0.001	0.095
Others	0.28	0.44	0.15	0.18	0.03			
Unsaturated (U)	76.10^{a}	73.54^{b}	70.32^{c}	69.38^{c}	0.47	<0.001	<0.001	0.040
Saturated (S)	23.65^{c}	26.16^{b}	29.55^{a}	30.48^{a}	0.49	<0.001	<0.001	0.056
U:S	3.23^{a}	2.82^{b}	2.39^{c}	2.28^{c}	0.07	<0.001	<0.001	0.009

[a-d] Means with different superscripts in the same row are significantly different (P<0.05); [1] Data represent mean values of 10 replicates per treatment; [2] Grower Phase (11 to 21 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 10 g/kg CST; CST20 = basal diet containing 20 g/kg CST; CST30 = basal diet containing 30 g/kg CST. Finisher Phase (22 to 42 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 15 g/kg CST; CST20 = basal diet containing 30 g/kg CST; CST30 = basal diet containing 45 g/kg CST

higher (P=0.003) in CST30 group in comparison to CST0 and CST10. Dietary inclusion of CST (20 g/kg grower and 30 g/kg finisher phase) resulted with an increase (P=0.020) of stiffness in tibia samples when compared with birds fed a diet containing 0 g/kg CST (CST0).

The effects of graded levels of CST on tibia ash percentage and mineral concentrations are shown in *Table 8*. Birds fed CST10 diet significantly lower (P=0.011) tibia ash percentage in comparison to those fed with CST0. In addition, tibia Ca (P=0.009) and P (P=0.009; linear, P=0.030) concentrations of CST10 and CST30 groups were lower than those in the CST0 group. With the increase in dietary CST, tibia Fe and Mn concentration exhibited a linear response (P<0.001). On the contrary, tibia Mg, K, Cu, and Zn levels were not influenced by the dietary fat treatment.

DISCUSSION

Improvements in genetic capacity have led to the ability for modern broilers to gain more weight by consuming less feed. This outcome resulted in precise ration formulations to meet their energy and nutrient requirements. As an essential ingredient, both animal and vegetable based fats are used in poultry diets for their high energy content, and also for their favourable effect on feed texture, nutrient digestibility, and fatty acid metabolism. However, these ingredients are more expensive than cereals or other major ingredients that are used in diets and ever-increasing prices have led the broiler producer to seek alternative cheap fat sources.

In the present study, dietary supplementation of calcium soaps of tallow had no significant effect on BWG and FI of the birds during the entire experimental period. On the contrary, overall FCR was found to be better in birds fed diets supplemented with CST0 and CST10 diet in comparison to birds fed CST20 diet, but similar to those fed with CST30. However, no differences were observed between CST0 and CST30 groups in terms of growth performance during

Table 6. *Effects of dietary fat treatments on histomorphological parameters of the duodenum, jejunum, and ileum on d 42*[1]

Intestine	Item	Dietary Treatment[2]				Statistics		Contrast	
		CST0	CST10	CST20	CST30	SEM	*P*-value	*L*	*Q*
Doudenum	Villus height (µm)	1565	1509	1529	1551	18.80	0.753	0.912	0.324
	Crypt depth (µm)	134.8	120.7	121.2	121.5	2.37	0.096	0.059	0.122
	VH:CD ratio[3]	11.69	12.64	12.86	12.90	0.27	0.365	0.122	0.404
	Villus width (µm)	155.6	149.9	156.3	141.5	2.25	0.068	0.067	0.291
	Villus S.A.[4] (mm^2)	0.767	0.710	0.751	0.689	0.01	0.237	0.153	0.947
Jejunum	Villus height (µm)	923.6	909.0	885.5	879.7	12.72	0.600	0.187	0.865
	Crypt depth (µm)	113.8^{a}	93.4^{b}	98.7^{b}	105.5^{ab}	2.24	0.005	0.272	0.001
	VH:CD ratio	8.18^{b}	9.82^{a}	9.04^{ab}	8.44^{b}	0.20	0.011	0.999	0.003
	Villus width (µm)	144.9	153.1	150.3	144.6	2.20	0.458	0.845	0.126
	Villus S.A. (mm^2)	0.421	0.436	0.418	0.399	0.01	0.447	0.248	0.294
Ileum	Villus height (µm)	588.6^{b}	716.3^{a}	642.4^{ab}	653.6^{ab}	14.42	0.014	0.304	0.031
	Crypt depth (µm)	99.3	108.9	104.6	112.3	2.16	0.170	0.074	0.819
	VH:CD ratio[4]	5.96	6.66	6.19	5.88	0.15	0.241	0.596	0.090
	Villus width (µm)	157.7	155.0	157.3	154.6	2.24	0.951	0.739	0.995
	Villus S.A. (mm^2)	0.289^{b}	0.348^{a}	0.318^{ab}	0.316^{ab}	0.01	0.047	0.418	0.038

[a,b] Means with different superscripts in the same row are significantly different (P<0.05)
[1] Data represent mean values of 10 replicates per treatment; [2] Grower Phase (11 to 21 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 10 g/kg CST; CST20 = basal diet containing 20 g/kg CST; CST30 = basal diet containing 30 g/kg CST. Finisher Phase (22 to 42 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 15 g/kg CST; CST20 = basal diet containing 30 g/kg CST; CST30 = basal diet containing 45 g/kg CST; [3] Villus height to crypt depth ratio; [4] Villus Surface Area; 2π × (villus width/2) × villus height

Table 7. *Effects of dietary fat treatments on femur and tibia parameters on d 42*[1]

Bone	Item	Dietary Treatment[2]				Statistics		Contrast	
		CST0	CST10	CST20	CST30	SEM	*P*-value	*L*	*Q*
Femur	Length, mm	73.22	73.78	73.59	72.89	0.39	0.863	0.743	0.434
	Width, mm	10.81	10.62	10.58	10.78	0.08	0.708	0.857	0.253
	UL, N	280.9	289.0	315.4	307.6	5.13	0.056	0.018	0.415
	DUL, mm	3.90	4.07	3.82	3.61	0.09	0.374	0.189	0.307
	YL, N	217.1	199.5	235.1	232.4	5.55	0.083	0.093	0.485
	DYL, mm	2.41	2.06	2.17	2.09	0.06	0.181	0.125	0.257
	Stiffness, N/mm	90.8^{c}	91.5^{bc}	105.5^{ab}	107.2^{a}	2.16	0.003	0.001	0.898
Tibia	Length, mm	101.8	101.9	102.0	102.0	0.44	0.997	0.850	0.925
	Width, mm	10.33	10.50	10.44	10.15	0.11	0.715	0.549	0.323
	UL, N	326.7	353.8	365.2	377.3	9.21	0.252	0.051	0.684
	DUL, mm	3.02	3.09	3.02	3.09	0.05	0.912	0.746	0.959
	YL, N	189.8	180.1	191.6	194.5	4.16	0.654	0.502	0.464
	DYL, mm	1.35	1.14	1.20	1.32	0.04	0.154	0.900	0.030
	Stiffness, N/mm	146.2^{b}	164.3^{ab}	175.7^{a}	163.5^{ab}	3.47	0.020	0.031	0.021

[a-c] Means with different superscripts in the same row are significantly different (P<0.05)
[1] Data represent mean values of 10 replicates per treatment; [2] Grower Phase (11 to 21 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 10 g/kg CST; CST20 = basal diet containing 20 g/kg CST; CST30 = basal diet containing 30 g/kg CST. Finisher Phase (22 to 42 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 15 g/kg CST; CST20 = basal diet containing 30 g/kg CST; CST30 = basal diet containing 45 g/kg CST; [3] UL: Ultimate Load, DUL: Displacement at Ultimate Load, YL: Yield Load, DYL: Displacement at Yield Load

the entire study. Our results are in agreement with the previous study results of Sanz et al.[13] who reported that dietary fat source (sunflower, tallow or lard) had no effect on feed intake, body weight gain, and final body weight. Similarly, Preston et al.[27] found no differences between experimental groups fed tallow or soy oil supplemented diets. It is generally known that saturated fatty acid-rich fats such as tallow, have lower AME value than vegetable oils which are rich in unsaturated fatty acids [12]. However, previous study revealed that dietary fat source had no

Table 8. *Effects of dietary fat treatments on tibia ash percentage and mineral concentration on d 42*[1]

Bone	Item	Dietary Treatment[2]				Statistics		Contrast	
		CST0	CST10	CST20	CST30	SEM	*P*-value	*L*	Q
Tibia	Ash, %	44.31[a]	41.11[b]	43.32[ab]	41.97[ab]	0.38	0.011	0.126	0.189
	Ca, g/kg	252.8[a]	234.7[b]	250.5[ab]	234.8[b]	2.60	0.009	0.073	0.795
	P, g/kg	126.1[a]	116.8[b]	123.6[ab]	115.7[b]	1.36	0.009	0.030	0.788
	Mg, g/kg	5.56	5.09	5.44	5.03	0.09	0.072	0.096	0.881
	K, g/kg	2.05	2.13	1.99	1.98	0.06	0.847	0.550	0.720
	Cu, mg/kg	0.60	0.60	0.60	0.50	0.02	0.127	0.058	0.171
	Fe, mg/kg	204.3[ab]	224.9[a]	159.5[bc]	139.9[c]	8.03	<0.001	<0.001	0.111
	Mn, mg/kg	4.86[a]	5.44[a]	3.47[b]	3.27[b]	0.19	<0.001	<0.001	0.152
	Zn, mg/kg	136.8	147.5	134.4	133.0	3.25	0.390	0.400	0.359

[a-c] Means with different superscripts in the same row are significantly different (P<0.05)
[1] Data represent mean values of 10 replicates per treatment; [2] Grower Phase (11 to 21 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 10 g/kg CST; CST20 = basal diet containing 20 g/kg CST; CST30 = basal diet containing 30 g/kg CST. Finisher Phase (22 to 42 d): CST0 = corn-soybean meal basal diet containing 0 g/kg CST; CST10 = basal diet containing 15 g/kg CST; CST20 = basal diet containing 30 g/kg CST; CST30 = basal diet containing 45 g/kg CST

effect on performance since the diets are balanced in terms of energy and protein [13]. On the contrary, it is generally known that fat utilization is affected by level of the included fat, basal diet composition, degree of saturation of the total dietary lipid fractions, and animal age [7,12]. Studies conducted with different dietary fats have suggested that the increasing level of Ca in diets tends to decrease AME value of supplemented fat by forming insoluble soap in the gut lumen [10,28,29] and depress broiler growth rate regardless of tallow supplementation level [10]. Tallow product used in this study was in the form of calcium soaps of fatty acids. However, dietary calcium concentration of the experimental diets was balanced by reducing the limestone level with the increasing level of calcium soaps of tallow in diets. Rising et al.[16] suggests that, rather than the added calcium, preformed calcium soaps of animal fats were utilized well by laying hens. However, as far as we know, no available data exists for the use of calcium soaps of tallow in broiler diets. According to our results, dietary supplementation of calcium soap of tallow up to 30 g/kg in grower and 45 g/kg in finisher diets of broiler chicken was well tolerated and had no negative effects on broiler growth performance in terms of BWG. Moreover, due to aforementioned synergistic effects of blending saturated and unsaturated fatty acids on broiler performance [3,12], combined use of CST with vegetable oils might have positive effects under commercial conditions. However, more research needs to be conducted to determine AME value of this product for precise diet formulation.

In modern broilers, the most significant amount of fat is deposited as abdominal fat. However, this discrete fat depot has no contribution to carcass quality and is considered a waste product for producers and consumers [30-32]. According to our results, carcass yield, liver weight, and abdominal fat weight were not influenced by dietary fat treatments. The results obtained in the present study are consistent with those of earlier studies which found no differences in abdominal fat deposition in broilers fed animal or vegetable fat supplemented diets [33-35]. However, several studies reported that broilers receiving saturated fat in their diets had higher abdominal fat weight than those fed diets containing unsaturated fat [3,13]. The lack of consistency between the studies might be related to the dietary fat level or fat type. As an important finding of the present study, dietary supplementation of CST significantly influenced the fatty acid composition of the abdominal fat. It is known that tissue fatty acid composition is typically modified by dietary fat source in pigs and poultry due to the direct absorption and deposition of fatty acids [35,36]. Crespo and Esteve-Garcia [37] reported more saturated fatty acids in the abdominal fat pad, thigh muscle, and breast muscle of birds fed a diet containing tallow in comparison to those fed with vegetable oil. From this point of view, increasing the saturated fatty acid content of the tissue might have an important impact on carcass quality by producing more stable fat against oxidation. Moreover, due to the direct impact of dietary fat treatments on abdominal fatty acid composition, it can be assumed that dietary addition of CST is efficiently utilized by broilers and deposited in the fat tissue.

Morphological changes in the small intestine, such as increased villus height, villus width, and villus height to crypt depth ratio (VH:CD), are important parameters that affect broiler performance by improving nutrient digestion and absorption [38]. Earlier studies with rats suggested that source and concentration of supplemented dietary fat influenced intestinal morphology [39]. In poultry, fat digestion and absorption mainly occurs in small intestines, especially in the duodenum and jejunum. According to our results, dietary supplementation of increasing level of CST had no detrimental effect on duodenum and jejunum villus height. Significant quadratic response in jejunum crypt depth and VH:CD ratio were observed with the increasing level of CST on d 42. Moreover, ileum villus height and

villus surface area increased in birds fed CST10 diet in comparison to those fed CST0 diet. As an important finding of the present study, feeding the fat blend that contained a low level of CST improved intestinal microarchitecture, which may be accompanied by a better FCR of these birds. These results corroborate with the results from Li et al.[40] who suggested that, supplementation of pig diets with soybean oil and coconut oil (1:1) increased villus height in comparison to soybean oil or coconut oil alone. Khatun et al.[41] showed that birds fed a diet containing 2% palm oil and 4% soybean oil diet had significantly higher villi in all small intestinal segments than those birds fed 6% palm oil alone. It can be assumed that the improvements in the small intestine morphology might be related to the synergistic effects of saturated and unsaturated fatty acids.

Leg abnormalities are highly important problems in fast-growing broilers, causing economic losses and reduced welfare [42]. A growing body of evidence indicates that dietary supplementation of saturated fats has an adverse effect on bone mineralization [43]. Our results showed that tibia ash content was reduced in birds fed CST10 diet in comparison to birds fed CST0. Moreover, increasing level of dietary CST had a negative effect on tibia Ca, P, Fe, and Mn concentrations. Results of the present study are consistent with Atteh and Leeson [29], who reported that dietary supplementation of palmitic acid significantly reduced bone ash and bone calcium content in broilers compared to oleic acid due to the increased excreted soap formation. More recently, Zhong et al.[44] suggested that supplementation of broiler diets with lard or palm oil reduced tibia calcium concentration in comparison to linseed oil. Contrary to our results, they reported that the combined use of palm oil and linseed oil, at the ratio of 60:40 or 40:60 (w:w), alleviated the adverse effect of saturated fatty acids on tibia calcium level [44]. As suggested by previous studies, structural stiffness of the bone improved with the increasing level of bone mineral content. However, at the same time, this increase negatively affects bone by making it more brittle [45,46]. Even though poor mineralization results in tibia samples were obtained in this study, it is not enough to evaluate bone strength, because bone mineral concentration needs to be evaluated along with the intrinsic and extrinsic biomechanical properties to determine bone strength and health [47]. Our results showed that femur and tibia stiffness improved with the increasing level of dietary CST concentration and it can be assumed that increased bone stiffness might help to protect against fractures [48] and improve the efficiency of leg movements in older chickens [49]. The reason for the improvement in stiffness is unclear, but it could be related to the collagen crosslinks, which are the major organic constituent of bone [50]. Rath et al.[51] revealed that crosslink content had a stronger correlation with bone strength than bone ash and density. As a fat source, the effect of dietary CST on broiler bone strength and mineral concentration has not previously been reported. Therefore, future studies should be performed to investigate the influence of saturated fatty acids on bone strength, taking into account the contribution of other determinants affecting bone strength and excreted soap formation and mineral retentions, especially Ca, P, Mg, and Zn, to determine bone quality and health.

Our results showed that dietary supplementation of CST had no detrimental effect on broiler performance parameters and might be used in broiler diets as an alternative fat source, like vegetable acid oil. However, more research needs to be conducted to determine apparent metabolisable energy value of calcium soap of tallow and to find out how this product affects fat metabolism, meat quality, and mineral retention.

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Research Article

Effect of Nano Zinc Oxide on Post-Thaw Variables and Oxidative Status of Moghani Ram Semen

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Abstract

The damages of freezing technique of sperm including motility, survival and membrane disorders involves the formation of ice crystals, oxidative stress, osmotic changes and the re-organization of lipid-protein in the cell membrane. Zinc plays basic role in membrane stability and physical properties of the attached fibers, sperm tail morphology and sperm motility. The aim of this study was to evaluate effect of Nano zinc oxide (Nano-znO) on post-thaw variables and oxidative status of Moghani ram semen. In order to collect semen of four Moghani rams, 3-4 years old with an average weight of 80-90 kg were used to twice a weekly. Sperm samples (in five replicates) were frozen in five groups: control group, 0.01, 0.1, 0.5 and 1 mg/mL of Nano-znO. The hypo-osmotic swelling test (HOST) was used to evaluate the integrity of the plasma membrane of the sperm. The results showed that the average of integrity of the plasma membrane of sperm and MDA production in the medium containing 0.1 mg/mL of Nano-znO was with the highest and lowest percentages, respectively ($P<0.05$). There was a significant difference in total motility for 0.1 and 1 mg/mL treatments compared to the control group and the use of 0.01 and 0.1 mg/mL treatments had a significant increase in the percentage of progressive motility compared to the control group but in 0.5 and 1 mg/mL treatments reduced progressive motility. Viability sperm in group 0.1 and group 0.01 was better than control group ($P<0.05$). The use of 0.1 mg/mL of Nano-znO in the ram semen diluted improved total motility, progressive motility and the percentage of survival. In conclusion using Nano-znO had a positive effect on qualitative properties of sperm and lead to a significant improvement in some antioxidant parameters of Moghani ram seminal plasma in the non-breeding season.

Keywords: *: Integrity, Motility, Nano zinc oxide, Peroxidation, Ram, Sperm, Viability*

Nano Çinko Oksitin Moghani Koç Semeninin Çözdürme Sonrası Değişkenler ve Oksidatif Durumuna Etkisi

Öz

Spermin dondurulma tekniğine bağlı olarak şekillenen mortalite, canlılık ve membran bozuklukları hasarları buz kristallerinin oluşmasını, oksidatif stresi, ozmotik değişiklikleri ve hücre zarında lipit-protein reorganizasyonunu içermektedir. Çinko membran stabilitesi, ataçlanmış fiberlerin fiziksel özellikleri, sperm kuyruk morfolojisi ve sperm motilitesi üzerinde önemli rol oynar. Bu çalışmanın amacı nano çinko oksitin (Nano-ZnO) Moghani koç semeninin çözdürme sonrası değişkenler ve oksidatif durumuna etkisini araştırmaktır. Ortalama ağırlıkları 80-90 kg olan 3-4 yaşlı dört Moghani koçundan haftada iki kere semen toplandı. Sperm örnekleri (beş tekrar olarak) kontrol, 0.01, 0.1, 0.5 ve 1 mg/mL Nano-ZnO ile dondurulan gruplara ayrıldı. Spermlerin plazma membran bütünlüklerini değerlendirmek amacıyla Hipo ozmotik şişme testi (HOST) kullanıldı. 0.1 mg/mL Nano-ZnO ile dondurulan spermanın sperm plazma membran bütünlüğü ortalaması en yüksek iken MDA üretimi ortalaması en düşük olarak belirlendi ($P<0.05$). Toplam motilite bakımından 0.1 ve 1 mg/mL uygulama grupları ile kontrol grubu arasında anlamlı fark tespit edildi. İlerleyici motilite yüzdesi 0.01 ve 0.1 mg/mL uygulama gruplarında kontrol grubu ile karşılaştırıldığında anlamlı derecede artma gösterirken 0.5 ve 1 mg/mL gruplarında düşme belirlendi. Sperm canlılığı kontrol grubuna göre 0.1 ve 0.01 gruplarında daha iyiydi ($P<0.05$). Koç semenin sulandırmada 0.1 mg/mL Nano-ZnO kullanmak total motiliteyi, ilerleyici motiliyeyi ve yüze hayatta kalma oranlarını iyileştirdi. Sonuç olarak, Nano-ZnO kullanmak sperm kalite özeliklerine pozitif etki gösterdi ve üreme sezonu dışında Moghani koç seminal plazmada bazı antioksidan parametrelerde anlamlı iyileşmelere neden oldu.

Anahtar sözcükler*: Bütünlük, Motilite, Nano Çinko oksit, Peroksidayon, Koç, Sperm, Canlılık*

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INTRODUCTION

Mammalian semen freezing has created a major development in the storage and protection of sperm cells and is a way of preserving the sex-cell protoplasm that can be used with biodiversity conservation [1]. Sperm banks can play an important role along with other reproductive technologies, in improving breeds and protecting endangered wild and native species. Freezing technique of sperm causes damage including motility, survival, membrane disorders and DNA abnormalities. The damage involves the formation of ice crystals, oxidative stress, osmotic changes, and the re-organization of lipid-protein in the cell membrane [2]. The formation of ice crystals in and around the cell in the freezing process is one of the most important destructive factors of the cell, which reduces sperm motility and fertility [3]. Rams sperm cells have high ratio of unsaturated fatty acids to saturated fatty acids, which makes membranes susceptible to peroxidation damage in the presence of oxygen free radicals. The sperm membrane is susceptible to lipid peroxidation because of containing a high amount of unsaturated fatty acids [4]. In this regard, the accumulation of free oxygen radicals due to metabolism leads to impairment in the membrane's strength, function, survival and fertility of the sperm. The most prominent effect of lipid peroxidation in cells is to disturb the order and function of cell membranes, so that the ion transfer process is changed and disrupted [4]. Zinc element plays basic role in membrane stability and physical properties of the attached fibers, sperm tail morphology and sperm motility. Lack of zinc may increase oxidative damage, resulting in poor sperm quality [5]. Also, zinc element in the sperm, reacts to tight dense fiber that reacts with sulfhydryl groups and prevents oxidation [6]. Some *in vivo* evidence suggests that zinc acts as *in vivo* as a superoxide cleaner produced by incomplete spermatozoids or leukocytes. Other tests have shown that zinc can clean up radicals induced by various factors, including ionizing radiation, and reduce MDA levels, so it is known as a highly antioxidant [7]. The most common combination of zinc element is its oxide form (ZnO) [8], which is preferred for two reasons: one that has the highest concentration of zinc [9], and the other is that it is absorbed high in the body and is also better tolerated by the target tissues [10]. Recently, Nano-zinc oxide has attracted much attention in animal studies [11,12]. Different nanoparticles are new forms of materials with high biologic properties and low toxicity, which seem to have high potential for passing through physiological barriers and access to specific target tissues [13]. The use of antioxidants, such as Nano-zinc oxide, can be important in reducing the production of free radicals and increasing sperm survival [4,14]. Therefore, the aim of this study was to evaluate effect of Nano zinc oxide on post-thaw variables and oxidative status of Moghani ram semen.

MATERIAL and METHODS

Animals, Semen Collection and Freeze-thawing Process

This experiment was performed at University of Mohaghegh Ardabili, Iran Ardabil (Latitude: 38.253736°; Longitude: 48.299990°; Elevation: 4423 ft) and lasted from May to June. The animals (3-4 year old rams) were maintained in open front barns; under uniform nutritional conditions, so that levels of nutrition remained equal as each ram was fed a daily with *ad libitum* diet according to the National Research Council (NRC) containing a 20% concentrate (75% barley, 25% corn, soya and bran) and 80% alfalfa hay. Semen was collected, twice a week for 4 weeks, from four mature Moghani breed rams during the non-breeding season (from mid-January to mid-February 2017) that had been trained to serve an artificial vagina (42-43°C). This study has been approved by local ethical committee of Mohaghegh Ardabili University (Permission No: 51-1842 date: 2016-11/08). The training period lasted four to six weeks. An ovariectomized ewe was used as a teaser and rams were placed in a pen next to the collection area for semen collection of a ram using an artificial vagina. The semen collection procedure could be visualized by each ram [15]. Immediately after collection, the ejaculates were immersed in a warm water bath at 37°C until freezing process. Before the freezing process, macroscopic and microscopic examination was performed for all samples, which included amount of the volume of ejaculate, apparent colors, sperm concentration, and native sperm activity. The native sperm activity was evaluated by subjective method according to the motile sperms percentage in the native ejaculate (Student Microscope Model SM 5 with phase contrast by INTRACTO MICRO, spol. s r. o., lens 10 × /0, 25 PHD). In this experiment, the semen samples showed natural color (color cream), and semen samples with more than 70% of progressive motility and a more than 2.5×10^9 of concentration were used for freezing process.

The basic extender consisted of 3.07 g Tris (hydroxymethyl-aminoethane, Merck 64271, Germany), 1.64 g citric acid (BHD 1081, England), and 1.26 g fructose (BDH 28433, England) in 100 mL distilled water, containing 5.0% (v/v) glycerol (Merck, 2400, Germany) and 20% (v/v) egg yolk with 150 ppm α- tocopherol [16]. The experimental treatments were supplementation of the base extender with 0, 0.01, 0.1, 0.5 or 1 mg/mL of Nano zinc oxide. Nano Zinc Oxide was purchased from Iranian agent of US Research Nanomaterial, Inc. Port Co., Ltd., USA. The sizes of elemental ZnO particles ranged from 10 to 30 nm, stock: US3590, in the form of white powder and Purity: 99%, APS: 10-30 nm, Color: white, Crystal Phase: single crystal, Morphology: nearly spherical, SSA: 20-60 m^2/g, True Density: 5.606 g/cm^3.

Extenders were centrifuged at 15.000×g for 30 min. The supernatants were aspirated and filtrated through a 0.45 mm membrane filter (Millipore, S.A., Molsheim, France). At

each semen collection time, eight straws per extender (16 replicates) were then stored in liquid nitrogen. Two straws per extender per semen collection were thawed at 37°C for 30 sec after 24 h, for evaluation of the post-thawing sperm characteristics. The mean values of the sperm characteristics obtained for the two straws were used in statistical analysis of the data (16 replicates for each cryopreservation extender).

The semen diluent was prepared and kept in a water bath at 37°C, on the day of semen collection. Semen samples were pooled and diluted (1:4) before freezing. Diluted semen samples were cooled to 5°C over 120 min, transferred into 0.25 mL straws, equilibrated for 2 h at 5°C and frozen in liquid nitrogen vapor (4-6 cm from the liquid nitrogen surface level) for 10 min. They were then stored in liquid nitrogen for 24 h. The straws were thawed in water bath at 37°C for 30 sec and the frozen-thawed semen assessed to semen characteristics.

Evaluation of Sperm Motility Characteristics

To evaluate the sperm motility characteristics, after the freezing-thawing process, a CASA computer analysis system equipped with an x-ray contrast microscope (CKX41; Olympus, Tokyo, Japan) was used, with 5 µL. The semen samples were preheated (37°C) and analyzed by Computer computer-assisted analysis sperm (CASA) after coating with lamina. The evaluation was based on a count of at least 100 sperm [16]. The sperm motility, and sperm motion variables that were estimated by these systems included total motile, progressively motile, straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (LIN = VSL/VCL), and straightness (STR = VSL/VAP), lateral head displacement, beat cross frequency (BCF) *(Fig. 1)*. Eosin-nigrosin was used to evaluate the number of live and dead sperm. The basis of this staining is that the eosin color penetrates into the dead sperm, while the sperm do not color *(Fig. 2)*. From each sample 200 sperm were counted and the percentage of colored sperm (dead) and sperm (no color) were calculated.

Evaluation of the Integrity of the Plasma Membrane of the Sperm

Hypo-osmotic swelling test (HOST) was used to assess the integrity of the plasma membrane of the sperm [17]. For this purpose, 30 µL of semen samples were incubated with 100 µL of hypo osmotic medium. The hypo-osmotic solution was prepared by dissolving 0.735 g of Tri-sodium citrate dehydrate and 1.351 g of fructose in 100 mL of double-distilled water. First, 500 µL of hypoosmotic solution was mixed with 50 µL of each specimen and incubated at 37°C for 30 min, then 0.2 mL of the mixture was poured onto the slurry and was spread out [18]. The resultant lens was evaluated under a contrast phase microscope (CKX41; Olympus, Tokyo, Japan) and magnified at ×400. From each slide, 200 sperm were counted and the percentage of tangled sperm (healthy membrane sperm) were calculated *(Fig. 3)* [18]. In this experiment, kinky sperm were identified as healthy sperm because the main function of the tail is motility, since there is no fertility without motility [15]. MDA was used to evaluate oxidative stress as major bioactive marker of lipid peroxidation.

Evaluation of Lipid Peroxidation

MDA concentration was measured as a lipid peroxidation

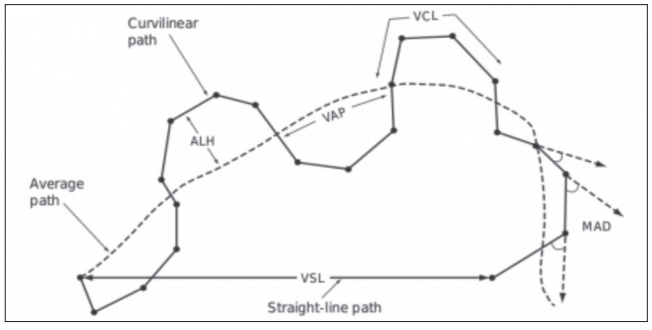

Fig 1. The characteristics of sperm motility

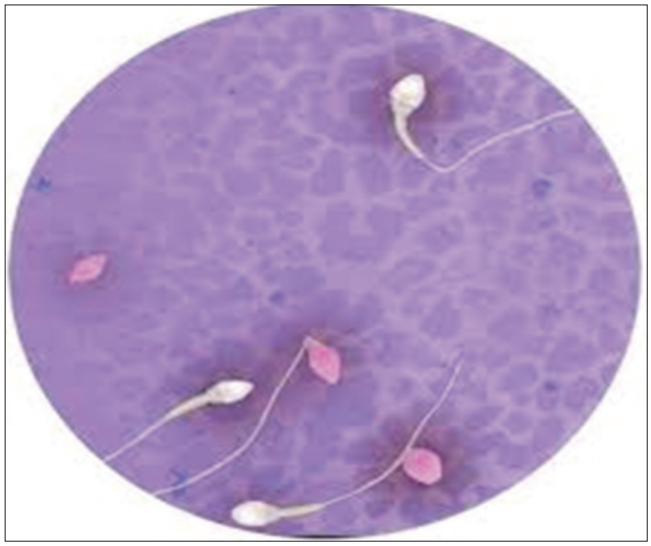

Fig 2. Eosin test: Pink stained sperms are non-viable sperms as compared to white ones with intact membranes

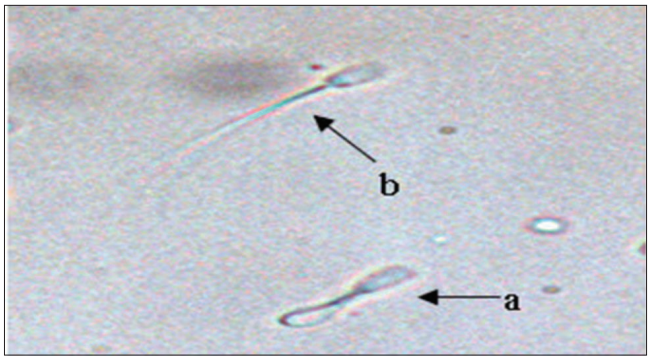

Fig 3. Plasma membrane integrity of the sperm. **a:** healthy sperm and **b:** dead sperm

index in semen samples using the TBARs method and by spectrophotometry [19]. At 95°C, the MDA molecule reacts with two molecules of TBB (TBA) and forms a pink complex. To measure the concentration of MDA, 200 µL of each sample was mixed with 1 mL of EDTA, 1 mL of TCA, and centrifuged at 3.000 rpm, then mixed with 1 mL of butylated hydroxy toluene (BHT), and heated at 95°C for 40 min. The samples were centrifuged for 10 min at 7.000 rpm after cooling in ice. Samples were recorded at 532 nm with spectrophotometry and MDA concentration in nmol/dL.

Methods of Data Analysis and Statistical Model

The normalization of the data was evaluated using SAS software [20]. Values are reported as mean ± SD. Statistical significance between groups was computed by analysis of variance and Duncan's test was used to compare the meanings. Data was analyzed using one way ANOVA test with SAS software [20] and using the GLM procedure in a completely randomized analysis and $P<0.05$ was considered significant. Statistical model research design was as:

$$Y_{ij} = \mu + A_i + e_{ij}$$

where: Y_{ij} is the observation, μ is the population mean, A_i is the effects of experimental treatments and e_{ij} is the residual error.

RESULTS

The effects of different levels of Nano zinc oxide on the motility parameters are presented in *Table 1*. The total motility percentage for the 0.01 and 0.1 mg/mL treatments was different compared with the control group and treatment with 1 mg/mL caused a significant decrease in total motility. The use of 0.01 and 0.1 mg/mL treatments was resulted in a significant increase in progressive motility than the control group. The use of the 0.1 mg/mL Nano zinc oxide level had the greatest impact compared to other levels in VCL (Curvilinear Velocity), VSL (Straight line velocity) and VAP (Average path velocity). The effects of different levels of Nano zinc oxide on survival of sperm are presented in *Table 2*. The use of 0.1 and 0.01 mg/mL Nano zinc oxide levels resulted in the survival of sperm at the highest level compared to other levels and also the use of 1 mg/mL lead to in the most significant reduction compared to the control group.

The effects of different levels of Nano zinc oxide on the average percentage of integrity of the plasma membrane of sperm and the amount of MDA production as lipid peroxidation indicator are presented in *Table 3*. The amount of MDA production was lower at 0.1 mg/mL Nano zinc oxide and was significantly different from the control group ($P<0.05$). The average percentage of integrity of the plasma membrane of sperm was highest in the medium containing 0.1 mg/mL Nano zinc oxide, which was significantly different from other groups ($P<0.05$).

DISCUSSION

The resulting papers reported that some protection and distribution to spermatozoa for high fertility and genetic value suggest a freeze–thawing process or cryo-

Table 1. *Effect of Nano zinc oxide on ram sperm motility parameters (mean ± SD)*

Treatments (mg/mL)	Total Motile (%)	Progressively Motile (%)	VCL (µ/s)	VSL (µ/s)	VAP (µ/s)
0	44.4±2.68 ^{b}	17.6±1.35 ^{b}	102.6±3.28 ^{b}	43.9±1.51 ^{b}	53.6±1.83 ^{b}
0.01	54.2±2.68 ^{a}	23.4±1.35 ^{a}	114.9±3.28 ^{a}	48.6±1.51 ^{ab}	58.6±1.83 ^{ab}
0.1	58.4±2.68 ^{a}	26.0±1.35 ^{a}	117.6±3.28 ^{a}	52.3±1.51 ^{a}	60.8±1.83 ^{a}
0.5	42.2±2.68 ^{bc}	18.8±1.35 ^{b}	111.3±3.28 ^{ab}	46.2±1.51 ^{b}	55.7±1.83 ^{ab}
1	35.0±2.68 ^{c}	17.2±1.35 ^{b}	101.1±3.28 ^{a}	43.8±1.51 ^{b}	55.1±1.83 ^{ab}

The same letters in each column represent significantly different at 5% level
VCL (Curvilinear Velocity), VSL (Straight line velocity) and VAP (Average path velocity)

Table 2. *Effect of Nano zinc oxide on survival of ram sperm (mean ± SD)*

Treatments (mg/mL)	Survival of Ram Sperm (%)
0	51.0±2.18^{b}
0.01	64.3±2.18^{a}
0.1	66.4±2.18^{a}
0.5	52.6±2.18^{b}
1	46.1±2.20^{b}

The same letters in each column represent significantly different at 5% level

Table 3. *The effect of Nano zinc oxide on lipid peroxidation and plasma membrane integrity*

Treatments (mg/mL)	Malondialdehyde (MDA)	Plasma Membrane Integrity
0	3.6±0.41^{ab}	33.5±1.89^{cd}
0.01	3.0±0.41^{bc}	46.7 ±1.89^{b}
0.1	2.1±0.41^{c}	56.9 ±1.89^{a}
0.5	3.5±0.41^{ab}	38.8± 1.89^{c}
1	4.6±0.41^{a}	28.7 ±1.89^{d}

The same letters in each column represent significantly different at 5% level

preservation [21-23]. In the study conducted by Dissanayake et al.[24] on human sperm, it was shown that adding 1.2 µmol per mL of zinc to the culture medium significantly reduced the percentage of human sperm counts between zero to four hours, this means that with time declined the percentage of motile spermatozoa. However, this researcher added the amount of 0.6 µmol/mL to the culture medium was observed that sperm motility increased compared to the control group. Also, the percentage of sperm recovery was higher in group 0.6 µmol/mL, and there was a significant difference with the control group [24]. This is likely to be related to the function of the element in Zn such that Zinc is associated with ATP, plays a role in shrinking and regulating the energy of its phospholipids, and therefore has a direct impact on sperm motility [25]. In a research on buffalo sperm, it has been shown that zinc in the semen plasma has a direct relationship with the survival and progressively motile of sperm [26]. In another study on buffalo sperm, it has been shown that the presence of semen in the plasma directly affects the survival and progressively motile of sperm [26]. In the present experiment, the levels above 0.1 mg/mL (administration at high concentration) have not a positive effect on the parameters of sperm motility and viability, which it can be expressed as a toxic level for the sperm dilution environment *(see Tables)*. Our results indicated that any Zn imbalance in seminal plasma or spermatozoa may have a negative impact on seminal abnormalities and/or oxidative stress development and therefore may be considered as a risk cause for ram fertility problems [26].

Animal studies have shown that [27] zinc effect on oxidative damage in rat testicular was investigated and the results showed that iron concentration in testes in zinc deficient male diets was higher and there was a significant difference in diets with zinc. Observed oxidative damage may occur due to the increased free radicals associated with the accumulation of iron in the tissue or the reduction of zinc-dependent antioxidant processes [27]. The addition of zinc element in human sperm samples produced a significant difference in lipid peroxidation and hypo-osmotic swelling test (HOST) with the control group [28]. Although the evidence for the antioxidant properties of zinc is compelling, the mechanisms are still unclear. But the important effects are generally thought to involve two mechanisms: protection of protein sulfhydryls or reduction in the formation of ·OH from H_2O_2 through the antagonism of redox-active transition metals, such as iron and copper [29]. At the our study, the level of 0.1 mg/mL Nano zinc oxide showed that the production level of malondialdehyde was in minimum which showed that there was less peroxidation in Moghani ram sperm after freeze-thawing process, and indicated that there was not stress oxidative or peroxidation in sperm cryopreservation medium containing Nano zinc oxide. Lipid peroxidation is the oxidative degradation of lipids. It is the process in which reactive oxygen species (ROS) or free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage due to higher production of the oxidative cryo-injury resulting from sperm cooling, freezing and thawing. Furthermore, the cryopreservation process induces ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) [30] and when spermatozoa are attacked by ROS, a loss in polyunsaturated fatty acids (PUFAs) from the plasma membrane is occurred and their survival and fertilizing ability is declined [31]. These results are consistent with previous data showing that Zinc can clean up radicals induced by various factors, including ionizing radiation, and reduce the amount of lipid peroxidation, and therefore it is known as a high-protection antioxidant [7]. Also, in other study found that zinc oxide nanoparticles could maintain the integrity of the cell membrane against oxidative damage, increase the amount of antioxidant enzymes, reduce the amount of malondialdehyde, improve the anti-oxidant activity, and reduce the amount of free radicals [12]. In this study, the antioxidant properties of zinc oxide nanoparticles were helpful in reducing the production of malondialdehyde, and the addition of zinc oxide to the environment in diluent improved the integrity of the membrane. According to the results, the use of 0.01 and 0.1 mg/mL Nano zinc oxide improved sperm survival and sperm motility characteristics. As a result, the use of appropriate levels of zinc supplementation in ram semen diluent can be useful in maintaining the quality of the sperm during the freeze-thawing process.

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Research Article

Molecular Survey of Hepatozoonosis in Natural Infected Dogs: First Detection and Molecular Characterisation of *Hepatozoon canis* in Kyrgyzstan

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Abstract

Canine hepatozoonosis is a tick-borne protozoan disease and widespread in Europe, Africa, Asia and America. There is not any available data about the presence of *Hepatozoon* infections in dogs in Kyrgyzstan. In the study we aimed that investigate the presence of *Hepatozoon canis* and the prevalance of *Hepatozoon* infections in dogs from Kyrgyzstan using polymerase chain reaction (PCR) and sequence analysis. To determine the prevelance of hepatozoonosis in dogs, a total of 170 blood samples were applied to PCR to amplify a fragment of 666 bp found in 18S SSU rRNA gene of *Hepatozoon* spp. The PCR results shown that *Hepatozoon* infection rate was 28.8% (49/170) in dogs. Eleven representative positive samples were sequenced to classification of the species. The nucleotide sequences were compared to the *H. canis* sequences which registered in GenBank using the basic local alignment search tool. Results of sequence analyse of 11 amplicons indicated that 8 were 100% identical and the other 3 sequences shared 99% similarity with *H. canis*. The sequences were deposited in Genbank with accession numbers from MG917709 to MG917719. It was the first record of *H. canis* in dogs in Kyrgyzstan.

Keywords: *Hepatozoonosis, Hepatozoon canis, Dog, PCR, Sequencing, Kyrgyzstan*

Doğal Enfekte Köpeklerde *Hepatozoon* Enfeksiyonlarının Moleküler Yöntemlerle Araştırılması: *Hepatozoon canis*'in Kırgızistan'da İlk Tespiti

Öz

Köpeklerde hepatozoonozis kenelerle nakledilen ve Afrika, Avrupa, Asya ile Amerika'da yaygın olarak görülen protozoan bir hastalıktır. Kırgızistan'da köpeklerde *Hepatozoon* enfeksiyonlarının varlığına dair bir bilgiye ulaşılamamıştır. Bu çalışmada, polimeraz zincir reaksiyonu (PZR) ve sekans analizi ile Kırgızistan'da köpeklerde *Hepatozoon* enfeksiyonlarının yaygınlığının ve *Hepatozoon canis*'in varlığının belirlenmesi amaçlanmıştır. *Hepatozoon* enfeksiyonlarının prevalansını belirlemek için 170 kan örneğine, *Hepatozoon* spp. 18S SSU rRNA geninin 666 bp'lik kısımını amplifiye etmek üzere PZR uygulanmıştır. PZR sonucunda *Hepatozoon* spp. enfeksiyon oranı %28.8 (49/170) olarak ortaya çıkmıştır. On bir PZR pozitif DNA örneğinin DNA dizilimi belirlenmiş ve elde edilen DNA dizilimleri BLAST programı kullanılarak GenBank'ta kayıtlı diğer dizilimlerle karşılaştırılmıştır. Bunlardan 8'inin GenBankasında kayıtlı *H. canis* DNA dizilimleri ile %100 oranında eşleştiği, diğer 3 dizilimin ise %99 oranında bir benzerliğe sahip olduğu belirlenmiştir. DNA dizilimleri GenBankasına MG917709-MG917719 numaraları altında kaydedilmiştir. Bu çalışma Kırgızistan'da köpeklerde *H. canis*'in belirlendiği ilk çalışma niteliğindedir.

Anahtar sözcükler: *Hepatozoonozis, Hepatozoon canis, köpek, PZR, Sekans analizi, Kırgızistan*

INTRODUCTION

Canine hepatozoonosis is a tick-borne protozoan disease and widespread in Europe, Africa, Asia and America. The first hepatozoonosis case was determined in India, 1905, named as *Leukocytozoon canis*. After the parasite was

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detected in neutrophils, it transferred to the genus of *Hepatozoon* [1,2]. There are more than 340 species in the genus of *Hepatozoon* and two of them infect dogs, *Hepatozoon canis* and *Hepatozoon americanum* [1,3]. On the other hand, some new genotypes have been found in dogs with the molecular-genetic based studies in recent years [4,5]. The disease is transmitted by the ingestion of the vector ticks. *Rhipicephalus sanguineus* in Asia, Europe, Africa and Brazil, *Amblyomma maculatum* in South America are known as vector of canine hepatozoonosis [6-8].

Clinical signs of hepatozoonosis in dogs depend on caused species, the nutritional and the others individual factors. *H. canis* infections can change from subclinical to severe and fatal disease. Subclinical and mild disease is common and generally have low parasitemia but 100% of neutrophils can be infected with *H. canis* in the severe disease which characterised by lethargy, fever and extreme weight loss. *H. americanum* infections causes fever, generalised pain or hyperaesthesia, myositis, muscle atrophy, weakness, depression, reluctance to rise and mucopurulent ocular discharge [2,9,10].

Diagnosis of canine hepatozoonosis may be done with seen of intracytoplasmatic gamonts in neutrophils and monocytes by microscopic examination of the thin blood smears. Secondly the histopathologically meront and monosoic cysts can be investigated. The biopsy of skeletal muscle is the gold standard for the diagnosis of *H. americanum* infections in dogs because gamonts of the parasite are rarely seen in thin blood smears. Serological methods such as indirect immunofluorescence and enzyme-linked immunosorbent assay have been used diagnosis of *Hepatozoon* infections in dogs. But serological methods are generally preferred in epidemiological studies to detection of chronic infections [2,9,10].

In the recent years, molecular-genetic based diagnostic methods such as polymerase chain reaction (PCR) and DNA sequencing have been used for survey of the presence, the characterisation and detection of prevalence of *Hepatozoon* species in dogs [11-14]. These methods have been accepted to be more sensitive and specific than microscopic and serological methods for the diagnosis of hepatozoonosis and the other blood parasites [15-22]. We have not found available data about canine hepatozoonosis in dogs in Kyrgyzstan. In this study we aimed that investigate the presence of *H. canis* and to determine the prevalence of *Hepatozoon* infections in dogs from Kyrgyzstan using polymerase chain reaction (PCR) and sequence analysis.

MATERIAL and METHODS

Study Area and Collection of Blood Samples

Kyrgyzstan Republic is a Central Asia Union country and it borders Kazakhstan to the north, Uzbekistan to the west, Tajikistan to the southwest and China to the southeast. The country is landlocked and mountainous, located in the Northern Hemisphere in the center of the Eurasian continent, as well as it is far away from large water bodies (the seas and oceans) and close of the desert. Bishkek, formerly Pishpek or Frunze is the largest city and the capital of the country [23] *(Fig. 1)*. The blood samples were collected from dogs living in a shelter in Bishkek with cooperation with the shelter's and Kyrgyz-Turkish Manas University Veterinary Teaching Hospital's staffs.

The sampling was carried out from May 2016-October 2017. The dogs have been accepted without clinical signs with behavioral inspection of the dogs, but particular clinical examination was not conducted. A total of 170 blood samples were obtained. The blood samples were collected in tubes containing ethylendiamine tetraacetic acid (EDTA) and stored -20°C until use for DNA extraction.

Fig 1. Map of Kyrgyzstan and location of Bishkek

Total DNA Extraction from Blood Samples and Polymerase Chain Reaction (PCR)

Total DNA extraction from blood samples was performed using a PureLink Genomic DNA mini kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. The DNA samples were stored at -20°C until use for PCR. To determine the presence *Hepatozoon* spp. in the DNA samples, a PCR analysis was carried out using Hep-F (5'-ATACATGAGCAAAATCTCAAC-3') and Hep-R (5'-CTTATT ATTCCATGCTGCAG-3') primers which amplify the partial 18S small subunite ribosomal RNA (18S SSU rRNA) gene of *Hepatozoon* species [24]. Sterile water (DNase, RNase free) and *H. canis* positive control DNA were used as negative and positive control in PCR, respectively. *H. canis* positive control DNA was provided from Department of Parasitology, Faculty of Veterinary Medicine, Selcuk University. The PCR was practiced in a touchdown termocycler in a total reaction volume of 25 µL according to Aydin et al.[4].

Eleven PCR positive sample products were purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany). The purified PCR samples were sequenced in a commercial company (Iontek, Istanbul, Turkey). The sequences of the partial 18S SSU rRNA gene of *Hepatozoon* spp. obtained in the study were submitted to basic local alignment search tool (BLAST) similarity search. After the sequences were identified they have been deposited in GenBank.

RESULTS

A total of 170 blood samples were analyzed with PCR to amplify a fragment of 666 bp located on 18S SSU rRNA gene of *Hepatozoon* spp. using Hep-F and Hep-R primer pairs *(Fig. 2)*.

Hepatozoon spp. was detected in 49 blood samples of investigated 170 dogs with PCR. Out of 170 field samples, 121 were found as negative in terms *Hepatozoon* spp.

To identify and confirm the PCR positive results, randomly selected representative 11 PCR positive samples were purified and sequenced. A BLAST search performed to compare the sequences alignments with the other *Hepatozoon* spp. sequences registered in GenBank under the accession numbers from MG917709 to MG917719.

Nucleotide sequences of partial 18S SSU rRNA gene of *Hepatozoon* spp. obtained in the study (MG917709 to MG917719) were aligned with the GenBank registered 18S SSU rRNA gene sequences of *H. canis* (KX712126, KX880505, KY197000). Sequence alignment of the PCR positive samples were identified as *H. canis*. BLAST analyse showed that 8 isolates were shared 100% similarity with *H. canis* isolates while the other 3 isolates were shared 99% similarity from Czeck Republic (accesion number: KX712126), Iran (accesion number: KX880505), Turkey (accesion number: KY197000).

DISCUSSION

Canine hepatozoonosis, a worldwide protozoon parasitic infection, has been reported in many countries of Africa, America, Asia and Europe such as Nigeria [25], Italy [14], Thailand [26], Croatia [27], Brazil [28], Argentina [11], and Turkey [13]. There are not any available data on presence *Hepatozoon* infections in dogs in Kyrgyzstan. The DNA based molecular diagnostic techniques such as PCR, RLB are widely used for detection and identification of blood parasites from animals. These techniques have superiority in specifity and sensitivity, and they permit to identification new genotypes and/or species [11-22]. Otronto et al.[14] showed that PCR the most sensitive assay for the detection of *H. canis* infection in dogs. They suggest that PCR can be used in epidemiological studies as a convenient diagnostic test. Using PCR, this study revealed hepotozoonozis has a high prevalence in dogs in Kyrgyzstan. 49 out of 170 blood samples were found as positive for *Hepatozoon* spp. with PCR. The result showed that hepatozoonosis has a high prevalence in dogs in Kyrgyzstan.

Hepatozoon canis and H. americanum are primary agents of canine hepatozoonosis. *H. canis* is more prevalent species than *H. americanum* in dogs. *H. canis* is seen in Europe,

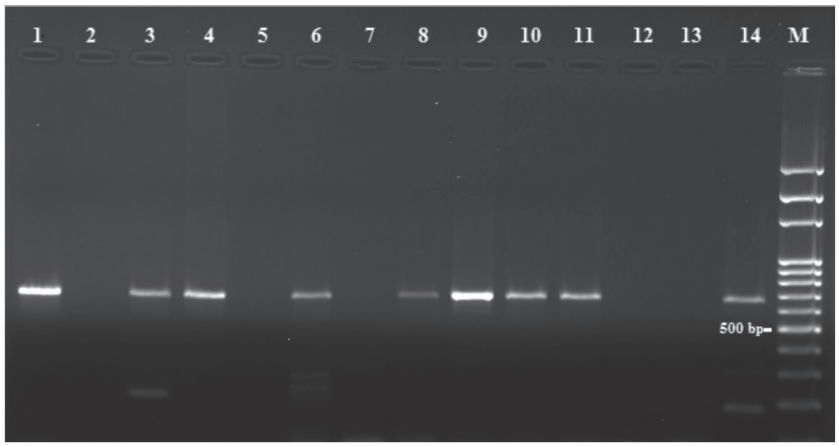

Fig 2. Agarose-gel electrophoresis of *Hepatozoon* spp. specific polymerase chain reaction products using Hep-F and Hep-R primers. Lane 1, *Hepatozoon canis* positive control DNA from dog; lane 2, negative control distilled water; lane 3,4,6,8,9,10,11,14, positive dog blood samples; 5,7,12,13 negative dog blood samples

Asia, Africa and also America while *H. americanum* is limited in America continent [1-3,29]. In this study 49 samples were detected positive with *Hepatozoon*-genus specific PCR. The partial sequences were determined of 11 PCR positive samples. The positive samples were identified as *H. canis* according to sequence alignments. BLAST analysis of the sequences showed that 8 *isolates* were shared 100% similarity with *H. canis* isolates while the other 3 isolates were shared 99% similarity from Czeck Republic (accesion number: KX712126), Iran (accesion number: KX880505), Turkey (accesion number: KY197000). It was the first detection of *H. canis* in dogs from Kyrgyzstan using molecular methods. There is need more comprehensive studies to detection of the infection status in Kyrgyzstan.

Currently, there are no protective vaccines for the prevention of canine hepatozoonosis. Therefore, the only powerful method to prevent the canine hepatozoonosis is the control of vector ticks. On the other hands wild carnivores such as jackals have been found naturally infected with *H. canis* [30]. This situation enhances the importance of epidemiological knowledge of *Hepatozoon* infections in terms of the development and application of appropriate control strategies. This survey reveals the high prevalence of canine hepatozoonosis in dogs in Kyrgyzstan, and *H. canis* is the primary agent of the diseases in the country. There is still need more prevalent epidemiological studies on canine hepatozoonosis in the country.

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Research Article

Performances, Ileal and Cecal Microbial Populations and Histological Characteristics in Broilers Fed Diets Supplemented with Lignocellulose [1]

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Abstract

The effect of dietary lignocellulose on broilers performance, intestinal microbiota and morphology, pH of digesta and litter humidity after 28 and 42 days of the experiment was evaluated. A total of 384 Cobb500 chickens (initial weight: 41.88±1.56 g) were divided into 4 groups with 24 replications and fed with control diet (C), a control diet with added 0.4% of lignocellulose (T1), a diet with added 0.6% of lignocellulose at the expense of soybean meal and maize (T2), and a diet supplemented with 0.6% of lignocellulose at the expense of soybean meal (T3). T2 treatment significantly influenced body weight, weight gain (WG), feed intake (FI) and feed conversion ratio (FCR). T2 and T3 treatment increased average LAB and *Bifidobacterium* spp. count, and decreased the number of *Escherichia coli* in the ileum and cecum, while differences in cecal *Clostridium perfringens* count among 0.4% and 0.6% treatments were not observed. Feeding the lignocellulose diet did not affect the relative weights of empty proventriculus, gizzard or intestines, but led to a decrease in pHs. T3 treatment caused an increase of the villi heights and significantly lower moisture content in the litter. Even though the addition of lignocellulose into broilers diet positively influenced performances, changes in intestinal microbiota and villi heights, based on the results of the present study, supplementation with 0.6% lignocellulose is recommended.

Keywords: *Bifidobacterium spp., Broilers, Intestinal histomorphology, Lignocellulose, Production results*

Lignoselüloz İlaveli Diyetle Beslenen Etlik Piliçlerde Performans, İleal ve Sekal Mikrobiyal Popülasyon İle Histolojik Özellikler

Öz

Diyetteki lignoselülozun 28 ve 42 gün uygulama sonrası broiler tavuklarda performans, barsak mikrobiyatı, morfolojisi ve içerik pH'sı ile dışkı nemi üzerine etkileri değerlendirildi. Toplam 384 adet Cobb500 tavuk (ilk ağırlıkları: 41.88±1.56 g) 24 tekrar olmak üzere 4 gruba ayrıldı ve kontrol diyet (C), %0.4 lignoselüloz ilaveli kontrol diyet (T1), soya fasulyesi ve mısır yerine %0.6 lignoselüloz ilaveli diyet (T2), soyafasulyesi yerine %0.6 lignoselüloz ilaveli diyet (T3) ile beslendi. T2 uygulaması vücut ağırlığı, ağırlık kazanımı, yem tüketimi ve yem konversiyon oranını anlamlı derecede etkiledi. T2 ve T3 uygulamaları ileum ve sekumda ortalama LAB ve *Bifidobacterium* spp. sayısını artırırken *Escherichia coli* sayısında azalmaya neden oldu. %0.4 ile %0.6 uygulamaları arasında sekum *Clostridium perfringens* miktarında ise fark belirlenmedi. Lignoselüloz ilaveli diyetle besleme proventrikulus, taşlık ve barsakların orantısal organ ağırıklarını etkilemezken pH'da azalmaya neden oldu. T3 uygulaması, villus uzunluklarında artmaya ve anlamlı derecede dışkının neminde azalmaya neden oldu. Broiler diyetine lignoselüloz ilavesi performans, barsak mikrobiyatası ve villus uzunluğunda pozitif etkilere neden olmuştur. Elde edilen sonuçlar doğrultusunda %0.6 oranında lignoselüloz kullanılması önerilmektedir.

Anahtar sözcükler: *Bifidobacterium spp., Broiler, Barsak histomorfolojisi, Lignoselüloz, Üretim sonuçları*

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INTRODUCTION

Addition of dietary fiber to poultry diets could be beneficial in enhancing gut health, which consequently affects broilers performances and meat quality. Soluble dietary fiber contains anti-nutritional factors which can limit digestion and absorption of major nutrients [1-3], leading to bad animal growth performances [4].

Cellulose and lignin are the main components of plant cell walls, 40-50% and 20-30%, respectively, and therefore, are some of the most widely available organic materials in nature [5]. According to previous reports, these non-fermentable fibers are considered to have effects on the energy balance of broilers [6-8]. Bogusławska-Tryk et al.[8] showed that different concentrations of lignocellulose in broiler diet promoted the growth of *Lactobacillus* spp. and *Bifidobacterium* spp. and reduced the number of *Escherichia coli* and *Clostridium* spp. Furthermore, Farran et al.[9] reported that dietary lignocellulose affects litter quality, and that inclusion of 0.8% lignocellulose in broiler diet significantly improved ready-to-cook carcass yield, lowered abdominal fat pad and increased protein digestibility compared to the control.

To the best of our knowledge, only a few studies reported the effect of including lignocellulose in broiler diets, so there is still a lack of information about effects of this compound on broiler performance and intestinal characteristics, especially intestinal microbiology during the first stages of production. Therefore, the aim of the present study was to evaluate the effects of dietary lignocellulose and basal diet modification in terms of maize and soybean meal content on broiler performance over 42 days, and the pH of digesta, litter humidity, lactic acid bacteria (LAB), *Bifidobacterium* spp., *Clostridium perfringens* and *E.coli* in intestinal contents and morphological and histological intestinal characteristics in 28-day-old broilers.

MATERIAL and METHODS

The experimental protocol was approved by the Veterinary Directorate of the Serbian Ministry of Agriculture, Forestry and Water Management (Approval No. 01-990/2) and the Ethics Committee of the Faculty of Veterinary Medicine, University of Belgrade.

Birds, Housing and Feeding

A total of 384 one-day-old Cobb500 chickens of mixed sexes were divided into control and 3 treatment groups with 6 replicates per group, each of which consisted of 16 chickens. Birds were reared on a commercial poultry farm, following guidelines set by the Cobb Broiler Management Guide [10] from 1 to 42 days of age. The chickens were housed in floor pens and provided with continuous light (24 hours per day) in an environmentally controlled room according to the technological recommendations for the breed. Houses had chopped straw as litter material, approximately 4 cm thick. Feed and water were offered *ad libitum* throughout the experiment.

The feeding program was applied as fallow: starter (up to 13 days; crushed pellets), grower (14-28 days; pellets 3.5 cm) and finisher (29-42 days; pellets 3.5 cm) mixtures. Four experimental diets were used *(Table 1)*: a control without additives (C), diet T1 consisting of the control diet plus added 0.4% of a commercial lignocellulose preparation which contains about 70% acid detergent fiber (ADF) and 24% acid detergent lignin (ADL) (Arbocel® R, J. Rettenmaier & Söhne GmbH+CO. KG, Rosenberg, Germany); diet T2 with added 0.6% Arbocel® R at the expense of 0.3% soybean meal and 0.3% maize, and; diet T3 with 0.6% Arbocel® R at the expense of 0.6% soybean meal. The lignocellulose supplementation was only in starter and grower mixtures for the treatment groups (up to day 28), while finisher diets did not differ between groups. Experiment with differences in protein content in the basal diet between groups was designed in order to evaluate if the addition of lignocellulose in different concentrations can replace the reduction of maize and/or soybean meal. The proximate composition of all feed mixtures was analyzed according to AOAC procedures [11] *(Table 1)*.

On day 28 of the trial, after body weight measurements, 12 birds (2 birds per pen) from each group were electrically stunned and immediately killed by severance of the jugular veins.

Performance of Broilers

The body weight of broilers was measured on electronic scales at the start and after every week of fattening. The quantity of feed eaten was recorded on a pen basis and the feed conversion ratio (FCR) was calculated from the data obtained for feed intake and weight gain. The amount of feed wastage and body weight of broilers that died during the study were taken into account when determining the feed conversion ratio.

pH Analysis

The pHs of fresh gizzard and jejunal digesta were measured using a hand-held pH-meter Testo 205 (Testo AG, Lenzkirch, Germany).

Microbiological Analyses

Contents from the ileum and cecum were collected by squeezing the intestine gently, after which samples were transported in sterile beakers under cold conditions to the laboratory and analyzed for LAB, *Bifidobacterium* spp., *Cl. perfringens* and *E. coli*. The samples were diluted with Ringer's solution, and homogenized for 3 min. Then digesta homogenate was serially diluted to 10^{-9} and 0.1 or 1 mL of appropriately diluted suspension was inoculated directly on the surface of the plates with

Table 1. *Composition and chemical analysis of the experimental diets for broilers*

Ingredients (%)	Starter (up to 13 days)				Grower (days 14-28)				Finisher* (days 29-42)
	C	T1	T2	T3	C	T1	T2	T3	
Maize	40.6	40.6	40.3	40.6	45.99	45.99	45.69	45.99	44.66
Soybean meal (44% CP)	31.1	31.1	30.8	30.5	21.86	21.86	21.56	21.26	17.75
Soy grits	7	7	7	7	12	12	12	12	12
Wheat	15	15	15	15	15	15	15	15	20
Soybean oil	1.71	1.71	1.71	1.71	0.78	0.78	0.78	0.78	1.68
Chalk	1.44	1.44	1.44	1.44	1.26	1.26	1.26	1.26	1.24
Monocalcium phosphate	1.02	1.02	1.02	1.02	0.86	0.86	0.86	0.86	0.66
Salt	0.2	0.2	0.2	0.2	0.19	0.19	0.19	0.19	0.19
Sodium bicarbonate	0.17	0.17	0.17	0.17	0.15	0.15	0.15	0.15	0.17
Lysine	0.13	0.13	0.13	0.13	0.16	0.16	0.16	0.16	0.14
Methionine (liquid)	0.33	0.33	0.33	0.33	0.27	0.27	0.27	0.27	0.22
Threonine	0.02	0.02	0.02	0.02	0.04	0.04	0.04	0.04	0.02
Adsorbent	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	-
Choline chloride	0.1	0.1	0.1	0.1	0.08	0.08	0.08	0.08	0.07
Coccidiostat	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	-
Lignin sulfonate	-	-	-	-	0.2	0.2	0.2	0.2	0.2
Premix**	1	1	1	1	1	1	1	1	1
Arbocel® R	-	0.4	0.6	0.6	-	0.4	0.6	0.6	-
Chemical Composition (%)									
Moisture	10.47	10.51	10.58	10.42	10.78	10.75	10.80	10.46	10.91
Total ash	5.85	5.88	5.97	5.91	5.65	5.74	5.80	5.69	5.12
Crude protein	22.07	21.92	21.74	21.79	20.81	20.77	20.42	20.08	18.46
Crude fiber	3.43	3.79	3.98	3.96	3.25	3.67	3.85	3.82	3.07
Total lipids	5.42	5.23	5.19	5.18	5.54	5.32	5.29	5.22	6.38
Calcium	1.01	0.99	0.98	0.99	0.91	0.90	0.89	0.88	0.82
Phosphorus	0.65	0.64	0.66	0.66	0.58	0.59	0.60	0.60	0.57
NFE***	52.76	52.67	52.54	52.74	53.97	53.75	53.84	54.73	56.06

Control group (no supplementation); T1 - broilers supplemented with 0.4% lignocellulose; T2 - broilers supplemented with 0.6% lignocellulose with reduction of 0.3% soybean meal and 0.3% maize; T3- broilers supplemented with 0.6% lignocellulose with reduction of 0.6% soybean meal.
** Finisher was the same for all experimental groups; ** Mineral-vitamin premix provided per kg of diet: Vitamin A 13.500 IU, Vitamin D_3 5.000 IU, Vitamin E 80 IU, Vitamin K_3 4 mg, Vitamin B_1 4 mg, Vitamin B_2 6 mg, Vitamin B_6 5 mg, Vitamin B_12 0.025 mg, Vitamin C 25 mg, Biotin 0.15 mg, Niacin 60 mg, Calcium pantothenate 16.5 mg, Folic acid 2 mg, Iodine 1 mg, Selenium 0.3 mg, Iron 40 mg, Copper 20 mg, Manganese 100 mg, Zinc 80 mg, Antioxidant 125 mg, 6-phytase (4a1640) EC3.1.3.26 - 500 FTU; **** N-free extractives

agar for enumeration of the different bacteria. LAB were enumerated on MRS agar (Hi Media, India) following incubation at 30°C for 72 h, *E. coli* on TBX agar (Oxoid, UK) and plates were incubated for 24 h at 37°C. *Cl. perfringens* were enumerated on Perfringens Agar Base plus supplements TSC and SFP (Oxoid, UK), and plates were incubated for 72 h at 35°C, under anaerobic conditions. For enumeration of *Bifidobacterium* spp., Bifidobacterium Selective Count Agar Base plus Bifido Selective Supplement A and B (Hi Media, India) were used, and plates were incubated for 72 h at 35°C under anaerobic conditions. Bacterial colonies were counted immediately after removing plates from the incubator and the bacterial numbers were expressed as $\log_{10}$ CFU per gram of digesta.

Morphological and Histological Analyses

After being emptied, proventriculus, gizzard and intestines were weighed on a balance with a precision of ±0.2 g. The intestine samples (1 cm segments) were taken immediately after slaughter, washed in physiological saline and fixed in 10% buffered formalin. After fixation and shaping, intestinal samples were dehydrated in increasing concentrations of ethyl alcohol, cleared with xylene, paraffin infiltrated and embedded in paraffin blocks. Sections of 2 µm in thickness were placed on glass slides and stained with Mayer's hematoxylin and eosin, and with a combination of Periodic acid Schiff's stain and Alcian blue (PAS-AB) [12,13]. Histological sections were examined using a light microscope Olympus

BX53 with the objective magnifications x4 and x10. Morphometric examinations were carried out using the Olympus cellSens software (www.olympus-lifescience.com), and included the following measurements: the villus height and width, and crypt depth of the small intestine (duodenum, jejunum and ileum) [14].

Litter Sample Measurements

On day 28 of the study, five samples from the full depth of the litter were collected from each pen (4 near the corners and one from the middle). The litter samples for each group were pooled, thoroughly mixed and the moisture content was determined in duplicate by drying at 103±2°C until a constant weight [15].

Statistical Analyses

Obtained data were statistically processed in GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Results are presented in tables and figure as means and standard error of means (SEM). The significance of differences among the mean values was identified by One-way ANOVA with Tukey's post hoc test. Statistical significance was considered at a level of $P<0.05$.

RESULTS

The mortality rate was low (1.0%), with deaths only in the first week, and not related to any of the treatments. The performances of broilers are shown in *Table 2*. At the end of week 3 of the trial, the body weight of T2 group differed significantly from the control and T1 group. Subsequently, on days 28 and 42 of the study, T2 broilers had greater body weight compared to birds on other diets ($P<0.05$).

In weeks 2 to 4, 0.6% lignocellulose supplementation in T2 broilers resulted in their increased weekly weight gain compared to other groups of broilers ($P<0.05$).

In week 4 of the study, feed intakes of the groups fed with 0.6% lignocellulose were significantly higher than those of C and T1 groups of broilers, while in the last two weeks of the study, during which lignocellulose was not added to the feed mixture, feed consumption of all treatment groups was lower than that of the control.

When broilers were fed with grower mixture (week 3), the feed conversion in T2 group was significantly lower than in the control and T3 group, and in the last phase, all groups that had received lignocellulose in feed had a better FCR than did the control ($P<0.05$).

Table 2. *Performances of four groups of broiler chickens during the study*

Parameter	Day	Group Mean				SEM	P Value
		C	T1	T2	T3		
Body weight (g)	Day 1	41.72	42.16	41.71	41.93	0.27	0.1516
	Day 7	163.00^{a*}	170.00^{bc}	174.00^{b}	167.80^{ac}	2.56	<0.0001
	Day 14	376.30	380.10	394.60	383.10	10.14	0.1453
	Day 21	772.10^{a}	782.10^{a}	826.20^{b}	785.90^{ab}	19.39	0.0040
	Day 28	1191^{a}	1218^{a}	1343^{b}	1253^{a}	30.04	<0.0001
	Day 42	2428^{a}	2423^{a}	2611^{b}	2495^{a}	53.87	<0.0001
Weekly weight gain (g)	Day 1-7	121.30^{a}	127.90^{bc}	132.20^{b}	125.90^{ac}	2.52	<0.0001
	Day 7-14	213.30	210.10	220.60	215.30	7.63	0.3925
	Day 14-21	395.70^{a}	401.90^{a}	431.60^{b}	402.80^{a}	9.46	<0.0001
	Day 21-28	418.90^{a}	435.40^{a}	516.50^{b}	467.20^{c}	11.10	<0.0001
	Day 28-42	1284	1245	1310	1292	30.96	<0.0711
Weekly feed intake (g)	Day 1-7	129.10^{a}	131.90^{a}	139.60^{b}	129.90^{a}	2.11	<0.0001
	Day 7-14	302.60	292.80	302.30	297.80	14.43	0.7473
	Day 14-21	590.10	587.80	621.40	593.50	17.71	0.0487
	Day 21-28	668.60^{a}	674.20^{a}	786.70^{b}	737.30^{c}	12.24	<0.0001
	Day 28-42	2295^{a}	2084^{b}	2186^{c}	2182^{bc}	87.71	<0.0001
Weekly feed conversion ratio	Day 1-7	1.07^{a}	1.03^{b}	1.06^{a}	1.03^{b}	0.011	<0.0001
	Day 7-14	1.42	1.39	1.37	1.38	0.067	0.7735
	Day 14-21	1.49	1.46	1.44	1.47	0.043	0.4221
	Day 21-28	1.60^{a}	1.53^{ab}	1.52^{b}	1.58^{a}	0.026	0.0046
	Day 28-42	1.79^{a}	1.67^{b}	1.67^{b}	1.69^{b}	0.026	<0.0001

** Different lower-case superscript letters after means in the same row indicate differences ($P<0.05$) among groups*

Table 3. *Effect of lignocellulose supplementation on mean relative weight (% of body weight) of different organs and digesta pH in different segments of the gastrointestinal tract of broilers*

Parameters	Group Mean				SEM	P Value
	C	T1	T2	T3		
Weight (% BW)						
Proventriculus	0.58	0.57	0.47	0.55	0.088	0.2860
Gizzard	1.48	1.60	1.39	1.36	0.212	0.3728
Intestines	5.33	5.16	5.84	5.47	0.603	0.4484
pH						
Gizzard	4.31 ^{a*}	4.20 ^{b}	4.15 ^{c}	4.19 ^{b}	0.016	<0.0001
Jejunum	4.90 ^{a}	4.75 ^{b}	4.59 ^{c}	4.69 ^{d}	0.017	<0.0001

** Different lower-case superscript letters after means in the same row indicate differences (P<0.05) among groups*

Table 4. *Bacterial counts in ileal and cecal digesta of broilers*

Bacterial Group	Group Mean				SEM	P Value
	C	T1	T2	T3		
LAB						
Ileum	5.85^{a*}	6.21^{b}	6.81^{c}	6.71^{c}	0.178	<0.0001
Cecum	6.89^{a}	7.21^{b}	7.52^{c}	7.50^{c}	0.143	<0.0001
Bifidobacterium						
Ileum	5.61^{a}	6.32^{b}	6.82^{c}	6.75^{c}	0.174	<0.0001
Cecum	6.63^{a}	7.03^{b}	7.38^{c}	7.31^{bc}	0.177	<0.0001
E. coli						
Ileum	6.32^{a}	5.81^{b}	5.45^{c}	5.49^{c}	0.106	<0.0001
Cecum	7.05^{a}	6.62^{b}	6.26^{c}	6.30^{c}	0.094	<0.0001
Cl. perfringens						
Ileum	5.19^{a}	4.86^{b}	4.59^{bc}	4.55^{c}	0.153	<0.0001
Cecum	5.60^{a}	5.01^{b}	4.89^{b}	4.81^{b}	0.226	0.003

** Different lower-case superscript letters after means in the same row indicate differences (P<0.05) among groups*

Feeding the lignocellulose diet for four weeks did not result in significant increases in the relative weights of empty proventriculus, gizzard or intestines *(Table 3)*.

Significant differences between pH of gizzard and jejunal digesta were noted between broilers fed different experimental diets. Gizzard and jejunum pHs were lowered by the increased fiber content of the lignocellulose diet. Namely, the inclusion of 0.4 and 0.6% of lignocellulose led to significant decrease in jejunal pH of treatment groups (pH 4.75, 4.59 and 4.69, respectively, in T1, T2 and T3 broilers). Also, gizzard pH of treatment groups was significantly lower compared to the control, but without difference between groups T1 and T3, and ranged from pH 4.15 to 4.31.

The results of average microbial counts in broilers' ileum and cecum are presented in *Table 4*. The inclusion of lignocellulose in broiler diets significantly affected all bacterial groups in both intestinal segments. The lowest numbers of LAB in the ileum and cecum of broilers were found in the control group (P<0.05). LAB counts were significantly higher (P<0.05) in the ileum and cecum of broilers fed 0.6% lignocellulose than in the birds fed 0.4% lignocellulose, while significant differences between T2 and T3 broilers were not observed.

In the ileal and cecal digesta, the counts of *Bifidobacterium* spp. were significantly increased (P<0.05), for the broilers fed diets supplemented with 0.4% and 0.6% lignocellulose compared to the birds which received the control diet. The highest numbers of *Bifidobacterium* spp. were measured in the birds fed 0.6% lignocellulose (T2 group).

The numbers of *E. coli* were significantly lower (P<0.05) in the ileum and cecum of broilers fed lignocellulose than in the control birds, and the lowest average number of this pathogen was recorded in the birds fed 0.6% lignocellulose.

Clostridium perfringens counts in the ileum were significantly higher (P<0.05) in the control group than in the broilers fed diets with lignocellulose, while in the cecum, no dose-

Table 5. *Small intestinal morphology of broilers fed experimental diets (mean±SEM; µm)*

Intestinal Segment	Group Mean				SEM	P Value
	C	T1	T2	T3		
Duodenum						
Villus height	1330^{a*}	1380^{a}	1434^{a}	1574^{b}	69.38	<0.0001
Villus width	96.73^{ab}	105.70^{b}	105.70^{b}	108.10^{c}	5.61	0.0246
Crypt depth	134.00^{a}	132.10^{a}	156.60^{b}	168.20^{b}	9.34	<0.0001
Jejunum						
Villus height	871.40^{a}	873.40^{ab}	892.60^{ab}	984.80^{b}	61.48	0.0301
Villus width	77.22^{a}	81.49^{a}	87.64^{a}	102.10^{b}	6.24	<0.0001
Crypt depth	131.90^{a}	137.00^{ab}	145.20^{b}	156.30^{c}	5.53	<0.0001
Ileum						
Villus height	630.10^{a}	702.90^{b}	731.40^{b}	741.20^{b}	37.34	0.0001
Villus width	70.30^{a}	82.55^{b}	88.92^{c}	91.55^{bc}	4.47	<0.0001
Crypt depth	131.20^{a}	140.70^{ab}	145.60^{b}	145.60^{b}	6.88	0.0104
Villus height: Crypt depth ratio	10.47^{a}	11.29^{a}	10.29^{a}	8.55^{b}	2.62	<0.0001

** Different lower-case superscript letters after means in the same row indicate differences (P<0.05) among groups*

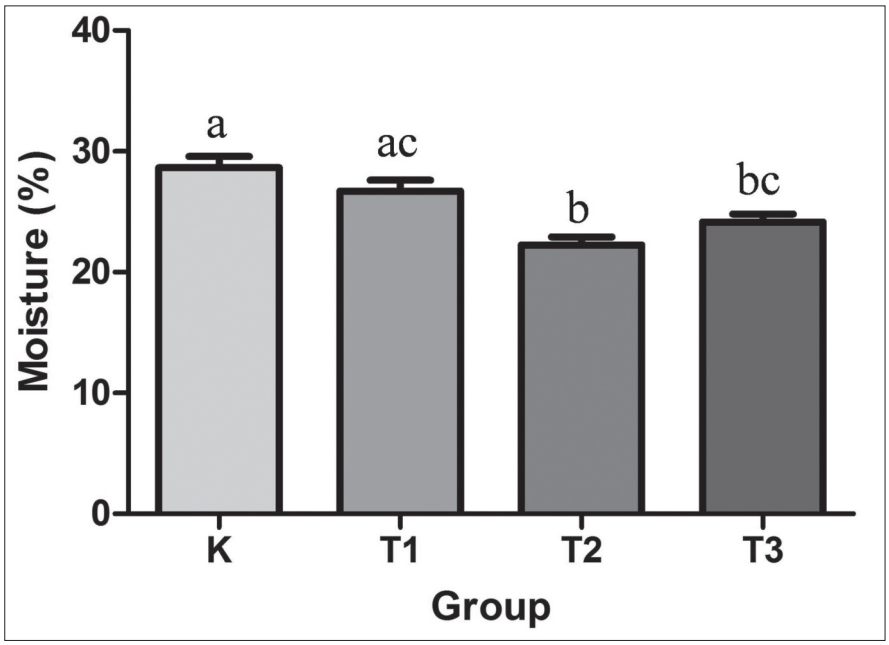

Fig 1. Impact of different lignocellulose level in broiler diets on litter humidity (%), day 28

dependent effect of lignocellulose addition was observed. No differences (P>0.05) were found in the mean cecal population of *Cl. perfringens* among dietary treatments involving addition of 0.4% or 0.6% lignocellulose.

Small intestinal morphology of broilers fed experimental diets is shown in *Table 5*. Villi of the small intestine segments of the broilers fed 0.6% lignocellulose (T2 and T3) were higher and wider compare to those broilers fed 0.4% lignocellulose (T1) and control birds. Crypt depth was also affected with addition of the lignocellulose, with significant increase of crypt depth (P<0.05) in birds with the higher percentage of dietary lignocellulose (T2 and T3) than in the control birds.

The impact of lignocellulose inclusion in broiler diets for four weeks on litter moisture content is presented in *Fig. 1*. The moisture content in the litter of broilers fed with 0.6% lignocellulose was significantly lower than in the litter from control birds, with the lowest moisture content (22.25%) in litter from the T2 broilers.

DISCUSSION

Many literature data relating to the impact of cellulose and lignin, in the pure form or from the different sources, on production performance and the possibilities of using lignocellulose as a growth promoter in poultry production

are not consistent [16] which required further investigation that could clarify its effect. In our study, the dietary insoluble fiber affected different production parameters of the experimental birds, where the addition of 0.6% lignocellulose with the concomitant reduction of 0.3% soybean meal proved to be the most effective. Previously, Sarikhan et al.[17] determined that supplementation of an insoluble fiber concentrate at the level of 0.25-0.75% in a broiler diet from weeks 3 to 6 had a positive impact on birds' growth and feed efficiency. Increased digestibility of fats and proteins induced by the addition of insoluble fibers such as lignocellulose could lead to better broilers' growth performance [18,19] which was observed in the present study. Insoluble fiber supplementation can prolong transit time of ingested feed from the crop to the gizzard, and increase gizzard function and proteolytic digestive enzyme activities in the proventriculus and pancreas [4,20-22]. In addition, the low pH in the gizzard of broilers fed with lignocellulose observed in this study could improve the solubility and absorption of mineral salts and favor pepsin activity [23,24]. However, some studies showed that inclusion of lignocellulose in diets had no effect on broilers', layers' and roosters' growth performances [4,8,22], or that an excess of dietary fiber reduced feed intake, nutrient digestibility and growth performance [20,25]. Thus, a minimum amount of dietary fiber in broilers' diet is required and according to Jiménez-Moreno et al.[20] should be in the range from 2.56 to 3.50%. Our study showed that the addition of 6 g/kg lignocellulose preparation with 3.98% of the crude fiber in the starter, and 3.85% in the grower feed mixture resulted in the best broiler performances. Namely, there is a limit in reducing the content of the protein part in the feed mixture to which the addition of lignocellulose enables a positive effect on the production results which should not be higher than 0.3% soybean.

Morphology characteristics of different parts of the digestive tract reported in the study conducted by Kimiaeitalab et al.[26] are in agreement with our results. Contrary, Jiménez-Moreno et al.[20] and Oke and Oke [27] found that the increase of relative weight and length of digestive tract correlate with the higher amount of insoluble fibers in the diet which caused GIT dilation and an increase of the chyme in the gizzard [24].

The inclusion of adequate amounts and types of fiber could modify the pH of the GIT of broilers [28]. The changes in the pH of the gizzard digesta observed in this study could be the result of stimulated gizzard function and increased production of HCl in the proventriculus [24], although we did not find any increase of gizzard relative weight. Lower jejunal pH in our treatment groups could be attributed to bacterial fermentation and consequently higher concentration of short-chain fatty acids [8]. Also, Jiménez-Moreno et al.[20] suggested that the crude fiber could stimulate bile salt and bicarbonate secretion, which would result in lowering the pH of digesta.

Intestinal health and balance between beneficial and pathogenic intestinal bacteria is crucial for maintaining health, leads to better broiler performances, improves feed conversion ratios, and most importantly, reduces the growth of pathogenic bacteria that pose a food safety risk for consumers [29]. This is why broilers diets supplemented with different antibiotic replacements (phytobiotics) including the insoluble crude fibers are applied to improve broiler intestinal health.

Despite numerous reports on the influence of pure cellulose and purified lignin when added to broiler diets on the intestinal bacterial population, there are not many data concerning the effect of lignocellulose. In the present study, the addition of lignocellulose in the broiler diets increased the number of beneficial bacteria, LAB and *Bifidobacterium* spp., and decreased the number of pathogenic bacteria, *E. coli* and *Cl. perfringens*, in ileal and cecal digesta of 28-day-old broilers. These results are in accordance with those previously reported by Bogusławska-Tryk et al.[8] and Abazari et al.[30]. Insoluble fibers such as cellulose have an abrasive effect which is exhibited by not allowing pathogenic bacteria to adhere to the intestine mucosal surfaces [8,21]. Moreover, lignin contains many phenolic monomers that possess biological effects, including an antibacterial effect. However, in the present study, the lower number of pathogenic bacteria can not be attributed to the antibacterial effect of phenols, since chemical structures from purified lignin differ from native lignin, which is, as in the lignin used in this study, strongly intermeshed with cellulose [31]. The number of the LAB was higher in broilers fed diets with lignocellulose, and many LAB are known to release bacteriocins which inhibit or prevent further proliferation of pathogenic microorganisms in the intestine [32]. This could be the cause of the lower numbers of *E. coli* and *Cl. perfringens* we measured in broilers on lignocellulose diets. Lignocellulose supplementation at the 0.6% level was more effective than the lower level (0.4%) supplementation. The effect of lignocellulose on *Lactobacillus* and *Bifidobacterium* spp. is dose-dependent as previously reported by Cao et al.[33]. However, Bogusławska-Tryk et al.[8] did not find significant differences between the microbiota of broilers fed with 0.25, 0.5 or 1% lignocellulose.

Diet is one of the main factors influencing birds gut morphology including the villus height: crypt depth ratio which is used as an important indicator of absorption in the small intestine [21]. Addition of the lignocellulose caused a major decrease of the crypt depths in the small intestine, and consequently, increase of the villi heights. The higher lignocellulose level had more effect, especially with concomitant reduction of 0.6% soybean meal. Iji et al.[3] reported that jejunal and ileal crypts depths increased in broilers fed a diet with the addition of highly viscous non-starch polysaccharides fibers compared to the control group after 28 days fattening period, while Baurhoo

et al.[31] found that high protein soybean and corn-based diet containing up to 3% insoluble fiber exhibit a positive effect on the development of the digestive tract and growth performance of broiler chickens. As previously mentioned, increased beneficial bacterial counts found in the small intestine probably improved vascularization which led to villi development [34] and consequently increased absorption. Furthermore, increased absorption causes greater use of nutrients and digestion capacity explaining better growth performances seen in the groups with lignocellulose supplementation. Similarly, Jiménez- Moreno et al.[20] who investigated the effect of a diet supplemented with pea hulls (2.5-7.5%) reported that inclusion of lower levels of insoluble fibers improved performances and the villus height:crypt depth ratio, but the addition of 7.5% pea hulls decreased this ratio.

The poor quality of the litter in poultry production is a serious deficiency that causes various health problems and economic losses [35]. Preliminary studies showed that 0.8-1.0% lignocellulose exhibited a positive impact on a fecal consistency and litter quality in chickens and laying hens [9,16], which was in accordance with T2 and T3 treatment in the present study.

The results from this study show that better broiler production results can be expected if the protein content in broiler diets is reduced, by 0.33% in starter feed and 0.39% in grower feed, due to 0.6% supplementation with lignocellulose, which produces a resultant 0.55% increase in the crude fiber content of the diet. This implies that the inclusion of lignocellulose can reduce the percent of expensive, high-protein ingredients in the feed mixture in a cost-effective manner. Overall, the presence of 0.4% and 0.6% lignocellulose in broiler diets decreased populations of *C. perfringens* and *E. coli* and increased populations of LAB and *Bifidobacterium* spp. in both ileal and cecal segments, and a dose-dependent effect was observed. Addition of the lignocellulose in the present study caused a decrease of the crypt depths in all segments of the small intestine, and consequently, increase of the villi heights. The higher lignocellulose level (0.6%) had more effect compared to supplementation with the lower concentration of ligno-celluloses.

Conflict of Interest

The authors declare no conflict of interest.

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Research Article

Using Mineral Elements to Authenticate the Geographical Origin of Yak Meat

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Abstract

Labeling systems for niche market food products is becoming increasingly important to address consumers' expectations. Yak meat is 'green' product from natural extensive rangeland on Qinghai-Tibetan plateau. A trace technique is essential for consumers to know the origin of yak meat. In the current study, mineral fingerprints were investigated for their potential to classify yak meat according to geographical origin. The concentration of more than 50 mineral contents in 24 yak meat samples from three regions on Qinghai-Tibetan plateau were analyzed by ICP-MS. Multivariate statistical analyses were used to identify the most relevant indicators of origin. Seven elements (Na, As, Ni, Se, Rb, Cd and Ti) were selected for further routine analyses based on the significant origin differences ($P<0.05$). The three minerals (Se, Rb, Ti) were selected by statistics analysis and established discriminant model for yak meat traceability. Linear discriminate analysis gave an overall correct classification rate of 91.7% and cross-validation rate of 87.5%. These results demonstrate the usefulness of multi-element fingerprints as indicators for authenticating the geographical origin of yak meat.

Keywords: *Yak meat, Mineral elements, Geographical origin, Authentication*

Yak Etinin Coğrafi Kökenini Doğrulamak Amacıyla Mineral Elementlerin Kullanılması

Öz

Pazardaki gıda ürünlerinin kökenini belirtmek için etiketleme sistemleri, tüketicilerin beklentilerini karşılamak için giderek daha önemli bir hal almaktadır. Yak eti, Qinghai-Tibet platosu üzerindeki doğal geniş meralardan elde edilen 'yeşil' bir üründür. Tüketicilerin yak etinin kaynağını bilmeleri için bir takip tekniği gereklidir. Bu çalışmada, yak etinin coğrafi kökenine göre sınıflandırılma potansiyeli açısından mineral parmak izleri araştırıldı. Qinghai-Tibet platosundaki üç bölgeden gelen 24 yak eti örneğinde 50'den fazla mineral içeriğin konsantrasyonu ICP-MS ile analiz edildi. En önemli kaynak göstergelerini tanımlamak için çok değişkenli istatistiksel analizler kullanıldı. Önemli orijin farklılıklarına bağlı olarak, rutin analiz için yedi element (Na, As, Ni, Se, Rb, Cd ve Ti) seçildi ($P<0.05$). Üç mineral (Se, Rb, Ti), yak eti izlenebilirliği amacıyla istatistik analiz sonucu seçildi ve ayırıcı model olarak belirlendi. Doğrusal ayırıcı analiz, %91.7 doğru sınıflandırma oranı ve %87.5 çapraz doğrulama oranı vermiştir. Bu sonuçlar çoklu element parmak izlerinin, yak etinin coğrafi kökenini doğrulamada bir gösterge olarak yararlı olduğunu göstermektedir.

Anahtar sözcükler: *Yak eti, Mineral maddeler, Coğrafi orijin, Doğrulama*

INTRODUCTION

The yak *(Bos grunniens)* is a ruminant which lives in harsh conditions in the mountains of Central Asia. More than 90% of the world's total yak population are currently herded in Chinese territories, mainly on the Qinghai-Tibetan plateau in area of altitude ranging from 2500 m to 6000 m [1,2]. Through thousands of years of evolution, the yak has

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adapted to survive in a cold and anoxic environment [3]. Products from yaks (meat, milk) and functions of yak farming (workload, energy, etc) are numerous and closely related to the daily life of the local human population. The yak meat is the main source of protein and the most important economic income for farmers [4]. It is of good quality with a fine texture, high protein and low fat content, and rich in amino acids compared with that of Chinese beef cattle [5,6].

Recently, the demand for yak meat is increasing in most areas of China since it is extensively produced in this scenic area far from the pollution and its reputation for good quality, high protein and low fat content, favorable amino acid and fatty acid profile compared with *Bos Taurus* cattle [2]. Moreover, face to the rapid development of the intensive beef cattle industry in the grain production areas in China, the yak meat production needs to be financially supported by the differentiated product label [7]. The labeling system for differentiated meat products has been developed in Europe [8], many of these labels have defined the geographic restriction of the production location, and some of them has even been developed exclusively for geographic origin such as Protected Geographical Indication. Now, it is difficult to clarify the food origin in Chinese market except organic food which is labeled. Therefore, it is necessary to develop routine analytical methods to authenticate the geographic origin of yak meat with the ultimate goal to set up a similar label for yak meat product.

Efforts have been made to develop analytical tools to quantify specific compounds in the product or the animal tissues that can act as tracers of the animal's geographic origin. Methods involving the measurement of quantifiable components at both an elemental and molecular level have been applied, including stable isotopes [9,10]; and trace elements [11]. Since specific feed stuffs are associated with a particular geographical region [12], the "markers" related to feeding background such as fatty acids [13], volatile compounds [14] and carotenoids [15,16] could also be useful for assignment of geographical origin [17]. However, most of yaks were fed exclusively by grass from natural grassland on the Qinghai-Tibetan plateau, the "markers" related to grass-feeding could not be applied to authenticate the geographical origin of yak. Successful authentication of geographical origin of beef has been achieved by analyzing mineral elements [11]. Since the production system and the chemical composition of yak meat differ from that of beef cattle, it is necessary to verify the reliability of this method in yak meat. The objective of this study was to primary analyze the mineral composition of yak meat from three major yak-producing regions on Qinghai-Tibetan plateau, and to investigate the feasibility labeling yak meat origin by its mineral elements concentrations of yak meat to authenticate its geographical origin.

MATERIAL and METHODS

Sample Origin

All animals were used for according to the Guide for the Care and Use of Laboratory Animals of Qinghai Province [18]. Twenty-four, 3-4 years old, male yaks were randomly selected from Da-tong (DT, n=10), He-nan (HN, n=8) and Lu-qu (LQ, n=6) County on Qinghai-Tibetan plateau. Da-tong County is located in the eastern part of Qinghai Province, the soil type includes alpine frozen soil, gray-drab soil and alpine meadow soil [19]. The grassland type in DT is mainly alpine meadow, and mountain meadow [19]. He-nan County is located in southeast part of Qinghai Province where the soil is constituted by alpine desert soil, alpine meadow soil, chernozem, mountain meadow soil, gray-drab soil and bog soil. Alpine meadow is the main grassland vegetation in HN [20]. Lu-qu County is located in the Gannan Tibetan Autonomous Prefecture of Gansu Province, in southeastern of Qinghai-Tibetan Plateau is constituted by alpine meadow soil, alpine shrub meadow soil, swamping meadow soil and mountain steppe soil [21].

Sample Preparation

The yaks were slaughtered in September 2013. Meat samples of 500 g were collected from the *longissimus* muscle of yak carcasses, and stored at -20°C prior to processing. Each sample was cut into small pieces for freeze-drying over 48 h before being pulverized in a ball mill. The crude fat of yak muscle powder was extracted with petroleum ether in a soxhlet apparatus, and the residue, mostly de-fatted proteins, was used for further analysis [10,22].

Mineral Analysis

The detailed procedures for biochemical methods are described by references [10,22]. Briefly, the samples were analyzed after microwave digestion using MARS microwave digestion system (CEM Corp. North Carolina, America). A 0.2 g of de-fatted yak sample, with 6 mL of 65% HNO_3 and 2 mL of 37% HCl were added into a Teflon PTFE digestion tube and digested for 40 min by increasing the power to 1600W and the temperature to 180°C in a stepwise fashion. The digested solution was diluted to 50 mL with ultra-pure water (Resistivity, in MW × cm at 25°C:18.2) and stored in plastic tube before analysis. Contents of more than 50 mineral elements (Na, Mg, Al, K, Ca, Sc, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Y, Mo, Ru, Rh, Pd, Ag, Cd, Sn, Sb, Te, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Lu, Yb, Hf, Ir, Pt, Au, Tl, Pb, Th, U) were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, 7500a, Agilent, America). Optimisation of the method using this instrument was done for higher sensitivity and lower detection limits. The optimised operation conditions for analysis of the diluted samples were as follows: radio frequency power of 1600 W, plasma gas flow rate of 1.12

L/min, auxiliary gas flow rate of 0.5 L/min, nebulisation chamber temperature at 2°C, the oxide indice of 0.45%, and dual current indice of 1.01%.

Analysis of each sample was quantified by using external standard. The Environmental Calibration Standard (a series of ICP-MS Standards) supplied by Agilent was used as a standard solution and the determination coefficient of standard curve was higher than 0.99. The internal standards Ge, In, Bi and other elements were used to ensure the stability of the instrument. The samples were analysed twice in each run when the Relative Standard Deviation (RSD) of internal standards was higher than 3%.

Statistical Analysis

The statistical analysis of the data was performed using the SPSS 19.0 package for windows. Data were first analyzed for the homogeneity of their variance using the test of Shapiro. Differences between means were analyzed using the Kruskal-Wallis H test since most of the data did not follow the normal distribution.

The mineral elements, in the de-fatted yak meat, which differed according to the regions on the northeastern of Qinghai-Tibetan plateau were analyzed using principle components analysis (PCA). In order to better visualize the relative distribution of the defatted yak meat samples according to their geographical origin, cluster analysis (CA) was performed using the first three principal component normalization scores. The clustering analysis was performed using the Euclidean distance and clustering methods using the sum of squares. To ascertain the discriminating efficiency of each element in yak meat, stepwise discriminant analysis was carried out on the basis of the mineral element compositions found to be significantly different among the regions.

RESULTS

The means of seven (Na, As, Ni, Se, Rb, Cd, Ti) of the 50 elements analysed in de-fatted yak meat, were significantly different among the regions (P<0.05) *(Table 1)*. The Na, As and Ti concentrations were significantly higher in DT County than HN and LQ County. And no differences were shown between HN and LQ County. The Ni and Cd concentrations in HN County were lower than DT County, but similar with LQ County. The Se and Rb concentrations in HN and DT County were higher than in LQ County, but no differences between HN and DT County.

Following PCA, the first three factors explained 77.40% of the total variability *(Table 2)*. The contents of Cd and Ti had the highest weight on the first PC (explaining 46.80% of the variability). Na and Se were the most important variables explaining variation in the second PC (16.20% of variability). Rb content showed the highest weight on the third PC (explaining 14.40% of variability).

Scatter plots of scores on the first two principal components presented in *Fig. 1*. The three regions reflecting differences in patterns of mineral composition were easily distinguished. Compared with the other two regions, samples from DT is more dispersed. The largely horizontal distribution of DT and LQ samples reflect their classification with regards to differences in Na and Se in PC2. On the other hand, the vertical distribution of samples from HN represents variation due to differences in Cd and Tl on PC1.

Table 2. *Correlations of the first 3 principal components with the original variables and variance explained by these principal components*

Mineral Element of De-Fatted Muscle	PC1	PC2	PC3
Na	0.175	0.859	-0.119
As	0.668	0.309	0.093
Ni	0.706	0.291	-0.374
Se	0. 216	0.811	0.250
Rb	0.114	0.093	0.943
Cd	0.869	-0.055	0.179
Ti	0.817	0.454	0.138
Variance contribution, %	46.8	16.20	14.40
Cumulative variance contribution, %	46.8	63.00	77.40

Table 1. *Mineral elements compositions of de-fatted muscle of yak sample from different regions (mg/kg, DM)*

Element	HN[1]		DT[2]		LQ[3]		P-value
	Mean±SD	CV	Mean±SD	CV	Mean±SD	CV	
Na	1533±161.7^{b}	0.105	2104±704.2^{a}	0.335	1540±199.40^{b}	0.130	0.032
As	0.032±0.012^{b}	0.375	0.055±0.017^{a}	0.309	0.030±0.010^{b}	0.333	0.036
Ni	0.050±0.011^{b}	0.220	0.079±0.032^{a}	0.405	0.063±0.012^{ab}	0.190	0.002
Se	0.040±0.027^{a}	0.675	0.056±0.016^{a}	0.286	0.008±0.004^{b}	0.5	0.000
Rb	5.602±1.517^{a}	0.271	4.826±3.316^{a}	0.687	2.153±0.770^{b}	0.358	0.037
Cd	0.002±0.000^{b}	0	0.005±0.002^{a}	0.4	0.003±0.001^{ab}	0.333	0.012
Ti	0.001±0.000^{b}	0	0.003±0.001^{a}	0.333	0.001±0.001^{b}	1	0.000

[1] HN, He-nan County; [2] DT, Da-tong County; [3] LQ, Lu-qu County; [a,b] Superscripts within rows indicate significant difference at 0.05 level

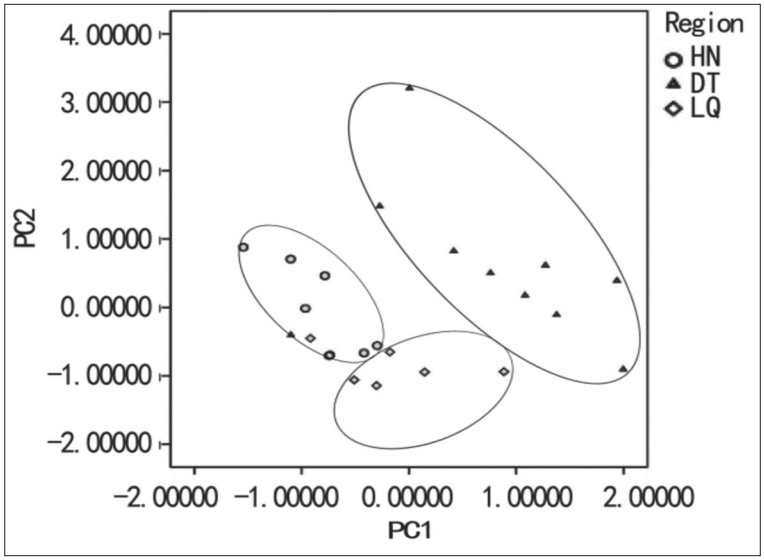

Fig 1. Scatter plots of de-fatted muscle of yak samples scores on the first three principle components. HN, He-nan County; DT, Da-tong County; LQ, Lu-qu County

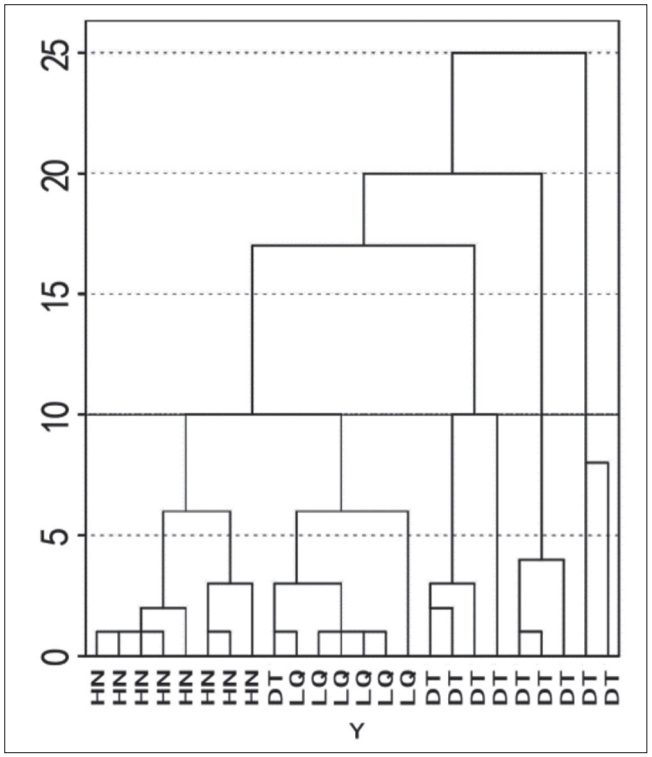

Fig 2. Dendrogram of Cluster analysis. HN, He-nan County; DT, Da-tong County; LQ, Lu-qu County

Yak samples from different regions were separated into six clusters based on the dendrogram cut at a distance of 10 *(Fig. 2)*. The first cluster was mainly composed of samples from He-nan County (n=8). The second clusters were composed of samples from Lu-qu County (n=8). Finally, the rest of cluster mainly comprised the samples from Da-tong County (n=9). Overall, the cluster results were generally in agreement with the actual origin of samples, which implied that multi-element information could be suitably utilised to classify yak samples from the different regions.

By statistics analysis, three elements (Se, Rb, Ti) were selected to establish a classification model using a stepwise discriminant procedure. The model establish as follows:

$Y_{HN} = -7.316+151.354Se+1.347Rb-1433.200Ti$
$Y_{DT} = -9.619+133.994Se+0.545Rb+2312.683Ti$
$Y_{LQ} = -2.272+10.447Se+0.203Rb+1071.610Ti$

Samples from LQ had an overall correct classification rate and cross-validation rate of 100%; HN and DT samples had an overall correct classification rate of 87.5% and 90%, respectively *(Table 3)*. A satisfactory classification was obtained with an overall correct classification rate of 91.7% and a cross-validation rate of 87.5%. These results were consistent with those obtained by PCA and CA, and reconfirmed the feasibility of multi-element analysis for yak geographical origin traceability.

DISCUSSION

The elemental analysis in this study demonstrated that mineral element content of yak meat from three regions on Qinghai-Tibetan plateau were different due to their geographic origin *(Table 1)*. A number of techniques have been used to determine the origin of feed source and these techniques are included in a review [23]. Perhaps the most common technique is the measurement of stable isotopes in meat using isotope ratio mass spectrometry (IRMS). Authenticating meat origin using IRMS has been endorsed because the environmental and management factors that affect the stable isotope ratios of bioelements that end up in animal tissue directly reflect where that meat was derived. The stable isotopes of C and N was used to successfully classify beef from different regions in China [10]. However, routine use of IRMS has been limited as there are large costs associated with setting up reference databanks [24]. Others have combined multi-element and stable isotope analysis to improve classification, but indicated that using multi-mineral analysis was as good as stable isotopes for identifying meat origin [11].

The use of mineral elements to classify meat from different origins has previously been demonstrated for beef [11], lamb [22], pork [25] and poultry [11] meat. Those studies used mineral composition to trace meat back to different countries. The feasibility of discriminating meat source over vast geographical boundaries, ie different countries, is achieved because of large differences in geological profiles, soil formation and climate. The results of this study demonstrated that yak meat source can be discriminated at a much smaller scale, ie between regions within

Table 3. *Classification of yak samples in different regions and percentage of observations correctly classified*

Predicted Group Membership [a]						
Original Group			**Predict Group Membership**			**Total**
			HN[1]	**DT[2]**	**LQ[3]**	
Original	Number	HN	7	0	1	8
		DT	0	9	1	10
		LQ	0	0	6	6
	Original count %		87.5	90	100	91.7[b]
Cross-validated	Number	HN	7	0	1	8
		DT	1	8	1	10
		LQ	0	0	6	6
	Cross-validated count %		87.5	80	100	87.5[c]

[1] HN, He-nan County; [2] DT, Da-tong County; [3] LQ, Lu-qu County; [a] The number of correctly classified observations are tabulated diagonally; [b] 91.7% of empirical grouped observations correctly classified; [c] 87.5% of cross-validated grouped observations correctly classified

a province. This is made possible by the unique farming system in these areas where the farm practice is essentially organic. Because there is virtually no importation of fertilizer or supplemental feed, which would otherwise introduce external sources on dietary minerals, it is possible to discriminate yak meat from different areas in Qinghai province.

The mineral composition of meat is influenced by the environment [22] and the geological processes which influence the soil profile. There was a research demonstrated the environmental influence and geographical variation in mineral content of pastures grown in extensive grassland regions of southern Patagonia [26]. In this research the soil characteristics differed markedly among the three Counties [27]. For example, Da-tong County is characterized by its saline, chestnut, alpine meadow soils and high arsenic content [28]. Two minerals which characterized yak meat to this area [29]. The soil in Lu-qu County is poor salinization [21], which was reflected by the lack of variation in Na in the PCA analysis *(Fig. 1)*. The content and availability of minerals in soils and plants influence the intake and utilisation by grazing animals.

The classification of the different yak meat samples to their origin had a combined cross-validation rate of 87.5%. While we acknowledge the small sample size of the present study, the ability of PCA and LDA to clearly identify yak samples from different origins, provides some confidence in using minerals to authenticate yak meat origin. In agreement with our research, beef geographical origin based on multi-element analysis was also able to discriminate beef from four regions in China, with 98.4% correct classification [30]. In previous studies, there were investigated the suitability of element signatures for authenticating poultry meat and dried beef samples from different origins [11,31]. Those authors concluded that element composition in meat samples could be used to discriminate samples from different countries. However, they noted that classification confidence declined when a large proportion of the diet was imported and the deposition of mineral elements in lean tissue no longer reflected the environment. Similarly, it is recognized that the way in which the meat is processed, i.e. curing, deboning, seasoning, can lead to changes of mineral contents. Because of the unique environment in which yak meat is farmed and the organic practices used, the risk of misclassification is relatively small. But we would highlight the potential impact of factory processing of yak meat beyond the farm gate which could reduce confidence in both origin and perceived 'purity' of marketed products.

In summary, this study provides evidence that yak meat can be authenticated according to its geographical origin using mineral contents. Se, Rb, Ti were selected from more than 50 minerals by statistics analysis and established discriminant model for yak meat traceability. Multivariate statistical analysis gave an overall correct classification rate of 91.7% and cross-validation rate of 87.5%.

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Research Article

Survey on the Presence of the *Mx* and *MHC* Resistance Alleles to Avian Influenza Virus Infection Compared with its Outbreaks Among Chicken Breeds in Egypt

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Abstract

A parallel survey for the incidence of AI in chicken breeds in Egypt including 12 breeds was performed, together with the screening for the presence of their *Mx* and *MHC* alleles. Four years' worth of records of occurrences AI types extending from 2014 to 2017 were used. Also genotyping for the 310 birds under the study using PCR, RFLPs and DNA sequencing was performed. The Baldi (native) chickens were the most affected with all types of AI, especially H5 and H9. In addition, AI was observed in the commercial broilers rather than the layers. Regarding the *Mx* genotypes, although the only detected genotype was the resistance one (Mx/A+) among the Baldi, Cobb and Ross, they were the most affected with AI. Regarding the *MHC* haplotypes, neither the B21 (resistance allele to highly pathogenic AI [HPAI]) nor B13 (susceptible allele) was detected among the studied birds. The homozygous genotypes B4/B4 and B11/B11 were the most common, and new *MHC* alleles were recorded. We recommend recording the names of chicken breeds in AI outbreaks, which will facilitate identification of susceptible breeds in AI outbreaks rather than experimental infection. Further study should be carried out on the most affected breeds with AI to explore their role in the disease epidemiology and whether it is recommended to limit their use during winter, when AI outbreaks occur.

Keywords: *Chickens, HPAI, MHC gene, Mx gene*

Kanatlı İnfluenza Virus Enfeksiyonunda *Mx* ve *MHC* Dayanıklı Allellerinin Mevcudiyeti ve Mısır'daki Tavuk Cinslerindeki Salgınlarla Karşılaştırılması

Öz

Bu çalışmada, Mısır'da toplam 12 tavuk cinsinde Kanatlı İnfluenza insidansı ile birlikte *Mx* ve *MHC* allellerinin varlığı araştırıldı. Çalışmada, 2014 ile 2017 yılları arasındaki dört yıllık periyotta meydana gelen Kanatlı İnfluenza vakaları kullanıldı. PCR, RFLP ve DNA sekans analizi kullanılarak 310 tavuğun genotiplendirilmesi gerçekleştirildi. Baldi (yerel) tavukları H5 ve H9 başta olmak üzere tüm kanatlı influenza tiplerinden en çok etkilenen idi. Ayrıca, Kanatlı influenzası ticari broiler tavuklarda yumurtacılardan daha ziyadesi ile gözlemlendi. *Mx* genotipi ile ilgili olarak, Baldi, Cobb ve Ross cinsleri arasında dirençli olan tek genotip (Mx/A+) olmakla beraber, bu cinsler kanatlı influenzasından en çok etkilenenlerdi. *MHC* haplotipi ile ilgili olarak, ne B21 (oldukça patojenik Kanatlı İnfluenza dayanıklı allel) ne de B13 (duyarlı allel) çalışmadaki kanatlılar arasında tespit edildi. Homozigot genotipler B4/B4 ve B11/B11 en yaygın olup yeni MHC allelleri de belirlendi. Kanatlı İnfluenza salgınlarında tavuk cinslerinin adlarının kayıt edilmesi deneysel enfeksiyonlardan daha ziyadesi ile salgınlarda duyarlı cinslerin belirlenmesinde faydalı olacaktır. Kanatlı influenzasına en duyarlı cinslerin tespitine yönelik ileri çalışmalara hastalığın epidemiyolosindeki rolünün açığa çıkarılması amacıyla ihtiyaç duyulmakta olup bu cinslerin hastalık salgınlarının meydana geldiği kış aylarında kullanımının kısıtlanması tavsiye edilebilir.

Anahtar sözcükler: *Tavuk, HPAI, MHC geni, Mx geni*

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INTRODUCTION

Avian influenza virus (AIV) is a highly contagious viral infection that causes high mortality in chickens. AI A subtypes H5N1 and H9N2 are endemic in Egypt, and the outbreaks of H5N1 have been associated with human infections, especially in 2014-2015. Several genetic markers have been associated with birds' survival from AIV outbreaks in endemic regions [1,2]. The chicken major histocompatibility complex *(MHC)* is known to have a strong association to disease resistance and susceptibility to numerous pathogens [2,3], and this association has been identified in some laboratory and commercials chicken flocks. A set of 27 *MHC* haplotypes were identified in the white Leghorn breed, but little *MHC* information is known for other chicken breeds [4]. The homozygous and heterozygous B21 allele *(MHC* haplotype) is associated with a 100% survival rate from AI outbreaks in Thai indigenous chickens, whereas B13 has a 100% mortality rate [5]. Chazara et al.[6] studied the LEI0258 marker which is located in the B region of the chicken *MHC* and is becoming the reference marker for *MHC* genotyping in chickens.

Mx proteins confer resistance to different virus families; Lee and Vidal [3]; Watanabe [7] demonstrated the *Mx* antiviral activity in response to influenza viruses. In addition, Ko et al.[8] reported one non-synonymous substitution (S631N) in the chicken *Mx* associated with resistance to AIV infection. In addition, Sartika et al.[9] used the mismatching PCR-RFLP method in the *Mx* gene to determine whether chickens carry positive or negative virus activity. Furthermore, the genetic distribution and polymorphism analysis of the *Mx* gene locus as a genetic marker for AI resistance in many indigenous chicken breeds has been documented in several studies [9,10]. The aim of this study was to screen for the presence of different *Mx1* and *MHC1* alleles in chicken breeds in Egypt, especially those reported to be associated with the resistance to AI, as well as the incidence of different types of AIV outbreak in chicken breeds present in Egypt, including 2 native and 10 commercial broiler and layer breeds.

MATERIAL and METHODS

Data Collection

Four years' worth of records of AI A occurrences were obtained from the reference laboratory for veterinary quality control on poultry production, Animal Health Research Institute, Damanhur branch. The records comprise types of AI outbreaks, including both the highly pathogenic AI (HPAI; H5 and H9) and low pathogenic AI (LPAI; H7 and common influenza) from 2014 to 2017 (January-May for each year). The data included in the record represent the number of affected poultry farms from five different Egyptian governorates, the record also includes the breed of the bird.

The virus diagnosis at the reference laboratory depends on the extraction of RNA from pooled homogenate obtained from each flock using and qPCR protocol for virus diagnosis.

Birds Used and DNA Extraction

Blood samples were collected from 310 birds representing 12 chicken breeds from five different Egyptian governorates. The used birds were either native breeds, such as Baldi and Fayoumi (layers) or foreign commercial broilers (Ross, Cobb, Sasso, Hubbard and Arbor) or layers (Hy-line, H&N, ISA, Tetra and Lohman), the number of birds from each breed and their locations are listed in *Table 1*. The blood samples were collected from the brachial vein in the wing area in sterile tubes containing EDTA, transferred to the lab and kept frozen at -20°C. All the birds were handled in accordance with the recommendations of the Committee on the Ethics of Animal Experiments of Kafrelsheikh University, Egypt. Genomic DNA was extracted from whole blood using thermo scientific kits according to the manufacturer's protocol. The purity of genomic DNA was assessed on 2% agarose.

Genotyping of Mx Gene

PCR-RFLP mismatched primers were used for genotyping of the G/A SNP as described by Ommeh et al.[11]. The used primer, annealing temperature, and PCR product size are listed in *Table 2*. *Rsa I* (Thermo Scientific) was employed to cut at the polymorphic (G/A) site, yielding one visible fragment of either 101 bp for allele A or at 73 bp for allele G. PCR conditions for conventional PCR was done using an initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, annealing temperature *Table 2* for 1 min and 72°C for 1 min, and completed by a final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis through 2% agarose. *Rsa I*(1 U/µg) reaction mixture was carried out according to the manufacturer's protocol, and the fragments were separated on 4% agarose.

Table 1. *Breeds used in this study, their location and number*

No	Chicken Breed	Site of Collection	Number
1	Baldi	Aldelngat, Elbeheira Fowa, Kafr-Elsheik	35 30
2	Sasso	Jankles, Elbeheira	25
3	Cobb	Kafr-Elhenawy, Itay, Elbeheira	30
4	Happered	Alalmia, Alexandria	30
5	Arbor	Almahmodia, Elbeheira	20
6	Ross	Abo-Elmatamir, Elbeheira	20
7	Lohman	Elmansoura- Eldaqalia	20
8	ISA Brown	Gharbia	20
9	H&N	Gharbia	20
10	Tetra	Elmansoura- Eldaqalia	20
11	Fauomy	Damanhour- Elbeheira	20
12	Hayline	Damanhour- Elbheira	20

DNA Sequencing

Mx gene sequences of a representative one bird from each breed under study were performed. The PCR products of *Mx* gene were purified using MEGA quick-spin total fragment DNA purification kit (Intron biotechnology) according to manufacturer instruction. The purified products were sent to LGC Company (Germany). The sequence results were analyzed using Chromas 1.45 (http://www.technelysium.com.au). Sequence comparisons were performed using the BLAST program from National Center of Biotechnology information website http://www.ncbi.nlm.nih.gov. The alignment of obtained sequences done by Clustalw version 1.8.

Genotyping of MHC Gene

The amplified PCR products were used to identify the LEI0258 marker of *MHC +class I αI* and *αIV* (BF) using the genomic DNA [12,13], the PCR reaction mixture and conditions as described in the amplification of *Mx* gene. The primer sequence and the annealing temperature are listed in *Table 2*. The PCR fragments were separated on 4% agarose. Different *MHC* haplotypes were identified as described by Fulton et al.[13].

RESULTS

Based on the four years records, the native breed (Baldi) showed a higher incidence of all types of avian influenza during 2014-2017 *(Fig. 1)*, also, it is the most affected breeds with HPAI (H5 and H9). Cobb broilers were the second affected by AI especially H9, then Sasso breed. Sporadic appearance of the AI in some other breeds as Hubbard during 2014, Ross in 2015 and Arbor during 2016. No AI outbreaks were recorded in Fayoumi. Additionally, no reports of AI among the studied layer breeds were recorded

The genomic DNA of 310 samples was successfully amplified for *Mx* gene with a size product of 100 bp. *Rsa1* was used for identification of resistant and sensitive chicken *Mx* gene based on the fragment size. The homozygous individuals (A/A) with one band at100 bp (resistant *Mx*); heterozygous (A/G) with two bands (100 bp and 73 bp); and a third genotype (G/G), homozygous sensitive *Mx* allelic gene) with one band at 73 bp. Regarding the distribution of these allele among the chicken breeds, AA is the only genotype found in all selected birds of the 10 out of 12 studied breeds, AA frequency in Hy-line is 0.6, AG genotype is 0.3 and GG genotype is 0.1. AA genotype in Fayoumi

Table 2. *Primers used in this study*

Gene	Sequence	Anealing Temp	Size of PCR Product
Mx primer	5′-GAGTACCTTCAGCCTGTTTT-3′ 5′-TGCAAAAACATCTTCAAGTCTCTG-3′	60 C	101 bp
MHC-BFαI	5′-GTGGACGGGGAACTCTTC-3′ 5′TCTGGTTGTAGCGCCG-3′	58 C	Variable
MHC-BFαIV	5′GTGGACGGGGAACTCTTC- 3′ACCGCCGGTCTGGTTGTA-3′	58 C	Variable

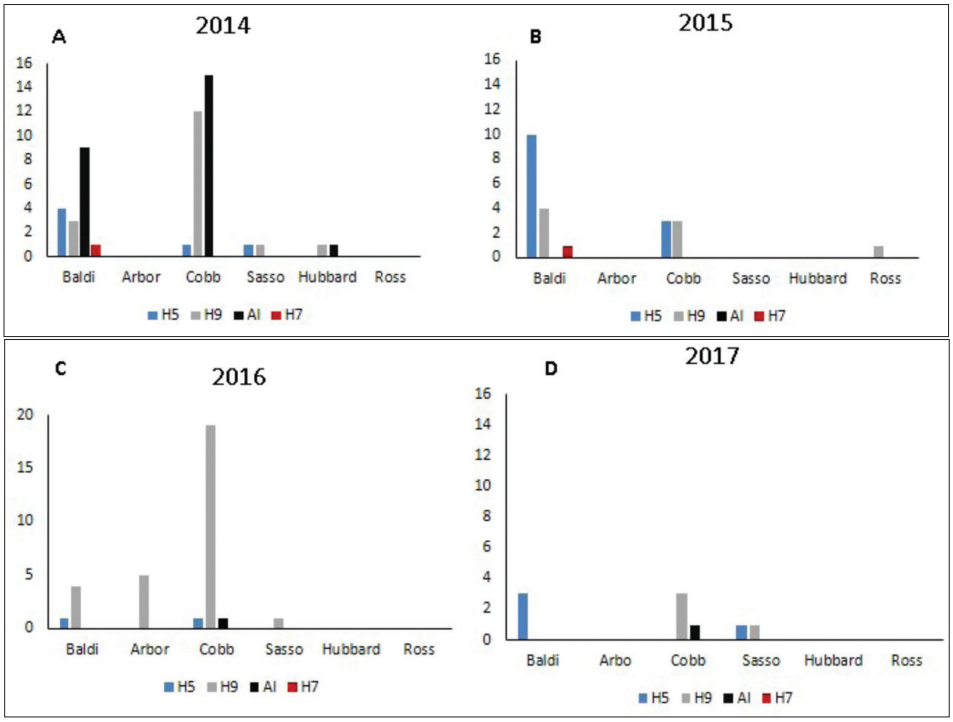

Fig 1. Incidence of different types of Avian influenza A (H5, H9, AI and H7) among chicken breeds in Egypt from 2014-2017

Table 3. *Distribution of resistant and sensitive Mx genotypes in various breeds of chicken based on PCR-RFLP product*

Groups	Breed	Mx Genotype		
		AA/Mx^{++}	AG/Mx^{+-}	GG/Mx^{--}
1	Baldi	65 (1.0)	0	0
2	Fayoumi	10 (0.5)	10 (0.5)	0
3	Hy-Line	12 (0.6)	6 (0.3)	2 (0.1)
4	Cobb	30 (1.0)	0	0
5	Ross	20 (1.0)	0	0
6	Sasso	25 (1.0)	0	0
7	Hubbard	30 (1.0)	0	0
8	Lohman	20 (1.0)	0	0
9	H&N	20 (1.0)	0	0
10	ISA	20 (1.0)	0	0
11	Arbor	20 (1.0)	0	0
12	Tetra	20 (1.0)	0	0

Resistance allele: A/Mx^{+}. Sensitive allele: G/Mx^{-}

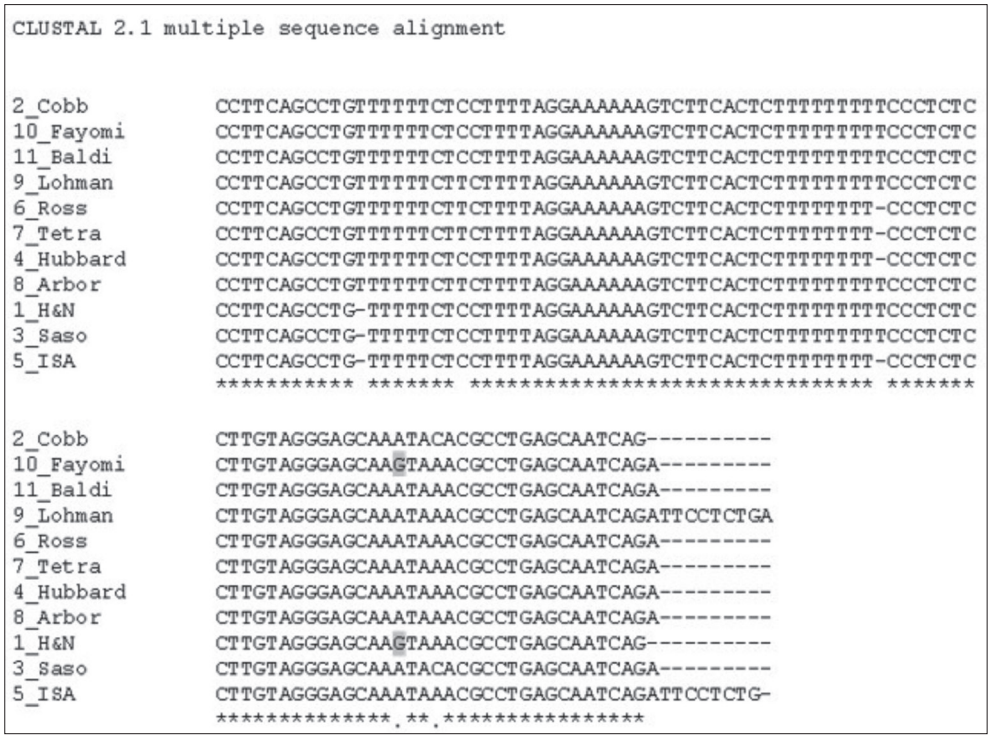

Fig 2. Sequence alignment of *Mx* gene from different chicken present in Egypt

is 50% and AG genotype is 50% *(Table 3)*.

Sequence alignment of Mx gene among the studied chicken breeds indicated the presence of alternative A or G *(Rsa1* recognition site) allele at the nucleotide number 73 of the amplified sequences. The A allele is present at Baldi, Sasso, Ross, H&N, Arbor, Hubbard, ISA, Cobb, tetra, and Lohman. G allele is present at Fayoumi and Hy-line *(Fig. 2)*.

Following the amplification and identification of the LEI0258 alleles of various B haplotypes of *MHC* in different chicken breeds, they are listed in *Table 4*. For *MHC-BFaI* haplotype, three genotypes were repeated among the studied chicken breeds; B 4 (182 bp) was the most common allele present either in the homozygous (B4/B4) in Fayoumi, Sasso, Hubbard, and Arbor, or heterozygous with other new alleles B-N1 750 bp or BN2 800 bp. among the other breeds. Regarding the *MHC-BfaIV haplotypes,* homozygous genotype (B11/B11) was the only genotype detected among Fayoumi, Hy-line, Sasso, Hubbard, Tetra, and Cobb. Heterozygous genotype, B11/B12, B11/B24, B11/B12.3 were detected among Baldi, H &N, Ross and Arbor chickens *(Table 4)*. Neither B21 allele (resistance allele to HPAI) nor B13 alleles (susceptible allele) were detected among the studied breeds

Table 4. *Distribution of LEI0258 alleles of defined MHC (MHC-BFaI and BfaIV) haplotypes in different chicken breeds under study*

Breed		*MHC-BFaI* Haplotype			*MHC-BFaIV* Haplotype			
		B4/B4 182/182	B4/B-N1 182/750	B4/B-N2 182/800	B11/B11 193/193	B11/B24 193/309	B11/B12 193/478	B11/B12.3 193/513
1	Baldi	26	26	13	33	32	0	0
2	Fayomi	20	0	0	12	0	0	0
3	H-Line	16	4	0	18	2	0	0
4	Cobb	20	10	0	10	20	0	0
5	Ross	0	20	0	5	0	20	0
6	Sasso	25	0	0	20	1	5	0
7	Hubbard	30	0	0	25	0	0	5
8	Lohman	0	20	0	0	20	0	0
9	H & N	7	13	0	13	7	0	0
10	ISA	0	20	0	20	0	0	0
11	Arbo	20	0	0	10	10	0	0
12	Tetra	0	15	5	20	0	0	0

DISCUSSION

Although, many reports suggests that native chicken breeds are more resistant to AIV infection, however, no data available supporting this suggestion [14]. Our data indicate that the Baldi chickens (native Egyptian breed) was the most affected with all types of AIV especially H5 and H9 along the records extends from 2014-2017. Also, Sims et al.[15]; Ellis et al.[16] demonstrated that 'local' Chinese breeds of chicken, exposed to an infectious dose of HPAI H5N1 die such as observed in some naturally-infected commercial flocks. On the other hand, Fayoumi another important native layer breed, with an absence record of avian influenza viruses. Additionally, a high incidence of Avian influenza was also recorded in only the commercial broilers as Cobb chickens which is the second affected by avian influenza, then Sasso, Hubbard, and Arbor. No reports of AI among the studied layer breeds were recorded. Reversely, Bertran et al.[17] suggested that broiler breed is less susceptible to the H5N2 virus than the layer breed.

Regarding *Mx* genotypes, variation in *Mx* allele frequency among the two studied native breeds (Baldi and Fayoumi) was observed, and the resistance genotype is the only demonstrated one among 10 out of 12 breeds, however, in the Hy-line and Fayoumi, both have resistance and sensitive alleles. Fadhil and Mercan [18] demonstrated that the resistant *Mx* gene allele (A/Mx+) frequency was 98% and the sensitive allele frequency (G/Mx-) was 2%, in Gerze Turkish chicken breed while in pure line chicken breed, *Mx* sensitive allele frequency was 48% and the resistant allele frequency was 52%. Hassanane et al.[19] demonstrated that the average allele frequency of the resistant A allele in some Egyptian chicken breeds was higher than the sensitive G allele, and the resistance allele A is the only allele present in Egyptian Baldi chickens which is in agreement with our results.

Many reports have investigated the two allele A/G substitution polymorphism coding amino acid position 631 of *Mx* gene and its role in resistance to AI [7,20]. However, Sironi et al.[21] indicated that the *Mx* genotype did not affect the clinical status or the time course of infection after viral experimental infection of five chicken lines with one of HPAI virus. Furthermore, Sironi et al.[22] in a genomic study to the response of chicken to HPAI virus, concluded that neither the genotype at the *Mx* gene or *MHC-B* locus, involved in variations to response to AIV infection. However, many reports have been documented the role of chicken's major histocompatibility complex (*MHC*) haplotype on the resistance or susceptibility of HPAI [5]. Hunt et al.[23] challenged a series of *MHC* congenic white leghorn chicken lines with a low dose of (H5N1) virus, they demonstrated that none of the lines were completely resistant to the lethal effects of the challenge.

Regarding the *MHC* haplotypes in our study, neither B21 allele (resistance allele to HPAI) nor B13 alleles (susceptible allele) indicated by Boonyanuwat et al.[5] were detected among the birds included in this study. Additionally, few number of alleles are detected and the homozygous genotypes B4/B4, B11/B11 are the most common especially among Fayoumi, Sasso, and Hubbard. Heterozygous genotypes were detected among the other breeds with newly detected haplotypes. Shavakand [24] detected less variation in microsatellite markers (LEI0258), situated within the *MHC* region, in the industrial chicken populations compared to non-industrial populations.

Comparison between the data of AI incidence with the genotypes of chickens under the study, Baldi breeds, Cobb and Ross were the most affected, although they only have the resistance genotype (Mx/A+). On the other hand, Fayoumi chickens another native chicken breed with no reports of avian influenza have both resistance and

susceptible genotypes with 50% of each. Matsuu et al.[25], observed absence of a significant association between the *Mx* and *MHC* class I genes polymorphisms in these loci and resistance to HPAIV.

In a survey about HPAI introduction into Europe by EFSA supporting publication [26], at the period 2005-2015, the species affected by HPAI outbreaks were frequently reported as ducks, mixed ducks and geese, and undetermined species of backyard flocks; however, no data about the chicken breeds are included. Taking into consideration the consumer demand and the related economic losses of Avian Influenza outbreak [27], we suggest that the determination of the most susceptible chicken breed, would have great impact in controlling the disease and subsequently the economic losses.

In a conclusion, we recommend recording the names of chicken breeds in AI outbreaks, which will facilitate identification of susceptible and resistance breeds in AIV outbreaks rather than experimental infection. Further study should be carried out on the most affected breeds with AI to explore their role in the disease epidemiology and whether it is recommended to limit their use during winter, when AI outbreaks occur.

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Research Article

Use of CART and CHAID Algorithms in Karayaka Sheep Breeding

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Abstract

The aim of this study was to determine the effect of some factors (sex, birth type, farm type, birth weight and weighting time) on weaning weight through CART and CHAID data mining algorithms. The classification and regression trees are modern analytic techniques that construct tree-based data-mining algorithms. Regression trees are used for the purpose of preliminary selection of the traits affecting the continuous dependent variable. The studied data were consisted of 366 records from Karayaka sheep breed. The CHAID algorithms results revealed that; predictors such as weighting time, sex and farm type statistically influenced weaning weight Regression tree diagram constructed by CART algorithm depicted that birth type was effect the weaning weight, and in this tree weighting time of single born lambs was affected the birth type. The predicted values and original values were correlated (P<0.05). As a result, it could be suggested that CHAID algorithm was found more useful biologically than CART.

Keywords: *CART, CHAID, Karayaka, Weaning weight*

CART ve CHAID Algoritmalarının Karayaka Koyun Islahında Kullanımı

Öz

Bu çalışma, sütten kesim ağırlığı üzerime bazı faktörlerin (cinsiyet, doğum tipi, işletme tipi, doğu ağırlığı ve ölçüm zamanı) CART ve CHAID veri madenciliği algoritmaları ile belirlenmesini amaçlamaktadır. Sınıflandırma ve regresyon ağaçları veri madenciliği kapsamında olan modern analitik yöntemler sınıfında yer almaktadır. Regresyon ağaçları, sürekli bağımlı değişkeni etkileyen özelliklerin ön seçimi amacıyla kullanılmaktadır. Çalışmada Karayaka koyun ırkına ait 366 kayıt veri olarak kullanılmıştır. Sonuç olarak; CHAID algoritmasına göre ölçüm zamanı, cinsiyet ve işletme tipi sütten kesim ağırlığı üzerinde önemli derecede etkili bulunmuştur. CART algoritmasına ait sonuçlar ise sütten kesim ağırlığı üzerine doğum tipinin etkili olduğunu göstermiştir. Bu ağaçta tekiz kuzuların ölçüm zamanının doğum tipinden etkilendiği anlaşılmıştır. Tahmin edilen ve gözlenen değerler yüksek ilişkili bulunmuştur (P<0.05). Sonuç olarak, CHAID algoritmasının CART algoritmasına göre biyolojik olarak daha kullanışlı olduğu belirlenmiştir.

Anahtar sözcükler: *CART, CHAID, Karayaka, Sütten kesim ağırlığı*

INTRODUCTION

In general, the aim of animal breeding is to genetically improve populations of livestock so that they produce more efficiently under the expected future production circumstances. Genetic improvement for economic traits is achieved by selecting the best individuals of the current generation and by using them as parents of the next generation [1]. To achieve the aim of animal breeding, evaluation of the data is very important manner.

Necessary data for animal breeding is generally consisting of many factors which make the data multidimensional. When the number of factor increases, interpretation of the results become difficult. In this case, overlooking the important details will be unavoidable. Fail to satisfy of conventional analysis tools on complex data, new approaches such as data mining have begun to use. Data mining is an entire process of applying a computer-based methodology, including new technologies, to discover knowledge from data [2].

The classification and regression trees are modern analytic techniques which are member of data-mining. They allow for building graphic easily-comprehensible models used to describe and to predict the phenomenon expressed in both the nominal and the ordinal scale. The classification and regression trees can also be used for the purpose

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of preliminary selection of the traits which have a statistical effect on the dependent variable [3]. Classification and regression trees are machine-learning methods for constructing prediction models from data. The models are obtained by recursively partitioning the data space and fit a simple prediction model within each partition. The partitioning can be represented graphically as a decision tree. Classification trees are designed for dependent variables that take a finite number of unordered values, with prediction error measured in terms of misclassification cost. Regression trees are for dependent variables that take continuous or ordered discrete values, with prediction error typically measured by the squared difference between the observed and predicted values [4-6].

Some studies reported significant information on usability of data mining algorithms in sheep and goat breeding. In recent times, many studies have been reported on applying CART (Classification and Regression Trees), CHAID (Chi-square Automatic Interaction Detection), exhaustive CHAID and MARS (Multivariate Adaptive Regression Splines) algorithms for various animal species in animal science for regression type problems [7-10]. However, the application of the mentioned algorithms is still scarce for Karayaka sheep. Therefore, in this study, we aimed to compare the CART and CHAID algorithms of classification and regression trees to predict weaning weight by means of the significant ones among sex, birth type, farm type, birth weight and weighting time in Karayaka sheep.

MATERIAL and METHODS

In this study, to predict weaning weight through CART and CHAID tree-based algorithms, 366 records of Karayaka sheep breed were taken from three different farms in the year 2017. Sex, birth type, farm type, birth weight and weighting time (age as days) variables were used as possible explanatory variables to predict weaning weight. Descriptive statistics for dependent and explanatory variables were given in *Table 1*.

This method was modified and extended by using some algorithms to minimize an estimate of misclassification error [11].

The classification and regression tree algorithm contains three important tasks. The first task is how to segment data at each step?, the second task is when to stop segmentation?, and the last one is how to predict the value Y for each X in a segment? [5,11,12]. The classification and regression trees begin with a single root node for response variable. The tree is constructed by splitting the whole data into nodes or sub-groups by using all the independent variables. This process goes on until the requirements of homogeneity are met on any child node [13,14]. It is aimed with obtaining terminal nodes in order to increase proportion of variance among nodes [15].

The most popular algorithms used in decision trees are CART and CHAID tree-based algorithms. These algorithms are nonparametric methods which allow using nominal, ordinal and continuous variables [12]. The term "Regression tree" is used for the tree that its response variable is continuous [16].

The response variable is a continuous and explanatory variables can be continuous or categorical in CART algorithm and it creates binary split. In the CHAID algorithm, the response variable can be continuous and categorical. But, explanatory variables are categorical variables only (can be more than 2 categories) and it can create multiple splits [17,18]. The explanatory variable has continuous structure will turn into categorical structure with use of CHAID algorithm [19]. CART algorithm has a characteristic of continuation of CHAID algorithm [19,20]. The aim is to produce homogeneous data sets as much as possible. CART algorithm produce more homogeneous groups than CHAID using pruning [21,22]. Gini, Twoing and Ordered Twoing impurity or diversity measures can be used for categorical response variables, but for continuous

Table 1. *Descriptive statistics*

Parameter		N	%	Birth Weight (Mean±Std. Deviation)	Weaning Weight (Mean±Std. Deviation)	Weighting Time (Mean±Std. Deviation)
Sex	Male	198	54.1	3.42±0.48	23.28±4.58	100.49±13.76
	Female	198	45.9	3.23±0.54	22.22±4.71	101.43±13.58
	Total	366	100	3.33±0.51	22.79±4.66	100.92±13.66
Birth type	Single	332	90.7	3.45±0.35	23.62±4.05	100.53±13.96
	Twin	34	9.3	2.10±0.09	14.63±0.45	104.70±9.70
	Total	366	100	3.33±0.51	22.79±4.66	100.92±13.66
Farm type	Farm 1	95	26.0	3.12±0.75	21.3±5.37	97.87±11.67
	Farm 2	110	30.0	3.4±0.45	23.89±4.33	107.09±11.32
	Farm 3	161	44.0	3.41±0.34	22.93±4.21	98.51±14.87
	Total	366	100	3.33±0.52	22.79±4.66	100.92±13.67

variables, Least-Squared Deviation (LSD) or Least Absolute Deviation (LAD) can be used [19,23].

The best algorithm produces minimum SD_{Ratio} (Standard Deviation Ratio), MAD (Mean Absolute Deviation), RMSE (Root Mean Square Error) and coefficient of determination (R^2) criteria for goodness of fit. Standard Deviation Ratio, MAD and RMSE can be written as [24,25];

$$SD_{Ratio} = \sqrt{\frac{\frac{1}{n-1}\sum_{i=1}^{n}\left(\varepsilon_i - \bar{\varepsilon}\right)^2}{\frac{1}{n-1}\sum_{i=1}^{n}\left(Y_i - \bar{Y}\right)^2}},$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}\left(Y_i - \hat{Y}_i\right)^2}{n}},$$

$$MAD = \frac{1}{n}\sum_{i=1}^{n}\left|Y_i - \hat{Y}_i\right|$$

$$R^2 = \left[1 - \frac{\sum_{i=1}^{n}(Y_i - \hat{Y}_i)^2}{\sum_{i=1}^{n}(Y_i - \bar{Y}_i)^2}\right].$$

All statistical analysis was performed using IBM SPSS 20.0 via Ondokuz Mayıs University license. The best algorithm will produce the lowest goodness of fit value for mentioned statistics.

RESULTS

Decision tree diagrams to estimate weaning weight by CART and CHAID algorithms are depicted in *Fig. 1* and *Fig. 2*, respectively. CART was seen to produce more branches compared with CHAID tree-based algorithm. Both diagrams reflected environmental factors that can be effective on weight trait, more understandably.

For CART algorithm, Node 0 was split into two smaller subgroups as single birth (23.629±4.052; n=332) and twin birth (14.634±0.456; n=34) according to birth type. These results showed that the effect of single birth was higher than twins on weaning weight as expected. Nodes derived for single lambs had heavier average than those derived for twin lambs.

For single birth, the branch was divided into two smaller subgroups according to age predictor as Node 3 (the subgroup of the single lambs with the age≤91.5 [18.863±2.545 kg; n=94]) and Node 4 (the subgroup of the single lambs with the age>91.5 (25.512±2.796 kg; n=238).For twin birth lambs, binary branching was occurred with Nodes 5 and 6. Node 5 represented the subgroup of the twin born lambs with the age≤108.0 (14.372±0.362 kg; n=20), whereas the subgroup of the twin born lambs with the age>108.0 (15.008±0.283; n=14) was named Node 6. From *Fig. 1*, it was understood that the heaviest lamb average was obtained with the subgroup of the single lambs with birth weight>3.195 kg and age>112.500 days (28.344 kg).

Pearson correlation coefficient between weaning weight and predicted weaning weight for CART Algorithm was estimated as 0.938 (P<0.01).

For CHAID algorithm weaning weight 22.794±4.663 with n=366 began with Node 0 and the tree had 30 child nodes. The age variable divided into six child nodes at first depth of the tree structure. Predictive performances of the CART and CHAID algorithms are presented in *Table 2*. The influential predictors of the CHAID algorithm were found as age, birth weight and birth type, and farm. Nodes 1, 2, 7, 11, 12, 13, 15, 16, 17, 19, 20-30 were terminal nodes. Node 0 was divided by age predictor into six smaller subgroups i.e. (Nodes 1-6) at the first tree depth. Node 3 was split into three smaller subgroups i.e. (Nodes 7-9) at the second tree depth according to birth weight. Node 4 was branched into two smaller subgroups. Each of Nodes 5 and 6 was divided into four smaller subgroups (Nodes 12-15) and (Nodes 16-19) at the second tree depth according to birth weight. At the third tree depth, Nodes 8-10 were divided into two or three smaller subgroups according to farm. Nodes 14 and 18 were exposed to a binary partition at the third tree depth. At the first tree depth, Node 19 (the subgroup of those with age>114 days birth weight>3.5 kg) produced the heaviest average body weight with 29.327 kg, as expected.

Pearson correlation coefficient between weaning weight and predicted weaning weight for CHAID Algorithm was estimated as 0.937 (P<0.01).

Pearson correlation coefficient between weaning weight and predicted weaning weight for CHAID Algorithm.

DISCUSSION

Results displayed that the partition at the first depth in the tree structures was based on birth type in CART algorithm and weighting age in CHAID algorithm. So, the most predictor variables were different according to the specified algorithms. CART algorithm produced five levels of branching where CHAID algorithm produced three levels of branching. It could be said that CHAID algorithm could be interpreted more easily [12].

For these algorithms, the estimation of SD_{ratio} value smaller than 0.40 was an indicator of the good fit means [12,26,27]. Both MAD and RMSE produced nearly equal values, coefficient of determination were found equal for both algorithms.

To predict weaning weight from sex, birth type, farm type,

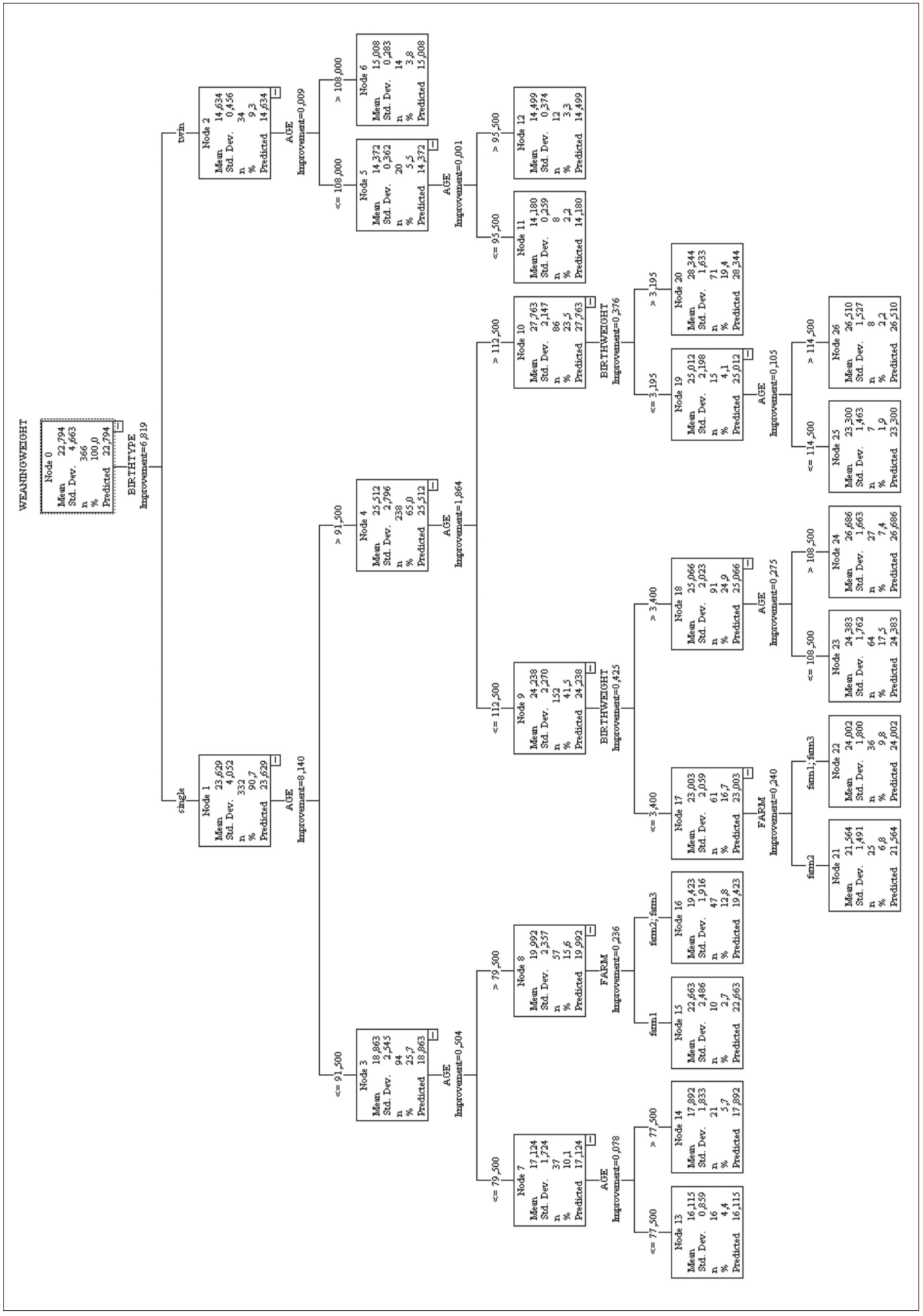

Fig 1. The decision tree diagram obtained by CART

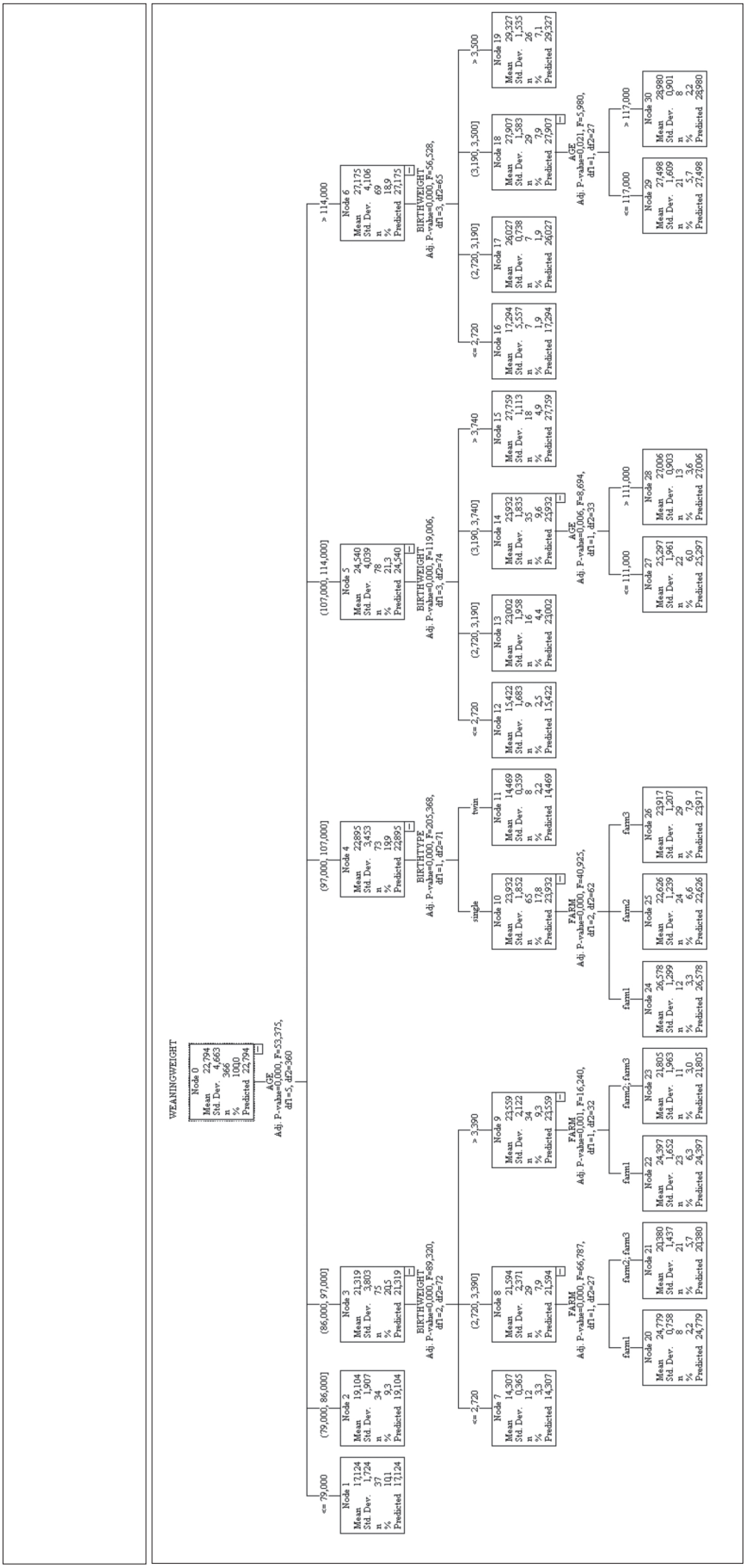

Fig 2. The decision tree diagram obtained by CHAID algorithm

Table 2. *Comparison criteria for CART and CHAID algorithms*

Algorithm	SD_{ratio}	MAD	RMSE	R^2
CART	0.347	1.195	1.612	0.88
CHAID	0.348	1.192	1.623	0.88

birth weight and weighting time (age) variables, CART and CHAID algorithms can be used interchangeable. The sex variable was excluded from the model in both algorithms. This result was biologically interesting for sheep breeding. Both two methods had similar fitting criteria; however, interpretation of CHAID algorithm because of less branching was more user friendly than CART algorithm. CART algorithm had sub branching over the same variable, which obstruct the interpretation. Similar findings also supported the statements of some earlier authors [10,12,15,25].

Also, the-tree based algorithms could be applied as a remarkable alternative for the data of response surface designs [10].

Regression tree aims to repeatedly partitioning the population into different child nodes where the variation of response variable is minimum within and maximum between the child nodes. Also, it aims to balance predictive accuracy and complexity with interpretation of model. Advantage of easy interpretation for both response and predictor variables with visual diagrams are superiority of regression tree. Due to nonparametric properties, it does not require any parametric assumptions. Results of this study showed that CART and CHAID algorithms can be used interchangeable.

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Research Article

A Fuzzy Logic Application to Predict Egg Production on Laying Hens [1]

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Abstract

Fuzzy logic has a great potential for researchers and it has been developed over the last two decades. In animal science, there are limited numbers of studies on fuzzy logic approach. This study was carried out to examine the Fuzzy logic applications for prediction of the egg production data. Egg production records were obtained from the commercial poultry farm in Izmir, Turkey. Egg production traits of brown laying hens at 22 to 40 weeks of age were analyzed with Fuzzy logic system. In this study, Fuzzy logic model was developed for the prediction of egg production values of three classes; top, middle and lower (bottom for the cage effect) production. For this purpose 120 data lines representing 4 inputs consisting of cage, age at sexual maturity (ASM), body weight at sexual maturity (BWSM), body weight at mature age (BW) and 1 output, egg production (EP) that collected daily and individually were used in a Fuzzy logic model. The similarities between predicted and original production records were investigated, the coefficient of determination (R^2) was found as 0.89 which was also shown the prediction's success rate. The probability of egg production at ASM of 168 days, BWSM of 1500 g and BW of 1820 g was found 98.97% while egg production's probability at ASM of 157 days, BWSM of 1720 g and BW of 1940 g was determined as 97.97%. These results were also concluded that layers reached at sexual maturity later have lower egg production. The results illustrated that Fuzzy model could provide an effective and accurate prediction for classifying egg production of laying hens. However, since the applications of fuzzy logic related to the prediction of egg production are limited, this work will be pioneered by future studies.

Keywords: *Artificial intelligent, Egg production, Fuzzy inference, Fuzzy logic, Poultry*

Yumurtacı Tavuklarda Yumurta Veriminin Tahminlenmesinde Bulanık Mantık Uygulaması

Öz

Yaygın bir çalışma alanına sahip olan bulanık mantık, son 20 yılda gelişmiştir. Hayvancılıkta bulanık mantık yaklaşımını kullanan az sayıda araştırma vardır. Bu çalışma, yumurta verimi kayıtlarının tahmininde bulanık mantık uygulamalarını incelemek amacıyla yapılmıştır. Yumurta verim kayıtları İzmir, Türkiye'de bulunan ticari kümes hayvanlarından elde edilmiştir. 22-40 haftalık yaştaki kahverengi yumurtacı tavuklara ait yumurta verim özellikleri bulanık mantık yöntemi ile analiz edilmiştir. Bu araştırmada bulanık mantık modeli; yüksek, orta ve düşük (kafes etkisi için alt) şeklinde üç sınıfta gruplandırılan yumurta verim değerlerini tahminlemek için geliştirilmiştir. Bu amaçla bulanık mantık modelinde kafes etkisi, eşeysel olgunluk yaşı (ASM), eşeysel olgunluktaki canlı ağırlığı (BWSM), ergin yaştaki canlı ağırlığı (BW) olmak üzere toplam 4 girdiyi temsil eden 120 veri satırı ile 1 çıktı değişkeni, günlük ve bireysel yumurta verimi (EP) kullanılmıştır. Belirtme katsayısı (R^2) 0.89 olarak bulunmuştur. Bu aynı zamanda tahminlemenin başarısını göstermektedir. Eşeysel olgunluk yaşı (ASM) 168. gün, eşeysel olgunluktaki canlı ağırlık (BWSM) 1500 g ve ergin yaştaki canlı ağırlık (BW) 1820 g olduğunda yumurtlama veriminin olasılığı %98.97 olarak bulunmuş iken eşeysel olgunluk yaşı (ASM) 157. gün, eşeysel olgunluktaki canlı ağırlık (BWSM) 1720 g ve ergin yaştaki canlı ağırlık (BW) 1940 g olduğunda yumurtlama veriminin olasılığı %97.97 olarak tespit edilmiştir. Elde edilen sonuçlar aynı zamanda geç yaşta eşeysel olgunluğa erişen yumurtacılarda yumurta veriminin daha düşük olduğunu göstermektedir. Bulanık mantık ile tahminlenen yumurta verimleri ile orijinal kayıtlar arasındaki benzerlikler araştırılmıştır. Bulgular, yumurta veriminin sınıflandırılmasında bulanık modelin etkili ve doğru bir tahmin sağlayabileceğini göstermiştir. Bununla birlikte, yumurta veriminin tahmini ile ilgili bulanık mantık uygulamalarının sınırlı olması yapılan bu çalışmayı daha sonra yapılacak çalışmalara öncü kılacaktır.

Anahtar sözcükler: *Yapay zeka, Yumurta verimi, Bulanık çıkarım, Bulanık mantık, Kanatlı*

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INTRODUCTION

In animal breeding studies, several methods have been developed to predict future yield by using available information of traits. Linear and nonlinear models are used as classical tools for this purpose. Artificial intelligent methods have also been introduced as an alternative to these classical approaches in recent years. Both mentioned approaches are used for mathematical description.

There are several applications of fuzzy logic in agriculture. In particular, in animal production studies it was used for the determination of oestrus cyclus, prediction of mastitis and culling levels [1-3]. Fuzzy logic also used in disease, weed and pest management as well as, soil analysis and developing expert systems for crops [4].

Nowadays, egg production has an important role in poultry industry. Besides genotypic structure; temperature, lighting, feeding and diseases have also direct effects on egg production [5]. North and Bell [6] implied that there was an increase in the 8th or 9th weeks of egg production while afterwards the production decreases at a constant rate [7]. Mashhadi et al.[8] invented an incubator, Olaniyi et al.[9] designed poultry feed and water systems, Abreu et al.[10] modelled the broiler performance in heat stress, and Bamigboye and Titus [11] applied temperature controlled systems in poultry houses by using Fuzzy logic method.

As far as we reached, there was no study carried out with the Fuzzy logic to predict egg production. So, in this study, for the first time, Fuzzy logic was examined to predict egg production.

MATERIAL and METHODS

Material

Egg production data from a commercial sire line in the 6th generation were the material of the study. Brown layer hens were individually kept in three tiers double sided battery cages in an environmentally controlled hen house *(Fig. 1)*.

Table 1. *Descriptive statistics of scaled traits the developed Fuzzy Logic Model*

Traits	Rules Data Set (n=90)		
	Minimum	Maximum	Mean
ASM	125	198	152
BWSM	960	2160	1529
BW	1220	2540	1762
EP	37	131	108.81

ASM: age at sexual maturity, BWSM: body weight at sexual maturity, BW: body weight at mature age, EP: egg production

Egg production data were collected from the 22nd to 40th weeks of age defined as early part of whole egg production record. For each individual, egg production to the 40th weeks of age (EP) was recorded and age at sexual maturity (ASM), body weight at sexual maturity age (BWSM), body weight at mature age (BW) were individually recorded, as well. The minimum, maximum and average values of the scaled input and output parameters are shown in *Table 1*. The modeling was performed by MATLAB (MatLab 2015b, The Mathworks, USA) software.

Methods

Fuzzy logic is a machine learning method that uses of human knowledge and experience to process this information into rule bases and to obtain the result that each rule base corresponds to a specific mathematical function. It is an artificial intelligence-based method that provides a realistic and flexible perspective to people, especially in decision-making processes. It depends on Aristotle's laws. In spite of Aristotle's "with or without

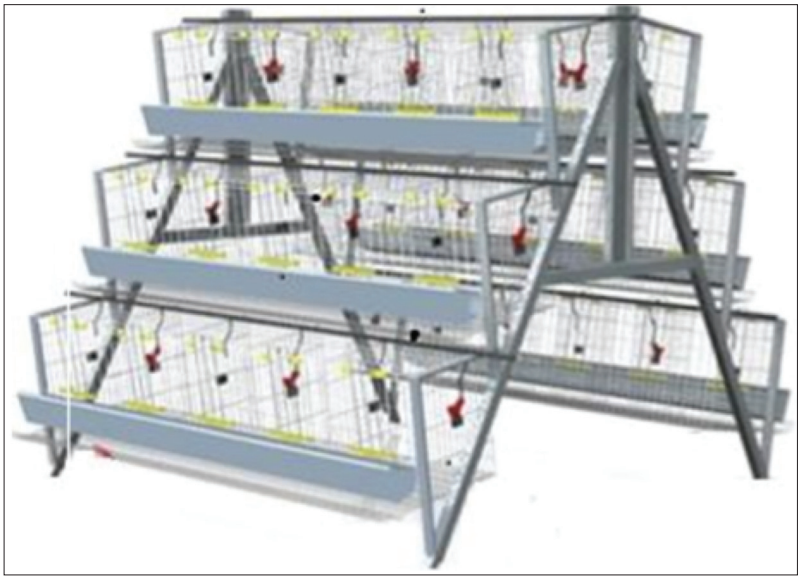

Fig 1. Three tiers battery cages double sided

laws"; Plato, on the other hand, has taken this situation forward and described a third situation in which right and wrong are internal, apart from being "right" and "wrong" [12,13]. Zadeh [14] explains the applicability of fuzzy logic to uncertainty-based systems and gave the name "Fuzzy Logic" in the name of the theory that these values are expressed in the range between 0.0 and 1.0 [14,15]. Zadeh [16] shows the concept of Fuzzy logic, which predicts to get rid of the limited movement and thought of the t wo-valued logic, with the Fuzzy set theory, which contains the truth values of an idea in infinite numbers between exact and definite truth [17].

Fuzzy logic; modeling in solving the problems due to multiple evaluations has advantages such as proximity to reality [18]. The first step of fuzzy logic is to define the problem and determine the appropriate parameters then forming the membership functions. Triangular, trapezoidal and Gaussian type membership functions from the membership functions in the Fuzzy library are compatible with the existing data set [19,20]. In this study, triangular type which gives the most appropriate estimation precision was used. Following the creation of membership functions, a chart of rules containing the solution of the problem was created. Then, inference methods, Sugeno, Mamdani and Tsukamoto were determined [21-23]. In this study Mamdani inference method was used.

The rule structure of this method is given as below,

If $X_1 = A_1$ and $X_2 = B_1$ then $Z_1 = C_1$

If $X_1 = A_2$ or $X_2 = B_2$ then $Z_2 = C_2$

where, X_1 and X_2 represent input variables; Z_1 and Z_2 represent output variables. A_1, B_1, A_2 and B_2 are membership functions, and C_1 and C_2 are the resultant set of fuzzy results for each rule. The "and" and then the "or" processors are used before the threshold values of the rules are calculated in the method of Mamdani inference [21,24].

In the last stage, the method of transforming the fuzzy numbers generated by a Fuzzy logic model into the classical numbers is determined [25]. An important step in fuzzy modeling and fuzzy decision making is the process of defuzzification which determines the best non-fuzzy performance value. Several methods for such defuzzification are available, including the mean of maxima (MOM), α-cut and center of area (COA) [26]. The MOM method that computes the average of those having the highest fuzzy outputs was used as the defuzzification method. The defuzzification value is computed as following [27].

$$Z = \sum_{j=1}^{n} \frac{Z_j}{n}$$

In the equation Z_j represents the output variable, n is the number of quantized and Z represents the defuzzication value that reaches their maximum memberships.

RESULTS

In the present study, both scaled (ASM, BWSM and BW) and discrete (cage) input variables were used for prediction of egg production to show more detailed solutions and relationships between egg production and scaled input variables.

It is necessary to determine the classes of the selected parameters and the range of the class before the fuzzification process which is the first step of the fuzzy system formation is started. Quality classes of input variables were determined by options of consulting experts. *Table 2* shows the quality classes and class ranges of the input variables; Cage, ASM, BWSM and BW.

Fig. 2 shows the Fuzzy logic model with 4 inputs on the left and 1 output on the right. Input variables consist of cage, age of sexual maturity (ASM), body weight of sexual maturity (BWSM) and body weight (BW). The output variable is egg production (EP).

Input and output variables of membership functions are shown in *Fig. 3-7*. All of the membership functions are displayed and edited to integrate the Fuzzy inference system, including both input and output variables.

After the membership functions were obtained in practice, a rule table was created. The Fuzzy rules were created by using the training algorithms with input-output variable pairs, and the Fuzzy rules were indicated in the IF-THEN form that was expressed the relation between machining performance and machining parameters [28,29]. It contains the real experiment results generated according to the characteristic change of the input parameters. Rules table consists of 76 rules. A part of the rule table is shown in *Table 3*.

Table 2. *Fuzzy sets for inputs*

Quality Class	Cage	ASM	BWSM	BW
Lower	0<x<1300	0<x<100	0<x<1100	0<x<900
Middle	1100<x<3100	80<x<3100	900<x<1600	800<x<1900
Top	3000<x<4128	140<x<198	1400<x<2160	1800<x<2540

ASM: age at sexual maturity, BWSM: body weight at sexual maturity, BW: body weight at mature age

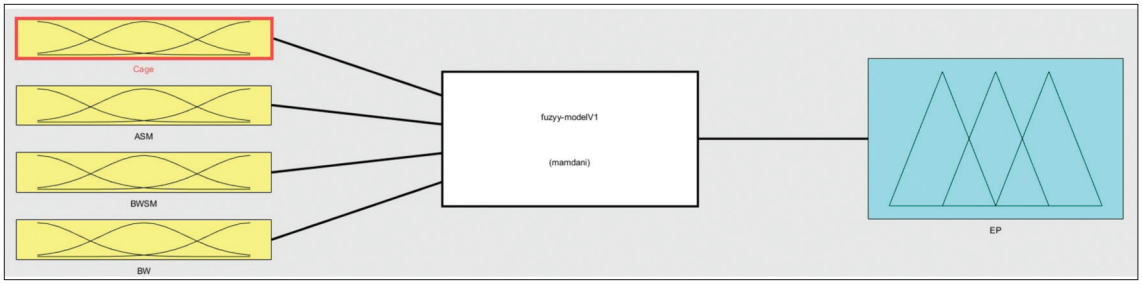

Fig 2. Fuzzy logic model

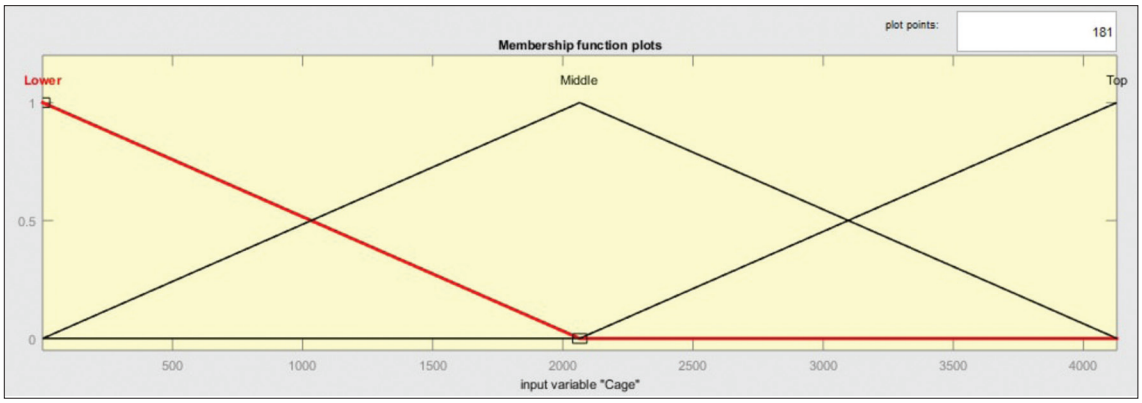

Fig 3. Cage membership function

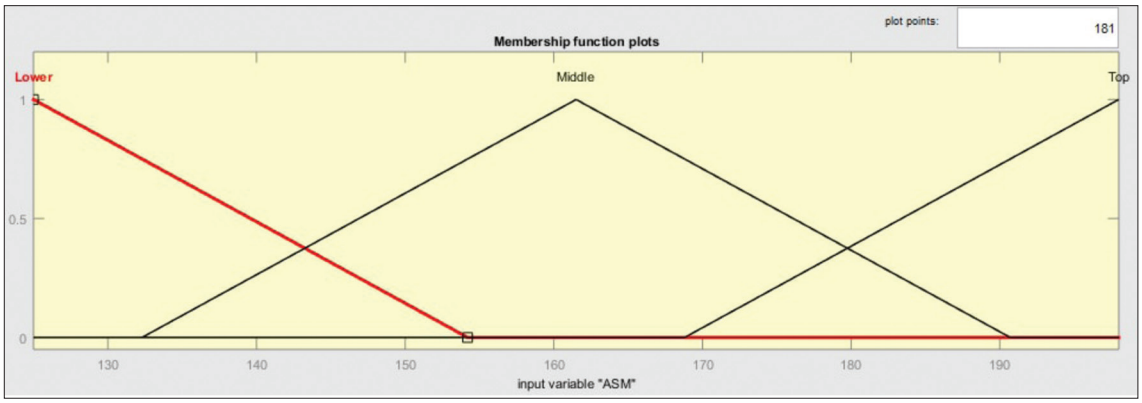

Fig 4. Age at sexual maturity membership function

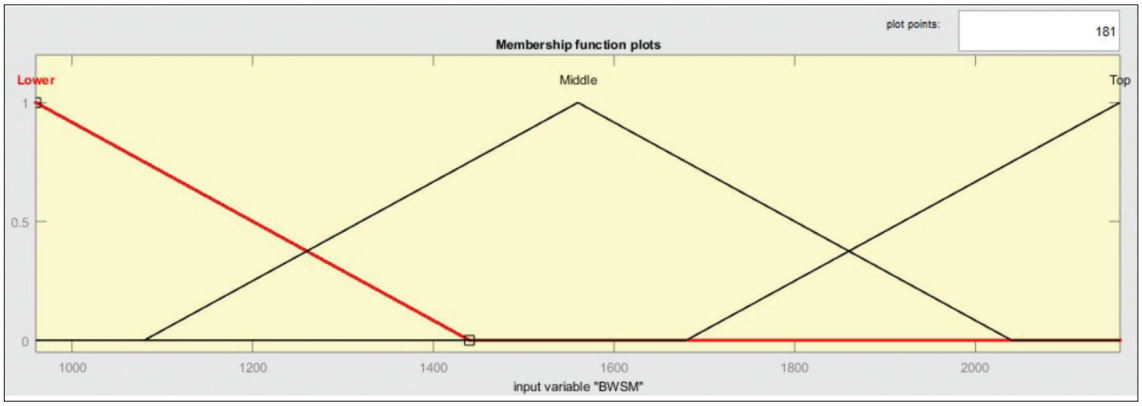

Fig 5. Body weight of sexual maturity membership function

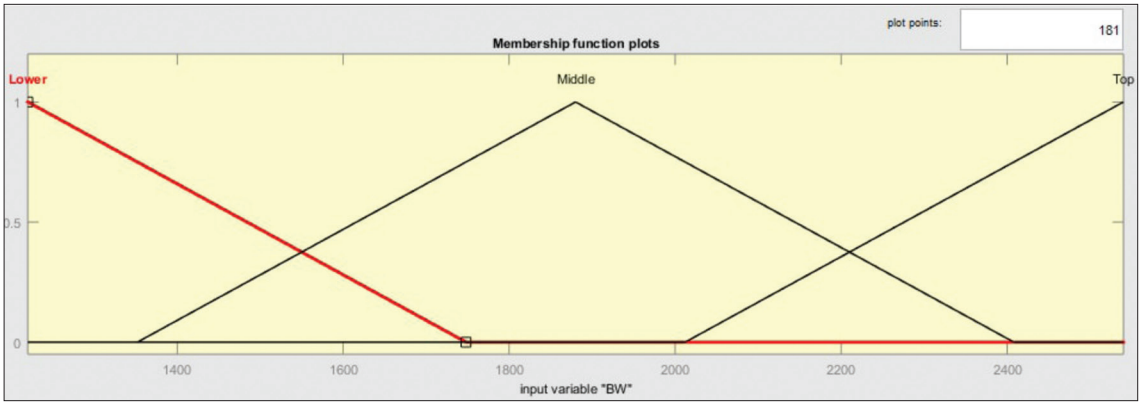

Fig 6. Body weight membership function

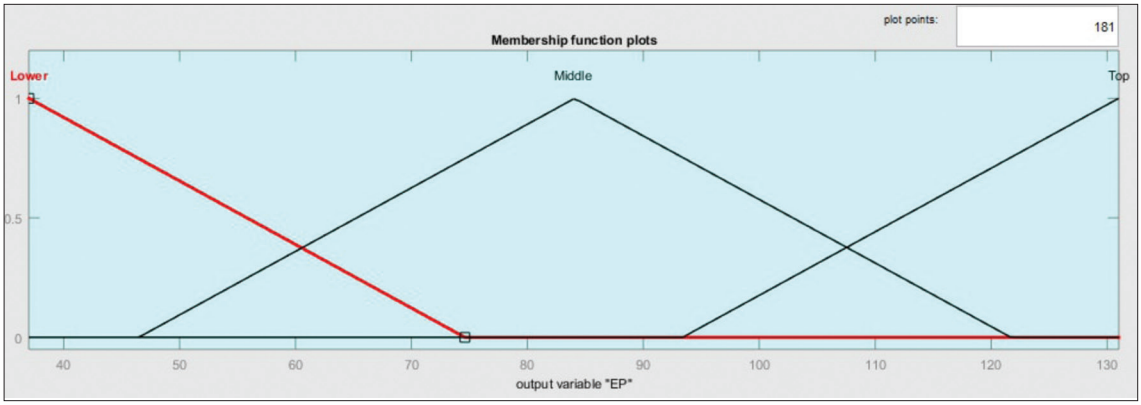

Fig 7. Egg production membership function

Table 3. *Fuzzy rule list*

Rule No	Rules
1	If (Cage is Lower) and (ASM is Middle) and (BWSM is Top) and (BW is Top) then (EP is Top) (1)
2	If (Cage is Lower) and (ASM is Middle) and (BWSM is Middle) and (BW is Middle) then (EP is Top) (1)
3	If (Cage is Middle) and (ASM is Middle) and (BWSM is Middle) and (BW is Middle) then (EP is Top) (1)
4	If (Cage is Lower) and (ASM is Top) and (BWSM is Middle) and (BW is Top) then (EP is Middle) (1)
5	If (Cage is Top) and (ASM is Middle) and (BWSM is Middle) and (BW is Middle) then (EP is Top) (1)
6	If (Cage is Top) and (ASM is Middle) and (BWSM is Middle) and (BW is Middle) then (EP is Top) (1)
7	If (Cage is Lower) and (ASM is Top) and (BWSM is Middle) and (BW is Middle) then (EP is Top) (1)
8	If (Cage is Top) and (ASM is Middle) and (BWSM is Top) and (BW is Top) then (EP is Top) (1)
9	If (Cage is Top) and (ASM is Middle) and (BWSM is Middle) and (BW is Middle) then (EP is Top) (1)
10	If (Cage is Lower) and (ASM is Top) and (BWSM is Lower) and (BW is Middle) then (EP is Top) (1)
11	If (Cage is Lower) and (ASM is Middle) and (BWSM is Middle) and (BW is Middle) then (EP is Top) (1)
12	If (Cage is Top) and (ASM is Top) and (BWSM is Middle) and (BW is Middle) then (EP is Top) (1)
13	If (Cage is Top) and (ASM is Middle) and (BWSM is Top) and (BW is Top) then (EP is Top) (1)
14	If (Cage is Top) and (ASM is Middle) and (BWSM is Middle) and (BW is Top) then (EP is Top) (1)
15	If (Cage is Lower) and (ASM is Middle) and (BWSM is Middle) and (BW is Middle) then (EP is Middle) (1)
16	If (Cage is Top) and (ASM is Middle) and (BWSM is Top) and (BW is Middle) then (EP is Top) (1)
17	If (Cage is Lower) and (ASM is Top) and (BWSM is Middle) and (BW is Top) then (EP is Middle) (1)

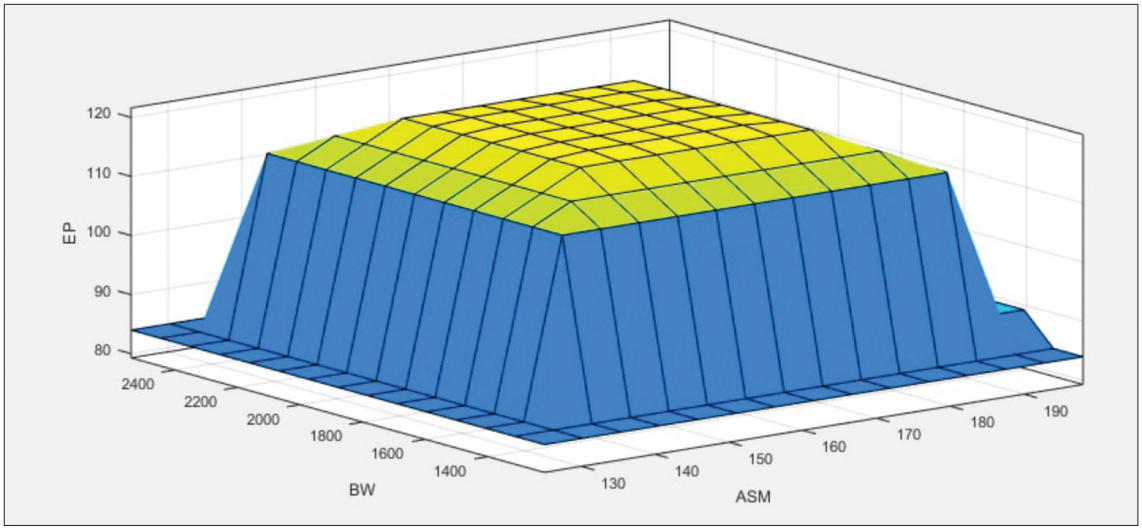

Fig 8. ASM-BW-EP correlation

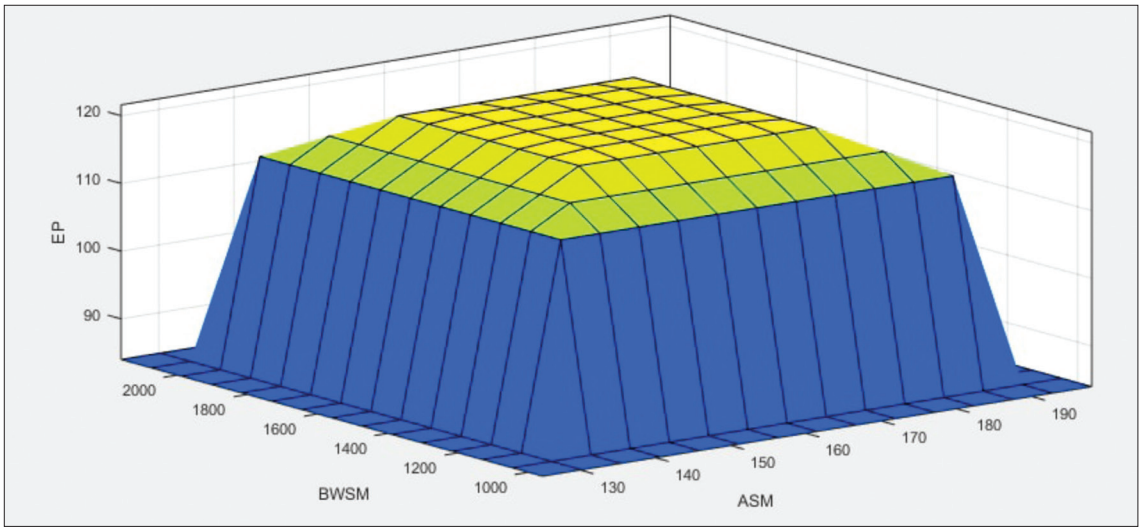

Fig 9. ASM-BWSM-EP correlation

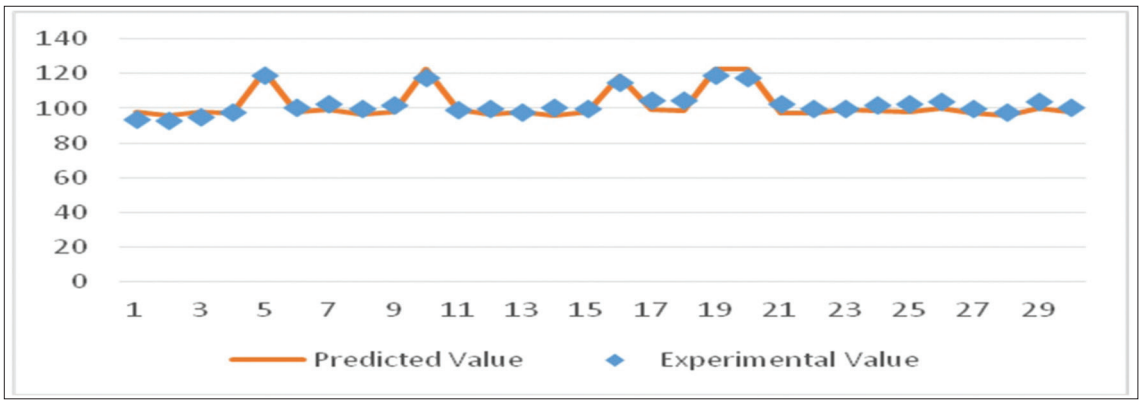

Fig 10. Comparisons of predicted results and observed results

There is surface viewer that shows a three dimensional (3-D) surface from two scaled input variables and one output variable of a Fuzzy inference system *(Fig. 8,9)*. The relationship among ASM, BW and EP is shown in *Fig. 8*

Table 4. *The analysis of results by using Fuzzy Logic Model*

Cage	ASM	BWSM	BW	Observed EP	Predicted EP	Classifications
299	155	1400	1700	94	97.96	TOP
3957	172	1540	1700	58	56.48	MIDDLE
687	171	1500	1720	95	97.96	TOP
883	163	1480	1760	98	97.25	TOP
342	167	1360	1600	101	96.57	TOP
687	171	1500	1720	73	72.68	MIDDLE
3192	154	1600	1860	87	88.54	TOP
170	168	1500	1820	99	98.97	TOP
291	146	1580	1800	100	96.58	TOP
650	164	1600	1520	67	68.00	MIDDLE
266	152	1440	1560	95	96.07	TOP
661	157	1720	1940	97	97.97	TOP

ASM: age at sexual maturity (days), BWSM: body weight at sexual maturity (g), BW: body weight at mature age (g), EP: egg production

while *Fig. 9* shows the relationship between ASM, BSWM and EP. It observed that the egg production as an output variable was effective at both values.

Fig. 10 compares the actual data with the data obtained from the Fuzzy logic model. Similarities between both data were investigated by the coefficient of determination (R^2). It showed that there was 89% success rate of the prediction, so the Fuzzy logic model was efficient in predicting the egg production. So, based on this high value of R^2, it can be said that, Fuzzy logic model is a powerful tool for the prediction of egg production. On the other hand, fuzzy models can also be used as an alternative tool to predict the whole record production from early part of egg production as a selection criterion to improve the whole egg production.

Some of the results by Matlab are given in *Table 4*.

Table 4 shows that the probability of egg production at sexual maturity of 168 days of age, body weight at sexual maturity of 1500 g and body weight of 1820 g was 98.97%. The probability of egg production at sexual maturity of 157 days of age, body weight at sexual maturity of 1720 g and body weight of 1940 g was 97.97%. Moreover, the probability of egg production at sexual maturity of 172 days of age, body weight at sexual maturity of 1540 g and body weight of 1700 g was 56.48%. It was obvious that layers reached at sexual maturity later have lower egg production.

DISCUSSION

Artificial intelligence studies have sought scientists to be able to think of machines as human brains. However, it has been observed that mental abilities such as intuition, non-monotonic and reasonable reasoning cannot be resolved with the classical logic understanding; therefore different studies have been made. The Decision support system is the one of those alternatives [24,29].

Fuzzy logic uses to achieve optimum solution of problems in engineering and agriculture. Similar to the findings of this study, researchers reported that Fuzzy logic could be used successfully in poultry. Fuzzy logic is a parallel structure to human thinking and in this parallelism the ability to recognize and determine systems has led to its rapid application areas. Poultry production is a well-developed and important industry in worldwide. The production of poultry meat and egg has been increased globally in years. However, there are still limited studies on Fuzzy logic systems in poultry, but no studies on egg production. Peruzzi et al.[30] used egg's physical characteristics such as egg weight, egg sphericity, eggshell thickness, and yolk per albumen ratio in Fuzzy logic modeling to estimate hatchability. Mehri [31] studied the factors affected hatchability in laying hens from 29 to 56 weeks of age. They used four inputs with 28 data lines including egg weight, egg sphericity, eggshell thickness and yolk/albumin ratio.

Artificial intelligence and its subfields such as machine learning are applied widespreadly in any scientific area in nowadays for any purposes such as pattern recognition or image classification. Fuzzy logic is an equal or sometimes faster method than the other applications for image classification since it is based on existed rules [32].

In animal science and veterinary medicine, there are limited numbers of studies about fuzzy logic. Some of those studies are related with diseases prediction, assessment of risks, designing equipments and controllers [33,34]. Artificial intelligence approaches like Fuzzy Logic can be initiated the expert's vision since they are included the information of experts. Because of this feature, fuzzy logic can be an alternative method for animal science related studies [24].

In conclusion, egg production is a complex process consisting of relationships among hormones, biochemical reactions and animal physiology with low heritability which means it is hard to predict and improve by individual selection. Low heritability also means that the trait is highly controlled by environmental factors more than the genotype. A Fuzzy logic model can be used for optimization of those effects. The method also shows its usefulness for predicting egg production in early weeks of the production period. Developing a Fuzzy logic model for prediction of egg production in earlier ages in hens has also an economic importance. It may give a chance to farmer to remove hens having low productivity at the beginning of the production period. Therefore, it can also be helpful for breeders in establishing new selection methods for traits having low heritability degree like egg production. Finally, it's thought that the Fuzzy logic has potential for fast, sensitive and realistic prediction values.

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Research Article

Improvement in Pellet Production Parameters and Pellet Quality Characteristics with Sepiolite Supplementation in Dairy Cattle Concentrate [1]

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Abstract

The aim of this study was conducted to determine the effects of sepiolite usage on pellet production parameters and pellet quality characteristics for dairy cattle concentrate feed under regular industrial conditions. In the experiment, 14 mt pellet feeds for control and two treatment groups with 7 batch each were produced in a commercial feed factory. Each batch was 2 mt. Control group feed produced contained 87.37% dry matter, 18.06% crude protein, 6.95% crude fibre and 5.34% ether extract. For the treatment groups, 1% and 1.5% sepiolite (Exal T) were used as top dressed in the mixer. Pelleting disc having 6.5 mm hole diameter was used in the factory. Energy consumption during pellet manufacturing was decreased with sepiolite supplementation ($P<0.001$) and pellet durability index (PDI) was enhanced with 1.5% sepiolite ($P<0.05$). These findings demonstrated that 1.5% sepiolite in dairy cattle concentrates as top dressed may be used as a binder to improve pellet quality and to reduce energy consumption during pellet production in the feed mill.

Keywords: *Energy consumption, Dairy cattle concentrate, Pellet durability, Sepiolite*

Süt Sığırı Konsantre Yemlerinde Sepiyolit Kullanımı İle Pelet Üretim Parametreleri ve Pelet Kalite Özelliklerinin İyileştirilmesi

Öz

Bu çalışmanın amacı, endüstriyel koşullar altında süt sığırı konsantre yemlerinde sepiyolit kullanımının pelet üretim parametreleri ve pelet kalite özelliklerine olan etkisini belirlemektir. Denemede, kontrol ve 2 deneme grubunun her biri için yedişer parti olmak üzere toplam 14 tonluk pelet yem ticari bir yem fabrikasında üretilmiştir. Her parti 2 tondur. Üretilen kontrol grubu yemi %87.37 kuru madde, %18.06 ham protein, %6.95 ham selüloz ve %5.34 eter ekstraktı içermektedir. Deneme grupları yemine, %1 ve %1.5 sepiyolit (Exal T) karıştırıcıda dökme olarak (top dressed) ilave edilmiştir. Fabrikada 6.5 mm delik çaplı peletleme diski kullanılmıştır. Pelet üretimi süresince sepiyolit ilavesiyle enerji tüketimi azalmış ($P<0.001$) ve pelet dayanıklılık indeksi (PDI) ise %1.5 sepiyolit ilavesiyle artmıştır ($P<0.05$). Sonuçlar, dökme olarak %1.5 sepiolit ilavesinin, süt sığırı konsantre yemlerinde pelet kalitesini iyileştirmek ve yem fabrikasında pelet üretimi sırasında enerji tüketimini azaltmak için bir bağlayıcı olarak kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Enerji tüketimi, Pelet dayanıklılığı, Sepiyolit, Süt sığırı konsantre yemi*

INTRODUCTION

One of the most common feed processing techniques is pelleting of feeds. The main target of pelleting is to agglomerate smaller feed particles by the use of heat, mechanical pressure and moisture [1]. The history of the pellet is based on the nineteenth century [2]. However, it was reported that pelletization dates back to the time of

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Napoleon where army horses were fed a type of feed that was agglomerated by an expeller [3]. Today, pellet feeding is widely used due to the fact that it improves animal performance and feed conversion ratio compared with feeding with meal form [4]. Some researchers [5,6] reported that broilers fed pellet feed performed greater feed intake, better feed conversion ratio and thus greater weight gain than that of mash feed. In pig, pelleted feed compared with mash, resulted in a decreased feed intake of 2%, increased weight gain of about 7% and improved feed utilisation of around 8% [3]. In ruminant, feed processing affects the rate of transit through the gastrointestinal tract and rate of passage of feed components [7]. When animal feed is pelleted, contribution to performance is important due to reducing selective feeding, decreasing feed wastage, reducing time and energy consumption, destructing of pathogenic organisms and improving palatability [4].

During this pellet formation, some factors affecting pellet quality are ingredients and nutrient composition of concentrate diet and its properties, process technology as well as certain pellet binders. In particular, components such as starch, protein, fat, fiber and sugar are important factors determining the hardness and durability of the feed. Starch has a function as an adhesive or binding agent in feed production [8]. After gelatinization, when starch includes in diets, it has affirmative effect on the pellet hardness and durability [9]. Sugars that in the form of molasses may positively effect of that as a binder [8]. Protein can act as a binding agent and during denaturation processing may positively affect the hardness and durability of the feed pellets. However, it is reported that the addition of fats in the diets has a negative effect on pellet hardness and durability. The addition of fats to the diets that serves as a lubricant between the mash and die occurs a low pressure during pressing [3]. Moreover, pellet binders such as bentonite, carboxymethylcellulose and lignosulphonates can be used to improve pellet hardness and durability by reducing the gaps to make the pellets more compact and durable [8]. The pellet binder property to improve durability especially in high fat diets, sepiolite is a good technological additive due to its properties such as reducing energy cost in pellet, increasing pellet durability, reducing the amount of dusting during the production and transport of feeds [10]. Sepiolite is a hydrated magnesium silicate ($Si_{12}.Mg_8.O_{36}.(H_2O)_{4.8}H_2O$) which belong to phyllosilicates. Sepiolite (E562) is currently authorised as binders, anti-caking agents and coagulants for all animal species according to Regulation (EC) No 1831/2003 of the European Union [11]. It is also stated that the use of sepiolite at different levels as a feed additive also makes positive contribution to the performance and quality parameters of animals [12-14]. Burçak and Yalçın [15] showed that 2% sepiolite supplementation added to the rations of lamb increased the IgG level in the blood serum and thus enhanced immunity. Yalçın et al.[16] reported that sepiolite addition at 1% to dairy cattle feed and fattening cattle feed as top-dressed decreased energy consumption during pelleting and enhanced pellet durability index (PDI). Sepiolite usage at 1% in layer diets significantly reduced energy consumption at the level of 16.14% and increased PDI [17]. However, there are limited studies with supplementation of different levels of sepiolite and other clay minerals in diets about pellet quality during the pelleting processes. Therefore, the purpose of this experiment was to evaluate the effects of different levels of sepiolite usage to concentrate feeds of dairy cattle on some pellet production parameters and pellet quality characteristics.

MATERIAL and METHODS

Commercial concentrate feed for dairy cattle was used in this experiment. Manufacturing pellet feeds were produced in a commercial feed factory. Commercial dairy cattle concentrate feed contained mainly wheat bran (200 kg/t), sunflower seed meal (180 kg/t), rice bran (170 kg/t), canola seed meal (120 kg/t), broken rice (110 kg/t), corn gluten feed (70 kg/t), corn embryo meal (50 kg/t), condensed molasses solubles (40 kg/t) and corn (20 kg/t). Concentrate feeds for one control and two treatment groups were manufactured in this study. Sepiolite (Exal T, Tolsa Turkey Company-Polatlı, Türkiye) was added to the treatment concentrate feeds at 1% and 1.5% as top dressed to the mixer. Pellet concentrate feeds were produced with 7 batch (each batch was 2 t) and pellet diameter was 6.5 mm. Water was not used in the pellet manufacturing processes. Mixer capacity of the feed mill was 2 t and hole length of pellet disc was 90 mm.

In this commercial factory the data of steam temperature (°C), electric current (ampere) and pellet production time (min/10 t) were measured. Energy consumption of pelleting machine (electric power in kilowatts, kW) was calculated as multiplying electric current (in ampere) with voltage supply (volts) and then dividing by 1000. Voltage supply of feed pellet machine was 380 volts.

Seven samples from the mixer, after the conditioner and pelleted feed after cooling were collected from each group dairy cattle feed. Moisture content was analysed in all of the samples collected. Crude protein, crude fibre, ether extract, ash and starch analysis of control pelleted feeds were determined [18]. Metabolizable energy level was calculated according to the formula proposed by TSI [19]. Mineralogical composition was analysed by D8 Advance Diffractometer AXS (Bruker, Germany) and chemical composition was determined by Atomic Absorption Spectrometer (Varian Atomic Absorption Spectrometer AA240, Varian Inc., The Netherlands). PDI values of pelleted concentrate feeds were measured with a Pfost Box Equipment using the sieve having the hole diameter of 4.75 mm [20]. Quadruplicate measurements were done with each sample.

Data were analysed using the ANOVA procedure of the SPSS 23.0 (SPSS Inc., Chicago, IL, USA). The experimental

unit was 7. The normality of data distribution was checked using the Kolmogorov-Smirnov test. The effects of graded levels of dietary sepiolite supplementation on different variables were analyzed using polynomial contrasts. Significant differences among groups were tested by Tukey test. Level of significance was taken as P<0.05 [21].

RESULTS

Sepiolite used in this trial was comprised of 73% of clay minerals (of which 65% was sepiolite), 21% dolomite and 6% calcite. Heavy metal analysis of sepiolite showed that it contained 1.6 mg/kg As, <1 mg/kg Cd, 1.72 mg/kg Pb and 0.02 mg/kg Hg. Chemical composition of sepiolite was given in *Table 1* and the nutrient composition of basal dairy cattle concentrate feed was shown in *Table 2*. Crude protein was 18.06% and metabolizable energy was 2.681 kcal/kg in concentrate feed. Production parameters during pelleting of dairy cattle concentrate feed were given in *Table 3*. Addition of 1% and 1.5% sepiolite to the mixer as top dressed significantly (P<0.001) decreased energy consumption during pelleting. There was also linear reduction in energy consumption with increasing sepiolite dose (P<0.001). Moisture content and PDI value of feeds during pellet manufacturing were shown in *Table 4*. PDI values of concentrate pellets were increased with 1.5% sepiolite supplementation (P=0.020). There was also linear increasing in PDI value with increasing sepiolite dose (P=0.006).

Table 1. *Chemical composition of sepiolite*

Chemical Composition	Content, %
SiO_2	42
Al_2O_3	1.1
MgO	20
CaO	13.90
Fe_2O_3	0.50
Na_2O	0.32
K_2O	0.44
Mn_2O_3	0.01

Table 2. *Nutrient composition of basal dairy concentrate feed*

Nutrient Composition	Content
Dry matter, %	87.37
Crude protein, %	18.06
Crude fibre, %	6.95
Ether extract, %	5.34
Starch, %	16.40
Sugar, %	7.00
Ash, %	6.75
ADF, %	13.97
NDF, %	29.51
Metabolizable Energy, kcal/kg	2.681

DISCUSSION

Sepiolite clay used in this study had high content of sepiolite (65%). Heavy metal analysis of sepiolite also suggested no contamination of heavy metals according to the Turkish Communique of 2014/11 [22] that was harmonized by the Directive of 2002/32/EC [23]. Many factors in feed pelleting processes may affect pellet quality, such as feed moisture, feed nutritional composition, ingredient particle size, conditioning temperature and time, compression rate of pellet die, cooling process, etc.[24-29].

In the present study, the conditions of amount of production, mixer capacity, disc hole diameter and disc hole length were same in the manufacturing of pellet concentrate feeds for control group and treatment groups. Sepiolite is a feed additive used as a binder and anticaking agent up to 2% in all feeds for all animal species [11]. Sepiolite supplementation at the rate of 1-1.5% to the mixer as top dressed significantly (P<0.001) reduced energy consumption at the level of 13.81-18.45% with increasing steam temperature at the level of 24.39-37.73%. Similar results were obtained with the layer concentrate pellets using 1% sepiolite [17]. Yalçın et al.[17] reported that 1% sepiolite supplementation in layer diet reduced energy consumption at 16.14% and increased steam temperature at 36.74%. Yalçın et al.[16] also indicated that energy consumption during pelleting for dairy cattle feed and fattening cattle feed is 9.63% and 5.27% lower (P<0.01) in group feed supplemented sepiolite than control group. In

Table 3. *Production parameters during pelleting of dairy cattle concentrate feed*

Production Parameters	Sepiolite			SEM*	Significance	
	0%	1%	1.5%		Combined	Linear
Steam temperature, °C	31.86^{c}	39.63^{b}	43.88^{a}	1.330	<0.001	<0.001
Electric current, Ampere	126.52^{a}	109.06^{b}	103.18^{c}	2.656	<0.001	<0.001
Energy consumption**, kW	48.08^{a}	41.44^{b}	39.21^{c}	1.009	<0.001	<0.001
Production time, min for 14 t	79	78	78			

** Pooled standard error of mean; ** Energy consumption was calculated using 380 of voltage in pelleting machine*
^{a,b,c} means a row followed by different letters differ significantly (P<0.01)

Table 4. *Moisture content and PDI value of feeds during pellet manufacturing*

Feed Characteristics	Sepiolite			SEM*	Significance	
	0%	1%	1.5%		Combined	Linear
Moisture in the mixer, %	13.12	13.08	13.07	0.010	0.132	0.094
Moisture after conditioner, %	14.18^{b}	14.21^{b}	14.54^{a}	0.048	<0.001	<0.001
Moisture after pellet cooling, %	13.00	13.06	13.16	0.031	0.106	0.038
PDI, %	95.80^{b}	96.12^{ab}	96.43^{a}	0.096	0.020	0.006

** Pooled standard error of mean; ^{a,b} Means a row followed by different letters differ significantly (P<0.01)*

the present study, similar production time was obtained for the control group and treatment groups. Similarly, sepiolite supplementation to the layer diets [17] and dairy cattle concentrate [16] did not affect pellet manufacturing time. However, in some studies sepiolite supplementation reduces the pellet production time by 8.47% in fattening cattle feed [16] and 10.60% in broiler starter diets [30]. These differences among the studies may be due to the ingredients and chemical composition of diets, disc hole diameter, disc hole length and steam temperature.

Moisture content of dairy concentrate feeds in mixer was 13.12, 13.08 and 13.07% for control and treatment groups, respectively. Sepiolite supplementation at the level of 1.5% increased moisture content of concentrate feed after conditioner (P<0.001). There was a linear relation (P<0.001) with moisture content and dose of sepiolite. This increase may be due to the increase in steam temperature. Higher moisture content achieved after conditioning had lubricating effect and decreased the specific energy consumption [23]. No significant effect was seen in moisture content of concentrate pellet feeds among groups. However, there was a linear relation (P<0.05) with moisture content and dose of sepiolite.

Pellet physical characteristics, which are usually used for determination of pellet quality, are hardness and durability. In this study, adding 1.5% sepiolite improved pellet durability when compared to the control group (P<0.05). A linear effect of PDI with sepiolite dosage was seen (P<0.05) in *Table 4*. Sepiolite is a binder used in feed technology to improve physical pellet quality. In line with our results, Angulo et al.[10] reported that sepiolite improved the performance of pelleted diets. Similarly, since sepiolite acts as a filler and thereby decreases porosity in pelleted feed, it improved pellet durability [16,17]. However, Pappas et al.[31] indicated that palygorskite, a clay with similar physical properties to sepiolite [32] did not statistically affect pellet durability. When sepiolite increased the pelleting temperature, increasing durability and hardness of the pellets could cause more friction in the die. During this pellet formation, the feed can be exposed to high friction temperatures. Actually, when too much heat is applied, temperature of pellets is higher on account of frictional heat and of this effect will be impaired pellet quality [33]. Cutlip et al.[34] reported that high temperature conditioning increased PDI and decreased total fine particles. Similarly, Abdollahi et al.[35] indicated that if conditioning temperature increases from 60 to 90°C, pellet hardness and durability of broiler diets was improved. However, Vukmirovic et al.[36] stated that increasing the moisture content of diets to 15.97% and 21.88% resulted in decreasing pellet durability during pelleting process.

In present study, crude protein and starch content of diet was 18.06% and 16.40, respectively *(Table 2)*. Diet ingredients also strongly effect pellet durability. In our study, with together sepiolite usage, increasing protein and starch content of diet might have been caused an increase in pellet quality. Similarly, Briggs et al.[37] found an affirmative relationship between pellet durability and increased protein content of broiler diets. Wood [9] also reported that binding properties were based on the denaturation of the protein rather than gelatinisation of the starch during steam conditioning. However, Zimonja and Svihus [38] reported that pellet durability was lower (P<0.05) for gelatinised starch containing diets than non-starch diets.

In conclusion, 1 and 1.5% sepiolite supplementation decreased energy consumption during pellet manufacturing and 1.5% sepiolite supplementation increased pellet durability index. Therefore, from the results obtained in this study it is concluded that 1.5% sepiolite supplementation to dairy cattle concentrate feeds can be useful to improve pellet durability and to reduce energy consumption in the feed mill.

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Short Communication

Analysis of Pilus Biogenesis Genes in Bacteria Expressing Type IV Pili

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Abstract

The structural and sequences similarities of type-IV pili proteins were analysed in *Pseudomonas aeruginosa, Vibrio cholerae, Neisseria meningitidis* and *Neisseria gonorrhoeae.* Pathogenic *Neisseria* species possess pili-genes that code structural and assembly proteins of type-IV pili. A collection of assembly proteins reveal homology amongst *P. aeruginosa* and *Neisseria*. While in *V. cholerae,* these proteins ensure functional counterparts. The pilus retraction and assembly ATPases, PilB/PilF and PilT, PilU are homologous in Neisseria and *P. aeruginosa,* whereas *V. cholerae* holds only one ATPase called TcpT. In this work only type-IV pilus machinery of *P. aeruginosa* shows high likeness with pathogenic *Neisseria*.

Keywords: *Type IV pilus genes, Sequence similarity, Pilus machinery, Schematic overview*

Tip IV Pili Eksprese Eden Bakterilerde Pilus Biogenez Genlerinin Analizi

Öz

Pseudomonas aeruginosa, Vibrio cholerae, Neisseria meningitidis ve *Neisseria gonorrhoeae* bakterilerinde Tip IV pili proteinlerinin yapısal ve sekans benzerlikleri incelendi. Patojenik *Neisseria* türleri tip IV pilinin yapısal ve montaj proteinlerini kodlayan pili genlerine sahiptir. Bir grup montaj proteinleri *P. aeruginosa* ve *Neisseria* arasında homoloji göstermektedir. *V. cholerae*'da ise bu proteinler fonksiyonel karşılıkları sağlamaktadır. Pilus retraksiyon ve montaj ATPaz'ları, PilB/PilF ve PilT, PilU *Neisseria* ve *P. aeruginosa*'da homolog olup *V. cholera* sadece TcpT isimli bir ATPaz'a sahiptir. Bu çalışmada sadece *P. aeruginosa*'nın tip-IV pilus organeli patojenik *Neisseria* ile yüksek benzerlik göstermiştir.

Anahtar sözcükler: *Tip IV pilus genleri, Sekans benzerliği, Pilus organeli, Şematik bakış*

INTRODUCTION

Bacteria express hair-like appendages on their surface called pili or fimbriae, which facilitate the interaction between bacterial and the host cell surfaces [1]. One the most essential pili is Type IV, involved in adhesion to host cells, twitching and gliding motility, DNA uptake, formation of biofilm and microcolonies, signals transduction and escaping from the immune response [2]. Type IV pili has type IVa and type IVb subtypes differentiated on the basis of structure and function of their assembly system. Type IVa pili are expressed in *Neisseria*, *Pseudomonas* and *Dichelobacter,* found in plants, animals and humans. Type IVb pili are expressed in bacteria such as *Salmonella enterica serovar Typhi, Enterotoxic Escherichia coli*, *V. cholerae* and *P. aeruginosa* [2]. Type IV pilus mediated migration of bacteria make a polysaccharide-like Psl-fibres and with the loss of Psl-fibre a reduction of biofilm biomass occur [3,4].

Neisserial Type IV pili play an important role in the pathogenesis of disease. The PilD, PilF, PilM, PilN, PilO, PilP are the major proteins required for the functional assembly of type IV pili in this species [5]. PilV and PilX are minor pilin protein required for *Neisseria* type IV assembly and are involved in adherence of type IV pili to human cells. PilV also mediates PilC exposure and modification within the type IV pili [6]. The structure of *Neisseria* type IV pili consists of approximately 500-2000 major PilE subunits [7].

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Toxin-coregulated pilin (TCP) is the major subunit of *V. cholerae* biogenesis [8]. The *tcpA* gene is located in the cluster of genes required for the assembly and regulation of type IV pilus biogenesis. In this cluster of genes, downstream of the *tcpA* gene, are *tcpB, tcpQ, tcpC, tcpR, tcpD, tcpS, tcpT, tcpE, tcpF* and *tcpJ,* while the genes located upstream of *tcpA* are *tcpH, tcpP, tcpI* [9]. Among all the *TcpJ* are involved in TcpA processing, while *tcpC* gene plays an important role in the pilus biogenesis and involved in resistance to complement [10].

The Typ4 pili systems in *P. aeruginosa* include additional, non-core minor pilins [2] called FimU, PilV, PilW, and PilX, encoded in an operon with the large putative adhesin, PilY1, and the non-core minor pilin, is PilE [11]. PilVWX and PilY1 depend on one another for incorporation into pili and they form a sub-complex required for PilE to be recovered in the pilus fraction [12]. *P. aeruginosa* has only one non-core minor pilin (PilE), while *Neisseria meningitidis* has three, and are used for direct binding to DNA [13].

Few studies revealed the similarities among genes in different species. For instance Helaine et al.[14] reported that the PilXNm protein in *N. gonorrhoeae* is possible orthologous to *P. aeruginosa* PilE, based on same location at their respective minor pilin operons and their sequence identity. Similarly PilVNm, of *Neisserial* genome, is also the orthologue of *P. aeruginosa*-PilE indicating that *N. meningitidis* possibly encodes two PilE equivalents. A cluster of genes in *V. cholerae* shows homology to the pilus encoding genes of *P. aeuroginosa* and *Neisseria* is the *pil* genes. The nomenclature of these genes is based on *P. aeruginosa* related genes, as *P. aeruginosa* was the first identified organism with type IV pili [15]. We, in the present study have analysed the similarity and homology of proteins involved in type IV pili assembly and functions in between *Neisseria meningitis*, *N. gonorrhoeae*, *P. aeruginosa* and *V. cholerae*. Moreover we have compared the type IV pilus machinery between bacterial species and summarized the function of type IV pili in biofilm formation.

MATERIAL and METHODS

Bacterial Strains and Species

The type IV pilus biogenesis machinery of bacterial strains used in this study is described in *Table 1* and *Table 2*. KEGG (Kyoto Encyclopedia of Genes and Genome) database was used for the collection of protein sequences.

Sequence Alignment and Structure and Function of Proteins

KEGG database (koyoto encyclopedia of genes and genomes), ClustalX, ClustalW and Clustal Omega were used for multiple sequence alignment of proteins. All the protein sequences of type IV pilus biogenesis were aligned in pairwise between the strains and different species to observe similarity between proteins. String database is used for the function and structure of protein and their direct or indirect interactions.

RESULTS

Structural Similarities Between Type IV Pili Biogenesis Pil Proteins

Eleven pilus biogenesis proteins were analysed for similarity among *P. aeruginosa*, *V. cholera* and *N. meningitides*. The analysed proteins are: PilQ-an outer membrane pore, PilD-a pre-pilin peptidase, PilP-secretin dynamic associated protein, PilM, PilN, PilO-the pilus assembly proteins, PilT-pilus retraction ATPase, PilU-a pilus ATPase, PilC-a platform protein, PilF-a pilotin, and PilB-a pilus ATPase. The proteins are described below and the similarities demonstrated by schematic figures.

As shown in *Table 1*, the analysed sequence comparison of Pil genes within the Neisseria strain, the first column contains the strains name we have compared, and in upper first row Pil gene name has been mentioned. For this analysis we keeps Nmc *(N. meningitidis* FAM18) as a standard and compared with Pil gene of other 16 strain of Neisseria, After analysis the sequence similarity by Clustal X, W, and Omega we observed almost 100% sequence similarity within Pil genes (Nma, Nmp, Nmh, Nmd, Nmm, Nms, Nmq, Nmz) of these strains, Except PilD gene which was not present in some of the Neisseria strain of my study. The PilC gene (Nma, Nme, Nmn, Nmt, Nmw, Nmz, Ngo) between Nmc (Standard) shows only 18% sequence similarity. While PilE gene between Nmc, Nmt and Nmi shows 24% and 26% similarity, *Table 1*. The type IV pili protein in *P. aeruginosa* which were compared are shown in *Fig. 1A,* which shows the overview of the genomic map of the Pil and their associated genes which are responsible for type IV pili in *P. aeruginosa*.

In *Table 1*, we have mentioned the observed sequence similarity between Neisseria standard strain Nmc *(N. meningitidis* FAM18) and *V. cholerae* strain. The gene name of *V. cholerae* strains has been listed in first column of the table while the upper first column contain Pil genes name. After analysing the sequence similarity it has been investigated that there is no significant similarity between Nmc and *V. cholerae* strains. The highest similarity 62% has been shown in PilT gene between Nmc and *V. cholerae* strains. While a PilD gene does not exist in *V. cholerae* strains of Vcj and Vcl, *Table 1*. The position of type IV pili proteins and the genomic map of type IV pili and their associated genes in *V. cholerae* M66-2 are clear in *Fig. 1B* and *Fig. 1C*.

Table 2 contain the comparison of the Sequence similarities within the standard strain Nmc *(N. meningitidis* FAM18) and *Pseudmonas* strains. In the first column the Pseudomonas strains name while in the first row the Pil genes name has

Table 1. *The percentage of protein sequences similarities between Nmc (Neisseria meningitidis FAM18) key factors of type IV pilus machinery and TCP key factors of V. cholerae. (Nil) in the table below shows that these protein sequences are not found in strains of V. cholerae By using KEGG database*

Key Protein	*V. cholerae* Strain							
	Vch	Vce	Vcj	Vco	Vcr	Vcm	Vci	Vcl
TcpA PilE	Nil	24%	50%	24%	24%	24%	Nil	Nil
TcpC PilQ	Nil	17	Nil	17	17	17	Nil	Nil
TcpS PilP	Nil	15	Nil	15	15	15	Nil	Nil
TcpQ PilW	Nil	50	Nil	50	50	50	Nil	Nil
TcpJ PilD	28	28	Nil	28	28	28	28	Nil
TcpE PilG	Nil	17	Nil	17	17	17	Nil	Nil
TcpT PilF	Nil	26	Nil	26	26	26	Nil	Nil

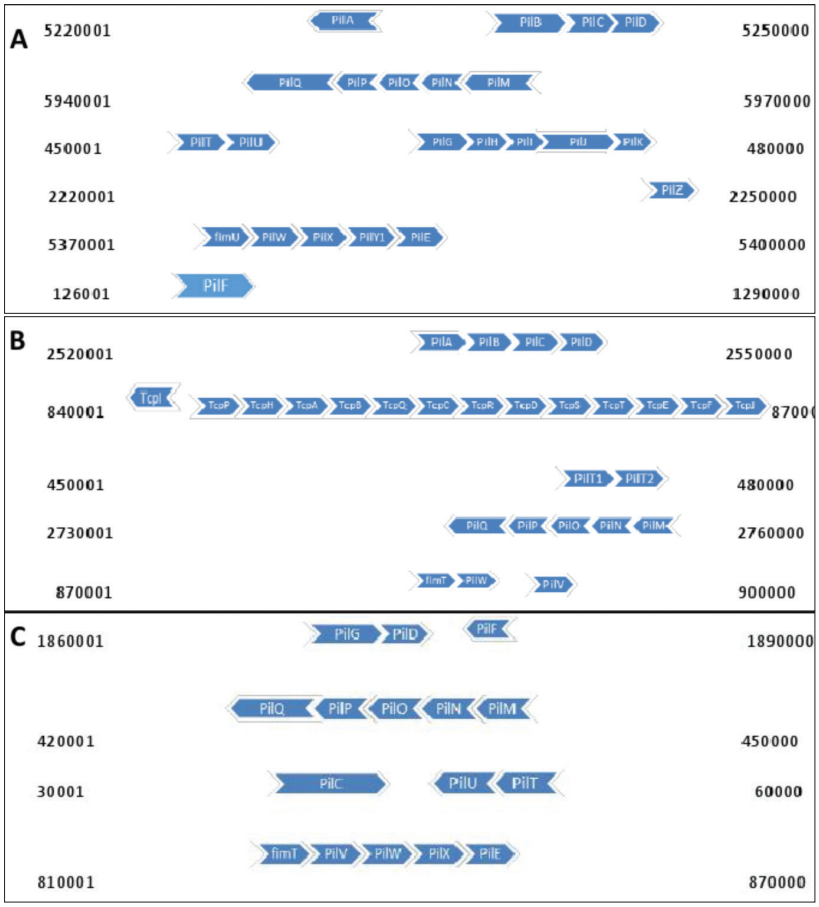

Fig 1. Schematic overview of type IV pili protein in *P. aeruginosa*. A- The figure shows the overview of the genomic map of the Pil and their associated genes which are responsible for type IV pili in *P. aeruginosa* UCBPP-PA14. B). Diagram has been originated from KEGGs database (koyoto encyclopaedia of gene and genome); B- Figure shows Schematic overview of type IV pili proteins in Vibrio cholera and the genomic map of type IV pili and their associated genes in *V. cholerae* M66-2. The position and location of Pil genes has been mentioned. Diagram has been originated from KEGGs database (koyoto encyclopaedia of gene and genome); C- The Schematic overview of type IV pili protein in Neisseria. The schematic overview of genomic map of the type IV pili responsible genes in Neisseria meningitidis (FAM18) are shown with respect to its position and location has been mentioned. Diagram has been originated from KEGGs database

been listed. From this analysis we observed that PilC gene has no similarity except 18% similarity has been shown within Pdk and Nmc, while PilC of Pae and Paf are pseudo gene. The highest sequence similarity is 66% and was observed in PilT and Nmc of *Pseudmonas* strains, while the rest Pil gene sequence similarity is in the range of 18% to 50%, *Table 2*.

Comparison of Pilus Machinery

The major subunits PilE of *Neisseria sp.* and PilA of *P. aeruginosa* have a conserved N-terminal region with a short signal peptide, which is not present in the major subunit TcpC of *V. cholerae* [3,10]. We have shown the protein sequences similarities between the key factors of type IV pilus machinery of Nmc *(Neisseria meningitidis* FAM18) and TCP (key factors of *Vibrio cholera)*. We have found no significant similarity *(Table 1)*. Similarly the protein sequences similarities between Pau *(P. aeruginosa* UCBPP-PA14) key factors of type IV pilus machinery and key factors of *V. cholerae* TCP shows no significant similarity *(Table 2)*.

DISCUSSION

Many bacterial species express type IV pili. In the current study type IV pili of *P. aeruginosa, V. cholerae, N. meningitidis and N. gonorrhoeae* were discussed. Type IV pili are flexible and long filaments attached to the bacterial cell. The type

Table 2. *This table shows the percentage of protein sequences similarities between Pau (P. aeruginosa UCBPP-PA14) key factors of type IV pilus machinery Pil proteins and key factors of V. cholerae TCP. (Nil) in the table below shows that these protein sequences are not found in these strains of V. cholerae By using KEGG database Bacterial strains are listed in Table 1*

Protein Names	Strains of *V. cholerae*							
	Vch	Vce	Vcj	Vco	Vcr	Vcm	Vci	Vcl
TcpA PilA	Nil	18%	30%	24%	24%	18%	Nil	Nil
TcpC PilQ	Nil	20	Nil	20	20	20	Nil	Nil
TcpS PilP	Nil	22	Nil	14	14	22	Nil	Nil
TcpQ PilF	Nil	50	Nil	50	50	50	Nil	Nil
TcpJ PilD	26	26	Nil	26	26	26	26	Nil
TcpE PilC	Nil	22	Nil	22	22	22	Nil	Nil
TcpT PilB	Nil	25	Nil	25	25	25	Nil	Nil

IV pilus system is located in the cell membranes/cell wall of bacteria. Many similarities between type IV pili and the type II secretion have been observed. Many proteins are involved in the biogenesis of type IV pili and many proteins play different roles in type IV pilus machinery within different bacteria [3,5,15]. Type IV pilus biogenesis play a very important role in the pathogenicity of bacteria and it has been observed that type IV pilin and their assembly is the main target for vaccine and drugs. It is also noticed that type IV pili are able to evoke the immune system of the host. Type IV pili play many roles in causing the disease (i) attachment of bacteria to the surface of host cell. Attachment initiate micro colony formation and initiate host cell signal transduction. (ii) Type 4 pili expressing bacteria are able to move in a special type of movement called as twitching motility and twitching motility is the cause of rapid colonization on the new surfaces and it is also noticed that twitching motility lead to complex process of fruiting bodies and biofilm formation [3,5,15]. Many proteins have the same function in type IV pilus biogenesis in different bacterial species. It is important to identify similarities between structures of those proteins which are involved in the type 4 pili biogenesis, since it will help in finding novel treatment against severe bacterial diseases caused by type pili expressing bacteria.

For the investigation of type IV pili and its associated functions in *P. aeruginosa* primary model systems has been important. Interestingly, in a single strain of *P. aeruginosa* three types of type IV pili have been identified; Type IVa, Type IVb and Tad. The Type IVb pili have been reported in *P. aeruginosa* strains with Pathogenicity Island PAPI-1 or PkLC102, while Tad and Type IVa pili are common to all *P. aeruginosa* strains. In *P. aeruginosa,* the Type IVa pili and Type IVb pili systems depend on the PilD prepilin peptidase, while it has been observed that Tad has its own prepilin peptidase called FppA [15].

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Case Report

Thelazia callipaeda (Railliet and Henry, 1910) Case in a Dog: First Record in Turkey

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Abstract

A 2.5 years old male Golden Retriever breed dog with the itching, runny eyes, and continuous unease complaints was brought to a private veterinary medical centre in the Thrace region of Turkey, in September 2017. It was observed that there was a purulent conjunctivitis in the left eye and there was a mobile structure under the membrane nictitans after the examination. A drop of local anesthetic was dripped into the eye, and the mobile structure was removed with the help of a forceps. This removed structure was taken into the solution of 70% alcohol on suspicion of parasite. Both the extracted material and the blood samples were sent to the Department of Internal Medicine, Afyon Kocatepe University, Faculty of Veterinary Medicine for evaluation and it was evaluated with parasitologist. The parasite was cleared by taken into a 0.9% physiological saline and kept in the lactophenol for two days for transparency. Then, the transparent parasite was determined as *Thelazia callipaeda* after microscopic examination. The ocular form of thelaziasis caused by *T. callipaeda* in a dog has been reported for the first time in Turkey with this case report. By this report, first case of ocular thelaziasis reported seen a dog in Turkey and it was aimed to point out that this parasite can lead to significant eye problems in animals.

Keywords: *Dog, Ocular thelaziasis, Thelazia callipaeda, Golden Retriever*

Bir Köpekte *Thelazia callipaeda* (Railliet ve Henry, 1910) Olgusu: Türkiye'de İlk Kayıt

Öz

Türkiye'nin Trakya Bölgesi'nde bir özel veteriner tıp merkezine 2017 yılı Eylül ayında gözde kaşıntı, akıntı ve sürekli huzursuzluk şikâyetleri ile 2.5 yaşında erkek Golden Retriever ırkı bir köpek getirilmiştir. Yapılan muayene sonrasında sadece sol gözde purulent bir konjunktivitisin olduğu ve membrana nictitansın altında hareketli bir yapının olduğu gözlenmiştir. Göze bir damla lokal anestezik damlatılarak hareketli yapı gözden bir pens yardımıyla çıkarılmıştır. Çıkarılan bu yapı parazit olması şüphesi ile %70'lik alkol içine alınmıştır. Alınan numune ve kan numuneleri değerlendirilmek üzere Afyon Kocatepe Üniversitesi Veteriner Fakültesi İç Hastalıkları Anabilim Dalı'na gönderilmiş ve parazitolog eşliğinde değerlendirilmiştir. Parazit %0.9'luk fizyolojik tuzlu suya alınarak temizlenmiş ve şeffaflaşması için iki gün laktofenolde bekletilmiştir. Şeffaflaştırılan parazitin mikroskobik muayenesi sonrasında *Thelazia callipaeda* olduğu belirlenmiştir. *T. callipaeda*'nın köpekte oluşturduğu oküler thelaziasis vakası Türkiye'de ilk olarak bu olgu sunumu ile bildirilmektedir. Bu rapor ile Türkiye'de bir köpekte rastlanılan ilk oküler thelaziasis vakası bildirilmiş ve parazitin hayvanlarda önemli göz problemlerine sebep olabileceğine dikkat çekmek istenmiştir.

Anahtar sözcükler: *Köpek, Oküler thelaziazis, Thelazia callipaeda, Golden Retriever*

INTRODUCTION

Thelazia species, which are important nematode parasites that can be inoculated in the eye, are on the Spirurida order and in Thelaziidea family [1]. There are different types of *Thelazia* in domestic and wild animals such as in cattle: *Thelazia rhodesii, T. gulosa* (Syn. *T. alfortensis)*, *T. skrjabini*, in buffalo: *T. bubalis*, in sheep, cat, dog, human: *T. californiensis*, in camel: *T. leesei*, in horse: *T. lacrymalis*, in dog, rabbit, human: *T. callipaeda*, and in pig: *T. erschowi* [2]. It has been reported that these parasites could be found in the eyelids, membrana nictitans, and lacrimal channels, and sometimes in the nose and pharynx [3]. Eye involvement is high and infection induces from mild (conjunctivitis, epiphora and

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ocular discharge) to severe (keratitis and corneal ulcers) ocular manifestation in animals as well as humans [4].

Thelaziasis is known a zoonosis. Dog's thelaziasis caused mainly by *T. californiensis* and *T. callipaeda*. The two species are also important for humans [3]. However, *T. californiensis* has been reported also in sheep, deer, jackals and bears [5].

Adult worms look like creamy white threads [6]. Male adults are 4.5-13 mm in length and 0.25 to 0.85 mm in diameter, while the females are longer, from 6.2 to 17 mm and from 0.3 to 0.85 mm in diameter [7]. There are fine lines and protrusions in the cuticle of *Thelazia* species. There is a hexagonal mouth capsule and six festons on the inside edge of the mouth capsule. The presence of tail wings and lengths of spiculations in males varies according to species [6]. The vulva position is used as the diagnostic criterion. *T. callipaeda's* vulva is located at the front of the esophagus region vulva also has a short cover [6]. While other *Thelazia* species are vivipar, *T. callipaeda* is ovovivipar [3].

In the life cycle of *T. callipaeda*, flies in the Diptera order act as vectors. Although Shi et al.[8] have suggested that *Musca domestica* may seldom be a vector, Otranto et al.[9] reported that *Phortica* spp. in the family Drosophilidae. *(P. variaegata* and *P. okadai)* should be the important vector for *T. callipaeda*, and that *M. domestica* could not be vector by both the natural infections they encountered and the experimental studies they performed.

In the parasite life cycle, the first period of larvae in the lacrimal secretion of the infected eye (very short life span, 1-2 hours) is taken during the feeding of the vector flies. They pass through the intestines of the flies to their abdomen and stay there for 1-2 days. On the third day, in the female flies they are moving to the adipose tissue and in the male flies to the testicles. They change moult twice and become L_3 in 14-21 days. They reach the mouth organelles through the body cavity of the flies. The flies transfer larvae to the environment during feeding with lacrimal secretions around the eyes of the last hosts. There is no migration period in the last host. By changing moult twice (in 35 days) they become adults [10]. Prepatent time is 3-6 weeks. Infection is seen in seasons when flies are active, so it is depending on the season. Infections peak in two periods, beginning of the summer and the ending of the summer [11,12].

By this case report, it was aimed to point out that this parasite can lead to significant eye problems in animals.

CASE HISTORY

A 2.5 years old male Golden Retriever breed dog with the itching, runny eyes, and continuous unease complaints was brought to a private veterinary medical centre in the Thrace region of Turkey, in September 2017. It was observed that there was a purulent conjunctivitis in the left eye *(Fig. 1)* and there was a mobile structure under the membrane nictitans after the examination.

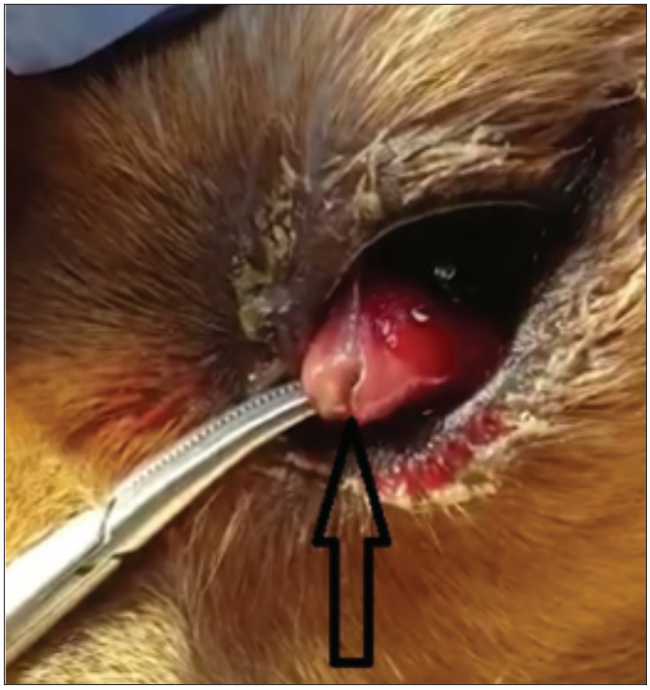
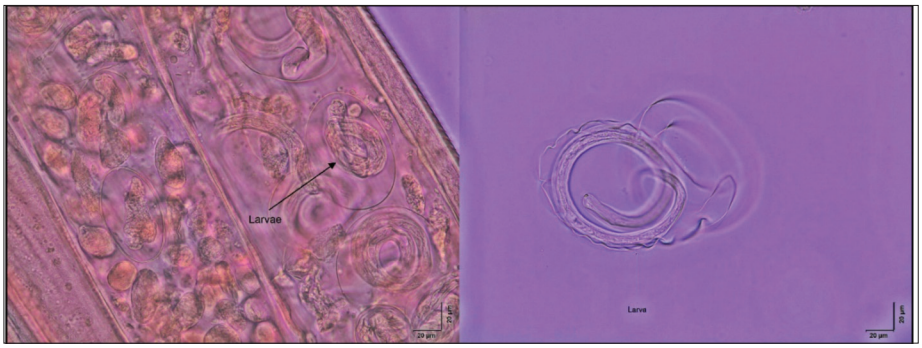


Fig 1. Membrane nictitans and parasitic appearance in infected dog

A drop of local anesthetic was dripped into the eye, and the mobile structure was removed with the help of a forceps. There was only one worm-parasite. This parasite was taken into the solution of 70% alcohol. Both the extracted material and the blood samples were sent to the Department of Internal Medicine, Afyon Kocatepe University, Faculty of Veterinary Medicine for evaluation and it was evaluated with parasitologist. The parasite was cleared by taken into a 0.9% physiological saline and kept in the lactophenol for two days for transparency. After this processes, morphological examinations were carried out on a light microscope (Olympus CX31) by parasitologist and pictures were taken (Olympus Imaging System Olympus LC30). The morphological features of the parasite were determined and the species was diagnosed by using the related literature [3,6,7,10]. There was no pathological result in the blood test. After cleaning and clarification, the front, and back of the parasite examined in the light microscope separately and measured. The size of the parasite was 11.34 mm, and the width was 0.3 mm. It was noted that it had a hexagonal buccal capsule on the front end. The large part (upper part) of the buccal capsule (upper part) was 0.04, and the narrow part (base) is 0.03 mm. A prominent esophageal structure, esophagus, and intestinal junction were observed. It has been observed that vulva is localized in the anterior part of the esophagus-intestine junction (OIJ). It is noted also that the parasitic cuticle is the transverse stripe (TCS). This transverse stripe structure was also measured as 0.02 mm *(Fig. 2)*.

Fig 2. Front end of the *T. callipaeda* (female) *(upper left)* (x10), Buccal capsule (BC) (X40), Esophagus (O), Transverse Cuticular stripe (TCS) *(upper right)* (x40), Vulva and Esophagus-Intestinal Junction (OIJ) *(lower left)* (x40), back end of the *T. callipaeda* (female) *(lower right)* (x4)

Fig 3. Larvae *(left)* in the distal uterus and external larvae of *T. callipaeda (right)* (x40)



In the parasite's uterus, grown larvae and larvae in the development phase were observed *(Fig. 3, left)*. The egg-shell in the sheath style was determined outside of the freed larvae *(Fig. 3, right)*.

Upon diagnosis of thelaziasis, the dog was treated with ivermectin (200 µg/kg, S.C. injection, Ivomec®, Merial, Turkey) two times with an interval of two weeks. It was stated that the eyes of two other dogs belonging to the animal owner have similar clinical symptoms and brought to veterinary center for treatment. It has also been mentioned that there was conjunctivitis in animal owner for about 6 months. For the possibility of zoonosis, the owner was informed and suggested to consult by a specialist physician.

DISCUSSION

Railliet and Henry first identified *T. callipaeda* in 1910 in the eyes of a dog in Pakistan [11]. Then Evans and Rennie reported in Myanmar, while Stuckey reported *T. callipaeda* in dogs in China. The medical records were followed by other countries such as Far East countries (Former Soviet Countries, India, China, Thailand, Taiwan, Indonesia, South Korea, and Japan) [3,13] and Europe [4,14,15]. The first human cases (4 cases) reported from Italy and France [16]; and *T. callipaeda* reported found in Italy in cat and foxes [17]. *T. callipaeda* is endemically present in poor, rural areas, and communities with low health and socio-economic standards as in this case.

Since information on *T. callipaeda* is rare and less known, the diagnosis of infections of this zoonotic species is omitted [7]. To the best of our knowledge, in Turkey, there is not a case report about this species. There are only prevalence studies on cattle, sheep, and horses related to *Thelazia* species in Turkey [18-22]. The prevalence in different regions of Turkey was reported as in cattle 5.5% and 22% [18,21] and in buffaloes 1.2% [19] where the causative agent was *T. rhodesii*. Doganay and Oge [22] have done studies on the prevalence of sheep.

Literature reported that adult female *T. callipaeda*`s length may be 6.2-17 mm and width may be 0.3-0.85 mm [7]. The parasite's measure has been determined 11.34 mm (lenght), 0.3 mm (width) in this case. These measurements coincide with the values given in the literature. It has been reported this species in both sexes has a serrated cuticle [16] and the buccal capsule has a hexagonal profile [3,7,16] 0.036 mm width and 0.030 mm depth on the front end [12]. It has

been observed in findings, this parasite cuticle's serrated and the buccal capsule has hexagonal profile *(Fig. 2, upper right)*. It was determined upper part of length 0.04 mm and 0.03 mm the base part of length of the buccal capsules of the parasite. This finding supports the information of the shape of the cuticle and the buccal capsule in the literature.

The most important diagnostic criteria for the identification of the adult female *T. callipaeda* is the position of the vulva. Vulva located anterior to the oesophagus region [3,6,10]. Localisation of the vulva at the anterior of the oesophagus-intestinal junction separates this species from other species [12,16]. It was observed that the parasite is located at the anterior part of the vulva and in front of the oesophagus-intestinal junction in this case *(Fig. 2, lower left)*. These findings have been corroborated *T. callipaeda* of this parasite.

According to Naem [3] *T. callipaeda* was ovoviviparous; Otranto ve Dutto [16] reported that mature female nematodes had embryonated eggs in the proximal uterus and larvae in the distal uterus. It has also been reported that a shell membrane around the first period larvae of the parasite is seen [12]. In the taken pictures, the parasite had larvae in the distal uterus and freed first-stage larvae outside a shell membrane were seen *(Fig. 3, right)*.

The researches on the vectors of *T. callipaeda* have been determined that the most important vector was *Phortica varieagata*. *T. callipaeda* infections are encountered in environments where vector flies are suitable for ecological living conditions. *Phortica* species, also known as fruit flies, are living in forest habitats with relative humidity of 50-70%. They prefer tree shades (Central Europe, Austria, the Czech Republic, Ukraine, Poland, Slovakia and Hungary) [15]. In two different studies conducted in Turkey, it was reported that these flies live in Zonguldak [23] and in Pehlivankoy/Kırklareli [24]. In this case report, the subject lives on the European side of Turkey at latitude 41.16° and longitude 27.79° (Tekirdag, Corlu district). This city is a neighbor to Kırklareli where previous researches on the vector have been made. In Corlu, the summers are warm and dry, and the winters are warm and rainy. In winter, there is more precipitation than in summer months. The average annual rainfall is 577 mm, and the average temperature is 12.7°C. No cases reported from Turkey formerly to be explained by two reasons: the vector flies rarity and misdiagnosis of zoonotic eye complaints. In addition, it is needed to include this pathogen in the differential diagnosis of bacterial and allergic conjunctivitis. The clinical manifestations usually occur in the form of a single-eye infection [7] as in this case.

As a conclusion, the ocular form thelaziasis caused by an adult female *T. callipaeda* in a dog has been reported firstly in Turkey, despite it is a widespread zoonotic pathogen in the animal world. By this case report, it was aimed to raise awareness of the eye problems, in order to control of its spread within population of domestic animals.

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Case Report

Desmoplastic Small Round-Cell Tumor in a Dog [1]

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Abstract

A 13-year-old, neutered female Husky dog was brought to the clinic with the complaints of anorexia, vomiting, abdominal distension and respiratory distress. It was suddenly died during intervention. At the necropsy, a mass was seen in the abdominal cavity. The mass had 16x15x9 cm in diameters. Histological examination revealed clusters of cells with slightly eosinophilic and scanty cytoplasm, small round hyperchromatic nuclei, and inconspicuous nucleoli encompassed by hypocellular extensive desmoplastic connective tissue stroma comprising few spindle-shaped connective tissue cells. In immunohistochemical examination the cytoplasms of tumour cells were detected to be mildly positive by nestin. The tumour cells were negative for α-SMA (Alpha-Smooth Muscle Actin), vimentin and pancytokeratin, but stromal cells were positive for α-SMA and vimentin. Despite the presence of partially incompatible immunohistochemical findings, the tumor in this case was diagnosed as desmoplastic small round-cell tumor because its aggressiveness, localization, and histopathology was similar to that observed for this tumor in humans. Previously this tumor has not been identified in animals.

Keywords: *Clinicopathology, Desmoplastic tumor, Dog, Immunohistochemistry*

Bir Köpekte Küçük Yuvarlak Hücreli Dezmoplastik Tümör

Öz

Husky ırkı, 13 yaşlı, kısırlaştırılmış dişi bir köpek, anoreksi, kusma, karın şişliği ve solunum sıkışması şikayetiyle kliniğe getirildi. Hayvana müdahele sırasında aniden öldü. Nekropside karın boşluğunda 16x15x9 cm boyutlarında bir kitleyle karşılaşıldı. Histolojik incelemede hiposelüler, az sayıda mekik şekilli bağ doku hücrelerinden oluşan geniş desmoplastik bağ doku stromasıyla adacıklara ayrılan; hafif eozinofilik dar sitoplazmalı, küçük yuvarlak-oval hiperkromatik çekirdekli, çekirdecikleri fark edilmeyen hücre kümeleri gözlendi. İmmunohistokimyal incelemede tümör hücre sitoplazmalarının nestin yönünden hafif pozitif olduğu saptandı. Tümör hücreleri α-SMA (Alpha-Smooth Muscle Actin), vimentin ve pansitokeratin negatif olup stroma α-SMA ve vimentin pozitifti. İmmunohistokimyasal yönden kısmen farklı bulgular göstermekle beraber gerek lokalizasyonu gerekse histomorfolojik açıdan insanlardakine benzerliklerinden dolayı bu tümöre desmoplastik küçük yuvarlak hücreli tümör teşhisi konmuştur. Daha önce bu tümör hayvanlarda tanımlanmamıştır.

Anahtar sözcükler: *Dezmoplastik tümör, Klinikopatoloji, İmmunohistokimya, Köpek*

INTRODUCTION

Desmoplastic small round-cell tumor was first described in humans by Gerald and Rosai in 1989 [1]. It is a very aggressive and rare tumor and is usually reported in young male adults. It typically affects the peritoneum (88%) [2,3]. While its etiopathogenesis is still unknown, it is histologically composed of nests and large clusters of small round cells with scanty cytoplasm and hyperchromatic nuclei separated by fibrosclerotic desmoplastic stroma [2,4]. Immunohistochemistry revealed that the tumor was positive for epithelial (cytokeratin [95%] and Epithelial Membrane Antigen [EMA] positivity), mesenchymal (desmin and vimentin positivity [81%]), and neural (NSE, CD57 and synaptophysin positivity) markers [2,4,5]. However, the tumor was also positive for vimentin and cytokeratin (AE1/AE3) [6] markers and negative for α-SMA [4]. We aimed to examine morphopathological and immunohistochemical

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characteristics of such a tumor present in a dog; the dog's tumor characteristics were consistent with those of this rare human tumor in terms of localization, histopathology, and aggressiveness.

CASE HISTORY

A 24 kg, 13-year-old, neutered female Husky breed dog referred to Veterinary Teaching Hospital with a history of anorexia, vomiting, abdominal distention and respiratory distress. The dog was receiving a medication for gastritis at the time of referral investigation. Physical examination revealed severe abdominal distress and pain in right abdominal region. Poor general health status, mucosal pallor and increased capillary refill time (>3s) were also remarkable. The dog was hypothermic (36.9°C) and tachypneic (respiratory rate = 40-53 breaths per minute) with increased respiratory effort. Femoral arterial pulses were slightly weak. It could not be possible to auscultate the heart rate because of the severe tachypnea. A serum biochemistry panel showed hypoalbuminemia, hypoproteinemia and increased blood levels of ALP and GGT *(Table 1)*.

Necropsy revealed a 16×15×9 cm-sized, round shaped, elastic, lobular mass lesion with a grayish–red color between the liver, spleen, and bowel; the lesion was adhered to the dorsal peritoneum of the abdomen and cranial pole of the right kidney *(Fig. 1)*.

Tissue samples were fixed in 10% buffered formalin solution. After fixation, the tissues were dehydrated to enable embedding with paraffin. The tissues were dehydrated gently by immersion in increasing concentrations of alcohol (70%, 80%, 96%, 100%). The dehydrating agent was then cleared by incubation in xylene prior to paraffin embedding. Next, 5 µm thick sections were cut from paraffin-embedded blocks, deparaffinized in xylol, and stained with Harris' Hematoxylin and Eosin after being passed through a series of 100%, 96%, 80%, and 70% alcohol treatments. After Harris' hematoxylin and eosin (H&E) staining, the sections were examined by light microscopy. Histological examination revealed clusters of cells with slightly eosinophilic and scanty cytoplasm, small round hyperchromatic nuclei, and inconspicuous nucleoli encompassed by hypocellular extensive desmoplastic connective tissue stroma comprising few spindle-shaped connective tissue cells *(Fig. 2a, b)*. High numbers of mitotic figures were encountered in all microscopic fields *(Fig. 2c)*. Moreover, foci of bleeding and necrosis were observed in some fields *(Fig. 2d)*.

Immunohistochemistry was carried out using indirect immunoperoxidase method (ABC-P) method using primary antibodies against cytokeratin, α-SMA, vimentin, and nestin. Primary antibody dilution rate, incubation duration, incubation temperature, antigen retrieval, and endogenous biotin block are shown in *Table 2*. After deparaffinization and dehydration, peroxidase activity was blocked for 30 min by peroxidase blocking reagent (Novocastra Peroxidase Detection Systems, Ready to use). A SensiTek HRP (ScyTec Laboratories, Super block LOT: 24062; Biotinylated antibody LOT: 24205; HRP LOT: 24242®) kit was used in accordance with the manufacturer's instructions. Either heat or trypsin was used for antigen retrieval, and 3,3'-diaminobenzidine (DAB) (DAB-Substrate Kit, Invitrogen, 896320A®) was used as a chromogen. The heated sections were applied egg white and milk powder for the blocking of endogenous

Table 1. *Results of routine blood work in the dog*

Complete Blood Count	**Results**	**Reference Ranges**
WBC (10^9/L)	15.8	6-17
LYM (10^9/L)	2.4	0.9-5
MONO (10^9/L)	1.1	0.3-2.5
NEUT(10^9/L)	10.4	3.5-12
EOS (10^9/L)	1.9	0.1-19
LYM %	15.3	12-30
MON %	7.0	2-13
NEU %	66.1	35-70
EOS %	11.4	0.1-19
RBC	6.17	5.5-8.5
HGB	14.8	12-18
HCT	35	37-55
MCV	56.7	60-72
MCH	24	19.5-25.5
MCHC	42.3	32-38.5
RDWa	33.9	35-53
RDW %	18.3	12-17.5
PLT (10^9/l)	433	200-500
MPV (fl)	7.6	5.5-10.5
Serum Biochemistry		
Glucose (mg/dL)	110.5	65-118
Urea (mg/dL)	12.5	15-59.9
Creatinine (mg/dL)	0.62	0.5-1.5
Total Protein (g/dL)	4.6	5.4-7.1
Albumin (g/dL)	2.7	3.1-4.0
Total Bilirubin (mg/dL)	0.13	0.1-0.3
Direct Bilirubin (mg/dL)	0.08	-
ALP (IU/L)	169.9	20-156
ALT (IU/L)	38.7	21-102
AST (IU/L)	52.6	23-66
GGT (IU/L)	145	6-28
Creatine Kinase (IU/L)	154	<200
Na (mmol/L)	133	140-154
K (mmol/L)	5	3.8-5.6

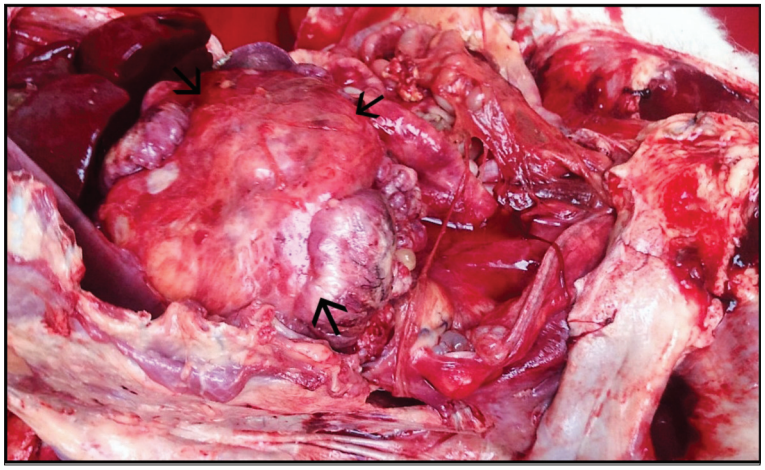

Fig 1. Macroscopical view of desmoplastic tumor *(arrows)*

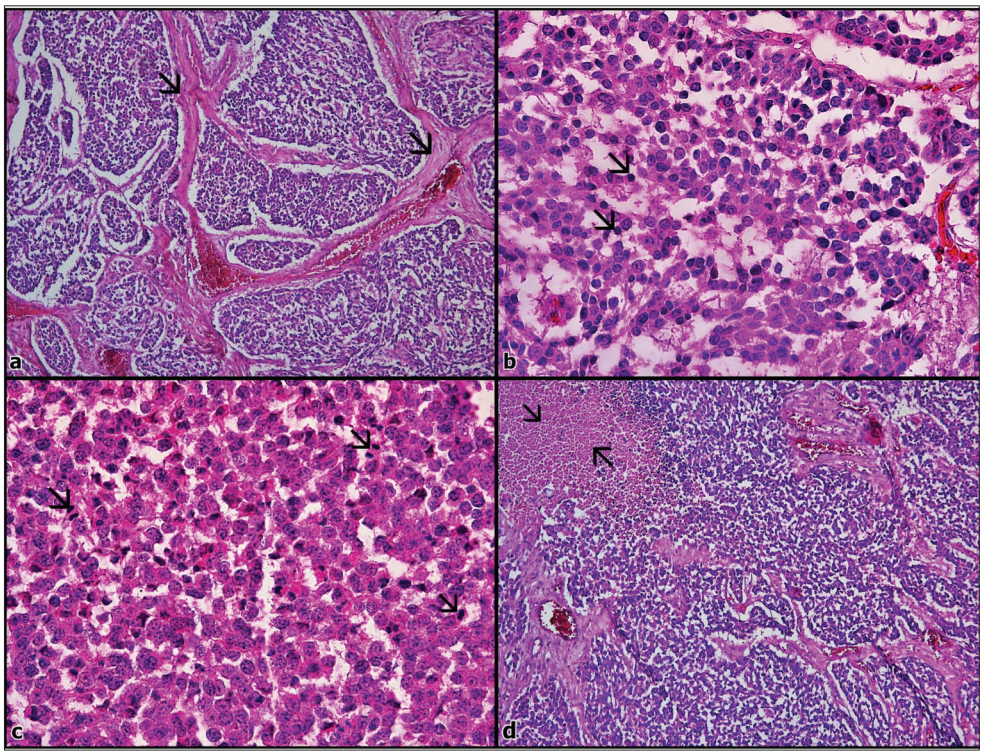

Fig 2. Histopathological findings **a-** Fibrosclerotical desmoplastic stroma forming cell islands *(arrows)*, x100, HE. **b-** Tumor cells having small round-oval hyperchromatic nuclei *(arrows)*, x400, HE. **c-** Numerous mitotic figures (arrows), x400, HE. **d-** Extensive areas of necrosis *(arrows)*, x100, HE

Table 2. *The dilution rate of the primary antibodies, the duration of the incubation period, the incubation temperature, antigen retrieval and endogenous biotin block*

Primary Antibodies	Antigen Retrieval	Endogenous Biotin Block	The Dilution Rate of the Primary Antibodies/Duration/ Temparature	Chromogen
Nestin (Acris, AP07829PU-N)®	Citrate Buffer (pH 6.0)+0.1% Tween; 700 watt, 3x5 min/heat	Egg white and milk powder (7)	1/100 PBS Overnight; +4°C	DAB (Invitrogen, 896320A)®
Vimentin antibody (Dako, Vim3B4)®	5 min 0.1% trypsin 37°C	-	1:100 PBS 75 min; 37°C	DAB (Invitrogen, 896320A)®
α-SMA antibody (Sigma, 120M4768)	10 min , 0.1% trypsin 37°C	-	1:200 PBS 45 min; 37°C	DAB (Invitrogen, 896320A)®
Pancytokeratin antibody (AE1/AE3+5D3) (Abcam, ab86734)®	5 min, 0.1% trypsin 37°C	-	1:100 PBS 1 h; 37°C	DAB (Invitrogen, 896320A)®

biotin [7]. Immunohistochemical examination of stroma cells revealed positive staining for vimentin and α-SMA *(Fig. 3a)*. Tumor cells revealed negative staining for α-SMA, pan-cytokeratin *(Fig. 3b)* and vimentin *(Fig. 3c)*. The cytoplasm of tumor cells revealed slightly positive staining for nestin *(Fig. 3d)*.

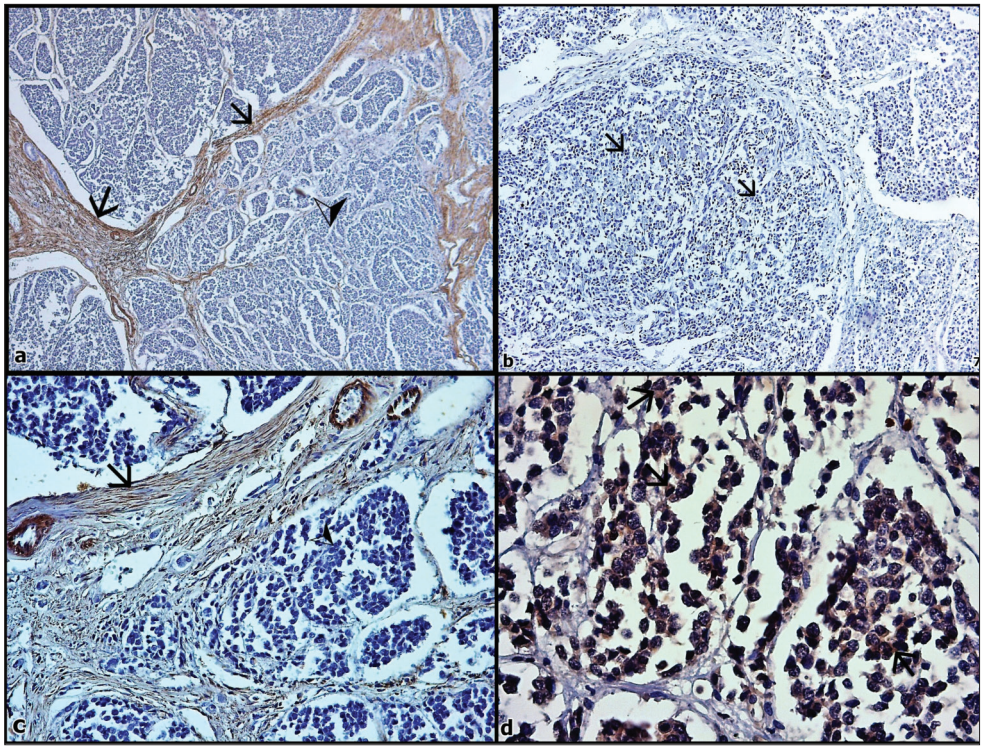

Fig 3. Immunohistochemical findings **a-** Tumor cells were negative with alpha SMA (arrow head) but stromal cells were positive *(arrows),* x50, ABC-P. **b-** Tumor cells were negative pancytokeratin *(arrow),* x100, ABC-P. **c-** Tumor cells were negative with vimentin *(arrow head)* but stromal cells were positive (arrow), x200, ABC-P. **d-** Cytoplasms of tumor cells were detected to be mildly positive by nestin *(arrow),* x400, ABC-P

DISCUSSION

Involvement of the peritoneal surfaces and aggressiveness are the prominent characteristics of desmoplastic small round-cell tumors [2,3]. Tumor evaluation in the present case also indicated desmoplastic small round-cell tumor owing to the involvement of the peritoneum and the tumor having reached a large size.

Histopathological examination of the tumor in the present case revealed presence of cells with slightly eosinophilic and scanty cytoplasm, small round hyperchromatic nuclei, and inconspicuous nucleoli forming islands through the connective tissue stroma; these microscopic features were similar to those observed for desmoplastic small round-cell tumors [2,4].

Etiopatogenesis of desmoplastic small round-cell tumor is still unknown and diagnosis can be achieved only by immunohistochemistry and sitogenetic studies [4]. Although immunohistochemical studies reported positive staining for epithelial, mesenchymal, and neural markers in desmoplastic small round-cell tumors [2,4,5], tumor cells in this case tested negative for α-SMA, vimentin, and pancytokeratin and only the tumor cytoplasm revealed positive staining for nestin. Xie and Shen [6] reported that 87% of these tumors revealed positive staining for cytokeratin (AE1/AE3); however, the present case revealed negative staining. Koniari et al.[4] reported negative staining, for α-SMA, a finding consistent with that observed in the current case.

Despite the presence of partially incompatible immunohistochemical findings, the tumor in this case was diagnosed as desmoplastic small round-cell tumor because its aggressiveness, localization, and histopathology was similar to that observed for this tumor in humans. Moreover, this is the first report of desmoplastic small round-cell tumor diagnosed in an animal.

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