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Research Article

Determination of Some Oxidative Stress and Inflammation Markers in Serum, Blood and CSF in Cattle with Head-Eye Form of Malignant Catarrhal Fever

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Abstract

The aim of this study was to determine changes in total sialic acid (TSA), malondialdehyde (MDA), nitric oxide (NO), inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) levels on sera and CSF and reduced glutathione (GSH) levels on blood in cattle with Malignant Catarrhal Fever (MCF). For this purpose 17 cattle which clinically diagnosed "head-eye form" of MCF and clinically healthy 10 cattle were evaluated. Blood and cerebrospinal fluid (CSF) were taken from the animals on the MCF diagnosed group MDA, GSH, NO, eNOS, iNOS, and TSA values were 25.65±0.42 µmol/L, 37.21±1.12 mg/dL, 30.61±0.41 µmol/L, 4.05±0.09 U/L and 10.98±0.35 U/L, 88.33±1.03 mg/dL, on the control group 13.77±0.55 µmol/L, 60.06±1.73 mg/dL, 11.27±0.4 µmol/L, 3.12±0.18 U/L, 5.55±0.3 U/L and 63.60±1.86 mg/dL respectively, and all parameter changes between the groups were determined to be statistically significant (P<0.001). On the CSF, no statistically significant difference between taken from MCF diagnosed group and healthy group. NO value and iNOS activity obtained from control groups CSF were relatively higher than the same group's serum whereas eNOS activities were found to be low. The study group consisting of MCF diagnosed cattle's serum were found to have NO value, eNOS and iNOS activities relatively higher than the same group's CSF values. As a result, it was concluded that there is a need for more comprehensive studies for better understanding the reason of failure to obtain the significant changes of animals diagnosed MCF that determine in blood but not in CSF.

Keywords: *Malignant catarrhal fever, GSH, Nitric oxide, eNOS, iNOS, Cattle*

Koriza Gangrenosa Bovum Baş-Göz Formlu Sığırlarda Serum, Kan ve BOS'ta Bazı Oksidatif Stres ve İnflamasyon Belirteçlerinin Tespiti

Özet

Bu çalışmanın amacı Koriza Gangrenosa Bovum'lu sığırlarda serum ve beyin omurilik sıvısı (BOS), total sialik asit (TSA), malondialdehit (MDA), nitrik oksit (NO), indüklenebilir nitrik oksit sentetaz (iNOS) ve endotelyal nitrik oksit sentetaz (eNOS) ile kan redükte glutatyon (GSH), düzey değişikliklerinin belirlenmesidir. Bu amaçla klinik olarak Coryza Gangrenosa Bovum'un (CGB) "Baş-Göz Formu" teşhisi konulan 17 adet ve kontrol amacı ile klinik olarak sağlıklı sığırlardan oluşan 10 adet sığır değerlendirildi. Hasta ve kontrol gruplarındaki hayvanlardan kan ve BOS alındı. CGB teşhisi konulan grupta MDA, GSH, NO, eNOS, iNOS ve TSA değerlerinin sırası ile 25.65±0.42 µmol/L, 37.21±1.12 mg/dL, 30.61±0.41 µmol/L, 4.05±0.09 U/L ve 10.98±0.35 U/L, 88.33±1.03 mg/dL kontrol grubunda ise 13.77±0.55 µmol/L, 60.06±1.73 mg/dL, 11.27±0.4 µmol/L, 3.12±0.18 U/L, 5.55±0.3 U/L ve 63.60±1.86 mg/dL olduğu ve tüm parametre değişikliklerinin gruplar arasında istatistiksel olarak önemli (P<0.001) olduğu belirlendi. Sağlıklı ve CGB teşhisi konulan gurubun BOS'larından elde edilen değerler arasında istatistiksel olarak önemli bir farkın bulunmadığı tespit edildi. Kontrol grubunun BOS'undan elde edilen NO değeri ve iNOS aktivitesinin aynı grubun serumlarından elde edilen değere göre nispeten yüksek, eNOS aktivitesinin ise düşük olduğu, CGB'li sığırlardan oluşan çalışma grubunun serumlarından elde edilen NO değeri, eNOS ve iNOS aktivitelerinin ise aynı grubun BOS'larından elde edilen değerlerden nispeten yüksek olduğu belirlendi. Sonuç olarak CGB'li hayvanlarda yangısal süreç ve oksidatif strese bağlı olarak kanda ortaya çıkan anlamlı değişikliklerin BOS'ta elde edilememesinin daha iyi anlaşılabilmesi için yeni ve daha kapsamlı çalışmalara ihtiyaç olduğu kanısına varıldı.

Anahtar sözcükler: *Koriza Gangrenosa Bovum, GSH, Nitrik oksit, eNOS, iNOS, Sığır*

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INTRODUCTION

Malignant catarrhal fever (MCF) is usually fatal lympho-proliferative disorder of cattle and other hoofed animals [1-3]. Disease is caused by alcelaphine herpes virus-I (circumstances-I) and Ovine herpesvirus-II (OHV-II) carried by sheep as reservoir [4,5]. Agents located on *Macavirus* genus [6]. OHV-II leads to subclinical infection in sheep and sheep serves as the reservoir for the disease occurring in cattle [1]. Clinically fever, muco-purulent rhinorrhea, corneal opacity [7,8], erosive stomatitis (especially erosion of the buccal papillae ends) and gastroenteritis, tear flows, blepharospasm, upper respiratory tract erosion, encephalitis, cutaneous exanthema and enlarging in lymph nodes were observed. The reservoir sheeps infect environment with agent with nasal discharge. The maximum virus excretion to environment takes place when the sheeps are 6-9 months of age. Contact with sheeps should be prevented through the control of the disease [9].

Sialic acid (SA), increases quickly after the inflammation and injury process [10,11]. Until today, some researchers reported increased serum SA concentrations during the course of many diseases [12-19].

Malondialdehyde (MDA) content increases due to the induction of lipid peroxidation. This process activates antioxidant defense mechanisms [20]. Reduced glutathione (GSH) and MDA concentrations can be used as indicators of oxidative stress in some diseases but no studies determined the oxidative stress in MCF on sera or CSF previously [21,22]. In inflammations, via stimulation of inducible nitric oxide synthase (iNOS), nitric oxide (NO) production increases. This situation leads to NO mediated tissue injury [23].

On many physiological and pathological processes nitric oxide acts as a biologically active molecule with different effects [24]. Nitric oxide is a cytotoxic factor which is generated from the terminal guanidine nitrogen atom of L-arginine by NO synthase and released by a variety of cells [25-28]. Its plays a primary defence against some pathogens [29,30]. But it has also been reported to be immunosuppressive [31,32].

A large spectrum of pathologic events cause oxidative stress in farm animals [33]. Oxidative stress and lipid peroxidation may end up with cellular and tissue damage if the production of reactive oxygen species are excessive [34,35]. Both enzymatic and non-enzymatic anti-oxidative mechanisms used in the elimination of these reactive oxygen species [34,36,37].

This study was therefore designed to determine changes in TSA, MDA, NO, iNOS and eNOS levels on sera and CSF and GSH levels on blood in cattle with MCF.

MATERIAL and METHODS

Animals

A total of 27 animals, age 2-4 years used in the study, consisting of 17 patients with clinically diagnosed MCF, which were referred to the clinic of the Internal Medicine Department of Kafkas University, Faculty of Veterinary Medicine and 10 healthy cattles. In this study, control group were provided from the faculty farm animals which found to be healthy during routine examination. The study was approved from the Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK/2016-137).

Blood (n=27) and CSF (n=17) samples were taken from all the animals. Blood samples were taken from vena jugularis and CSF were taken from L6-S1 intervertebra with Tuohy epidural needle® (Perican epidural needle, 18G-80 mm. Braun, Germany). The blood samples were centrifuged 3.000 rpm x 10 min and serum was obtained. The whole blood samples with EDTA were taken for GSH analyses. The obtained samples stored at -20°C until analyses are done.

NO, MDA, iNOS, eNOS, TSA and GSH Analysis

Serum TSA levels were measured by the method of Sydow [38], serum MDA concentrations were determined by the method of Yoshoiko et al.[39], serum NO was determined according to the method of Miranda et al.[40]. Also levels of GSH was measured according to the method of Beutler et al.[41] iNOS and eNOS activities were determined commercial ELISA kits (MyBiosource®). Same procedures (excluding GSH) were performed during the measurement of the CSF and serum samples.

Statistical Analysis

The data obtained in this study were evaluated by SPSS® (SPSS 20, USA) programme using the t test.

RESULTS

The serum MDA, NO, eNOS, iNOS and TSA levels and whole blood GSH levels are shown in *Table 1*, CSF; MDA, NO, TSA levels and eNOS, iNOS activities are shown in *Table 2* which are obtained form study.

Clinical Examination Findings

In the clinical examination of the group consisting of diseased animal, typical clinical symptoms; keratitis on the cornea which starts from periphery and directs to center, keratoconjunctivitis and increased opacity, photophobia, mukoprulent lacrimation and nasal discharge, high fever (40-41°C), redness of the mouth and nasal mucosa, necrosis and erosive lesions in the mouth and buccal papillae, growth in all lymph nodes that are palpable, dysphagia

Table 1. *Analysis results of serum and blood in control and amimals with MCF*

Parameter	Control (mean±std error)	MCF (mean±std error)	P
MDA µmol/L	13.77±0.55	25.65±0.42	P<0.001
GSH mg/dL	60.06±1.73	37.21±1.12	P<0.001
NO µmol/L	11.27±0.4	30.61±0.41	P<0.001
eNOS U/L	3.12±0.18	4.05±0.09	P<0.001
iNOS U/L	5.55±0.3	10.98±0.35	P<0.001
TSA mg/dL	63.60±1.86	88.33±1.03	P<0.001

Table 2. *Analysis results of CSF in control and animals with MCF*

Parameter	Control (mean±std.error)	MCF (mean±std.error)	P
MDA µmol/L	19.38±0.43	19.48±0.69	P>0.05
NO µmol/L	21.22±0.58	20.84±0.69	P>0.05
eNOS U/L	1.95±0.07	1.93±0.07	P>0.05
iNOS U/L	7.71±0.26	8.07±0.26	P>0.05
TSA mg/dL	104.15±1.88	102.55±1.85	P>0.05

and the findings which are reported by researchers. Clinical symptoms associated with encephalitis such as somnolence, staying head put on a place, staggering gait, indifference to the environment were not evident in patients which were clinically diagnosed with head-eye form of MCF.

In addition, from anamnesis received from animal owners, it was determined as infected animals are being hosted along with sheep.

Biochemical Findings

The serum levels of MDA, NO, eNOS, iNOS and TSA were significantly higher (P<0.001) in animals with MCF than control group animals and whole blood GSH values were detected significantly lower (P<0.001) *(Table 1)*.

On the animals with MCF, levels of MDA and iNOS in CSF were detected high whereas levels of NO, eNOS ve TSA were detected low than control group animals. This changings were not statistically significant (P>0.05) *(Table 2)*.

DISCUSSION

This study aimed to demonstrate on sera, blood and CSF some indicators of oxidative stress and inflammation, which were determined different bacterial and viral diseases, in cases of MCF in cows that parameters are not fully investigated yet.

Clinical symptoms determined in this study like keratitis on the cornea which starts from periphery and directs to center, keratoconjunctivitis, corneal opacity, photophobia, high/acute fever (39.5-41.0ºC), disphagie, redness of the mouth and nasal mucosa, necrosis and erosive lesions in the mouth and buccal papillae, growth in the lymph nodes of infected animals with consequent excessive mucopurulent lacrimation and nasal discharge in agreement with those reported for MCF but clinical symptoms associated with encephalitis were not determined in patients [1-3,7-9]. Furthermore from anamnesis taken from animal owners, it was determined as infected animals are being hosted along with sheep which are involved in the occurrence of the disease in cattle as reservoir [1,4,5].

In this study, serum TSA level in MCF cases was found higher than the control animals as reported previously by some researchers in different diseases [15-19]. Increased sialic acid levels are reported in mammals during different pathological situations [11]. SA is present in all biological membranes. When the pathological situations mentioned above occur, SA is released from the cell membrane to circulation and rise in SA is observed [11,13,42,43]. TSA changes in CSF were not statistically significant. This is may be due to that symptoms of encephalitis do not occur sufficiently in patients.

Another indicator of cellular damage and lipid peroxidation during the course of MCF may be increased MDA, and decreased GSH. The MDA results of our study are similar to foot and mouth disease in recent years [19,44]. According the present study finding that MCF causes tissue damage in various organs and systems. Lower GSH levels on the animals with MCF than healthy animals is thought to be caused by oxidative stress on the animals due to disease, similar to the previous reports on oxidative stress situations [45,46].

NO is a free radical which is produced via inducible nitric oxide synthase (iNOS) from activated leukocytes by pro-inflamatuary cytokins [47-50]. It is known that NO plays an important role in the primary defense mechanism against several pathogens and NO production can be induced by various viruses which inhibit virus replication [44,51,52]. In present study, NO levels in MCF cases was determined higher than control animals in serum samples, therefore our study results indicate that herpesvirus can induce the production of NO.

NO level and iNOS activity obtained from the control group's CSF were relatively high compared to the serum levels of the same group and eNOS activity was low. NO level, eNOS and iNOS activities obtained from study group's serum with MCF detected relatively higher than the same group's CSF levels. Serum levels of NO, eNOS and iNOS were found to increase significantly in the cattles diagnosed MCF's head and eye form compared to healthy animals, this situation were found to be consistent with the studies reported for these markers in pathological events [53-56]. Besides all these on the MCF group, iNOS, induced by such situations like inflammation, and eNOS, known to locate in vascular endothelia, were not observed as the expected

increased level in CSF. At this point, it was concluded that there is a need for new studies for a better understanding that blood-brain barrier, or a different mechanism may be effective and why statistically significant changes on the blood of animals with MCF caused by inflammatory processes and oxidative stress were not seen on the CSF. Changes in the inflammatory and oxidative stress parameters observed in the serum, are not observed in CSF of animals diagnosed with head eye form may suggest that the encephalitis did not developed/occured.

This is the first study to our knowledge in which some oxidative and inflammation parameters was evaluated on sera, blood and CSF in the MCF. In this study some oxidative stress parameters can significantly increased in the serum, but not in the CSF, produced from diseases and oxidative damage to tissues along with other mechanisms might have taken part in the pathogenesis of CSF and further detailed studies at cellular level are needed to fully understand the pathogenesis and clinical expression of the disease in cattle, an important source of infection.

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Research Article

Monitoring of *Nosema* Infections Levels During Hygienic Honey Bee Breeding Programs in Turkey

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Abstract

The objective of this study was to follow *Nosema* infection levels and species under hygienic bee breeding program for resistance to American foulbrood (*Paenibacillus larvae*). The incidence of *Nosema* parasite infection levels and detection of the species of *Nosema* were evaluated in 5 periods during 2012-2014 for Mugla honey bees known as an ecotype of *Apis mellifera anatoliaca* in the hygienic bee breeding program. During the hygienic breeding program, no organic or synthetic chemical treatments were applied against nosemosis in the colonies. The incidences of *Nosema* spores were followed in 123 colonies at five time periods. Although the correlations were negative for between spores-temperature (r = -0.115; P>0.01) and positive for spores- humidity (r = 0.013; P>0.01) but not significant statistically. Molecular diagnosis showed that only *N. ceranae* spores were detected from samples during 5 seasons. In conclusion, nosema infection levels decreased under hygienic bee breeding programme but further monitoring studies should be performed in order to decide whether the nosema spores decrease due to hygienic behavior. To our knowledge, this is the first long- term and unique study for observation of *Nosema* during breeding program in Turkey so far.

Keywords: *Nosema ceranae, Nosema apis, Breeding program, Mugla ecotype, Turkey*

Türkiye'deki Hijyenik Bal Arısı Islah Çalışması Süresince *Nosema* Enfeksiyon Düzeyinin Takibi

Özet

Bu çalışmada, Amerikan Yavru Çürüklüğü (*Paenibacillus larvae*) hastalığına dirençli olan hijyenik balarısı ıslah programında *Nosema* enfeksiyon düzeyinin takibi amaçlanmıştır. Islah programında *Apis mellifera anatoliaca* ekotipi olarak adlandırılan Muğla balarısında 2012-2014 yılları arasında 5 dönem boyunca *Nosema* türleri ve nosema enfeksiyon düzeyi belirlenmiştir. Islah çalışması süresince kolonilere nosema için her hangi bir ilaç uygulaması yapılmamıştır. Beş dönem boyunca 123 kovanın nosema sporu bulaşıklığı takip edilmiştir. Spor sayısı- sıcaklık arasında negatif korelasyon (r = -0.115; P>0.01) ve spor sayısı nispi nem arasında pozitif korelasyon (r = 0.013; P>0.01) bulunmasına karşın ilişki istatistiksel olarak önemli değildir. Moleküler tanımlamada, beş sezon boyunca alınan örneklerde yalnızca *N. ceranae* sporu tespit edilmiştir. Sonuç olarak, hijyenik arı yetiştirme programı süresince nosema enfeksiyon seviyeleri azaldığı gözlenmiştir. Fakat nosema sporlarının azalmasının nedeninin hijyenik davranışa bağlı olup olmadığına dair karar verebilmek için başka hijyenik çalışmalarda da gözlemler yapılmalıdır. Bu çalışma bugüne kadar Türkiye'deki ıslah program süresince *Nosema* düzeyinin takibini içeren en uzun süreli ve tek çalışmadır.

Anahtar sözcükler: *Nosema ceranae, Nosema apis, Islah programı, Muğla ekotipi, Türkiye*

INTRODUCTION

The Microsporidia have more than thousand species (160 genera and 1300 species) and Nosema species being Microsporodian are parasitic for invertebrates [1-4]. There were two microsporidian species of genus *Nosema*, *Nosema apis* and *Nosema ceranae* in honey bees. It was supposed that *N. ceranae* was specific for *Apis cerana* and *N. apis,* a pathogen specific for the *Apis mellifera,* gave rise to nosemosis previously [5,6].

However, other studies illustrated that *N. ceranae* could infect *A. mellifera.* In the last decade, *N. ceranae* has expanded its distribution in the world and the replacement of *N. apis* by *N.ceranae* was reported by many researchers [7-10]. The serious colony losses referred to colony collapse disorders (CCD) were observed in the last decade and attracted great

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attention of scientists, but the reasons are still unclear [11]. The studies indicated that *Nosema* was considered the possible suspect for colony losses. Furthermore, the researchers thought that many factors such as beekeeping practices, host susceptibility and various combinations of pathogens resulted in colony losses [12,13]. Also researchers indicated that synergistic effects of various pesticides and *N. ceranae* combination increased honey bee mortality [14-16]. Recent studies illustrated that *N. ceranae* is the most prevalent bee pathogen and it does not show any prior clinical symptoms [7,8,17]. The one possible explanation for the higher pathogeny of *N. ceranae* is that it has better adaptation than *N. apis* to different temperature conditions [18,19]. Another finding is that *N. ceranae* infection exerts a higher immune suppression than *N. apis* [20] and *N. ceranae* infection X imidacloprid affect the immune response [14]. On the other side, if honey bee colonies have enough protein and energy reserves, honey bees can tolerate *N. ceranae* infection as the host energy intake is increased by the parasite. Otherwise, it experiences energy stress if the host does not have enough energy reserve [21,22]. *Nosema* is still considered as the possible suspect for colony losses causing economic loss. Many survey studies indicated that *Nosema* infections led to colony losses worldwide [9,10,13,23]. Universities and several governmental institutions in many countries have performed breeding and selection programs supporting to increase honey, pollen production and gentleness. Recently, breeding programs for increased disease resistance including hygienic behavior of colonies to American foulbrood [24,25], and *Varroa destructor* were performed in many countries [26-30]. In Turkey, breeding programs have generally been applied for conservation of subspecies or native populations and used widely for enhancing desirable traits *(www.tagem.gov.tr)*. In contrast, the breeding programs for disease resistance have not been widely applied before. The negative effects of diseases could often be compensated by pharmaceuticals, and other management techniques.

This study was a part of a hygienic behavior breeding program against American foulbrood which has been coordinated by universities and Mugla Beekeepers Association. The incidence and levels of *Nosema* infections and the type of *Nosema* were determined in 5 periods during 2012-2014 for Mugla ecotype in the program which recorded the hygienic test values in colonies. In here, *Nosema* spore monitoring results and types of *Nosema* are given. More detailed results on breeding program will be prepared for publications. The reason why *Nosema* spores have been observed during this breeding program is that selected populations are very important, and so *Nosema* spores were observed during five periods in order to see the effect of *Nosema* spores in any colony loss of selected population considering the pathogen effect of nosema.

The objective of this study was to follow *Nosema* infection levels and species under hygienic selection program for resistance to American foulbrood. To our knowledge, this is the first long- term study for observation of *Nosema* during breeding program in Turkey so far.

MATERIAL and METHODS

The colonies used in the present study were screened in order to detect the presence of *Nosema* spores using microscopic method. During the hygienic breeding programme, no organic or synthetic chemical treatments have been applied against nosemosis. The honey bee samples were taken from each colony in both spring and autumn in 2012-2014 and kept in alcohol for spore counts in 123 colonies.

Determination of Nosema Infection Levels

Twenty older foragers from each colony were sampled from in order to determine the number of spores per bee in a pooled sample. Homogenates were prepared according to the OIE terrestrial manual [31]. *Nosema* spp. spores were microscopically determined from each homogenate at 400x magnification. The spores were counted on the haemocytometer [31] and the average number of spores per bee was calculated [32].

Molecular Detection of Nosema spp.

Total DNA was extracted from each homogenate using DNA isolation kit (Fermentas K512). Isolated DNA was analyzed by multiplex PCR in order to confirm the *Nosema* species of the spores as previously described using 321APIS FOR/321APIS REV and 218MITOC FOR/218MITOC REV primers specific for *N. apis* or *N. ceranae* respectively [33]. PCR amplification was detected in agarose gel (1.5%) electrophoresis and visualized under UV after ethidium bromide staining [33]. Molecular detection of *Nosema* spp. was performed in all samples for each season. The positive PCR products were compared with the controls for *N. ceranae* and *N. apis* provided by Etlik Veterinary Control Central Research Institute (Ankara, Turkey).

Statistical Analysis

ANOVA tests were used to determine the variation in *Nosema* prevalence and the degree of infections over the seasons. Multiple comparison tests were applied to spore counts by Tukey B and correlation test were done using SPSS for Windows Version 16.0 (SPSSInc., Chicago, IL, USA). Selected three positive PCR products from positive samples for *N. ceranae* were sequenced and the sequence similarity analyses were performed using BLAST database search.

RESULTS

The Prevalence of Nosema Spores

During the hygienic behavior breeding program, prevalence of *Nosema* spores was followed in 123 colonies.

During the 2012-2014 period of breeding program, we observed seasonality in spore densities in colonies. The first year (2012) of the breeding program, *Nosema* sporecounts were very high in May and November *(Fig. 1)*. The highest percentage of infected colonies was observed in November 2012 (76%). In the second year, *Nosema* spores were decreased dramatically and the lowest percentage of infected colonies was observed in November 2013 (18%) and nearly similar percentage was observed in May 2014 (19%). Descriptive statistics results of spore counts for sampling seasons were given in *Table 1*. Spore numbers from sampled seasons were decreased during years in breeding programs. The lowest mean numbers of spores were observed in November 2013. Analysis of variance was performed to determine of significance among variables (spore numbers in sampled seasons). Variance analysis illustrated that the differences among spores variables were highly significant (P<0.001). Multiple comparison; Tukey B test was conducted to determine differences which arise from variables. According to multiple comparisons test, spore numbers in May 2012 and November 2012 were different from each other and May 2013-November 2013-May 2014 spore numbers *(Table 1)*.

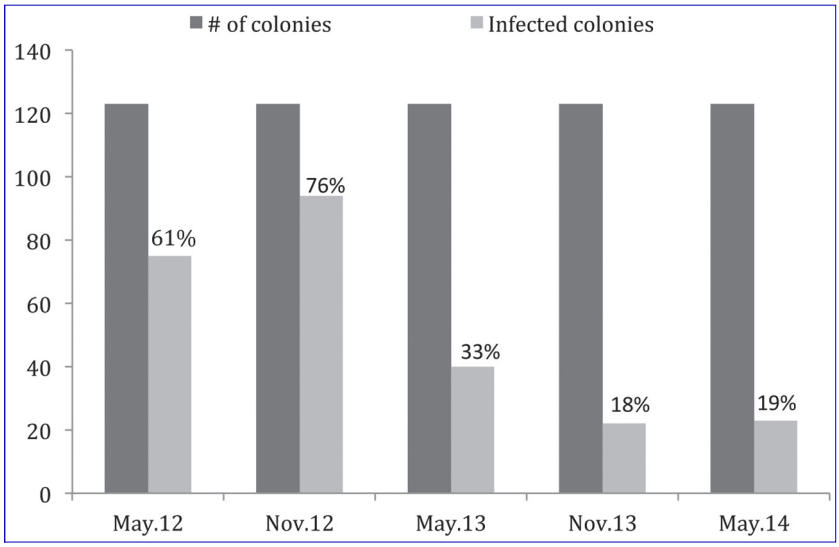

Fig 1. Studied and infected colonies in years 2012-2013-2014

Table 1. *Descriptive statistics results of spores for five seasons*

Group	Mean	N	St. Dev.
May 2012	52. 10^4b	123	12.10^5
November 2012	106.10^4a	123	13.10^5
May 2013	7. 10^4c	123	15. 10^4
November 2013	5.10^4c	123	16. 10^4
May 2014	6. 10^4c	123	18. 10^4

The group means having the different letter (a, b and c) in the same column were different from each other, P≤0.05

Average temperature and relative humidity values were obtained from Turkish State Meterological Service (on electronic data base: *http://tumas.mgm.gov.tr/wps/portal/*) for months during selection periods. Correlation tests were performed between spores- temperature and humidity spores. Negative correlation was detected between spores temperature (r = -0.115; P>0.01) and positive correlation spores- humidity (r = 0.013; P>0.01). But the correlations were not statistically significant. During the selection program, mean spore numbers, mean temperature (Temp.), and relative humidity (RH%) were given in *Fig. 2*.

Molecular Diagnosis of Nosema spp.

DNA isolation was done from *Nosema* spores positive samples using commercial isolation kit. Multiplex PCR were performed to detect of *Nosema* types, *N. apis* and *N. ceranae (Fig. 3)*. Molecular diagnosis illustrated that only *N. ceranae* spores were detected from samples during 5 seasons. Selected positive samples for *N. ceranae* were sequenced. BLAST database search illustrated that the nucleotide sequences of amplification products from the *Nosema* infested honeybees were 99% identical with *N. ceranae* sequence from many countries deposited in GenBank database in this study.

DISCUSSION

The results in here were interpreted with hygienic behavior values which increased in colonies during 3 years of selection. Selection on queens with an artificial insemination showed a steady increase in hygienic bees from 43% in 2012 to 63% in 2013 and 91.7% in 2014. The percentage of bees with hygienic behavior was significantly different among the years (P<0.001) [34]. The results revealed that hygienic behavior increased, and on contrary, infected colony numbers and spore loads significantly decreased in the 5 periods. During breeding program, 3-4% over wintering colony losses were observed through the years, and no suddenly bee death was observed. Honey production was not measured. During these periods, no *Nosema* symptoms were observed in colonies.

The significant differences have been detected in prevalence of *Nosema* spp. from 1990s until now by many studies in the world. The first period examination of *Nosema* spore distribution was carried

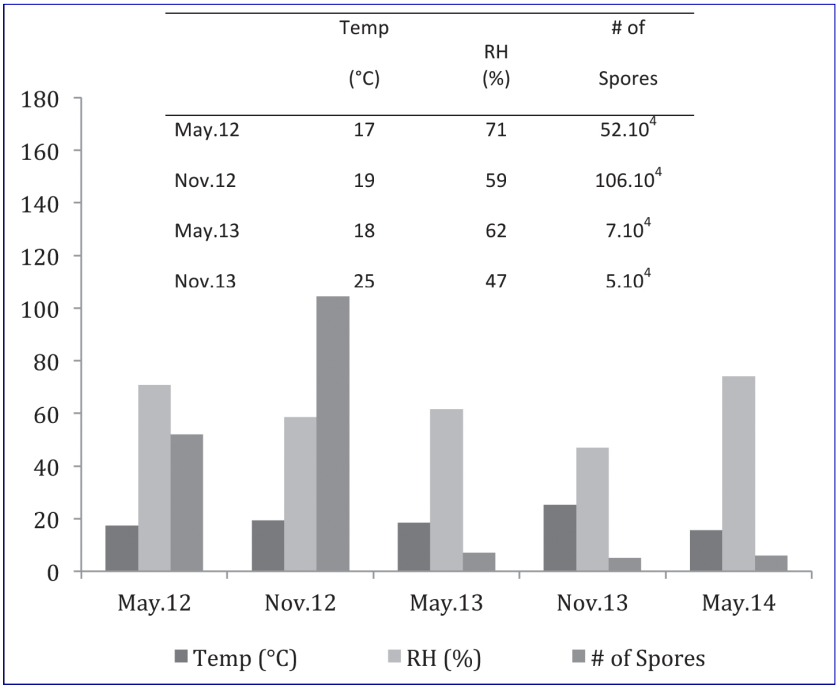

Fig 2. Average temperature (Temp.), relative humidity (RH%), and mean spores numbers under selection

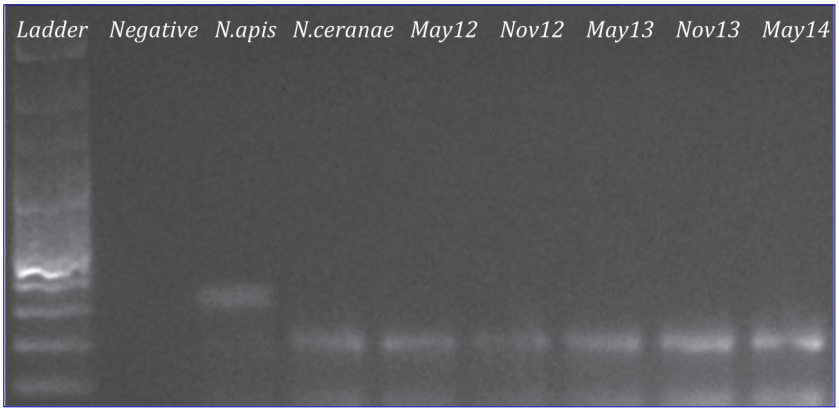

Fig 3. PCR results of positive samples on gel: Line 1: 100bp DNA ladder, line 2: negative control, line 3: *N. apis*, line 4: *N. ceranae*, line 5- 9: Positive samples

out between 1999 and 2002 [10]. During this period, the smallest numbers of *Nosema* positive samples were detected during the summer and the spores looked like *N. apis*. In the other years among 2003-2005, *Nosema* positive samples in all months showed tendency to increase of spores [10]. In Spain, the high number of colony losses had been related with *N. ceranae* more common than *N. apis* [9,13]. The coexistence of both *N. ceranae* and *N. apis* in colonies with the higher prevalence of *N. ceranae,* a sign of a developmental advantage over *N. apis,* and different developmental preferences according to prevalence under the different climatic regions in Spain has been revealed through the other studies [35]. The majority of colonies were found to be infected with mixed *Nosema* spp. in another survey study performed in Sweden in 2007 [17]. With intend of determining the prevalence of *Nosema* spp. in Germany, the long term studies has been made during the five years [36]. Each spring and autumn periods, samples were collected from these colonies and there were no relation between colony losses and detectable levels of infection with *N. apis* or *N. ceranae* in this report [36]. Although, the samples collected in Finland from the 1990s infected with *N. apis*, collected samples from colonies after 2000s infected with either *N. ceranae* or association with *N. apis* [8]. Although the climatic conditions are quite similar, the prevalence of *N. ceranae* has been determined to be higher in Finland than in Sweden or Norway [7]. The *Nosema* spores were detected during four years and the infection level altered both winter and summer periods during this four year period as a decrease in winter 2009-2010 and 2011-2012 in Poland [37]. *N. ceranae* has been reported in France since 2002 and a high variability in spore was observed in studied colonies [38]. The study completed in order to determine the prevalence of *Nosema* spores in Slovakia during the period of two years (2009-2010) reported that the prevalence of *N. ceranae* gradually increased whereas the prevalence of *N. apis* decreased [39]. In Iran, the percentage of *Nosema* positive samples changed according to regions and seasons and *N. ceranae* was the only *Nosema* species in studied colonies [40-43]. The study completed in Eastern part of world illustrated that the percentage of *N. apis* and *N. ceranae* varied from 28% to 61% in China and 33% and 73% in Taiwan, respectively [2]. All these studies above contained seasonal variation of *Nosema* spore loads but not during breeding projects in long term.

In Turkey, the survey studies for colony losses and diseases have shown that the presence of *Nosema* spores in different regions. The percentage of *Nosema* changed from 2% to 9% during years and regions according to these studies but the species of *Nosema* spores were not distinguished [44,45]. Up to now, many studies have reported the presence of *N. ceranae* and *N. apis* from different regions of Turkey [46-48]. Also the replacement of *N. apis* by *N. ceranae* was indicated [49-51].

Nosema ceranae is the only species in sampled colonies in our study. The sequence results for present study also showed high level identity of *N. ceranae* sequences from many European countries and also Australia, Iran, Lebanon, and Thailand according to BLAST database search.

This study is the unique in that the levels of *Nosema* infections were followed in colonies under selection for resistance to another pathogen. In the world, bee breeders in Denmark informed that they have aimed to improve resistance to *Nosema* spp. in their breeding stocks [52].

The infected colony numbers and spore loads were observed in three years. During the five seasons, the number of infected colonies and spores were decreased. In studied colonies, there were negative correlation observed between spores-temperature and positive correlation spores-relative humidity. But these correlations were not significant statistically. In conclusion the *Nosema* infection levels decreased under hygienic bee breeding programme for American Foulbrood disease but further monitoring studies should be performed in order to decide whether the *Nosema* spores decrease due to hygienic behavior. The level of *Nosema* spores observed in colonies under breeding programme for resistance to another pathogen is the unique and long term study in Turkey

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Research Article

Associations Between *GH, PRL, STAT5A, OPN, PIT-1, LEP* and *FGF2* Polymorphisms and Fertility in Holstein-Friesian Heifers [1]

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Abstract

In this study, it was aimed to investigate polymorphisms in seven genes (*GH, PRL, STAT5A, OPN, PIT-1, LEP* and *FGF2*) related to reproductive traits in dairy heifers. Frequency distributions of the genotypes between fertile and repeat breeder heifers groups were investigated. Allele effects on fertility were also analyzed. Blood samples were taken from a total of 160 Holstein-Friesian heifers and they were divided into two groups according to their artificial insemination numbers (AI). The heifers becoming pregnant after the first AI were used as the fertile heifers (FH, n=80) and the heifers with 3 or more equal AIs were accepted as the repeat breeder heifers (RBH, n=80). All the animals were genotyped by the PCR-RFLP method for seven genes and the association works were performed for 145 animals (RBH, n=79; FH n=66). For all loci investigated, two alleles and three genotypes were found for overall population with the exception that PRL locus had two alleles and two genotypes. The chi-square test (χ^2) revealed that the whole population and the two groups separately were at Hardy-Weinberg equilibrium. The genotype distributions of *PIT-1* and *STAT5A* conspicuously differed between the FH and the RBH groups; however, these differences were not found significant. Association of *GH*-AB genotype was found significant on AI number for the first pregnancy. Mixed effect logistic regression model was used to investigate the allele effects on fertility. No linkage disequilibrium was detected between the investigated loci.

Keywords: *Polymorphism, Fertility, Infertility, Dairy Heifers*

Holstein-Friesian Düvelerde Fertilite ile *GH, PRL, STAT5A, OPN, PIT-1, LEP* ve *FGF2* Polimorfizimlerinin İlişkileri

Özet

Bu çalışmada sütçü düvelerde reprodüktif özellikler ile ilişkili yedi gendeki (*GH, PRL, STAT5A, OPN, PIT-1, LEP* ve *FGF2*) polimorfizimlerin araştırılması amaçlanmıştır. Genotip frekanslarının fertil ve repeat breeder düve gruplarındaki dağılımı araştırılmıştır. Ayrıca fertilite üzerine allel etkisi de incelenmiştir. Toplam 160 Holstein-Friesian düveden kan alınmış ve bu düveler tohumlama sayılarına (ST) göre iki gruba ayrılmıştır. İlk tohumlamada gebe kalan düveler fertil düve (FH, n=80) olarak kullanılmış ve üç veya daha fazla ST'si olan düveler repeat breeder düve (RBH, n=80) olarak kabul edilmiştir. Tüm hayvanlar yedi gen bakımından PCR-RFLP metodu ile genotiplendirilmiş ve ilişkilendirme çalışmaları toplam 145 hayvanda yapılmıştır (RBH, n=79; FH, n=66). İki allel ve iki genotipin bulunduğu PRL hariç incelenen tüm lokuslarda iki allel ve üç genotip belirlenmiştir. Ki-kare sonuçları (χ2) tüm populasyonun ve ayrı ayrı grupların Hardy-Weinberg dengesinde olduğunu ortaya koymuştur. *PIT-1* ve *STAT5A* lokuslarının genotip frekanslarının dağılımları FH ve RBH grupları arasında belirgin biçimde farklı olmasına rağmen bu farklılık istatistiksel anlamda önemli bulunmamıştır. GH-AB genotipinin fertilite üzerine etkisi önemli bulunmuştur. Fertilite üzerindeki allel etkisini incelemek için karışık etkili lojistik regresyon analizi kullanılmıştır. İncelenen lokuslar arasında bir bağlantı dengesizliği belirlenmemiştir.

Anahtar sözcükler: *Polimorfizm, Fertilite, İnfertilite, Sütçü düve*

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INTRODUCTION

Although female reproduction is essential for the prolificacy of animal production, decreasing reproductive performance is one of the major problems in dairy industry [1,2]. As it is well known, the use of conception rate as an indicator of reproductive performance has decreased in the last decades [1]. Repeat breeder heifers (RBH) cause economic losses due to both the high insemination cost and the increased age of heifers at first calving, which is a source of complex health problems. Identifying dairy cattle with superior genetic potential for improved fertility might increase dairy farm profitability.

This dramatic reduction in reproductive performance of dairy cows is unlikely that this decline could be reversed only through improved management conditions [3]. In the minimization of these problems, the use of molecular markers may provide rapid genetic gains [4].

As Khatib et al.[5] noted, it might be useful to use the whole pathway rather than a single gene in a selection scheme. This seems logical due to the well-known multi-locus combined effect variations observed in quantitative traits. We selected seven different mutations in different genes involved growth, development and other essential actions for maintaining the pregnancy or other reproductive performance and examined their associations with the conception number for each pregnancy [6-16].

We targeted heifers for not only their economic importance but also avoiding evaluation of endocrinologic and oestrus problems found in repeat-breeder heifers [17]. The number of studies on heifer reproductive performance is limited [18]. The aim of the present study is to investigate the frequency distributions of seven loci, considered to be assocaiated with the reproductive traits, in the fertile and the repeat breeder Holstein heifer groups. We also aimed to examine the associations between these polymorphisms and fertility in dairy heifers.

MATERIAL and METHODS

The study was approved by the Ethics Committee of Uludag University (UUHADYEK), (approval date: 04.06.2013; no: 2013-11/1). This study was carried out in seven different lactating dairy farms located in the Marmara region of Turkey with an average 400-800 milking cows. The reproductive management of the dairy heifers in the farms was based on the artificial insemination following the estrus detection after spontaneous or $PGF_2\alpha$ (one or two doses of $PGF_2\alpha$ apart from 14 days) induced estrus. The first insemination age of the heifers was average 15 months in all the dairy farms. The artificial inseminations (AI) were performed by the farm veterinarians.

The Holstein-Friesian heifers (n=160) between 14-28 months of age were included in the study and the heifers were divided into two groups: fertile and repeat breeder. The heifers that became pregnant after the first artificial insemination (AI) were determined as the fertile group (FH, n=80) and the heifers with 3 or more equal AIs were placed in the repeat breeder group (RBH, n=80). The first and the second pregnancy checks were performed on the 30^{th} and 60^{th} days following the AI in the fertile heifers. If the embryonic loss was detected on the 60th day of the pregnancy check, the heifers were excluded from the fertile group. The blood samples were obtained from the coccygeal vein for DNA isolation.

A total of 160 Holstein Friesian heifers were analyzed for polymorphisms in the seven genes and seven different gene regions by using the PCR-RFLP method. However, the association works were performed for a total of 145 animals, 79 from the RBH group and 66 from the FH group. Fifteen of the 160 animals could not been genotyped for all loci. Due to this limitation these animals were not included to association analysis. The total DNA was extracted by using a genomic DNA purification kit (K0512, Fermentas, Lithuania) according to the instruction manual. The quantity and quality of the DNA were checked with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The primers and restriction enzymes used for PCR-RFLP analysis are given in *Table 1*.

The PCR amplifications were performed in reaction mixtures of 25 µl containing 12.5 µl of 2× PCR Master Mix (K0172, Fermentas, Lithuania), 0.5 µM of each primer and 25-75 ng of genomic DNA. The amplification was performed by using a Techgene Thermal Cycler (Techne, Cambridge, UK). The restriction enzyme digestions were performed according to the manufacturer's protocols. The digested restriction fragments were directly analyzed via electrophoresis on 2% and 2.5% agarose gels in 1XTBE buffer, stained with SafeView™ Classic (Applied Biological Materials Inc., Canada) and visualized under UV light.

The allele and genotype frequency calculations as well as the chi-square (χ^2) test were carried out by using the Popgene32 [26] program. The linkage disequilibrium between the investigated loci was analyzed according to Weir [27] by using the Popgene32 [26] program. The differences in the genotype frequency distribution between the FH and the RBH groups were analyzed by the likelihood ratio chi-square (χ^2) [28].

Mixed effect logistic regression in framework of generalized linear mixed model was applied to estimate the parameter of linear predictor contains vet and farms as random effects in addition to a set of our fixed explanatory variables (*OPN, STAT5A*, *GH*, *PIT1*, *PRL*, *FGF2*, *LEP*). Parameter estimates of both random and fixed effects were obtained by using the PROC GLIMMIX in SAS [29]. We used a CONTRAST option to test the hypotheses for the comparison of alleles within genotypes.

RESULTS

The distrubutions, numbers of the AIs and the ages of the heifers were shown in *Table 2* and numbers of the AIs and the ages of the heifers were greater (P<0.01) in the repeat breeder heifers (4.5±0.18 and 20.9±0.50) than in the fertile heifers (1.00±0.00 and 15.7±0.52, respectively). All the loci investigated were found to be polymorphic with two alleles and three genotypes for each with the exception that the *PRL* loci had two alleles and two genotypes *(Table 3)*. We found limited variation on the *LEP* locus with a quite low frequency of B allele. There was only one animal carrying BB genotype at this locus.

The observed allele and genotype frequencies and the expected heterozygosity values as well as the χ^2 values and the number of investigated individuals from each group are given for each investigated loci in *Table 3* and *Table 4*. The population was found to be in Hardy-Weinberg equilibrium for the investigated loci. The linkage disequilibrium anaylsis showed that there was no linkage disequilibrium between these loci.

The genotype distributions of some genes were different in the two groups *(Table 3)*. While genotype AB of *GH* was higher in RBH group, genotype AB and GC of *PIT-1* and *STAT5A* loci were higher in the FH group *(Table 3)*. While these differences were not significant for *PIT-1* and *STAT5A* loci, *GH* locus was found to be differ between groups (P=0.05). Heterozygote genotype (AB) at *GH* locus seems to be unfavorable for AI number for the first pregnancy. The heterozygote genotype (AB) at *GH* locus frequency of the FH group was different from that of the RBH group. Odds Ratios with 95% Wald confidence limits graphic and logistic regression graphics are given in *Fig. 1* and *Fig. 2*, respectively. The index plots of the Pearson residuals and the deviance residuals in *Fig. 2* indicate that no cases are poorly accounted for by the model, causing instability in all parameter estimates and goodness of fit. In addition, according to both logistic regression and association analysis, farms and inseminaters effects were found insignificant on fertility.

DISCUSSION

Two alleles and three genotypes were found for SNPs located between in exon 6 and intron 5 of the *PIT1* gene *(Table 3, Table 4)*. Similar to other studies on the B allele, a positive effect on growth and development

Table 1. *Gene locations of loci, size of PCR products, primer sets and restriction enzymes (RE) used for RFLP analysis*

Loci	Primers (5′ → 3′)	R. E	Location within Gene	PCR product size (bp)	References
FGF2	F: CATAGTTCTGTAGACTAGAAG R:CTCTAAAGAAGGATTAAGTCAAAATGGGGCTGGTA	*Csp6*I	Intron 1	207	[19]
OPN	F: GCAAATCAGAAGTGTGATAGAC R: CCAAGCCAAACGTATGAGTT	*Bse*NI	Intron 4	290	[20]
PIT1	F:AAACCATCATCTCCCTTCTT R:AATGTACAATGTGCCTTCTGAG	*Hinf*I	Between Intron 5-Exon 6	447	[21]
STAT5A	F:GAGAAGTTGGCGGAGATTATC R: CCGTGTGTCCTCATCACCTG	*Bst*EII	Exon 8	820	[22]
GH	F:CCCACGGGCAAGAATGAGGC R:TGAGGAACTGCAGGGGCCCA	*Msp*I	Intron 3	329	[23]
PRL	F:CCAAATCCACTGAATTATGCTT R:ACAGAAATCACCTCTCTCATTCA	*Rsa*I	Exon 4	294	[24]
LEP	F: AGTGTCTCTTGGGGCATTTT R: CCTGGGCTCCTATCTTTCTG	*Sau3A*I	Between Intron 2-Exon 3	1147	[25]

Table 2. *The numbers of the AIs and the ages of the heifers according to groups*

Farms	N		Age of Heifers*		Numbers AI of Heifers	
	FH	RBH	FH	RBH	FH	RBH
Farm 1	7	11	16.55±1.46	23.10±3.00	1.00±0.00	4.18±1.40
Farm 2	20	15	15.58±1.16	22.87±5.05	1.00±0.00	5.20±1.93
Farm 3	9	11	14.64±1.17	17.91±2.97	1.00±0.00	4.54±0.93
Farm 4	14	12	14.38±0.95	19.01±1.66	1.00±0.00	5.00±2.33
Farm 5	10	12	15.35±1.49	18.87±0.63	1.00±0.00	4.50±0.90
Farm 6	10	12	14.70±0.26	18.80±1.39	1.00±0.00	4.16±1.93
Farm 7	10	7	15.47±0.70	18.88±1.89	1.00±0.00	3.86±0.80

** Month,* ***FH:*** *Fertile Heifers;* ***RBH:*** *Repeat Breeder Heifers,* ***AI:*** *Artificial Insemination*

***Table 3**. Allele and genotype frequencies observed (Ho) and expected heterozygosity (He) as well as chi-square test values for all loci investigated for overall population*

Locus	N	Allele Frequency (%)					Genotype Frequency (%)									Ho	He	χ^2
		A	B	G	C	T	TC	TT	CC	GC	GG	AG	AA	AB	BB			
OPN	160				52.81	47.19	48.13	23.13	28.75							0.481	0.500	0.226^{ns}
STAT5A	160			50.94	49.06				23.13	51.88	25.00					0.519	0.501	0.193^{ns}
GH	160	83.75	16.25										70.00	27.50	2.50	0.275	0.273	0.0084^{ns}
PIT1	146	25.34	74.66										5.48	39.73	54.79	0.373	0.380	0.316^{ns}
PRL	159	11.01		88.99							77.99	22.01				0.220	0.196	2.356^{ns}
FGF2	146	34.25		65.75							43.84	43.84	12.33			0.438	0.452	0.132^{ns}
LEP	160	89.69	10.31										80.00	19.38	0.63	0.194	0.185	0.320^{ns}

***Table 4.** Allele and genotype frequencies, observed (Ho) and expected heterozygosity (He) and chi-square test values for all loci investigated according to groups*

Groups	Locus	N	Allele Frequency (%)					Genotype Frequency (%)									Ho	He	χ^2
			A	B	G	C	T	TC	TT	CC	GC	GG	AG	AA	AB	BB			
RBH	*OPN*	80				50.63	49.38	43.75	27.5	28.75							0.438	0.500	1.247^{ns}
	STAT5A	80			52.50	47.50				23.75	47.50	28.75					0.475	0.499	0.181^{ns}
	GH	80	80.63	19.38										62.5	36.25	1.25	0.363	0.312	2.055^{ns}
	PIT1	79	26.58	73.42										3.80	45.57	50.63	0.456	0.390	2.216^{ns}
	PRL	80	10.00		90.00							80.00	20.00				0.200	0.180	0.988^{ns}
	FGF2	79	37.98		62.03							39.24	45.57	15.19			0.456	0.471	0.084^{ns}
	LEP	80	88.75	11.25										78.75	20	1.25	0.200	0.200	0.000^{ns}
FH	*OPN*	80				55.00	45.00	52.5	18.75	28.75							0.525	0.495	0.294^{ns}
	STAT5A	80			49.38	50.63				22.50	56.25	21.25					0.563	0.500	1.254^{ns}
	GH	80	86.88	13.13%										77.5	18.75	3.75	0.188	0.228	2.529^{ns}
	PIT1	67	23.88	76.12%										7.46	32.84	59.70	0.328	0.364	0.628^{ns}
	PRL	79	12.03		87.98							75.95	24.05				0.241	0.212	1.476^{ns}
	FGF2	67	29.85		70.15							49.25	41.79	8.96			0.418	0.419	0.000^{ns}
	LEP	80	90.63	9.38										81.25	18.75		0.188	0.170	0.856^{ns}

as well as on growth hormone expression was reported and found to be predominant [30]. The allelic frequency of this locus is in line with previous studies [30-32]. Although the allelic frequencies of this locus in the FH and the RBH groups were similar, the genotype distribution was different *(Table 4)*. The number of AB genotype animals was higher than the number of those in the RBH group for *PIT1* loci such as the heterozygote genotype of *GH (Table 4)*.

The associations between the A→G SNP in the *FGF2* gene and fertilization and embryonic survival were reported by Khatib et al.[19]. They found a higher embryonic survival rate among the embryos produced by the GG genotype compared to the dams with the AG and AA genotype. In another study [18], no relationship was found between SNP 11646 and the reproductive, productive and health traits in cows. In the present study, we found the favorable G allele of *FGF2* gene to be predominant in both the overall population and two separate investigated groups *(Table 3, Table 4)*.

As it is seen in *Table 3* and *Table 4*, the frequency of the C allele was slightly higher than the others [20,33]. Furthermore, genotype and allele frequency distrubitions were not differed between groups for *OPN* locus in our study. The allelic frequency of this locus was found to be similar in the studies where the frequency of the T allele was higher, with the exception of Jersey cows having a much higher C allele frequency [34].

Two alleles and three genotypes were also detected in exon 4 of the *PRL* locus resulting in the A→G nucleotide substitution in the synthesized protein *(Table 3, Table 4)*. The G allele and the GG genotype were found to be predominant. The frequency of the G allele was found to vary between 0.61-0.914 in previous studies [35,36]. The reverse was observed for only the Shimal and Jersey breeds with frequencies of 0.49 and 0.294, respectively [35,36].

Polymorphisms in *STAT5A* and their associations with the reproductive and other economically important traits were investigated [5,15]. Of them, the C→G transversion in

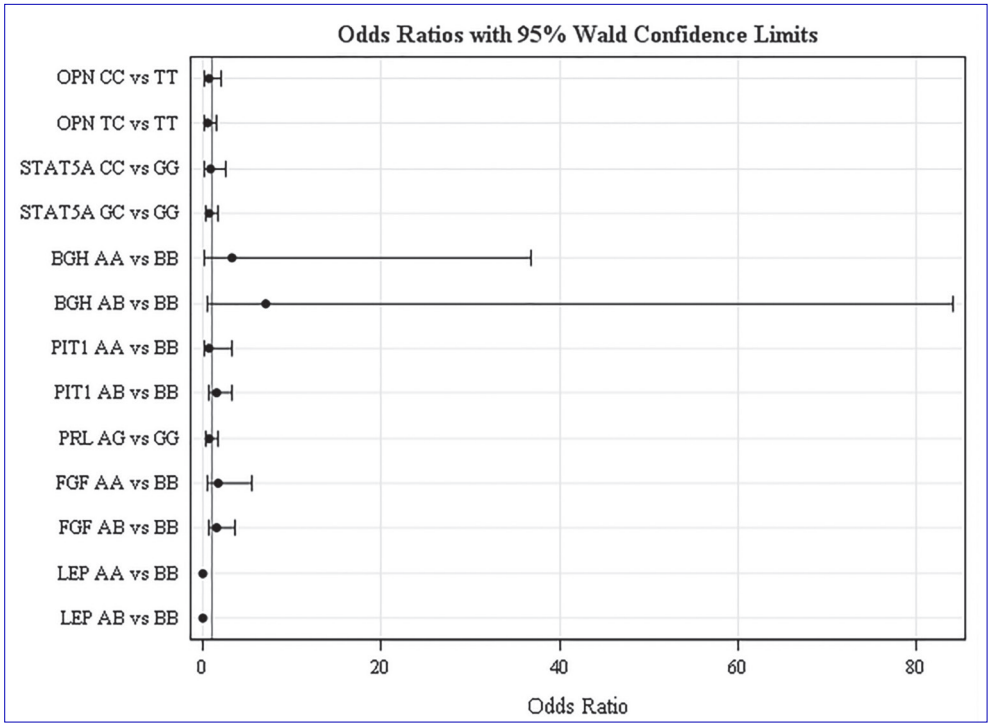

Fig 1. Odds ratios with %95 wald confidence limits

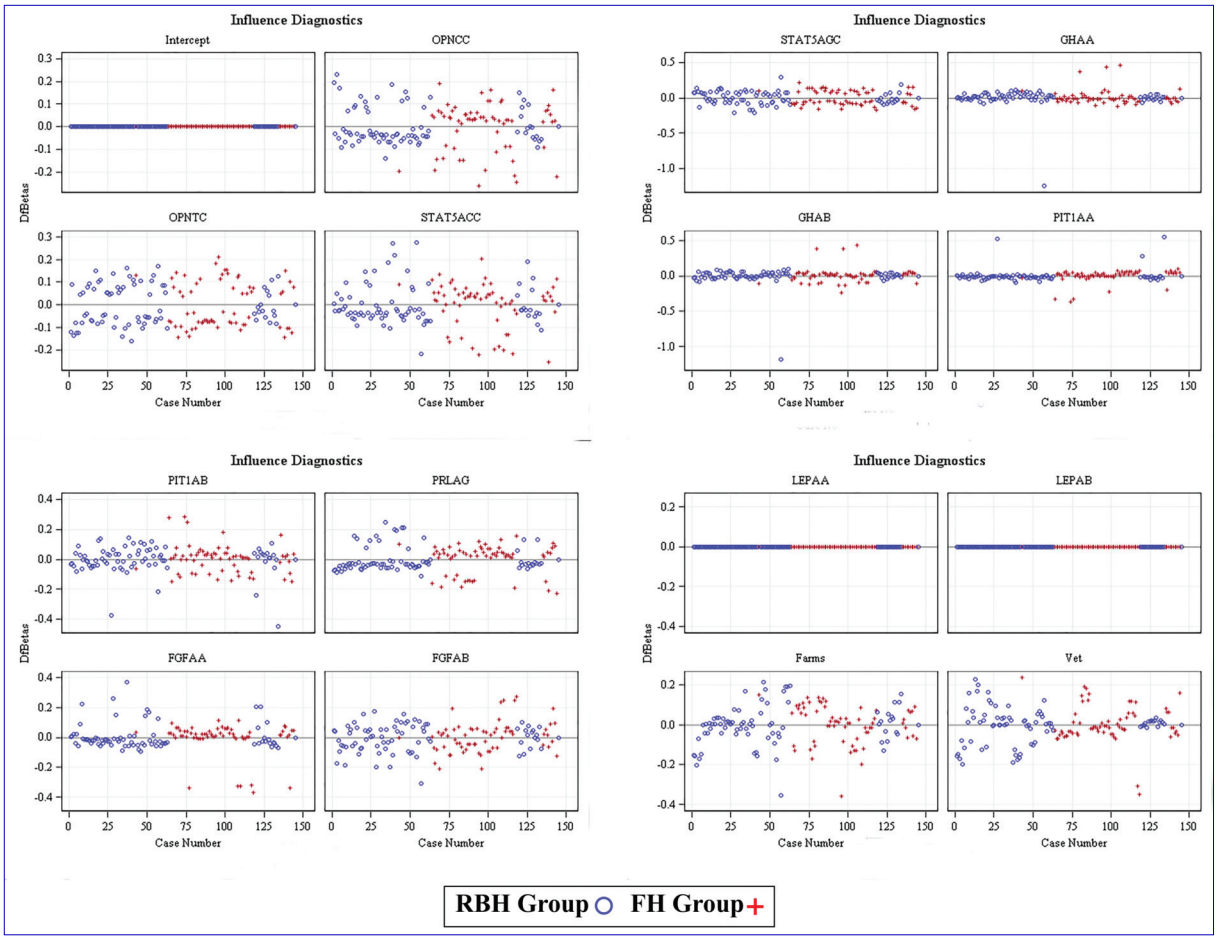

Fig 2. Influence diagnostics in logistic regression

Exon 8 of the *STAT5A* gene was found to be associated with the influence on embryonic survival in cattle [5,37] and previous reports on the expression of this gene support this finding [38]. In our study, the frequencies of the C and G alleles were the same for the two groups *(Table 4)*. On the other hand, while the C allele was present in the FH group, the unfavourable G allele frequency was higher in the RBH group. The genotypic frequencies for GC and GG also seemed different, but this difference was not significant *(Table 4)*.

Due to its key role in energy metabolism, the *LEP* gene was also investigated a lot. In the promoter and protein coding regions, several SNPs and microsatellite were reported. Polymorphisms in promoter region of the gene were found associated with the food intake leptin concentations, energy metabolisms and the reproductive parameters [39,40]. One of the most frequently investigated polymorphism was *Sau3AI* RFLP in the region located between intron II and exon II of the *LEP* gene. In the majority of previous studies, this polymorphism was not found influential on the investigated traits such as the age of puberty, BCS, milk production and properties [25,41,42]. We did not find any relationships between AI numbers for the first pregnancy, either. The results for allele and genotype frequencies were also in line with those studies. Allele A was predominant while the frequency of allele B was quite low. According to these results, it can be stated that the allele and genotype distributions were not suitable for the association analysis and this polymorphism was not suitable for being a marker in the selection scheme.

A strong relationship was suggested between the growth hormone circulation and the calving interval [43]. Therefore, polymorphisms on this gene seem to be potential selection criteria for improving reproductive performance. Mullen et al.[44] found six SNPs in 5'UTR region of *GH* in Irish Holstein Frisian cows and reported that some of these polymorphisms were associated with the reproductive traits. Some restriction fragment length polymorphims were also reported on Bovine *GH* locus [45-47]. One of these ploymorphisms in intron III of the gene creates *Msp*I recognizing site and was intensively studied due to its location near a transcription-binding site [23]. Various investigations revealed associations between *GH-Msp*I polymorphism and both famele and male reproductive traits [48-50]. At the same time, it was observed that the distribution of this polymorphism obviously differed between geographic regions [51,52]. The A allele was predominant among breeds from Europe while the frequency of B allele was higher among Bos indicus cattle [51,52]. The B allele was found related to meat quality and the lower frequencies of the allele among Holstein-Frisian cows were explained with this finding [23]. In line with this, the A allele was also found to be predominant in the Holstein Friesian heifer population investigated in our study *(Table 3, Table 4)*. The allelic frequency of the A allele was similar for the heifer groups *(Table 4)*. On the other hand, the genotypic distribution of *GH-Msp*I polymorphism differed between groups. While the frequency of the AB genotype in the RBH group was much higher than the FH group in our study, the BB genotype was observed in the RBH group at very low frequency *(Table 4)*. According to the statistical analyses, the difference between the frequency distributions of the groups was significant. Significant associations were found between the testis quality and the *GH-Msp*I polymorphism in previuos studies carried out on male fertility [48,49]. Arango et al.[50] reported a strong relationship between *GH-Msp*I genotypes and weight in the first estrus and first calving. Associations between the AB genotype and the milk components and the somatic cell counts were also reported [45,47]. In the literature, we've encountered no studies on associations between reproductive performance and *GH-MspI* polymorphism in heifers. Our results were in line with those of some previous studies revealing the opportunity of *GH-Msp*I polymorphism in the selection scheme.

No relationships were found between the *PRL, STAT5A, OPN, PIT-1*, *LEP* and *FGF2* polymorphisms and the AI numbers for the first pregnancy except for the *GH-Msp*I locus. On the other hand, the genotype distributions of *PIT-1* and *STAT5A* loci were also quite different between these two heifer groups. These differences were not statistically significant.

It can be suggested from these results that studies covering more individuals with an extended dataset should be performed to determine more accurate relationships. These polymorphisms may be important for the improvement of reproductive traits. Gene regions effect reproductive and productive traits should be also investigated in native breeds to reveal genetic composition of these breeds as some investigation groups have already done [53,54].

To enhance animal reproductive performance management of environmental conditions may be more expensive or unsustainable. It would be better to produce genetically valuable herds for a more profitable dairy industry. The loci investigated in this study were located on the strong candidate genes for reproductive performance. We observed differences in *PIT-1* and *STAT5A;* nevertheless, we could not prove these differences statistically. Studies with more animals from each heifer group will reveal the accuracy of these loci. On the other hand, we found a possible effect of polymorphism in *GH-Msp*I locus on the fertility in Holstein heifers, which is in line with previous studies finding associations between the *GH-Msp*I locus polymorphism and the reproductive traits. We suggest that *GH-Msp*I locus may be used as the selection criterion in breeding programs and phenotypic effects on herds should be monitored. There is also a need for studies to be made by using more animals, phenotypic data and epigenetic tools to prove this possible relationship.

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Research Article

Antimicrobial Susceptibility Profiles and Coagulase Gene Polymorphism of *Staphylococcus aureus* Isolated from Bovine Subclinical Mastitis [1] [2]

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Abstract

The purpose of the study was to isolate *Staphylococcus aureus* from bovine subclinical mastitis, determine their antibiotic susceptibilities and investigate the coagulase gene polymorphism by using a PCR-based restriction fragment length polymorphism (RFLP) method. Milk samples from 463 CMT positive udders from 237 cows cultured. The antimicrobial susceptibility of the isolates were determined by disc diffusion method. A total of 82 out of the 83 isolates (98.8%) were found to be resistant at least one out of the 16 antibiotics studied. In this experiment 53 isolates (63.8%) were found to be resistant to penicillin; 52 (62.67%) to trimethoprim/sulphamethoxazole; 51 (61.5%) to ampicillin; 40 (48.2%) to erytromycin; 29 (34.9%) to tetracycline; 18 (21.6%) to ciprofloxacin, 16 (19.3%) to clindamycin, 13 (15.6%) to chloramphenicol; 8 (9.6%) to gentamicin; 5 (6.0%) to cefoxitin; 4 (4.9%) to vancomycin; 3 (3.6%) to cephalotin; 2 (2.4%) nafcillin; one (1.2%) to oxacillin and one to (1.2%) furazolidon. No imipenem resistance was seen in the *S. aureus* isolates. The coagulase gen polymorphism were examined by PCR amplification of coagulase gene followed by *Alu*I digestion of repeating 81 bp DNA sequences. After nested PCR, double bands were produced in 8 of the isolates while there were single band in remaining 75 isolates. Following *Alu*I digestion, isolates that formed single band in length of approximately 300 bp showed 3 different groups.

Keywords: *Bovine subclinical mastitis, Staphylococcus aureus, Antibiotic susceptibility, Coagulase gene polymorphism*

Subklinik Mastitli İneklerden İzole Edilen *Staphylococcus aureus* İzolatlarinin Antibiyotik Duyarlılık Profillerinin Çıkarılması ve Koagulaz Geni Polimorfizmine Göre Tiplendirilmesi

Özet

Bu çalışmanın amacı subklinik mastitisli sığırlardan *Staphylococcus aureus*'u izole etmek, bunların antibiyotiklere duyarlılığını belirlemek ve bir PCR tabanlı restriksiyon fragment lengh polimorfizmi (RFLP) yöntemi kullanarak koagulaz gen polimorfizmi araştırmaktır. 463 sığırdan CMT pozitif olan 237 sığır memesinden süt örnekleri alınarak ekim yapılmıştır. İzolatların antimikrobiyal duyarlılığı disk difüzyon yöntemi ile belirlenmiştir. Toplam 83 izolatın 82'si (%98.8) uygulanan 16 antibiyotikten en az bir antibiyotiğe dirençli bulundu. Bu çalışmada 53 izolat (%63.8) penisiline, 52 izolat (%62.67 trimethoprim/sulphamethoxazole, 51 izolat (%61.5) ampisiline, 40 izolat (%48.2) eritromisine, 29 izolat (%35.0) tetrasikline, 18 izolat (%21.7) siprofloksasine, 16 izolat (%19.3) klindamisine, 13 izolat (%15.6) kloramfenikole, 8 izolat (%9.6) gentamisine, 5 izolat (%6.0) sefoksitine, 4 izolat (%4.9) vankomisine, 3 izolat (%3.6) sefalotine, 2 izolat (%2.4) nafsiline ve 1'er (%1.2) izolat ise oksasilin ve furazolidona dirençli bulundu. *S. aureus* izolatlarında imipenem dirençliliği görülmedi. Koagulaz gen polimorfizmi koagulaz genin tekrarlanan 81 bp DNA dizisinin *Alu*I sindirimini müteakiben koagulaz genin amplifikasyonu ile incelenmiştir. Nested PCR'den sonra izolatların 8'inde çift bant görülmüş kalan 75 izolatta ise tek band vardı. *Alu*I sindirimini müteakiben yaklaşık 300 bp uzunluğunda tek bant oluşturan izolatlar 3 farklı grup göstermiştir.

Anahtar sözcükler: *Sığır subklinik mastitis, Staphylococcus aureus, Antibiyotik duyarlılığı, Koagulaz gen polimorfizmi*

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INTRODUCTION

Staphylococcus aureus has a wide range host spectrum and can cause serious infections by its methicillin resistant isolates. Long-term antibiotic usage is important in development of resistance against methicillin and other beta-lactam antibiotics. *S. aureus* is one of the most important pathogen for cattle mastitis and it is prevalent all around the world. Despite of strict control measures, control and eradication of *S. aureus* caused intramammary infections are quite difficult and continue as an economical problem. Antimicrobial therapy is one of the measures can be taken in order to control of staphylococcal mastitis. Detection of antibiotic susceptibilities of clinical isolates is necessary not only for treatment but also for preventing spread of resistant isolates. Regional evaluation of antibiotic susceptibilities of *S. aureus* may help veterinary surgeons [1,2]. Although most of the current *S. aureus* isolates have different genotypic and phenotypic characteristics, few are known about geographical distribution of those isolates and types of the pathogens in the herd [3]. Previously, distinct classification methods such as phage typing had been applied to both human and cattle originated *S. aureus* isolates [4,5]. Afterwards, methods such as plasmid analysis, ribotyping, pulsed-field gel electrophoresis, PCR- based fingerprinting, amplification of specific gene regions, and binary typing technics were started to be applied [1,4,6-8]. In recent years, there are some publications about genetic diversity of *S. aureus* isolates in Turkey recovered from subclinical cattle mastitis cases [2,9-13]. The aim of this study was to evaluate the biochemical capacity of the antibiotic resistances of *S. aureus* isolates recovered from subclinical cattle mastitis cases in the Middle Western Anatolia and perform molecular typing on coagulase gene polymorphism.

MATERIAL and METHODS

Bacteriological Studies

Milk samples were collected from 16 different dairy farms located in four different districts of Middle Western Anatolia between January-June 2010. Milk samples were collected in the mid-lactation period. California Mastitis Test (CMT) positive 463 milk samples were collected from 237 cows. The samples were inoculated onto Nutrient agar supplemented with 7% sheep blood, incubated at 37°C for 24-48 h. Eighty three *S. aureus* has been isolated and identified by the conventional tests such as Oxidase, catalase and coagulase positive (slide and tube), susceptibility to furazolidone, hemolysis, pigment formation, O/F, Baird Parker Agar (BP), Egg yolk tellurit, Mannitol Salt Agar (MSA), DNase Agar [14,15].

Gram positive cocci were further identified with conventional biochemical test and API Staph (Bio Merieux, France). The isolates were kept at -70°C in Trypticase Soy Broth (TSB) containing 15% glycerine in order to further use in molecular studies. Antimicrobial suscebtibility tests of the isolates were performed in accordance with National Committee for Clinical Laboratory Standards-NCCLS [16]. The isolates were tested against to the following antibiotics: penicillin (10 IU), gentamicin (10 µg), vancomycin (30 µg), clindamycin (2 µg), trimethoprim/sulfamethoxazole (1.25 µg/23.75 µg), cefalotin (30 µg), imipenem (10 µg), nafcillin (1 µg), furazolidone (100 µg), ampicillin (10 µg), tetracycline (30 µg), oxacillin (1 µg), chloramphenicol (30 µg), cefoxitin (30 µg), erythromycin (15 µg) and ciprofloxacin (5 µg). *S. aureus* ATCC 25923 was used as control strain [15]. Chi square test was used to evaluate the significance between antimicrobial sensitivities or resistances of *S. aureus* isolates.

Molecular Studies

DNA extractions from *S. aureus* isolates were performed by using genomic DNA purification kit (Bio Basic Inc., Totonto, Canada). In addition to the protocol, 11 U lysostaphin was added during lysis phase. Multiplex PCR developed by Maes et al.[17], were performed for the comfirmation of *S. aureus* identification, and for the detection of methicillin resistance. Briefly 2 µL of mecA and nuc primers (10 µmol), 3 µL of 16S rRNA specific primers (10 µmol), 5 µL of dNTP mixture (2.5mM), 5 µL of 10xPCR buffer, 4 µL of $MgCl_2$ (25mM), 0.4 µL of Taq polymerase (TaKaRa, Tokyo, Japan) and 2.5 µL of template DNA were added to PCR mixture and made up to 50 µL by adding distilled water. Amplification conditions consisted of 10 min at 94°C, followed by 23 cycles of 1 min at 94°C, 1 min at 51°C, and 2 min at 72°C, with a final step of 5 min at 72°C. The amplified DNA fragments were evaluated following the gel electrophoresis on a 1.5% agarose (Bio Basic Inc., Totonto, Canada) gel stained with ethidium bromide. Nested PCR followed by *Alu*I restriction enzyme dependent RFLP method was used to determine the polymorphism in coagulase gene regions of *S. aureus* isolates [18]. Primers to replicate the coagulase gene region described previously by Goh et al.[18] were used in nested-PCR assays. PCR mixture was prepared by adding 2 µL of each COA1 and COA4 primers (10 µmol), 5 µL of dNTP mixture (2.5mM), 5 µL of 10xPCR buffer, 4 µL of $MgCl_2$ (25mM), 0.4 µL of Taq polymerase (TaKaRa, Tokyo, Japan) and 26.6 µL ultra distilled water to obtain a 50 µL of final mixture, subsequently 5 µL of DNA extract was added and amplified. Fifty µL of similar mixture was prepared for the second cycle of nested PCR but this time 2 µL of COA2 and COA3 primers (10 µmol) were used as primers and 1.5 µL PCR product obtained from previous amplification as target DNA. DNA amplification was performed by pre-denaturation at 95°C for 5 min followed by 40 amplification cycles of 95°C for 30 sec, 55°C for 2 min, 72°C for 4 min and final extension at 72°C for 5 min. Ten µl of nested PCR product was digested by *Alu*I restriction enzyme (TaKaRa, Tokyo, Japan) according to the manufacturer's recommended protocol. Both PCR

product and restriciton digest fragments were detected by electrophoresis through a 3% agarose gel with Φx 174-Hae III Marker (TaKaRa, Tokyo, Japan) and 100 bp marker (Bio Basic Inc., Totonto, Canada).

Note: Ethics committee approval has been taken from AKU HADYEK on 01.04.2010 with the number 81.

RESULTS

Bacteriological Studies Results

Eighty-three *S. aureus* were isolated from milk samples. According to the results of susceptibility tests, 82 out of 83 isolates (98.8%) were resistant at least one of 16 antimicrobial agents involved in the study. In this experiment 53 isolates (63.8%) were found to be resistant to penicillin; 52 (62.67%) to trimethoprim/sulphamethoxazole; 51 (61.5%) to ampicillin; 40 (48.2%) to erytromycin; 29 (34.9%) to tetracyclin; 18 (21.6%) to ciprofloxacin, 16 (19.3%) to clindamycin, 13 (15.6%) to chloramphenicol; 8 (9.6%) to gentamicin; 5 (6.0%) to cefoxitin; 4 (4.9%) to vancomycin; 3 (3.6%) to cephalotin; 2 (2.4%) nafcillin; one (1.2%) to oxacillin and one to (1.2%) furazolidon. No imipenem resistance was seen in the S. aureus isolates. There were significant differences between antimicrobial sensitivities of *S. aureus* isolates (χ^2=459.03; P<0.01). Fourty one isolates were multidrug resistant (resistant to four and/or more antimicrobial agents). The antimicrobial susceptibilities of those isolates were shown in *Table 1*.

Table 1. *Antimicrobial susceptibilities of S. aureus isolates*

Antibiotic Disc	Disc Content (µg)	Susceptible (%)	Moderate (%)	Resistant (%)
Penicilin	10 Units	36.2	0.0	63.8
SXT*	25	33.7	3.6	62.7
Ampicillin	10	37.3	1.2	61.5
Erythromycin	15	47.0	4.8	48.2
Tetracycline	30	59.0	6.0	34.9
Ciprofloxacin	5	68.6	9.6	21.6
Clindamycin	2	72.3	8.4	19.3
Chloramphenicol	30	79.5	4.9	15.6
Gentamicin	10	84.3	6.1	9.6
Cefoxitin	30	94.0	0.0	6.0
Vancomycin	30	85.5	9.6	4.9
Cefalotin	30	95.2	1.2	3.6
Nafcillin	1	96.3	1.2	2.5
Oxacillin	1	98.8	0.0	1.2
Furazolidon	100	95.2	3.6	1.2
Imipenem	10	100	0.0	0.0
P	-	<0.01		<0.01

**Trimethoprim/sulfamethoxazole*

Molecular Studies Results

As the result of multiplex PCR, presence of nuc gene was determined and *S. aureus* identifications were comfirmed in 83 isolates which were identified by conventional methods previously. One isolate which was determined to have phenotypic resistance to meticillin was also genotypically positive in presence of mecA. All 83 *S. aureus* isolates showed differences in coagulase gene region polymorphism. After nested PCR, double bands were produced in 8 of the isolates while there were single band in remaining 75 isolates. Following *Alu*I digestion, isolates that formed single band in length of approximately 300 bp were also showed 3 different groups. Fragments of 81 bp or multiplies were evaluated for RFLP type classification *(Table 2)*. As the result of RFLP typing out of 55 isolates from district A (4 farms), 44 isolates were type II and 11 isolates were type I. Out off 15 isolates from district B (5 farms) eight of them were type I, four of them were type II and three of them were type III in RFLP profile. Isolates from district C (3 farms) were distributed into type II and type IV profiles. And finally the isolates from district D (4 farms) were belonging to type I and type IV. The antimicrobial resistnace profiles within the groups were shown in *Table 3*.

As a consequence, according to antibiogram results, staphylococci have gained resistance to some commonly used antibiotics. That's why it is recommended that it should not be used antibiotic without making an antibiogram.

DISCUSSION

β-Lactam antibiotics are commonly used in cattle mastitis treatment. Penicillin resistance may be related to national policies about usage of antimicrobial drugs and differencies about animal raising systems [5]. The highest penicillin resistance was reported to be in Ireland (71.4%) and England (67.3%) within the European countries followed by 50% in USA. It was rather low in Denmark (18.7%) and in Norway (2%) which were the Scandinavian countries [5,19]. It was also shown by Sori et al.[20] that resistance against penicillin was quite high (87.2%) in South-West Ethiopia. There are studies about cattle mastitis showing that penicilin resistance is high in staphylococcus bacteria from isolates in different regions of Turkey. Guler et al.[2] reported the highest resistance was against penicilin and ampicillin as high as 63.3%. In Aydin region resistance against penicillin, SXT and erythromycin were 81%, 17% and 7% respectively [21]. In another study from different region, resistance of *S. aureus* isolates against penicillin G, tetracycline, erythromycin and oxacillin were 85.4%, 39.6%, 5.2% and 3.1%, respectively in mastitic cow milk [10]. The results of this study showed that penicillin resistance (63.86%) in Middle Western Anatolia of Turkey was higher than other antibacterials. This situation is relatively similar to that in other countries such as Ireland, England and USA. It is quite higher than those in Denmark

Table 2. *Typing of isolates based on PCR and AluI digestion*

Number of Isolates (%)	RFLP Type	Sizes of PCR Products (approx.bp)	*Alu*I Profiles*		
			81 (bp)	162(bp)	243(bp)
21 (25.3%)	Type-I	300	+	+	+
51 (61.4%)	Type-II	300	+	-	+
3 (3.6%)	Type-III	300	-	-	+
8 (9.6%)	Type-IV	290, 870	+	-	+

** Results for only fragments of 81 bp or multiples are shown*

Table 3. *Distribution of S. aureus isolates within dairy farms, antimicrobial sensitivities and RFLP types*

Origin of the Samples (number of farms)																	
	Antimicrobial Agent	A(4)				B(5)				C(3)				D(4)				Total
		RFPL Type				RFPL Type				RFPL Type				RFPL Type				
		I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV	
Number of antimicrobial resistant isolates	P	6	33	-	-	5	4	1	-	-	-	-	1	1	-	-	2	53
	GM	3	2	-	-	1	-	-	-	-	1	-	-	-	-	-	-	7
	VA	2	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	4
	DA	1	7	-	-	4	-	-	-	-	-	-	1	2	-	-	1	16
	SXT	7	30	-	-	5	-	2	-	-	2	-	4	-	-	-	2	52
	CF	1	1	-	-	-	-	-	-	-	-	-	1	-	-	-	-	3
	IPM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	NAF	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1
	FX	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1
	AMP	7	29	-	-	5	2	2	-	-	1	-	3	1	-	-	1	51
	TE	3	15	-	-	6	2	1	-	-	-	-	1	-	-	-	2	30
	OX	-	2	-	-	1	-	-	-	-	-	-	2	-	-	-	-	5
	C	1	8	-	-	1	-	1	-	-	1	-	-	1	-	-	1	14
	CTX	-	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6
	E	5	24	-	-	7	2	-	-	-	2	-	1	1	-	-	1	43
	CIP	4	8	-	-	1	-	-	-	-	2	-	1	-	-	-	1	17
RFPL Type	I	11	-	-	-	8	-	-	-	-	-	-	-	2	-	-	-	21
	II	-	44	-	-	-	4	-	-	-	3	-	-	-	-	-	-	51
	III	-	-	-	-	-	-	3	-	-	-	-		-	-	-		3
	IV	-	-	-	-	-	-	-	-	-	-	-	6	-	-	-	2	8

***P:** penicillin, **GM:** gentamicin, **VA:** vancomycin, **DA:** clindamycine, **SXT:** trimethoprim/sulfamethoxazole, **CF:** cefalotin, **IPM:** imipenem, **NAF:** nafcillin, **FX:** furazolidone, **AMP:** ampicillin, **TE:** tetracycline, **OX:** oxacillin, **C:** chloramphenicol, **CTX:** cefoxitin, **E:** erythromycin, **CIP:** ciprofloxacin, **-;** no growth*

and Norway. Besides the penicillin, resistance against trimethoprim/sulphamethoxazole and ampicillin were considerably high. These antimicrobial agents are usually preferred for the treatment of mastitis cases in Turkey. Coagulase production is an important phenotypic specification to identify *S. aureus*. Gene coagulase is the most important virulance factor for *S. aureus*. It is reported that both counts and localisations of *Alu*I restriction regions in 3'ends of gene coagulase contain a sequence of 81 base pairs which is different between *S. aureus* isolates [18]. The classification of *S. aureus* isolates depending on gene coa is a simple method for molecular typing and can be assumed as a validation test [18,22]. Aslantas et al.[9] reported that RFLP models of gene coagulase of *S. aureus* isolates from cattle mastitis showed a great diversity. They determined that coagulase gene polymorphism of *S. aureus* isolates from mastitic cow milk by RFLP method using *Alu*I enzyme produced 9 different genotype strains. Rodrigues da Silva and Silva [22], reported that there were 49 different types of RFLP samples after digestion with *Alu*I enzyme. Raimundo et al.[23] examined coa gene type of 151 samples of cattle *S. aureus* isolates from 76 farms

by using Coag2 and Coag3 primers and found 6 types of PCR. Su et al.[3] investigated coa gene diversity in *S. aureus* isolates from 4 countries. They reported 5 genotypes were dominant for each country. However, dominant types changed according to the geographical regions. Karahan and Cetinkaya [24] reported that 83.9% were produced single band and 16.1% produced 2 bands after coa gene amplification in PCR results of 161 coa positive *S. aureus* isolates. They found that 23 different types of restriction profiles in RFLP results by using *Alu*I enzyme. Guler et al.[2] investigated 125 *S. aureus* isolates which had antibiotic resistance and found that there were 4 types of coagulase gene. In the present study, 90.4% of the isolates produced single band and 9.6% of them produced double bands after coa gene amplification. In this study, 51 out of 83 *S. aureus* isolates were obtained from 7 dairy farms and each of them produced bands approximately 300 bp in length. After RFLP those isolates produced 2 bands (81, 243 pb). Remaining 32 isolates were obtained from 9 different dairy farms and produced 2 types of PCR products of 300 and 290, 870 bp. It was also found that 24 samples to have 2 different types of 300 bp product after RFLP (81, 162, 243 bp and 243 bp) while PCR products of 290, 870 bp in length were produced 2 bands of 81, 243 bp in length after RFLP. This study showed that there were 4 different types of *S. aureus* as Type I, II, III and IV in Middle Anatolia region upon classification by PCR-RFLP. Besides Type II was the most common one to be present in 61.4% of the isolates. Eventually, 33 out of 51 Type II isolates were found to be resistant to penicilin. 6 out of 8 Type IV isolates were resistant to trimethoprim/sulfamethoxazole and, 13 out of total 21 Type I isolates were resistant to ampicillin amongst antibiotics involved in the trial. All of 83 isolates were sensitive to imipenem. However, only 1 isolate were resistant to nafcillin and furazolidon *(Table 3)*.

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Research Article

Inactivation Effect of Probiotic Biofilms on Growth of *Listeria monocytogenes*

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Abstract

Probiotic lactic acid bacteria and their biofilms have antagonistic activity against food spoilage organisms and pathogenic bacteria. Recently, researchers focused on the use of probiotic biofilms for inhibition of pathogenic bacteria. The aim of this research is to improve probiotic biofilms with optimal prebiotic concentration and to determine their inactivation effect on both planktonic cells and biofilm growth of *Listeria monocytogenes*. Biofilm formations were detected by using microplate method. Prebiotic ingredients were used to form biofilm with highest viable probiotic cell counts and optimal concentrations of prebiotic ingredients were determined according to the response surface method. Biofilm produced by *Lactobacillus casei* Shirota and *Lactobacillus rhamnosus* contained 9.46 and 9.66 log cfu/mL viable cell counts, respectively. Optimal prebiotic concentrations were found 3% casein peptone-0% fructo-oligosaccharides (FOS) for biofilm formation with highest viable cell counts by *L. casei* Shirota and 1.5% casein peptone-1.5% FOS for biofilm formation with highest viable cell counts by *L. rhamnosus*. Probiotic biofilms exhibited inactivation against growth of *L. monocytogenes* and caused a reduction of 0.66- 2.01 log cfu/mL for planktonic *L. monocytogenes* and 0.40-1.69 log cfu/mL for *L. monocytogenes* biofilm. Planktonic cells of *L. monocytogenes* were observed to be more susceptible to probiotic biofilms than biofilm of *L. monocytogenes*. Biofilm of *L. rhamnosus* showed higher inhibition effect on *L. monocytogenes* growth than *L. casei* Shirota. These findings showed that biofilms of probiotic *Lactobacillus* strains used in this study may be excellent candidate for controlling of pathogenic bacteria.

Keywords: *Biofilm, Probiotic, L. monocytogenes, Inhibition of pathogens*

Listeria monocytogenes'in Gelişimi Üzerine Probiyotik Biyofilmlerin İnaktivasyon Etkisi

Özet

Probiyotik laktik asit bakterilerinin ve biyofilmlerinin gıdaları bozucu organizmalara ve patojen bakterilere karşı antagonistik etkileri bulunmaktadır. Son zamanlarda araştırmacılar patojen bakterilerin inhibisyonu için probiyotik biyofilmlerin kullanımı üzerine yoğunlaşmışlardır. Bu çalışmanın amacı, ideal prebiyotik konsantrasyonuyla probiyotik biyofilmleri geliştirmek ve *Listeria monocytogenes*'in hem planktonik hücrelerinin hem de biyofilmleri üzerine probiyotik biyofilmlerin inaktivasyon etkisini belirlemektir. Biyofilm oluşumları mikroplak yöntemi kullanılarak uygulanmıştır. Prebiyotik katkılar en yüksek canlı probiyotik hücre sayılı biyofilmleri oluşturmak için kullanılmış ve prebiyotik katkıların ideal konsantrasyonları cevap yüzey tekniğine göre belirlenmiştir. *Lactobacillus casei* Shirota ve *Lactobacillus rhamnosus* tarafından üretilen biyofilmler sırasıyla 9.46 log kob/ml ve 9.66 log kob/mL canlı hücre sayısı içermiştir. *L. casei* Shirota ve *L. rhamnosus* tarafından en yüksek canlı hücre sayılı biyofilm oluşumu için ideal prebiyotik konsantrasyonları sırasıyla %3 kazein pepton-%0 FOS ve %1.5 kazein pepton-%1.5 FOS olarak bulunmuştur. Probiyotik biyofilmler *L. monocytogenes* gelişimine karşı inaktivasyon sergilemiştir ve *L. monocytogenes*'in planktonik hücrelerinde 0.66-2.01 log kob/mL'lik ve biyofilmlerinde 0.40-1.69 log kob/mL'lik bir azalışa neden olmuştur. *L. monocytogenes*'in planktonik hücreleri probiyotik biyofilmlerine *L. monocytogenes*'in biyofilmlerinden daha duyarlı bulunmuştur. *L. rhamnosus* biyofilmi *L. monocytogenes* gelişimi üzerine *L. casei* Shirota'dan daha yüksek bir inhibisyon etkisi göstermiştir. Bu bulgular bu çalışmada kullanılan probiyotik türlerin biyofilmlerinin patojen bakterilerin kontrolünde çok iyi birer aday olabileceğini göstermiştir.

Anahtar sözcükler: *Biyofilm, Probiyotik, L. monocytogenes, Patojen inhibisyonu*

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INTRODUCTION

Prebiotics are chemical food ingredients, which support colonization of probiotics and in recent years there has been considerable interest in the usage of prebiotics due to beneficial effects on human health and food industry [1-3]. Several fermented food products contain lactic acid bacteria with probiotic properties and are accepted as safe due to protective role of probiotics [4]. Probiotic bacteria produce antimicrobial compounds against various pathogens and thus might form a natural barrier against pathogen in the gastrointestinal tract or preserve food [5]. Probiotic *Lactobacillus* are also able to adhere to various surfaces. Adhesion of probiotic *Lactobacillus* species prevents colonization of pathogenic bacteria and plays an important role as a protective barrier [3,6,7]. Furthermore the effectiveness of probiotics is strain-specific [8].

Listeria monocytogenes, a foodborne pathogen, has been a great concern due to its capacity to survive and grow in a wide variety of food substrates and environmental conditions [5,9-11]. The prevalence of *L. monocytogenes* varies mostly depending on the product and processing environments [8,12,13]. *L. monocytogenes* can form biofilms and produce extracellular polymeric substances on various food contact materials. The ability of *L. monocytogenes* to form biofilm on different surfaces poses a major concern for food industry because biofilms show more resistance to antimicrobial compounds [9,14,15]. *L. monocytogenes* has good adhesion ability and requires only a short contact time for attachments. The adhesion of *L. monocytogenes* to various surfaces such as stainless steel, plastic, glass and rubber cause to the decrease of its sensitivity to disinfectants [5,16].

Biofilm is the sessile form of microbial life, characterized by adhesion of microorganisms to biotic or abiotic surfaces, with consequent production of extracellular polymeric substances [9,17]. Microbial biofilm may be unfavorable or favorable and undesirable or desirable in food plant and human gastrointestinal systems. For example, biofilms formed by probiotic *Lactobacillus* strains in the gastrointestinal tracts may have a protective role and valuable characteristic for host with competitive inhibition of pathogen colonization. Probiotic biofilms can favor beneficial bacterial colonization. Biofilm or adherent structured microbial communities of *L. monocytogenes* in gastrointestinal tracts or food processing environments has negative effect on human health and product quality [18,19]. In several studies it was reported that adherence of lactic acid bacteria to the surface may prevent the adherence and the biofilm formation of *L. monocytogenes*. The use of probiotic biofilms can be considered as an alternative approach for reducing growth of pathogenic bacteria as regards human health and food safety [4]. However, the optimal functionality and expression of health-promoting physiological functions of probiotics is dependent on survivability and colonization in gastrointestinal tract and fermented foods [20]. Transition from planktonic cells to biofilm of the most bacteria depends on bacterial community and environmental conditions [21]. Some factors affecting the biofilm formation are equipment, temperature, nutrients and water. Presence of prebiotics in growth medium of probiotic microorganisms improves the formation of probiotic biofilms [12].

There are many studies focused on the biofilm formation of the pathogenic bacteria and the inactivation of pathogens with chemical compounds. However, biofilm formation was not studied extensively in nonpathogenic bacteria such as different *Lactobacillus* species [19]. Additionally, only a few studies have focused on the use of probiotic biofilms to inhibit the growth of *L. monocytogenes* [11]. The objective of the present study was to determine optimal prebiotic concentration for the formation of probiotic biofilms with highest viable cell concentration and to evaluate inactivation capacities of two different biofilms produced by *L. casei* Shirota and *L. rhamnosus* on growth of *L. monocytogenes*.

MATERIAL and METHODS

All experiment was carried out three times, with duplicate samples per trial and results were expressed as average.

Microorganisms and Prebiotics

In this study, *Lactobacillus rhamnosus* (Danisco USA INC.) and *Lactobacillus casei* Shirota (Yakult-RIUM/The Netherlands) were used as probiotic cultures and *Listeria monocytogenes* (ATCC 7644-Remel/USA) was used for inactivation experiment as pathogenic bacteria. As prebiotic ingredients, casein peptone (CP) (Merck-Germany) and fructo-oligosaccharides (FOS) (Sinerji Food-Turkey) were used.

Quantification of Biofilms Produced by L. monocytogenes and Probiotic Lactobacillus Strains

The quantification of biofilm production of *L. monocytogenes* and probiotic culture were performed as described previously by Bondi et al.[22]; Kubato et al.[23] and van der Veen and Abee [24] with some modifications. Biofilm assay was performed using 12-well microtiter plates. In order to standardize the number of bacteria, overnight grown cultures were used for all experiments. Three ml of each previously obtained probiotic suspensions *(L. rhamnosus* or *L. casei* Shirota) in MRS Broth (Merck, Germany) and *L. monocytogenes* suspensions in Tryptic Soy Broth (Merck-Germany) were added into each well and microtiter plates were incubated for 48 h at 30°C to allow the adhesion and formation of mature biofilm on the well bottoms. After 48 h the suspensions were removed and the wells washed three times with 2 mL of sterile saline

solution (NaCl 0.85%) (Merck-Germany). After that each biofilm in well was resuspended in 1 mL of saline solution by pipetting rigorously and serial diluted in saline solution for quantification of the biofilm formation expressed as unit of log cfu/well. Probiotic cells from biofilm were plated on MRS Agar (Merck-Germany) and plates were anaerobically incubated for 2 days at 30°C. *L. monocytogenes* were plated on BHI Agar (Oxoid-United Kingdam) and plates were aerobically incubated for 2 days at 30°C. In addition to quantification, for confirmation of biofilm formation, attached bacteria in well were stained with 3 mL of a 0.1% (V/V) crystal violet solution (Merck, Darmstadt, Germany) for 30 min. and washed three times with 3.5 mL water to remove unbound crystal violet. After drying, attached crystal violet was dissolved in 3.5 mL of absolute ethanol (Merck, Darmstadt, Germany) and absorbance was measured at 600 nm. If absorbance is more than 0.1, result is accepted as biofilm positive.

Optimization of Prebiotic Ingredients in Probiotic Biofilms

To carry out the response surface modeling, regression was performed on the experimental results to construct mathematical models. Variables and responses were defined as prebiotic ingredients and viable cell counts in biofilms of probiotic, respectively. The response surface method was employed in a similar way to the work by Chen et al.[25].

Detection of The Inactivation Effect of Probiotic Biofilms on L. monocytogenes Growth

Three ml of tryptic soy broth containing 0.1% *L. monocytogenes*, 0.5% *L. monocytogenes*, and 0.1% mix of *L. monocytogenes* and probiotic culture (0.1% *L. rhamnosus* or 0.1% *L. casei* Shirota) were added onto attached probiotics (probiotic biofilm) in well and these microtiter plates incubated at 30°C for 24 h. After incubation, both planktonic cell enumeration and the viable count of adherent *L. monocytogenes* in probiotic biofilm were performed for inactivation test. To find inactivation of adherent *L. monocytogenes* in probiotic biofilms washed three times with saline solution and resuspended in 1 mL of saline solution by pipetting rigorously. The attached cells were serial diluted in saline solution and plated Oxford Listeria Selective Agar (Merck-Germany) incubated for 24 h at 30°C. After 24 h, both planktonic cells and adherent cells of *L. monocytogenes* were enumerated on Oxford Listeria Selective Agar (Merck-Germany) after incubation for 24 h at 30°C. Whereas to find inactivation of planktonic *L. monocytogenes* cells, 1 mL of medium was removed from each well and suspended in 1 mL of saline solution by pipetting rigorously. The planktonic cells were serial diluted in saline solution and plated on Oxford Listeria Selective Agar (Merck-Germany), then incubated for 24 h at 30°C [11].

RESULTS

Biofilm Formation of L. monocytogenes and Probiotic Cultures

Table 1 showed that *L. monocytogenes*, *L. casei* Shirota and *L. rhamnosus* had ability of biofilm formation. Viable cell counts in biofilm of *L. rhamnosus* (9.66 log cfu/mL) were found higher than biofilm of *L. casei* Shirota (9.46 log cfu/mL). Biofilm of *L. monocytogenes* contained lowest viable cell counts (8.01 log cfu/mL).

Proportion for the Formation of Probiotic Lactobacillus Biofilms

Response surface methodology was used in the present work to develop a prediction model for establishing the optimal concentrations of prebiotics on viable cell growth in probiotic biofilms. As represented in *Table 2*, responses were obtained according to 13 combinations of prebiotics. Viable cell counts in probiotic biofilms were enumerated for each prebiotic combination and optimal prebiotic concentrations were calculated according to the obtained responses. We observed that the ability of probiotic *Lactobacillus* strains to form biofilms varied dependent on the prebiotic proportion.

In this study both of probiotic *Lactobacillus* strains utilized casein peptone. However, FOS has no effect on growth of *L. casei* Shirota. Optimal prebiotic concentration for *L. casei* Shirota were 3% peptide and 0% FOS and these results were found significant at 94% acceptable level. In this rate it is possible that *L. casei* Shirota counts in well are 9.89 log cfu/ml. Optimal prebiotic rate for *L. rhamnosus* were 1.5% casein peptone and 1.5% FOS and these results were found significant at 80% acceptable level. In this rate it is possible that *L. rhamnosus* counts in well are 10.81 log cfu/mL. The result of possibility from response surface detected that *L. rhamnosus* is more adhesive than *L. casei* Shirota in agreement with Collado et al.[3].

The Inactivation Effect of Probiotic Biofilms on L. monocytogenes Growth

In this study, inactivation efficiency of probiotic biofilms was tested against *L. monocytogenes* in different rates. For this experiment, 0.1% *L. monocytogenes*, 0.5% *L. monocytogenes*, and 0.1% mix of *L. monocytogenes* and probiotic culture (0.1% *L. rhamnosus* or 0.1% *L. casei* Shirota) were added onto probiotic biofilm and reduction

Table 1. *Biofilm formation of strains*

Strains	Biofilm Formation	Viable Cell Counts (log cfu/mL)	Absorbance
L. casei Shirota	+	9.46	0.477
L. rhamnosus	+	9.66	0.46
L. monocytogenes	+	8.01	0.44

Table 2. *The variables and responses of experiment*

Combination	Variables		Response	
	FOS (% 0-3)	Casein Peptone (% 0-3)	*L. casei* Shirota (log cfu/mL)	*L. rhamnosus* (log cfu/mL)
1	3	0	9.51	10.85
2	1.5	1.5	9.81	11.00
3	0	1.5	9.85	10.53
4	1.5	1.5	9.59	10.54
5	0	0	9.60	11.01
6	3	1.5	9.78	11.22
7	1.5	0	9.64	11.37
8	3	3	9.82	11.02
9	0	3	9.94	10.30
10	1.5	1.5	9.83	10.18
11	1.5	1.5	10.00	11.12
12	1.5	1.5	9.79	10.41
13	1.5	3	9.90	11.02

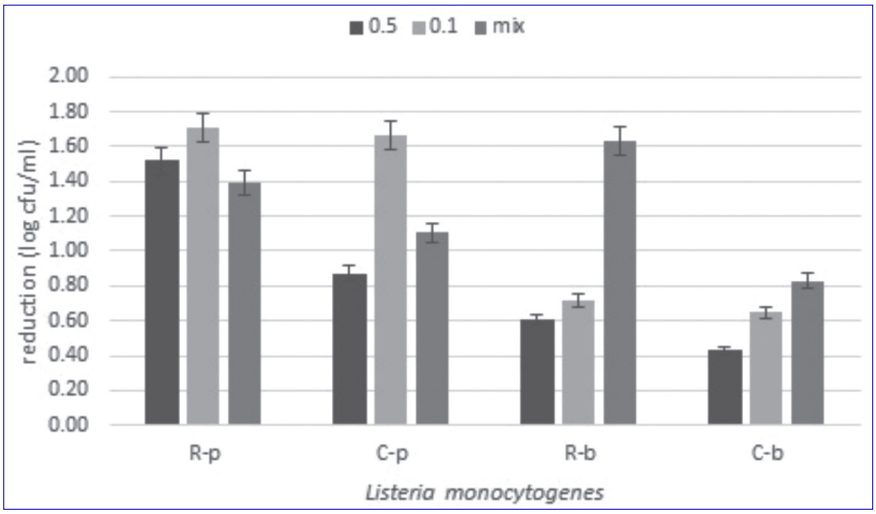

Fig 1. Reduction in viable cell counts of *L. monocytogenes*
R-p: Planktonic *L. monocytogenes* cells in well containing *L. rhamnosus* biofilm, C-p: Planktonic *L. monocytogenes* cells in well containing *L. casei* Shirota biofilm, R-b: *L. monocytogenes* attached to *L. rhamnosus* biofilm, C-b: *L. monocytogenes* attached to *L. casei* Shirota biofilm, 0.5: The effect of growth medium containing 0.5% *L. monocytogenes*, 0.1: The effect of growth medium containing 0.1% *L. monocytogenes* mix: The effect of growth medium containing 0.1% *L. monocytogenes* and 0.1% probiotic culture *(L. rhamnosus* or *L. casei* Shirota)

in viable cell counts of *L. monocytogenes* was compared. *Fig. 1* showed the inactivation effect of *L. casei* Shirota biofilm and *L. rhamnosus* biofilm on the growth of *L. monocytogenes*. As seen from our results, probiotic biofilms had not only inhibition effect on planktonic cells of *L. monocytogenes* but also biofilm of *L. monocytogenes*. Each probiotic biofilm exhibited inactivation efficiency in different levels and caused different reduction in viable cell counts of *L. monocytogenes*. The reduction in planktonic cells of *L. monocytogenes* varied from 0.66 to 2.01 log cfu/mL whereas the reduction in *L. monocytogenes* attached to biofilm changed between 0.40 and 1.69 log cfu/mL. These findings proved the hypothesis of Gomez et al.[4] that planktonic cells of *L. monocytogenes* were more susceptible than biofilm of *L. monocytogenes*. Similarly, Guerrierri et al.[11] determined that *L. plantarum* 35d biofilm, *L. plantarum* 396/1 biofilm and *Enterococcus casseliflavus* IM 416K1 biofilm caused more reduction in planktonic cells of *L. monocytogenes* than adherent cells of *L. monocytogenes*. As mentioned before in our results, *L. rhamnosus* and *L. casei* Shirota had different efficiency in terms of biofilm formation. In addition to these differences in the ability of biofilm formation of probiotics, it was detected that *L. rhamnosus* and *L. casei* Shirota showed different inactivation properties against pathogenic bacteria. Biofilm of *L. rhamnosus* showed higher inhibition effect on both planktonic cells and adherent cells of *L. monocytogenes* than biofilm of *L. casei* Shirota. These results confirmed that inhibition effect of probiotic biofilms varied according to the strains [4,11].

Additionally, 0.1% *L. monocytogenes* addition to probiotic biofilms led to the highest inactivation in planktonic cells of *L. monocytogenes,* whereas highest anti-adherence activities of probiotic *Lactobacillus* strains against biofilm formation of *L. monocytogenes* were obtained with addition of mix culture of 0.1% *L. monocytogenes* and 0.1% probiotic culture *(L. rhamnosus* or *L. casei* Shirota) to probiotic

biofilms. This situation showed that culture addition in different rates to probiotic biofilms differently affected level of inactivation. As known from literature, probiotic *Lactobacillus* strain may adhere more easily than pathogens [17]. Similarly, in our experiment with mix culture, the addition of probiotic culture plus *L. monocytogenes* caused to competition and prevented adherence of *L. monocytogenes* to probiotic biofilm. When 0.5% of *L. monocytogenes* was added to probiotic biofilm, probiotics led to 0.4 and 0.6 log cfu/mL reduction in attachment of *L. monocytogenes*. These results are considered that as addition of *L. monocytogenes* increased to probiotic biofilms, adherence of *L. monocytogenes* to probiotic biofilm reduced.

DISCUSSION

Many bacteria could form biofilm by adhering to the various surfaces thanks to their aggregation ability [4]. Adherence of probiotic cultures is desirable properties for displacement of pathogens. However these beneficial effects of probiotic bacteria can be observed by having an adequate mass through aggregation. Hydrophobicity and aggregation ability of probiotics can give prediction about detection of the most useful and highly adhesive probiotic *Lactobacillus* strains [3]. Furthermore, the specific composition of the medium contribute to biofilm formation of each species [24]. As it is known that both effect of growth medium and strains were very important on biofilm formation by microorganisms. Lebeer et al.[26] reported that prebiotic may have biofilm-promoting effect. The ability of probiotic culture to metabolize prebiotics is a species-dependent feature. For this reason, the proper selection of probiotics and prebiotics for symbiotic is highly important [17]. As a matter of fact, the present treatment with prebiotics in different concentrations differently affected the biofilm formation of probiotic *Lactobacillus* strains.

In general, probiotic *Lactobacillus* strains may adhere more easily than pathogens [17]. This hypothesis proved in our study that viable cell counts in probiotic biofilms were found higher than *L. monocytogenes* biofilm *(Table 1)*. Additionally, as can be seen our results, the attachment of *L. rhamnosus* to wells (biofilm formation) were found higher than *L. casei* Shirota. In accordance with our results, Collado et al.[3] determined that *L. rhamnosus* had higher adherence abilities to well than other lactobacilli species such as *L. casei, L. acidophilus, L. plantarum* and *L. salivarus.*

Recently, researchers and industry have been focused on novel strategies using natural products to control the pathogens in food industry. The use of lactic acid bacteria showing the highest biofilm formation in food products as starter or probiotic cultures can be a very promising approach for the control of pathogenic bacteria. Especially successful results with lactobacilli biofilms were obtained to control the growth of *L. monocytogenes* [4].

As reported in the previous studies, microorganisms in biofilms are more resistant to antimicrobial agents than planktonic cells [4,11].

Inhibitory mechanisms of probiotic *Lactobacillus* strains on biofilm formation of *L. monocytogenes* based on the competition, exclusion and displacement [21]. Similarly, Aoudia et al.[20] reported that biofilm growth in probiotic *Lactobacillus* strains had an antagonistic effect against *L. monocytogenes*. Similarly, many researchers concluded that probiotic *Lactobacillus* strains or lactobacilli was capable to reduce biofilm formation of *L. monocytogenes* [4,21,27,28].

According to our results, the application of probiotic biofilms can be an alternative method to reduce the growth of pathogens. Probiotic *Lactobacillus* strains such as *L. casei* Shirota and *L. rhamnosus* might have protective role against adhesion by *L. monocytogenes* inside the gastrointestinal tract of patients and onto food contact surfaces. However the inhibition is strain-dependent and varies according to conditions in growth medium. Our data indicated that optimal probiotic adherence to surfaces made possible with prebiotics-promoting probiotic growth. Biofilm formation ability of probiotic *Lactobacillus* strains might interfere with the ability of the pathogenic species to infect the host and can prevent the colonization of food-borne pathogens. It was obtained new information about the use of potential probiotic cultures biofilms for the inactivation of *L. monocytogenes*. However, more experiments are needed to determine the efficacy of probiotic *Lactobacillus* strains in inhibiting *L. monocytogenes* when different nutrients and environmental conditions are present. Additionally, this study should be supported with *in vivo* experiments because gastrointestinal adhesiveness of certain species may be different than adhesiveness to microtiter plates.

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Research Article

Genotypic, Antimicrobial Resistance and Virulence Profiles of Thermophilic *Campylobacter* Isolates in Broilers [1]

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Abstract

The objective of this study was to investigate the prevalence, antimicrobial resistance and genetic determinants of resistance of *Campylobacter jejuni* and *Campylobacter coli* isolated from commercial broiler farms in Adana and Hatay provinces, Turkey. The assessment of the genetic diversity among the isolates was determined by *fla*A based RFLP-Polymerase Chain Reaction (PCR) with restriction enzyme *Dde*I and sequence analysis of short variable regions (SVRs) of *fla*A and *fla*B-SVR genes. Antimicrobial susceptibility of the isolates was performed by disk diffusion method and tetracycline *(tet*O), ampicillin *(bla*$_{OXA-61}$), aminoglycoside *(aph-3-1)* and multidrug efflux pump *(cme*B) resistance genes were investigated by PCR as well. The genes conferring resistance to ciprofloxacin was screened by PCR and following DNA sequencing. The presence of ten virulence *(fla*A, *vir*B11, *rac*R, *cad*F, *cia*B, *dnaJ* and *pld*A) and toxin genes *(cdt*A, *dt*B, *cdt*C) among the isolates was also investigated using PCR. Out of 220 cloacal swabs, 218 (99.1%) *Campylobacter* spp. including *C. jejuni* (n=194; 89%) and *C. coli* (n=24; 11%) were isolated. While all the isolates were susceptible to chloramphenicol, gentamicin and erythromycin, resistance rates for *C. jejuni* and *C. coli* isolates to nalidixic acid, ciprofloxacin, ampicillin, tetracycline and amoxicillin-clavulanic acid were determined as 86.6-100%, 86.6-100%, 45.9-45.8%, 43.3-50% and 2.6-0.0%, respectively. The *tet*O, *bla*$_{OXA-61}$, *cme*B and *aph-3-1* genes detected in *C. jejuni* and *C. coli* isolates were 45.3-54.2%, 36.1-75%, 1.5-83.3% and 0.5-0%, respectively. The prevalence of *fla*A, *cdt*A, *cdt*B, *cdt*C, *rac*R, *cad*F, *cia*B, *dnaJ* and *pld*A in *C. jejuni* and *C. coli* isolates was 100-100%, 95.4-100%, 94.3-100%, 89.7-54.2%, 89.2-79.2%, 92.8-100%, 67-16.7%, 85.6-75% and 80-66.7%, respectively. *vir*B11 gene was not detected in any of *Campylobacter* isolates. All *fla*A and *fla*B-SVR alleles displayed same PCR-RFLP patterns. The results of this study revealed high prevalence, pathogenic potantial, genetic diversity and antimicrobial resistance of *Campylobacter* spp. in broiler flocks, which highlights urgent need for implementing effective contol measures to reduce emergence and spread of antimicrobial resistant *Campylobacters*.

Keywords: *Thermophilic Campylobacter, Antimicrobial Resistance, Virulence, Genotyping*

Broilerlerden İzole Edilen Termofilik *Campylabacter*'lerin Genotipik, Antimikrobiyal Direnç ve Virulens Profilleri

Özet

Bu çalışmanın amacı, Adana ve Hatay illerindeki ticari broyler çiftliklerinde *Campylobacter jejuni* ve *Campylobacter coli*'nin prevalansını, antimikrobiyal direnç ve genetik belirleyicilerini araştırmaktır. İzolatlar arasındaki genetik çeşitliliğin değerlendirilmesi, *fla*A geninin *Dde*I restriksiyon endonükleaz enzimi ile kesilmesine dayalı RFLP-Polimeraz Zincir Reaksiyonu (PZR) ve *fla*A ve *fla*B-SVR genlerinin kısa değişken bölgelerinin (SVR'ler) dizi analizi ile belirlendi. Ayrıca, izolatların antimikrobiyal duyarlılıkları disk difüzyon yöntemi ile yapıldı ve tetrasiklin *(tet*O), ampisilin *(bla*$_{OXA-61}$), aminoglikozid *(aph-3-1)* ve çoklu dirence aracılık eden efluks pompası *(cme*B) direnç genlerinin varlığı ise PZR ile araştırıldı. Siprofloksasine dirence neden olan mekanizmalar PZR ve takiben DNA dizi analizi ile araştırıldı. İzolatlar arasında 10 virulans *(fla*A, *vir*B11, *rac*R, *cad*F, *cia*B, *dnaJ* and *pld*A) ve toksin geninin *(cdt*A, *cdt*B, *cdt*C) varlığı PZR ile incelendi. İncelenen 220 kloakal sıvabtan 194'ü *C. jejuni* (%89) ve 24'ü *C. coli* (%11) olmak üzere 218 (%99.1) *Campylobacter* spp. izole edildi. Tüm izolatlar kloramfenikol, gentamisin ve eritromisin'e duyarlı iken; *C. jejuni* ve *C. coli* izolatlarının nalidiksik asit, siprofloksasin, ampisilin, tetrasiklin ve amoksisilin-klavulanik asite direnç oranları sırasıyla %86.6-100, %86.6-100, %45.9-45.8, %43.3-50 ve %2.6-0.0 olarak belirlendi. *C. jejuni* ve *C. coli* izolatlarında *tet*O, *bla*$_{OXA-61}$, *cme*B ve *aph-3-1* genlerinin prevalansı sırasıyla %45.3-54.4, %36.1-75, %1.5-83.3 ve %0.5-0 tespit edildi. *C. jejuni* ve *C. coli* izolatlarında *fla*A, *cdt*A, *cdt*B, *cdt*C, *rac*R, *cad*F, *cia*B, *dnaJ* ve *pld*A genlerinin prevalansı ise sırasıyla %100-100, %95.4-100, %94.3-100, %89.7-54.2, %89.2-79.2, %92.8-100, %67-16.7, %85.6-75 ve %80-66.7 olarak belirlendi. *vir*B11 geni *Campylobacter* izolatlarının hiçbirinde tespit edilmedi. Tüm *fla*A ve *fla*B -SVR allelleri aynı PCR-RFLP patternleri gösterdi. Bu çalışmanın sonuçları broyler sürülerinde *Campylobacter* spp. prevalansının, patojenik potansiyelinin, genetik çeşitliliğinin ve antimikrobiyal direnç oranlarının yüksek olduğunu göstermektedir. Bu durum antimikrobiyallere dirençli *Campylobacter*'lerin ortaya çıkışını ve yayılımını azaltmak için etkili kontol önlemlerinin acilen uygulanması gerektiğini vurgulamaktadır.

Anahtar sözcükler: *Termofilik Campylobacter, Antimikrobiyal direnç, Virulens, Genotiplendirme*

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INTRODUCTION

Of the 25 *Campylobacter* species described to date, *Campylobacter jejuni* and *Campylobacter coli* are the most frequently reported foodborne pathogens responsible for human gastroenteritis in both developing and developed countries [1]. Although thermophilic *Campylobacters* can colonize on the intestinal flora of many animal species, poultry (especially commercial chicken) are the main reservoir of *Campylobacter* spp. and responsible for most of human campylobacteriosis cases [2] through improper handling and consumption of raw chicken meat [3]. One of the important vehicles for the transmission of *campylobacter* to humans is improper handling and consumption of chicken meat [3].

Human campylobacteriosis cases are mostly self-limiting, however, in some cases may result in severe health consequences such as Guillain-Barre syndrome, reactive arthritis and irritable bowel syndrome [4]. In severe cases, fluoroquinolones, macrolides and tetracyclines are the drugs of choise. However, over the years, increasing trend of resistance to these antimicrobials in *C. jejuni* and *C. coli* have been reported by various studies in Turkey [5-7] and in the world [8-11].

In addition to antimicrobial resistance, *Campylobacter* spp. produces several virulence factors playing an important role in the pathogenesis of infection. The most important virulence genes of campylobacteriosis were: *fla*A, *cad*F, *rac*R and *dna*J genes responsible for adherence and colonization; *vir*B11, *cia*B and *pld*A genes responsible for invasion and survival within the host cells and *cdt*A, *cdt*B and *cdt*C genes responsible for the expression of cytolethal distending toxins [12]. Studies regarding the prevalence of virulence genes in *Campylobacter* obtained from broiler are very scarce in Turkey, and there have been only a study in order to determine *cdt* toxin genes in limited number of *C. jejuni* isolates of broiler origin [13].

Many molecular methods have been used for molecular characterization of *C. jejuni* and *C. coli* strains. But, each molecular method has different discriminatory power to determine the genetic relatedness of the *campylobacter* isolates [14]. Of these methods, *fla*A gene based molecular techniques are widely used for epidemiological studies of thermophilic *Campylobacter* spp. due to its rapidity, relative simplicity, low cost and easy method with an acceptable discriminatory power [15]. One of these methods, *fla*A gene based PCR-RFLP is one of the widely used method for discrimination of *C. jejuni* and *C. coli* isolates [16]. Sequence analysis of short variable regions (SVRs) of *fla*A and *fla*B genes is another widely used method for genotyping of *Campylobacter* isolates, particularly in short-term and localized epidemiological studies [17-19].

The aims of this study were to (i) determine the prevalence and antimicrobial resistance of thermophilic *Campylobacter* spp. in commercial broiler flocks in Adana and Hatay provinces and its resistance mechanisms, (ii) to investigate genetic diversity of all *C. coli* and representative *C. jejuni* isolates using *fla*A based PCR-RFLP and *fla*A-SVR and *fla*B-SVR sequence-based typing and (iii) to determine the presence and frequency of ten virulence genes.

MATERIAL and METHODS

Study Area and Sample Collection

A cross-sectional study was conducted to determine the prevalence of *Campylobacter* spp. from May 2015 through September 2015. A total of 220 cloacal swabs from 11 commercial broiler farms were collected from Hatay and Adana provinces. The study was approved by Mustafa Kemal University Animal Ethic Commitee (2014-8/5).

Bacterial Isolation

The cloacal swabs were taken using Amies Transport Medium with charcoal (LP Italiana, 118598, Italy) and transported to laboratory in a cold chain for further analysis. The cloacal swabs were directly streaked on modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid, CM0739, England) containing CDDA selective supplement (Oxoid, SR0155, England) for primary isolation. The plates were incubated at 41.5°C for 36-48 h under microaerophilic conditions. One presumptive colony from each mCCDA plate and subcultured onto blood agar (Merck, 110886, Germany) supplemented with 5% defibrinated sheep blood to obtain pure culture. The isolates, microscobically curved Gram negative rods with characteristic seagull-winged morphology, catalase and oxidase positive were accepted as *Campylobacter* spp. and stored within cryobeads (Biomériuex, France) in deep freeze (-80°C) until use.

DNA Extraction and PCR Analysis for Identification of Genus/Species Level

Chromosomal DNA was obtained by boiling method as previously described [20]. Briefly, one colony was suspended in 200 µL RNase and DNase free water and heated 100°C for 10 min and centrifuged at 10.000 g for 10 min. Supernatant was transferred to another sterile eppendorf tube and used as template DNA.

The isolates were identified to genus/species level by multiplex polymerase chain reaction (mPCR) as described by Wang et al.[20] using primers spesific for *Campylobacter* spp., *C. jejuni* and *C. coli*.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of *C. jejuni* and *C. coli* isolates was determined by disk diffusion method according to Clinical Laboratory Standards Institute guidelines (CLSI, 2008) [21]. Following antimicrobial disks (Bioanalyse, Turkey)

were used: ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), chloramphenicol (30 µg), tetracycline (30 µg), gentamicin (10 µg), streptomycin (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg) and erythromycin (15 µg). The inocula was prepared from colonies of overnight agar plates and suspended within steril 0.85% sodium chloride to obtain MacFarland 0.5 turbity. The bacterial suspension was inoculated on Mueller-Hinton agar (Merck, Germany) plates containing 5% (v/v) defibrinated sheep blood. Antimicrobial disks were placed on the dry medium and incubated at 37°C for 24 h in anaerobic jar under microaerophilic condition. Inhibition zones were recorded and evaluated following CLSI (2008) criteria. *C. jejuni* (NCTC 12500) and *C. coli* (NCTC 12525) were used as control strains for antimicrobial susceptibility testing.

Detection of Antimicrobial Resistant Genes

All *Campylobacter* spp. were tested for the presence of *tet*O (tetracycline), *aph-3-1* (aminoglycoside), bla_{OXA-61} (ampicillin) and *cme*B (multi-drug efflux pump) genes by mPCR as previously reported by Obeng et al.[22].

Analysis of the Molecular Mechanisms of Fluoroquinolone Resistance

Mutations in the quinolone resistance determining region (QRDR) of *gyr*A gene was determined by PCR amplification of *gyr*A gene and sequencing, using primers as described by Gibreel et al.[23].

Genotyping by *fla*A-SVR and *fla*B-SVR Sequencing

PCR amplification of fragments of *fla*A and *fla*B genes comprising the SVRs were performed following the procedures described by Lévesque et al.[24] and Korczak et al.[25], respectively. Primers described by those authors were used, amplifying regions of 641 base pairs (bp) and 602 bp of the *fla*A and *fla*B genes, respectively. The *fla*A and *fla*B allele types were determined by comparing the nucleotide sequences with those in the PubMLST *Campylobacter* database *(http://pubmlst.org/campylobacter/)*.

*fla*A-RFLP Assay

*fla*A-RFLP analysis of the isolates (24 *C. coli* and 55 *C. jejuni*, 5 strain from each flock) were performed as described elsewhere by Nachamkin et al.[6]. The *fla*A amplicons were digested for 18 h at 37°C with *Dde*I (Fermentas, *Lithuania*). Then, DNA fragments were separated using 2.5% agarose gels in TBE buffer at 200 V for 1 h, and visualized under UV light.

Detection of Virulence Genes

Presence of virulence genes *(fla*A, virB11, *cad*F, *dna*J, *cia*A, *fla*A, and *pld*A) and cytolethal distending toxin genes *(cdt*A, *cdt*B and c*dt*C) were investigated by PCR as previously described by Nachamkin et al.[16], Bang et al.[26], Konkel et al.[27], Bacon et al.[28] and Datta et al.[29].

RESULTS

Occurence and Distribution of Campylobacter spp.

Out of 11 broiler flocks examined, all were positive for thermophilic *Campylobacter* spp. *(Table 1)*. Two hundred and eighteen (99.1%) *Campylobacter* spp. was isolated from cloacal swabs taken. Of these isolates, 194 (89.1%) were *C. jejuni* and 24 (10.9%) were *C. coli* by PCR analysis.

Antimicrobial Susceptibility Testing

The results of antimicrobial susceptibility of *C. jejuni* and *C. coli* isolates are given in *Table 2*. All isolates were found to be susceptible to erythromycin, chloramphenicol and gentamicin. *Campylobacter* isolates showed highest resistance to ciprofloxacin and nalidixic acid at the rate of 86.6-86.6% and 100-100%, respectively. High resistance rates were also detected to tetracycline (43.3% for *C. jejuni* and 50% for *C. coli*) and ampicillin (45.9% for *C. jejuni* and 45.8% for *C. coli*). Low resistance rate for amoxicillin/

Table 1. *Distribution of Campylobacter spp. in broiler flocks*

Flocks	Sampling Location	Broilers' Age (days)	Numbers of Samples	Species	
				C. jejuni (%)	*C. coli* (%)
I	Adana	33	20	19 (95)	1 (5)
II	Adana	35	20	14 (70)	5 (25)
III	Hatay	42	20	20 (100)	0 (0)
IV	Hatay	40	20	20 (100)	0 (0)
V	Adana	38	20	20 (100)	0 (0)
VI	Adana	36	20	19 (95)	0 (0)
VII	Hatay	37	20	20 (100)	0 (0)
VIII	Hatay	41	20	20 (100)	0 (0)
IX	Hatay	40	20	20 (100)	0 (0)
X	Adana	35	20	14 (70)	6 (30)
XI	Adana	37	20	8 (40)	12 (60)
Total			220	194 (88.2)	24 (10.9)

Table 2. *Antimicrobial resistance of C. jejuni and C. coli isolates*

Antimicrobial	No of Isolates (%)	
	C. jejuni (n=194)	*C. coli* (n=24)
Nalidixic acid	168 (86.6)	24 (100)
Ciprofloxacin	168 (86.6)	24 (100)
Ampicillin	89 (45.9)	11 (45.8)
Tetracycline	84 (43.3)	12 (50)
Amoxicillin/clavulanic acid	5 (2.6)	0 (0)
Chloramphenicol	0 (0)	0 (0)
Gentamicin	0 (0)	0 (0)
Erythromycin	0 (0)	0 (0)

clavulanic acid was only detected in *C. jejuni* as being 2.6% *(Table 2)*. Multidrug resistance (MDR) phenotype was observed in 70 (36.1%) *C. jejuni* and in 8 (33.3%) *C. coli* isolates, repectively. The most common MDR phenotype encountered among isolates were NA/CIP/TE/AM *(Table 3)*.

Table 3. *Antimicrobial resistance profile of C. jejuni and C. coli isolates*

Resistance Profile*	No. of Resistant Isolates (%)	No. of Resistant Isolates (%)	
		C. jejuni	*C. coli*
Pan-susceptible	2 (0.92)	2 (1.03)	0
TE	6 (2.8)	6 (3.1)	0
AM, TE	1 (0.5)	1 (0.5)	0
NA, CIP	78 (35.8)	69 (35.6)	9 (37.5)
NA, CIP, TE	18 (8.3)	14 (7.2)	4 (16.7)
NA, CIP, AM	32 (14.7)	29 (14.9)	3 (12.5)
NA, CIP, TE, AM	76 (34.9)	68 (35.1)	8 (33.3)
NA, CIP, AM, AMC	3 (1.4)	3 (1.5)	0
NA, CIP, TE, AM, AMC	2 (0.9)	2 (1.0)	0

** TE: tetracycline, AM: ampicillin, CIP: ciprofloxacin, NA: nalidixic acid, AMC: amoxicillin-clavulanic acid*

Table 4. *Distribution of antimicrobial resistance genes in C. jejuni and C. coli isolates*

Species	Resistance Phenotype and the Occurence of Related Gene	No of Isolates (%)			
		*tet*O	bla_{OXA-61}	*aph-3-1*	*cme*B
C. jejuni (n=194)	Resistant with genes	85 (43.8)	41 (21.1)	0	3 (1.0)
	Resistant without genes	11 (5.7)	44 (22.7)	0	0
	Susceptible with genes	3 (1.5)	29 (14.9)	1 (0.5)	0
C. coli (n= 24)	Resistant with genes	12 (50.0)	9 (37.5)	0	20 (83.3)
	Resistant without genes	0	2 (8.3)	0	0
	Susceptible with genes	1 (4.2)	9 (37.5)	0	0

Antimicrobial Resistance Genes

Of the 96 tetracycline resistant *C. jejuni* isolates, 85 were found to carry *tet*O gene, whereas *tet*O gene was found in all tetracycline resistant *C. coli* isolates. However, three *C. jejuni* and one *C. coli*, despite carrying *tet*O gene, were found to be susceptible to tetracycline. Similarly *aph-3-1* gene was detected in one phenotypically susceptible *C. jejuni* isolate. Among the ampicillin resistant 85 *C. jejuni*, 41 was found to carry bla_{OXA-61}, 29 of the ampicillin susceptible isolates were found to encode bla_{OXA-61}. While bla_{OXA-61} was found in 11 ampicillin resistant *C. coli* isolates, 9 isolates that harbored bla_{OXA-61} were susceptible to ampicillin. Although *cme*B gene was found in nearly all of *C. coli* (n=20) isolates, *cme*B was found in low number of *C. jejuni* (n= 3) isolates *(Table 4)*.

Fluoroquinolone Resistance Mechanism of *Campylobacter* spp.

Sequence analysis of 219 bp amplicon of *gyr*A gene revealed Thr86Ile mutation in all ciprofloxacin resistant isolates.

*fla*A Gene PCR-RFLP Analysis

Restriction analysis of 1.7 kb *fla*A PCR amplicons with *Dde*I revealed two-five bands ranging from 100 to 1000 bp. Two different band profiles of 24 *C. coli* isolates were observed. Analysis of 55 *C. jejuni* isolates selected randomly from the flocks (five isolates from each flock) gave 11 band profiles *(Fig. 1)*.

*fla*A-SVR and *fla*B-SVR Typing

The results of *fla*A-SVR sequence typing of the 24 *C. coli* and 55 *C. jejuni* isolates are given in *Table 5* and *Table 6*. Ten *fla*A alleles and 8 *fla*B alleles were detected among *C. jejuni* isolates, two *fla*A and *fla*B alleles were detected among *C. coli* isolates. In *C. coli* isolates, three different

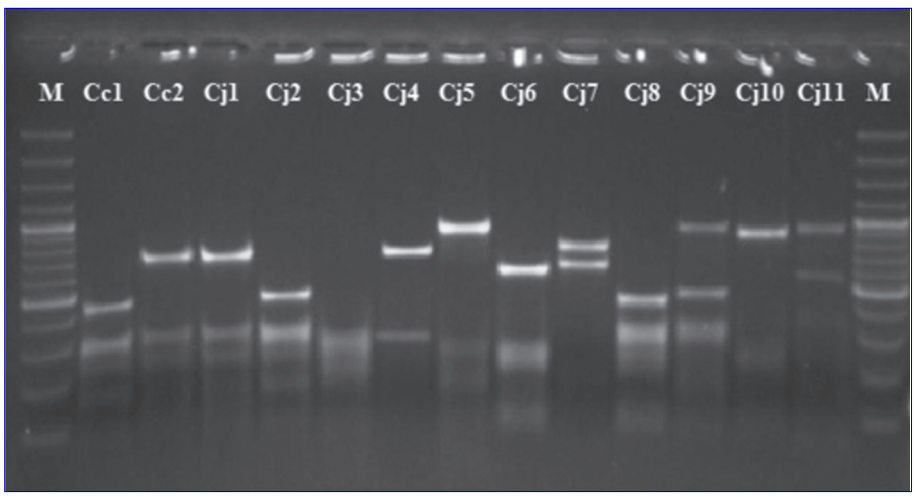

Fig 1. RFLP-PCR profiles obtained from *C. coli* and *C. jejuni* isolates. Lane M. 100 bp molecular marker, Lane Cc1-Cc2: RFLP profiles determined in *C. coli* isolates, Lane Cj1-Cj11: RFLP profiles determined in *C. jejuni* isolates

nucleotide sequences (allele numbers) for *fla*A and three for *fla*B were identified, corresponding to a total of two genotypes. *fla*A and *fla*B sequences were identical in four isolates (allele 116). The most common *fla*A alleles were 61 with 20 isolates (83.3%) and 116 with four isolates (16.7%). *fla*B allele 107 and 116 were detected in 20 isolates (83.3%) and in 4 isolate (16.7%). In *C. jejuni* isolates, 11 different nucleotide sequences (allele number) for *fla*A and eight different nucleotide sequences for *fla*B were identified, corresponding to a total of 12 genotypes. *fla*A and *fla*B sequences were identical in 11 isolates (allele 116). The most common *fla*A alleles were 116 with 12 isolates (21.8%), 67 with 16 isolates (29.1%), 36 with 10 isolates (18.2%), 44 with 5 isolates (9.1%), 42 with 5 isolates (9.1%), 67 with 5 isolates (9.1%). Among *fla*B alleles, 116 (17 isolates, 30.9%), 2 (11 isolates, 20%), 87 (6 isolates, 10.9%), 6 (10 isolates, 18.2%), 65 (5 isolates, 9.1%) were the most frequently detected.

Table 5. *fla-SVR alleles and PCR-RFLP profiles determined in C. coli isolates according to flocks*

No of isolates	Species	Flock	*fla*A-SVR Alleles	*fla*B-SVR Alleles	RFLP Profile
1	*C. coli*	I	116	116	Cc2
2	*C. coli*	II	61	107	Cc1
3	*C. coli*		116	116	Cc2
6	*C. coli*	X	61	107	Cc1
12	*C. coli*	XI	61	107	Cc1

Table 6. *fla-SVR alleles and PCR-RFLP profiles determined in C. coli isolates according to flocks*

No of Isolates	Species	Flock	*fla*A-SVR Alleles	*fla*B-SVR Alleles	RFLP Profile
1	*C. jejuni*	I	61	107	Cj1
4	*C. jejuni*		116	116	Cj2
3	*C. jejuni*	II	116	116	Cj2
2	*C. jejuni*		44	119	Cj3
5	*C. jejuni*	III	44	65	Cj4
5	*C. jejuni*	IV	67	2	Cj5
5	*C. jejuni*	V	36	6	Cj6
5	*C. jejuni*	VI	36	6	Cj6
5	*C. jejuni*	VII	42	87	Cj7
5	*C. jejuni*	VIII	116	116	Cj2
4	*C. jejuni*	IX	67	116	Cj8
1	*C. jejuni*		54	121	Cj9
2	*C. jejuni*	X	63	121	Cj10
1	*C. jejuni*		67	116	Cj8
2	*C. jejuni*		67	2	Cj5
4	*C. jejuni*	XI	67	2	Cj5
1	*C. jejuni*		31	87	Cj11

Virulence Gene Patterns

Prevalence of virulence genes detected in the *Campylobacter* isolates is given in *Table 7*. All isolates had at least two virulence genes investigated and in varying prevalence rates between 16.7% and 100%. *vir*B11 gene was not present in any of the isolates. The prevalence of *fla*A, *vir*B11, *cdt*A, *cdt*B, *cdt*C, *rac*R, *cad*F, *cia*B, *dna*J and *pld*A in *C. jejuni* and *C. coli* isolates was 100-100%, 0-0%, 95.4-100%, 94.3-100%, 89.7-54.2%, 89.2-79.2%, 92.8-100%, 67-16.7%, 85.6-75% and 80-66.7%, respectively.

DISCUSSION

This study has shown that broiler flocks are colonized with *Campylobacter* spp. at the high rate (99.1%). In Turkey, studies on the prevalence of *Campylobacter* spp. in broiler flocks are very limited. In these studies, samples were taken from different slaughterhouses and the prevalence of thermophilic of *Campylobacter* spp. was reported as 91.8% [7] and 52.5% [6]. Variations in the prevalence rates can be attributed to the sample type, sampling procedures, isolation methods, lack of biosecurity measures applied on farms, presence of other animals around poultry houses, seasonal and climate changes, fly population, use of ventilators, slaughter age, stock density, age and number of houses on a farm, use of old litter, farm equipment and farm workers [4,6].

C. jejuni has been reported as the most prevalent species colonizing broiler flocks [4]. Similarly, *C. jejuni* was the dominant species isolated in this study. This finding is consisted with previous studies that *C. jejuni* is the predominant species isolated from broilers [6,7]. Although reasons for the variations are not exactly known, variations seen between prevalence of *C. jejuni* and *C. coli* can be attributed to different factors such as season, production practices and environment [4].

Table 7. *Prevalence of virulence genes detected in C. jejuni and C. coli isolates*

Strain	No of Isolates	Number of Virulence Genes (%)									
		*fla*A	*vir*B11	*cdt*A	*cdt*B	*cdt*C	*rac*R	*cad*F	*cia*B	*dna*J	*pld*A
C. jejuni	194	194 (100)	0 (0)	185 (95.4)	183 (94.3)	174 (89.7)	173 (89.2)	180 (92.8)	130 (67)	166 (85.6)	156 (80)
C. coli	24	24 (100)	0 (0)	24 (100)	24 (100)	13 (54.2)	19 (79.2)	24 (100)	4 (16.7)	18 (75)	16 (66.7)
Total	218	218 (100)	0 (0)	209 (95.9)	207 (96.7)	187 (85.8)	192 (89.7)	204 (93.6)	134 (61.5)	184 (84.4)	172 (78.9)

Increasing resistance rates observed in *Campylobacter* isolates to antimicrobial agents has been reported in all over the world [30]. In this study, nearly all isolates (99.1%, 216/218) showed resistance to one or more of antimicrobial agents tested. Particularly, high percentage of resistance to ciprofloxacin is alarming, given the fact that resistant *Campylobacters* can be transmitted to humans via contaminated poultry meat. No resistance fluoroquinolones was determined in *campylobacter* isolates of broiler origin untill 1992 [31]. First fluoroquinolone resistance was observed in strains isolated in 1992 (1.4% for enrofloxacin and 1.2% for ciprofloxacin), and resistance of *Campylobacter* spp. to fluoroquinolones significantly increased in 2000 (75.5% for enrofloxacin and 73% for ciprofloxacin) [32]. In this study, 86.6% of *C. jejuni* and 100% of *C. coli* isolates were resistant to ciprofloxacin. Our results was comparable with findings of Abay et al.[5], but higher than findings of Cokal et al.[6]. The high fluoroquinolone resistance rate may be attributed to unrestricted use of flouroquinolones in poultry production, as it was highly used as a growth promoting agent between 1989 and 2006 in Turkey.

The resistance of *Campylobacter* to fluoroquinolones is mainly associated with point mutations in the QRDR of DNA gyrase *(gyr*A). The most encountered mutation in fluoroquinolone resistant *Campylobacter* isolates are Thr86Ile substitution, which confer resistance to both ciprofloxacin and nalidixic acid [30]. In this study, all ciprofloxacin resistant *Campylobacters* have Thr86Ile substitution in the QRDR of *gyr*A. Recently, Kurekci and Pehlivanlar Önen [33] have also identified the *gyr*A mutation of Thr86Ile among all *Campylobacter* spp. isolated from chicken meat sold in Turkey. Authors also identified Ala40Ser mutation in a *C. jejuni* strain [33].

In this study, all isolates were susceptible to erythromycin, chloramphenicol and gentamicin. Similar results were also reported by previous studies indicating that *Campylobacter* isolates are still sensitive or less resistant to these antimicrobials in Turkey [5-7].

Resistance to ampicillin and other β-lactams have widely been reported among *Campylobacter* isolates from humans, poultry and food of animal origins [5,7,22,33]. A novel class D β-lactamase gene, bla_{OXA-61}, described by Alfredson and Korolik [34] has been held responsible for resistance to the β-lactams including ampicillin, piperacillin, and carbenicillin. In this study, *C. jejuni* (45.9%) and *C. coli* (45.8%) isolates showed higher resistance rates to ampicillin. Resistance rate to ampicillin was higher than those findings of Abay et al.[5], but lower than findings of Yıldırım et al.[7]. In contrast to these findings, Cokal et al.[6] found all *C. jejuni* and *C. coli* isolates susceptible to ampicillin. This result might be attributed to the frequent use of β-lactams.

Resistance rate to tetracycline was slightly higher in *C. coli* (50%) than in *C. jejuni* (43.3%). Our findings is consistent with previous studies [5,7]. But, higher tetracycline resistance rate was reported by Cokal et al.[6].

In this study, 97 out of 108 (89.8%) tetracycline-resistant isolates were found to carry *tet*O and 23 (29.5%) of the isolates with MDR phenotype (n=78) had *cme*B, and 51.5% of 97 ampicillin resistant isolates carried bla_{OXA-61}. The higher presence of *tet*O in tetracycline resistant isolates was consisted with previous studies conducted by Obeng et al.[22], Abdi-Hachesoo et al.[35], Pratt and Korolik [36]. However, *tet*O was not detected in 11 tetracycline resistant *C. jejuni* isolates in this study. Recently, Abdi-Hachesoo et al.[35] reported presence of *tet*A gene in 18% (15/83) of *Campylobacter* spp. from poultry carcasses in Iran. Also, Guevremont et al.[37] reported that detection of *tet*O gene in tetracycline resistant isolates might vary according to primer sets used. The prevalence of *cme*B in *C. jejuni* isolates showing MDR phenotype was found to be low. Similar results have been previously reported by Obeng et al.[22] and Kashoma et al.[38]. Cagliero et al.[39] indicated that higher sequence variation can exist in *cme*B gene, could lead false negative results when presence of *cme*B gene is examined by PCR. As reported by Olah et al.[40], primers used in the study are probably located in regions subjected to modification. Similarly, in this study, most of ampicillin resistant *Campylobacter* isolates did not harbour bla_{OXA-61} gene, which is chromosomally encoded beta-lactamase. This could be explained by presence of other resistance mechanisms such as reduced uptake due to alterations in outer membrane porins and efflux pump system [41].

Investigation of *Campylobacter* isolates for the presence of ten virulence genes revealed high prevalence of virulence genes. Although numerous studies are available on the investigation of virulence genes in *Campylobacter* spp. isolated from broiler and chicken carcasses, so far, there was a only one study to examine *cdt* genes in a small number (23 isolates) of *C. jejuni* isolates of broiler origin in Turkey by Fındık et al.[13], who reported prevalence of *cdt*A, *cdt*B and *cdt*C genes as 95%, 100% and 90%, respectively. The prevalence of *cdt*A, *cdt*B and *cdt*C genes obtained in this study were comparable to findings of Fındık et al.[13], except prevelance rate of *cdt*C gene (54.2%) in *C. coli* isolates.

*fla*A gene are one of important virulence genes involved in colonization, which was detected in all *Campylobacter* isolates in present study. This result is in agreement with the study of Datta et al.[29], who reported this gene in 100% of *C. jejuni* isolates.

The *rac*R, *dna*J, *cad*F, *pld*A and *cia*B genes have important role in invasion and colonization of *Campylobacters*. Each of these genes were found in 16.7-100% of *Campylobacter* isolates in this study. Chansiripornchai and Sasipreeyajan [42] reported *dna*J, *cad*F, *pld*A and *cia*B virulence genes as 100,

76, 31 and 41% in *C. jejuni* isolates from broilers. In contrast to this study, higher prevalence rate for *rac*R, *dna*J, *cad*F, *pld*A and *cia*B was reported by Datta et al.[29] as 85.7%, 100%, 100%, 100% and 100%, respectively.

To the best of my knowledge, this is the first study to investigate the genetic diversity of *C. jejuni* and *C. coli* isolates in broiler flocks using fla-SVR analysis and compare its results with PCR-RFLP in Turkey. In this study, the results showed a distinct association between *fla*-SVR alleles and RFLP-PCR profiles for both *C. jejuni* and *C. coli* isolates. It has been reported that *fla*A gene based typing methods are not suitable for long-term epidemiological studies due to possible intra- and inter-genomic recombination between flagellin genes, but is a useful tool for *Campylobacter* genotyping, particularly initial screening of *Campylobacter* isolates [15,17,18,43]. Similarly, the results indicated that *fla*-SVR typing alone or in combination with other PCR-based methods can be used preliminary screening of *campylobacter* isolates in epidemiological studies.

In conclusion, the current study revealed that fla-SVR typing could be used successfully for the discrimination of *C. jejuni* and *C. coli* strains. The high prevalence of virulence and toxin genes among *Campylobacter* isolates of broiler origin suggests potential virulence for humans. In addition, high prevalence of ciprofloxacin resistant *Campylobacter* in broilers, which is accepted as one of the drugs of choise for the treatment of human campylobacteriosis, is major concern due to foodborne transmission of antimicrobial resistant *Campylobacter* to humans. Thus, this study highlights the need of establishment of prudent measures and continous efforts to reduce colonization and spread of antimicrobial resistant *Campylobacter*.

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Research Article

Investigation of Probiotic Features of Bacteria Isolated from Some Food Products

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Abstract

Probiotics are used as natural supplements for good health and treatment of various diseases. Probiotics affect health in a positive way due to their activities in the gastrointestinal tract. There is a growing interest in using probiotic bacteria for their protective effects against diseases and an emerging trend towards consuming healthy foods. The aim of the present study was to reveal species with alternative probiotic properties from some food products, which are already known to have probiotic properties and whose natural properties are preserved. Probiotic characteristics of isolated bacteria strains from 130 food samples which include 10 boza, 40 cheese, 20 kefir and 60 raw milk samples were microbiologically analyzed in the present study.A total 144 strains including 127 *Enterococcus faecium,* 7 *Lactobacillus plantarum,* 5 *Lactobacillus para-plantarum* and 5 *Lactobacillus brevis* were typed with characterizing by mass spectroscopy (MALDI-TOF MS) to have probiotic effects.Then, all the tests required to comply with the probiotic properties of these bacteria were applied sequentially. Of the 144 bacterial strains identified, only 35 were resistant to gastric pH. In the next step, only 8 isolates from 35 isolates were able to survive under bile salt conditions. It has been determined that only 6 of bile salt-resistant isolates have the hydrophobicity ability. The remaining 6 isolates were examined for antimicrobial resistance and the presence of extended-spectrum beta-lactamases (ESBL) resistance and ESBL were not detected. At the end of analysis, only 6 (4.1%) bacteria of 144 isolates were found to have probiotic properties. Three of them were *Lactobacillus brevis* isolated from boza and 3 of them were *Lactobacillus plantarum* species isolated from kefir. However, no probiotics could be isolated from other food samples such as milk and cheese. Therefore, the present study demonstrated that probiotic bacteria could be produced as an alternative to industrial probiotics through non-transgenic microorganisms isolated from natural food products such as kefir and boza.

Keywords: *Probiotic Bacteria, Boza, Milk, Cheese, Kefir, Probiotic properties*

Bazı Gıda Ürünlerinden İzole Edilen Bakterilerin Probiyotik Özelliklerinin Araştırılması

Özet

Probiyotikler, çesitli hastalıkların tedavisi ve sağlık için doğal takviyeler olarak kullanılırlar. Probiyotikler gastrointestinal sistemde yaptıkları faaliyetler sonucunda sağlığı olumlu yönde etkilemektedirler. Günümüzde hastalıklara karşı koruyucu etkileri ve sağlıklı gıdaların tüketimine yönelik yoğun ilginin ortaya çıkışına bağlı olarak probiyotik bakterilere ilgi artmıştır. Bu araştırmada probiyotik özellikleri olduğu bilinen ve doğal özellikleri korunmuş bazı gıdalardan probiyotik özelliklere sahip türlerin ortaya çıkartılması amaçlanmıştır. Araştırmamızda 10 boza, 40 peynir, 20 kefir ve 60 çiğ süt olmak üzere toplam 130 gıda örneği mikrobiyolojik bakımdan incelenmiştir. Sonuçta; 127 *Enterococcus faecium,* 7 *Lactobacillus plantarum,* 5 *Lactobacillus para-plantarum* ve 5 *Lactobacillus brevis* olmak üzere toplam 144 probiyotik etkisi gösterebilecek bakteri kütle spektofotometre (MALDI-TOF MS) ile karakterize edilerek tiplendirilmiştir. Daha sonra bu bakterilerin probiyotik özelliklere uygunluğu konusunda gerekli olan tüm testler sırasıyla uygulanmıştır. Karakterize edilen 144 izolattan sadece 35 'inin mide pH'sına dayanıklı olduğu saptanmıştır. Bir sonraki basamakta ise yine 35 izolatdan sadece 8'i safra tuzu koşullarında canlılıklarını devam ettirebilmiştir. Safra tuzuna dayanıklı izolatlardan sadece 6'sının hidrofobisite yeteneğine sahip olduğu belirlenmiştir. Kalan 6 izolatın antimikrobiyel direnç durumları incelenmiş dirençliliğe ve ESBL varlığına rastlanmamıştır. İncelemeler sonunda 144 izolattan sadece 6 (4.1%) sının probiyotik özelliklere sahip olduğu görülmüştür. Bunlardan; 3'ü *Lactobacillus brevis* bozadan ve 3'ü *Lactobacillus plantarum* kefirden izole edilmişlerdir. Kefir ve bozadan probiyotik özellik gösteren bakteri izole edilirken diğer gıda örnekleri olan süt ve peynirden probiyotik özellik gösteren bakteri izole edilememiştir. Sonuç olarak, kefir ve boza gibi doğal gıda ürünlerinden izole edilen transgenetik olmayan mikroorganizmalar içinden probiyotik bakterilerin endüstriyel probiyotiklere alternatif olarak üretilebileceği bu araştırmada saptanmıştır.

Anahtar sözcükler: *Probiyotik bakteri, Boza, Süt, Peynir, Kefir, Probiyotik özellik*

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INTRODUCTION

Probiotics are microorganisms that improve the microbial balance of human and animal intestines and thus are beneficial for the digestive system. These may be used as a natural supplement for both the healthy development of the body and the treatment and prevention of diseases. The probiotic bacteria colonize on the surface of the intestine by competing against pathogen microorganisms for nutrients in the gastrointestinal system, thereby positively affect the health [1,2]. A study has reported that probiotics prevent *Escherichia coli* associated with diarrhea and death in newborns [3]. It has been reported that probiotics produce volatile fatty acids as a result of fermentation by digesting dietary fibers: oligosaccharide structures that remain undigested in the colon. In addition, the formation of butyric acid inhibits colon cancer [4]. Probiotics have been found to contribute to lactose digestion due to the production of lactase enzyme; stimulation of immune system with the enhancement of IgA production; and to reduce allergens by inhibiting the passage of antigen-presenting substances into the circulatory system. Similarly, several reports highlight the importance of preventive effects of probiotics on heart diseases, hypertension, and urogenital diseases due to anti-oxidant effects, the act of cell wall components like angiotensin 1 enzyme inhibitors, and colonization on urinary and vaginal surfaces [5]. Additionally, it has been reported that *Helicobacter pylori* exert gastritis and ulcer-inhibiting effects by the production of inhibitors and preventive effect on hepatic encephalopathy formation by inhibiting the urease-producing intestinal flora and reducing the serum ammonia levels [6,7]. Various studies report the use of *Lactobacillus rhamnosus,* a combination of *L. rhamnosus* and *Lactobacillus acidophilus* for the treatment of children with diarrhea, the prevention of intestinal diseases, colon cancer and for the treatment of heart diseases [8-10].

Therefore, it is essential to develop new probiotic strains with different effects against different diseases and to use them in preventive medicine.

There has been an increasing interest in the use of probiotics due to their protective effects against diseases. An expenditure of $28 million was reported on research related to probiotic market and consumption in USA in 2011 [11]. The increasing interest in probiotics has accelerated the studies on the development of new probiotic products.

The therapeutic results of probiotics have been found to treat diseases such as colon cancer, ulcers and gastritis, and allergies, diabetes. In one study, it was reported that a commercial culture mixture obtained from *Lactobacillus* species had the antiproliferative effect by inhibiting tumor cells causing colon cancer [12]. A study has reported the beneficial effects of *Lactobacillus* species, especially *L. rhamnosus* and *L. acidophilus* against the infections caused by *Helicobacter pylori* [13]. In an *in vivo* study with probiotic *L. brevis*, an anti-allergic effect on anaphylaxis reduction was observed [14]. Another study provided the evidence that hemoglobin A1C and fasting blood glucose decreased in diabetic patients after treatment with probiotic supplements [15].

Probiotics obtained from nutrients should be able to resist stomach acidity and bile salts and reach the intestinal system alive to exert their beneficial effects. Further, they should be able to colonize and survive on the epithelial cell surfaces of intestinal mucosa [16].

The current study aimed to discover the alternative species of bacteria with probiotic properties along with preserved natural characteristics. Bacteria isolated from boza, cheese, kefir, and raw milk samples were characterized by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) (VITEK® MS) to examine the probiotic properties of the single species. The relationship between these parameters was established according to Pearson's nonparametric statistical correlation.

MATERIAL and METHODS

Materials

A total of 130 food samples consisting of 10 boza, 40 cheeses, 60 raw milk, and 20 kefir were obtained from Marmara, Central and Eastern Anatolia regions of Turkey between 2014 and 2016. The food samples were listed in *Table 1*.

Methods

Isolation of Bacteria

de Man, Rogosa and Sharpe (MRS) agar, MRS broth, M17 agar and M17 broth media were prepared and used to isolate and identify the pure cultures of probiotics [17].

Identification with MALDI-TOF MS

The microorganisms were identified by using a system formed by comparison with a reference spectrum obtained from colonies formed on M17 and MRS agar. Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) (VITEK® MS) (bioMerieux, France) was utilized to identify the protein profiles of cell structures of the microorganisms [18].

Measurement of Acid Tolerance

In order to determine acid tolerance, the pH value of MRS and M17 broths was reduced to 2.5 by using hydrochloric acid (Sigma Aldrich, USA) for creating similar environment to stomach acidity conditions. The viability

of cultures was then monitored at pH 2.5. Colony growth on solid media and broth turbidity were evaluated as presence of the development [19].

Determination of Bile Salt Tolerance of Isolates

For the bile salt tolerance test, 0.3% (w/v) Oxgall (Bile bovine, Sigma-Aldrich, USA) showing the antimicrobial effect and containing conjugated and deconjugated bile components was inoculated (1%) to 7 mL of MRS and M17 broths. The viability was analyzed by colony counting and broth turbidity after 48-72 h incubation at 37°C [20].

Determination of Hydrophobicity of Isolates

Active cultures in MRS and M17 broths were centrifuged for 15 min at 10.000 rpm. The resulting pellet was washed twice with phosphate buffer, dissolved in 0.1 M KNO_3 (pH 6.2) buffer, added to 96-well plates, and OD was set to 600 nm using a spectrophotometer (A_0). The cell suspension (1 mL) was mixed with 0.3 mL of xylene and incubated at room temperature for 4 h. Subsequently, the OD of the aqueous phase was measured again at 600 nm (A_1) and the microbial adhesion of isolates to hydrocarbons was determined using the formula $[(A_0-A_1)/A_0] \times 100$ [21].

Antibiotic Susceptibility Test

Disc diffusion method was utilized for antibiotic susceptibility analysis. Antibiogram verification and determination of MIC (Minimal Inhibitory Concentration) were performed by using Micronaut-S beta-lactamase VII Plate (Merlin Diagnostika, Germany) according to the phenotypic determination to identify the presence of ESBL with MIC parameters [22].

Statistical Analyses

Statistical analyses were performed by SPSS Inc. Software (22.0 Version, SPSS Inc., Chicago, IL). In the statistical analysis, Pearson's correlation was used to examine whether all the data correlated with each other.

RESULTS

Isolation of bacteria from a total of 130 food samples, including boza, cheese, kefir, and raw milk with MALDI-TOF MS resulted in as of *L. brevis, L. plantarum, L. para plantarum,* and *E. faecium* species. Among the 144 identified probiotic isolates (five *L. brevis*, seven *L. plantarum*, five *L. para plantarum,* and 127 *E. faecium*), 35 (five *L. brevis*, five *L. plantarum*, three *L. para plantarum*, and 22 *E. faecium*) passed the gastric pH resistance test. Out of the 35 isolates, eight isolates (four *L. brevis*, three *L. plantarum*, and one *E. faecium*) could resist stomach pH and maintain the viability in bile salt conditions in the gastrointestinal tract, whereas only six isolates (three *L. brevis* and three *L. plantarum*) displayed hydrophobicity. The remaining six isolates (three *L. brevis* and three *L. plantarum*) were analyzed for antimicrobial resistance according to the instructions of the Institute for Clinical and Laboratory Standards, and resistance or ESBLs were not detected. The study concluded that only six (4.1%) of a total of 144 probiotic bacteria exhibited probiotic properties. *L. brevis* and *L. plantarum*, the bacteria isolated from kefir and boza, were able to companced the criteria of probiotics [23,24].

The relationship between test parameters was determined according to Pearson's nonparametric statistical correlation, which revealed that there was a significant correlation between acid and bile salt tolerance of the isolates ($P<0.05$). Results are listed in *Table 2, Table 3, Table 4, Fig. 1, Fig. 2* and *Fig. 3*.

DISCUSSION

Only six (4.1%) isolates with probiotic properties were detected among 144 isolates obtained from food sources. Three of them were *L. brevis* strains isolated from boza, and the others were *L. plantarum* strains from kefir. In the previous studies, *Lactobacillus* spp. isolates with similar probiotic properties to our study were isolated from kefir and boza samples [23,24].

Similarly, Yadav (2016) isolated 54 strains belonging to *L. plantarum* which were obtained from a local fermented food from grain, stomach acidity and bile salts were checked. It was determined that all isolates showed poor resistance. Only 24 isolates (44%) were able to show good resistance. Six (11%) species that could remain viable were analyzed for probiotic properties, and *L. plantarum RYPR1* (1.9%) exhibited satisfying results [25].

In the present study, the bacteria obtained from raw

Table 1. *Distribution of food samples*

Region	Type of Food			
	Boza[a]	Cheese[b]	Raw Milk[c]	Kefir[d]
Marmara	10	10 *	10 *	5 *
Central Anatolia	-	5 * + 5 **	17 * + 13 **	10 *
Eastern Anatolia	-	9 * + 11 **	10 * + 10 **	5 *

[a,b,c,d] natural, non industrial type and non using starter culture
** Cow Milk, ** Goat Milk*

Table 2. *Distribution of isolates identified with MALDI-TOF MS (VITEK® MS)*

Isolate Name	Source			
	Boza	Cheese	Raw Milk	Kefir
Enterococcus faecium	-	43	83	1
Lactobacillus brevis	5	-	-	-
Lactobacillus plantarum	-	3	1	3
Lactobacillus para plantarum	-	3	-	2

Table 3. *Acid-tolerant isolates and bile salt tolerances of isolates, hydrophobicity results*

No	Product	Sample No	Isolate Code	Microorganism	Acid Tolerance	Bile Salt Tolerance	Hydrophobicity Ability
					Viability (+/-)	Viability (+/-)	Hydrophobicity (+/-)
1	Boza	10	5	*Lactobacillus brevis*	+	+	-
2	Boza	3	78	*Lactobacillus brevis*	+	-	-
3	Boza	1	79	*Lactobacillus brevis*	+	+	+
4	Boza	9	81	*Lactobacillus brevis*	+	+	+
5	Boza	8	84	*Lactobacillus brevis*	+	+	+
6	Cheese	32	60B	*Enterococcus faecium*	+	+	-
7	Cheese	25	81B	*Enterococcus faecium*	+	-	-
8	Cheese	35	69B	*Enterococcus faecium*	+	-	-
9	Cheese	23	24B	*Enterococcus faecium*	+	-	-
10	Cheese	30	65B	*Enterococcus faecium*	+	-	-
11	Cheese	29	80B	*Enterococcus faecium*	+	-	-
12	Cheese	38	43C	*Enterococcus faecium*	+	-	-
13	Cheese	27	77C	*Enterococcus faecium*	+	-	-
14	Cheese	21	54C	*Enterococcus faecium*	+	-	-
15	Cheese	26	G76	*Enterococcus faecium*	+	-	-
16	Cheese	31	G4	*Enterococcus faecium*	+	-	-
17	Cheese	33	G37	*Enterococcus faecium*	+	-	-
18	Cheese	46	E54	*Lactobacillus paraplantarum*	+	-	-
19	Cheese	22	13B	*Lactobacillus paraplantarum*	+	-	-
20	Cheese	48	8C	*Lactobacillus paraplantarum*	+	-	-
21	Cheese	33	A21	*Lactobacillus plantarum*	+	-	-
22	Raw milk	58	21B	*Enterococcus faecium*	+	-	-
23	Raw milk	98	70B	*Enterococcus faecium*	+	-	-
24	Raw milk	69	23B	*Enterococcus faecium*	+	-	-
25	Raw milk	72	8B	*Enterococcus faecium*	+	-	-
26	Raw milk	77	A19	*Enterococcus faecium*	+	-	-
27	Raw milk	64	G11	*Enterococcus faecium*	+	-	-
28	Raw milk	59	G37a	*Enterococcus faecium*	+	-	-
29	Raw milk	81	G1	*Enterococcus faecium*	+	-	-
30	Raw milk	98	E14	*Enterococcus faecium*	+	-	-
31	Raw milk	85	E75	*Enterococcus faecium*	+	-	-
32	Raw milk	96	4C	*Lactobacillus plantarum*	+	-	-
33	Kefir	111	44C	*Lactobacillus plantarum*	+	+	+
34	Kefir	112	74C	*Lactobacillus plantarum*	+	+	+
35	Kefir	128	12C	*Lactobacillus plantarum*	+	+	+

milk and cheese samples did not show any probiotic properties. It has been reported that *E. faecium* obtained from animal milk and cheese had good acidification and strong bile salt tolerance in the previous studies [26,27]. However, these studies may be considered as incomplete in terms of probiotic properties due to the lack of study about the ability of colony formation in the intestinal system.

In this study, the intestinal adhesion abilities, acid and bile tolerance of the isolates were examined to determine the probiotic properties of bacteria isolated from the food samples. A similar study was conducted by Sanni [28] for bacterial isolates from some regional food products derived from grain, in which *L. plantarum* showed a good and fast acid production capability. *L. plantarum* also showed similar results in our study *(Table 3)*.

A similar study was conducted by Banwo [29] for *E. faecium* isolated from raw milk, and the technological and

Table 4. *Antibiotic disc confirmation zones (mm) of the samples, antibiogram confirmation and MIC (µg/ml) results*

No	CAZ ZON	CAZ CV	CTX ZON	CTX CV	CPD ZON	CPD CV	CAZ	CAZ MIC	CAZ CV MIC	CTX	CTX MIC	CTX CV MIC	ESBL
3	16	18	24	25	23	23	R	32	>32/4	S	≤1	≤0.5/4	-
4	-	-	-	-	-	-	?	-	-	S	≤1	≤0.25/4	-
5	-	-	-	-	-	-	S	≤1	≤0.25/4	S	≤1	≤0.25/4	-
33	-	-	-	-	-	-	S	≤1	≤0.25/4	S	≤1	≤0.25/4	-
34	-	-	-	-	-	-	S	≤1	≤0.25/4	?	-	-	-
35	18	18	15	15	18	18	S	≤1	≤0.25/4	S	≤1	≤0.25/4	-

CAZ: Ceftazidime, CTX: Cefotaxime, CPD: Cefpodoxime, CV: Clavulanate
The isolates were identified as ESBL (-)

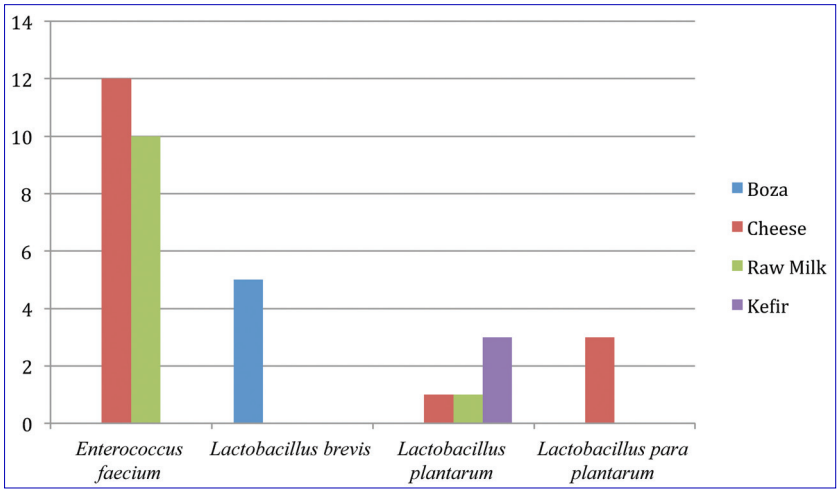

Fig 1. Distribution of isolates exceeding stomach resistance

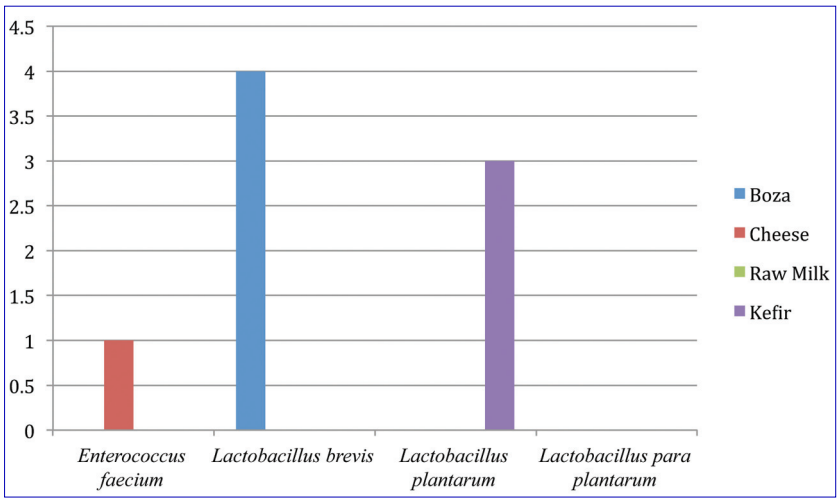

Fig 2. Distribution of bile-tolerant isolates

food safety characteristics of the species were investigated. *E. faecium* species have been detected in respect to resistance to bile salts and sensitive to antibiotics. In our study, *E. faecium* species isolated from raw milk did not show enough bile acid resistance.

In a study carried out by Gulel [30], lactobacilli strains isolated from kefir were able to resist both acid and bile salts, but their hydrophobicity remained low. In our study, the lactobacilli strains isolated especially from kefir and boza showed good hydrophobicity.

Probiotic bacteria must resist gastric acidity and bile salts and adhere to the epithelial surface of the intestinal mucosa. These properties are fundamental criteria for a bacterium to be a probiotic [31]. However, the relevant bacteria must be tested for antibiotic resistance and antibiotic resistance genes to ensure the safety for human consumption. In the studies carried out by Sanni and other researchers, the detection of antibiotic resistance status of the microorganisms seems to be missing [32-34]. It would be useful to consider these criteria, which should be examined in terms of food safety, among the probiotic properties. Thus, the probiotic character of examined the microorganisms should be the end result.

Acid tolerance is one of the most important criteria for probiotic bacteria as they are destroyed by the acidity of the stomach [35]. Probiotic bacteria are more resistant to stomach acidity than other microorganisms and are usually exposed to stomach acid with pH between 2.5 and 3.5 before arriving the colon. Acidic conditions are one of the important physiological challenges encountered by probiotic bacteria [20]. In our study, 13 strains of the 16 Lactobacilli showed resistance to pH 2.5. Besides, 22 (17%) of 126 *E. faecium* strains were able to show resistance to pH 2.5. However, there are technological methods recommended to analyze the probiotic bacteria for their

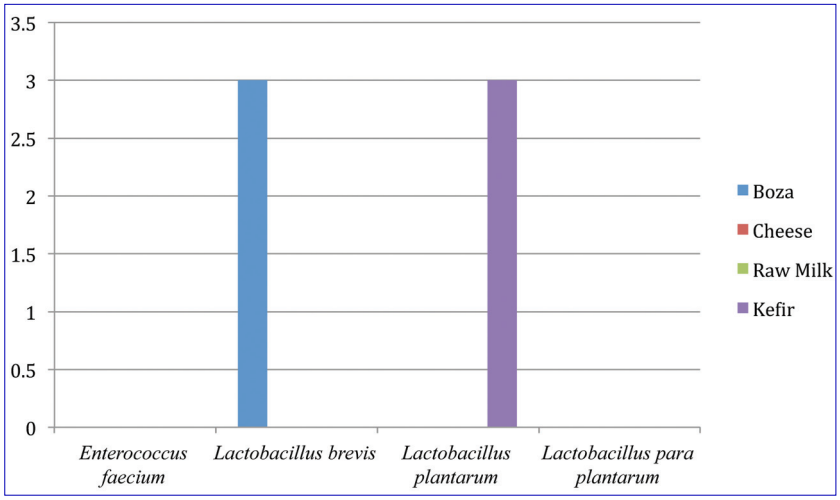

Fig 3. Distribution of isolates displaying high hydrophobicity

ability to pass through the stomach without being destroyed. The most commonly used method is microencapsulation. In principle, the powdered form of isolates is covered with a suitable material enabling bacteria to pass through the acidic environment of the stomach without getting killed [36]. In a study by Mishra and Prasad [37], three strains (43%) of seven lactobacilli were reported to be resistant to pH 2.0 or 3.0. It is indicated that the differences in pH resistance of different species and even of the same species are attributed to the differences in the multiplication stage of the bacteria [38].

The probiotic bacteria pass through the acidity of the stomach and then come into the contact with bile [39]. Bile salt tolerance is another important criterion used in the selection of probiotic bacteria [30]. Therefore, bacteria to be used as probiotics need to be resistant to bile to maintain their viability in the small intestine, a part of the gastrointestinal tract [40]. The present study found that *L. brevis* obtained from boza and *L. plantarum* obtained from kefir showed resistance to bile salts. *E. faecium* obtained from cheese and raw milk did not show enough resistance. In particular, the earlier studies on *L. brevis* and *L. plantarum* confirm the findings of our study. In the studies carried out by Ronka [41], Ramos [42], and Golowczyc [43], *L. brevis* and *L. plantarum* isolates exhibited good resistance to bile. However, in another study, 86 of the 122 *E. faecium* species isolated from traditional cheese samples (about 70%) were reported to be highly resistant to the medium containing 0.3% bile. In addition, *E. faecium* was reported to be more resistant to the harsh conditions of the gastrointestinal tract than other probiotic bacteria [44].

An important criterion for the selection of probiotic bacteria is their ability to colonize by attaching to the epithelial surfaces on the intestinal mucosa. A positive correlation has been observed between adhesion of bacterial cells and bacterial cell surface hydrophobicity [45,46]. In our study, *L. plantarum* obtained from kefir and *L. plantarum* obtained from boza showed high hydrophobicity. The probiotic properties of *L. plantarum* isolated from traditional Iranian dairy products and *L. brevis* obtained from Brazilian origin products were analyzed by Nejati [47] and Ramos [42] respectively, and the hydrophobicity abilities were found high. These studies conform to our findings on the high hydrophobicity of *L. brevis* and *L. plantarum* isolates obtained from different food samples [48].

According to the criteria established by Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) [31], bacteria with resistance to antibiotics and able to transfer the antibiotic resistance genes are considered unsafe for health and cannot be used as probiotics [49]. Therefore, transfer of antibiotic resistance genes by probiotics, especially to pathogenic bacteria is the most important risk factor and needs to be controlled [50]. Earlier studies have shown that transfer of antibiotic resistance genes to pathogenic bacteria from the *Lactobacillus* species found in the intestinal flora may be possible in limited numbers [51,52]. In our study, antibiotic resistance and especially the presence of ESBL were not observed in any bacterium. However, *Lactobacillus* strains carrying the genetic vancomycin resistance gene may be reliably used as probiotics, as no evidence has been shown for the transfer of this gene to other strains [53].

In a study by Gulel [30], although the *Lactobacillus* strains isolated from the kefir showed high resistance to nucleic acid synthesis inhibitors and cytoplasmic membrane inhibitors, a lower resistance to cell wall inhibitors and most of the protein synthesis inhibitors was observed. In our study, none of the isolated *Lactobacillus* strains displayed antibiotic resistance to nucleic acid synthesis, cytoplasmic membrane, and cell wall and protein synthesis inhibitors. In a study carried out by Zheng [54], *L. plantarum* isolated from kefir was susceptible to gentamicin, erythromycin, and chloramphenicol inhibitors, whereas it showed resistance to vancomycin.

In a study carried out by Forssten [55], the presence of ESBL was determined by administering a probiotic blend of *Lactobacillus* strains during antibiotic treatment, and ESBL negative results were obtained. *L. plantarum* and *L. brevis* strains isolated from kefir and boza yielded ESBL-negative results in a similar manner.

Kefir and boza have been produced by the fermentation of mixed cultures, including *Lactobacilli* species, could be regarded as beneficial microorganisms [23,24]. Especially, the *in vivo* studies on kefir have reported beneficial effects on health [56-59]. The result of our study show that the

bacteria obtained from kefir and boza displayed probiotic properties. This explains the beneficial effects of probiotic-containing boza and kefir on health. However, the bacteria from cheese and milk samples did not show enough probiotic properties. These observations indicated that kefir and boza consist of more bacteria with probiotic bacteria as compared to cheese and milk.

The present study determined that *L. brevis* and *L. plantarum* isolated from kefir and boza were able to compansate the set probiotic criteria. The study indicates that the probiotic bacteria may be obtained as an alternative to industrial probiotics through non-GMO (non-genetically modified organism) isolated from natural fermented food products such as kefir and boza. Besides, probiotics of Turkish origin were identified from the bacteria isolated from kefir and boza samples.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effect of Betaine Supplementation on Performance Parameters, Betaine-homocysteine S-methyltransferase Gene Expression in Broiler Chickens Consume Drinking Water with Different Total Dissolved Solids

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Abstract

The objective of the current work was to evaluate the effect of drinking water with various levels of total dissolved solids (TDS) and betaine supplementation on the performance and gene expression of betaine-homocysteine S-methyltransferase (BHMT) in broiler chickens. In a completely randomized design with a 3×2 factorial arrangement, chicks were assigned to six treatments with four replicates and 15 chicks per each. The treatments were included of three levels of total dissolved solids (400, 2.000 and 3.500 ppm) and two levels of betaine supplementation (0 and 0.2% of diet). Weight gain decreased and feed conversion ratio and water intake increased as TDS of water increased. Betaine supplementation had no effect on weight gain and water intake during the grower period, but had a significant effect on gain and feed conversion ratio in the finisher period. Mortality rate and excreta moisture content increased as TDS of water increased. Excreta moisture content decreased with betaine supplementation. Gene expression of BHMT decreased significantly with increases in TDS level and betaine supplementation increased its expression (28 folds) as compared with the non-additive group. It was concluded that consumption of drinking water with higher than 2.000 ppm TDS adversely and betaine supplementation positively affect the performance of broiler chickens. The lowest feed conversion ratio was seen in chicks fed 400 and 2.000 ppm TDS with betaine supplementation.

Keywords: *Betaine, Blood parameters, Broiler chick, Gene expression, Performance, Total dissolved solids*

Betain İlavesinin Değişik Miktarlarda Toplam Çözünmüş Katı Madde Tüketen Broiler Tavuklarda Performans Parametrelerine ve Betain-hemosistein S-metiltransferaz Gen Ekspresyonuna Etkisi

Özet

Bu çalışmanın amacı, içme suyu içerisinde değişik oranlarda toplam çözünmüş katı madde (TDS) ve betain ilavesinin broiler tavuklarda performans ve Betain-hemosistein S-metiltransferaz (BHMT) gen ekspresyonu üzerine etkisini değerlendirmektir. Tamamen rastgele olarak 3x2 faktöriyel dizayn kullanılarak, civcivler her birinde 15 hayvan ve 4 tekrar olmak üzere altı uygulama grubuna ayrıldı. Deneysel uygulamalar üç farklı dozda toplam çözünmüş katı madde (400, 2.000 ve 3.500 ppm) ve iki farklı dozda betain (diyetin %0 ve %2'si) takviyesinden oluştu. Suyun TDS'sinin artışıyla orantılı olarak kilo artışı azalırken yem dönüşüm oranı ve su tüketimi artış gösterdi. Betain ilavesi büyüme döneminde kilo artışı ve su tüketiminde etki etmezken bitirme döneminde kazanım ve yem konversiyon oranında anlamlı derece etki gösterdi. Suyun TDS'i arttıkça mortalite ve dışkı nem oranında artış tespit edildi. Dışkı nem oranı betain takviyesi ile azalma gösterdi. BHMT gen ekspresyonu TDS seviyesindeki artışla ilişkili olarak anlamlı oranda azalırken betain ilavesi betain uygulanmayan grupla karşılaştırıldığında 28 kat artış gösterdi. Sonuç olarak içme suyu içerisinde 2.000 ppm'den daha yüksek oranda TDS'in tüketilmesi broiler civcivlerde performansı olumsuz yönde etkilerken betain takviyesi pozitif yönde etki gösterdi. En düşük yem konversiyon oranı betain takviyesi ile birlikte 400 ve 2.000 ppm TDS'li civcivlerde gözlemlendi.

Anahtar sözcükler: *Betain, Kan parametreleri, Broiler civciv, Gen ekspresyonu, Performans, Toplam çözünmüş katı madde*

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INTRODUCTION

Water, as an important nutrient, is supplied for broiler chickens in sufficient access and amount, but the quality of water and its importance on the health and production has been considered less or neglected. Total dissolved solids (TDS), especially calcium, magnesium, and sodium salts, in the drinking water considered as the important factors that affect water quality. The presence of these salts in drinking water could change the osmotic status of cells and body [1] and have negative impacts on optimal regulation of intracellular macromolecules [2]. Hence, high levels of salts in water cause negative effects on water intake [3] and on feed intake [4,5] and consequently health [6,7] and growth performance [8]. In an interesting study, feed efficiency and body weight gain were affected negatively at TDS level of 3448 ppm and the highest weight gain was observed at the level of 2610 ppm [9].

To solve this problem, several management and nutritional procedures were suggested. Of which, reducing dietary salt content, purifying the drinking water and etc., that are less effective or cost consuming. Nowadays, alternative procedures that increase the animals' tolerance to high levels of TDS recommended. Tolerance of livestock animals to high levels of TDS differs and depends on species, classes of animals in each species, physiological status, dietary salt content, feed additives and season [4,8]. Therefore, feed additives like betaine that reduce the cells osmolality and rearing of livestock strains with tolerance to high levels of TDS are two executable alternative procedures to overcome the problem.

Betaine is a tri-methyl derivative of glycine and a naturally occurring compound in animal body and plant tissues [10]. Petronini et al.[11] speculated that betaine can accumulate in the cells exposed to osmotic and ionic stress and increase the water-binding capacity of cells and as an osmolyte can assist the water homeostasis in the cells, especially in the intestinal epithelium cells [12]. Supplementation with natural betaine reduced the impact of coccidia challenge on intestinal lesion scores and positively affected nutrient digestibility and feed efficiency in broilers [13]. Betaine content of body tissues in rats depends on the level of BHMT gene expression [14]. Increase in osmolality due to high concentration of salts in drinking water caused a decrease in the level of BHMT gene expression and consequently increased the level of tissue betaine level and tolerance to this condition in rat [15].

The effect of betaine on health and performance of broiler chicks exposed to salinity water (mainly sodium salt) was evaluated [16], but the effect of drinking water with high TDS (from calcium, magnesium and sodium salts) on performance and gene expression of BHMT in broilers was not defined. In addition, in the literature the effect of strain in this subject remained unclear. Therefore, the main objective of the current work was to evaluate the effect of drinking water with various levels of TDS and betaine supplementation on the performance and gene expression of BHMT in broiler chickens.

MATERIAL and METHODS

Chickens and Experimental Design

Chicks used in this study received human care and the experimental protocol was approved by the Research Committee of Islamic Azad University, Science and Research Branch (approval date: 20.06.2014; no: 27999).

A total of 360 one-day-old Ross 308 male broiler chicks (43±1.2 g) was obtained from a local hatchery and allocated to 24 wire-floored pens covered with weed shaving. In a completely randomized design with a 3×2 factorial arrangement, chicks were assigned to 6 experimental treatments with four replicates and 15 chicks per each. The treatments were included of three levels of total dissolved solids (400, 2.000 and 3.500 ppm) and two levels of betaine supplementation (0 and 0.2% of diet). Water samples was taken from natural wells in the Qazvin Province of Iran and analyzed for different quality parameters. Then, water with different total dissolved solids was provided *(Table 1)* and kept in the large plastic tanks and given to chicks based on the treatments using automatic Plasson drinker. In Qazvin province, the lowest level of TDS in drinking water of natural source is 400 ppm and the highest level is 3.500 ppm. As TDS level of 3448 reduced and around 2.000 increased the performance, these ranges of TDS were selected. Drinking of water during the first week was the same for all chicks and thereafter drinking water with different TDS was initiated. The diets of chicks were formulated based on the strain guides for nutrition. All chickens were fed the same starter, grower and finisher diet based on Ross recommendation [17]. Feed and water

Table 1. *Characteristics and elements in waters supplied for broilers*

Treatments	Parameters						
TDS mg/L	EC (µ mhos/cm)	pH	Na mEq L^{-1}	Ca mEq L^{-1}	CL mEq L^{-1}	Mg mEq L^{-1}	HCO_3 mEq L^{-1}
400	626	7.31	63.2	47.5	198	34.6	152
2000	3250	7.35	350	128	475	48.06	121
3500	5360	8.17	560	171	590	53.01	143

were provided for *ad libitum* intake. Chicks were kept in the clean and sanitary house building and under temperature and humidity controls. The house temperature was raised to 32°C in the first week and declined gradually to 21°C at the end of the experiment (days 42 of age).

Sample Collection and Measurement

In the initial day 28 and 42 of age, body weight (BW) and also total feed intake (FI) were recorded, and then feed conversion ratio (FCR) was calculated by dividing corrected weight gain on feed intake. Correction for hen day was done by daily collection of dead chicks in each pen. Carcass weights of dead chicks were included in the calculations of feed conversion ratio.

At day 28 and 42 of age, excreta moisture contents were determined in each pen by collecting total excreta samples immediately after excretion. The samples were mixed together in each pen and one homogeneous sub-sample was weighed, oven dried at 105°C for 24 h, and reweighed to determine moisture content.

At days 28 and 48 of age, the blood samples of eight chicks per treatment (two chicks per pen) from wing vein were obtained in heparin-gel containing vacuum tubes. Plasma was separated after centrifugation at 2.500 × g for 15 min and stored at -20°C for further analysis. Calcium, potassium and sodium concentration in the plasma of chicks were measured by atomic absorption spectrophotometry [18].

At day 42 of age, eight chicks per treatment (two chicks per pen) were sacrificed by cervical dislocation, then liver was removed. Liver sample was taken, then frozen quickly in liquid nitrogen and stored at -80°C until analysis for gene expression of BHMT [19].

BHMT Gene Expression

The mRNA relative abundance of BHMT was determined by q-PCR [19]. The frozen liver was crushed in a sterile mortar, and the powder was applied for total RNA extraction using a suitable kit (Fermentas Gene JET RNA purification). cDNA for BHMT gene was synthesized based on reverse transcription technique using kit (Revert Aid TM first stand cDNA). Generation analysis and Melting Curve was performed by using Real time PCR Step One™ Real-Time PCR Systems (Applied Bio systems, Foster City, CA). Quantitative PCR was performed with a specific primer pairs (forward: 5′-GCCTGAAACAGGGCAAAAGG-3′; reverse: 5′-TCCCTGTGAAGCTGACGAAC-3′) using Quanti Fast SYBER Green PCR kit (QIAGEN). B-actin (forward: 5'-CCACCGCAAATGCTTCTAAAC-3', reverse: 5'-AAGACTGCT GCTGACACCTTC-3') was chosen as a reference gene. Amplification of liver BHMT gene was performed for 35 cycles, which consisted of an initial activations step (95°C, 5 min), denaturation cycle (95°C, 30s) and combined annealing (58°C, 30s) and extension (72°C, 45s). The relative expression ratio of BHMT as a target gene was normalized to B-actin gene using method as previously described by Livak and Schmittgen [20]. Quantification for each treatment group was performed in triplicates.

Statistical Analysis

The statistical analysis was performed with ANOVA of SAS for Windows version 9.1 (SAS Institute Inc., Cary, NC) [21]. At first, the normality of data distribution was checked using the Kolmogorov–Smirnov test. ANOVA GLM (general linear model) procedure and Tukey test was used to compare the means. Statistical significance was accepted when $P<0.05$.

RESULTS

The effect of treatments on feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR) and water intake at period 7 to 28 and period of 29 to 42 of age are presented in *Table 2*. There were no main effects of TDS and betaine on feed intake at two periods and main effect of strain was significant. There were differences among main and interactions effect of TDS on body weight gain, feed conversion ratio and water intake. Weight gain decreased and feed conversion ratio and water intake increased ($P<0.05$) as TDS of water increased. Betaine supplementation had no effect ($P>0.05$) on weight gain and water intake during grower period ($P<0.05$), but had significant effect on gain and feed conversion ratio in finisher period ($P<0.05$). The lowest feed conversion ratio was seen in chicks fed 400 and 2.000 ppm TDS with and without betaine supplementation.

The main effects of TDS and betaine supplementation on mortality rate and excreta moisture content measured at days 28 and 42 of age are shown in *Table 3*. Mortality rate and excreta moisture content increased ($P<0.05$) as TDS of water increased. Main effect of betaine supplementation was not differ ($P>0.05$) for mortality rate. Excreta moisture content significantly decreased with betaine supplementation. The interactions were not significant and data not shown.

Table 4 shows the main effects of TDS and betaine supplementation on blood sodium, potassium and calcium concentration. There were no main effects ($P>0.05$) on these measured parameters in the blood, except for main effect of TDS on sodium concentration. There were no differences for interactions and data not shown

The main effects of TDS *(Fig. 1A)* and betaine supplementation *(Fig. 1B)* on the relative abundance of mRNA for BHMT gene were significant. Gene expression of BHMT decreased significantly with increases in TDS level and betaine supplementation increased its expression (28 folds) as compared with non-additive group.

Table 2. *Effects of treatments on performance parameters of broiler chickens at total period*

Treatments	Feed Intake (g)		Body Weight (g)		Feed Conversion Ratio		Water Intake (cm³)	
	days 8-28	days 29-42	days 8-28	days 29-42	days 8-28	days 29-42	days 8-28	days 29-42
TDS main effect								
400	1680	2240	1162^{a}	2210^{a}	1.55^{b}	1.80^{b}	150^{b}	279^{b}
2000	1722	2268	1169^{a}	2295^{a}	1.52^{b}	1.75^{b}	151^{b}	282^{b}
3500	1701	2212	1118^{b}	1910^{b}	1.67^{a}	2.05^{a}	160^{a}	298^{a}
SEM	35.2	48.3	61	74	0.35	0.42	4.28	6.41
P-value	0.885	0.648	0.030	0.015	0.027	0.038	0.019	0.034
Betaine main effect								
0	1711	2254	1122	2170^{b}	1.59	1.88^{a}	154	289
0.2	1693	2240	1180	2295^{a}	1.53	1.73^{b}	153	284
SEM	32.31	47.39	70.1	81.3	0.32	0.39	5.66	8.62
P-value	0.145	0.291	0.075	0.036	0.312	0.024	0.215	0.449
TDS × Betaine								
0×400	1764^{b}	2268^{b}	1218^{b}	2544^{a}	1.58^{c}	2.04^{b}	160^{b}	292^{a}
0.2×400	1701^{b}	2184^{b}	1290^{a}	2655^{a}	1.51^{c}	1.85^{c}	161^{b}	290^{a}
0×2000	1827^{a}	2324^{a}	1287^{a}	2768^{a}	1.51^{c}	1.78^{c}	168^{a}	291^{a}
0.2×2000	1869^{a}	2338^{a}	1304^{a}	2686^{a}	1.50^{c}	1.74^{c}	153^{b}	278^{b}
0×3500	1785^{b}	2366^{a}	1187^{b}	2426^{b}	1.75^{a}	2.20^{a}	165^{b}	306^{a}
0.2×3500	1701^{b}	2184^{b}	1312^{a}	2558^{a}	1.75^{a}	1.98^{b}	172^{a}	311^{a}
SEM	47.1	56.8	40.6	64.2	0.14	0.18	3.2	5.8

Within the same column, means with different superscripts are significantly differ (P<0.05)

Table 3. *The effect of treatments on mortality and excreta moisture content*

Treatments	Mortality (%)		Excreta Moisture Content (%)	
	d 28	d 42	d 28	d 42
TDS main effects				
400	5.15^{c}	2.18^{b}	80.3^{b}	81.8^{b}
2000	7.11^{b}	3.04^{a}	82.7^{ab}	82.3^{b}
3500	8.49^{a}	3.23^{a}	84.4^{a}	85.9^{a}
SEM	0.69	0.31	0.69	2.33
P-value	0.036	0.014	0.036	0.036
Betaine main effects				
0	6.23	2.24	84.5^{a}	84.2^{a}
0.2	5.71	2.11	81.9^{b}	82.2^{b}
SEM	0.31	0.28	2.88	2.41
P-value	0.936	0.864	0.031	0.039

Within the same column, means with different superscripts are significantly differ (P<0.05)

DISCUSSION

This study was carried out to evaluate the impact of drinking water with different TDS levels and betaine supplementation on performance, excreta moisture content, and gene expression of BHMT that affect health and welfare of broiler chicks.

In the present study, the level of TDS had no effect on feed intake, although TDS level of 4.300 ppm trends to decrease feed intake. Drinking water with higher levels of TDS (6.000 ppm) caused the negative effect on feed intake significantly [4,5]. Decrease in feed intake may be related to higher water intake as shown in *Table 2*. The high level of TDS in water caused an increase in water output from

Table 4. *The effect of treatments on plasma level of sodium, potassium and calcium*

Treatments	Sodium mEq		Potassium mEq		Calcium mEq	
	d 28	d 42	d 28	d 42	d 28	d 42
TDS main effects						
400	149[b]	150	5.36	5.33	10.20	10.31
2000	155[ab]	155	5.81	5.47	10.45	10.69
3500	161[a]	156	6.40	5.68	9.86	10.01
SEM	2.71	2.89	0.13	0.2	0.25	0.31
P-value	0.025	0.13	0.186	0.150	0. 367	019
Betaine main effects						
0	156[a]	157	5.79	5.15	10.22	10.52
0.2	132[b]	151	7.04	6.38	10.08	10.17
SEM	3.16	2.75	0.24	0.19	0.31	0.33
P-value	0.011	0.12	0.064	0.066	0.493	0.16

Within the same column, means with different superscripts are significantly differ (P<0.05)

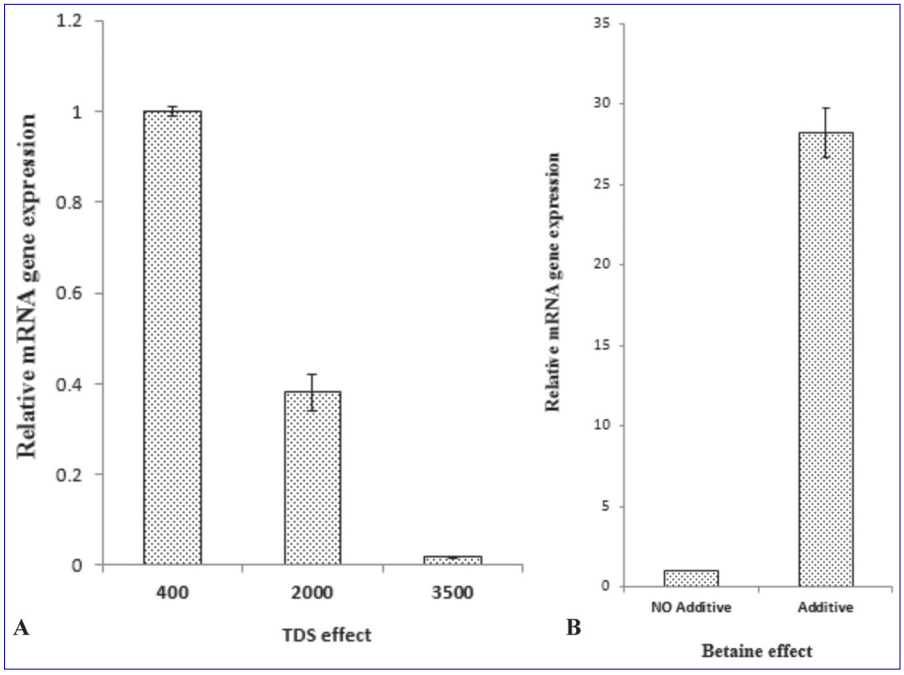

Fig 1. The effect of treatments on gene expression of BHMT

kidneys for excretion of anions and cations, hence chicks drink more water and again more salts, and consequently chicks increases its water intake. Increases in water intake impact negatively on feed intake.

Increase in TDS from 400 to 2.000 ppm increased body weight gain of broilers insignificantly. In consistent with our finding, a study [3] reported that increasing the water TDS level up to 2.375 ppm improved body weight and feed conversion ratio In this study, the decrease in body weight gain with increasing the level of TDS in drinking water exceed 2.000 ppm might be related to the increase water intake, the anion-cation imbalance [6], also lower nutrient digestion and absorption [22]. The finding of this study are in agreement with study of Pourreza et al.[23] and Ahmed [5] who speculated a decrease in body weight of broiler chicks as water TDS increased over 3.000 ppm.

In this study, betaine supplementation had no effect on feed intake of broilers and water consumption, in contrast, Wang et al.[24] found an increase in feed intake of meat ducks by betaine supplementation. Betaine supplementation had no effect on weight gain and feed conversion ratio at the grower period, but increased weight gain and decreased feed conversion ratio at the finisher period. In agreement to our finding, Honarbakhsh et al.[3] found improvements in growth performance of broilers with increases in dietary betaine levels. In contrast to our results, authors [25,26] reported no effect of dietary betaine supplementation on growth performance. The improvement in growth performance at the finisher period might be due to the osmolytic property of betaine [27] and its impact on energy expenditure for pumping ions in cells especially those exposed to hyperosmotic media [28]. Also, increase in the water retention capacity of tissues may be increased weight gain in broilers received dietary betaine as explained by Eklund et al.[29]. Moreover the osmolytic property of betaine impact positively on the growth, survival and contractile activity of intestinal cell,

thereby increase the pancreatic secretion and digesta mixing and consequently enhance nutrient digestibility [30].

In the present study, the negative effect of total dissolved solids on mortality percentage showed that young chicks (days 8-28 of age) are more susceptible to total dissolved solids in drinking water than older birds (days 29-42 of age). The most mortality in broilers of our study were caused by ascites. It has been revealed that ascites induced by salt stress in broilers was the result of osmotic difference between the plasma and tissue [31]. Betaine had no effect on mortality.

The results of this study showed an increase in water consumption and consequently excreta and excreta moisture percentage with increases in total dissolved solids. In line with our finding, Watkins et al.[7] and Honarbakhsh et al.[3] reported an increase in the excreta moisture as levels of water TDS increased. The greater litter moisture or litter wetness scores with elevated dietary Na also has been found by several researchers [5,32,33]. When salt consumption, especially sodium salts, exceeds the normal level, the secretion of renin decrease and formation of angiotensin II increase. Angiotensin II is a stimulant of water consumption which results in reduction in kidney water reabsorption [1]. Consequently it results in elevation of water excretion to excreta and increase the excreta moisture content. Betaine supplementation decreased the moisture content of excreta. In agreement, Honarbakhsh et al.[3] found that betaine supplementation could reverse the compromising effect of using high water TDS on excreta moisture.

There was no significant difference among different total dissolved solids levels, betaine supplementation and strains for the plasma levels of potassium and calcium, but differences exist for sodium level. In agreement to our finding, Balnave et al.[22] and Pourreza et al.[23] found a significant increase in blood levels of sodium by increasing TDS levels in drinking water. In another study, Talha et al.[34] showed an increase in sodium and calcium levels as TDS levels increased.

The gene expression of BHMT reduced as the level of TDS in drinking water increased. The reduction in BHMT gene expression may be caused to accumulate betaine in tissues and is a natural response to increase the tolerance of cells to hyper-osmosis induced by high levels of TDS. The enzyme BHMT activity causes a reduction in tissue betaine level as it catalyzes the transport of the methyl group from the betaine to homocysteine and syntheses methionine [29]. The result obtained in this study for the effect of TDS on BHMT gene expression are consistent with finding of Hoffman et al.[13]. Betaine supplementation increased BHMT gene expression, thereby reduce the excess betaine in the tissue for methionine synthesis. This finding was in line with report of Puchala et al.[35] in calves.

It was concluded that consumption of drinking water with higher than 2.000 ppm TDS adversely and betaine supplementation positively affect the performance of broiler chickens. Therefore, betaine supplementation can work as an osmolyte in diets of broiler which faced with high levels of TDS in drinking water. The lowest FCR was seen in chicks fed 400 and 2.000 ppm TDS with betaine supplementation.

Conflict of Interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Research Article

Effect of Chitosan Oligosaccharides on Antioxidant Function, Lymphocyte Cycle and Apoptosis in Ileum Mucosa of Broiler

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Abstract

The purpose of this study was to investigate the effects of chitosan oligosaccharides (COS) on the antioxidative function, lymphocyte cycle and apoptosis of ileum mucosa in broiler. 640 AA broilers were randomly allocated into four groups, which were fed with diets supplemented 0, 200, 350 and 500 mg/kg of COS for six weeks, respectively. The results showed that compared with the control group, the activities of glutathione peroxidase and superoxide dismutase, the ability of total antioxidant capacity and inhibit hydroxy radical as well as the contents of glutathione in the 350 and 500 mg/kg COS groups were significantly increased, while the levels of malonedialdehyde were significantly decreased. The percentages of S and gap 2/mitosis (G_2M) phases and proliferating index of ileum mucosal lymphocytes in the 350 and 500 mg/kg COS groups were increased, but, the percentages of apoptotic ileac lymphocytes were not significantly different compared with the control group. No significant difference in the levels of antioxidant function mentioned above, cell cycle phase distribution and the percentages of apoptotic ileac lymphocyte existed between the 350 and 500 mg/kg COS groups. Conclusion: dietary chitosan oligosaccharides supplements with 350 mg/kg and 500 mg/kg could improve the antioxidant function and accelerate lymphocytes proliferation, but had non-influence on lymphocytes apoptosis in the ileum mucosa of broiler.

Keywords: *Chitosan oligosaccharides, Lymphocyte, Antioxidant function, Cell cycle, Apoptosis*

Kitosan Oligosakkaritlerin Broiler İleum Mukozasında Antioksidan Fonksiyon, Lenfosit Döngüsü ve Apoptozis Üzerine Etkileri

Özet

Bu çalışmanın amacı; kitosan oligosakkaritlerin (COS) broiler ileum mukozasında antioksidan fonksiyon, lenfosit döngüsü ve apoptozise olan etkilerini araştırmaktır. 640 AA broiler rastgele olarak dört gruba ayrılarak sırasıyla 0, 200, 350 ve 500 mg/kg COS içeren diyetle altı hafta süresince beslendi. Kontrol grubu ile karşılaştırıldığında 350 ve 500 mg/kg COS içeren diyetle beslenen gruplarda glutatyon peroksidaz, ve süperoksit dismutaz aktiviteleri, total antioksidan kapasite ile hidroksit radikalini inhibe etme kapasitesi ve glutatyon miktarı anlamlı derecede artarken malondialdehit seviyesi azaldı. S yüzdesi ve gap 2/mitoz (G_2M) fazları ve ileum mukozal lenfositlerinin çoğalma indeksi 350 ve 500 mg/kg COS içeren diyetle beslenen gruplarda artarken, apoptotik iliak lenfositlerin yüzdesi anlamlı derece kontrol grubundan farklılık göstermedi. 350 ve 500 mg/kg COS içeren diyetle beslenen gruplar arasında yukarıda bahsi geçen antioksidan fonksiyon, hücre döngü faz dağılımı ve apoptotik iliak lenfosit yüzdeleri bakımından anlamlı fark tespit edilmedi. Sonuç olarak, diyette kitosan oligosakkaritlerin 350 ve 500 mg/kg oranında ilavesi broilerlerin ileum mukozasında antioksidan fonksiyonu geliştirerek lenfosit çoğalmasını hızlandırırken, lenfositlerde apoptozis üzerine etki etmemektedir.

Anahtar sözcükler: *Kitosan oligosakkaritler, Lenfosit, Antioksidan fonksiyon, Hücre döngüsü, Apoptozis*

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INTRODUCTION

In recent decades, chitosan has been used as one of additives in forage due to its various biological activities, including antitumor [1], antioxidative [2], immunepotentiating [3] and some other health benefits [4], but its high molecular weight and high viscosity may limit its use *in vivo* [5]. Chitosan oligosaccharides (COS) are the depolymerized products of chitosan, having similar or even better biological activities [6]. COS received wide-spread attention because of its solubility [7,8], low toxicity to eukaryotes [4], and immune-enhancing effects [9], along with improvement in health status of human being and animals [10-13].

The antioxidant activity of COS has been demonstrated *in vitro* and *in vivo*. In murine models, COS could reversed the decrease of glutathione (GSH) levels, and catalase (CAT) activity, and the increase of malonedialdehyde (MDA) levels in the liver, lung and kidney from LPS-induced mice [14]. In Alzheimer's disease rat, COS treatment increased hippocampal superoxide dismutase (SOD) activity level [15], and in another *in vitro* study, COS exerted antioxidative effects on pancreatic islet cells in streptozotocin-induced diabetes in rats [16]. HJ Yoon [17] reported that the production of creatinine and MDA was increased and SOD was decreased in the glycerol-induced acute renal failure rats, and COS recovered aforesaid oxidative damage in kidney [17]. COS not only has antioxidative activities in aforementioned disease models, but also has a similar effect in normal animal such as *Penaeus monodon*, in which the total antioxidant status (TAS) and glutathione peroxidase (GSH-Px) activities of digestive gland in the COS diet group were higher than those in the control [18].

It has been shown that COS can affect cell cycle and inhibit tumor growth. In the study of Liu et al.[19], pre-incubation of COS with ECV304 cells for 24 h resulted in the induction of cell cycle arrest in G_1/S+M [19]. Han et al.[20] reported that COS could inhibit the proliferation of human lung cancer line HepG2 cells, and induce their G_2/M phase arrest [20]. In another study, COS significantly inhibited the proliferation of three types of human gastric cancer cells (BGC823 cells, MKN45 cells and SGC7901 cells) after treatment for 48 h and the inhibition rate was positively correlated with the concentration of COS [21]. But, Jiang et al.[22] demonstrated that the proliferation index and the expression of cyclin D1 of normal Schwann cells treated with 0.25, 0.5 and 1.0 mg/mL COS were increased when compared with those of control [22].

Moreover, COS can affect the apoptosis process. It could induce various cells apoptosis, such as human colon cancer cells HT-29 [23], SW480 cells [24], AGS human gastric cancer cells [25], hepatocellular carcinoma cells [26], and human myeloid leukemia HL-60 cells [27]. Besides, 50-200 μg/mL COS treatment reversed the increasing of apoptotic articular chondrocytes by IL-1β-induced and downregulated the expression of Bax and caspase-3, and upregulated the expression of Bcl-2 of chondrocytes [28].

Intestinal tract is the body's structure which contacts with various antigens including bacteria, virus, parasites, sitotoxin and medicines. As part of the intestinal tract, the ileum is the major component of the gastrointestinal tract and its mucosal immune system plays an important role in the intestinal immune function. The lymphocytes of mucosa take part in mucosal immunity, their proliferation and apoptosis are one of the bases of the mucosal immune system operation. Early researches have shown that effects of COS on antioxidant role, cell cycle and apoptosis were mainly focused on tumors or disease models, and information concerning these areas on normal intestine was not available. The aim of this study was to investigate the effects of COS on antioxidant function, lymphocyte cycle and apoptosis in ileum mucosa of broiler by the methods of biochemistry and flow cytometry.

MATERIAL and METHODS

Animals and Diets

Six hundred and forty one-day-old male AA chicken were randomly and equally divided into four groups with eight replicate per group, that is, control group, COS-A group, COS-B group, COS-C group (the degree of deacetylation of COS exceed 95%, the molecular weight of COS was less than 2000 DA, ZTH Tech. Co., Beijing, China). All animal studies were approved by the Animal Ethics Committee of Sichuan Agricultural University (Approval no. 2012-024). Chickens were housed in coops with electrically heated units and provided with water and diet *ad libitum* for 42 days.

In all experiments, the basal diet was a typical corn-soybean diet which was formulated to meet standards of National Research Council [29]. COS was mixed into the corn-soybean basal diet to constitute the experimental diets with 200 mg/kg, 350 mg/kg and 500 mg/kg of COS for COS-A, COS-B and COS-C groups, respectively *(Table 1)*.

Detection of Antioxidant Function in Ileum Mucosa

At 21 and 42 days of age during the experiment, eight broilers of each group were sacrificed and ilea were immediately removed and chilled to 0°C in normal saline (Ileum was defined as the segment before the ileocecal junction equalto the length of the ceca [30]). An approximately 4-cm length of tissue was collected from the middle of ileum. Then, each sample was dissected longitudinally and washed in normal saline. The mucosae were carefully scraped from the inner surface of the each sample. Each sample was weighed, immediately transferred into a centrifuge tube, added nine-volumes of ice-cold 0.85% NaCl solution and homogenized. The homogenized solution was immediately centrifuged at 3500×g for 10

Table 1. *Composition of the experimental diets*

Composition(%)	Control Group	COS-A Group	COS-B Group	COS-C Group
Corn	54.02	54.02	54.02	54.02
Soybean meal	38.19	38.19	38.19	38.19
Soybean oil	3.53	3.53	3.53	3.53
Salt	0.40	0.40	0.40	0.40
Choline chloride	0.15	0.15	0.15	0.15
DL-metionine	0.20	0.20	0.20	0.20
Dicalcium phosphate	1.88	1.88	1.88	1.88
Calcium carbonate	1.20	1.20	1.20	1.20
Multivitamin[1]	0.03	0.03	0.03	0.03
Trace element premix[2]	0.20	0.20	0.20	0.20
COS (mg/kg)	0	200	350	500

Multivitamin[1]: *Vitamin A, 12.500 IU/kg; Vitamin D, 3.000 IU/kg; Vitamin E, 18.75 IU/kg; Vitamin K3, 3 mg/kg; pantothenic acid, 15 mg/kg; folic acid, 1.05 mg/kg; nicotinamide, 30 mg/kg; biotin, 0.14 mg/kg; Trace element premix[2]: $FeSO_4 \cdot H_2O$, 364.7 mg/kg; $CuSO_4 \cdot 5H_2O$, 32 mg/kg; $MnSO_4 \cdot H_2O$, 377.4 mg/kg; $ZnSO_4 \cdot H_2O$, 289.9 mg/kg; $K(IO_3)_2$, 18.4 mg/kg; Na_2SeO_3, 35 mg/kg*

min at 4ºC and the supernatant was preserved for future detection.

According to Bradford method [31], the concentration of total protein in the supernatant of mucosa homogenate was detected. The activities of SOD, GSH-px; and ability of total antioxidant capacity (T-AOC); and ability to inhibit hydroxy radical (IHR), and contents of MDA and GSH in the supernatant were detected by biochemical methods following the instructions of the corresponding reagent kits (SOD: Cat. NO. A001-1; GSH-px: Cat. NO. A005; ability of T-Aoc: Cat. NO. A015; ability to IHR: Cat. NO. A018; MDA: Cat. NO. A003-1; GSH: Cat. NO. A006, All of these kits were purchased from Nanjing Jiancheng Bioengineering Institute of China, Nanjing, China). The absorbance of SOD, GSH-Px, T-AOC, MDA, GSH and IHR was measured at 520, 450, 532, 412, 420 and 550 nm using microtiter plate reader (Thermo, Varioskan Flash, USA), respectively.

Detection of Lymphocytes Cell Cycle of Ileum Mucosa

The mixture of Intra-Epithelial Lymphocytes (IELs) and Lamina Propria Lymphocytes (LPLs) were isolated and detected by Flow Cytometer (FCM). Briefly, at 21 and 42 days of age during the experiment, eight broilers of each group were humanely killed. The ilea were immediately removed and placed in petri dishes containing chilled (4°C) RPMI-1640 (Catalog No. SH4007-13, LOT: MXL0747; Hyclone, Logan, UT, USA). Then ilea were opened longitudinally and washed twice in phosphate buffered saline (PBS) to remove fecal contents. They were transferred to preheated (37ºC) 10 mL glass tube containing 5 mL nutrient (D-Hank's, EDTA, DTT). The glass tubes were incubated at 37°C for 40 min with gentle stirring. The tissue slurry was filtered through a wet 300-mesh nylon mesh in order to remove undigested tissue pieces. The cell suspension was centrifuged for 10 min at 400×g, and supernatant was discarded, and 3 mL of 40% Percoll (Lot: 10036869, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) (4 parts 100% Percoll and 6 parts 10× PBS) was added to the cell pellet, then layered onto 4 mL of 70% Percoll (7 parts 100% Percoll and 3 parts 10×PBS), and centrifuged at 400×g for 30 min. IELs was collected from the two 40%/70% interface areas, combined and washed by centrifugation in supplemented RPMI-1640. The IELs concentration was adjusted to 1.0×10^6 cells/mL with PBS.

Twenty-five mL RPMI-1640 and 60 U/mL of type IV collagenase (Sigma Chemical, St. Louis, MO, USA), 50 µL/mL gentamicin and 1% Fetal calf serum (FCS) were added to a 50 mL tube and prepared for use, then the ileal segments were washed twice with 25 mL RPMI-1640 medium and then transferred to a preparatory 50 mL tube after EDTA treatment (as described in the isolation of IELs). The tubes were incubated horizontally at 37°C for 30 min in a shaking-water bath. The contents of each tube were transferred to petri dishes and 200 µL FCS were added. The ileal mucosa was compressed with a syringe plunger over a plastic mesh. Single cell suspensions containing lamina propria cells were filtered through organdy mesh and then centrifuged 10 min at 2500× g. LPLs were collected and centrifuged in a discontinuous 40/70% Percoll gradient at 600× g for 30 min. Cells collected from the interface were washed and suspended in RPMI-1640 medium with 1% FCS, and then centrifuged at 200× g for 5 min. The cell density was diluted to 1.0×10^6 cells/mL with PBS. Then 1 mL mixture of IELs and LPLs were transferred to a 5-mL centrifuge tube and centrifuged at 200×g for 5 min. The supernatant was discarded, and 1 mL PI staining solution (5 µL/mL propidium iodide, 0.5% Triton X-100, 0.5% RNase, PBS) was added. The cells were gently vortexed and incubated for 20 min at room temperature (25°C) in the dark. 2 mL PBS were added. The cells were re-suspended in 0.5 mL PBS and the cell phases were analyzed by flow cytometry (FACSCalibur, BD, Franklin Lake, NJ, USA).

The proliferating index (PI) was calculated through the following formula:

$$PI = (S+G_2M) \times 100\% / (G_0G_1+S+G_2M)$$

Detection of Lymphocyte Apoptosis of Ileum Mucosa

The aforementioned proper concentration (1.0×10^6 cells/mL) mixture of IELs and LPLs (100 µL) was transferred to a 5-mL flow tube and 5 µL of Annexin V-FITC (Cat. No. 51-65874X, BD Pharmingen, Santiago, CA, USA) and 5 µL of propidium iodode (Cat. No. 51-66211E, BD Pharmingen, Santiago, CA, USA) were added. The samples were slightly vortexed and incubated for 15 min at room temperature (25°C) in the dark. 400 µL PBS was added to each sample and percentages of apoptosis were determined by flow cytometry (FACSCalibur, BD, Franklin Lake, NJ, USA).

Statistical Analysis

The significance of difference between four groups was analyzed by variance analysis, and SPSS 17.0 for Windows was used for statistics calculation. The results were presented as mean± standard deviation (X±S), and a value of P<0.05 was considered significant results data.

RESULTS

The changes of antioxidant function in the ileum mucosa were shown in *Fig. 1*. Compared with the control group, the values of GSH-Px, SOD, GSH, T-AOC and IHR in the COS-B and COS-C groups were significantly increased (P<0.05 or P<0.01), while the levels of MDA in the COS-B and COS-C groups were significantly decreased (P<0.05 or P<0.01). However, no significant changes of antioxidant function in the COS-A group were noted (P>0.05) when compared with the control group except for values of T-AOC and IHR which were significantly increased (P<0.05 or P<0.01). Furthermore, no significant difference in the levels of antioxidant function existed between the COS-B and COS-C groups (P>0.05) *(Fig. 1)*.

As shown in *Table 2*, compared with the control group, the percentages of G_0G_1 phase lymphocytes in the COS-B and COS-C groups were obviously decreased (P<0.05 or P<0.01), while the percentages of G_2M phase, S phase and PI value were increased (P<0.05 or P<0.01) at 21 and 42 days of age except for the percentage of G_2M phase at 21 days of age. However, the percentages of G_0G_1, G_2M and S phase as well as PI value in the COS A group were not significantly different from those in the control group (P>0.05) except for the percentages of S phase (P<0.05). In addition, no significant difference in the percentages of lymphocyte cycle existed between the COS-B and COS-C groups (P>0.05) *(Table 2)*.

As shown in *Table 2*, no significant changes in the percentage of apoptotic lymphocytes in ileum mucosa among four groups were observed (P>0.05).

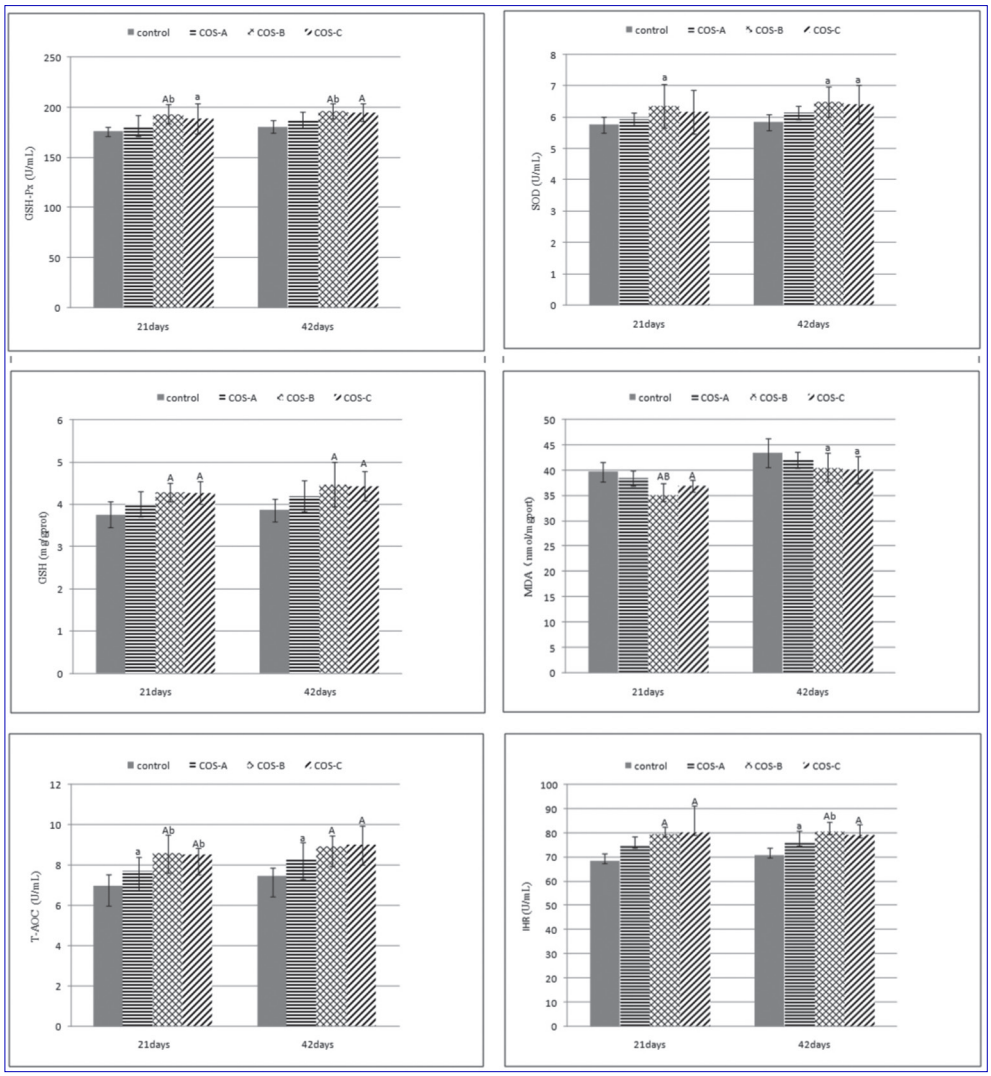

Fig 1. Effects of the GSH-px, SOD activities; GSH and MDA contents; T-AOC and IHR ability in the ileum

Table 2. *Effects of COS on lymphocyte cycle and apoptosis of ileum mucosa*

Time	Group	G_0G_1 Phase (%)	G_2M Phase (%)	S Phase (%)	PI (%)	Apoptosis (%)
21 days	Control	83.05±0.56	7.19±0.6	9.76±0.38	16.95±0.56	7.57±0.58
	COS-A	81.8±5.8	6.32±2.38	12.07±5.21	18.39±5.78	6.91±0.6
	COS-B	$77.55±1.92^{Ab}$	$8.39±0.67^{B}$	$14.06±1.37^{a}$	$22.45±1.92^{Ab}$	6.42±0.81
	COS-C	$77.03±0.29^{ab}$	$8.92±0.27^{b}$	$14.06±0.1^{a}$	$22.97±0.29^{ab}$	7.67±1.61
42 days	Control	85.03±0.31	7.22±0.33	7.75±0.1	14.97±0.31	5.7±0.44
	COS-A	84.24±1.00	7.19±0.17	$8.57±0.85^{a}$	15.76±1.00	5.54±0.87
	COS-B	$83.5±0.43^{A}$	$7.87±0.52^{ab}$	$8.64±0.23^{a}$	$16.50±0.43^{A}$	5.14±0.62
	COS-C	$83.44±0.31^{A}$	$7.99±0.44^{ab}$	$8.58±0.22^{a}$	$16.56±0.31^{A}$	5.4±1.37

Data are presented with the means ± standard deviation (n = 8). $^{a}P<0.05$, $^{A}P<0.01$, compared with the control group; $^{b}P<0.05$, $^{B}P<0.01$, compared with the COS-A group

DISCUSSION

Oxidative stress results when production of ROS exceeds the capacity of cellular antioxidant defenses to remove these toxic species [32], and antioxidant exerts its role in vivo or in food mostly via inhibiting generation of ROS, or scavenging free radicals [33]. Some antioxidant enzymes, such as SOD and CAT, are considered to be the first line of cellular defense against oxidative damage by scavenging free radical [34,35]. Hydroxy radical can cause oxidative stress and GSH is regarded as an early biological marker of oxidative stress [35]. The MDA production induces alteration of membrane fluidity and increase of membrane fragility [36-38]. Early research has shown that LPS could result in oxidative damage, in which the GSH levels and the CAT activity decreased while the MDA levels increased in mice after LPS injection [14]. However, pre-injection 100 mg/kg COS could smooth out the oxidative stress [14]. COS is a potent radical scavenger, which has the high radical scavenging activity [33]. It has been demonstrated that COS exerted antioxidant effects on pancreatic islet cells in streptozotocin-induced diabetes in rats, that is, COS recovered the maladjusted T-AOC and SOD activity as well as MDA levels [39]. In brief, COS can effectively scavenge of ROS [33] and it is a potent therapeutic agent against cancer and antioxidant additive, and has potential for application as a dietary supplement or nutraceutical [40,41]. As part of small intestine, ileum mucosa is one of the important structures that interface with external environments, but it is vulnerable to oxidative damage due to the large workload and high rate oxidative metabolism of intestine which result in abundant ROS [42]. In this study, dietary COS supplements with 350 mg/kg and 500 mg/kg could significantly increase SOD, GSH-Px activities and GSH concentration as well as improve the ability of T-AOC and IHR. These results indicated that COS played an important role in the antioxidant function in the normal ileum mucosa of broilers and could protect animals from oxidative stress. The mechanism of this might be related to the fact that being a potent radical scavenger, COS has effectively scavenging activity of ROS [33].

The cell cycle is a ubiquitous and complex process, and it can be subdivided into G_1, S, G_2 and M phases. The S phase of cell cycle is define as the period during which DNA is replicated; G_2: the period between completion of DNA synthesis and mitosis; M: from prophase to telophase. Our results show that compared with the control group, the percentages of S and G_2M phase and PI index of lymphocytes in the COS-B and COS-C groups were generally increased, suggesting dietary COS supplements with 350 mg/kg and 500 mg/kg could accelerate cell proliferation of lymphocytes in the ileum mucosa. This was accord with previous studies, in which COS enhanced immunity via accelerating T-cell differentiation and maintain T-cell activity [43], and treatment of primary Schwann cells with COS promoted cell proliferation as determined by cell cycle analysis [22]. The cell cycle is controlled by numerous mechanisms ensuring correct cell division, for example, cyclin-dependent kinases (CDK) (a family of serine/threonine protein kinases), which belongs to a family of serine/threonine protein kinases that are activated at specific points of the cell cycle [44]. The three D type cyclins (cyclin D1, cyclin D2, cyclin D3) bind to CDK4, CDK6 and CDK-cyclin D complexes are essential for entry in G_1 [45]. Cyclin A binds with CDK2 and this complex is required during S phase [46]. Previous studies demonstrated that COS could downregulated Cdk-2 and cyclin A of HepG2 cells to inhibited cell proliferation [47], and COS also could increased the cyclin D1 expression of normal neural glia cells [22]. Whether the mechanism of COS affects lymphocytes cycle in the ileum mucosa observed in this study was also related to cyclin-dependent kinases (CDKs), further studies are needed since numerous factors control the progression of the cell cycle.

Early researches have shown that COS has the tumor inhibitory effect, inducing various tumor cells apoptosis [26,27,48-52]. Also, COS had anti-apoptosis effect in IL-1β-induced chondrocytes apoptosis on osteoarthritis model

rats [28]. However, the information about the effects of COS on the cell apoptosis of normal animals is rarely available. In the present study, no significant changes in the lymphocytes apoptosis were observed among the control and three COS groups, suggesting that COS had no effect on the lymphocyte apoptosis of broiler's ileum mucosa. The mechanism of different effects of COS on apoptosis between tumor and normal cells, such as lymphocyte observed in this study may partially related to the different electric fields of different cells. It has reported that altering the electric fields can induce cell apoptosis [53,54], and tumor cells membranes have more negative charges than normal cells [55]. Unlike most polysaccharides, COS has positive charges on surface, and this chemical feature allows COS to bind strongly to negatively charged surfaces and responsible for many of observed biological activities [56,57]. However, the mechanism for this needs further studying, because properties of COS, such as DP (degree of polymerization), DA (degree of acetylation), charge distribution and nature of chemical modification to the molecule strongly influence its observed biological activities [4].

According to the results of the present study and the aforementioned discussion, it is concluded that dietary COS supplement with 350 mg/kg and 500 mg/kg could improve the antioxidant function and accelerated lymphocytes proliferation, but had non-influence on lymphocytes apoptosis in the ileum mucosa of broiler.

Conflict of Interest

The authors declare no conflict of interest.

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Research Article

Investigation on Diagnosis and Metabolic Profile of Ovarian Cysts in Dairy Cows

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Abstract

A clinical study was performed to evaluate the diagnostic methods for ovarian cysts (OC), and to determine the metabolic profiles of animals with OC in the region of Mitidja in the North of Algeria. A total of 504 non-pregnant lactating cows were used in this study. Ultrasonography was performed by EXAGO scanner and was combined with assessment of serum P4. Biochemical serum parameters were assayed by spectrophotometry and insulin and cortisol serum measurement was performed by electrochemiluminescence. The results showed an overall incidence of 11.9% of OC. The incidence of OC was higher among cows in third lactation. Holstein breed was the most affected by OC compared with other breeds ($P<0.001$). There were no effects of average BCS (Body Condition Scoring) and milk production on the incidence of OC ($P>0.05$). OC were single in 91% of cases. They were found mainly on the right ovary (66.66%). Seasonality had a significant influence on incidence rate of OC with higher incidence rates during winter and spring (71.66%); while, 28.33% of OC were detected during the summer and autumn ($P<0.05$). OC were associated with low serum concentrations of glucose, insulin and urea as well as high levels of cortisol. Ultrasound examination and progesterone assays were proposed as the most effective diagnostic combination to diagnose OC. In conclusion, in addition to hormonal imbalances, metabolic disorders are involved in the formation and/or persistence of OC. Therefore, the use of metabolic indicators in understanding and exploration of OC is of great interest.

Keywords: *Cow, Ovarian cyst, Metabolic profile, Ultrasonography*

Sütçü İneklerde Ovaryum Kistlerinin Tanısı ve Metabolik Profili Üzerine Bir Çalışma

Özet

Ovaryum kistlerinin tanısında kullanılan metotları değerlendirmek ve Cazayir'in kuzeyindeki Mitidja bölgesindeki ovaryum kistli hayvanların metabolik profillerini belirlemek amacıyla klinik bir çalışma gerçekleştirildi. Toplam 504 gebe olmayan laktasyondaki inekler bu çalışmada kullanıldı. EXAGO tarayıcı kullanılarak ultrasonografi uygulandı ve serum P4 verileri ile birlikte değerlendirildi. Biyokimyasal serum parametreleri spektrofotometri ile belirlenirken insülin ve kortizol serum ölçümleri elektrokimyasal görüntüleme ile gerçekleştirildi. Ovaryum kist insidansı %11.9 olarak belirlendi. Ovaryum insidansı üçüncü laktasyondaki ineklerde daha fazla oranda tespit edildi. Diğer türler ile karşılaştırıldığında Holstein ırkında ovaryum kisti daha fazla oranda gözlemlendi ($P<0.001$). Ovaryum kisti üzerine Vücut Kondisyon Skorunun bir etkisi gözlemlenmedi ($P>0.05$). Ovaryum kisti, vakaların %91'inde tek taraflı olarak tespit edildi. Kistler çoğunlukla (%66.66) sağ ovaryumda yer almaktaydı. Mevsimin ovaryum kistlerinin ortaya çıkmasında önemli bir etkiye neden olduğu, kış ile ilkbahar aylarında (%71.66) insidansın arttığı yaz ve sonbaharda ise düşük seviyede (%28.33) olduğu belirlendi ($P<0.05$). Ovaryum kistlerinin düşük glukoz, insülin ve üre ile yüksek kortizol serum konsantrasyonları ile ilişkili olduğu belirlendi. Ultrason incelemesi ile birlikte progesteron testi uygulamasının ovaryum kistlerinin tanısındaki en etkili kombinasyon olduğu önerildi. Sonuç olarak, hormonal düzensizliğe ilave olarak metabolik bozukluklar ovaryum kistlerinin oluşmasında ve devamlılığında rol oynamıştır. Bu nedenle, metabolik indikatörlerin kullanılması ovaryum kistlerini anlama ve incelemede büyük önem arz etmektedir.

Anahtar sözcükler: *İnek, Ovaryum kisti, Metabolik profil, Ultrasonografi*

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INTRODUCTION

Cystic ovarian disease (COD) is one of the most common reproductive disorders in cattle, causing reproductive failure in dairy cattle [1]. Ovarian cysts have been defined as fluid filled structures > 25 mm in diameter that persist in the ovaries for at least 10 days in the absence of a corpus luteum and are classified as follicular or luteal according to the degree of luteinization and P4 (progesterone) secretion [2]. The reported incidence of ovarian follicular cysts in dairy cattle showed a range from 5.6% to 18.8% [1].

Treatment efficiency of cows with ovarian cysts (OC) requires an early diagnosis at the end of the waiting period or during the reproductive period [3], taking into consideration the fact that during the first weeks post-partum, over 60% of the OC may regress spontaneously [1]. Moreover, the choice of a therapeutic strategy must also depend on the degree of accuracy (predictive value) of the diagnosis of OC (follicular cyst [FC] or luteinized cyst [LC]) because incorrect diagnosis leads to inadequate treatment [4,5]. Indeed, it is very common that veterinary practitioners diagnose ovarian cyst based on a single visit to the affected animals without reassessing the diagnosis a few days later [5], which does not provide an accurate picture of the problem because one visit indicates only the size of OC. Moreover, without hormonal analysis, it is impossible to determine if the cyst is functional or not. Similarly, diagnosis of OC is traditionally based on the history of the animal affected and the result of rectal palpation. However, the criteria suggested in the literature to distinguish types of OC are divergent and poorly defined. For example, although nymphomania characterizes FC and anoestrus is noted in LC, it is also recognized that cows with either type of cyst may show variable behavior [4]. Therefore, it seems essential to use ultrasound or P4 analysis rather than rectal palpation [1]. According to Rauch et al.[5], positive predictive value for FC or LC diagnosed by rectal palpation is 66%, it increases to 74% for FC and 85% for LC when ultrasound examination is performed. This same value also increases by performing P4 assay [5].

The present study aimed to identify the difficulties of OC diagnosis in practice and to compare the usefulness of each diagnostic method (rectal palpation, ultrasonography and P4 assay) to distinguish OC types. Furthermore, metabolic profile and BCS of cystic cows that were over 60 days post-partum (PP) were investigated.

MATERIAL and METHODS

Animals

The study was performed on 504 nonpregnant lactating cows (Holstein, Montbeliard, Fleckvieh and crossed breeds), selected from 12 herds of the Mitidja (in the North of Algeria), aged between 4 to 12 years, housing and feeding conditions were similar for all the animals, lacking of any other disorder. The study was conducted between 3 April 2013 and 5 August 2015. Animals included in the present study were more than 60 days post-partum (60-215 days PP in range), and were presumed to have OC after the first rectal palpation of veterinary practitioners. Only cows bearing ovarian structures with a diameter larger than 25 mm, in the absence of any corpus luteum and persisting for at least 10 days were considered as having OC [2]. The complaint of breeders was the successive failures of artificial insemination (AI). Control group (n=30) were cycling dairy cows that were also more than 60 days PP but without OC. These cows expressed estrus behavior, and had corpus luteum identified a few days later by rectal palpation and ultrasound examination.

Ultrasound Examination and BCS

Ultrasonography was performed by EXAGO scanner (ECM, Noveko International Inc., Angouleme, France) and equipped with 5-7.5 MHz linear transducer. Body condition scoring was evaluated according to Edmonson et al.[6].

Biochemical and Hormonal Analyses

Blood samples from each animal were collected by jugular venipuncture on the day of ultrasound examination. Collection of all samples was performed before feeding (except for BHB, 1-4 h after feeding) [7]. After collection, blood serum was separated from coagulated blood by centrifugation (3.000 rpm/20 min) and stored at -20°C until analysis.

Blood serum parameters including glucose, total protein, triglycerides, cholesterol, urea, creatinine, aspartate aminotransferase (AST), alanineaminotransferase (ALT), gamma glutamyl transpeptidase (GGT) and β-hydroxy-butyrate (BHB) were assayed by spectrophotometry on automated clinical chemistry analyser Architect plus, ci 4100 (Architect c Systems, Abbott Diagnostics, Germany). Insulin and cortisol Serum measurement was performed on another analyzer Cobas e411 by electrochemiluminescence (Roche Diagnostics GmbH, Germany). The minimum detection limit was 0.5 nmol/L for cortisol and 0.2 µU/mL for insulin.

Ultrasound examination was combined with assessment of serum P4 which was determined on Architect plus, 4100 by competitive immunoassay using chemiluminescence technology. According to the manufacturer, the minimum detection limit was 0.1 ng/mL. A serum cut-off value of 1 ng/mL was considered in the classification of OC (FC: P4 <1 ng/mL; LC: P4 ≥1 ng/mL) [5,8,9]. Cysts with thin walls ≤3 mm, a uniformly anechogenic fluid, with a P4 concentration <1 ng/mL were considered FC. In contrast, cysts with walls ≥3 mm, showing a visible echogenic rim or echogenic spots with a P4 concentration ≥1 ng/mL were considered LC [8,9].

Statistical Analysis

Statistical analysis was performed using the STATISTICA software (Version 10, Stat Soft France, 2003). Statistical differences in the concentrations of metabolic parameters between the cycling and the cystic cows were carried out using Student's t-test. Chi-squared test were used to analyze the characteristics of cystic animals and their effects on OC incidence. Stage of lactation, BCS, mean milk yield, behavior, breed, and season were considered as covariates. A logistic regression was constructed using the different risk factors as fixed effects and herd as the random effect. The effects of each variable were measured using an odds ratio along with 95% confidence intervals. Pearson's coefficient was used to calculate correlations between different measurements. Data are expressed as percentage or mean ± standard deviation. The results were considered significant when $P<0.05$.

RESULTS

Incidence of Ovarian Cysts

Among the 504 animals examined, 60 cows were affected by OC (11.9%). Identification of OC type based on rectal palpation of the veterinary practitioners, ultrasound examination and P4 serum level, is presented in *Table 1*. Based on rectal palpation, veterinary practitioners identified 60% FC and 40% LC. Ultrasound examination showed different percentages of OC (FC: 51.66%; LC: 48.33%) whereas P4 analysis combined with ultrasonography revealed 55% LC and 45% FC ($P<0.05$) *(Fig. 1, Fig. 2)*.

General Characteristics of Cows with OC

Table 2 presents the characteristics of cystic cows including incidence of OC according to the stage of lactation, reproductive behavior and the breed of the cow. Parity was excluded as all cystic animals were multiparous. Dairy production and BCS between cycling and cystic cows was also compared in this table.

Incidence of OC was higher among cows in the third lactation, followed by those in the fourth lactation (41.66% and 36.66%, respectively). Regarding other ranks of calving (2nd and 5th lactation), OC were noted in 18.33% and 3.33%, respectively ($P<0.001$). According to the farmers and veterinary practitioners, anoestrus was the predominant sexual behavior associated with the two types of OC (93.33%). Nymphomania was noted in only four cases (6.66%) ($P<0.001$). Holstein-Friesian cows were more affected than Montbeliard cows (61.66% *vs* 38.33%; $P<0.001$). The other breeds (Fleckvieh and crossed) were not affected in the present study. The average BCS of both cystic cows and cycling cows were not significantly different (3.1 *vs* 3.08; $P>0.05$). Similarly, milk production did not differ between the two groups (cycling and cystic cows) ($P>0.05$).

Description of Ovarian Cysts

The characteristics of ovarian cysts diagnosed in this study were elucidated in *Table 3*. Ovarian cysts were single in 91% of cases. Polycystic ovaries were found in 8.33% of animals ($P<0.001$). In most cases, OC were diagnosed on the right ovary (66.66%). The left ovary was affected in 28.33% of the cases whereas the two ovaries were simultaneously affected in 5% of the cases ($P<0.001$). The majority of OC were noted during the winter season (71.66%) while 28.33% were detected during the warm season ($P<0.01$).

Table 4 presents the results of the multivariate analysis, which uses cow status (cystic cows versus control animals) as the dependent variable and stage of lactation, season, behavior and breed as the independent variables. The analysis shows that the odds to have OC increased if the cow was in the third or fourth lactation. Prim'Holstein cow was the most exposed to develop OC with anoestrus as the predominant behavior. Summer and winter had the most negative effect on OC development.

Table 1. *Type of ovarian cysts according to the diagnostic methods*

Type of OC	Rectal Palpation n (%)	Ultrasonography n (%)	P4 Assessment n (%)
FC	36 (60)	31 (51.66)	27 (45) **
LC	24 (40)	29 (48.33)	33 (55) *
Total	60	60	60

** P<0.05; ** P<0.01*

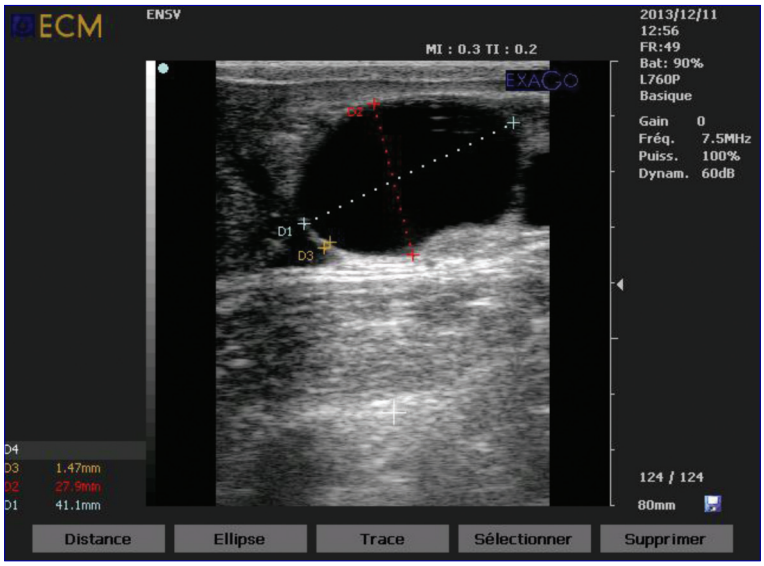

Fig 1. Follicular cyst

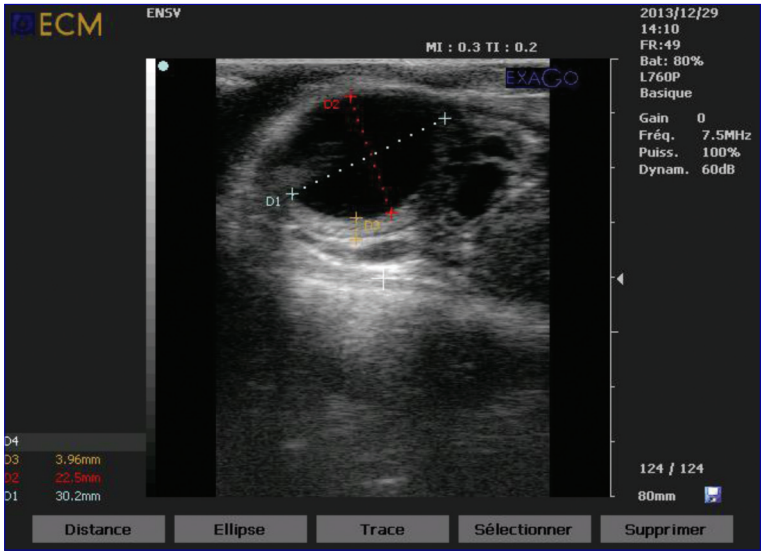

Fig 2. Luteal cyst

Table 2. *Characteristics of cows with OC*

Characteristic (n=60)		**n**	**%**
Stage of lactation	2	11	18.33
	3	25	41.66
	4	22	36.66
	5	2	3.33***
Behavior	Nymphomania	4	6.66
	Anoestrus	56	93.33***
Breed	Prim'Holstein	37	61.66
	Montbéliard	23	38.33***
BCS	Cystic cows	3.1	
	Cyclic cows	3.08^{ns}	
Dairy production (L/day)	Cystic cows	12.88	
	Cyclic cows	12.5^{ns}	

ns: *Not significant; *** P<0.001*

Table 3. *Description of ovarian cysts*

Ovarian Cysts (n=60)		**n**	**%**
Position	Right	40	66.66
	Left	17	28.33
	Right and left	3	5***
Number	Single	55	91.66
	Polycystic	5	8.33***
Season	Winter	23	38.33
	Spring	10	16.66
	Summer	21	35
	Autumn	6	10**

*** P<0.01; *** P<0.001*

Concentrations of Metabolic Parameters

The results about blood metabolic parameters are summarized in *Table 5*. They are expressed as mean ± standard deviation. To interpret these results, the reported and/or established values of these metabolites in previous studies were also mentioned.

Table 5 demonstrated that cystic cows had significantly low serum concentrations of glucose than cycling ones. However, the mean values were within the accepted normal range in the two groups. Insulin and urea concentrations were very low in cystic cows and were below the reference range while in cycling animals, the values were in the reference range. In contrast, cortisol concentrations were higher in cystic cows compared with cycling ones, but the values remained within the reference range. Although the mean values of BHB were in the normal range, there was a significant difference between the two groups of animals.

In general, concentrations of total protein, cholesterol, triglycerides, creatinine and the activity of liver enzymes (AST, ALT, and GGT) were all within the accepted range and the statistical test revealed no significant differences between the two groups of animals.

Correlations among blood metabolites, metabolic hormones, breed, stage of lactation, season, BCS and mean milk yield are summarized in *Table 6*. There were significant associations between glucose and insulin (P<0.01), glucose and cholesterol (P<0.05), glucose and cortisol (P<0.01).

Table 4. *Results of logistic regression model of cows with OC. Odds ratios along with 95% confidence intervals were calculated according to different parameters*

Parameter		**Odds Ratio**	**95% CI**	**P**
Stage of lactation	2	0.61	0.22 to 1.75	0.3
	3	1.9643	0.75 to 5.12	0.01
	4	1.3509	0.53 to 3.46	0.04
	5	0.1724	0.03 to 0.95	0.04
Behavior	Nymphomania	0.0714	0.02 to 0.22	<0.0001
	Anoestrus	14	4.51 to 43.5	<0.0001
Breed	Prim'Holstein	0.5850	0.22 to 1.53	0.02
	Montbéliard	1.7095	0.65 to 4.47	0.01
Season	Winter	0.93	0.38 to 2.28	0.03
	Spring	0.8	0.26 to 2.45	0.8
	Summer	1.2564	0.4888 to 3.2294	0.01
	Autumn	1	0.232 to 4.3098	0.3

Table 5. *Metabolic profile of cystic cows compared with cystic animals and reference range*

Parameter	Cystic Cows (n=60)	Cyclic Cows (n=30)	Reference Range
Glucose (mmol/L)	3.66±0.37	4.67±1.28 ***	2.1-5.56 [7,10-14]
Insulin (µU/mL)	1.65±1.03	5.02±1.3 ***	4.92-11.25 [14-16]
TP (g/L)	74.17±9.94	77.6±8.67 ns	70-94 [10,12,13]
TG (mmol/L)	0.19±0.10	0.19±0.13 ns	0.06-0.2 [7,10,11,17-22]
Chol (mmol/L)	3.61±0.88	3.22±1.43 ns	1.3-8.0 [10,13,15,19]
Cortisol (nmol/L)	51.71±15.74	30.41±13.87 ***	11.59-92.46 [9,10,17]
AST (U/L)	84.15±28.51	83.69±35.20 ns	56-176 [12,13,22]
ALT (U/L)	22.88±4.7	22.57±7.26 ns	11-40 [21,22]
GGT (U/L)	22.81±6.2	23.46±6.11 ns	17-51 [12,13]
Urea (mmol/L)	1.39±0.23	4.02±3.2 ***	3.3-6.06 [10,11,18,22]
Creatinine (µmol/L)	114.33±29.9	111.26±27.02 ns	88.4-240 [7,19,22]
BHB (mmol/L)	0.61±0.35	0.33±0.3 ***	<0.8 [7,10,16]

TP: *total protein;* ***TG:*** *triglycerides;* ***Chol:*** *cholesterol;* ***AST:*** *aspartate aminotransferase;* ***ALT:*** *alanine aminotransferase;* ***GGT:*** *gamma glutamyl transpeptidase;* ***BHB:*** *β-hydroxy butyrates,* ***ns:*** *Not significant; *** P<0.001*

Table 6. *The relationship among parameters in cows with ovarian cyst*

Parameter	Parameter																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1-glu	1																
2-insu	0.42	1															
3-TP	0.06	0.03	1														
4-TG	0.22	-0.1	0.08	1													
5-Chol	0.36	0.09	-0.58	0.42	1												
6-Cort	0.4	0.06	0.01	0.11	0.01	1											
7-AST	-0.1	-0.3	-0.02	-0.25	-0.23	-0.4	1										
8-ALT	-0.3	-0.7	-0.03	-0.3	-0.3	-0.12	0.14	1									
9-GGT	-0.02	-0.4	-0.04	-0.01	-0.1	-0.04	0.06	0.11	1								
10-Urea	-0.2	-0.36	0.23	0.04	0.02	0.05	-0.22	-0.22	-0.04	1							
11-Crea	-0.06	-0.48	0.07	-0.3	-0.3	-0.03	-0.06	-0.14	-0.14	0.12	1						
12-BHB	-0.56	-0.3	-0.47	0.01	-0.44	-0.14	-0.12	-0.09	-0.04	-0.09	-0.02	1					
13-MMP	-0.22	-0.7	0.18	-0.2	-0.32	0.07	-0.15	-0.02	-0.17	-0.11	0.03	0.1	1				
14-BCS	-0.32	0.06	0.09	-0.1	-0.11	-0.03	-0.23	-0.03	-0.32	-0.02	0.1	-0.08	-0.02	1			
15-Se	-0.4	-0.23	-0.23	-0.14	-0.09	-0.22	-0.06	-0.11	-0.07	0.01	-0.14	-0.14	-0.1	-0.02	1		
16-ST	-0.01	-0.4	-0.4	-0.3	-0.1	-0.32	-0.07	-0.14	-0.05	0.04	-0.22	-0.1	0.22	0.08	-0.09	1	
17-Br	-0.3	-0.09	-0.25	-0.4	-0.22	-0.09	-0.04	-0.12	0.01	-0.16	-0.09	0.03	0.08	-0.22	-0.001	-0.01	1

Glu: *glucose;* ***insu:*** *insuline;* ***TP:*** *total proteins;* ***TG:*** *triglycerides;* ***Crea:*** *creatinine;* ***MMP:*** *mean milk production;* ***Se:*** *season;* ***ST:****stage of lactation;* ***Br:*** *breed*

DISCUSSION

In the present study, the incidence of OC was 11.9% for a total of 504 animals examined. This result is very close to that determined by Brito and Palmer [23] and remains below that observed by Gümen et al.[24]. The divergence of results might therefore be explained by the difference of diagnosis methods, times and criteria of OC diagnosis, animal factors (breed, age, parity and lactation stage) other factors such as breed, age, parity, season, nutrition, breeding management, and monitoring periods.

The current study evaluated the effectiveness of three methods of OC diagnosis in dairy cows. These included: Rectal palpation by veterinary practitioners (more than 10 years of experience), ultrasound examination and measurement of serum P4. The results showed that veterinary practitioners identified 36 FC and 24 LC whereas ultrasound diagnosed 31 FC and 29 LC. In contrast,

hormonal analysis combined with ultrasound examination identified 27 FC and 33 LC. Therefore, the diagnosis had become more accurate. Progesterone concentrations noted were similar to those reported in the literature [4,25]. Data supposed that 9/60 (15%) of animals received inadequate treatment by because of treatment of FC by PGF2a, which is generally used to induce luteolysis. Indeed, OC misdiagnosis leads to incorrect therapeutic choice which delays conception. The probability of a false classification of OC is larger with the structures diagnosed firstly as being FC, in agreement with Leslie and Bosu [4]. These results reiterate the importance of ultrasound examination and hormonal diagnosis in the identification of OC type.

LC was more frequent than FC. This result supports those found by Leslie and Bosu [4] and differs from those suggested by Carroll et al.[25]. Our result is reasoned by the fact that the LC are FC at a late stage and the complaint of breeders followed upon the successive failures of the artificial insemination (AI) which can exceed more than 100 days, therefore, it's plausible that FC are transformed into LC if they are not treated in the opportune period. Most cases of OC have been associated with anoestrus in accordance with previous data [4,26,27]. In fact, it is very difficult or impossible to identify types of OC based on the clinical appearance of affected animals and on one rectal palpation. In this study, all cows affected by OC were multiparous. Within these animals, the incidence was higher for those in third lactation, followed by those in the fourth lactation. Data demonstrated that the OC were associated with higher milk production [28]. Among the OC, 91.66% were single and 8.33% were multiple. A high incidence of polycystic ovary was noted by Silvia et al.[8]. The right ovary was more affected than the left ovary. This is consistent with studies conducted by different authors [29,30]. The Holstein-Frisian cow was more affected by OC followed by the Montbeliard. This fact is consistent with what has been reported in the literature [23]. The BCS did not differ between the two groups of animals (cycling and cystic). The mean values obtained were within the reference range which is 2.5-3.4 [31]. This result was in agreement with those of Vanholder et al.[32] and Yousefdoost et al.[14]. Opsomer et al.[27] reported that the BCS at calving is not associated with the risk of OC development. In the contrast, increasing milk production in early lactation is considered a predisposing factor in the formation of OC [28]. In this study, no association between milk production and OC were recorded. It is important to note that our protocol targeted cows that were over 60 days PP and therefore outside the period of risk. Ovarian cysts were common in winter and summer, respectively. This is consistent with the result of López-Gatius et al.[28]. Lack of exercise, vitamins and minerals in winter and heat stress in summer are the main factors favoring the appearance of OC [28,33]. As shown above, stage of lactation, breed and season are clearly related to OC development.

In this study, samples were taken towards the end of peak lactation, as from the reproductive period. In this period, the animals can be expected to have recovered from a negative energy balance and loss of BCS, resulting in a restoration of blood levels of different metabolites (Non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), glucose, insulin, Insulin like Growth Factor 1 (IGF-1)). Therefore, this work was carried out in order to study the relationship between metabolic profile and the persistence and/or formation of OC in dairy cows that were over 60 days PP. To interpret the biochemical parameters, analysis of liver enzymes seems essential to reveal any hepatic dysfunction that can affect the metabolism of the animal.

Cows with OC were characterized by lower glucose levels than normal cows although the values noted were within the normal reference range. These data support those noted by Braw -Tal et al.[34] and Khan et al.[35]. In the same context, Vajdi et al.[36] reported that glucose levels were significantly different between animals that become pregnant after first AI (artificial insemination) and those with failed to first AI. However, it seems that glucose may be associated with infertility when it is well below its usual values [37].

Insulin levels were significantly different between the 2 groups of animals. Insulin levels were below normal accepted reference values previously established. Our results are similar to those of Vanholder et al.[32] and Hein et al.[16]. In another study, Obese et al.[38] found that insulin concentrations were higher in animals that had an early resumption of cyclicity compared to those who had a delay. The importance of insulin in follicular growth, maturation and ovulation, and in the stimulation of steroidogenesis has been widely accepted. Insulin also stimulates expression of LH receptors in granulosa cells, and indirectly by stimulation of IGF-1 receptor [32]. Therefore, hypoinsulinemia may not only reduce the production of androgens and estradiol, but also alters the follicle's ability to acquire LH receptors, which compromises development and ovulation and promotes persistence of follicles as anovulatory structures [16]. Previous studies have revealed that insulin secretion is impaired even after the administration of glucose in cystic cows [27]. However, although the phenomenon of 'insulin resistance' is linked to the polycystic ovary syndrome associated with metabolic disorders including diabetes [39], the authors were unable to demonstrate such relationship in the case of OC in cows [27]. More recently, Hein et al.[16] observed low expression of insulin receptor (IR and IRS1) in cystic cows.

The present study found no significant difference in the concentrations of total cholesterol, total protein, triglycerides, creatinine and the activity of liver enzymes (AST, ALT, and GGT) between the groups of animals. Similar results were noted by Vajdi et al.[36], Khan et al.[35] and Ghoneim et al.[40].

Urea concentrations between cycling and cystic cows were significantly different. Cystic animals had low concentrations of urea serum. In fact, several authors have studied the consequences of low urea levels on reproductive function in dairy cows; Miettinen [37] noted that reproductive function is impaired as soon as urea concentrations are less than 2.5 mmol/L. Enjalbert [41] mentioned that reproductive disorders appear only in cases of severe and prolonged nitrogen deficiency although it is rare. Kaur and Arora [42] reported that a low urea levels may reflect a lack of ammonia in the rumen resulting from insufficient protein intake. According to these authors, low intake caused by a lack of nitrogen has long been known in ruminants and had been particularly well studied with low nitrogen fodder. Following this deficiency, microbial protein synthesis, appetite, diet digestibility and efficiency of the use of metabolizable energy decrease. It results in hypoglycemia and low insulin, inhibiting hypothalamic GnRH secretion, pulsatile secretion of LH and P4 synthesis. However, the results differ from those noted in previous research [14], which have found that cystic cows had high concentrations of urea compared to cycling cows. Result discrepancies can be explained by diet received by cows, the time of sampling relative to food intake and the physiological stage of the animal.

The average concentrations of cortisol were significantly higher in cystic cows compared to cycling animals although the values were within the accepted normal range. Our results are consistent with those of Khan et al.[35] in buffaloes but differ from Probo et al.[9] in cattle and Ghoneim et al.[40] and El -Bahr et al.[43] in camels. This divergence may be explained by the difference in breeding conditions, climatic factors, stage of lactation and food intake. For these criteria, we may also add the way of OC development (spontaneous or induced by ACTH injection). According to Dobson and Smith [44], any change in the environment (especially PP) may be at the origin of a stress which increases the cortisol concentrations. This induces a reduction in the secretion of estradiol and a rise in serum P4. Thus, the concentrations of LH and FSH are reduced by negative feedback, the preovulatory LH surge is not observed and OC develop.

Serum concentrations of BHB were significantly higher in cystic cows although its values were in the reference range. Vanholder et al.[32] and Jackson et al.[45] reported similar results. According to Probo et al.[9], low BHB may indicate a low mobilization of fat reserves. Despite the involvement of BHB in the OC pathogenesis [5], the results of the present work have demonstrated no influence of this parameter in OC maintaining.

This study demonstrated that ultrasound examination combined with assessment of serum P4 was the best method to distinguish OC type. LC was predominant and the majority of cystic cows were in the third lactation. Anoestrus was the most common behavior observed by these cows. Stage of lactation, season and breed constitute the risk factors of OC development. Clearly, other risk factors need to be considered when trying to understand the problem of OC. Metabolic disorders are involved in the formation and/or persistence of OC. This is supported by the high incidence of OC in dairy cows that had over 60 days PP. In this study, the OC were associated with low serum concentrations of glucose, insulin and urea as well as high concentrations of cortisol. Although we have tried to explain the involvement of each of these parameters in the OC pathogenesis, the mechanism exact leading to the formation of OC remains obscure. The role of nutrition in the changes of the concentrations of these metabolites is widely reported. The use of biochemical indicators in understanding and exploration of OC is of great interest.

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Research Article

Lentivirus-mediated bta-miR-193a Overexpression Promotes Apoptosis of MDBK Cells by Targeting BAX and Inhibits BVDV Replication

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Abstract

MicroRNAs (miRNAs) are a class of naturally occurring, short, endogenous, noncoding RNA molecules (~22 nt) involved in a wide variety of regulatory pathways, including cell growth, development, differentiation, proliferation, and apoptosis, as well as viral defense, hematopoiesis, organ formation, and metabolism. Previous studies showed that bta-miR-193a (miR-193a) was upregulated in Madin-Darby bovine kidney (MDBK) cells infected with bovine viral diarrhea virus (BVDV) strain NADL; however, the role of miR-193a in apoptosis-associated regulation remains unclear. In this study, we found that miR-193a is a novel regulator of MDBK apoptosis and that lentiviral infection exhibited a positive effect on miR-193a expression. Additionally, we observed that the miR-193a-target sequence was present in the 3′-untranslated region of B-cell lymphoma-2-associated X protein (BAX) mRNA, with miR-193a overexpression resulting in reduced BAX mRNA and protein levels. Furthermore, we observed that miR-193a promoted apoptosis and inhibited BVDV strain NADL replication according to quantitative reverse transcription polymerase chain reaction results. These findings confirmed miR-193a as a positive regulator of apoptosis and provided a theoretical basis for the important role of miRNAs in regulating BVDV replication.

Keywords: *Lentivirus, miR-193a, Apoptosis, BAX, BVDV strain NADL*

Lentivirus Aracılı bta-miR-193a Overeksprasyonu MDBK Hücrelerinin Apoptozisini Artırır ve BVDV Replikasyonunu Baskılar

Özet

MikroRNAlar (MiRNA) doğal olarak bulunan, kısa, endojen, kodlama yapmayan RNA molekülleri (~22 nt) olup, hücre büyümesi, gelişmesi, farklılaşması, çoğalması ve apoptozis gibi çok çeşitli düzenleyici yolaklarda ve ayrıca viral savunmada, hematopoieziste, organ şekillenmesinde ve metabolizmada görev yapmaktadır. Yapılan çalışmalar Bovine Viral Diare Virus (BVDV)'un NADL suşu ile enfekte Madin-Darby Bovine Böbrek (MDBK) hücrelerinde bta-miR-193a (miR-193a)'nın ekspresyonunun upregule edildiğini göstermiştir. Ancak, apoptozis ilişkili regulasyonda miR-193a'nın rolü bilinmemektedir. Bu çalışmada, miR-193a'nın MDBK apoptozisinde görev yapan bir regülatör olduğu ve lentivirus enfeksiyonunun miR-193a ekspresyonunda pozitif bir etki gösterdiği tespit edilmiştir. Ayrıca, miR-193a hedef sekansının B-hücre lenfoma-2-ilişkili X protein (BAX) mRNA'sının 3′-translasyon yapılmayan bölgesinde mevcut olduğu ve miR-193a overekspresyonunun azalmış BAX mRNA ve protein seviyesi ile ilişkili olduğu belirlendi. Kantitatif ters transkripsiyon polimeraz zincir reaksiyonu ile belirlendiği üzere miR-193a apoptozisi artırdı ve BVDV suş NADL'nin replikasyonunu inhibe etti. Elde edilen sonuçlar miR-193a'nın apoptozisin pozitif regülatörü olduğunu onaylayarak BVDV replikasyonunun düzenlenmesinde miRNA önemli rol oynadığı hakkında teorik temel oluşturmuştur.

Anahtar sözcükler: *Lentivirus, miR-193a, Apoptozis, BAX, BVDV suş NADL*

INTRODUCTION

Apoptosis plays an important role in regulating cell death and is involved in many important physiological processes, including the normal development of the immune system [1], new and old cell replacement [2], embryonic development [3], and hormone-dependent atrophy [4]. Anti-apoptotic members of the B-cell lymphoma 2 (Bcl-2) family

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include Bcl-2, Bcl-w, and Bcl-xL [5-7], whereas pro-apoptotic effectors include Bcl-2 homologous antagonist/killer (BAK) and Bcl-2-associated X protein (BAX). However, apoptosis may exhibit opposite effects on viral pathogenesis by preventing or enhancing viral transmission from infected cells [8,9]. Bovine viral diarrhea virus (BVDV) strain NADL down-regulates Bcl-2 expression by activating the endoplasmic reticulum (ER) transmembrane RNA-like endoplasmic reticulum kinase [10]. Host cells infected with cytopathic BVDV subsequently undergo unregulated apoptosis [11].

MicroRNAs (miRNAs) are endogenous, ~22-nt, small, noncoding RNAs that negatively regulate gene expression at the post-transcription level by blocking translation of or degrading target mRNAs [12-14]. miR-193a is a critical regulatory factor that targets anti-apoptotic myeloid leukemia cell sequence-1, which mediates cell proliferation and apoptosis [15-20]. Additionally, miR-193a overexpression inhibits 5-bromo-2′-deoxyuridine incorporation and induces activation of caspase-3/7, resulting in apoptotic cell death in A2780 cells [21]. Moreover, transfection of HA22T/VGH hepatocellular carcinoma cells with miR-193a results in increased apoptosis and reduced proliferation, and combined treatment with miR-193a and sorafenib results in proliferation inhibition [22]. Furthermore, ectopic expression of miR-193a leads to reduced cell proliferation, increased differentiation, and induction of apoptosis in acute myeloid leukemia blasts by targeting tyrosine-protein kinase Kit, DNA (cytosine-5)-methyltransferase 3A, cyclin D1, and mouse double minute-2 homolog [23,24].

Previous findings suggested that miR-193a is significantly upregulated in BVDV strain NADL-infected MDBK cells [25]; however, the biological roles of miR-193a in BVDV strain NADL-infected MDBK cells remain unknown. In this study, we observed that miR-193a effectively promoted apoptosis associated with BAX downregulation. These results provided a novel perspective in methods for the prevention of BVDV spreading.

MATERIAL and METHODS

Cells and Plasmids

The MDBK and HEK-293T cell lines were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and grown in Dulbecco's modified Eagle medium (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Hyclone; GE Healthcare, Pittsburgh, PA, USA). The plasmids of the lentiviral-packing system (pLentiLox 3.7/pLL3.7, CMV-VSVG, pMDLg/pRRE, and pRSV-REV) were supplied by Dr. Bin Jia [26].

Target Prediction

Targets of miR-193a were predicted using different miRNA-target-prediction algorithms, including Microcosm Targets *(http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/genome.pl)* and TargetScan *(http://www.targetscan.org/vert_71/)*.

Lentiviral Production and Identification

To generate miR-193a-overexpressing, inhibiting, and negative-control lentiviruses, pLL3.7-pre-miR-193a, pLL3.7-pre-miR-193a IN, and pLL3.7 empty plasmids were co-transfected into HEK-293T cells using the helper plasmids (CMV-VSVG, pMDLg/pRRE, and pRSV-REV) and a high-efficiency transfection reagent (Cat. No. BW11002; Biowit, Hangzhou, China). At 48 h post-transfection, the lentiviral particles increased in number, and the viral titer was subsequently measured using the Reed-Muench method [27]. Viral titer was determined based on expression of the enhanced green fluorescent protein [28] as visualized by a fluorescence microscope (model TE2000; Nikon, Tokyo, Japan). Lentiviruses overexpressing pre-miR-193a and pre-miR-193a inhibitor were named lv-pLL3.7-pre-miR-193a and lv-pLL3.7-pre-miR-193a IN, respectively, and lentivirus packaged with pLL3.7 empty vector (negative-LV) served as a negative control. Quantitative real-time PCR (qRT-PCR) was employed to monitor the miR-193a expression at 48 h post-transfection. The primers for *Bos taurus* pre-miR-193a, pre-miR-193a inhibitor, miR-193a, and 5S rRNA (serving as an internal control) were designed using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA). The primers used are listed in *Table 1*, and qRT-PCR was performed as described in the proceeding sections.

Dual Luciferase-reporter Assay

The sequences of 3′-untranslated regions (UTRs) and corresponding BAX mutations were cloned into the *Sac*I and *Xho*I restriction sites in the dual luciferase-reporter vector pmirGLO (Promega, Madison, WI, USA). The primers for *BAX* and *GAPDH* (serving as an internal control) are listed in *Table 1*. The relative light units (firefly luciferase/Renilla luciferase) were determined using a dual luciferase-reporter assay system (E1910; Promega) in HEK-293T cells co-transfected with the indicated 3′-UTR and miRNA combinations at 48 h post-transfection as described previously [29].

qRT-PCR

Cells treated with lv-pLL3.7-pre-miR-193a, lv-pLL3.7-pre-miR-193a IN, or negative control (NC) for 48 h were harvested and subjected to total RNA extraction using a total RNA-extraction kit (TIANGEN Biotech, Beijing, China). First-strand cDNA was synthesized from 2 μg of total RNA using a reverse-transcription kit (TIANGEN Biotech) according to manufacturer instructions. The synthesized cDNA was used for qRT-PCR to analyze the mRNA levels of BAX using a LightCycler 480 (Roche, Indianapolis, IN, USA). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method [30,31].

Table 1. *Primers for amplifying and real-time quantitative PCR*

Primers	Primers sequences (5′→3′)
pre-miR-193a-F	CCGTTAACGGGAGCTGAGAGCTGGGTCTTTG
pre-miR-193a-R	CCCTCGAGGGGGGCCGAGGACTGGGA
pre-miR-193a-R-IN	CCCTCGAGGGGGGCCGAGGAACTGGCCTACAAAGTCCCAGTGAACCGACACCTTCATCT
miR-193a-RT	AACTGGCCTACAAAGTCCCAGT
5S rRNA-F	GCCCGATCTCGTCTGATCT
5S rRNA-R	AGCCTACAGCACCCGGTATT
Bax 3′UTR-F	CGGAGCTCTTATGGCATTTTTCAGGGGG
Bax 3′UTR-R	CGCTCGAGCACAATTTAACTCGCCAC
Bax 3′UTR mutation-F	CGGAGCTCTTATGGCATTTTTCAGGGGG
Bax 3′UTR mutation-R	CGCTCGAGCACAATTTAACTCGCCTGACAGGCTGTGTCGGCACTGGTTACCCTCAG
GAPDH-qRT-PCR-F	GTCACCAGGGCTGCTTT
GAPDH-qRT-PCR-R	TGTGCCGTTGAACTTGC
Bax-qRT-PCR-F	CCCCGAGAGGTCTTTTTC
Bax-qRT-PCR-R	TGAGCACTCCAGCCACAA
5′UTR-qRT-PCR-F	TAAACGTGGTAACACAAGCTAGAGATA
5′UTR-qRT-PCR-R	GTCAACCCGTCAACAAGGTAAAG

Western Blot Analysis

MDBK cells were infected with lv-pLL3.7-pre-miR-193a, lv-pLL3.7-pre-miR-193a IN, or NC. At 48 h post-infection, cells were collected and treated with cell lysis buffer (Beyotime, Haimen, China). Total proteins were extracted and subjected to concentration determination using the BCA protein quantification assay kit (TIANGEN Biotech). Western blot analysis was performed as previously described [32]. For Western blots, the primary antibodies used were polyclonal anti-BAX (1:1000; ab32503; Abcam, Cambridge, MA, USA) and monoclonal anti-β-actin (1:2500; AP0060; Bioworld Technology, Louis Park, MN, USA). The horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin G (H+L; 1:5000; BS13278; Bioworld Technology) was used as the secondary antibody. Western blots were analyzed using ImageJ software (v10.2; National Institutes of Health, Bethesda, MD, USA).

Flow Cytometry Analysis

At 48 h post-infection with lv-pLL3.7-pre-miR-193a, lv-pLL3.7-pre-miR-193a IN, or NC, apoptosis was determined by flow cytometry using the BD FACSCalibur system (BD Biosciences, San Jose, CA, USA) after AnnexinV-APC/7-AAD (KGA1025; KeyGEN BioTECH, Nanjing, China) staining according to manufacturer instructions.

Detection of BVDV Replication

To determine the effects of miR-193a on BVDV NADL replication, BVDV NADL replication was measured by qRT-PCR. miR-193a mimics, miR-193 inhibitor, and NC miRNA mimics were purchased from GenePharma (Shanghai, China) and transfected into MDBK cells for 48 h, respectively. The cells were harvested after infection with BVDV strain NADL for 24 h and subjected to total RNA extraction using the Total RNA-extraction kit (TIANGEN Biotech) and reverse transcribed into cDNA. Levels of BVDV NADL mRNA and the presence of the 5′-UTR were determined by qRT-PCR.

Statistical Analysis

SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for single-factor analysis of variance statistics for the experimental data. Data are shown as the mean ± standard error. Asterisks indicate statistical significance as determined by Student's *t* test, with $P<0.05$ or $P<0.01$ indicating significance.

RESULTS

Analysis of Lentivirus-mediated miR-193a Expression

To identify the effects of pre-miR-193a-overexpressing and -inhibiting lentiviruses on miR-193a expression in MDBK cells, vectors with the targeted pre-miR-193a were designed and cloned into pLL3.7 lentiviral vectors, followed by co-transfection of the recombinant plasmids into HEK-293T cells with the helper packing plasmids. Lentiviral particles were collected at 48 h post-transfection *(Fig. 1A)*. The lentivirus was then used to infect MDBK cells, and miR-193a expression was determined by qRT-PCR. As shown in Figure 1B, miR-193a expression increased significantly following transfection with lv-pLL3.7-pre-miR-193a as compared with levels observed in cells transfected with lv-pLL3.7-pre-miR-193a IN or NC (lv- pLL3.7) at 48 h post-infection ($P<0.05$; $P<0.01$). By contrast, lv-pLL3.7-pre-miR-193a IN infection significantly reduced miR-

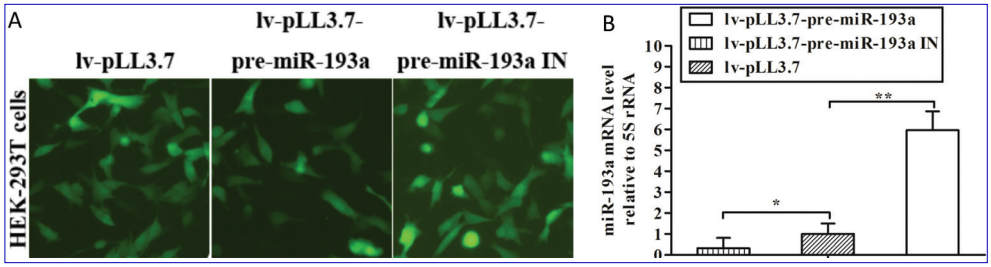

Fig 1. Analysis of lentivirus-mediated miR-193a expression (A) pLL3.7-pre-miR-193a, pLL3.7-pre-miR-193a IN, and pLL3.7 plasmids were transfected into HEK-293T cells along with helper plasmids. At 48 h post-transfection, a large number of positive cells (green fluorescence) were observed by fluorescence microscopy. (B) Analysis by qRT-PCR of miR-193a expression in MDBK cells at 48 h post-infection with lentivirus. Data showed miR-193a expression was significantly upregulated in MDBK cells infected with lv-pLL3.7-pre-miR-193a as compared with cells infected with lv-pLL3.7 at 48 h post-transfection. ** $P<0.01$. By contrast, miR-193a expression was significantly downregulated in MDBK cells treated with lv-pLL3.7-pre-miR-193a IN. $P<0.05$

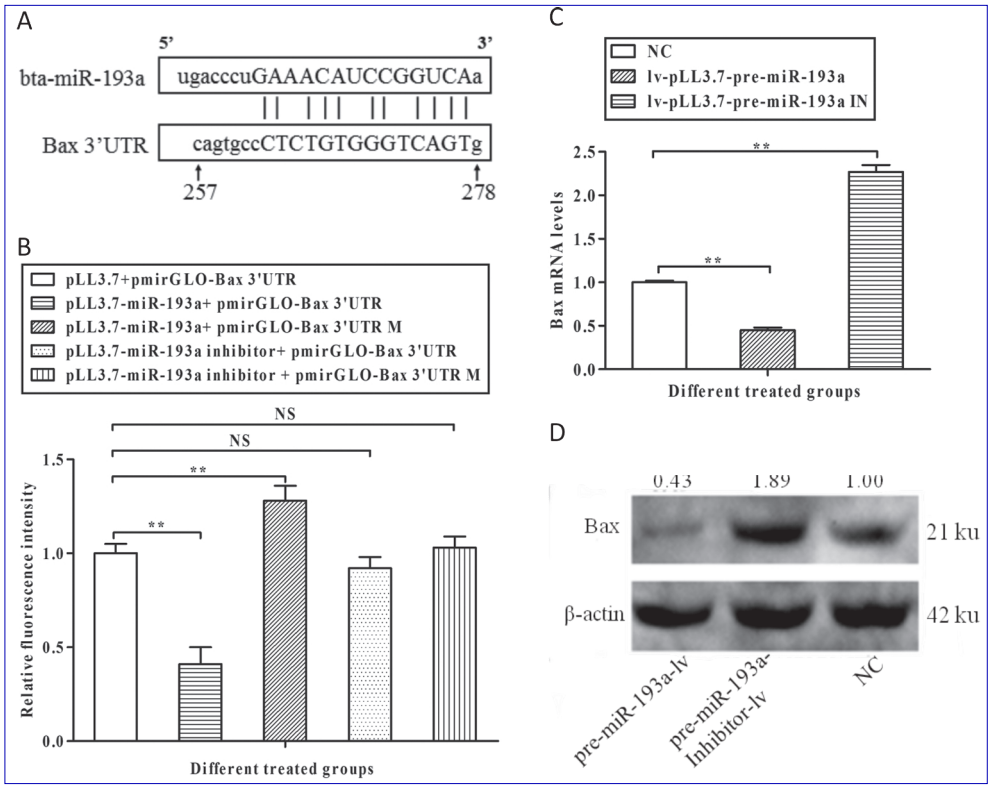

Fig 2. miR-193a targets BAX and downregulates BAX expression. (A) Schematic representation of the 3′-UTR of BAX mRNA showing the miR-193a-binding site. (B) A dual luciferase-reporter assay was performed in HEK-293T cells. Luciferase-reporter plasmids with miR-193a-target sites or corresponding mutants were co-transfected along with pre-miR-193a lentiviral vectors into HEK-293T cells. Fluorescence was visualized at 48 h post-transfection using a dual luciferase-assay system. Data represent the mean ± standard deviation *(error bars)*. ** $P<0.01$ ($n = 3$). (C) BAX mRNA levels were analyzed by qRT-PCR in cells transfected with lv-pLL3.7-pre-miR-193a, lv-pLL3.7-pre-miR-193a IN, or lv-pLL3.7. Data represent the mean ± standard deviation *(error bars)*. ** $P<0.01$ ($n = 3$). (D) Western blot analysis of BAX protein levels in the indicated MDBK cells. NC-lv represents negative-control lentivirus. Western blots were analyzed using ImageJ software ($n = 3$)

193a expression levels ($P<0.01$) *(Fig. 1B)*. These findings suggested that lentivirus-delivered miR-193a exhibited a positive effect on miR-193a expression.

miR-193a Targets the BAX mRNA 3′-UTR and Downregulates BAX Expression

The function of identifying the target sequence of miR-193a is significant to the regulation of BVDV strain NADL replication and apoptosis of infected cells. To determine whether the 3′-UTR of BAX mRNA was the functional target of miR-193a, the potential miRNA-binding site and corresponding mutations to the 3′-UTR were cloned into a dual luciferase-reporter vector (pmirGLO) *(Fig. 2A)*. The pre-miR-193a lentiviral vector and the pmirGLO-BAX 3′-UTR

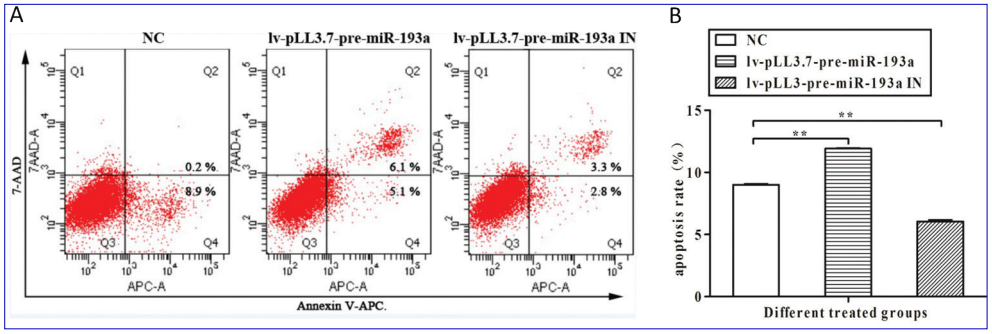

Fig 3. Infection with lv-pLL3.7-pre-miR-193a significantly promotes apoptosis in MDBK cells. (A) Representative flow cytometry images were used to assess apoptotic cells after double staining with Annexin V-APC and 7-AAD ($n = 3$). (B) Data were obtained from three independent experiments and represent the mean ± standard deviation *(error bars)*. ** $P<0.01$

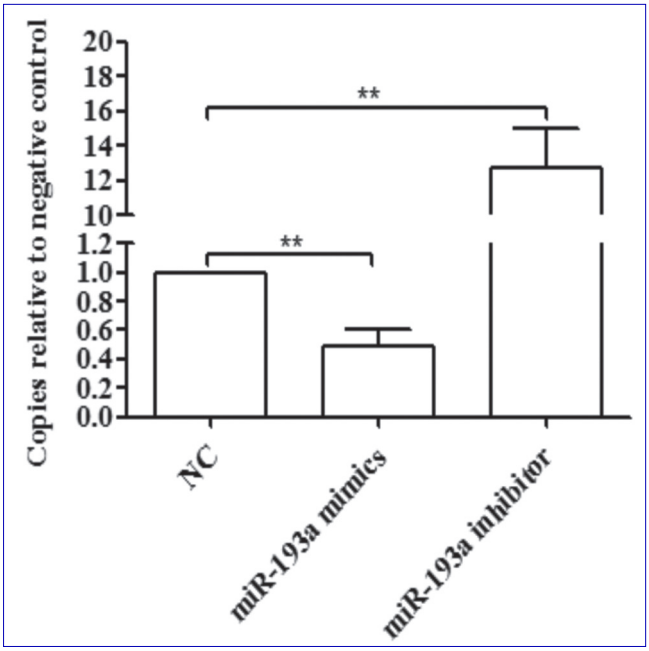

Fig 4. qRT-PCR analysis of BVDV replication in miR-193a-overexpressing and -inhibiting cells. Data represent the mean ± standard deviation *(error bars)*. ** $P<0.01$ ($n = 3$)

reporter plasmids were co-transfected into HEK-293T cells along with an internal control vector pLL3.7 + pmirGLO-BAX 3′-UTR, resulting in significant decreases in relative fluorescence activity. We observed no significant effect by the mutation on the target sequence as compared with the control vector *(Fig. 2B)*. We then determined whether miR-193a overexpression affected BAX expression according to qRT- PCR and western blot analyses. The data indicated that BAX expression was significantly inhibited by lv-pLL3.7-pre-miR-193a infection *(Fig. 2C* and *D)* ($P<0.01$). These findings showed that miR-193a directly targeted the BAX 3′-UTR and downregulated BAX expression.

miR-193a Promotes Apoptosis in MDBK Cells

To determine the relationship between miR-193a and apoptosis, we determined the effects of miR-193a over-expression on MDBK apoptosis by flow cytometry. As shown in *Fig. 3A*, the rate of apoptosis was significantly elevated in cells infected with lv-pLL3.7-pre-miR-193a as compared with the rate observed in cells infected with lv-pLL3.7-pre-miR-193a IN or lv-pLL3.7 (NC) *(Fig. 3B)*. These results suggested that miR-193a overexpression reduced BAX expression and promoted MDBK apoptosis.

Overexpression of miR-193a Reduces BVDV NADL Replication

As shown in *Fig. 4*, compared with the control group transfected with miR-193a mimics, copies of BVDV strain NADL in MDBK cells transfected with miR-193a mimics were significantly reduced ($P<0.01$). By contrast, copies of BVDV NADL were significantly increased in MDBK cells transfected with miR-193a inhibitors ($P<0.01$). These results suggested that miR-193a inhibited BVDV strain NADL replication in MDBK cells.

DISCUSSION

Previous studies reported miR-193a involvement in regulating apoptosis [21,24,33], cell proliferation [34], and differentiation [23,35]. In a previous study, we showed that BVDV NADL-infected MDBK cells exhibited upregulated levels of miR-193a. In this study, we found that miR-193a expression was regulated by miRNA-precursor-expression lentiviruses, with our data confirming that miR-193a directly targeted the 3′-UTR of BAX mRNA, thereby down-regulating BAX expression and leading to increased levels of apoptosis in MDBK cells.

BAX is a member of the Bcl-2 family and is the major pro-apoptotic protein involved in bidirectional regulation of apoptosis [36,37]. BAX overexpression results in apoptosis induction in multiple cell types and plays an important role in the neuronal cell death [38,39]. However, BAX is also a potent inhibitor of neuronal cell death in mice infected with the Sindbis virus and protects newborn mice from neuronal apoptosis [40]. According to previous studies, the Bcl-2 family, including Bcl-2, Bcl-X, and Mcl-1, are important

regulators of programmed cell death and apoptosis [41]. When intracellular Bcl-2 expression promotes apoptosis, if intracellular BAX levels are in excess, These proteins form a homologous structure resulting in a BAX-BAX dimer [42]. There are three types of apoptotic pathways related to caspases, including the mitochondrial cytochrome C pathway, the ER pathway, and the death-receptor pathway [43]. BAX is involved in the caspase-associated death-receptor-signaling pathway. The death-receptor factors fasciclin domain (Fas)1 and Fas transmit signals to the apoptosis-inducing complex, inducing free caspase-8 to form other apoptosis-inducing complexes. The BH3-interacting domain protein (Bid) is transformed into tBid when caspase-8 concentrations are too low. This is followed by activation of multi-domain BAX and BAK variants by tBid, oligomer formation on the mitochondrial outer membrane, and alterations in mitochondrial permeability and release of cytochrome C to activate caspase-9 and caspase-3 and induce apoptosis [44,45]. In a previous study, bta-miR-29b attenuates apoptosis by directly targeting caspase-7 and NAIF1 and suppresses bovine viral diarrhea virus replication in MDBK cells [32]. Here, A similar finding was that we confirmed the presence of a miR-193a-binding site in the BAX mRNA 3′-UTR according to results of a dual luciferase-reporter assay. The resulting downregulation of BAX levels promoted induction of apoptosis in lv-pLL3.7-pre-miR-193a-infected MDBK cells and inhibition of viral replication.

In conclusion, our results indicated that BAX is a critical target of miR-193a and plays a central role in the apoptosis pathway, with BAX downregulation inducing apoptosis. Moreover, miR-193a plays an important role in BVDV strain NADL replication. The mechanism associated with miR-193a-mediated apoptosis and inhibition of BVDV replication involves its interaction with mRNA of the pro-apoptotic gene BAX, induction of apoptosis, and engulfing of apoptotic debris by phagocytes for lysosomal degradation of the BVDV strain NADL virus. Our findings provided a theoretical basis for the important role of miRNA in apoptotic regulation and offer a potential target for the prevention and control of BVDV strain NADL infections.

Acknowledgments

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Conflicts of Interest Statement

The authors have declared no conflicts of interest.

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Research Article

Identification and Characterization of *Clostridium perfringens* Isolated from Necrotic Enteritis in Broiler Chickens in Tiaret, Western Algeria

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Abstract

The present study was carried out to investigate the presence of *Clostridium perfringens (C. perfringens)* in broiler chickens from various locations in Tiaret province, western Algeria, and to characterize the bacterium isolates for the presence of *cpa, cpb, etx, iA* and *netB* gene. A total of 180 samples representing intestinal contents of broiler chickens showing enteric disorder symptoms and lesions suspected to be Necrotic Enteritis (NE) were analyzed by conventional methods and polymerase chain reaction (PCR). *C. perfringens* was isolated at the rate of 34.44% (62/180), and its presence was confirmed by cultural and biochemical characterization. 83.87% (52/62) *C. perfringens* isolates were toxigenic and 16.13% (10/62) were non-toxigenic. Multiplex PCR was performed to toxinotype the 52 toxigenic isolates, and the results showed that all isolates were positive for the gene *cpa* and negative for *cpb, etx* and *iA*. This indicates that all the toxigenic isolates were *C. perfringens* type A (52/52). Uniplex PCR for detection of *NetB* toxin gene was carried out on 22 type A isolates, and these results showed none of the isolates as positive for the gene *netB*. This result indicates that the *C. perfringens* type A was the most predominant etiology of NE without carrying the *netB* gene.

Keywords: *Clostridium perfringens, Necrotic enteritis, Broiler, Toxinotyping, NetB*

Batı Cezayir'in Tiaret Bölgesinde Nekrotik Enteritli Broiler Tavuklardan İzole Edilen *Clostridium perfringens*'in İdentifikasyonu ve Karakterizasyonu

Özet

Bu çalışma Batı Cezayir'in Tiaret Bölgesinin değişik alanlarındaki broiler tavuklarda *Clostridium perfringens (C. perfringens)* mikroorganizmalarının varlığını araştırmak ve *cpa, cpb, etx, iA* ve *netB* genlerinin mevcudiyeti açısından bakteri izolatlarını karakterize etmek amacıyla yürütülmüştür. Enterik bozukluk semptomları ve lezyonları göstererek Nekrotik Enterit (NE) şüpheli olduğu düşünülen toplam 180 broiler tavuğa ait bağırsak içeriği örneği klasik metot ve polimeraz zincir reaksiyonu (PCR) ile incelendi. *C. perfringens* %34.44 (62/180) oranında izole edildi ve mikroorganizmanın kültürel ve biyokimyasal karakterizasyonu teyit edildi. *C. perfringens*'in %83.87 (52/62) izolatı toksijenik ve %16.13'ü (10/62) non-toksijenik olarak belirlendi. 52 toksijenik izolata toksinotiplendirme amacıyla Multipleks PCR uygulandı ve elde edilen bulgular tüm izolatlarda *cpa* geni için pozitif *cpb, etx* ve *iA* genleri için negatif olduğunu gösterdi. Bu durum tüm toksijenik izolatların *C. perfringens* tip A (52/52) olduğuna işaret etti. 22 tip A izolata *NetB* toksin genini belirlemek amacıyla unipleks PCR uygulandı ve sonuçlar izolatların hiç birinin *netB* geni için pozitif olmadığını gösterdi. Bu sonuçlar *netB* geni taşımaksızın *C. perfringens* tip A'nın Nekrotik Enteritin predominant etiyolojik etkeni olduğunu göstermiştir.

Anahtar sözcükler: *Clostridium perfringens, Nekrotik enteritis, Broiler, Toksinotiplendirme, NetB*

INTRODUCTION

Clostridium perfringnens is a Gram positive spore-forming anaerobic bacterium that plays an important role in the etiology of NE disease, which is the cause of the greatest economic losses in the poultry production industry [1,2]. It has been estimated that NE costs the poultry industry 2 billion dollars per year as result of reduction

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performance, disease treatment and preventive measures [3,4].

C. perfringens is responsible for synthesis and secretion of more than 17 different extracellular toxins, and it has been classified into 5 toxinotypes (A, B, C, D and E) on the basis of their ability to produce the major lethal toxins alpha *(α)*, beta *(β)*, epsilon *(ℰ)* and iota *(ι)* [5-7]. NE disease is caused mainly by type A strains, which produce the alpha toxin, and to a lesser extent, type C strains, which produce both alpha and beta toxin [8,9]. The alpha toxin was long considered the main cause responsible of the induction of the disease, while, a novel pore-forming toxin NetB has been demonstrated in type A strains [2,10,11].

NE can present as an acute clinical disease characterized by severe intestinal necrosis, leading to a sudden 50% increase in flock mortality rates [12,13]. NE can also arise as a sub-clinical infection, associated with chronic damage of the intestinal mucosa, causing problems such as lower performance and reduced weight gain [14,15]. In the past, NE has been controlled in poultry flocks with antimicrobial growth promoters in commercial poultry feed [16,17]. However, since the ban of these supplementations due to policy changes, NE has reemerged as a costly disease in poultry industry [2,16].

In Algeria, poultry meat is the primary source of protein, especially for Tiaret province population where broiler breeding was developing through the last decade. The detection and the characterization of *C. perfringens*, which is known as causative agent of NE in the poultry industry, and one of the most frequently isolated bacterial pathogens in foodborne disease outbreaks in humans [18], remains unknown in this region.

In this study, we aimed to investigate the presence of *C. perfringens,* at various locations in Tiaret province (western Algeria) and to characterize the bacterium isolates for the presence of *cpa*, *cpb*, *etx*, *iA* and *NetB* gene by PCR technology.

MATERIAL and METHODS

Sampling

A total of 180 samples were collected aseptically from freshly sacrificed broiler chickens (2-8 weeks old) reared in 70 poultry farms (average of 2500 broiler chickens by farm) at different locations in Tiaret province, western Algeria, from August 2015 to July 2016. The samples were collected after postmortem examination and the sections of intestine displaying gross lesions suspected to be NE were collected. Samples were taken to the laboratory in an ice box as soon as possible.

Isolation and Identification of Clostridium perfringens

The intestinal content of the collected samples were inoculated into tubes of freshly prepared cooked meat medium (Oxoid,UK) for enrichment and incubated in an anaerobic jar (Oxoid,UK) for 24 h at 37°C in anaerobic atmosphere provided by AnaeroGen atmosphere generation system. 0.1 mL of inoculated fluid media was streaked onto perfringens agar base containing 400 µg/mL of cycloserine (TSC) without egg emulsion (Oxoid, UK) and incubated anaerobically [19]. After 24-48 h incubation at 37°C, typical black colonies presumed to be *C. perfringens* were taken out with the help of the loop, re-streaked onto two plates of 5% defibrinated sheep blood agar and egg yolk agar, and incubated anaerobically for 24 h at 37°C [20]. *C. perfringens* isolates were identified via morphological and biochemical characterization as previously recommended by Koneman *et al.*[21] and Macfaddin [22].

Determination of Toxigenic Clostridium perfringens Isolates

- Mouse Bioassays (Lethality Test)

After an anaerobic incubation for 24 h at 37°C, the cultures of isolated *C. perfringens* strains in cooked meat medium were centrifuged at 3.000 rpm for 15 min, and the cell-free culture supernatants were recovered. 0.3 mL from the clear supernatant fluid was I/V inoculated in the tail vein of each Swiss mouse (25-40 g), and injected mice were observed over a period of three days for nervous symptoms or death [23]. One mouse was injected with broth culture without bacteria as a control.

- Nagler's Reaction (Toxin - Antitoxin Half Plate)

This test was carried out according to the method of Smith and Holdeman [24]. It was performed by spreading *C. perfringens* type A antitoxin serum (National Institute for Biological and Standard Control, UK) on half of the egg yolk agar plate and allowed to dry in the incubator for half an hour. The cultures were then streaked across the plate, beginning at the non-antitoxin coated portion and ending to the side containing the antitoxin. The cultures were incubated anaerobically for 24 h at 37°C.

All isolated toxigenic strains were stored in thyoglycolate medium (Oxoid, UK) with 30% glycerol at -20°C for subsequent toxin genotyping.

The experiment on animals was carried out according to the National Regulations on Animal Welfare.

Genotyping of the Toxigenic Clostridium perfringens Isolates

- DNA extraction and PCR

The DNA was extracted from toxigenic *C. perfringens* isolates using QIAamp DNA Mini Kit (QIAGEN, USA), as indicated per the manufacturer's instructions. Specific oligonucleotide primers sequences corresponding to alpha, beta, epsilon and iota toxin genes of *C. perfringens* and NetB toxin gene, as reported by Yoo *et al.*[25] and

Table 1. *Details of oligonucleotide primers sequences used in this study*

Gene	Primer	Sequence	Product Size (bp)
cpa (α toxin)	F	GTTGATAGCGCAGGACATGTTAAG	402
	R	CATGTAGTCATCTGTTCCAGCATC	
cpb (β toxin)	F	ACTATACAGACAGATCATTCAACC	236
	R	TTAGGAGCAGTTAGAACTACAGAC	
etx (ε toxin)	F	ACTGCAACTACTACTCATACTGTG	541
	R	CTGGTGCCTTAATAGAAAGACTCC	
iA (ι toxin)	F	GCGATGAAAAGCCTACACCACTAC	317
	R	GGTATATCCTCCACGCATATAGTC	
NetB (NetB toxin)	F	GCTGGTGCTGGAATAAATGC	560
	R	TCGCCATTGAGTAGTTTCCC	

Keyburn *et al.*[10], respectively were procured from Midland Certified Reagent Company, (Oligos, USA).The details of primers are given in *(Table 1)*.

The PCR reaction mix for alpha, beta, epsilon and iota toxins was prepared as follows: 8 µL of extracted DNA template from bacterial cultures, 25 µl EmeraldAmp GT PCR master mix (TAKARA, USA), 1 µL of each alpha, beta, epsilon and iota forward and reverse primers (20 pmol μL^{-1}), and 9 µl of PCR-grade water, to a total volume of 50 µL. For NetB toxin, the reaction mixture was prepared as follows: 6 µl of extracted DNA template from bacterial cultures, 12.5 µL EmeraldAmp GT PCR master mix (TAKARA, USA), 1 µL NetB forward and reverse primers (20 pmol μL^{-1}), 4.5 µL of PCR grade-water, to a total volume of 25 µL.

The PCR amplification for detection the toxins (α, β, ε and ι) was programmed in TRIO thermal cycler (Biometric, Germany) as follows: initial denaturation step at 94°C for 5 min, followed by 35 cycles of amplification. Each cycle comprised denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 45 sec. There was then a final extension for 10 min at 72°C. For detection of *netB* gene PCR, an initial denaturation step at 94°C for 5 min was followed by 35 cycles. Each cycle comprised denaturation at 94°C for 30 sec, annealing 58°C for 45 sec, and extension at 72°C for 45 sec. There was a final extension for 10 min at 72°C. Finally, 30 µL of the amplified product for alpha, beta, epsilon and iota toxin genes and 20 µL of the amplified products for NetB toxin gene were electrophoresed in 1.5% agarose gel and stained with ethidium bromide. Standard DNA fragments (Gel Pilot100-bp DNA molecular weight marker; QIAGEN, USA) were used as molecular weight markers to indicate the sizes of the amplification products. Amplified bands were visualized and photographed by a gel documentation system (Alpha Innotech, Germany), and the data was analyzed through computer software (Automatic Image Capture Software, ProteinSimple formerly Cell Biosciences, USA).

The positive DNA samples for alpha, beta, epsilon, iota and NetB toxins were obtained from reference laboratory for veterinary quality control on poultry production. (Animal Health Research Institute of Cairo, Egypt). Distilled water was used as negative control.

RESULTS

Necropsy Findings

At necropsy, all the 180 broiler chickens, from which the intestinal contents were collected, showed gross lesions suspected to be NE. Lesions were seen in the middle of the small intestine that had friable wall and distended with gases. Intestinal mucosa was covered by a tan to yellow necrotic membrane with or without hemorrhagic foci *(Fig. 1)*.

Bacteriological and Biochemical Examination

Out of the 180 intestinal samples examined, 62 *C. perfringens* isolates (34.44%) were detected. The isolates produced 1 to 2 mm typical black colonies on TSC agar medium, as shown in *Fig. 2*. Also produced were smooth, round, glistening colonies surrounded by double zone of haemolysis on sheep blood agar medium, as shown in *Fig. 3*. Biochemical characterization revealed that all the isolates were positive for lecithinase activity on egg yolk agar medium, sugar fermentation, milk digestion, while indole production, catalase and oxidase tests were negative.

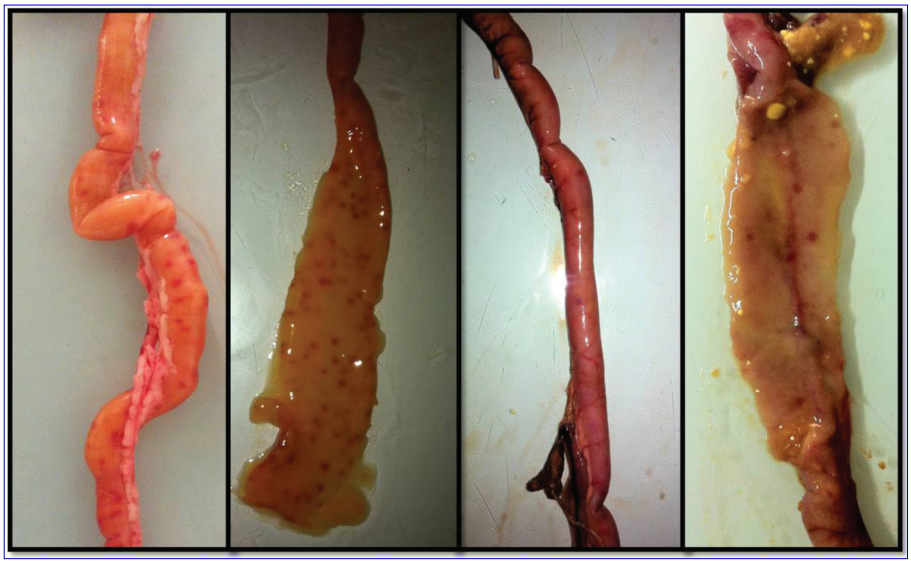


Fig 1. Parts of chicken intestine from field cases with lesions suspected to be NE

Fig 2. Typical black colonies presumed to be *C. perfringens* on TSC agar medium

Fig 3. *C. perfringens* colonies surrounded by double zone of haemolysis on sheep blood agar medium

Toxigenic Activities of Clostridium perfringens Isolates

52 isolates out of 62 *C. perfringens* isolates (83.87%) were toxigenic, as indicated by the death of the inoculated mice in mouse bioassays, and positive reaction on Nagler's tests expressed by zone of opacity surrounding the toxigenic *C. perfringens* colonies on the half of the plate without antitoxin while no change was observed on the other half containing the antitoxin *(Fig. 4)*. The remaining 10 *C. perfringens* isolates (16.13%) were non-toxigenic.

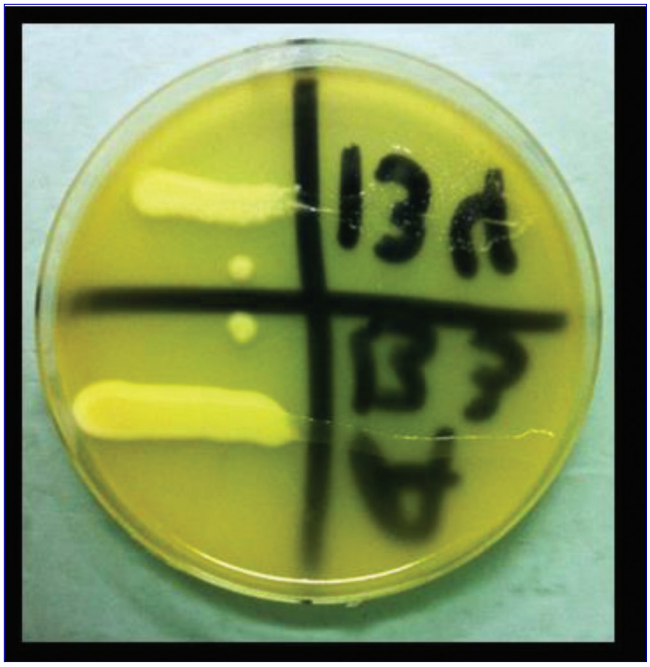


Fig 4. Nagler's test by half antitoxin plate

Multiplex PCR for the Genotyping of Toxigenic C. perfringens Isolates

The genotyping of the 52 toxigenic isolates revealed that all the isolates carried the gene *cpa* (402 bp), coding for the alpha toxin as illustrated in *Fig. 5*, and none of the isolates carried the genes *cpb* (236 bp), *etx* (541 bp) and *iA* (317 bp) coding for beta, epsilon and iota toxins, respectively. This indicates that all the toxigenic isolates were *C. perfringens* type A 100% (52/52).

Uniplex PCR for the Detection of netB Gene

A selection of 22 toxigenic isolates were confirmed to be *C. perfringens* type A and analyzed by uniplex PCR to detect the presence of the *netB* (560 bp) gene. None of the isolates were found positive for this gene that expresses for the NetB toxin, as illustrated in *Fig. 6*.

DISCUSSION

C. perfringens has been demonstrated in several regions in the world. It is one of the most common causes of severe gastro-intestinal infection and necrotic enteritis in poultry [1,13,26]. However, no studies on the detection and molecular characterization of *C. perfringens* inducing NE in broiler chickens were carried out in Algeria.

The presence of *C. perfringens* was investigated in different broiler chicken flocks located at Tiaret province. *C. perfringens* was isolated in 62 from 180 samples analyzed

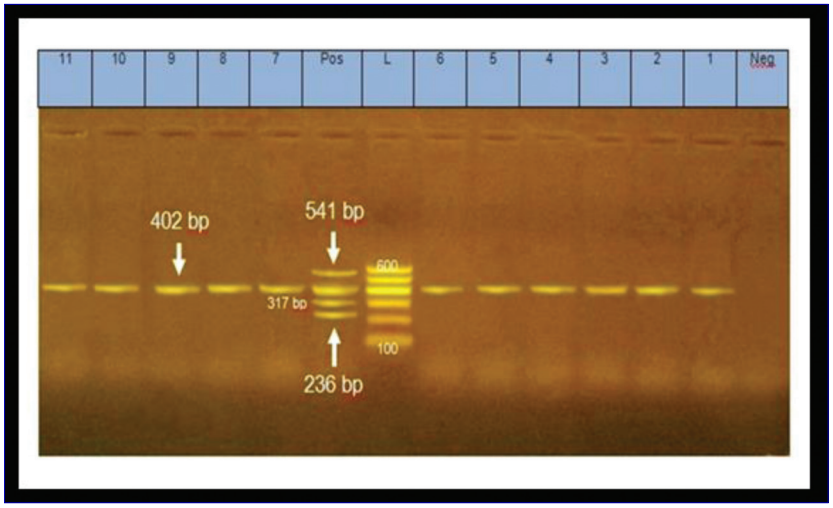
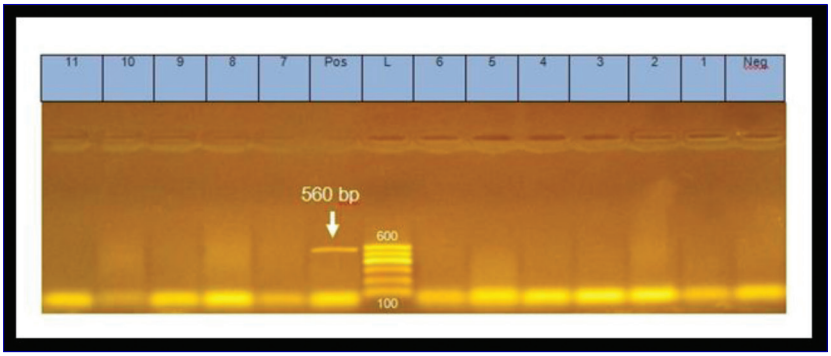


Fig 5. Typing of *C. perfringens* toxin genes by Multiplex PCR. Pos: positive control; Neg: negative control; L: DNA ladder (molecular weight marker 100-bp); lane 1-11: cpa positives *C. perfringens* toxigenic isolates

Fig 6. Uniplex PCR identification of *netB* gene. Pos: positive control; Neg: negative control; L: DNA ladder (molecular weight marker 100-bp); lane 1-11: netB negatives *C. perfringens* type A isolates



at the rate of 34.44%, this finding indicates that not all the intestinal lesions observed in the field were due to *C. perfringens* infection. Other pathogens may be incriminated in the etiology of these lesions. It is also believed that the sampling was carried out on farms receiving curative antibiotics which lead to the destruction of the intestinal microbial population, thus explaining the low isolation rate of *C. perfringens* observed in our study. Several studies have reported different isolation rates; Svobodova *et al.*[27] isolated *C. perfringens* at the rate of 18.39%, and Schocken-Iturrino *et al.*[28] analyzed 560 intestinal contents and reported that *C. perfringens* was found in 94 samples at the rate of 16.78%. Manfreda *et al.*[29] have detected *C. perfringens* in 87 from 149 samples analyzed (58.40%), while the lowest frequency of isolated *C. perfringens* was reported by Kalender and Ertas [30], who found that only 5% of intestinal contents were positive for *C. perfringens*. This variation may be due to the different methodologies used for the isolation, selection of samples (number and nature of samples, from healthy and/or diseased birds) and poultry farm management (the use or not of antibiotics as growth promoters in feed).

C. perfringens is considered a commensal organism of normal chicken intestinal flora [31], for this reason, we should differentiate between toxigenic and non-toxigenic isolates. Our results revealed that, out of the 62 isolates that were previously identified morphologically and biochemically as *C. perfringens*, 52 isolates (83.87%) were toxigenic. The high rate of toxigenic *C. perfringens* isolates recorded in our study confirm the role of this bacterium in the occurrence of the NE disease due to the high production of toxin that is responsible of the destruction of intestinal mucosa.

Multiplex PCR is a rapid and effective method for typing of *C. perfringens* toxins. The typing of 52 toxigenic isolates revealed that all the isolates were *C. perfringens* type A, in agreement with previous investigations carried out by Keyburn *et al.*[32], Crespo *et al.*[33], Svobodova *et al.*[27], Drigo *et al.*[34] and Trinh *et al.*[35]. However, there were no *C.perfringens* type C and D that were isolated from broiler chickens which demonstrated NE, disagreeing with the results of Shane *et al.*[36] and Heier *et al.*[37], who isolated *C.perfringens* type D and type C from broiler chickens suffering from NE.

Several studies have reported the role of other toxins in the induction of NE. The most important of these is Necrotic Enteritis toxin B (NetB), a pore forming toxin capable of causing lesions typical of NE [10]. Since the discovery of this new virulence factor, the presence of *netB* gene in *C. perfringens* isolates was investigated in different regions of the world. According to our study none of the selected toxigenic *C. perfringens* type A isolates were positive for NetB toxin. These results agree with Datta *et*

al.[38], who investigated the presence of NetB toxin in 26 isolates of *C. perfringens* type A and reported that none of the isolates were positive for this toxin. Similar results were reported by Thomas *et al*.[39], who recorded tested isolates for the presence of *netB* gene were negative. In contrast to our finding, several studies demonstrated the existence of *netB* gene in *C. perfringens* isolates. Johansson *et al*.[40] investigated the prevalence of this toxin and reported that more than 90% of all isolates from cases of NE carried *netB* gene. In addition, the presence of this gene was examined in 36 isolates of *C. perfringens* and was detected in 19 isolates at the rate of 52.8% from diseased flocks by Talooe *et al*.[41]. The lack of *netB* gene in our study may be explained by the insufficient number of samples analysed or the inexistence of this gene in our region.

Our study indicated the presence of *C. perfringens* in broiler chicken flocks in Tiaret province, and type A was the most predominant etiology in the occurrence of NE, an important bacterial disease of poultry. Hence, considerable attention should be paid in the prevention of this disease as *C. perfringens* type A is considered one of the most important causes of foodborne disease in human.

All the selected toxigenic *C. perfringens* type A isolates were negatives for the *netB* gene. This finding represents the first report on the detection of *netB* gene in Algerian field isolates of *C. perfringens*. We suggest further study to find other toxins that are the cause of NE, as in the case of NetB negative isolates. Future investigations should be carried out on an important number of *C. perfringens* isolates and on other regions in Algeria.

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Research Article

Antiviral Activity of Recombinant Porcine Interferon-α Against *Porcine Transmissible Gastroenteritis Virus* in PK-15 Cells

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Abstract

A recombinant porcine interferon alpha (rPoIFN-α) has been developed and patented previously (Chinese patent number ZL200810020180.4). In the current study, we investigated the inhibitory effects of the rPoIFN-α on the propagation of *porcine transmissible gastroenteritis virus (TGEV)* at different doses in porcine kidney cell line (PK-15). To quantitatively determine the inhibition of viral growth by rPoIFN-α, TCID50 assay, plaque formation assay, real-time qRT-PCR, western blot and immunofluorescence assay were adopted to evaluate the changes of viral infectious particles, viral genome copy numbers and viral protein expression levels respectively. The results demonstrated that all the three batches of the rPoIFN-α tested inhibited *TGEV*-induced cytopathic effect in PK-15 cells with very similar potency. rPoIFN-α inhibited *TGEV* proliferation more strongly than human IFN-α product. The inhibitory activity of rPoIFN-α on *TGEV* growth in culture was dose dependent, and the activity was gradually reduced with the decreasing of the concentration of rPoIFN-α.

Keywords: *Recombinant porcine interferon-α (rPoIFN-α), Porcine transmissible gastroenteritis virus (TGEV), Immunofluorescence assay, Real-time qRT-PCR, TCID50 assay, Western blot*

PK-15 Hücrelerinde *Domuz Transmissible Gastroenteritis* Virüsüne Karşı Rekombinant Domuz İnterferon-α'nın Antiviral Aktivitesi

Özet

Bir rekombinant domuz interferon alfa (rPoIFN-α) geliştirilmiş ve patenti daha öncesinde alınmıştı (Çin patent numarası ZL200810020180.4). Bu çalışmada, rPoIFN-α'nın farlı dozlarının *domuz transmissible gastroenteritis virüs (TGEV)*'ün üremesi üzerindeki baskılayıcı etkisi domuz böbrek hücre kültüründe (PK-15) araştırıldı. rPoIFN-α ile viral büyümenin baskılanmasını kantitatif olarak belirlemek için TCID50 testi, plak oluşum testi, gerçek zamanlı qRT-PCR, western blot ve immunfloresan teknikleri viral enfeksiyöz partiküllerin değişimlerini, viral genom kopya sayılarını ve viral protein ekspresyon seviyelerini belirlemek amacıyla uygulandı. Araştırma sonuçları test edilen üç rPoIFN-α'nın da PK-15 hücrelerinde *TGEV* ile oluşturulmuş sitopatik etkisinin aynı derecede olduğunu göstermiştir. rPoIFN-α, *TGEV* proliferasyonunu insan IFN-α ürününden daha güçlü olarak inhibe etti. Kültürde *TGEV* büyümesine rPoIFN-α'nın baskılayıcı aktivitesi doza bağımlı olup aktivite rPoIFN-α'nın azalan dozu ile göreceli olarak azalma gösterdi.

Anahtar sözcükler: *Rekombinant domuz interferon-α (rPoIFN-α), Domuz transmissible gastroenteritis virüs (TGEV), İmmunfloresan tekniği, Gerçek zamanlı qRT-PCR, TCID50 testi, Western blot*

INTRODUCTION

Porcine transmissible gastroenteritis virus (TGEV) is an enveloped virus that contains a large, positive-sense single-stranded RNA genome, belonging to the genus of *Alphacoronavirus* in the family of *Coronaviridae* [1]. The genomic size of *coronaviruses* ranges from approximately 28.6 kilobases. *TGEV* causes transmissible gastroenteritis

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(TGE) in pigs, and its mortality is close to 100% in young pigs. This disease is the major infectious disease that restricts the healthy development of pig breeding industry and results in huge economic losses to animal husbandry [2]. At present, however, there is no vaccine available for *TGEV* infection. Although antiviral agents such as ribavirin may be used to treat *TGEV* infection, severe side effects that come with ribavirin have been found in piglets including the toxicity to erythrocytes, bone marrow cells, as well as the epithelial cells of the gastroentestine and pancreas, which greatly restricted its use in animals [3].

Interferons (IFNs) are cytokines that are crucial for preventing viral replication at the site of infection and for coordinating adaptive immune responses that lead to the development of long-lasting, specific immunity. IFNs are composed of three physiologically distinct types I, II, III [4]. IFN-α belongs to type I, which plays an important role in innate immunity against viral infections [5,6]. The antiviral activities of porcine IFN-α (PoIFN-α) have been widely observed in response to infections with *Foot-and-mouth disease virus* (*FMDV*) [7-9], *Porcine respiratory and reproductive syndrome virus (PRRSV)* [10,11], *Pseudorabies virus (PRV)* [12], *Vesicular stomatitis virus (VSV)* [13], *Classical swine fever virus (CSFV)* [14], and *Influenza viruses* (IFV) including the swine origin *influenza virus A (H1N1)* [15-17]. There have been a great deal of studies that demonstrated antiviral activity and adjuvant function of recombinant PoIFN-α in various models of infection, suggesting that recombinant PoIFN-α might be a potential antiviral agent for the control of swine virus infections [18-22]. Both human IFN-α and natural porcine IFN-α have been shown to have antiviral activity in response to *TGEV* infection in vitro [23,24]; however, to our knowledge, no detailed report on the anti-*TGEV* activity of recombinant PoIFN-α (rPoIFN-α) is available.

We have successfully produced rPoIFN-α with high biological activity (Chinese patent ZL200810020180.4). In order to investigate the inhibitory effects of this rPoIFN-α on the propagation of *TGEV* in PK-15 cells, we employed five different methods, including $TCID_{50}$ assay, plaque formation assay, real-time qRT-PCR, western blot and immunofluorescence assay, to analyze the inhibitory effect of rPoIFN-α on the proliferation of *TGEV*. We hope the data from this research could lay a foundation for clinical trials of rPoIFN-α.

MATERIAL and METHODS

Drugs, Cells and Virus

The rPoIFN-α in this study was produced by our team (Chinese patent number: ZL200810020180.4). Briefly, *PoIFN-α* gene was cloned into a prokaryotic expression vector pET32a, which was then transformed into *E. coli* BL21 (DE3) strain before IPTG was added to induce the expression of the recombinant protein. The product yielded was purified with a two-step chromatographic procedure (Ni^{2+} affinity chromatography and DEAE anion exchange chromatography), and its biological activity was achieved as high as 1.1×10^6 IU/mL.

Three batches (2013001, 2013002 and 2013003) of rPoIFN-α were included in the study. Their titers was 2.01×10^4 IU/vial, 2.06×10^4 IU/vial and 2.02×10^4 IU/vial, respectively. Human interferon standard (HuIFN, batch number 07/01, 1.1×10^4 IU/vial) was provided by the National Institute for the Control of Pharmaceutical and Biological Products of China. Pig kidney epithelia cell line (PK-15 cells, ATCC® CCL-33) was cultured in Dulbecco Minimal Essential Medium (D-MEM) (Gibco BRL, MD, USA) supplemented with 10% heat-inactivated newborn bovine serum (Gibco BRL, MD, USA), 100 μg/mL of streptomycin and100 IU/mL of penicillin, 2 mmol/L L-glutamine, 75 g/L $NaHCO_3$, pH 7.2. PK-15 cell suspension was adjusted to 1.0×10^5/ml and 0.1 ml was transferred to each well of a 96 well cell culture plate before the incubation at 37°C in a 5% CO_2 atmosphere incubator.

TGEV was gifted by Professor Zhi-Wen Xu (Sichuan Agricultural University, Yaan, Sichuan Province, China) and identified by the viral CPE in PK-15 cells, RT-PCR and sequence analysis. Viral titers were determined as 10^{-5} $TCID_{50}$/mL with the calculation formula of Reed and Muench [25].

TGEV Titration ($TCID_{50}$ Assay)

The inhibition effects of rPoIFN-α on the growth of *TGEV* were determined by the changes of *TGEV* titers in PK-15 cells. The cells were plated onto 96-well plates at 1.0×10^4/well followed by the incubation for 24 h in a 5% CO_2 atmosphere incubator at 37°C. When the cell monolayer reached to 90% confluency, the cells were infected with 100 $TCID_{50}$ *TGEV* and treated with two-fold serially diluted rPoIFN-α at 1 h post-*TGEV* infection. The antiviral activity of the rPoIFN-α was expressed as $TCID_{50}$ in PK-15 cells, defined as the amount of the virus that produces CPE in 50% of PK-15 cells inoculated. At the same time, normal cell control, the virus control, human interferon-α control were included in the experiment. $TCID_{50}$ was determined by the Reed-Muench method as previously described [26,27].

Plaque Assay

PK-15 cells in 6-well plate were pretreated with serially diluted rPoIFN-α and incubated at 37°C for 24 h in a 5% CO_2 atmosphere incubator. The culture medium was then removed and 100$TCID_{50}$ of *TGEV* in 100 μL were added to each well and incubated at 37°C for 1 h with 5% CO_2. After the culture was washed twice with PBS, agarose nutrient broth (DMEM containing 3% calf serum and 0.75% agarose) was added at 1 mL per well. The culture was further incubated at 37°C for 5 days with daily monitor and record of the plaque appearance time, shape/size and

numbers. The total plaque numbers were counted after staining with crystal violet.

Quantification of TGEV by Real-time qRT-PCR after Application of rPoIFN-α

PK-15 cells (1.0×10^4/well) were pretreated with serially diluted rPoIFN-α at 37°C for 24 h in a 5% CO_2 incubator. The culture supernatant was removed, the cells were washed with PBS before 100$TCID_{50}$ *TGEV* in 100 µL was added and incubated at 37°C for 1 h. The culture was replaced with DMEM containing 2% heat-inactivated newborn bovine serum after washing twice with PBS. The plates were incubated at 37°C for 24 h in a 5% CO_2 incubator before total RNA was extracted with Trizol reagent (Invitrogen, Inc.). Each RNA sample was reverse transcribed using Reveraid First Strand cDNA Synthesis Kit (ThermoFisher, Waltham, MA, USA). Sequences of the PCR primers for the amplification of the 258 bp fragment of *TGEV* S gene were (forward) 5′-GTATTGGGATTATGCT-3′ and (reverse) 5′-CCACAATTTGCCTCTG-3′. The cycling condition was composed of 95°C for 5min, followed by 35 cycles of 95°C for 50 s, 48°C for 30 s, 72°C for 30 s and a final cycle at 72°C for 10 min. PCR product was cloned into pCR®-T easy vector (Invitrogen, Inc.). RNA fragments coding for *TGEV* S protein were prepared by in vitro transcription with the plasmid DNA as template. The concentration of the transcripts was determined by spectrophotometer (NanoDrop 2000, Wilmington, US) after the template DNA was removed. Standard curves for the qRT-PCR were generated using serial dilutions of the RNA fragments (within a range from 5-200 ng/µL) to convert Ct values into arbitrary values. These values were then normalized with the mean values of the house-keeping gene - porcine β-actin (forward primer 5′-GAGAAGCTGTGCTACGTCGC-3′ and reverse primer 5′-CCAGACAGCACTGTGTTGGC-3′) [28]. The copy number of the viral genome in the experimental samples was determined by interpolating the threshold cycle values using the standard curve. The qRT-PCR reactions were carried out in 20 µL volume containing dNTP, SYBR Green I (Roche, Basel, Switzerland), primers (0.2 µM each) and target cDNA. PCR amplification included an initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 10 s, and elongation 72°C for 15 s. After the cycling was completed, a melting curve was constructed to confirm the authenticity of the amplified products. A negative control sample that contained no template RNA was run with each experiment.

Western-blot Analysis of rPoIFN-α Inhibition on TGEV Spike Protein Expression

PK-15 cells were pretreated with rPoIFN-α as described methods. The total cellular protein was extracted using Radio Immunoprecipitation Assay Lysis Buffer (Beyotime, Shanghai, China). Protein concentration was determined using BCA Protein Assay Kit (Beyotime, Shanghai, China). Fifty µg of protein were loaded and electrophoresed on 15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Subsequently, the proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corp, Atlanta, GA, USA). The membrane was blocked with 5% nonfat milk at room temperature for 1 h, and then incubated with *TGEV* spike protein antibody (JBT-9181, Hannotech, Korea) overnight at 4°C followed by HRP-conjugated secondary antibodies at room temperature for 1 h. The signals in the membrane were detected using ECL reagent (ThermoFisher, Waltham, MA, USA).

Immunofluorescence Assay

PK-15 cells were cultured on cover slips and treated as indicated in methods. Cells were then fixed with 4% formaldehyde for 30 min, and incubated in blocking buffer (1% bovine serum albumin in PBS, 0.1% Triton-X100) for 1 h. Subsequently, the slides were incubated with anti-*TGEV* monoclonal antibody (Abcam, ab20301) overnight at 4°C, and then incubated with the FITC labeled anti-mouse IgG (Abcam, ab6785) for 1 h at room temperature. The slides were mounted and images were acquired by using a fluorescence microscope (OLYMPUS IX73, Japan).

Statistical Analysis

All data were presented as mean ±SEM from three independent experiments as triplicate. The results were analyzed by One-way analysis of variance (ANOVA) using the SPSS manager software (version 18.0, licence serial: 10034432, CODE:c66b5316e05ac32a8434). A value of $P<0.05$ was considered significant. $P<0.01$ was considered highly significant.

RESULTS

The Influence of rPoIFN-α to TGEV Titers

The inhibition effects of rPoIFN-α on *TGEV* proliferation were determined by the reduction of $TCID_{50}$ in PK-15 cells. We compared the antiviral effect of rPoIFN-α to that of human IFN-α standard by determining *TGEV* titers with the formula of Reed and Muench [25]. The results showed that the inhibition of rPoIFN-α on the multiplication of *TGEV* gradually reduced as the dose of rPoIFN-α in PK-15 cells was decreased from 1:2 to $1:2^8$ *(Fig. 1)*. The inhibition effect of human IFN-α was comparable to that of rPoIFN-α although the inhibition appeared not as well as rPoIFN-α on *TGEV* in PK-15 cells.

Plaque Formation Assay to Detect Changes on the Virus Numbers of TGEV Infections

The plaque formation assay was carried out with three different batches of rPoIFN-α lyophilized product, and the results are shown in *Fig. 2* and *Table 1*. It showed that all the 3 batches of rPoIFN-α demonstrated dose-depend

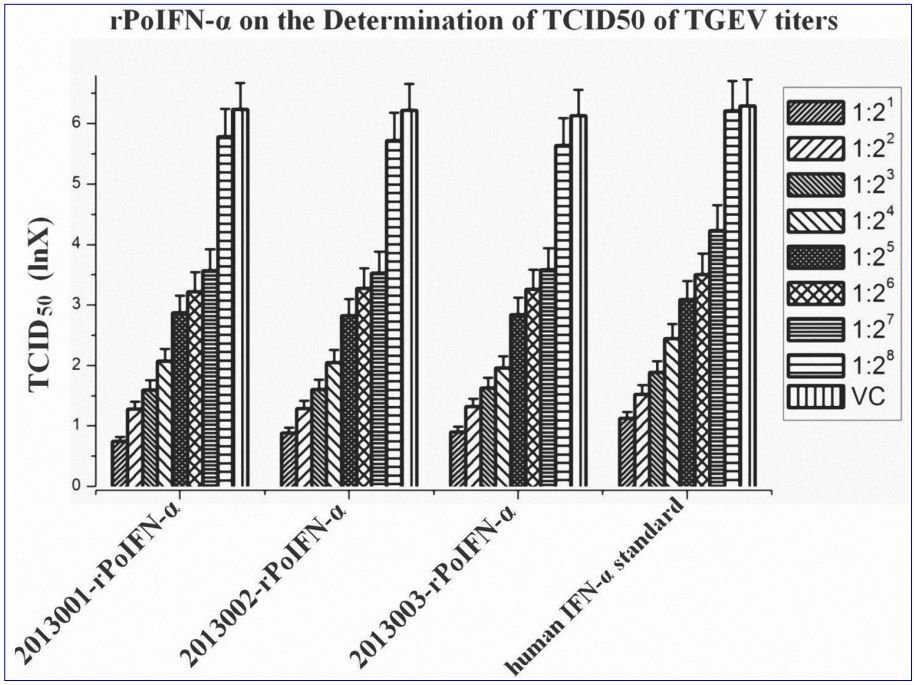

Fig 1. Different batch, different doses of rPoIFN-α on the Determination of $TCID_{50}$ of *TGEV* titers. Data are expressed as the average ± standard deviation (X±SD) which are shown above. "VC" means "virus control"

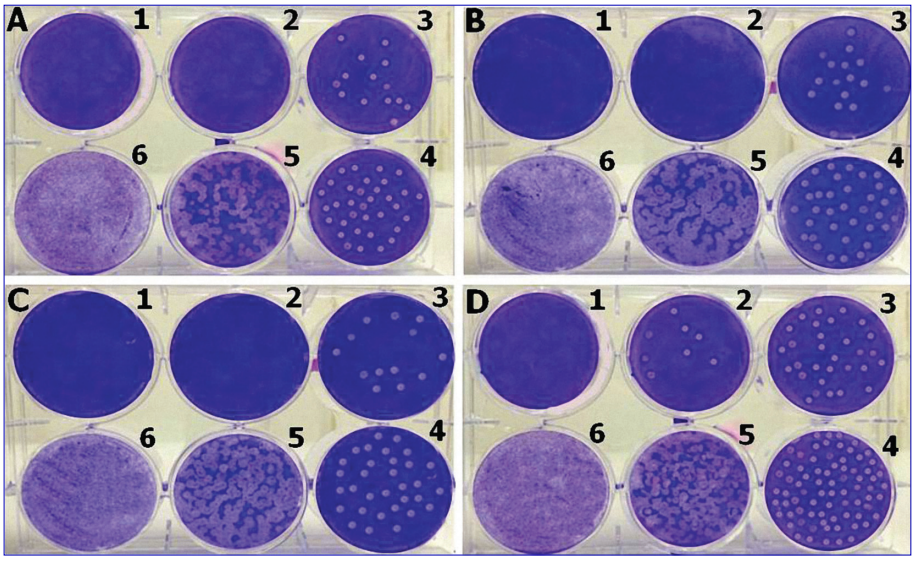

Fig 2. Plaque formation assay with different batches and doses of rPoIFN-α

A: rPoIFN-α batch 2013001. B: rPoIFN-α batch 2013002. C: rPoIFN-α batch 2013003. D: Human IFN-α standard. Wells 1: PK-15 cell control. Wells 2~5: With rPoIFN-α in the dilutions of 1:32, 1:64, 1:128 and 1:256. Well 6: 100$TCID_{50}$ virus control

inhibition on the formation of virus plaques in PK-15 cells. Viral plaques started to appear when rPoIFN-α was diluted to $1:2^6$, and became too many to count when rPoIFN-α reached to $1:2^8$ dilution. The inhibitory effect of human interferon on *TGEV* was similar to that of rPoIFN-α, but the number of plaques was more than those with rPoIFN-α, which suggested that the effect on *TGEV* proliferation by rPoIFN was significantly higher than that of human interferon.

The Inhibition of rPoIFN-α on TGEV Multiplication by qRT-PCR Assay

As shown in *Fig. 3* and *Table 2*, the viral copy numbers decreased with the increase of rPoIFN-α dilution ratio, which suggested that rPoIFN-α had a significant inhibition effect on the multiplication of *TGEV*. The inhibition effect of human IFN-α was not as good as rPoIFN-α on *TGEV* in PK-15 cells.

Inhibition of rPoIFN-α on the Expression of TGEV Spike Protein by Western Blot

To evaluate rPoIFN-α as an inhibitor against *TGEV* replication, the expression of *TGEV* spike protein was investigated by western blot in *TGEV* infected PK-15 cells in which rPoIFN-α was diluted from 1:16 to 1:256. Inhibition with rPoIFN-α was more pronounced than that with human IFN-α. The highest inhibition level appeared at the dilution of 1:32 ($P<0.01$). As expected, the expression level of *TGEV* spike protein in the culture without rPoIFN-α treatment was the highest among all the samples *(Fig. 4A, 4B)*.

ImmunofluorescenceAssayfor Testing the Inhibition of rPoIFN-α to TGEV in vitro

In *Fig. 5*, it was shown that the number of *TGEV* fluorescence positive cells increases gradually with the increase of the dilution factor of the three rPoIFN-α products, indicating the inhibition effect of *TGEV* is gradually decreased when the dilution of rPoIFN-α exceeded 1:32.

DISCUSSION

IFNs are a group of cytokines, initially identified by their ability to induce resistance to viral infection, it is currently also recognized as pro-inflammatory molecules and potent modulators of both innate and adaptive immune responses. Recent studies have shown that IFNs play a key role in the immune response to *TGEV*. As An et al.[29] reported, *TGEV* infection induced interferon signal transducer and activator of transcription 1 STAT1 phos-

Table 1. *The results of plaque formation assay*

rPoIFN-α Dilution	The Numbers of Plaque			
	rPoIFN-α (2013001)	rPoIFN-α (2013002)	rPoIFN-α (2013003)	Human IFN-α Standard
2^1	0	0	0	0
2^2	0	0	0	0
2^3	0	0	0	0
2^4	0	0	0	0
2^5	0	0	0	8
2^6	14	16	13	30
2^7	34	35	32	75
2^8	N	N	N	N

N: *Too many plaques to count*

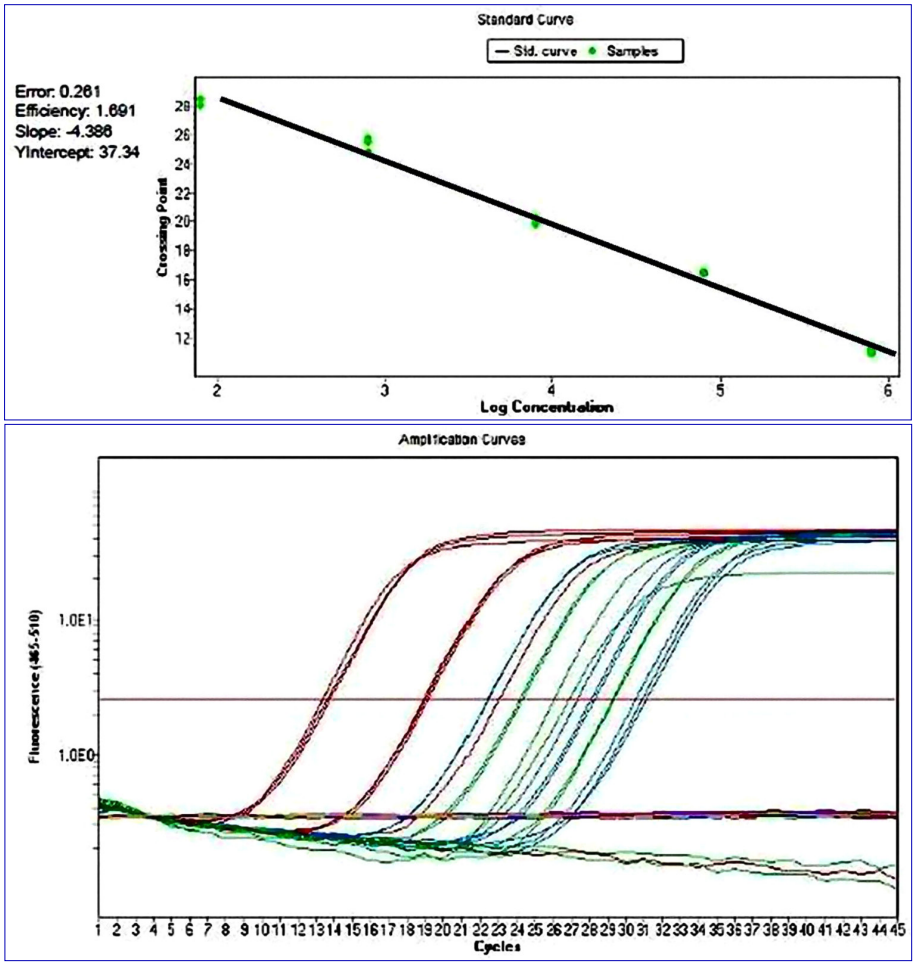

Fig 3. Standard curve (A) and copy numbers (B) of *TGEV* S gene determined by qRT-PCR

(A) standard curve of *TGEV* S gene real-time quantitative RT-PCR (B) detection results of copy numbers of *TGEV* S gene real-time quantitative RT-PCR

phorylation and nuclear translocation, as well as interferon-stimulated genes (ISGs) expression. Jordan et al.[30] found that titres of *TGEV* were reduced between 6 and 15 h post-infection in swine testis cells if the cells were treated with 1000 units/mL or 2500 units/mL of IFN. Lee et al.[31] demonstrated that the combined administration of the swIFN and swIL-18 cytokines using attenuated *Salmonella enterica serovar Typhimurium* as an oral carrier provided enhanced protection against intestinal tract infection with *TGEV*. Zhu et al.[32] modified rare codons encoding for 6 amino acids of porcine interferon-α and expressed the modified PoIFN-α gene in *Pichia pastoris*. The authors reported that the modified interferon-α showed more potent protection than that of the original protein in *VSV* or *TGEV* infected cells, the magnification factors reaching 100 for the *TGEV* and 300 for the *VSV*. The higher antiviral activities of the modified IFN-α gene was attributed to its higher expression and higher concentration of the cytokine.

IFN-α is encoded by a family of closely related intronless genes in all mammalian species [33]. They are mainly produced by virus infected peripheral blood leukocytes, or lymphoblastoid and myeloblastoid cell lines [34]. The porcine IFN-α (PoIFN-α) gene family is located on chromosome 1 [35]. Currently there are 17 different PoIFN-α subtypes (PoIFN α1-α17) with different antiviral activities and different expression profiles, among them PoIFN-α1 showed highest antiviral activity and anti-inflammatory activity at 10 IU/mL [36].

Proteins of PoIFN-*α* subtypes consist of 158 to 166 amino acid residues with monomer active form and most

Table 2. *TGEV copy numbers with different batch of rPoIFN-α at different doses*

Dilution of rPoIFN-α	Copy Numbers of TGEV			
	2013001 Batch of rPoIFN-α	2013002 Batch of rPoIFN-α	2013003 Batch of rPoIFN-α	National Human Interferon-α Standard
2^1	3.94E0	7.34E0	1.27E0	8.67E0
2^2	1.53E2	3.67E2	5.32E2	6.58E2
2^3	1.01E3	9.43E2	1.00E3	9.98E2
2^4	9.74E3	7.90E3	9.45E3	7.24E3
2^5	9.91E4	5.43E4	9.55E4	1.56E4
2^6	4.50E5	8.87E4	7.07E5	9.86E5
2^7	4.58E5	1.47E5	5.51E5	9.29E5
2^8	8.02E5	5.74E5	9.81E5	6.05E6
NC*	0	0	0	0

** **NC:** negative controls*

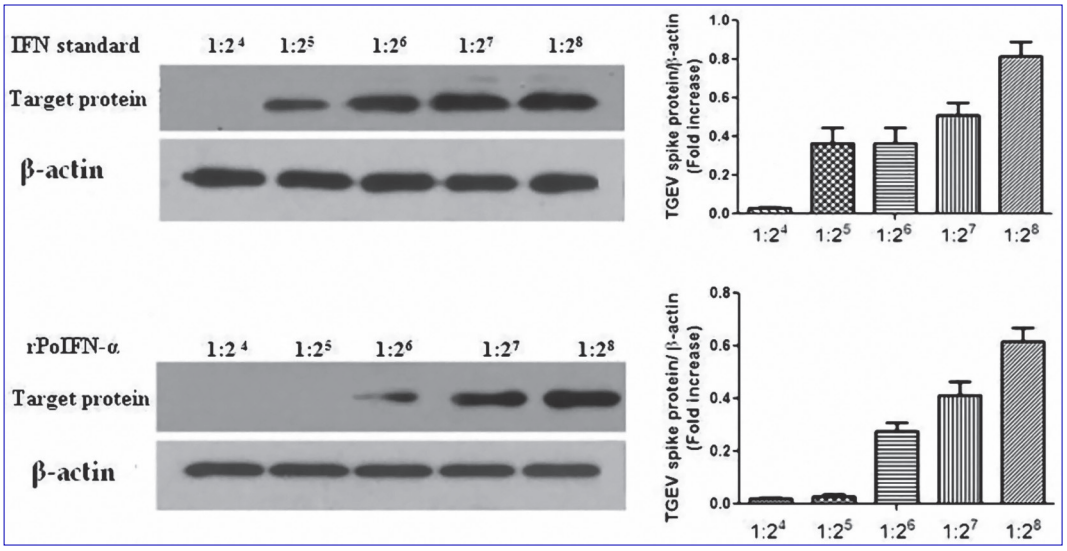

Fig 4. The expression of *TGEV* spike protein as Interferon standard and rPoIFN-α inducement for Serial dilution. The results were analyzed with Gel-Pro-analyzer manager software

of them are not glycosylated. The PoIFN-*α* subtypes have very high homology and share 96-99.8% identity at the nucleotide level and 91.1-100% at the amino acid level [37]. Multi-sequence alignment revealed a C-terminal deletion of 8 residues in 6 subtypes. It was found that the antiviral activity of intact PoIFN-*αs* are 2-50 times higher than those subtypes with C-terminal deletions in WISH cells and 15-55 times higher in porcine kidney PK-15 cells. Interestingly, the highest degree of nucleotide divergence was found in the leader region of porcine IFN-*α* genes, which might include signals for intracellular storage of both dimers and monomers of some IFN-*α* subtypes during constitutive expression [38].

Comparative studies have showed that antiviral activity of porcine type I IFNs is virus- and cell-dependent. Sang et al.[39] reported that although most IFN-*α* subtypes retained the greatest antiviral activity against both *PRRSV* and *VSV* in porcine PK-15 cells and monkey MARC-145 cells, some IFNs including IFN-*α* 7/11 exhibited minimal or no antiviral activity in those target cell-virus systems. Also, Sosan et al.[40] found that most PoIFN subtypes *except* PoIFN-*α*5 and 7 showed excellent inhibition activity on the proliferation of classical swine fever virus. In the study performed by Cheng et al.[37], PoIFN expression was compared in 3 different systems including poly(I).poly(C)-DEAE-dextran induced PK-15 cells, pseudorabies virus infected PK-15 cells, and an attenuated strain of *swine fever virus* infected PK-15 cells. It was observed that expression of PoIFN-*α* was time-dependent in the former two systems, but was not such time-dependent in the third system.

So far, many IFN-α genes have been cloned and expressed in eukaryotic or prokaryotic cells [22,41,42]. Lefèvre et al.[22] expressed recombinant porcine IFN-α in the form of inclusion body in *E. coli* and the antiviral activity of refolded rPoIFNα was 6-fold greater than the natural porcine leukocyte interferon in the protection of porcine

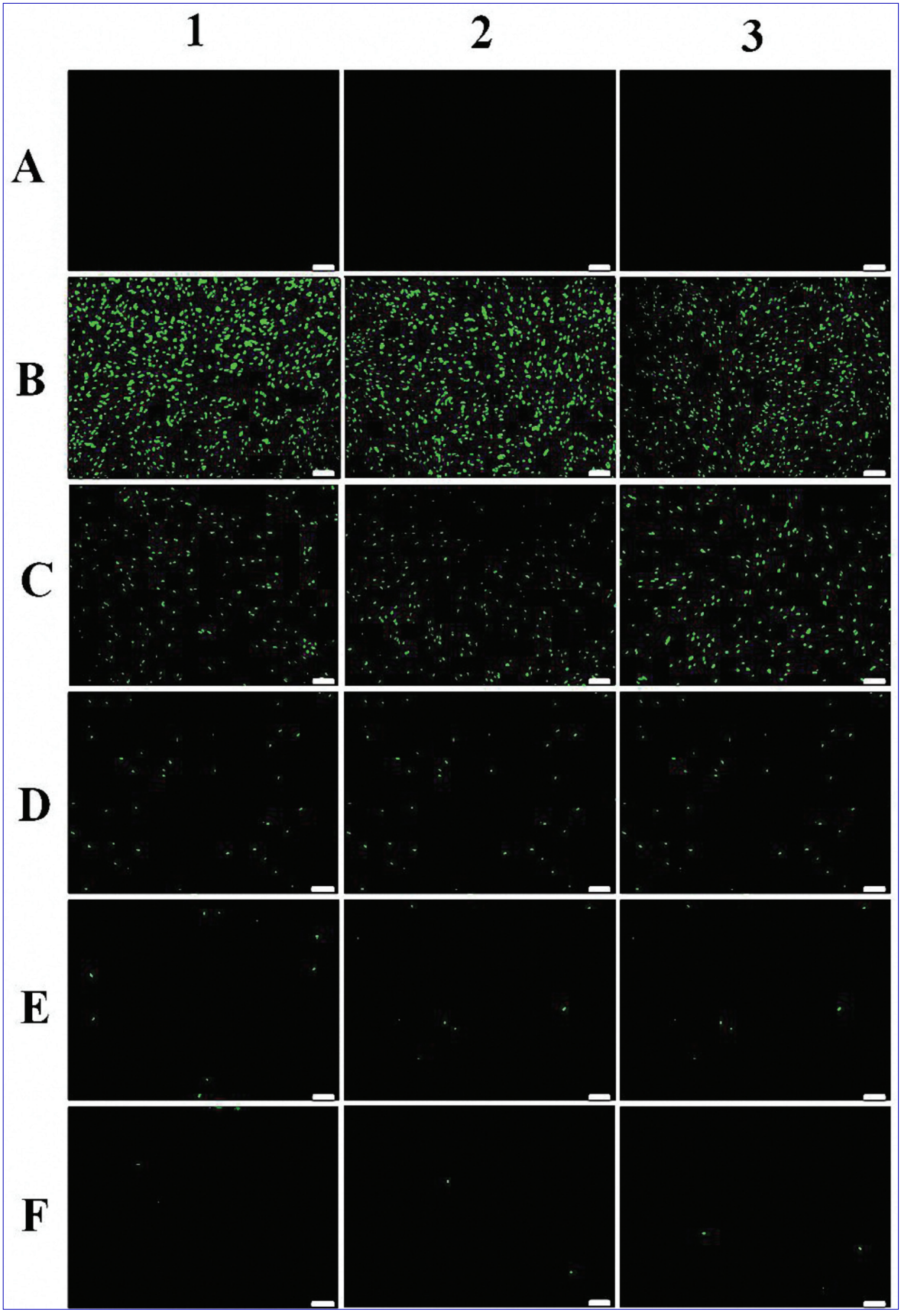

Fig 5. Immunofluorescence assay with three batches of rPoIFN-α on *TGEV* culture. 1: 2013001 batch of rPoIFN-α; 2: 2013001 batch of rPoIFN-α; 3: 2013001 batch of rPoIFN-α; A. cell control group. B. Virus control group. C. rPoIFN-α at 1:256 dilution. D: rPoIFN-α at 1:128 dilution. E. rPoIFN-α at 1:64 dilution. F. rPoIFN-α at 1:32 dilution

cells against *VSV* infection. Kim et al.[43] produced a recombinant mixture of *adenoviruses* bicistronically expressing porcine IFN-*α* and porcine IFN-γ and found it synergistically enhanced anti-*FMDV* effects compared with that of the *adenovirus* expressing a single IFN. More recently, a recombinant non-naturally occurring consensus porcine interferon-α (CoPoIFN-α) was designed by scanning 17 porcine IFN-α nonallelic subtypes and assigning the most frequently occurring amino acid in each position. It was revealed that the antiviral activity (units/mg) of CoPoIFN-α was higher than that of natural PoIFN-α in MDBK, PK-15 and MARC-145 cells [44]. In order to develop an IFN that might be used as an oral antiviral agent in animal health, PoIFN-α was successfully cloned and expressed in *Lactobacillus casei* with a vector that contains the inducible *lac* promoter and the secretion signal from an S-layer protein of *Lactobacillus brevis* [45].

Because the conventional production of interferon from natural leucocytes has disadvantages including low expression in healthy hosts and difficult extraction and purification procedures with high cost, large-scale preparation of rPoIFN-α with potent biological activities has become necessary. We achieved high level expression of the soluble form of bioactive rPoIFN-α in *E. coli* by selection of an appropriate expression vector pET32a. This vector contains Trx gene, which improves the solubility and activity of the rPoIFN-α protein [46,47]. The expression product of rPoIFN-α reached 32% of total bacterial proteins leading to the yields of 48 mg of recombinant PoIFN-α per liter of bacterial culture (data not shown). In addition, the His-tag carried by pET32a enables subsequent protein purification through Ni^{2+} affinity column. Our rPoIFN-α product was purified using essentially two-step chromatographic procedure which achieved biological activities as high as 1.1×10^6 IU/ ml. Furthermore, our rPoIFN-α is lyophilized and can be preserved at room temperature for a long period of time without carrier protein. The lyophilized product can be easily reconstituted in sterile saline or PBS. Therefore, comparing with native PoIFN-α , the rPoIFN-α we produced has many advantages in practical applications.

In summary, all the three batches of rPoIFN-α could inhibit the *TGEV*-induced cytopathic effect with consistent stable quality. The results of plaque formation assay, qRT-PCR, western blot and immunofluorescence assay showed that the rPoIFN-α had good inhibitory effect on the proliferation of *TGEV* in vitro. Thus, the current study suggested that the rPoIFN-α we produced has great potential for use as a novel antiviral agent in pig healthcare.

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Research Article

Evaluation of Pulmonary Infection Risk in Dogs with Pulmonary Contusion [1]

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Abstract

There is a dilemma about usage and selection of the antibiotics following pulmonary contusion. Thus, this study aimed to evaluate pulmonary infection risk in dogs with pulmonary contusion. In total, eleven dogs with pulmonary contusion included in the study. Diagnosis of the pulmonary contusion and its degrees were determined based on the clinical and radiological findings. Under general anesthesia, bronchoscopy and bronchoalveolar lavage (BAL) was applied on 0, 24 and 48th h and BAL samples were collected. Cytological examination, culture and antibiotic susceptibility analysis of the BAL samples were performed. Clinically, mild, moderate and severe degree pulmonary contusions were diagnosed, but radiological results pointed out severe degree of pulmonary contusion in all dogs. Bronchoscopy showed the hemoptysis in all dogs except one. Cytologically, all preparations had the inflammatory results at 0th hour and these were increased at 24 and 48th hours. Additionally, evidence of infection was determined in the cytological preparations of four dogs (case 1, 2, 5 and 8) taken at 0, 24 and 48th hours. These cytological results were compatible with microbiological results of case 1, 2, 5 and 8. *Pasteurella multocida* in case 1 and 2, and *Escherichia coli* in case 5 and 8 was cultured microorganism from the BAL samples, separately. While *P. multocida* was resistant to gentamicin, erythromycin and oxytetracycline; *E. coli* was resistant only sulfamethoxazole/trimethoprim. In conclusion, it may be stated that pulmonary contusion triggers inflammation process, and if there is a pathogenic opportunistic flora in the lower respiratory tracts, the pulmonary infections might accompany with inflammation process. Thus, if it is able, antibiotic usage must be planned considering to the results of BAL culture and antibiogram tests.

Keywords: *Bronchoalveolar lavage, Bronchoscopy, Cytology, Dog, Microbiology, Pulmonary contusion*

Pulmoner Kontüzyonlu Köpeklerde Pulmoner Enfeksiyon Riskinin Değerlendirilmesi

Özet

Pulmoner kontüzyon sonrası antibiyotik kullanımı ve seçimi hakkında bir çelişki vardır. Bu nedenle bu çalışma pulmoner kontüzyonlu köpeklerde pulmoner enfeksiyon riskinin değerlendirmesini amaçladı. Çalışmaya pulmoner kontüzyonlu 11 köpek dahil edildi. Pulmoner kontüzyon tanısı ve derecelendirilmesi klinik ve radyolojik bulgular temelinde belirlendi. Genel anestezi altında, 0, 24 ve 48. saatlerde köpeklerde bronkoskopi ve bronkoalveolar lavaj (BAL) uygulandı ve BAL numunesi alındı. BAL numunelerin sitolojik muayenesi, kültür ve antibiyotik duyarlılık analizleri yapıldı. Klinik olarak hafif, orta ve şiddetli derece pulmoner kontüzyon tanıları konuldu, ancak radyolojik bulgular tüm köpeklerde şiddetli derecede pulmoner kontüzyonu belirtti. Bronkoskopi, biri hariç tüm köpeklerde hemoptiziyi gösterdi. Sitolojik olarak, tüm preparatlar 0. saatte enflamasyon bulgularına sahipti ve bu bulgular 24 ve 48. saatlerde artıyordu. Ek olarak, enfeksiyon varlığı, dört köpeğin (olgu 1, 2, 5 ve 8) 0, 24 ve 48. saatlerde alınan sitoloji preparatlarında belirlendi. Bu sitolojik bulgular olgu 1, 2, 5 ve 8'in mikrobiyolojik bulguları ile uyumluydu. Sırasıyla, *Pasteurella multocida* olgu 1 ve 2'de, *Escherichia coli* olgu 5 ve 8'de BAL numunelerinden kültüre edilen mikroorganizmalardı. *P. multocida* gentamisin, eritromisin ve oksitetrasiklin'e dirençli iken, *E. coli* sadece sulfametoksazol/trimetoprim'e dirençliydi. Sonuç olarak, pulmoner kontüzyononun yangısal süreci tetikleyebildiği ve eğer alt solunum yollarında patojenik fırsatçı bir flora var ise akciğerdeki yangısal sürece enfeksiyonun da eşlik edebileceği söylenebilir. Bu yüzden, antibiyotik kullanımı olanaklar elveriyor ise BAL kültür ve antibiyogram sonuçları göz önüne alınarak kurgulanmalıdır.

Anahtar sözcükler: *Bronkoalveolar lavaj, Bronkoskopi, Köpek, Mikrobiyoloji, Pulmoner kontüzyon, Sitoloji*

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INTRODUCTION

Thoracic trauma results in pulmonary contusion, pneumothorax, hemothorax, rib fractures, intercostal muscle ruptures, diaphragmatic hernia and external thoracic wall injuries in dogs [1-5]. Pulmonary and myocardial contusions are severe traumatic injuries that they may occur if the blunt trauma affects the thorax [3,6].

Pulmonary contusion is the most common traumatic lesion in the dogs, accounting for approximately 50% of all thoracic injuries [6-9]. It is informed as an anatomic and pathologic lesion of the lung occurring after a compression-decompression injury of the thoracic wall [4,7]. Pulmonary contusion classifies as mild, moderate and severe according to pulmonary injury and associated findings (pneumothorax, rib fractures etc.) [5-7,9-11]. Pulmonary contusion has 20-50% mortality rate in dogs, and its progression leads to unresponsive dyspnea and died; thus, patients had blunt thoracic trauma should be examined carefully, and history, physical and radiological examinations, bronchoscopic findings and microbial analysis of BAL samples should be assessed in conjunction with the cytological results to clarify the exact diagnosis and estimate the prognosis of the dogs [7,12-15]. Additionally, cytological examination and bacterial cultures of intraluminal secretions may be performed before antibiotic usage to assist the diagnosis and evaluate the possible establishing lower respiratory tract disease [16,17].

Identification of the normal reference range of cell counts is impeded by a lack of standardization due to BAL fluid handling techniques and high variability of the clinically normal dogs [13]. Increased numbers of the neutrophils and phagocytosis of the organisms or macrophages, which are containing intracellular bacteria, is the evidence of the bacterial infections [13,15,16]. This condition can together with the acute inflammation, because acute inflammatory process often associated with bacterial infections [13,15]. Pulmonary contusion is a risk factor for pulmonary infection in dogs, which begins the cellular inflammatory process and activate the normal bacterial flora [13,18]; thus, BAL fluid should be analyzed in terms of enzymatic, cytological, microbiologic and histopathologic changings to determinate the exact diagnosis in dogs [17].

Antibiotic usage is recommended by surgeons to prevent the possible secondary infections to be encountered following pulmonary contusion; however, there is still a dilemma about usage and selection of the antibiotics in pulmonary contusion cases [9,18]. If bronchopneumonia develops following pulmonary contusion, the antibiotic usage is indicated [7,17]. On the other hand, there is no reported data on secondary pulmonary infections following pulmonary contusion in dogs, which is more common complication in humans [7]. Thus, to demonstrate the pulmonary infection risk, this study aimed to investigate cytological and microbiological analysis results of the BAL fluids in dogs with pulmonary contusion.

MATERIAL and METHODS

This study plan was approved by Local Ethic Committee of Uludag University (Decision no: 2013-01/05).

Material of the study consisted of 11 owned dogs (different breed, sex and age) presented in different times to Uludag University, Faculty of Veterinary Medicine, Department of Surgery Clinics with suddenly onset thoracic trauma. These dogs were included in the study by taking to permissions of the owners after diagnoses of the pulmonary contusion.

Initial Approaches and Diagnosis

Based on the history, physical examination, laboratory analysis and radiological results, pulmonary contusion was diagnosed in the dogs. Severity of these results helped the grading of the pulmonary contusion both clinically and radiologically; thus, vital parameters, general condition, thoracic auscultation, respiratory tracts examinations, breathing model and radiological findings were attentively evaluated for the study plan.

According to severity of the traumatic results and associated injuries, emergency medical protocols such as diuretics (furosemide), fluid therapy (lactate ringer and mannitol), and hemostatic (tranexamic acid) were applied to the dogs. When the dogs were stable, clinical, laboratory (routine hematology and blood gas analysis) and radiological examinations were completed to reach the exact diagnosis of pulmonary contusion and its degree.

For blood gas analysis, a specific 2 mL injector (containing 100 µL lithium heparin) was inserted to femoral artery with sterile technique, and about 2 mL arterial blood was aspirated into the injector. Body temperature of the case was recorded, and the arterial gases were instantly sent to laboratory to achieve an exact blood gas analysis results. Blood gas kits (Irma® blood analysis system kits, USA) and an analyzer (Truepoint®, IRMA, USA) were used to determinate the pH, pO_2 and pCO_2 values.

The effected hemithorax and lung lobe(s) from the trauma were identified by radiological examinations. Close monitorisation (SPO_2, pulsation and respiration controls: frequency and tidal volume) and ECG examinations were performed in the dogs. In ECG examinations, cardiac arrhythmias, amplitude of ECG waves and duration of intervals were measured on lead II (50 mm/s; 10 mm (mV; Esoate®, Italy). Furthermore, intensive care (instant oxygenation with mask or after endotracheal intubation) was taken to manipulate the complications, and thoracocentesis, tube thoracostomy and surgery were also carried out, if required.

Anesthesia

The dogs were anesthetized for tracheobronchoscopic examinations. After catheterization of the vena cephalica parva, the dogs were premedicated and induced with injectable anesthetic protocols (10 mg/kg ketamine HCl + 0.5 mg/kg diazepam combination, iv), and then they were intubated with a proper size of sterile endotracheal tube according to body weight and tracheal size compliance. The maintenance of the anesthesia was provided by iv administration of the same combination.

BAL Procedure

The same surgeon was performed all tracheobronchoscopy procedures as described previously [12]. A fiber optic flexible endoscope (5.2 mm diameter, 85 cm long) (Karl Storz®, Germany), a recorder (Tele Pack Vet X, Karl Storz®, Germany) and their endoscopic equipment were used during this procedure. A mouth gag was placed to fix the chin and an assistant fixed the head of the dog to prevent the bronchoscope from the trauma for any reason. The endoscope was inserted into the endotracheal tube and trachea, right and left bronchus and bronchioles were examined for the possible pathological findings. These endoscopic findings were recorded and bronchoalveolar lavage (BAL) procedure was performed to obtain specimens from lower respiratory tracts. For flushing to bronchial three, 0.5 mL of sterile saline (0.09% NaCl) per kg bodyweight was instilled through bronchoscope port, and then the fluid was aspirated slowly with gentle pressures through suction canal of the bronchoscope. The obtained fluid samples were collected into a sterile tube connecting to the suction system. The samples were taken to the laboratory for cytological examination, microbiological culture and antibiotic susceptibility. As performed in 0th h, BAL procedures were repeated at 24 and 48th h in dogs under general anesthesia.

Cytology

BAL fluids were centrifuged 1.000xg for 5 min, and the preparation was made from the precipitate of the fluids. This precipitate laid on the lam by a pipet and slides were performed. These preparations were stained by Diff-quick stain. All slides were evaluated in a blindly, ten randomly selected areas at higher magnification (x400 magnification) were evaluated and number of epithelial cells, macrophages, neutrophils and other cellular components (eosinophils and mast cells) were recorded and averaged in each time period. According to the microscopic findings, evidence of the inflammation and infection at 0, 24 and 48th h were evaluated.

Microbiology

BAL fluid samples taken 0, 24 and 48th h were processed within 2 h as soon as obtained from dogs. The samples were cultured for bacteria and yeasts with 3 pair on 5% defibrinated sheep blood (one of them was incubated in aerobically, one of them micro-aerobically and the other was incubated anaerobically), MacConcey (MCA, Oxoid) agar and Sabouraud dextrose (SAD, Oxoid) agar (aerobically incubated at 37°C and 28°C), respectively. Pleuropneumonia-like organism base agar (PPLO, Oxoid) was used for *Mycoplasma* isolation with incubation at 35°C in 5% CO_2. After incubation for 48-72 h, plates were examined for growth. Standard biochemical methods and commercial miniaturized identification systems (BBL Crystal Panel™) were used for identification of pure cultures [19]. Furthermore, the long time period was awaited for growing of *Mycoplasma* and mycotic microorganism to achieve the definitive microbiological results, as well.

Bacterial susceptibility testing was performed according to standards established by the Clinical Laboratory Standards Institute (CLSI) using Kirby-Bauer disc diffusion procedure [20]. Fourteen antibiotics were used for *P. multocida* isolates; azithromycin (AZM, 15 µg, Oxoid CT0906B), chloramphenicol (C, 30 µg, Oxoid CT0013B), doxycycline (DO, 30 µg, Oxoid CT0018B), enrofloxacine (ENR, 5 µg, Oxoid CT0639B), erythromycin (E, 15 µg, Oxoid CT0020B), gentamicin (CN, 10 µg, Oxoid CT0024B), ofloxacin (OFX, 5 µg, Oxoid CT0446B), penicillin G (P, 10units, Oxoid CT0043B), streptomycin (S, 25 µg, Oxoid CT0048B), sulfamethoxazole/trimethoprim 19:1 (SXT, 25 µg, Oxoid CT0052B), kanamycin (K, 30 µg, Oxoid, CT0026B), amoxicillin clavulanic acid (AMC, 30 µg, Oxoid CT0223B), ampicillin (AMP, 10 µg, Oxoid CT0003B), oxytetracycline (OT, 30 µg, Oxoid CT0041B). Twelve antibiotics were used for *E. coli* isolates; sulphamethoxazole/trimethoprim 19:1 (SXT, 25 µg, Oxoid CT0052B), ceftriaxone (CRO, 30 µg, Oxoid CT0417B), gentamicin (CN, 10 µg, Oxoid CT0024B), ampicillin (AMP, 10 µg, Oxoid CT0003B), ceftazidime (CAZ, 30 µg, Oxoid CT0412B), tobramycin (TOB, 10 µg, Oxoid CT0056B), amoxicillin clavulanic acid (AMC, 30 µg, Oxoid CT0223B), amikacin (AK, 30 µg, Oxoid CT0107B), ciprofloxacin (CIP, 5 µg, Oxoid CT0425B), cloksacilin (OB, 5 µg, Oxoid CT0016B), cefoperazone (CFP, 75 µg, Oxoid CT0249B), cefotaxime (CTX, 30 µg, CT0166B). The reference bacterial strains *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used as quality control strains following the recommendations of CLSI.

Considering to antibiotic susceptibility results of the BAL samples, a selective antibiotic would be administered to all dogs both prophylactically and as a treatment protocol as described previously [21].

Statistical Analysis

Average and standard deviations of blood gas analysis results (pH, pO_2 and pCO_2) were estimated in a statistical program (SPSS 23.0, IBM®, USA).

Considering to microbiological results into consideration, Fisher's exact test (SPSS 23.0, IBM®, USA) was applied to number of the cases, which had positive or negative culture results ($P<0.05$).

RESULTS

Clinical, Laboratory and Radiological Results

The causes of the thoracic trauma in dogs were traffic accident (n=7), fighting (n=3) and falling dawn (n=1). These dogs had different clinical appearance. According to severity of the clinical examination (changings in the vital parameters, abnormal lung sounds during auscultation, evidence of the hemoptysis, cardio-respiratory system abnormalities and etc.) and laboratory analysis results, pulmonary contusion was determined as mild in 3 dogs, moderate in 5 dogs and severe in 3 dogs *(Table 1)*. Because the cases were acutely presented to our clinics, there was no markedly abnormal changings in hematological parameters; however, there was minimal changes in the blood gas analysis results performed at 0th h (pH: 7.237±0.08; pO_2: 89.01±45.07 mmHg; pCO_2: 51.51±16.14 mmHg). The other associated thoracic pathologies were flail chest in case 7, penetrated wounds in case 3 and 7, costal fracture in case 7 and skin lacerations in case 7 and 11. All dogs had respiratory system problems (increased respiration rate, dyspnea, abnormal respiratory sounds, hemoptysis etc.), which were related to degree of pulmonary condition. In addition, thoracic region of the dogs were pointed out the thoracic trauma because all dogs had moderate respiratory dyspnea.

ECG examinations of the cases were showed some abnormalities: small complex QRS's (n=3), sinus tachycardia (n=6), electrical alternans (n=3), sinus arrhythmia (n=2), atrial premature complexes (n=2) increased amplitude of R wave (n=1) and P pulmonale (n=2). Specifically, considering to severity of the thoracic trauma, ECG abnormalities were small complex, sinus tachycardia and electrical alternans in case 3 and 5; sinus arrhythmia, P pulmonale and atrial premature complex in case 7.

Radiological findings of the dogs were changing due to influence of the trauma, but all dogs had third degree pulmonary contusion findings as described previously [6] *(Table 1)*. These dogs had increased radiopacity in a hemithorax (case 1, 4, 6, 7 and 8) or both hemithoraxes (case 2, 3, 5, 9, 10 and 11). The increased fluid density in the lung because of the hemorrhage and atelectasis of the lung lobes secondary to pneumothorax *(Fig. 1)* and air-bronchogram pattern appearance due to more severe lung contusion indicated the pulmonary contusion in the cases. The other radio-abnormalities were also pneumothorax in case 2, 3, 5, 7 and 10, pneumomediastineum in case 3, and flail chest and fractured ribs in case 7 that observed secondarily to the pulmonary contusion.

After diagnosis of the cases, as well as the medical therapy, some surgical manipulations were carried out as treatment regimens under general anesthesia. Thoracocentesis was applied in case 2, and pneumothorax was treated with tube thoracostomy in case 3, 5 and 7. Surgery was performed in case 7 to reconstruct the thoracic wall injury. However, in order to perform the study plan, antibiotic regimens were not applied to the dogs during the first 48 h. After the last sample was taken by BAL at 48th h, ampicillin sulbactam (20 mg/kg, bid, for 5 days) was started to the cases considering to previously reported

Table 1. *Signalments, clinical and radiological degree of the pulmonary contusion and broncoscopic findings of the dogs*

Case no	Signalments	Degree of the Clinical Findings	Degree of the Radiological Findings	Bronchoscopic Findings
1	Mixed-breed, ♂, 1 year-old, 5.4 kg	moderate	3	Hemoptysis, left bronchial hemorrhage, cyanotic left bronchial mucosa
2	Mixed-breed, ♂, 8 month-old, 13 kg	mild	3	Bilateral cyanotic bronchial mucosa
3	Anatolian shepherd, ♂, 10 month-old, 45 kg	severe	3	Hemoptysis, bilateral bronchial hemorrhage and cyanotic bronchial mucosa
4	Anatolian shepherd, ♂, 2 month-old, 9.8 kg	moderate	3	Hemoptysis, bilateral bronchial hemorrhage and cyanotic bronchial mucosa
5	Anatolian shepherd, ♀, 4 year-old, 48 kg	moderate	3	Hemoptysis, right bronchial hemorrhage, bilateral cyanotic bronchial mucosa
6	Staffordshire Bull Terrier, ♀, 3 month-old, 9.4 kg	mild	3	Hemoptysis, right bronchial hemorrhage, bilateral minimal cyanotic bronchial mucosa
7	Mixed-breed, ♀, 5 year-old, 5.5 kg	severe	3	Hemoptysis, right bronchial hemorrhage and cyanotic bronchial mucosa
8	Anatolian shepherd, ♂, 3 year-old, 55 kg	moderate	3	Hemoptysis, bilateral bronchial hemorrhage and minimal cyanotic bronchial mucosa
9	Mixed-breed, ♂, 5 month-old, 5.5 kg	moderate	3	Hemoptysis, left bronchial hemorrhage and minimal cyanotic bronchial mucosa
10	Pitbull Terrier, ♂, 5 month-old, 7 kg	mild	3	Hemoptysis, right bronchial hemorrhage and minimal cyanotic bronchial mucosa
11	White Terrier, ♂, 7 year-old, 12 kg	severe	3	Hemoptysis, left bronchial hemorrhage and minimal cyanotic bronchial mucosa

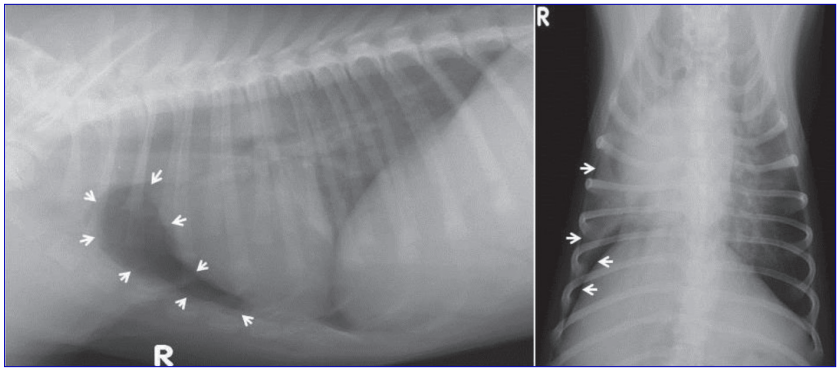

Fig 1. Radiograms of case 10 taken at 0th h, the ventrodorsal radiograph points out increased fluid density in the lung due to 3rd degree pulmonary contusion. There is minimal evidence of the pneumothorax, atelectatic caudal lung lobe border *(right arrows)* and free air accumulation *(left arrows)*. The right lateral radiograph shows the free air accumulation and air-bronchogram pattern view due to more severe lung contusion

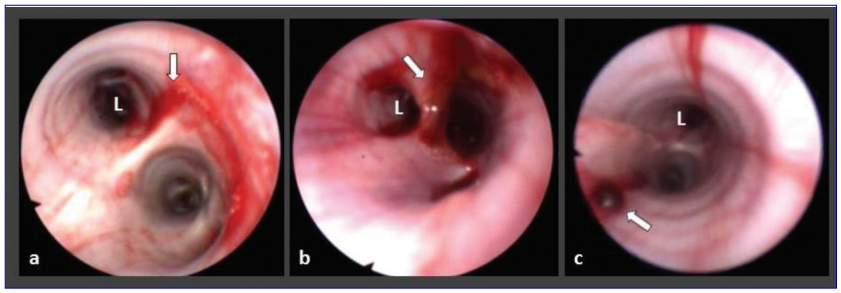

Fig 2. Bronchoscopic views of the case 1, hemorrhage *(arrow)* in the left (L) bronchus at 0th h (a), fibrin formation *(arrow)* at 24th h (b), and mucoid-hemorrhagic sputum *(arrow)* upcoming from the left (L) bronchus to the tracheal lumen at 48th h (c)

data [20], which had found compatible with the antibiotic susceptibility results afterwords.

Bronchoscopic Results

At presentation; during bronchoscopic evaluations, mucosal surface of the trachea had bloody appearance in all dogs except case 2, which was pointed out the hemoptysis. There was unilateral *(Fig. 2a)* or bilateral bronchial hemorrhage and also cyanosis in the bronchial mucosa of the cases *(Table 1)*.

At 24th h; there was a bloody appearance in the tracheal lumen and on the effected bronchial mucosa. Fibrin formations on the mucosal surface *(Fig. 2b)* and foamy hemorrhagic fluid accumulations were markedly observed.

At 48th h; broncoscopic evaluations of the cases revealed minimal hemorrhagic spots on the mucosa, minimal fibrin formation, muco-purulent and mucoid-hemorrhagic sputum in the lumen of trachea and effected bronchus *(Fig. 2c)*.

Cytological Results

Taking the cytological findings into consideration, inflammation was determined in all dogs; however, case 1, 2, 5 and 8 had both inflammatory and infective findings in the preparations. Comparative evaluations of the preparations at 0, 24 and 48th h in dogs implied that;

- Inflammation

0th hour: All preparations of the cases had intense erythrocyte, neutrophil (80-85%), bronchial epithelial cells, macrophage (10%), muco-protein fibers and mast cells (0.5-1%).

24th hour: There were more severe inflammatory findings. Neutrophil counts (85-90%) had increased and many degenerated respiratory epithelial cells were noticed in all cases.

48th hour: Degenerated respiratory epithelial cells, increased number of macrophages (80%) and lower neutrophil count (30-40%) were evident at that time in all cases *(Fig. 3)*.

Infection

0th hour: Existence of various shaped bacteria was found in four cases (case 1, 2, 5 and 8).

24th and 48th hour: The intensity of bacterial cells was increased *(Fig. 4)* and phagocytized bacteria by macrophages were evident.

Microbiological Results

P. multocida was cultured from the BAL samples of case

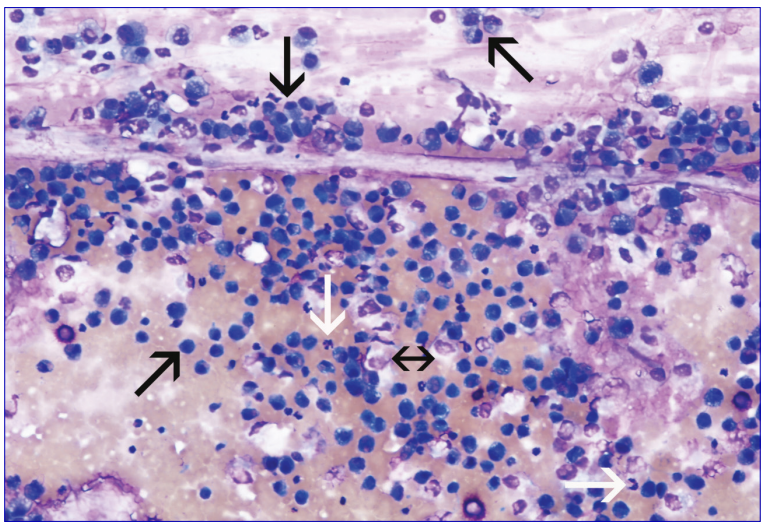

Fig 3. Severe inflammatory results of case 3 at 48th h, cytological examination view of the BAL samples demonstrates respiratory epithelial cells (double-headed arrow), neutrophil leukocytes *(white arrow)* and alveolar macrophages *(black arrows)* (200X magnification)

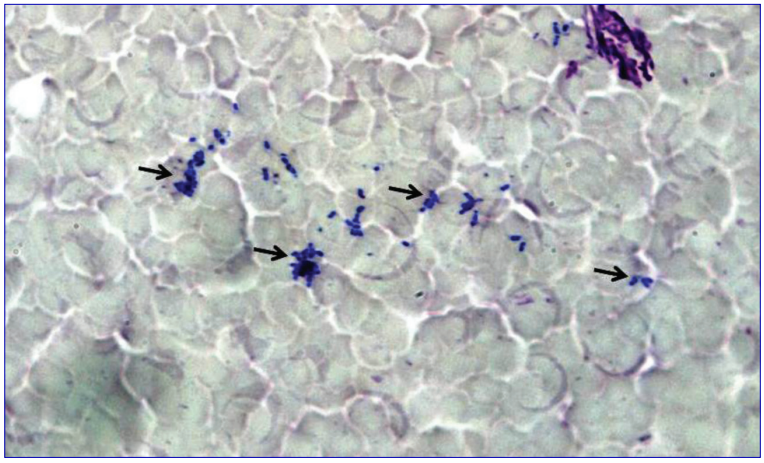

Fig 4. The encountered very intense bacterial cells *(arrows)* in the cytological preparation of the case 1 (1000X magnification)

1 and 2, and *E. coli* was the other cultured microorganism from the BAL samples of case 5 and 8. There was no *Mycoplasma* and mycotic microorganisms in the cultures. Microbiological results were obtained 0th h BAL sample of case 1, 5 and 8, but it was cultured at 24th h in case 2. According to bacterial susceptibility testing, *P. multocida* isolates were resistant to gentamicin, erythromycin, oxytetracycline and *E. coli* isolates were resistant only to sulfamethoxazole/trimethoprim 19:1.

Considering to these antibiotic susceptibility results, which were time-consuming procedure, the previously planned antibiotic regimen of ampicillin sulbactam (20 mg/kg, im., bid for 5 days) were repeated to all dogs.

Statistical Analysis Results

Statistical analysis results pointed out that there was no significant difference in terms of cultured microorganisms between the numbers of the cases (P= 0.2774).

DISCUSSION

Pulmonary contusion results in structural and functional changes in the lung tissues, which may lead to respiratory failure. In the management of the pulmonary contusion, antibiotic therapy is not indicated unless bronchopneumonia develops. If bacterial pneumonia develops at the early stage, bacterial culture of the tracheal fluid is recommended [7,18]. This study was planned to evaluate the pulmonary infection risks of the dogs with pulmonary contusion, and our results showed to some supportive findings of the infections following pulmonary contusion in the studied dogs. In addition to clinical and radiological examinations, bronchoscopic examinations of the respiratory tracts were made to determinate the traumatic results of the respiratory tracts and to take BAL samples for cytological and microbiological analysis at 0, 24 and 48th h. Cytological evaluations targeted to presence of the inflammation and infection as well as microbiological culture and antibiotic susceptibility analysis to answer the possible questions about to pulmonary trauma in dogs. Because inflammatory process begins in the lung parenchyma following trauma, the organ might be labile to opportunistic bacterial infections due to immunocompromised status of the parenchyma [22,23]. Thus, this study was planned to answer the questionable issues about antibiotic usage in dogs with pulmonary contusion.

Physical examination of the patients with mild to moderate pulmonary contusion exhibit some superficial respiratory system abnormalities and findings (tachypnea, dyspnea, orthopnea, hypoxia and hemoptysis), and auscultation of the thorax include abnormal respiratory sounds such as increased bronchial and broncho-vesicular sounds [5,7-9]. If the pulmonary contusion degree is mild, only tachypnea may be observed in pulmonary contusion cases. Severe degree pulmonary contusion includes cyanosis, dyspnea and shock. Hemoptysis is a rare clinical appearance, but if it is clinically detected, the prognosis of the case should be evaluated [9]. In this study, we clinically evaluated changings of the vital parameters, abnormal lung sounds and evidence of hemoptysis as well as cardio-respiratory system abnormalities, while pulmonary contusion was graded in the dogs. These clinical findings of the pulmonary contusion were different in dogs due to severity of the trauma, and we clinically diagnosed mild, moderate and severe degree of pulmonary contusion in the dogs. Hematoma and edema in the lung parenchyma following contusion increases the airway pressure and may responsible to irregular gas exchange in the alveoli [9]. Clinical deterioration is the appearance of the cellular damage in the patient that it may be seen at the first 24-48th h after trauma [24]. Progressive lung dysfunction and hypoxia may occur at the early stage of the pulmonary contusion due to hypoventilation, gas-diffusion abnormality, intra-pulmonary shunt and discordance of ventilation-perfusion [7,25]. In presented cases, hematological results were not congruent with the pulmonary contusion, but blood gas analysis results had minimal parametric difference. The time between the trauma and formation of the clinical findings is about 1-28 days [26]. It was clearly seen in this study that there was a meaningful difference between the clinical and radiological grading of the pulmonary contusion in dogs. Although all degrees of the pulmonary contusion were clinically observed, radiological results of the dogs pointed out that all dogs had 3rd degree pulmonary contusion. Radiological findings of the pulmonary contusion are uncertain in the first 4-6 h [27]. The interstitial and severe alveolar model pulmonary findings and air-bronchogram pattern radiopacities are usually observed in the radiographs taken initially [2,6,7,9].

Irregular, complex alveolar-interstitial model spots are the evidence of the pulmonary contusion [6,7,27]. After 24-36 h from the trauma, the lesions seen on the radiographs may be matching to the patients' healthy status [6-8]. In the cases with pulmonary disease, asymmetrical radiological lesions and diffuse pattern radio-abnormalities may be seen in dogs [22]. Radiologically, diagnosis of the dogs was third degree pulmonary contusion that the dogs had unilateral or bilateral increased fluid density in the ventrodorsal radiographs because of the hemorrhage and atelectasis of the lung lobes and air-bronchogram pattern appearance due to more severe pulmonary contusion and associated thoracic pathologies. Treatment of the pulmonary contusion changes according to degree of the pulmonary injury. In the mild degree, there is no advised treatment protocol; thus, restriction of the cases in a box is recommended until the clinical symptoms terminate. The cases with moderate degree should be treated with diuretics and colloids. If there is a severe respiratory problem, oxygenation by mask or mechanical ventilation following intubation, and treatment protocols should be planned to manage the vital parameters. Fluid therapy is required to improve the tissue perfusion and optimize the cardiac output. Corticosteroid usage has a confliction, because it may exaggerate to pulmonary disturbance. Bronchodilatators and analgesics may be given to regulate the respiration [9]. In this study, to complete the study plan, the dogs were medically treated following to emergency medical protocols. For this purpose, furosemide, lactate ringer and mannitol, and tranexamic acid were applied to the dogs. Moreover, thoracocentesis, tube thoracostomy and surgery were performed, if required.

Bronchoscopy is a valuable procedure in evaluation of canine respiratory disease [12,14,28]. It is usually used in referral hospitals to characterize respiratory disease processes and provides valuable visual information and documentation of the diseases and theirs characterize [23]. Bronchoscopy is also more sensitive for detecting airway pathology than diagnostic imaging techniques [14,23]. Thus, bronchoscopic evaluation was considered in dogs with pulmonary contusion. It permitted to visual assessment of the traumatized lower respiratory tracts and facilitated collection of deep respiratory samples by BAL for cytological and microbiological examination as described previously [14,15,28]. At 0th h, in bronchoscopic evaluations, bloody appearance (hemoptysis) in both tracheal and bronchial lumens and cyanosis in the bronchial mucosa was determined in the dogs. At 24 and 48th h, the results were not severe and there were only fibrin formation, mucopurulent and mucoid-hemorrhagic sputum in the trachea and effected bronchus.

BAL is a minimally invasive technique that is widely used in veterinary medicine [12-15], because it is the most effective procedure to take samples from respiratory tracts of immunocompromised patients and patients with opportunistic bacterial pneumonia, pulmonary hemorrhage or neoplasm [22,23,29]. By flushing to saline from the airways, samples are collected from bronchi, bronchioles and alveolar spaces for laboratory analysis [12-15,22]. Considering to simplicity and effectivity of the technique, BAL was applied by bronchoscopy at 0, 24 and 48th h under general anesthesia and the samples were investigated in pathology and microbiology laboratories, separately.

Diagnosis of the bacterial pneumonia is made on the basis of microbial culture results of BAL samples, and treatment should be planned by BAL cytology, microbial culture and susceptibility results [12,15-17,22,29]. Inflammation identifies according to total nucleated cell counts in BAL samples [12,13]. Inflammatory process is classified as neutrophilic, eosinophilic, lymphocytic, macrophagic, mixed or suppurative (>12% neutrophil) [12,16,17,22]. In the presented study, inflammation was evaluated by cytological analysis of BAL fluids at 0th h, and its progression was controlled at 24 and 48th h. The obtained results showed that there were intense erythrocyte, neutrophil, bronchial epithelial cells, macrophage, muco-protein fibers and mast cells at 0th h. It was considered that this cell intensity was due to hemopthysis (intrabronchial bleeding). However, the inflammation was more severe (neutrophilic, neutrophil count: 80-85%) and suppurative as described previously [12,13,16,22] at 24 and 48th h. There were also many degenerated respiratory epithelial cells in the cytological preparations. These conditions was interpreted that the inflammation was continuing and there is an immune response by the immune defense system. The presence of the bacteria in the cytological preparations is definitive diagnosis for the infectious disease in the respiratory tracts or parenchyma [12,13,15]. The presence or absence of the hemorrhage, etiologic agents, and other causes should compare for diagnosis in both suppurative and inflammatory diseases, and exact diagnosis is possible, only if infectious organisms are presented in cytology [13,15,22]. The cytological diagnosis of the infection in case 1, 2, 5 and 8 were made by determination of the existence of various shaped bacteria in the preparations. There were also phagocytized bacteria in the macrophages. It was deduced that these suppurative condition was together with the inflammatory process for case 1, 2, 5 and 8.

In general, Gram - and anaerobic bacteria is isolated from the lower respiratory tracts of the animals [17,18,29]. *Mycoplasma* is cultured in the normal pharyngeal flora of healthy dogs, and it can be isolated tracheo-bronchial lavages due to contamination. In a retro-spective study, *Pseudomonas* sp. is the more common encountered bacteria. *Staphylococcus, Streptococcus, Flavobacterium, Pseudomonas, Enterococcus, Bacillus, Bordettella bronchiseptica* and *Actinomyces* have been isolated in dogs with chronic bronchitis and dogs with lower respiratory tract infections [15,16]. In another study, the

culture results of the BAL fluids in diseased dogs include *E. coli, Streptococcus, Klebsiella, Mycoplasma* and *Enterobacter sp.*[15,17]. In the current study, *P. multocida* in case 1 and 2, and *E. coli* in case 5 and 8 were the cultured organisms from the BAL samples. Microbiological results were obtained 0th h samples in case 1, 5 and 8, but 24th h in case 2. According to these microbiological results, it has been concluded that pathogenic and opportunistic microorganisms, together with the inflammatory process, may be active with the pulmonary contusion due to immune system disturbance; hence, microorganisms could be cultured following to the pulmonary trauma in the bronchial lumen.

According to the patients' general status and suspected underlying disease, prophylactic antibiotic may be given with appropriate dosages [18,21]. Antibiotics should be considered on basis of the suspected pathologies and applied after culture and antibiotic susceptibility results [15,18,21,29,30]. In presented study, because pulmonary infection risk was evaluated, the antibiotic was not applied to dogs due to study plan at 0, 24 and 48th h. However, considering to antibiotic susceptibility results of a previous study [21], a selective antibiotic (ampicillin sulbactam) was applied to all dogs both prophylactically and as treatment protocol.

As a conclusion, thoracic trauma may result in pulmonary contusion, and clinical degree of the pulmonary contusion may not be compatible with the radiological degrees. Regardless of the pulmonary contusion degree, it should be noticed that pulmonary contusion triggers the inflammation process. If there is a pathogenic opportunistic flora in the lower respiratory tracts, the pulmonary infections may start together with the inflammation process according to our study results. Hence, antibiotic usage must be planned considering to culture and antibiogram test results of BAL fluids.

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Research Article

Management of Feline Glaucoma with Surgical Interventions: Some Less Preferred But Beneficial Options

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Abstract

Glaucoma is an eye disease which, particularly in cats, cannot always be controlled with medical treatment, and in most cases requires surgical treatment methods. It is a known fact that blindness occurs within 1-30 months even in feline glaucoma patients where intraocular pressure (IOP) is controlled with medical treatment and vision is present. This study comprised 11 eyes with glaucoma, in a total of 10 cats (3 female, 7 male). The selected and performed surgical methods were; cyclocryosurgery in 6 patients, trabeculectomy in 3 and chemical ablation in 1 patient. The patients were re-examined at regular intervals in the post-operative period and changes in intraocular pressure (IOP) and the degree of pain and vision were assessed. Nine cats with vision prior to surgery were assessed on post-operative Day 60 for criteria including; IOP, presence of vision and pain. The blind cat was assessed with respect to IOP and pain. The results of trabeculectomy performed in 3 cats with vision in the pre-operative period were unsuccessful. In the patients that received cyclocryosurgery (6 cases) and chemical ablation (1 case), IOP was controlled and pain eliminated. In conclusion, since we know that feline glaucoma almost always ending with blindness, surgical interventions like cyclocryosurgery and chemical ablation should be considered especially when the IOP can not be controlled with medical therapy.

Keywords: *Feline, Glaucoma, Trabeculectomy, Cyclocryosurgery, Chemical ablation*

Kedilerde Glokomun Cerrahi Yaklaşımlarla Kontrol Altına Alınması: Az Tercih Edilen Ancak Faydalı Bazı Seçenekler

Özet

Glokom özellikle kedilerde, çoğu zaman tıbbi tedavi ile kontrol altına alınamayan, olguların büyük bir kısmında cerrahi tedavi yöntemlerinin kullanılmasını gerektiren bir hastalıktır. Tıbbi tedavi ile göz içi basıncı (GİB) kontrol altına alınabildiği ve görmenin var olduğu glokom hastası kedilerde dahi sonraki 1-30 ay içerisinde körlük şekillendiği bilinmektedir. Çalışmamıza 3 dişi, 7 erkek toplam 10 kediye ait 11 glokomlu göz dahil edildi. Hastalardan altısında siklokriyocerrahi, üçünde trabekülektomi, bir tanesinde ise kimyasal ablasyon operasyon yöntemi olarak seçildi ve uygulandı. Hastalar postoperatif dönemde belli aralıklarla kontrol edilerek, GİB 'deki değişimler, ağrı ve görme drumu açısından değerlendirildi. Operasyon öncesi görebilen 9 kedi post operatif 60. Gün GİB, görüş varlığı ve ağrı gibi kriterlere bakılarak değerlendirildi. Kör olan bir kedi ise GİB ve ağrı açısından değerlendirildi. Preopertif dönemde görme var olan 3 kedide trabekülektomi ile başarılı sonuçlar elde edilemedi. Siklokriyoterapi (altı olgu) ve kimyasal ablasyon (1 olgu) yapılan hastalarda ise GİB kontrol altına alınabildiği gibi ağrı da ortadan kaldırılabildi. Sonuç olarak, kedilerde glokomun çoğu zaman körlükle sonuçlandığı için, medikal tedavi ile GİB kontrol altına alınamayan hastalarda siklokriyocerrahi ve kimyasal ablasyon gibi cerrahi girişimlerin de tedavi seçeneği olarak düşünülmesinin uygun olabileceği kanısına varılmıştır.

Anahtar sözcükler: *Kedi, Glokom, Trabekülektomi, Siklokriyocerrahi, Kimyasal ablasyon*

INTRODUCTION

Glaucoma is described as, the progressively worsening loss of vision as a result of (IOP) remaining at a much higher level than where the optic nerve and retina can function normally [1]. Today, veterinary ophthalmologists still indisputedly consider it to be the most challenging eye disease to treat [2].

Since it is a multifactorial disease with many different fenotypes and varying etiology, its classification is equally difficult. Depending on its etiology, stage and iridocorneal angle morphology, glaucoma can be classified as; primary, secondary or congenital; early non-congestive, acute congestive or chronic; open-angled, narrow/closed-angled or glaucomas progressing with goniodysgenesis, respectively [3]. However, this classification has limited

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significance for a surgeon developing a suitable treatment protocol for each individual patient. In any case, 95-98% of glaucoma cases observed in cats are of secondary character and originate from factors causing changes in aqueous humor flow dynamics, such as uveitis, tumour, trauma and intraocular haemorrhage [4]. The normal IOP value determined using applanation tonometry in cats is 18.4 (±0.67) mm Hg [5].

According to data collected by the Veterinary Medical Database over a period of 20 years, glaucoma causes loss of vision in 1 in 367 cats [1]. Also, feline glaucoma is different to canine glaucoma in that it develops insidiously and is a constantly deteriorating disease, where cats are usually presented by their owners for clinical examination in the late period [4,6]. The main target at this point is to alleviate the perception of pain by lowering IOP, therefore, improving the quality of life [7].

In cats, if it is not possible to bring down IOP to under 25 mmHg with medical treatment, selection of a surgical method is indicated. The main criteria in selecting the surgical method to be used is whether or not vision is present in the eye with glaucoma [7]. In cats that can still see, cyclodestructive procedures, which decrease aqueous humor production, or filtering procedures, which increase aqueous humor drainage are used. Surgical interventions such as chemical ablation and enucleation are preferred in cats that have total loss of vision [8].

Using present clinical medical treatment methods, IOP can not be lowered to less than 10-15 mmHg and the painful phase remains persistent [1]. Also, medical therapy is beneficial in the early stages of the disease in controlling glaucoma, however, for the long term control of IOP, surgical intervention is almost always necessary [9]. In spite of the general knowledge, even in feline glaucoma patients that have vision and IOP under control by medical treatment, blindness could be ocur within 1-30 months [7]. In addition, there is only a relatively small number of clinical studies carried out on the surgical treatment of glaucoma in cats [6]. The aim of this study is to present information regarding the contribution of trabeculectomy and cyclocryosurgery, administered in addition to medical treatment, to the process of controlling glaucoma in cats, as well as the clinical results of chemical ablation performed in a patient with total loss of vision.

MATERIAL and METHODS

In this study, 11 glaucomatous eyes of 10 cats (3 female, 7 male) were treated surgically. The age range was between 24-48 months. Breed distribution was; 2 Siamese, 1 Persian and 7 mixed breed. Cyclocryosurgery in 6 patients, trabeculectomy in 3 and chemical ablation in 1 patient were performed.

Patient Selection

Cats with IOP higher than 25 mmHg were considered to be glaucoma patients. None of the patients had received glaucoma treatment previously. All patients were initially given medical treatment. For medical therapy, an agent containing carbonic anhydrase inhibitor and beta-blocker (Cosopt®, Merck Sharp & Dohme) was prescribed for use twice daily. IOP values of the patients were measured 3 and 7 days later.

First Examination

Examination of the eye was carried out using an indirect ophthalmoscope, slit lamp, retinal camera (Kowa, RC-2, Japan) and applanation tonometer (Tono-Pen XL®, Reichert). Vision was considered to be present in cats that followed the movements of a cotton wool ball and displayed the menace response. The mean value of five consecutive measurements performed using the applanation tonometer was determined and recorded.

Surgical Procedure Selection and Anaesthesia

In the follow-up examination of the patients on Day 7, either trabeculectomy or cyclocryosurgery was performed in patients whose vision was present, while at the same time, where IOP could not be lowered to less than 25 mmHg. In patients where IOP could not be lowered to less than 40 mmHg after one week of medical treatment, trabeculectomy was selected as the surgical procedure. Cyclocryosurgery was carried out in cats where IOP could be lowered to under 30 mmHg following a 7-day medical treatment. Chemical ablation was performed in a cat with no vision but with moderate buphthalmos. Pre-operative complete blood count and some serum biochemical parameters (glucose, AST, ALT, BUN, creatinin) were examined. Patients suitable for anaesthesia were sedated using 1 mg/kg IV xylazine hydrochloride (Rompun®, Bayer). Anaesthesia induction was done by administering ketamine hydrochloride (Ketalar®, Bayer) at a dose of 20 mg/kg IM.

Trabeculectomy

The anaesthetised patient was placed in lateral recumbency with the affected eye uppermost. Initially, a limbus-based conjunctival flap in the 12 o'clock position was elevated. A ½ thickness square scleral flap with a dimension of approximately 4x4 mm was elevated from the sclera directly below the conjunctiva and the trabuculum was reached. At his stage, gauze soaked in mitomycin-c (Mitomycin-c®, Onko) at a concentration of 0.25 mg/mL was placed on the sclera for approximately 3 min and sclera was irrigated with 10 mL isotonic NaCl solution. Then a trabecula with a dimension of approximately 1x3 mm was removed *(Fig. 5)*. In the next stage, iridectomy was performed. The scleral flap was attached with only 2 sutures using 8/0 (Vicryl®, Ethicon) absorbable suture material. In the final stage, the conjunctival flap was

sutured using 8/0 (Vicryl®, Ethicon) absorbable suture material with simple interrupted sutures and the procedure was completed.

Cyclocryosurgery

The anaesthetised patient was placed in lateral recumbency with the affected eye uppermost. For the cyclocryosurgery procedure, the 1.5 mm-diameter cryoprobe of the nitrous oxide cyclocryosurgery device (Metzen) was used. The cryoprobe was placed on the sclera 2-3 mm caudal to the limbus and the cooling process was begun *(Fig. 4)*. The maximum temperature reached by the probe tip during the cooling process was -76°C. The cooling process was continued until a sphere of ice formed at the cryoprobe's point of contact. The cooling process was applied to 6-8 different points between the 12 and 9 o'clock positions. No extra care was taken to prevent the sphere of ice from coinciding with the 3 and 9 o'clock positions.

Chemical Ablation

The anaesthetised patient was placed in lateral recumbency with the affected eye uppermost. The 0.75 mL chemical ablation emulsion was prepared by filling a syringe with 20 mg gentamycin sulphate (Gensif® 80 mg ampul, AVICENNA) and 1 mg dexamethasone (Dekort® 8 mg ampul, Deva). Initially, a 22-gauge 2 mL syringe was used to enter the front camera through the limbus and approximately 0.8 mL aqueous humor was removed. The prepared emulsion was injected into the globe via the same route and the chemical ablation procedure was completed.

Post-operative Assessment

The cats were called back for check-ups on post-operative days 1, 7, 14, 30 and 60 and assessed for presence of complications, state of vision, pain and changes in IOP. Vision was considered to be present in cats that followed the movement of a cotton wool ball and displayed the menace response. Surgery was considered to be successful in patients that had an IOP below 25 mmHg on post-operative day 60 with no evidence of pain and that had vision. The pre-operatively blind case that received chemical ablation was considered to be successful on account of the IOP value dropping to under 25 mmHg on post-operative day 60 and that no pain was evident.

RESULTS

The study included 11 eyes with glaucoma from a total of 10 cats; of which 3 (30%) were female and 7 (70%) were male. The age range was 24-48 months (mean 35 months). Breed distribution was; 2 Siamese, 1 Persian and 7 mixed breed. Glaucoma was due to secondary to uveitis in 7 patients, trauma in 2 patients and of congenital origin in 1 patient. Any acute symptoms related with the uveitis (aqueous flare, periperal iridal hypemia, dyscoria etc.) were not determined during first clinical examination in uveitis induced secondary glaucoma patient cats. In the first examination, while 9 of the cats had vision, 1 was blind. Glaucoma had developed in the right eye in 3 patients, in the left eye in 6 patients and bilaterally in 1 patient. Mean IOP in the affected eyes was 33.6 mmHg in the initial examination. Following one week of medical treatment, mean IOP was determined to be 29.7 mmHg, while the difference between mean IOP values was found to be 3.9 mmHg. Surgically, trabeculectomy was performed in 3 cats, cyclocryosurgery in 6 cats and chemical ablation in 1 cat *(Table 1)*.

When obtaining the history from patient owners, it was recorded that 7 cats (case no.s 1, 3, 6, 7, 8, 9, 10) had suffered from Herpes virus infection, with subsequent clinical complaints relating to the eye. In the clinical examination of these 7 cats, there were no clinical findings such as lens luxation, tumour, corneal oedema, rubeosis iridis or aqueous flare. Glaucoma diagnosis was made based on the presence of clinical findings such as episcleral congestion, pain, raised IOP and buphthalmus *(Fig. 1)*. In the author's opinion, glaucoma in these patients had occurred due to the blockage in the flow route of the aqueous humor secondary to uveitis. In 1 (case no. 2) of the 3 cats that developed glaucoma following trauma, the iris was found to be bulging during clinical examination. The narrowing of the iridocorneal angle in this patient was evident even in clinical examination. Therefore it was assessed as a closed-angle glaucoma due to trauma. Eyedrops could not be administered to this patient (case no. 2) due to aggression so trabeculectomy was performed without the medical treatment option. Bilateral aniridia was determined on clinical examination in one patient (case no. 5) diagnosed with bilateral congenital glaucoma *(Fig. 2)*. On fundus photography, none of the patients displayed optic disk cupping *(Fig. 3)*.

In the follow-up examination on post-operative day 1, a slight hyphema had developed in all of the patients that had received trabeculectomy *(Fig. 6)*. Two of these patients (case no.s 1 and 3) were prescribed eye drops containing dexamethasone to be administered 1 drop 4 times daily, and eye drop containing an ciprofloxacin to be administered 1 drop 6 times daily. On post-operative day 7, the hyphema was seen to have resolved in these two cases. In the follow-up examination of one of the patients (case no. 2) on post-operative day 7, no significant decrease was determined in the IOP and, since medical treatment was not possible for this patient, the related eye was removed. In the follow-up examinations of case no.s 1 and 3 on post-operative day 60, although vision was present, it was determined that IOP could not be lowered to under 25 mmHg and that the pain sensation continued. In 3 cats receiving trabeculectomy, the result was considered to be unsuccessful.

Table 1. *Preoperative informations belongs to patients, IOP variations during medical treatment and preferred surgical procedures*

Case No	Age (month)	Breed	Sex	Etiology	Clinical Examination Findings	Evaluation of Vision	Side	IOP 1st Day (mm Hg)	IOP 3rd Day (mm Hg)	IOP 7th Day (mm Hg)	Surgical Procedure
1	24	Mix	♂	Uveitis	P, EC, Bup+	Sighted	Unilateral Right	44	36	41	Trabeculectomy
2	36	Mix	♂	Traumatic	Iris bombe, P, EC, Bup+	Sighted	Unilateral Left	41	-	-	Trabeculectomy
3	36	Mix	♀	Uveitis	P, EC, Bup+	Sighted	Unilateral Left	43	36	40	Trabeculectomy
4	30	Mix	♂	Traumatic	P, EC, Bup+	Sighted	Unilateral Left	29	27	26	Cyclocryosurgery
5	24	Siamese	♂	Congenital	Aniridia, P, EC, Bup+	Sighted	Bilateral	R29/L32	R29/L30	R28/L27	Cyclocryosurgery
6	36	Mix	♂	Uveitis	P, EC, Bup+	Sighted	Unilateral Right	30	28	28	Cyclocryosurgery
7	48	Persian	♀	Uveitis	P, EC, Bup+	Sighted	Unilateral Right	29	30	28	Cyclocryosurgery
8	42	Mix	♀	Uveitis	P, EC, Bup+	Sighted	Unilateral Left	32	30	29	Cyclocryosurgery
9	48	Mix	♂	Uveitis	P, EC, Bup+	Sighted	Unilateral Left	31	27	26	Cyclocryosurgery
10	24	Siamese	♂	Uveitis	P, EC, Bup ++	Blind	Unilateral Left	34	28	27	Chemical ablation

*P, pain; **EC,** episcleral congestion; Bup+, mild buphthalmos; Bup ++, moderate buphthalmos*

Table 2. *Postoperative variations of IOP, postoperative complications and visibility after surgery*

Case No	Procedure	Preoperative IOP (mm Hg)	Postoperative Complications	Postoperative 1st Day IOP (mm Hg)	Postoperative 7th Day IOP (mm Hg)	Postoperative 14th Day IOP (mm Hg)	Postoperative 30th Day IOP (mm Hg)	Postoperative 60th Day IOP (mm Hg)	Evaluation of Vision After Surgical Intervention
1	Trabeculectomy	41	Hyphema	40	30	36	34	38	Sighted
2	Trabeculectomy	41	Hyphema	37	38	-	-	-	-
3	Trabeculectomy	40	Hyphema	32	29	36	38	36	Sighted
4	Cyclocryosurgery	26	-	24	18	18	16	18	Sighted
5	Cyclocryosurgery	R28/L27	-	R28/L24	R20/L22	R14/L17	R14/L14	R12/L14	Sighted
6	Cyclocryosurgery	29	-	24	20	18	16	16	Sighted
7	Cyclocryosurgery	28	-	24	22	18	16	18	Sighted
8	Cyclocryosurgery	29	-	28	29	29-Reop	20	16	Sighted
9	Cyclocryosurgery	26	-	26	20	20	16	16	Sighted
10	Chemical ablation	27	-	30	20	14	18	14	Blind

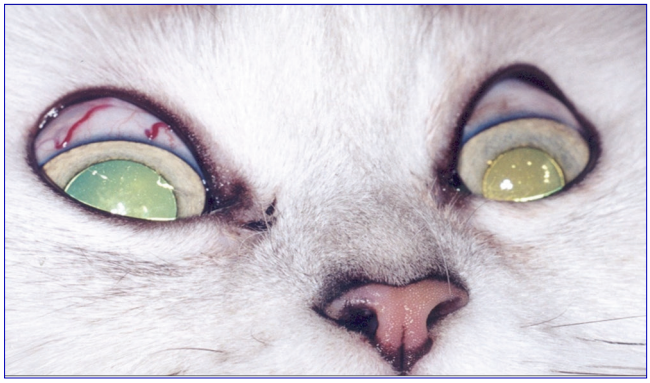

Fig 1. Appearance of eye at initial examination. Episcleral congestion, mydriazis and mild buphthalmos in right eye (case no 7)

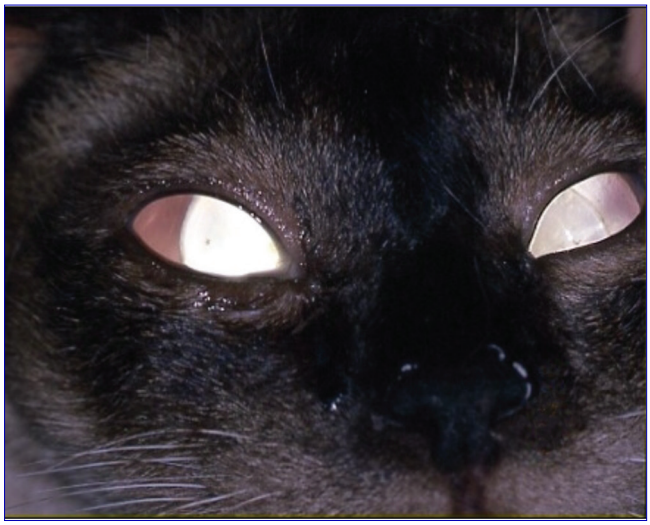

Fig 2. Clinical appearance of bilateral aniridia in case no 5

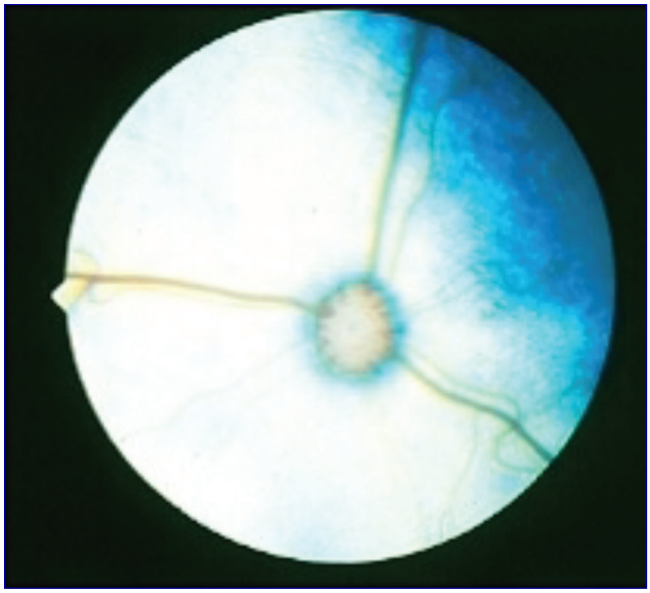

Fig 3. Fundus photography of case no 6, optic disc cupping was not present

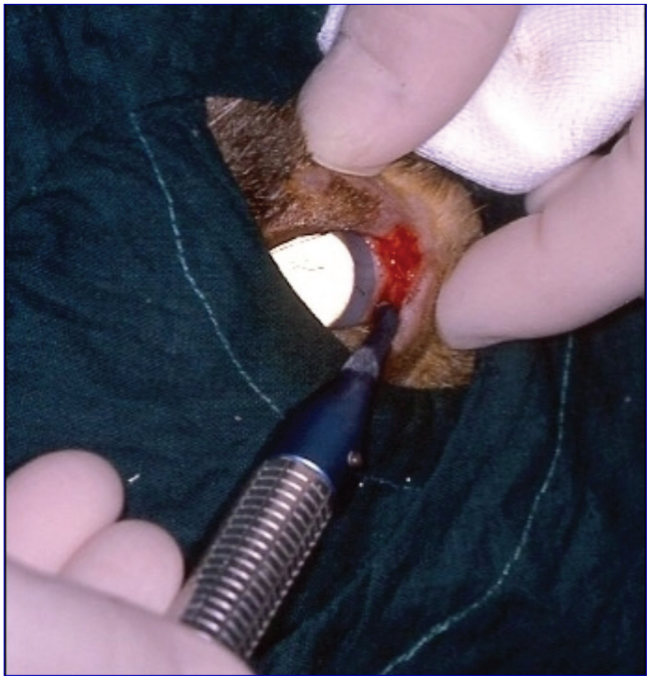

Fig 4. Cyclocryosurgery. Touch point of cryoprobe 2-3 mm caudal to the limbus

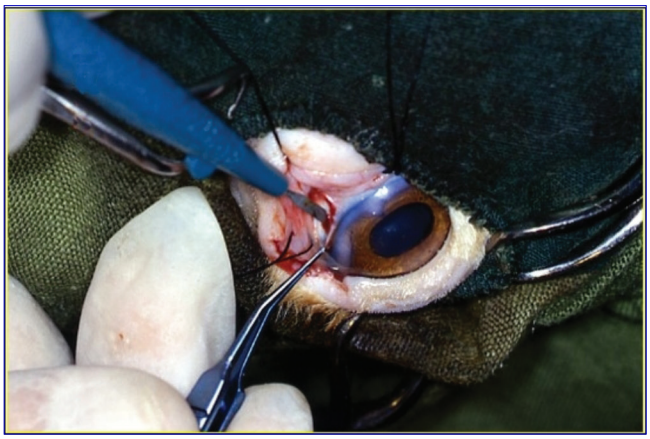

Fig 5. Stage of 1×3 mm trabecula removing during trabeculectomy

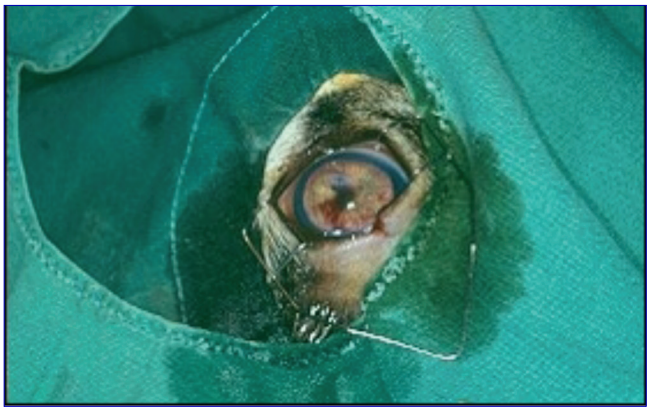

Fig 6. Postoperative hyphema complication after trabeculectomy (case no 1)

In patients that received cyclocryosurgery, there were no post-operative complications such as partial necrosis in the iris, blindness or retinal detachment. Only in one cat (case no. 8) almost no reduction occurred in IOP in the post-operative period and the state of pain continued. The same procedure was repeated in this case 14 days after the first operation. In the second operation, the cryoprobe made contact with 8 different points between the 3 and 12 o'clock positions where ice spheres were formed and the procedure was completed. In the follow-up examination on post-operative day 60, IOP levels were below 20 mmHg in all patients receiving cyclocryosurgery and the mean value of these 7 cats was 15.7 mmHg. Considering the mean IOP value of patients receiving cyclocryosurgery was 27.5 mmHg in the pre-operative period, a 42% reduction in mean IOP values was demonstrated. As well as the continued presence of vision in patients in this group, pain was also eliminated. A successful result was achieved in patients receiving cyclocryosurgery *(Table 2)*.

The cat receiving chemical ablation had an IOP value of 14 mmHg on post-operative day 60 and pain was not present. Due to the fact that this cat was already blind in the pre-operative period and since targets such as, the eye remaining *in situ* for cosmetic reasons, elimination of pain and lowering IOP were met, the result was considered to be a success *(Table 2)*.

DISCUSSION

Glaucoma is an eye disease which, particularly in cats, cannot always be controlled with medical treatment, and in most cases requires surgical treatment methods [10]. However, there is not an overall single method that would benefit all glaucoma patients [1].

Different filtration procedures to increase aqueous humor drainage have been described for the surgical treatment of glaucoma. Transscleral iridencleisis applied together with cyclodialysis and posterior sclerotomy, trabeculectomy, glaucoma shunts and gonioimplants are used for this purpose [2,3,9,11,12]. For many years, trabeculectomy has been used and accepted as the gold standard among filtration procedures in human medicine. Studies carried out in human medicine in recent years have focused on comparing long-term results of surgical inter-ventions performed using GDD and trabeculectomy [13]. The use of glaucoma drainage devices is increasing day by day. However, the fact that feline glaucoma generally occurs secondary to uveitis, which causes glaucoma drainage tubes to be blocked by the inflammatory filtrate, limits its use. Other factors contributing to the difficulty of using these devices include, the conformation of the orbit in cats, its relatively taut structure and the presence of buphthalmus [4]. On the other hand, not many studies exist on the use of the trabeculectomy procedure in the treatment of secondary glaucoma in cats. In this study, trabeculectomy was preferred in order to achieve a significant and rapid drop in patients with an IOP higher than 40 mmHg. However, in these patients neither could IOP be lowered to below 25 mmHg, nor could pain be eliminated.

Cyclocryosurgery and cyclophotocoagulation are procedures used to lower IOP by decreaseing aqueous humor production and to control glaucoma, and could be used like filtration procedures in patients that still have vision [2]. It has been suggested that, in order to avoid causing damage to the long ciliary arteries while administering cyclocryosurgery, the cryoprobe should not make contact at the 3 and 9 o'clock positions [7,9]. In the present study, however, this idea warning of the risk of necrosis patches developing in the iris was disregarded. Also serious complication risk of cyclodestructive procedures are considered to be low [14].

Although the use of nitrous oxide and liquid nitrogen for cyclocryosurgery serve the same purpose, there are some differences. When liquid nitrogen is used, a temperature of -185°C is produced at the tip of the cryoprobe, leading to the ciliary epithelium to cool down to -23°C. With the use of nitrous oxide, a temperature of between -60°C and -80°C is produced at the tip of the cryoprobe and the ciliary epithelium cools down to -15°C. The aim in cryosurgery is to generate not complete but only partial necrosis in the ciliary epithelium in order to decrease aqueous humor production [9]. The temperature in the ciliary epithelium required to produce this condition has been reported as between -12°C and -15°C. In the author's opinion, the use of nitrous oxide in this study may have been effective in the lack of post-operative complications, by producing an optimal degree of cilionecrosis in the ciliary epithelium.

In one of the patients receiving cyclocryosurgery, since IOP did not show any indication of decreasing in the post-operative period, cyclocryosurgery was repeated 14 days after the first operation. Recordings taken 45 days after the second operation in this patient revealed that IOP had been controlled and pain eliminated. The reason for the necessity of a second operation in this patient may be due to the fact that adequate cilionecrosis could not be produced in the first instance because of the change in the anatomical position of the ciliary epithelium [4] caused by buphthalmus in some situations.

Despite the fact that previous studies indicate that cyclocryosurgery in cats does not produce results as successful as those in dogs with respect to controlling IOP [11]. In the present study, the results obtained from cyclocryosurgery performed using nitrous oxide were successful.

Chemical ablation is a method which decreases IOP, eliminates pain and, when compared to enucleation, produces a more successful cosmetic result by the chemical destruction of the ciliary epithelium in patients with no vision due to glaucoma. Although it is suggested that this method causes intravitreal sarcoma developing in later stages [15], the relationship between chemical ablation using gentamycin and sarcoma has not been clearly identified [4].

Despite no ophthalmic abnormalities being detected in the eye examination, patients with an IOP higher than the reference range are considered to have primary glaucoma. In this study, glaucoma was determined to have developed secondary to uveitis in 7 patients (case no.s 1, 3, 6, 7, 8, 9, 10). However, no ophthalmic abnormality such as lens luxation, aqueous flare, corneal oedema, tumour etc. was observed in any of the patients. The reason the author is able to state that glaucoma had developed secondary to uveitis in these patients was the fact that, all the cats had a history of Herpes virus infection and that glaucoma had not developed bilaterally in any of the cases. In the author's opinion, the inflammatory products generated during uveitis had blocked the iridocorneal angle, permanently preventing aqueous humor drainage, leading to the development of secondary glaucoma.

Studies indicate that the most common surgical intervention used in glaucoma patients is enucleation [6]. Yet, in a disease where it has been proven that patients can lose their vision within 1-30 months even in situations where IOP can be controlled with medical treatment [7], the fact that surgical methods should be used more often is an obvious statement. Results obtained, particularly with cyclocryosurgery, in the patient population most of which still have vision, are favorable and may be beneficial for use in feline glaucoma patients.

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Research Article

Risperidone as a Promising Treatment of Choice in Dog Owner Directed Aggression

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Abstract

Owner-directed aggression is reported as the most frequent of the aggression cases observed. Some factors underlying aggressive behavior are, adopting before the end of socialization period, genetic and hereditary parameters, inbreeding, environment, excessive punishment, sex, age, size, somatic reasons, territorial threats and owner dependant factors. The choices to deal with aggressive dogs is usually limited by relinquishment or euthanasia. The aim of this study is to evaluate the effects of risperidone (atypical antipsychotic) in decreasing the risk of danger in dogs presenting owner directed aggression. Sixteen household dogs from various age, sex and breed with the complaint of showing aggressive behavior were examined and after elimination of somatic reasons a scoring scale based on the considerations for assessing danger and risk of injury was prepared with the owner. Treatment was comprised of owner informing on the management and behavioral approach for each individual case and medical treatment with Risperdal oral solution 1 mg/1 mL,100 mL sid (1 mg/m^2). Examination, history taking and scoring were repeated after 1 month of therapy. Risperidone created satisfactory results in decreasing the risk of danger, also will prevent many household dogs from relinquishment or euthanasia.

Keywords: *Dog, Owner directed aggression, Risperidone, Dog bite, Antipsychotic*

Köpeklerde Sahibine Yönelik Agresyonda Başarı Vaad Eden Bir Tedavi Seçeneği Olarak Risperidon

Özet

Sahibine yönelik agresyon, en sık gözlenen agresyon vakalarındandır. Agresif davranış biçiminin altında yatan faktörlerden bazıları, sosyalizasyon dönemi tamamlanmadan anneden ayrılma, genetik ve herediter parametreler, aile içi çiftleşme, çevre, aşırı cezalandırma, cinsiyet, yaş, büyüklük, somatik nedenler, çevresel tehditler ve sahip kaynaklı nedenlerdir. Agresif köpek sahiplerinin çözümü genellikle ötenazi veya hayvanı terk etmek olmaktadır. Bu çalışmanın amacı, sahibe yönelik agresyon gösteren köpeklerde risperidon'un (atipik antipsikotik) tehlike riski üzerindeki etkilerini ortaya koymaktır. Agresif davranış şikayeti ile getirilen farklı yaş, cinsiyet ve ırktan 16 ev köpeği, muayeneleri ve fiziksel nedenlerin eliminasyonunu takiben tehlike ve yaralanma riskinin belirlenmesi kriterleri bazında hasta sahibi ile birlikte skorlanmışlardır. Tedavi, hasta sahibinin genel ve davranışsal yaklaşım açısından bilgilendirilmesi ile Risperdal oral solüsyon 1 mg/1 mL, 100 mL sid (1 mg/m^2) kullanımı ile şekillendirilmiştir. Muayene, anamnez ve skorlama işlemleri 1 aylık tedaviyi takiben tekrarlanmıştır. Risperidonun, tehlike riskini düşürmede etkin sonuçlar sağladığı ortaya konmuştur. Bu sonuç aynı zamanda pek çok ev köpeğininde terk edilme ve ötenaziden uzak tutulmasına neden olacaktır.

Anahtar sözcükler: *Köpek, sahibine yönelik agresyon, Risperidon, köpek ısırma, antipsikotik*

INTRODUCTION

Aggression is a major canine behavioral disorder [1] and an important public issue, but bite and attact cases are usually remain unreported [2]. Human directed aggression is the main presentation of canine aggression with 54%-67% of all aggression cases [3]. In United States, nearly 885.000 dog bite victims seek medical care, 30.000 undergo reconstructive procedures, and between 10 and 20 are reported fatalities [4]. Approximately 2-4% of all dog bite cases require hospital involvement [5]. The target is usually the family members, in which the dog fights for a higher hierarchical position in the family [1], as well as adults [6], men [6], children [7,8] and the elderly [8]. Children has a higher

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risk for attacking to the vital areas as the face and neck [4].

Dog aggression usually take place at home [9], and during interaction with the dog [6,9] nearby childrens provocation [7,9]. Infact, studies on dog bite cases of humans especially children, suggest that serious human directed aggression is most likely to occur in the home from known or family dogs [10,11]. More than half of severe aggression cases are reported to be associated with a family member taking away food or other goods important for the dog [5].

Owner-directed aggression is reported as the most frequent of the aggression cases observed diagnostic category (35.34%) [1].

There are many factors originating aggressive behavior such as adopting before the end of socialization period, genetic and hereditary parameters, inbreeding, enviroment, excessive punishment, sex, age, size, somatic reasons, territorial threats and owner factors [12]. Surprisingly, owner dependent factors are superior to dog dependent factors in aggresivity [13]. However, these factors do not provide a presicion about an individual dog's risk of aggression to people based on these parameters [14].

Alas, the choices to deal with dogs presenting aggression is usually limited by relinquishment or euthanasia.

Current managing strategies for dog aggression starts with the diagnosis and classification of aggression type followed by environmental manipulation, physiologic intervention, behavioral techniques and medical therapy [12,15]. Pharmacological intervention aims to produce a stable change in the perception of a stimulus and the resulting emotion, leading to the correction of the behavioural problem. It is very important to evaluate the subject's pathological state based on history and examinations, to identify the functional impairment of the pivotal neurotransmitter systems involved in the disorder in order to select a suitable pharmacological treatment [15].

Level of rigor, risk of injury and danger for the household together with foreigners including pedestrians risk of attact during walks must be concerned at the first stage. The main target of the aggressivity management should be reducing the risk of danger to a reasonable level. Therefore, especially in dogs with high risk and danger potential, pharmacological treatment is essential to avoid irreversible damages.

Psychoactive drugs are efficient to regulate the behavior and underlying mechanisms and they are commonly prescribed in clinical practice for serious behavioural problems [15,16]. Thymoregulators, antidepressants and neuroleptics are used to treat different types and degrees of aggressivity, but in cases presenting high risk antipsychotics are good choices to reduce the injury potential [16,17].

Risperidone, an atypical antipsychotic, is a serotonin type 2 (5-HT_2) and dopaminergic D_2 receptor antagonist with high potency, also used in human medicine in schizophrenia, bipolar mania and irritability associated with autistic disorder [16]. In dogs it is proposed for diminishing behavioral arousal and in sociopathy stage 1 when the warning phases are still complete, social directed regression, sociopathies, impulsive aggression, hallucinatory type signs [17], with considerable safety [16-18], but there is not much literature on the clinical therapeutic results in dog aggression.

The aim of this study is to observe the therapeutic affects of risperidone in dog aggression cases with considerable risk which is also an important public health issue.

MATERIAL and METHODS

Animal Material and Examinations

The material of this study consisted of 16 household dogs from various breeds, age and gender which referred to the faculty clinics with the complaint of showing aggressive behavior *(Table 1)*. Elimination of somatic disorders were performed and the patients were evaluated from the behavioral aspect *(Table 2)*. After aggression was diagnosed, assessment of risk of injury and danger was achieved by a scoring scale based on the considerations

Table 1. *General characteristics of the dogs*

Factors	Condition	
Household population	1 Member	6 Families
	2 Members	3 Families
	3-5 Members	6 Families
	6- Members	1 Families
Kids and adolescent number	9 in 7 families	
Owner experience with dogs	First dog	11
	Previously owned one or more dogs	5
Dog gender	Male	6
	Female	10
Dog age	29.9±21.9 months of age	
Dog race	Labrador retriever	1
	Terrier	2
	Poodle	1
	Rotweiler	1
	Kangal	2
	Cocker spaniel	2
	Mix	5
	German shepherd mix	1
	Boxer	1
Neuter status	Neutered	2
	Intact	14

Table 2. *Behavioral Complaints and Concurrent Behavior Problems*

Dog	Aggression to Owners (less- more/1-5)	Aggression to Foreigners (less- more/1-5)	Concurrent Behaviour Problems	Presence of Dominant Posture and Behaviour
1	4	2	Tail chasing, Anxiety Play related aggression	+
2	5	2	-	+
3	5	4	Anxiety	+
4	4	2	Fear aggression	+
5	3	3	-	+
6	3	2	Tail chasing, Hallucitative episodes	+
7	4	3	-	+
8	4	3	Territorial aggression	+
9	3	1	-	+
10	3	2	-	+
11	4	1	-	+
12	4	1	Territorial aggression	+
13	3	1	Separation anxiety	+
14	4	1	-	+
15	3	2	-	+
16	3	3	Territorial aggression	+

Table 3. *Danger and risk of ınjury scores before and after therapy*

Parameter			Before Therapy (X±Sx)	After Therapy (X±Sx)	Significance
Predictabılity	Identifiable stimuli and situations		3.56±1.09	1.56±0.96	***
	Consistent response to aggression eliciting stimuli		3.75±0.93	1.38±0.81	***
	Benignness of stimuli that trigger aggression		3.63±0.96	1.13±0.34	***
	Existence of warning signals		3.31±1.14	1.75±1.13	***
	Latency to attack		3.19±1.05	1.19±0.40	***
	Avaliability of pertinent historical info		2.94±1.76	2.81±1.87	
Potential to cause damage	Size and strength of animal		3.44±1.15	3.44±1.15	
	Degree of bite inhibition		3.19±0.91	1.13±0.5	***
	Intensity of focus/level of arousal		3.56±0.81	1.31±0.6	***
	Target for aggresion		3.25±0.77	1.25±0.58	***
	Type of aggression		1.5±0.63	0.56±0.51	***
The human element	Comprehension of danger		3.56±1.03	1.75±1.29	***
	Ability to understand management and treatment		3.68±1.08	1.75±1.44	***
	Ability to provide safe control		3.88±0.96	1.43±0.81	***
	Verbal control of pet		3.69±0.95	1.38±0.72	***
	Dependability	History of compliance and consistency	2.81±1.56	2.56±1.75	
		Family size. life style	2.56±1.55	2.56±1.55	
		Ages of family members	2.31±1.62	2.31±1.62	
	Experience with animals		4.25±1.24	4.25±1.24	
Complexity of the situation	Number of types of aggression		1.44±0.63	0.56±0.51	***
	Number of situations/stimuli that trigger aggression		3.68±0.95	1.31±0.60	***
	Number of concurrent behaviour problems		0.63±0.89	0.06±0.25	*
	Opportunity for confrontations		3.63±0.96	1.31±0.60	***

*Statistically significant at * P≤0.05 and *** P≤0.001 level*

for assessing danger and risk of injury [16]. For each case, owner was questionered in order to score each parameter of the scale. Treatment was comprised of owner informing on the management and behavioral approach for each individual case and medical treatment with Risperdal® oral solution 1 mg/1 mL,100 mL (Eczacıbaşı - İstabnul) sid (1 mg/m^2) [16,17]. Examination, history taking and scoring were repeated after 1 month of therapy *(Table 3)*.

Scoring

Considerations for assessing danger and risk of injury has four major parameters (predictibility, potential to cause damage, the human element, complexity of the situation) according to Landsberg et al.[16]. Scoring was recorded according to these parameters 1 to 5 (subjective parameters were scored as good to worse conditions, from 1 to 5).

Statistical Analyses

Student t test was used in comparing pre and post therapy scores of the groups. Duncan test was used in determination of difference between groups.

RESULTS

Results were presented in Table 3. Significant progress in predictibility, decrease in the potential to cause damage and the complexity of the situation demonstrates a favorable effect for risperdal in dominance cases with high risk of danger. As a matter of fact, owners mentioned a great difference in behavior and uttered a higher and safer life quality for the mutual lifetime.

DISCUSSION

Literature states that, human directed aggression is usually observed in smaller breeds and small breed dogs has a higher risk of biting as the result of their nervous and disobedient nature [5,19,20]. The advantage of this condition is that the risk of danger is less for small breeds as more likely to be tolerated by the owner. In the long term, this may have led to a genetic predisposition in smaller dogs [21].

In the present study half of the cases were small breeds, but deduction of a consequence from 16 dogs will not be a suitable attempt in that respect.

Breed associated aggression is a focus of interest in behavior medicine and variations among results may be due to breed distribution on the area basis and the genetic lines of each breed [20,22]. Nevertheless, consensus to a great degree is achieved on most frequently reported breeds with human directed aggression in a serious manner**;** these are Dachshunds, Chihuahuas, English Springer Spaniels, Jack Russell Terriers, Australian Cattle Dogs, American Cocker Spaniels and Beagles [1,5,20,21]. The least aggressive breeds were reported as Retrievers, Labrador Retrievers, Bernese Mountain Dogs, Brittany Spaniels, Greyhounds and Whippets [5].

In the present study only two Cocker spaniels are belonging to the group of most aggressive dogs, and surprisingly one Labrador retriever belonging to the less aggressive group was present among our cases. Again, the number of cases do not allow drawing a consequence. Besides, authors opinion is that, although undeniable genetic influences are present for aggression, local education level and understanding of the dog nature together with breed preference are significant factors. For example, Kangal dog is known as a calm and well behaving local breed, but the Kangal dogs in the present study were household dogs contradictory to their nature. Unfortunately, national preference for Kangal dog is very high neglecting the physiologic and behavioral nature, resulting with a high yield of aggressivity, so concluding a genetic predisposition would be misleading for this breed.

According to literature, aggression is most frequently observed between adolescence to social maturity (6 months - 4 years) [23-25]. Similarly, dogs in the present study were 29.9±21.9 months of age.

Male sex was reported to be superior with regard to dominance [1,14,25,26]. In the present study only 6 dogs were male and 10 were female. Only dogs showing owner directed aggression were included in this study, so a larger scale regarding generally aggression would allow different observations.

Controversial reports are published on the effect of neutering on aggression. While some authors suggest that neutered dogs of either sex were calmer than the intact ones [1,7,24], others suggest that incidence of aggression increases with neutering. Anyway, hormonal influences also must be considered in the clinical examinations [27,28]. Interestingly only 2 dogs were neutered in the present study and 14 dogs were intact. This may be due to low dog neutering tendency of local people.

There is a positive correlation reported between the number of people in the household and aggression and disobedience and is believed to be another important factor [24,29]. Observations in this study were concordant with these suggestions as aggression level and risk of danger increased with the household population.

Owners' past experience with dogs also is an important factor. First time dog owners complain with more problems [30], where the experienced ones were reported to have less problems [29]. Dogs of first time or less experienced owners are reported to present dominance aggression more frequently [13]. Similarly, 11 of 16 owners (68.75%) were living their first experience and unsurprisingly were not familiar to dog nature.

The complexity of the situation is an important factor

forming the risk level. Presence of different types of aggression or behavioral disorders and the number of stimuli that triggers aggression complicates the situation. Concurrent behavioral disorders are reported in similar cases including a frequent observation of anxiety [20,25]. In the present study half of the cases presented one or more behavioral disorders and/or aggression types together with anxiety. The other half possessed owner directed aggression as the single disorder suggesting that this aggression may be a learnt response to situations and to specific perceived threats occurring in particular contexts, yielding the environmental and living conditions nearby the human factor [14,31,32].

Management of owner directed aggression has multiple contexts, but identifying the underlying reasons has major importance. Then, modification of the human factor including the consistency of the owner, informing about the dog nature and communication rules, is an important step, because dogs usually learn to present aggression in response. Positive reinforcement, desentisization, playing activities, rewarding, limiting the boundries of dominance patterns are important modification attempts [21,32,33].

However, presence of risk of danger must be strictly considered for human welfare. Keeping the dog under close control and away from public places will not be enough to secure family members. In cases with considerable risk of danger, pharmacological treatment is essential. Satisfactory results had been achieved with risperidone in dogs for diminishing behavioral arousal and in sociopathy stage 1 when the warning phases are still complete, social directed regression, sociopathies, impulsive aggression and hallucinatory type signs with considerable safety [16-18,34]. Our results are concordant with previous work. Satisfactory effect in decreasing the risk of danger had been achieved in dogs with owner directed aggression.

In conclusion, understanding the underlying motivation in a case of owner directed aggression is the first step of management, but in cases with high danger potency, safety of the family and foreigners must be considered at the first step. The results obtained in the present study reveal that risperidone creates satisfactory results in decreasing the risk of danger. In addition, from the view of animal welfare, pharmacological treatment with risperidone will prevent many household dogs from relinquishment or euthanasia.

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Research Article

Prevalence, Enterotoxin Production and Antibiotic Resistance of *Bacillus cereus* İsolated from Milk and Cheese [1] [2]

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Abstract

Bacillus cereus is a type of bacteria that can cause severe food poisoning. The aim of this study was to determine the incidence of *B. cereus* in various full-fat milk and cheese samples and to assess the HBL (haemolysin BL) and NHE (nonhaemolytic enterotoxin) production and the resistance to several antimicrobial agents of the isolates. A total of 259 samples of full-fat milk (raw, pasteurized and UHT) and cheese obtained from different retail markets in Bursa province between July and December 2013 were analysed. Isolation of *B. cereus* was performed using Bacara agar according to the method suggested by FDA. Twenty six (10.04%) out of 259 samples were found to be contaminated with presumptive *B. cereus* based on their colony morphology and microscopic appearance, by counts that ranged from $1x10^1$ to $1.1x10^3$ CFU/mL in raw and pasteurized milk and from $4x10^1$ to $3.8x10^5$ CFU/g in cheese. Thirteen isolates of *B. cereus* were identified by API system. However, further analysis using MALDI-TOF-MS confirmed 19 isolates as *B. cereus*. Thirteen out of 19 (68.4%) isolates showed evidence of only NHE toxin production while six out of 19 (31.6%) isolates were positive for both NHE and HBL production. All isolates were resistant to penicillin G, although they were susceptible to oleondamycin, erythromycin and streptomycin. There were seven different patterns of multiple antibiotic resistance in this study. In our study, 84.2% (n = 16) of *B. cereus* isolates exhibited multiple antibiotic resistance.

Keywords: *Bacillus cereus, Milk, Cheese, Enterotoxin, Multiple antibiotic resistance*

Süt ve Peynirden İzole Edilen *Bacillus cereus*'un Prevalansı, Enterokoksin Üretimi ve Antibiyotik Direnci

Özet

Bacillus cereus ciddi gıda zehirlenmesine neden olabilen bir bakteri türüdür. Bu çalışmanın amacı, çeşitli süt ve peynir örneklerinde *B. cereus* insidansını belirlemek ve elde edilen izolatların HBL (hemolizin BL) ve NHE (nonhemolitik enterotoksin) üretimini ve çeşitli antimikrobiyal ajanlara direncini belirlemektir. Temmuz - Aralık 2013 tarihleri arasında Bursa ilinde farklı satış yerlerinden toplam 259 adet tam yağlı süt (çiğ, pastörize ve UHT) ve peynir örneği analiz edilmiştir. *B.cereus* izolasyonu FDA tarafından önerilen metoda göre Bacara agar kullanılarak yapıldı. Analize alınan çiğ ve pastörize süt örneklerinde $1x10^1$ ile $1.1x10^3$ kob/mL ve peynirde $4x10^1$ ile $3.8x10^5$ kob/g arasında değişen sayılarda olmak üzere, toplamda 259 örneğin 26 adetinin (%10.04) koloni morfolojileri ve mikroskobik görünüşlerine de dayalı olarak *B. cereus* ile kontamine olduğu tespit edilmiştir. Örneklerden elde edilen 13 adet *B. cereus* izolatı API sistemi ile identifiye edilmiştir. Bununla birlikte, MALDI-TOF-MS kullanılarak yapılan analizde, 19 adet izolat *B. cereus* olarak identifiye edilmiştir. Bu 19 izolatın 13'ünün (%68.4) sadece NHE toksini bakımından, altı izolatın da (%31.6) NHE ve HBL toksinleri bakımından pozitif olduğu gözlenmiştir. Tüm izolatlar oleondamisin, eritromisin ve streptomisine duyarlı olmalarına rağmen penisilin G'ye dirençlidir. Bu çalışmada, çoklu antibiyotik direnci gösteren yedi farklı model bulunmuştur. Çalışmamızda, *B. cereus* izolatlarının %84.2'si (n = 16) çoklu antibiyotik direnci göstermiştir.

Anahtar sözcükler: *Bacillus cereus, Süt, Peynir, Enterotoksin, Çoklu Antibiyotik Direnci*

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INTRODUCTION

B. cereus is a Gram-positive, aerobic or facultative anaerobic, rod-shaped, one of the most important endospore-forming spoilage microorganism in dairy environment and also responsible for foodborne outbreaks around the world [1]. Outbreaks caused by this pathogen have been reported in different parts of the world [2-5]. It has been associated with almost all categories of proteinaceous food products including raw milk, pasteurized milk and dairy products such as cheese, butter and cream [6]. *B. cereus* can contaminate the raw milk, pasteurized milk and dairy products via dirty teats, soil, feed and processing equipment and also post-pasteurization contamination of milk can occur [7,8].

There are two types of severe foodborne diseases caused by *B. cereus*, the emetic (heat-stable) and the diarrheal type (heat-labile) [9-12]. The emetic and the diarrheal syndromes can occur when the bacterial cell concentration reaches a level of 10^5 to 10^8 CFU/g and 10^5 to 10^7 CFU/g, respectively [13,14]. The emetic syndrome caused by an intoxication is characterised by an acute attack of nausea and emesis occurring within 1-5 hours after consumption, caused by cereulide, a heat stable, ring-structured dodecadepsipeptide toxin [15,16]. The diarrheal syndrome characterised by abdominal pain and diarrhea, with an incubation period of 4-16 h and symptoms that last for 12-24 h, can be caused by the enterotoxin-complexes nonhaemolytic enterotoxin (NHE), haemolysin BL (HBL) and enterotoxin FM (EntFM) and the single protein cytotoxin K (CytK) [17-19].

In recent years, antimicrobial resistance is one of most serious health threats worldwide and there have been a dramatic increase in the number of foodborne bacterial pathogens resistant to a variety of antibiotics. The wide-spried use of antibiotics in farming and through food chain contributes an important source of antimicrobial resistance [20,21]. Many previous reports have shown that *B. cereus* isolates obtained from different foods have resistance to several antibiotics [22-25].

Our study was planned to assess the prevalence and level of *B. cereus* contamination in full-fat milk (raw, pasteurized, UHT) and cheese; to determine the NHE and HBL enterotoxin production characteristics of the isolates and to examine antibiotic resistance and any possible multiple antibiotic resistance of the isolates.

MATERIAL and METHODS

Sample Collection

Between July and December 2013, a total of 259 full-fat milk and cheese samples including 53 raw milk, 50 pasteurized milk, 50 ultra-high temperature (UHT) milk and 106 cheese (kashar, white pickled, braided, stick, old, village, ricotta) were collected from Bursa province of Turkey. Raw milk samples were provided from several dairy farms while the other samples were purchased from different retail markets and neighbourhood bazaars. The samples were analysed on the day of arrival to laboratory under refrigerated conditions.

Sample Preperation

The detection of *B. cereus* in the samples was achieved according to the Standard Method of the U.S. Food and Drug Administration's (FDA) Bacteriological Analytical Manual (BAM) [26]. First, 10 mL or g portions of each sample were homogenized with 90 mL of sterile saline peptone water (0.1%, w/v) for 1 min in a Stomacher 400 (Seward, London, UK). Tenfold serial dilutions of homogenates were made in 0.1% peptone water as the diluents [27].

Isolation and Enumeration of B. cereus

Detection and enumeration of cultured bacteria were performed through plating on selective solid medium. For this purpose, 0.1 mL (and/or 0.5 mL) of each dilution was spread on (spread plate method) a plate of Bacara agar (bioMerieux, France) plates followed by incubation under aerobic conditions at 30°C for 24 h. After incubation, colonies with a pink/orange colour grown on Bacara agar were considered to have positive lecithinase activity and were subsequently enumerated. The results were expressed as colony forming units (CFU) per milliliter or gram of analysed sample. Three characteristic colonies were picked from each plate were isolated on Tryptone Soya Agar (Oxoid, UK) at 30°C for 24 h. The morphology of the cultures was also examined microscopically. The isolates which were rod-shaped and with central or subterminal spores were considered as presumptive *B. cereus* [28]. The purified isolates were transferred in cryo-tubes containing Nutrient Broth (Oxoid, UK) with 20% (v/v) glycerol and stored at -80°C for identification, further MALDI-TOF-MS analysis and the ability of toxin production.

Identification and Further Confirmation of Presumptive B. cereus Isolates

Identification of *Bacillus'* species were performed using a API 20E and API 50CHB test strips (bioMerieux, France) according to manufacturer's instructions. Confirmation of isolated bacteria was made using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) (Microflex LT, Bruker Diagnostics, Germany), in collaboration with the Duzen Laboratories Group, Istanbul, Turkey.

Screening of Enterotoxic B. cereus

Haemolytic enterotoxin (HBL) and non-haemolytic enterotoxin (NHE) production was assessed using the Duopath® Cereus Enterotoxins Test Kit (Merck, Belgium) according to the manufacturer's instructions. The LODs

(limit of detection) of this test were 6 ng/mL for NHE and 20 ng/mL for the HBL [29].

Antibiotic Resistance Testing

Antibiotic resistance of the isolates was tested by the Kirby-Bauer disc diffusion method [30]. Mueller-Hinton Agar (Oxoid, UK) was used for this test. The disks used (Oxoid, UK) and antibiotic concentrations were as follows: oleandomycin (15 µg), tetracycline (30 µg), polymyxin B (300 U), chloramphenicol (30 µg), erythromycin (15 µg), penicillin G (10 U), cephalothin (30 µg), ampicillin (10 µg), kanamycin (30 µg), vancomycin (30 µg), streptomycin (10 µg), and neomycin (30 µg). *B. cereus* ATCC 10876 was used as control strain. According to the inhibition zone measured, the isolates were classified as resistant, intermediate or susceptible as recommended by Bauer et al.[30].

RESULTS

In total, 259 samples consisting of 153 milk (raw milk, pasteurized milk, UHT milk) and 106 cheese were analysed. Of these, 26 (10.04%) were observed to be contaminated with *B. cereus* on the basis of the morphological and microscopically features. These isolates were obtained from raw milk, pasteurized milk and cheese samples while none of UHT milks contained bacteria. The bacterial counts varied from $1x10^1$ to $1.1x10^3$ CFU/mL in milk samples, and $4x10^1$ to $3.8x10^5$ CFU/g in cheese. The incidence and contamination levels of presumptive *B. cereus* in the samples are shown in *Table 1*.

Table 2 presents API (20E and 50CHB) and MALDI-TOF-MS identification results. Overall, 19 (73.1%) out of 26 presumptive isolates were confirmed as *B. cereus* by MALDI-TOF-MS whereas 13 (50%) of these isolates were initially identified by API test. Consequently, MALDI-TOF-MS identified 6 isolates as *B. cereus* that were not identified as such by API identification systems.

In the present survey, 19 isolates characterized as *B. cereus* were also analysed for enterotoxin production potential. Thirteen (68.4%) of the isolates tested showed the evidence of only NHE toxin production while six isolates (31.6%) were positive for both NHE and HBL production reaction *(Table 2)*.

The antibiotic resistance profiles of the tested isolates are presented in *Table 3* and *Table 4*. All isolates were resistant to penicillin G, 63.2% to ampicillin, 57.9% to polymixin B, 57.9% to cephalothin, 15.8% to kanamycin

Table 2. *The results of testing for confirmation and toxin-producing capability of presumptive B. cereus isolates (n = 26)*

Sample	Identification		Toxin Production	
	API	MALDI-TOF-MS	NHE	HBL
Raw milk	-	+	+	+
Raw milk	-	-	NT	NT
Pasteurized milk	-	-	NT	NT
Pasteurized milk	+	+	+	-
Pasteurized milk	-	+	+	-
Pasteurized milk	-	-	NT	NT
Pasteurized milk	-	-	NT	NT
Pasteurized milk	+	+	+	-
Pasteurized milk	-	-	NT	NT
Pasteurized milk	+	+	+	-
Pasteurized milk	-	-	NT	NT
Pasteurized milk	+	+	+	-
Pasteurized milk	+	+	+	+
Pasteurized milk	-	-	NT	NT
Pasteurized milk	+	+	+	+
Kashar cheese	+	+	+	-
Kashar cheese	-	+	+	-
Kashar cheese	+	+	+	-
Kashar cheese	+	+	+	+
Kashar cheese	+	+	+	+
White pickled cheese	-	+	+	-
White pickled cheese	+	+	+	-
Stick cheese	-	+	+	-
Village cheese	+	+	+	-
Ricotta cheese	-	+	+	-
Old cheese	+	+	+	+

NT: *not tested (MALDI-TOF-MS negative isolates (n = 7) were not tested for toxin production),* ***NHE:*** *nonhaemolytic enterotoxin,* ***HBL:*** *haemolysin BL, + positive, - negative*

Table 1. *Incidence and the counts of presumptive B. cereus from milk and cheese*

Sample Type	No. of Samples Analysed	No. and Percentage (%) of Contaminated Samples by *B. cereus*	*B. cereus* Count (CFU/ml-g)		
			Minimum	Maximum	Mean±SD
Raw milk	53	2 (3.8)	$1x10^1$	$2.2x10^2$	$1.2x10^2$±$1.5x10^2$
Pasteurized milk	50	13 (26)	$1x10^1$	$1.1x10^3$	$2.1x10^2$±$3x10^2$
UHT milk	50	-	-	-	-
Cheese	106	11 (10.4)	$4x10^1$	$3.8x10^5$	$3.5x10^4$±$1.2x10^5$
All	259	26 (10.04)	$1x10^1$	$3.8x10^5$	$1.5x10^4$±$7.5x10^4$

SD: *standard deviation*

Table 3. *Antibiotic resistance profiles by disc diffusion method of B. cereus isolates (n = 19)*

Sample	Antibiotics											
	OL	TE	PB	C	E	P	KF	AMP	K	VA	S	N
Raw milk	S	S	R	S	S	R	R	R	S	R	S	S
Raw milk	-[1]	-	-	-	-	-	-	-	-	-	-	-
Pasteurized milk	-	-	-	-	-	-	-	-	-	-	-	-
Pasteurized milk	S	S	I	S	S	R	I	I	S	S	S	S
Pasteurized milk	S	S	I	S	S	R	I	S	S	S	S	S
Pasteurized milk	-	-	-	-	-	-	-	-	-	-	-	-
Pasteurized milk	-	-	-	-	-	-	-	-	-	-	-	-
Pasteurized milk	S	S	R	S	S	R	R	R	R	S	S	I
Pasteurized milk	-	-	-	-	-	-	-	-	-	-	-	-
Pasteurized milk	S	S	R	S	S	R	R	R	I	S	S	S
Pasteurized milk	-	-	-	-	-	-	-	-	-	-	-	-
Pasteurized milk	S	S	I	S	S	R	R	R	S	S	S	S
Pasteurized milk	S	I	R	I	S	R	R	R	R	S	S	S
Pasteurized milk	-	-	-	-	-	-	-	-	-	-	-	-
Pasteurized milk	S	S	I	S	S	R	R	R	S	S	S	S
Kashar cheese	S	S	R	S	S	R	I	I	I	S	S	S
Kashar cheese	S	S	R	S	S	R	I	I	I	S	S	S
Kashar cheese	S	S	I	S	S	R	S	R	S	S	S	S
Kashar cheese	S	S	R	I	S	R	R	R	R	S	S	S
Kashar cheese	S	S	I	S	S	R	I	S	I	S	S	S
White pickled cheese	S	S	R	S	S	R	R	I	S	S	S	S
White pickled cheese	S	S	R	S	S	R	R	R	I	S	S	S
Stick cheese	S	S	I	S	S	R	I	R	S	S	S	S
Village cheese	S	S	R	S	S	R	I	S	S	S	S	S
Ricotta cheese	S	S	I	S	S	R	R	R	I	S	S	S
Old cheese	S	I	R	S	S	R	R	R	I	I	S	I

[1] MALDI-TOF-MS negative isolates (n= 7) were not tested for antibiotic resistance; ***OL:*** *oleandomycin,* ***TE:*** *tetracycline,* ***PB:*** *polymixin B,* ***C:*** *chloramphenicol,* ***E:*** *erythromycin,* ***P:*** *penicillin G,* ***KF:*** *cephalothin,* ***AMP:*** *ampicillin,* ***K:*** *kanamycin,* ***VA:*** *vancomycin,* ***S:*** *streptomycin,* ***N:*** *neomycin,* ***R:*** *resistant,* ***IM:*** *intermediate resistant,* ***S:*** *susceptible*

and 5.3% to vancomycin. None of the isolates showed resistance to other antibiotics including oleondamycin, tetracycline, chloramphenicol, erythromycin, streptomycin and neomycin. Moreover, all of the isolates were found to be susceptible to oleondamycin, erythromycin and streptomycin. As shown in *Table 5*, seven different patterns of multiple antibiotic resistance has been observed in this study. Sixteen out of 19 *B. cereus* (84.2%) isolates exhibited resistance to multiple antibiotics.

DISCUSSION

Several studies have also demonstrated the occurrence of *B. cereus* (vegetative cells or spores) in milk from Turkey and other countries. The incidence of *B. cereus* in raw milk was recorded as 25% by Larsen and Jørgensen [31] and as 10.6% by Němečková et al.[32]. In comparison to those studies, a lower incidence rate (3.8%) of *B. cereus* in raw milk was observed in the present study. It is likely to be due to improper hygienic conditions and poor farm management practices during feeding, milking and milk storage. A study conducted by Lin et al.[33] reported that the incidence of *B. cereus* in raw milk from holding tanks, raw milk from balance tanks, pasteurized milk from high-temperature short time pipes, pasteurized milk from holding tanks and the final product was 80%, 85%, 85%, 76% and 90%, respectively.

Twenty-six percentage (13/50) of pasteurized milk samples analysed in this study had *B. cereus* in counts ranging from $1x10^1$ to $1.1x10^3$ CFU/mL. The presence of *B. cereus* in pasteurized milk samples could perhaps be due to high initial load of spores in the milk used for production, inadequate pasteurization or post-contamination due to unsanitary conditions. On the other hand some other authors reported much higher *B. cereus* incidence levels. Te Giffel et al.[34] reported that 40% of pasteurized milk

Table 4. *Number of resistant and susceptible B. cereus isolates to antibiotics*

Antibiotics	No. of *B. cereus* Isolates (*n*= 19)		
	No. and Percentage (%) of Resistant Isolates	No. and Percentage (%) of Intermediate Isolates	No. and Percentage (%) of Susceptible Isolates
Oleandomycin	0	0	19 (100)
Tetracycline	0	2 (10.5)	17 (89.5)
Polymixin B	11 (57.9)	8 (42.1)	0
Chloramphenicol	0	2 (10.5)	17 (89.5)
Erythromycin	0	0	19 (100)
Penicillin G	19 (100)	0	0
Cephalothin	11 (57.9)	7 (36.8)	1 (5.3)
Ampicillin	12 (63.2)	4 (21.1)	3 (15.8)
Kanamycin	3 (15.8)	7 (36.8)	9 (47.4)
Vancomycin	1 (5.3)	1 (5.3)	17 (89.5)
Streptomycin	0	0	19 (100)
Neomycin	0	2 (10.5)	17 (89.5)

Table 5. *Multiple antibiotic resistance patterns observed among B.cereus isolates (n = 19)*

Source	No. (%) of Multiple Resistant Isolates	Resistance Patterns
Raw milk	1 (5.3)	PB, P, KF, AMP, VA
Pasteurized milk (*n*= 2), kashar cheese (*n*= 1)	3 (15.8)	PB, P, KF, AMP, K
Pasteurized milk (*n*= 1), White pickled cheese (*n*= 1), old cheese (*n*= 1)	3 (15.8)	PB, P, KF, AMP
Pasteurized milk (*n*= 2), Ricotta cheese (*n*= 1)	3 (15.8)	P, KF, AMP
White pickled cheese	1 (5.3)	PB, P, KF
Kashar cheese (*n*= 2), Village cheese (*n*= 1)	3 (15.8)	PB, P
Kashar cheese (*n*= 1), stick cheese (*n*= 1)	2 (10.5)	P, AMP

PB: *polymixin B,* ***P:*** *penicillin G,* ***KF:*** *cephalothin,* ***AMP:*** *ampicillin,* ***K:*** *kanamycin* ***VA:*** *vancomycin*

samples were contaminated with *B. cereus* and that the contamination levels were less than 5 CFU/mL in 77% of the samples. In a study conducted in India, in approximately 10% of the milk and milk samples the level of *B. cereus* contamination was more than 10^5 CFU/g [35]. Zhou et al.[36] informed that 92 isolates obtained from 54 samples of packaged pasteurized full-fat milk were identified as *B. cereus*. In Poland, milk collected from a farm have been found to contain *B. cereus*. Morever, the authors suggested that a considerable level of contamination by this bacteria of milk was also found in milk after pasteurization [37]. In another study [38] 12 isolates of *B. cereus* were identified from pasteurized milk.

Varying incidence rates of *B. cereus* in dairy products were also reported by different workers. Prevalence rates of 33.33% in raw pooled milk samples and 37.83% in pasteurized milk samples were reported by Rather et al.[39]. Absence of *B. cereus* in UHT milk in our study was similarly observed by Pacheco-Sanchez and Massaguer [40] on 6500 packed whole processed UHT milk samples. Merzouqui et al.[24] detected *B. cereus* contamination in 51.6% of milk and dairy products. The incidence of *B. cereus* in Port Salut Argentino cheese was notified as 50% [41]. A study performed by Cosentino et al.[42] on dairy products (pasteurized milk, UHT milk and cheese) indicated the presence of *Bacillus* spp. in 265 (70%) of 378 samples tested. By Khudor et al.[43], the incidence of *B. cereus* in milk, soft cheese, curls cheese and yogurt samples was reported as 32.7%, 16.6%, 18% and 26%, respectively. A previous study [22] demonstrated contamination with *B. cereus* in 31% of 215 dairy products included soft fresh cheese, soft ripening cheese, cottage cheese, cream cheese, butter and cream. Reyes et al.[44] recorded a 45.9% incidence of *B. cereus* in dried milk products. In India, Bedi et al.[35] reported an overall incidence of 53.8% of *B. cereus* in milk and various of dairy products. In Turkey, Gundogan and Avci [23] investigated the occurrence of *B. cereus* in raw milk, white cheese and ice cream samples from different dairy processing plants and determined that the contamination rates with *B. cereus* were 90% in raw milk, 70% in white cheese and 20% in ice cream samples. In the current study, the presence of *B. cereus* in raw milk, pasteurized milk and cheese samples could perhaps be due to high initial load of spores in the milk used for production, inadequate pasteurization or post-pasteurization contamination due

to unsanitary conditions such as dirty teats, soil, feed and processing equipments. The spores also formed by this microorganism may resist pasteurization of milk.

In our study, initial identification of species level of *B. cereus* isolates was confirmed by API 20E and API 50CHB test systems and further with MALDI-TOF-MS analysis. The results of these tests did not always match. Performance of the MALDI-TOF-MS technique was higher for identification than API systems. Rapid and reliable identification of pathogenic microorganisms is important for the surveillance, prevention and control of foodborne illnesses [45]. In recent years, MALDI-TOF-MS has been demonstrated as a powerful method for identification of bacteria in routine laboratories [46]. Previous studies also described the performance of MALDI-TOF-MS for bacterial identification [47-51].

B. cereus causes two types of food poisoning, the emetic and diarrheal syndromes and produces one emetic toxin and four other enterotoxins: haemolysin BL (HBL), nonhaemolytic enterotoxin (NHE), cytotoxin K (CytK) and enterotoxin FM (EntFM). The HBL and NHE enterotoxins are considered as the primary virulence factors of diarrhea after infection by *B. cereus* [52,53]. Of 19 *B. cereus* isolates obtained from our study, 68.4% (13 isolates) were positive for NHE toxin production and 31.6% (6 isolates) for both NHE and HBL production. Enterotoxigenic characterization of *B. cereus* in milk and various dairy products has been examined by several authors. Te Giffel et al.[34] found that the 27% of the *B.cereus* isolates from pasteurised milk in household refrigerators produced haemolysin BL enterotoxin. Of the 37 *B. cereus* isolates 70-76%, were found to be enterotoxigenic (as determined by three different methods). Zhou et al.[36] determined that 33.7% of *B. cereus* isolates from pasteurized full-fat milk contained three enterotoxic HBL complex encoding genes *hbl*A, *hbl*C and *hbl*D. In another investigation, 28 of 94 (29.8%) isolates of *B. cereus* from dried milk products were positive for diarrheal enterotoxin production [44]. The results of the study performed by Cosentino et al.[42] exhibited the toxin-production ability of 72% of *B. cereus* isolates from dairy products by a reversed passive latex agglutination assay. Svensson et al.[54] suggested that mesophilic isolates compared with psychrotrophic *B. cereus* isolated from the farm, in silo tanks and pasteurized milk had higher enterotoxin (HBL and NHE) production potential. In Turkey and other countries, the detection of enterotoxigenic *B. cereus* isolates in milk and dairy products can pose a major public health threat because the toxins NHE and HBL could potentially be able to cause diarrhea.

In comparison to our results, studies conducted by Schlegelova et al.[22] demonstrated that 18 of 96 *B. cereus* isolates from dairy products exhibited resistance to streptomycin, four isolates to erythromycin, two isolates to neomycin and one isolate to tetracycline. Earlier reports also specified ampicillin and penicillin resistant *B. cereus* isolates from a variety of foods [23,24,55]. Our findings are in agreement with those reported by Khudor et al.[43] who observed that all *B. cereus* isolates from milk, cheese and yogurt had resistance to penicillin, but the susceptibility to neomycin. However, the same researchers found that 45.1% of the isolates were resistant to tetracycline, 6.4% to erythromycin and 3.2% to streptomycin. Susceptibility to oleondamycin, erythromycin and neomycin of all isolates, as observed in our study, is contrary to those reported by these authors. Merzouqui et al.[24] isolated *B. cereus* from milk, dairy products, spices and rice salads and screened the antibiotic susceptibility profiles of the isolates. They indicated susceptibility to chloramphenicol (67.2%) and erythromycin (84.4%), which are consistent with the outcome of our study. However, tetracycline resistance (90.6%) determined in their study is contrary to that found in our study which revealed susceptibility to this antibiotic (89.5%) of *B. cereus* isolates. A study performed in Turkey [55] showed that none of the 34 *B. cereus* isolates from ice cream samples was resistant to vancomycin, whereas one isolate of *B. cereus* from raw milk had resistance to vancomycin.

In conclusion, this study revealed that the presence of *B. cereus* in full-fat milk (raw and pasteurized) and cheese. In addition, our findings presented the potential of *B. cereus* isolates to produce the enterotoxins HBL and NHE as a health hazard. *B. cereus* counts of $3.8x10^5$ CFU/g in cheese samples can be sufficient to cause illness by this bacterium. Although the numbers of *B. cereus* in the samples were relatively lower than the actual number of cells required to cause illness by this bacterium, it is important to consider that milk and milk products may be easily contaminated and bacterial counts may rapidly rise due to poor milking, equipment cleaning and sanitizing procedures and improper cooling. We also detected the occurrence of multiple antibiotic resistant *B. cereus* isolates. Therefore, antimicrobial agents should be responsibly and prudently used in veterinary medicine. As a result, to ensure the efficient pasteurization and cooling of milk, and to avoid the post-pasteurization contamination are of primary importance to prevent foodborne illnesses caused by consumption of milk and dairy products contaminated with *B. cereus*.

Conflict of Interest Statement

The authors declared that they have no conflict of interest.

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Research Article

Telomeric Attrition with Increasing Age in Short- (Chihuahua Dog) and Long- (Asian Elephant) Life Span Animals

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Abstract

Here, we explored the rate of telomere attrition with increasing age by real-time quantitative PCR (qPCR) in a short- (Chihuahua dog) and long- (Asian elephant) lived species. A total of 122 Asian elephants (female = 106, male = 16) ranging from 24-840 months of age, and 89 Chihuahuas (female = 65, male = 24) 1-179 months of age were used in this study. We found that young (pre- and peri-pubertal) Asian elephants had a higher relative telomere length (RTL) compared to dogs. A low, but significant negative relationship between RTL and increasing age was observed in both Chihuahuas (R^2=0.0490, P=0.0017) and Asian elephants (R^2=0.0177, P=0.0210). The estimated rate of telomere loss for males and females of both species ranged from -0.0023 to -0.0065, with no clear differences between gender or species. Results suggest that Asian elephants may start with longer telomeres than Chihuahuas, as RTL was higher, but then the rate of telomere attrition proceeds at a similar rate in both species. Age accounted for only a small percentage of the variation in RTL in both Chihuahua dogs and Asian elephants, however. Thus, its use as a biological tool for age estimation would appear to be limited for these species.

Keywords: *Age, Asian elephant, Dog, Telomere*

Kısa (Chihuahua köpek) ve Uzun (Asya fili) Ömürlü Hayvanlarda Artan Yaş İle Birlikte Telomerik Yıpranma

Özet

Bu çalışmada, kısa (Chihuahua köpek) ve uzun (Asya fili) ömürlü hayvanlarda artan yaş ile birlikte telomerik yıpranma oranı gerçek zamanlı kantitatif PCR (qPCR) kullanılarak araştırıldı. Yaşları 24-840 hafta arasında değişen toplam 122 Asya fili (106 dişi ve 16 erkek) ile 1-179 aylık Chihuahua (65 dişi ve 24 erkek) çalışmada kullanıldı. Genç Asya filleri (pre- ve peri-puberte) köpekler ile karşılaştırıldığında daha fazla orantısal telomer uzunluğuna sahip olduğu tespit edildi. Hem Chihuahua (R^2=0.0490, P=0.0017) hem de Asya fillerinde (R^2=0.0177, P=0.0210) orantısal telomer uzunluğu ile artan yaş arasında düşük ama anlamlı negatif yönlü bir ilişki gözlemlendi. Her iki tür için erkek ve dişi hayvanlardaki tahmini telomer kayıp oranı 0.0023 ile 0.0065 arasında değişirken tür veya cinsiyet yönünden bir fark tespit edilmedi. Elde edilen sonuçlar daha fazla orantısal telomer uzunluğuna sahip olan Asya fillerinin Chihuahua köpeklerden daha uzun telomer ile başladıklarını ancak daha sonra telomer yıpranmanın her iki türde de benzer oranda şekillendiğini gösterdi. Hem Chihuahua köpeklerde hem de Asya fillerinde orantısal telomer uzunluğundaki varyasyonun sadece küçük bir yüzdesi yaşa bağlıdır. Bu sebeple, yaş tayininde bir biyolojik araç olarak kullanılması bu türlerde sınırlı gözükmektedir.

Anahtar sözcükler: *Yaş, Asya fili, Köpek, Telomer*

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INTRODUCTION

Field biologists and veterinarians often work with animals for which there are no data on birth dates. Yet, knowledge of the age structure of populations or individuals is important for understanding factors affecting survival and how to improve species management, both *ex situ* and *in situ*. Unfortunately, age determination is difficult for most species, especially on living animals. Some methods involve using age-specific characteristics like tooth eruption and dental wear [1,2], skeletal morphology [1,3], body morphometrics [4] and bone ossification [5], although most of these are applicable only after death. In addition, radiocarbon dating and aspartic acid racemization can serve as a tool for age estimation [6,7], but again, not for living animals. An accurate estimate of age that could be easily obtained with minimal impact would therefore benefit biologists working on natural populations.

The aging process, or senescence, is related to progressive and irreversible cellular changes, like a molecular clock, and that after a certain number of divisions, cells reach a replicative limit [8,9]. One theory is that this cellular senescence is caused by the gradual decrease in the length of the telomere [10,11]. Telomeres are comprised of several canonical repeated nucleotides. Located at each end of a chromosome, they protect the chromosome from deterioration or damage [12]. Telomere length shortening occurs normally in somatic cells during DNA replication [13], throughout the life of an individual [14-20]. Thus, measures of telomere length have been used to estimate age in several mammalian species, including mice [19], dogs [15,21,22], sea lions [20] and humans [18,23]. However, results have been inconsistent; correlations between telomere length and age have been found in some [14,16], but not all studies [15]. Thus, additional studies across a broader array of species is needed to determine if measures of telomere length could be used as a means of age estimation. Today, there are three methods used to assess telomere length: southern blotting or terminal restriction fragments (TRFs) [14,16,24], flow fluorescence *in situ* hybridization (FISH) [25] and quantitative PCR (qPCR) [17-20]. TRF requires a large amount of DNA (0.5-5 µg/individual) and is time-consuming (3-5 days), whereas flow FISH limits the type of tissues for analysis [14,16,24]. The qPCR technique may be more useful because it can be applied to a variety of tissues, is easier to use and relies on a high throughput system [17,24].

In this study, we used a qPCR-based technique to examine telomere lengths in two species with vastly different lifespans: Chihuahua dogs represent short- (~10 years) and Asian elephants represent long- (~70 years) lived species. Elephants were of interest because of their endangered status and limited information on aging biology, plus they have a long lifespan similar to human. Dogs are the most popular companion animal, but have a limited lifespan. Our hypothesis is that relative telomere length is related to age in both long- and short-lived animals.

MATERIAL and METHODS

Animal and Samples

Animals in this study included 89 Chihuahuas *(Canis familiaris)* (female=65, male=24) ranging in age from 1-179 months) and 122 captive Asian elephants *(Elephas maximus)* (female=106, male=16) aged 24-840 months *(Table 1)*. Animal age was known for each individual based on interviews with owners and existing record. Two to three blood samples were collected between May and August 2015 from each individual for hematology and serum chemistry analyses, and for DNA extraction. All animals were deemed healthy based on a normal physical examination and unremarkable serum chemistry results,

Table 1. *Age distribution of Chihuahuas and Asian elephants*

Chihuahuas				Asian Elephant			
Age (year)	**Male**	**Female**	**Total**	**Age (year)**	**Male**	**Female**	**Total**
<1	8	9	17	<10	4	14	18
1-2	2	10	12	10-20	5	15	20
2-3	3	5	8	20-30	2	21	23
3-4	3	15	18	30-40	2	29	31
4-5	2	7	9	40-50	3	20	23
5-6	3	6	9	50-60	0	3	3
6-7	2	4	6	60-70	0	4	4
7-8	0	1	1	total	16	106	122
8-9	1	4	5	-	-	-	-
>9	0	4	4	-	-	-	-
Total	24	65	89	-	-	-	-

including liver function (alkaline phosphatase and alanine aminotransferase) and kidney function (blood urea nitrogen and creatinine), and normal complete blood counts (hematocrit and hemoglobin levels, red blood cell count, white blood cell count and platelet count). Animals had no history of infectious disease or injury in the 3 months prior to the sample collection. This study was approved by the Animal Use Committee of the Faculty of Veterinary Medicine, Chiang Mai University, Thailand, in 2015 (S17/2558 and S25/2558).

DNA Extraction and Real Time Quantitative PCR

Blood was extracted according to manufacturer instructions of the genomic DNA Extraction kits (catalog number: 1RBC-RT011, RBC Bioscience, Taiwan). DNA was measured qualitatively and quantitatively using agarose gel electrophoresis and absorbance at A260 to be given 50 ng/μL of stock DNA. Subsequently, DNA (50 ng) was used for estimation of telomere length of individuals by qPCR as described by Cawthon [17]. Briefly, the final concentrations of reagents in the PCR were 1x real-time master mix (catalog number: MBL-BIO-98005, Bioline), containing telomere primer concentrations of 270 nM of tel1:5'-GGTTTTTGAGG GTGAGGGTGAGGGTGAGGGTGAGGGT-3', and 900 nM of tel 2: 5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3' in a total volume of 10 μL [17]. The acidic ribosomal phosphoprotein PO or 36B4 (single copy gene) primer concentrations were 400 nM of forward: 5'-CAGAGTGAYGTGCAG CTGAT-3' and 400 nM of reverse: 5'-AGCACTTCAGGG TTGTAGATGCTGCC-3'; the primer pair was designed based on the multiple alignment of the 36B4 gene from various species, including mouse, African elephant, dog, human and cat, and confirmed by Sanger sequencing. The cycling profile for the telomere (T) PCR was as follows: 40 cycles of 95°C for 15 s, 65°C for 2 min. For 36B4 as single-copy gene (S) there followed 30 cycles of 95°C for 15 s, 68°C for 1 min. After that, cycle threshold (C_t) values were acquired from qPCR to be used for calculating the relative telomere length (RTL). The RTL was derived from the following formula: $2^{-\text{delta(ct(telomere)-ct(36B4 gene))}}$ and was expressed as T/S [17-20]. The rate of telomere loss was obtained from 1/slope of linear regression model (RTL/year) [26].

Statistical Analysis

Differences in RTL between young (pre- and peri-pubertal) Asian elephants (0-15 years) [27] and Chihuahua dogs (0-24 months) and between males and females of each species were determined using Student's t-tests**.** Correlations between relative telomere length and age were determined as the coefficients of determination (R^2) by a linear regression model in R program [17-20]. *P*-values were based on slopes being zero or not using analysis of variance *(ANOVA)*. The rate of telomere loss was obtained from the slope of the linear regression model and expressed as RTL/year. Differences were considered significant if $P<0.05$.

RESULTS

In this study, multiple alignment of acidic ribosomal phosphoprotein PO or the 36B4 gene (used as a single-copy gene for calculating RTL) from different species, including mouse, African elephant, dog, human and cat *(Fig. 1)*, was performed based on a 129 bp-sized amplicon. The RTL determined by qPCR was greater in Asian elephants than Chihuahua dogs, with no difference between sexes within species for the youngest animals (pre- and peri-pubertal) *(Fig. 2)*. When considering in total for both male and female, a general decline in RTL with increasing age was then observed *(Fig. 3)*. The overall coefficients of determination (R^2), which indicate the relationship between age and RTL, were significant ($P<0.05$) for both species, with a very low correlation of 0.0490 and 0.0117 in Chihuahua dogs and Asian elephants, respectively *(Table 2)*; although when gender was considered separately, the only significant correlation between RTL and age was in female Chihuahuas *(Table 2, Fig. 3A)* in contrast to female Asian elephants *(Table 2, Fig. 3B)*. However, our study found no correlation between age and RTL in males in both species *(Table 2, Fig. 3A,B)*. The estimated rate of telomere loss for males and females of both species was variable and ranged between -0.0023 and -0.0065 *(Table 2)*.

DISCUSSION

Based on our knowledge, this is the first study to measure telomere length in Asian elephants by qPCR and compare age-related telomere loss between a long- and short-lived species. Our results demonstrated that RTL was greater in Asian elephants compared Chihuahua dogs, and that telomere length attrition was linked to increasing age in both species, although correlations were low and accounted for only about 5 and 2% of the variation, respectively.

There are few studies of telomere shortening with age in dogs, although numbers of subjects were generally limited [15]. Investigations of telomere length in three dog breeds, Labrador retriever (n=22), miniature schnauzer (n=17) and beagle (n=8), by telomeric restriction fragment analysis found a negative relationship between age and telomere length across but not within breeds [15]. By contrast, we did find a significant negative correlation between age and telomere length in Chihuahua dogs (n=89). A shortening of telomeres with cell replications of *in vitro* canine fibroblasts has been detected [22], and Fick et al.[21] showed a connection between telomere loss with age in 15 dog breeds (n=175), similar to our results. Furthermore, Fick et al.[21] reported that different dog breeds varied in telomere length, which contributed to average lifespan. Moreover, dog breeds with shorter telomeres exhibited higher susceptibility to cardiovascular, gastrointestinal, musculoskeletal and respiratory disorders.

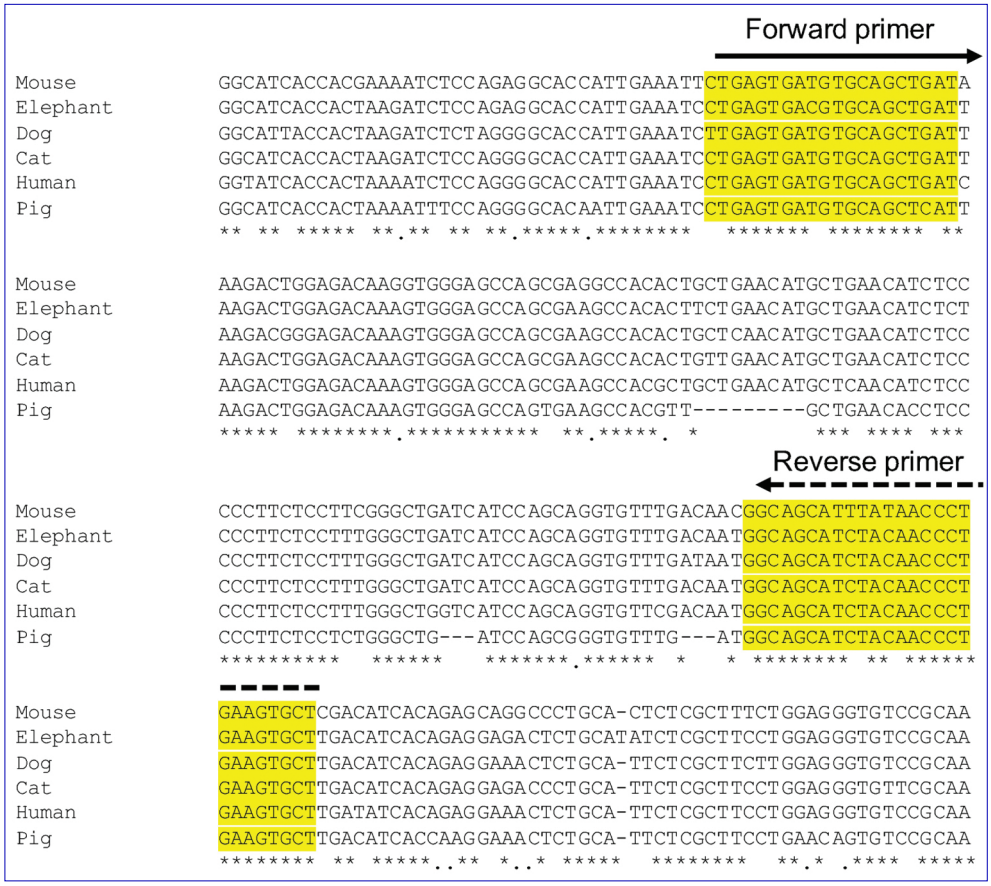

Fig 1. Multiple alignment of acidic ribosomal phosphoprotein PO or the 36B4 gene from various species. Below the nucleotide sequence is a key denoting conserved nucleotide (*) and semi-conservative mutation (.)

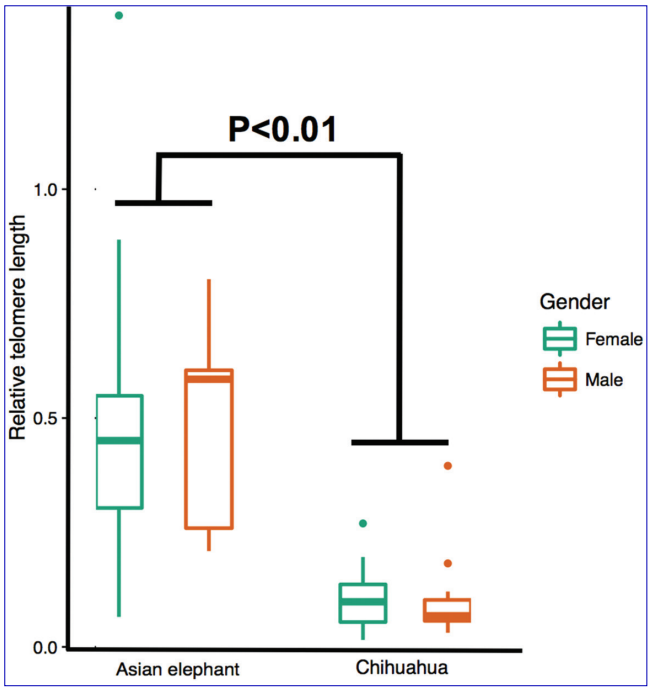

Fig 2. Comparison of relative telomere length before maturity in male and female Asian elephants (<15 years of age) and Chihuahua dogs (<2 years of age). Box plots present the median, 25% and 75% confidence intervals, and non-outlier minimum and maximum whiskers

Similar patterns of telomere length shortening with increasing age have been shown in other mammalian species, like human, mouse and sea lion [15,17-20]. Karlsson et al.[23] reported high variability in the degree of telomere repeats in humans, however, even within the same age group (~20 years). Other studies in birds, such as terns *(Stena hirundo)*, wandering albatrosses *(Diomedea exulans)* and Leach's storm-petrel *(Hydrobates pelagicus)*, also found high variability in telomere length with age at hatching [14,16]. In addition to the variation in telomere length at birth, environmental and lifestyle factors can affect the acceleration of telomere loss in cells. For example, in rats *(Rattus norvegicus)*, poor nutrition during the growth phase resulted in accelerated telomere shortening [28]. In shag *(Phalacrocorax aristotelis)*, a long-lived bird species, a faster growth rate was linked to a higher rate of telomere loss [29]. Thus, variation in telomere length across individuals may be a consequence of (i) growth rates of somatic, especially in early age [30], (ii) variation in telomere length at birth [14,16,31] and (iii) differences in telomere attrition rate [31,32]. The animals in this study had vastly differing backgrounds, which could explain the inhomogeneous nature of telomere lengths observed among individual Chihuahuas and Asian elephants, although in both species RTL did decline with age.

Table 2. *Summary of the relationship between telomere length and age in Chihuahua dogs and Asian elephants*

Parameter	Chihuahua Dog			Asian Elephant		
	All (n=89)	Female (n=65)	Male (n=24)	All (n=122)	Female (n=106)	Male (n=16)
[1]Adjusted R^2	0.0490	0.0537	0.0167	0.0177	0.0109	0.1427
RTL/year	-0.0048	-0.0048	-0.0065	-0.0023	-0.0023	-0.0065
P-value	0.0017*	0.0352*	0.4389	0.0210*	0.1442	0.0825

RTL = relative telomere length; [1]Adjustment of R^2 based on the number of variables in the model

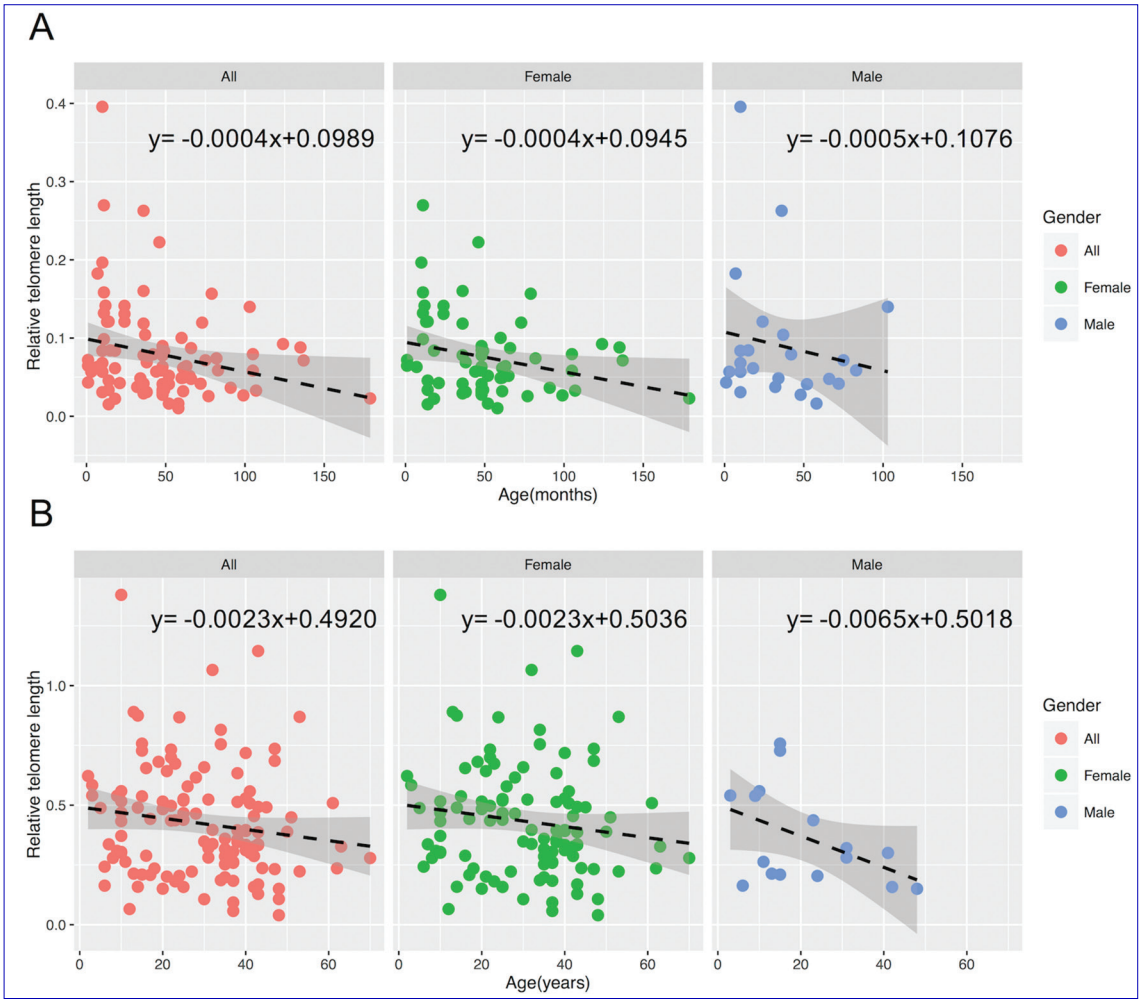

Fig 3. Correlation of relative telomere length and age from blood samples of Chihuahua dogs **(A)** and Asian elephants **(B)** using real-time quantitative PCR. Data are relative telomere length in which the C_t of telomere region **(T)** was normalized by that of 36B4 gene as single copy gene. Linear regression and 95% confidence intervals are shown as dashed lines with grey shading

The negative relationship between RTL and age was higher in Chihuahuas (R^2=0.049) than Asian elephants (R^2=0.0177), although there did not appear to be a difference in the estimated rate of telomere loss between these two species despite markedly differing lifespans. Within a species, expected life span may be related to the number of telomeric DNA repeats, something that has been found in dogs [21]. Canine telomeres range from 11 to 29 kbp [21]; however, the number of telomere repeats in Asian elephants is unknown. Presumably, the telomere length of Asian elephants is longer than that of Chihuahua dogs because they had a higher RTL. However, these relationships will not be completely understood until the actual telomere length in Asian elephants is determined. A slower loss of telomere DNA repeats has been observed in long-lived birds and mammal species [15,16,19,31]. On the other hand, telomere length and average lifespan has shown no connection across other vertebrate groups [16,31]. Hence, although the initial telomere length important appears to be important, the rates of telomere erosion and telomere restoration may also be related to life expectancy [16,31].

Sex differences in telomere length in mammalian species may be due to heterogametic influences, which account for some of the observed sex-bias in mortality [33]. In our study, however, no gender differences between age and RTL were observed in Asian elephants. By contrast, in Chihuahua dogs, we found a significant correlation between RTL and age in females. This result contrasts with that of Fick et al.[21] though, who found a relationship in male, but not female dogs. That study did not consider breed in data analyses, so differential results might be due to a species difference. In rats, shorter telomere lengths in males than females have been reported, but in both sexes, telomere attrition increased with age in most tissues [34]. However, these observations have not been consistently observed in other species; for example, Australia sea lion *(Neophoca cinerea)* [20]. In women, average telomere length in females was higher, and telomere length attrition was less than that of males [35]. However, there was no correlation of RTL and age in males of both species, which might be due to a smaller number of males. Additionally, in both species, the oldest individuals were female.

In conclusion, identifying and understanding the connection between telomere length and senescence or aging could potentially help in age estimation in some species. However, in our study, age accounted for only a small percentage of the variation in RTL in both Chihuahua dogs and Asian elephants (<5%). Thus, its use as a forensic tool for age discrimination would appear to be limited in these species. Rather, more research is needed to determine how numbers of telomere repeats at birth, rate of telomere loss and environmental factors affect overall length on an individual basis in these and other species, and if any are related to aging and/or mortality.

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Authors' Contributions

K.N. designed and conducted all the experiments. K.B. assisted in the experiments, performed the statistical analysis and support of information for discussion. W.K. and I.K. performed all the experiments. K.N. and S.C. collected dog blood sample C.S and C.T. collected elephant blood sample. S.P. gave a statistical advice. K.N., K.B., J.B. and C.T. assisted in discussions and writing of the manuscript. All authors read and approved the final manuscript.

Competing Interest

We have no competing interests.

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Research Article

Effect of Fish Oil Addition to the Skim Milk-egg Yolk Extender on the Quality of Frozen-thawed Bali Bull Spermatozoa [1]

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Abstract

This study was conducted to investigate the effect of fish oil addition to the semen extender on post-thaw quality of Bali bull spermatozoa. Fish oil was added at the doses of 0 (control), 50, 100, 150 and 200 mg/100 mL to the skim milk-egg yolk extender. Supplementation of fish oil at the dose of 100 mg/mL significantly (P<0.05) increased both live sperm rate and motility, but its dose of 200 mg/mL caused a significant (P<0.05) decrease in the live sperm rate when compared with the control group. A significant (P<0.05) increment was observed in abnormality rate of frozen-thawed spermatozoa in comparison with the control group when all doses of fish oil except 50 mg/100 mL were added. In conclusion, addition of 100 mg fish oil to 100 mL of skim milk-egg yolk extender could be beneficial for the improvement of the quality of Bali bull spermatozoa after freeze-thawing.

Keywords: *Bull semen, Fish oil, Cryopreservation, Live sperm, Motility*

Süt Tozu-Yumurta Sarısı Sulandırıcısına Balık Yağı İlavesinin Dondurulmuş-Çözdürülmüş Bali Boğa Spermatozoonlarının Kalitesi Üzerine Etkisi

Özet

Bu çalışma sperma sulandırıcısına katılan balık yağının Bali boğa spermatozoonlarının çözdürme sonrası kalitesi üzerine etkisini araştırmak amacıyla yapıldı. Süt tozu-yumurta sarısı sulandırıcısına 0 (kontrol), 50, 100, 150 ve 200 mg/100 mL dozunda balık yağı ilave edildi. 100 mg/100 mL balık yağı ilavesi kontrol grubu ile karşılaştırıldığında hem canlı sperm oranını hem de motiliteyi önemli derecede (P<0.05) artırdı, ancak 200 mg/100 mL'lik doz canlı sperm oranında önemli (P<0.05) bir azalmaya neden oldu. 50 mg/100 mL'lik doz hariç balık yağının tüm dozları ilave edildiğinde kontrol grubuna kıyasla dondurulmuş-çözdürülmüş spermatozoonların anormallik oranında önemli (P<0.05) bir artış gözlendi. Sonuç olarak, süt tozu-yumurta sarısı sulandırıcısının 100 mL'sine 100 mg balık yağı ilavesi Bali boğa spermatozoonlarının dondurma-çözdürme sonrası kalitesinin artırılması için faydalı olabilir.

Anahtar sözcükler: *Boğa sperması, Balık yağı, Kriyoprezervasyon, Canlı sperm, Motilite*

INTRODUCTION

Artificial insemination (AI) is an important tool for genetic improvement and has effectively been used in several livestock species including cattle, sheep and goats. One of the factors affecting the success of AI is the semen cryopreservation. The good post-thawed semen quality depends on the composition of the cryopreservation media. The process of semen cryopreservation is a stage of procedure that causes several forms of spermatozoa damages. One of the most significant components in semen processing is the ingredients of extender that provide protection to the sperm against cold shock [1]. The injuries in post-thawed spermatozoa are generally associated with osmotic changes, membrane alteration, inter-and intra-cellular ice crystal formation during cryopreservation process [2,3]. Therefore, cryoprotectants are supplemented to the semen extender to reduce damaging effects of cryopreservation processes [4]. Considerable evidences suggest that lipidic composition of the sperm membrane is

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responsible for the detrimental effect of cryopreservation [5]. Before semen freezing, percent docosahexaenoic acid was greater in fatty acid treatment than that in the group without fatty acid and reduced significantly in both groups after thawing [6].

Fish oil contains omega 3 fatty acid and is a major source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [7]. However, it has been reported [8] that fish oil contains 1970 mg saturated fatty acid (SFA), 35.5% poly-unsaturated fatty acid (PUFA), 180 mg eicosapentaenoic acid (EPA) and 120 mg docosahexaenoic acid (DHA). Many researchers have assessed the effect of fish oil addition on semen quality parameters in domestic animals. They have reported that fish oil caused increments in sperm motility and reductions in abnormal sperm in boars [9], improved fertility in turkeys [10] as well as increased total of sperm count in rams [11]. On the other hand, the addition of fish oil as a source of omega-3 fatty acids to diet of boars has been demonstrated to possess no significant improvement effect on the quality of frozen-thawed sperm [12]. Similarly, incorporation of DHA as an omega-3 fatty acid in the diet has been found to improve significantly the motility, live sperm and normal morphology of fresh semen [13,14]. Addition of DHA from fish oil to the egg yolk extender was found to be effective for increase the progressive motility and membrane integrity in post-thawed boar semen [15]. Although there are many evidences about the influence of DHA enriched extenders on freezing of boar semen, however, there are limited numbers of reports about the effect of fish oil and/or DHA enriched extender on the quality of frozen-thawed bull semen [16,17]. The objective of this study was to evaluate the effect of fish oil addition to the extender on live sperm rate, motility and abnormality of post-thawed Bali bull spermatozoa.

MATERIAL and METHODS

Semen Collection

Semen was collected by artificial vagina from four Bali bulls raised in Center Insemination Banjarbaru, province of south Kalimantan-Indonesia. All the bulls of at least 4-6 years of age and average weight of 625-650 kg were raised under a similar grazing system (various kinds of grass) and supplemented with concentrat at the rate of 4.5 kg/head/day. Two ejaculates were obtained from each bulls every week for one month. Immediately after semen collection, the ejaculates were immersed in the warm water bath at 37°C until their assessments in the laboratory. Then, semen parameters were assessed based on the macroscopic and microscopic characteristics. Macroscopic evaluations included volume, pH and color. Microscopic evaluations included live sperm rate, motility and abnormality. The main extender consisted of 10% skim milk, 5% egg-yolk, 1% glucose, 8% glycerol, Streptomicine (1 mg/mL) and Penicilin (1000 IU/mL).

This study was designed to compare the effect different concentrations of fish oil *(liquid)* with doses 0 (control), 50 mg, 100 mg, 150 mg and 200 mg in the 100 mL skim milk-egg yolk extender. The cryoprotective extender for the control group was the same as that for the treatment groups except that it was not supplemented with fish oil [(natural, 1000 mg) containing Omega-3 Marine; Triglycerides (300 mg) as EPA (180 mg) and DHA (120 mg) from Nature's Care (Manufacture Pty, Ltd. Minna Close Belrose, Australia)].

Freezing, Thawing and Evaluation

Semen was diluted to obtain a final concentration of 25 x 10^6 sperm/mL. Diluted semen was packed into 0.25 ml straws (Biovet, France). After sealing, straws were placed horizontally on a cold rack (5°C) and lowered into nitrogen vapors (-50°C), 4 cm above the surface of liquid nitrogen. After 3 min, when the temperature reached -120°C, the frozen straws were transferred into goblets of appropriate size and transferred into a liquid nitrogen tank. After two weeks of storage, the straws per treatment were randomly chosen, thawed in water at 37°C for 30 sec and evaluated for post-thawed semen quality including live sperm rate, motility and abnormality.

To evaluate sperm motility, a small droplet (≈10 µL) of post-thawed semen was placed in the center of a pre-warmed slide and covered with cover slip. It was transferred to a heated microscope stage at 37°C and subjectively calculated by phase contrast microscopy (400× magnification). For assessment of live sperm percentage, a modification of the eosin-nigrosin procedure described by Evans and Maxwell [18] was used. 10 µL of post-thawed semen was mixed with 30 µL of eosin-nigrosin stain. A thin smear was prepared and 200 spermatozoa was observed under a microscope (400 x). The same eosin-nigrosin stained slides were also used to define sperm abnormality for which 200 spermatozoa were examined for defects associated with tail region, mid-piece and sperm head.

Statistical Analysis

The data are expressed as mean ± SEM. A one-way analysis of variance (ANOVA) with tukey's multiple comparison test was applied to determine differences among the treatments using SPSS statistical software (version 16.0). Differences with P value 5% were considered to be statistically significant.

RESULTS

The effect of addition of different doses of fish oil on post-thawed live sperm rate, motility and abnormality is shown in *Table 1* and *Fig. 1*. Addition of 100 mg fish oil to the 100 mL extender significantly ($P<0.05$) increased both live sperm rate and motility when compared to the control and other treatments. However, the treatment with 200 mg fish oil caused significant ($P<0.05$) reduction in live sperm

Table 1. *Post-thawed sperm parameters including different dose fish oil Bali bulls*

Treatments	Sperm parameters		
	Live Sperm Rate (%)	Motility (%)	Abnormality (%)
Control	54.64±2.41^{b}	42.50±2.50^{ab}	18.83±23.06^{a}
50 mg/100 mL	57.32±2.15^{bc}	43.33±1.44^{b}	21.17±50.49^{ab}
100 mg/100 mL	60.36±1.76^{c}	46.67±1.42^{c}	23.66±17.01^{b}
150 mg/100 mL	53.34±2.25^{b}	43.33±1.37^{b}	22.67±48.05^{b}
200 mg/100 mL	47.30±2.02^{a}	40.01±0.21^{a}	22.33±09.21^{b}

^{a,b,c} *Values in the same column with different superscripts indicate significant difference at P<0.05, (n= 24)*

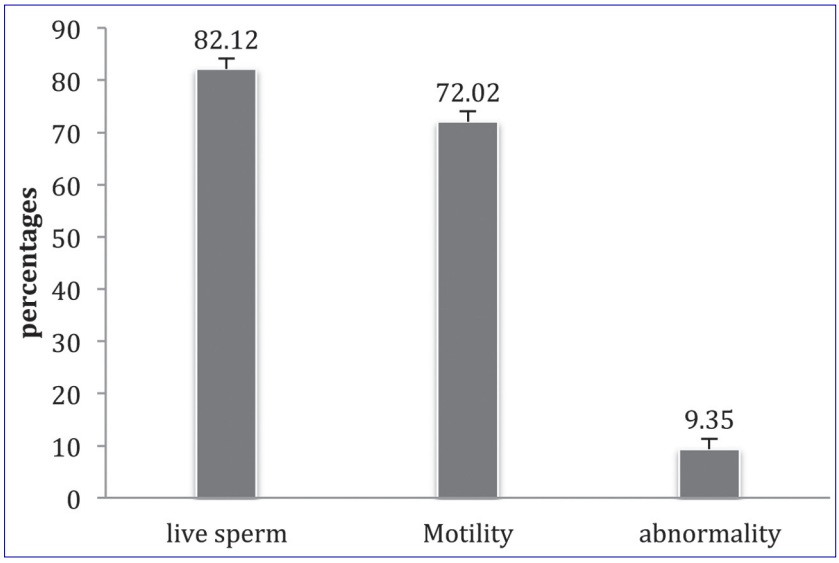

Fig 1. Caracteristic fresh semen of Bali bulls spermatozoa (Mean±SEM) (n= 24)

rate in comparison with the control and other treatments. In addition, 200 mg fish oil supplementation also caused significant (P<0.05) reduction when compared with the other treatment groups but not control group. A significant (P<0.05) increment was observed in abnormality rate of frozen-thawed spermatozoa in comparison with the control group when all doses of fish oil except 50 mg/100 mL were added.

DISCUSSION

In the present study was evaluated the effects of fish oil supplementation on post-thaw semen quality in Bali bull spermatozoa. We hypothesized that fish oil supplement can enhanced semen quality in terms of live sperm rate and motility but not on sperm abnormality. The results obtained from this study are generally in agreement with the findings of previous studies conducted in other species such as turkeys [10], pigs [8] and rams [10], in which fish oil supplement improved semen quality. Meanwhile, reactive oxygen species (ROS) produced from cryopreservation can also induce acrosome reaction and spermatozoa damage [19]. The toxicity effects of ROS eventually results in protein ionization and inactivation, peroxidation of lipids, in particular polyunsaturated fatty acids, and DNA damage which destabilize spermatozoa plasma membrane [20,21].

Live sperm rate and motility of semen are important factors because spermatozoa must travel from the vagina and uterus if semen is deposited by natural mating and AI, respectively. Cryopreservation, freezing and thawing processes may induce spermatozoa damages especially to the plasma membrane and organelles [22,23]. The determination of live sperm rate and motility is important for the evaluation of post-thaw quality of semen. In the present study, it was observed that addition of 100 mg fish oil to the 100 mL extender significantly increased both live sperm rate and motility, but treatment with 200 mg fish oil caused significant reduction in live sperm rate in comparison with the control and other treatments. In addition, 200 mg fish oil supplementation also caused significant (P<0.05) reduction when compared with the other treatment groups but not control group. This situation is shown that the dose of 100 mg fish oil provides an ideal concentration in diluent so as to provide comfort to the spermatozoa, thus preventing damage to the process freezing, cryopreservation and thawing. These results are confirmed by previous reports by Abdi-Benemar et al.[24], who revealed that all frozen-thawed sperm characteristics are significantly enhanced with a surge in DHA levels from fish oil. One of the components of fish oil is PUFA with long chains and has been found in the spermatozoa of various species including ram, man and bull. These fatty acids improve the fluidity of the sperm plasma membrane which is then responsible for increased resistance of the sperm to cold conditions [25]. On the other hand, DHA can be presented as a cryoprotectant that will be able to penetrate the plasma membrane of the sperm and thus act intracellularly. Therefore, modifications in fatty acid composition of surrounding diluents with fish oil supplement can cause changes in the fluidity and elasticity of the sperm plasma membrane by incorporation its fatty acids in the lipids of sperm [26]. In the present study, 150 and 200 mg fish oil supplementations were observed to decrease the percentages of live sperm and motility. This result are

supported by the data previously reported by Kandelousi et al.[27], who stated that higher levels of omega-6 fatty acids result in decreased sperm concentration, motility, and altered morphology.

In the present study, a significant increment was observed in abnormality rate of frozen-thawed spermatozoa in comparison with the control group when all doses of fish oil except 50 mg/100 mL were added. Majority of sperm abnormalities detected in this research were minor or secondary defects like simple coiled or bent tails that usually encounter in response to change in temperature, but not the defects of major type such as acrosome defect, defects associated through the sperm head and mid-piece, which are irreversible and genetically related [28].

In conclusion, the results indicate that addition of 100 mg fish oil to 100 mL of skim milk-egg yolk extender could be beneficial for the improvement of the post-thaw quality of Bali bull spermatozoa including live sperm and motility. Therefore, fish oil addition at the dose of 100 mg per 100 mL skim milk-egg yolk extender can be recommended to bovine semen production centers.

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Research Article

Antimicrobial Resistance and Virulence Characteristics in Enterococcus Isolates from Dogs [1]

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Abstract

Antimicrobial resistant enterococci are among the leading causes of nosocomial infections. Transmission of antimicrobial-resistant enterococci from animals to humans has been shown. For this reason, continuous monitoring of antimicrobial resistance in different animal species is of importance both for animal and human health. In this study, it was aimed to investigate the antimicrobial resistance profiles, resistance mechanisms implicated and virulence traits of 107 enterococci isolated from 125 rectal swab samples taken from dogs. The highest resistance rate was determined against tetracycline (65.4%), followed by ciprofloxacin (19.6%), erythromycin (19.6%), chloramphenicol (8.4%) and ampicillin (3.7%). Fourteen (12.1%) enterococci showed multidrug resistance (MDR) phenotype. The *tet*M gene was predominantly detected among tetracycline isolates. Of 21 erythromycin resistant isolates, 18 harbored the *erm*B gene. The frequently detected virulence genes was *ccf* (54.2%), *efa*A_{fs} (52.3%), *cpd* (45.8%) and *gel*E (44.9%). These results indicate that high level of antimicrobial resistance and virulence genes exist among enterococci from dogs and pose a potential public health concern.

Keywords: *Dog, Enterococcus spp., Antimicrobial resistance, Virulence genes*

Köpeklerden İzole Edilen Enterokok Suşlarının Antimikrobiyal Direnç ve Virulans Özellikleri

Özet

Antimikrobiyal dirençli enterokoklar nozokomiyal enfeksiyonların önde gelen nedenleri arasında bulunmaktadır. Antimikrobiyal dirençli enterokokların hayvanlardan insanlara geçişi gösterilmiştir. Bu nedenle, farklı hayvan türlerinde antimikrobiyal direncin sürekli olarak izlenmesi hem hayvan hem de insan sağlığı için önemlidir. Bu çalışmada, köpeklerden alınan 125 rektal sıvab örneğinden izole edilen 107 enterokok suşunun antimikrobiyal direnç profilleri, dirence aracılık eden mekanizmalar ve virülans özelliklerinin araştırılması amaçlandı. En yüksek direnç oranı tetrasikline (%65.4) karşı belirlenirken; siprofloksasin (%19.6), eritromisin (%19.6), kloramfenikol (%8.4) ve ampisiline (%3.7) ise değişen oranlarda direnç tespit edildi. On dört (%12.1) enterokok suşunda çoğul direnç fenotipi (MDR) görüldü. Tetrasiklin dirençli izolatlarda *tet*M geni ağırlıklı olarak saptandı. Eritromisin dirençli 21 izolatın 18'inin *erm*B geni taşıdığı tespit edildi. İzolatlar arasında *ccf* (%54.2), *efa*A_{fs} (% 52.3), *cpd* (%45.8) ve *jel*E (%44.9) virulens genleri sıklıkla belirlendi. Bu sonuçlar köpeklerden izole edilen enterokok suşlarında yüksek düzeyde antimikrobiyal direncin ve virülans genlerinin mevcut olduğunu ve potansiyel bir halk sağlığı sorunu olduğunu ortaya koymaktadır.

Anahtar sözcükler: *Köpek, Enterococcusspp., Antimikrobiyal direnç, Virulens genleri*

INTRODUCTION

Although enterococci are known as commensal agents residing on intestinal microbiota of animals and common in environmental sources, they emerged as an important agents of nasocomial and community-acquired infections due to their ability for developing high level of antimicrobial resistance [1,2]. Overuse and misuse of antimicrobials in pet animals also increases the likelihood of developement of resistance to these agents [3-5]. Enterococci can develop antimicrobial resistance mainly by two mechanisms: (i) target mutation and (ii) horizontal gene transfer which includes transformation, conjugation and transduction [6]. Furthermore, enterococci may serve as a resevoir to transfer

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resistance genes to other resident bacteria [4,5,7].

Enterococci have the ability to produce numerous virulence factors playing an important role of pathogenesis of diseases caused by these bacteria [8]. These virulence factors include gelatinase *(gel*E), enterococcal surface protein *(esp),* aggregation substance*(agg*A), cytolysin *(cyl*A, *cyl*B, *cyl*M), cell wall adhesins *(efa*A_{fs} and *efa*A_{fm}), sex pheromones *(cpd, cob, ccf, eep)* [9].

Pet animals are owned by people at increasing rates in Turkey, and this has made these animals a member of the family. Pet animals are known to transfer antimicrobial-resistant bacteria to humans due to their close physical contact with humans [4]. This situation requires continuous surveillance to track antimicrobial resistance among commensal, zoonotic and pathogenic bacteria from these animals, and to monitor changes in antimicrobial resistance over time. Therefore, the objective of this study was to investigate the prevalence of *Enterococcus* spp. and their antimicrobial resistance proflies and to determine the antimicrobial resistance and virulence genes by polymerase chain reaction (PCR).

MATERIAL and METHODS

Sampling

A total of 125 rectal swab samples from dogs admitted to private veterinary clinics in Hatay were collected from January 2014 to June 2014 for different purposes (medical check up, vaccination, treatment etc.). Rectal swabs were transferred to laboratory in Stuart transport medium. Age of dogs was ranging from two month to 13 year (median age 2 years), including 17 breeds. Among the dogs sampled, 61.6% (n=77) were males and 38.4% (n=48) were females. The study was approved by the Animal Ethical Committe of Mustafa Kemal University (2013/7-6).

Isolation and Identification

Rectal swabs were subjected to pre-enrichment procedure in Enterococcosel™ Broth (BD, USA) at 37°C for 24 h. Following enrichment procedure, a loopful of broth was streaked on Vancomycin Resistant Enterococci (VRE) agar (Oxoid, CM0985, UK) plates with and without vancomycin (6 mg/L). Plates were incubated at 37°C for 24 h and one typical colony randomly selected and passaged to blood agar (Merck, 110886, Germany) supplemented with 5% defibrinated sheep blood for obtaining pure culture. These isolates were presumptively identified as *Enterococcus* spp. by Gram staining and catalase test. Identification of the isolates was done by 16S rRNA sequencing by using universal primers [10,11]. The PCR products were sequenced from both ends and compared with published nucleotide sequences in GenBank using the BLAST program *(http://blast.ncbi.nlm.nih.gov/)*. The sequencing data were compared with the previously published sequences in GenBank using the BLAST program *(http://blast.ncbi.nlm.nih.gov/)* and a similarity score of 97% or higher was accepted as criterion for establishing species-level identification.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the isolates was determined using disk diffusion method in accordance with Clinical Laboratory Standards Institute guidelines (CLSI) [12] on Mueller Hinton Agar (MHA) plates. Antibiotic disks used were: ampicillin (10 µg), chloramphenicol (30 µg), vancomycin (30 µg), erythromycin (15 µg), tetracycline (30 µg), teicoplanin (30 µg), ciprofloxacin (30 µg), trimethoprim-sulfamethaxozole (1.25 µg /23.75 µg) and gentamicin (120 µg). *Enterococcus faecalis* ATCC29212 was used as control strain. The isolates resistant to at least three different antimicrobial classes were deemed as multidrug resistant (MDR).

Detection of Antimicrobial Resistance Genes

Presence of antimicrobial resistance genes responsible for phenotypic resistance in disk diffusion assay was investigated by PCR. Erythromycin and tetracycline resistant isolates were screened for the presence of *tet*K, *tet*L, *tet*M, *tet*O, *tet*S, *erm*A, *erm*B and *mef*A/E [13], high level gentamicin resistant isolates for *aac(6)-Ie-aph(2)-Ia, aph(2)-Ib, aph(2)-Ic, aph(2)-Id, aph(3)-IIIa* and *ant(4)-Ia* [14], chloramphenicol resistant isolates for *cat* [15], vancomycin resistant isolates for *van*A, *van*B, *van*C1/2, *van*D, *van*E and *van*G [16]. Following resistant reference strains were used as controls: *E. faecium* BM4147 *(van*A), *E. faecalis* V583 *(van*B), *E. gallinarum* BM 4174 *(van*C1), E. *casseliflavus* ATCC 25788 (*van*C2), *S. pyogenes* BM137 *(tet*M, *erm*B), *S. pyogenes* UR1092 *(erm*A), *S. aureus* R-16794 *(tet*K).

Detection of Virulence Genes

The genes responsible for the expression of gelatinase *(gel*A), cytolysin *(cyl*A, *cyl*M and *cyl*B), cell wall adhesins *(efa*A_{fs} and *efa*A_{fm}), enterococcal surface protein *(esp),* sex pheromones *(cpd*, *cob*, *cad*, *ccf* and *eep)* and the aggregation substance *(agg*A) were investigated as previously described by Eaton and Gasson [17], Marques and Suzart [18] and Shankar et al.[19].

RESULTS

Isolation and Identification of Enterococcus spp.

Overall, 107 (85.6%) *Enterococcus* spp. were isolated from 125 rectal swab samples. *E. faecalis* was the most frequently detected species (96; 89.7%), followed by *E. faecium* (9; 8.4%), *E. hirae* (1; 0.9%) and *E. durans* (1; 0.9%).

Antimicrobial Resistance Profiles of Enterococcus spp.

Of 107 enterococci isolates, 15 (14.02%) were susceptible to all antimicrobial tested. None of the isolates were resistant to vancomycin, teicoplanin and high level gentamicin

(HLG). Various rates of resistance were observed to tetracycline (70; 65.4%), ciprofloxacin (21; 19.6%), erythromycin (21; 19.6%), chloramphenicol (9; 8.4%) and ampicillin (4; 3.7%). MDR were detected among 14 (13.1%) enterococci. MDR to six, five, four and three antimicrobials was detected in one (0.9%), one (0.9%), five (4.7%) and six (5.6%) isolates, respectively *(Table 1)*.

Presence of Resistance Genes Among Resistant Enterococci

Antimicrobial resistant isolates were screened for the presence of resistance genes acquired by horizantal gene transfer. Of the 70 tetracycline resistant isolates, *tet*M was the most common, found in 45 isolates (64.3%), both *tet*L and *tet*M in 12 (17.1%), *tet*O in 4 (5.7%) isolates, *tet*K in 2 (2.9%) and *tet*L in 2 (2.9%) isolates, which encode proteins involved in ribosomal protection and efflux, respectively. Of 21 erythromycin resistant isolates, 18 harbored *ermB* gene. *cat* gene was detected only two resistant isolates *(Fig.1, Fig. 2)*.

Characterization of Virulence Genes

The percentage of virulence genes among the tested

Table 1. *Resistance phenotypes determined in Enterococcus spp.*

Resistance Phenotype*	*E. faecalis*	*E. faecium*	*E. durans*	*E. hirae*
AMP, TE, E, CIP, C	1			
TE, E, CIP, C	1			
AMP, TE, E		3		
TE, E, CIP	6			
TE, E, C	2			
TE, CIP	3			
TE, C	3			
TE, E	6			
CIP	9			1
E	3			
TE	41	3	1	
C	2			
Susceptible	19	3		
Total	96	9	1	1

* ***AM:*** *ampicillin,* ***TE:*** *tetracycline,* ***CIP:*** *ciprofloxacin,* ***E:*** *eryhtromycin,* ***C:*** *chloramphenicol*

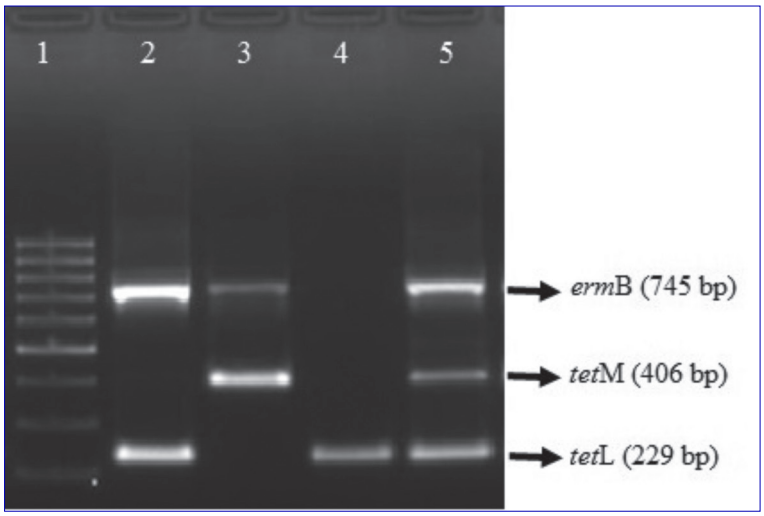

Fig 1. Agarose gel electrophoresis of tetracycline and macrolide resistance genes. Lane 1: 100 bp molecular marker, Lane 2: *erm*B and *tet*L, Lane 3: *erm*B and *tet*M, lane 4: *tet*M, Lane 5: *erm*B, *tet*L and *tet*M

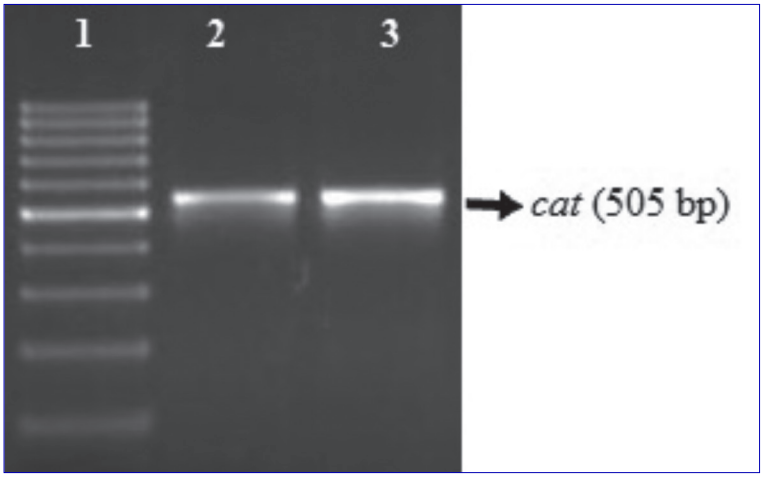

Fig 2. Agarose gel electrophoresis of *cat* gene detected in chloramphenicol resistant isolates. Lane 1: 100 bp molecular marker, Lane 2-3: *cat* gene

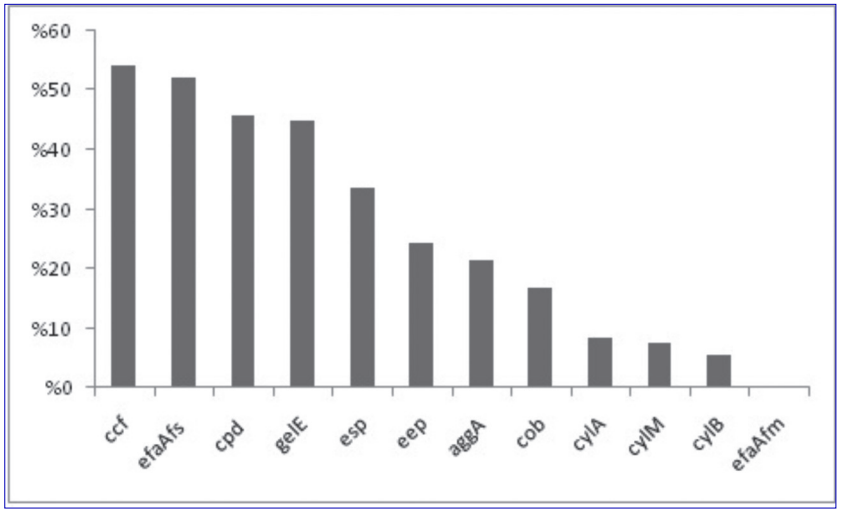

Fig 3. Distribution of virulence genes among enterococci

strains is shown in *Fig. 3*. One hundred and four (97.2%) isolates carried one or more virulence genes. *efa*A_{fm} was not detected in any of the isolates. The *efa*A_{fs} gene, encoding cell wall adhesin in enterococci, was found in 52.3% of the isolates. Sex pheromone genes, *ccf, cpd*, *cob* and *eep*, were detected in 54.2%, 45.8%, 16.8% and 24.3% of the isolates, respectively. Frequency of *cyl*A, *cyl*M and *cyl*B genes were 8.4%, 7.5% and 5.6%, respectively. The *gel*E, *esp* and *agg*A genes were observed in 44.9%, 33.6% and 21.5% of the isolates, respectively.

DISCUSSION

Antimicrobial resistance is a major concern in human and animal health throughout the world. Even tough it is known that enterococci are ubiquitous microorganisms found different habitats including gastrointestinal tract of human and animals, enterococci recently emerged as a leading cause of multiresistant, nosocomial infections in humans [20,21]. A recent study showed that possible transmission of resistant enterococci species between dog, human and hospital environments [22].

Among 107 enterococcal isolates, *E. faecalis* (89.7%) was the most frequently isolated species followed by *E. faecium* (8.4%). In contrast, *E. faecium* was reported to be most common species isolated from dogs in previous studies carried out in Turkey [23,24]. *E. faecalis* has been reported to be predominantly isolated from urinary tract infections of both dogs KuKanich and Lubbers [25] and humans [26] as a causative agent.

Increased use of antimicrobials in pet animals lead to the emergence of resistant bacteria [4]. The rate of antimicrobial resistance observed among enterococci in this study was lower than those reported of Türkyılmaz et al.[23] in Aydın, but higher than findings of Boynukara et al.[24] in Van, suggesting that resistance rates for enterococci vary regionally and influenced by antibiotic usage. However, Boynukara et al.[24] did not mention the origin of the dogs in their study. Resistance rate to tetracycline (65.4%), which is a widely used in veterinary field in Turkey, was higher than those from findings (41.1%) of Boynukara et al.[24], but lower than findings (70.3%) of Türkyılmaz et al.[23]. The erythromycine resistance rate (19.6%) was similar to findings (21.1%) of Boynukara et al.[24]. But, Türkyılmaz et al.[23] reported higher erythromycin resistance rate (69.2%). In previous studies, no acquired vancomycin resistance mediated by transferable *van*A or *van*B gene was reported in Turkey among enterococci [23,27,28]. Similarly, no resistance was observed against this agent in the current study. However, intrinsic resistance encoded by *van*C gene has been reported among *E. gallinarum* and *E. casseliflavus* species from dogs [23,27,28]. Resistance to ciprofloxacin, which is a clinically important drug, was found high (19.6%), in contrast to findings of Boynukara et al.[24], who reported a resistance rate of 6.7%. The rate of chloramphenicol resistance (8.4%) found in this study was comparable to findings (12.1%) of Türkyılmaz et al.[23]. Ampicillin is a drug of choise to be used for the treatment of multi-drug resistant enterococcal infection in combination with aminoglycosides when the isolates did not exhibite high level aminoglycoside resistance [29]. Furthermore ampicillin resistant enterococci are usually resistant to other classes of antimicrobilals used in dogs [30]. Even tough overall prevalence of ampicillin resistance was low (3.7%), detection of ampicillin resistance poses a significant risk for both human and animal health, and further investigation are, therefore, necessary to determine the true prevalence of ampicillin resistant enterococci in dogs using selective media. In a recent study, Çelik et al.[31] reported higher prevalence (20.9%) of ampicillin resistant enterococcus using selective media.

In current study, the most common resistance gene detected was the *tet*M in tetracycline resistant enterecocci isolates. Previous studies carried out in Turkey showed predominance of *tet*M gene in tetracycline resistant enterococci from different sources such as meat [32], cheese [33] as well as dogs [23]. Reason of wide dissemination of *tet*M gene is that this gene is frequently carried by *Tn*946 in enterococci [5]. Main resistance mechanism against macrolides is the modification of 23S rRNA by *erm* methylases among enterococci [34]. In the current study, *erm*B was the only gene detected in macrolide resistant enterococci. Similarly, Türkyılmaz et al.[23] reported high prevalence of *erm*B gene. The *cat* gene responsible for chloramphenicol resistance was found at a very low

rate (22.2%), suggesting presence of acquired other resistance genes or mechanisms responsible for this resistance phenotype. In a study carried out by Yılmaz et al.[32], lower prevalence rate (4.8%) of *cat* gene among chloramphenicol resistant enterococci from meat was reported. In contrast, Türkyılmaz et al.[22] detected *cat* gene in 63.6% of chloramphenicol resistant enterococci.

Enterococci have the ability to produce various virulence factors contributing the course and severity of infection [35]. The *efa*A_{fs} and *efa*A_{fm} are cell wall adhesins associated with infective endocarditis. In this study, *efa*A_{fs} was detected 58.3% of *E. faecalis* isolates. *efa*A_{fm} was not present in any *E. faecium* isolate. Iseppi et al.[36] found *efa*A_{fs} gene in 33.3% of *E. faecalis* isolates and *efa*A_{fm} gene in 50% of *E. faecium* isolates.

Cytolysin is a bacteriocin-type exotoxin, exerts its effects towards erythrocytes, leukocytes and macrophages [8]. *cyl*A, *cyl*M and *cyl*B genes were detected in 8.4%, 7.5% and 5.6% of the isolates, respectively. A similar finding was also reported by Iseppi et al.[36], who detected these genes in 8.3%, 8.3% and 8.3% of *E. faecalis* isolates. In this study, the authors detected only *cyl*M gene (7.1%) in *E. faecium* isolates. Gülhan et al.[37] investigated cytolysin activity of *E. faecalis* and *E. faecium* from humans and pet animals, and detected no cytolytic activity in dog isolates, except one *E. faecium* isolate from cat.

*gel*E is zinc-dependent metalloendopeptidase, is capable of hydrolysing gelatine, elastin, collagen, haemoglobin [8]. Geletinase has also been reported to play an important role in the developement of endocarditis and decrease neutrophil accumulation to the site of infection by splitting complement C5a fragment, which has anaphylotoxin activity [38]. In the current study, the *gel*E was detected in 44.9% of the isolates. However, Iseppi et al.[36] reported higher prevalence rates 57.1% and 80.3% of *E. faecium* and *E. faecalis* isolates, respectively. Gülhan et al.[37] found 26.6% and 60% of *E. faecium* and *E. faecalis* isolates positive for gelatinase activity.

Aggregation substance (AS) is enterococcal surface protein that contributes the formation of mating aggregates facilitating bacterial conjugation [8]. In the current study, 21.5% of the isolates were positive for *agg*A gene. In contrast, Iseppi et al.[36] did not find this gene, and speculated that the isolates had a reduced capability to transfer resistance virulence genes by mating. A very low rate was also reported by Gülhan et al.[37], who detected a positivity rate of 1.6% and 6.7% for *E. faecium* and *E. faecalis* isolates, respectively.

Enterocci have the ability to produce sex pheromones (*cpd*, *cob*, *ccf*, *cad*) facilitating conjugative plasmid transfer betwen cells [8]. Sex pheromone genes, *ccf, cpd*, *cob* and *eep*, were detected in 54.2%, 45.8%, 16.8% and 24.3% of the isolates. In a recent study, higher prevalence of sex pheromones was reported among enterococci from meat samples by Yılmaz et al.[32].

The *esp* gene is known to be involved in biofilm formation. Furthermore, biofilm production has been shown to play an important role in the exchange of antibiotic resistance genes between cells and to increase their resistance to antibiotics [8]. The *esp* gene was detected in 33.6% of the isolates. In contrast, Iseppi et al.[36] did not find this gene among the isolates. Also, Oliveira et al.[39] reported a lower prevalence rate (10%) among enterococci from dogs with periodontal disease.

In conclusion, the current study reveals that dogs are colonized with potentially virulent and antibiotic resistant enterococci. Therefore, the possibility of transmission of enterococci from dogs to humans, particularly those in the risk group, can not be excluded. Also, further studies are needed to elucidate the risk of transmission to humans by analyzing the clonal relationship in enterococci from dogs and dog owners.

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Short Communication

Effect of 7-dehydrocholesterol and Cholesterol-loaded Cyclodextrins on Bull Sperm Motility During Short Term Storage [1] [2]

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Abstract

The study was conducted to determine the effect of 7-dehydrocholesterol loaded cyclodextrin (7-DHCLC) and cholesterol-loaded cyclodextrin (CLC) on changes of bull sperm motility during short term storage (+4°C). Collected ejaculates were pooled and divided into 7 groups, as following one control (C); three 7-DHCLC and the other three CLC concentrations. Diluted semen samples were transferred and stored at +4°C and sperm motility was analysed in determined intervals during three days storage period with computer aided sperm analyse (CASA) system. Motility decreased in all groups; however, 7-DHCLC and CLC groups motility decreased in the next two days gradually. But, CLC 1.5 mg/120x10^6 group were identified as the best groups for protecting motility in short time preservation. In conclusion, adding different rates of 7-DHCLC and CLC to semen extender preserved motility for three days in bull semen stored at +4°C.

Keywords: *Bull sperm, Cholesterol, Motility, Short term storage, 7-dehydrocholesterol*

7-dehidrokolesterol ve Kolesterol İle Doyurulmuş Siklodekstrinlerin Kısa Süreli Saklama Süresince Boğa Spermasının Motilitesine Etkisi

Özet

Bu çalışma, kısa süreli saklama süresince (+4°C) 7-dehidrokolesterol doyurulmuş siklodekstrin (7-DHCLC) ve kolesterol ile doyurulmuş siklodekstrinin (CLC) boğa spermasının motilitesinin korunmasındaki etkisinin değerlendirilmesi için yürütülmüştür. Alınan ejekulatlar birleştirilerek 7 gruba ayrıldı, bu gruplardan biri control (C), üçü 7-DHCLC'nin diğer üçü ise CLC'nin farklı konsantrasyonlarını içeren sulandırıcılarla sulandırıldı. Sulandırılan sperma +4°C'ye transfer edildilerek saklandı ve sperma motilitesi üç gün boyunca bilgisayar destekli sperm analiz cihazında (CASA) değerlendirdi. Motilitenin tüm gruplarda azaldığı görüldü fakat, 7-DHCLC ve CLC gruplarında motilite azalmasının daha yavaş olduğu tespit edildi. Fakat, CLC 1.5 mg/120×10^6 grubunun spermanın kısa süreli saklanmasında motiliteyi koruyan en iyi grup olduğu belirlendi. Sonuç olarak, sperma sulandırıcısına değişik oranlarda 7DHCLC ve CLC'nin eklenmesi +4°C'de boğa sperma motilitesini üç gün boyunca koruduğu tespit edildi.

Anahtar sözcükler: *Boğa sperması, Kolesterol, Motililite, Kısa süreli saklama, 7-dehidrokolesterol*

INTRODUCTION

The plasma membrane of the sperm cells differs from many other cell membranes in its lipid composition. It contains huge amount of polyunsaturated fatty acids (PUFA), especially diPUFA (phospholipids esterified with two PUFA), which is found only in retina, brain and sperm [1]. PUFA in sperm membrane are very vulnerable to oxidative

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stress during sperm preservation and a lot of studies have been carried out on usage of antioxidant during short or long term storage of sperm [2-4]. Stabilization of sperm membrane lipids may be an alternative to antioxidant usage.

These overpowering lipids in many membranes are built with phospholipids and cholesterol. Membranes also contain carbohydrate-lipid complexes (glycolipids), with the most common cholesterol and phospholipids that build the capacity of the lipid substance of the membrane. When the temperature of the membranes is lowered, the phospholipids are converted from liquid phase to the gel state. Each type of lipid species performs this phase change at different temperatures; therefore, the membrane being composed of complex different lipids, undergoes the phase change over a relatively extensive temperature range. Cholesterol provides the fluidity of membranes, along with the fatty acid chains of the phospholipids, when the temperature decreases [5].

Plasma membrane can be affected at any stage of semen preservation; causes such as cell death, which are seen as the result of the formation of ice crystals, may induce many stresses on sperm [5]. The spermatozoon membrane acts as a physical barrier and its main function is to protect the cell from damage during low temperatures. Damage contains loss of lipids from the membrane, peroxidation of membrane lipids as a result of formation of reactive oxygen species (ROS) and phase transition, from the fluid phase to the gel phase, as temperature is decreased and membrane destabilization occurs due to lateral lipid rearrangement. These damages can be decreased by adding lipids, in the form of egg yolk, to the sperm prior to cooling and freezing. Egg yolk is the main membrane protective agent in semen extenders [6]. The main disadvantage is of having a protective agent of an animal source in extenders used for short term preservation or cryopreservation is the risk of disease transportation [7] and microbial contamination which allows production of endotoxins [8]. This has raised a problem to international semen transport because of biosecurity issues and for this reason we are working on alternatives to egg yolk [9].

Cholesterol can easily interaction from the plasma membranes of cells using cyclodextrins. Cyclodextrins are cyclic oligosaccharides which are main degradation yields of starch. Methyl-beta-cyclodextrin which is one of the most used cyclodextrins, can solubilise hydrophobic molecules, such as cholesterol [5] and can transport cholesterol into or out of membranes down a concentration gradient. 7-dehydrocholesterol is a cholesterol conjugate and consists of cholesterol upper stage (intermediate product) in biochemical diagram which means that it is one of the cholesterol conjugates and 7-DCLC formed before cholesterol is produced. Amorim et al.[10] cryopreserved bull semen with cholesterol conjugates (heptanoate, palmitate, pelorganate and stearate) and obtained increased percentages of sperm motility after thawing. However, to our knowledge, there are no studies demonstrating semen preservation with 7-DHCLC in any animal species.

In the light of the above, these experiments were conducted to determine the effect of 7-DHCLC and CLC treatment on bull sperm motility at short term preservation.

MATERIAL and METHODS

Chemicals

All chemicals used in this study were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Semen Collection

Ejaculates were collected from three Simmental bulls (2-4 years of age), regularly used for breeding purpose which were held in the International Center for Livestock Research and Training Ankara-Turkey under uniform breeding conditions. Totally, 30 ejaculates were taken from the three bulls using artificial vagina. The ejaculates from bulls were kept at 34°C with aid of water bath until their evaluation in the laboratory. The evaluation was performed within 15 min after collection; each ejaculate was evaluated to determine percentages of total and progressive motility as well as concentration. Only ejaculates containing sperm with >75% motility and >1.0×10^9 sperm/mL concentration were used in current study. Ejaculates provided these criterias were pooled in order to decrease individual differences.

Cyclodextrin Preparation

Methyl-beta- cyclodextrin was loaded with 7-dehydrocholesterol and cholesterol as explained by Purdy and Graham [5].

Semen Processing

The total semen volume was obtained from the graded conical tube soon after collection, and its concentration was determined using photometer (IMV, France). Fresh total and progressive motility was evaluated subjectively by a phase contrast microscopy (Olympus BX43) at 37°C. For short-time semen preservation, we used Tasdemir et al.[4]'s Tris-based extender (T) without cryoprotectant and egg yolk. Each pooled ejaculate was equally split into seven groups. These groups were: one as control (C); three different (1.5, 2.5, 3.5 mg/120×10^6) 7-DHCLC and three different (1.5, 2.5, 3.5 mg/120×10^6) CLC treatments. Control were diluted T extender without any additives. 7-DHCLC and CLC groups were initially incubated with CLC or 7-DHCLC for 15 min at 22°C, prior to diluted with T, then diluted with T to final concentration of 100×10^6 sperm/ml. Diluted semen cooled slowly in the water bath (22°C), inside the cold box (4°C) for four h. Then semen samples

were transferred to the refrigerator at +4°C and kept in the throughout the replications.

Motility Analysis

Computer aided sperm analysis (CASA) system (SCA®, Barcelona, Spain) was used to examine of motility and kinetic parameters. The sperm motility properties were set as static, slow >50 µm/s, medium >75 µm/s, fast >100 µm/s. Five µl of rewarmed semen was put onto slide and covered with a coverslide then motility were analysed with a 10 x objective at 37°C. Progressive motility (%) and total sperm motility (%) were recorded. For each evaluation, at least 200 to most 300 sperm were analysed in six microscopic fields. Sperm motility was evaluated for three days (0, 1, 2, 6, 24, 48 and 72. h) in short tem storage.

Statistical Analysis

Descriptive statistics for each variable obtained from ten experimental replications were calculated and presented as mean ± standard error of mean. The main effect of "treatment", "time" and the interaction term of "treatment x time" upon the fertility parameters were modelled by using the GLM procedure for repeated measures of SPSS 14.01 (SPSS Inc., Chicago, IL, USA). Post hoc testing was carried out for significant interactions and was performed using simple effect analysis. A probability value of less than 0.05 was considered significant, unless otherwise noted.

RESULTS

The CASA result of progressive and total motility of the pooled semen, over the three days are given in *Table 1* and *Table 2*. There was no significant difference between 7-DHCLC and CLC groups in terms of progressive motility ($P>0.05$); however statistically significant difference was detected when this were compared with control groups ($P<0.001$) at 1 h. Sperm motility rates reduced in each group; however, CLC and 7-DHCLC 1.5 mg/120×10^6 were maintained motility higher than control group.

DISCUSSION

Storage process causes damage to both intracellular and extracellular sperm membranes starting with dilution and continuing until warming procedures. Plasma membranes are considered to be in the lamellar liquid crystalline phase at normal body temperatures with the

Table 1. *Mean (±SE) CASA progressive motility of semen treated with CLC or 7-DHCLC (1.5, 2.5 or 3.5 mg/120x10^6)*

Type	Group	Time						
		0 hour	1 hour	2 hour	6 hour	24 hour	48 hour	72 hour
Progressive	CLC 1.5	31.2±4.12^{a,A}	30.4±7.13^{ab,A}	30.02±7.01^{a,AB}	29.5±8.10^{a,AB}	25.4±5.81^{a,B}	17.67±2.92^{a,C}	11.28±1.56^{a,D}
	CLC 2.5	34.1±3.31^{a,A}	26.9±4.32^{ab,A}	26.27±4.66^{ab,B}	25.1±6.38^{ab,AB}	16.1±4.03^{b,C}	8.15±5.20^{bc,D}	5.05±3.88^{bc,E}
	CLC 3.5	36.1±6,23^{a,A}	28.4±5.53^{ab,A}	27.72±5.14^{ab,B}	25.5±2.74^{ab,B}	17.7±3.27^{b,C}	9.88±4.62^{b,D}	5.22±2.63^{bc,E}
	7DHCLC 1.5	33.2±4.21^{a,A}	31.1±3.11^{ab,A}	30.68±3.06^{a,B}	23.8±2.78^{ab,C}	14.9±6.56^{bc,D}	9.23±3.96^{bc,E}	6.05±2.21^{b,F}
	7DHCLC 2.5	38.4±5.00^{a,A}	27.0±3.00^{ab,A}	26.63±2.91^{ab,A}	22.0±6.20^{b,B}	16.9±3.88^{b,C}	8.38±4.59^{bc,D}	6.43±3.39^{b,D}
	7DHCLC 3.5	36.0±2.45^{a,A}	24.0±3.75^{ab,B}	23.58±3.75^{b,A}	22.6±4.88^{b,BC}	16.0±5.59^{bc,D}	11.00±3.93^{bc,E}	7.53±2.76^{b,F}
	Control	34.3±3.26^{a,A}	15.4±1.19^{c,B}	15.23±1.14^{c,B}	15.3±1.19^{c,B}	10.7±0.91^{c,B}	5.25±0.31^{c,C}	2.73±1.63^{c,D}

^{a,b,c} Different superscripts within the same column demonstrate significant differences among groups ($P<0.001$)
^{A,B,C} Different superscripts within the same line demonstrate significant differences during storage period in same group ($P<0.001$)

Table 2. *Mean (±SE) CASA total motility of semen were treated with CLC or 7-DHCLC (1.5, 2.5 or 3.5 mg/120x10^6)*

Type	Group	Time						
		0 hour	1 hour	2 hour	6 hour	24 hour	48 hour	72 hour
Total	CLC 1.5	78.1±5.38^{a,A}	77.2 ± 8.86^{a,A}	77.05±8.93^{a,A}	72.48±12.49^{a,AB}	60.7±7.23^{a,B}	46.87±6.04^{a,C}	41.10±2.15^{a,D}
	CLC 2.5	75.3±6.23^{a,A}	67.6 ± 7.73^{b,B}	67.48±7.78^{b,B}	63.5±11.60^{ab,BC}	52.6±15.71^{ab,C}	29.13±8.57^{b,D}	28.27±9.65^{b,D}
	CLC 3.5	73.2±4.35^{a,A}	65.8±6.13^{bc,B}	65.63±6.15^{bc,B}	53.5±18.43^{bc,C}	37.0±6.12^{c,D}	25.37±3.25^{b,E}	22.53±2.68^{b,E}
	7DHCLC 1.5	77.2±3.45^{a,A}	74.9±4.74^{a,A}	74.75±4.49^{a,A}	63.4±15.04^{ab,B}	46.4±13.49^{bc,C}	30.82±15.07^{b,D}	25.32±8.06^{b,E}
	7DHCLC 2.5	72.1±4.34^{a,A}	60.4±4.67^{bc,B}	60.07±4.66^{c,B}	60.3±5.10^{ab,B}	55.5±6.24^{ab,B}	28.93±9.75^{b,C}	24.40±6.89^{b,D}
	7DHCLC 3.5	70.5±6.2^{1a,}A	63.8±6.65^{c,B}	62.25±4.66^{bc,B}	61.6±4.80^{ab,BC}	51.5±11.02^{ab,C}	29.67±7.93^{b,D}	24.75±6.21^{b,E}
	Control	70.3±4.38^{a,A}	42.4±1.37^{d,B}	42.08±1.40^{d,B}	42.1±1.36^{c,B}	39.9±1.21^{c,B}	8.58±1.99^{c,C}	6.98±2.21^{c,C}

^{a,b,c} Different superscripts within the same column demonstrate significant differences among groups ($P<0.001$)
^{A,B,C} Different superscripts within the same line demonstrate significant differences during storage period in same group ($P<0.001$)

acyl chains relatively irregular. At low body temperatures, the lamellar gel phase is formed, in which case the lipid acyl chains are highly regular. In a cell membrane, the mixture of sterols, proteins and lipids causes complex thermal phase behaviour [11]. Membrane complexity increase stress susceptibility that result in membrane disorderliness, membrane lipid/protein structure, and osmotic changes across the membrane. Therefore, sperm membranes may be altered its intrinsic structure resulting in cell death.

In this research, mixed semen groups was observed during 3 days at 4°C and the highest total motility was 77.2±8.86% in CLC 1.5; the lowest total motility was 42.4±1.37% in control (P<0.001) at 1 h. In progressive motility, there were no significant differences between 7-DHCLC and CLC groups (P>0.05); but these groups were statistically different from control (P<0.001) at 1 h. The highest progressive motility was 30.4±7.13% in CLC 1.5; the lowest motility was 15.4±1.19% in control. These motility results were similar to Vyas et al.[12] who was collected 166 ejaculates in 5 hybrid bull semen and at 0 h motility was 71.03%. After semen collection, they kept samples in refrigerator and evaluated the motility at 24, 48, 72 and 96 h and observed 60.69, 50.47, 41.75, 30.13% respectively. Motility rates reduced in each group also during our experiment with similar rates; however the groups CLC and 7-DHCLC 1.5 mg/120×10^6 decreased their rates in 2 days gradually. Similar results were obtained by Franceshini et al.[13] investigated the effect on the motility of the pig sperm storage at 4°C for 4 days and reported motility decrease.

In our research, we added 7-DHCLC and CLC to T solution for external preservation. It's well known that cholesterol modulates the fluidity of membranes by interacting with the fatty acyl chains of the phospholipids similarly with egg yolk in extender [5] and maintains the phospholipids in a random, lamellar arrangement as temperature decreases. However, cholesterol to phospholipid ratio is an important determinant of membrane fluidity and stability at low temperature. Besides, different animal species exhibit different sperm membrane composition, such as different cholesterol/phospholipid ratio and degree of hydrocarbon chain saturation, which can affect how the sperm responds to cooling and, subsequently, can confer different sperm sensitivities to low temperature across various species [14].

In conclusion, adding different rates of CLC and 7-DHCLC in T can be used for maintain bull sperm alive for 3 days at +4°C. Although CLC 1.5 mg/120×10^6 treatment was identified as the best for short time preservation, 7-DHCLC reduced cell damage at low temperatures compared to control. 7-DHCLC can be used in tris extender instead of cholesterol loaded cyclodextrin (CLC). Also, 7-DHCLC and CLC may be considered a good alternative to egg yolk, which is animal sourses, in bull semen short term preservation.

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Short Communication

Hematologic and Biochemical Reference Intervals for Captive Asian Elephants *(Elephas maximus)* in Thailand

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Abstract

Species specific blood value reference intervals are needed for the proper diagnosis, and treatment of disease, appropriate for specific populations, because age, sex, management, exercise and geographical location can all affect hematological values. The aim of this study was to establish a set of hematology and blood chemistry reference intervals for captive Asian elephants. Blood samples were collected from 149 healthy Asian elephants in 15 tourist camps in Northern Thailand. Hematological and biochemical parameters were determined. The results showed similarity of haematological and blood chemistry range to others previously published. There were no sex differences for most hematological parameters except some parameters were different i.e. MCV, MCHC, BUN, AST, and ALP. The hematology and blood chemistry reference intervals of our study can be used as the reference for hematological analysis in Thailand, and several Asian elephant range countries and zoos.

Keywords: *Asian elephant, Blood parameter, Blood chemistry, Hematology, Reference intervals*

Tayland'da Evcil Asya Filinin *(Elephas maximus)* Kan ve Biyokimyasal Referans Aralıkları

Özet

Türlerin spesifik kan değerleri referans aralıklarının bilinmesi uygun teşhis ve hastalıkların tedavisi için gerekli olup yaş, cinsiyet, bakım, egzersiz ve coğrafi bölge kan değerlerini etkilemektir. Bu çalışmanın amacı evcil Asya filinin hematoloji ve kan kimyası değerlerini ortaya koymaktır. Kuzey Tayland'daki 15 turist kampında yer alan 149 adet sağlıklı Asya filinden kan örnekleri toplandı. Kan ve biyokimyasal parametreler belirlendi. Sonuçlar daha önceden bildirilmiş olan kan ve biyokimyasal değerler ile benzerlik gösterdi. Çoğu kan parametreleri için cinsiyetin bir farkı gözlenmezken MCV, MCHC, BUN, AST ve ALP gibi bazı parametrelerde fark gözlemlendi. Mevcut çalışmada elde edilen hematoloji ve kan kimyası referans aralıkları Tayland'da ayrıca Asya filinin bulunduğu ülke ve hayvanat bahçelerinde kan analizleri için referans olarak kullanılabilir.

Anahtar sözcükler: *Asya fili, Kan parametreleri, Kan kimyası, Hematoloji, Referans aralıkları*

INTRODUCTION

Accurate hematologic and biochemical reference intervals are useful for evaluating the health status of animals and for proper diagnosis of disease and evaluation of treatment efficacy. The Asian elephant *(Elephas maximus)* is an endangered species, and in Thailand is an important part of the country's history and culture. Today, they are also important economically, as most captive elephants in Thailand are used primarily for tourism. The need for proper veterinary care of tourist camp elephants is increasing, and more veterinarians are relying on evaluations of blood

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tests to assist in diagnosis and treatment of disease.

Although hematology and blood chemistry data exist for both African and Asian elephants, overall the ranges tend to be very broad [1-4], which makes interpretation difficult. Furthermore, values may not be relevant across all populations because factors such as age, sex, management, exercise, as well as geographical location can affect values (e.g., horse [5], human [6]). Yaqub [7] reported that in farm animals, in the same species which locate in different farm could be found the unique of baseline or haematologic and blood chemistry. Therefore, the elephant in different country or feeding management like captive elephant in zoos and private camps may be different in hematologic and biochemistry value. Most blood parameter data in Asian elephants are based on samples collected from zoo elephants in North America and Europe [8], where the geographic, climate and management conditions are different from Thailand. There are some data on Asian elephants in range countries; e.g., India [2] and Sri Lanka [9,10], but there still are considerable differences in geography, where differs in the nutrition, which can lead to different blood parameters [11]. Physical exercise due to work and management, can cause stress between those elephants, which may affect the blood profiles [9,12]. Moreover, De Mel [9] found hematological ranges differed across several populations of Asian elephants in Sri Lanka. The aim of this study was to begin establishing a set of hematology and blood chemistry reference intervals for Asian elephant used in tourist camps in Thailand, and other Asian elephant range countries.

MATERIAL and METHODS

Animals

One hundred forty nine Asian elephants (41 males, 108 females) aged 3–60 years, from 15 tourist elephant camps in Chiang Mai, Thailand (latitude, 18°47′N; longitude, 98°59′E). These elephants were originally from various parts of Thailand. The age could not be identified in these elephants due to unclear birth date history. All elephants in this study were classified as healthy based on a physical examination by veterinarians experienced with elephants. All elephants performed work daily, either trekking or giving tourist rides or in an elephant show (not more than 6 hours per day). Most elephants were chained at night. For nutrition management, camps provided similar types and amounts of roughage, fruit, sugar cane and some vegetables. Bulls were provided a lower energy diet such as less roughage, winter melon or banana trunk, during the musth period which is a circannual period of anatomical, physiological and behavioural changes in mature Asian and African elephant bulls. During musth period, the bull shows temporal gland secretions (TGS), continuous urine dribbling (UD), increased aggression, and elevated serum androgen concentrations.

Sample Collection, Hematology and Blood Chemistry Analysis

Blood samples were collected from the auricular vein of these elephants during annual health checks. Blood samples were collected from the auricular vein of these elephants during annual health checks, mostly in the morning before activities. Blood was divided into 1) EDTA tube and gently mixed, and to 2) non-coagulation tube and was allowed to clot for ~1-2 h at room temperature before the serum was separated by centrifugation (1500 g) for 5 min. All samples were submitted to the Veterinary Diagnostic Laboratory of Faculty of Veterinary Medicine, Chiang Mai University, Thailand within 24 h of collection. Blood samples were analyzed by the Auto Hematology Analyzer (Mindray BC5300, Mindray Medical, Thailand) and by the Biochemical Analyzer Vitalab Flexor XL (Vital Scientific NV, Netherlands) except for white blood corpuscle differential count, which was manually assessed by a hematologist in the Veterinary Diagnostic Laboratory of the Faculty of Veterinary Medicine, Chiang Mai University, Thailand. Hematology parameters i.e. packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), hemoglobin, Red Blood Cell count (RBC count), White Blood Cell count (WBC count), differential blood count, platelet count, and biochemical parameters i.e. Blood Urea Nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were analyzed.

Statistical Analysis

R program version 3.1.1 [13] was used for statistical analysis. Reference interval with 90% confidence intervals for each parameter was calculated using "referenceIntervals" package [14]. The option for outlier detection method was cook's distance. The student T-test was used to determine differences of blood parameters between males and females. The level of statistical significance was set at $\alpha<0.05$.

RESULT

Reference range data for hematology and biochemistry evaluations of domestic Asian elephants in current study are shown in *Table 1*. Hematological parameters revealed no sex-associated clinically significant differences, except for MCV (P=0.011) and MCHC (P=0.002), which were higher in females than males, respectively. By contrast, BUN (P=0.001), AST (P=0.001) and ALP (P=0.049) were higher in males than females, respectively. White blood cell counts indicated that segmented neutrophils (47.5%) and lymphocytes (43.1%) predominated in this elephant population. The proportion of monocytes (monocyte and lobular monocyte) and eosinophils were found to be 7.4% and 2.05%, respectively, while observations of band neutrophils and basophils were rare.

Table 1. *Reference Intervals of hematology and blood chemistry values for domestic Asian elephants in northern Thailand for both sexes*

Parameter	Unit	Range (male, n= 41)	Range (female, n=108)	P-value
PCV	%	29.4-40.7	27.8-43	0.572
Hemoglobin	g/dL	9.8-15.2	10.1-15.6	0.162
RBC count	x 10^6 cell/µL	1.9-3.2	1.9-3.1	0.188
MCV*	fl	104-123.8	105.7-127.2	0.009
MCHC*	g/dL	29.9-38.9	32.1-38.7	0.015
WBC	cell/µL	7924.3-21890.3	7202.5-23220.5	0.657
Banded Neutrophil	cell/µL	Not found	Not found	-
Segmented Neutrophil	cell/µL	967.3-13425.8	828.7-13514.3	0.966
Lymphocyte	cell/µL	1672.4-11179.5	1064.1-12032.8	0.793
Monocyte	cell/µL	0-2391.4	0-3298	0.609
Bilobed monocytes	cell/µL	Not found	Not found	-
Eosinophil	cell/µL	0-866.8	0-1170	0.057
Basophil	cell/µL	0-142.6	0-36.3	0.067
Platelet	x 10^3 cell/µL	101.6-577.7	105.3-598.7	0.583
Reticulocyte	%	Not found	Not found	-
BUN*	mg/dL	3.1-27.2	4.2-19.7	0.004
Creatinine	mg/dL	0.7-2.2	0.9-1.8	0.358
AST*	U/L	4.8-56.3	10.1-39.6	0.013
ALT	U/L	0-4.9	0-5.6	0.826
ALP*	U/L	0-281.5	0-225.4	0.033
TP	g/dL	6.5-8.9	6.6-9.3	0.148

Asterisk showed significant difference of blood parameters between sexes (P<0.05)

DISCUSSION

In this study, most of the hematology and blood chemistry values were within the range of other reports for Asian elephants. There were some parameters which higher than other like the WBC count. This could be due to the inclusion of samples from different locations and management conditions, or from both healthy and unhealthy elephants.

Analysis of WBC found that segmented neutrophils and lymphocyte predominated as in previous reports [2,3,9]. However, we found that monocytes were in higher proportion than eosinophils, which were the same as Dastjerdi [15] reported that monocytes were the main WBC in juvenile elephants. This was contrasted with de Mel [9] and Salakij [3] who reported finding more eosinophils than monocytes. The variation of blood parameters of each study might be influent by various of laboratory error such as pre-analytical error; for instance, blood collecting method, blood sample preserving procedure before handing to laboratory, storage period until examination, and sample transportation. Process before handling to lab and storage period until examination also important due to damaged erythrocytes may swell during storage and transport, and this can increase the MCV which measured by automatic counters [16]. We assessed WBC counts manually because elephant cells, particularly monocytes that are bi-lobed, differ from those of humans, and using an automated human hematology analyzer can lead to unreliable results [17]. Also automated cell counting may report decreased values because of platelet or WBC aggregation, or fragile WBC.

Gender has been reported to be one of the factors that can affect hematological values in numerous species [18], although Silva and Kuruwita [19] and Salakij [3] also found no sex-associated clinically significant hematology differences in Asian elephants. In our study, MCV and MCHC were significantly higher in females than in males. In our study, the reproductive status of study elephants was unknown at the time of sampling, but camp records indicate most were not pregnant. Salakij [3] found leukocyte count and fibrinogen were significantly higher in males than females, although this difference was not observed in the present study. De Alwis [10] reported no significant difference of biochemical parameters between sexes; however, we found that male elephants had higher BUN levels than females. This may be due to a higher protein intake in bulls, which generally receive more food than females. The higher AST levels in bulls may be due to muscle activity; male elephants are stronger and work harder than females, especially in activities such as trekking (more tourist can be accommodated in the saddle), or kicking footballs

or logging demonstrations as part of the daily shows (personal communication). For ALP, which also was higher in males, Niemuller [20] suggested that musth bulls have higher concentrations of this enzyme than non-musth elephants. So in our study, the significant sex-different in ALP may indicate a musth condition for some bulls.

Gromadzka-Ostrowska [2] found hematocrit values, WBC, and neutrophil numbers of elephants in India were slightly higher, while RBC were lower during the winter due to poorer food quality. However, blood samples had been collected, and food with supplement were provide to these tourist elephants throughout the year; therefore, the influence of seasonal variation to hematological value was not high in these elephants. An animal's activities or type of work can affect health.

In this study, most of the referral ranges for hematology and blood chemistry values were similar to previous reports [17,19] which suggests they should be more appropriate for assessing health status in domestic Asian elephants in Thailand. There were gender difference for some of the blood parameters evaluated; therefore, sex of the animal should be taken into consideration for proper interpretation of blood data. Because of results of previous studies in other mammals, we suggest that further research in elephants should evaluate the effect of age and season on health parameters.

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Case Report

Trancient Horner's Syndrome as a Result of Nasopharyngeal Polyp Traction-Avulsion in a Cat [1]

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Abstract

A 5-month old, female, half-breed Russian Blue cat was admitted to Animal Hospital of Near East University with a complaint of noisy breathing. Nasopharynx examination revealed a mass in the left region. The findings of external ear canal and tympanic membrane, and cavum tympani were normal according to video otoscopic examination and direct tympanic bulla radiography, respectively. Mass was removed by traction-avulsion and histopathological examination showed chronic inflammatory polyp. Horner's syndrome was recovered within a month in post-operative period.

Keywords: *Nasopharyngeal polyps, Feline inflammatory polyps, Horner's syndrome*

Bir Kedide Nasofarengeal Polip Traksiyon-Avulziyonu Sonucu Gelişen Geçici Horner Sendromu

Özet

Yakın Doğu Üniversitesi Hayvan Hastanesi'ne, gürültülü soluma şikayeti ile gelen 5 aylık, dişi, Russian Blue melezi bir kedide yapılan farenks muayenesi sonucu sol nazofarengeal bölgede kitle tespit edildi. Videootoskopik muayenede dış kulak yolu ile kulak zarının, direkt bulla radyografisinde ise kavum timpaninin normal olduğu belirlendi. Kitle; traksiyon-avülziyon yöntemi ile alındı ve yapılan histopatolojik muayenede kronik inflamatorik polip olduğu belirlendi. Postoperatif 1. gün gelişen Horner sendromunun, topikal sempatomimetik midriyatik kullanımı ile bir ay içinde düzeldiği gözlendi.

Anahtar sözcükler: *Nazofarengeal polipler, Kedi yangısal polipleri, Horner sendromu*

INTRODUCTION

Non-carcinogenic feline inflammatory polyps (FNPs) are benign fibrous masses that are invasively pedunculated with inflammatory cells. It is reported that middle ear and nasopharynx FNPs in cats originate from tympanic bulla or Eustachian tube epithelium lines [1,2]. They may either reach pharynx via the eustachian tube, or the external ear canal with rupture of the tympanic membrane [2]. The dominant idea is that its etiology has infection or congenital reasons [3]. Due to the anatomical different in feline middle ear, it is known that there is frequently Horner's syndrome and rarely vestibular syndrome taking place following removal of polyps originated in the middle ear via the method of traction and/or ventral bulla osteotomy (VBO). It was reported that excessive curettage of promontory may lead to this in VBO [4]. In treatment of FNPs, primary recommendation is traction and avulsion of the polyp from the ear canal or in the nasopharyngeal way, where hemorrhage is minimal under anesthesia. It was reported that there is a 50% change of polyp relapse this way. There is a dominant consideration that postoperative medical therapy increases the success rate [2,5]. Other complications include vestibular disturbances, polyp re-growth, otitis media, hemorrhage, wound drainage, hypoglossal nerve damage, damage to auditory ossicles and vascular structures, and facial nerve paralysis [6].

Publication of the case presentation was approved because of beneficial effect of topical sympathomimetic

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mydriatic using in the treatment of Horner's syndrome after FNPs removed.

CASE HISTORY

A mass was detected on the left nasopharyngeal region as a result of the pharynx examination on a Russian Blue hybrid, 5-month-old, female cat brought to YDU Veterinary Hospital with complaints of rough heavy breathing. Normal signs were found in the external ear canal and the ear drum with the video otoscopy examination, and in the cavum tympani with the direct bulla radiography. Following the Xylazine HCl (Vetaxyl 20 mg, Vetagro, 2 mg/kg IM) premedication; induction was achieved with Ketamine HCl (10% Ketamine, Dutchfarm, 10 mg/kg IM). The polyp was removed with traction avulsion after endotracheal intubation *(Fig. 1)*.

In the histopathologic examination, it was found that this was a chronic inflammatory polyp. Phenylephrine HCL (2.5% Mydfrin ophthalmic solution, Alcon, 3x2 drops) was given to fix the Horner's syndrome that occurred following the traction-avulsion of the polyp *(Fig. 2)*, while Methylprednisolone acetate (Prednol tablet, Mustafa Nevzat, 2 mg/kg/day orally for 1 month) was given to prevent relapse of the polyp. It was observed that the Horner's syndrome that developed in the 1st operative day was fixed in a period of 1 month *(Fig. 3)*. The results of the hemogram, blood count, blood biochemistry, urine analysis, feline leukemia virus and feline immunodeficiency virus tests on the cat were in normal ranges.

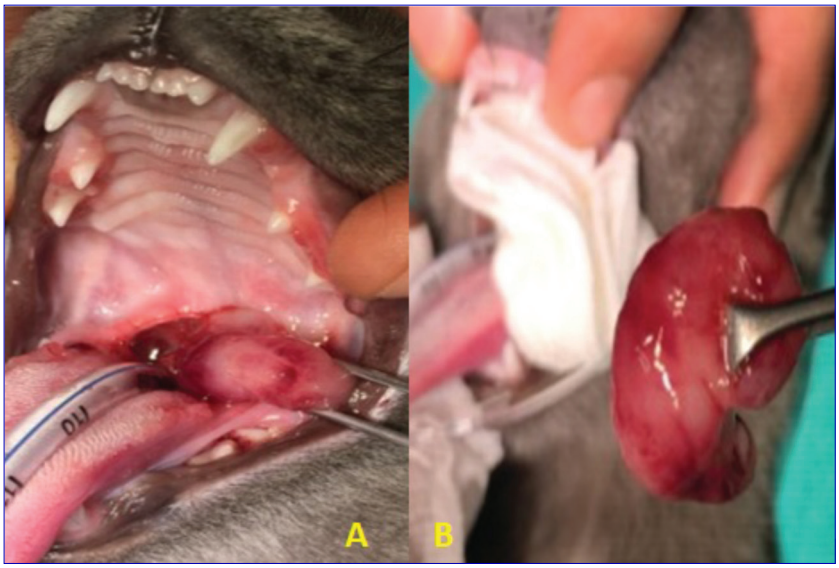

Fig 1. Removal of nasopharyngeal polyp by traction-avulsion (A). Inflammatory and ulcerated macroscopic view of polyp (B)

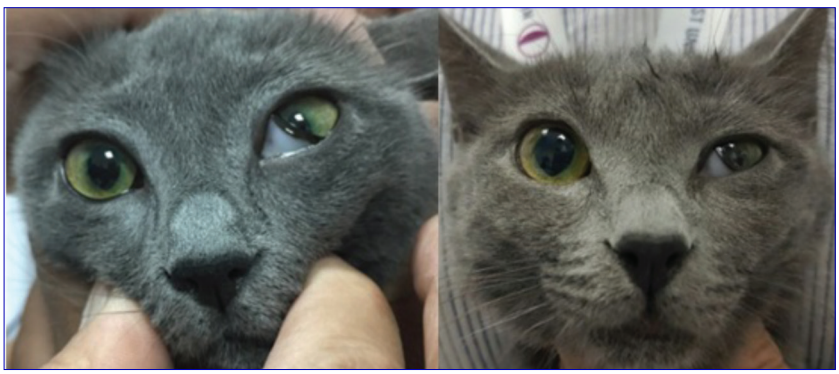

Fig 2. Post-operative Horner's syndrome in the left eye (ptosis, miosis, enophthalmos, protrusion of palpebra tertia)

DISCUSSION

It is reported that inflammatory cat polyps are usually seen under the age of 2 years unilaterally, there is no tendencies based on breed or sex [2,3] and these have pink, peduncular and ulcerous macroscopic appearances [5]. In the pharynx examination of our case, it was observed that the polyp was on one side, it was ulcerous and had a chronic inflammatory structure. Cats with FNPs may show symptoms of otitis externa and/or otitis media, as well as symptoms of upper airway obstruction [1]. It is known that symptoms include heavy breating, change of voice, nasal discharge, difficulty in swallowing, nutation, sneezing, vestibular signs, Horner's syndrome and blood-containing discharge from the external ear canal [2,5]. It is reported that the best diagnostic plan is oropharyngeal examination under anesthesia [2,7], otoscopic examination [5], and lateral radiography or computer tomography where soft tissue opacity may be monitored in the nasopharynx [2,8]. Diagnosis of inflammatory polyp was made after the pharynx and histopathology examination on our case with difficulty in swallowing and heavy breathing complaints. Ear canal video otoscopy and bulla radiography were carried out for the differential diagnosis of otitis media. Taking the factor of age into account, nasopharynx or middle ear originated polyp cases may be considered in cats with complaints of upper airways, otitis externa and otitis media. It should be considered to take bulla radiography with the purpose of examining the connection between the external ear canal and the middle ear via video otoscopy in addition to pharynx examination. These methods are required in cases where the advanced diagnosis of CT is not available. It is reported that rate of relapse in FNPs is 50%. In their

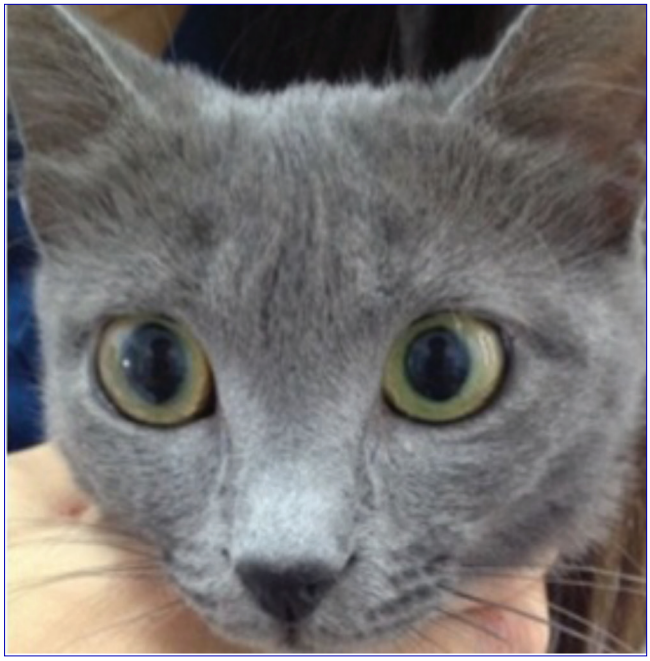

Fig 3. Recovery of the Horner's syndrome within one month post-operatively

operative treatment, it is recommended to perform traction and avulsion of the polyp through the ear canal or in a nasopharyngeal way. It was reported that corticosteroid (1 to 2 mg/kg/day for 14 days) in the postoperative period reduces relapse in cats by 11% [2,5]. VBO was recommended for operative treatment of polyps that originate from the middle ear [3]. Positive outcomes were obtained from the corticosteroid application following the traction-avulsion method, and no mid-term relapse was encountered.

It is known that the most important postoperative complication that may follow removal of polyps is temporary Horner's syndrome by 40%, this is caused by the damage on the oculosympathetic tract during operation, and the complication is solved in weeks or months in most cases [2,3,5]. It was reported that the success rate of the traction-avulsion method cats with only nasopharyngeal polyps is four times higher than those with ear canal polyps [9]. It was reported that 25% of cats that are affected by temporary Horner's syndrome in the postoperative period are affected long-term. In another study, in polyps removed with the method of traction-avulsion only, not only were relapses seen, but also the Horner's syndrome that occurred was solved in 7-10 months [7]. In the case, there were typical sympathetic denervation signs of Horner's syndrome (ptosis, miosis, enophthalmos, and elevation of the third eyelid), and it was thought that application of topical phenylephrine contributed to the recovery in a short time like 1 month.

As a result, it was concluded that combining the traction-avulsion method with VBO in polyps originating from the middle ear increased success, and usage of the traction-avulsion method by itself in polyps with no connection to the middle ear is sufficient, bloodless method and a technique that leads to minimal tissue damage. It was concluded that success rate is increased by making the diagnosis after bulla radiography, video otoscopy and pharynx examination in cases where CT is not available for dismissing the possibility of middle ear connection, usage of sympathomimetic mydriatics in treatment of Horner's syndrome in the postoperative period, and postoperative corticosteroid usage in case of relapse.

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Review

A Review of Machine Learning Applications in Veterinary Field

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Abstract

Machine learning is a sub field of artificial intelligence which allows forecasting through learning past behaviors and rules from old data. In today's world, machine learning is being used almost in any fields such as education, medicine, veterinary, banking, telecommunication, security, and bio-medical sciences. In human health, although machine learning is generally preferred particularly in predicting diseases and identifying respective risk factors, it is obvious that there are a limited number of publications where this method was applied on veterinary or indicates whether it is correct and applicable. In this review, it was observed that the neural network, logistic regression, linear regression, multiple regression, principle component analysis and k-means methods were frequently used in examined publications and machine learning application in veterinary field upward momentum. Additionally, it was observed that recent developments in the field of machine learning (deep learning, ensemble learning, voice recognition, emotion recognition, etc.) is still new in the field of veterinary. In this review, publications are examined under clustering, classification, regression, multivariate data analysis and image processing topics. This review aims at providing basic information on machine learning and to increase the number of multidisciplinary publications on computer sciences/engineering and veterinary field.

Keywords: *Machine learning, Artificial intelligence, Veterinary, Computer sience*

Veteriner Hekimlik Alanında Makine Öğrenmesi Uygulamaları Üzerine Bir Derleme

Özet

Makine öğrenmesi yapay zekanın bir alt çalışma alanı olup eski verilerden geçmiş davranışların ve kuralların öğrenilerek ileriye doğru tahminlerin yapılmasına olanak sağlar. Makine öğrenmesi günümüzde eğitim, tıp, veterinerlik, bankacılık, telekomünikasyon, güvenlik, biyomedikal gibi hemen hemen her alanda kullanılmaktadır. İnsan sağlığında özellikle hastalıkların önceden tahmin edilmesi ve ilgili risk faktörlerinin tespit edilmesinde makine öğrenmesi yöntemleri genellikle tercih edilmekle birlikte hayvan sağlığında doğruluğu ve aynı zamanda ilgili alanda kullanılabilir olup olmadığını belirleyen bu yöntemin uygulandığı çalışmaların sınırlı olduğu görülmektedir. Bu derlemede incelenen çalışmalarda sinir ağları, lojistik regresyon, lineer regresyon, çoklu regresyon, temel bileşen analizi ve k-ortalamalar yöntemlerinden sıklıkla yararlanıldığı ve veterinerlik alanında yapılan makine öğrenmesi çalışmalarının son yıllarda ivme kazandığı gözlemlenmiştir. Ayrıca makine öğrenmesi alanındaki son gelişmelerin (derin öğrenme, kolektif öğrenme, ses tanıma, duygu tanıma, vb.) veterinerlikte yeni yeni uygulandığı gözlemlenmiştir. Bu derlemede çalışmalar kümeleme, sınıflandırma, regresyon, çok değişkenli veri analizi ve görüntü işleme başlıkları altında incelenmiştir. Bu derlemenin amacı makine öğrenmesi ile ilgili temel bilgileri vermek ve bilgisayar bilimleri/mühendisliği ile veterinerlik alanındaki ortak çalışmaları arttırmaktır.

Anahtar sözcükler: *Makine öğrenmesi, Yapay zeka, Veterinerlik, Bilgisayar bilimleri*

INTRODUCTION

Machine learning deals with data analysis which aims to build analytical models in an automated way. Machine learning helps computers to discover insight information by learning from data through iterative algorithms, even if they are not programmed to search for them [1].

Thanks to machine learning methods, the machines have become capable of contributing to the brain power of the humanity as well as their contribution to manpower. These methods support us in creating assumptions on the future by analyzing a large amount of data for any practice and they help us in decision making. Therefore, the importance and contribution of machine learning methods are increasing more and more each day [2].

As new detection and diagnostic modalities are developed and data types getting complexer and multi-modal analysis getting more important which causes a

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remarkable increase of collected data in livestock, it is obvious that machine learning is required more than ever with great potential for future use. Multi-dimensional and complex datasets confronted by researcher may be interpreted through new tools enabled by machine learning.

There is a close relation between machine learning and fields such as artificial intelligence, data mining, statistics and especially probability theory, pattern recognition and computer sciences in general. Undoubtedly, there are many applications currently used in fields such as health and medicine [3], drug design [2], banking [4,5], education [6,7], telecommunications [8], software development [9], biomedicine, security, geology, astronomy. Other fields of use are fingerprint recognition [10], iris recognition [11], face recognition [12], handwriting and signature recognition [13], medical data identification [14], internet search engines [15] etc. *(Fig. 1)*.

In medicine, especially computer aided disease diagnosis and exploration of risk factors and their relations to diseases, very good results have been achieved by machine learning techniques. Machine learning techniques are distinguished with their successful results in relation to human health and animal health studies and they may provide new frontiers to solve problems which are known to be persistent in the field.

In several medical studies which are important for human health such as breast cancer prediction [16], estimation of cancer types [17], estimation of survival in patients with severe burns [18], identification of risk factors that trigger heart attacks [19], early detection of cancer [20], heart and vascular disease diagnosis [21], anomaly detection [22], polysomnographic [1], machine learning methods were benefited from.

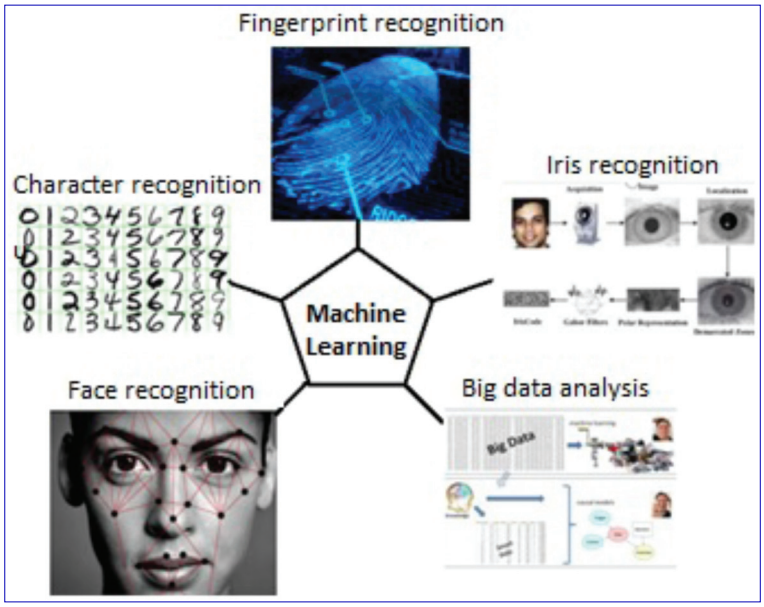

Fig 1. Application fields of machine learning

Machine learning is obviously one of the areas where most of researches were conducted recently. Thus, the importance of exploring this area is increasing. For analyzing various aspects of livestock farming which is closely related to human health since the very early days of history, such as the estimation and determination of animal diseases and respective risk factors, machine learning applications are required. Machine learning on veterinary is a new application method and it has drawn some attention recently and some studies were conducted in this area [23-27].

In this review, machine learning methods were explained briefly, in order to foster the implementation of machine learning methods in the field of veterinary and to provide basic information to researchers. Applications of clustering, classification, regression, feature analysis and image processing on veterinary were investigated. We suggest that the researchers may acquire a basic idea on machine learning through this study, so that they refer to machine learning methods for solving problems on animal health. In this way, the number of machine learning applications on veterinary shall increase.

MACHINE LEARNING TECHNIQUES

Machine learning is a way of programming computers where a performance criterion is optimized thanks to recent data or past experience. We define a model with some variables and through training data or past experience a computer program is taught to optimize the parameters of this model. The model may either be designed as a predictive one to predict the future, or a descriptive model to acquire knowledge from the dataset, or both [28]. *Fig. 2* outlines one way in the steps might be incorporated into an end to end machine learning system for analyzing data from a science or engineering application.

Some advantages of the machine learning are as follows [29]:

- In some cases, whenever we specify the input/output it may be impossible to estimate the relations among various data and to indicate their relationship. Thus, the machine is expected to estimate their relationship by setting its own microstructure.

- If the amount of information is too much it is likely to be handled by machines rather than humans.

- The machine is able to adapt to change easily.

- Machine learning methods reveal concealed relation-ships and links through a large data stack.

Fig. 3 shows the process of the machine

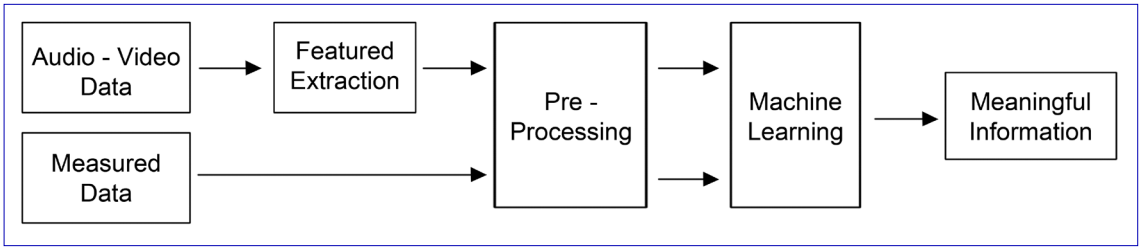

Fig 2. General piplines of machine learning

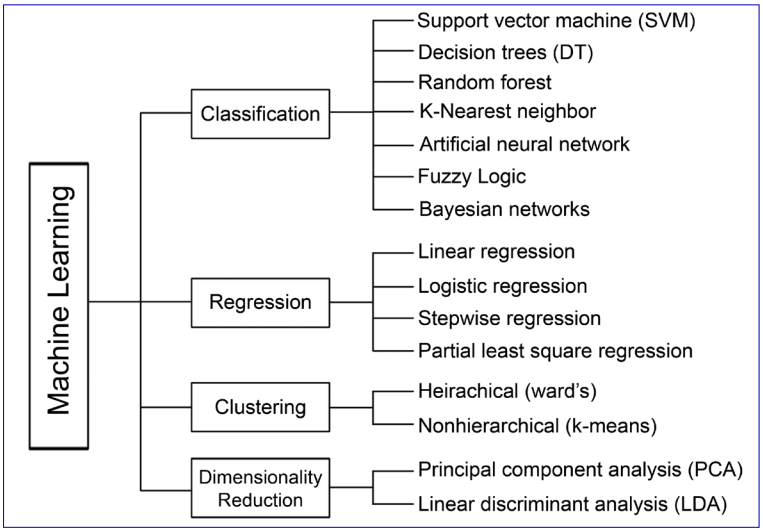

Fig 3. Techniques of machine learning and commonly used algorithms

learning and commonly used algorithms in computer science field. Techniques of machine learning model as categorized into supervised and unsupervised methods. The supervised machine learning techniques uses algorithms to reason from external instances which then generate universal theorems in order to predict future instances. This typeofmachinelearningconstructsabriefmodelforallocation of class labels to predict features. The resulting classifier is used to assign class labels to testing instances, the significance of the predicting feature is known, but there is no data about the value of the class label. The unsupervised methods do not utilize surrounding data to generate objective output or benefits.

Classification is a process to establish a model which is used to describe and discriminate between data classes. A group of training data is analyzed to derive a model. For objects with unknown class labels, the categorical (discrete, unordered) class label is predicted by this model. The process of data classification consists of a learning step and a classification step. A classifier is generated at the first step, i.e. the learning step (or training phase) which describes a preset group of data classes. We test the accuracy of this model at the second step and if it is positive, we use it for classifying the new data [6].

Regression analysis is the most frequently used among statistical methodologies for numeric prediction. While categorical labels are predicted through classification, continuously valued functions are modeled by regression. Rather than predicting (discrete) class label, regression is used for missing or unavailable numerical values [30,31].

Clustering analyzes data objects without consulting class label, unlike classification and regression, which are utilized in analysis of class labeled (training) datasets. Generally, it is quite usual for class labeled data to be missing at the early stage. Class labels may be created for a dataset through clustering. By maximizing and minimizing the interclass similarity the objects may be grouped or clustered. Clustering of objects take place in order to compare the similarity of objects within a cluster to one another, but without similarities to objects in other clusters [2].

Clustering methods are divided in two categories. These are hierarchical clustering and non-hierarchical clustering, also known as partitioning. The hierarchical methods (often known as k-means clustering methods) produce a set of nested clusters in which each pair of objects or clusters is progressively nested in a larger cluster until only one cluster remains. The non-hierarchical methods divide a dataset of *N* objects into *M* clusters, with or without overlap. Each object is a member of the cluster with which it is most similar, however the threshold of similarity has to be defined [32].

Image processing is used as a method to work on an image, in order to enhance it or to find out some useful information about it. Image processing grows rapidly today with its many application areas. It is established as a core research area within fields of engineering and computer sciences. Image processing uses two methods. These are analogue and digital image processing methods. For hard copies such as printed materials and photographs analogue image processing is used. Image analysts implement many interpretation techniques when these methods are used [33].

Multivariate data analysis is a statistical method for

Table 1. *Machine learning applications on veterinary field*

Classification Studies				
Author	**Year**	**Method**	**Objective**	**Results**
Akıllı et al.[35]	2016	Fuzzy Logic	They have designed a decision support system based on fuzzy logic and tried to determine the compatibility between the system and expert decision.	They have reported that the decision support system designed using the records on reproduction and milk production efficiency of Holstein Friesian dairy cows was conducting an accurate classification with a success rate of 92.6% and that fuzzy logic based decision support systems shall be successful on livestock farming.
Hempstalk et al.[36]	2015	DT, NB, BN, LogR, SVM, PLSR, RF, RotF	They have attempted to estimate the success of insemination and conception in dairy cows.	In this study, they have used 8 different machine learning methods and they have reported that the logistic regression method has had the best performance in general.
Küçükönder et al.[24]	2014	ANN, RBF Network, NB, KStar, Ridor	They have studied the classification on fertilization of Japanese quail eggs according to factors such as season, natural selection and frequency of settlement and to determine the influences of these factors.	In this study, 85% of quail eggs were determined to be fertile and 15% of them to have lower reproduction capacities with a success rate of 99.73%. It was observed that the Ridor algorithm for classification of eggs according to their fertility (as fertile or unfertile) has generated better results with lower error rate and the fertility rate was determined as 85.71%.
Lewis et al.[37]	2011	BN	They have tried to indicate that Bayesian network is an analytical method for complex animal health data.	As a result of this study they have reported that the statistical inference of Bayesian network model offers a richer analytical tool in comparison to any standard statistical technique.
Karabag et al.[38]	2009	DT	They have tried to determine the influential factors on hatching ability for Chucar partridges eggs.	They have determined overall hatching, fertility and hatching ability as 56.2%, 79.2%, and 71.0% respectively and they have reported that the classification tree method has predicted the external egg traits such as, egg weight, egg volume, egg length and width was a significant factor on hatching ability with an accuracy of 75.6%.
Pelaez and Pfeiffer[39]	2008	LogR, DT, FA	They have tried to classify cattle herds according to the presence of infectious disease.	In this study, they have reported that there was high risk in regions where the cattle population is dense and in many regions of Wales which are closed and where the cattle movement is frequent.
Regressional Studies				
Author	**Year**	**Method**	**Objective**	**Results**
Gokçe et al.[31]	2014	Simple/ multiple regression	They have tried to analyze the relationship between serum lactoferrin concentrations and serum IgG concentrations in lambs.	They have reported that there was a significant linear correlation (R^2=0.375) between serum lactoferrin concentrations as a predictor of passive immunity and serum IgG concentrations in lambs during different days of neonatal period (1st, 2nd, 4th, 7th, 14th and 28th day), but that it was insufficient for calculating the IgG concentration.
Gokçe et al.[40]	2013	Chi-square, Odds, RR	They have tried to investigate the influence of some factors on diseases and death of sheep at neonatal period and afterwards.	They have reported that the most important risk factors of lamb diseases and deaths were lambing season, number of births per dam, birth weight and dam's health status.
Gokçe et al.[41]	2013	MSRA, Simple/ multiple regression	They have tried to determine the relationship between passive immunity and growth performance in lambs during and after the neonatal period and to assess the impact of some factors on the growth performance.	They have reported that passive immunity in lambs has varied significantly in the early 12 weeks, that growth performance was depending on the birth weight, type of birth, gender, healthiness, dam's age and lambing season by indicating that growth performance was reduced if the dam's age was ≤2 years or it was a twin birth or a female lamb was born or the lamb was born in the winter or it has got ill or if the birth weight ≤3 kg.
Teke et al.[42]	2013	LinR, MLP, SMOreg	They have tried to model the live weights of Holstein Friesian breed using their body sizes.	They have reported that Linear Regression model, Multilayer Perceptron and SMOreg had success rates of 97.94%, 97.72% and 99.17% respectively and that it was possible to predict live weights through data mining with a high reliability.
Ghotoorlar et al.[25]	2012	ANN	They have tried to develop an automatic scoring system complying with the subjectively calculated lameness score.	23 features of 105 freely moving dairy cows were used and the cows were divided into 5 groups according to their movement score. They have reported that group No.1 and No.4 had the highest sensitivity and specificity value.
Takma et al.[43]	2012	Multiple regression, ANN	They have tried to research the effects of duration of lactation, calving year and service period in cows on lactation milk yield and their adaptability.	They have reported that the ANN model was more appropriate than multiple regression model in estimation of milk yield of Holstein Friesian cows and that it might be an alternative method to regression analysis, because it produces results with fewer errors.

Table 1. *Machine learning applications on veterinary field (Continue...)*

Clustering Studies				
Author	**Year**	**Method**	**Objective**	**Results**
Bank et al.[44]	2015	K-means	They have tried to characterize some gut bacteria which were found in piglets within the first week of their birth.	They have reported that there is a larger variation between the disturbed bacterial composition of neonatal piglets and diarrhoeic piglets and that diarrhoea was affecting the first week of neonatal piglets.
Nantima et al.[45]	2015	Ward's	They have tried to identify the risk factors associated with the occurrence and spread of African swine fever among smallholder pig farmers	They have used to ward's hierarchical clustering method to determine the number of clusters. According to their analyses they have observed some significant differences among the three cluster. for instance, households in cluster 1 that had purchased the least number of pigs reduced their risk to ASF compared to the other two clusters. Cluster 3 that was most vulnerable had the majority of the households practicing free range which increased ASF risk into these farms. Although cluster 2 had more households feeding swill. few of these households were affected by ASF outbreaks.
Dupuy et al.[46]	2013	K-means, Hierarchical clustering, PCA, MFA	They have tried to group the cows using carcass and health-related data.	They have obtained 12 consistent clusters according to slaughtering year and slaughterhouse and they have reported that the combination of their clustering methods with multiple factor analysis were appropriate for larger and complexer slaughterhouse data.
Petit et al.[47]	2010	K-means	They have tried to group the wild animals according to syndromes using wildlife necropsy data.	They have reported that they have obtained 9 clusters and that these clusters reflect the most obvious and frequent diseases. They have also indicated that k-means was a useful tool.
Dogan [48]	2002	K-means	He has tried to demonstrate that clustering analysis method might be applied in studies on animal breeding using some body sizes of the Arabian fillies.	He has reported that the height at withers, heart girth and cannon bone circumference of fillies were lower and that the differences among genders were rather caused by the genetic structure due to the gender than age and he has indicated that clustering analysis would be appropriate especially during selection at animal breeding.
Gürcan et al.[23]	2002	Hierarchical clustering	They have tried to classify the German Meat Merino and Karacabey Merino Sheep genotypes using live weight, body size, and fiber diameter.	After the clustering analysis it was discovered that two genotypes have similar body sizes and for both genotypes it was observed that the subgroup consisting of 1.5 and 2.5 year old sheep indicated heterogeneity, whereas the subgroup consisting from 3.5, 4.5 and 5.5 year old sheep showed homogeneity. Besides they have reported that 98.9% of the herd was found in the same cluster when both genotypes were assessed together and that there were no significant difference between the two in terms of body size.
Multivariate Data Analysis Studies				
Author	**Year**	**Method**	**Objective**	**Results**
Gokçe et al.[26]	2013	MSRA, GLM	They have tried to research some risk factors influencing the passive immunity and birth weight in lambs and to figure out the relationship between the passive immunity and birth weight.	They have reported that some farm administration applications and animal characteristics were related to birth weight and passive immunity and also that birth weight was effective on passive immunity.
Yunusa et al.[49]	2013	PCA	They have tried to analyze the morphological structure of Nigerian Uda and Balami sheep.	They have reported that the most important features for describing both breeds were traits relating to cranial measurements and bone development.
Akçay et al.[50]	2012	PCA	They have tried to evaluate carcass parts of broiler chicken.	10 criteria were used in the study, after the principal component analysis they have determined that the first five principal components were corresponding to the 80.4% of the total variance and that the first principal component was able to describe 42.3% of the total variance. Moreover, they have reported that PCA may be used as a tool in assessing and understanding the total variance in livestock farming
Casanova et al.[51]	2012	PCA	They have tried to classify the cows of French and Spanish breeds according to their morphological properties.	They have concluded that the first principal component was face and skull lengths describing 49.9% of the variance and the second principal component was skull and head width describing 19.2% of the variance.
Meyer [52]	2007	PCA	They have tried to demonstrate that it was possible to reduce the number of properties through PCA using carcass data and ultrasound data of Angus cows.	They have reported that 14 traits were used in the study and that 7 of them sufficed for selection index calculations and estimation of breeding values and that it was possible to halve the number of traits to estimate the breeding values directly through principal components.
Lee et al.[53]	2006	LDA, MFCCs	They have tried to automatically identify animals (30 kinds of frog calls and 19 kinds of cricket calls) from their sounds.	LDA used to reduce the feature dimension and increase the classification accuracy. They have reported that the average classification accuracy is 96.8% for 30 kinds of frog calls and 98.1% for 19 kinds of cricket calls.
Caraviello et al.[54]	2006	DT, BN, Instance-based algorithms	They have tried to determine the variables affecting pregnancy in cows and the variables affecting first-service conception rate.	Through the decision tree algorithm, they have obtained an accurate classification for the pregnancy status at a rate of 71.4% and for the first-service conception a rate of 75.6% in terms of Holstein cows on large dairy farms.
Bilgin and Esenbuga [55]	2005	Canonical correlation analysis	They have tried to evaluate the relationship between the body sizes and and carcass weight of Morkaraman sheep.	They have reported that it is possible to estimate carcass traits of male Morkaraman lambs from their live body sizes and that it may be useful to implement low cost live body measurements in earlier years for carcass weights as a detailed selection criterion.

Table 1. *Machine learning applications on veterinary field (Continue...)*

Image Processing Studies				
Author	**Year**	**Method**	**Objective**	**Results**
Bozkurt et al.[27]	2013	ANN	They have tried to determine the feeding performance and carcass properties of Brown Swiss and Holstein Friesian breeds in a feedlot system.	They have reported that according to models obtained from digital image analysis and ANN, body length and heart girth were the best predictive variable for estimating the live weight; they have reported that the best predictive variable for estimation of warm carcass weight was the carcass length.
Mcevoy et al.[56]	2013	ANN, PLSDA	They have tried to determine the region including the hip joint on radiographic images of dogs.	They have reported that veterinary images have the potential of being utilized for educational purposes in classification and grouping.
Bilgin et al.[57]	2011	Image Processing Method	They have tried to demonstrate that nose prints of Kangal breed dogs were different from each other.	The resulting values were very different and remote from each other according to statistical data obtained. They have declared that this was caused by the uniqueness of images.
Slósarz et al.[58]	2011	ANN	They have attempted to estimate the fat content within the muscular parts of lambs.	They have reported that there was a significant relation between the body weight and the lamb's age before slaughter, while the relationship between the body weight and intramuscular fat content was weak.

ANN: *Artificial neural networks,* ***BN:*** *Bayes networks,* ***DT:*** *Decision tree,* ***FA:*** *Factor analysis,* ***GLM:*** *General linear model,* ***LDA:*** *Linear discriminant analysis,* ***LogR: Logistic regression****,* ***MFA:*** *Multiple factor analysis,* ***MFCCs:*** *Mel-frequency cepstral coefficients,* ***MLP:*** *Multilayer perceptron,* ***MSRA:*** *Multivariable stepwise regression analysis,* ***NB:*** *Nive bayes,* ***Odds:*** *Odds ratio,* ***PCA:*** *Principal component analysis,* ***PLSDA:*** *Linear partial least squares discriminant analysis,* ***PLSR:*** *Partial least squares regression,* ***RotF:*** *Rotation forest,* ***RR:*** *Relative risk,* ***SMOreg:*** *support vector machine for regression,* ***SVM:*** *Support vector machine*

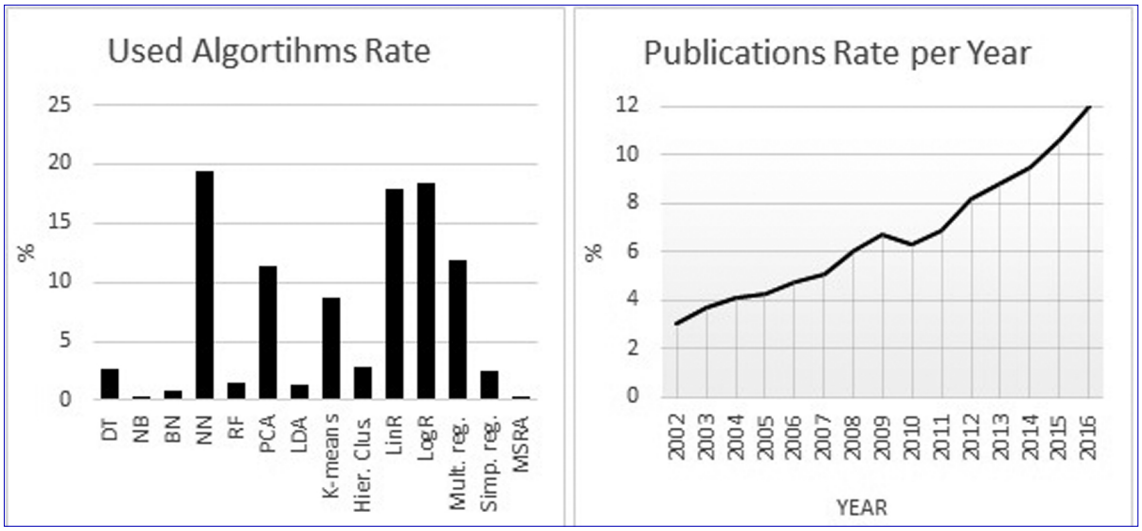

Fig 4. The distrubition of publications according to machine learning methods and publication frequency rate per year

analyzing data consisting of multiple variables. Multiple variables typically come into play in many cases of daily life, products to be chosen or decisions to make and they help modeling the real life. The available data is abundant, but difficulties arise in terms of clarifying the picture and making intelligent decisions based on it [34].

MACHINE LEARNING APPLICATIONS ON VETERINARY

While the articles scanning we selected keywords: "analysis of animal diagnosis", "diagnosis of disease veterinary", "evaluation of risk factor analysis for animal diseases", "animal medical data analysis", "disease decision support animal", "predict animal diagnosis machine learning", "analysis small ruminant diagnosis machine learning", "animal passive immune system machine learning", "naïve bayes in veterinary", "machine learning predict animal disease", "predict animal disease using decision tree", etc. and their combinations are used. The research questions may not cover all machine learning area in detail. We tried to get only a snapshot of machine learning literature. This study is not a subject specific literature review. We grouped 30 publications are classified into six different topic (clustering, classification, regression, multivariate data analysis and image processing) according to *Table 1*.

We searched Science Driect digital databases to get frequency of machine learning algortihms and publications rate per individual year. We selected keywords: (Veterinary

OR animal) AND (machine learning algorithms which is related in this study). The percentage of publications according to machine learning methods and publications frequency rate per year (2002 - 2016) is shown in *Fig. 4*. The most frequently used methods are NN, LogR, LinR, multiple regression, PCA and k-means. Also we have identified that the literature on machine learning application in veterinary field up and upward momentum with the proliferation.

CONCLUSION

Machine learning is a field of study associated with artificial intelligence, in order to enable a machine to perform a task on its own and with the best performance and this is a discipline based on extracting information and learning from data. A learning computer is able to generate predictions about future thanks to machine learning methods. Therefore, it contributes a great deal to scientific researches by being widely used especially in the field of medicine.

Today, precautions to avoid diseases on animal health and particularly in herd health and herd management in farms, determination of risk factors and development of appropriate protection-control programs are being assessed to be quite important and popular. Multi-disciplinary studies carry a significant potential for contribution to this field of research.

In this paper, related works concerning machine learning applications from veterinary field are reviewed. For this purpose 30 publications are classified into clustering, classification, regression, multivariate data analysis and image processing. The main goal of this review is to introduce a baseline and an idea to researchers who would work on this field. Thus, it is envisaged that the machine learning applications on veterinary shall increase and the latest developments on machine learning shall be applied to solving the problems on animal health.

We have identified that the literature on machine learning application in veterinary field up and upward momentum with the proliferation. And the neural network, logistic regression, linear regression, multiple regression, principle component analysis and k-means methods are most frequently used.

It was observed that recent developments in computer science/engineering have not adequately addressed the problems encountered in veterinary field. Such as deep learning, ensemble learning, voice recognition, emotion recognition, etc. is still new in the field of veterinary.

In this study, we focused machine learning applications in veterinary field. This study is not a subject specific literature review. Therefore as for future works, provide a review on computer aided disease diagnosis in veterinary. Also, introducing the tools, package and successful implemented programs in this field is proposed.

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Letter to the Editor

Translatory Facet Joint Locking in a Cat
(Bir Kedide Translasyonel Facet Eklem Kilitlenmesi)

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Dear Editor,

Spinal trauma resulting from the traffic accidents is a common etiology in cats and dogs. Severity of the trauma may cause to traction, compression, extension, flexion and torsion causing vertebral column injury. These forces act on the vertebral column and lead to different types of fracture and luxation [1]. Anatomically, the facet joints limit the segmental range of motion, and absorb the energy that results from the ventrally directed shearing forces [2,3].

The facet joints together with ligaments determine the direction and degree of displacement of the vertebrae one to another [3]. A locked facet joint is a type of facet joint dislocation [2,4]. This luxation causes by a skipping of the cranial articular process over the superior articular process of the vertebra below and becomes locked in the position [4]. The facet joint locking is a spinal cord deformity that it develops after a significant trauma, which is encountered either "rotatory" or "translator" locking in humans [2]. A plan radiograph is almost sufficient for the diagnosis but computed tomography and magnetic resonance imaging may be performed [2,3]. The treatment methods applied may be conservative (provocative mobilization with movements) or operation; however, the conservative method is less effective than surgery [2]. In the literature, acute cervical and lumbar facet joint locking is reported in humans, but thoracic facet joint locking is not familiar described because of rib cage coupling [4]. According to our knowledge, this condition has not been reported in companion animals yet; thus, we aimed to report radiological and surgical findings of a translatory facet joint locking in a cat.

A 7 month-old, female, domestic shorthair cat was presented to Uludag University, Faculty of Veterinary Medicine, Department of Surgery Clinics after a traffic accident. Clinically, vital parameters of the cat were normal; however, she had minimal hind-limb sensation to the reflexes of the skin pinches, which was suspected as upper motor neuronal lesion, and decreased motor reflexes (patellar, pedal reflexes etc) due to lower motor neuronal lesion, as well. In addition, when the cat hold up by an assistant, it was seen that the left front limb had second degree lameness and the cat was not bearing her weight on this leg, compared to right one. Palpation of this leg revealed a scapular fracture, and palpation of the vertebral column determined an irregularity of the spinal process on the cranial part of thoracic vertebras. The other orthopedic and neurologic examination findings were normal. Lateral and ventrodorsal radiographes pointed out a minimal dislocated scapular fracture and the dorsal dislocation of third thoracic vertebra at the level of facet joints. The caudal articular process of the facet joint of 3rd vertebra was jumping of the cranial articular process and the superior articular process of the vertebra was below and become locked. There was no rotational vertebra at the dislocation site, as well *(Fig. 1)*.

Based on the clinical and radiological examination results, the facet joint locking and a scapular fracture were diagnosed in the cat. Due to conservative treatments (flexional and extensional vertebral movements), the luxation was not replaced; thus operation was decided in this cat by the permission of the owner.

Sedation and induction of the cat was provided with xylazine HCl (1 mg/kg, im) and ketamine HCl (8 mg/kg, im) respectively. After endotracheal intubation, general anesthesia was applied with 2% concentration of isoflurane. The cat was positioned sternoabdominaly, and operation site was prepared for aseptic surgery. The dorsal midline incision and dissections were performed to expose the dislocated area. It was evident that facet joints of 3rd thoracic vertebra were out of the joint, and

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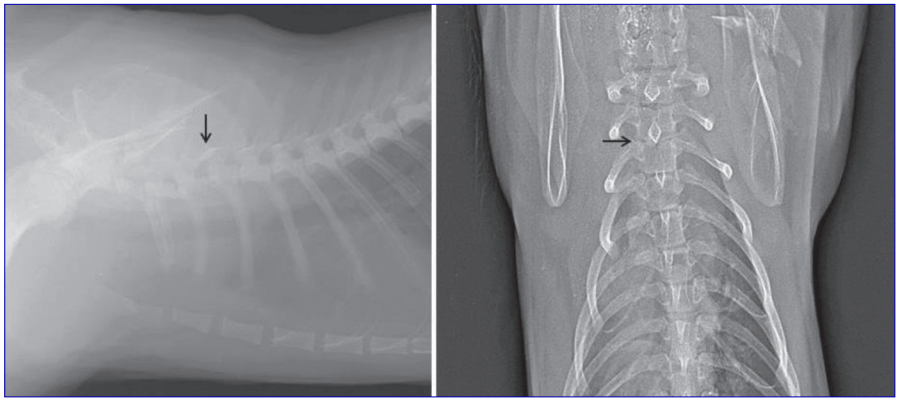

Fig 1. The views of dorsal dislocation of 3rd thoracic vertebra at the level of facet joints *(arrows)* in lateral and ventrodorsal radiographies

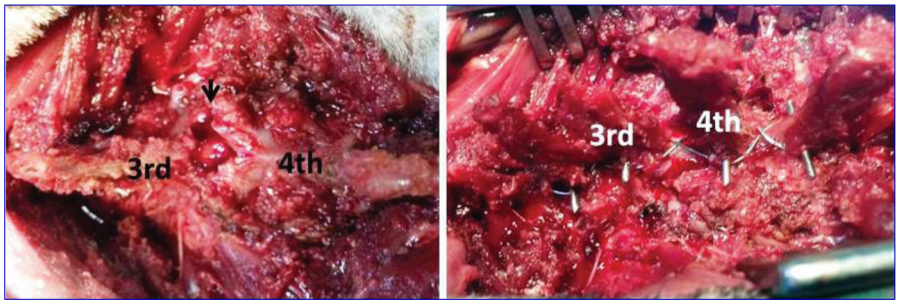

Fig 2. In the intraoperative views, left figure shows dorsal luxation parts of facet joints *(arrow)* following dissection and minimal dorsal laminectomy, and right figure demonstrates applied stabilization technique between the vertebras

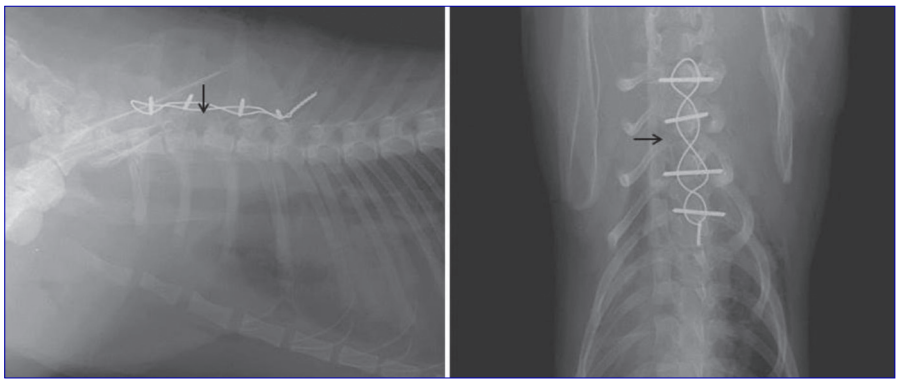

Fig 3. These views present repositioning of the intervertebral space *(arrows)* and vertebral column in the lateral and ventrodorsal radiographs

3rd vertebra was luxated dorsally. A minimal dorsal laminectomy and a conventional modified fixation technique were performed *(Fig. 2)*. As a stabilization technique, a kirschner pin was transversally inserted to spinal processes of 1st, 2nd, 3rd and 4th thoracic vertebra, and then a tension band was applied between these pins, which were passing to spinal processes of the effected thoracic vertebra from 1st to 4th *(Fig. 3)*. Spinal cord injury was not determined during surgery. Surgical incisions were closed routinely. Postoperative radiographs showed that the luxation part of the vertebral column was placed in normal location and fixation was ensured. At postoperative period, cefazolin Na (20 mg/kg, im, bid) and tolfenamic acid (2 mg/kg, sc, qd) were administered as antibiotic and analgesic agents, respectively. The bandage was applied to left extremity for scapular fracture and routine controls were planned. The cat was discharged.

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